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Arnaud van den Broeck, Damien Nissou, Elisabeth Brambilla, Béatrice Eymin, Sylvie Gazzeri

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Complete List of Authors:	Van Den Broeck, arnaud; INSERM U823, Bases moleculaires de la progression des cancers du poumon nissou, damien; INSERM U823, Bases moleculaires de la progression des cancers du poumon Brambilla, Elisabeth; INSERM U823, Bases moleculaires de la progression des cancers du poumon Eymin, Beatrice; INSERM U823, Bases moleculaires de la progression des cancers du poumon Gazzeri, Sylvie; INSERM U823, Bases moléculaires de la progression des cancers du poumon
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Arnaud Van Den Broeck[1,2], Damien Nissou [1,2], Elisabeth Brambilla[1,2], Beatrice Eymin[1,2] and Sylvie Gazzeri[1,2]

[1] Equipe Bases Moléculaires de la Progression des Cancers du Poumon, Centre de Recherche INSERM U823, Institut Albert Bonniot, 38042 Grenoble Cedex 09, France, [2] Université Joseph Fourier, 38041 Grenoble Cedex 09, France,

Request for reprints:

Sylvie Gazzeri

Equipe Bases Moléculaires de la Progression des Cancers du Poumon, Centre de Recherche INSERM U823, Institut Albert Bonniot BP170, 38042 Grenoble Cedex 09 France

Phone: 33 4 76 54 94 76, Fax: 33 4 76 54 94 13, Sylvie.Gazzeri@ujf-grenoble.fr

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Abstract

The Tip60 and E2F1 proteins are key players of the cellular response induced by genotoxic stresses. Here, new insights into the involvement of both proteins during the DNA damage response are provided. We show that Tip60 interacts with E2F1 and promotes its acetylation. We identify the Lysine residues 120/125 of the E2F1 protein as the prime target sites of Tip60 and show that acetylation at these sites promotes the accumulation of E2F1. Importantly, we demonstrate that cisplatin induces the accumulation of E2F1 in a Tip60-dependent manner. However, and in contrast to PCAF and p300, Tip60 is not required for the induction of apoptosis in cisplatin-treated cells. Instead, Tip60 and E2F1 are involved in the upregulation of the Excision Repair Cross-Complementation group 1 (ERCC1) protein expression, an enzyme involved in the repair of cisplatin-induced DNA lesions. These findings identify Tip60 as a direct regulator of E2F1 and support their cooperative role in DNA repair.

Introduction

Tip60 is a member of the MYST family of HAT and was originally identified as a HIV-1 Tat interactive protein [1]. Tip60 acetylates core histones and modulates chromatin structure, influences gene transcription and contributes to the DNA damage response [2]. Tip60 also catalyzes acetylation of non-histone proteins involved in transcription control and activation of DNA damage checkpoint pathways [3]. Acetylation of p53 by Tip60 plays a critical role in the p53-dependent apoptotic response following DNA damage [4,5]. Tip60's acetyltransferase activity is also rapidly activated by ionizing radiation and this contributes to the acetylation and activation of the Ataxia Telangiectasia Mutant (ATM) protein kinase and further phosphorylation of p53 and Chk2 [6]. Therefore through its ability to acetylate proteins, Tip60 is a key actor of the DNA damage response.

Mounting evidence also implicates the transcription factor E2F1 as an important component of the DNA damage response. E2F1 protein is phosphorylated and stabilized by ATM/ATR and Chk2 kinases ([7-10], both of which are integral components of the DNA damage signalling pathway. The high level of E2F1 protein allows the binding and activation of a different spectrum of genes involved in the induction of apoptosis such as Apaf-1, ARF, Chk1, ATM and p73 (for review[10]). E2F1 may have an additional role in replication control and DNA repair. Indeed, it is becoming apparent that E2F1 co-localizes and/or interacts with the MRN checkpoint protein complex [11] as well as with TopBP1 and the BRCA1-repair complex [12] in response to DNA damage. Furthermore, recent studies demonstrate that E2F1 contributes to UV-induced Nucleotide Excision Repair (NER) by promoting accessibility of NER factors to sites of damage [13,14].

In response to genotoxic stress, E2F1 is stabilized through post-transcriptional modifications and it has been shown that acetylation can induce an E2F1-dependent apoptotic response in damaged cells [15]. Although the p300 and P/CAF HATs have been involved in

E2F1 acetylation and accumulation following exposure to some DNA damaging agents [15,16], the role of the Tip60 HAT has never been investigated. In this study, we demonstrate that Tip60 acetylates the E2F1 protein and promotes its accumulation in response to cisplatin. Importantly, such accumulation does not trigger apoptosis. Instead, the Tip60/E2F1 complex promotes the accumulation of the Excision Repair Cross-Complementation group 1 (ERCC1) enzyme. These data point to a new aspect of E2F1 and Tip60 function in DNA repair.

Material and methods

Cell culture, transfection and treatments

H358 and H69 human lung carcinoma cell lines and SAOS2 osteosarcoma cells were cultured as described [17,18]. H69 cells express high level of E2F1 and Tip60, H358 cells express moderate level of both proteins and SAOS2 cells express very low level of E2F1 and Tip60. Transient transfection experiments of plasmids were carried out using Fugene 6 (Roche Diagnostic) according to manufacturer's instructions. The amounts of expression vectors were normalized with the corresponding empty vectors. Cells were harvested 24 or 48h post-transfection. pcDNA3-HA-tagged Tip60, pcDNA3-HA-tagged Tip60^{G380} and production of His-Tip were previously described [18]. pcDNA3-E2F1 was a gift from D.Trouche. E2F1 truncated mutants pGEX-E2F1$^{1-191}$ and pGEX-E2F1$^{1-284}$ were a gift from K. Helin, pGEX-E2F1$^{41-108}$ and pGEX-E2F1$^{41-127}$ from W. Krek and pGEX-E2F1$^{1-120}$ and pGEX-E2F1$^{89-191}$ from M. Giacca. Transfection of siRNA was carried out using oligofectamine (Invitrogen) according to manufacturer's instructions. The siRNAs used were as follow : Tip60 forward 5'-AUAGUACAGUGUCUUAUGGUCAAGG-3' and reverse 5'-CCUUGACCAUAAGACACUGUACUAU-3'; p300 forward 5'-CAGAGCAGUCCUGGAUUAG-3' and reverse 5'-CUAAUCCAGGACUGCUCUG-3'. siRNA against PCAF were purchased from invitrogen (PCAF Stealth^{TM}RNAi). For all

experiments, the siRNA oligonucleotide duplex used as control was 5'-UCGGCUCUUACGCAUUCAA-3'. The cells were analyzed 72h post-transfection. For experiments with cisplatin, cells were transfected for 72h with siRNAs and Cisplatin (Calbiochem) (50μM) was added for the indicated time. For the study of E2F1 acetylation, cells were incubated with TSA (Sigma Aldrich) (100ng/ml) for 18h before lysis.

In vitro translations and pull-down assays

In vitro translation and pull-down assays were performed essentially as described previously using beads coated either with GST or GST-E2F1 fusion proteins and equivalent amounts of *in vitro*-translated wild-type Tip60 protein [19].

***In vitro* acetylation assays and site-directed mutagenesis**

In vitro acetylation assays were performed as described previously using GST and a panel of E2F1 GST fusion fragments spanning the full length protein and equivalent amounts of recombinant His-tagged $Tip60^{212-513}$ [18]. Site-directed mutagenesis was carried out with the QuickChange II Site-directed Mutagenesis Kit (Stratagene) using pGST-E2F1 and pcDNA3.1-E2F1 as templates. Mutation was confirmed by DNA sequencing.

Immunoblotting and immunoprecipitation

Immunoblotting was performed by using whole cell extracts prepared in RIPA buffer. For immunoprecipitation of acetylated E2F1, TSA (100ng/ml) was added to RIPA buffer and 500 μg of proteins were used. For co-immunoprecipitation experiments from transient transfections, cells were lysed in TNE buffer (150mM NaCl, 50mM Tris pH 7.4, 0,5% NP40, 1% Triton X-100, 1mM EDTA, 1mM EGTA) supplemented with protease inhibitors and processed as described previously [18]. For co-precipitation of endogenous proteins, nuclear

extracts were prepared by lysing the cells in 5 volumes of pre-cold hypotonic buffer (10mM Tris-HCl pH 7,5, 10mM KCl, 1,5mM $MgCl_2$, and 0,5mM DTT) supplemented with 1% NP40 for 10 min on ice. Nuclei were pelleted by centrifugation for 15 min at 3000 rpm and lysed in a buffer containing 150mM NaCl, 50mM Tris pH 7.4, 0.5% NP40, 1% Triton X-100, 1mM EDTA, 1mM EGTA, supplemented with protease inhibitors. Three milligrams of nuclear extracts were subjected to immunoprecipitation with anti-E2F1 antibody. The antibodies used are as follows: anti-Ha (HA-11; Covance), anti-E2F1 (KH95; BD Pharmingen, C20; Santa Cruz), anti-acetylated lysine (Cell Signaling), anti-ERCC1 (FL-297, Santa Cruz), anti-PCAF (E8, Santa Cruz), anti-p300 (N-15, Santa Cruz) and anti-actin (A2066, Sigma).

Quantitative RT-PCR and RT-PCR analyses

Total RNA was extracted using RNeasy Mini Kit (Qiagen) and subjected to quantitative RT-PCR as previously described [20]. The specific primers used for mRNA amplification were as follows: *Tip60* forward: 5'-AGGGCACCATCTCCTTCTTT-3'; *Tip60* reverse: 5'-GTTAGGATGCAGGCCACATT-3'; *E2F1* forward: 5'-AGACCCTGCAGAGCAGATGGTTAT-3'; *E2F1* reverse: 5'-TCGATCGGGCCTTGTTTGCTCTTA-3'; *ERCC1* forward: 5'-TGTCCAGGTGGATGTGAAAG-3'; *ERCC1* reverse: 5'-AGGATCAATGTGCAGTCGG-3'; *GAPDH* forward: 5'-GCAGATCCCTCCAAAATCAA-3'; *GAPDH* reverse: 5'-ATCCACAGTCTTCTGGGTGG-3'.

Apoptosis analysis

Apoptosis was studied on a total cell population using the PE-conjugated monoclonal active caspase3 antibody apoptosis kit (BD Pharmingen) according to the manufacturer's protocol.

Stained cells were analysed by flow cytometric analysis using a FACScan flow cytometer (BD Biosciences).

Results

Endogenous E2F1 protein binds to Tip60

It had been previously shown that overexpressed Tip60 and E2F1 proteins are able to form a complex [21]. Therefore, we examined whether both proteins could also interact in our cells. H358 lung adenocarcinoma cells were cotransfected with HA-Tagged Tip60 and/or E2F1 expression vectors and coimmunoprecipitation experiment was performed using anti-E2F1 antibody. Immunoblotting with anti-HA antibody showed that Tip60 is present in E2F1 immunoprecipitates confirming that exogenous Tip60 and E2F1 proteins interact (Figure 1A). To investigate the existence of a Tip60/E2F1 complex at a more physiological level, co-immunoprecipitation experiments were conducted in nuclear extracts of H69 small cell lung carcinoma cells that express high level of both proteins. Endogenous Tip60 was detected in E2F1 immunoprecipitates, but not in immunoprecipitates obtained with a control IgG antibody (Figure 1B). Finally, to determine whether Tip60 interacts directly with E2F1, in vitro binding experiments were carried out. Figure 1C showed that « *in vitro* » translated Tip60 binds to GST-E2F1 but not to GST alone. We conclude from these data that E2F1 is a direct Tip60 binding partner.

Tip60 acetylates E2F1 at lysines 120/125

E2F1 has been reported to recruit Tip60 (and other members of the Tip60 complex) to target chromatin in vivo [21]. However, the ability of Tip60 to acetylate E2F1 has never been investigated although it has been demonstrated that the E2F1 protein is acetylated by the p300/CBP and P/CAF HATs [22]. To analyse if E2F1 was acetylated by Tip60 *in vivo*, we

transfected H358 cells with HA-Tip60 expression vector. An anti-acetylated lysine antibody was then used to precipitate endogenous acetylated E2F1 which was detected by western blotting using anti-E2F1 antibody. The data showed that the level of acetylated E2F1 increases in cells transfected with HA-Tip60 compared to cells transfected with a control plasmid (Figure 2A). Similar results were obtained when immunoprecipitation was performed with anti-E2F1 antibody followed by immunoblotting with anti-acetylated lysine antibody (Figure 2B). Interestingly, we noticed that the level of the E2F1 protein increases in the presence of Tip60. On the other hand the anti-acetylated lysine antibody precipitated less E2F1 in cells depleted for Tip60 by the use of specific siRNA compared to cells transfected with control siRNA (Figure 2C). Together, these results indicate that Tip60 acetylates E2F1 *in vivo*. To go further, we wanted to identify the lysines modified by Tip60 on the E2F1 protein. Using *in vitro* acetyltransferase assays (HAT) with C^{14}-Acetyl-CoA and a series of E2F1-GST fusion fragments spanning the full length E2F1 protein, we demonstrated that recombinant Tip60 acetylates the N-terminal half of E2F1(89-284) in a region restricted to amino acids 109 to 127 (Figures 3A and B). As a cluster of three lysines resides in this region, we then generated a series of mutant proteins in which two out of the three lysine residues were mutated to arginine, an amino acid resistant to acetylation. Acetylation of these mutants by recombinant Tip60 was then tested using *in vitro* HAT assays. As shown in Figure 3C, arginine substitution at lysines 120/125 (K120/125R) abolished E2F1 acetylation whereas mutating the 117/120 (K117/120R) or 117/125 (K117/125R) residues resulted in a reduced capacity of Tip60-mediated acetylation. Therefore, these results indicate that lysines 120 and 125 are the prime-candidate sites for Tip60 acetylation. To confirm these data, we tested whether the K120/125R E2F1 mutant was defective for acetylation by Tip60 *in vivo*. SAOS2 cells that express very low level of endogenous E2F1 were transfected with HA-Tip60 along with wild type E2F1 or mutant K120/125R E2F1 expression vectors. Anti-acetylated lysine

antibody was used to precipitate the E2F1 or the K120/125R E2F1 protein which was detected by immunoblotting using anti-E2F1 antibody. As expected, only the wild type form of E2F1 was precipitated (Figure 3D). Overall, these data demonstrate that Tip60 acetylates E2F1 on its lysines residues 120/125.

Acetylation by Tip60 stabilizes E2F1 protein

Because we had observed that the expression level of E2F1 was modified when Tip60 was expressed (Figure 2B), we postulated that Tip60 could control E2F1 expression. Transfection of HA-Tip60 in H358 cells resulted in a significant increase of the E2F1 protein level (Figure 4A). On the other hand, depriving cells of Tip60 using siRNAs diminished the expression of the E2F1 protein (Figure 4B). In both experiments, the E2F1 transcripts were not significantly affected by Tip60 as measured by quantitative PCR (Supplementary Fig1). To test whether E2F1 accumulation occurred as a result of Tip60-dependent acetylation, we used a mutant Tip60 (Tip60G380) with impaired HAT activity [23]. H358 cells were transfected with HA-Tip60 or HA-Tip60G380 and western blots experiments were performed. As compared to wild type Tip60 whose transfection clearly increased the level of E2F1, the Tip60 mutant did not have a significant effect on E2F1 expression (Figure 4C) although it was expressed at levels equal to wild type Tip60, and was still able to interact with E2F1 (Figure 4D). These data support the fact that Tip60 HAT activity is required for the accumulation of the E2F1 protein. In that case, expression of the E2F1-K120/125R mutant might not change in the presence of Tip60. To confirm this, SAOS2 cells were co-transfected with HA-Tip60 and/or WT E2F1 or mutant E2F1-K120/125R and immunoblotting was carried out with anti-E2F1 antibody. As expected, expression of mutant E2F1-K120/125R did not vary in the presence of Tip60 whereas expression of WT E2F1 was upregulated (Figure

4E). Collectively, these results show that acetylation by Tip60 on lysine residues 120/125 stabilizes the E2F1 protein.

The Tip60/E2F1 complex controls ERCC1 accumulation in response to cisplatin

Both Tip60 and E2F1 play active roles in the cellular response induced by genotoxic agents [3 ,5,10,15,24]. It has been reported that E2F1 is stabilized by radiations and DNA damaging agents [9,25]. Furthermore, acetylation by PCAF and to a lesser extend by p300 promotes the accumulation of E2F1 [22,26], and PCAF-dependent acetylation of E2F1 is important in activating pro-apoptotic genes following doxorubicin exposure [15]. These data prompted us to examine whether regulation of E2F1 by Tip60 could play a role in the response to genotoxic stress. Platinum-based chemotherapy is commonly used for the treatment of lung cancer. Treating with cisplatin the H358 cellular model that derives from a lung adenocarcinoma induced a time-dependent upregulation of the E2F1 protein (Figure 5A) but did not change the level of the E2F1 transcripts (Supplementary Fig2). In these conditions, apoptosis was observed after 24 hours of cisplatin treatment (Figure 5B). Abrogation of PCAF expression by specific siRNA in H358 cells exposed for 24 hours to cisplatin prevented E2F1 accumulation (Figure 5C) as well as apoptosis (Figure 5D). This was in agreement with the role of PCAF in the stimulation of the E2F1-dependent apoptotic response following genotoxic stress [15], in that case cisplatin. In the same conditions of cisplatin treatment, a less significant effect on E2F1 expression and apoptosis was observed when expression of p300 was abolished using specific siRNA (Supplementary Fig3). In contrast, the neutralisation of Tip60 using siRNA did not affect apoptosis in cells exposed for 24h to cisplatin (Figure 5D), although it was able to prevent E2F1 accumulation (Figure 5E). These data therefore demonstrate that Tip60 is implicated in the stabilisation of E2F1 expression in response to DNA damage but is not required for apoptosis occurrence. E2F1

was recently involved in DNA repair [13]. Interestingly, we observed that Tip60 was important for the accumulation of E2F1 soon after cisplatin exposure and before apoptosis takes place (Figure 5F). These data suggested that a Tip60/E2F1 crosstalk acts at the early times of the DNA damage response.

Cisplatin forms DNA adducts leading to intrastrand or interstrand cross-links which disrupt the structure of the DNA molecule. The excision repair cross-complementation group1 (ERCC1) enzyme plays a rate-limiting role in the repair of platinum-DNA adducts [27-29]. Increased levels of the ERCC1 protein were observed when H358 cells were exposed to cisplatin (Figure 6A). Interestingly, we noticed that ERCC1 upregulation correlates with E2F1 accumulation (Figure 5A). Based on these data we postulated that the Tip60/E2F1 cross talk could control ERCC1 accumulation following cisplatin exposure. To respond, H358 cells were first transfected with siRNA against Tip60 and exposed for 2 hours to cisplatin. Beside its repressive effect on E2F1 expression, the neutralisation Tip60 expression also prevented the accumulation of ERCC1 expression induced by cisplatin (Figure 6B). In the same conditions, the abrogation of PCAF or p300 expression did not significantly affect ERCC1 expression (Supplementary Fig4). To confirm the direct involvement of Tip60 in the control of ERCC1 expression, we investigated the impact of Tip60 overexpression on ERCC1 protein expression level in cells exposed or not to cisplatin for 2 hours. The data showed an increase of the ERCC1 protein level in cells transfected with HA-Tip60 and treated with cisplatin, as expected (Figure 6C). To evaluate the role of E2F1 in the Tip60-dependent control of ERCC1 expression, SAOS2 cells were transfected with plasmids encoding wild type or K120/125R mutant E2F1, and protein extracts were subjected to western blotting with an anti-ERCC1 antibody. Overexpression of wild type E2F1 induced an accumulation of the ERCC1 protein compared to cells transfected with an empty vector, whereas no significant effect was observed with the E2F1-K120/125R mutant (Figure 6D). Together, these data strongly

suggested that a Tip60/E2F1 crosstalk controls ERCC1 accumulation in response to cisplatin. Of note, no significant variation of the ERCC1 transcript was observed following cisplatin exposure or when H358 cells were transfected with siRNA against Tip60 or with the wild type or mutant E2F1 expression vector (Supplementary Fig5), suggesting that post-transcriptional modifications account for variation of ERCC1 protein expression level in these conditions.

Discussion

DNA damage and repair mechanisms are a subject of intense investigations. They constitute major defenses for the maintenance of genomic stability and play a key role in the successful treatment of cancer. E2F1 and Tip60 are multi-functional damage responsive proteins. Recently, their involvement in the repair of DNA lesions has also emerged. In this study, we demonstrate a direct link between the E2F1 and Tip60 proteins, and provide data that support a role of the E2F1/Tip60 complex in the control of key regulators of DNA repair mechanisms.

Earlier studies have unravelled acetylation as an important post-translational modification of the E2F1 transcription factor during the DNA damage response. In this respect, the p300/CBP and PCAF acetyltransferases are key regulators of E2F1 activity as they promote its stabilization through reversible acetylation at lysines residues 117/120/125 [22,26]. Acetylation at these sites specifically activates the DNA binding activity and transactivation potential of E2F1, and modifies the target promoter selection, preferentially boosting E2F-driven transcription of pro-apoptotic genes after doxorubicin treatment [15]. In this study, we demonstrate that E2F1 is also acetylated and stabilized by the Tip60 acetyltransferase and show that E2F1 accumulates in a Tip60-dependent manner after DNA damage. Surprisingly, we provide evidence that the lysine residues 120/125 of E2F1 are also

the prime target sites of Tip60 HAT activity. However, and in contrast to the PCAF acetyltransferase, acetylation/accumulation of E2F1 by Tip60 does not promote an E2F1-dependent apoptotic program but induces the accumulation of the Excision Repair Cross-Complementation group1 (ERCC1) protein after cisplatin exposure. Of note and also in contrast to PCAF, acetylation by Tip60 does not modify the DNA binding activity of E2F1 (data not shown). Altogether, these results support the idea that E2F1 acetylation favors either DNA repair or apoptosis depending on the selected HAT. Alternatively, we cannot exclude the possibility that DNA damaging agents could first induce the acetylation of E2F1K120/125 by Tip60 to promote DNA repair, and then stimulate subsequent E2F1K117 acetylation by P/CAF to induce apoptosis if DNA lesions cannot be repaired.

Nucleotide Excision Repair (NER) is a highly conserved DNA repair pathway that repairs DNA lesions induced by cisplatin. ERCC1 is a critical gene within the NER pathway through its ability to excise the damaged portion of the DNA [30]. Overexpression of ERCC1 is associated with the repair of cisplatin-induced DNA damage and the clinical resistance to platinum-chemotherapy, especially in non-small cell lung cancer patients [31,32]. A recent report in non treated prostate cancer cells shows that Tip60 controls the expression of several DNA repair genes including *ERCC1 [33]*. In this study, we demonstrate that Tip60 is absolutely required for the accumulation of the ERCC1 protein induced by cisplatin treatment, thereby highlighting Tip60 as an critical regulator of DNA repair genes. Furthermore, we provide some data that strongly support the notion that a Tip60/E2F1 cross talk is important for the accumulation ERCC1 in response to cisplatin. Together, these results fit well with previous studies identifying a direct correlation between cisplatin resistance and expression of E2F1 [34] and Tip60 [33] in tumor cell lines. Very recently, expression of E2F1 was shown to enhance NER following UV exposure [13]. Our results add further support to a critical role of E2F1 in the repair of bulky DNA lesions. Interestingly, we show that Tip60

does not modulate the level of ERCC1 transcripts in H358 cells exposed to cisplatin. Therefore these results demonstrate a non-transcriptional role of the Tip60/E2F1 complex on ERCC1 expression and suggest that post-transcriptional modifications account for regulation of ERCC1 expression. Further experiments are now needed to characterize the molecular mechanisms that control the expression of the ERCC1 protein in that case.

It was previously reported that the Tip60 HAT complex is recruited to E2F1-target genes and contributes to histone acetylation in response to mitogenic signals [21]. In this study, we demonstrate a functional link between E2F1 and Tip60 during the process of DNA repair. Therefore, it is tempting to speculate that Tip60 could act in physiological conditions as a transcriptional cofactor of E2F1 to drive the expression of cell cycle target genes. In a context of genotoxic stress, the Tip60/E2F1 complex could behave as a sensor of DNA damage and regulate the expression of genes involved in DNA repair. The Tip60/E2F1 complex could thus facilitate cell cycle progression while guaranteeing genome stability.

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Figure legends

Fig. 1. Endogenous E2F1 interacts with Tip60

H358 cells were cotransfected with pcDNA3-E2F1 and/or pcDNA3-HA-Tip60 expression vectors. Whole cell extracts were immunoprecipitated with anti-E2F1 antibody and immunoblotted with anti-HA or anti-E2F1 antibody. Irrelevant IgG was used as a negative control for immunoprecipitation. Actin was used as a loading control for western blotting. (**B**) Endogenous E2F1 was immunoprecipitated from H69 nuclear-enriched extracts with anti-E2F1 antibody or irrelevant IgG as a negative control. E2F1 and Tip60 were revealed by western blotting using anti-E2F1 and anti-Tip60 antibodies respectively. (**C**) GST or GST-E2F1 fusion protein was incubated with ^{35}S-labelled *in vitro* translated (IVT) Tip60 in a pull-down experiment and analysed by SDS-PAGE followed by autoradiography.

Fig.2. Tip60 acetylates E2F1

(**A,B**) H358 cells were transfected with control or HA-Tip60 expression vector. (**A**) Anti-acetylated lysine antibody was used to precipitate endogenous acetylated E2F1 which was detected by western blotting using anti-E2F1 antibody. (**B**) Anti-E2F1 antibody was used to precipitate endogenous acetylated E2F1 which was detected by western blotting using anti-acetylated lysine antibody. The Ac-K blot was stripped and re-probed with anti-E2F1 antibody to confirm the presence of an overlapping E2F1 band. Expression of Tip60 was analysed by western blotting using anti-HA antibody. (**C**) H358 cells were transfected with control siRNA (Ctl) or with siRNA raised against Tip60. Anti-acetylated lysine antibody was used to precipitate endogenous acetylated E2F1 which was detected by western blotting using anti-E2F1 antibody. Tip60 transcripts were quantified by RT /QPCR. *Gapdh* was used as an internal control.

Fig. 3. Lysines 120/125 of E2F1 are the prime-candidate sites for Tip60 acetylation

(**A**) Shematic representation of the E2F1 fragments used for the experiments. Minimal acetylated domain of E2F1 is represented as a grey bar. (**B**) *In vitro* acetyltransferase assays with C^{14}-Acetyl-CoA, *in vitro* translated Tip60 and a panel of recombinant GST-E2F1 fragments. Acetylated proteins were resolved by 10% SDS-PAGE. (**C**) GST-E2F1 proteins mutated for two out of three lysines (K117/K120/K125) were studied for acetylation by Tip60 as in B. Signal intensities from autoradiography and coomassie were quantified using the ImageJ software and the relative densitometric areas for WT and mutant forms of E2F1 were determined according to coomassie staining in each condition. (**D**) pcDNA3-HA-Tip60 was transfected together with wild type or mutant K120/125R E2F1 expression vector into SAOS2 cells. Anti-acetylated lysine antibody was used to precipitate acetylated E2F1 which was detected by western blotting using anti-E2F1 antibody. Actin was used as a loading control.

Fig. 4. Acetylation by Tip60 upregulates E2F1 protein

(**A**) H358 cells were transfected with pcDNA3.1 or pcDNA3.1-HA-Tip60 expressing vectors. Western blotting was carried out using anti-E2F1 or anti-HA antibody. Actin was used as a loading control. (**B**) H358 cells were transfected with control siRNA (Ctl) or with siRNA against Tip60. Expression of endogenous E2F1 was studied by western blotting. Actin was used as a loading control. Neutralisation efficiency of Tip60 expression was studied by RT/QPCR. *Gapdh* was used as an internal control. (**C**) H358 cells were transfected with pcDNA3.1, pcDNA3.1-HA-Tip60 or pcDNA3.1-HA-Tip60G380 expression vectors. Western blotting was carried out with anti-E2F1 or anti-HA antibody. Actin was used as a loading control. (**D**) H358 cells were cotransfected with pcDNA3-E2F1 and/or pcDNA3-HA-Tip60G380 expression vectors. Whole cell extracts were immunoprecipitated with anti-E2F1 antibody and immunoblotted with anti-HA or anti-E2F1 antibody. Irrelevant IgG was used as

a negative control for immunoprecipitation. (**E**) SAOS2 cells were transfected with pcDNA3.1, pcDNA3.1-E2F1 or pcDNA3.1-E2F1K120/125R in the presence or absence of pcDNA3.1-HA-Tip60. Western blotting was carried out with anti-E2F1 or anti-HA antibody (upper panel). Actin was used as a loading control. Signal intensities of E2F1 and actin were quantified using the ImageJ software and the relative densitometric areas for WT and mutant forms of E2F1 were determined according to actin in each condition (lower panel).

Fig. 5. Tip60-dependent accumulation of E2F1 by cisplatin does not promote apoptosis

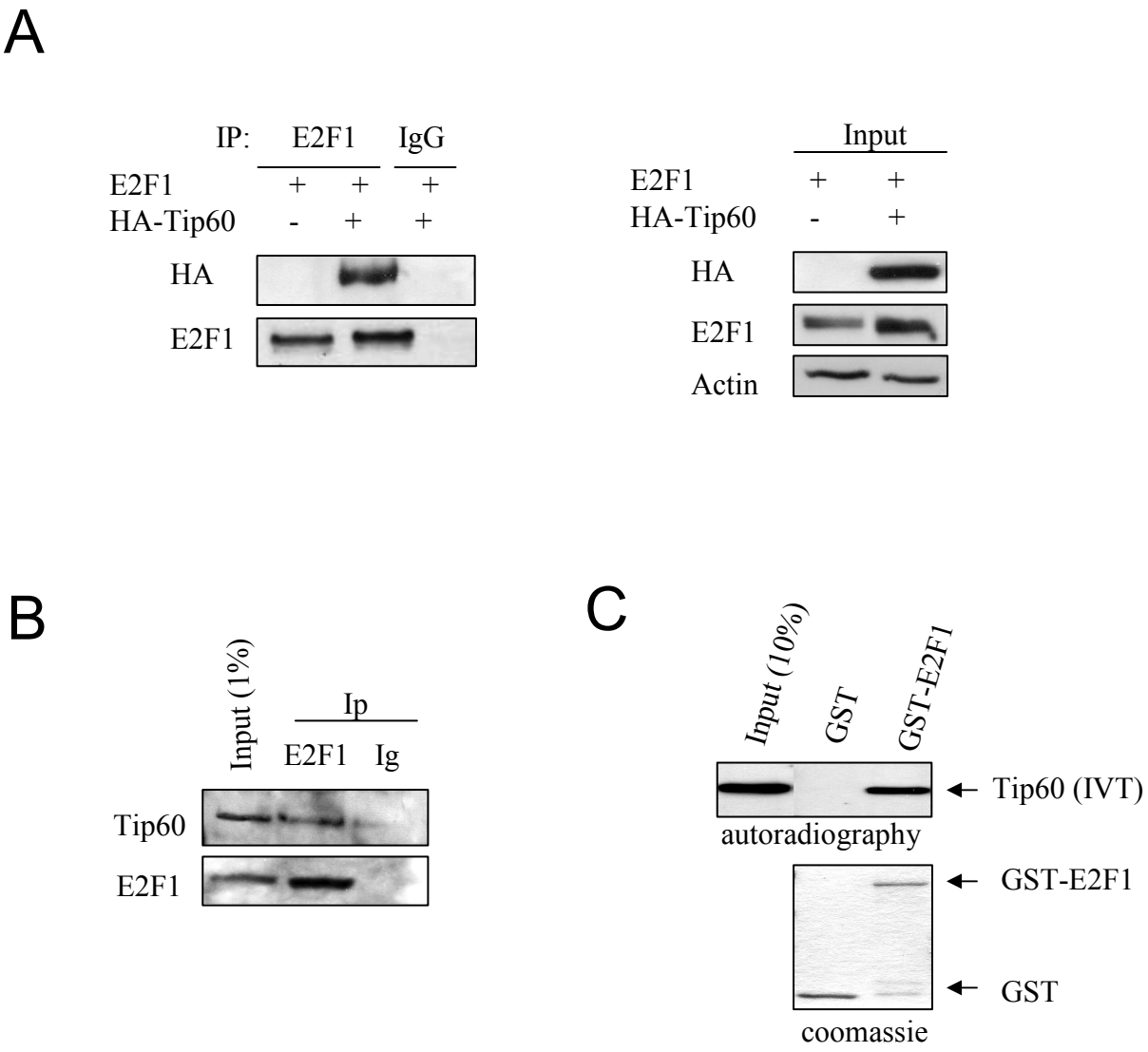
(**A, B**) H358 cells were exposed for the indicated times to 50μM cisplatin. Expression of E2F1 was studied by western blotting with anti-E2F1 antibody (**A upper panel**). Actin was used as a loading control. Signal intensities of E2F1 and actin were quantified using the ImageJ software and the relative densitometric areas for E2F1 were determined according to actin in each condition (**A, lower panel**). Apoptosis was evaluated by active caspase 3 staining followed by FACS analysis (**B**). Data represent the mean ± S.D. of three independent experiments. (**C**) H358 cells were transfected for 48h with either control (Ctl) or PCAF siRNA and treated with 50μM cisplatin for an additional 24 hours. Western blotting was performed using anti-PCAF or anti-E2F1 antibody. Actin was used as a loading control. (**D**) H358 cells were transfected for 48h with either control (Ctl), PCAF or Tip60 siRNA and treated with 50μM cisplatin for an additional 24 hours. Apoptosis was evaluated by active caspase 3 staining followed by FACS analysis. Data represent the mean ± S.D. of three independent experiments. (**E**) H358 cells were transfected for 48h with either control (Ctl) or Tip60 siRNA and treated with 50μM cisplatin for an additional 24 hours. Western blotting was performed using anti-E2F1 antibody (left panel). Actin was used as a loading control. Neutralisation efficiency of Tip60 expression was studied by RT/QPCR. (right panel). *Gapdh* was used as an internal control. (F) H358 cells were transfected for 72h with either control

(Ctl) or Tip60 siRNA and treated with 50μM cisplatin for an additional 2 hours. Expression of E2F1 was studied by western blotting (upper panel). Actin was used as a loading control. Neutralisation efficiency of Tip60 expression was studied by RT/QPCR. (lower panel). *Gapdh* was used as an internal control.

Fig. 6. Tip60 and E2F1 control ERCC1 accumulation in response to cisplatin

(**A**) H358 cells were exposed for the indicated times to 50μM cisplatin. Expression of ERCC1 was studied by western blotting with anti-ERCC1 antibody (upper panel). Actin was used as a loading control. Signal intensities of E2F1 and actin were quantified using the ImageJ software and the relative densitometric areas for ERCC1 were determined according to actin in each condition (lower panel). (**B**), H358 cells were transfected for 72h with either control (Ctl) or Tip60 siRNA and treated with 50μM cisplatin for an additional 2 hours. Expression of E2F1 and ERCC1 was studied by western blotting (upper panel). Actin was used as a loading control. Neutralisation efficiency of Tip60 expression was studied by RT/QPCR. (lower panel). *Gapdh* was used as an internal control. (**C**) H358 cells were transfected with HA-Tip60 expression vector for 72h and treated or not with 50μM cisplatin for an additional 2 hours. Western blotting was performed using anti-HA or anti-ERCC1 antibody. Actin was used as a loading control. (**D**) SAOS2 cells were cotransfected with plasmids encoding wild type or K120/125R mutant E2F1. Protein extracts were subjected to western blotting with anti-E2F1 or anti-ERCC1 antibody. Actin was used as a loading control.

Figure 1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E2F1 + + +
HA-Tip60 - + +
HA
E2F1
Input
E2F1 + +
HA-Tip60 - +
HA
E2F1
Actin
B
Input (1%)
Ip
E2F1 Ig
Tip60
E2F1
C
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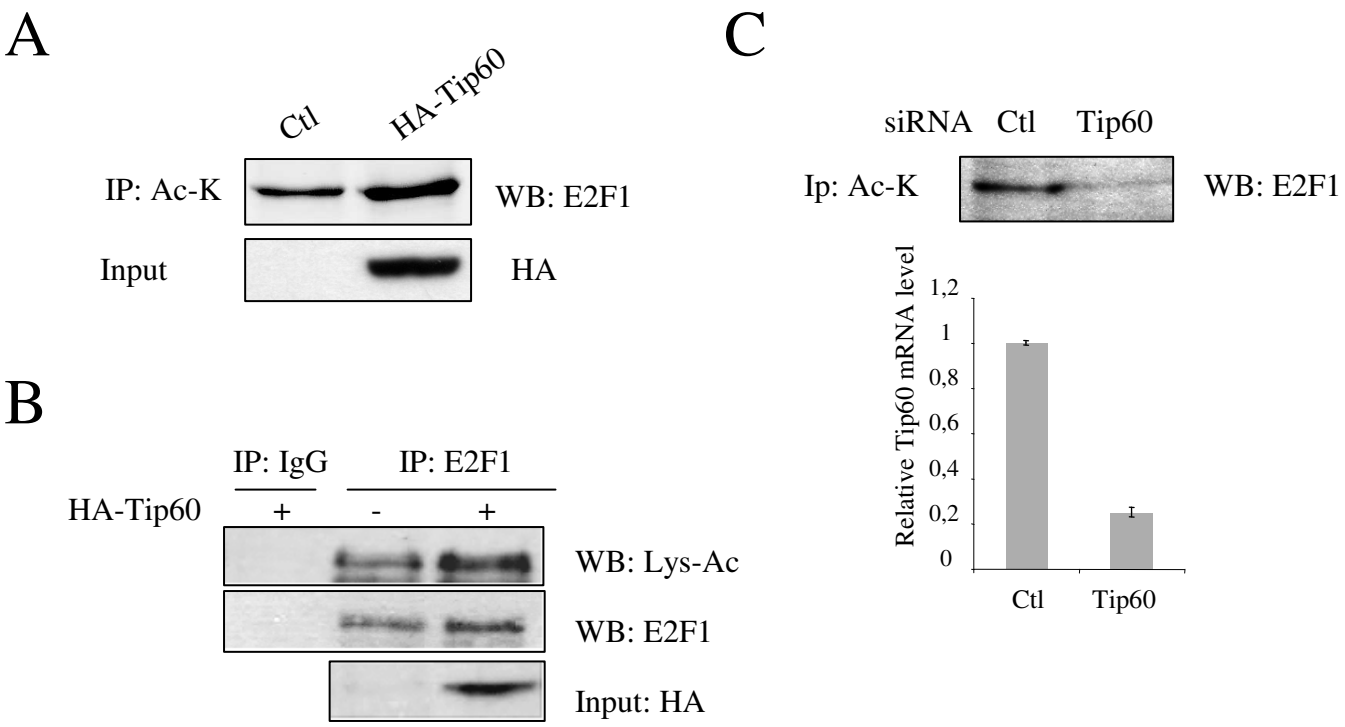
autoradiography
GST-E2F1
GST
coomassie

Figure 2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HA
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+
-
+
WB: Lys-Ac
WB: E2F1
Input: HA
C
siRNA Ctl Tip60
Ip: Ac-K
WB: E2F1
Relative Tip60 mRNA level
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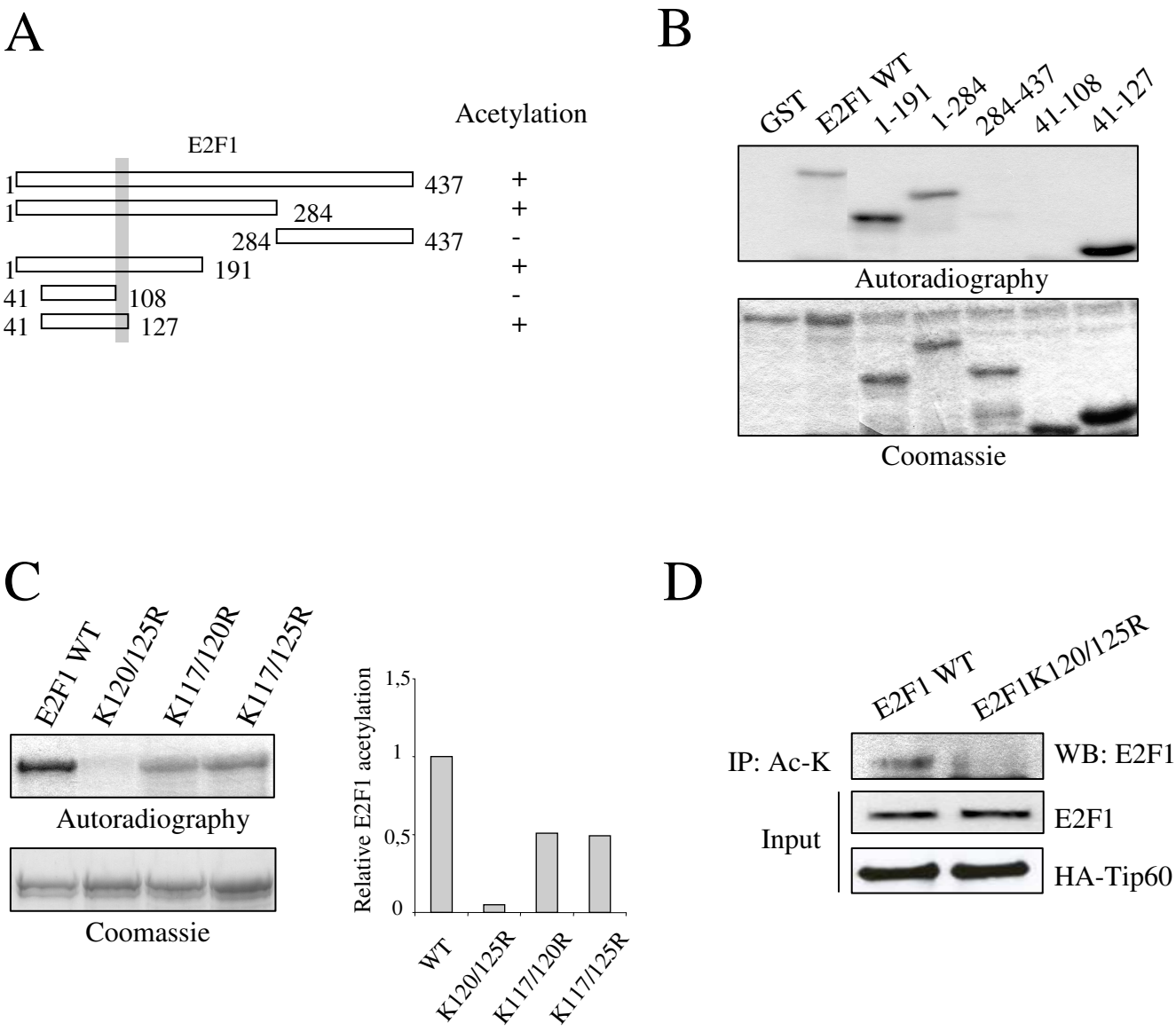
0,4
0,2
0
Ctl
Tip60

Figure 3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Acetylation
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437
+
1
284
+
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437
-
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191
+
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108
-
41
127
+
B
GST
E2F1 WT
1-191
1-284
284-437
41-108
41-127
Autoradiography
Coomassie
C
E2F1 WT
K120/125R
K117/120R
K117/125R
Autoradiography
Coomassie
Relative E2F1 acetylation
1,5
1
0,5
0
WT
K120/125R
K117/120R
K117/125R
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E2F1 WT
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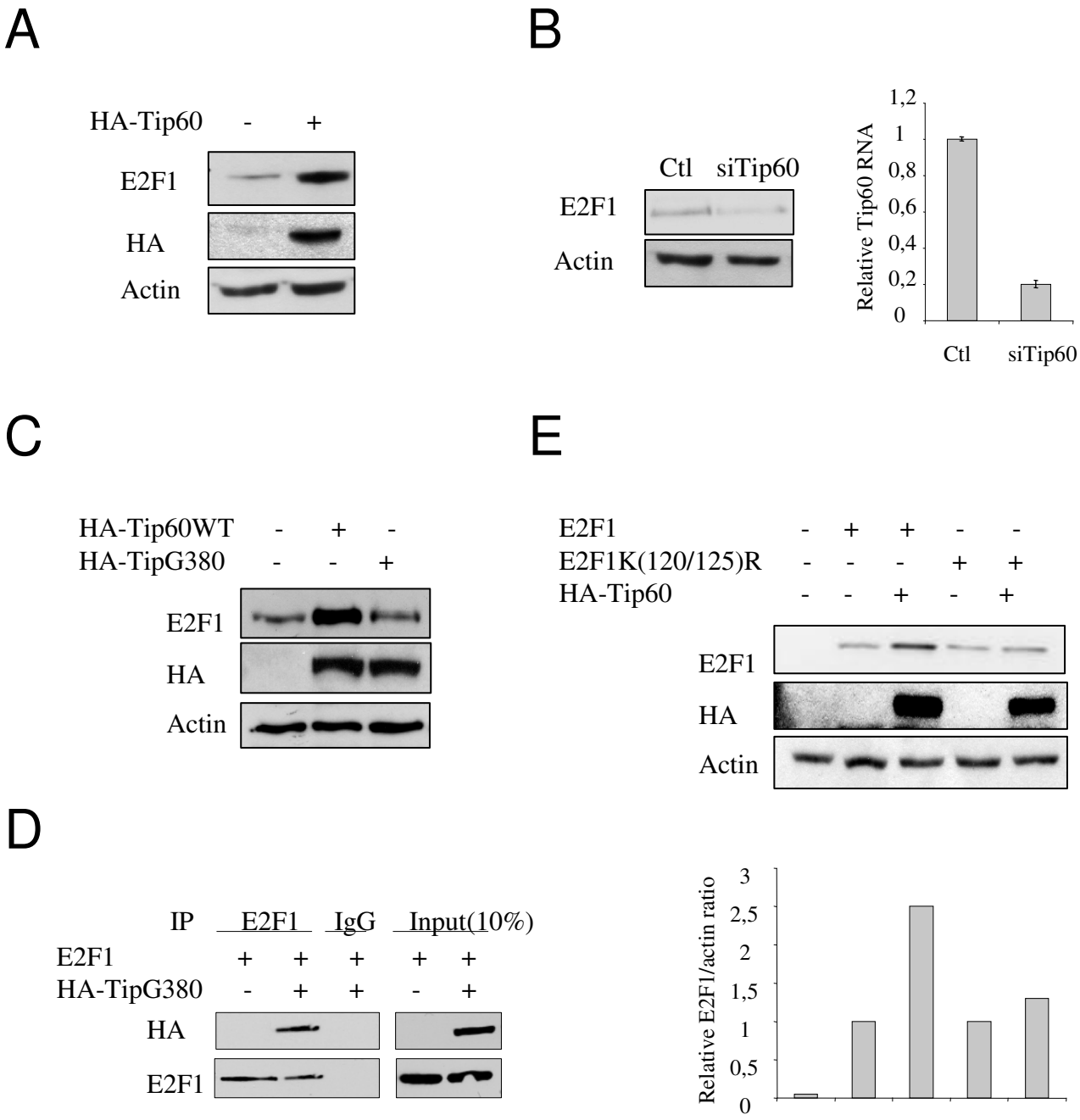
WB: E2F1
Input
E2F1
HA-Tip60

Figure 4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HA-Tip60 - +
E2F1
HA
Actin
B
Ctl siTip60
E2F1
Actin
Relative Tip60 RNA
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0,8
0,6
0,4
0,2
0
Ctl
siTip60
C
HA-Tip60WT - + -
HA-TipG380 - - +
E2F1
HA
Actin
E
E2F1 - + + - -
E2F1K(120/125)R - - - + +
HA-Tip60 - - + - +
E2F1
HA
Actin
D
IP E2F1 IgG Input(10%)
E2F1 + + + + +
HA-TipG380 - + + - +
HA
E2F1
Relative E2F1/actin ratio
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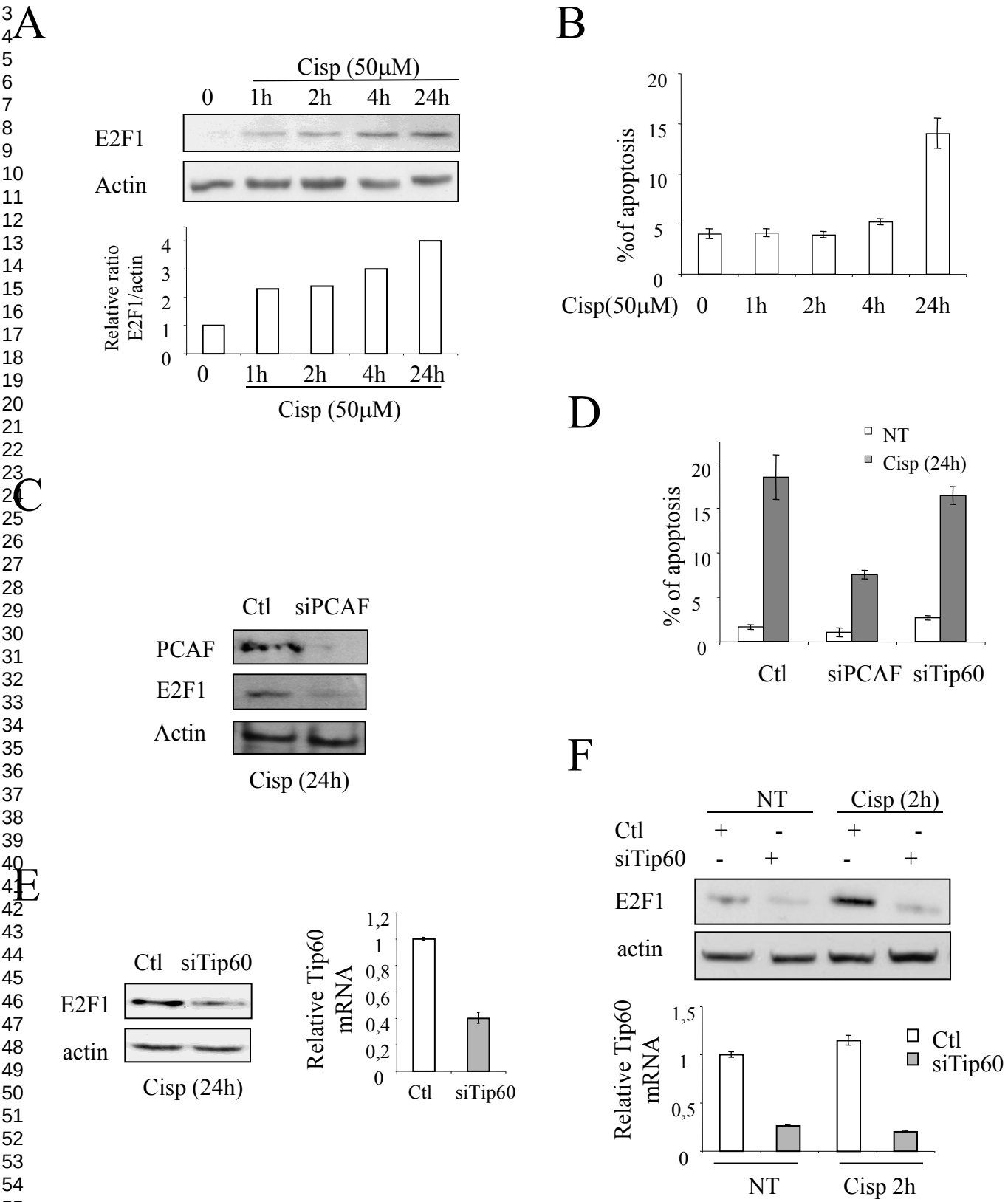
2,5
2
1,5
1
0,5
0

Figure 5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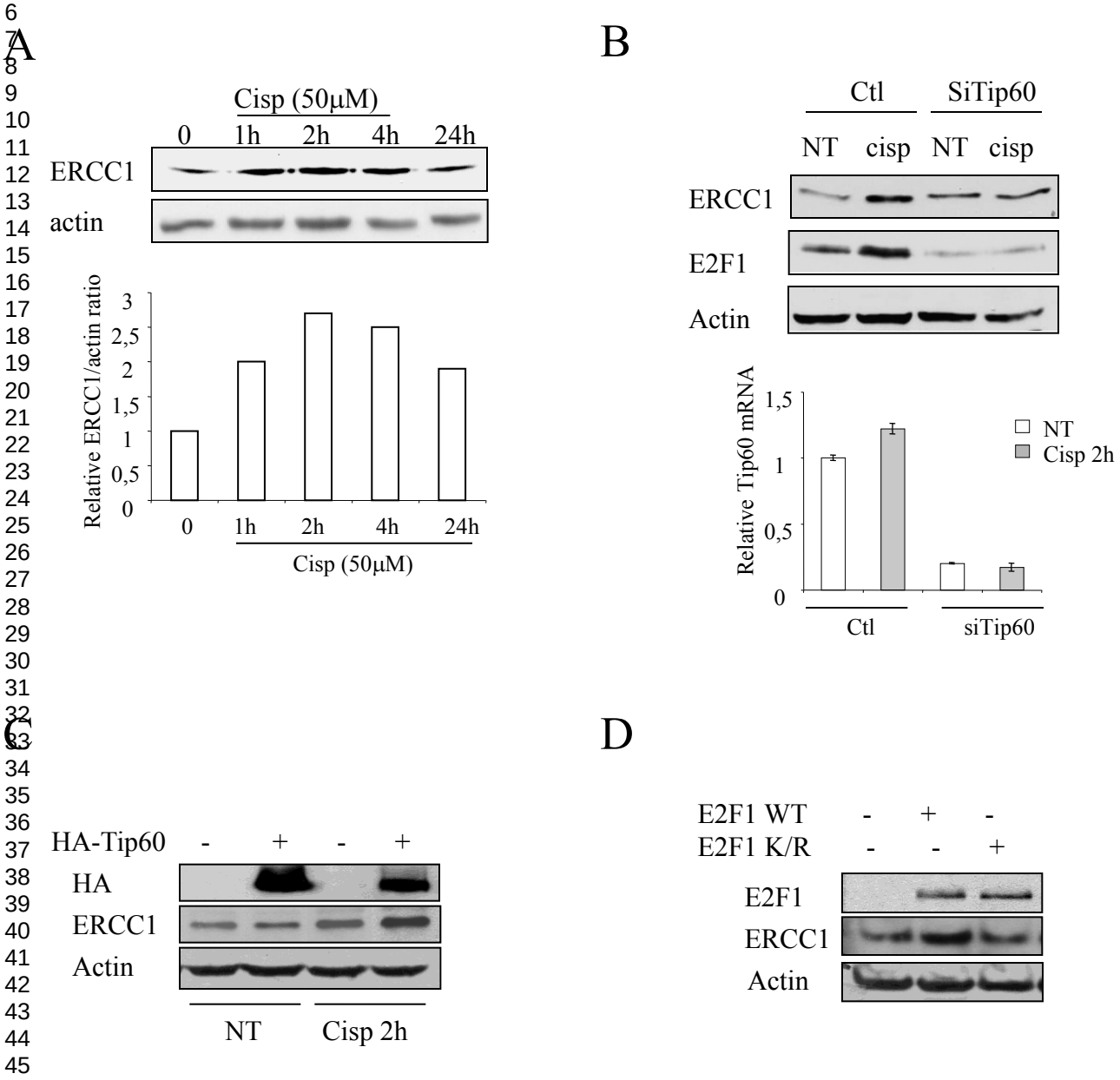
Cisp (50µM)
0 1h 2h 4h 24h
E2F1
Actin
Relative ratio E2F1/actin
0 1h 2h 4h 24h
Cisp (50µM)
B
%of apoptosis
Cisp(50µM) 0 1h 2h 4h 24h
C
Ctl siPCAF
PCAF
E2F1
Actin
Cisp (24h)
D
NT
Cisp (24h)
% of apoptosis
Ctl siPCAF siTip60
E
Ctl siTip60
E2F1
actin
Cisp (24h)
Relative Tip60 mRNA
Ctl siTip60
F
NT Cisp (2h)
Ctl + - + -
siTip60 - + - +
E2F1
actin
Relative Tip60 mRNA
Ctl
siTip60
NT Cisp 2h

Figure 6


A
Cisp (50µM)
0
1h
2h
4h
24h
ERCC1
actin
Relative ERCC1/actin ratio
3
2,5
2
1,5
1
0,5
0
Cisp (50µM)
B
Ctl
SiTip60
NT
cisp
NT
cisp
ERCC1
E2F1
Actin
Relative Tip60 mRNA
1,5
1
0,5
0
NT
Cisp 2h
Ctl
siTip60
C
HA-Tip60
-
+
-
+
HA
ERCC1
Actin
NT
Cisp 2h
D
E2F1 WT
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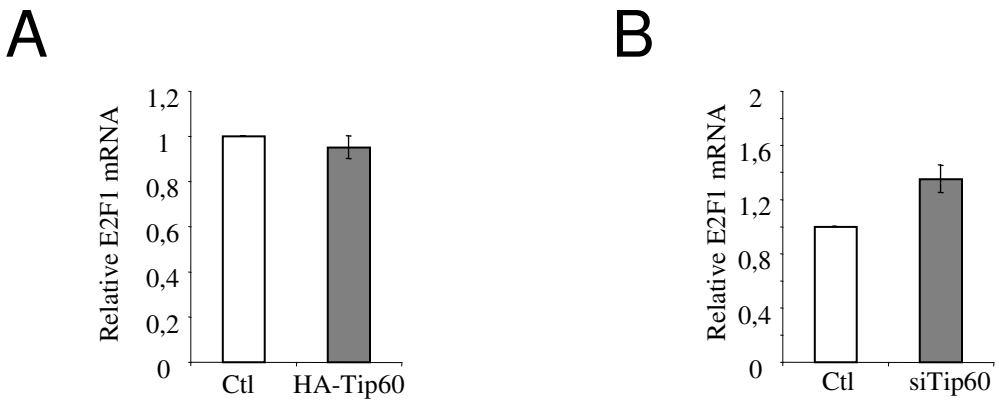
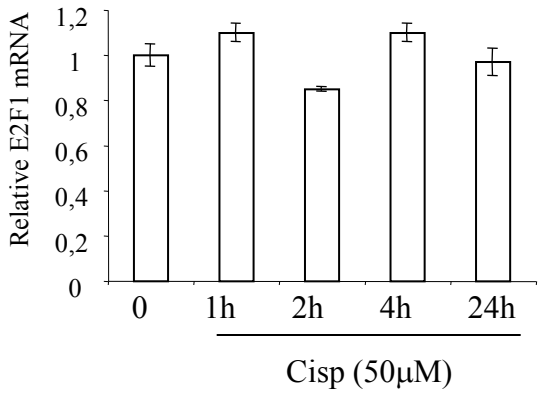
+
-
E2F1 K/R
-
-
+
E2F1
ERCC1
Actin

Supplementary Figure 1



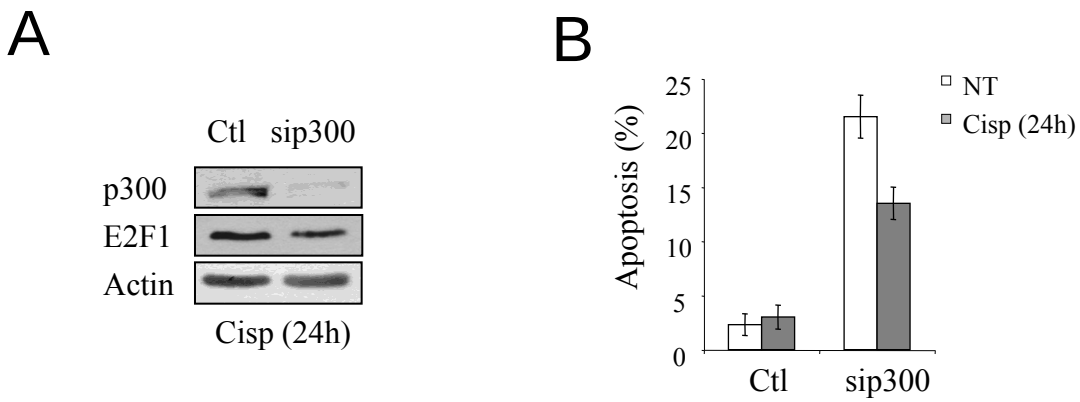
Tip60 does not affect the level of E2F1 transcripts. H358 cells were transfected with either HA-Tip60 expression vector (**A**) or siRNA raised against Tip60 (**B**). E2F1 transcripts were quantified by RT /QPCR. *Gapdh* was used as an internal control.

Supplementary Figure 2



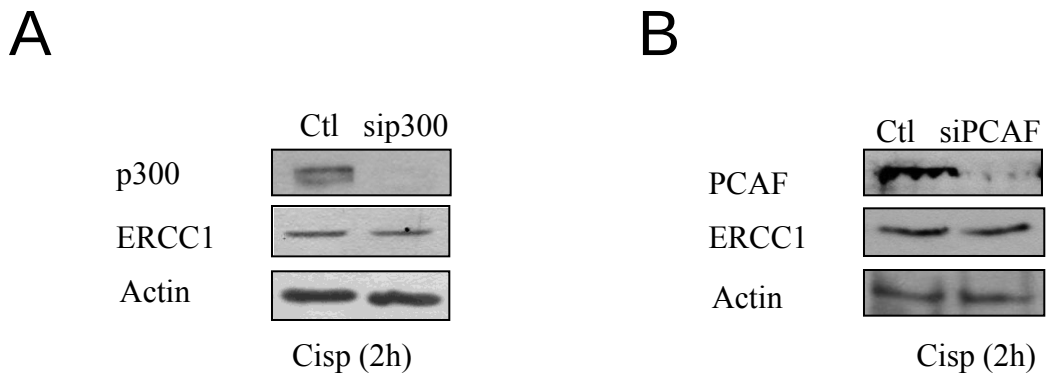
Cisplatin does not affect E2F1 mRNA expression. H358 cells were exposed for the indicated times to 50µM cisplatin. Expression of E2F1 mRNA was studied by RT/QPCR. *Gapdh* was used as an internal control

Supplementary Figure 3



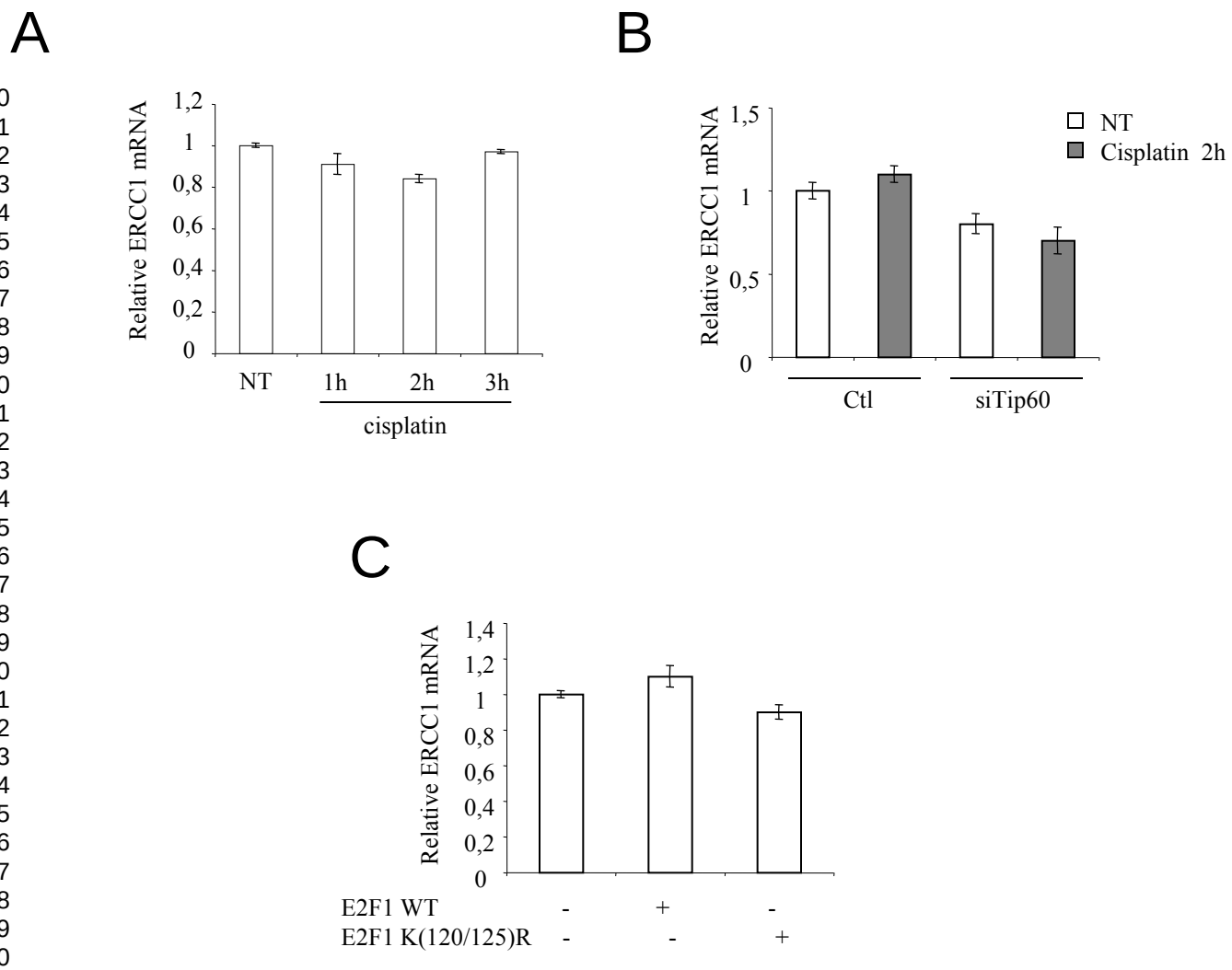
Neutralization of p300 prevents E2F1 accumulation and apoptosis in response to cisplatin.
H358 cells were transfected for 48 hours with either control (Ctl) or p300 siRNA and exposed to 50μM cisplatin for an additional 24 hours. (A) Western blotting was performed using anti-E2F1 or anti-p300 antibody. Actin was used as a loading control. (B) Apoptosis was studied by Active Caspase 3 staining followed by FACS analysis.

Supplementary Figure 4



PCAF and p300 are not involved in ERCC1 accumulation in response to cisplatin. H358 cells were transfected for 72 hours with either control (Ctl), p300 (A) or PCAF (B) siRNA and exposed to 50μM cisplatin for an additional 2 hours. Western blotting was performed using anti-PCAF, anti-p300 and anti-ERCC1 antibodies. Actin was used as a loading control

Supplementary Figure 5



Cisplatin, Tip60 and E2F1 do not affect the level of ERCC1 transcripts. (**A**), H358 cells were exposed for the indicated times to cisplatin (50μM). (**B**), H358 cells were transfected for 72h with control (Ctl) or Tip60 siRNAs and exposed for 2 hours to cisplatin (50μM). (**C**) H358 cells were transfected for 48h with wild type or K(120/125)R mutant E2F1 and exposed for 2h to cisplatin (50μM). In all cases, expression of ERCC1 transcripts was studied by RT/QPCR. *Gapdh* was used as an internal control.