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Specific Role for p300/CREB-binding Protein-associated Factor Activity in E2F1 Stabilization in Response to DNA Damage*

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E2F1, a member of the E2F family of transcription factors, plays a pivotal role in controlling both physiological cell-cycle progression and apoptotic cell death in response to DNA damage and oncogene activation. In response to genotoxic stresses, E2F1 is stabilized by signals that include ATM-dependent phosphorylation. We recently demonstrated that DNA damage induces also E2F1 acetylation, which is required for its recruitment onto apoptotic gene promoters. Here we show that E2F1 is stabilized in response to doxorubicin and cisplatin treatments even in the absence of either ATM-dependent phosphorylation or p53 and cAbl, two major transducers of DNA damage signaling. We found that acetylation of E2F1 is, instead, required to stabilize the protein in response to doxorubicin. Finally, we report that the formation of E2F1-p300/CREB-binding protein-associated factor (P/CAF) complexes is preferentially induced in doxorubicin-treated cells, and that P/CAF acetyltransferase (HAT), but not p300 HAT activity, is required for a significant E2F1 stabilization and accumulation. Our results unveil a differential role of P/CAF and p300 in acetylation-induced stabilization of E2F1, thus supporting a specific role for P/CAF HAT activity in E2F1-dependent apoptosis in response to DNA damage.

E2F1 belongs to the E2F family of transcription factors. Seven different E2F members (E2F1 through E2F7) (1–4) and three DRTF protein (DP)[1] proteins have been identified so far. As a heterodimeric complex with the DP-1 protein, E2F1 binds to promoters containing E2F-responsive elements and activates transcription through its C-terminal activation domain. Initial studies implicated E2F1 in the control of cell-cycle progression, because many E2F1 target genes encode S-phase regulatory proteins as well as proteins regulating cell-cycle progression and DNA synthesis. However, transcriptional activation of cell-cycle-related genes is only one facet of E2F1 activity. Recent data obtained both from mouse and cellular models and from microarray analysis demonstrate that E2F1 has important roles also in modulating antiproliferative processes such as senescence and apoptosis (5).

E2F1 activity is regulated at different levels. First, the transactivation potential of E2F1 is negatively affected by the interaction with the retinoblastoma gene product pRb. The E2F1-bound Rb inhibits transcription actively by contact with promoter-bound proteins (6, 7) and by recruiting a histone deacetylase complex (8–10). The E2F1-Rb interaction is limited by Rb phosphorylation, which is mainly mediated by the cyclin D/cyclin-dependent kinase-4 at the G_1/S transition (11). A second level of control of E2F1 activity concerns the regulation of its DNA-binding ability. Indeed, E2F1 interacts directly with cyclin A, which results in phosphorylation of DP-1 in S phase, causing down-regulation of E2F binding to DNA (12, 13).

A final level of regulation of E2F1 relates to the control of its abundance by a number of mechanisms. After mitogenic stimuli, one or more E2F species activate(s) the E2F1 promoter in late G_1, resulting in an increase in E2F1 RNA and protein synthesis. Conversely, transcription of the E2F1 gene decreases in late S, because of the negative regulation of the DNA-binding activity of E2F1–3 by cyclin A/cyclin-dependent kinase-2 (14, 15).

E2F1 protein levels are also regulated by the ubiquitination-dependent proteasome degradation. The epitope recognized by the ubiquitin-proteasome pathway is located in the carboxyl terminus, near the acidic activation domain of E2F1 (16, 17). Both post-translational modifications, such as phosphorylation by the transcription factor IIH (TFIIH) kinase and protein-protein interactions with pRb have been shown to increase E2F1 stability through the inhibition of the ubiquitination process (18).

Distinct modalities of E2F1 regulation might be responsible for opposite outcomes of its activation, cell-cycle progression, or apoptosis. A prompt response to genotoxic stresses has been reported to induce stabilization of E2F1 through its phosphorylation by the ataxia-telangiectasia mutated (ATM) kinase, the ATM and RAD3-related (ATR) kinase (19) and the checkpoint kinase 2 (20, 21). We recently demonstrated that DNA damage also causes the acetylation-dependent activation of E2F1 apoptotic potential by boosting E2F1-driven transcription of the proapoptotic target gene p73 (22). Although E2F1 can be acetylated by both p300/CREB-binding protein (CBP)-associated factor (P/CAF) and CREB-binding protein/P300 acetylases *in vitro* at the same lysine residues (23, 24), the relationship between this post-translational modification and the stabilization of E2F1 in response to DNA damage has not

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[1] The abbreviations used are: DP, DRTF protein; Rb, retinoblastoma; ATM, ataxia-telangiectasia mutated; ATR, ATM- and RAD3-related kinase; P/CAF, p300/CREB-binding protein-associated factor; CREB, cAMP-response element-binding protein; MEF, mouse embryo fibroblast; HA, hemagglutinin; GFP, green fluorescent protein; AT, ataxia-telangiectasia; TERT, telomerase reverse transcriptase.

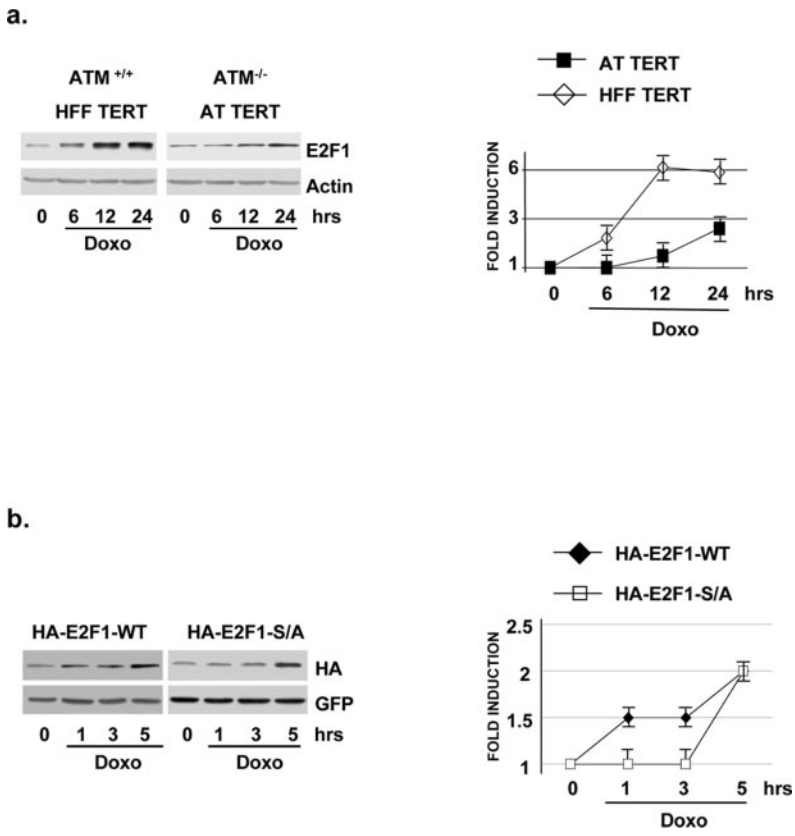

FIG. 1. **E2F1 stabilization occurs in the absence of ATM/ATR-dependent phosphorylation.** *a*, Western blot analysis for E2F1 in total extracts from primary fibroblasts derived from a patient affected with ataxia-telangiectasia (*AT*) stably expressing the catalytic subunit of telomerase (*AT TERT*) and in the control cell line (*HFF TERT*) (26) after treatment with 2 μM doxorubicin (*Doxo*) up to 24 h. *b*, human glioblastoma T98G cells co-transfected with GFP and either WT-HA-E2F1 or HA-E2F1-S/A and treated 24 h after transfection with 2 μM doxorubicin for the indicated times. Immunoblottings were performed with either anti-E2F1 or anti-HA antibodies as indicated (*upper panels*). Anti-actin and anti-GFP were used to normalize protein loading and efficiency of transfection, respectively (*lower panels*). *a* and *b*, *right panels* summarize the densitometric analysis of E2F1 levels from three independent experiments (±2 S.D.). Results are expressed as fold induction with respect to untreated cells.

been explored. We describe here a specific role for P/CAF in the acetylation-induced accumulation of E2F1 in response to DNA damage.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—Human fibroblast cell lines AT-TERT and HFF-TERT, mouse embryo fibroblasts (MEFs) 3T3 ABL$^{-/-}$, 3T3 P53$^{-/-}$, and 3T3 E2F1$^{-/-}$, U2OS and HEK293 cells were cultured in Dulbecco's modified Eagle's medium with heat-inactivated 10% fetal bovine serum. Human glioblastoma cell line T98G was cultured in RPMI 1640 medium with heat-inactivated 10% fetal bovine serum, sodium pyruvate, and non-essential amino acids. MEFs 3T3 ABL$^{-/-}$ and 3T3 P53$^{-/-}$ were kindly provided by Dr. J. Y. J. Wang. AT-TERT and HFF-TERT were kindly provided by Dr. J. Y. J. Wang and Dr. L. Wood. MEFs 3T3 E2F1 $^{-/-}$ were kindly provided by Dr. L. Yamasaki. Transfections were performed either with LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's instructions or with the standard $CaPO_4$ method.

Plasmids—The wild-type E2F1 expression vector was generated by subcloning the PCR-generated full-length E2F1 cDNA into the HApCDNA3 vector. The expression vectors pCDNA-HA-E2F1-S31/A (HA-E2F1-S/A) and pCDNA-HA-E2F1-K117,12,125R (HA-E2F1-R) were generated by PCR-mediated site-directed mutagenesis and verified by sequencing. The PMCV E2F1, the wild-type and ΔHAT (Δ 579–608) PCI P/CAF and the wild type and ΔHAT (LY-RR) PCI p300 expression vectors were kindly provided by V. Sartorelli and P. L. Puri (25).

Immunoprecipitations and Immunoblotting—The following antibodies were purchased from Santa Cruz Biotechnology: anti-E2F1 (C-20), anti-hemagglutinin (HA) monoclonal (F-7) and polyclonal (Y-11), anti-green fluorescent protein (GFP) (FL), anti-actin (I-19), and the agarose-conjugate anti-HA monoclonal (F7). Monoclonal anti-acetyl-lysines were purchased from Upstate Biotechnology. Cells were lysed with RIPA lysis buffer (50 mM Tris, pH 7.6, 1% Nonidet P-40, 140 mM NaCl, 0.1% SDS), and the insoluble pellet was discarded after centrifugation. Protein concentration was determined by the BCA protein assay reagent (Pierce). Extracts were immunoprecipitated with the indicated antibodies and protein A/G plus (Santa Cruz Biotechnology).

Pulse-chase—Subconfluent T98G cells in 10-cm dishes were transfected with 4 μg of either HA-E2F1-WT or with HA-E2F1-R. 12 h after transfection, cells were trypsinized and replated to normalize for the efficiency of transfection. 24 h after transfection, cells were starved for 1 h in methionine-cysteine-free medium and subsequently incubated with 200 μCi/ml of Promix (containing [^{35}S]methionine-cysteine, Promix-Amersham) for 30 min. Medium with an excess of unlabelled methionine-cysteine (1 mg/ml) with or without doxorubicin 2 μM (Sigma) was then added, and cells were collected at the indicated times. The ^{35}S-labeled HA-E2F1 in the anti-HA immunoprecipitate from each time point was quantified by PhosphorImager and normalized to that of the zero time point.

Cycloheximide Chase—Transfected U2OS cells were seeded in 35-mm dishes. Two days after transfection, cells were treated with 2 μM doxorubicin for 8 h, and then Me_2SO-solubilized cycloheximide (Sigma) was added to the culture medium at a final concentration of 50 μg/ml for the indicated times.

RESULTS

E2F1 Is Induced in Response to DNA Damage in the AT-Tert Human ATM-defective Cell Line—Genotoxic stress has been shown to induce both phosphorylation and acetylation of E2F1 protein (19, 22). To investigate the relative contribution of these two post-translational events in E2F1 stabilization induced by genotoxic stresses, we first assessed the role of the ATM/ATR pathway in E2F1 activation in response to DNA damage. To this purpose, we analyzed ATM-defective primary fibroblasts derived from a patient affected with ataxia-telangiectasia (AT) and immortalized by the stable expression of the catalytic subunit of telomerase (AT-TERT) (26). We found that treatment of AT-TERT cells with doxorubicin still leads to stabilization of the E2F1 protein, although to a lesser extent and with a delayed kinetics, with respect to control HFF-TERT cells (Fig. 1*a*). To rule out the hypothesis that the ATM and RAD3-related kinase ATR might be responsible for the delayed accumulation of E2F1 observed in our cells, we compared the kinetics of stabilization of wild-type E2F1 *versus* the E2F1 mutant (HA-E2F1-S/A) that cannot be phosphorylated by both ATM and ATR. We observed that exogenously expressed wild-type E2F1 was already accumulated after 1 h of doxorubicin treatment, whereas induction of the exogenously expressed HA-E2F1-S/A mutant still occurred, although it was delayed (Fig. 1*b*). Taken together, our data imply that additional post-

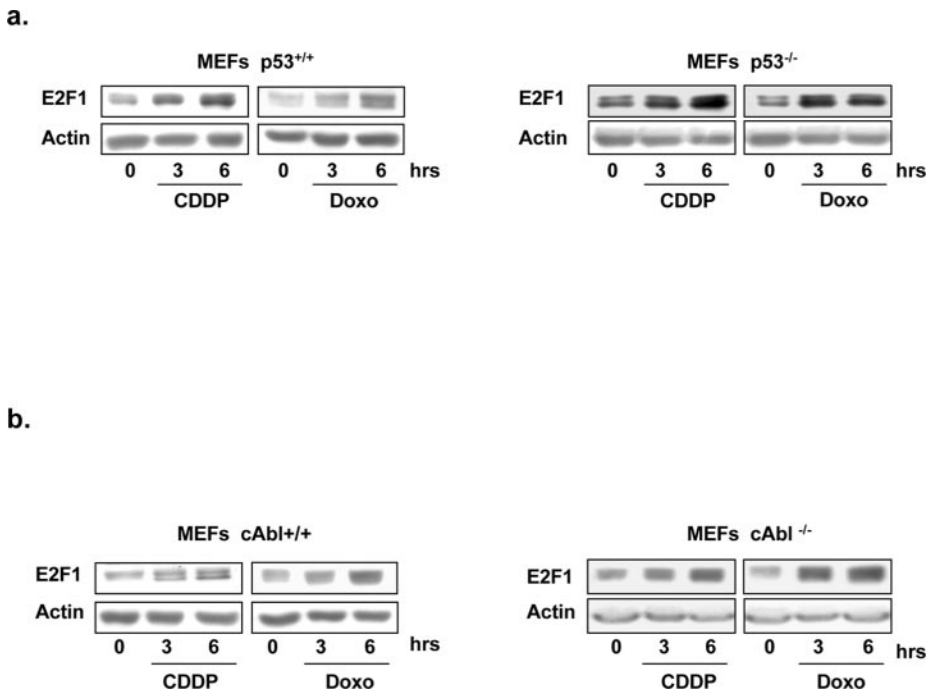

FIG. 2. **Cisplatin and doxorubicin treatments stabilize E2F1 independently of p53 and cAbl.** MEFs $p53^{-/-}$ and $cAbl^{-/-}$ and their $p53^{+/+}$ and $cAbl^{+/+}$ respective controls were treated with either 25 μM cisplatin (*CDDP*) or 2 μM doxorubicin for up to 6 h. To reduce background, nitrocellulose filters were saturated with 2% porcine gelatin in Tris-buffered saline with Tween (Sigma) prior to saturation with 2% milk in Tris-buffered saline with Tween. Anti-actin antibody was used in the same blots as loading control.

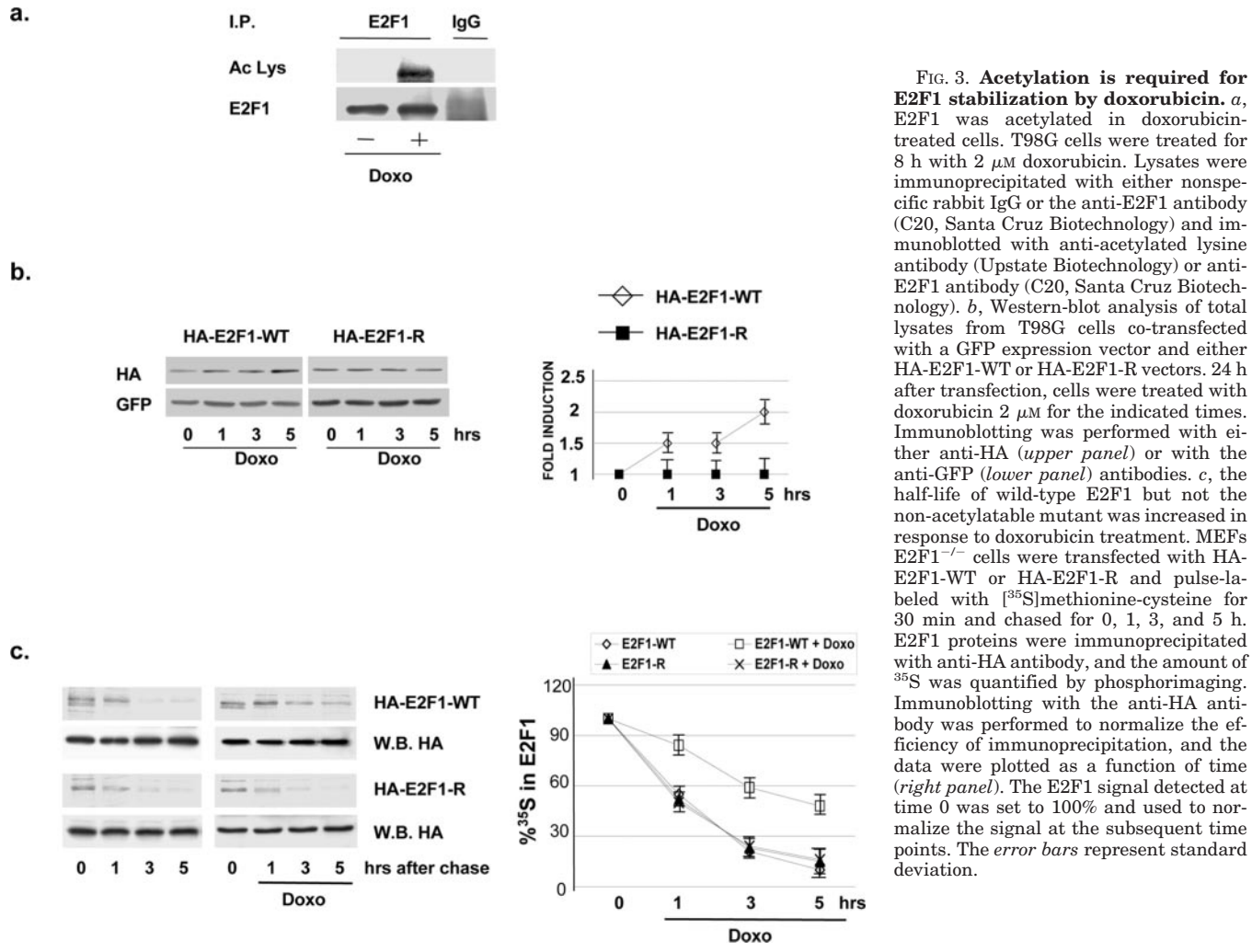

FIG. 3. **Acetylation is required for E2F1 stabilization by doxorubicin.** *a*, E2F1 was acetylated in doxorubicin-treated cells. T98G cells were treated for 8 h with 2 μM doxorubicin. Lysates were immunoprecipitated with either nonspecific rabbit IgG or the anti-E2F1 antibody (C20, Santa Cruz Biotechnology) and immunoblotted with anti-acetylated lysine antibody (Upstate Biotechnology) or anti-E2F1 antibody (C20, Santa Cruz Biotechnology). *b*, Western-blot analysis of total lysates from T98G cells co-transfected with a GFP expression vector and either HA-E2F1-WT or HA-E2F1-R vectors. 24 h after transfection, cells were treated with doxorubicin 2 μM for the indicated times. Immunoblotting was performed with either anti-HA (*upper panel*) or with the anti-GFP (*lower panel*) antibodies. *c*, the half-life of wild-type E2F1 but not the non-acetylatable mutant was increased in response to doxorubicin treatment. MEFs $E2F1^{-/-}$ cells were transfected with HA-E2F1-WT or HA-E2F1-R and pulse-labeled with [^{35}S]methionine-cysteine for 30 min and chased for 0, 1, 3, and 5 h. E2F1 proteins were immunoprecipitated with anti-HA antibody, and the amount of ^{35}S was quantified by phosphorimaging. Immunoblotting with the anti-HA antibody was performed to normalize the efficiency of immunoprecipitation, and the data were plotted as a function of time (*right panel*). The E2F1 signal detected at time 0 was set to 100% and used to normalize the signal at the subsequent time points. The *error bars* represent standard deviation.

translational events, other than ATM/ATR-dependent phosphorylation, might contribute to E2F1 stabilization in cells exposed to the DNA-damaging drug doxorubicin.

E2F1 Stabilization in Response to DNA-damaging Drugs Is Independent of P53 and cAbl—Searching for additional pathways involved in DNA-damage-dependent stabilization of

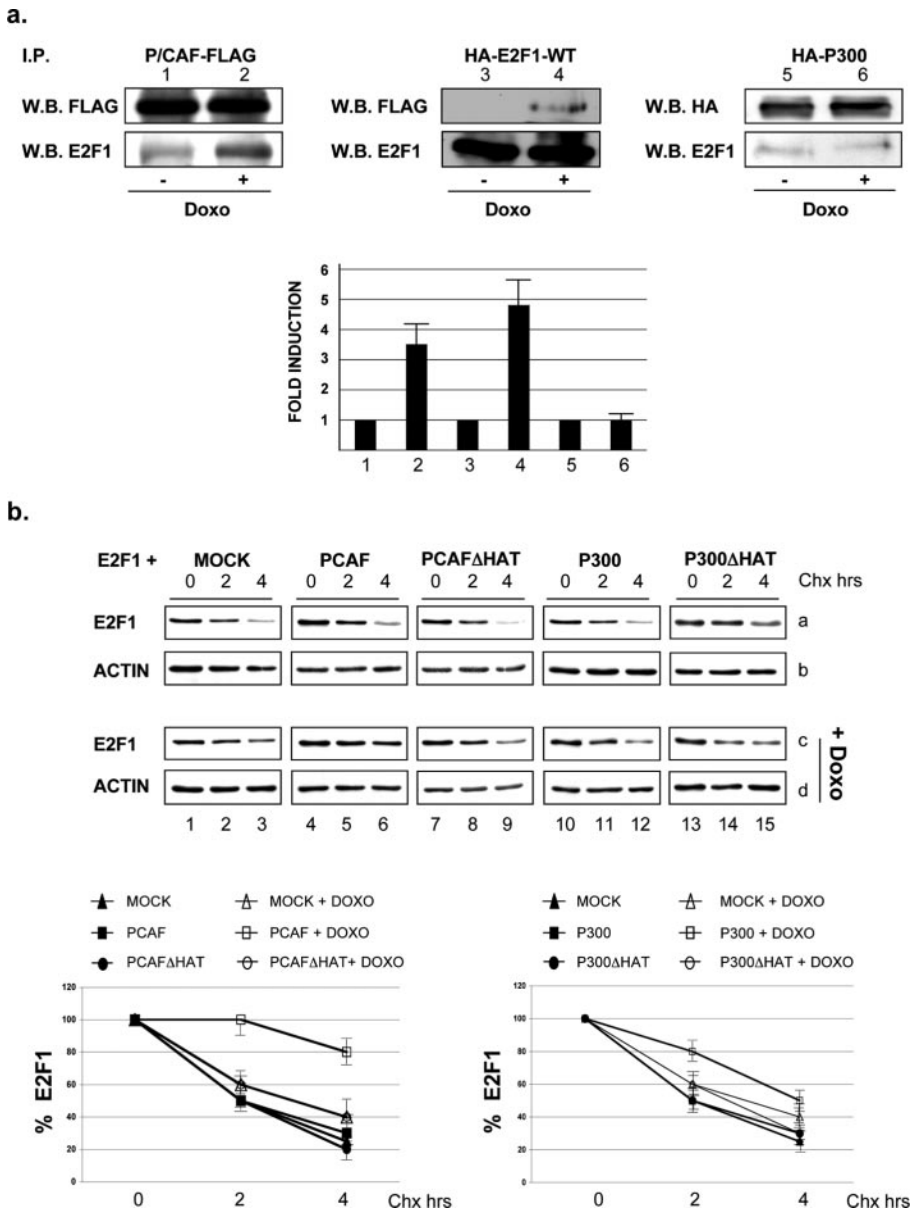

FIG. 4. **P/CAF binds to E2F1 and increases its half-life in a HAT-dependent manner after doxorubicin treatment.** *a*, E2F1$^{-/-}$ MEFs were transfected with either HA-E2F1-WT and P/CAF-FLAG (*lanes 1–4*) or with PCMV-E2F1 and HA-P300 expression vectors (*lanes 5–6*) and treated 12 h after transfection with 2 μM doxorubicin for an additional 8 h. Anti-FLAG and anti-HA immunoprecipitates were immunoblotted with either anti-FLAG or anti-E2F1 antibodies (*lanes 1–4*) or with anti-HA and anti-E2F1 antibodies (*lanes 5–6*). The *lower panel* summarizes the densitometric analysis from three independent experiments. Results are expressed as fold induction with respect to untreated cells. *b*, P/CAF acetylase activity is required to induce E2F1 stabilization in response to doxorubicin treatment. Subconfluent U2OS cells in 100-mm dishes were transfected with the LipofectAMINE Plus method (Invitrogen). Twelve hours later, cells were harvested and seeded in 35-mm dishes to normalize for the efficiency of transfection. The day after cells were treated with 2 μM doxorubicin for 8 h, cycloheximide (50 μg/ml) was added, and cells were harvested at the indicated times. Anti-actin antibody was used to normalize for protein loading. *Lower panels* summarize the densitometric analysis of E2F1 levels from three independent experiments ($\pm$2 S.D.). Results are expressed as percent remaining with respect to time zero.

E2F1, we analyzed the role of p53. Indeed, increasing evidence suggests that the integrity of the Rb pathway could influence the activity of p53 and *vice versa*. E2F1 cooperates with p53 to induce apoptosis by increasing ARF transcription and thereby blocking murine double minute 2 (MDM2)-mediated p53 degradation (27). In addition, it has been shown that p53 interacts directly with the E2F1/DP1 complex through the cyclin A-binding domain located in the N terminus of E2F1. Cyclin A inhibits E2F1 binding to p53 *in vitro* and abrogates the ability of E2F1 to cooperate with p53 to induce apoptosis *in vivo* in response to DNA damage (28). Moreover, p53 lies upstream of E2F1 in the apoptotic pathway induced by UV treatment in mouse keratinocytes (29). As shown in Fig. 2*a*, endogenous E2F1 accumulates to a very similar extent in p53$^{-/-}$ MEFs as compared with wild-type MEFs exposed to either cisplatin or doxorubicin, thus ruling out the requirement of p53 protein in this context. Next we analyzed the requirement of the cAbl tyrosine kinase for E2F1 stabilization in response to DNA damage. cAbl is involved in multiple DNA damage signaling pathways. In response to IR treatment, cAbl induces the phosphorylation of the proapoptotic p73 protein and controls its accumulation and apoptotic activity in response to cisplatin and doxorubicin (30–33). As shown in Fig. 2*b*, endogenous E2F1 protein accumulates in response to both treatments in cAbl$^{-/-}$ MEFs, thus excluding a role for this kinase in this context.

E2F1 Acetylation Is Required for Its Stabilization in Response to DNA Damage—E2F1 can be acetylated *in vitro* at lysine residues 117, 120, and 125 by P/CAF and, to a lesser extent, p300 acetyltransferases (23, 24). Acetylation of these residues leads to increased DNA-binding affinity, induction of its transcriptional activity, and accumulation of the protein. Because doxorubicin treatment leads to the accumulation of acetylated E2F1 protein species (Fig. 3*a*; Ref. 22), we hypothesized that DNA damage-induced acetylation might be responsible for the E2F1 stabilization we observed in the absence of ATM/ATR-dependent phosphorylation. To assess whether E2F1 acetylation is required for its stabilization after genotoxic stress, we first analyzed the behavior of a non-acetylatable E2F1 mutant protein (HA-E2F1-R) (24). As shown in Fig. 3*b*, exogenously expressed wild-type E2F1 is clearly accumulated 5 h after doxorubicin treatment, whereas the HA-E2F1-R mutant is not accumulated at all at the time points investigated. Doxorubicin treatment does not change cellular levels of E2F1 mRNA (data not shown; Refs. 24, 34). Therefore, we examined

whether acetylation might affect protein half-life in cells exposed to DNA-damaging drugs. The levels of exogenously expressed wild-type E2F1 and HA-E2F1-R proteins were monitored in a pulse-chase experiment, either in the presence or the absence of doxorubicin. Fig. 3*c* shows that HA-E2F1-WT half-life is significantly prolonged in response to doxorubicin treatment, whereas the half-life of the HA-E2F1-R mutant remains unaffected. Altogether, these results indicate that acetylation of E2F1 is required to stabilize the protein in response to DNA damage.

Different Role for P/CAF and P300 in E2F1 Stabilization after DNA Damage—In vitro studies indicate that P/CAF is more efficient in acetylating E2F1 with respect to p300. However, the relative role of the two acetyltransferases in influencing E2F1 stability following genotoxic stress was not determined. To address this issue, we performed co-immunoprecipitation experiments to investigate the ability of P/CAF and p300 to form complexes with E2F1 in response to DNA damage. As shown in Fig. 4*a*, immunoprecipitation of exogenously expressed FLAG-tagged P/CAF in E2F1$^{-/-}$ MEFs treated with doxorubicin results in increased levels of co-immunoprecipitated exogenous HA-E2F1 (*lanes 1–2*). Likewise, exogenously expressed HA-E2F1 co-immunoprecipitates FLAG-P/CAF only in treated cells (Fig. 4*a*, *lanes 3–4*). Unlike P/CAF, p300 complex formation with E2F1 is not modulated by doxorubicin treatment (Fig. 4*a*, *lanes 5–6*).

To investigate the relative contributions of P/CAF and p300, and, specifically, of their HAT activity in E2F1 stabilization in response to DNA damage, we performed a cycloheximide chase on transfected U2OS cells treated with doxorubicin. As shown in Fig. 4*b*, exogenously expressed P/CAF (*lanes 4–6*, *row a*) and p300 (*lanes 10–12*, *row a*) do not alter E2F1 half-life in untreated cells. Conversely, in doxorubicin-treated cells, the half-life of E2F1 is significantly prolonged in the presence of P/CAF (compare *lanes 4–6* to *lanes 1–3*, *row c*). Stabilization of E2F1 is likely due to the activation of the P/CAF acetyltransferase activity, because the P/CAF-ΔHAT mutant (*lanes 7–9 versus lanes 1–3*, *row c*), deleted of the acetyltransferase domain, is unable to stabilize E2F1, when compared with wild-type P/CAF. On the other hand, p300 (*lanes 10–12 versus lanes 1–3*, *row c*) only slightly increased E2F1 half-life in the presence of doxorubicin, whereas its HAT-defective mutant (*lanes 13–15 versus lanes 1–3*, *row c*) showed behavior similar to the control empty vector both in treated and untreated cells.

DISCUSSION

Many transcription factors have been identified as substrates for P/CAF and p300/CREB-binding protein (35). Particularly, acetylation regulates the transcriptional activity of p73 and p53 in response to genotoxic stress (36). We recently demonstrated that DNA damage induces E2F1 apoptotic potential (22). This is achieved by the selective relocalization of E2F1 from cell-cycle progression genes to the promoter of the pro-apoptotic p73 gene, where E2F1 accumulates into transcriptionally active complexes that include the acetyltransferase coactivator P/CAF. We found that E2F1 acetylation is required in this context (22).

Here we report that in addition to specifying its transcriptional potential, DNA damage-induced acetylation of E2F1 is also important for its accumulation. Indeed, the finding that E2F1 is stabilized in the ATM-defective cell line AT-TERT strongly suggests that additional pathways, other than ATM/ATR-dependent phosphorylation, are required to stabilize E2F1 in response to genotoxic stress. We ruled out a role for p53 and cAbl, two of the major transducers of DNA-damage signaling, in inducing E2F1 accumulation in this context. We found that DNA damage-induced acetylation of E2F1 is the major determinant of its accumulation. We further assessed the relative role of the two acetyltransferases P/CAF and p300, both previously reported to be involved in E2F1 acetylation. Although E2F1 binding to p300 is unaffected by DNA damage, the affinity between P/CAF and E2F1 is strongly increased in doxorubicin-treated cells. Accordingly, only the HAT activity of P/CAF, and not that of p300, is required to induce a prolongation of E2F1 half-life in the presence of genotoxic stress.

The mechanisms that lead to the increased stability of the acetylated E2F1 protein still remain elusive. Acetylation of p53 has also been reported to induce its stabilization by preventing its MDM2-dependent ubiquitination (37). Similarly to p53, E2F1 is degraded through the ubiquitin-proteasome pathway. Phosphorylation at the Ser-403 in S phase by the TFIIH kinase targets E2F1 for its rapid ubiquitination and degradation (18). One mechanism might be that acetylation of E2F1 competes with the TFIIH-dependent phosphorylation, thus subtracting the fraction of E2F1 targeted for elimination. Furthermore, binding to Rb also protects E2F1 from its ubiquitination (14, 15). A differential interaction of E2F1 with Rb in response to DNA damage may impart distinct fates to the protein. This possibility is also suggested by the recent identification of an additional binding site for E2F1 on Rb, which putatively controls E2F1-apoptotic functions and is regulated by DNA damage (38). These observations and the results we reported here, together with the fact that Rb functions are regulated by acetylation as well (39), suggest that acetylation might coordinately control Rb/E2F1 interaction in response to DNA damage and, in this way, E2F1 stability and apoptotic activity.

In conclusion, the findings reported here support previous observations (22), indicating that E2F1 acetylation plays a pivotal role in the recruitment of its apoptotic potential and are consistent with a two-step model of E2F1-driven apoptotic response to DNA-damaging agents. In this model, DNA damage first enhances P/CAF-dependent acetylation of E2F1, resulting in its stabilization and formation of stable protein complexes with P/CAF. The increased levels of acetylated E2F1 are then available for its selective recruitment onto the p73 gene promoter to trigger the apoptotic response. Whether this mechanism is impaired in human cancers, frequently associated to genetic impairments of the Rb/E2F1 pathway, or might be exploited to improve the therapeutic index of genotoxic anticancer drugs, will represent the goal of our future investigations.

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