

E2F1 and E2F2 induction in response to DNA damage preserves genomic stability in neuronal cells

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Abbreviations: ODN, oligodeoxynucleotide; NCS, neocarzinostatin; ASCAT, CAT antisense; ASE2F1, E2F1 antisense; ASE2F2, E2F2 antisense; wt E2F DO, wild-type E2F decoy oligodeoxynucleotide; mut E2F DO, mutant E2F decoy oligodeoxynucleotide.

E2F transcription factors regulate a wide range of biological processes, including the cellular response to DNA damage. In the present study, we examined whether E2F family members are transcriptionally induced following treatment with several genotoxic agents, and have a role on the cell DNA damage response. We show a novel mechanism, conserved among diverse species, in which *E2F1* and *E2F2*, the latter specifically in neuronal cells, are transcriptionally induced after DNA damage. This upregulation leads to increased E2F1 and E2F2 protein levels as a consequence of *de novo* protein synthesis. Ectopic expression of these E2Fs in neuronal cells reduces the level of DNA damage following genotoxic treatment, while ablation of E2F1 and E2F2 leads to the accumulation of DNA lesions and increased apoptotic response. Cell viability and DNA repair capability in response to DNA damage induction are also reduced by the E2F1 and E2F2 deficiencies. Finally, E2F1 and E2F2 accumulate at sites of oxidative and UV-induced DNA damage, and interact with γ H2AX DNA repair factor. As previously reported for E2F1, E2F2 promotes Rad51 foci formation, interacts with GCN5 acetyltransferase and induces histone acetylation following genotoxic insult. The results presented here unveil a new mechanism involving E2F1 and E2F2 in the maintenance of genomic stability in response to DNA damage in neuronal cells.

Introduction

The E2F family of transcription factors is encoded by 8 genes, *E2F1–E2F8*, which give rise to 9 different proteins. The 2 E2F3 proteins –E2F3a and E2F3b– are the product of alternative use of promoters of the E2F3 locus.^{1–2} Traditionally, E2F family members have been subdivided into 2 groups based on their transcriptional activities, structures and interactions with the pocket proteins Retinoblastoma protein (pRB), Retinoblastoma-like protein 1 (p107) and Retinoblastoma-like protein 2 (p130). E2F1, E2F2 and E2F3a, that only bind pRB, constitute the ‘activator’ E2Fs due to their ability to activate transcription of E2F target genes. E2F3b and E2F4–E2F8 are considered the ‘repressor’ E2Fs since they are capable of repressing the expression of mostly overlapping sets of target genes. E2F3b, E2F4 and E2F5 exert their repressive function in association with a pRB family member, while E2F6–E2F8 repress transcription in a pocket protein-independent manner as they lack the pocket protein binding domain.^{3–6}

Despite the fact that E2Fs were originally described to play a pivotal role in cell cycle control,^{7–8} it has become clear that they participate in the regulation of a plethora of biological processes, including the cellular response to DNA damage. It was first reported that E2F1 protein levels increase upon treatment with several DNA damaging agents.^{9–12} Further studies revealed that E2F4 levels decrease whereas E2F3a, E2F7 and E2F8 are upregulated following DNA damage.^{13–15}

E2F1 is the best studied family member with respect to its regulation and function following genotoxic stress. Its participation in the DNA damage response can be described from 3 different angles.¹⁶ First, E2F1 undergoes posttranslational modifications in response to DNA damage. E2F1 is phosphorylated on serine 31 by ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) kinases,¹² and on serine 364 by Checkpoint kinase 2 (Chk2).¹⁷ It is also acetylated on lysines 117, 120 and 125 by either p300/CREB-binding protein (p300/CBP) or p300/CREB-binding protein-associated factor (P/CAF)

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acetyltransferases.¹⁸⁻¹⁹ These modifications contribute to E2F1 stabilization and explain the elevated E2F1 protein levels observed after genotoxic insult.^{12,17,18} Second, E2F1 transactivation ability following DNA damage is regulated through its interaction with specific protein partners. E2F1-pRB complexes repress transcription when they are associated with nucleosome remodeling proteins, methyltransferases, or histone deacetylases,²⁰⁻²² but activate it upon interaction with histone acetyltransferases.²³⁻²⁴ Finally, E2F1 is recruited to sites of DNA lesions and promotes the recruitment of repair factors, suggesting a role for E2F1 in DNA repair.²⁵⁻²⁷

It was also shown that UV irradiation induces the transcription of *E2F1*, resulting in increased E2F1 protein levels.²⁸ Therefore, 2 parallel mechanisms contribute to E2F1 induction in response to DNA damage: the posttranslational modifications and consequent protein stabilization, and the enhanced transcription that leads to *de novo* protein synthesis. Recent observations have also demonstrated that other E2F family members are transcriptionally induced upon treatment with doxorubicin.¹⁴ These findings suggest that DNA damage itself, and not a signal generated by a particular genotoxic agent, is at the origin of the upregulation of *E2F1* gene transcription. The aim of the present work is to examine whether various E2F family members are transcriptionally induced following treatment with several genotoxics in different species—with special interest on a neuronal cell based system—and have a role on the cell DNA damage response.

Here, we show that *E2F1* and *E2F2*, the latter specifically in neuronal cells, are transcriptionally induced upon DNA damage. This upregulation contributes to the augmentation of E2F1 and E2F2 protein levels, which are active in their transcription regulation functions. Importantly, ectopic expression of these E2Fs in neuronal cells reduces the accumulation of DNA damage following treatment with genotoxic agents. Conversely, ablation of E2F1 and E2F2 leads to increased levels of DNA damage and apoptotic response, and also reduces cell viability and DNA repair capability upon genotoxic stress. Moreover, we show that E2F1 and E2F2 accumulate at sites of oxidative and UV-induced DNA damage, and associate with γ H2AX DNA repair factor. Finally, as it was formerly established for E2F1,^{25,27} we demonstrate that E2F2 promotes Rad51 foci formation, interacts with GCN5 acetyltransferase and induces histone acetylation following genotoxic insult. In summary, the evidence presented here establishes a new mechanism involving E2F1 and E2F2 in the response to DNA damage and the maintenance of genomic integrity in neuronal cells.

Results

E2F1 and E2F2 are induced upon DNA damage in neuronal cells

In order to evaluate the response of *E2F* genes to DNA damage, we first performed a time course study of the mRNA levels of *E2F1-5* after exposure of cells to several genotoxic agents differing in their mechanism of action and the resulting lesions. The genotoxics used were neocarzinostatin (NCS)—a radiomimetic

drug that generates double strand breaks—, hydrogen peroxide (H_2O_2)—known to produce oxidative stress and consequently single-strand breaks (SSBs) and base damage— and UV-C (UV) irradiation—which causes essentially pyrimidine dimers—. Northern blot assays on HepG2 cells revealed an increase of *E2F1* mRNA levels upon treatment with each of the genotoxic agents used (Fig. S1A). No such changes were detected for the other family members studied. *E2F1* mRNA raise was also observed in HEK293 cells (Fig. S1B). Next, we used the same approach on neuronal cell lines and found that both *E2F1* and *E2F2* transcripts were augmented after treatment with each of the DNA damaging agents in SH-SY5Y (Fig. 1A), Neuro-2a, HN9 and PC12 cells (Fig. S2A-C). Finally, *E2F1* and *E2F2* transcript levels were increased in rat primary hippocampal neuron cultures irradiated with UV (Fig. S2D), strengthening the notion that the *E2F2* mRNA augmentation in response to DNA damaging agents is characteristic of neuronal cells. Together, these results indicate that in response to DNA damage there is an *E2F1* mRNA increase in all cell types, while *E2F2* mRNA raise would be restricted to neuronal cells. As proposed, this response appears to be independent of the type of DNA damage induced and shared by several species. The specificity of the probes used in Northern blot assays is shown in Fig. S3. Next, to rule out the possibility that *E2F1* and *E2F2* mRNA increase observed after the genotoxic stress was a consequence of transcript stabilization, we studied the effect of blocking transcription through actinomycin D treatment. To examine this, cells were incubated with actinomycin D for 3 h, exposed to genotoxic agents and harvested for a Northern blot assay at the time the maximum mRNA levels had been observed. Transcriptional inhibition prevented *E2F1* and *E2F2* mRNA accumulation induced by the 3 types of DNA damage (Fig. 1B). These data suggest that the upregulation of *E2F1* and *E2F2* mRNA by genotoxics is due to enhanced transcription.

In light of these findings, we examined whether the increases in *E2F1* and *E2F2* mRNA levels resulted in an elevation of their protein levels. Western blot assays on SH-SY5Y cells revealed an increase in E2F1 and E2F2 proteins upon exposure to each of the genotoxics tested (Fig. 1C). Surprisingly, while the induction of E2F1 was expected due to its increased transcription and the reported stabilization of the protein,^{12,17,18} the induction of E2F2 was detected not only in neuronal cells, but also in HEK293 cells (Fig. S4) where we did not observe an increase in *E2F2* mRNA level in response to the DNA damaging treatments (Fig. S1B), suggesting a stabilization of the protein after the genotoxic insult. To test this notion, we evaluated whether ectopically expressed E2F2 could be stabilized by UV light. Western blot assays on SH-SY5Y cells transfected either with E2F1-GFP or E2F2-GFP vectors and exposed to UV showed a significant increase in the exogenously expressed E2F1 or E2F2 detected with anti-GFP tag antibody (Fig. 1D, E). Since the expression of these E2Fs is driven by a promoter that does not respond to UV treatment (Fig. 1F), these results suggest that E2F2 levels are increased by a posttranslational mechanism in response to DNA damage, and confirm E2F1 stabilization by genotoxic stress as previously reported.^{12,17,18}

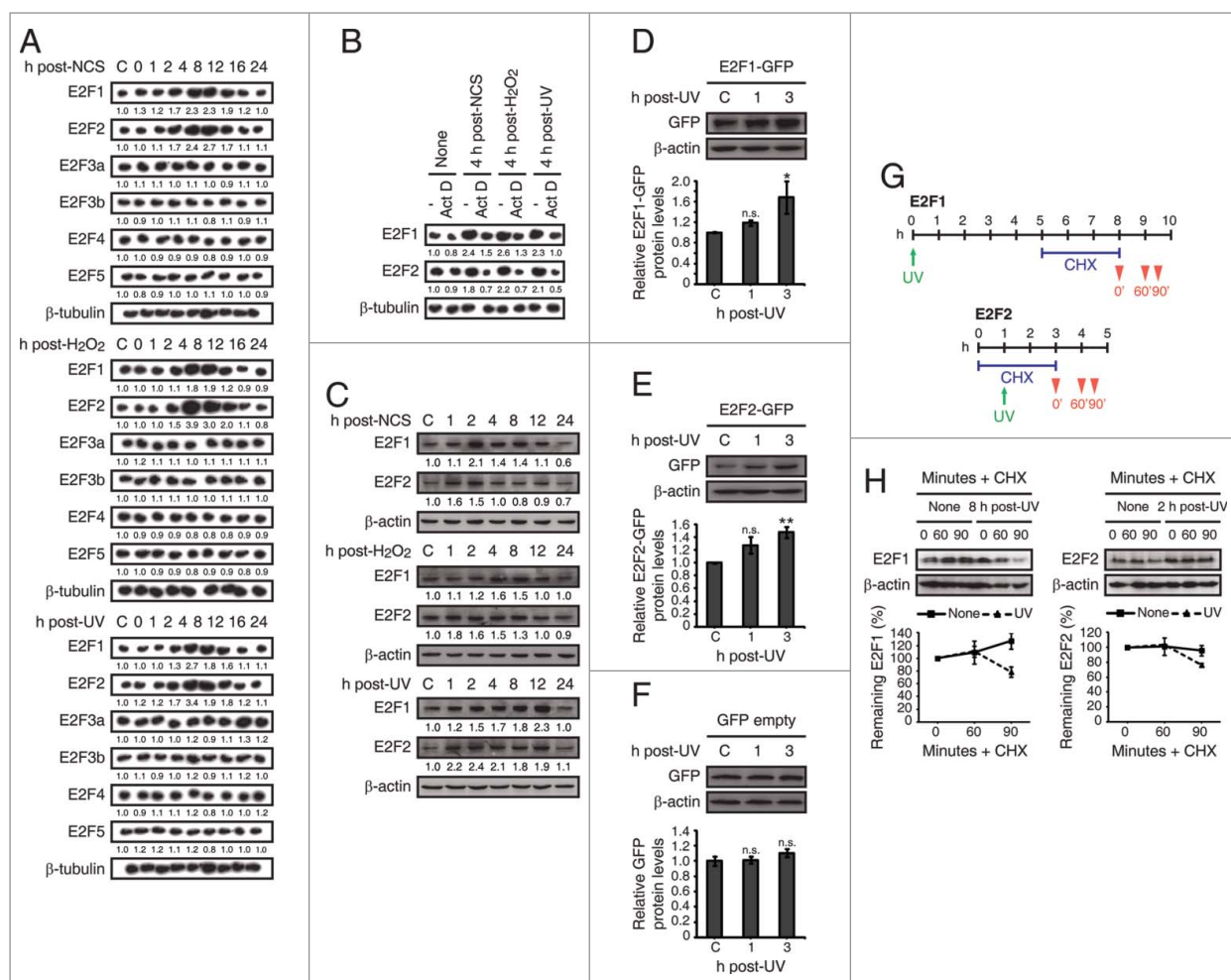


Figure 1. *E2F1* and *E2F2* mRNA and protein levels increase following DNA damage in neuronal cells. **(A and B)** Northern blot analysis of SH-SY5Y cells treated with NCS, H₂O₂ or UV and harvested at the specified times. Total RNA was extracted from cells and subjected to Northern blot with the [³²P]-labeled probes shown in the left margin. In **(B)**, cells were pre-incubated 3 h with 1 μM actinomycin D (Act D). The numbers under the bands indicate *E2F1–5* quantitation normalized to β-tubulin and control in **(A)**, and *E2F1* and *E2F2* quantitation normalized to β-tubulin and None (-) condition in **(B)**. **(C)** Western blot of *E2F1* and *E2F2* in SH-SY5Y cells treated with NCS, H₂O₂ or UV and harvested at the indicated times. The numbers under the bands indicate *E2F1* and *E2F2* quantitation normalized to β-actin and control. **(D–F)** Immunoblot of GFP in SH-SY5Y cells expressing *E2F1*-GFP **(D)**, *E2F2*-GFP **(E)** or pEGFP-C1 empty vector **(F)** and harvested at the indicated times post-UV. **(G and H)** Western blot of *E2F1* and *E2F2* in SH-SY5Y cells pre-incubated 3 h with 10 μM cycloheximide (CHX) and harvested at the specified times after genotoxic treatment, as shown in **(G)**. In **(D–F, H)**, data represent the mean ± S.E.M. of at least 4 independent experiments for **(D and E)** and *n* = 3 for **(F and H)**. In **(D–F)**, *P*-values were calculated by one-way ANOVA, Dunnett's: **P* < 0.05, ***P* < 0.01, n.s. not significant. C, control mock-treated cells.

Finally, to assess whether *E2F1* and *E2F2* increases upon genotoxic stress were a consequence—at least partially—of *de novo* protein synthesis, we examined the effect of inhibiting this process by treating cells with cycloheximide. Cells pre-incubated for 3 h with cycloheximide were harvested and analyzed by Western blot assays 0, 60 and 90 minutes after the times maximum proteins levels had been detected post-genotoxic treatment: 8 h for *E2F1* and 2 h for *E2F2* (**Fig. 1G**). Results revealed that *de novo* protein synthesis inhibition blocked *E2F1* and *E2F2* induction in response to UV irradiation (**Fig. 1H**). *E2F1* increase in mock-treated cells might be due to the unspecific stabilization by cycloheximide of some mRNAs.²⁹ Therefore, these results indicate

that *E2F1* and *E2F2* are *de novo* synthesized following DNA damage.

***E2F1* and *E2F2* induced by DNA damage are transcriptionally active**

To address whether the induced *E2F1* and *E2F2* are active in their transcription regulation functions, we initially transfected neuronal cells with p*E2F*-CAT or pΔ*E2F*-CAT reporter plasmids and treated them with the DNA damaging agents. p*E2F*-CAT encodes the chloramphenicol acetyltransferase (CAT) reporter gene driven by adenovirus E2 core promoter and 4 copies of the *E2F* DNA binding sequence, while pΔ*E2F*-CAT lacks them.³⁰

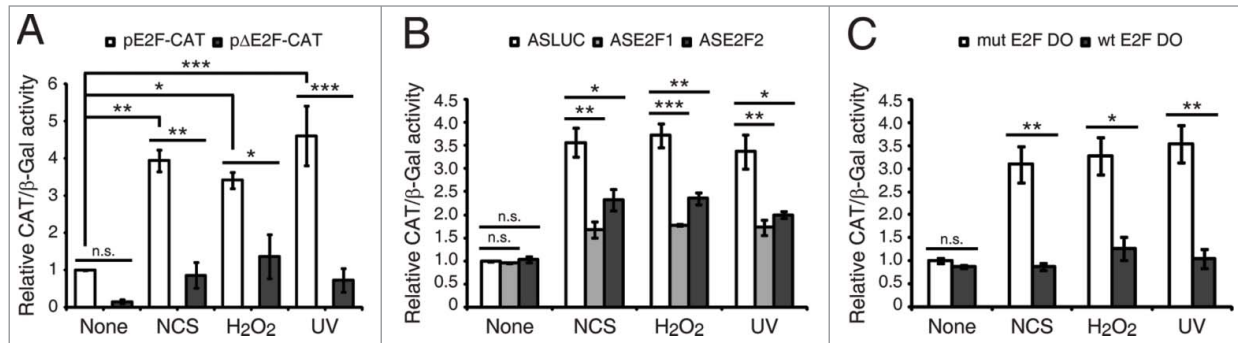


Figure 2. DNA damage induced E2F1 and E2F2 are transcriptionally active. **(A)** CAT activity of Neuro-2a cells transfected with pE2F-CAT or pΔE2F-CAT along with pCEFL-β-galactosidase, and harvested 24 h post-genotoxic treatment. **(B and C)** CAT activity of Neuro-2a cells transfected with pE2F-CAT, pCEFL-β-galactosidase and 1 μM of the indicated ODN, and harvested 24 h after DNA damage. In all cases, CAT activity was normalized to β-galactosidase activity. In **(B and C)**, results are expressed relative to None-ASLUC or None-mut E2F DO conditions. Data represent the mean ± S.E.M. of 3 independent experiments performed in triplicate. *P*-values were obtained using one-way ANOVA with Tukey's posttest in **(A)**, one-way ANOVA with Dunnett's posttest in **(B)** and Student's *t*-test in **(C)**: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, n.s. not significant. DO, decoy oligodeoxynucleotide.

When compared to mock-treated control cells, we observed an increase in pE2F-CAT activity in cells exposed to DNA damage (Fig. 2A). Consequently, we sought to distinguish between the contributions to this increased transactivation capability of the *de novo* synthesized E2F1 and E2F2 from that of the stabilized E2F after genotoxic treatment. To this purpose cells were transfected with pE2F-CAT along with E2F1 or E2F2 antisense oligodeoxynucleotides (ODNs) –ASE2F1 and ASE2F2 respectively– (Fig. S5), which were designed to block protein synthesis, or with wild-type E2F decoy ODN (wt E2F DO) containing E2F consensus sequences that would sequester cellular E2F away from its target gene promoters,³¹ hence abolishing both contributions. After subjecting these cells to DNA damaging agents, we observed that transfection with ASE2F1 or ASE2F2 led to an impaired CAT induction in response to the 3 genotoxic agents tested (Fig. 2B). Interestingly, a more pronounced diminution on the reporter activity was detected with wt E2F DO (Fig. 2C). Taken together, these data indicate that both transcription followed by

de novo protein synthesis and protein stabilization contribute to the pools of transcriptionally active E2F1 and E2F2.

E2F1 and E2F2 transcriptional induction in response to genotoxic stress requires ATM/ATR and MEK kinases

The cellular response to genomic instability implies the activation of a network of transduction pathways. To investigate which pathways are involved in *E2F1* and *E2F2* mRNA induction after genotoxic treatment, SH-SY5Y cells were incubated with specific inhibitors before exposure to the DNA damaging agents and harvested 4 h later, which was the time the maximum induction had been detected (Fig. 1A). Inhibition of both ATM and ATR kinases, ATM kinase alone or MEK kinase –with caffeine, KU-55933 or PD-98059 respectively– abrogated *E2F1* and *E2F2* mRNA increase after genotoxic stress (Fig. 3). Inhibition of PI3K or JNK kinases with LY-294002 or SP-600125 didn't have an effect on *E2F1* and *E2F2* transcriptional induction in response to DNA damage. Similar results were obtained with Neuro-2A and HN9 cells (Fig. S6). Therefore, these results imply that ATM/ATR and MEK kinases activities are required for the *E2F1* and *E2F2* transcriptional upregulation following DNA damage.

E2F1 and E2F2 induction prevents increased cellular DNA damage upon genotoxic stress

In view of these findings, we addressed the possibility that E2F1 and E2F2 may play a functional role in the neuronal cell response to genotoxic stress. Phosphorylation of histone H2AX on serine 139 (γH2AX) is a well-known indicator of genomic injury.³² We first analyzed the levels of DNA damage by measuring the intensity of γH2AX in cells that

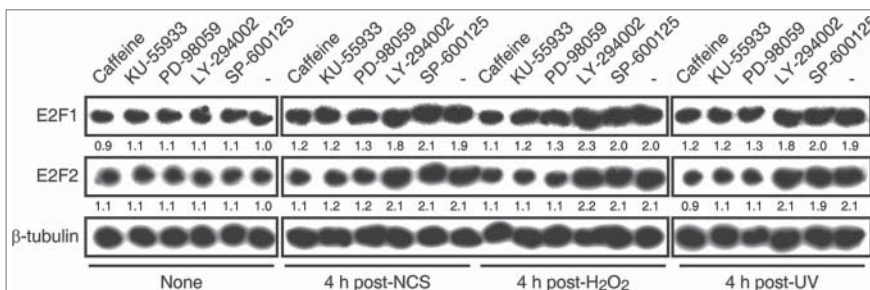


Figure 3. *E2F1* and *E2F2* transcriptional upregulation requires ATM/ATR and MEK kinases activity. SH-SY5Y cells incubated 1 h with 5 mM caffeine, 10 μM KU-55933, 10 μM PD-98059, 50 μM LY-294002 or 25 μM SP-600125 and harvested after a 4 h treatment with NCS, H₂O₂ or UV. Total RNA was extracted and subjected to Northern blot analysis with the [³²P]-labeled probes shown in the left margin. The numbers under the bands indicate *E2F1* and *E2F2* quantitation normalized to β-tubulin and None (-) condition.

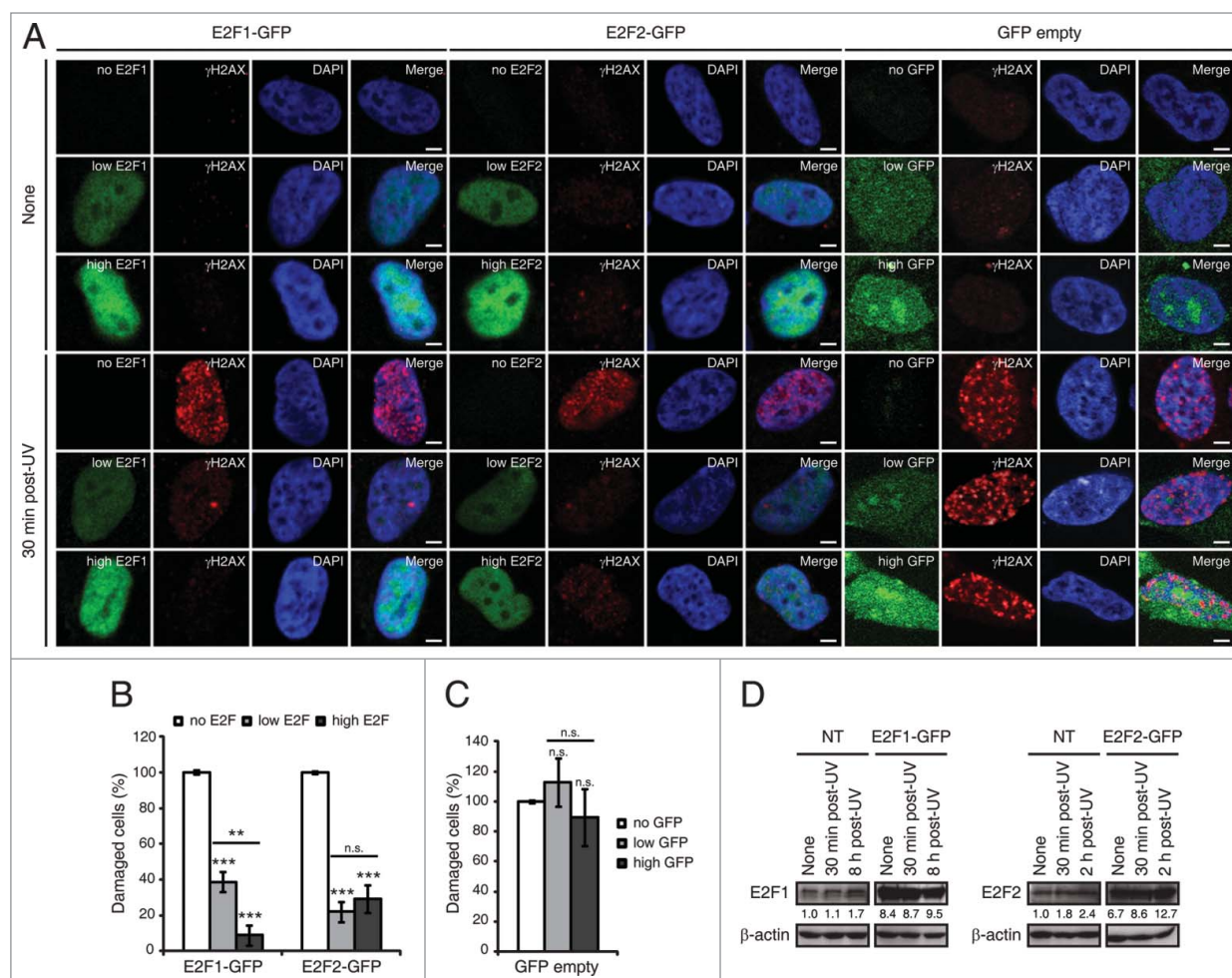


Figure 4. E2F1 and E2F2 upregulation reduces γ H2AX intensity following UV irradiation. **(A)** SH-SY5Y cells expressing E2F1-GFP, E2F2-GFP or pEGFP-C1 empty vector, fixed 30 minutes post-UV and immunostained with anti- γ H2AX antibody. Nuclei were visualized with DAPI staining. Scale bar, 10 μ m. **(B and C)** Percentage of damaged cells obtained by measurement of γ H2AX intensity levels. Quantifications were carried out classifying cells according to the E2F expression level: *no E2F*, *low E2F* or *high E2F* in **(B)**, or to the GFP expression level: *no GFP*, *low GFP* or *high GFP* in **(C)**. Results are expressed relative to mock-treated *no E2F* condition in **(B)** or mock-treated *no GFP* condition in **(C)**, which represent the 10% of the maximum γ H2AX intensity detected, and UV treatment was normalized to mock-treatment for each of the E2F **(B)** or GFP **(C)** intensity levels. Data represent the mean \pm S.E.M. of at least 4 independent experiments, in which 250 to 400 cells were analyzed for each condition. *P*-values were calculated by one-way ANOVA, Tukey's: ***P* < 0.01, ****P* < 0.001, n.s. not significant. **(D)** Immunoblot of E2F1 or E2F2 in SH-SY5Y cells transfected with E2F1-GFP or E2F2-GFP respectively, and in control not transfected (NT) cells. Cells were harvested at the indicated times post-UV. E2F bands correspond to the endogenous protein in NT and to the exogenously expressed protein in E2F-GFP. The numbers under the bands indicate E2F1 and E2F2 quantitation normalized to β -actin and NT-None condition.

overexpressed E2F1 or E2F2. To do this, cells were transfected either with E2F1-GFP, E2F2-GFP or GFP empty vector, fixed 30 minutes after UV irradiation and immunostained with anti- γ H2AX antibody (Fig. 4A). For the data analysis, cells were classified according to the E2F fluorescence intensity observed –no E2F, low E2F or high E2F–, which reflects the protein's expression level. Significantly lower γ H2AX intensity levels were observed in irradiated cells when E2F1 or E2F2 were upregulated (Fig. 4B). Besides, it is interesting to note the inverse correlation between E2F1 expression and γ H2AX staining. No reduction on γ H2AX intensity levels was detected by different expression levels of the GFP empty vector –no GFP, low GFP or high GFP– (Fig. 4C), indicating

that the observed decrease is indeed a consequence of E2F1 and E2F2 upregulation. Finally, overexpression was confirmed by E2F1 and E2F2 immunoblot of the endogenous and exogenously expressed proteins. There was a 8.4-fold and 6.7-fold increase in E2F1 and E2F2 exogenous proteins respectively compared to the endogenous proteins in basal conditions. These increases were higher than the levels observed for the endogenous proteins at the time post-UV cells were fixed (30 minutes) and also at the time maximum protein levels had been previously observed post-UV irradiation: 8 h for E2F1 and 2 h for E2F2 (Fig. 4D). Thus, these results suggest a role for E2F1 and E2F2 in protecting neuronal cells against the accumulation of DNA damage in cells exposed to UV.

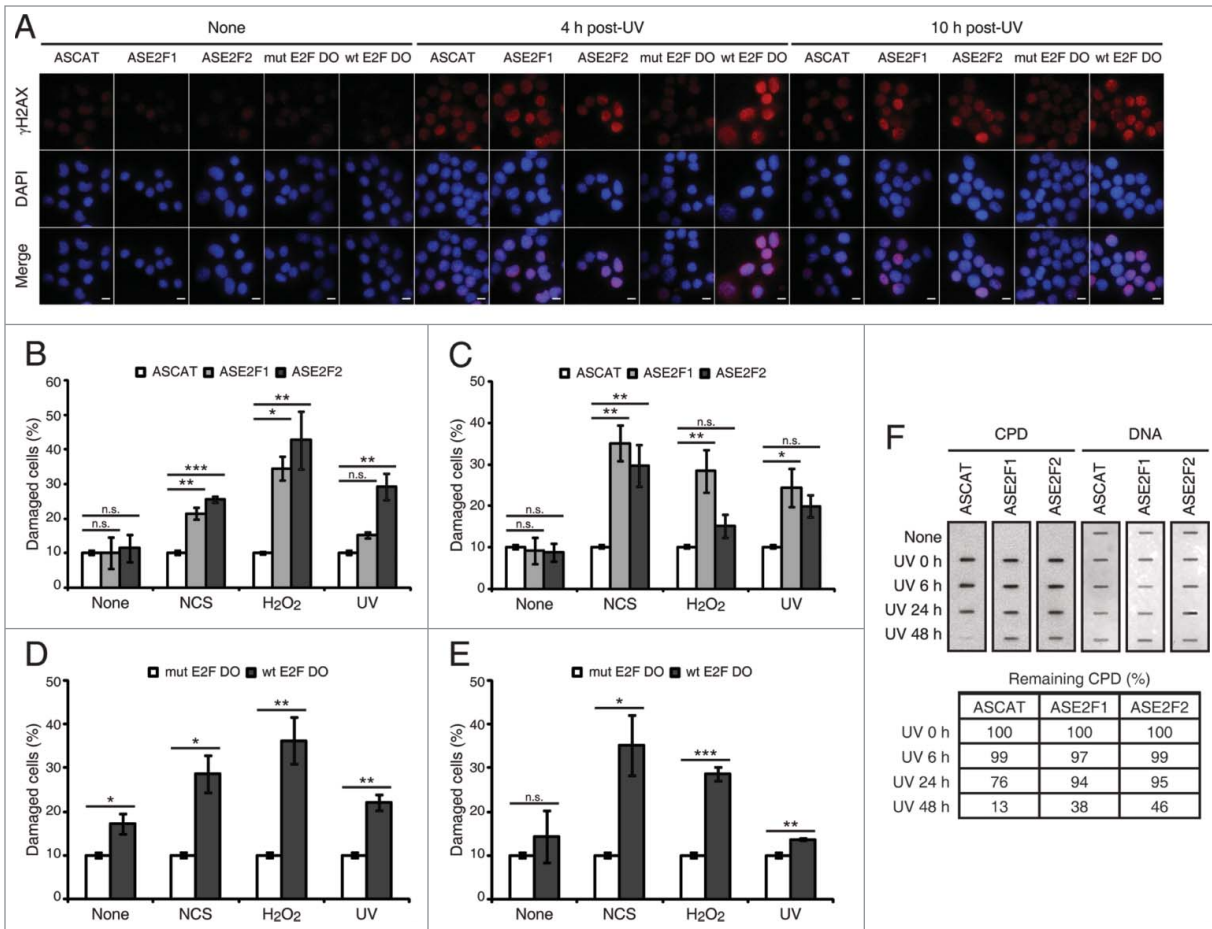


Figure 5. Blockade of E2F1 and E2F2 induction increases γ H2AX intensity and reduces DNA repair capability in response to DNA damage. **(A–E)** Neuro-2A cells transfected with 1 μ M of the indicated ODN, exposed to NCS, H₂O₂ or UV **(A)** for 4 h **(B and D)** or 10 h **(C and E)**, fixed and immunostained using anti- γ H2AX antibody. Nuclei were visualized with DAPI staining. Scale bar, 10 μ m. In **(B–E)**, data represent the mean \pm S.E.M. of at least 3 independent experiments, in which 300 to 1000 cells were analyzed for each condition. The percentage of damaged cells was obtained by measurement of γ H2AX intensity levels. Quantifications were carried out so that data is expressed relative to the control ODNs ASCAT or mut E2F DO, which represent the 10% of the maximum γ H2AX intensity detected. *P*-values were obtained by one-way ANOVA followed by Dunnett's posttest in **(B and C)** and Student's *t*-test in **(D and E)**: **P* < 0.05, ****P* < 0.01, *****P* < 0.001, n.s. not significant. **(F)** SH-SY5Y cells transfected with 1 μ M of the indicated ODN, UV-irradiated and harvested immediately (0 h) or at 6, 24 or 48 h post-irradiation. Genomic DNA was slot-blotted and analyzed by immunoblot for CPD photoproducts. Methylene Blue staining for total DNA was used as a loading control. The table indicates the average of 2 independent experiments of the percentage of remaining CPD photoproducts, obtained by CPD quantitation and normalization to total DNA. DO, decoy oligodeoxynucleotide.

Next, we decided to discriminate between the *de novo* protein synthesis from the stabilization contributions to this E2F protection capability against DNA damage. To assess this, cells were transfected with ASE2F1, ASE2F2 or wt E2F DO, exposed to genotoxics and analyzed for γ H2AX immunostaining (Fig. 5A; Fig. S7). Either at 4 h or 10 h post-genotoxic stress we observed an increased percentage of damaged cells in the presence of ASE2F1 or ASE2F2 (Fig. 5B and C), as well as with wt E2F DO (Fig. 5D and E). It is worth noting that the temporal analysis using the antisense ODNs revealed that *E2F1* and *E2F2* transcriptional induction plays a role in regulating the level of DNA damage in neuronal cells after genotoxic insult at different time points: E2F2 at an early and E2F1 at a later phase after DNA damage. Moreover, our data suggests that *de novo* protein synthesis is the major contribution in this

regulation. To summarize, these results demonstrate that the E2F1 and E2F2 increase upon genotoxic treatment, either from *de novo* protein synthesis or protein stabilization, reduces the accumulation of DNA damage.

Finally, to assess whether E2F1 and E2F2 affect DNA repair capability in global genomic repair, we measured the levels of UV induced-cyclobutane pyrimidine dimers (CPDs) in total DNA extracted from neuronal cells that have been transfected with ASE2F1 or ASE2F2 and exposed to UV light. Downregulation of E2F1 and E2F2 led to a delayed removal rate of CPDs (Fig. 5F). While removal of CPD lesions started at 24 h post-UV irradiation and were almost completely repaired at 48 h post-UV in control cells (ASCAT), significant amounts of CPD lesions were still detected at this time point in E2F1 and E2F2 downregulated cells (ASE2F1 and ASE2F2). Therefore, these

results show that E2F1 and E2F2 enhance the efficient removal of UV-induced CPD lesions.

E2F1 and E2F2 upregulation reduces apoptotic response after genotoxic injury

Cell death triggered by apoptosis is a common consequence of the excessive accumulation of DNA damage. We therefore investigated whether E2F1 and E2F2 downregulation affected the apoptotic response after genotoxic insult. To this purpose, we first measured caspase-3 activity in neuronal cells exposed to each of the 3 genotoxics. The results revealed that all treatments led to an increase in caspase-3 activity (Fig. S8). Next, we transfected cells with ASE2F1, ASE2F2 or wt E2F DO and treated them with H₂O₂, the genotoxic agent that triggered the maximum caspase-3 activity. Higher caspase-3 activity levels were observed in the presence of ASE2F1 and ASE2F2 or wt E2F DO after H₂O₂ exposure (Fig. 6A and B). To confirm these findings, we also analyzed caspase-3 cleavage through a Western blot assay in cells transfected with the ODNs and exposed to H₂O₂. Cells treated with ASE2F1, ASE2F2 or wt E2F DO had increased levels of cleaved caspase-3 (Fig. 6C), suggesting that impairment of E2F1 and E2F2 induction after genotoxic treatment triggers caspase-3 activation. Taken together, these findings point out a role for E2F1 and E2F2 in protecting neuronal cells from apoptosis induced by DNA damage.

E2F1 and E2F2 confer increased cellular resistance to DNA damaging agents

We next investigated a potential physiological function of E2F1 and E2F2 in neuronal cell response to genotoxic stress. Our findings implicating E2F1 and E2F2 in the reduction of the apoptotic response and in the protection of neuronal cells from DNA damage after genotoxic stress, raise the hypothesis that impairment of E2F1 and E2F2 induction might also affect cell viability in response to DNA damage. To test this, we measured cell survival by MTT reduction assay at different times during the 7 d following UV irradiation on cells that have been previously transfected with ASE2F1, ASE2F2 or wt E2F DO. A significant sensitization of cells transfected with ASE2F2 was observed, but not of those transfected with ASE2F1 (Fig. 7A). Cells transfected with wt E2F DO also showed a reduced percentage of viable cells (Fig. 7B).

To further analyze E2F1 and E2F2s long-term effects and biological relevance on cell viability, we performed a clonogenic assay. Neuronal cells were transfected with ASE2F1, ASE2F2 or

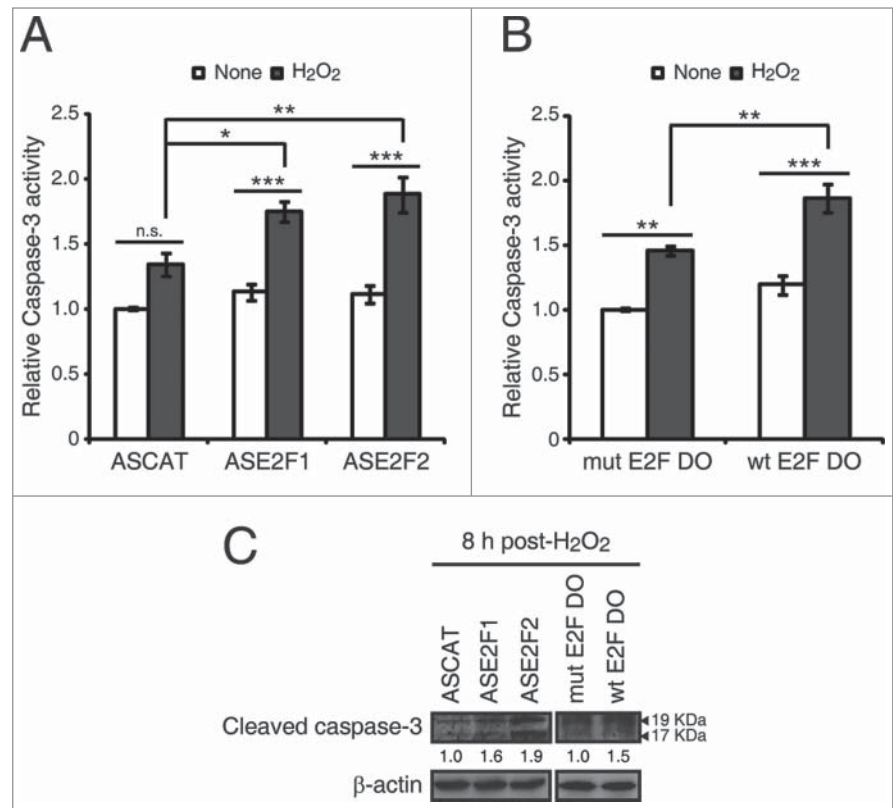


Figure 6. E2F1 and E2F2 reduce apoptotic response after genotoxic stress. SH-SY5Y cells transfected with 1 μM of the specified ODN and treated with H₂O₂. (A and B) Cell lysates examined for caspase-3 activity 24 h post-H₂O₂. (C) Western blot of anti-cleaved caspase-3 8 h post-H₂O₂. The numbers under the bands indicate cleaved caspase-3 quantitation normalized to β-actin and control ODN. In (A and B), data represent the mean ± S.E.M. of 4 independent experiments performed in duplicate. One-way ANOVA, Tukey's: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, n.s. not significant. DO, decoy oligodeoxynucleotide.

wt E2F DO and treated with each of the 3 genotoxics (Fig. 7C). Ten days later, we observed a reduced colony formation capability in cells transfected with ASE2F1 or ASE2F2, and also in cells treated with wt E2F DO (Fig. 7D and E). In summary, the experiments described above unveil a role for E2F2, and to a minor extent for E2F1, in cellular resistance to different genotoxic stresses.

E2F1 and E2F2 accumulate at sites of DNA lesion

To investigate the mechanism of action of E2F1 and E2F2 following DNA damage, we examined whether these proteins localize to the sites of DNA injury in neuronal cells. As a first approach, we analyzed whether E2F1 and E2F2 were recruited to chromatin upon genotoxic stress. To do this, we subjected cells to subcellular fractionation following genotoxic insult, and collected the cytoplasmic and chromatin fractions which were analyzed by electrophoresis. Immunoblots against E2F1 and E2F2 revealed an increase in E2F1 and E2F2 proteins in the chromatin insoluble fraction after treatment with each of the genotoxic agents, which peaks at 30 minutes post-DNA damage (Fig. 8A). Besides, quantification of the cytoplasm and chromatin-

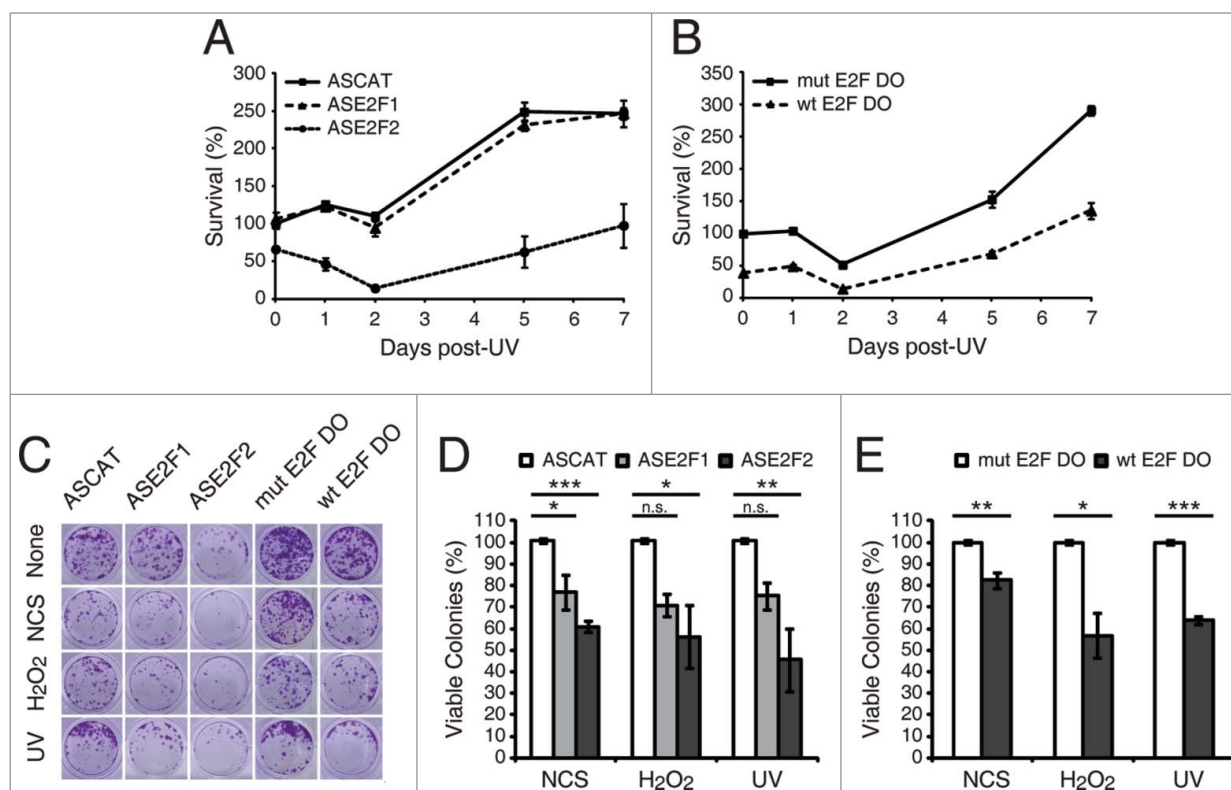


Figure 7. E2F1 and E2F2 confer cellular resistance to genotoxic stimuli. **(A and B)** Cell survival assessed by MTT reduction assay in SH-SY5Y cells transfected with 1 μ M of the specified ODN and exposed to UV irradiation. Data is representative of 4 independent experiments carried out in octuplicate. **(C–E)** Clonogenic assay in SH-SY5Y cells transfected with 1 μ M of the indicated ODN and treated with the DNA damaging agent. In **(D and E)**, results are expressed relative to the control mock-treated cells for each ODN, and data represent the mean \pm S.E.M. of 4 independent experiments performed in quadruplicate. *P*-values were calculated using one-way ANOVA with Dunnett's posttest in **(D)** and Student's *t*-test in **(E)**: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, n.s. not significant. DO, decoy oligodeoxynucleotide.

associated E2F relative percentages shows an enrichment of E2F1 and E2F2 in the chromatin fraction versus the cytoplasmic fraction following genotoxic injury.

To address the possibility that E2F2—as well as E2F1—accumulates at sites of DNA lesion, we performed microirradiation experiments with a 405 nm laser coupled with the photosensitizer Ro 19–8022 that promotes local formation of oxidative DNA damage,³³ in neuronal cells previously transfected with E2F1-GFP or E2F2-GFP. We observed recruitment of E2F1 and E2F2 to sites of DNA damage, but only in the presence of the photosensitizer (Fig. 8B–E; Movies S1, S2). These results suggest that both proteins localize to the sites of induced oxidized bases but not in the SSBs generated by the 405 nm laser.³⁴ Hence, in order to determine if E2F1 and E2F2 are recruited to sites of UV-induced lesions, we carried out a modified chromatin immunoprecipitation assay on cells transfected with E2F1-HA or E2F2-HA, after exposure to UV light. E2F1 and E2F2 were capable of pulling down DNA fragments that contained the CPD DNA photoproduct, characteristic of UV-induced DNA damage (Fig. 8F). JNK was used as a negative control of precipitation. Taken together, these results indicate that E2F1 and E2F2 accumulate at sites of oxidative and UV-induced DNA damage.

Finally, to study whether E2F1 and E2F2 interact with factors of the DNA repair machinery such as γ H2AX, we performed co-immunoprecipitation assays. Whole-cell extracts from neuronal cells harvested 1 h post-NCS treatment were immunoprecipitated with anti-E2F1 or anti-E2F2 antibodies and analyzed by immunoblot against γ H2AX. Results showed that γ H2AX co-immunoprecipitates with endogenous E2F1 and E2F2 (Fig. 8G). E2F4 family member was used as a negative control of co-immunoprecipitation since it didn't associate with γ H2AX in response to NCS-induced DNA damage. These results indicate an E2F1- γ H2AX and E2F2- γ H2AX interaction following genotoxic injury.

E2F2 promotes Rad51 foci formation and induces histone acetylation following DNA damage

E2F1's role at sites of DNA lesion has been described in previous work, but E2F2's nontranscriptional function is still unknown. It has been established that E2F1 promotes the recruitment of DNA repair factors, such as Rad51, to sites of DNA double-strand breaks.²⁵ Rad51 is the central recombinase in homologous recombination pathways.³⁵ To determine if E2F2 is also involved in Rad51 recruitment to sites of DNA lesion, we performed immunofluorescence assays in neuronal

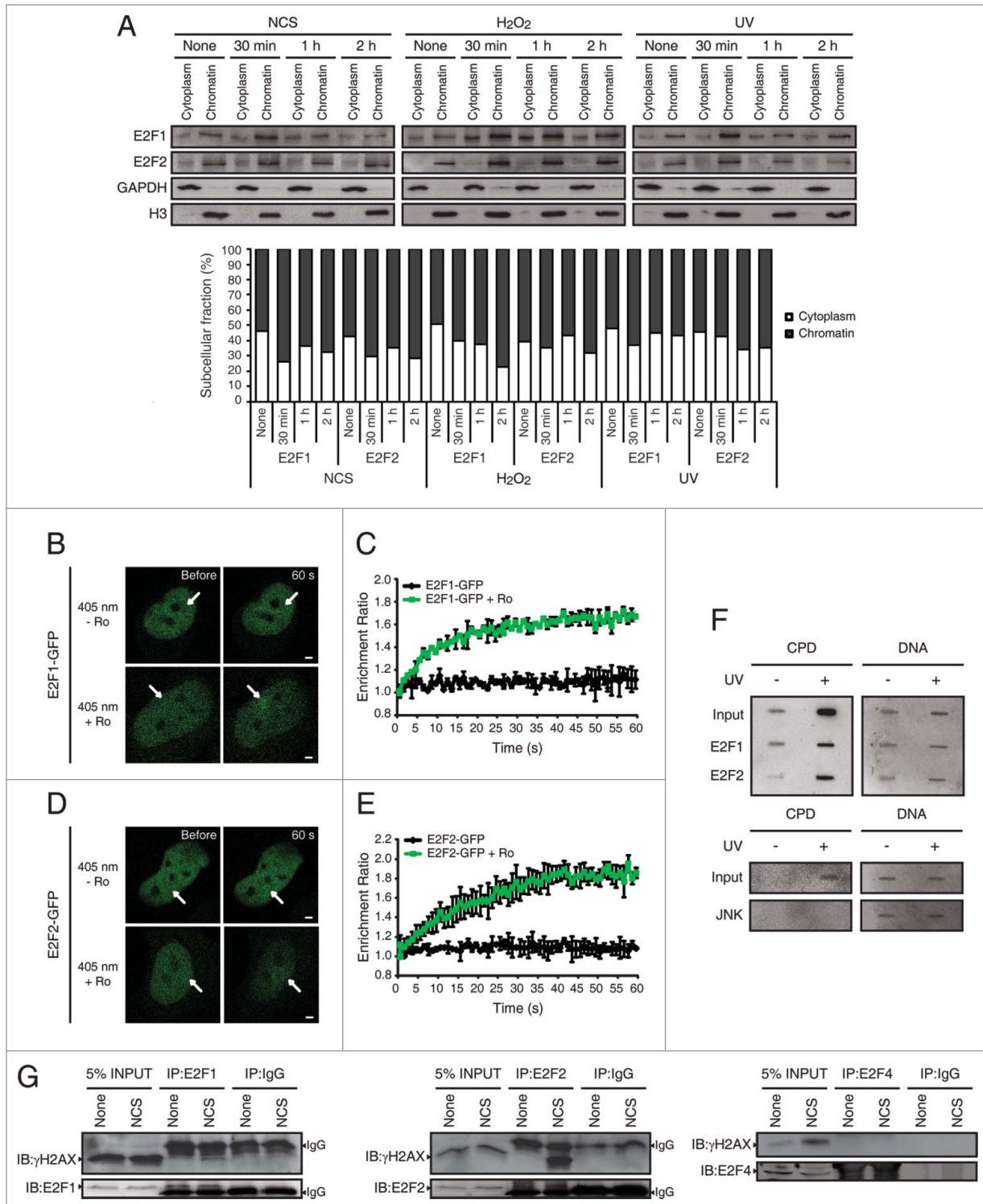


Figure 8. Accumulation of E2F1 and E2F2 at sites of DNA damage. **(A)** E2F1 and E2F2 Western blot of cytoplasmic and chromatin fractions of SH-SY5Y cells treated with genotoxic agents for the indicated times. GAPDH and H3 were used as cytoplasmic and chromatin specific markers respectively. Data represents the cytoplasm and chromatin-associated E2F relative percentages for each condition, obtained by normalization to GAPDH and H3 correspondingly. **(B–E)** Live-cell imaging of SH-SY5Y cells expressing E2F1-GFP or E2F2-GFP microirradiated with a 405 nm laser and pre-incubated or not with the photosensitizer Ro 19–8022 (Ro). In **(C and E)**, data represent the mean \pm S.D. of 2 independent experiments, in which 10 cells were analyzed for each condition. Arrows indicate the site of microirradiation. Scale bar, 2 μ m. **(F)** SH-SY5Y cells expressing E2F1-HA or E2F2-HA (upper panel) or JNK-HA (lower panel) were UV-irradiated or mock-treated, fixed, lysed and ChIP was carried out with anti-HA antibody. Pulled-down DNA was slot-blotted and analyzed by immunoblot for CPD photoproducts. Methylene Blue staining for total DNA was used as a loading control. **(G)** Co-immunoprecipitation assays of whole-cell lysates from SH-SY5Y cells harvested 1 h post-NCS treatment. Immunoprecipitation (IP) was performed with anti-E2F1, anti-E2F2 and anti-E2F4 antibodies and associated proteins were detected by immunoblot (IB). Non specific IgG isotype antibody served as IP control.

cells transfected with ASE2F2, fixed 1 h post-NCS treatment and analyzed for Rad51 immunostaining (Fig. 9A). Downregulation of E2F2 impaired Rad51 foci formation in response to NCS-induced DNA damage (Fig. 9B). To further confirm this result, Rad51 redistribution to sites of DNA damage was detected by biochemical fractionation with Triton X-100.³⁶ Neuronal cells with ASE2F2 harvested 1 h post-NCS treatment were fractionated into Triton soluble fraction, containing soluble cytoplasmic proteins, or Triton insoluble fraction that contained chromatin-bound proteins. Equal amounts of proteins from each fraction were analyzed by immunoblot against Rad51. Results revealed a decrease in Rad51 recruitment to chromatin in the Triton insoluble fraction in DNA-damaged cells transfected with ASE2F2 (Fig. 9C). Taken together, these results indicate that E2F2, as it was reported for E2F1, is also implicated in genomic stability maintenance through the recruitment of DNA repair factors to DNA double-strand breaks.

Previous work has demonstrated that E2F1 interacts with GCN5 acetyltransferase, promoting its recruitment to sites of DNA damage, and that both E2F1 and GCN5 are necessary to induce H3K9 acetylation in response to UV irradiation.²⁷ To evaluate if E2F2 is involved in a similar epigenetic mechanism, we first performed co-immunoprecipitation assays to determine

if E2F2 also interacts with GCN5 following UV-induced DNA damage. Results showed an association between E2F2 and GCN5 upon UV exposure (Fig. 9D). GCN5 has been implicated in the acetylation of both H3 and H4 histones after UV irradiation.³⁷ To assess whether loss of E2F2 affected global H4 acetylation in response to UV-induced DNA damage, we analyzed acetylated H4 protein levels by Western blot in neuronal cells downregulated for E2F2 and harvested 2 or 30 minutes following UV light exposure. We observed that H4 acetylation induction in response to UV irradiation is impaired in E2F2 downregulated cells (Fig. 9E). Therefore, these results suggest that E2F2, like E2F1, is involved in an epigenetic mechanism that promotes histone acetylation upon DNA damage, which in turn would facilitate repair by increasing DNA repair machinery accessibility to sites of damage.

Discussion

In this work we report that *E2F1* and *E2F2*, the latter specifically in neuronal cells, are transcriptionally induced in response to DNA damage. This novel mechanism, which is common to the response to various genotoxic stresses and is conserved in

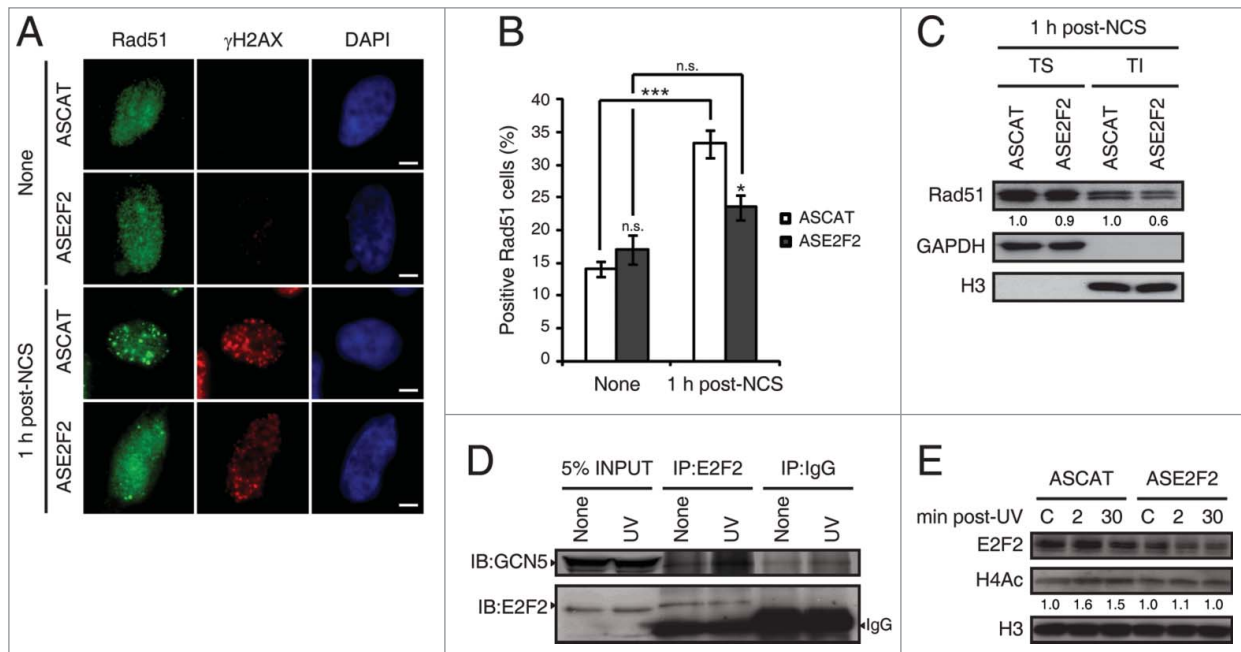


Figure 9. E2F2 promotes Rad51 foci formation and induces histone acetylation in response to DNA damage. **(A and B)** SH-SY5Y cells transfected with 1 μ M of ASE2F2, fixed 1 h post-NCS treatment and immunostained using anti-Rad51 and anti- γ H2AX antibodies. Nuclei were visualized with DAPI staining. Scale bar, 10 μ m. In **(B)** data represent the mean \pm S.E.M. of 4 independent experiments, in which 100 to 250 cells were analyzed for each condition. *P*-values were calculated using one-way ANOVA with Tukey's posttest: **P* < 0.05, ****P* < 0.001, n.s. not significant. Cells with 5 or more Rad51 foci were considered as positive Rad51 cells. **(C)** Rad51 immunoblot of Triton soluble (TS) and insoluble (TI) fractions of SH-SY5Y cells transfected with 1 μ M of ASE2F2 and harvested 1 h post-NCS treatment. GAPDH and H3 were used to detect soluble cytoplasmic and chromatin-bound proteins respectively. The numbers under the bands indicate Rad51 quantitation normalized to GAPDH or H3 in TS or TI fractions correspondingly, and ASCAT control condition for each fraction. **(D)** Co-immunoprecipitation assay of whole-cell lysates from SH-SY5Y cell harvested 30 minutes post-UV irradiation. Immunoprecipitation (IP) was performed with anti-E2F2 antibody and associated proteins were detected by immunoblot (IB). Non specific IgG isotype antibody served as IP control. **(E)** Western blot of acetylated H4 (H4Ac) in SH-SY5Y cells transfected with 1 μ M of ASE2F2 and harvested 2 or 30 minutes following UV light exposure. The numbers under the bands indicate H4Ac quantitation normalized to H3 and ASCAT-control condition. C, control mock-treated cells.

several species, contributes to increase E2F1 and E2F2 protein levels. Therefore, there are 2 parallel mechanisms that lead to the upregulation of E2F1 and E2F2 following DNA damage: the posttranslational modifications of the already synthesized E2F and consequent protein stabilization and, on the other hand, the transcriptional *E2F* gene induction and *de novo* protein synthesis. The resulting E2F1 and E2F2 act to promote DNA repair, leading to a reduced apoptotic response and an increased cell survival capability, thereby conferring resistance to genotoxic insult and cooperating in the maintenance of the genome integrity. It should be emphasized that we show for the first time that E2F2 is upregulated following genotoxic stress and plays a critical role in the DNA damage response.

E2F1 response to DNA damage has been subject of interest for many years. It has been well established that E2F1 undergoes posttranslational modifications –such as phosphorylation and acetylation– resulting in protein stabilization.^{12,17,18} Here, we show evidence that suggests that E2F2 is also stabilized by a post-translational mechanism in response to genotoxic stress. Further studies to determine the types of modifications and enzymes responsible for these modifications are required.

Our findings highlight a transcriptional mechanism for *E2F1* and *E2F2* induction upon DNA damage, which depends on ATM/ATR and MEK kinases activities. A recent study has reported an increase of the mRNAs of the 3 activating E2Fs –*E2F1*, *E2F2* and *E2F3a*– in Saos2 cells upon overnight doxorubicin treatment.¹⁴ We were unable to detect any *E2F3a* induction with the cell lines and genotoxic insults used. Besides, we only observed *E2F2* mRNA upregulation in neuronal cells. We don't know the reason for the discrepancy between the results, although we can speculate differences are due to the cell type analyzed and the dose of the DNA damaging agent used. Anyway, since we performed a broad study on the time course changes of the mRNA levels of *E2F1–5* in a variety of cells (diverse tissues and species) after exposure to several genotoxic agents, we propose that in response to DNA damage there is a general mechanism resulting in *E2F1* and *E2F2* transcriptional induction, the latter being restricted to neuronal cells. We presume that *E2F2* neuronal specificity upregulation might rely on EGR-1 activity. EGR-1 transcription factor, which is expressed in the nervous system, suits as a potential candidate since it has been shown to be upregulated –mRNA and protein– in response to DNA damage, and to behave as a prosurvival factor following ionizing and UV radiation.³⁸⁻⁴² Considering these evidence, we suggest EGR-1 as an upstream regulator of E2F1 and E2F2 induction upon DNA damage. Although EGR-1 has many potential binding sites in both E2F1 and E2F2 promoters, we speculate that in E2F2 promoter in neuronal cells it might associate with coactivators necessary to promote its transcription upon genotoxic stress. Additional studies are required to evaluate this hypothesis and elucidate the mechanism underlying the basis of neuronal specificity of *E2F2* transcriptional induction in response to DNA damage.

E2Fs transcriptional functions depend on the signals elicited by a particular type of DNA damaging agent.⁴³⁻⁴⁴ Many lines of evidence have indicated that the E2F1 protein increase in

response to genotoxic insult is associated to DNA damage-induced apoptosis.^{12,17,43,45} Although it was always believed that the induction of apoptosis is a unique function of E2F1, it was demonstrated that E2F2 and E2F3a can also activate pro-apoptotic genes.⁴⁶⁻⁴⁷ In contrast, here we show that the induced E2F1 and E2F2 reduce the apoptotic response following DNA damage. This is consistent with the dual role these E2Fs may play upon genotoxic stress, either promoting apoptosis or cell survival, and reinforces the notion that this depends on the cell type and the source and dose of DNA damage.⁴⁸ In line with our study, previous work has indicated a prosurvival role for E2F1. Transgenic E2F1 *-/-* mice show enhanced levels of keratinocyte apoptosis following UV-B radiation, whereas basal layer keratinocyte specific overexpression of E2F1 suppresses UV-B-induced apoptosis. Besides, inhibition of apoptosis induced by UV-B is correlated with increased efficiency on removal of DNA photoproducts.^{49,50} In addition, experiments have shown that in response to DNA damage and ATM phosphorylation, E2F1 apoptotic activities are inhibited by binding to TopBP1, and that this interaction results in E2F1 relocalization to BRCA1-containing repair complexes.⁵¹ E2F1 also participates in the recruitment of the Mre11 recombination/repair complex to replication forks.⁵² Furthermore, E2F1 regulates the transcription of the base excision repair gene XRCC1 and thus contributes to DNA repair.⁵³ Collectively, these findings point out a role for E2F1 in DNA damage checkpoints and/or repair. Consistently, our results provide evidence that E2F1 and E2F2 upregulation upon genotoxic insult protects neuronal cells from the accumulation of DNA damage. We can speculate that the observed reduction in the apoptotic response following genotoxic stress is a consequence of an increased DNA repair efficiency.

Interestingly, we show that E2F1 and E2F2 upregulation contributes to the maintenance of the genome stability. Activator E2Fs can behave both as oncogenes and tumor suppressors depending on the cellular context.^{54,56} The oncogene activity is probably due to their ability to promote cell proliferation, while the tumor suppression is believed to be a consequence of their pro-apoptotic functions. The fact that we observed that E2F1 and E2F2 reduce apoptosis, localize to the sites of DNA lesions and stimulate DNA repair in response to genotoxic stress, is in agreement with earlier evidence that supports that E2F1 tumor suppressor activity is in some cases unrelated to its apoptotic regulation but rather an outcome of its nontranscriptional functions that facilitate DNA repair. E2F1 is localized to sites of DNA double-strand breaks and UV-induced DNA damage, and promotes the recruitment of DNA repair factors and chromatin modifying enzymes.^{25,27,51} Our results add a new component to this puzzle, E2F2, which as E2F1, is recruited to sites of oxidative and UV-induced DNA damage, interacts with γ H2AX DNA repair factor and GCN5 acetyltransferase, induces histone acetylation and promotes Rad51 foci formation. We also demonstrate that upregulation of E2F1 and E2F2 protects against the accumulation of DNA damage in cells exposed to UV light, whereas downregulation of either E2F1 or E2F2 leads to increased levels of γ H2AX following NCS, H₂O₂ or UV exposure, and impairs the removal of CPD lesions after UV treatment. Therefore, these

results suggest 2 possible roles for E2F1 and E2F2 in DNA damage repair. First, a nontranscriptional function in which these E2Fs localize to sites of DNA lesion upon genotoxic stress, and promote the recruitment of DNA repair factors and chromatin modifying enzymes. Second, a transcriptional role involving the expression of prosurvival genes in response to DNA damage. Given that we show that the induced E2F1 and E2F2 are transcriptionally active, further experiments designed to determine which are the E2F1 and E2F2 target genes that promote cell survival following genotoxic insult are required.

DNA damage is a causal factor in neurodegenerative syndromes such as Alzheimer and Parkinson diseases, with patients having an impaired DNA repair ability in their neural tissues.⁵⁷ E2F1 and E2F2 represent potential targets for therapies to ameliorate the neurological symptoms of these diseases. Thereby, a deeper understanding of the fine molecular mechanisms that regulate these transcription factors' participation in the DNA damage cellular response is needed.

Materials and Methods

Cell culture, genotoxic agents and transfections

Neuro-2a murine neuroblastoma, HN9 murine hippocampal, HepG2 human hepatoma, and HEK293 human embryonic kidney cells were grown in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 1 mM sodium pyruvate at 37°C in a 5% CO₂ humidified atmosphere. The human neuroblastoma SH-SY5Y and rat pheochromocytoma PC12 cells were maintained in DMEM (45%) and HAM F-12 (45%) (Life Technologies) at 37°C in 5% CO₂ supplemented as indicated above. Rat primary hippocampal neuron cultures were obtained from Wistar embryos of 18–19 gestation days as previously described,⁵⁸ and were grown in Neurobasal medium (GIBCO) containing N2 supplement, B27 supplement, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 1 mM sodium pyruvate at 37°C in 5% CO₂.

When indicated, cells were treated with 1 µM actinomycin D (Sigma-Aldrich), 10 µM cycloheximide (Sigma-Aldrich), 50 ng/ml neocarzinostatin (Sigma-Aldrich), 100 µM hydrogen peroxide, or were irradiated in open dishes with UV-C (UV) 40 J/m², 254 nm (range 240–280 nm) at room temperature from a Philips ultraviolet lamp (TUV15WG15T8) calibrated to deliver 2.5 J/m² sec. After UV exposure the medium was replaced and cells were maintained at 37°C in 5% CO₂.

Cells were transfected either with polyethylenimine (PEI, Polysciences) or Lipofectamine 2000 reagent (Life Technologies), according to the manufacturer's instructions.

Oligodeoxynucleotides and plasmids

Single-strand oligodeoxynucleotides (ODNs) were synthesized with phosphodiester linkage by Bio-Synthesis (Lewisville, TX). Antisense ODN sequences are: ASE2F1: 5'-CCCAGAGCAGGGCTCCAGCGC-3' and ASE2F2: 5'-TCTGTGGG GCTCATCGCG-3'. ASCAT: 5'-TGAACTCACCCAGGG

ATTG-3' and ASLUC: 5'-GCATACGACGATTCTGTGATTTG-3' were used as internal controls. Circular dumbbell double-stranded decoy ODN³¹ wild-type E2F decoy: 5'-ATGCGC-GAAACGCGTTTTTCGCGTTTTTCGCGCATAGTTTTTCT-3' and mutant E2F decoy: 5'-ATCTAAACGCGTTTTTCGCGTT-TAGATTATAGTTTTTCT-3' were annealed and ligated for 24 h at 16°C with 1 unit of T4 DNA ligase (Life Technologies). The underlined sequences correspond to the E2F consensus binding sites. In all cases, cells were transfected at a final concentration of 1 µM ODN.

pE2F-CAT and pΔE2F-CAT plasmids were kindly provided by M. Imperiale.⁵⁹ The human E2F1 expression plasmid E2F1-GFP was generously supplied by D. Johnson.²⁶ To obtain E2F2-GFP fusion protein, the open reading frame of human E2F2 was inserted into a pEGFP-C1 vector (Clontech) at the BglIII/XbaI sites. E2F1-HA and E2F2-HA plasmids were a gift from M. Campanero.⁶⁰

RNA extraction and Northern blotting analysis

Total cellular RNA was isolated from cultures as described previously.⁶¹ Briefly, for Northern blotting analysis, 10 µg of total RNA were denatured, electrophoresed in 1% glyoxal-agarose gels, and transferred to nylon membranes (Hybond N⁺, GE Healthcare). Membranes were sequentially hybridized with the indicated [³²P]-labeled probes and radioactivity was detected using a PhosphorImager (FujiFilm BAS-1800II). Densitometric analysis was performed using the NIH ImageJ software.

Western blotting analysis

Cells were lysed with RIPA buffer (50 mM Tris-HCl pH 7, 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM EDTA pH 8 and 1X protease inhibitor cocktail (Calbiochem)) and equal amounts of protein were resolved on SDS-PAGE. After transfer to a nitrocellulose membrane (Hybond-ECL, GE Healthcare), analysis by immunoblotting was performed using 1:100 E2F1 (sc-251), 1:100 E2F2 (sc-9967), 1:1000 cleaved caspase-3 (Cell Signaling 9664), 1:3000 β-actin (sc-47778), 1:1000 GAPDH (sc-32233), 1:1000 Histone H3 (sc-8654-R), 1:1000 phospho-Histone H2A.X Ser139 (Upstate 05-636), 1:200 E2F4 (sc-866), 1:1000 Rad51 (sc-8349), 1:1000 GCN5 (sc-20698) and 1:2000 affinity purified polyclonal anti-GFP antibodies. Secondary antibodies were from Sigma-Aldrich. The signal was visualized with enhanced chemiluminescence reagent (GE Healthcare) and LAS-1000 Image Analyzer (Fujifilm). Densitometric analysis was performed using the NIH ImageJ software.

Chloramphenicol acetyltransferase assay

Neuro-2a cells were transfected with pE2F-CAT or pΔE2F-CAT along with pCEFL-β-galactosidase for chloramphenicol acetyltransferase (CAT) assay. After 18 h, cells were exposed to the genotoxic agents and harvested 24 h later. CAT activity was determined as previously described⁶² and normalized to β-galactosidase activity.

Immunofluorescence

SH-SY5Y or Neuro-2a cells were seeded onto glass coverslips and allowed to attach 24 h before transfection with the specified plasmid or ODN. Genotoxic treatment was performed 18 h later and cells were fixed and immunostained as described,⁶³ using 1:500 anti-phospho-Histone H2A.X Ser139 (Upstate 05–636) and 1:2000 Alexa Fluor 555 anti-mouse (Life Technologies) antibodies, or 1:1000 anti-Rad51 (Calbiochem PC130) and 1:2000 Alexa Fluor 488 anti-rabbit (Life Technologies) antibodies, and 1 $\mu\text{g}/\mu\text{l}$ DAPI to visualize nuclei. SH-SY5Y cells image acquisition was performed with a Leica confocal microscope SPE, and Neuro-2a slides were analyzed using an Eclipse E600W Nikon microscope and images were acquired with a Coolpix 5000 Nikon digital camera. γH2AX intensity measurements and Rad51 foci number determinations were performed with CellProfiler cell image analysis software.

Slot-blot DNA repair assay

SH-SY5Y cells were UV-irradiated and harvested at different time points post-irradiation. Genomic DNA was isolated and equal amounts (200 ng) of DNA were spotted onto a nylon membrane (Hybond N⁺, GE Healthcare) with a slot-blot device (Life Technologies). DNA was denatured by incubation of the membrane in 0.4 M NaOH for 20 minutes at room temperature. The filter was further baked at 80°C for 2 h. UV-induced DNA lesions were detected by immunoblot with 1:500 CPD antibody (Kamiya Biomedical, clone KTM53). The membrane was also stained with Methylene Blue (Merck Millipore) for loading control, according to the manufacturer's protocol.

Caspase-3 activity assay

SH-SY5Y cells were transfected with the specified ODN when indicated and exposed to genotoxic agents. Cells were harvested 24 h later and incubated with lysis buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8, 10 mM EGTA and 0.5 mM PMSF) at 37°C for 1 h with vigorous vortexing every 15 min, and centrifuged at 10000 $\times g$ for 15 min. The activity of caspase-3 in 150 μl cell lysate was determined using 150 μM of the synthetic caspase-3 substrate Ac-DEVD-pNA (Sigma-Aldrich) in reaction buffer (100 mM HEPES pH 7.5, 0.5 mM EDTA pH 8, 20% glycerol and 5 mM dithiothreitol) in a final volume of 300 μl and incubated at 37°C for 10 h. Color development was measured at 405 nm and caspase-3 activity was estimated as $A_{405}/\mu\text{g}$ protein h.

MTT assay

SH-SY5Y cells were transfected with the specified ODN as described above. After 18 h, UV irradiation was carried out and cells were further incubated for the indicated times. Cell activity was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay as described previously.⁶³

Clonogenic assay

SH-SY5Y cells plated as single cell in 24-well plates (aprox. 100 cells/well), were transfected with the indicated ODN and

18 h later were treated with the specified genotoxics. After 10 days, colonies were stained as previously described.⁶⁴

Chromatin isolation

Chromatin isolation was carried out as described,⁶⁵ with minor modifications. Briefly, 1×10^7 SH-SY5Y cells were resuspended in 300 μl of buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT and 1X protease inhibitor cocktail (Calbiochem)), 0.1% Triton X-100 was added and the cells were incubated on ice for 5 min. Nuclei were obtained in the pellet after low-speed centrifugation (4 min, 1300 $\times g$, 4°C). The supernatant was clarified following high-speed centrifugation (15 min, 16,000 $\times g$, 4°C) to collect the cytoplasmic soluble fraction. Nuclei were washed in buffer A, and lysed in 200 μl of buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT and 1X protease inhibitor cocktail (Calbiochem)) 30 min on ice. Insoluble chromatin was obtained by centrifugation (5 min, 1700 $\times g$, 4°C), washed in buffer B, centrifuged again and the final chromatin pellet was resuspended in 200 μl of Laemmli buffer and sonicated twice for 15 sec in a Fisher Sonic Dismembrator Model 300 sonicator at 50% power. Cytoplasmic and chromatin fractions were analyzed by immunoblotting (see *Western blotting analysis*).

Live-cell imaging and microirradiation

SH-SY5Y cells were transfected with E2F1-GFP or E2F2-GFP plasmids. Live-cell imaging was carried out as previously described,³⁴ with a Nikon A1 inverted confocal microscope with an environmental chamber that allows the control of temperature, humidity and gas conditions. Microirradiation was performed for ~ 6 sec in preselected regions of 1 μm^2 , with a 405 nm diode laser at 10% power. Confocal image series of a mid z -section were acquired every 1 sec with the 488 nm laser at 5% power, during the 6 sec before irradiation and for 1 min following irradiation. Fluorescence intensities of the microirradiated region were expressed relative to the immediate post-irradiation intensity for the recruitment kinetics analysis. Five minutes before irradiation, 5 μM Ro 19–8022 photosensitizer was added to the medium when indicated.

Chromatin Immunoprecipitation and Slot-blot

SH-SY5Y cells were transfected with E2F1-HA, E2F2-HA or JNK-HA plasmids, UV-irradiated or mock-treated and 30 min later in vivo cross-linking and chromatin immunoprecipitation (ChIP) was performed as previously reported,⁶⁶ with some modifications. In brief, cells were fixed with 1% formaldehyde for 10 min at room temperature and further incubated for 5 min with 0.125 M glycine solution at room temperature. Cells were lysed in RIPA buffer and sonicated 12 times for 15 sec in a Fisher Sonic Dismembrator Model 300 sonicator at 50% power. ChIP was carried out at 4°C overnight with 4 μl of HA antibody (Covance, clone 16B12) along with 20 μl of protein A/G PLUS-agarose beads (sc-2003). The precipitated DNA was reverse cross-linked, purified and quantified. UV-induced DNA lesions were detected as described for the *Slot-blot DNA repair assay*.

Co-immunoprecipitation

Whole-cell lysates (1 mg of protein diluted to 1 ml RIPA buffer) from SH-SY5Y cells treated with the DNA damaging agent and harvested at the indicated times were subjected to immunoprecipitation at 4°C overnight with 1 µg of E2F1 (sc-251), E2F2 (sc-9967) or E2F4 (sc-866) antibodies, along with 20 µl of protein A/G PLUS-agarose beads (sc-2003). Isotype IgG control antibody (sc-2025) served as control. The beads were washed 3 times in PBS, 20 µl of Laemmli buffer was added, and the samples were analyzed by immunoblotting (see *Western blotting analysis*).

Triton X-100 cell fractionation

SH-SY5Y cells were transfected with the indicated ODN and 18 h later were exposed to UV irradiation and harvested at the indicated times. Biochemical fractionation into Triton X-100 soluble and insoluble fractions was performed as previously described.⁶⁷ Equal amounts of proteins from each fraction were analyzed by immunoblotting (see *Western blotting analysis*).

Data analysis

Data analysis was performed with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Statistical differences were assessed by analysis of variance (ANOVA) with Tukey *post hoc* analysis for multiple comparisons or with Dunnett *post hoc* analysis for multiple comparisons to one control group. Student's

t-test was applied when only 2 independent groups were compared. *P*-values of <0.05 were considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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