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**14. ABSTRACT**

A majority of cell lines respond to DNA-damaging stimuli such as irradiation by growth arrest, DNA repair and eventually apoptosis of damaged cells. The GADD45 gene family members are involved in these functions, and in cells where TP53 is normal, induction by p53 is the major mechanism for the transcriptional up-regulation of GADD45. However, in the absence of wild-type (wt) p53, as in a majority of cancer cells, we show that Egr1 immediate early transcription factor plays a major role in stress response. Egr1-null mouse embryo fibroblasts (MEFs) do not respond to UV-C by the induction of GADD45 and are resistant to DNA damaging stress, while in wt MEFs, GADD45 is inducible, resulting in cell death. Egr1 acts as a rapidly inducible transcriptional regulator of GADD45a and b in normal and cancer cell lines independently of p53, thereby substituting as a tumor suppressor in place of p53. Since Egr1 is rarely mutated, this factor becomes an important mediator of cancer therapy by irradiation and chemotherapy.

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Final report for DOD-1-01-0165
Original PROJECT was "Cripto: A Target for Breast Cancer Treatment"

As reported fully in June 2004, the IDEA grant was not successful in the original mission of finding a peptide that would block its activity of Cripto in breast cancer.

We reported last year that we had worked on another important gene that is inducible by radiation and chemotherapy drugs such as etoposide, that are used to treat breast cancer patients. The gene GADD45 has three isoforms with similar properties and we studied the induction of GADD45 by the transcription factor Egr1 (that is the regulator that binds to the GADD45 promoter to up-regulate its transcription) and hence GADD45 protein expression. GADD45 is a DNA repair protein that is one of the genes recently found to be in a group of such genes that are induced by chemotherapy drugs. It is known that cancer cells are not able to make normal repairs because of the genes that have become disrupted, mutated or inactivated genes during unscheduled mitoses and therefore have become deficient in making repairs. The result is that some cells always survive to establish new growth that is now resistant to further treatments. This study is to determine if Egr1 induces the expression of GADD45 and whether Egr1 could be a target for gene therapy.

We reported the outline of our studies in June 2004, and now this is a version that we have submitted for publication by Cell Death and Differentiation. It has not yet been reviewed, but it is supplied in full here, as part of the final report.


# GADD45 is rapidly transcriptionally up-regulated by Egr1 in response to DNA damage

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ABSTRACT
A majority of cell lines respond to DNA-damaging stimuli such as irradiation by growth arrest, DNA repair and eventually apoptosis of damaged cells. The GADD45 gene family members are involved in these functions, and in cells where TP53 is normal, induction by p53 is the major mechanism for the transcriptional up-regulation of GADD45. However, in the absence of wild-type (wt) p53, as in a majority of

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cancer cells, we show that Egr1 immediate early transcription factor plays a major role in stress response. Egr1-null mouse embryo fibroblasts (MEFs) do not respond to UV-C by the induction of GADD45 and are resistant to DNA damaging stress, while in wt MEFs, GADD45 is inducible, resulting in cell death. Egr1 acts as a rapidly inducible transcriptional regulator of GADD45a and b in normal and cancer cell lines independently of p53, thereby substituting as a tumor suppressor in place of p53. Since Egr1 is rarely mutated, this factor becomes an important mediator of cancer therapy by irradiation and chemotherapy.

## INTRODUCTION
In a normal cell, over-expression of any oncogene such as *H-Ras*, or the activation of p38 MAPK, activates the *TP53* gene and this leads to growth arrest and apoptosis. Any DNA damaging agent such as UV or ionizing irradiation or anticancer chemotherapy drug treatment activates the p53 protein product and subsequently the regulation of its target genes, including the group called growth arrest and DNA damage-induced genes (GADD). GADD45 is one of the genes that have been found invariably upregulated in many kinds of irradiated cells and tissues [1]. At least 10 DNA damage inducible genes have been recorded including three isoforms of GADD45 (a, b and g) that have been shown to be transcriptionally induced by p53. However, active p53 is not *required* for GADD45 transcriptional regulation because combinations of high levels of GADD45 and mutant p53 protein have been reported for several cancer cell lines derived from breast, CNS, lung, lymphoid, prostate and so the question arises that other transcription factors may elicit GADD45 expression.

GADD45 plays a role in repairing DNA damage by regulating cell growth arrest in the G2 phase of cell cycle, as demonstrated after the microinjection of GADD45 expression vector into human primary fibroblasts [2]. Gadd45a, like p53, was shown to be a key component protecting skin against UV-induced tumors by causing apoptosis [3]. However, other results indicate that tumor cells can abrogate the growth inhibitory function of the GADD45a gene, especially in the presence of an abnormal status of p53 [4]. Several clinical studies have also shown GADD45 over-expression to be a prognosticator of poor outcome, suggesting that the expression of GADD45 is associated with an accelerated progression to malignancy and with resistance to adjuvant therapy. Down-regulation of GADD45a and g expression by NF-kB is an essential step for various cancer types to escape apoptosis and this occurs by blocking c-Jun kinase activation [5]. Therefore, it is important to understand the signals leading to GADD45 expression and the mechanism of its functions that result in apoptosis and whether this function can be used in order to direct cancer cells to apoptosis during cancer therapy.

When p53 is mutated or inactivated, alternative transcription factors must act in its place because the cell cycle checkpoint still operates and apoptosis can ensue. Several transcription factors have been shown to be involved in the transcriptional regulation of GADD45 after stresses such as UV irradiation. This includes p53 family members, p73 and p63 [6-8], OCT1 [9], BRCA1[10], NF-Y [11], c-Myc [5, 12], FOXO3a [13] and WT1[14, 15]. WT1 is interesting because the binding sites for WT1 and Egr1 may be identical, and indicates that Egr1 could also regulate GADD45a. In addition, while WT1 is not rapidly inducible by DNA damaging stimuli, Egr1 is exquisitely sensitive to induction especially by DNA damaging stimuli. Egr1 is an immediate early response gene encoding a transcription factor which is normally low in expression in most normal tissues. Moreover, when cells are exposed to DNA damaging stimuli, Egr1 is rapidly induced and can give rise to growth arrest, DNA repair and is usually followed by apoptosis. This precipitated our study on the influence of Egr1 on the expression of GADD45a and GADD45b, which have high-affinity Egr1 binding sites in their promoters.

We tested here whether GADD45a and GADD45b are regulated by endogenous Egr1 after induction by several different kinds of stimuli and if so, how the level of response varied with dose and time. We show that Egr1 plays a tumor suppressor role by the transcriptional activation of the GADD45a and b, leading eventually to the apoptosis of cancer cells in the absence of wild type p53.

## RESULTS AND DISCUSSION
### Egr1 is induced earlier than GADD45a following UV-C irradiation
The GADD45 promoter contains several putative Egr1 binding sites; therefore the aim of this study was to determine whether GADD45 is a target gene of Egr in cells affected by DNA damaging stimuli. UV irradiation of

DU145 prostate cancer cells led to the maximum induction of Egr1 protein expression 2h after UV treatment, while GADD45a showed maximum expression 3h after induction (**Figure 1A**). Also, both Egr1 and GADD45a showed maximum protein expression at a UV dose of $40J/m^2$ (**Figure 1B**). Egr1 and GADD45a proteins were also induced in DU145 cells upon treatment with the tumor promoter, TPA (**Figure 1C**). Moreover, the rapid induction of Egr1 mRNA peaks 1h after induction by UV-C (**Figure 1D**) while GADD45a mRNA is induced by UV irradiation to reach a maximum at 2h, thus the induction of Egr1 expression precedes GADD45a. As Egr1 and WT1 have similar promoter binding sites, we also analyzed WT1 protein expression after UV-C induction in DU145 cells since it has been reported that WT1 induces GADD45a [15]. The levels of WT1 remained unchanged at different doses of UV-C treatment or were somewhat reduced at various time points after UV induction (**Figure 1A,B, line 2**) indicating that WT1 was not involved under these conditions. Similar results were obtained for the induction of Egr1 and GADD45a in MCF7 breast cancer cell line, which expresses wt-p53 where maximum expression occurred at a UV dose of 40 $J/m^2$ (data not shown). This indicates that the effect of Egr1 on the induction of GADD45a is common to several cell types whether p53 is inactivated or not. Factors reported to induce GADD45 include p53, WT1, CBP and p300 [16, 17] and although there is no p53 binding site in the promoter, there is a site in the first intron. It is possible that p53 could interact with WT1 to bind to the WT1 binding site (see Fig 4A) at the −211 to −182, GC-rich 20-bp site in the promoter:- TCGGCACCGCCCC<u>CGCCCCCGC</u>CCCCTCGG, that was identified as a WT1 site by Zhan et al [15]. This is the site that contains a perfect EGR1 /WT1 binding sequence (underlined). However, under the conditions used here, WT1 was not induced and because p53 is mutant in DU145 cells, this TF pair is unlikely to be active in the induction of GADD45a. We can largely rule out the involvement of p300/CBP also, because Egr1 inhibits the transcription of these co-factors after UV-C stimulation [16].

## Egr1 is required for GADD45a expression

Mouse embryo fibroblasts (MEFs) that lack Egr1 expression, derived from Egr1 null mouse embryos [18] were tested for GADD45a inducibility after treatment of null and wild-type MEFs with UV-C. **Figure 2A** shows that UV-C induced Egr1 and GADD45a protein expression in wt-MEFs, while Egr1-null MEFs were unable to induce Egr1 and GADD45a expression. Similarly, upon induction with TPA, wt-MEFs induced GADD45a mRNA expression, while Egr1-null cells failed to induce GADD45 expression (**Figure 2B**). These findings are consistent with our earlier results using antisense Egr1 oligonucleotides, that were designed to reduce the expression of mouse or human Egr1 specifically [19]. These results support the hypothesis that Egr1 is required for the induction of GADD45a and other pro-apoptotic genes in response to UV treatment. In addition, we also showed earlier that while Egr1-null MEFs are resistant to apoptosis brought about by UV irradiation, Egr1+/+ MEFs are highly sensitive [20] and this is caused partly by the direct induction of PTEN phosphatase by Egr1. In summary, Egr1 expression elicited by DNA damaging stimuli leads to apoptosis of both normal and cancer cells.

## Egr1 binds directly to the GADD45a promoter

Since GADD45a was originally identified by us as a possible target gene regulated by Egr1 using a promoter microarray study, revealed by a positive signal for hybridization with Egr1-immunoprecipitated chromatin (ChIP) cross-linked DNA, we expected that we should be able to verify this finding by "conventional" ChIP assay. Therefore, to assess whether direct binding of Egr1 to the GADD45a promoter sequences occurs in intact DU145 cells, we performed chromatin crosslinking with formaldehyde followed by immunoprecipitation with antibodies to Egr1. The sonicated chromatin fragments were processed as described earlier [21] and PCR was used to detect DNA specific to GADD45a promoter that had been pulled down by the anti-Egr1. **Figure 3** demonstrates that the expected 264 bp DNA, a portion of the promoter that contains the putative Egr1 binding sites is recognized in UV and IR-induced cells but not in serum or no ntreated (NT) cells, indicating that induction with genotoxic stresses is required for Egr1 binding to the GADD45a promoter. The same DNA ChIP samples were also used for amplification using primers to detect cyclophilin (CPH) as a negative control. This demonstrated that Egr1 can bind directly to consensus binding sequences in the GADD45a promoter and it does so in live cells that express endogenous Egr1 after DNA-damaging stimuli.

**Egr1 stimulates a GADD45a promoter-reporter construct**

The full length GADD45a promoter contains several putative Egr1 binding sites and was cloned from genomic DNA and sequenced (Fig 4A). To determine which sites might be responsible for Egr1 stimulation, a series of deletions were made and compared to full-length (2.3kb DNA) 5' regulatory sequence containing 2kb of GADD45a promoter and 29 bp of 5'UTR, ligated to luciferase using the expression plasmid pGL3-basic vector. Using transfection into 293T cells with Renilla luciferase as an internal control, the expression of pGL3-luciferase was measured and expressed as a fold-change of normal to truncated promoter activities, when Egr1 was expressed ectopically, or after Egr1 was induced by irradiation with UV-C at 40 J/m$^2$. The results in **Figure 4B,** show that very strong induction of the GADD45a promoter occurs by both exogenous and endogenous Egr1 expression. Also of interest was the finding that 450bp of promoter sequence was sufficient to induce most of the activity and hence this region contains the most active Egr1 binding sites. Mutation of the potential Egr1 binding sites (EBS) in the GADD45a promoter by site-directed mutagenesis was used to change four nucleotides in each of the putative EBS shown in **Figure 4C** (white ovals). The mutant form 3 (wherein sites 1 and 2 were both mutated) was the most potent in reducing the promoter activity, whereas mutation in site 4 (which contains a group of 3 overlapping EBS (recognized as a WT1 binding site) has negligible effect on the promoter activity (**Figure 4D**). We concluded that the most effective Egr1-activated sites are sites 1 and 2 in the 5'UTR of GADD45a. We noted that the relative levels of each set of the inducing conditions, that is, ectopic Egr1 (Fig 4D, middle) versus UV-induced (right) in 293T cells had a similar fold change relative to the vector-transfected cells expressing each reporter construct (left). One exception was the last bar (Mut4) showing that the Egr1 binding sites at ~-200 were unimportant while the two sites closest to the start of translation are required for full UV-responsive activity. This is consistent with the finding of reduced WT1 in DU145 after UV-C (Fig. 1A,B). In summary, the results here demonstrate that the GADD45a promoter reporter has two Egr1 sites in the 5'UTR domain, that respond best to Egr1 induction in p53-null cells. Egr1 was effective whether transiently transfected to be expressed as ectopic protein or when endogenously induced by stresses, such as UV, IR or TPA. This was demonstrated as transcriptional induction (Fig 4) as well as by mRNA levels and protein levels (Fig 1). In addition, the putative binding sites on the promoter construct were shown to bind Egr1, demonstrated by chromatin immunoprecipitation after UV or IR treatment but not after serum stimulation (Fig.3).

With the multiple activities of the GADD45a gene product in growth checkpoint, cell cycle retardation, DNA repair responses and apoptosis, it is important that its induction is rapid and strong. We suggest that only Egr1 can provide this kind of response among the several Transcription Factors that have been shown to take part in the regulation of the GADD45a gene. It has been reported that Oct1[22], NF-Y [23] and FOXO [13] can be induced by stresses, but this is indirect and likely to be slower than the 1 h maximal induction time for Egr1mRNA. In addition Egr1 like p53 can interact with and become activated by CBP and p300 or with APE/REF-1[24], thus strengthening the response to stress and suggesting that when p53 is mutated, then Egr1 can make the appropriate responses in the activation of relevant target genes [16]. These include p21[25], ARF[26], TGFb1[27], p53 and p73 [28], and many more genes that also have Egr1 binding sites that have not yet been verified or recognized and therefore, not yet established. GADD45a is one of these genes. Of note, a recent finding using skin keratinocytes is that UV-B irradiation induces NF-kB which then transcriptionally induces Egr1 to further elicit GADD45a expression and cell death [29], thus extending our finding to other human tissues.

In view of its extreme sensitivity to induction by stresses, we suggest that Egr1 is a major supporter of p53 functions and can also replace p53. The ability of Egr1 and also p73 and p63 to substitute for p53 for some activities emphasizes the importance of these functions and the co-ordinate effects of these two families of genes. Our results overall indicate that intricate relationships occur at the transcriptional level between the proteins that regulate the cell's responses to stress, especially genotoxic stress that leads to apoptosis. This has important relevance to understanding the mechanisms that underlie responses to chemotherapy and hence predictions of outcome.

## Materials And Methods

## Cells, Serum Stimulation, and UV Irradiation

Prostate cells DU145, breast cells MCF7, MEFs Egr1$^{-/-}$ and Egr1$^{+/+}$ cells were cultured in DME containing 10% FBS, penicillin and streptomycin at 37°C and 5% $CO_2$. For serum stimulation, cells were incubated in 0.5% CS for 24 hr and then 20% serum was added for the indicated time. For ultraviolet-C (UV-C) irradiation (40 J/m$^2$) in a Stratalinker (Stratagene, La Jolla, CA) as well as for mock treatment, the growth medium was aspirated and then replaced after treatment.

## Antibodies and Reagent

Rabbit polyclonal IgG antibodies, Egr1 (C-19, 588) and GADD45a (C-20) and mouse monoclonal IgG$_{2a}$ antibody, GADD45a (4T-27) were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin monoclonal antibody was from Sigma as was phorbol ester tumor promoter, TPA (Sigma Corpn., St Louis, MO), 12-O-tetra-decanoylphorbol-13-acetate and was used at a concentration of 100 μg/ml.

### Western Blot and Immunoprecipitation

For Western blots, cells were lysed in ice-cold RIPA buffer (50 mM Tris-HCl (pH7.4), 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 was performed with ~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). Endogenous Egr1 samples were precleared with protein A-agarose beads (Santa Cruz Biotechnology) prior to immunoprecipitation with Egr1 (C-19) or GADD45a (C-20) rabbit antibody. Immunocomplexes bound to agarose beads were washed four times in the same buffer. Immunoblotting was performed by resolving whole-cell lysates or immunocomplex by NuPAGE 10% Bis-Tris Gel (Invitrogen Co., Carlsbad, CA) and blotting onto Immobilon-P membranes (Millipore Corporation, Billerica, MA). The membranes were probed with the appropriate antibodies. GADD45a was detected by IP with a rabbit antibody followed by blotting with a mouse anti-GADD45 a in order to separate the 18kD protein from the immunoglobulin used in the IP. Antibody-antigen complexes were detected by the Amersham ECL kit (Amersham Biosciences Corp, Piscataway, NJ).

### Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously [21] with modifications. Egr1-chromatin was immunoprecipitated using Egr1 (Santa Cruz Biotechnology, 588) antibody. PCR identification of the captured GADD45a promoter sequences was performed using Advantage-GC Genomic Polymerase Mix Kit (BD Biosciences Clontech). The GADD45a promoter contains several Egr1 binding sites, but only one site seems to be a high affinitybinding site. PCR primers were designed around this Egr1 binding site Sense 5' CGGCCCAATTAGTGTCGTGCG 3'; Antisense 5' TATTGCAAACTGCAGGTCGCC 3'. Two consecutive rounds of 35 cycles each of PCR were performed, using the captured fragments as templates. PCR products were confirmed by sequencing.

### Quantitative Real time PCR (Q) RT-PCR

Total RNAs were isolated using TRIzol reagent (Invitrogen) and RNeasy Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's protocol. Reverse Transcription was performed using the SuperScript First-Strand system for RT-PCR (Invitrogen, Carlsbad, CA). 2mg of total RNA was reverse transcribed from each sample and one-tenth of it was used as a template for QRT-PCR reaction using SYBR Green I (Applied Biosystems) according to the manufacturer's instructions. The data were normalized against GAPDH.

expression. Specific primers were designed as below: Egr1, 5'-GACCGCAGAGTCTTTTCCTG-3' and 5'-AGCGGCCAGTATAGGTGATG-3'; GADD45a, 5'- GGAGGAAGTGCTCAGCAAAG-3' and 5'-ATCTCTGTCGTCGTCCTCGT-3'; GAPDH 5'-GGCCTCCAAGGAGTAAGACC-3' and 5'-AGGGGTCTACATGGCAACTG-3'. Cyclophilin A (CPH as a control; 5'CTCCTTTGAGCTGTTTGCAG-3' and 5'-CACCACATGCTTGCCATCC-3'. QRT-PCR was performed on the Stratagene Mx3000p instrument (Stratagene, San Diego, CA).

## GADD45 promoter reporter studies.

Advantage®-GC Genomic Polymerase Mix Kit (BD Biosciences Clontech., Palo Alto, CA)
together with human genomic DNA (BD Biosciences Clontech) were used to synthesize the GADD45a promoter series by PCR. The following primers were used: for the 2.3 kb promoter, primer 5' TCCGAGCTCGGA.GGCAAAGGACCAGGAATTG 3'; for 0.6 kb 5' primer TCCGAGCTCGGA.AGGCCACCTCTAGCCTCTTG 3'; for 0.45 kb; 5' primer TCCGAGCTCGGA.GCTCCTCTCAACCTGACTCC 3', a Sac I site is underlined; the 3' primer was:- 5' GAAGATCTTC.ATTGCAAACTGCAGGTCGCC 3', the Bgl II site underlined. The fragments were subcloned into the pGL3-Basic luciferase reporter plasmid, thus creating three pGL3-GADD45a reporter genes (confirmed by sequencing). The pGL3-GADD45a reporter gene mutation constructs were made by directed mutagenesis using the Quick-Change Multi Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA). The mutation strategy was that 4 nt of each Egr1 binding site (9 nt) were mutated into the restriction endonuclease site in order to destroy the active binding site. All mutation constructs were confirmed by sequencing. The purified plasmid DNA (added in final equal amounts in all dishes) was transfected into HEK293T cells using transfectamine according to the manufacturers instructions. All measurements were performed two to three times in triplicate and the averaged results were used to calculate the ratio of the normalized luciferase signal from each expression plasmid versus the empty vector.

## Key Research Accomplishments
The work that we proposed was an important aspect of breast cancer progression. The importance of Cripto is clearly increasing, and this line of research is worth supporting. However, our efforts were not productive and not for the lack of effort.
The work on GADD45A is in a much better place with consistent and interesting results that are worth pursuing. This work needs several more experiments and some more studies that compare other transcription factors that might affect GADD45A regulation

## Reportable Outcomes
1.  The PI was invited to present the studies made on **Cripto** in her laboratory, as well as one describing the work on Egr1 (also supported by the DOD) at the University of Texas at San Antonio. The former was a 2 hour teaching seminar to the graduate students and postdoctoral trainees at the Institute of Biotechnology.
2.  A very favorable response was made to our published review reported last year.
3.  Three publications on Cripto from the past two years by the PI were sent with last years report:
    a) Adamson, E. D., Minchiotti, G., and Salomon, D. S. (2002). Cripto: a tumor growth factor and more. [Review] [74 refs]. Journal of Cellular Physiology *190*, 267–278.
    b) Parisi S. D'Andrea D. Lago CT. **Adamson** ED. Persico MG. Minchiotti G. (2003). Nodal-dependent Cripto signaling promotes cardiomyogenesis and redirects the neural fate of embryonic stem cells. (2003). J.Cell Biology. 163(2):303–14.
    c) Liguori GL, Echevarria D, Improta R, Signore M, **Adamson E,** Martinez S, Persico MG. (2003) Anterior neural plate regionalization in cripto null mutant mouse embryos in the absence of node and primitive streak. Dev Biol. 264(2):537–49.

## Conclusions

The structure of Cripto and especially the disulfide bridge loops were not known at the time and have recently been published. The educated prediction that we made was incorrect and the mutant forms of Cripto that we made were not useful. In this case, the collaboration of larger teams of workers in several different fields combined to make excellent advances on this important gene that we could not match. We therefore worked on another protein (GADD45a) that was also highly relevant to breast and prostate cancer. Publications resulting will acknowledge the support of the BCRP program of the DOD.

## Acknowledgements

## Figure Legends

**Figure 1. Egr1 is induced before GADD45a after UV irradiation or TPA treatment of prostate cancer DU145 cells. A,** To detect low levels of GADD45 a, DU145 cell lysates were first immunoprecipitated using a rabbit antibody to GADD45a at a range of times after a 40 $Jm^{-2}$ stimulus. Sepharose A beads were added to the lysates to adsorb the antibody complex and the washed complexes were analyzed by immunoblotting. GADD45a was detected using a mouse anti-GADD45a antibody. In parallel, cell lysates were separately analyzed by immunoblotting for Egr1, WT1 and actin to indicate equal loading. **B,** a similar analysis to A after a range of radiation doses; **C,** Tumor promoter TPA induced Egr1 and GADD45a after 1-2 h of treatment: **D,** mRNA levels were measured in DU145 cells induced with 40 $Jm^{-2}$ UV treatment and harvested for measurement by QRT-PCR after the indicated times.

**Figure 2, Egr1 is required for GADD45a induction after UV-C irradiation. A,** Mouse embryo fibroblasts prepared from Egr1 null fibroblasts were unable to induce Egr1 or GADD45a after UV-C treatment analyzed as described in the legend to Figure 1A, while Egr1+/+ MEFs showed strong upregulation of Egr1 at 1h and GADD45a after 2-3h. Actin is shown as a loading control. **B,** GADD45a mRNA levels measured by QRT-PCR in Egr1 null MEFs were little affected by TPA treatment, while Egr1 +/+ MEFs gave three-fold induced levels.

**Figure 3, Egr1 binds to the GADD45a promoter after UV or IR induction** of DU145 cells but not after serum or untreated cells. Chromatin immunoprecipitation was performed as described in the methods section. The samples in number 3 lanes were immunoprecipitated with anti-Egr1 in cells where Egr1 bound to the promoter. Lanes 1 are positive controls and below cyclophilin was shown as a negative control in contrast with TPA-induced DU145 cells.

**Figure 4, GADD45a promoter responds to the binding of Egr1 at two sites** in the 5'UTR by upregulating the transcriptional activity. **A,** A pGL3-GADD45a promoter luciferase reporter construct was constructed in three different lengths.
**B,** The normalized transcriptional activity of the three promoter constructs was measured 48h after transfection into HEK293T cells, of empty expression vector, or an Egr1 expression vector or after UV-C irradiation at 40 $Jm^{-2}$. **C,** The proximal putative Egr1 binding sites were mutated as shown by the clear ovals. **D,** the relative luciferase signal was measured in Mut 1, Mut 2, Mut 3 (1+2), and Mut 4 reporter constructs in cells that were also transfected with empty vector (pcDNA) or with the Egr1 expression vector or with Egr1 induced by UV-C.

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1. Snyder, A.R., and Morgan, W.F. (2004). Gene expression profiling after irradiation: clues to understanding acute and persistent responses? Cancer Metastasis Rev 23, 259-268.

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A

DU145 cells

control | 30 min | 1 hr | 2 hr | 3 hr | 4 hr | 5 hr

← Egr-1
← WT1
← GADD45α
— Actin

B

Control | 20J/m² | 40J/m² | 80J/m²

← Egr-1
← WT1
← GADD45α
← Actin

C

Control | TPA

← Egr-1
← GADD45α
← Actin

D

Relative Fold Change

| | Control | 30min | 1h | 2h | 3h | 4h | 5h |
|---|---|---|---|---|---|---|---|
| ···◆··· Egr1 | 1.00 | 2.14 | 2.58 | 1.52 | 1.04 | 0.96 | 0.93 |
| —◆— GADD45a | 1.00 | 1.04 | 2.61 | 2.78 | 1.70 | 1.35 | 1.95 |

Figure 1
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A



Egr1-/- MEFs

Control | 1 h | 2 h | 4 h

Egr1+/+ MEFs

Control | 30 min | 1 h | 2 h | 3 h | 4 h | 5 h

← Egr-1
←GADD45α
← Actin

B



Relative mRNA level (Fold Change)

Egr+/+ UT | Egr+/+ TPA | Egr-/- UT | Egr-/- TPA

Figure 2
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NT | UV | IR | Serum

1 2 3 | 1 2 3 | 1 2 3 | 1 2 3

TPA

1 2 3

Gadd45α
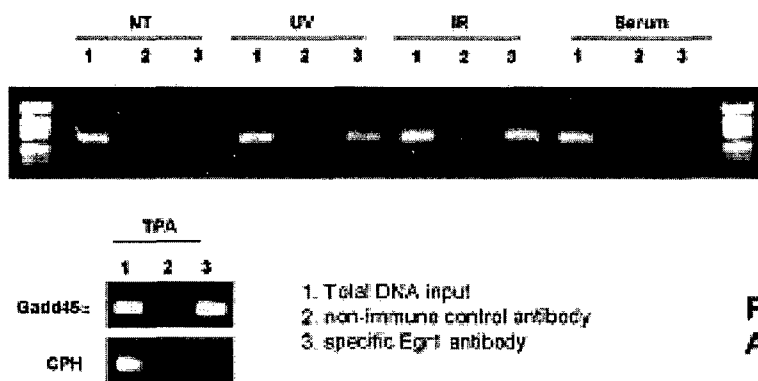
CPH

1. Total DNA input
2. non-immune control antibody
3. specific Egr1 antibody
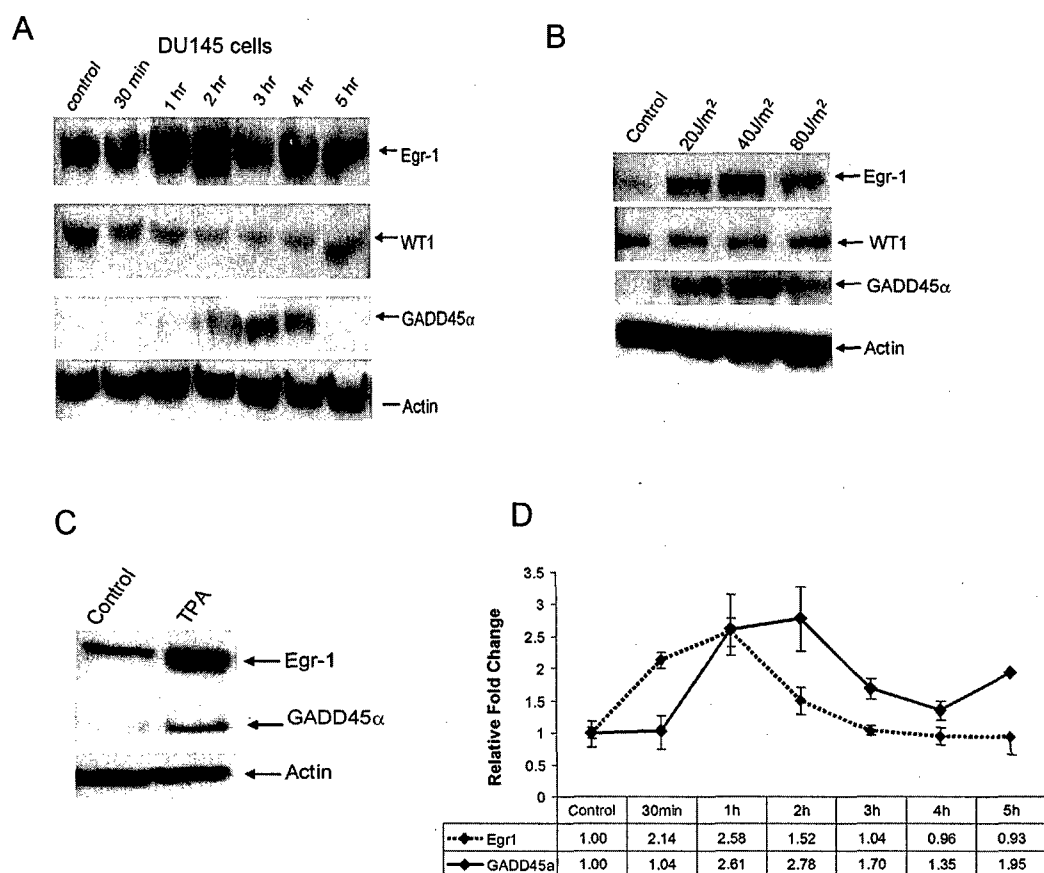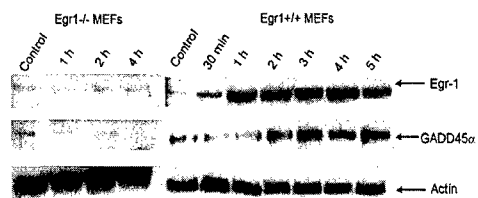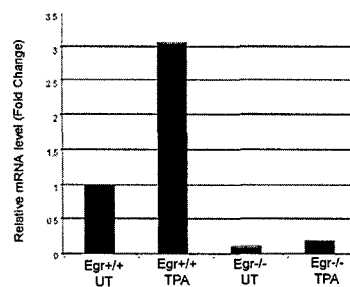
**Figure 3
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Figure 4
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