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# p300 Modulates ATF4 Stability and Transcriptional Activity Independently of Its Acetyltransferase Domain*

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**ATF4 plays a crucial role in the cellular response to stress and multiple stress responses pathways converge to the translational up-regulation of ATF4. ATF4 is a substrate of the SCF$^{\beta TrCP}$ ubiquitin ligase that binds to $\beta$TrCP through phosphorylation on a DSG*XXX*S motif. We show here that ATF4 stability is also modulated by the histone acetyltransferase p300, which induces ATF4 stabilization by inhibiting its ubiquitination. Despite p300 acetylates ATF4, we found that p300-mediated ATF4 stabilization is independent of p300 catalytic activity, using either the inactive form of p300 or the acetylation mutant ATF4-K311R. ATF4 deleted of its p300 binding domain is no more stabilized by p300 nor recruited into nuclear speckles. In consequence of ATF4 stabilization, both p300 and the catalytically inactive enzyme increase ATF4 transcriptional activity.**

ATF4, a member of the ATF/CREB bZIP transcription factor family, plays a crucial role in response to stress, because multiple intracellular stress pathways (endoplasmic reticulum stress, amino acid deprivation, and exposure to oxidant or reactive metals) converge on a single event, phosphorylation of eIF2$\alpha$, which leads both to a general inhibition of protein synthesis but also to the translational up-regulation of the mRNA encoding ATF4 (1–4). Thus the targets of ATF4 are of paramount importance in a generalized stress response. In fact, higher eucaryotes have conserved through activation of ATF4 the same fundamental mechanism used by yeast through up-regulation of GCN4 in response to amino acid starvation (5). In addition, ATF4 is important for cell proliferation and differentiation, because ATF4 knock-out mice display abnormal lens formation (6) and defects in cell proliferation in fetal liver, embryonic lens and hair follicles, as well as an overall reduction in size of the animals (7). ATF4 is a critical regulator of osteoblast differentiation and function (8) and bone resorption (9). ATF4 is also involved in long term memory induction (10). Hence ATF4 is a master transcription factor for which temporal expression and activity are under tight cellular control. ATF4 interacts with several general transcription factors such as TBP, TFIIB, and RAP30 (11). The transcriptional selectivity of ATF4 is modulated by the formation of heterodimers with multiple C/EBP bZIP or AP-1 family members (12–14). CBP and p300 acetylate ATF4 in its bZIP domain (15) and enhance its transcriptional activity (11, 15).

Acetylation of histone and non-histone proteins is emerging as a central process in transcriptional activation. Nuclear histone acetyltransferases (HATs)[5] act as transcriptional co-activators that have been shown to acetylate different transcription factors, including p53, $\beta$catenin, MyoD, E2F-1, ATF4, and SREBP1 (15–19). The consequences of acetylation on protein function vary from one protein to another depending on where within the protein the acetylation takes place. Acetylation has also been reported to modulate protein-protein interactions, to inhibit nuclear export (20), and to alter protein stability.

We have shown that ATF4 degradation is mediated by the E3 ubiquitin ligase SCF$^{\beta TrCP}$ (21) that we first identified as the E3 ubiquitin ligase responsible for the degradation of CD4 induced by the human immunodeficiency virus type 1 protein Vpu (22). For most of the $\beta$TrCP substrates identified up to now, including ATF4, serine phosphorylation of a DSG*XX*(*X*)S motif is required for interaction with $\beta$TrCP and subsequent degradation by the proteasome. Although phosphorylation of $\beta$TrCP substrates is a crucial step for the regulation of their stability, it is probably not the unique event that regulates their destruction.

Here, we show that the HAT p300 is associated with ATF4 and interferes with its stability. p300 increased ATF4 stability, independently of its acetyltransferase activity and thus enhances ATF4 mediated transcription. Our results provide a new mechanism for the regulation of ATF4 stability and expression.

## MATERIALS AND METHODS

*Plasmid Construction and Mutagenesis*—Vectors for FLAG-P/CAF, FLAG-p300, and FLAG-p300ΔHAT were kindly provided by Y. Nakatani (23). 1XAARE-TK-LUC plasmid-expressing luciferase (24) was kindly provided by P. Fafournoux. In this construct, a 19-bp segment of the CHOP promoter from −313 to −295 (AACATTGCATCATCCCCGC) containing the positive element AARE was cloned 5′ of the minimal herpes simplex virus promoter for thymidine kinase (−40 to +50).

pAS1B vectors for HA-ATF4, HA-ATF4ΔbZIP, and HA-ATF4-S219N, and plasmid expressing Myc-$\beta$TrCP were described previously (21). HA-ATF4Δ1–85 was obtained by subcloning in pAS1B; GST proteins were obtained by subcloning in pGEX4T-3.1. ATF4 lysine mutants were obtained by PCR mutagenesis and FLAG-ATF4 by subcloning in pSG (Sigma).

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[5] The abbreviations used are: HAT, histone acetyltransferase; E3, ubiquitin-protein isopeptide ligase; GST, glutathione *S*-transferase; siRNA, small interference RNA; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; PML, promyelocytic leukemia; CBP, CREB-binding protein; SCF, Skp1-Cullin-F-box.



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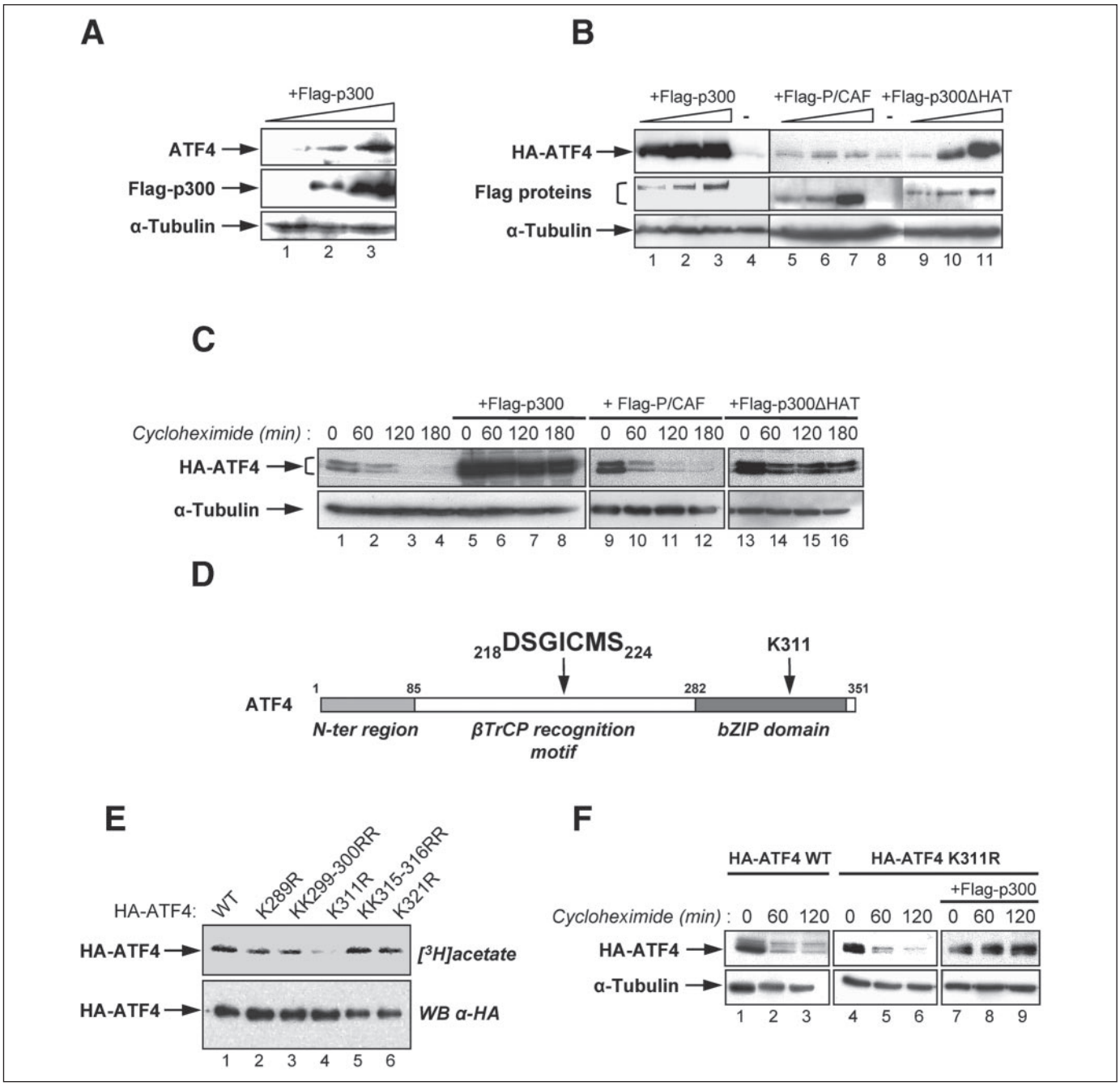


FIGURE 1. **ATF4 stability is enhanced by p300 independently of its catalytic activity.** *A*, p300 increases endogenous ATF4 protein level. 293T cells untransfected (*lane 1*) or transfected with 10 or 20 $\mu$g of FLAG-p300 expressing vector (*lanes 2* and *3*) were analyzed by SDS-PAGE and Western blotting using indicated antibodies. *B*, ATF4 protein level is both increased by p300 and its inactive mutant. Lysates from 293T cells expressing HA-ATF4 and 5, 10, or 20 $\mu$g of FLAG-p300, FLAG-P/CAF, or FLAG-p300ΔHAT (FLAG proteins) were analyzed by SDS-PAGE and Western blotting using anti-HA, anti-FLAG, or anti-$\alpha$-tubulin antibodies. *C*, p300 and its inactive mutant p300ΔHAT, but not P/CAF, can stabilize ATF4. After co-transfection of HA-ATF4 with FLAG-p300, or FLAG-p300ΔHAT, or FLAG-P/CAF, 293T cells were treated with cycloheximide, lysed at indicated times, and analyzed by Western blotting. *D*, schematic representation of the ATF4 structure. *E*, lysine 311 of ATF4 is the major site of acetylation *in vivo*. HA-ATF4 lysine mutants were expressed in 293T cells, acetylated with [$^3$H]acetate, immunoprecipitated using anti-HA antibody, and analyzed by SDS-PAGE and autoradiography for acetylation (*upper panel*) or by Western blot using anti-HA antibody for estimation of total expression of HA-ATF4 (*lower panel*). *F*, mutation of the major acetylation site in ATF4 does not inhibit stabilization by p300. After co-transfection of HA-ATF4 or HA-ATF4 K311R with empty or FLAG-p300 vectors, 293T cells were treated with cycloheximide, lysed at indicated times, and analyzed by SDS-PAGE and Western blotting using indicated antibodies.

*Lysis, Immunoprecipitation, and Western Blotting*—24 h after transfection, cells were harvested and lysed in 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris HCl (pH 7.5), 1 mM EDTA. When indicated, 50 $\mu$g/ml cycloheximide was added before lysis. For immunoprecipitations, cell lysates were pre-cleared with mouse, rabbit, or goat non-immune antibodies and protein A- or G-agarose for 90 min, and supernatants were incubated overnight with 5 $\mu$g/ml mouse monoclonal anti-FLAG (M2, Sigma), 5 $\mu$g/ml of mouse monoclonal anti-Myc (9E10, Roche Applied Science), or 5 $\mu$g/ml rabbit anti-ATF4 (C-20, Santa Cruz Biotechnology), and then incubated with proteins A/G-agarose beads (Sigma) for 1 h. Immune complexes were eluted with Laemmli buffer, separated by SDS-PAGE, and revealed by Western blotting. Antibodies used for Western blotting were rabbit anti-ATF4 (C-20) or goat polyclonal anti-ATF4 (C-19, Santa Cruz Biotechnology), rat anti-HA-PER (3F10, Roche

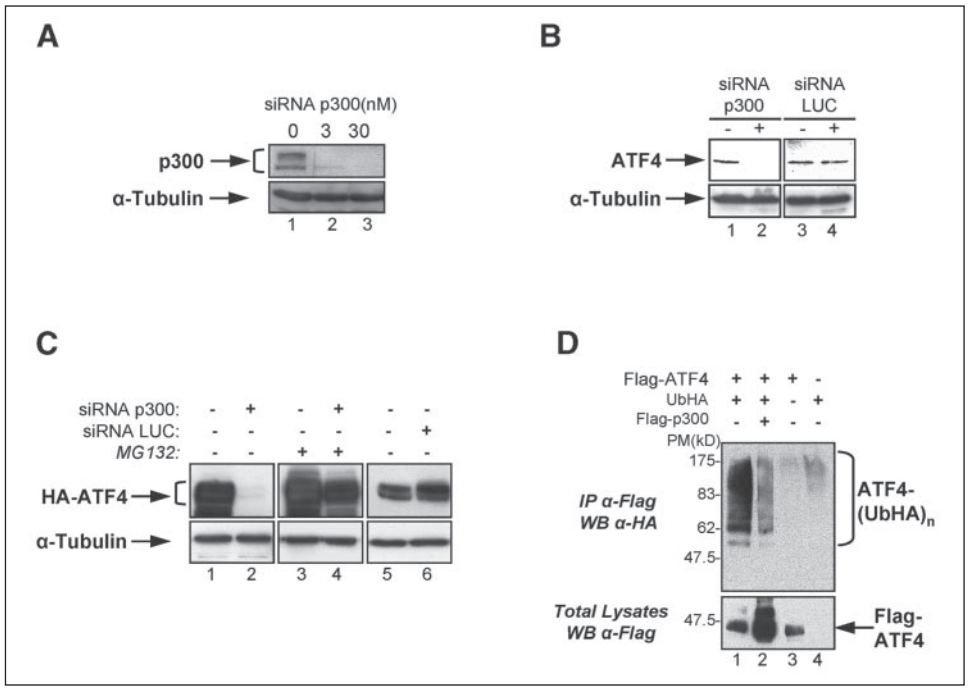


FIGURE 2. **Silencing of p300 reduces ATF4 expression, and overexpression of p300 inhibits ATF4 ubiquitination and enhances its expression.** *A*, silencing of p300 by siRNA. HeLa cells were transfected by p300 siRNA and p300 was detected by Western blotting using anti-p300 antibodies. *B*, silencing of p300 expression by siRNAs results in the disappearance of endogenous ATF4. HeLa cells were transfected (+) or not (−) by p300 or luciferase (*LUC*) siRNAs, and analyzed by Western blotting using anti-ATF4 or anti-α-tubulin antibodies. *C*, p300 interferes with ATF4 targeting to the proteasome. HeLa cells were transfected with p300 siRNA or Luc siRNA, as indicated, together with HA-ATF4. Cells were treated or not with the proteasome inhibitor MG132 and analyzed by Western blotting using anti-HA or anti-α-tubulin antibodies. *D*, overexpression of p300 decreased ATF4 ubiquitination. 293T cells were co-transfected with FLAG-ATF4, ubiquitin-HA, and FLAG-p300 (*lane 2*), or only with FLAG-ATF4 and ubiquitin-HA (*lane 1*) or FLAG-ATF4 (*lane 3*) or ubiquitin-HA (*lane 4*), immunoprecipitated with anti-FLAG antibodies, and probed with anti-HA (for detection of ubiquitinated ATF4, *upper panel*) or anti-FLAG antibodies (*bottom panel*). Because of its high molecular mass, FLAG-p300 is not visible in the part of the gel selected for detection of FLAG-ATF4 ubiquitinated species.

Applied Science), mouse anti-FLAG-PER (M2, Sigma), rabbit anti-p300 (C-20, Santa Cruz Biotechnology), mouse anti-Myc (9E10, Roche Applied Science), anti-actin (sc1616, Santa Cruz Biotechnology), and mouse anti-α-tubulin (DM1A, Sigma).

*GST Pull-down Assays*—GSTATF4 and ATF4 mutant fusion proteins were expressed in BL21 *Escherichia coli* and purified according to standard protocols. 293T cells transfected with 10 μg of FLAG-p300 vector were lysed in 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100 (pH 8). Lysates were incubated overnight at 4 °C with 5 μg of purified GST proteins. The beads were then washed with 50 mM Tris-HCl, pH 7.6, 300 mM NaCl, 5 mM EDTA 1% Triton X-100 (pH 8). Bound proteins were separated by SDS-PAGE and revealed by Western blotting using anti-FLAG antibody.

*Small Interfering RNAs*—RNA oligonucleotides (from Eurogentech) anti-p300 (forward: 5′-cagagcaguccuggauuagtt-3′; reverse 5′-cuaauccaggacugcucugtt-3′) and luciferase (25) were annealed and transfected in HeLa cells using oligofectamine. Twenty-four hours later, cells were co-transfected with siRNA and plasmid vectors using Lipofectamine (Invitrogen), and then harvested after another 24 h.

*In Vivo ATF4 Acetylation Assay*—293T cells were transfected with HA-ATF4 or ATF4 mutants for 24 h, washed with phosphate-buffered saline, incubated in medium minus methionine/cysteine supplemented with 2% fetal bovine serum for 1 h at 37 °C, then in medium containing [$^3$H]NaAc at 1 mCi/ml for 1 h at 37 °C. Cells were washed in phosphate-buffered saline, resuspended in lysis buffer (120 mM NaCl, 50 mM Tris, pH 7.5, 0.5% Triton, pH 8, 1 mM dithiothreitol, 1 mM EDTA, 400 nM trichostatin A, protease inhibitors), and immunoprecipitated using anti-HA antibody (12CA5, Roche Applied Science).

*Ubiquitination Assay*—293T cells were co-transfected with expression vectors and HA-ubiquitin plasmid. Cell pellets were resuspended in denaturing lysis buffer (50 mM Tris, pH 7.5, 0.5 mM EDTA, 1% SDS, and 1 mM dithiothreitol), boiled for 10 min, and diluted in 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM dithiothreitol, 20 μM MG132 with protease inhibitors, then immunoprecipitated with anti-FLAG antibody. Ubiquitin species were revealed by Western blotting using anti-HA antibodies.

*Immunostaining of Cells*—The protocol for immunostaining the cells is described in Lassot *et al.* (21). Briefly, HeLa cells were fixed, permeabilized, and incubated with anti-FLAG (M2, Sigma), anti-RNAPollII (8WG16, BabCO), or anti-PML (PG-M3, Santa Cruz Biotechnology) antibodies, washed in phosphate-buffered saline, and incubated with anti-mouse Cy3 antibodies (Jackson ImmunoResearch), or directly incubated with anti-HA-fluorescein isothiocyanate antibody (3F10, Roche Applied Science). For the second staining, cells were incubated with anti-HA-fluorescein isothiocyanate antibody. Confocal or direct microscopy was carried out under fluorescent light.

*Luciferase Assays*—293T or HeLa cells were plated in 12-well flat bottom plates on the day prior to transfection at a density of $2 \times 10^4$ cells/well in DMEM. Transfections were performed using the calcium phosphate co-precipitation method with the Mammalian Transfection Kit (Stratagene) or with oligofectamine (Invitrogen). We used the luciferase reporter 1XAARE-LUC containing sequence placed 5′ to the gene for luciferase. Cells were co-transfected with 1 μg of 1XAARE-LUC, 30 ng of pRL-TK-Renilla (PRL-TK from Promega), and various amounts of plasmids expressing HA-ATF4 or HA-ATF4 mutants, FLAG-p300 and FLAG-p300ΔHAT, or siRNA against p300 as indicated. 24 h post-transfection, cells were lysed and luciferase and *Renilla* activities were measured with luciferase assay reagent (Dual-Luciferase Reporter Assay System, Promega) using a Lumat LB9507 luminometer (EG&G instruments). Graphs indicate the average of three independent experiments.

## RESULTS

*p300 Enhances ATF4 Stability Independently of Its Acetyltransferase Activity*—Because Gachon *et al.* (15) reported recently that ATF4 was acetylated by p300, we investigated the potential role of p300 on ATF4 stability. We found that expression of increasing amounts of p300 leads to the accumulation of endogenous ATF4 (Fig. 1*A*) and transfected HA-ATF4 (Fig. 1*B*, compare *lanes 1–3* to *lane 4*). This effect was specific for p300, because another acetylase, P/CAF, was unable to promote ATF4 accumulation (Fig. 1*B*, compare *lanes 5–7* to *lane 8*). Surprisingly, p300ΔHAT, an inactive mutant of p300, was still able to accumulate

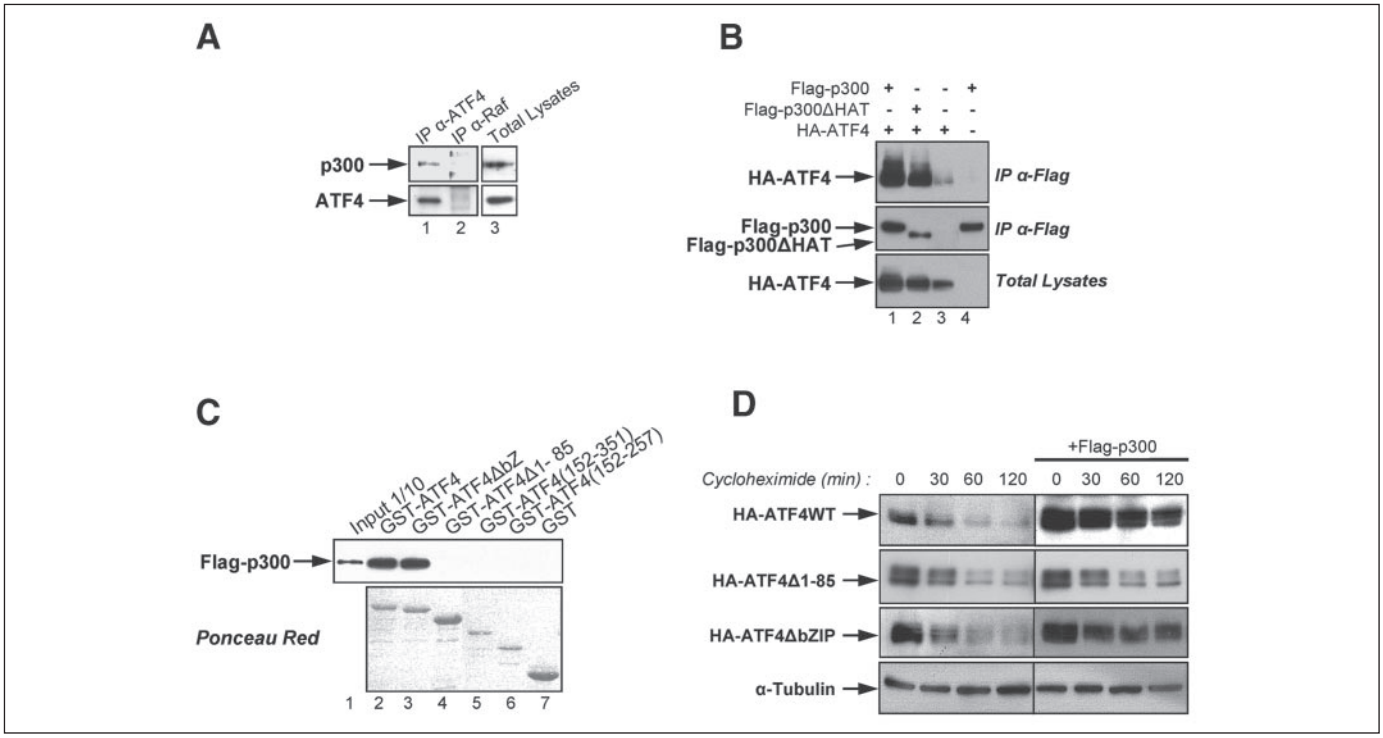


FIGURE 3. **The N-terminal region of ATF4-(1–85) is required for ATF4 binding to p300 and for p300-mediated ATF4 stabilization.** *A*, endogenous p300 and ATF4 are associated in cells. Lysates from 293T cells were immunoprecipitated with anti-ATF4 antibody (*IP α-ATF4*) or with the control antibody anti-Raf (*IP α-Raf*) and analyzed by Western blot using anti-p300 (*top*) or anti-ATF4 (*bottom*) antibodies. *B*, the p300 inactive mutant p300ΔHAT still interacts with HA-ATF4. 293T cells were co-transfected with HA-ATF4 and FLAG-p300 (*lane 1*), FLAG-p300ΔHAT (*lane 2*), the empty vector (*lane 3*), or with FLAG-p300 alone (*lane 4*), immunoprecipitated with anti-FLAG antibodies and analyzed by SDS-PAGE, and Western blotting using anti-HA or anti-FLAG antibodies. *C*, ATF4 residues 1–85 are required for p300-ATF4 interaction. Lysates from 293T cells expressing FLAG-p300 were used in GST pull-down experiments with bacterially expressed ATF4 wt or various ATF4 deletion mutants fused to GST (*lanes 2–6*). FLAG-p300 bound to the GST-ATF4 constructs was detected by Western blot with anti-FLAG antibodies. 10% of the cell lysate used in the pull-down experiments was loaded in the *Input lane 1*. *D*, stabilization of ATF4 by p300 depends on ATF4 residues 1–85 but not on the ATF4 bZIP region. 293T cells were co-transfected with wild type HA-ATF4, HA-ATF4Δ1–85, or HA-ATF4 ΔbZIP and plasmids expressing FLAG-p300 (*right panels*) or the corresponding empty vector (*left panels*). Cells were treated with cycloheximide at the indicated times, lysed, and probed with anti-HA or anti-α-tubulin antibodies.

ATF4 (Fig. 1*B*, compare *lanes 9–11* to *lane 8*), however less efficiently than wild-type p300. We also found, by studying kinetics of ATF4 degradation after cycloheximide treatment, that ATF4 was stabilized by both p300 (Fig. 1*C*, compare *lanes 5–8* to *1–4*) and its inactive mutant p300ΔHAT (Fig. 1*C*, compare *lanes 13–16* to *1–4)* but not by P/CAF (Fig. 1*C*, compare *lanes 9–12* to *1–4*). Indeed, deduced from the scanning of the Western blot shown in Fig. 1*C*, we found that ~31% of ATF4 was present after 60 min of cycloheximide treatment, whereas ~90% of ATF4 was still present in the same conditions in the presence of p300, 85% in the presence of p300ΔHAT, and 36% in the presence of P/CAF.

To confirm that p300 regulates ATF4 stability independently of its enzymatic activity, we decided to test the stability of an ATF4-unacetylated mutant. Gachon *et al.* had shown that ATF4 is acetylated in the bZIP domain, but they did not map a particular residue (15). We performed an *in vivo* acetylation assay using several lysine mutants in the bZIP domain of ATF4 (see Fig. 1*D* for ATF4 structure), and we found that mutation of the lysine residue 311 (K311R) drastically reduced ATF4 acetylation (Fig. 1*E*, *lane 4*). The degradation kinetics of the ATF4K311R mutant was similar to that of wild-type ATF4 (Fig. 1*F*, compare *lanes 4–6* to *1–3*), and this mutant was still stabilized by p300 (Fig. 1*F*, compare *lanes 7–9* to *4–6*). Overall, these results show that the acetylase p300 is able to stabilize ATF4 independently of its enzymatic activity.

*p300 Inhibits ATF4 Ubiquitination and Degradation*—Because overexpression of p300 leads to ATF4 stabilization, we wanted to check whether inactivation of p300 would destabilize ATF4 and favor its targeting to the ubiquitin proteasome pathway by the $SCF^{\beta TrCP}$ complex, the E3 ubiquitin ligase for ATF4. For that purpose, we used small interfering RNAs (siRNAs) to inactivate endogenous p300, as previously described (26). The efficiency of p300 gene silencing by siRNA treatment was assessed by Western blot using anti-p300 antibodies (Fig. 2*A*). Treatment of HeLa cells with siRNA-inactivating p300 caused a striking diminution in the expression of endogenous ATF4 (Fig. 2*B*, *lanes 1* and *2*) as well as transfected HA-ATF4 (Fig. 2*C*, *lanes 1* and *2*) up to an almost complete disappearance of both proteins. By contrast, treatment with a control siRNA duplex (siRNA LUC) had no effect (Fig. 2*B*, *lanes 3* and *4* and Fig. 2*C*, *lanes 5* and *6*). These results confirm that p300 plays an important role in the control of ATF4 stability. Such a decrease in the level of ATF4 was rescued when cells were treated with MG132 (Fig. 2*C*, *lanes 3* and *4*). Indeed, deduced from the scanning of the Western blot, we found that ~81% of ATF4 was still present when p300 siRNA-treated cells were incubated with MG132, whereas ATF4 was not detected in siRNA p300-treated cells in the absence of MG132. These results suggest that stabilization of ATF4 mediated by p300 interferes with ATF4 targeting to the proteasome. Confirming this hypothesis, we observed less ATF4-ubiquitinated species in the presence of overexpressed FLAG-p300 (Fig. 2*D*, *top panel*, compare *lane 2* to *lane 1*), whereas the non-ubiquitinated forms of ATF4 were stabilized (Fig. 2*D*, *bottom panel*, compare *lane 2* to *lane 1*). Thus, p300 appears to interfere strongly with the ubiquitin-proteasome pathway to stabilize ATF4 protein.

*The N-terminal Domain of ATF4 Is Required for Interaction with p300*—To gain insight into the mechanisms accounting for ATF4 stability, we then asked whether p300 interact with ATF4. By co-immunoprecipitation using anti-ATF4 antibodies, we found that endogenous

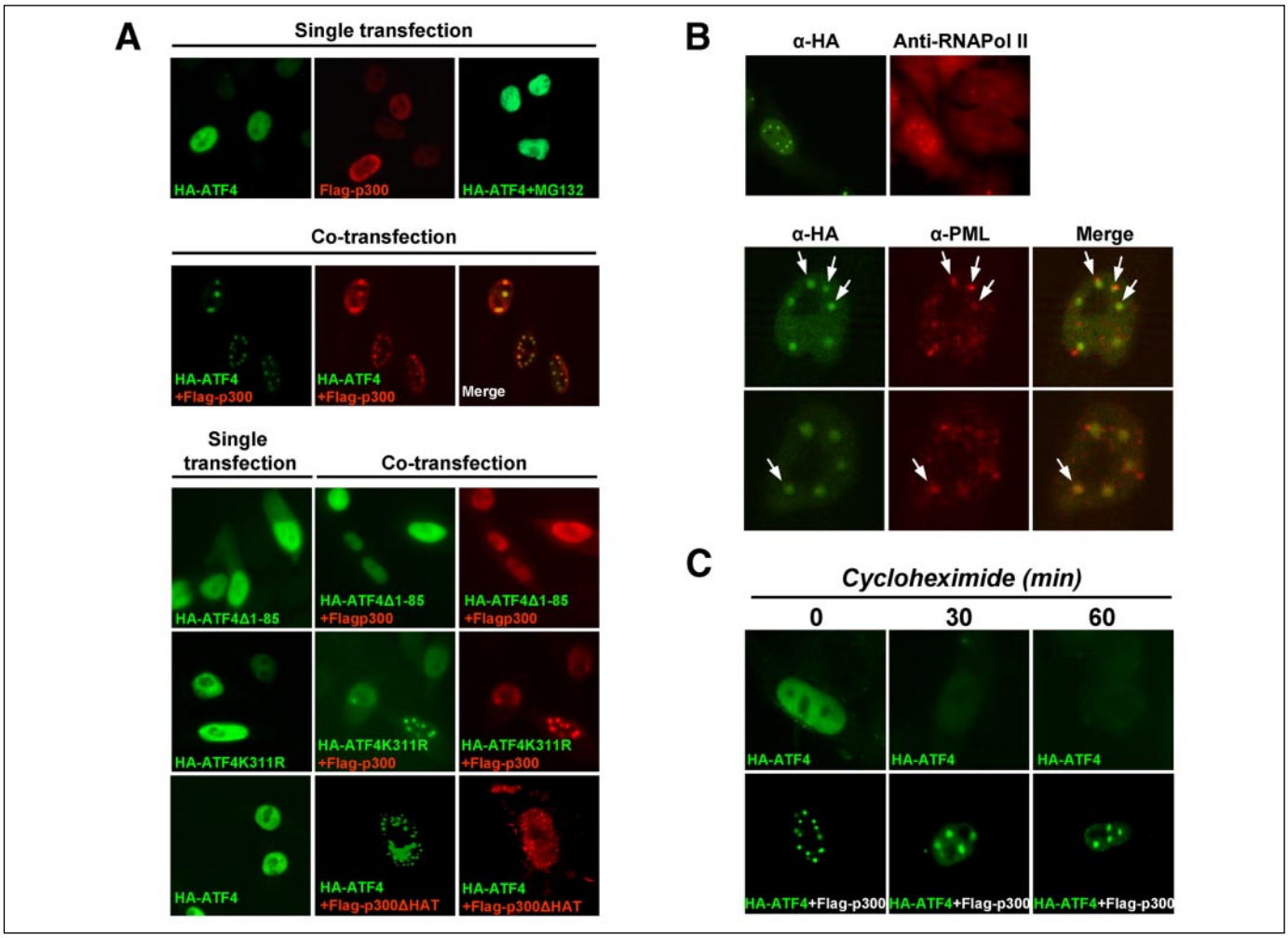


FIGURE 4. **p300 recruits ATF4 in nuclear speckles independently of p300 acetyltransferase activity.** *A*, p300 and ATF4 co-localize in nuclear speckles. HeLa cells were transfected together or separately with HA-ATF4 or ATF4 mutants as indicated, and FLAG-p300 or FLAG-p300ΔHAT, treated or not with MG132 (*top right panel*), fixed, and stained with primary anti-HA (coupled to fluorescein isothiocyanate) or anti-FLAG antibodies (*red*), and analyzed by confocal microscopy. *B*, nuclear speckles co-localized with RNA polymerase II and partially with some PML nuclear bodies. HeLa cells transfected with HA-ATF4 and FLAG-p300 were fixed and stained with primary anti-RNApolII or anti-PML antibodies and with anti-HA (coupled to fluorescein isothiocyanate), and analyzed by confocal microscopy. *C*, ATF4 is protected against degradation in nuclear speckles. HeLa cells were transfected with HA-ATF4, alone or with FLAG-p300, treated with cycloheximide for the indicated times, and then analyzed by immunofluorescence using anti-HA antibodies.

p300 was indeed associated with endogenous ATF4 (Fig. 3*A*, *lane 1*). This interaction was specific, because neither p300 nor ATF4 were found co-immunoprecipitated using an unrelated anti-Raf antibody (Fig. 3*A*, *lane 2*). The association between ATF4 and p300 was confirmed in cells transfected with HA-ATF4 and FLAG-p300 by co-immunoprecipitation of HA-ATF4 using anti-FLAG antibodies (Fig. 3*B*, *lane 1*). Interestingly, we found that the p300 inactive mutant FLAG-p300ΔHAT, which has conserved the ability to stabilize ATF4 (see Fig. 1), still associated with HA-ATF4 (Fig. 3*B*, *lane 2*). In an attempt to further correlate the capacity of p300 to stabilize ATF4 with its ability to interact with ATF4, we decided to map the ATF4 domain required for interaction with p300 in GST pull-down experiments, and then to analyze the effect of p300 on the stability of ATF4 mutants deleted of this domain. We found that ATF4 deleted of residues 1–85 (ATF4Δ1–85) or other larger deletion mutants (ATF4-(152–351) and ATF4-(152–257)), like GST alone used as a control, did not interact with p300 (Fig. 3*C*, *lanes 4–7*). In correlation, by contrast with wild-type ATF4 (see Fig. 1*B*), the stability of the ATF4Δ1–85 mutant was not enhanced by p300 (Fig. 3*D*). On the contrary, the ATF4 mutant deleted of the bZIP domain, which still interacted with p300 like ATF4 wild-type (see Fig. 3*C*, *lane 3*), remained as sensitive as ATF4 wild type to the stabilization mediated by FLAG-p300 (Fig. 3*D*). These results suggest that p300 needs to interact with ATF4 to promote ATF4 stabilization.

*p300 Recruits ATF4 into Nuclear Speckles and Protects It from Proteasome Degradation*—When expressed separately, both FLAG-p300 and HA-ATF4 showed a uniform nuclear staining (Fig. 4*A*, *top two left panels*). However, when co-expressed simultaneously, FLAG-p300 and HA-ATF4 were co-localized in nuclear speckles (Fig. 4*A*, *second row*). Importantly, the simple stabilization and enhancement of HA-ATF4 expression obtained by addition of the proteasome inhibitor MG132 was not sufficient to promote the recruitment of HA-ATF4 into nuclear speckles (Fig. 4*A*, *right top panel*). HA-ATF4Δ1–85 that was unable to interact with p300 (see Fig. 3*C*) was not recruited into nuclear speckles when overexpressed with FLAG-p300 (Fig. 4*A*, *third row*). By contrast, the mutant ATF4K311R, which could not be acetylated but still interacted with p300, could be recruited into nuclear speckles (Fig. 4*A*, *fourth row*). Hence, the recruitment of overexpressed HA-ATF4 into nuclear speckles when co-expressed with FLAG-p300 correlates with the ability of ATF4 to interact with p300 and not with its high level of expression. The recruitment of HA-ATF4 into nuclear speckles by p300 seemed also independent of p300 enzymatic activity, because an inactive mutant of p300, p300ΔHAT, was still able to delocalize ATF4 in nuclear speck-

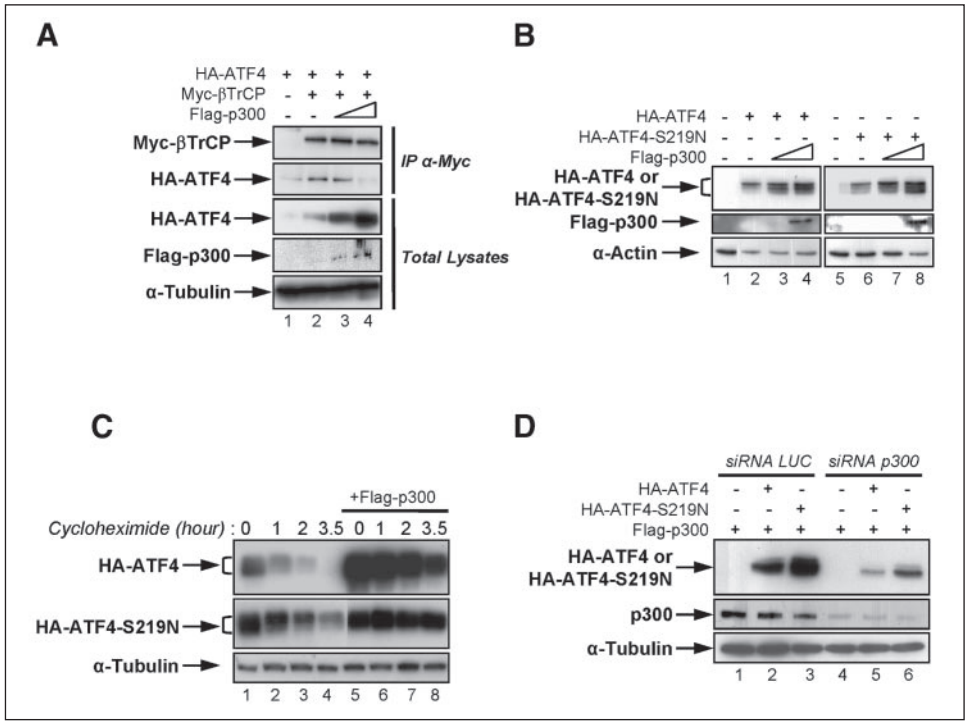


FIGURE 5. **The ATF4-S219N mutant, defective in its ability to interact with βTrCP, is also stabilized by p300.** *A*, p300 inhibits the ATF4-βTrCP interaction. HeLa cells were transiently transfected with or without Myc-βTrCP, HA-ATF4, and FLAG-p300 expression vectors. Lysates were immunoprecipitated using Myc antibodies and probed with Myc (*top panel*) or HA antibodies (*second panel*) or directly probed with HA, FLAG, or anti-tubulin antibodies (*three bottom panels*). *B*, the presence of p300 increases ATF4-S219N protein level. Lysates from cells expressing or not HA-ATF4 or HA-ATF4-S219N and 0, 10, or 20 μg of FLAG-p300 were analyzed by SDS-PAGE and Western blotting using anti-HA, anti-FLAG, or anti-actin antibodies. *C*, overexpression of p300 stabilizes the ATF4-S219N mutant. After co-transfection of HA-ATF4 or HA-ATF4-S219N with FLAG-p300, cells were treated with cycloheximide, lysed at indicated times, and analyzed by Western blotting. *D*, silencing of p300 expression by siRNAs de-stabilizes both wild-type and S219N ATF4 proteins. HeLa cells were transfected (+) or not (−) by p300 or luciferase (*LUC*) siRNAs, together with HA-ATF4 or HA-ATF4-S219N, and analyzed by Western blotting using HA, p300, or anti-tubulin antibodies.

les (Fig. 4*A*, *fifth row*). Interestingly, p300 mediated redistribution of ATF4 in nuclear speckles where ATF4 co-localized with the RNA polymerase II (Fig. 4*B*, the *two upper panels*). This suggests, in addition to the transcriptional studies in Fig. 6, a functional role of p300 in ATF4 transcriptional activity. These structures co-localized also partially with PML (Fig. 4*B*, *middle* and *lower panels*).

In an attempt to determine whether the stability of HA-ATF4 was somewhat affected when delocalized in speckles, we analyzed by immunofluorescence the degradation kinetics of HA-ATF4 in the presence and absence of p300. Interestingly, in the presence of FLAG-p300, HA-ATF4 could still be observed in nuclear speckles 60 min after treatment of HeLa cells with cycloheximide, whereas under the same conditions but in the absence of FLAG-p300, HA-ATF4 became barely detectable after 30 min of cycloheximide treatment (Fig. 4*C*). These results suggest that ATF4 could be protected from degradation when expressed in nuclear speckles.

*Role of βTrCP in p300-mediated ATF4 Stabilization*—We have previously shown that ATF4 degradation is mediated by the $SCF^{\beta TrCP}$ ubiquitin ligase. We therefore wondered whether the presence of p300 would inhibit the recruitment of βTrCP to ATF4. We transfected increasing amounts of FLAG-p300 in cells together with Myc-βTrCP and HA-ATF4 and performed an immunoprecipitation of βTrCP using anti-Myc antibodies. ATF4 was co-immunoprecipitated with βTrCP (Fig. 5*A*, *lane 2*, *second panel*), and this interaction was inhibited in the presence of p300 (Fig. 5*A*, *lanes 3* and *4*, *second panel*). We subsequently hypothesized that p300-mediated ATF4 stabilization could be the result of a direct competition between p300 and βTrCP for ATF4 binding. If this was the case, p300 overexpression would not affect the stability of the ATF4-S219N mutant, which does not interact with βTrCP. On the contrary, we found that both wild-type ATF4 (Fig. 5*B*, *lanes 1–4*; Fig. 5*C*, *top panel*) and ATF4-S219N (Fig. 5*B*, *lanes 5–8*; Fig. 5*C*, *middle panel*) were stabilized by p300 overexpression. Furthermore, silencing of p300 by siRNA de-stabilized both wild-type ATF4 (Fig. 5*D*, compare *lanes 5* to *2*) and ATF4-S219N proteins (Fig. 5*D*, compare *lanes 6* to *3*). As expected, ATF4-S219N expression is enhanced relatively to ATF4 wild type due to its inability to interact with βTrCP (Fig. 5*D*, compare *lanes 3* to *2* and *6* to *5*). Altogether, theses results suggest that the inhibition of the ATF4/βTrCP interaction in the presence of p300 is not the result of a competition between βTrCP and p300 for ATF4 binding.

*Effect of p300 on ATF4 Transcriptional Activity*—We then asked whether ATF4 transcriptional activity was increased as a result of ATF4 stabilization by p300. We used a luciferase reporter gene system sensitive to amino acid starvation, through an ATF4 activation pathway, to study the effect of p300 on stress-mediated ATF4 transcriptional activity. In this system, expression of the luciferase gene is under the control of the AARE element of the CHOP promoter, a target of ATF4 (24), and expression of ATF4 enhanced the basal (+leucine) and stress-mediated (−leucine) transcriptional activities of the reporter gene (Fig. 6*A*, compare *lanes 1* to *3* and *lanes 5* to *7*).

The overexpression of p300 enhanced the basal and stress-mediated transcriptional activities of the CHOP promoter resulting both from endogenous ATF4 (Fig. 6*A*, *lanes 1* and *2* and *lanes 5* and *6*) and from overexpressed ATF4 (Fig. 6*A*, *lanes 3* and *4* and *lanes 7* and *8*). In agreement, p300 inactivation using siRNA leads to a strong reduction of endogenous or transfected ATF4 transcriptional activities, probably due to an enhanced instability of ATF4 (Fig. 6*B*).

Importantly, the inactive mutant of p300, p300ΔHAT, still increased the transcriptional activity of ATF4 (Fig. 6*C*). These results suggest that stabilization of ATF4 by p300 leads to an increase of its transcriptional activity that is not dependant on the acetyltransferase activity of p300 as already described for the human T-cell lymphotrophic virus, type I promoter (15). Confirming this result, the transcriptional activity of the unacetylated ATF4K311R mutant (see Fig. 1*E*), was comparable to the transcriptional activity of wild-type ATF4 (Fig. 6*D*). Controls were performed using ATF4Δ1–85, which has lost the transcriptional activator domain (11), and the binding to p300 and ATF4ΔbZIP, which has lost ATF4 DNA binding domain. These two mutants display reduced transcriptional activity (Fig. 6*D*). In addition, although the presence of p300 increased the transcriptional activity of ATF4 and ATF4-K311R in a dose-dependent manner, p300 had no effect on ATF4Δ1–85 and

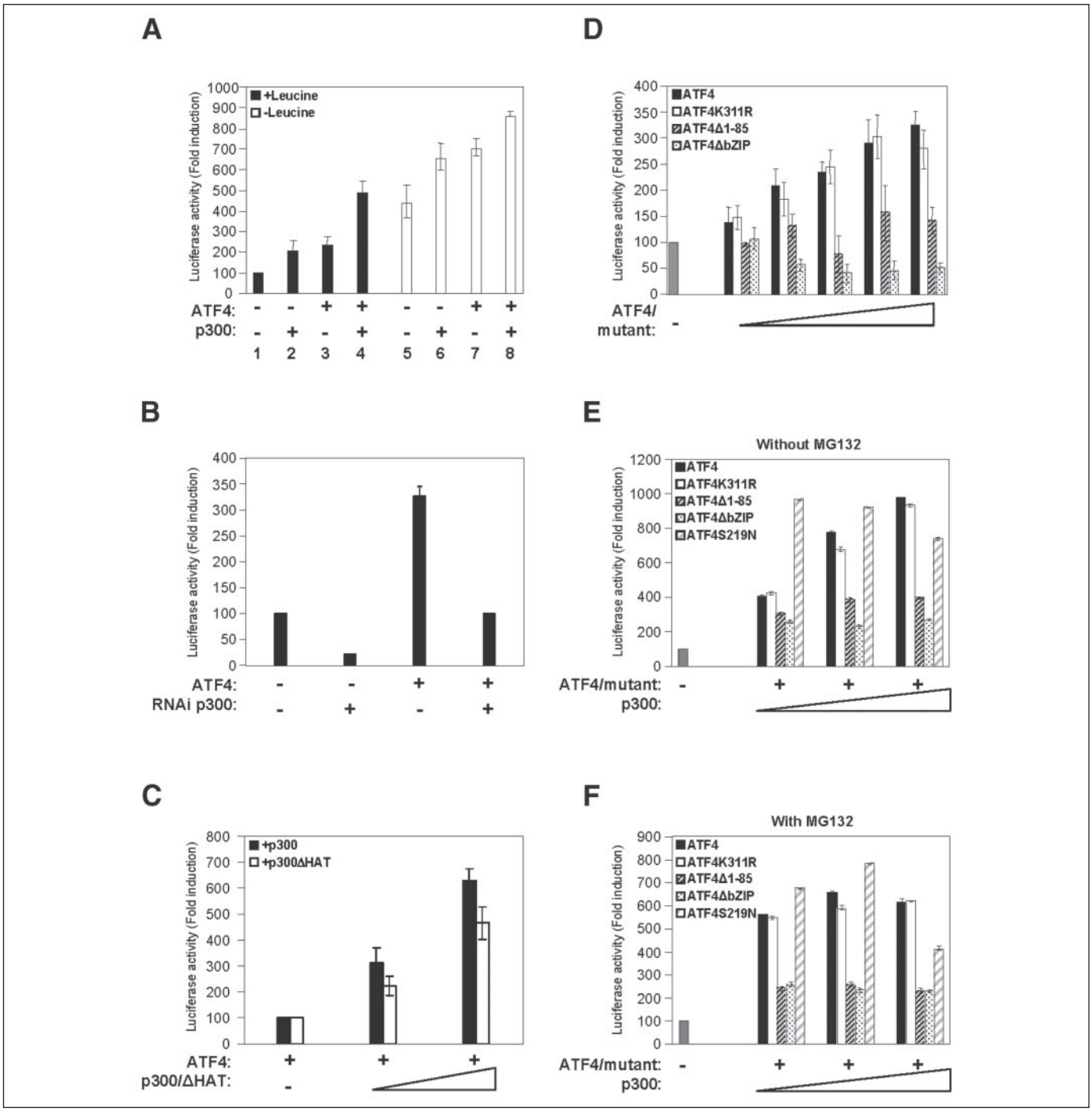


FIGURE 6. **p300 enhances ATF4-mediated transcription independently of its acetyltransferase activity.** *A*, p300 potentiates ATF4 transcriptional activity. 293T cells were transiently co-transfected with or without ATF4 and p300 expression vector, and a reporter plasmid expressing luciferase under the control of an ATF4 target promoter (1XAARE-TK-LUC vector) and incubated in DMEM containing leucine (+leucine) or DMEM without leucine (−leucine) for 12 h. Averages of three independent experiments are represented. *B*, silencing of p300 expression by siRNAs reduces ATF4 transcriptional activity. HeLa cells were co-transfected (+) or not (−) by p300 siRNAs and with or without ATF4 and a 1XAARE-TK-LUC vector. *C*, p300ΔHAT still enhances ATF4 transcriptional activity. The experiment was performed as in *A* using p300 or p300ΔHAT expression vectors. Cells were incubated in DMEM containing leucine. *D*, transcriptional activity of ATF4K311R mutant is comparable to ATF4 wild type. The experiment was performed as in *A* using increasing amounts (0.01 to 0.5 $\mu$g) of ATF4 mutants expression vectors. *E*, p300 increases the transcriptional activity of ATF4 wild-type and ATF4-K311R. The experiment was performed as in *A* using p300 and ATF4 mutants expression vectors. *F*, experiment was performed as in *E* except that cells were incubated in the presence of 20 $\mu$M MG132 for 4 h before harvesting.

ATF4ΔbZIP (Fig. 6*E*). In the presence of MG132, wild-type ATF4 and ATF4K311R transcriptional activities were increased to a high level, which could not be further increased by p300 overexpression (Fig. 6*F*). Such a saturation effect was also obtained with the ATF4-S219N mutant, which was highly stabilized compared with wild-type ATF4 (see Fig. 5*D*), even in the absence of p300 and MG132 (Fig. 6*E*: without MG132, Fig. 6*F*: with MG132). In consequence, despite p300-induced ATF4-S219N stabilization (see Fig. 5), p300 addition had no effect on the transcriptional activity of this mutant. Altogether our data suggest that ATF4 transcriptional activity is regulated by p300-mediated stabilization but is independent of p300 enzymatic activity.

## DISCUSSION

In this work we show that the expression of ATF4 can be regulated at the level of its stability by the acetylase p300. Multiple lines of evidence support this conclusion: (i) ATF4 is stabilized upon overexpression of p300, (ii) this effect is specific, because other HAT like P/CAF had no effect, (iii) silencing of p300 by RNA interference resulted in a marked increase in instability of ATF4, (iv) ATF4 ubiquitination was inhibited by p300 overexpression, (v) the effect of p300 on ATF4 stability correlated with its ability to interact with ATF4: a mutant of ATF4 deleted from the N-terminal region required for binding to p300 was insensitive to this protein.

Importantly, we could gain insights into the mechanism involved in this novel regulation of ATF4 stability by p300: (i) the acetylation of ATF4 is not involved in this regulation, because ATF4 mutated in the main acetylation site remained sensitive to the effect of p300, (ii) the enzymatic activity of p300 is not required for its effect on ATF4 stability, (iii) the presence of p300 inhibits the ATF4/βTrCP interaction, and (iv) the ATF4-S219N mutant, which has lost its ability to interact with βTrCP, is also stabilized by p300.

Altogether these results support the notion that ATF4 stability is in fact controlled by two domains. The first one is, as we previously described, the DSG*XXX*S motif, whose phosphorylation by an unknown kinase is required for subsequent recognition by the $SCF^{\beta TrCP}$ and degradation by the proteasome (21); the second one is the N-terminal domain 1–85 that binds to p300.

In addition, based on our data we propose a molecular basis for the cell specificity of ATF4 accumulation in osteoblasts observed by Yang and Karsenty (27). Indeed, whereas the *ATF4* gene was expressed ubiquitously, they observed ATF4 protein accumulation only in some tissues. Profile expression of βTrCP contrasts with ATF4 protein expression, and they concluded that it may be that ATF4 is protected from degradation in osteoblasts and a few other cell types, by an unknown mechanism (27).

*p300-mediated Redistribution of ATF4 in Nuclear Speckles*—Strikingly, we observed a redistribution of ATF4 in nuclear speckles as a consequence of p300 transfection. This redistribution was not simply due to the stabilization of ATF4, because it was not observed when accumulation of ATF4 was obtained in the absence of p300 transfection, by treatment of cells with proteasome inhibitors. Such recruitment of ATF4 in nuclear speckles correlated with the binding of p300 to the N-terminal domain of ATF4. A large number of nuclear proteins, including transcription factors, co-activators, and co-repressors, have been localized to various sub-nuclear particles. Formation of such structures has been reported with p300 and several other of its partners such as Mdm2 (28), βcatenin (29, 30), SREBP1 (16), or EVI1 (31). Structures formed by βcatenin and p300 are able to partially recruit PML and cooperate in transactivation of a subset of βcatenin-responsive pathways (29, 30). Interestingly, the nuclear speckles we observed upon co-transfection of p300 and ATF4 co-localize with RNA polymerase II and partially with PML, proteins previously described in such structures. The relevance of these structures would have to be further confirmed by looking at endogenous ATF4.

It also remains to be determined whether such structures could play an active role in eliciting some protection of ATF4 from degradation by the nuclear proteasome. In favor of this hypothesis we found that, in cells treated with cycloheximide, ATF4 was more stable when it was localized in such nuclear structures than when it was uniformly present in the nucleus.

Because p300 inhibits the ATF4-βTrCP interaction but has no effect on the ATF4-S219N mutant, which is unable to interact with βTrCP, we favor the hypothesis that p300-mediated ATF4 stabilization is more likely due to sequestration of ATF4 into the nuclear speckles, where it is unavailable for βTrCP binding, rather than to a competition between p300 and βTrCP for ATF4 binding.

*Regulation of ATF4 Stability Is Independent of p300 Enzymatic Activity*—There are several examples of protein stabilization by p300 that depend on acetylation. In these cases, p53, Smad7, and SREBP1 (16, 32, 33) ubiquitination and acetylation occur at a common set of lysine residues, and acetylation controls protein stability by competing with ubiquitination. The recruitment of histone deacetylase 1 by Mdm2 promotes p53 degradation by removing these acetyl groups (16, 32–34). Mdm2 was the first reported example of a protein stabilized by p300 independently of acetylation (28). However, Mdm2 is not acetylated by p300. So ATF4 is the first example reported of a protein acetylated by p300 that can be stabilized by p300 independently of its acetyltransferase activity. However, we noticed that, in the presence of p300ΔHAT, ATF4 stabilization or the increase of ATF4 transcriptional activity were slightly less effective than with wild-type p300. It thus appears that the acetyltransferase activity of p300, even though it is not an absolute requirement, might partially contribute to ATF4 stabilization.

Hence, the stabilization of ATF4 induced by p300 is probably not due to a competition between acetylation and ubiquitination on the same residues. The lysine residue 311, localized in the bZIP domain, is the major acetylation site of ATF4, but not of ubiquitination, because both the mutants ATF4ΔbZIP and ATF4K311R are normally ubiquitinated.[6] Although the ubiquitinated lysine residues in ATF4 are not yet mapped precisely, the ubiquitination sites are likely located close to the DSG*XXX*S destruction motif, in the vicinity of residues 200–210, as it is the case for IκBα and βcatenin, two other βTrCP substrates (35, 36). Therefore, the question is raised of a novel function of p300 mediated not by its enzymatic activity but by its ability to interact with ATF4.

Recently, it has been reported that p300 had E3/E4 ubiquitin ligase activity (37). We also cannot exclude that this activity is indirectly involved in the mechanism of ATF4 stabilization. For example, p300 might ubiquitinate and degrade a potential activator of ATF4 degradation.

*Functional Redundancy between p300 and CBP?*—We found that CBP, like p300, interacts with the N-terminal domain of ATF4 (data not shown), in agreement with a previous study showing that CBP is unable to interact *in vivo* with the C-terminal region of ATF4 (38); in addition, both CBP and p300 are able to enhance ATF4 transcriptional activity (herein and Gachon *et al.* (38) and Liang and Hai (11)). However, an increasing amount of data favors the hypothesis that the two proteins, despite their high degree of homology, are not redundant and have unique roles *in vivo*. Our study suggests that ATF4 stabilization is the result of only p300, because the siRNA we used target specifically p300 and not CBP (data not shown), and they totally destabilized ATF4. Recent studies suggest that the functional differences between the two acetyltransferases could be due to association with different proteins or differences in substrate specificity (39).

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