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RESEARCH ARTICLE

Downregulation of E2F1 during ER stress is required to induce apoptosis

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ABSTRACT

The endoplasmic reticulum (ER) has recently emerged as an alternative target to induce cell death in tumours, because prolonged ER stress results in the induction of apoptosis even in chemoresistant transformed cells. Here, we show that the DNA-damage-responsive pro-apoptotic factor E2F1 is unexpectedly downregulated during the ER stress-mediated apoptotic programme. E2F1 decline is a late event during the ER response and is mediated by the two unfolded protein response (UPR) sensors ATF6 and IRE1 (also known as ERN1). Whereas ATF6 directly interacts with the E2F1 promoter, IRE1 requires the involvement of the known E2F1 modulator E2F7, through the activation of its main target Xbp-1. Importantly, inhibition of the E2F1 decrease prevents ER-stress-induced apoptosis, whereas E2F1 knockdown efficiently sensitises cells to ER stress-dependent apoptosis, leading to the upregulation of two main factors in the UPR pro-apoptotic execution phase, Puma and Noxa (also known as BBC3 and PMAIP1, respectively). Our results point to a novel key role of E2F1 in the cell survival/death decision under ER stress, and unveil E2F1 inactivation as a valuable novel potential therapeutic strategy to increase the response of tumour cells to ER stress-based anticancer treatments.

KEY WORDS: E2F, ATF6, ER Stress, UPR, Apoptosis

INTRODUCTION

Cancer refers to a large number of diseases characterised by the development of abnormal cells that divide in an uncontrollable manner and have the ability to escape cell death programmes. Multiple cancer-related genomic alterations that drive unrestrained cell proliferation bypass many checkpoints controlling cell cycle, DNA repair, differentiation and apoptosis. Thus, abnormalities in the apoptotic machinery often occur in tumours, causing a chemotherapy-resistant phenotype of transformed cells. DNA damage, together with intrinsic and extrinsic apoptotic pathways, have represented the main targets for clinical cancer therapy so far. In recent years, the endoplasmic reticulum (ER) has been extensively studied as an alternative opportunity to induce cancer cell death (Boelens et al., 2007; Corazzari et al., 2007). The ER compartment contributes to overall intracellular Ca^{2+} storage and is responsible for the folding of transmembrane, secretory and ER-resident proteins, and post-transcriptional protein modifications (Helenius, 1994). Many intra- and extracellular stimuli result in imbalance of the ER functions, causing the accumulation of unfolded or misfolded proteins and protein aggregates that are detrimental to cell survival, a state termed 'ER stress'. Eukaryotic cells have evolved an adaptive response to ER stress, commonly termed the unfolded protein response (UPR), consisting of: (1) general protein translation attenuation, (2) upregulation of ER-resident chaperones and (3) activation of a degradative pathway (ER-associated degradation – ERAD) to eliminate unfolded proteins by proteasomal degradation. All these activities are orchestrated by the cooperative action of three ER transmembrane proteins acting as stress sensors – PERK (also known as EIF2AK3), ATF6 and IRE1 (also known as ERN1), together with the ER-resident main chaperone GRP78 (also known as Bip). The aim of the ER stress response is to reduce the stress and thereby restore the folding capacity of this apparatus (Rutkowski and Kaufman, 2004). Although the UPR is generally viewed as a cytoprotective response, prolonged ER stress can activate cell death through both mitochondrial-dependent and -independent pathways (Breckenridge et al., 2003; Rao et al., 2002).

The Bcl-2 protein family plays a pivotal role in the regulation of cell death, including during ER-stress-mediated apoptosis. More than 20 members with either pro-apoptotic or anti-apoptotic functions are part of the family. In particular, members of the BH3-only subgroup inhibit the anti-apoptotic and activate the pro-apoptotic members of the family to induce cell death. Recent evidence supports an involvement of Bcl-2 family members in the maintenance of ER homeostasis and in stress signal transduction pathways, with the BH3-only proteins Bim, Puma and Noxa (also known as BCL2L11, BBC3 and PMAIP1, respectively) playing a pivotal role in ER-stress-mediated apoptosis (Li et al., 2006; Puthalakath et al., 2007; Reimertz et al., 2003). Several reports also support the hypothesis of an involvement of the transcription factor E2F1 in the regulation of both Puma and Noxa during the execution of the ER stress programme, but the molecular mechanism is still largely unclear (Futami et al., 2005; Park et al., 2006).

E2F1 belongs to the E2F family of transcription factors, which is composed of at least eight members that can be divided into two distinct subgroups of activators and repressors, on the basis of their structural and functional similarities (DeGregori and

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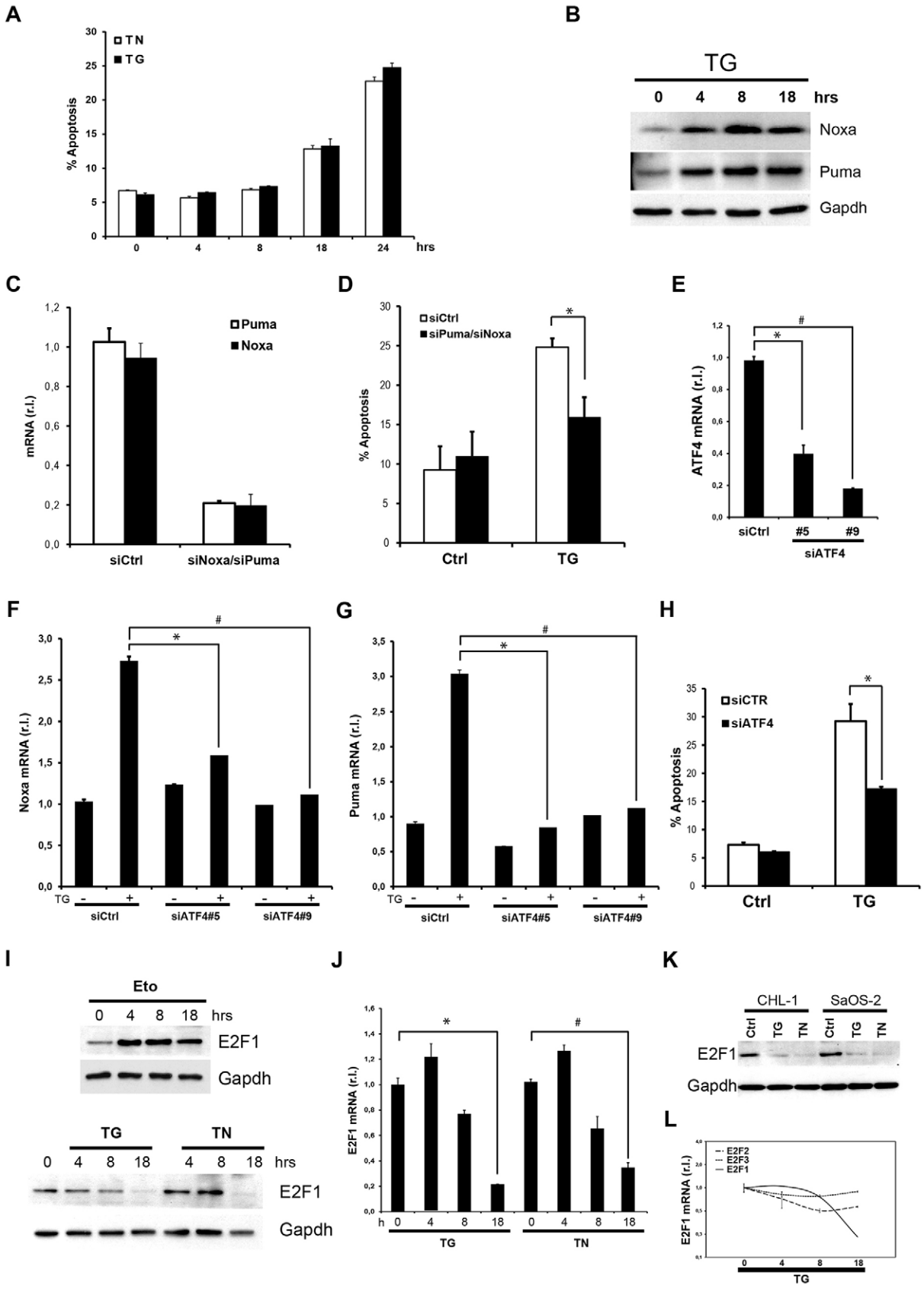

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Fig. 1. ER stress-mediated apoptosis results in ATF4-mediated early expression of Puma and Noxa, and late downregulation of E2F1. (A) Flow cytometric analysis of propidium-iodide-stained 2F cells incubated with thapsigargin (TG) or tunicamycin (TN), as indicated, shows increased cell death induction in a time-dependent manner. (B) Western blotting analysis shows the upregulation of Puma and Noxa protein levels in a time-dependent manner in 2F cells treated as in A. Gapdh is shown as a protein loading control in all blots. All western blotting data are representative of experiments performed three times. (C) 2F cells were transiently co-transfected with specific oligos for Noxa (siNoxa) and Puma (siPuma), and the expression of both factors was evaluated by qRT-PCR. r.l., relative levels. (D) 2F siCtrl and siNoxa/Puma cells were exposed to thapsigargin (24 h), and flow cytometric analysis of propidium-iodide-stained cells shows a decreased apoptosis induction in cells lacking both Noxa and Puma factors. (E) 2F cells were transiently transfected with specific oligos for ATF4 (siATF4#5; siATF4#9) and the expression of ATF4 was evaluated by qRT-PCR, confirming a specific downregulation of ATF4. Scrambled oligo was used as a negative control (siCtrl). (F,G) Cells in E were treated for 4 h with thapsigargin or left untreated, and the expression of Noxa (F) or Puma (G) was evaluated by qRT-PCR, revealing impaired thapsigargin-mediated early upregulation of both factors in the absence of ATF4. (H) 2F siCtrl and siATF4 cells were treated with thapsigargin and the induction of apoptosis was evaluated at 24 h post-treatment, revealing a decreased cell sensitivity to cell death induction in the absence of ATF4. (I,J) Western blotting (I) and qRT-PCR (J) analysis of E2F1 expression of 2F cells treated with thapsigargin, tunicamycin or etoposide (Eto), as indicated, shows a clear downregulation of E2F1 under ER stress conditions. (K) E2F1 protein levels were evaluated in melanoma CHL-1 and osteosarcoma SaOS-2 cells treated with thapsigargin or tunicamycin for 24 h or left untreated, by western blotting analysis, confirming data reported in I. (L) The expression of E2F1, E2F2 and E2F3 was evaluated in 2F cells treated or untreated with thapsigargin, as indicated, by qRT-PCR. All quantitative data show the mean±s.d. and are representative of three independent experiments performed in triplicate. $^{*}P<0.05$, $^{\#}P<0.05$ (Student's *t*-test).

Johnson, 2006). The E2F transcription factors modulate progression through the cell cycle by regulating the expression of genes required in the G1 to S phase transition (Dyson, 1998). A large number of studies clearly support the role of E2F1 and other family members as both tumour promoters and suppressors. It is now clear that this bifunctional role appears to be linked to the cell context as well as to the presence and the activity of other members of the E2F family and the E2F-interacting tumour suppressor pRb (Tsantoulis and Gorgoulis, 2005). E2F1 plays a key role in DNA-damage-induced apoptosis in a p53-dependent and -independent manner (Bates et al., 1998; Hiebert et al., 1995). However, it is still unclear whether E2F1 is involved in other stress signalling pathways, including ER stress.

In the present work, we demonstrate that upregulation of Puma and Noxa is strictly required for the apoptotic execution phase of ER stress. The early upregulation of both proteins is dependent on ATF4 activity, whereas late downregulation of E2F1 during the UPR is required to sustain the expression of both the BH3-only proteins, regulating the life/death switch during prolonged ER stress response. Moreover, we identified ATF6 and E2F7 as the key regulators of UPR-mediated E2F1 downregulation.

RESULTS

ER stress-induced apoptosis is mediated by ATF4-dependent upregulation of Puma and Noxa and by E2F1 downregulation

Although the role of the BH3-only proteins Puma and Noxa in the ER-stress-mediated apoptotic pathway is well known, the molecular mechanisms controlling the expression of such factors under conditions of ER stress remain only partially elucidated. To address this issue, we induced ER stress-mediated apoptosis in the human fibrosarcoma cell line 2FTGH (hereafter referred to as 2F cells), by using two well-known inducing agents – thapsigargin and tunicamycin (Kass and Orrenius, 1999) (Fig. 1A), and confirmed the upregulation of both Puma and Noxa in a time-dependent manner (Fig. 1B).

Next, 2F cells were transiently transfected with specific small interfering (si)RNA oligos for both factors (Fig. 1C) and cells were exposed to thapsigargin (24 h). Apoptotic rates evaluated with respect to control-siRNA-treated (siCtrl) cells revealed that the absence of the two proteins resulted in the inhibition of ER-stress-mediated apoptosis, indicating that both Puma and Noxa are required for the execution of ER-stress-mediated apoptosis (Fig. 1D).

To confirm the involvement of ATF4 in the ER stress-mediated upregulation of these BH3-only proteins (Armstrong et al., 2010; Galehdar et al., 2010; Qing et al., 2012), the expression of ATF4 was inhibited by transiently transfecting specific siRNA oligos into 2F cells (Fig. 1E), and the transcriptional levels of both Noxa and Puma were analysed upon thapsigargin treatment (4 h) in comparison to those of siCtrl cells. As reported in Fig. 1F,G, the expression of both factors was abrogated when thapsigargin-mediated ATF4 upregulation was inhibited. Moreover, the inhibition of ATF4 expression also resulted in decreased apoptosis induction after thapsigargin treatment, compared with that of control (Fig. 1H), confirming an active role of this transcription factor in the ER-stress-mediated cell death pathway.

Because E2F1 regulates the expression of BH3-only proteins under DNA-damage-induced apoptosis (Hershko and Ginsberg, 2004), we asked whether it could also contribute to the regulation of Noxa and Puma during the UPR. To test this hypothesis, we evaluated E2F1 mRNA and protein levels in 2F cells after 4, 8 and 18 h of thapsigargin and tunicamycin treatment, using etoposide as a non-ER stress-mediated stimulator of apoptosis. Unexpectedly, there was a significant decrease in E2F1 mRNA and protein levels between 8 and 18 h of thapsigargin or tunicamycin treatment (Fig. 1I, lower panel; Fig. 1J). By contrast, etoposide treatment promoted an upregulation of E2F1 (Fig. 1I, upper panel), suggesting that this downregulation represents a specific feature of the ER stress response. Downregulation of E2F1 under ER stress conditions was, in fact, confirmed in other cells, such as CHL-1 melanoma and SaOS-2 osteosarcoma cell lines, after an 18-h exposure to thapsigargin or tunicamycin (Fig. 1K).

In parallel, we evaluated the expression of the other two members of the same E2F1 subgroup of the E2F family, E2F2 and E2F3. Thapsigargin treatment only marginally affected the expression of these transcription factors, indicating that E2F1 is a specific target of ER stress among the E2F1 subgroup family members and also excluding compensatory effects by E2F2 and E2F3 during ER stress-induced apoptosis (Fig. 1L). Collectively, these data suggest that ER perturbation leading to activation of the UPR and cell death results in E2F1 downregulation due to its transcriptional repression.

Downregulation of E2F1 increases ER stress-mediated apoptosis

In order to evaluate whether E2F1 downregulation represents a key event for the induction of ER stress-mediated apoptosis or merely represents a consequence of ER stress or apoptosis induction, we tested whether the absence of E2F1 affects the apoptotic outcome induced by ER stress. To this end, we downregulated the expression of E2F1 by RNA interference (RNAi) and assessed the rate of cell death after the induction of

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ER stress. E2F1-specific siRNA oligonucleotides (siE2F1) were transiently transfected into 2F or SaOS-2 cells, and E2F1 expression was evaluated by measuring either protein or mRNA levels by western blotting or quantitative (q)RT-PCR analysis, respectively. Two independent siE2F1 oligos efficiently abrogated the expression of target protein (>75%), in both 2F and SaOS-2 cell lines (Fig. 2A,C). Then, 2F and SaOS-2 cells transiently transfected with control siRNA and siE2F1 (#1 or #2) were exposed to thapsigargin for 24 h, and cell death was quantitatively assessed by measuring the sub-G1 cell population of propidium-iodide-stained cells by flow cytometry. Interestingly, E2F1 downregulation significantly increased the apoptotic rate of both cell lines in response to thapsigargin exposure compared to that of control, whereas basal cell viability was not affected (Fig. 2B,D).

Finally, to show that the results were not affected by a compensatory upregulation of E2F2 or E2F3, we evaluated the levels of these transcription factors in control-siRNA- and siE2F1-transfected 2F cells by qRT-PCR. As shown in Fig. 2E, the absence of E2F1 does not impact on E2F2 or E2F3 expression. Collectively, these results indicate that E2F1 downregulation observed during ER stress is a necessary event to execute the apoptotic programme.

Xbp-1 regulates the expression of E2F1 through E2F7 under conditions of ER stress

To explore the mechanism(s) responsible for E2F1 transcriptional regulation under conditions of ER stress, we evaluated the involvement of transcription factors known to regulate the expression of this protein, such as E2F7 (Liu et al.,

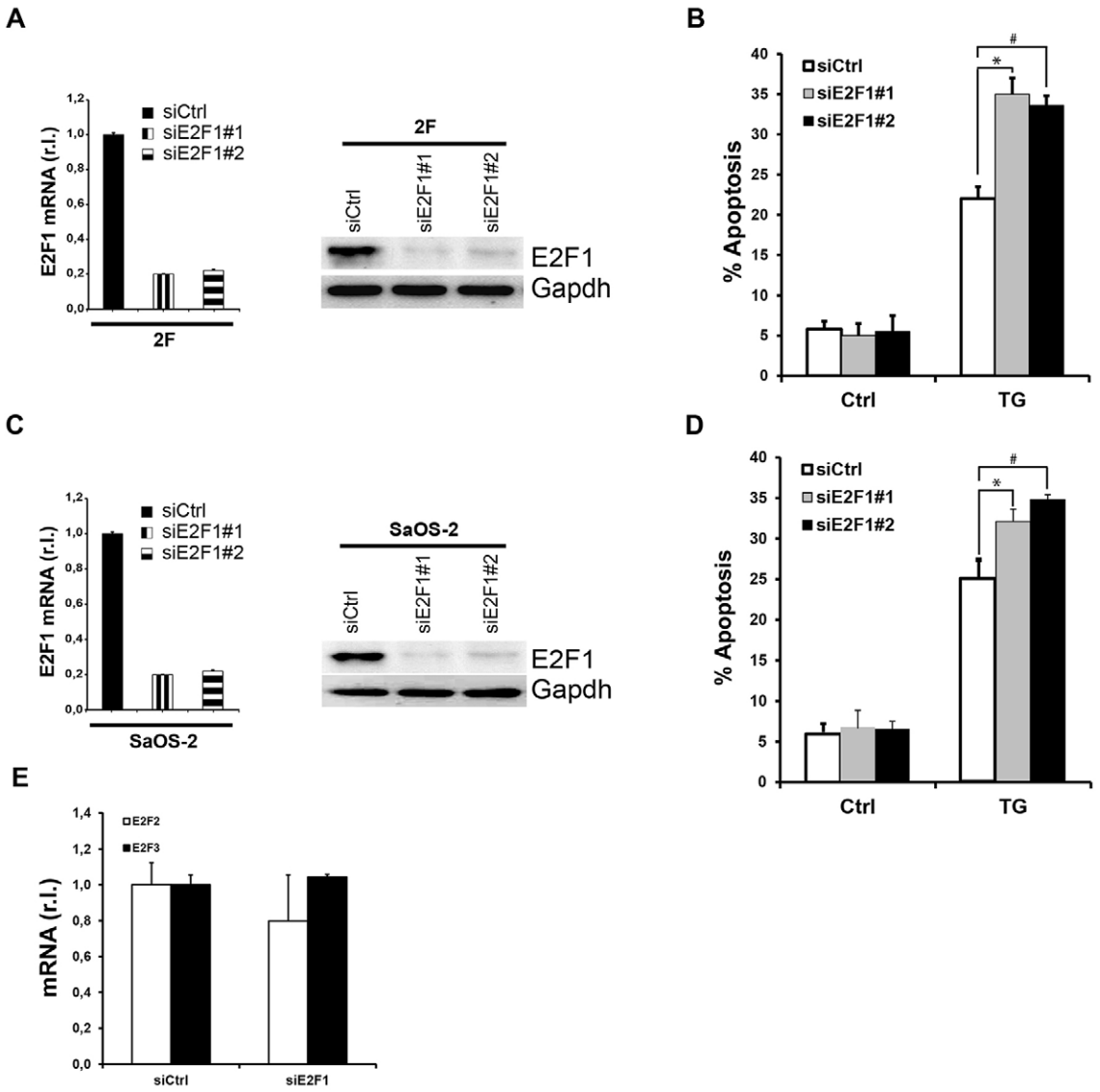

Fig. 2. E2F1 downregulation and UPR or apoptosis response. (A,C) E2F1 expression was downregulated by RNAi (siE2F1#1 and #2) and E2F1 mRNA (left panel) and protein (right panel) levels were revealed by qRT-PCR or western blotting analysis, respectively, in 2F (A) or SaOS-2 (C) cells. Scrambled oligo was used as a negative control (siCtrl). Gapdh is shown as a protein loading control. Western blotting data are representative of experiments performed three times. r.l., relative levels. (B,D) Cell death was measured by flow cytometric analysis at 24 h post-thapsigargin (TG) treatment under the same experimental conditions in 2F (B) or SaOS-2 (D) cells, indicating an enhanced susceptibility of siE2F cells to thapsigargin treatment. (E) The expression level of E2F2 or E2F3 was evaluated in both siCtrl and siE2F1 2F cells by qRT-PCR, revealing no compensative upregulation of either factor in the absence of E2F1. Quantitative data show the mean±s.d. and are representative of three independent experiments performed in triplicate. $^{*}P<0.05$, $^{\#}P<0.05$ (Student's *t*-test).

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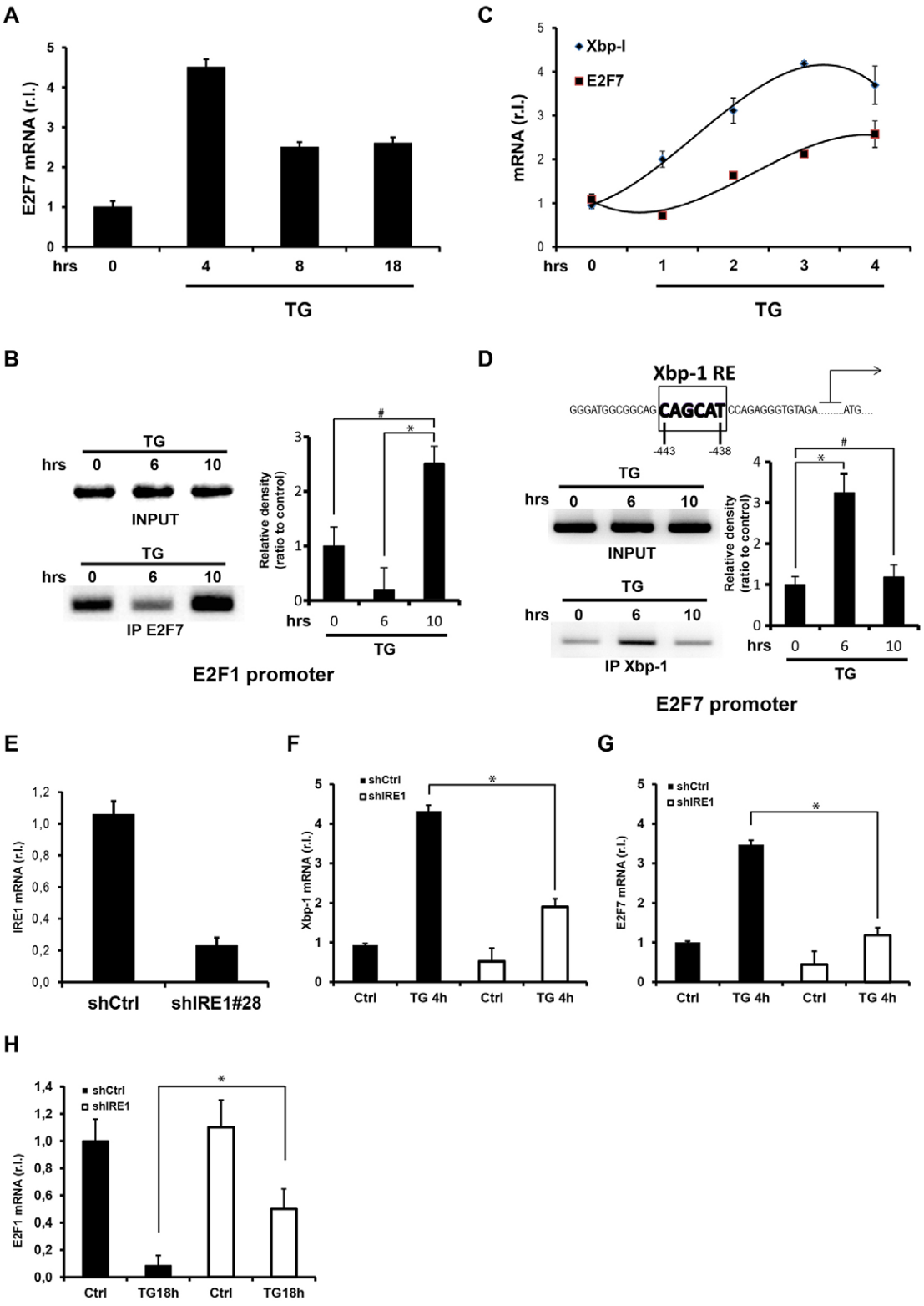

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2013; Panagiotis Zalmas et al., 2008), together with those activated under ER stress conditions, such as ATF6 (Haze et al., 2001).

It is well established that E2F7 is able to control the expression of E2F1 and, in turn, influence the ability of cells to respond to DNA damage (Panagiotis Zalmas et al., 2008). Thus, we evaluated the kinetics of expression of E2F7 in 2F cells treated with thapsigargin or left untreated. This evaluation was performed within 18 h of thapsigargin treatment, corresponding to the time during which we observed a

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Fig. 3. E2F7 contributes to E2F1 downregulation under conditions of ER stress. (A) 2F cells were treated with thapsigargin (TG) as indicated and qRT-PCR analysis shows the upregulation of E2F7. r.l., relative levels. (B) ChIP analysis of E2F7 transcription factor binding to the E2F1 promoter reveals an enhanced interaction at 10 h post-thapsigargin treatment. Left panel, representative analysis of an experiment performed three times. INPUT, total DNA. IP, immunoprecipitation. Right panel, graph showing densitometric analysis of three independent experiments. (C) The expression patterns of E2F7 and Xpb-1 were monitored in thapsigargin-exposed 2F cells by qRT-PCR, indicating an earlier upregulation of Xbp-1 compared with that of E2F7. (D) Schematic representation of the putative Xbp-1 responsive element (RE) in the E2F7 promoter region (upper panel). ChIP analysis of Xbp-1 transcription factor binding to the E2F7 promoter was evaluated (lower panel), indicating an enhanced interaction 6 h post-thapsigargin treatment. Lower left panel, representative analysis of an experiment performed three times. Lower right panel, graph showing densitometric analysis of three independent experiments. (E) 2F cells were infected with lentiviral particles carrying a specific shRNA for IRE1 (shIRE1#28), and the expression of IRE1 was evaluated by qRT-PCR. (F–H) 2F cells in E were treated with thapsigargin or left untreated, and Xbp-1 (F), E2F7 (G) or E2F1 (H) expression was evaluated by qRT-PCR analysis, showing an inhibited thapsigargin-dependent upregulation of all factors in shIRE1 cells compared with shCtrl ones. All quantitative data show the mean±s.d. and are representative of three independent experiments performed in triplicate. $^{*}P<0.05$, $^{\#}P<0.05$ (Student's *t*-test).

collapse of E2F1 expression (Fig. 1I–K). As reported in Fig. 3A, we observed a clear upregulation of E2F7 under thapsigargin treatment, with a peak of expression at 4 h. Therefore, to assess whether E2F7 binds to the E2F1 promoter during ER stress response, we performed a chromatin immunoprecipitation (ChIP) assay in 2F cells treated with thapsigargin for 0, 6 and 10 h, using a specific anti-E2F7 antibody. As shown in Fig. 3B, we confirmed that E2F7 is bound to the E2F1 promoter ($t=0$) (Panagiotis Zalmas et al., 2008); after thapsigargin exposure, we observed an initial slight dissociation of E2F7 from the E2F1 promoter (6 h), in line with a slight E2F1 mRNA upregulation reported in Fig. 1J after 4 h of thapsigargin exposure, whereas we registered a clear increased interaction between E2F7 and the E2F1 promoter at 10 h of thapsigargin exposure, correlating with the previously observed E2F1 expression decrease (Fig. 1I–L).

We thus focused on E2F7 expression regulation under ER stress conditions and performed a computational analysis of the E2F7 promoter region and identified the presence of a putative responsive element (RE) site for Xbp-1 (−443 to −438; Fig. 3D, upper panel), a factor known to be upregulated early during the UPR (Iwakoshi et al., 2003). We compared the kinetics of expression of Xbp-1 and E2F7 within 4 h of thapsigargin exposure, a time period corresponding to the peak of E2F7 expression, in 2F cells. This analysis revealed that Xbp-1 upregulation precedes that of E2F7, consistent with our hypothesis that Xpb-1 regulates the expression of E2F7 during ER stress (Fig. 3C). ChIP analysis of the E2F7 promoter with an anti-Xbp-1 antibody confirmed the interaction between Xbp-1 and the putative RE on the E2F7 promoter during the UPR response (Fig. 3D).

To confirm that Xbp-1 is upstream of E2F7, we inhibited the upregulation of Xbp-1 under ER stress conditions, by silencing the expression of IRE1 (Fig. 3E). We observed a clear inhibition of both Xbp-1 and E2F7 upregulation (Fig. 3F,G, respectively) in thapsigargin-treated cells expressing short hairpin (sh)RNA against IRE1 (shIRE1 cells), indicating that Xbp-1 is directly responsible for E2F7 expression regulation under ER stress conditions that, in turn, repress the expression of E2F1, as shown in Fig. 3H.

ATF6 is involved in E2F1 downregulation during ER stress-mediated cell death

ATF6 is constitutively synthesised as a type II transmembrane glycoprotein anchored to the ER membranes, and it is activated by proteolysis in response to ER stress (Haze et al., 1999; Haze et al., 2001). During the UPR, ATF6 translocates to the Golgi where is cleaved by site-1 (S1P) and site-2 (S2P) proteases (Ye et al., 2000). The N-terminal fragment (active form of ATF6) is thereby released from the membrane, enters the nucleus and interacts with target gene promoters by direct binding to a mammalian ER stress response element (ERSE) (Yoshida et al., 2000; Yoshida et al., 2001).

In order to evaluate the potential role of ATF6 in the E2F1 downregulation during ER stress-mediated apoptosis, we inhibited ATF6 activation using the serine protease inhibitor AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride] and evaluated E2F1 mRNA and protein levels after thapsigargin treatment, by qRT-PCR and western blotting analysis, respectively. AEBSF (Okada et al., 2003) inhibited the ER stress-induced proteolysis of ATF6, thus preventing its transcriptional activity, as demonstrated by the inhibition of both its self-upregulation (Fig. 4A, left panel) and upregulation of its main target Grp78 (Fig. 4A, middle panel). Interestingly, as shown in Fig. 4A (right panel) and Fig. 4B, AEBSF impaired the ER stress-mediated decrease in E2F1 expression at both mRNA (TG 18 h – 85% E2F1 repression, compared to 50% repression in the presence of AEBSF) and protein levels (TG 18 h – 90% E2F1 repression, compared to 40% repression in the presence of AEBSF). Moreover, the presence of AEBSF was also accompanied by a significant reduction in thapsigargin-induced cell death (Fig. 4C; TG – 24% apoptosis, compared to 14% in TG+AEBSF treated cells), indicating a potential direct correlation between the ER-stress-induced ATF6-mediated cell death and E2F1 downregulation.

To gain additional support for the direct role of ATF6 in the regulation of E2F1 expression during UPR-mediated cell death, a computational analysis of the E2F1 promoter region was performed and a putative ERSE site for ATF6 binding (−64 to −45) was identified (Fig. 4E, upper panel) close to the two known regulative E2F REs (E2F-A and E2F-B) (Araki et al., 2003). Therefore, a ChIP assay was performed using an ATF6-specific antibody. As shown in Fig. 4D, ATF6 binds to the E2F1 promoter after thapsigargin treatment in a time-dependent manner, thus correlating with the E2F1 mRNA decrease observed during thapsigargin treatment (Fig. 1J).

To further characterise the role of the putative ERSE sequence in the control of E2F1 expression, this site was mutated in a reporter plasmid carrying the luciferase gene under the control of the human E2F1 promoter containing the region 242 bp upstream of the transcription initiation site (ERSE-Mut; Fig. 4E, lower panel) (Araki et al., 2003). The wild-type ERSE (ERSE-WT) or ERSE-Mut E2F1 luciferase promoter constructs were transiently transfected into 2F cells, and luciferase reporter activity was analysed either before or 8 h after thapsigargin exposure. As reported in Fig. 4F, mutation of the ERSE site resulted in higher basal activity of the ATF6 promoter compared to that observed with ERSE-WT, and enhanced rather than decreased the activity of the promoter after thapsigargin exposure, completely abrogating the repression from ATF6. Combined with the ChIP analysis, these data confirm that active ATF6 binds to the ERSE motif on the E2F1 promoter, thus repressing the expression of E2F1 during UPR.

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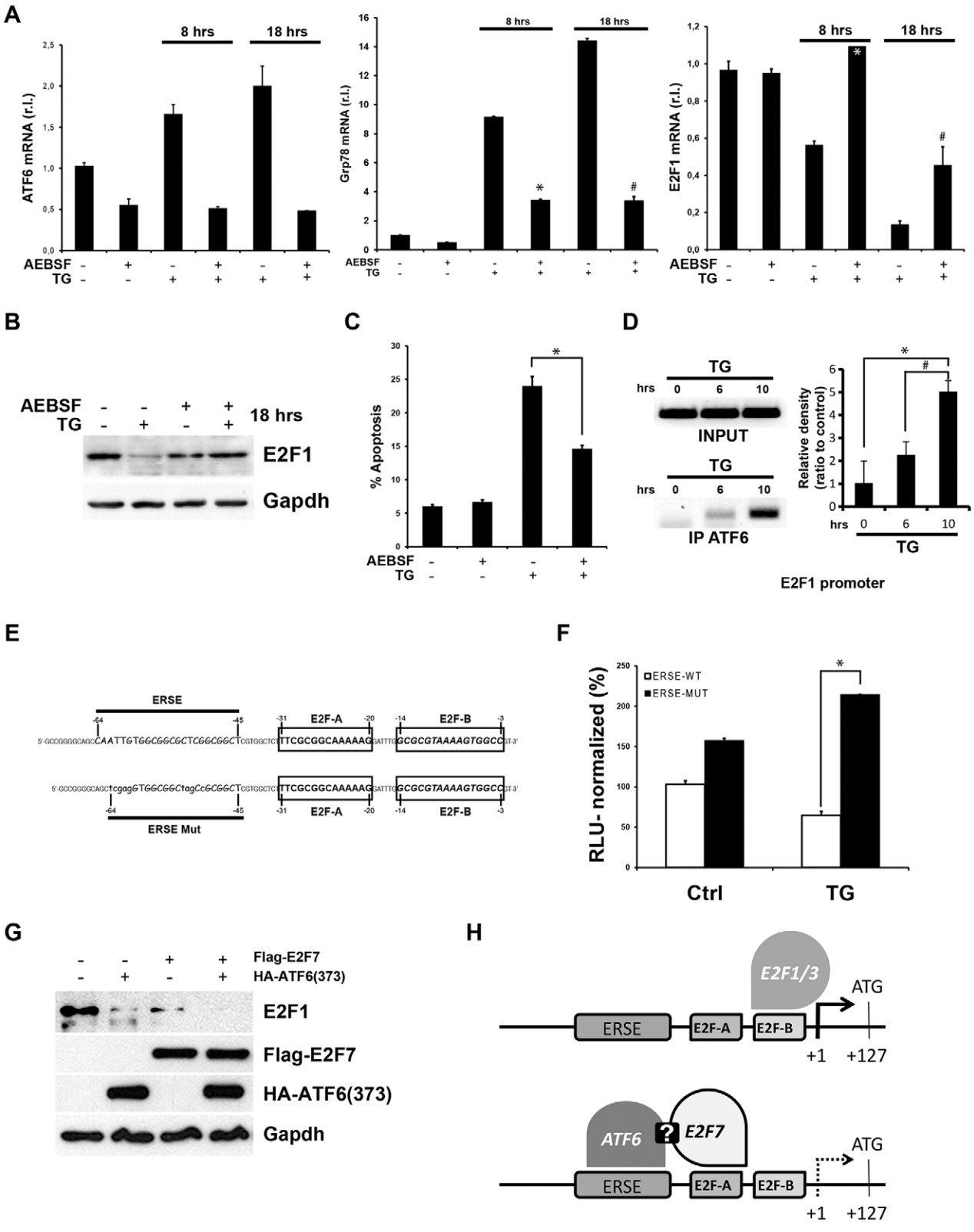

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Finally, to confirm that both E2F7 and ATF6 are concomitantly involved in the transcriptional repression of E2F1 under ER stress conditions, we transiently overexpressed E2F7 and active ATF6 either alone or in combination and evaluated the levels of E2F1 protein. As shown in Fig. 4G, E2F7 and active ATF6 were found to downregulate the expression of E2F1 when expressed

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Fig. 4. ATF6 contribution to E2F1 gene expression regulation during ER stress. (A) 2F cells were pre-treated for 1 h with AEBSF and exposed to thapsigargin (TG), as indicated. ATF6 (left panel), Grp78 (middle panel) or E2F1 (right panel) expression levels were evaluated by qRT-PCR, showing an inhibited thapsigargin-induced upregulation of both ATF6 and Grp78, and a decreased repression of E2F1, in the presence of AEBSF. r.l., relative levels. (B,C) E2F1 protein levels were measured by western blotting analysis in the presence or absence of both thapsigargin and AEBSF as indicated (B), confirming results reported in A. Western blotting data are from a representative experiment performed three times. Cell death was evaluated under the same experimental condition at 24 h post-treatment, by flow cytometric analysis (C), revealing a partial inhibition of thapsigargin-induced cell death in the presence of AEBSF. (D) ChIP analysis was performed to verify the binding of ATF6 to the E2F1 promoter. Results indicate a time-dependent increase in ATF6 binding to E2F1 promoter. Left panel, representative analysis of an experiment performed three times. INPUT, total DNA. IP, immunoprecipitation. Right panel, graph showing densitometric analysis of three independent experiments. (E) Schematic representation of the ERSE responsive element (RE) (ERSE, upper panel) and the mutant (ERSE Mut, lower panel) in the E2F1 promoter region. The E2F binding sites (RE) are also indicated (E2F-A and E2F-B). (F) *Renilla* and luciferase constructs containing the E2F1 promoters carrying the putative ATF6-binding site wild-type (ERSE-WT) or mutant (ERSE-MUT) were co-transfected into 2F cells. Cells were treated for 8 h with thapsigargin or left untreated as indicated. The luciferase assay reveals an abrogated ATF6-dependent repression of the E2F1 promoter. Relative luciferase units (RLU), normalised to *Renilla*, are shown as a percentage of the control (untreated cells). (G) 2F cells were transiently transfected with plasmids encoding Flag–E2F7 and/or HA–ATF6(373), and protein levels of E2F1, E2F7 or ATF6 were evaluated by western blotting analysis, revealing a complete repression of E2F1 expression in cells overexpressing both ATF6 and E2F7. (H) Schematic representation of the E2F1 promoter region carrying the ATF6 (ERSE) and E2F7 (E2F-A) binding sites. The question mark indicates a possible direct or indirect cooperative interaction between ATF6 and E2F7. All quantitative data show the mean±s.d. and are representative of three independent experiments performed in triplicate. *$P<0.05$, $^{\#}P<0.05$ (Student's *t*-test). In A, the asterisk (*) refers to AEBSF+TG compared to thapsigargin alone after 8 h, whereas the hash (#) refers to AEBSF+TG compared to thapsigargin alone after 18 h.

separately, whereas their coexpression resulted in complete abrogation of E2F1 expression. Collectively, these data indicate that ER-stress-mediated E2F7 upregulation and ATF6 activation are responsible for E2F1 transcriptional repression (Fig. 4H).

Downregulation of E2F1 does not affect the UPR

To further elucidate the role of E2F1 downregulation during ER stress-mediated apoptosis, we monitored the induction of the UPR by measuring the expression of key markers in thapsigargin-treated cells upon silencing of E2F1. Our results showed that E2F1 knockdown only slightly increases the capacity of the cell to mount an UPR under conditions of ER perturbation. In fact, thapsigargin treatment (4 h) of siE2F1 cells resulted in a modest increase in the levels of the analysed ER stress markers compared with those of the siCtrl cells – these markers included ERp57 (also known as PDIA1; Fig. 5A, upper left panel) (Frickel et al., 2004), ERdj5 (also known as DNAJC10; Fig. 5A, upper right panel) (Cunnea et al., 2003) and Xbp-1 (Fig. 5A, lower left panel) (Iwakoshi et al., 2003). It also resulted in enhanced Gadd153 (also known as DDIT3) expression (Fig. 5A, lower right panel) (Wang et al., 1996), possibly owing to the removal of known repression exerted by E2F1 on Gadd153 expression (Pan et al., 2010).

Given that E2F1 regulates the expression of pro-apoptotic BH3-only proteins during DNA-damage-induced cell death (Hershko and Ginsberg, 2004), we asked whether its downregulation could have had an influence on the regulation of Noxa and Puma under ER stress conditions. To this aim, we evaluated the mRNA levels of Puma and Noxa in 2F cells in which the expression of E2F1 was impaired by transient transfection of specific siRNA. As shown in Fig. 5B, the absence of E2F1 did not prevent the upregulation of these BH3-only proteins under ER stress conditions but, unexpectedly, it resulted in an increase in the expression levels of both Noxa and Puma, which could explain the enhanced susceptibility of these cells to apoptosis induction (Fig. 2), and possibly indicates that this factor negatively regulates the transcription of both of the BH3-only proteins.

These data also indicate that E2F1 and ATF4 might regulate the expression of these proteins at different levels. To demonstrate the independent regulation of the two BH3-only proteins by ATF4 and E2F1, 2F cells were transiently transfected with specific siRNA oligos for ATF4 or E2F1 individually or concomitantly, and the expression of Puma and Noxa was evaluated in the presence or absence of thapsigargin (4 h). As shown in Fig. 5C, (1) the absence of E2F1 per se confirmed enhanced basal expression of the two proteins but did not affect their thapsigargin-mediated upregulation, (2) the silencing of ATF4 abrogated the thapsigargin-mediated upregulation of both Puma and Noxa but did not affect their basal expression and, finally, (3) the concomitant absence of both E2F1 and ATF4 resulted in both enhanced basal expression and inhibition of thapsigargin-mediated expression of the two BH3-only pro-apoptotic proteins, indicating that Noxa and Puma are independently regulated by E2F1 and ATF4.

E2F1 expression is inhibited at the point of no return of the life/death switch during the ER stress response

Timely downregulation of E2F1 in advanced stages of the UPR could be required for the switch from the pro-survival to pro-death programme. To test this hypothesis, we evaluated the expression pattern of the main pro-apoptotic and anti-apoptotic actors of this process, in 2F cells exposed to thapsigargin in a timecourse experiment (0–18 h). As reported in Fig. 6A, we confirmed the upregulation of both E2F7 and ATF6 after thapsigargin exposure, highlighting an overlapping pattern of expression after 6 h of treatment, a time when E2F1 levels start to decrease, further confirming their cooperation in the transcriptional repression of E2F1. The early ATF4-mediated upregulation of Noxa and Puma (2–6 h) is followed by a sharp decrease in their expression until E2F1 is downregulated, after which the expression of Noxa and Puma remains constant until the end of the treatment (compare Fig. 6B,C). In parallel, we also observed an early increased expression of the pro-apoptotic factors Bax, Gadd153 and TRB3 (also known as TRIB3) (Fig. 6D), whereas expression of anti-apoptotic Bcl-2 decreased after 6–8 h of thapsigargin exposure (Fig. 6E, left panel). Similar to Puma and Noxa, the early upregulation of Bax, Gadd53 and TRB3 (2–6 h) is followed by a decrease in their expression until E2F1 is downregulated, after which their expression remains constant until the end of the treatment. This is particularly true for Bax and Gadd53. Moreover, the initially increased level of the anti-apoptotic Mcl-1 (Fig. 6E, right panel), which is induced by ATF4 – as shown by the lack of upregulation in absence of this transcription factor (Fig. 6F) – returned to basal levels after 6–8 h of thapsigargin exposure.

Finally, we performed a treatment and recovery experiment in which 2F cells were exposed to tunicamycin for the indicated

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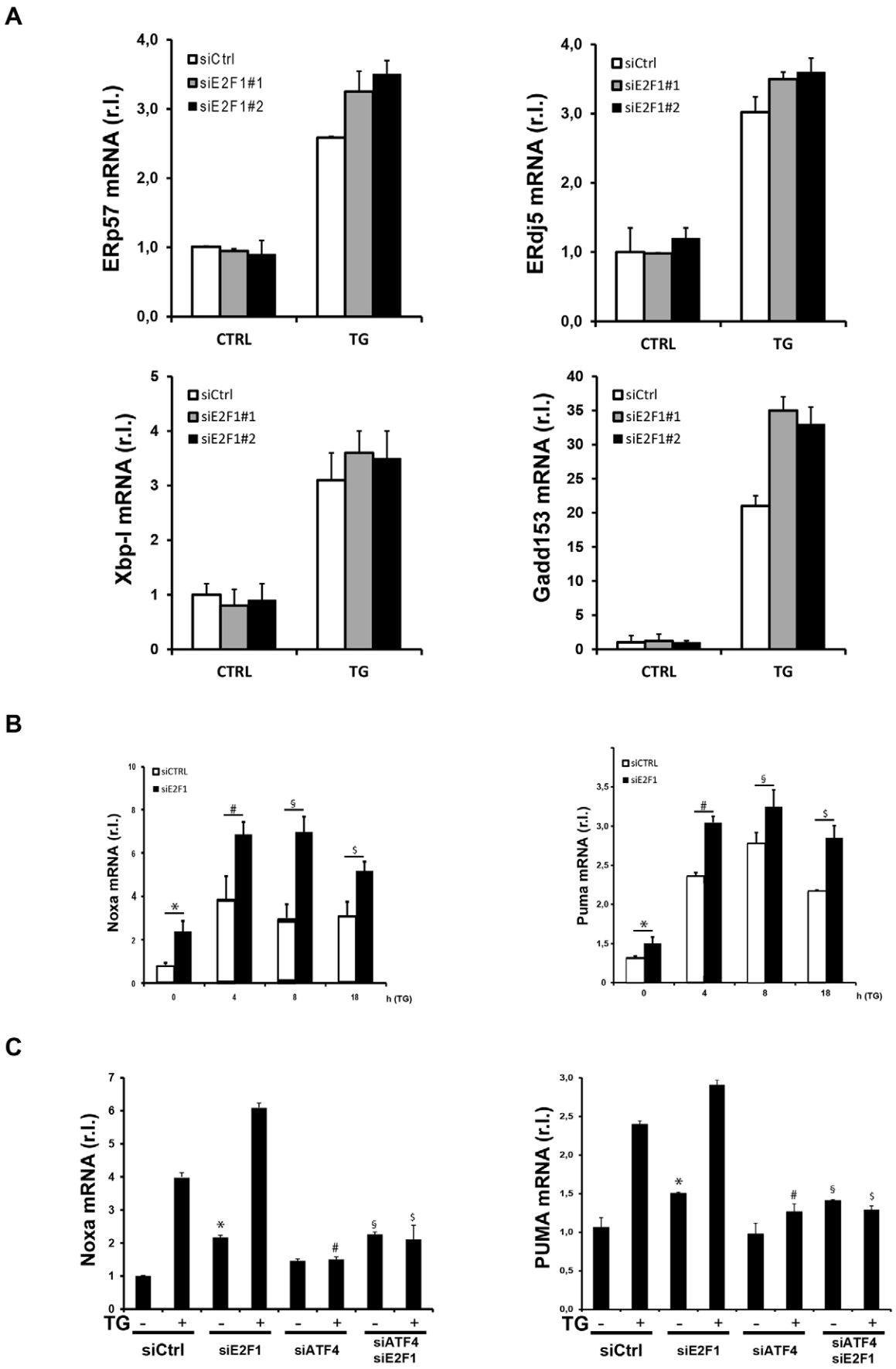

Fig. 5. See next page for legend.

Fig. 5. E2F1 does not interfere with the UPR and controls Puma and Noxa expression independently of ATF4. (A) E2F1 expression was downregulated in 2F cells by RNAi and mRNA levels of ERp57, ERdj5, Xbp-1 or Gadd153 were evaluated by qRT-PCR in treated [thapsgargin (TG), 4 h] or untreated cells, indicating no significant change in their expression, except for Gadd153. r.l., relative levels. (B) Levels of Noxa (left panel) or Puma (right panel) mRNA were also evaluated after thapsigargin exposure, as indicated, under the same experimental conditions as in A. (C) E2F1 and/or ATF4 expression were downregulated in 2F cells by RNAi, and mRNA levels of Noxa (left panel) or Puma (right panel) were evaluated by qRT-PCR in thapsigargin-treated (4 h) or untreated cells, indicating no overlapping signalling. All data show the mean±s.d. and are representative of three independent experiments performed in triplicate. $^{*}P<0.05$, $^{\#}P<0.05$, $^{\S}P<0.05$, $^{\$}P<0.05$ (Student's *t*-test). In C, *siE2F1−TG compared to siCtrl−TG; $^{\#}$siATF4+TG compared to siCtrl+TG; §siATF4/siE2F1−TG compared to siCtrl−TG; $^{\$}$siATF4/siE2F1+TG compared to siCtrl+TG.

time, then tunicamycin was removed and the cells were allowed to recover in culture until 24 h. Tunicamycin was used rather than thapsigargin because of its reversible effects on ER. We then evaluated both the induction of apoptosis (0–24 h) and mRNA levels of E2F1 (0–18 h), by flow cytometric analysis of propidium-iodide-stained cells or by qRT-PCR, respectively. As reported in Fig. 6G, we observed a complete cell recovery from ER stress between 0 and 8 h of tunicamycin treatment, whereas progressive apoptosis induction was observed after 10 h of tunicamycin exposure, paralleled by a progressive and dramatic E2F1 downregulation. Overall, these data support the hypothesis that the regulation of levels of anti-apoptotic versus pro-apoptotic factors during the UPR is responsible for the life/death cell decision. In this context, the inhibition of E2F1 expression during sustained ER stress contributes to the induction of apoptosis by regulating the expression of pro-apoptotic BH3-only proteins.

DISCUSSION

The ER represents a compartment that is able to sense several cellular stresses and, as a last resort, to trigger cell death (Rutkowski and Kaufman, 2004). However, the ER stress condition primarily represents a pro-survival cell response, resulting in the activation of a number of adaptive responses, collectively termed the UPR, to bring the cell back to homeostasis. In fact, the UPR inhibits pro-death signals unless the stimulation is prolonged and/or the damage exceeds the adaptive response, resulting in the induction of apoptosis (Verfaillie et al., 2013; Walter and Ron, 2011).

However, many human cancers show alterations in the UPR, allowing them to adapt to chronic stress to avoid cell death. Therefore, components of the UPR might represent a useful therapeutic target for cancer therapies. Thus, understanding the molecular mechanisms regulating the life/death response switch under conditions of ER stress is essential to allow the identification of novel strategies to overcome cancer cell death resistance.

Here, we show that two key events contribute to an efficient induction and execution of ER-stress-mediated cell death: (1) the upregulation of pro-apoptotic BH3-only family members Noxa and Puma and (2) the downregulation of the transcription factor E2F1.

We found that, under ER stress conditions, ATF4, one of the early transcription factors activated during the UPR, upregulates Noxa and Puma at both the mRNA and protein level. The key role of these players is shown by the fact that both their direct silencing or that of ATF4 results in a strong inhibition of ER-stress-mediated cell death. The residual level of apoptosis observed in the absence of these BH3-only proteins suggests that other ER stress-activated pro-apoptotic factors might be involved in this pathway, such as Bim (Puthalakath et al., 2007). These results are in line with data recently reported by Qing and colleagues, showing that glutamine deprivation induces apoptosis in Myc-overexpressing neuroblastoma cells through ATF4-dependent Puma and Noxa upregulation (Qing et al., 2012).

It is important to note that, although the pro-apoptotic signalling involving ATF4, Puma and Noxa is an early-activated system (2–4 h after the stimulus), the execution phase of the apoptotic pathway is a later event (18 h after stimulus), suggesting that a secondary signal is required to switch between the pro-survival and pro-death response. Indeed, at variance with those apoptotic pathways in which E2F1 expression is required for their execution, unexpectedly, we found that E2F1 levels decrease during ER-stress-mediated apoptosis, and that this downregulation importantly contributes to the time-regulated execution of the cell death pathway.

E2F1 is known to exert different effects on cell growth and apoptosis depending on the cell context. During cell cycle progression, transient activation of E2F1 promotes cell growth by driving the G1 to S phase transition, under the control of pRb, whereas its deregulated activity leads to uncontrolled cell proliferation, a hallmark of cancer. Interestingly, E2F1 activation and upregulation following DNA damage induces a cell death response by both upregulating pro-apoptotic genes and inhibiting anti-apoptotic survival signals (Blattner et al., 1999; Ginsberg, 2002; Stanelle and Pützer, 2006) in a p53-dependent and -independent manner (Croxton et al., 2002; Eischen et al., 2001).

Analysis of the E2F1 expression pattern during the UPR suggests that its downregulation is a result of specific transcriptional repression. Moreover, siRNA-mediated E2F1 downregulation increases the amount of cell death in response to ER stress, indicating that the phenomenon is not merely a consequence of UPR execution, but represents a prerequisite to efficiently induce the ER-stress-mediated death pathway. In this context, E2F1 might thus control the switch between the two ER stress branches – the pro-survival and pro-apoptotic one. In fact, E2F1 knockdown results in enhanced basal expression of Noxa and Puma, but not in cell death induction per se.

Collectively, these data suggest that, under ER stress conditions, the expression of Puma, Noxa and Gadd153 is promptly induced by ATF4 as an early event, together with the upregulation of pro-survival factors (such as Mcl-1), so that anti-apoptotic signals will keep pro-apoptotic ones at bay to ensure the initial survival activity of UPR. However, prolonged ER stress leads inevitably to cell death, a condition requiring a secondary signal to switch from pro-survival to pro-death UPR pathways. In fact, although sustained ER stress results in an initial decline in ATF4, Puma, Noxa and Gadd153 expression, the specific repression of E2F1 transcription, coinciding with the point of no return as evidenced by the induction of apoptosis, is associated with a new sustained expression of the above-mentioned pro-apoptotic factors together with the downregulation of anti-apoptotic proteins, such as Bcl-2 and Mcl-1. Although the inhibition of Mcl-1 expression is a direct consequence of ATF4 inactivation (Hu et al., 2012), the transcriptional repression of Bcl-2 is a result of Gadd153 activity that, free from its negative regulator (Ohoka et al., 2005), might contribute to the induction of the pro-death pathway. The contribution of E2F1 to the control of the UPR life-to-death switch is also corroborated by our data

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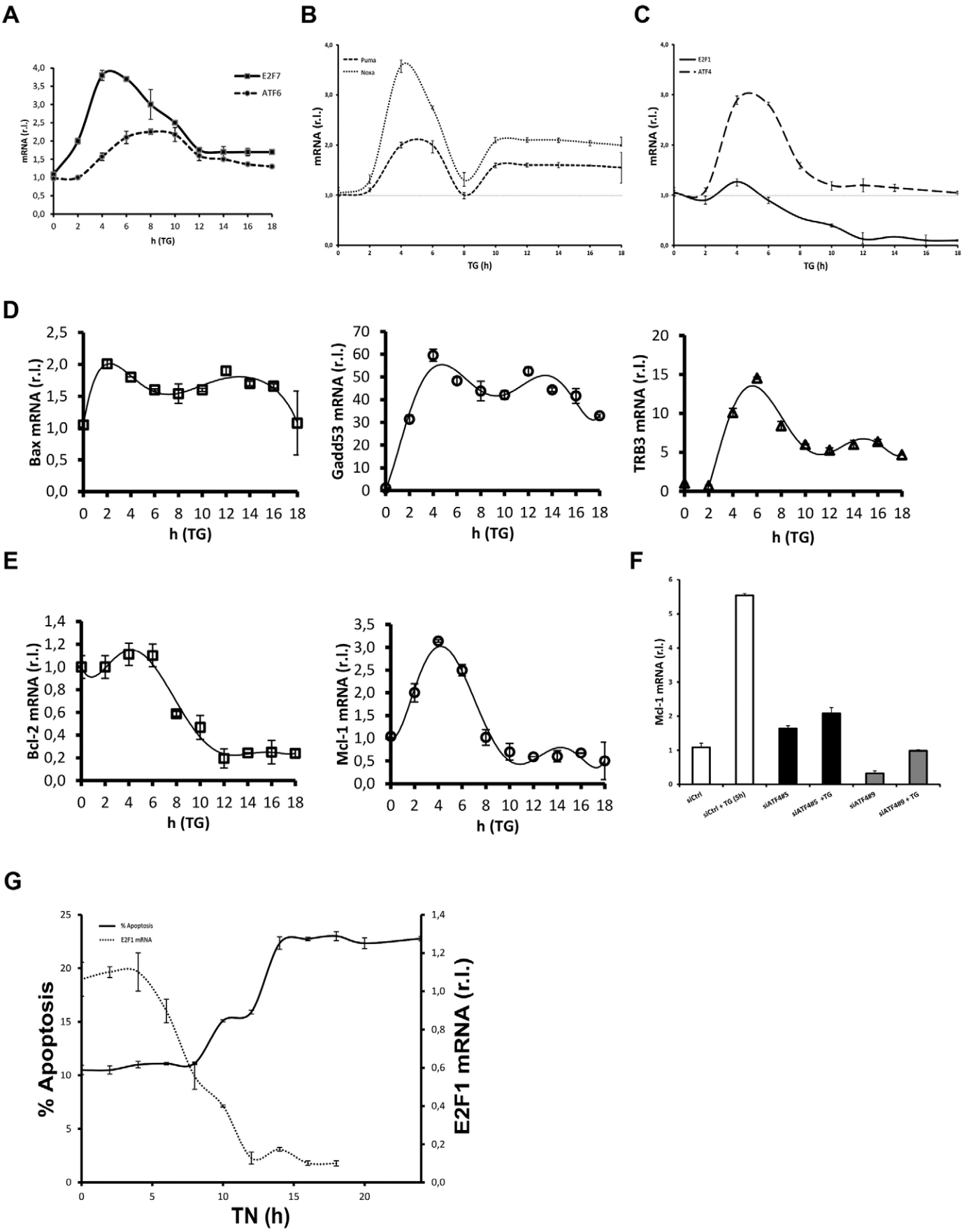

Fig. 6. See next page for legend.

Fig. 6. E2F1 modulation contributes to life/death cell decisions under ER stress conditions. (A–E) 2F cells were exposed to thapsigargin (TG) as indicated (0–18 h), and expression of E2F7 and ATF6 (A), Puma and Noxa (B), ATF4 and E2F1 (C), Bax, Gadd53 and TRB3 (D), Bcl-2 and Mcl-1 (E) were evaluated at each time-point by qRT-PCR. r.l., relative levels. (F) 2F cells were transiently transfected with two siRNA oligos to downregulate the expression of ATF4 (siATF4#5 and #9) and were treated with thapsigargin for 5 h or left untreated. The expression level of Mcl-1 was evaluated by qRT-PCR. Scrambled oligo was used as a negative control (siCtrl). (G) For the exposure/recovery experiment, 2F cells were exposed to tunicamycin (TN); at each time-point, tunicamycin was removed and cells were maintained in culture for a total of 24 h; expression of E2F1 (dotted line) and apoptosis induction (solid line) were evaluated by qRT-PCR or flow cytometry, respectively. The results indicate an increased apoptosis induction concomitant to E2F1 downregulation. All data show the mean±s.d. and are representative of three independent experiments performed in triplicate.

showing decreased cell death induction if the downregulation of E2F1 is inhibited.

Our hypothesis is also supported by recent evidence that shows that the activity of E2F1 might affect the ER stress response by inhibiting the expression of the ER master chaperone Grp78 (Racek et al., 2008), together with the identification of E2F1 as a transcriptional regulator of autophagy, a typical pro-survival stress response (Polager et al., 2008; Wang et al., 2010). Thus, owing to the close link existing between ER stress, autophagy and apoptosis induction, and the reported evidence establishing that reduction of E2F1 expression inhibits stress-induced autophagy (Polager et al., 2008), it is conceivable that ER-stress-mediated E2F1 downregulation might contribute to the life/death cell decision under prolonged ER stress.

In contrast to our results, Park and collaborators observed that E2F1$^{-/-}$ murine embryonic fibroblast (MEF) cells are resistant to apoptosis triggered by ER stress (Park et al., 2006). A possible explanation for such contrasting data might reside in the experimental model used to inhibit the expression of E2F1. Indeed, RNAi represents a means of acute gene silencing, whereas the gene knockout is, instead, a chronic state that can give rise to issues of adaptation or compensation.

Furthermore, we identified ATF6 and E2F7 to be responsible for the tuning of E2F1 expression during UPR. We demonstrated that ATF6 controls the expression of E2F1 during the UPR through direct binding to an ERSE site within the E2F1 gene promoter. To our knowledge, this is the first evidence of repressor activity for ATF6 during the ER-stress-mediated cell death programme. Indeed, besides the well-established positive transcriptional activity of ATF6 exerted during the UPR programme, it is now becoming evident that ATF6 might also repress the transcription of some target genes, such as CFTR, probably interacting with other transcription factors (Bartoszewski et al., 2008).

We also found that E2F7, a known transcription factor regulating E2F1 expression, is involved in the ER-stress-mediated downregulation of E2F1. We identified the IRE1–Xbp-1 UPR axes responsible for direct E2F7 early upregulation during the ER stress response, thus modulating, in turn, the expression of E2F1.

The kinetics of interaction of both E2F7 and active ATF6 with the E2F1 promoter correlate with the transcriptional repression of E2F1. The close proximity of the two responsive elements, ERSE and E2F-A, suggests that ATF6 and E2F7 might work in a coordinated way, possibly by direct interaction or requiring additional partners.

Collectively, our data indicate that disruption of ER stress homeostasis, coupled to E2F1 gene expression modulation, might represent a new valuable target for the development of novel therapeutic strategies against chemoresistant tumour malignancies. In this respect, it is conceivable that tumour cells could be treated with a combination of compounds able to negatively regulate the expression or activity of E2F1, such as eugenol (Ghosh et al., 2005) or CDK4/6 inhibitors (Zhussupova et al., 2014), plus compounds triggering an ER-stress-mediated apoptotic process, such as fenretinide (Corazzari et al., 2007).

MATERIALS AND METHODS

Cell culture and flow cytometry

The human fibroblastic 2FTGH (2F), osteosarcoma (SaOs-2) and melanoma (CHL-1) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and 1% penicillin-streptomycin solution (Sigma-Aldrich) at 37℃ under 5% CO_2. Cells were treated with 10 µg/ml thapsigargin (Sigma-Aldrich), 10 µg/ml tunicamycin (Sigma-Aldrich) or 10 µg/ml etoposide (Sigma-Aldrich) in DMSO, with an equal volume of vehicle used to treated control cells. AEBSF (Sigma-Aldrich) was added to a final concentration of 500 µM. Flow cytometry of fixed and propidium-iodide-stained cells was used to estimate the level of cell death by measuring the percentage of cells in the sub-G1 fraction as described previously (Corazzari et al., 2003).

RNA interference

siRNA oligoribonucleotides corresponding to the indicated human cDNAs were purchased as follows: E2F1, E2F7 and Puma from Invitrogen, ATF4 from Qiagen, Noxa from Dharmacon. Non-targeting scrambled siRNA (siCtrl) was used as the negative control. shRNA vectors corresponding to the human IRE1 cDNA were purchased from Invitrogen. A non-targeting shRNA with a scrambled targeting sequence (shCtrl) was used as the negative control. A total of 25×10^4 cells/well were transfected with 100 pmol siRNA in six-well plates by using RNAi Max (Invitrogen) as recommended by the supplier. Transfection was repeated on two consecutive days to increase transfection efficiency. At 24 h after transfection, cells were trypsinised, plated at 30×10^4 cells/well in six-well plates and treated with the indicated agents. RNA was checked by quantitative RT-PCR (qRT-PCR) and western blotting analysis at 48 h after transfection.

Cell transfection

Flag–E2F7 was kindly provided by Dr Nicholas B. La Thangue (Panagiotis Zalmas et al., 2008), whereas HA-ATF6(1-373) was purchased from Addgene (plasmid 27173, from Dr Yan Wang) (Wang et al., 2000). A total of 25×10^4 cells/well were transfected with 1 µg of total DNA in six-well plates by using Lipofectamine LTX (Invitrogen) for 8 h, as recommended by the supplier. At 24 h after transfection, cells were trypsinised, plated at 30×10^4 cells/well in six-well plates and treated as indicated.

Lentivirus generation and infection

To generate lentivirus, 10 µg of the lentiviral vectors (shRNA-pLKO) were co-transfected with 2.5 µg of an expression plasmid for the vesicular stomatitis virus G protein and psPAX2 plasmid, containing gag, pol and rev genes, into a 293T packaging cell line by using the calcium phosphate method. At 48 h later, the supernatant containing the lentiviral particles was recovered and supplemented with polybrene (4 µg/ml). Cells were infected by incubation with lentiviral-containing supernatant for 6–8 h.

qRT-PCR

RNA was extracted by using Trizol reagent (Invitrogen). cDNA synthesis was performed using the reverse transcription kit (Promega) according to the manufacturer's recommendations. Quantitative PCR reactions were performed with the Rotor-Gene 6000 (Corbett Life Science) thermocycler. The Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) was used to produce fluorescently labeled PCR

products during repetitive cycling of the amplification reaction and the melting curve protocol was used to check for probe specificity as described previously (Corazzari et al., 2007). Primer sets for all amplicons were designed using the Primer-Express 1.0 software system and are reported in supplementary material Table S1.

The result of the fluorescent PCR was expressed as the threshold cycle (CT). The ΔCT is the difference between the CT for the specific mRNA and the CT for the reference mRNA, L34. To determine relative mRNA levels, 2 was raised to the power of ΔΔCT (the difference between the ΔCT from treated cells and the CT from untreated cells). L34 mRNA level was used as an internal control because this gene was shown to be stable with cell induction (Corazzari et al., 2007).

Western blotting

Total proteins were extracted from cells by using the cell lytic buffer (Sigma-Aldrich), resolved by electrophoresis through 10% SDS-PAGE gels (10–20 μg per line) and electroblotted onto nitrocellulose membrane (Protran, Schleicher and Schuell). Blots were incubated with the indicated primary antibodies (supplementary material Table S2) in 5% non-fat dry milk in PBS plus 0.1% Tween-20 overnight at 4℃. Detection was achieved using horseradish-peroxidase-conjugated secondary antibody (1:5000; Jackson ImmunoResearch) and blots were visualised with ECL plus (Amersham Bioscience).

ChIP assay

2F cells (3×10^7/treatment) were treated with 10 μg/ml thapsigargin for 6 or 10 h, then crosslinked in 1% formaldehyde for 10 min at room temperature and quenched with 125 mM glycine for 5 min. Cells were washed twice with PBS, sonicated for 30 min (Diagenode, Belgium, 30-s pulses, 30-s rests) and immunoprecipitated with magnetic beads and 8 mg of primary antibody (Abcam mouse monoclonal antibody against ATF6 or Santa Cruz Biotechnology rabbit polyclonal antibody against XBP-1) overnight at 4℃ with rotation. Next, complexes were eluted, sequentially washed in low salt buffer, LiCl buffer and TE buffer (5 min each, with rotation), then incubated with 0.5 mg/ml Proteinase K at 50℃ for 3 h with rotation. Eluted DNA was purified and subjected to PCR analysis with primers directed against the E2F1 or E2F7 promoter (supplementary material Table S3). PCR products (E2F1, 124 bp; E2F7, 120 bp) were resolved on 2% agarose gels.

ERSE mutant

The human wild-type E2F1 promoter reporter plasmid [E2F1-Luc (−242)] was kindly provided by Prof. Masa-Aki Ikeda (Araki et al., 2003). Mutations in the putative ERSE sequence on the E2F1 promoter region of the wild-type E2F1-Luc (−242) were introduced by site-directed mutagenesis. A primer set carrying mutations of the putative ERSE sequence, introducing restriction sites for *Nhe*I and *Xho*I, was used to amplify the E2F1-Luc vector, using *Pfu* DNA polymerase (Pomega) (supplementary material Table S4). After amplification, *Dpn*I was used to degrade the wild-type vector. *Xho*I or *Nhe*I digestion and vector sequencing were used to identify positive mutants.

Luciferase assay

For the luciferase assay, 2 μg of pGL2-E2F1-Promoter [ERSE-WT or pGL2-E2F1(ERSE-Mut)-Promoter (ERSE-Mut)] was transiently co-transfected together with 0.2 μg of *Renilla* luciferase vector into 2.5×10^5 2F cells using Lipofectamine LTX as indicated by the supplier (Invitrogen). At 24 h post-transfection, 9×10^4 cells were plated in a 12-well plate and treated for 8 h with 10 μg/ml thapsigargin or left untreated. Firefly and *Renilla* luciferase were quantified by using the Dual Luciferase Reporter Assay System (Promega) as indicated by the supplier. All assays were performed at least three times in quadruplicate and independently.

Promoter analysis

The identification of putative transcription factor binding sites (TFBS) in E2F7 and E2F1 promoter DNA sequences was performed by using MatInspector software (Genomatix).

Statistical analysis

All experiments were performed at least three times. All data reported are representative of three independent experiments performed in triplicate; western blotting and ChIP images are from a representative experiment performed three times. GraphPad Prism 5 was used for statistical analysis. Statistical significance was determined using the Student's *t*-test. A *P*-value of equal to or less than 0.05 was considered significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.C. conceived of the project, designed and performed experiments and analysed the data. V.P., P.G., P.B. and D.D.Z. performed experiments. V.P., G.M.F. and M.P. contributed to the study and experimental design as well as data analysis. This manuscript was written by M.C., V.P., G.M.F. and M.P. All authors reviewed the manuscript.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.164103/-/DC1

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