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A network of p73, p53 and Egr1 is required for efficient apoptosis in tumor cells

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p73, a transcription factor rarely mutated in cancer, regulates a subset of p53 target genes that cause cells to respond to genotoxic stress by growth arrest and apoptosis. p73 is produced in two main forms; only TAp73 reiterates the roles of p53, while ΔNp73 can be oncogenic in character. We show that the TAp73 form produced by *TP73* P1 promoter has five distinct Egr1-binding sites, each contributing to the transcriptional upregulation of TAp73 by Egr1 in several cell types. In contrast, *TP73* P2 promoter transcribes ΔNp73, is not induced by Egr1, but *is* induced by TAp73 and p53. Induction of TAp73 by genotoxic stress requires Egr1 in mouse *in vivo*. Newly discovered non-consensus p53-binding sites in p73, p53 and Egr1 promoters reveal inter-regulating networks and sustained expression by feedback loops in response to stress, resulting in prolonged expression of the p53 family of genes and efficient apoptosis.
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Egr1 (early growth response 1), also known as NGFI-A, ZIF268, Krox24, or TIS8) has been endowed with the ability to function in numerous capacities, including differentiation,[1] growth;[2] growth inhibition[3,4] and apoptosis[5] depending on the cell type and the stimulus. The *Egr1* gene is characterized by its rapid and transient upregulation in response to numerous stresses. In addition, Egr1 upregulates the transcription of the p53 promoter to induce the apoptosis of cancer cells.[6] Thus, in response to stress, Egr1 displays a remarkable functional similarity to p53 and p73. For instance DNA-damaging chemotherapy leads to growth arrest and imperfect DNA repair that may be followed by programmed cell death (apoptosis). To provide this responsiveness, the most important gene is *TP53,* a tumor suppressor gene. However, *TP53* is frequently mutated and its protein product, p53mut, has altered activities in cancer cells.[7] Then, stress responses fall on the related gene *TP73* that has similar features to *TP53* with some distinctions. There are large differences in the protein products of the *TP73* gene compared with *TP53*, in their promoters and by alternate splicing of exons that lead to the production of 17 different proteins for *TP73* (reviews[8–10]).

TP73 P1 promoter produces a set of transcription-activating forms named TAp73. Promoter P2 produces ΔNp73 forms that were thought to have non-transactivating roles although they now appear to have transcriptional activity on target genes that give rise to both anti-apoptotic[11–13] and pro-apoptotic activities.[14,15] It was noted earlier that ΔNp73 protein acts as a dominant negative of p53 action on its target genes[16] through the oligomerization of the ΔNp73 subunits with p53 or TAp73 subunits.[17]

A search for EBS and p53BS in the regulatory regions of the p53 family of genes revealed novel, specific, non-consensus p53 responsive elements (RE) in the p53 and Egr1 promoters that are bound and transactivated by the p53 family members including ΔNp73 forms. Egr1 can now be classified as a key transcriptional regulator of this group of genes. Moreover, we show that the promoters of *Egr1*, *TP53* and *TP73* all respond to Egr1, p53, TAp73 and ΔNp73 proteins and elicit feedback loops between these four genes. In contrast, the p63 member of the p53 gene family does not respond to Egr1 and lacks Egr1-binding sites (EBS) in its promoter.

The existence of the regulatory network described here is important because the most effective function of tumor suppressor genes in cancer cells is to activate apoptosis. The versatility of this cross-regulation permits a wide range of graded responses of cells to stresses such as radiation and chemotherapy, a finding with therapeutic implications.

Results

Cells that differentially express Egr1, p53 and p73 exhibit different sensitivities to etoposide. Three different types of cancer cell lines were used to measure levels of apoptosis following treatment with the chemotherapeutic drug etoposide (ETO). Only 20% of MCF7 breast cancer cells died compared with 80% in M12 prostate cancer cells and U2OS osteosarcoma cells (Figure 1a). Each of these cell lines expresses different levels of Egr1, p53 and p73 proteins as indicated below the graph. MCF7 cells express the lowest protein levels

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Abbreviations: ChIP, chromatin immunoprecipitation; EBS, Egr1 binding site; ETO, Etoposide; MEFs, mouse embryo fibroblasts; RE, responsive element; RT-PCR, real-time polymerase chain reaction; TAD, transcription activation domain; UV-C, ultraviolet radiation at 254 nm
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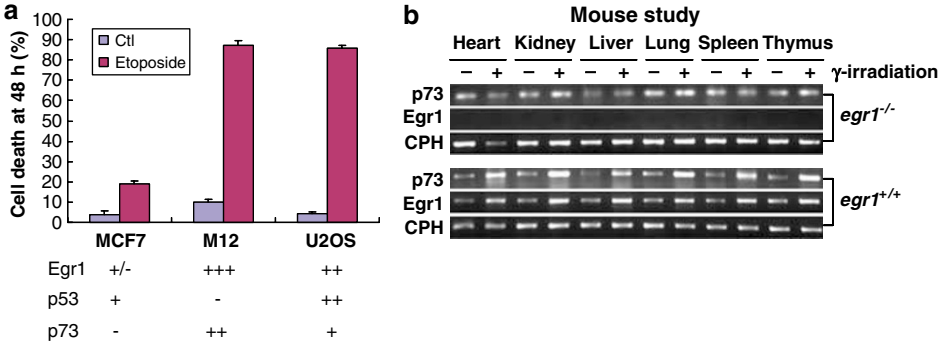

Figure 1 Genotoxic stress leads to apoptosis in cell lines and to increased Egr1 and p73 expression in vivo. (**a**) MCF7 breast, M12 prostate and U2OS fibrosarcoma cells induced with ETO (20 μg/ml) chemotherapy drug, show induced apoptosis levels consistent with the additive expression of Egr1, p53 and p73. (**b**) Tissues dissected from (Egr1$^{+/+}$) or knockout (Egr1$^{-/-}$) mice 2.5 h after gamma-irradiation at 5 Gy, were analyzed by semiquantitative RT-PCR to reveal the induction of Egr1 and p73 mRNAs. One example is shown of duplicate studies that gave similar results

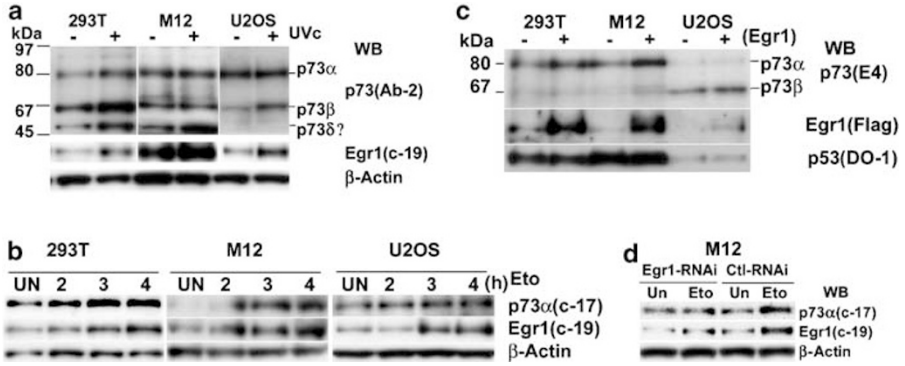

Figure 2 Immunoblots indicate that Egr1 and p73 are induced by UV irradiation or ETO treatment of a variety of cell types. (**a**) UVc irradiation (40 J/M^2) of three cell types induced Egr1 and p73 protein isoforms measured 4 h later. (**b**) ETO (20 g/ml) added to three cells types induced Egr1 and p73α in a time-course study. (**c**) Transfection of an Egr1 expression plasmid into three cell lines induced p73α and β protein isoforms. (**d**) Egr1 RNAi reduces the induction of Egr1 and p73α in ETO treated M12 prostate cancer cells, while a control RNAi has no effect

compared with the other two cell lines, correlating with lower apoptosis levels.

Egr1-null mice fail to induce p73 in response to radiation. Mice with Egr1 inactivated by homologous recombination (Egr1$^{-/-}$) or wild-type (WT) (Egr1$^{+/+}$) were analyzed for p73 expression 2.5 h following γ-irradiation (5 Gy). Mice were killed and the mRNA levels of six tissues were measured by semi-quantitative real-time-polymerase chain reaction (RT-PCR). We show in Figure 1b that irradiation-induced Egr1 in the WT mouse was accompanied by increased p73 mRNA in all tissues. The presence of Egr1 was required since the Egr1-null tissues showed no irradiation-induced p73. Instead, p73 levels were somewhat reduced. We previously observed that p53 expression is absent in Egr1$^{-/-}$ MEF (mouse embryo fibroblasts).[18,19] Thus, Egr1 appears to be *required* for induction of both p53 and p73.

Promoter upregulation by Egr1 results in p73 protein expression. As p73 occurs in many isoforms, we determined which forms are accumulated as a result of promoter transactivation by Egr1. In 293T cells, M12, and U2OS cells treated with UV to induce Egr1 (Figure 2a), three p73 isoforms (α, β and δ) were induced to varying levels. In M12 prostate cells, only p73δ was upregulated while in U2OS cells, only p73β was induced. Clearly, expression levels and isoform of p73 that are upregulated upon Egr1 induction depend on the cell line. In Figure 2b, Egr1 and p73 protein levels were measured at increasing times of ETO treatment. In 293T cells, Egr1 protein remained elevated for 2–4 h after treatment and p73α was elevated accordingly. Similarly, the addition of an Egr1 expression vector increased the expression of p73 in three cell lines tested (Figure 2c). We conclude that Egr1 regulates p73 expression following genotoxic stress. Vector-based Egr1-RNAi reduced Egr1 expression and inhibited ETO-induced levels of p73α (Figure 2d).

Egr1 upregulates the transcription of the TAp73gene but not the ΔNp73 form. We constructed luciferase reporter pGL3-TAp73 containing *TP73* P1 promoter. The P1 promoter contains five potential high-affinity EBS named a, b, c, d and e (Supplementary Figure S1). Promoter P2 was cloned into pGL3 to serve as a reporter gene for ΔNp73 isoforms and contains a single EBS (named f). Transfection of pGL3-TAp73 into 293T cells together with expression plasmids for Egr1 shows that Egr1 transactivated both promoters by

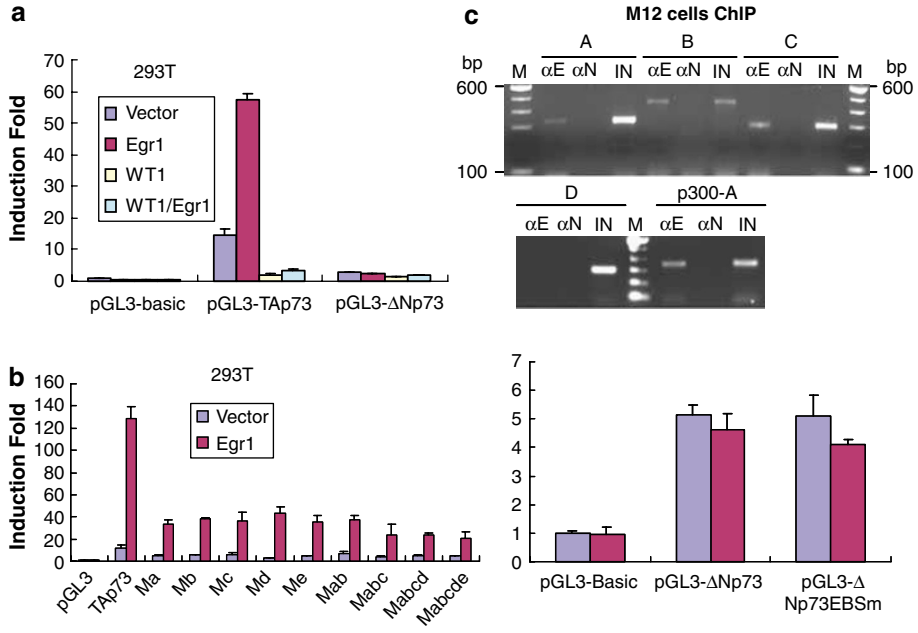

Figure 3 TAp73 promoter (P1) binds Egr1 and is upregulated; the ΔNp73 promoter (P2) does not respond to Egr1 (see Supplementary Figure S1). (**a**) TAp73 promoter luciferase reporter is strongly induced by Egr1 but not by the related WT1 protein or by a dominant negative expression vector WT1/Egr1 measured 24 h after transfection. (**b**) Mutation of each of the five EBS showed that all sites are activated by Egr1 expression. In contrast, Egr1 did not transactivate the ΔNp73 promoter or the mutated EBS in the ΔNp73 promoter. Triplicate results were in close agreement and were averaged for all the transcription studies presented here. (**c**) ChIP confirmed this result. The DNA captured by αEgr1 (αE) was purified and compared by PCR analyses on DNA captured from non-immune Ig (αN) as a negative control and also compared with input DNA (IN) purified from the cells as a positive control

four- to 12-fold compared with the empty expression vector (pcDNA) (Figure 3a and Supplementary Figure S2). As a control a 'dominant negative' Egr1 vector, WT1/Egr1 and WT1 (Wilm's Tumor protein1) that binds to the same EBS sequences, had a negative effect on promoter activity. In contrast, there was no significant difference in the luciferase activity of the P2 promoter reporter pGL3-ΔNp73 following transfection of pcDNA-Egr1, indicating that this promoter is not activated by Egr1. We confirmed this by mutating the putative EBS in P2 (Figure 3b, right) and each of the five EBS in P1 by individual and combined mutations (Figure 3b, left). Each mutated Egr EBS similarly reduced the promoter activity by an average of 75%. Only mutation of all five sites reduced the activity of the reporter gene by 85% although there was still some residual activity in P1 when all EBS were mutated. This analysis clearly shows that Egr1 upregulates the P1 promoter and may play a role in graded responses of *TAp73* governed by the expression level of Egr1 in the cell.

We applied chromatin immunoprecipitation (ChIP) to M12 cells to confirm that Egr1 is directly bound to the promoter during transactivation. The results of ChIP (Figure 3c) confirm that Egr1 was bound to DNA fragments A, B and C, but not to D, which is the inactive EBS in P2. As a positive control, ChIP detected p300, a known Egr1 target gene.[20] In summary, Egr1 binds to and transactivates the *TAp73* P1 promoter but not the *ΔNp73* P2 promoter.

P1 and P2 promoters of TP73 respond differentially to p53 and p73 proteins. We identified a new putative p53 RE (p53RE, Supplementary Figure S1) in the P1 promoter that was tested in 293T cells using a pGL3-TAp73 reporter transfected with vectors coding for p53, TAp73α β or ΔNp73α, β (Figure 4a). The known p53RE in the P2 promoter of ΔNp73 was far more responsive to p53 (80-fold) than the P1 promoter. We also observed that TAp73α and β strongly induced the single p53RE in P2, while ΔNp73 had no effect on its own promoter. When examined on a magnified scale (Figure 4b), TAp73α induction of the P1 promoter was threefold higher than the vector. p53 induction was weak but statistically significant, with $P<0.0038$ compared with empty vector. When the single p53RE in the P1 promoter was mutated (pGL3-TAp73REm) we observed that the activity of TAp73α and β was nearly abolished (Figure 4b, right). ChIP studies in ETO-treated U2OS cells confirmed that the p53RE-containing portions of P1 and P2 promoters bind to p53 and p73 (Figure 4c). We conclude that several p53 family proteins bind directly to the putative p53REs to effect and perpetuate transactivation within the family.

The Egr1 promoter is upregulated by Egr1, p53, TAp73 and ΔNp73. The *Egr1* promoter contains active EBS.[20] In addition, we identified four possible p53 REs (p53RE A1 to A4) in the non-coding sequences from −2 kb to +142 of the promoter and 5'UTR (Supplementary Figure S3). A reporter construct in pGL3 termed 'Egr1' contains all four p53 sites while 'Egr1F' contains additional flanking sequences. Fragments of the promoter named Egr1P1, Egr1P2 and Egr1P3 were cloned into pGL3 and co-transfected into 293T cells with either p53, TAp73 or ΔNp73 expression vectors. All promoters were activated similarly (2- to 2.5-fold) by TAp73

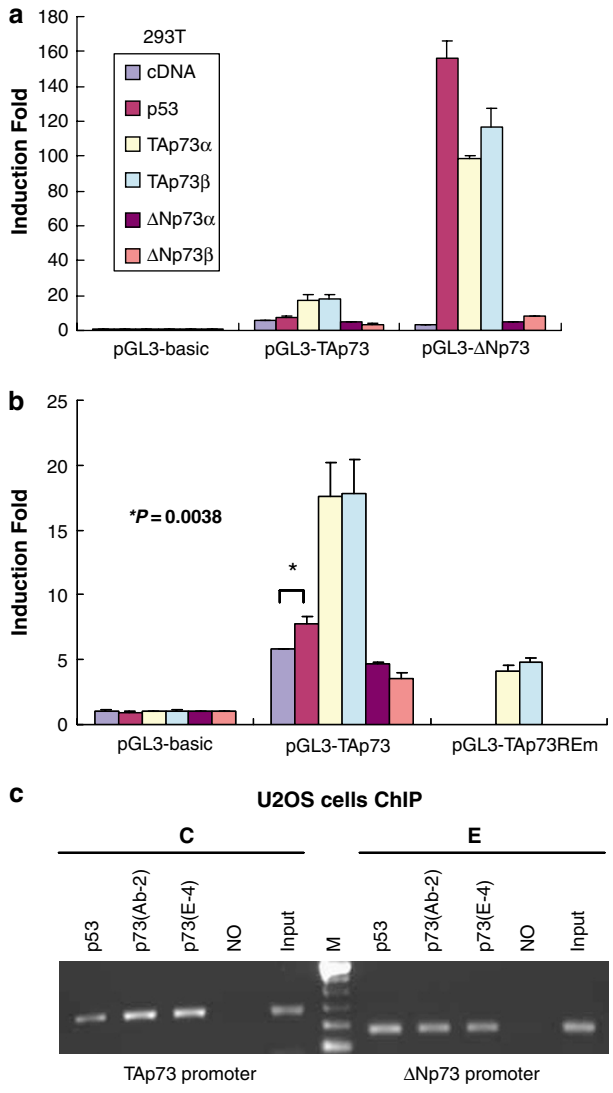

Figure 4 Analysis of the p53 REs in TAp73 and ΔNp73 promoters. (**a**, **b**). The pGL3-TAp73 and pGL3-ΔNp73 promoters, P1 and P2, respectively (structures are shown in the Supplementary Figure S1) were transfected into 293T cells to measure the luciferase signal 24 h later. Each promoter was tested for the effects of the expression of p53, TAp73α, TAp73β, ΔNp73α and ΔNp73β after co-transfection. The P1 promoter was weakly responsive to TAp73 (see replot in **b**), while ΔNp73 expression was inhibitory. In contrast, the P2 promoter was 60- to 80-fold induced by p53 and by TAp73, but not by ΔNp73. The right panel of **b** shows that the mutation of the p53RE in the P1 promoter reduces activity threefold. (**c**) ChIP confirmed that p53 and p73 proteins bind directly to the p53RE in intron 1 (P1) as well as to the p53RE in intron 2 (P2) in U2OS cells treated with ETO for 6 h before cross-linking (NO is the negative control)

and ΔNp73 whereas p53 was half as active (Figure 5a and Supplementary Figure S4). Thus, all members of the p53 family induce the *Egr1* promoter albeit to different degrees. The most effective reporter was Egr1P3 that contains only A2 and A3 p53RE.

To determine whether transactivation results in increased Egr1 protein levels, we performed immunoblots of M12 prostate cancer cells (contain inactive p53; Figure 5b) and MCF7 breast cancer cells (WT p53; Supplementary Figure S5), after transfection with expression vectors HA-tag ΔNp73α, TAp73α or p53. An increase of Egr1 protein was observed in both cell types. ChIP experiments confirmed that p53 and p73 are specifically bound to *Egr1* promoter in U2OS cells. Of the four putative p53RE, only p53A2 and p53A3 were bound (Figure 5c). Thus, only two sites are directly involved in transcriptional activation of *Egr1 in vivo*. We therefore mutated these two active p53RE in the corresponding pGL3-Egr1 luciferase reporter construct (Figure 5d). Co-transfection with plasmids expressing p53, TAp73 or ΔNp73 displayed reduced transactivation of p53RE-A2m and A3m compared to the WT promoter. p53RE-A3 was the most affected by mutation, which reduced promoter activity by six- to eightfold for all p73 isoforms. These results prove that two new p53RE in *Egr1* promoter are responsive to all members of the p53-family of proteins, resulting in increased Egr1 protein levels.

We next tested p73 isoforms for their ability to induce the *Egr1* promoter, to assess the role of p73 transactivating domain (TAD). TAp73α and ΔNp73α each induced *Egr1* transcription by ~3-fold even though ΔNp73α lacks a recognizable TAD (Figure 5e). However, the N-terminal domains of TAp73 and ΔNp73 are required, because expression of TAp73Δ1-62 (ΔTAD) and Δ1-127 (ΔTAD + ΔPXXP domains) failed to transactivate the *Egr1* promoter. Deletion of the unique 13 amino acids N-terminal of ΔNp73 indicated that it is required to transactivate the *Egr1* promoter and therefore acts as a TAD. Deletion of the PXXP motif in ΔNp73Δ1–78 made no further difference, indicating that the 13 amino-acid sequence is the crucial requirement for transactivation of *Egr1* promoter.

p53 transcription is upregulated by p53 family members. We tested the DNA sequence between –764 and +229 of human *TP53* for transactivation in pGL3-p53 reporter constructs (Supplementary Figure S6A and C). One EBS and three p53RE sites (p53F, p53M and p53Z) in *TP53* promoter have not been previously described. The 0.9 kb p53 promoter reporter was not induced by Egr1 in H4 cells (Supplementary Figure S7-left), but was induced by Egr1 as well as by WT1 and WT1/Egr1 in 293T cells (Figure 6a) and in Saos2 (Supplementary Figure S7-right). It is unusual that both WT1 and Egr1 transactivate *TP53* promoter equally well, when they usually have opposite effects on other target genes. In contrast, p53 induced *TP53* promoter by three- to ninefold, depending on the cell line and the size of promoter sequences used (Figure 6b and Supplementary Figure S6B, C and S8). The binding sites F and M contain CTG-CAG trinucleotide repeats that form hairpin structures (Supplementary Figure S6C) and form strong bonds with p53.[21] In 293T cells, *TP53* promoter was strongly induced by TAp73α and β but to a lesser extent by ΔNp73α and β (Figure 6b and Supplementary Figure S8). The induction of *TP53* transcription by p53 was also concentration-dependent in both 293T and Saos2 cells (Supplementary Figure S9) indicating that the transcription factor expression level plays a major role in the strength of the transactivation.

We tested the binding of the three proteins to *TP53* promoter by ChIP in U2OS cells (Figure 6c). Only p53F and p53M sites were bound to, while p53Z did not bind any of the three proteins (Figure 6c). We confirmed the transactivating

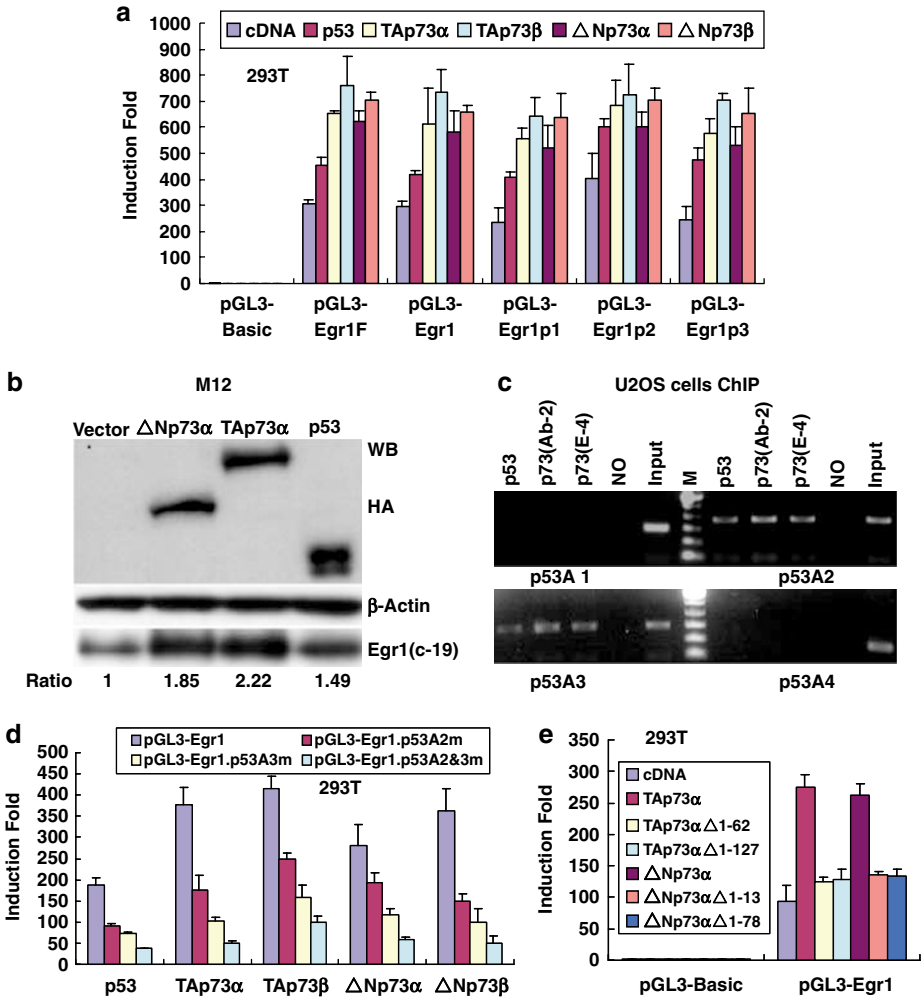

Figure 5 All members of the p53 family induce the Egr1 promoter by binding to two p53BS (see Supplementary Figure S3 for the structure and features of the Egr1 promoter). (**a**) The transactivation of the Egr1 promoter in 293T cells was studied using pGL3-Egr1. The Egr1 promoter was tested as five different fragments (Egr1, Egr1F, Egrp1, Egr1p2 and Egr1p3). TAp73β and ΔNp73β isoforms were the most active ($>$2-fold); the least active was p53. The Egr1F and Egr1 promoter fragments each carry four putative p53BS, but there was little difference in the fold increase in transactivation between the five different Egr1 promoter forms. (**b**) Immunoblot of Egr1 produced by expression vectors of the p53 family transfected into M12 cells. (**c**) ChIP was used to validate the transactivation result by showing that only two (A2 and A3) of the four putative p53RE were bound to their transactivator p53 family proteins. (**d**) Mutation of the A2 and A3 binding sites, singly or doubly clearly show that both sites are transactivating when not mutated, with A3 the most active p53RE. (**e**) wt TAp73 and ΔNp73 are equally active in up-regulation of the Egr1 promoter; in contrast N-terminal deletions of the expressed p73 proteins, TAp73$\alpha\Delta$1–62 and 1–127, ΔNp73$\alpha\Delta$1–13 and 1–78 are unable to induce the Egr1 promoter

function of these sites by mutational analysis of the promoter (Figure 6d). Only mutation at both p53F and p53M maximally inhibited transactivation of the pGL3-p53 reporter construct by TAp73α, β, ΔNp73α and β. The deletion mutants described in Figure 5e were also used to test their ability to transactivate the TAp73 and ΔNp73 promoter-reporters by transfection into 293T cells. Figure 6e shows that TAp73 induced *TP53* promoter sixfold, and ΔNp73 induced it twofold. Deletion mutants of TAp73 (Δ1–62 and Δ1–127) or ΔNp73 (Δ1–13 and Δ1–78) clearly reduced *TP53* transactivation in a step-wise manner, indicating a requirement for both the TAD and PxxP motifs. In contrast, the ΔNp73 promoter was activated only by intact TAp73 (Figure 6e). These studies show that the p53 protein is a functional transactivator of its own promoter *in vivo*.

Induction of Egr1 expression by ETO follows a time-course consistent with positive feedback via p73 and p53. We measured mRNA and proteins expressed during a time-course of ETO treatment. Figure 7a shows the time-course of Egr1 mRNA (left) and p53 mRNA (right) measured by qRT-PCR after ETO addition to MCF7 cells, in presence or absence of p53-RNAi. Egr1 mRNA level increased 26-fold at 1 h, peaked at 3 h before declining to a constant 10-fold induction for up to 11 h. In the presence of p53-RNAi, Egr1 levels were severely reduced. Levels of p53 mRNA increased in two peaks at 1 and at 11 h but were inhibited by p53-RNAi. A control RNAi gave the same results as Mock (data not shown). Figure 7b shows protein levels after immunoblotting. Interestingly, there were two peaks of Egr1 at 1 and 8 h while p53 peaked at 2 h and remained constant.

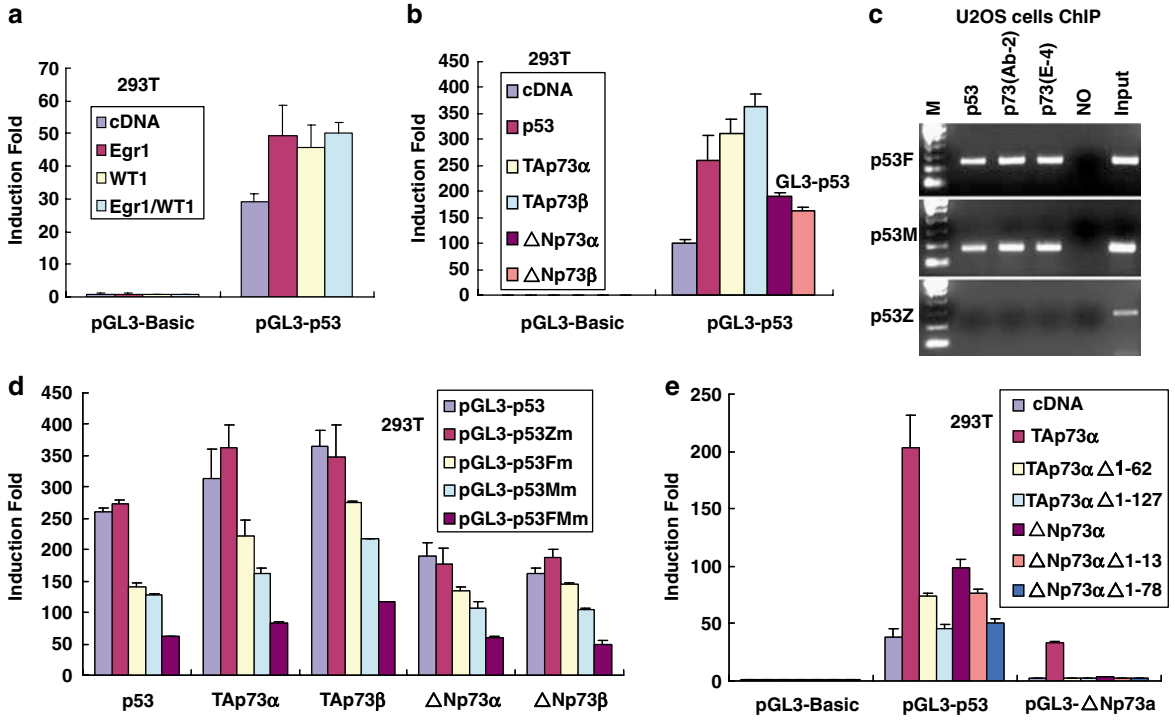

Figure 6 The p53 promoter is differentially activated by Egr1, and by p53, TAp73, ΔNp73 at two p53BS. (see Supplementary Figure S6A for details of the structure of the promoter). (**a**) In 293 T cells, transiently-expressed Egr1, WT1 and the dominant negative WT1/Egr1 all weakly induce the p53 promoter, (**b**) transfection of expression plasmids for the five members of the p53 family each induced the p53 promoter to different extents depending on the cell line. (**c**) ChIP was used to detect binding of the p53 and p73 proteins at the two of the three putative p53RE (F, M, positive binding) and (Z, no binding). The Z site is not responsive while both the F and M binding sites are required for p53 promoter responses to p53 and TAp73 and ΔNp73. (**e**) The same deleted forms of the expressed p53, TAp73 and ΔNp73 proteins as used in Figure 5e indicate that the amino-terminal sequences of TAp73 and ΔNp73 are required for transactivation

Use of phospho-specific antibodies against p53 phosphorylated at Ser46 (S46p-p53) indicated that p53 phosphorylation was high between 3 and 5 h. When p53-RNAi was added before treatment, both Egr1 and p53 protein levels were reduced.

Consistent with these results, ChIP shows that p53 bound steadily to *Egr1* promoter, as did S46p-p53 (Figure 7c). In addition, there was a small peak of p53 binding to *TP53* promoter matching the mRNA increase of Figure 7a. Interestingly, the timing of p53 phosphorylation at Ser46 correlated with the decreased levels of Egr1 seen in Figure 7b.

In M12 cells (p53 inactive), Egr1 mRNA was induced at two peaks at 1–3 h and 7 h. *Egr1* transactivation was inhibited in the presence of p73-RNAi, suggesting a feedback mechanism from p73 (Figure 7d-left). In contrast, p73 mRNA levels rose evenly to 4.5-fold after ETO treatment **(**Figure 7d-right) and this was completely eliminated by p73-RNAi. The changes in protein levels (Figure 7e) were less marked because both Egr1 and p73 are high in M12 cells. Egr1 expression peaked at 1 and 3 h while p73 was seen between 2 and 12 h. Inhibition of p73 expression by p73-RNAi resulted in inhibition of Egr1 (Figure 7e). There was a clear difference in timing of promoter binding observed in ChIP (Figure 7f). Egr1 bound *TAp73* promoter at all EBS at 2 h and again at 8 h, while p73 bound to both p53RE (A2 and A3) from 3 to 12 h. Binding of p73 to the p53RE of the C portion of the *Egr1* promoter followed a similar time-course. A similar analysis of U2OS cells is shown in Supplementary Data (Supplementary Figure S10). Thus, the expression of Egr1, p53 and p73 leads to a complex timing pattern.

In summary, Egr1 upregulates both p73 and p53, and Egr1 is itself upregulated by p53 and p73 in a feedback loop. This confirms earlier data that Egr1 induces the transcription of p53[6,18] and that p73 is regulated by p73 and p53.[22] We conclude that there is a transcriptional inter-regulatory network between Egr1, p53 and p73. Our hypothesis is that *Egr1* is the initial stress response gene that transactivates p73 and p53, which in turn create a network of positive feedback regulations.

Elevation of Egr1, p53 or p73 expression results in increased apoptosis. Figure 1a shows that ETO induced apoptosis in 80% of M12 and U2OS cells, but only in 20% of MCF7 cells, in which Egr1 and p53 are low and p73 is absent. This suggests that cooperation between the three proteins is required to achieve high levels of apoptosis. Increased expression of p53 (or p73) was required for high levels of apoptosis, which depended on the concentration of expression vector used (Supplementary Figure S9). Figure 8a shows that overexpression of Egr1, p53 or TAp73 increased apoptosis in U2OS cells, while ΔNp73 did not. Apoptosis further increased when two proteins were

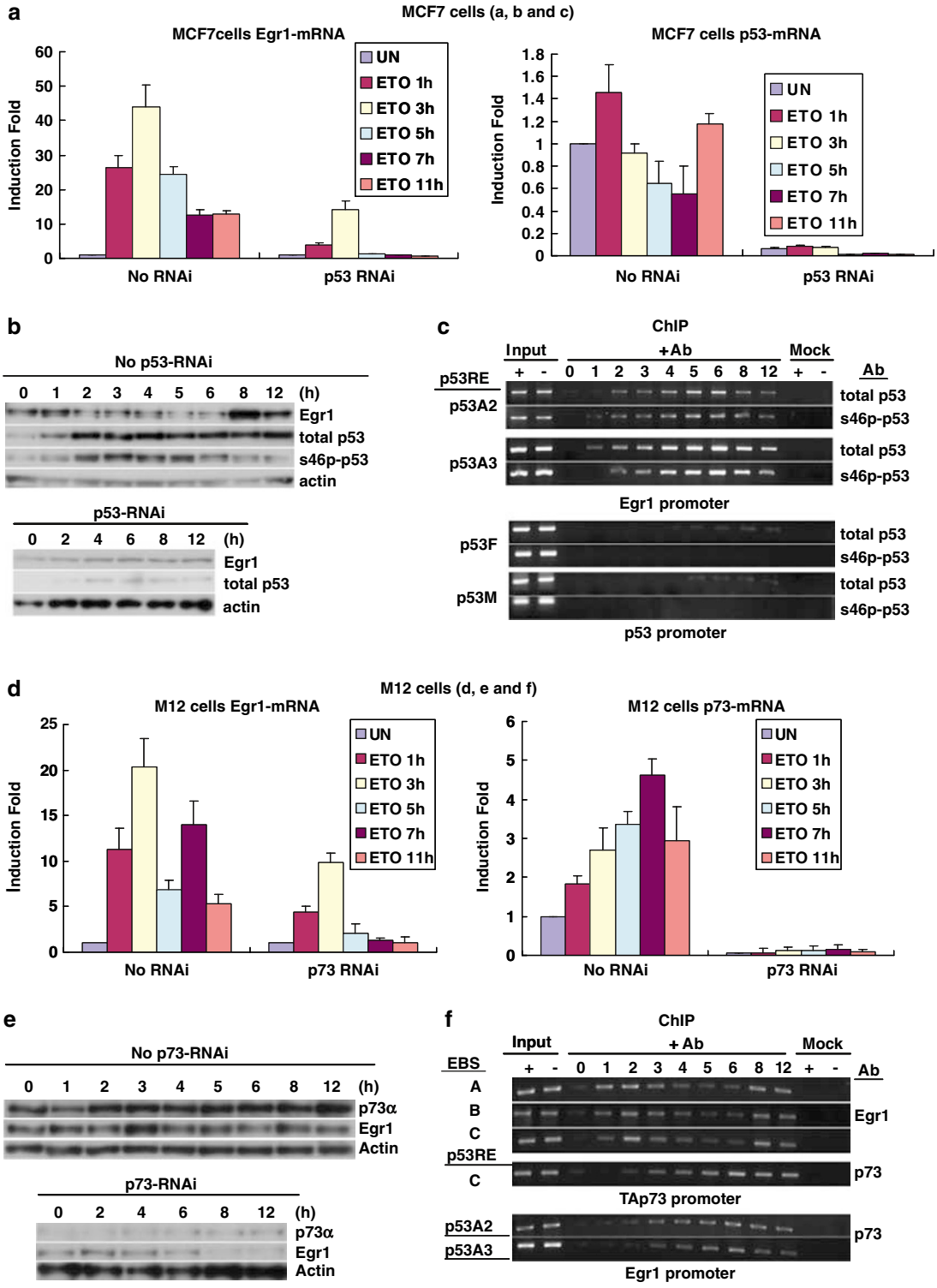

Figure 7 Two cell lines show differential expression of Egr1, p73 and p53 proteins during a time-course following ETO treatment. (**a**) MCF7 and (**d**) M12 cells show mRNA levels during the time-course. (**b**, **e**) Immunoblots using ECLplus, from MCF7 and M12 cells in the absence or presence of RNAi as shown, (**c**, **f**), ChIP studies on MCF7 and M12 cells show the differential binding of proteins to the relevant promoter fragments during the time-course. These are representative of at least two separate studies

co-expressed. RNAi to each protein decreased ETO-induced apoptosis (Figure 8b: reduction of 30% with Egr1-RNAi, 20% with p53-RNAi).

Co-transfection of two and three RNAi further reduced apoptosis, indicating that expression levels of the three genes contribute to the level of apoptosis achieved. In ETO-treated

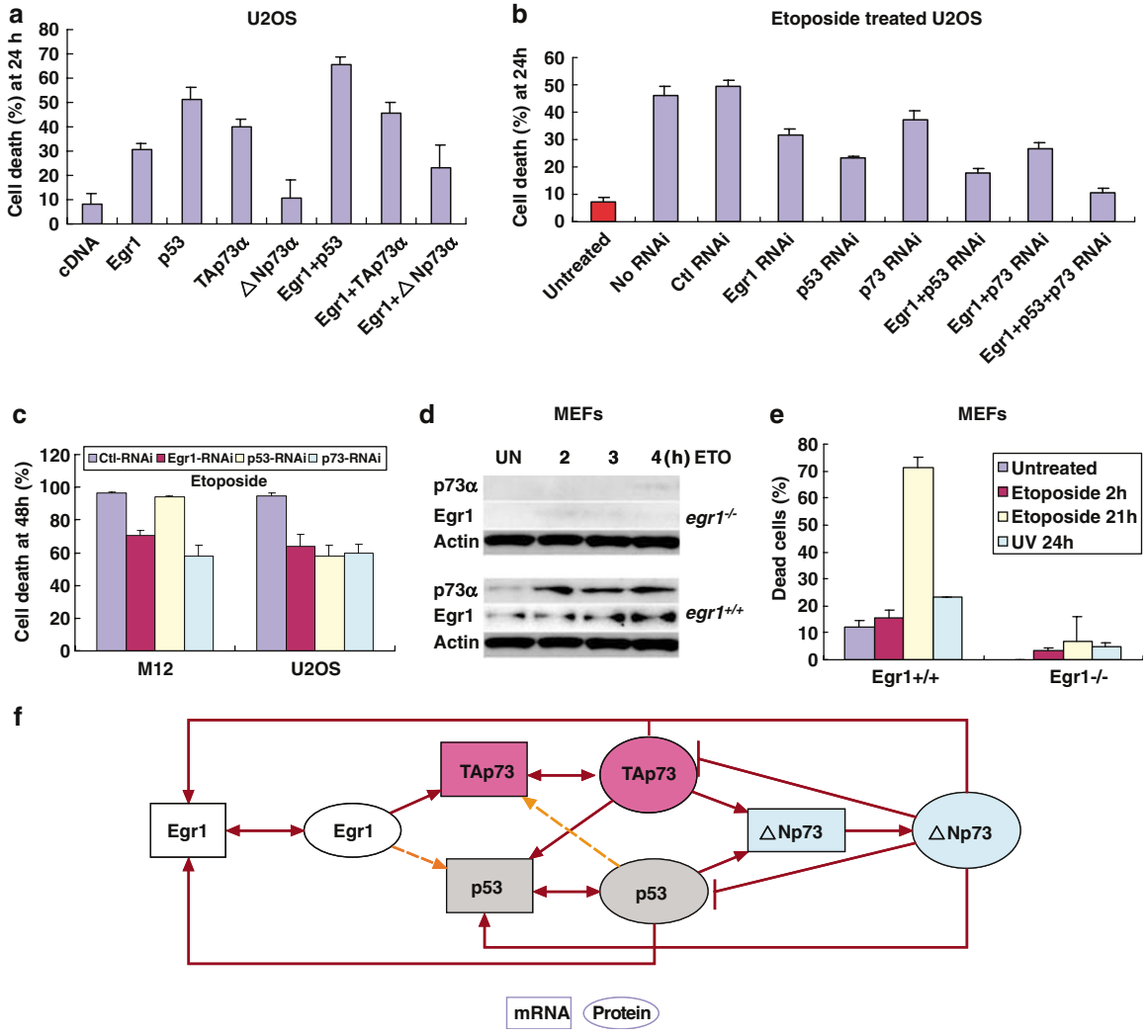

Figure 8 Apoptosis levels achieved by Egr1, p53 and p73 expression in several cell lines. The treated cultures were stained with Trypan blue to calculate the percentage of dead cells, in triplicate cultures repeated three times (**a**) U2OS cells transfected with Egr1 and /or p53 family members, (**b**) ETO-treated (20 μg/ml) U2OS cells were transfected with various RNAi, with measurement of apoptosis levels after 24 h. (**c**) M12 and U2OS cells were treated with ETO and apoptosis measured after 48 h. (**d**) Egr1 $^{+/+}$ and $^{-/-}$ MEFs were compared for the levels of proteins induced after addition of ETO during a time-course. (**e**) Egr1 $^{-/-}$ and $^{+/+}$ MEFs treated with ETO or UV (50 J/m^2) indicate that Egr1 is required for apoptosis as measured by nuclear fragmentation. (**f**) Summary Figure showing the interactions of proteins and promoters that regulate p53, p73 and Egr1 described in this paper. Arrows denote strong transactivating activity, dashed arrows indicate weak activity, T-bars indicate blocked activity

M12 cells Egr1-RNAi or p73-RNAi, but not p53-RNAi, decreased apoptosis (Figure 8c), consistent with the fact that p53 is inactive in these cells. Each RNAi achieved a 15–30% reduction in apoptosis of U2OS cells.

Egr1 $^{+/+}$ and Egr1 $^{-/-}$ MEFs [23,24] were tested next, at late passage. Egr1 $^{-/-}$ cells lack p73 as seen in Figure 8d and lack p53 as described previously[18] indicating that Egr1 is key to the interactive network between these genes. Figure 8e shows that in Egr1 $^{+/+}$ MEFs, ETO induced apoptosis in 70% of the cells and UV-C-induced apoptosis in 22% of the cells. In contrast, Egr1 $^{-/-}$ cells showed little apoptosis, allowing the conclusion that Egr1 is required for apoptosis.

A summary of the interactions between Egr1, p53, and the two major p73 subtypes at the transcriptional and protein levels is presented in Figure 8f. Our results indicate that p73 almost equals p53 as a physiologically effective inducer of apoptosis. In this interactive system the process of apoptosis can occur even in the absence of p53, if Egr1 and p73 are active. We conclude that all three interacting factors, Egr1, p53 and p73 are required for maximal induction of apoptosis *in vivo* and *in vitro*. More importantly, expression levels of each gene remain elevated for extended periods if two of the three are expressed together.

Discussion

Two important new findings are described in this paper. One is the interdependency on transcription factor Egr1 for the regulation of expression of p53 family members p53 and p73 in response to stress. In turn, proteins produced by each gene affect the others in feedback loops. Dependency on Egr1 for stimulatory effects *in vivo* was shown after genotoxic stress of

mice, followed by analysis of RNA levels in tissues. No induction of p73 occurred in mice that are null for *Egr1.* The second finding is the interdependency that occurs through previously unrecognized EBS as well as newly recognized non-consensus p53RE contained in *Egr1* and *TP53* promoters. Of four putative p53BS in the *Egr1* promoter, only two are active and bind p53 phosphorylated at S46 (Figure 7c). In contrast the S15-phospho-p53 form binds to the p53 promoter binding sites (Supplementary Figure S10). These p53RE (A2 and A3) are active, as proven by mutation of the sites. Similarly, we identified three new p53RE (called F, M and Z) in the p53 promoter (Figure 6d, Supplementary Figure S6C). Two of these sites with imperfect consensus sequences are transcriptionally active, while one with intact C + G/C + G motifs is *inactive.* We show in Supplementary Data-S6C, that only the non-consensus F and M p53RE contain trinucleotide triplet repeats of CTG-CAG that form hairpin loops, which provide strong binding sites for p53 family members (30). The two active p53RE in *TP53* promoter are upregulated by all members of the p53 family including p53, TAp73 and ΔNp73 (Figure 6b). The transactivating function of ΔNp73a (Figure 6e) requires the 13 amino-acid amino-terminal sequence, as a novel TAD, together with the PXXP domain (sequence 14–78). The responses of both *Egr1* and *TP53* promoters are similar for ΔNp73α and β isoforms (Figures 5a and 6b), whereas in other studies only β was active in upregulating p53 target genes,[25] suggesting that the specific sequence of the interacting p53RE on the promoter might play a crucial role. We conclude that the novel non-consensus p53RE in both the p53 and Egr1 promoters described here are specific for regulatory activity of ΔNp73 isoforms.

The new active sites in all four promoters are responsible for interdependency of these genes and fine tuning of cellular responses to various stimuli. Mutational analyses of previously undetected p53BS in the responsive promoters, together with verification of the binding of the proteins to these REs by ChIP supports our conclusions. The interpretation of the time-course studies after ETO treatment is simplest in MCF7 cells which lack p73. The first peak of Egr1 protein is an early response, while the sustained Egr1 mRNA is likely brought about by high levels of stabilized p53 through the MDM2 pathway.[26] This interpretation is supported by the maximal effect of RNAi-p53 (Figure 7a) during the time-course when the levels of active p53 protein are highest from 3 to 5 h, at the same time when maximal binding to the p53 RE in the Egr1 promoter is found (Figure 7c) and preceding the second peak of Egr1 protein at 8–12 h (Figure 7b),

S46p-p53 binds to p53 RE on *Egr1* promoter in U2OS cells whereas S15p-p53 binds to *TP53* promoter (Supplementary Figure S10C), indicating that post-translational forms of p53 have specific functions. We assume that ChIP analyses reveal which isoforms of p53 are transcriptionally active, when these forms are bound to the promoters of interest. This may explain the sustained p53 mRNA and protein levels from 2 to 12 h. It appears that several pathways contribute to the effects measured. Stability of p53 may also be sustained by the MDM2 pathway, but the results cannot show distinct peaks of activity because of the continuous feedback interactions and changes in levels, activities and molecular stabilities. Our results are consistent with the model of gene interrelationships shown in Figure 8f.

Athough Egr1 plays many roles in response to different stimuli, a major physiological response to increased Egr1 levels by genotoxic stress in most cell types is apoptosis.[27,28] In U2OS cells (Figure 1a) a combination of Egr1, p53 and p73 gave the highest level of apoptosis. In keeping with this, Figure 8b shows that all three RNAi species are effective in reducing apoptosis after ETO treatment and that combining RNAi to all three genes was required for maximal reduction of apoptosis. Importantly, this result was confirmed in Egr1$^{-/-}$ MEFs that do not express any of the three genes and are resistant to apoptosis (Figure 8d–e). We conclude that S20p-p53 plays no role in the transcriptional activity measured here but both S15p-p53 and S20p-p53 participate in p53 stabilization and nuclear localization through the MDM2 pathway.

Egr1 and p53 could synergize to increase PTEN expression,[29,30] which inhibits Akt, allowing retention of MDM2 in the cytoplasm. This in turn promotes p53 functions and sustains the sensitivity of cancer cells to chemotherapy. The synergism of Egr1 and p53 for transactivation of the same target genes such as PTEN is known (our unpublished data) and may also include p73. The final result is a more rapid apoptotic endpoint rather than DNA repair or cell cycle arrest.

We noted earlier that p53 target genes overlap those of Egr1.[31] The role of p53 in senescence, differentiation, apoptosis, growth control and DNA damage repair, echo similarly with those of Egr1.[5,6,19,32,33] We conclude from the present studies that transcriptional interdependence between Egr1 and p53/p73 largely explains these similarities. Other studies have reported changes in the stability of the proteins,[34,35] in their binding to p300[36] or in changes in protein localization during apoptosis.[37]

Here, we have reported mainly on the transcriptional properties of the four gene products Egr1, TAp73, ΔNp73 and p53. We hypothesize that p73 is more important than p53 in stress responses in prostate cancer cells where Egr1 is highly expressed and p53 and PTEN are often inactive. Thus, the forecast[38] that there must be 'a very tight relationship between the three members of the same family' is proved and extended here by the addition of Egr1 to the equation. These results have implications for the origins and progression of cancer.

Materials and Methods

Cells and culture, antibodies. H4, MCF7, 293T, Saos2, U2OS cells were cultured in DME containing 10% FBS, penicillin, and streptomycin, at 37°C and 5% CO2. M12 metastatic human prostate cancer cells were cultured as described earlier.[20] For ultraviolet-C (UV-C) irradiation (40 J/m^2) in a Stratalinker (Stratagene, La Jolla, CA, USA) as well as for mock treatment, the growth medium was aspirated and then replaced after treatment. Antibodies Egr1 (C-19), Egr1 (588), p53 (DO-1), p73 (E4), p73(S-20), p73α(C-17) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). E4 recognizes amino acids 1–80 of human p73 and C-17 reacts only to p73α. Antibodies to phosphorylated p53, Phospho-p53 (Ser 6, Ser15, Ser20 and Ser46) were from Cell Signaling Technology, Beverly, MA, USA. Antibody to p73 (Ab-2) (that recognizes all p73 isoforms), and ΔNp73 were from Oncogene Research Products (San Diego, CA, USA). Anti-(3-actin and anti-Flag M2 monoclonal antibodies were from Sigma (St Louis, MO, USA). Anti-HA (mouse IgG) was from Roche Applied Science (Indianapolis, IN, USA).

Mouse studies. Mice derived from two sources that are Egr1 null[23,24] were used to determine the effect of gamma-radiation (5 Gy) on gene expression

measured by semi-quantitative RT-PCR, 2.5 h later. Tissues were rapidly dissected and placed in 0.5 ml RNAeasy solution (Qiagen, Valencia, CA, USA). Total RNA was extracted and semi-quantitative RT-PCR was performed using 400 ng total RNA as template using the Superscript One-step RT-PCR with Platinum Taq kit (Invitrogen, Carlsbad, CA, USA). Primers to measure p73, Egr1 and Cyclophilin (CPH) were used (Supplementary Data-S11). The amplified cDNAs were resolved on a 2% agarose gel. The two strains of KO mice behaved identically in this study.

Plasmids. Three genomic DNA fragments with sizes of 2458, 1225 and 994 bp corresponding to the promoter and its 5′-upstream regulatory sequences of TAp73, ΔNp73 and p53, respectively, were amplified from human genomic DNA using Advantage-GC Genomic Polymerase Mix Kit (BD Biosciences Clontech, Palo Alto, CA, USA) and subcloned into a luciferase reporter, pGL3-Basic vector, thus creating pGL3-TAp73, pGL3-ΔNp73 and pGL3-p53 (confirmed by sequencing).

The pGL3-Egr1 Luciferase reporter constructs and expression vector for pcDNA3-Flag-Egr1were made as described.[20]

Mutations were introduced by directed mutagenesis according to Quick-Change Multi Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA, USA). All constructs were confirmed by sequencing. The mutation strategy was that 4nt of each p53RE or 3–4nt of each EBS (9 nt) were mutated and cloned into a restriction endonuclease site in order to identify the active binding sites.

We received many expression vectors from several researchers. Plasmids pCMV-neo-Bam-p53wt, were provided by B Vogelstein (Johns Hopkins University), pRC/CMV-p53wt from S Matsuzawa and pcDNA3.1-HA-hp53 from C Geisen, (both from The Burnham Institute). Plasmids pcDNA3-HA-TAp73a were provided by WG Kaelin (University of Toronto), pcDNA3-HA-TAp73β and pcDNA3-HA-ΔNp73a provided by Jean JY Wang, (University of California, San Diego). To generate pcDNA3-HA-ΔNp73(3, we replaced the fragment encoding C-terminal amino acids of pcDNA3-HA-TAp73β by excision of pcDNA3-HA-ΔNp73a with *Pml*I/*Xho*I. The pcDNA3-HA-TAp73aΔ1–62, pcDNA3-HA-TAp73aΔ1–127, pcDNA3-HA-ΔNp73aΔ1–13 and pcDNA3-HA-ΔNp73aΔ1–78 were provided by K Yoshihara, Nara Medical University, Japan. Primer sequences used for cloning the promoters and for mutated promoters are given in the Supplementary Information.

Transfection and Luciferase reporter assays. For DNA transfection, we seeded 293T, M12, U2OS and MCF7 cells at a density of 8–10 $\times$ 10^5 into 60 mm dishes the day before transfection in order to achieve 75–95% confluence. Transfection was performed with Lipofectamine™ 2000 (Invitrogen) following the instructions provided by the manufacturer, in a final volume of 1.5 ml of DMEM medium with 10% FBS without antibiotics. Typically 2 μg of DNA were mixed with 6 μl of Lipofectamine. Cells were collected 24 h after transfection for immunoblotting.

For luciferase assays, we added 2–3 $\times$ 10^3 H4 cells, 5–8 $\times$ 10^3 293T and Saos2 cells to each well of 96-well plates 1 day before transfection. Transfection was performed with Lipofectamine™ 2000 (Invitrogen). Typically 85 ng of total plasmid DNA (40 ng of reporter construct, 40 ng of the appropriate expression vector, plus 5 ng of pRL-SV40 for Egr1 target test or 5 ng of pRL-CMV for p53/p73 target test) were mixed with appropriate volume of Lipofectamine™ 2000. Cells were lysed 24 h after transfection and luciferase activities were assessed using Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) and an EG&G Berthold LB96P luminometer (PE Biosystem, Wellesley, MA, USA). Each data-point represents the mean-fold change compared to basic vector.

Light Cycler Quantitative RT-PCR. Total RNA was purified from cell lysates at different times after ETO addition (0, 1, 3, 5, 7 and 11 h). RNA purification and LightCycler QRT-PCR were performed as described previously.[20] Specific primers for cyclophilin A, Egr1, p53, p73 are given in the Supplementary Data.

Immunoblot analysis. Cells were lysed in 50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS and 1 $\times$ complete protease inhibitor cocktail (Roche). Lysate (22 μg) was separated on a NuPAGE 3–8% Tris-Acetate Gel (Invitrogen Co., Carlsbad, CA, USA) and analyzed by Western blotting. The membranes were probed with the appropriate antibodies.

Antibody-antigen complexes were detected using Amersham ECL or ECL$^+$ kit (Amersham Biosciences Corp, Piscataway, NJ, USA). Blots were photographed and quantified using Alpha Inotech Inc. equipment.

Chromatin immunoprecipitation. ChIP was performed as described previously[39] with modifications.[20] Cells were treated with ETO (20 μg/ml) for 2.5 h M12, (Figure 3c) or for 6 h (U2OS, Figures 4c, 5c and 6c) before addition of 1% formaldehyde to crosslink proteins to the chromatin. After immunoprecipitation with specific p73, Egr1 or p53 antibodies or a non-immune IgG, the DNA fragments collected after sonication were purified and tested for the presence of a promoter using specific primers and PCR for 35 cycles. Primer sequences are given in Supplementary Data-S11.

RNA interference experiments. We transfected cells with GenEclipse™ control, p53 or Egr1 vector-based siRNA (Chemicon International) and SignalSilence™ p73 siRNA (Cell Signaling Technology) according to the protocol provided by the manufacturer. M12, MCF7 and U2OS cells were seeded into 60 mm dishes at a density of 8 $\times$ 10^5, 5 $\times$ 10^5 and 5 $\times$ 10^5 cells, respectively, 1 day before transfection in order to achieve 75–95% confluence. At 24 h after transfection, ETO (Sigma, St Louis, MO, USA) was added at 20 μg/ml and cells were cultured for 24 or 48 h. For Western-blots cells were collected at time 0, 2, 4, 6, 8 and 12 h after ETO treatment.

Measurement of apoptosis. (a) Cells were treated with ETO and the percentage of dead cells was counted as Trypan Blue-stained cells together with total number of cells alive in triplicate dishes. (b) Egr1$^{+/+}$ and $^{-/-}$ MEFs were fixed in methanol, 2 and 21 h after ETO treatment or untreated. Adhering cells were stained with Hoechst dye according to the manufacturers instructions (Sigma-Aldrich, Milwaukee, WI, USA), and the ratio of fragmented nuclei (apoptotic) to normal nuclei was counted using a Nikon epifluorescence microscope. Counts were averaged over 10 fields. Cells in the supernatant were also counted. An average of 700–1000 cells were counted per sample and the experiment was repeated once. Photographic records were also made (data not shown). For UV stimulation, the Trypan Blue method was used.

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