

p73 cooperates with Ras in the activation of MAP kinase signaling cascade

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The *p73* gene is capable of inducing cell cycle arrest, apoptosis, senescence, differentiation and to cooperate with oncogenic Ras in cellular transformation. Ras can be considered as a branch point in signal transduction, where diverse extracellular stimuli converge. The intensity of the mitogen-activated protein kinase (MAPK) cascade activation influences the cellular response to Ras. Despite the fundamental role of p53 in Ras-induced growth arrest and senescence, it remains unclear how the Ras/MEK/ERK pathway induces growth arrest in the absence of p53. We report here that oncogenic Ras stabilizes p73 resulting in p73 accumulation and enhancement of its activity. p73, in turn, induces a sustained activation of the MAP kinase cascade synergizing with oncogenic Ras. We also found that inhibition of p73 function modifies the cellular outcome to Ras activation inhibiting Ras-dependent differentiation. Here, we show for the first time that there is a signaling loop between Ras-dependent MAPK cascade activation and p73 function.

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The p53 family members, *p73* and *p63*, share significant sequence homology with *p53*, but unlike *p53*, they give rise to multiple protein isoforms owing to alternative mRNA splicing and promoter utilization.¹ Increase in TAp73 α expression correlates with certain differentiation processes and has tumor suppression functions,² whereas Δ Np73 α can cooperate with oncogenic Ras in cellular transformation.³ The cellular outcome of p73 upregulation is dependent on the cellular context, but the full nature of this regulation remains unknown.⁴

Ras proteins were originally identified through their cell transformation properties, but since then they have been shown to regulate divergent functions such as cell proliferation, differentiation and apoptosis. The orchestration of a variety of signaling pathways to produce diverse and specific cellular outcomes requires the ability of Ras to activate a particular responsive module for each specific stimulus.⁵ Upon external stimuli, Ras becomes transiently activated relaying its signal to downstream effectors. The signaling intensity by the Raf/MEK/ERK module is known to play an important role in Ras-induced cell cycle arrest and cellular differentiation.⁶ In rat pheochromocytoma PC12 cells, sustained activation of extracellular signal-regulated kinase (ERK) is required for neuronal differentiation, whereas transient activation of ERK leads to increased proliferation.⁶ This scenario requires crosstalk between the contextual modulators and the mitogen-activated protein kinase (MAPK) cascade components.

The functional interaction between some of the pathways engaged by oncogenic Ras and the growth suppression function of p53 is well established.^{7,8} However, despite the fundamental role of p53 in Ras-induced growth arrest and senescence, p53-independent pathways for Ras outcomes have also been described. Ras-mediated growth arrest and p21^{WAF1} upregulation has been reported in p53-deficient cells.⁹ These observations suggest the existence of Ras-induced pathways leading to growth arrest, senescence and differentiation which are independent of p53.

In contrast to the previous avenue of data on the Ras-p53 crosstalk, there is little or no information about a possible Ras-p73 functional interaction. The capacity of p73 of inducing apoptosis, senescence, differentiation or transformation,^{3,10} makes the p73 gene a suitable candidate to be a modulator of Ras cellular outcome.

Here, we report for the first time, that there is a signaling loop between Ras-dependent MAPK cascade activation and p73 function.

Results

Constitutively active Ras induces p73 accumulation. To investigate a possible Ras-p73 crosstalk, we took advantage of the Ras-inducible UR61 model.¹¹ This is a PC12-derived cell line which contains a stably integrated transforming mouse *N-ras*(Lysine-61) gene under the control of a dexamethasone (Dex)-inducible promoter.

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Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; pp-ERK, phosphorylated ERK; Dex, dexamethasone; CHX, cycloheximide; p53-DD, dominant-negative p53; p73-DD, dominant-negative p73; p73-DD-L371P, inactive dominant-negative p73; Ph, phalloidin; Ad, adenovirus

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Treatment with Dex resulted in an increase in p73 protein, reaching maximal levels at 12 h (Figure 1a and Supplementary Figure 1a and b). To identify the specific p73 isoform expressed, we run *in vitro* translated proteins corresponding to the isoforms TAp73 α , TAp73 β and Δ Np73 α . We found that TAp73 α was the predominant isoform induced by activated Ras in this cell line (Figure 1a and Supplementary Figure 1a). Although UR61 cells expressed detectable basal levels of p63 and p53 under standard culture conditions (Supplementary Figure 1a), an increase in the expression was not detected upon Ras induction neither by RT-PCR (Figure 1b) nor by immunoblot (Supplementary Figure 1a). This observation

suggests that they may not play a role in Ras-induced differentiation in this cellular model.

The increased level of p73 in the presence of Ras was not due to enhanced p73 transcription, as Ras did not affect the levels of p73 RNA (Figure 1b). Furthermore, in transcriptional assays using the p73-P1-promoter-luciferase as a reporter, transfected H-RasV12 or induced N-RasK61 failed to stimulate p73-P1 luciferase activity, whereas ectopic E2F1 could activate this promoter (Figure 1c).

The former results are coherent with a post-transcriptional mechanism of Ras-mediated p73 accumulation. To elucidate the mechanism by which Ras augments p73 levels, we

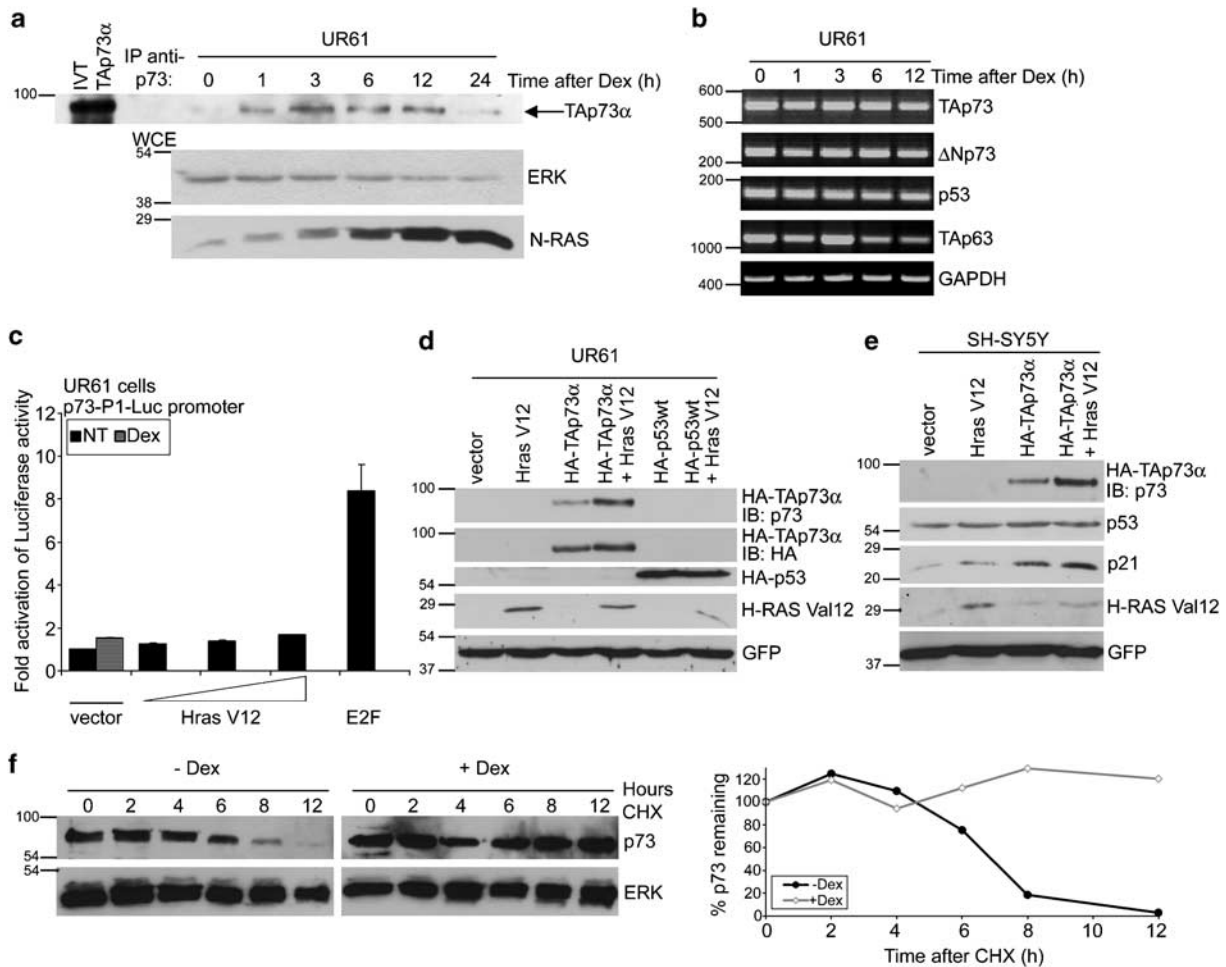


Figure 1 Constitutive active Ras stabilizes TAp73 α . Oncogenic ras induces accumulation of p73. (a) Immunoprecipitation/Western blot analysis showed upregulation of endogenous TAp73 following treatment of UR61 cells with dexamethasone (Dex). At the indicated time points, cell lysates were prepared and equal amount of protein was immunoprecipitated with anti-p73 antibody (ER15) and analyzed by Western blot. Equivalent amounts of input sample was run along and levels of induced N-RasK61 was analyzed by Western blot with an specific anti-N-Ras antibody. Total ERK expression was assessed as loading control. *In vitro* translated protein corresponding to the TAp73 α isoform was run along the samples. Activated Ras induces accumulation of p73 by a post-transcriptional mechanism. (b) Expression of different p53 family members was analyzed in Dex-treated UR61 cells by semiquantitative RT-PCR. (c) Transcriptional analysis of the P1-p73 promoter-luciferase reporter in UR61 cells in the presence or absence of Dex, transfected with increasing amounts of H-Ras V12 (0.5–2 μ g) or 2 μ g of E2F. Luciferase activity was normalized by the Renilla activity of the same lysate. The data represent the mean of at least three experiments. Error bars indicate S.E. Ras overexpression increases p73 at steady-state levels. (d) UR61 and (e) SH-SY-5Y cells were transiently transfected with empty vector, HA-TAp73 α or HA-p53 alone or together with constitutive active Ras (H-Ras V12). Equal amount of GFP expression vector was cotransfected with each sample. Cell lysates were prepared 24 h after transfection and equal amounts of protein were loaded. GFP expression was assessed as loading and transfection efficiency control. Oncogenic Ras prolongs p73 half-life. (f) p73 half-life determination. UR61 cells transfected with HA-TAp73 α were split into two and each cultured with either Dex (+ Dex) or DMSO (-Dex), after 6 h both cultures were treated with 20 μ g/ml of cycloheximide (CHX). Total cell lysates were used to detect p73 levels by immunoblotting. Densitometric analysis was performed to quantify protein expression and each sample value was normalized by its correspondent loading control. The graph shows the quantification of the remaining TAp73 α .

cotransfected Ras and TA-p73 α and asked whether Ras could also upregulate ectopic p73. In the presence of ectopic Ras, cotransfected TA-p73 α levels analyzed with either anti-p73 or anti-HA antibodies were higher (Figure 1d). In contrast, expression of H-RasV12 did not affect the levels of GFP, which was used to normalize the transfection efficiency. The same results were obtained in SH-SY5Y cells (Figure 1e), a human neuroblastoma cell line where oncogenic Ras results in neuronal-like differentiation.¹² These data indicate that Ras overexpression increases p73 steady-state levels in rat and human cells.

To quantify Ras-mediated stabilization of p73, we determined the p73 half-life in UR61 cells in the presence and absence of Ras. Cells transfected with TAp73 α were incubated with or without Dex and 12 h later, treated with cycloheximide (CHX). As shown in Figure 1f, in absence of activated Ras (–Dex), transiently expressed p73 had a half-life of about 6 h, whereas with Dex, p73 levels were maintained even at 12 h after CHX treatment (Figure 1f).

Oncogenic Ras enhances TAp73 activity. We observed that in the presence of Ras, p73 induction of p21^{WAF1}, a p53/p73 transcriptional target, was stronger (Figure 1e), whereas endogenous p53 levels were not affected by Ras expression (Figure 1e). Therefore, we sought to investigate whether Ras-mediated p73 stabilization resulted in increased p73 transactivation. Promoter reporter assays were performed in UR61 cells with constructs containing the luciferase gene driven by multiple p53 consensus binding sites (PG13 promoter) (Figure 2a and b) or by the full-length p21^{WAF1} promoter (Figure 2c). In UR61 cells, TAp73 α consistently activated the PG13 promoter weakly, whereas cotransfection of TAp73 and H-RasV12 dramatically enhanced TAp73 α transactivation (Figure 2a). Similar results were obtained in the inducible system (Supplementary Figure 2a). It is worth mentioning that neither ectopic expression of p53 nor p73 elicited a strong transcriptional response of the PG13 or p21^{WAF1} promoters in UR61 cells. However, ectopic expression of H-RasV12 (Figure 2a), or induced N-RasK61 (Supplementary Figure 2a), resulted in increased promoter activity. Previous reports,¹³ as well as our own data (Supplementary Figure 1), have shown that endogenous p53 levels do not increase upon Ras transfection in UR61 cells. Consequently, Ras-induced reporter activation could be accounted by the accumulation of endogenous p73, even though p53 involvement cannot be completely ruled out in this system.

To address whether Ras could modulate TAp73 transactivation in the absence of p53, we used human HCT116p53 $^{-/-}$

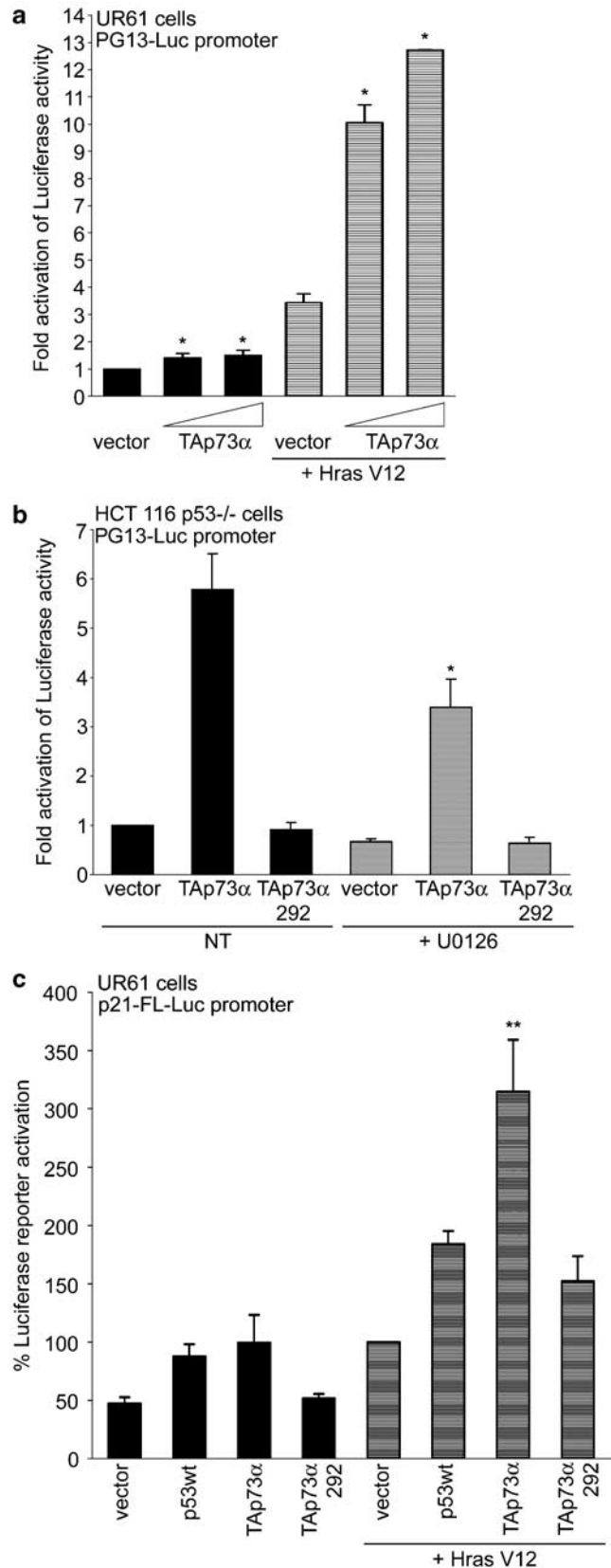


Figure 2 Oncogenic Ras enhances p73 activity. Cotransfection of activated Ras with TAp73 α resulted in enhanced p73 transactivation function in UR61 (a and c) or HCT116p53 $^{-/-}$ cells (b). Transcriptional analyses were performed in cells transiently transfected with the PG13-luciferase reporter promoter (a and b) or the p21^{WAF1} full-length promoter (p21-FL-Luc) (c), together with the indicated expression vectors. (b) Transfected cells were treated with 20 μ M of U0126 or DMSO (NT). Luciferase activity was normalized by the Renilla activity of the same lysate. The data are mean values from at least three independent experiments. Error bars indicate S.E. (* $P < 0.05$ ** $P < 0.005$)

cells, which harbor a constitutively active K-Ras mutation and lack p53.^{14,15} In these cells, TAp73 α overexpression elicited a strong upregulation of the PG13-luciferase activity (Figure 2b). Conversely, TAp73 α 292, a DNA-binding incompetent mutant, did not activate the promoter. Blockage of Ras signaling pathway with a MEK inhibitor, U0126 (10 μ M), decreased significantly, but not completely, TAp73 α -mediated activation of the reporter (Figure 2b). Consistently, Dex-enhanced TAp73 α activation of the PG13 promoter was dramatically inhibited upon U0126 treatment (Supplementary Figure 2a).

p21^{WAF1} plays an important role in Ras-induced cell cycle arrest.¹⁶ However, p53-independent pathways for Ras-mediated growth arrest and p21^{WAF1} upregulation have been reported.¹⁶ Therefore, we investigated whether activated Ras could modulate TAp73 upregulation of the p21^{WAF1} promoter in the UR61 system. In these cells, expression of TAp73 α , p53wt or H-RasV12 weakly induced the p21^{WAF1} promoter (2 \times induction) (Figure 2c), which parallel their effect on the PG13 promoter (Figure 2a). Nevertheless, coexpression of H-RasV12 and TAp73 resulted in a robust transactivation of this promoter (6 \times induction, Figure 2c). This synergistic effect seems to be specific for TAp73, as cotransfection of H-RasV12 with p53wt resulted in a mere additive induction of activity (4 \times induction, Figure 2c).

We used the HCT116p53^{-/-} cells to address whether Ras modulation of TAp73-mediated p21^{WAF1} transactivation was p53 independent. As expected, the strong upregulation of the p21^{WAF1} promoter elicited by TAp73 α was affected significantly by treatment with U0126 (Figure 3a). Furthermore, the levels of endogenous p21^{WAF1} induced by ectopic expression of TAp73 α , decreased drastically in cells treated with U0126 (Figure 3b). It is worth mentioning that we could observe a reduction of ectopically expressed p73 as a consequence of MEK inhibition on shorter film exposure (Figure 3b); suggesting again that Ras/MEK pathway stabilizes p73.

As MEK activation is strictly dependent on Ras, we wanted to explore the consequence of the knockdown expression of K-Ras by shRNA over TAp73-mediated p21^{WAF1} transactivation. Repression of K-Ras expression resulted in an attenuation on the p21 levels induced by transfected TAp73 α (Figure 3c). Furthermore, the levels of ectopically expressed

TAp73 decreased in the absence of K-Ras (Figure 3c). These results substantiate the role of Ras signaling in TAp73 protein stability and function.

p73 activates the MAP kinase signaling pathway and synergizes with Ras in this activation. Unexpectedly, we observed that ectopic expression of TAp73 resulted in

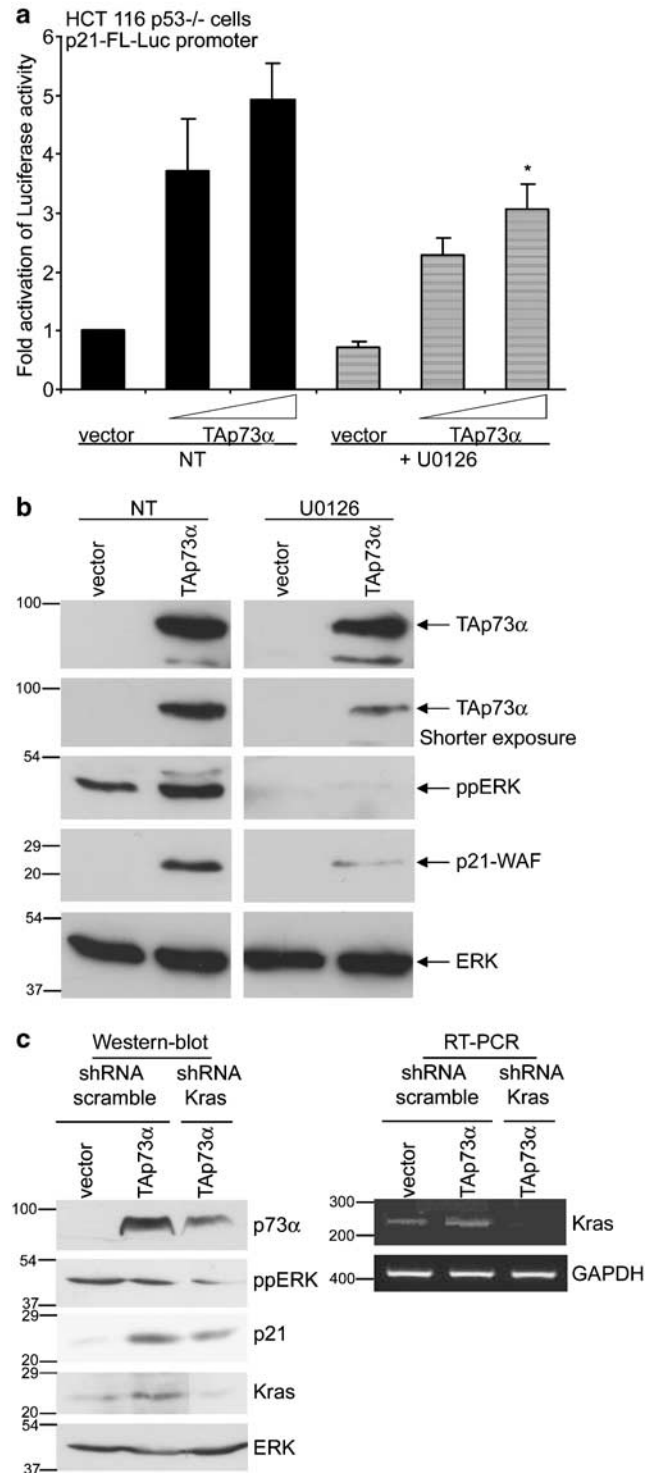


Figure 3 Ras functional inhibition results in an attenuation of the p21^{WAF1} expression induced by p73 in absence of p53. Inhibition of Ras signaling through the MAPK cascade by treatment with the MEK inhibitor U0126 leads to an abatement of p73 transcriptional function. **(a)** Transcriptional analysis of the p21-FL-Luc promoter in HCT116p53^{-/-} cells transiently transfected with the indicated expression vectors. Transfected cells were treated with 20 μ M of U0126 or DMSO (NT). Luciferase activity was normalized by the Renilla activity of the same lysate. The data are mean values from at least three independent experiments. Error bars indicate S.E. **(b)** HCT116 p53^{-/-} cells were transfected with either empty vector or TAp73 α expression plasmid and treated with DMSO (NT) or U0126 (20 μ M). Immunoblot analysis of p73, p21^{WAF1} and pp-ERK were performed as before with specific antibodies. K-Ras shRNA inhibits p21^{WAF1} induction by TAp73 α overexpression. **(c)** HCT116 p53^{-/-} cells either with shRNA against K-Ras or scrambled shRNA (scr-RNA) were, 18 h later, transiently transfected with empty vector or TAp73 α expression plasmid. Immunoblot analysis of p73, p21^{WAF1}, K-Ras and pp-ERK were performed 24 h later as before. Total ERK expression was used as loading control. Expression of endogenous K-ras and GAPDH were analyzed by semiquantitative RT-PCR

increased endogenous levels of phosphorylated-ERK (pp-ERK) (Figure 3b), suggesting that TAp73 could activate the MAPK cascade by itself. To address this question, we studied the ability of p73 to activate downstream targets of Ras activation in UR61 cells.

First, we analyzed the ability of p73 to activate Elk1, a phosphorylation substrate of activated ERK. UR61 cells were cotransfected with a Gal4-reporter plasmid (Gal4-Luc) and an expression vector for the chimeric Gal4-DNA-binding domain fused to the transactivation Elk1 domain (Elk1-Gal4),¹⁷ together with different p73 plasmids. TAp73 α and TAp73 β significantly activated the MAP kinase reporter (Figure 4a). This activation was reproduced in SH-SY5Y and Ntera2 cells (Supplementary Figure 3a and b). Both human cell lines are able of terminal differentiation along the neuronal pathway upon retinoic acid treatment.^{18,19} Thus, the p73-mediated enhancement of MAPK activity was consistent in the neuronal-derived cell lines.

To further address at which point p73 is activating this cascade, we studied the effect of TAp73 on endogenous ERK activation. As shown in Figure 4b, ectopic expression of TAp73 α resulted in increased pp-ERK, but it did not alter total ERK expression.

Considering that differentiation of PC12 cells by oncogenic Ras is accompanied by increased AP-1 (activator protein-1) activity,²⁰ and that the signaling pathway from Ras to AP-1 appears to be mediated through the Raf/MEK/ERK module,²¹ we decided to test whether overexpression of TAp73 could affect AP-1 activity in this model. Expression of TAp73 α and TAp73 β resulted in a robust stimulation of AP-1 activity (Figure 4c), confirming that TAp73 activation of the MAPK cascade could result in activation of Ras targets relevant to its cellular outcome.

Like UR61 cells, the human SH-SY5Y and Ntera2 cells contain a wild-type *p53* gene^{22–24} To determine whether p73 activation of the MAPK cascade was independent of *p53* status, we performed a similar experiment in SaOs-2, a *p53* null human osteosarcoma cell line with no oncogenic *Ras*,²⁵ and in HCT116p53 $^{-/-}$ cells. Ectopic expression of TAp73 α and β induced Elk1 activity (Figure 5a and b) in both cell lines.

In SaOs-2 cells, p73-mediated induction of Elk1 activity was weak, but nevertheless similar to the activation mediated by H-RasV12 in these cells (Figure 5a). On the other hand, TAp73 was capable of inducing a vigorous MAPK activation in HCT116p53 $^{-/-}$ cells, which contain mutated Ras (Figures 3b and 5b). This result led us to hypothesize that TAp73 activation of the MAPK cascade was stronger in the presence of oncogenic Ras. Furthermore, treatment of HCT116p53 $^{-/-}$ cells with U0126, or suppression of K-Ras expression by shRNA, abolished this activation (Figures 3b and c and 5b), confirming the cooperation between p73 and Ras.

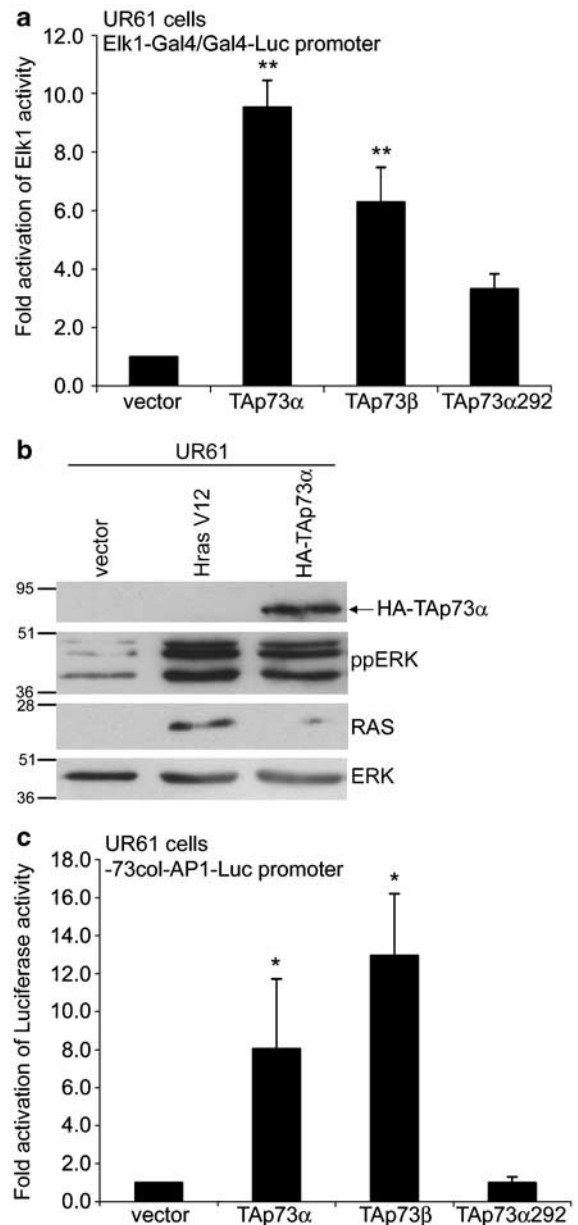
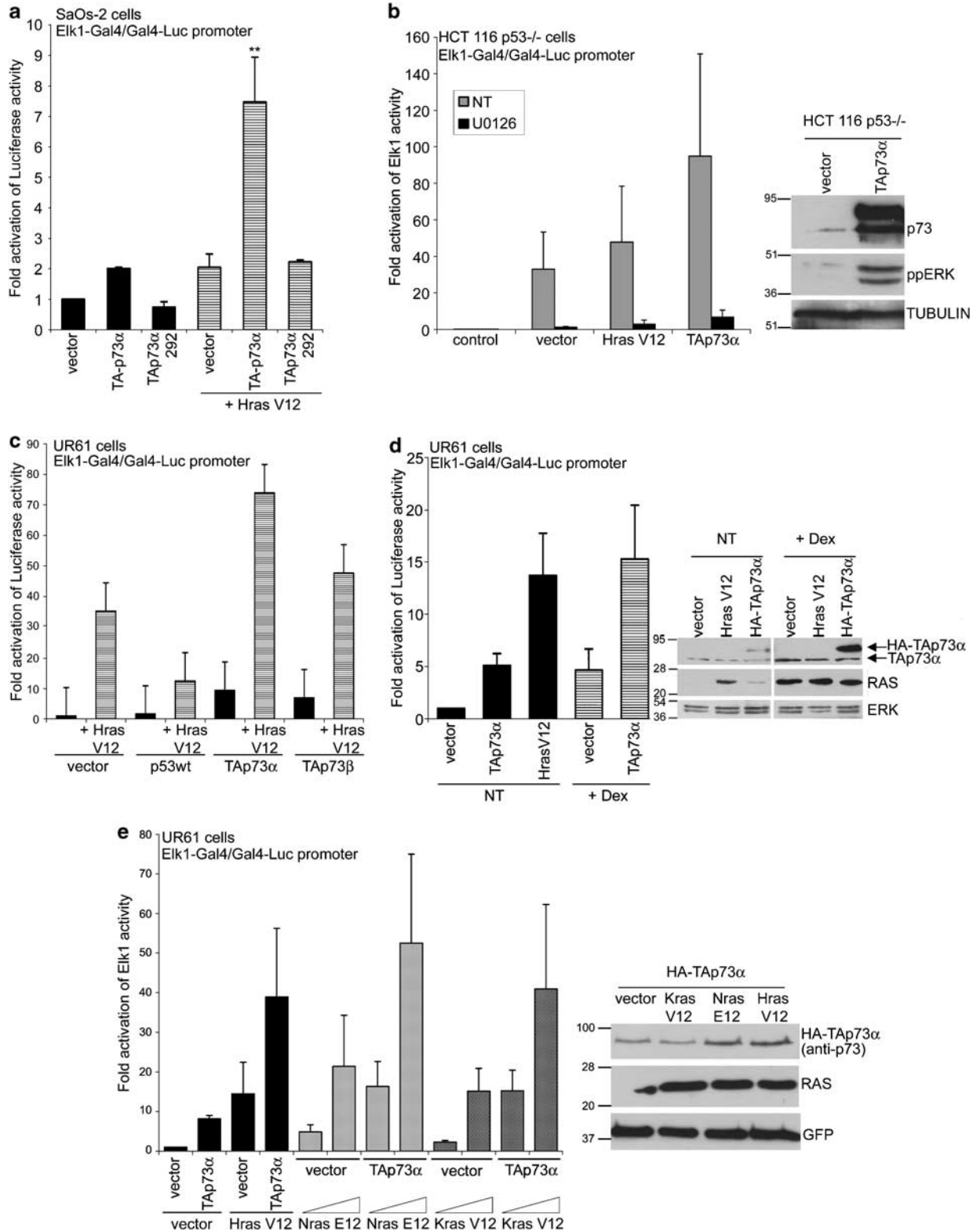


Figure 4 TA-p73 activates the MAP kinase signaling pathway. (a) MAP kinase reporter assay was performed transfecting the Elk1-Gal4/Gal4-Luc reporter system together with the indicated p73 expression vectors in UR61 cells. (b) TAp73 α increases ERK activation. UR61 cells were transiently transfected with the indicated expression vectors and analysis of phosphorylated ERK protein levels (pp-ERK) was performed by Western blot assay. Expression of transfected TAp73 α and H-RasV12 were analyzed with specific antibodies (ER15 and Ab-4 respectively). Total ERK expression was used as loading control. (c) AP-1 activation assay was performed in UR61 cells transiently transfected with both the AP-1 reporter plasmid (-73Col1-AP1-Luc) and the indicated plasmids. Luciferase activity was measured and normalized as before. The data represent the mean of at least three experiments. Error bars indicate S.E. (* $P < 0.05$, ** $P < 0.005$)

Figure 5 TAp73 α synergizes with oncogenic Ras in the activation of MAP kinase signaling cascade independently of p53. (a–e) MAP kinase reporter assay were performed as before in either the p53 null cells SaOs-2 (a), the HCT116p53 $^{-/-}$ cells (b) or in UR61 cells (c–e) with the indicated expression plasmids. (b) Transfected cells were treated with the MEK inhibitor U0126 (20 μ M) or DMSO as control (NT). Expression of transfected TA-p73 α and pp-ERK were analyzed by Western blot as described before. (d) Transfected cells were cultured in the presence of Dex (+ Dex) to induce N-Ras expression, or the vehicle (NT). Luciferase activity was measured and normalized as before. The data represent the mean of at least three experiments (\pm S.E. * $P < 0.05$). (d–e) Expression of endogenous (TA-p73 α) and transfected p73 (HA-TAp73 α) were analyzed by Western blot with the ER15 antibody. The levels of transfected H-RasV12 (d–e), K-RasV12, N-RasE12 (e) as well as the inducible N-RasK61 (d) were analyzed with anti-pan Ras antibody. Total ERK (d) or GFP (e) expression were used as loading control

To further investigate this cooperation, we studied Elk1 activation in UR61 cells. Consistent with our previous results, TAp73 α and TAp73 β by themselves induced a moderate activation of Elk1 when compared with H-RasV12, whereas

p53wt had no effect. However, TAp73 α cotransfected with Ras and to a lesser extent TAp73 β , but not p53wt, had a more than additive effect on Elk1 activation (Figure 5c). The attenuation of Ras-induced Elk activation observed in the



presence of p53 is consistent with a recent report where wild-type p53 directly interfered with Ras/MAPK cascade.²⁶ We tested whether TAp73 α could also enhance the signal triggered by the inducible N-RasK61. Ras induction resulted in a mild activation of Elk, similar to the activation triggered by ectopic TAp73 α alone (Figure 5d). However, treatment with Dex produced a dramatic increase in TAp73 α -induced Elk activity (Figure 5d), confirming the synergistic effect between TAp73 and Ras in the activation of the MAPK cascade. In this experiment, we can observe that the levels of endogenous (TA-p73 α) as well as ectopic (HA-TA-p73 α) p73 were augmented with Dex treatment (Figure 5d, insert), confirming our previous observation of Ras-mediated p73 stabilization.

Although the role of the three members of the Ras family (*H-Ras*, *N-Ras* and *K-Ras*) during embryonic development seems to be unique,²⁷ activated versions of all of them can induce neuronal differentiation in *in vitro*.¹⁶ We asked whether all the Ras isoforms could cooperate with p73 and observed that the synergistic effect was common to the three *Ras* genes (Figure 5e).

This cooperative effect between TAp73 and Ras is neither dependent of p53 status, as it is reproducible in SaOs-2 cells (Figure 5a), nor cell specific as it is consistent in human SH-SY5Y and NTERa2 cells, and in mouse embryonic fibroblast (Supplementary Figure 3a and 3b and data not shown). However, we failed to detect a cooperative effect in the lung carcinoma cell line H1299, where ectopic expression of oncogenic Ras did not activate this reporter system (data not shown).

Functional inactivation of p73 attenuates Ras activation of ERK. To define the role of p73 function in Ras activation of the MAPK cascade, siRNA oligonucleotides homologous to 5-prime unique TAp73 sequences,²⁸ missing in Δ Np73, were used to inhibit the accumulation of p73. In HCT116p53 $-/-$ cells, transfection of p73 siRNA, but not scrambled siRNA, led to decreased endogenous TAp73 without affecting the levels of total ERK (Figure 6a). TAp73 siRNA, but not control siRNAs, attenuated the high endogenous levels of pp-ERK (Figure 6a and b, $*P < 0.05$) as well as endogenous p21 (Figure 6a), confirming our hypothesis that p73 function contributes to Ras activation of the MAPK cascade.

We utilized the widely used dominant-negative p73 (p73-DD) mutant²⁹ to functionally inactivate endogenous p73 in UR61 cells. As *in vitro* translated p73-DD and human p63 proteins are capable of binding,²⁹ we analyzed whether p73-DD could bind the endogenous rat p63. Immunoprecipitation studies with an antibody (GC15) specific against p73-DD β ³⁰ failed to show interaction between p73-DD and endogenous rat p63, in conditions where p73-DD and endogenous TAp73 α complexes were detected (Figure 6c). Neither did we detect interaction between p73-DD and p63 using an antibody against p63 (Figure 6c). These data validate p73-DD as suitable reagents to specifically inactivate p73 in UR61 cells.

We then analyzed the effect of p73 inhibition in Ras-induced activation of the Elk1-reporter system. As shown in Figure 6d, p73-DDwt, but not the inactive dominant-negative p73 (p73-DD-L371P), decreases up to 40% ($*P < 0.05$) Ras-mediated activation. The apparent Elk1 activation elicited by p73-DD-

L371P was not statistically significant ($P > 0.5$). Similar results were obtained in HCT116p53 $-/-$ cells, where p73-DDwt, but not the mutant p73-DD-L371P (data not shown) reduced the levels of Elk1 activation. Taken together, these data indicate that functional inhibition of TAp73 results in an attenuation of endogenous Ras-mediated MAPK cascade activation.

p73 function is necessary for Ras sustained ERK activation and the subsequent differentiation.

As noted in the Introduction, both *p73* and *Ras* have been involved in cell differentiation in several models.^{3,16} In UR61 cells, induction of the activated *N-Ras* gene results in growth arrest and neuronal-like differentiation.¹¹ Following 24 h of treatment with Dex, 70% of the UR61 cells showed the morphological changes of neuronal-like differentiation (Figure 7a). As in PC12 cells,⁶ this process is dependent on the Ras/Raf/MEK/ERK activation, as the MEK inhibitor U0126 completely blocked it, whereas treatment with p38 (SB303580 and SB202190), or PI(3)K (LY294200) inhibitors did not affect the process (Figure 7b). In addition, p73 expression profile correlates with the kinetics of Ras-induced differentiation in these cells (Figure 1a).

To determine the role of p73 in Ras-mediated differentiation, we utilized p73 and p53 dominant-negative mutants. As shown in Figure 7c, p73-DDwt, but not a dominant-negative p53 (p53-DD), resulted in a significant decrease ($*P < 0.05$) in the number of differentiated cells compared with cells transfected with GFP alone. To confirm this result, UR61 cells were infected with recombinant bicistronic adenoviruses encoding GFP-p73-DDwt or the inactive GFP-p73-DD-L371P,²⁸ and treated with Dex. Infection with the inactive mutant failed to inhibit the differentiation process, whereas 90% of cells infected with p73-DDwt lacked any of the morphological changes associated with neuronal-like differentiation (Figure 7d top and middle panels, respectively). Confocal microscopy analysis showed that the GFP-p73-DDwt-infected cells remained viable, with a globular morphology and cytoplasmic blebs similar to those of undifferentiated cells, whereas the surrounding noninfected cells presented a neuronal-like phenotype with neurite extensions that finished in growth cones and lacked blebs (Figure 7d). Overall, these data demonstrate that p73 function plays a critical role in Ras-induced differentiation.

Sustained ERK activation is necessary for Ras-induced differentiation in PC12⁶ and UR61 cells (Figure 7b). Thus, we decided to elucidate the impact of p73 inactivation in Ras-induced ERK activation in our system. In vector-infected cells, pp-ERK was detected as early as 6 h and maintained up to 36 h (Figure 7e and f). Consistent with our previous results, pp-ERK was detected in Ad-p73-DDwt-infected cells, but this phosphorylation was not sustained and disappeared after 12 h of treatment, despite the continuous treatment with Dex (Figure 7e and f). The magnitude of N-RasK61 protein expression was not affected by the adenoviral infections, so the effect on ERK activation was not due to lack of activated Ras protein (Figure 7f). Altogether these data prove that functional inactivation of p73 modifies the cellular outcome of Ras activation, resulting in transient, rather than sustained, Ras-mediated ERK activation, and in the subsequent inhibition of differentiation.

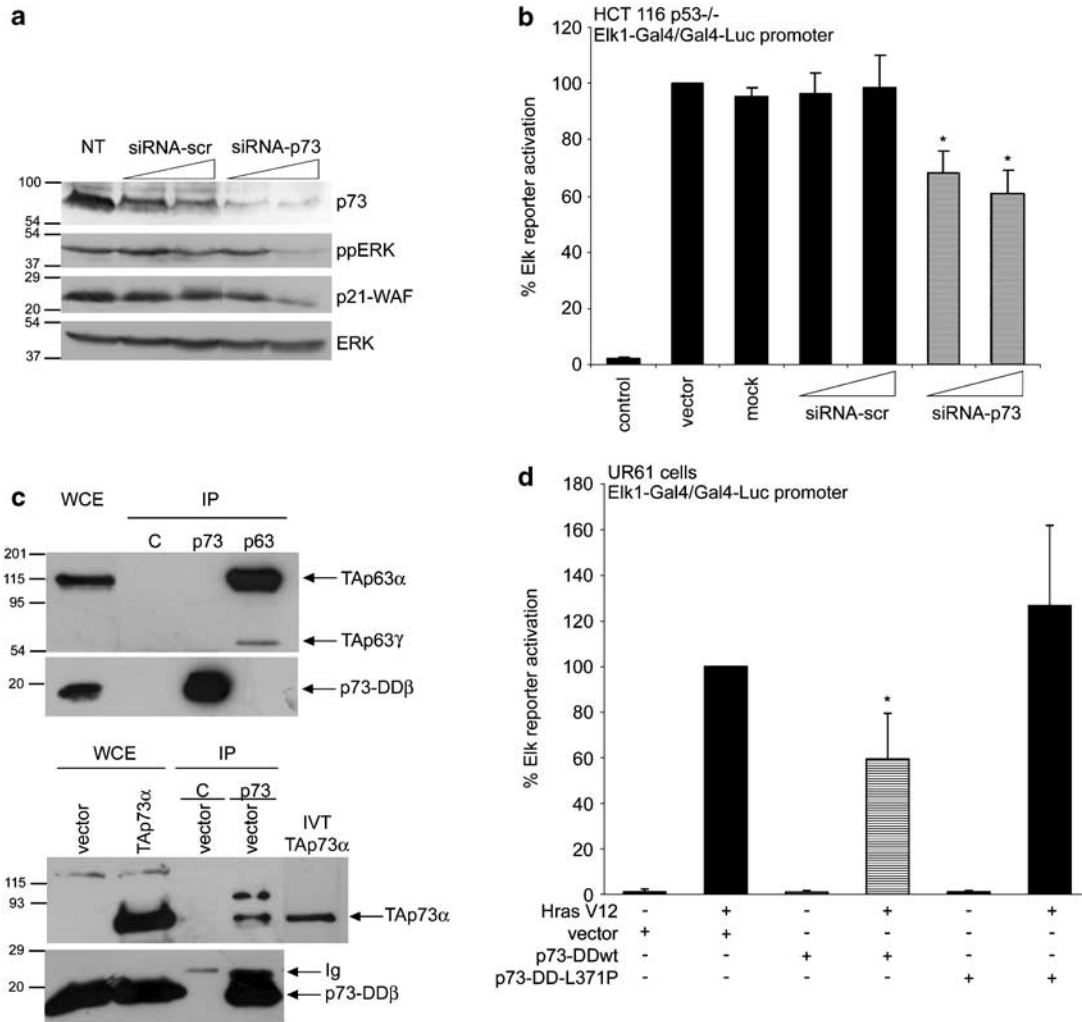


Figure 6 Functional inactivation of p73 attenuates Ras-induced ERK activation. p73 siRNA decreases endogenous p73 levels. (a) HCT116p53^{-/-} cells were transfected with siRNA against p73 or scrambled control and 72 h later p73, p21^{WAF1} and pp-ERK were performed as before. Total ERK expression was used as loading control. Genetic disruption of p73 attenuates Ras-induced MAPK cascade activation in the presence or absence of p53. (b) HCT116p53^{-/-} cells transfected with siRNA against p73, scrambled control or with annealing buffer only (mock) were transiently transfected 48 h later with the MAP kinase reporter plasmids to measure the endogenous levels of activated ERK. (c) Validation of the p73 dominant-negative mutants (p73DD) specificity in rat UR61 cells. Cellular lysates from UR61 cells transfected with human p73-DD/wt were immunoprecipitated with specific antibodies: anti-p73 (GC15), anti-p63 (4A4) or control antibody (anti-TAG 419). Cell lysate with ectopically expressed Tap73 α was used as marker. Protein complexes were analyzed by Western blot against p63 (4A4) or p73 (ER15). (d) MAP kinase reporter assay was performed cotransfecting the indicated vectors together with the reporter plasmids. The p73-DDwt expression vector harbors a p73-DD, whereas the p73-DD-L371P harbors an inactive point mutant. The values were compared with the value scored by transfected oncogenic H-RasV12. Luciferase activity was measured and normalized as before. The data represent the mean of at least three experiments (\pm S.E. * P <0.5)

Discussion

Ras has the capacity to elicit cell cycle arrest and, in primary cells, to induce replicative senescence.^{5,16} The accumulation and activation of tumor suppressor genes, like p53, is part of the fail-safe mechanism of a normal cell against the uncontrolled proliferation elicited by oncogenic stimuli.⁸ Although the crosstalk between Ras signaling pathway and p53 activation is well established, it remains unclear how the Ras/MEK/ERK pathway induces growth arrest in the absence of p53. Our studies offer one possible mechanism of Ras cellular outcome independent of p53. Our data show that Ras constitutive activation leads to the stabilization of p73. In addition, TAp73 can induce a sustained activation of the MAP

cascade and synergize with Ras modulating the strength of the response. We also present evidence that p73 function plays a decisive role in Ras-mediated neuronal differentiation.

Constitutive active Ras induces p73 accumulation and enhances p73 activity. Ras oncogenic activation leads to the stimulation of p53 activity.⁸ We report here a similar scenario, where constitutive Ras activation leads to stabilization of p73 and thereby augments p73 transcriptional activity. This effect is dependent on the cellular context, which is consistent with the tissue specificity of p73 tumor suppression function.² For example, in H1299 cells, activated Ras fails to stabilize p73 and to enhance its activity (B Fernandez-Garcia, unpublished results).

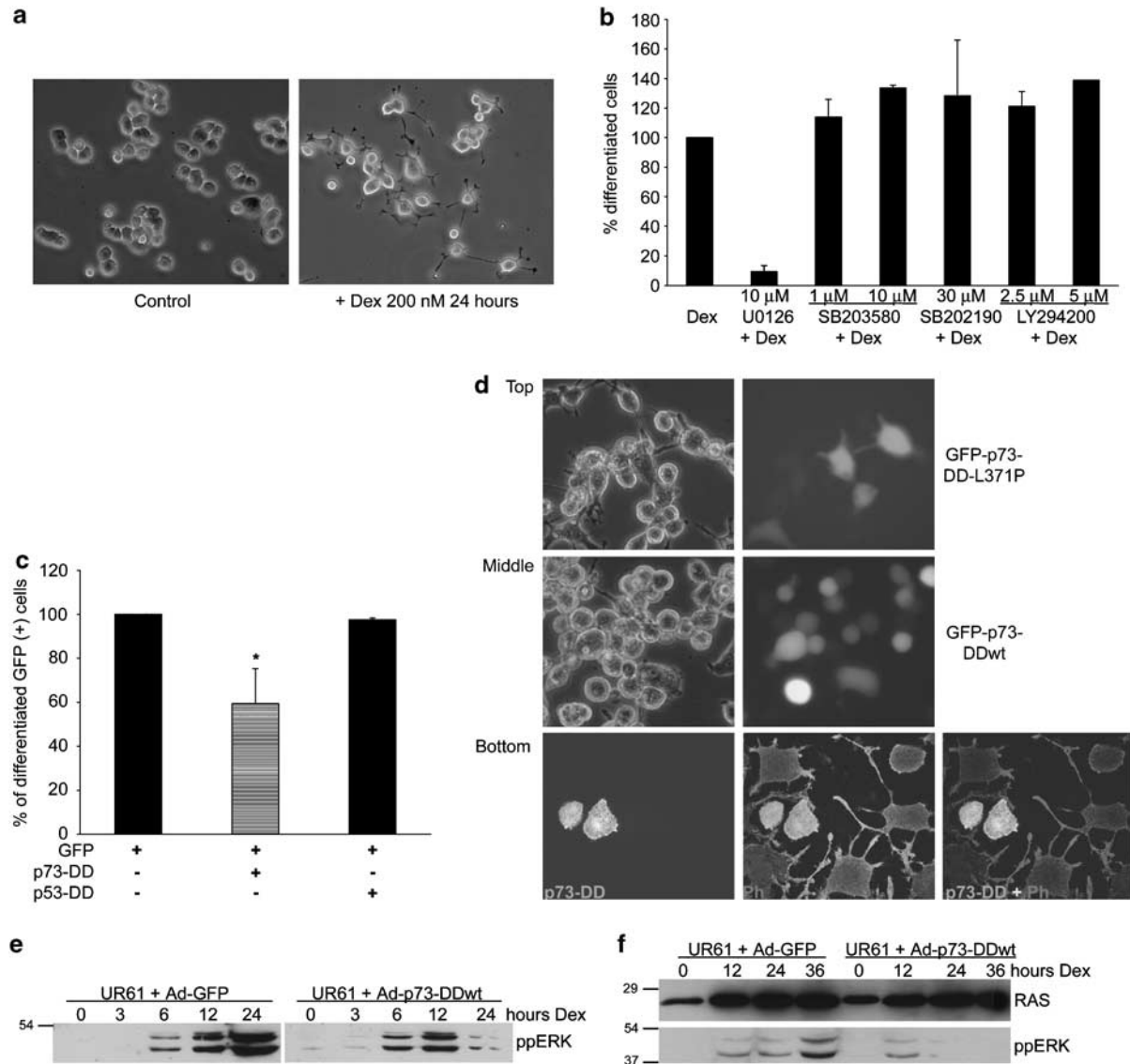


Figure 7 p73 function is necessary for Ras sustained ERK activation and the subsequent differentiation. Activation of the MAP kinase pathway is required for Ras-induced neuronal phenotype in UR61 cells. **(a)** Photomicrographs of UR61 cells incubated with vehicle control (DMSO) or Dex (Dex, 200 nM) 24 h after treatment. Although control-treated cells presented a globular morphology, the cells with Dex treatment showed a neuronal-like phenotype with numerous neurite extensions with growth cones. **(b)** UR61 cells were treated for 24 h with either Dex alone (Dex) or Dex plus one of the following inhibitors (10 μ M U0126, 1 and 10 or 30 μ M of SB203580 or SB202190, respectively, and 2.5 and 5 μ M of LY294200). The number of differentiated cells was scored by counting the number of cells with neurite extensions with a length at least two cell body diameters. The number of cells differentiated with Dex alone was considered 100%. Each experiment was performed at least three times. (\pm S.E. ** P < 0.005). p73 function is necessary for neuronal differentiation. **(c)** UR61 cells were transfected with GFP vector alone or together with expression plasmids containing either p73-DDwt or a p53 dominant negative (p53-DD). After 24 h of treatment, the percentage of GFP-positive cells that presented neurite extension was scored for each transfection. All values were compared with the value scored by the GFP-alone transfected cells. Each experiment was performed at least three times with similar results. (\pm S.E. * P < 0.05). **(d)** UR61 cells infected with a bicistronic adenoviral vector encoding GFP and p73-DD-L371P (top) or GFP and p73-DDwt (middle and bottom) were treated with Dex for 48 h and photomicrographs were taken by phase contrast and fluorescence microscopy (top and middle) or by confocal microscopy (bottom). For confocal analysis, the cells were stained with actin-binding phalloidin-TRITC (Ph) to visualize the cytoskeleton. p73 function is required for Ras sustained ERK activation during the differentiation process. **(e and f)** The expression profile of ERK activation during Ras-induced differentiation in UR61 cells was analyzed by Western blot. Cells were infected with a bicistronic adenoviral vector encoding GFP alone or GFP and p73-DDwt and treated with Dex. At the indicated times, cell lysates were prepared and analyzed as before

TAp73 expression levels are usually kept extremely low under normal conditions, making stabilization of p73 a critical control mechanism for its effects on the cell. Several lines of evidence indicate that stress-induced post-translational modifications of p73 such as phosphorylation and acetylation lead to a marked extension of its half-life. Furthermore, p73 stability is regulated, at least in part, by a proteasome-dependent

degradation pathway.^{4,31} We have failed to detect p73 acetylation in the presence of activated Ras in our system, suggesting that some of the other pathways are responsible for the stabilization (B Fernandez-Garcia, unpublished results).

p73 can modulate oncogenic Ras activation of the MAP kinase signaling cascade. Our results indicate that TAp73

synergizes with oncogenic Ras in the activation of the MAPK cascade leading to increased ERK activation. It has been proposed that oncogenic activation of the MAPK pathway in MEFs modifies the outcome of p53 activation³², on the other hand, the functional cooperation between p53 and TAp73 has been established.^{2,28,29} Therefore, a possible consequence of this synergy is that p73-mediated enhancement of ERK signaling results in further activation of p53, establishing cooperation between TAp73 activation and p53 function in the cellular response to oncogenic Ras. This possibility is currently under investigation in our laboratory.

The transcriptional function of p73 is required to activate the MAPK cascade. However, the cooperation with oncogenic Ras, seems to involve additional p73 interactions. The fact that TAp73 β shows a diminished ability to cooperate with Ras suggests that TAp73 α superactivation of the MAPK cascade does not depend only on TAp73 transcriptional functions, but also engages unknown interaction/s within the C-terminal region. As Δ Np73 α shares with TAp73 α the C-terminus region, it remains to be determined whether it also cooperates with oncogenic Ras in the activation of the MAPK cascade, and the cellular outcome of this cooperation. This scenario will be in accordance with a previous report that indicates that Δ Np73 cooperates with oncogenic Ras in cellular transformation.³ It remains unknown whether the mechanism of this cooperation relies on the ability of p73 to synergize with Ras signaling, or if it is only a reflection of Δ Np73 transdominant inhibition over p53.

Our data demonstrate that p73 acts upstream of MEK activation, but the precise point of the Ras-MEK cascade at which p73 is acting, is still unknown.

p73 function plays a critical role in Ras-mediated differentiation. Although Ras activation in cell lines exerts multiple effects, one of the most prevalent effects of Ras is to promote differentiation.¹⁶ The hypothesis proposed to explain this paradox relays in the existence of cell-specific modulators of the Ras signaling pathways. Here, we show that TAp73 can modulate the strength of the Ras-mediated MAPK cascade activation, as functional inactivation of p73 results in transient rather than sustained Ras-mediated ERK activation. Furthermore, lack of p73 function blocks Ras-induced differentiation in UR61 cells. In the light of these data, it is tempting to hypothesize that p73 can function as a contextual modulator of the cellular response to Ras activation. As sustained ERK activation is necessary for Ras-induced differentiation in UR61 cells, it is conceivable that p73 contributes to Ras-mediated differentiation by increasing the strength and duration of signaling by the Ras-MAPK cascade. It will be important to address whether p73 can mediate other Ras-induced cellular outcomes.

Materials and Methods

Cell lines, culture conditions and transfections. Rat pheochromocytoma cells UR61, human colorectal carcinoma cells HCT116 p53 $^{-/-}$, neuroblastoma cell line SH-SY5Y (provided by Dr. A Silva, Centro de Investigaciones Biológicas, Madrid, Spain) and human osteosarcoma cells SaOs-2 were maintained in DMEM medium supplemented with 10% fetal bovine serum, 200 mM, L-glutamine, in a 5% CO₂ humidified atmosphere at 37°C. The NTera-2

(NT-2) cells (provided by Dr. D Pleasure, University of Pennsylvania, Philadelphia, USA) were maintained in DMEM with 10% fetal bovine serum, 200 mM L-glutamine, in a 10% CO₂ humidified atmosphere at 37°C. All cell culture products were purchased from Sigma.

For protein analysis, the cells were transfected with the indicated expression vectors using jetPEITM (Polyplus Transfection) following the manufacturer's fast protocol. Cells were transfected with 4 μ g of the indicated plasmid together with 1 μ g pBB14 (Us9-GFP) and 10 μ l of JetPEI in 150 mM NaCl. Twenty-four hours later, cells were harvested and cellular extracts prepared as described below. In some experiments, the cells were treated 6 h after transfection with 10 μ M U0126 or vehicle (DMSO) and processed for Western blot analysis 24 h later.

To analyze the effect of p73-DD mutants, UR61 cells were transfected with 1.8 μ g of total DNA (0.2 μ g of pcDNA3-GFP and 1.2 μ g of vector (1 : 6), or 0.2 μ g of GFP together with 1.2 μ g (1 : 6) of one of the dominant-negative constructs: pcDNA3-p73 β DD, pcDNA3-p73 β L371P or pcDNA3-p53DD). The transfections were performed with Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, cells were treated with 200 nM Dex for 24 h and analyzed for neurite outgrowth as described below.

Neurite outgrowth analysis. UR61 cells were seeded at 60% confluence and next day treated with 200 nM Dex (Sigma). To analyze the effect of the different inhibitors, cells were treated with Dex together with one of various concentrations of the MAPK inhibitors (10 μ M of U0126 (Calbiochem), 2.5 or 5 μ M of LY294200 (Calbiochem), 30 μ M of SB202190 (Calbiochem) or 1 or 10 μ M of SB203580 (Sigma)). After 24–48 h of treatment, the differentiated cells were quantified using an inverted microscope (Nikon Eclipse TE300) by counting the cells with prolongations at least 2 times the diameter of soma. For each experiment, four quadrants with at least 100 cells were counted.

p73-DD mutants were transfected and treated as described above, and the GFP-positive cells that presented neurite extensions were counted, considering the number of differentiated cells transfected with pcDNA3-GFP alone as 100%.

Adenoviral plasmids and infections. The generation of the bicistronic adenoviral vectors pAd GFP-T7-p73DDwt, pAd GFP-T7-p73DD-L371P and pAd GFP-T7-vector has been described earlier.²⁸ As described before, tissue culture supernatants of 293A cells transfected with these plasmids were harvested and viral particles were purified by cesium chloride centrifugation. Equivalent amounts of infection particles were used to infect UR61 cells. Twenty-four hours after infection, cells were treated with Dex as described above, and 24–36 h later the neurite outgrowth of living cells was analyzed by capturing digital images on an inverted epifluorescence microscope (Nikon Eclipse TE300) or by laser confocal microscopy (see below). For protein expression analysis during the differentiation process of UR61, cells were plated, next day infected overnight with the adenoviral constructions and 24 h later treated with 200 nM Dex. Cellular extracts were prepared (see below) at the indicated times.

Immunostaining and confocal analysis. Cells were seeded on poly-D-lysine (Becton-Dickinson)-treated coverslips, 24 h later they were infected as described above and then treated with 200 nM Dex for another 24 h. Cells were fixed in 3% paraformaldehyde/2% sucrose, permeabilized with 0.2% Triton X-100 in PBS and incubated in Phalloidin-TRITC (Sigma) for 30 min.

Coverslips were mounted on Vectashield Mounting Medium (Vector Labs) and analyzed by confocal microscopy (Radiance 2000, Bio-Rad).

Western blot analysis. Cells were lysed in EBC lysis buffer (50 mM Tris pH 8, 120 mM NaCl, 0.5% NP-40) supplemented with protease inhibitors (Aprotinin, Leupeptin 20 μ g/ml, sodium orthovanadate 1 mM and PMSF 0.1 mg/ml, all from Sigma). Immunoblots were performed as described previously³³ and incubated overnight at 4°C in the following primary antibodies: mouse anti-p73 1 : 500 (Ab-2 and Ab-4 Oncogene), anti-p63 1 : 1000 (4A4 Becton-Dickinson), anti-p53 1 : 500 (Ab1 Oncogene), anti-pan-Ras 1 : 500 (Ab-4 Oncogene), anti-RasV12 1 : 500 (Ab-1 Calbiochem), anti-ppERK 1 : 10 000 (E-4 Santa Cruz), anti-ERK 1 : 10 000 (C-14 Santa Cruz), anti-p21-WAF 1 : 1000 (C-19 Santa Cruz), anti-HA 1 : 500 (Y11 Santa Cruz), anti-Kras 1 : 500 (F234 Santa Cruz) and anti-GFP 1 : 500 (FL Santa Cruz). Membranes were incubated with the appropriated HRP-coupled secondary antibodies (Pierce) and the enhanced chemiluminescence was detected with Super Signal West-Pico Chemiluminescent Substrate from Pierce.

Immunoprecipitation. For the analysis of p73 expression during the differentiation in UR61 cells, p73 immunoprecipitation was performed as described previously.³³ Cells were seeded and treated with Dex 200 nM at indicated times. Cells were lysed in EBC lysis buffer, incubated for 1 h with ER15 supernatant antibody in NET-N buffer and incubated for 30 min with protein A-sepharose. Western blot analysis was performed as described before.

The interactions between p73-DD and endogenous rat p73 or p63 were studied in UR61 cells stably transfected with p73DD β . The cells were treated with Dex (200 nM) and 12 h later cell lysates were prepared as described. Equal amounts of protein were incubated with specific antibodies (Anti-TAg (419 mAb) as control anti-p73 β (GC15) or anti-p63 (4A4)). Protein complexes were analyzed by Western blot as described above.

RNA interference analysis. The knockdown of Kras was performed by shRNA. The shRNA vectors were constructed by cloning the following oligos in the pSiren-ZsGreen vector following manufacturer's instructions (BD Biosciences).

K-Ras general-up: GATCCGAGGCTCAGGAGTTAGCAATTCAAGAGATTGC TAACTCCTGAGCCTGCTTTTTGCTAGCG;

K-Ras general 4-lo: AATTCGCTAGCAAAAAGCAGGCTCAGGAGTTAGCA ATCTCTTGAAATTGCTAACTCCTGAGCCTGCG;

K-Ras scrambled-up: AGCTTGGCTTAGGCTCGATGAGGCTTTCAAGAGAAG CCTCATCGAGCCTAAGCCTTTTTGCTAGCA;

K-Ras scrambled-lo: GATCTGCTAGCAAAAAGGCTTAGGCTCGATGAGGCT TCTCTTGAAGCCTCATCGAGCCTAAGCCA.

HCT 116p53^{-/-} cells were transfected with 8 μ g of pSiren-ZsGreen-scrambled or 8 μ g of pSiren-ZsGreen-K-Ras general using 16 μ g of JetPEI. After 24 h, cells were split and transfected with 8 μ g of pcDNA3 or pcDNA3-HA-Tap73 α plasmids and 16 μ g of JetPEI. Twenty-four hours later (48 h after pSiren transfection), cell lysates were obtained and analyzed as described above.

The knockdown of p73 was performed using anti-p73 siRNA oligos provided by Dr. M Irwin (Cancer Research and Developmental Biology, Toronto University, ON, Canada) and previously described.²⁸ HCT 116p53^{-/-} cells were transfected with siRNA at approximately 20–30% confluence using Oligofectamine (Invitrogen). For Western blot analysis, proteins were recovered 72 h later as described before. For luciferase reporter analysis, cells were transfected 48 h after oligofection with 1 μ g of Elk1-Gal4, 1 μ g of Gal4-Luc and 0.5 μ g of pRL-Null. Twenty-four hours later, cell extracts were prepared for luciferase analysis as described below.

In vitro translation. *In vitro* translation was performed with the TNT translation system (Promega) according to the manufacturer's protocol.

Luciferase reporter assay. Cells (2×10^6) were electroporated at 260 V and 1 mF (Bio-Rad Gene Pulser) in serum-free medium with one of the following reporters: p73-P1-Luc (1 μ g), p21-FL-Luc (4 μ g), -73Col-AP1-Luc (4 μ g), plus 0.5–2 μ g pRLNull (*Renilla* luciferase) and 3–9 μ g of the indicated expression vectors. The luciferase reporter p21-FL-Luc was described previously. We used a 73 bp fragment of the collagenase-I promoter (-73Col1-AP1-Luc) containing the AP-1 responsive site as the AP-1 reporter gene³⁴ that was provided by Dr. A Muñoz (Instituto de Investigaciones Biomédicas, Madrid, Spain).

For PG13-Luc reporter activity (provided by Dr. A Silva, Centro de Investigaciones Biológicas, Madrid, Spain), 1×10^6 UR61 cells were transfected with 0.5 μ g of the reporter, 0.5 μ g pRLNull and different amounts (0.5–1 μ g) of the expression vectors using jetPEITM following the manufacturer's fast protocol. When indicated, cells were treated with Dex (200 nM, Sigma) and the MAPK inhibitor (10 μ M U0126) 6 h after transfection.

Cellular extracts were prepared (Passive Lysis Buffer, Promega) 16 h after electroporation or 24 h in the case of jetPEI transfection, and the luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions in a Berthold's luminometer and normalized by the values of the *Renilla* luciferase.

MAP kinase reporter assay. Cells (2×10^6) were electroporated at 260 V and 1 mF (Bio-Rad Gene Pulser) in serum-free medium with 4 μ g of pSG424-Gal4-Elk1 (CMV promoter-driven GAL4-DNA binding domain-Elk fusion protein) and 4 μ g of pSG5 \times Gal4-Luc (GAL4 promoter-driven luciferase) plus 2 μ g pRLNull (*Renilla* luciferase) and 3–9 μ g of DNA of the indicated expression vectors. The MAP kinase reporter system was provided by Dr. Cobb, Southwestern Medical Center, TX, USA.

After 16 h of the electroporation, cells were lysed and the luciferase activity was determined as described above.

Reverse transcription-polymerase chain reaction. To analyze Kras knockdown in shRNA experiments, RNA was extracted with RNA-wiz reagent (Ambion) following the manufacturer's protocol. cDNA was prepared using the Ready-To-Go First Strand kit (APB) according to the manufacturer's protocol. The primers and conditions used to amplify GAPDH were published before.³⁵ To amplify K-ras, the primers used were: K-ras-sense ACTTGTGGTAGTTG GAGCTGGTGGC and K-ras-antisense GCAAATACACAAGAAAGCCCTCCC.

In differentiation analysis, UR61 cells were treated with 200 nM Dex at indicated times. RNA from cells and cDNA were prepared as described before. The primers and conditions used to amplify Tap73 and Δ Np73 were published before.^{36,37} To amplify p53 and p63, the primers used were: p53-sense GCTAAACGC TTGAGATGTTCCGGG and p53-antisense GCGCTGAGTCAGCCCCACTTT CTTG; p63-sense GCGCTTCATAGAAACCCATCTCATTCTCCTGG and p63-antisense GCGCGTTCTTTGCGCTGTCTGAGACTTG CTGCTTT.

RT-PCR products were analyzed in 3% agarose gels stained with ethidium bromide.

Plasmids. The cDNAs from human HA-Tap73 α , HA-Tap73 β , Tap73 α 292,³³ Myc- Δ Np73 α ,³⁷ HA-p53wt³⁸ and the dominant-negative forms p53-DD,³⁹ pcDNA3-T7-p73-DD β /wt and pcDNA3-T7-p73-DD β -L371P were described previously.²⁹

The expression plasmids containing the cDNAs for the oncogenic mutants H-Ras-G12V (H-Ras V12), K-Ras-G12V (K-Ras V12), and N-Ras-G12E (N-Ras E12) were a gift from Piero Crespo (Universidad de Cantabria, Santander, Spain).

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)