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PRINCIPAL INVESTIGATOR: Jianxiu Yu, Ph.D.

CONTRACTING ORGANIZATION: Burnham Institute
La Jolla, California 92037-1005

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Jianxiu Yu, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Burnham Institute
La Jolla, California 92037-1005

E-Mail: jyu@burnham.org

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Last year I finished the study of the related co-activators p300 and CBP. I have shown that the immediate early response TF, Egr1, acts upstream of p300/CBP to induce or to repress transcription, depending on the stimulus. I have shown that serum stimulation of prostate cells results in cell growth by the up-regulation of p300/CBP. In contrast, the stimulus of ultraviolet irradiation leads to cell cycle block and apoptosis through the inhibition of p300/CBP. This year, I studied the effect of Egr1 on the expression of the related p73 and p53 tumor suppressor genes, having found several Egr1 binding sites in their promoters. Endogenous and exogenous Egr1 expression causes the transcriptional activation of the TAp73 isoforms from promoter P1, and the modest activation of p53. The most notable finding was of positive feedback loops from both TAp73 and ΔNp73 isoforms and from p53 on Egr1 expression, strengthening our view that Egr1 is a tumor-suppressor by its genotoxic stress-responses that can substitute for the often mutant p53 in cancer cells. The interactive network between Egr1, p53, TAp73 and ΔNp73 explains how cancer cells make extensive responses to chemotherapy and irradiation even in the absence of wt p53.

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Egr1 target genes that regulate growth/survival of prostate cells.

Introduction and Background

Immediate early growth response–1 gene, *EGR1* (also called Zif268, NGFIA, Tis8 and Krox24) (Christy et al., 1988; LeMaire et al., 1988; Lim et al., 1987; Milbrandt, 1987; Sukhatme et al., 1988) encodes a zinc-finger transcription factor whose expression is elicited in response to a diverse variety of extracellular signals, including growth factors, cytokines, phorbol esters, irradiation, and stresses of many kinds in a rapid and transient manner. Egr1 is a protein that can bind to a GC-rich element in the promoters of a range of target genes. Growth factor stimulation of most cells leads to rapid induction of Egr1 within minutes that leads to the activation of downstream growth pathways in normal cells. Egr1 can also suppress growth when over-expressed or re-expressed in transformed cells (Huang et al., 1998b; Huang et al., 1995). The cellular responses to Egr1 are also duplicitous in relation to apoptosis. Egr1 can induce apoptosis either by stimulating p53 expression (Ahmed et al., 1997; Ahmed et al., 1996; Nair et al., 1997) or PTEN expression (Virolle et al., 2001). However, Egr1 can also promote survival in other cell types (de Belle et al., 1999; Huang et al., 1998a; Huang and Adamson, 1995; Nair et al., 1997) by slowing growth via p21/Cip1 induction. It appears that Egr1 can act as a tumor suppressor in some cells but as a growth stimulator in others. An example of the latter is in human prostate tumors where Egr1 is over-expressed (Eid et al., 1998; Mohiuddin et al., 1997; Thigpen et al., 1996) but is low or absent from normal prostate tissue. Moreover, the level of Egr1 increases with the degree of malignancy as measured by the Gleason score of the tumor (Gleason, 1988) (Eid et al., 1998). Mouse models using Egr1 knockout and transgenic mice (the TRAMP mouse model) support the conclusion that Egr1 is required for tumor progression. (Abdulkadir et al., 2001; Baron et al., 2003; Virolle et al., 2003). This is significant and specific to prostate tumor cells because in mammary, lung and glial tumors, Egr1, is not over-expressed.

In year 02 of my Fellowship. I finished the described SOW in May of 2004, and continued a similar type of study as follows. I focused on the similarities of Egr1 functions with those of p53. I have found that they have similar activities partially because they are transcriptionally inter-dependent. The work I have done measures the transcriptional effects of each of four promoters on the products of four genes in the relationship, Egr1, p53, TAp73 and DNp73. These are largely tumor suppressor genes that are activated after a stress stimulus and this is triggered by Egr1. However, I found that p73 is the hub of the cycle and is the strongest inducer of apoptosis of cancer cells after DNA-damaging stresses such as Etoposide or UV treatment.

SCIENTIFIC BODY

Conclusions from project 1(Egr1 regulates p300 and CBP co-activators) is now completed. In prostate cancer, a positive feedback loop from Egr1 and from growth factor products on the Egr1 promoter occurs to provide constitutive growth. This provides a high constitutive level of acetylated Egr1 which is more stable. Whether the transactivating and other properties of the stable acetylated form are different remains to be determined, but it does affect its choice of target genes. After UV irradiation, Egr1 is phosphorylated and remains active for a longer period of time than growth factor stimulated Egr1, thus allowing a range of genes to be regulated. These include growth arrest genes, DNA repair genes and apoptotic genes. A copy of this published paper is attached in the Appendix.

Abstract of the new paper just submitted

TP53 is often mutated in cancer, but *TP73* only rarely, and is known to regulate a subset of p53 target genes that cause cells to respond to stress by growth arrest and apoptosis. p73, however, produces two main forms; only TAp73 reiterates the roles of p53, while DNp73 has a distinct set of oncogenic properties. The P1 promoter of the *TP73* gene has five distinct Egr1 binding sites; all five contribute to the up-regulation of TAp73 by Egr1 in several cell types. In contrast, the P2 promoter regulating DNp73 is not induced by Egr1 but *is* induced by TAp73, and p53. Mutational analysis

confirmed these results. We show that stress induces mainly TAp73 via Egr1, but newly discovered active p53 binding sites in p73, p53 and Egr1 promoters reveal an auto-regulating network that controls complex stress responses of the four genes at the transcriptional level.

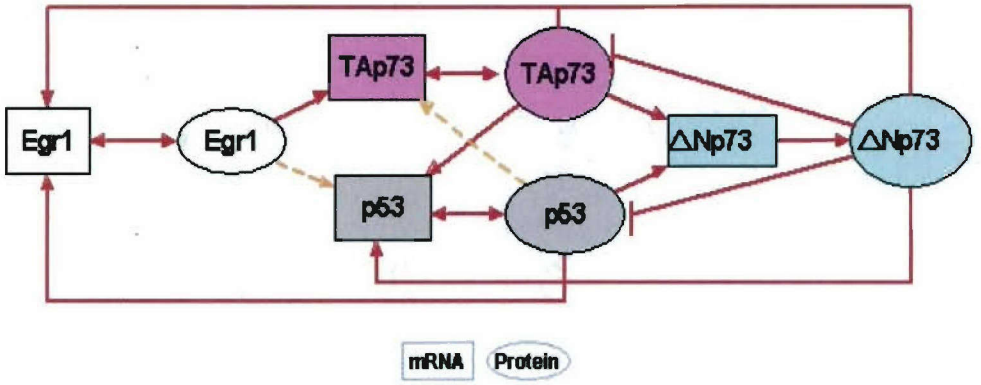

FIGURE 1

This major difference between Egr1 and p53 is likely highlighted by the fact that there are even more ways to regulate the activities of Egr1 post-transcriptionally than p53, such that mutation of the gene is not under pressure. This fact enlarges the importance of Egr1 in its myriad of roles and the variety of regulatory measures to modulate any large changes in its expression. A copy of the submitted paper is attached in the Appendix in lieu of putting the data here.

This is the list of studies in the SOW that were planned for the last year of the Fellowship.

Task 3. To determine the biological responses of prostate cancer cells that over-express p300 or CBP in prostate cancer cells. Can this be modulated by antisense Egr1 oligonucleotides? (months 13-24)

a. To prepare expression vectors to express p300 and CBP transiently and conditionally.
b. To transfect a transiently expressed vector and measure the effect on proliferation and migration in the presence and absence of antisense Egr1 oligonucleotides.
c. To express p300, CBP and EGFR conditionally in prostate cells in the presence and absence of antisense Egr1 oligonucleotides. Assays for the effect on cell growth rate, doubling time, migration rate, colony growth, soft agar growth and cell cycle.

To stimulate the cells with apoptotic stimuli to determine their sensitivity when expressing (or not) the p300 and CBP genes.

The studies described were completed in May, 2004 when our paper was accepted for publication in Molecular Cell. I chose to use some different techniques as well as those in the original plan in order to fit the theme of the research as it evolved. For example I determined the expression of a set of growth promoting genes that were up-regulated by serum addition to prostate cancer cells. Genes such as growth factors and survival genes were up-regulated. A different set of genes were up-regulated after UV-C irradiation to show that the target genes are changed to Bcl2, p53 and p73 apoptotic genes. As indicated I used antisense oligonucleotides and Egr1 knock out cells. In addition, I also used RNAi as an extra tool to reduce the expression of Egr1 and determine its effect. **We did not include work on the EGFR as indicated in the SOW because it did not fit in with the rest of the work.** However, I tested many more cell lines than planned because I analyzed the differences between the different prostate cancer cell types to determine if there were differences between the "normal" cell lines and the cancer cells. I did not see significant useable differences at the level of these established cell lines, therefore molecular profiling of patients at different stages of disease must be used next to develop possible therapeutic approaches.

The resulting article is attached: **Yu J**, de Belle I, Liang H and Adamson ED. (2004). Coactivating factors p300 and CBP are transcriptionally crossregulated by Egr1 in prostate cells, leading to divergent responses. **Mol Cell**. 15(1):83–94.

Adamson ED, **Yu J** and Mustelin T. (2005) Co-factors p300 and CBP catch Egr1 in their network (Review). **Prostate**. 2005 Jan 5; [Epub ahead of print in 2004]

KEY RESEARCH ACCOMPLISHMENTS

1. In year 01, I cloned full-length, and fragments of the EP300 and CREBBP genes for analysis of function
   - Determined that p300 and CBP are transcriptionally-regulated by Egr1, both up- and down-regulation occur, depending on the stimulus on the cells and the post-translational modification of Egr1. Growth factors up-regulate and stress stimuli inhibit.
2. In year 02, I cloned the promoters of p53, p73 and Egr1 and mutated forms.
   - Found that Egr1 regulates the transcription of members of the p53 family of genes, p73 but not the p63 gene.
   - Prepared a new paper on the inter-relationships between these important tumor suppressor genes in prostate cancer cells.

REPORTABLE OUTCOMES

1. We have a paper published on this data, **Yu J**, de Belle I, Liang H, Adamson ED (2004) Coactivating factors p300 and CBP are transcriptionally crossregulated by Egr1 in prostate cells, leading to divergent responses. **Mol Cell**. 15(1):83–94, 2004.
2. In year 02, I have made an analysis of the transcriptional control of the p53 family of genes including p53, TAp73 and DNp73 (the last two genes are encoded from the p73 genes but are derived different promoters and have different N-terminal sequences). The essential finding is that Egr1 regulates p73 and p53 gene expression through a feedback mechanism. This paper has just been submitted.
3. I am an author on a review article on the roles of Egr1 in prostate cancer where Egr1 is over-expressed in direct proportion to the degree of progression. The first author was my mentor Dr Eileen Adamson and together with Dr Tomas Mustelin at the Burnham Institute, this is currently published on line, and will be in paper format in The Prostate during 2005. Adamson ED, **Yu J** and Mustelin T. (2005) Co-factors p300 and CBP catch Egr1 in their network (Review). **Prostate**. Jan 5; [Epub ahead of print in 2004].

CONCLUSIONS

1. I have found that the tumor suppressor gene, p73 is a target gene of Egr1 that can be up regulated in its two different forms promoted by two different promoter elements to produce TAp73 from promoter P1 and DNp73 from promoter P2.
2. There is a complex of feedback loops that includes strong reactivation of Egr1 as well as p53.
3. Both wild type p53 and some mutant p53 genes can induce apoptosis while some mutant p53 genes are inactive on the four promoters p53, Egr1, P1 and P2 of p73 that I tested. In summary the apoptotic response of p73 predominates when Egr1 is induced by genotoxic stimuli and allows Egr1 and TAp73 to be the major players as tumor suppressors when p53 is mutant. The ability of DNp73 and p53 to select different gene targets that do not lead to apoptosis must be tested before this information can be useful in therapeutic applications.

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APPENDIX

1. **Yu J**, de Belle I, Liang H and Adamson ED. (2004). Coactivating factors p300 and CBP are transcriptionally crossregulated by Egr1 in prostate cells, leading to divergent responses. **Mol Cell**. 15(1):83–94.
2. **Yu J,** and Adamson ED. (2005). Transcriptional interdependence between p73, Egr1 and p53. (Submitted).

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Coactivating Factors p300 and CBP Are Transcriptionally Crossregulated by Egr1 in Prostate Cells, Leading to Divergent Responses

Jianxiu Yu, Ian de Belle, Hongyan Liang, and Eileen D. Adamson*
The Burnham Institute
Cancer Research Center
10901 North Torrey Pines Road
La Jolla, California 92037

Summary

Related coactivators p300 and CBP affect the transcriptional activities of many transcription factors (TF), producing multiple downstream effects. Here we show that immediate early response TF, Egr1, acts upstream of p300/CBP to induce or to repress transcription, depending on the stimulus. Cells induced with serum to increase endogenous Egr1 increase the transcription of p300/CBP only when Egr1 binding sites in the promoter are not mutated, causing the expression of downstream targets of Egr1 which leads to survival and growth. Induction of p300/CBP by Egr1 results in acetylation and stabilization of Egr1 and transactivation of survival genes but repression of Egr1 and p300/CBP in negative feedback loops. In contrast, induction of Egr1 by UV-C irradiation leads to repression of p300/CBP transcription: Egr1 is preferentially phosphorylated, leading to regulation of target genes that cause cell death. This complex balance of opposing effects appears to finely modulate important cellular life and death responses.

Introduction

Immediate early growth response-1 gene, *EGR1* (also called Zif268, NGFIA, Tis8, and Krox24) (Christy et al., 1988; LeMaire et al., 1988; Lim et al., 1987; Milbrandt, 1987; Sukhatme et al., 1988), encodes a zinc finger transcription factor whose expression is elicited in response to a diverse variety of extracellular signals, including growth factors, cytokines, phorbol esters, irradiation, and stresses of many kinds in a rapid and transient manner. Egr1 binds to GC-rich elements in the promoters of a range of target genes. Growth factor stimulation of most cells leads to rapid induction of Egr1 within minutes that leads to the activation of downstream growth pathways in normal cells. Egr1 can also suppress growth when overexpressed or reexpressed in transformed cells (Huang et al., 1995, 1998b). The cellular responses to Egr1 are also duplicitous in relation to apoptosis. Egr1 can induce apoptosis either by stimulating p53 expression (Ahmed et al., 1996, 1997; Nair et al., 1997) or PTEN expression (Virolle et al., 2001). However, Egr1 can also promote survival in other cell types (de Belle et al., 1999; Huang et al., 1998a; Huang and Adamson, 1995; Nair et al., 1997) by slowing growth via p21/Cip1 induction. It appears that Egr1 can act as a tumor suppressor in some cells but as a growth stimulator in others. An example of the latter is in human prostate tumors where Egr1 is overexpressed (Eid et al., 1998; Mohiuddin et al., 1997; Thigpen et al., 1996) but is low or absent from normal prostate tissue. Moreover, the level of Egr1 increases with the degree of malignancy as measured by the Gleason score of the tumor (Gleason, 1988; Eid et al., 1998). Mouse models using Egr1 knockout and transgenic mice (the TRAMP mouse model) support the conclusion that Egr1 is required for tumor progression (Abdulkadir et al., 2001; Baron et al., 2003a; Virolle et al., 2003).

The protein products of the paralog genes p300 and CBP (also known as CREB binding protein, CREBBP) are large and possess multiple binding domains for at least 40 transcription factors that allow them to act as multifunctional adaptor proteins with acetyl transferase activity for transcription factors and histones. Thus, they affect chromatin structure and can have multiple effects on downstream target genes (Frisch and Mymryk, 2002). The protein pair p300/CBP is important in both cell cycle progression and cellular differentiation and has been shown to interact with components of the RNA polymerase II holoenzyme, transcription factors, and nuclear hormone receptors and their coactivators. Mutations in the transcription factor binding domain of these genes occur in several types of tumors (Kasper et al., 2002). Transcription factors modulated by acetylation via p300/CBP include Myb, Creb, Ets-1, p53, c-Jun, c-Fos, MyoD, Stat1, Stat2, and AR. CBP and p300 associate with PCAF, and all have histone acetyltransferase (HAT) activity. The complex formed between CBP/p300 and PCAF is disrupted by the adenoviral protein E1A (Chakravarti et al., 1999), leading to suppression of p53 transactivation (Somasundaram and El-Deiry, 1997). The amino-terminal 80 amino acids of E1A also bind p300 and CBP to block the acetyltransferase activity and effectively negate their coactivation roles (Dorsman et al., 1997; Eckner et al., 1994). Thus, loss of complex formation with p300/CBP affects transactivation of the target genes.

The transcriptional regulation of p300/CBP has not yet been described. It has already been reported that both Egr1 and p53 activities are upregulated by the activity of p300/CBP and that there is a physical interaction of the p300/CBP proteins with p53 and Egr1 while coactivating their transcriptional activities (Lill et al., 1997; Shikama et al., 1999; Silverman et al., 1998). Our results show that the interactions between p300/CBP and Egr1 can lead to complex feedback loops and to inhibition of Egr1 activities under specific conditions. We demonstrate here that in cancer cells transfection of p300/CBP promoter reporter constructs are activated by exogenous and endogenous Egr1 induced by growth factors. In contrast, under conditions whereby Egr1 expression is highly induced, such as by UV-C irradiation, the reporter constructs are inhibited by Egr1. These effects are reversed in the presence of antisense Egr1 oligonucleotides, thus implicating Egr1 as the effective agent. This and other evidence presented below demonstrate that p300/CBP expression is remarkably respon-

*Correspondence: eadamson@burnham.org

sive to Egr1 levels, consistent with the frequently observed dual (opposite) effects of Egr1 expression in different cell types. In addition, we report the finding that the acetylation of Egr1 by p300/CBP accounts, in part, for the divergent effects of Egr1 and its specific effects on the responses of cells to various stimuli that induce Egr1.

Results

Egr1 Transactivates the Promoter and 5′-UTR Fragments of CBP and p300 Genes

The promoters and 5′-UTR sequences upstream of the translation start site of the p300 and CBP genes are highly GC rich and contain putative Egr1 binding sites (EBS). Using only the highest affinity binding sequences GNG(T/G)GGG(T/C)G (Hamilton et al., 1998) for Egr1, we found that there are six and seven sites in the p300 and CBP regulatory regions, respectively, which are labeled a through g in Figure 1A. p300 and CBP promoter-luciferase reporter genes (pGL3-p300 and pGL3-CBP) were constructed as described in the Experimental Procedures section to contain the 5′ noncoding region of the genes. The reporter construct was also made with putative EBS mutated at three or four consecutive bases to yield single site and multiple site mutated versions to determine which sites are active. Figure 1B indicates that, for the p300 promoter, all sites have some activity in H4 cells (Egr1 expression negative) after transfection of an Egr1 vector, such that p300mut-**abcdef** was not activated by Egr1. Activation values obtained for double mutants became lower as all sites were mutated. There was little distinction between "perfect consensus" sites and nonconsensus sites, with all EBS adding some activity to the 6-fold increase in p300 promoter activity (Figure 1B). Similarly, the CBP-luciferase construct was tested together with one mutated EBS, and similar results were obtained, except activation was lower overall by 5-fold (see Supplemental Figure S1A at http://www.molecule.org/cgi/content/full/15/1/83/DC1).

Egr1 Binds Directly to CBP/p300 Regulatory Sequences In Vivo

To assess whether direct binding of Egr1 to the CBP or p300 regulatory sequences occurs in intact cells, we performed chromatin crosslinking with formaldehyde followed by immunoprecipitation with antibodies to Egr1 (ChIP). To detect DNA binding under two different conditions, Egr1 levels were induced in DU145 prostate carcinoma cells by the addition of serum for 1 or 2 hr after UV-irradiated DU145 cells. The sonicated chromatin fragments were processed essentially as described (de Belle et al., 2000). PCR was used to detect DNA specific to CBP and p300 promoters that had been pulled down by the anti-Egr1. Figure 1C shows that the p300 promoter and 5′ regulatory sequences were represented by fragments A to D. All of these DNA fragments were shown to contain EBS that bound Egr1 since the anti-Egr1 immunoprecipitated a DNA fragment that was detected by PCR using specific primers. Similarly, the fragments B and C in CBP ChIP products contain Egr1 binding sites, since primers specific to that fragment showed a DNA product in the anti-Egr1 (αE) and the DNA input lanes in B and C but not for the A fragment which was upstream of the EBS in the promoter (Figure 1A, lower panel). The same DNA ChIP samples were also used for amplification using primers to detect cyclophilin as a negative control (data not shown). An additional control was provided by the inability of nonimmune serum to immunoprecipitate these DNA fragments. This demonstrated that Egr1 can bind directly to all of the binding consensus sequence-containing fragments that we tested, and it does so in live cells that express endogenous Egr1. We next asked whether this binding has an effect on the regulation of the gene when endogenous Egr1 is elevated by a stimulus such as serum or UV-C irradiation.

p300/CBP Are Upregulated by Egr1 in Serum-Induced Prostate Cancer Cells and in Mouse Embryo Fibroblasts

Metastatic prostate cancer cells, M12, were made quiescent by serum starvation and then were serum stimulated. mRNA levels were assayed by quantitative RT-PCR over a time course from 15 to 120 min. Figure 2A shows that the levels of Egr1 mRNA were maximal at 30–45 min while p300 and CBP mRNAs rose slowly and were maximal at 60 min. Western blots to assay protein levels over a longer time course were made in six cell lines. In wt mouse embryo fibroblsts (MEFs), Egr1 protein was maximal at 1 hr, while p300 and CBP peaked at 2–3 hr. As expected, if Egr1 was regulating transcription of the p300/CBP proteins, little or no changes were seen in Egr1 null MEFs (Figure 2B). Induced levels of proteins were seen in four different prostate cell lines that were serum starved and then stimulated with 20% serum, and the same pattern was observed (Figure 2C). We next tested whether the reduction of Egr1 levels with antisense oligonucleotides would affect the expression levels of p300/CBP in prostate cancer cells. Uptake of the highly specific oligonucleotides described previously (Virolle et al., 2003) by transfection of the malignant prostate cancer cell line M12 completely negated Egr1 expression at 0.2 μM and also drastically reduced p300 and CBP protein (Figure 2D) and mRNA expression (Figure 2E). We concluded that Egr1 induction by serum correlates with p300/CBP induction in many cell lines, while constitutive Egr1 normally maintains basal p300/CBP expression in prostate cells. These results led to the following study that defined the role of Egr1 in prostate cancer cells to show that the regulation is not simple and involves several feedback loops.

CBP/p300 Genes Are Directly Downregulated by Egr1 during UV Irradiation-Induced Signaling

We showed earlier that ultraviolet-C (UV-C) irradiation upregulates the production of Egr1 protein (Huang et al., 1996). We used UV-C irradiation to stimulate endogenous Egr1 levels in four prostate cell lines to show that 2 hr after irradiation with UV-C at 40 Jm^{-2} the mRNA levels of CBP and p300, quantified by real-time one-step RT-PCR, were downregulated in these four cell types. The inhibition of mRNA levels for CBP and p300 in M12 cells was the most affected. In M12 cells and P69 cells, p300 mRNA levels were 6.7-fold and 1.5-fold lower, $p < 0.01$, respectively. For CBP, M12 gave 2.7-

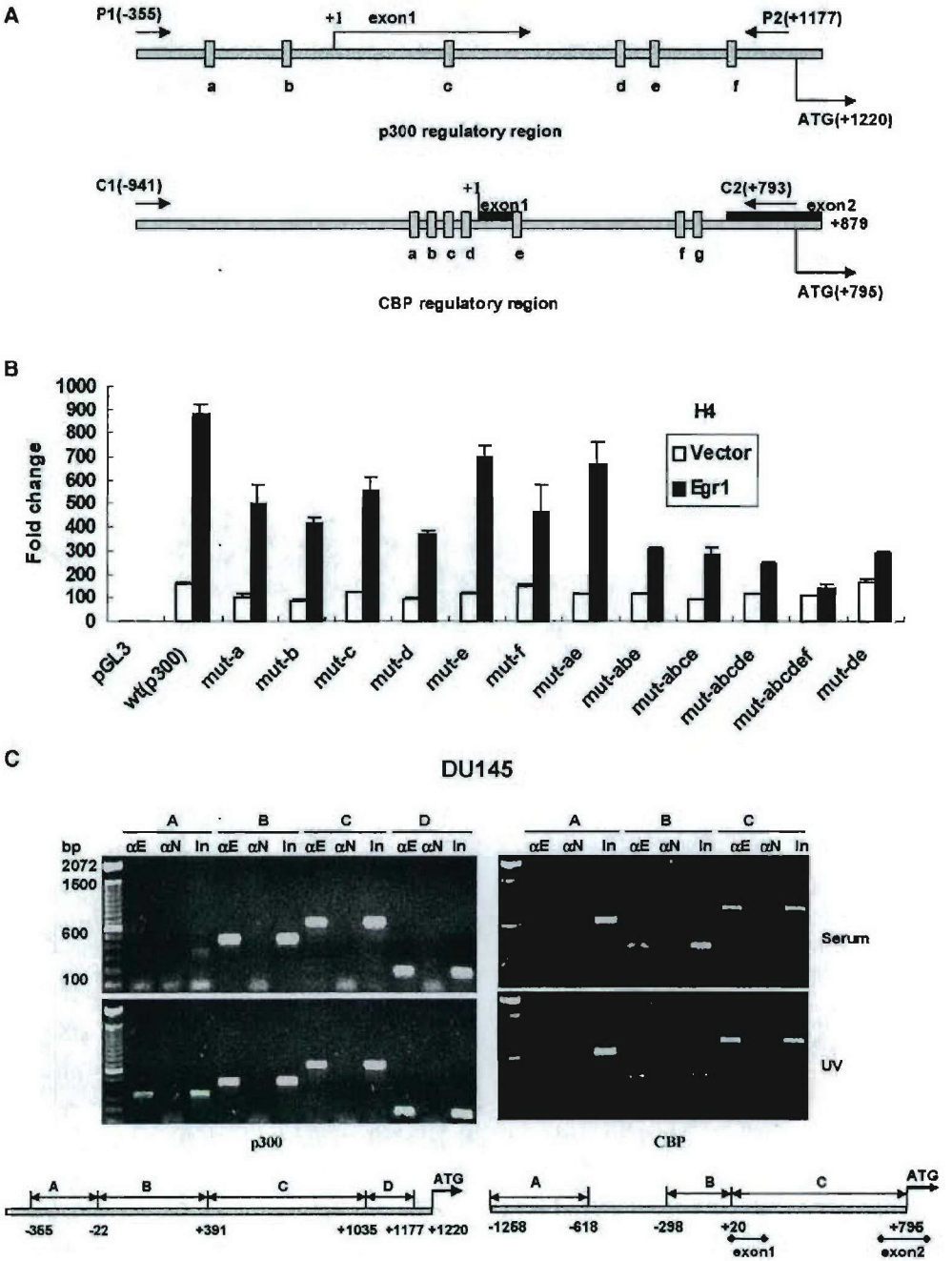

Figure 1. Binding and Regulation of the p300 and CBP Promoters by Egr1

(A) The highest-affinity binding sites for Egr1 in the p300 and CBP regulatory regions are marked a–g. The numbers indicate the position relative to the start of transcription. p300 and CBP regulatory fragments were amplified with primers P1 and P2 and primers C1 and C2 from genomic DNA, respectively.

(B) p300 promoter-luciferase reporter is induced by Egr1 in H4 cells: all binding sites are required for full activity. The constructs used were prepared as described in the Experimental Procedures. H4 cells were extracted 24 hr after transfection for analysis of luciferase expression. Each value represents the mean fold change ± SEM of three independent transfection experiments with three triplicates each.

(C) Conventional ChIP confirmed that Egr1 binds directly to the p300/CBP promoters in DU145 prostate cancer cells. DU145 cells treated with serum for 1 or 2 hr after irradiation with UV-C (40 J/M^2) were chromatin crosslinked and then immunoprecipitated with a specific Egr1 antibody (αE) or a nonimmune control IgG antibody [αN]). Primers spanning four subfragments of the p300 promoter (A–D) and three subfragments of the CBP promoter (A–C) were designed.

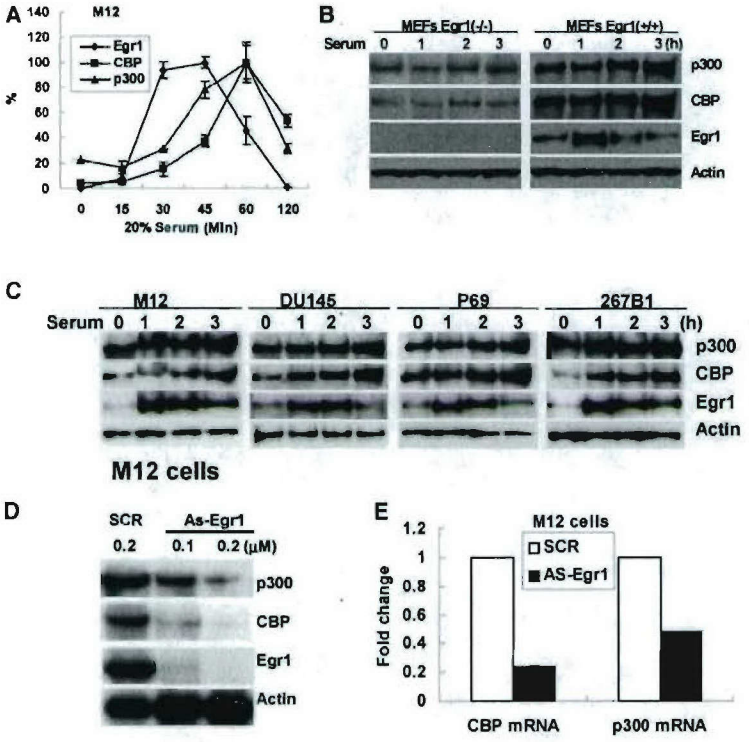

Figure 2. CBP/p300 Are Upregulated by Egr1 in Serum-Induced Prostate Cells and in Wild-Type MEFs but Not in Egr1$^{-/-}$ MEFs or in Antisense-Treated M12 Cells

(A) Total RNA of quiescent M12 cells that had been exposed to 20% serum at the times shown was extracted for analysis of CBP, p300, and Egr1 mRNA levels by quantitative RT-PCR.

(B) Quiescent MEFs Egr1$^{-/-}$ and MEFs Egr1$^{+/+}$ were harvested for analysis by immunoblotting before or after exposure to 20% serum for 1, 2, and 3 hr.

(C) Quiescent cells of M12, P69, DU145, and 267B1 were harvested for analysis by immunoblotting before or after exposure to 20% serum for 1, 2, and 3 hr. Expression of β-actin was monitored as an internal control.

(D) Inhibition of Egr1 expression by antisense Egr1 leads to inhibition of p300/CBP expression in untreated prostate cancer cells. M12 cells were lysed 24 hr after transfection with antisense Egr1 (AS, 0.1 μM or 0.2 μM) or scrambled (SCR, 0.2 μM) oligonucleotides. Western blot results showed that AS-Egr1 largely blocked EGR-1 expression, causing CBP/p300 protein reduction, indicating that CBP/p300 genes are normally upregulated by Egr1.

(E) In M12 cells transfected with 0.2 μM AS-Egr1, the mRNA levels for CBP and p300 decreased 4.1-fold and 2.1-fold, respectively, compared to SCR, consistent with the results of the Western blot.

fold and P69 gave 1.5-fold lower mRNA levels than untreated cells with $p < 0.01$ (Figure 3A).

The results of Western blot analyses in Figure 3B are consistent with the mRNA levels, showing that after irradiation with UV-C at 40 Jm^{-2}, Egr1 was elevated in Egr1$^{(+/+)}$ MEFs and all four prostate cell lines, while CBP and p300 proteins were severely decreased in all five cell lines and levels of the same proteins in Egr1$^{(-/-)}$ MEFs were not affected (Figure 3B). Since the protein levels of p300 and CBP after UV-C irradiation of cells are strongly reduced while Egr1 is elevated in this condition, we concluded that Egr1 appears to be responsible for direct transcriptional downregulation by binding to the promoters of CBP and p300 and inhibiting their transcription rate. As a further test, we applied antisense (AS) Egr1 oligonucleotides to UV-C-irradiated M12 cells to reduce the Egr1 expression level specifically without affecting other gene products. Figure 3C shows the resulting mRNA levels measured by QRT-PCR and protein levels by immunoblotting (Figure 3D) in the M12 cells after irradiation. In this case Egr1 protein levels were significantly reduced (Figure 3D) by the AS oligonucleotide treatment compared with the cells that were treated with scrambled oligonucleotides (SCR). In parallel, the level of CBP and p300 mRNAs and proteins were elevated, indicating that, in the absence of Egr1, both p300 and CBP genes are expressed at higher levels. These results are consistent with UV-C-induced Egr1 causing the transcriptional inhibition of the expression of p300/CBP.

What Is the Mechanism of This Differential Response to Elevated Egr1 Levels?

First, we set about proving that the Egr1 induced by serum and Egr1 induced by UV-C were different in their transactivating properties. Using dual luciferase reporter assays, we demonstrated that serum and exogenous Egr1 induce p300 and CBP promoter-luciferase activity in M12 (Figure 4A), H4, and 293T cells (Supplemental Figures S1B and S1C). Transfection of WT1 strongly decreased luciferase as did the Egr1 dominant-negative construct (WT1/Egr1) (Drummond et al., 1992; Rauscher, 1993) or UV-C irradiation. This result suggests that Egr1 is the agent responsible for upregulating p300 and CBP promoters, and that preventing activated Egr1 from binding blocks this effect.

We and others have shown that Egr1 becomes phosphorylated in activated cells (Cao et al., 1992; Huang and Adamson, 1994), and several kinases have been shown to interact with Egr1. Evidence in the literature suggests that Egr1 is physically associated with casein kinase2 (CK2), and in NIH3T3 cells this results in phosphorylation of Egr1 and accounts for decreased DNA binding and reduced transactivating activities of Egr1 (Jain et al., 1996; Srivastava et al., 1998) although Egr1 still binds to the p300/CBP promoters (Figure 1C). An immunoblot study in M12 cells untreated or treated with Apigenin or DRB (inhibitors of CK2) before UV-C, showing the levels of p300, CBP, Egr1, and actin, was perfectly consistent with a role for CK2 (Figure 4B).Tests on the p300/CBP reporter activities in UV-C-irradiated H4 cells that lack Egr1 expression (Supplemental Figure

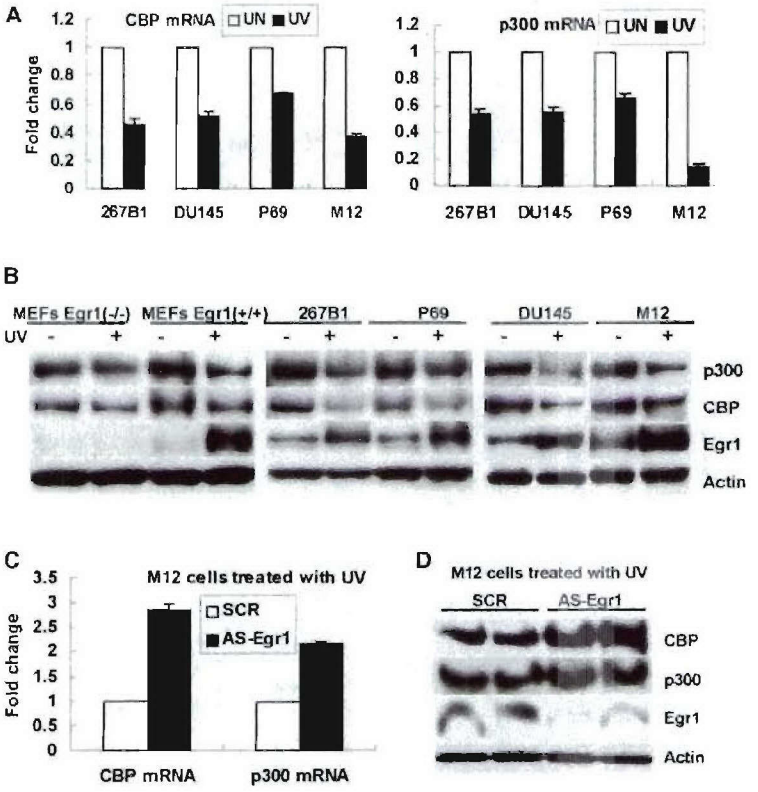

Figure 3. CBP and p300 Are Downregulated by Egr1 in UV-C-Irradiated Prostate Cancer Cells

(A) Analysis of CBP and p300 mRNA levels by QRT-PCR with total RNA extracted from nonstimulated and 2 hr after UV-C (40 Jm^{-2}) -stimulated cells of 267B1, DU145, P69, and M12.

(B) Immunblot analysis for CBP, p300, and Egr1 was performed with protein extracted from nonstimulated and irradiated cells 2 hr after UV-C (40 Jm^{-2}) stimulation of $Egr1^{-/-}$ and $Egr1^{+/+}$ MEFs and four prostate cell lines. Expression of β-actin was monitored as an internal control.

(C) Twenty-four hours after treatment of M12 cells with scrambled (SRC) or antisense (AS) oligonucleotides (0.1 μM), the cells were UV-C irradiated (40 Jm^{-2}); 2 hr later they were collected for QRT-PCR, and (D) 2.5 hr later were collected for protein assays by immunoblotting.

S1D) showed that there was no effect in the absence or presence of H7, an inhibitor of PKA, PKC, and PKG; genistein, an inhibitor of PKA, PKC, and tyrosine kinases; tyrphostin AG1112, also a tyrosine kinase inhibitor; or DRB and apigenin (Ser and Thr phosphokinase inhibitors of CK2). However, transfected exogenous Egr1 induces luciferase activity, and this is reduced after UV-C irradiation. This lower level remains unchanged with the addition of three kinase inhibitors, while CK2 inhibitors restored luciferase expression levels to that achieved by Egr1 alone, suggesting that CK2 plays a role in the inhibitory effect of Egr1 after UV-C treatment. Although nontoxic levels of these inhibitors only partially inhibit kinase activity, the results indicate that CK2 plays a role in the regulation of transcription by Egr1.

To determine whether Egr1 in cells is phosphorylated

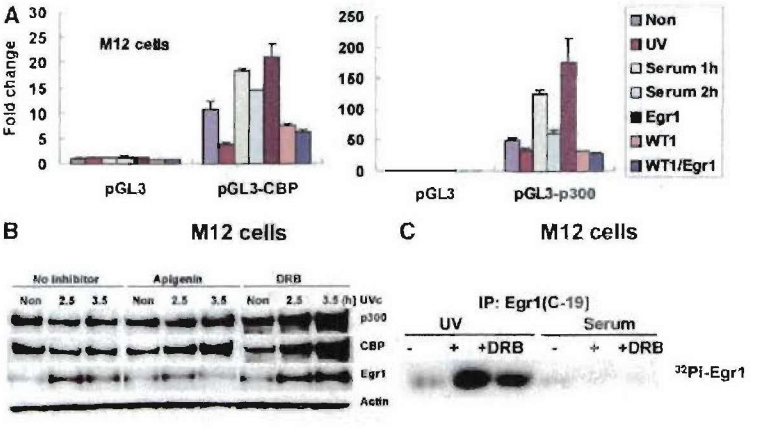

Figure 4. The Activities of CBP and p300 Promoters Are Induced by Egr1 or Serum, and Repressed by Dominant-Negative Egr1 or UV-C: CK2 Plays a Role in the Effects of UV-C

(A) pGL3-CBP and pGL3-p300 were transiently transfected into M12 cells with or without exogenous Egr1, WT1, and WT1/Egr1 (dominant-negative Egr1) expression or with exposure to ultraviolet-C (40 Jm^{-2}) or 20% serum. The activities of CBP and p300 promoters were assayed 2.5 hr later with the Dual-Luciferase Reporter Assay System as fold changes.

(B) M12 cells were treated with the CK2 inhibitors, DRB (50 μM), or Apigenin (50 μM) for 30 min immediately following exposure to UV (40 Jm^{-2}). Untreated irradiated controls and cells treated with inhibitors were analyzed by immunoblotting 2.5 and 3.5 hr later.

(C) M12 cells (1×10^6) cells in three dishes were starved in low serum for 24 hr, and 100 μM DRB (CK2 inhibitor) was added to one dish 30 min before serum was added to 20% followed by [^{32}Pi] for 1 hr (maximal response time). Two further dishes of M12 cells and one control were similarly prepared, 100 μM DRB was added to one dish 30 min before irradiation, and medium was removed briefly for UV-C irradiation at 40 J/M^2. Cells were labeled by addition of 0.5 mCi/ml ^{32}Pi for 2 hr (maximal response time). After labeling, the cells were collected for immunoprecipitation with Egr1-specific antibodies (C-19), followed by SDS-PAGE and autoradiography. The experiment was repeated once more with similar results.

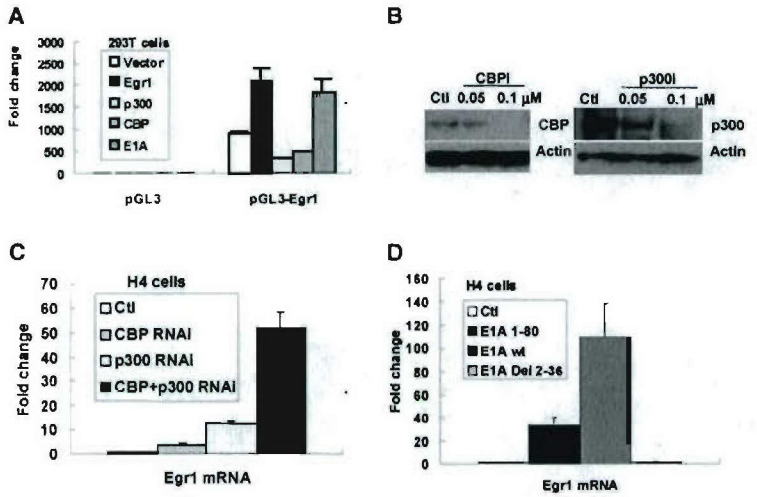

Figure 5. Feedback Regulation of Egr1 Transcription by Egr1 and by CBP/p300

(A) The pGL3-Egr1 luciferase reporter vector was transfected with vector, CBP, p300, or E1A expression plasmids in 293T cells. Twenty-four hours after transfection, luciferase activity of cell lysates was determined.

(B) p300-RNAi and CBP-RNAi were used to efficiently reduce the expression of the two cofactors.

(C) Analysis of Egr1 mRNA levels by QRT-PCR 24 hr after H4 cells were transfected with CBP-RNAi, p300-RNAi, or both RNAi.

(D) Analysis of Egr1 mRNA levels in H4 cells 24 hr after transfection of E1A, with E1A(1–80) to inhibit p300/CBP expression, or with E1A with the p300 binding site deleted (2–36). This shows that Egr1 upregulates its own promoter while p300/CBP downregulates the Egr1 promoter.

differentially according to the stimulus, M12 cells were subjected to radioactive inorganic [^{32}Pi]phosphate labeling after a stimulus of serum or UV-C and in the absence or presence of DRB. CK2 is known to be induced by UV-C in a mechanism involving p38 MAPK (Kato et al., 2003). The immunoprecipitated Egr1 after UV-C irradiation showed that Egr1 was strongly phosphorylated, and this was reduced to one-third by the inhibitor DRB (Figure 4C). In contrast, serum-stimulated cells were weakly phosphorylated in the presence or absence of DRB. We concluded that UV-C irradiation leads to the induction of phosphorylated Egr1 that acts to repress the transcription of p300/CBP.

Feedback Regulation of Egr1 Transcription by Egr1 and by p300/CBP

We noticed that the Egr1 promoter itself has several high-affinity Egr1 binding sites that could self-regulate its transcription. We tested this by using an Egr1 promoter-luciferase reporter construct in 293T cells transfected with Egr1 as well as p300 and CBP expression vectors. Figure 5A shows that Egr1 increased the transactivation of its own promoter 2-fold while p300 and CBP expression decreased Egr1-luciferase transcription also by about 2-fold. In contrast, the transfection of full-length E1A increased Egr1 transactivation, presumably by binding to p300/CBP to prevent the coactivators from inhibiting the Egr1 promoter reporter. This suggestion was further supported by transfecting RNAi against p300 and against CBP into H4 cells where Egr1 is not detectable, to show first that CBP and p300 genes are inhibited in translation (Figure 5B) as well as transcription (Supplemental Figure S2A). Then, we applied the RNAi to H4 cells to find that Egr1 mRNA was increased 3-fold and 12-fold, respectively, by RNAi to CBP or RNAi to p300, and the combination of both RNAi allowed the expression of 50-fold levels of Egr1 mRNA (Figure 5C). Moreover, EIA amino-terminal and full-length E1A vectors transfected into H4 cells increased Egr1 mRNA levels by 40- and 100-fold, respectively, while a dominant-negative E1A was inactive (Figure 5D). Egr1 protein also appeared after E1A transfection although in low amounts in these cells (Supplemental Figure S2B). These results indicate that removal of p300 and CBP allows the expression of Egr1 in cells that were previously unable to support Egr1 expression in response to various stimuli. Thus, a negative feedback loop by p300/CBP could be operating in some or most cell types in order to curtail any long-lasting effects of high Egr1 levels.

Egr1 Can Be Acetylated by p300/CBP In Vivo and Forms a Complex with p300/CBP with Negative Feedback Activity

p300/CBP proteins bind transcription factors (TF) at one of several cysteine- and histidine-rich (C/H) domains to form complexes for transcriptional coactivation. One of the requirements for stabilizing these complexes is the acetylation of the TF at a basic consensus sequence. A potential acetylation site (KDKK) occurs in mouse and human Egr1, close to the third zinc finger, forming the end portion of the basic nuclear localization signal (Matheny et al., 1994). This KDKK motif is very similar to that in p53 (KSKK) and other transcription factors that are acetylated by p300/CBP. We first tested whether Egr1 is acetylated using cells that were treated with Trichostatin A (TSA) to inhibit deacetylases. Culture of M12 prostate cells with TSA for 6 hr increased the effectiveness of the acetyl transferases of the cells so that the proportion of Egr1 that was acetylated was increased as shown using anti-acetylated lysine antibodies (Figure 6A, row 4) compared to the total immunoprecipitated Egr1 (Figure 6A, row 3). TSA at 2 μM had a maximal effect on increasing acetylated Egr1 levels (lane 4). As a result, the expression of p300 decreased in a dose-dependent manner (Figure 6A, top row). The levels of each protein in Figure 6A suggest that deacetylase depletion in the cells results in increased levels of acetylated proteins, as seen for Egr1 (Figure 6A, last row) and that this acetylated form of Egr1 is the species that inhibits p300 expression (Figure 6A, top row).

We then determined whether acetylation of Egr1 occurred at the KDKK site by mutating these residues singly or in combination with a Flag-Egr1 expression construct, after transient transfection into 293T cells. In

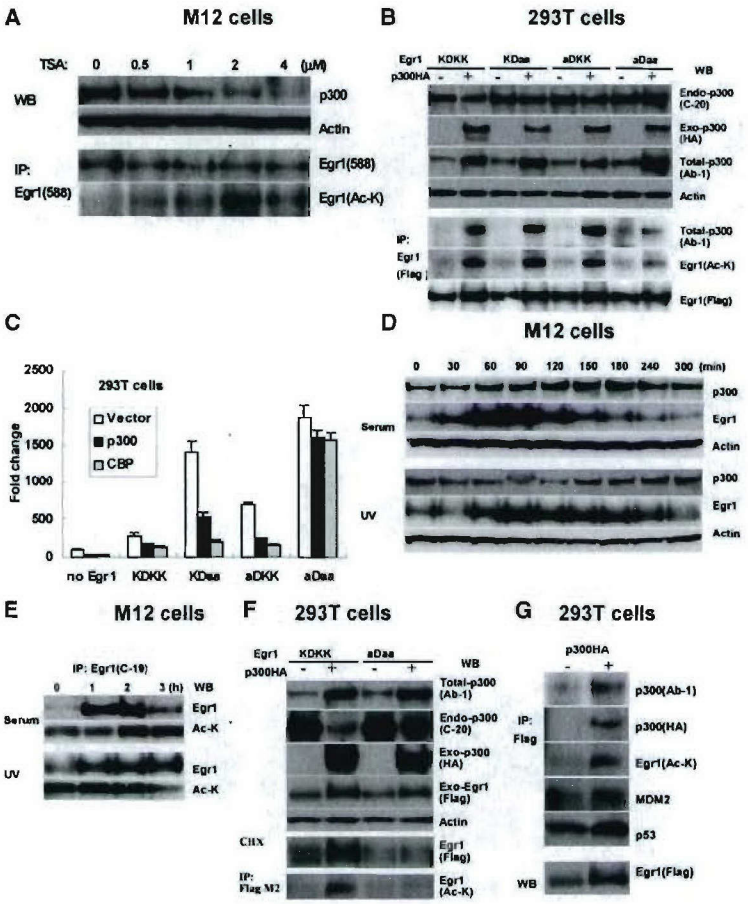

Figure 6. Egr1 Can Be Acetylated by p300 In Vivo, and Acetylated Egr1 Binds to p300

(A) M12 cells treated with increasing concentrations of trichostatin A (TSA) for 6 hr were collected for immunoblotting and immunoprecipitation. TSA (2 μM) leads to maximal acetylation of Egr1, without much effect on total levels.

(B) Transient transfections of Egr1 and its mutAc forms together with exogenous p300 expression in 293T cells show that acetylated Egr1 but not mutAc-Egr1 pulls down associated p300. Cells were collected 24 hr after transfection for analyses.

(C) The pGL3-p300 plasmid was transfected into 293T cells with either of the expression plasmids for wt-Egr1 (KDKK) or mutants of one, two, or three lysines in the acetylation domain, together with vectors expressing nil (cDNA), p300, or CBP as indicated. Cells were analyzed for luciferase activity as fold changes of the mean ± SEM of three independent experiments each in triplicate. Only wt (acetylated)-Egr1 represses transcription of the p300-promoter reporter.

(D) Immunoblot to show the time course of Egr1 and p300 protein expression after serum or UV stimulation of M12 cells.

(E) Immunoprecipitation using anti-Egr1 antibody followed by immunoblot detection of acetylated Egr1 (anti-Ac-K-103) after serum or UV-C stimulation of M12 cells.

(F) Plasmid pCMVβ-p300HA was transfected into 293T cells with either the wild-type KDKK-Egr1 or mutant aDaa-Egr1 expression plasmids. Twenty-four hours after transfection, cells were treated without or with cycloheximide, CHX (200 μg/ml) for 60 min (bottom panel) and lysed for Western blot analysis (upper panel) and immunoprecipitation to show that acetylated Egr1 binds to exogenous p300 much more efficiently than nonacetylated.

(G) The pcDNA-Flag-Egr1 without and with pCMV-β-p300-HA constructs was transfected into 293T cells, and 24 hr later coimmunoprecipitation with anti-Flag was used to pull down the Egr1 together with any complexed proteins. Immunoblots showed that p300 (Ab1), exogenous p300 (HA Ab), acetylated Egr1, MDM2, and p53 were all present in the complex when exogenous p300 was present. The bottom panel indicates that Egr1 remained at higher levels in cells that also contained exogenous p300, suggesting stabilization of the complex.

Figure 6B we used Flag-tagged Egr1 and HA-tagged p300 in order to use a combination of immunoblotting (upper panel) and coimmunoprecipitation (lower panel). Immunoblotting showed that each construct was expressed in addition to endogenous p300, but p300 was best expressed in the presence of the mutAc-Egr1 (KDKK to aDaa) (Figure 6B, row 3, lane 8). The same lysates immunoprecipitated with anti-Flag-tagged Egr1 and coprecipitated p300 (Figure 6B, lower panel) as a complex with wt-Egr1 or its two mutant forms. Only the triple Ac mutant Egr1-aDaa was unable to interact with p300 and was unable to pull down much p300 (Figure 6B, lane 8). But since the immunoblot shows that p300 was expressed best under these circumstances, we concluded that mutant Egr1 was still competent in inducing p300/CBP transactivation because all Egr1 forms were able to stimulate p300 or CBP transactivation compared with nonacetylated Egr1 (compare lower panel, row 2, lane 8, with lanes 2, 4, and 6). The nonacetylated mutant, Egr1-aDaa, was unable to coimmunoprecipitate with p300 while the acetylated forms were capable in this respect. The effect of serum is to induce p300/CBP via Egr1 which is then acetylated and now binds to p300/CBP. In contrast, it appears that phosphorylated Egr1 plays the role of inhibiting transcription of p300 following the UV irradiation of cells (Figures 4A and 4C).

The following results suggest that the complex of Ac-Egr1 with p300/CBP has a different effect on the transcriptional regulation of p300/CBP compared with Ac mutant Egr1. This was tested in 293T cells (which express endogenous wt-Egr1) by transfection of several expression vectors in order to compare the transcriptional effect of exogenous wt-Egr1 versus Ac mutant Egr1, transfected together with p300 or CBP expression vectors on the p300 promoter-luciferase construct. Figure 6C shows that even without extra Egr1 (no Egr1 bars) the expression of p300 or CBP was able to inhibit transactivation of the promoter. Similarly, the presence of wt-Egr1 (KDKK), inhibited transcription in a fashion that suggested that the p300/CBP is acetylating the Egr1 to cause the inhibition. This was supported by the transfection of Ac-single, or double or triple mutant forms of Egr1, the last of which was unable to inhibit

the transactivation of the p300 reporter. This suggests that there is a negative feedback loop on p300 transcription by the Ac-Egr1/p300/CBP complex but not by the nonacetylated Egr1, since this form increases the transcription of p300.

Conditions for the Acetylation of Egr1

We hypothesized that, after serum stimulation, new Egr1 protein is not acetylated at its peak (1 hr) expression level, but only after it has stimulated p300/CBP. When p300/CBP expression is high, it can then inhibit the transactivation of Egr1 as described in the section on feedback inhibition. We tested this using a time course of serum or UV stimulation in M12 cells (Figure 6D). In UV-irradiated cells Egr1 protein levels increase starting at 1 hr and remain high for 3–4 hr. Concomitantly, in UV-irradiated cells p300 levels decrease when Egr1 is at its maximal. Therefore, Egr1 might be unacetylated in UV-irradiated cells but be rapidly phosphorylated (Figure 4C) and hence become less able to transactivate (Jain et al., 1996). Immunoprecipitation of Egr1 in these samples shows that acetylated Egr1 is highest in serum-stimulated cells after 2 hr (Figure 6E, row 2) which is in temporal agreement with the highest levels of p300 in Figure 6D. In untreated cells, the initial levels of acetylated Egr1 are moderately high and decrease only after 3 hr in UV-C-treated cells (Figure 6E, lane 4) in accord with the results of Figure 6D. The untreated cells have high levels of acetylated Egr1, suggesting that p300 must keep acetylation high until UV irradiation causes Egr1 elevation, followed by reduction of p300 expression to low levels as Ac-Egr1 inhibits transcription. Thus, we conclude that acetylated Egr1 is stable or actively maintained at high levels in serum-stimulated cells, while a genotoxic stimulus causes a net gain of phosphorylated Egr1 (Figure 4C) that has an inhibitory effect on the transcription of p300 (Figures 4A and 4C).

Further Test of Egr1 Stabilization by Acetylation

We have shown that, in addition to acetylation of Egr1 by p300 or CBP, a complex of the two proteins may allow both to become more stable and more active. To test the conditions for Egr1 stability, 293T cells were transfected with Flag-tagged wt-Egr1 or mutAc-Egr1, with or without the addition of HA-tagged p300. All were well expressed as shown in the immunoblot in Figure 6F. When the cells were immunoprecipitated with anti-Flag, the immune complex contained acetylated Egr1 when wt-Egr1 was present in the cells (Figure 6F, lane 2, row 7). No mutAc-Egr1 was seen in the complex (lane 4, row 7). A sample was also treated with cycloheximide (CHX) to inhibit protein synthesis and allow the degradation of short-lived proteins. As a result (Figure 6F, row 6), only the Egr1 that had been acetylated (lane 2) was substantially preserved 1 hr after CHX addition and was even more stable when in the presence of excess p300. This is a direct demonstration of the improved stability of acetylated Egr1 when complexed with p300.

In the stabilized complex of p300 with Ac-Egr1, there may be other factors that affect transcription, such as p53; however, p53 is inactivated in 293T cells. We tested coimmunoprecipitated proteins using anti-Flag-Egr1 for other proteins that may also bind specifically to Egr1 or to the Ac-Egr1/p300 complex. Figure 6G shows that, while only acetylated-Egr1 binds well to p300, both MDM2 and p53 are also able to bind the Ac-Egr1 complex as well as non-Ac Egr1 in the absence of p300 (Figure 6G, lane 1, rows 4 and 5). It is possible that the binding of MDM2 to non-Ac-Egr1 is indirect through binding to p53 which is known to bind to Egr1 (Liu et al., 2001).

Nonacetylated/Phosphorylated Egr1 Is More Active in Regulating Genes toward Apoptosis while Acetylated Egr1 Is Required for the Regulation of Growth and Survival Genes

Starved cells in culture normally respond to 20% serum by stimulation of Egr1 followed by upregulation of its target genes leading to growth and survival (Baron et al., 2003a, 2003b; Virolle et al., 2003). In contrast, cells that are UV-C irradiated (40 JM^{-2}) induce Egr1, which leads to growth arrest, repair of DNA damage, and/or apoptosis (Virolle et al., 2001). We therefore studied eight known or suspected Egr1 target genes to determine whether highly acetylated Egr1 produced a different result compared with underacetylated Egr1 (UV-C). Target gene activity was measured by quantitative RT-PCR in M12 prostate cancer cells. Figure 7A shows that serum strongly induced the expression of FGF2, IGF2, and PDGFB, and was less active in inducing TGFβ1, BCL2, and NFκB (p50 and p65) while p73 was inhibited. The stimulus of UV-C, on the other hand reduced the expression of FGF2, IGF2, NFκB, and BCL2, while increasing the expression of PDGFB and p73 with a small effect on TGFβ1. This effect could be produced by underacetylated Egr1 or by phosphorylated Egr1. To demonstrate this point in a different way, a transient expression study was used in H4 cells by measuring the mRNA levels of the same genes after transfection with wt-Egr1 or mutAc-Egr1 with or without exogenous p300. The results in Figure 7B show that, for the seven target genes which wt-Egr1 induces (FGF2, IGF2, PDGFB, TGFβ1, NFκB p50 and p60, and Bcl2α), the non-Ac-Egr1 had little effect on five, in accord with the previous conclusion that serum-stimulated genes require acetylated-Egr1 for transactivation. For genes p53 and p73, mRNA levels were increased only by nonacetylated-Egr1 as seen in UV-C-induced cells. The nonacetylated form predominates in UV-C-treated cells, and upregulated p53 and p73 then results in an increase in susceptibility to apoptosis.

Discussion

This paper demonstrates that the transcription factor Egr1 is a key component in the early responses to environmental signals that control a large group of transcription factors through the regulation of the coactivators p300 and CBP (Figure 1). We have focused on the response to serum after serum starvation and the response to UV-C because the effects of Egr1 appeared to be diametrically opposed. Whereas serum-induced Egr1 caused upregulation of p300/CBP (Figure 2), UV irradiation led to downregulation of the promoter reporter construct (Figures 3 and 4). This was a surprising result, and we sought to determine the mechanism. As

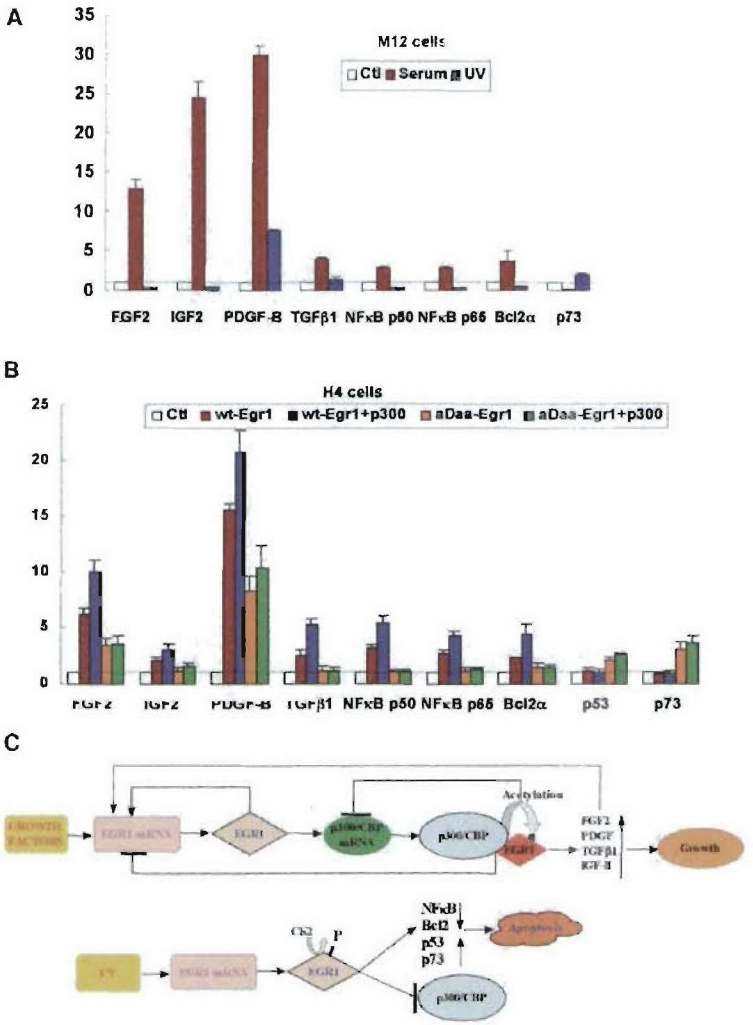

Figure 7. Responses of Egr1 Target Genes Differ in Serum-Stimulated Compared to UV-C-Stimulated Prostate Cells and According to the Level of Acetylated Egr1

(A) Total RNAs from normal growing M12 cells, quiescent cells exposed to 20% serum for 2 hr or 2.5 hr after irradiation with UV-C at 40 Jm $^{-2}$ were extracted for analysis of Egr1 target gene mRNA levels by quantitative RT-PCR.

(B) Transient transfection of Egr1 and its mutAc forms together with or without p300 expression vectors in H4 cells were analyzed 24 hr later. Total RNAs were extracted for analysis of Egr1 target gene mRNA levels by quantitative RT-PCR. Each value represents the mean ± SEM of three triplicates. p300 synergized with wt-Egr1 in stimulating seven of nine growth-related genes tested, but the nonacetylatable mutant Egr1 was inactive in five of the seven.

(C) The schematic diagram shows the interactions between Egr1 and p300/CBP. Two positive and two negative feedback loops were documented here.

a result, we found that many more complicating events occurred during the responses of cells to these extracellular stimuli. For example, a signal to upregulate Egr1 expression could be self-sustained by positive feedback of Egr1 protein on its own transcription (Figure 5A). In contrast, after the transactivation of the p300/CBP promoters by Egr1, a negative feedback of p300/CBP on Egr1 transactivation occurs (Figures 5C and 5D), and also a negative feedback loop operated by acetylated Egr1 or by its complex with p300/CBP on the transcription of p300/CBP occurs (Figures 6A–6E and the scheme is summarized in Figure 7C). When UV-C is the stimulus, however, Egr1 is induced as a phosphorylated entity (Figure 4C), and this form is repressive to transcriptional activity of the promoters of p300/CBP (Figures 4A and 4B). We cannot exclude the possibility that the ratio of acetylated to non-Ac-Egr1 or to phosphorylated Egr1 is the important factor for its choice of activities. Reduced levels of p300/CBP exclude the acetylation of Egr1 or reduce its level compared to levels of phosphorylated Egr1. The biological result of the serum-induced pathway is that acetylated Egr1/p300/CBP complexes activate growth and survival, while UV-C irradiation leads to gene regulations that slow growth or lead to apoptosis. This explains in part how dual roles of Egr1 are observed (Jean et al., 2001; Quinones et al., 2003) in cellular responses and physiology.

In summary, Egr1 responds to both genotoxic stress and to growth stimuli, with negative and positive actions on growth, respectively. The last attribute makes this transactivator different from its model, p53. Its modes of action and its controls are similar to p53, so much so that the activities of Egr1 could be seen as a general factotum or surrogate to p53 when the master tumor suppressor is disabled (Calogero et al., 2001). Moreover, p53 cannot function in the absence of Egr1 in mouse embryo fibroblasts (Krones-Herzig et al., 2003). It may be important to cancer control that the *EGR1* gene remains intact for the most part in cancer cells, and very few mutations have been described. Its importance to tumor suppression is clear for breast (Huang et al., 1997), fibrosarcoma (Huang et al., 1998b), brain (Calogero et al., 2001), and lung (Levin et al., 1995) tumors because in these tissues Egr1 is poorly expressed and acts as a growth and transformation suppressor when overexpressed. In contrast, in prostate cancer, Egr1 is an im-

portant component in the transformation and progression of the disease by its constitutive expression. This major difference is likely highlighted by the fact that there are even more ways to regulate the activities of Egr1 posttranscriptionally than p53, such that mutation of the gene is not under pressure. This fact enlarges the importance of Egr1 in its myriad of roles and the variety of regulatory measures to modulate any large changes in its expression. In prostate cancer, a positive feedback loop from Egr1 and from growth factor products on the Egr1 promoter occurs to provide constitutive growth. This provides a high constitutive level of acetylated Egr1 which is more stable. Whether the transactivating and other properties of the stable acetylated form are different remains to be determined, but it does affect its choice of target genes (Figure 7).

Prostate cancer tissue has high levels of p300/CBP proteins, and the levels increase with the Gleason score (Debes et al., 2003) as is the case for Egr1 (Abdulkadir et al., 2001; Baron et al., 2003a; Eid et al., 1998). High p300 levels are associated with prostate cancer growth and is a predictor of progression (Debes et al., 2003). This overexpression can now be explained by transcriptional activation of p300 by Egr1 and is sustained by growth factor targets of Egr1 and positive feedback. For practical applications, in prostate cancer the reduction of Egr1 might be effective in clinical treatments because this will reduce growth factor induction and positive feedback. Low Egr1 would still be inducible by irradiation of all kinds just as it is in breast cancer cells, and this would lead to repression of p300/CBP and apoptosis by the activation of Egr1 target genes that are induced after stress. These include FASL, PTEN, and TNFα—genes that are proapoptotic when activated. However, the number of countermeasures that the cells provide to regulate Egr1 make prediction of a general therapy regime difficult, all the more so because of its differential effects on different tissues. Further animal studies (Baron et al., 2003a) will help to decide whether antisense Egr1 might be an effective treatment for prostate cancer perhaps in an alternating combination with irradiation.

Experimental Procedures

Further details are given in the Supplemental Data at http://www.molecule.org/cgi/content/full/15/1/83/DC1.

Cells, Serum Stimulation, and UV Irradiation

Prostate cells 267B1, P69, M12, and DU145, 293T cells, HT1080 cells (clone H4) (Frisch, 1994), MEFs $Egr1^{(-/-)}$, and $Egr1^{(+/+)}$ cells were cultured as described in the Supplemental Data (Bae et al., 1998; de Belle et al., 2000; Jackson-Cook et al., 1996).

Antibodies and Reagents

Egr1 (C-19), Egr1 (588), p300 (C-20), CBP (A-22), pan-Acetyl (C2), and p53 (FL-393) rabbit polyclonal IgG antibodies, and p53 (Do-1) were mouse monoclonal IgG_{2a} antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). MDM2 (Ab-1), p73 (Ab-2), and p300 (Ab-1) monoclonal mouse IgG antibodies were from Oncogene Research Products (San Diego, CA). Acetylated-lysine polyclonal antibody and acetylated-lysine monoclonal antibody were from Cell Signaling Technology (Beverly, MA). Anti-β-actin, anti-Flag M2 monoclonal antibodies, and anti-Flag M2 agarose affinity gel were from Sigma (St. Louis, MO). Anti-HA (mouse IgG) was from Roche Applied Science (Indianapolis, IN). Reagents including trichostatin, cycloheximide, genestein, tyrphostin-AG1112, H-7 dihydrochloride, apigenin and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) were from Sigma (St. Louis, MO).

Plasmids

Three genomic DNA fragments with sizes of 1533, 1735, and 2250 bp corresponding to the promoter and its 5′-upstream regulatory sequences of p300, CBP, and Egr1, respectively, were used to create pGL3-p300, pGL3-CBP, pGL3-Egr1, pGL3-p300, and pGL3-CBP mutation constructs (confirmed by sequencing). An EcoRV-SmaI full-length, flag-tagged Egr1 from pCMVFLAG-Egr1 was inserted into the EcoRV site in pcDNA3 to generate pcDNA3-Egr1. Acetylation site mutations of (KDKK) of Egr1 expressing constructs were made as described in the Supplemental Data.

Western Blot and Immunoprecipitation

For Western blots, cells were lysed in ice-cold RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and complete protease inhibitor cocktail tablet [Roche Pharmaceuticals, Nutley, NJ]) to prepare whole-cell lysates. Immunoprecipitation (IP) and Co-IP were performed with 300 to ~500 μg lysates prepared using modified RIPA buffer (50 mM Tris-HCl [pH7.4], 150 mM NaCl, 1% NP-40, and complete protease inhibitor cocktail tablet [see the Supplemental Data]).

Chromatin Immunoprecipitation

ChIP was performed as described previously (de Belle et al., 2000) with modifications. Egr1-chromatin was immnunoprecipitated using Egr1 (Santa Cruz Biotechnology, 588) antibody.

DNA Transfection

H4 and 293T cells were used where high transfection efficiency was required. H4 cells have the advantage of being Egr1 protein negative, while 293T cells express Egr1 at a low level. H4 and 293T cells were seeded into 60 mm dishes at density of 2–3 × 10^5 and 8–10 × 10^5 cells 1 day before transfection in order to achieve 75%–95% confluence. The transfection was performed with the Lipofectamine 2000 (Invitrogen) following the instructions provided by the manufacturer in a final volume of 2.5 ml DMEM medium with 10% FBS without antibiotics. Typically, 2 μg of total plasmid DNA (1 μg of pcDNA3-Egr1 and 1 μg of pCMVβ-p300-vectors) was mixed with 6 μl of Lipofectamine. Cells were collected 24 hr after transfection for immunoblotting (IB) and immunoprecipitation.

Antisense Oligonucleotide Transfection

Egr1 phosphorothioate antisense oligonucleotide 5′-AGCGGCCAG TATAGGTGA-3′ and scrambled oligonucleotide 5′-TTCTTGCATCT GTCA-3′. This antisense reagent was shown to reduce Egr1 expression simultaneously with prostate cell growth and transformed character in vitro and in vivo (Virolle et al., 2003; Baron et al., 2003). M12 cells were seeded into 60 mm dishes at a density of 8 × 10^5 cells 1 day before transfection in order to achieve 75%–95% confluence. The transfection was performed with Lipofectamine 2000 (Invitrogen) following the instructions provided by the manufacturer, in a final volume of 2 ml RPMI medium without additives, for 6 hr at 37°C under 5% CO_2. Cells were washed once and maintained in a complete medium until the experiment was started.

RNA Interference Experiments

In order to "knock down" CBP/p300 expression, siRNAs were prepared with Silencer siRNA Cocktail Kit (RNase III; Ambion Inc., Austin, TX) according to the instruction manual. For more details see the Supplemental Data.

LightCycler Quantitative (Q)RT-PCR

Specific primers for cyclophilin A, CBP, p300, Bcl2a, Egr1, FGF2, IGF2, PDGFB, TGFβ1. NFκB (p50 and p65), p53, and p73 were designed as given in the Supplemental Data. Total RNAs were purified using TRIzol reagent (Invitrogen) and RNeasy Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's protocol. LightCycler QRT-PCR was performed on the LightCycler Instrument (Roche), with ~500 ng of total RNA as template using the LightCycler

RNA Amplification Kit SYBR Green I (Roche) according to the manufacturer's instructions.

Luciferase Assays

2–3 × 10^3 H4 cells, 5–8 × 10^3 293T cells, and 8–10 × 10^3 M12 cells were inoculated 1 day before transfection was performed with the Lipofectamine 2000 (Invitrogen). Luciferase activities were assessed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and an EG&G Berthold LB96P Luminometer (PE Biosystem, Wellesley, MA). The control Renilla luciferase vector was modified by replacement of the CMV promoter with the SV40 promoter to remove Egr1 binding sites.

Radioactive Inorganic Phosphate Labeling

M12 cells (1 × 10^6) cells in three 60 mm dishes were starved for 1 day in RPMI medium with 0.5% bovine serum. One hundred micromolars DRB was added to one dish 30 min before serum was added to 20% followed by [^{32}Pi] (0.5 mCi per dish) for 1 hr when the samples were harvested. Three dishes were similarly prepared with 100 μM DRB added to one dish 30 min before UV treatment, and medium was removed briefly for UV-C irradiation of 2 dishes at 40J/M^2. Cells were labeled by addition of 0.5 mCi/ml ^{32}Pi for 2 hr for maximal Egr1 expression. After labeling, the cells were collected for immunoprecipitation with Egr1-specific antibodies (C-19), followed by SDS-PAGE and autoradiography.

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Transcriptional interdependence between p73, Egr1 and p53

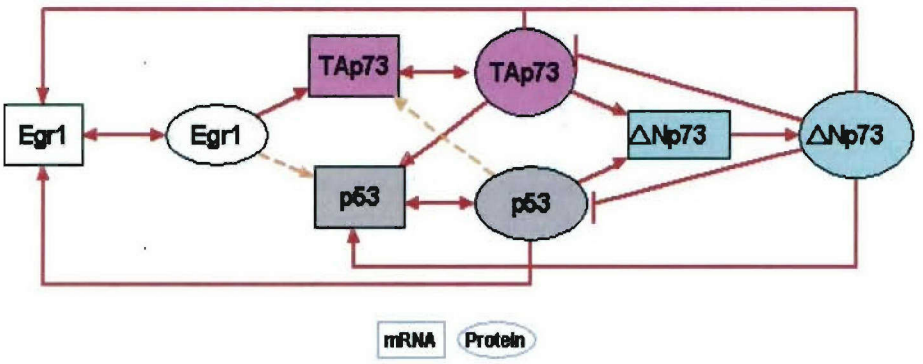
Jianxiu Yu and Eileen D. Adamson*

The Burnham Institute, Cancer Research Center, La Jolla, CA 92037

Key Words: Early Growth Response gene; luciferase reporters; transcriptional regulation; mutational analysis; p53 mutants, chromatin immunoprecipitation; feed-back loops; UV-C; etoposide; apoptosis
Running title: p73, Egr1 and p53 interdependence

*To whom correspondence should be addressed at
The Burnham Institute,
10901 N. Torrey Pines Rd,
La Jolla, CA 92037
Tel 858-646-3137
FAX 858-646-3198
eadamson@burnham.org

Abstract
TP53 is often mutated in cancer, but *TP73* only rarely, and is known to regulate a subset of p53 target genes that cause cells to respond to stress by growth arrest and apoptosis. p73, however, produces two main forms; only TAp73 reiterates the roles of p53, while ΔNp73 has a distinct set of oncogenic properties. The P1 promoter of the *TP73* gene has five distinct Egr1 binding sites; all five contribute to the up-regulation of TAp73 by Egr1 in several cell types. In contrast, the P2 promoter regulating ΔNp73 is not induced by Egr1 but *is* induced by TAp73, and p53. Mutational analysis confirmed these results. We show that stress induces mainly TAp73 via Egr1, but newly discovered active p53 binding sites in p73, p53 and Egr1 promoters reveal an auto-regulating network that controls complex stress responses of the four genes at the transcriptional level. (145 words)



Summary Figure (provided for readers}

Introduction

3786 words

Stress response includes growth arrest and DNA repair and may be followed by apoptosis. To provide this responsiveness, the most important gene is *TP53* which acts as a tumor suppressor gene in most cell types. However, *TP53* is frequently mutated and its protein product, p53, inactive in cancer cells. Two possibilities can prevail; mutated p53 activates a different set of target genes resulting in non-apoptotic responses[1,2]; or, the important function of stress response falls on the related genes *TP73*[3] and *TP63* that have similar features as *TP53* but with some distinct differences, for example, *TP73* and *TP63* genes are infrequently mutated in cancers. There are large differences in the structures of the *TP73* and *TP63* genes compared with *TP53*, particularly their promoters and the existence of multiple splicing of exons that each lead to the production of 17 different proteins from two different promoters for both *TP73* and *TP63* (see reviews[4-7]).

The P1 promoter of p73, produces a set of transcription-activating forms named TAp73. Promoter P2, produces ΔNp73 forms, that were thought to have mainly non-transactivating (TA) roles by splicing out of the TAD sequences in exon 2 and 3. However, this gene product now appears to have both anti-apoptotic activities[8-10]and pro-apoptotic activities[11,12]. In the early reports, it was noted that ΔNp73 protein acts as a dominant negative of p53 action on its target genes[13]. The predominant mechanism for this effect is the oligomerization of the ΔNp73 subunits with p53 or TAp73 subunits, such that the complex is no longer correctly structured for binding to the usual pro-apoptotic target genes.

The Egr1 promoter is marked by its responsiveness to numerous environmental and internal stress stimuli, by rapid and transient up-regulation. Therefore any gene that is regulated by Egr1 will also be stress responsive. It was reported earlier that Egr1 can up-regulate the transcription of the p53 promoter to induce the apoptosis of cancer cells[14] but we find that this up-regulation is a very small effect. We describe here how the Egr1, p53 and p73 promoters respond to Egr1, p53, TAp73 and ΔNp73 proteins to provide insight to the complex interactions between these four genes. The versatility of these inter-modulating genes permits a wide range of graded responses of cells to stresses such as to radiation and chemotherapy drugs, a finding that imparts great importance to planning in all therapeutic endeavors.

Results

Egr1 up-regulates the transcription of the TAp73 but not the ΔNp73 promoter

We cloned the *TP73* P1 promoter as described in the methods section, as pGL3-TAp73. The P1 promoter contains sequences that suggest that there are five high affinity Egr1 binding sites (EBS); a, b, c, d and e (See Supplementary Fig. S1 on line). Promoter P2 was cloned into pGL3 to serve as a reporter gene for ΔNp73 isoforms and has a single Egr1 binding site (EBS f). Transfection of pGL3-TAp73 into HEK293T, or into H4 cells together with expression plasmids for Egr1 and related genes, showed that wtEgr1 transactivated the promoters by 4-12-fold compared with the empty expression vector (pcDNA) (Fig. 1a). As a negative control the dominant negative Egr1 vector, WT1/Egr1 as well as WT1 (the Egr1-related zinc-finger Wilm's tumor protein 1 that binds to the same EBS sequences) had little effect on the promoter activity. In contrast, for the P2 promoter there was no significant difference in the luciferase activity of the reporter gene by the transfection of pcDNA-Egr1 versus the empty vector. The P2 promoter expressed from pGL3-ΔNp73 was not activated by Egr1. We confirmed this by mutating the putative EBS in P2 (Fig 1b, right) and then we tested each of the five EBS in P1 by individual mutation and in groups (Fig. 1b, left). The results demonstrated that each of the five mutated Egr EBS reduced the promoter activity by approximately the same amount, averaging 4-fold. Only mutation of all 5 sites reduced the activity of the reporter gene by 85%. It appears that there is some still some small residual activity in P1 even when all EBS were mutated. The analysis clearly shows that Egr1 up-

regulates the P1 promoter and also suggests that Egr1 may play a role in graded responses of *TAp73* governed by the level of Egr1 that pertains in the cell.

We used chromatin immunoprecipitation (ChIP) to confirm that Egr1 was directly bound to the putative promoter sites during transactivation. The EBS are grouped in three regions, A, B and C of the P1 promoter as indicated in Supplementary Fig. S1. Therefore primer oligonucleotides that flank the DNA of each of the three fragments were used to amplify the DNA captured by anti-Egr1 antibodies during ChIP. The antibodies were applied to sonicated fragmented chromatin derived from M12 prostate cancer cells treated with etoposide for 2.5 h to induce Egr1. The DNA captured by antibodies to Egr1 was purified and compared by PCR analyses with DNA captured from non-immune IgG as a negative control, and also compared with input DNA purified from the cells as a positive control. The results (Fig. 1c) confirmed that Egr1 was bound to all three DNA fragments A, B, and C but not to D, which is the inactive EBS in P2. As a positive control, ChIP using anti-p300 gave a band as previously reported[15]. In summary, Egr1 binds to and transactivates the *TAp73* but not the *ΔNp73* promoter.

Promoter up-regulation by Egr1 is translated into p73 protein products

Since p73 occurs in many isoforms, we wanted to know which proteins are accumulated, as a result of promoter transactivation by Egr1. In H4 cells, that do not express Egr1 in comparison with the H4-derived E9 clone that expresses constitutive Egr1[16] the results of immunoblotting (Fig. 2a) indicated that high levels of Egr1 in E9 cells correlates with a three-fold greater expression of TAp73γ, with little or no effect on the α or β isoforms or the ΔNp73 isoforms. In 293T cells, 267B1, M12 and U2OS cell lines treated with UV irradiation to induce Egr1 (Fig 2b), all three isoforms (α, β and γ) were induced in the irradiated cells. For normal prostate cells, 267B1, the induction was much smaller and in M12 metastatic prostate cells, only the p73γ form was strongly upregulated. Clearly, the p73 proteins are upregulated when Egr1 is induced but the isoforms and levels are quite variable depending on the cell line. Etoposide was next used to engender a stress response, during which Egr1 is induced. We show (Fig.2c) that in 293T, M12 and U2OS cells, Egr1 protein remains elevated for 2-4h after treatment and consistent with induction by Egr1, p73α was also elevated. Although wtp53 is also known to be induced by DNA damaging stimuli, 293T, 267B1 and M12 express inactive p53; only U2OS expresses wt-p53. The same three cell lines were induced by exogenous Egr1 to demonstrate the induction of p73α protein in two prostate lines and p73β in U2OS, with little effect on the level of p53 protein (Fig. 2d). To verify the role of Egr1 further, we used Egr1-RNAi to reduce the Egr1 expression level in M12 cells. Etoposide-treated cells expressing Egr1-RNAi eradicated the induced levels of p73α in comparison with control samples (Fig. 2e). These results indicate that Egr1 is required for the endogenous induction of p73 after genotoxic stress.

The p73 P1 and P2 promoters respond differentially to the expression of p53 and p73 family members

We tested a new putative p53 responsive element (p53RE, see Supplementary Fig. S1) in the P1 promoter using pGL3-TAp73 and pGL3-ΔNp73 reporters combined with vectors to transiently express p53, TAp73α, βor ΔNp73α, β in 293T cells. Simultaneous tests of the known p53RE in the P2 promoter driving ΔNp73, show that the latter is far more responsive to p53 than the P1 promoter and that TAp73α and β also strongly induce the single p53RE in P2, while ΔNp73 expression has little or no effect (Fig. 3a). When examined on an expanded scale (Fig. 3b), TAp73α induction of the P1 promoter was shown to be 3-fold more than the vector, or ΔNp73αor β, but p53 also induced weakly, with a probability of difference of p <0.0038. The single p53RE in the P1 promoter was mutated (GL3TAp73REm) to confirm that it is a responsive site, with the result that both TAp73α and β induced transactivation but only when the p53RE was intact since the activity was reduced 4-fold after mutation (Fig. 3b right). ChIP studies with two different antibodies confirmed that the p53RE-

containing portions of P1 and P2 promoters of etoposide-treated U2OS cells bind to the drug-induced proteins p53, TAp73 and ΔNp73 (Fig. 3c). In contrast, the non-immune IgG gave no DNA band. We concluded that several p53 family proteins bind directly to the putative p53REs to effect and perpetuate transactivation within the family. This is further sustained by Egr1 as described next.

The Egr1 promoter is up-regulated by Egr1, p53, TAp73 and ΔNp73

The Egr1 promoter itself is known to have active Egr1 binding sites[15]. We noted recently that there are four possible p53 responsive elements (p53RE A1 to A4) in the non-coding sequences from –2kb to +142 of the promoter and 5'UTR (see Supplementary Fig. S2). A promoter construct in pGL3 termed "Egr1" contains all four p53 sites while "Egr1F" contains some flanking sequences in addition. Fragments of the promoter named Egr1P1, Egr1P2 and Egr1P3 (containing 3, 3 and 2 p53RE respectively) were cloned into pGL3 and co-transfected into 293T cells with either p53, TAp73 or ΔNp73 as inducers. All promoters were activated similarly (2-3-fold) by the TAp73 and ΔNp73, with p53 about half as active (Fig. 4a). This result indicates that **all** members of the p53 family have some inducing effect on the Egr1 promoter but to different degrees. The most effective construct was Egr1P3 that contains only the A2 and A3 p53RE. A similar study in H4 cells that do not express Egr1 (Fig. 4b) confirmed that the TAp73α protein was more efficient than ΔNp73α and p53 in inducing up-regulation of the Egr1 promoter.

To determine whether transactivation results in elevated Egr1 protein levels, we immunoblotted extracts of M12 prostate cancer cells (inactive p53) and MCF7 breast cancer cells (wtp53), after transfection of the expression vectors, HA-tagged ΔNp73α, TAp73α or p53. There was an increase of 1.49 to 2.2-fold Egr1 protein 24 h later in both cell types (for MCF7, see Suppl. Fig. S4), indicating that transactivation by all three proteins was followed by translation of Egr1 (Fig. 4c). This effect keeps Egr1 protein levels high and this in turn replenishes TAp73 and ΔNp73 levels and so on. We confirmed the transcriptional upregulation of the Egr1 promoter by showing that p53, TAp73 and ΔNp73 proteins are specifically bound to the promoter, by ChIP of U2OS cells. Of the four putative p53RE, only p53A2 and p53A3 sites bound both proteins (Fig. 4d), revealing that only two sites are directly involved in transcriptional activation. We therefore mutated these two active p53RE and tested for Egr1 transcriptional activity as pGL3-Egr1 luciferase reporter genes (Fig. 4e). Co-transfection with plasmids expressing p53, or TAp73, or ΔNp73 gave reduced transactivation levels from p53RE-A2m and A3m compared to the normal reporter. p53RE-A3 was the most affected by mutation, reducing the original total activity by 6-8-fold for all four p73 isoforms tested. However, both p53RE A2 and A3 in the pGL3-Egr1 promoter are effective because after both sites were mutated there was little transactivation, consistent with the results of Fig. 4a and 4b. These results proved that two new p53RE are responsive to all members of the p53-family of proteins, thus producing Egr1 protein to continue the cycle.

We next tested deleted forms of expressed p73 proteins for their ability to induce the Egr1 promoter, since the p73 TAD domain is known to play roles in transactivation, for example, the HSP promoter[17], TAp73α and ΔNp73α each induced Egr1 transcription by 2-3-fold even though ΔNp73α lacks a recognizable transactivating domain (Fig 4f, left panel). However, the amino-terminal domains of both exogenously expressed TAp73 and ΔNp73 proteins are required, because TAp73Δ1-62 (ΔTAD) and Δ1-127 (ΔTAD+ΔPXXP domains) expression failed to transactivate the Egr1 promoter. Deletion of the unique 13 amino acid amino-terminal of ΔNp73 also showed its requirement for the transactivation of the Egr1 promoter and therefore acts as a TAD. Deletion of the PXXP motifs in the ΔNp73Δ1-78 fragment made no further difference, indicating that the 13 aa-sequence is the crucial requirement for the transactivation of p53RE in the Egr1 promoter. Interestingly, wtp53 was only able to induce the Egr1 promoter 1.4-fold (Fig. 4f, right panel) and mutant p53V143A lost all activity. However, mutants p53R175H, p53H179E, p53R249S and p53R273H had the similar activity as wtp53

in up-regulating the Egr1 promoter even though these mutations are in the DNA binding domain of p53. Some mutant forms of p53 have been reported to have a much improved activating effect on the Egr1 promoter [1] but under the conditions that we used here, up-regulation was less than 2-fold. Therefore, not only wt p53 but also some mutant forms of p53 are capable of continuing the transcriptional feedback loop.

p53 is transcriptionally up-regulated by members of the p53 family

We tested the DNA sequence between –764 to +229 of human *TP53* for transactivation when expressed as a pGL3-p53 construct (for the structure, see Supplementary Fig. S3). We noted at least one EBS and three p53RE sites (p53F, p53M and p53Z) in the p53 promoter that have not been described or tested before. The 0.9kb p53 promoter reporter was not induced by Egr1 in H4 cells, but was induced (about 1.4-2-fold) by Egr1 as well as by WT1 and WT1-Egr1 expression constructs in Saos2 and 293T cells (Fig. 5a). It is unusual that both WT1 (Wilms tumor protein) and Egr1 could transactivate the p53 promoter equally well in these cells, when they usually have opposite effects on other target genes. In contrast, p53 strongly induced the p53 promoter by 3-9-fold, depending on the cell line (Fig. 5b). In 293T cells, p53 was strongly induced by TAp73α and β, and less well induced by ΔNp73α and β (Fig. 5b) indicating that wtp53 synthesis can be maintained. The fold-induction of p53 transcription by p53 was also concentration-dependent in both 293T and Saos2 cells (see **Supplemental Fig. S5**) indicating that the level of the inducing transcription factor plays a major role in the strength of the transactivation.

We tested the physical binding of the three proteins to the p53 promoter by ChIP in U2OS cells (Fig. 5c). Only p53F and p53M sites were bound, while the p53Z site was unable to bind any of the three p53 family proteins, indicated by the lack of DNA bands in the immunoprecipitates from the p53Z putative p53RE (Fig.5c, bottom line). We determined the transactivating effect of these sites by mutational analysis (Fig. 5d). The result was perfectly consistent with the p53 binding-site ChIP result since mutation caused maximal inhibition of the transactivation of the pGL3-p53 reporter gene only when p53F and M together were mutated, reaching 4-fold reduction for TAp73α and β, as well as ΔNp73α and β. The same deletion mutants described in Figure 4f, were also used to test their ability to transactivate the p53 and ΔNp73 promoter-reporter by transfection into 293T cells. Cotransfection of deletion mutants of TAp73 (Δ1-62 and Δ1-127) or ΔNp73 deletion mutants (Δ1-13 and Δ1-78) clearly reduced the transactivation of p53 in a step-wise manner indicating requirement for both the TAD and PxxP motifs, that are important in DNA-protein interactions. The ΔNp73 promoter was only activated by intact TAp73 (Fig. 5e, left). We examined p53 promoter transactivation by wtp53 compared with five mutant p53 forms in 293T cells. This study revealed that two p53mut forms (p53V143A and H179E) were inactive while three forms retained transactivity (Figure 5e, right panel). In contrast, the ΔNp73 promoter was only activated by wtp53 and not by any mutants. Together, these deletion studies indicate that the p53 protein may still transactivate its own promoter even with mutations in the DNA binding domain (DBD) perhaps because a p53 tetramer can consist of a mixture of wt and mutant p53.

The elevation of Egr1 expression results in increased apoptosis in all cell types tested.

We saw in Figure 2, that Egr1 and p73 proteins are upregulated by Etoposide or UV treatment and also by exogenous Egr1 expression. We show (Fig. 6a) that the end result is almost 90% apoptosis of M12 and U2OS cells after 48 h, but only 20% in MCF7 cells where Egr1 and p53 are low and p73 absent (see Supplemental Fig. S4) and induction is also low. It has been shown that p73 and p53 must interact to achieve high levels of apoptosis[18] and even etoposide induction was not able to accomplish this. We show that the expression level is dependent on the amount of p53 expression vector used (see Supplemental Fig. S5 for a dose response analysis in two cells types). A similar result was noted when Egr1 was over-expressed in neuroblastoma cells that underwent apoptosis caused by p73[19]. When we

inhibited Egr1 expression with Egr1 RNAi in etoposide-treated M12 and U2OS cells, apoptosis fell from 96% to 60-70% further indicating that Egr1 regulates the apoptotic level of this interacting system (Fig. 6b). When RNAi to p53 was transfected into M12, the cells were not affected, however, Egr1 RNAi was effective at reducing apoptosis. In U2OS cells where wtp53 is active (Fig. 6b) apoptosis was reduced from 97% to 60% by RNAi to Egr1, p53 or to p73, indicating that each plays a similar role. Overall, the results described here indicate that p73 may equal if not exceed p53 as an effective inducer of apoptosis.

Discussion

Two important new findings are described in this paper. One is the interdependency of the zinc-finger transcription factor Egr1 in the activities and roles of the p53 family members, p53 and p73 (but not p63). The second finding is that a mechanism of the interdependency is through previously unrecognized active Egr1 binding sites (EBS) as well as p53 binding sites (p53RE) several of which have less than consensus sequences. The consensus for Egr1 is GCGG/TGGGCG, and this can be variable, while for p53RE the consensus RRRCWWGYYY, intervening bases from 0 to 14, followed by a repeat of the 10-nt binding site. Each half site has C at position 4 and G at 7. However, in P1 of TAp73 intron 1, we demonstrate a new functional p53RE, AGTCTAGCCT $(N)_{13}$ GGTCCTGCCC with three changes (underlined) that leave only 85% consensus. The p53RE in P2 has been previously described and shown to be active (see Figure 3b, and Supplemental Fig.S1).

We show that of four putative p53 binding sites in the Egr1 promoter, only two are active. These p53RE(A2 and A3) in Figure 4a and 4e (see also Supplemental Fig. S2) are active even when only 75% of sequence matched the consensus, proved by mutation of the sites (Fig 4e). The unusual feature of A2 and A3, is that the second half-sites have AG and CC instead of CG but these are both active at 75% and 80% consensus sequence, while two inactive sites (A1 and A4) also have 80% consensus. Similarly, we recognized three new p53RE (called F, M and Z) in the p53 promoter (see Fig 5d and Supplemental Fig. S3) and two of these sites with imperfect consensus sequences (CAGCAGGTCT t GCACCTCTTC) and (GATCCAGCTG (N_9) GCAAAAGCTT) are transcriptionally active, while one with intact CG/CG motifs is inactive even at 85% consensus. These studies identify the location of new active sites in all four promoters and these sites are responsible for the interdependency of these genes. Mutational analyses of previously undetected p53 binding sites in the responsive promoters, together with verification of the binding of the proteins to these responsive elements by ChIP supports our conclusions (Figs.1c, 3c, 4d and 5c).

We noted earlier that p53 target genes overlap those of Egr1[20]. Studies on the role of p53 in senescence, differentiation, apoptosis, growth control and DNA damage repair, echo similarly with Egr1[1,14,19,21-27]. We conclude from our studies here that transcriptional interdependence between Egr1 and p73 explains these similarities. Protein blots in Fig. 2 show that increasing Egr1 expression in a variety of cell lines increases p73 expression. In wt-p53-expressing U2OS cells, p53 was also induced (Fig 2d). When we reduced Egr1 with RNAi, p73 was also reduced (Fig 2e). Although Egr1 has only a weak effect on the transcription of p53, it has a much more robust effect on the transcription of TAp73, this can drive the TAp73 protein product to upregulate both the p53 and ΔNp73 promoters. All three p53 family protein products and Egr1 itself strongly induce the transcription of Egr1, thus providing a mechanism for sustained stress responses (summarized in Figure 6c) as long as the p53-related proteins are stable. Other studies have reported changes in the stability of the proteins[28,29], in their activity by binding to p300[30] or by changes in protein locations during apoptosis[31].

Here we have reported mainly on the transcriptional properties of the four gene products, Egr1, TAp73, ΔNp73 and p53. Our studies show that Egr1 upregulates the transcription of *TAp73* (Fig. 1) whose protein product is translated and appears to be the hub of a network since it upregulates the transcription of Egr1, TAp73, ΔNp73 and p53. p53 also is able to upregulate the transcription of all four promoters, although the effect on TAp73 is weaker, and ΔNp73 protein can only up-regulate p53 and Egr1 transcription. Thus, these positive feedback loops can sustain the effect of Egr1 in response to stress stimuli. As a result, the levels and activities of these four important tumor–related genes are finely tuned in an interacting relationship, as summarized in Figure 6c. Since Egr1 and p73 are rarely mutated, they can replace p53 and even exceed its effects, and therefore provide p53-independent responses that modulate cell fate. We show here that because Egr1 only weakly upregulates the p53 promoter, and if p53 is mutated in cancers, the production of transforming mutants[1] will be limited. In the prostate cancer cell lines used here, p53 is inactive and not mutated, and TAp73 becomes the most important regulator of the process of programmed cell death (Fig. 6b). We hypothesize that p73 is more important than p53 in the maintenance of stress responses in cancer cells. Although the in-built negative modifier of the *p73* gene, ΔNp73, can counteract apoptosis and induce oncogenic signals, this is minimized by the sustained levels of p73 expression with feed-back renewal from Egr1. On balance, the transactivating effects of ΔNp73 tested here are all much lower than TAp73 (Figs. 1a, 3b, 4b, 5b, 5d and 5e). Thus the overall effect is that TAp73 has a strong positive effect on apoptosis and this is rapidly activated after stress through the mediation of Egr1. Thus the forecast[32] that there must be "a very tight relationship between the three members of the same family" is proved here by the addition of Egr1 to the equation. These results have implications for the origins and progression of cancers.

METHODS

Cells and culture, antibodies

H4, H4E9, MCF7, 267B1, 293T, Saos2, U2OS cells were cultured in DME containing 10% FBS, penicillin, and streptomycin, at 37°C and 5% CO2. M12, highly metastatic prostate cancer cells, were cultured as described earlier[15]. For ultraviolet-C (UV-C) irradiation (40 J/m2) in a Stratalinker (Stratagene, La Jolla, CA) 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 (E-4), p73(S-20), p73α(C-17) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies p73 (Ab-2) and ΔNp73 were from Oncogene Research Products (San Diego, CA). Anti-β-actin and anti-Flag M2 monoclonal antibodies were from Sigma (St. Louis, MO). Anti-HA (mouse IgG) was from Roche Applied Science (Indianapolis, IN).

Plasmids.

Three genomic DNA fragments with sizes of 2458 bp, 1225 bp and 994 bp corresponding to the promoter and its 5′-upstream regulatory sequences of TAp73, ΔNp73 and p53, respectively, were amplified using Advantage-GC Genomic Polymerase Mix Kit (BD Biosciences Clontech., Palo Alto, CA) from human genomic DNA (BD Biosciences Clontech) and subcloned into a promoterless luciferase construct, 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[15].

The mutated constructs were made by directed mutagenesis according to the Quick-Change Multi Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA). All mutation constructs were confirmed by sequencing. The mutation strategy was that 3 or 4 nt of each Egr1 binding site (9 nt) were mutated into the restriction endonuclease site in order to identify the active binding sites.

We received many expression vectors from several researchers. Plasmids pCMV-neo-Bam-p53wt, pCMV-neo-Bam-p53V143A, pCMV-neo-Bam-p53R175H, pCMV-neo-Bam-p53R249S, pCMV-neo-

Bam-p53R273H were provided by B. Vogelstein (Johns Hopkins Univ), pRC/CMV-p53wt and pRC/CMV-p53H179E from S Matsuzawa, and pCDNA3.1-HA-hp53 was constructed by C..Geisen C, both from The Burnham Institute. Plasmids pCDNA3-HA-TAp73α were provided by WG Kaelin (Univ of Toronto), pCDNA3-HA-TAp73β and pCDNA3-HA-ΔNp73α provided by Jean J.Y. Wang, (Univ. Calif., San Diego). To generate pCDNA3-HA-ΔNp73β, we replaced the fragment encoding C-terminal amino acids of pCDNA3-HA-TAp73β by excision of pCDNA3-HA-ΔNp73α with Pml*I* /Xho*I*. The pcDNA3-HA-TAp73αΔ1-62, pcDNA3-HA-TAp73αΔ1-127, pcDNA3-HA-ΔNp73αΔ 1-13 and pcDNA3-HA-ΔNp73αΔ1-78 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, on line.**

Transfection and Luciferase reporter assays
For DNA transfection, we seeded 293T, M12, U2OS and MCF7 cells at a density of 8-10 x 10^5 into 60mm dishes for one day before transfection in order to achieve 75-95% confluence. The 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 24h after transfection for immunoblotting.

For luciferase assays, we added 2-3 x 10^3 H4 cells, 5-8 x 10^3 293T and Saos2 cells to each well of 96-well plates one day before transfection. The 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) and an EG&G Berthold LB96P luminometer (PE Biosystem, Wellesley, MA). Each value represents the mean fold change compared to the basic vector value.

Immunoblot analysis
We extracted protein from cells in 50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS and 1x complete protease inhibitor cocktail (Roche). We separated 22 μg of lysate protein on a NuPAGE 3-8% Tris-Acetate Gel (Invitrogen Co., Carlsbad, CA) and carried out western blotting using standard methods. The membranes were probed with the appropriate antibodies. Antibody-antigen complexes were detected by the Amersham ECL or ECL$^+$ kit (Amersham Biosciences Corp, Piscataway, NJ, USA).

Chromatin immunoprecipitation (ChIP).
ChIP was performed as described previously[33] with modifications[15]. In order to induce endogenous Egr1 expression, M12 cells were treated with etoposide (20 μg/ml) for 2.5h; U2OS cells were treated with etoposide (20 μg/ml) for 6 h before 1% formaldehyde was added to live cells to crosslink the chromatin. After immunoprecipitation with a specific p73, Egr1 or p53 antibody or a non-immune control IgG antibody, the DNA fragments collected after sonication were tested for the presence of specific portions of the promoter by specific primers and PCR for 35 cycles. Primer sequences are given in **Supplementary Data S6.**

RNA interference experiments and apoptosis assays.
We transfected GenEclipse™ p53 vector-based siRNA, GenEclipse™ Egr1 vector-based siRNA (Chemicon International) and SignalSilence™ p73 siRNA (Cell Signaling Technology) according to the protocol provided by the manufacturer.

24 h after transfection of Egr1-RNAi, p53-RNAi or p73-RNAi into M12 and U2OS cells, etoposide (Sigma, St Louis, Mo) was added at 20μg/ml and cells were cultured for 48h. The percentage of dead cells was counted as Trypan Blue-stained cells together with total number of cells alive or dead in triplicate dishes.

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FIGURE LEGENDS

Figure 1 TAp73 promoter (P1) binds Egr1 and is up-regulated; ΔNp73 promoter (P2) does not respond to Egr1 (see Supplemental Fig.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 48 h after transfection. **b**, Mutation of each of the five Egr1 binding sites (EBS) showed that all sites are activated by Egr1 expression and after mutation of at least 4 sites the activation is maximally reduced. In contrast, Egr1 did not transactivate the ΔNp73 promoter or the mutated EBS in the ΔNp73 promoter, indicating that only the TAp73 P1 promoter is responsive to Egr1. **c,** Chromatin immunoprecipitation confirmed this result since anti-Egr1 proved to be bound only to the EBS in the P1 promoter represented by PCR fragments A, B and C, and not to the D fragment found in P2. A positive control showed p300 binding (in a complex with Egr1) to the EBS-A site in the TAp73 promoter.

Figure 2 Immunoblots to show that p73 protein isoforms (α, β and γ) are differentially expressed at increased levels after Egr1 induction.
a, Egr1 null H4 and constitutively Egr1-expressing E9 (derived from HT1080 osteosarcoma cells) express Egr1-induced levels of p73γ. **b**, Four cell lines were induced with UV at 40J/m^2 to reveal specific changes in p73 protein isoform expression, 24 h later. **c**, Three cell lines were treated with etoposide at various times, the cells were lysed to analyze the p73 isoforms expressed. **d**, The same three cell lines were analyzed before and after transient expression of Egr1. **e**, The transfection into M12 cells of Egr1 RNAi before the treatment of the cells with etoposide to induce Egr1 expression, inhibited Egr1 induction as well as the p73, suggesting a causal effect of Egr1 on p73 expression. The anti-p73 antibodies were used to detect the more common α, β and γ isoforms and these varied with the cell line.

Figure 3. Analysis of the p53 response elements (RE) in TAp73 and ΔNp73
a and **b**. The pGL3-TAp73 and pGL3-ΔNp73 promoters, P1 and P2, respectively (structures are shown in the Supplementary Fig. S1) were transfected into 293T cells to measure the luciferase signal 24h 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 responsive to TAp73, but the induction of the activity was small but significant (see replot in b), while ΔNp73 expression was inhibitory. In contrast, the P2 promoter was 60-80-fold induced by p53 and by TAp73, but not by ΔNp73. The right panel of **b** shows that the mutation of the p53RE in P1 (located in the 288 bp fragment C) gives 3-fold reduced activity to the P1 promoter. **c**, ChIP confirmed that p53 and p73 proteins bind directly to the p53RE in intron1 (P1) as well as to the p53RE in intron 2 (P2) in U2OS cells treated with etoposide for 6h before cross-linking. (NO is the negative control where non-immune IgG was added to lysates, and input is total DNA in the U2OS cells used in this assay. This result confirms the direct binding of p53 family proteins to the responsive elements.

Figure 4. All members of the p53 family induce the Egr1 promoter by binding to two p53 binding sites (see Supplementary Fig.S2 for the structure and features of the Egr1 promoter).
a, The transactivation of the Egr1 promoter was studied using pGL3-Egr1. The Egr1 promoter was tested as 5 different fragments (Egr1, Egr1F, Egrp1, Egr1p2 and Egr1p3). All were transactivated by members of the p53 family expressed exogenously in 293T cells. Both the TAp73β and ΔNp73β isoforms were the most active (>2-fold); the least active was p53. The Egr1F and Egr1 promoter

fragments each carry 4 putative p53 BS, Egr1p1 has 3, Egr1p2 has 3 and Egr1p3 has 2 sites but there was little difference in the fold increase in transactivation between the five different Egr1 promoter forms. **b**, Induction of transfected pGL3-Egr1 and pGL3-Egr1F was tested also in H4 cells. Here TA-p73 was more effective in transactivation than ΔNp73. **c,** the transfection of the expression vectors of the three proteins in **b** were tested in prostate (M12) cells by immunoblotting, to measure the levels of Egr1 induced by each, confirming that TAp73 is the most effective inducer of Egr1 (top row). **d**, 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. **e,** Mutation of the A2 and A3 binding sites, singly or doubly clearly showed that both sites are transactivating when not mutated and gave step-wise reductions with each mutation, with A3 the most active p53RE. **f.** (left) wt TAp73 and ΔNp73 were equally active in up-regulation of the Egr1 promoter; in contrast N-terminal deletions of the expressed p73 proteins, TAp73Δ1-62 and 1-127, ΔNp73Δ1-13 and 1-78 were unable to induce the Egr1 promoter. **f.** (right), Mutation of p53V143A prevented its activation of the Egr1 promoter, but four other mutants were still transactivating in 293T cells.

Figure 5. The p53 promoter is weakly activated by Egr1; strongly induced by p53 and TAp73, and less strongly by ΔNp73 at two p53 binding sites. (see Supplementary Fig.S3 for details of the structure of the promoter).
a, In H4 cells, neither transiently-expressed Egr1 nor WT1 has any effect on the p53 promoter; in Saos2 and 293T cells Egr1, WT1 and the dominant negative WT1/Egr1 all weakly induce, **b**, In the same three cell types, transfection of expression plasmids for the 5 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 three p53RE (A, F, and Z). The presence of bands for the F and M binding sites, but lack of bands for the Z site indicated that only the F and M p53RE bind. **d**, Mutation of the F, M and Z p53RE in the pGL3-p53 reporter gene confirmed that 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** (left) the same deleted forms of the expressed p53, TAp73 and ΔNp73 proteins as used in Figure 4f indicated that the amino-terminal sequences of TAp73 and ΔNp73 are required for transactivation, **e** (right) four mutant forms of p53 can still activate the p53 promoter but two mutant forms are inactive. The ΔNp73 promoter was only activated by wtp53.

Figure 6. Etoposide induces Egr1 and cell death; RNAi analyses of the extent of cell death.
a, Three cell lines that express different levels of Egr1, p53 and p73 (indicated below the panel) were treated with etoposide and 48h later the cultures were stained with Trypan blue to calculate the percentage of dead cells, in triplicate cultures repeated three times. **b**, RNAi to each of the three genes was transfected into M12 and U2OS cells to indicate which genes had the most important apoptotic effect. **c,** 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.

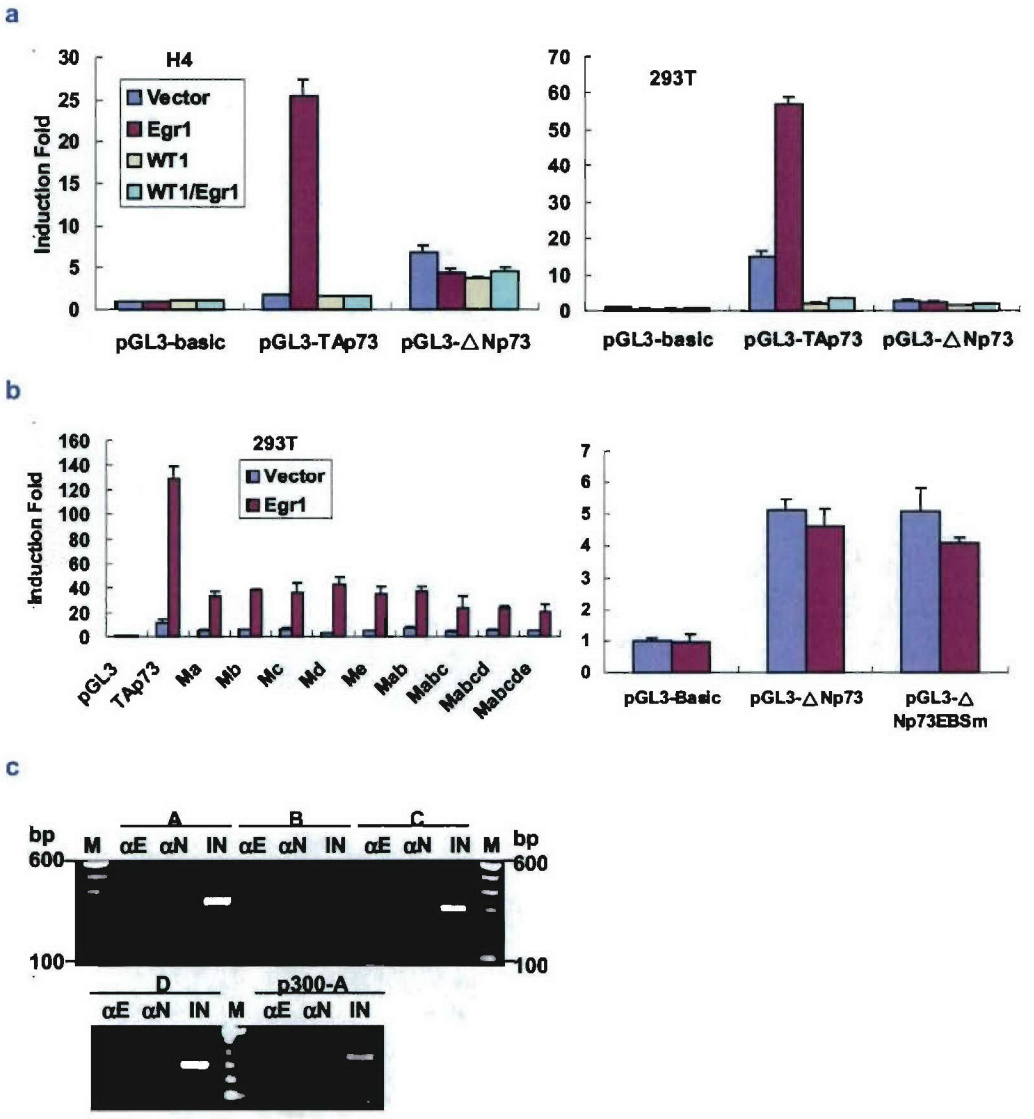


Figure 1. TAp73 promoter (P1) binds to Egr1 and is up-regulated; △Np73 promoter does not respond to Egr1.

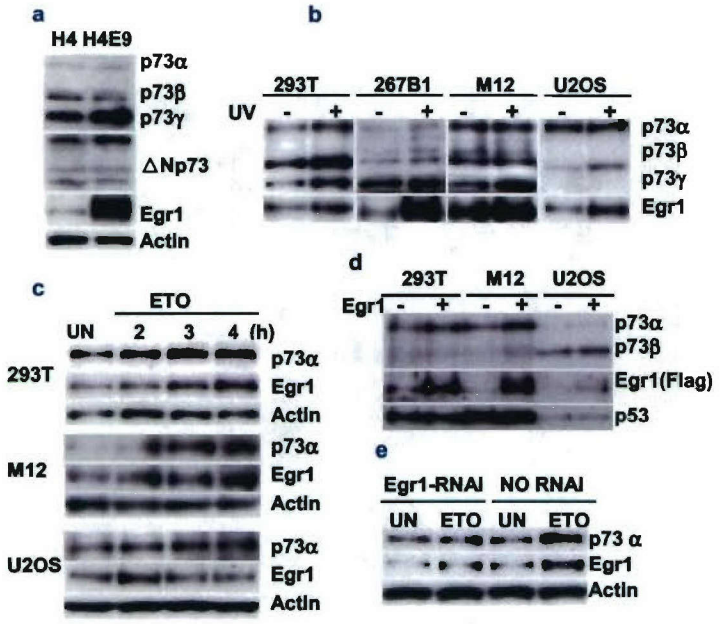


Figure 2. p73 protein isoforms (α, β and γ) are expressed at increased levels after Egr1 induction.

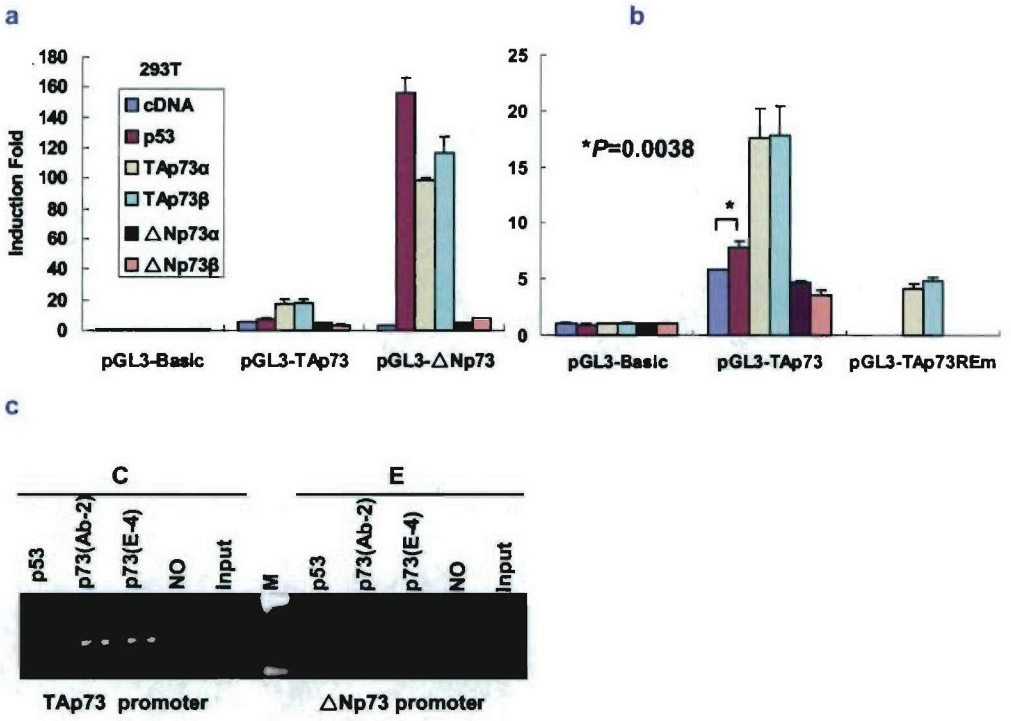


Figure 3. Analysis of the p53 response elements in p73 promoters.

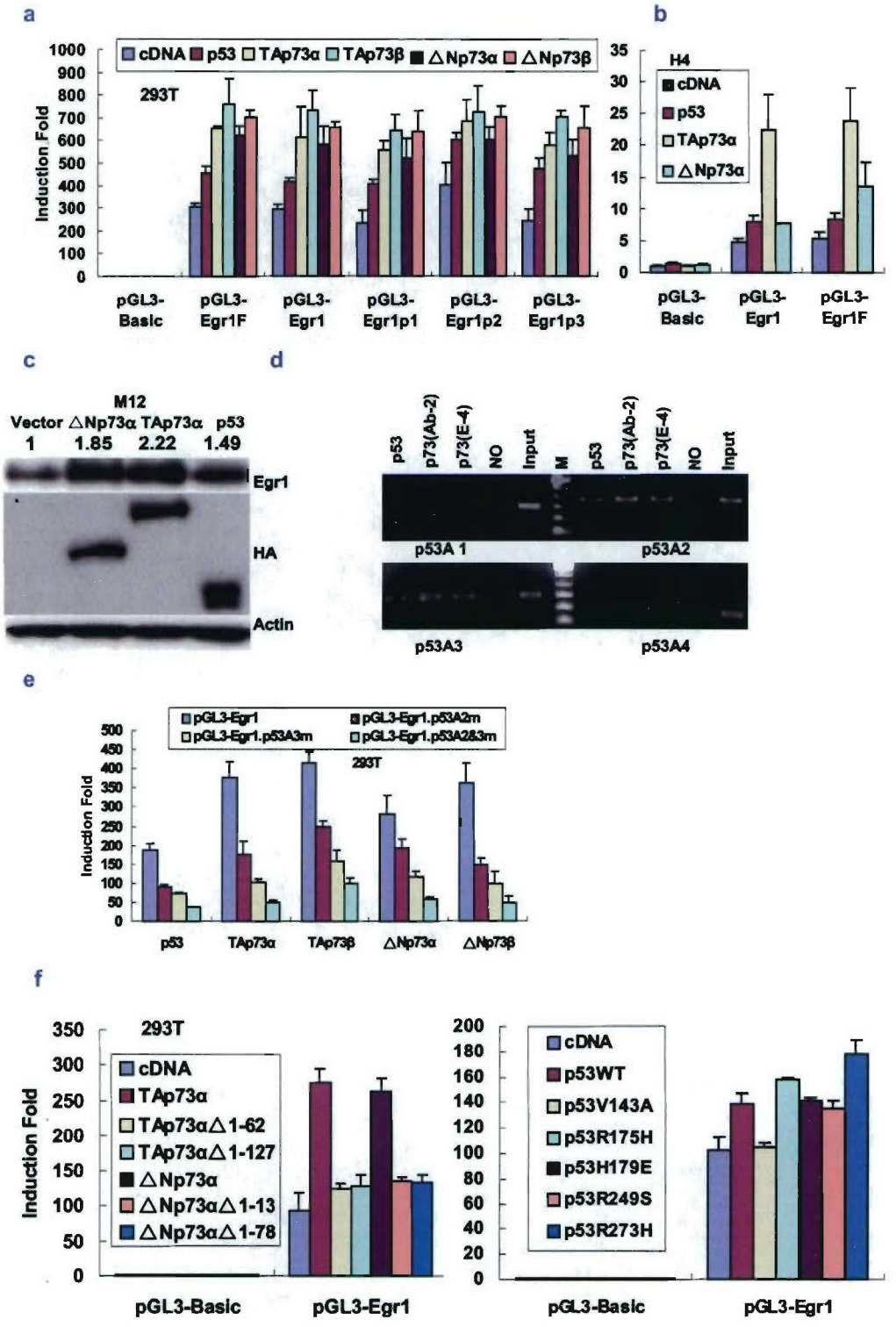


Figure 4. All members of the p53/p73 family induce the Egr1 promoter by binding two p53 binding sites, confirmed by ChIP and mutation analysis.

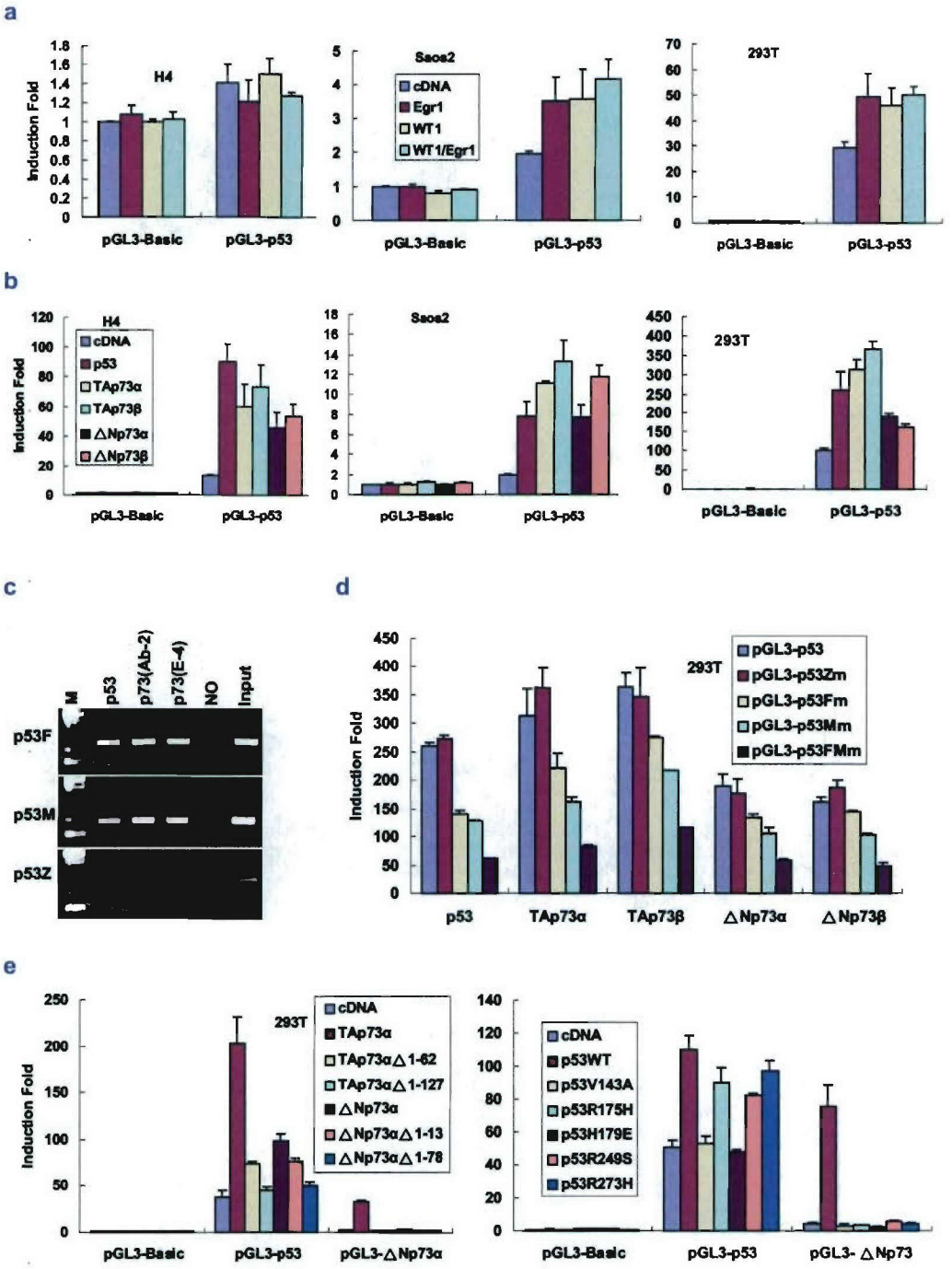
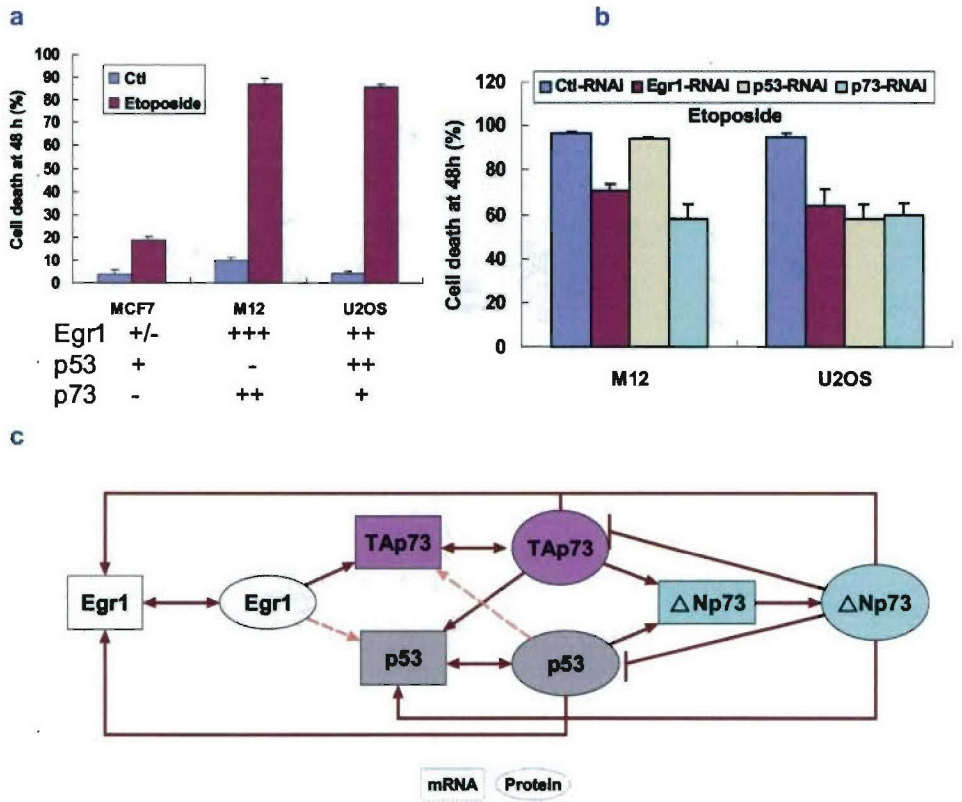


Figure 5. The p53 promoter is very weakly activated by Egr1; it is strongly induced by p53 and TAp73, and less strongly by ΔNp73 at two p53 binding sites.



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