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E4F1 Is an Atypical Ubiquitin Ligase that Modulates p53 Effector Functions Independently of Degradation

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SUMMARY

p53 is regulated by multiple posttranslational modifications, including Hdm2-mediated ubiquitylation that drives its proteasomal degradation. Here, we identify the p53-associated factor E4F1, a ubiquitously expressed zinc-finger protein first identified as a cellular target of the viral oncoprotein E1A, as an atypical ubiquitin E3 ligase for p53 that modulates its effector functions without promoting proteolysis. E4F1 stimulates oligo-ubiquitylation in the hinge region of p53 on lysine residues distinct from those targeted by Hdm2 and previously described to be acetylated by the acetyltransferase PCAF. E4F1 and PCAF mediate mutually exclusive posttranslational modifications of p53. E4F1-dependent Ub-p53 conjugates are associated with chromatin, and their stimulation coincides with the induction of a p53-dependent transcriptional program specifically involved in cell cycle arrest, and not apoptosis. Collectively, our data reveal that E4F1 is a key posttranslational regulator of p53, which modulates its effector functions involved in alternative cell fates: growth arrest or apoptosis.

INTRODUCTION

p53 is a key regulator of cell cycle control, apoptosis, and genomic stability in response to stress (Harris and Levine, 2005), and inactivation of the p53 pathway is a common feature of cancer cells (Vogelstein et al., 2000). Genomic insults lead to posttranslational modifications of p53 that enhance its transcriptional activity and stability (Brooks and Gu, 2003). Under normal growth conditions, interactions between p53 and several ubiquitin (Ub) E3 ligases (Alarcon-Vargas and Ronai, 2002; Brooks and Gu, 2006), such as Hdm2/Mdm2 (Haupt et al., 1997; Kubbutat et al., 1997), E6-AP (Scheffner et al., 1993), Cop1 (Dornan et al., 2004), Pirh2 (Leng et al., 2003), and Mule/ARF-BP1 (Chen et al., 2005), maintain low levels of the p53 protein by promoting its polyubiquitylation and degradation by the 26S proteasome-dependent pathway (Michael and Oren, 2003). The redundancy of these distinct E3 ligases toward p53 regulation, and the physiological settings that get them under way in vivo, remain largely unclear. Furthermore, some of the ubiquitylated lysines of p53 are subject to competitive mono- and polyubiquitylation (Li et al., 2003) and to several other modifications, including acetylation (Appella and Anderson, 2001; Ito et al., 2001; Kobet et al., 2000), methylation (Chuikov et al., 2004), neddylation (Xirodimas et al., 2004), and sumoylation (Bode and Dong, 2004; Gostissa et al., 1999; Melchior and Hengst, 2002; Rodriguez et al., 1999; Schmidt and Muller, 2002). Notably, competition between acetylation and ubiquitylation has been documented at several lysines in the C terminus of p53. While acetylation of human p53 on K372, K373, K381, and K382 by the acetyltransferase CBP/p300 coincides with p53 transcriptional activity, ubiquitylation of some of these residues by Hdm2 targets p53 for degradation (Ito et al., 2002; Cheng et al., 2003; Liu et al., 1999; Luo et al., 2001, 2000; Sakaguchi et al., 1998; Vaziri et al., 2001). p53 is also acetylated in position K320 by the CBP/p300-associated factor (PCAF) (Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998), a modification which has been proposed to direct p53 transcriptional response toward growth arrest or apoptosis (Sakaguchi et al., 1998; Terui et al., 2003, Knights et al., 2006).

Here, we report that E4F1 is an important modulator of this K320 ubiquitylation/acetylation-dependent p53 response. E4F1 is a ubiquitously expressed 120 kDa zinc-finger protein of the GLI/Kruppel family that was first

identified as a cellular target of the viral oncoprotein E1A13S, required for both transcriptional activation and repression of adenoviral genes (Fernandes and Rooney, 1997; Lee and Green, 1987; Lee et al., 1987; Raychaudhuri et al., 1987). Several recent observations suggest that E4F1 also plays important roles during normal cell proliferation and survival. E4F1$^{-/-}$ mouse embryos die at the peri-implantation stage and exhibit mitotic progression defects, chromosomal missegregation, and increased apoptosis (Le Cam et al., 2004). Conversely, ectopic expression of E4F1 in various cell lines results in inhibition of the G1-S-phase progression and cytokinesis defects that correlate with increased levels of the cell cycle inhibitor p21^{Cip1} and the transcriptional repression of the *cyclin A2* gene (Fajas et al., 2000, 2001; Fernandes et al., 1998, Rooney, 2001, Tessari et al., 2003). Notably, this E4F1-mediated cell cycle arrest is reduced in cells deficient for either pRB or p53 (Fajas et al., 2000; Sandy et al., 2000), and is enhanced in the presence of p14^{ARF} (Rizos et al., 2003) and RASSF1A (Ahmed-Choudhury et al., 2005), suggesting that E4F1 is involved in several essential oncogenic pathways. Importantly, E4F1 physically interacts with these four tumor suppressors: RASSF1A (Fenton et al., 2004), pRB (Fajas et al., 2000), p14^{ARF} (Rizos et al., 2003), and p53 (Sandy et al., 2000), as well as with other regulators/effectors of these pathways: the proto-oncogene Bmi1 (Chagraoui et al., 2006), the histone deacetylase HDAC1 (Colombo et al., 2003), and FHL2 (Paul et al., 2006).

Here we present evidence that E4F1 is also an atypical p53 Ub E3 ligase that regulates p53 effector functions. E4F1-mediated ubiquitylation of p53 occurs at sites distinct from those targeted by Hdm2, competes with PCAF-induced acetylation of p53, and does not target p53 for proteasomal degradation. It is noteworthy that these E4F1-stimulated p53-Ub forms are detectable on chromatin and that their stimulation coincides with the recruitment of p53 on growth arrest, but not on proapoptotic, target genes. Consistently, E4F1 directs the p53 response toward growth arrest at the expense of apoptosis, suggesting that E4F1 plays an important role in the cellular life-or-death decision controlled by p53.

RESULTS

E4F1 Has an Intrinsic Ubiquitin E3 Ligase Activity that Drives K48-type Ubiquitylation of p53

While investigating the mechanisms that control the stability of E4F1, we noticed that recombinant E4F1 was ubiquitylated in vitro (Figure 1A) and stimulated the formation of polyubiquitin chains in presence of recombinant Ub, E1, and E2, and without addition of any E3 ligase in the assay (Figure 3B and Figure S1E in the Supplemental Data). This suggested that E4F1 possess an intrinsic Ub E3 ligase activity. We next tested whether this activity was directed against its known interactors, including p53, p14^{ARF}, and pRb. Whereas we failed to detect such an effect on pRb and p14^{ARF} (data not shown), E4F1 efficiently stimulated p53 ubiquitylation in vitro with various sources of E4F1, p53, and Ub proteins, including recombinant E4F1 purified from bacterial extracts (Figure 1B) and insect cell lysates (Figures S1B–S1E), IVT E4F1 (Figure S1A), and cellular E4F1 immunoprecipitated from U2OS cells (Figure 1C). In vitro ubiquitylation assays with Ub mutants bearing mutations on all but one of its lysines indicated that lysine 48 was necessary for E4F1-stimulated oligoubiquitylation of p53 (Figure 1D). All other Ub mutants supported only monoubiquitylation, suggesting that p53 was ubiquitylated by E4F1 on a single lysine.

To investigate whether E4F1 could stimulate p53 ubiquitylation in cells, we established a conditional E4F1 overexpression system based on the Cre-dependent activation of a silent Lox-STOP-Lox E4F1-GFP expression vector (Figure S2D). This vector was stably integrated in U2OS, a cell line with wild-type p53. Removal of the STOP element was achieved by infecting cells with retroviral particles encoding a "self-excising" CRE recombinase (Silver and Livingston, 2001) and resulted in the ectopic (10- to 12-fold excess when compared with the endogenous E4F1), homogeneous, and stable expression of E4F1-GFP in the nucleus of more than 85% of the cells (Figure 1E and Figure S2E). Ubiquitylation levels of endogenous (Figure 1F) and ectopically expressed (Figure S2F) p53 were analyzed in parental and LSL-E4F1 cells transfected with 6XHis-tagged Ub construct. (Ni^{2+}-NTA agarose)-purified Ub-p53 conjugates were more abundant in cells expressing E4F1-GFP than in control cells. Identical results were obtained after transient transfection of U2OS cells with E4F1 and p53 expression constructs (Figure S2C). Conversely, SiRNA-mediated depletion of endogenous E4F1 resulted in a major decrease of the basal ubiquitylation levels of endogenous (Figure 1G) or ectopic (Figure S2B) p53. Taken together, these data indicate that E4F1 is a bona fide Ub E3 ligase for p53 in vitro and in vivo.

E4F1 Stimulates Ubiquitylation of p53 on Lysines Distinct from Those Targeted by Hdm2

Stimulation of Ub chain polymerization by Hdm2 was previously shown to depend on six lysines in the C terminus of human p53 (K370, K372, K373, K381, K382, and K386) (Krummel et al., 2005; Li et al., 2003; Nakamura et al., 2000; Rodriguez et al., 2000). It is noteworthy that a p53 mutant bearing K→R mutations of these six residues (6KR) remained fully sensitive to E4F1-mediated ubiquitylation (Figures 2A and 2C and Figure S3A) while showing greatly reduced ubiquitylation by Hdm2 (Figure 2B) in vitro. A screen for p53 deletion mutants that block E4F1-mediated ubiquitylation identified a region (aa 309–330) within the "hinge region" of p53 (Figure S3B) that links the DNA binding domain to the tetramerization domain (Aurelio et al., 1998; Kong et al., 2001). This region contains clustered lysines (K319, K320, and K321) whose K→R mutation rendered p53 resistant to E4F1-mediated ubiquitylation in vitro (Figure 2A) and in vivo (Figure 2C), while preserving its sensitivity to Hdm2 (Figure 2B). These data demonstrate that E4F1 stimulates the ubiquitylation

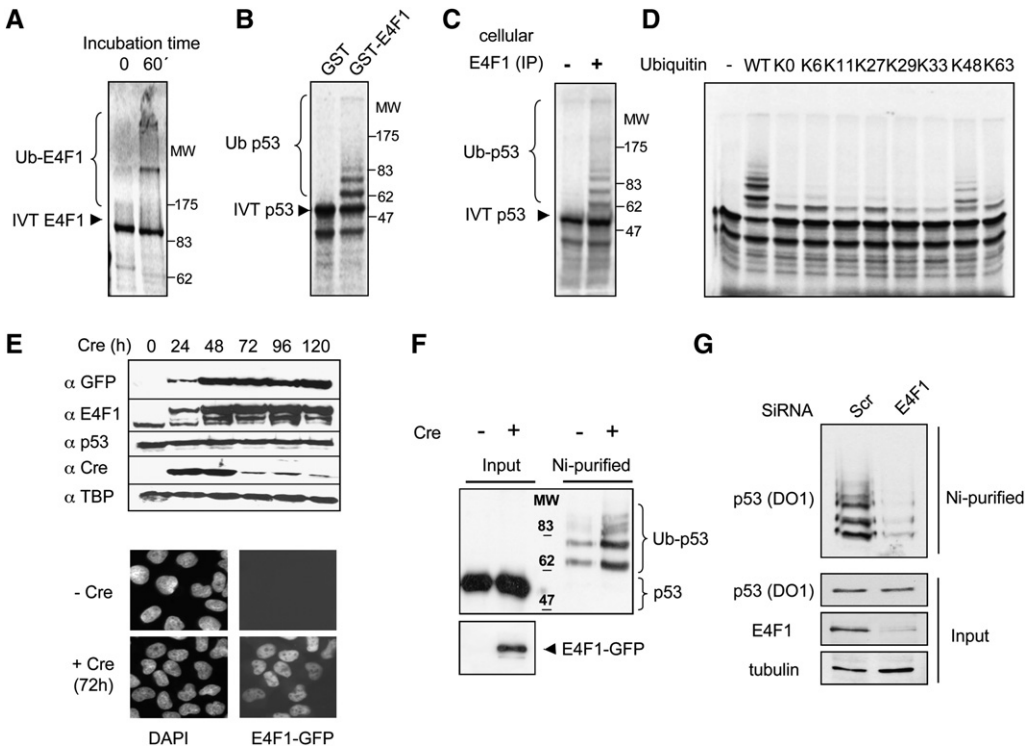

Figure 1. E4F1 Exhibits Intrinsic Ubiquitin E3 Ligase Activity on p53 In Vitro and In Vivo

(A) Autoubiquitylation activity of E4F1. Autoradiograms of in vitro ubiquitylation assays performed under standard conditions in presence of in vitro translated (IVT) E4F1 labeled by ^{35}S-methionine.

(B) Recombinant E4F1 stimulates p53 ubiquitylation in vitro. Autoradiograms of in vitro ubiquitylation assays performed with IVT ^{35}S-labeled p53 and GST-E4F1 or GST.

(C) Autoradiograms of in vitro ubiquitylation assays performed under standard conditions with IVT ^{35}S-labeled p53 and cellular E4F1 immunoprecipitated from E4F1- or mock-transfected U2OS cells.

(D) E4F1 stimulates K48-Ub branching. Autoradiograms of in vitro ubiquitylation assays performed with IVT ^{35}S-labeled p53, baculovirus expressed E4F1 and Ub (WT), or Ub mutants bearing mutations of all (K0) or all but one of its lysine residues at the indicated position. Equal amounts of Ub mutants were used in each assay (Figure S1F).

(E) Cre-induced E4F1-GFP expression in U2OS cells stably expressing the LSL-E4F1 construct. (Upper panels) Western blot analyses of nuclear extracts prepared from LSL-E4F1 cells at the indicated time points after infection with Cre retrovirus, probed with anti-GFP, -E4F1, -p53 (DO1), -Cre, and -TBP (loading control) antibodies (Abs). (Lower panels) Analysis of E4F1-GFP expression by fluorescence microscopy 3 days after infection with Cre (+) or control (–) retroviruses. Cells are shown at 40× magnification.

(F) E4F1 stimulates ubiquitylation of endogenous p53 in vivo. Western blot analyses of Ub-conjugated proteins in LSL-E4F1 cells transiently transfected for 24 hr with a 6XHis-Ub expression vector 4 days after infection by Cre (+) or control (–) retroviruses. One percent of cellular extracts were probed for the presence of E4F1-GFP and total endogenous p53 using anti-GFP and anti-p53 (DO1) Abs (input). These cellular extracts, normalized to contain equal amounts of total p53, were loaded on nickel (Ni+)-NTA columns. Ni+-purified 6XHis-Ub-conjugated proteins were probed for the presence of Ub-p53 forms using an anti-p53 (DO1) Ab (Ni-purified). Neither cells nor cellular extracts were treated with proteasome inhibitors.

(G) Depletion of endogenous E4F1 impacts on endogenous p53 ubiquitylation in vivo. Western blot analyses of Ub-conjugated proteins in U2OS cells treated for 72 hr with control scramble or E4F1 SiRNAs. His-Ub-conjugated forms of endogenous p53 present in these cells were purified and analyzed as described in Figure 1F.

of p53 on the lysine cluster K319–K321, i.e., at sites distinct from those targeted by Hdm2. Consistent with the notion that E4F1 ubiquitylates p53 independently of Hdm2, this ubiquitylation was still detected in U2OS cells treated with a Hdm2 SiRNA (Figure 2D).

Ubiquitin E3 Ligase Activity of E4F1 Requires a Region with Similarities to the SUMO E3 Ligase Domain of RanBP2

E4F1 contains a number of zinc finger motifs, including 9 C2H2 or C2HC Krüppel-like zinc fingers likely involved in DNA binding (Fernandes and Rooney, 1999). However, none of these zinc finger domains or other structured domains of E4F1 showed obvious sequence similarities or structural homologies with functional domains of known E3 ligases, i.e., RING, HECT, or U-Box domains (Pickart and Eddins, 2004). Nevertheless, a region located at the N terminus of E4F1 (aa 30–80) presented similarities (Figure 3A) with two regions (IR1 and IR2) that compose the catalytic core domain of the SUMO E3 ligase RanBP2 (Pichler et al., 2004; Tatham et al., 2005). E4F1 bearing a deletion within this region (Δ41–85E4F1) failed to

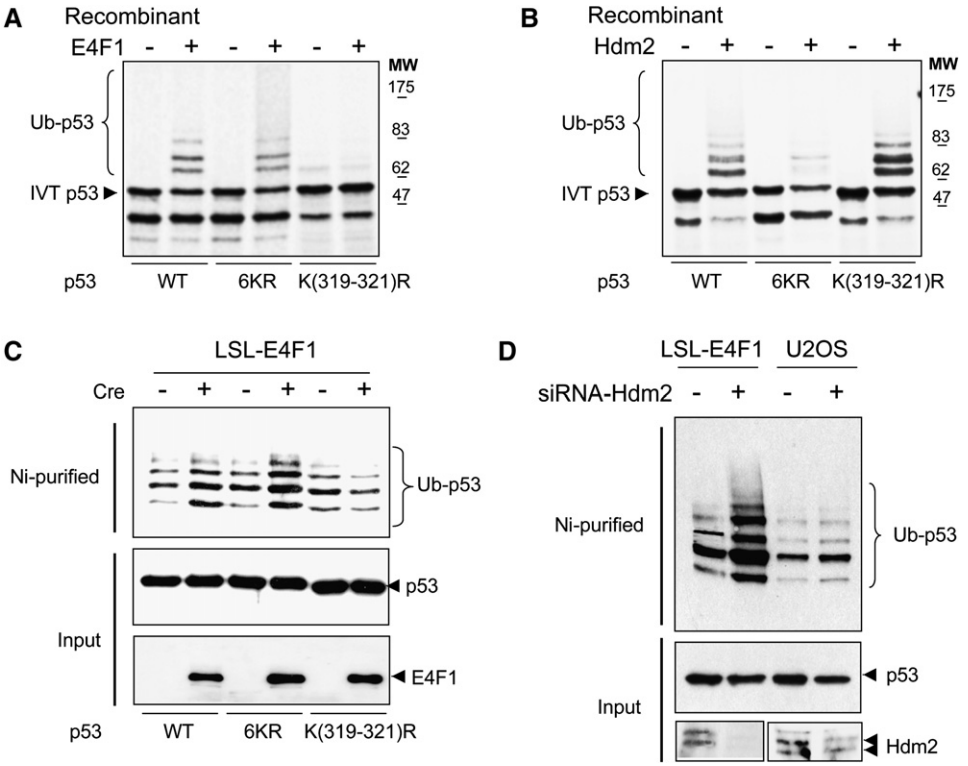

Figure 2. E4F1 Stimulates p53 Ubiquitylation on Lysine Motif K (319–321) In Vitro and In Vivo, at Sites Distinct from Those Targeted by Hdm2

(A) In vitro ubiquitylation assays performed in presence of purified baculovirus-expressed E4F1 (+) or purified mock extract (–), and with either IVT ^{35}S-labeled WT or the 6KR or K(319–321)R mutant p53 proteins.

(B) In vitro ubiquitylation assays performed as in (A) but with GST-Hdm2 (+) or control GST (–) instead of E4F1.

(C) E4F1 stimulates p53 ubiquitylation at K319–K321) in vivo. Western blot analysis of Ub-conjugated proteins in Cre-infected (+) or mock-treated (–) LSL-E4F1 cells transiently cotransfected with 6XHis-Ub and either WT-p53, 6KR-p53, or K(319–321)R-p53 expression vectors. His-Ub-conjugated forms of p53 were purified and analyzed as described in Figure 1F.

(D) Depletion of Hdm2 does not impact on the E4F1-stimulated ubiquitylation of p53 in vivo. Western blot analysis of Ub-conjugated proteins in Cre-transduced parental (U2OS) and LSL-E4F1 cells transfected with p53 and His-Ub expression vectors, and treated for 48 hr with either control (–) or Hdm2 SiRNAs (+). His-Ub-conjugated forms of p53 were purified and analyzed as described in Figure 1F.

promote polyubiquitin chain formation (Figure 3B) and p53 ubiquitylation in vitro and in vivo (Figures 3C and 3D). Importantly, both WT and Δ41–85E4F1 proteins were expressed at comparable levels in the nucleus (Figures 3D and 4B) and exhibited similar capacities to interact with p53 (Figure S4B).

Considering that E4F1 contains an atypical E3 domain, we also tested whether $p120^{E4F1}$ recombinant protein (full-length E4F1) could also function as a SUMO or Nedd8 E3 ligase for p53, but failed to detect such activity in experimental conditions where the SUMO 1-conjugating E3 PIAS1 and Hdm2 enhanced p53 sumoylation and neddylation, respectively (Figure 3E).

E4F1-Mediated Ubiquitylation Results Neither in Destabilization nor Relocalization of p53

Ubiquitylation of p53 has been previously associated with its proteasomal degradation and cytoplasmic/nuclear shuttling (Haupt et al., 1997; Kubbutat et al., 1997; Li et al., 2003). Surprisingly, E4F1-stimulated p53-Ub conjugates were easily detectable in absence of proteasome inhibitors, suggesting that E4F1 does not stimulate proteasome-dependent p53 degradation. Consistently, E4F1-GFP induction did not result in decreased levels of endogenous or ectopically expressed p53 (Figures 4A, 4B, and data not shown). On the contrary, evaluation of p53 protein decay in LSL-E4F1 cells treated with cycloheximide showed that E4F1 expression induced a moderate, but reproducible, stabilization of endogenous p53 (Figure 4A). Moreover, biochemical fractionation and immunofluorescence analysis indicated that cellular localization of endogenous p53 changed neither upon E4F1-GFP induction (Figure 4B and Figure S4A) nor upon depletion of endogenous E4F1 (Figure 4C).

Thus, in striking contrast to the ubiquitylation mediated by *hitherto* described p53 E3 ligases, E4F1-mediated ubiquitylation results in neither protein degradation nor changes in the cytoplasmic/nuclear shuttling of p53.

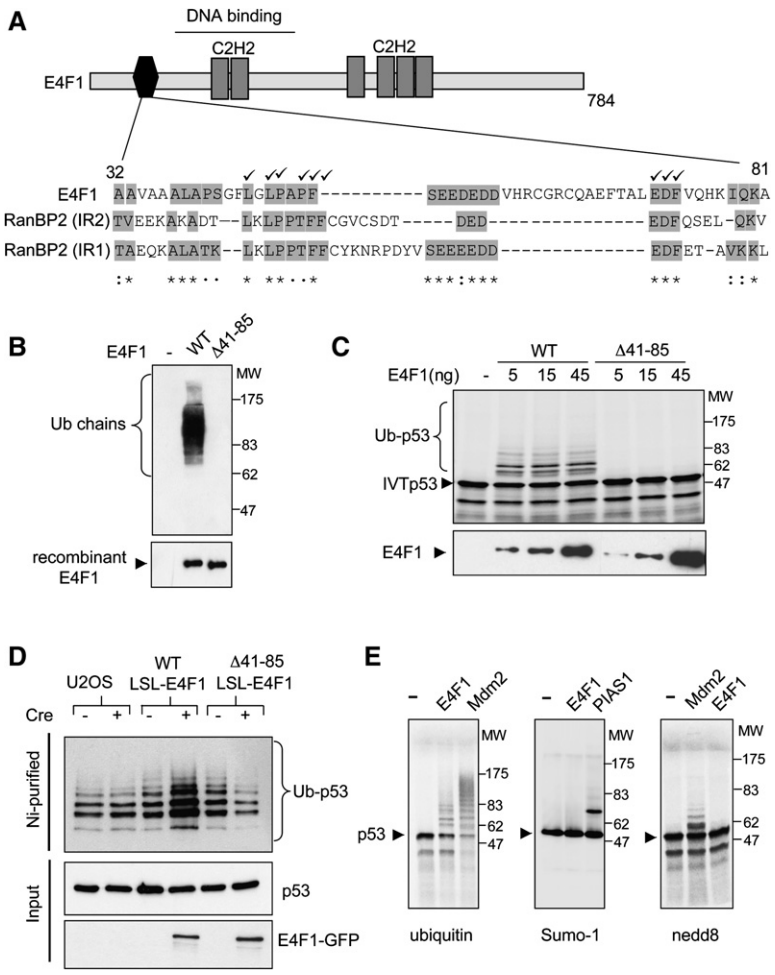

Figure 3. The Ubiquitin E3 Ligase Activity of E4F1 Requires a Region Showing Sequence Similarities with the IR1 and IR2 Regions of the SUMO E3 Ligase Domain of RanBP2

(A) Sequence alignments of the IR1 and IR2 domains from RanBP2 with the N-terminal region of E4F1 (aa 32–81). Residues whose mutation has been shown to affect RanBP2 E3 ligase activity are marked with a check.

(B) The aa 41–85 region of E4F1 is required for polyubiquitin chain formation in vitro. Western blot analysis of in vitro ubiquitylation assays performed in presence of similar amounts of baculovirus-expressed WT full-length E4F1 ($p120^{E4F1}$), Δ41–85E4F1 proteins, or mock-purified preparations (–). Poly-Ub chains are revealed with an anti-Ub Ab (upper panel). Immunoblotting with anti-E4F1 (AD1) Abs show that equal amounts of WT and Δ41–85 E4F1 proteins have been added in each assay (lower panel).

(C) E4F1-mediated ubiquitylation of p53 in vitro requires the aa 41–85 region of E4F1. Autoradiograms of in vitro ubiquitylation assays performed with ^{35}S-labeled IVT p53 and increasing amounts of baculovirus-expressed WT E4F1 ($p120^{E4F1}$) or Δ41–85E4F1 proteins (upper panel). Immunoblotting with anti-E4F1 (AD1) Ab showing the amounts of WT and Δ41–85 E4F1 proteins added in each assay (lower panel).

(D) E4F1-mediated ubiquitylation of p53 in vivo requires aa 41–85 region of E4F1. Western blot analysis of Ub-conjugated proteins in Cre-transduced (+) or mock-treated (–), parental, LSL-E4F1 and LSLΔ41–85E4F1 cells, transfected with 6XHis-Ub and p53 expression vectors. His-Ub-conjugated forms of p53 (ectopic + endogenous) were purified and analyzed as described in Figure 1F.

(E) Full-length $p120^{E4F1}$ does not exhibit SUMO or Nedd8 E3 ligase activity in vitro. Recombinant $p120^{E4F1}$, Hdm2, or PIAS1 proteins were incubated under standard ubiquitylation (left panel), sumoylation (middle panel), or neddylation (right panel) conditions in presence of baculovirus-expressed p53 protein. p53 conjugates were analyzed by western blotting with anti-p53 antibody (DO1).

p53 forms Ubiquitylated by E4F1 Are Localized on Chromatin

Another difference between p53 forms ubiquitylated by E4F1 or Hdm2 was revealed by assessing their association with chromatin using a small-scale chromatin fractionation assay (Wysocka et al., 2001). This assay was applied to cell lysates from U2OS cells expressing p53 and E4F1 or Hdm2, and showed that E4F1-stimulated Ub-p53 forms were predominantly found in chromatin-enriched fractions (P3), whereas Hdm2-stimulated Ub-p53 forms mainly localized in soluble fractions (S) (Figure 4D).

Similar results were observed in LSL-E4F1 cells, but not in LSLΔ41–85E4F1 cells, with endogenous Ub-p53 forms stimulated by E4F1 (Figure 4E). Importantly, these Ub-p53 forms present in P3 were solubilized by brief Micrococcal Nuclease (Mnase) treatment (Figure 4F), as was HCF1, a chromatin-associated protein monitored in parallel (Wysocka et al., 2001). These data indicate that the E4F1-stimulated Ub-p53 forms were associated with chromatin, and not associated in other insoluble structures. Although indirectly so, these observations suggest that E4F1-stimulated Ub-p53 forms could be transcriptionally active, and further support the notion that E4F1 and Hdm2 control distinct p53 fates.

E4F1 Induces a p53-Dependent Growth Arrest

Gene expression profiling assays performed in LSL-E4F1 cells identified several p53 target genes involved in cell cycle arrest whose expression was activated upon E4F1 expression, including *p21^{Waf1}*, *Gadd45α*, and *Cyclin G1* (E.J., L.L., and C.S., unpublished data). In contrast, those experiments did not reveal variations of p53 targets involved in apoptosis, such as *Fas, Killer/DR5, Bax, Noxa,* or *p53AIP1* (data not shown). RT-PCR analyses confirmed

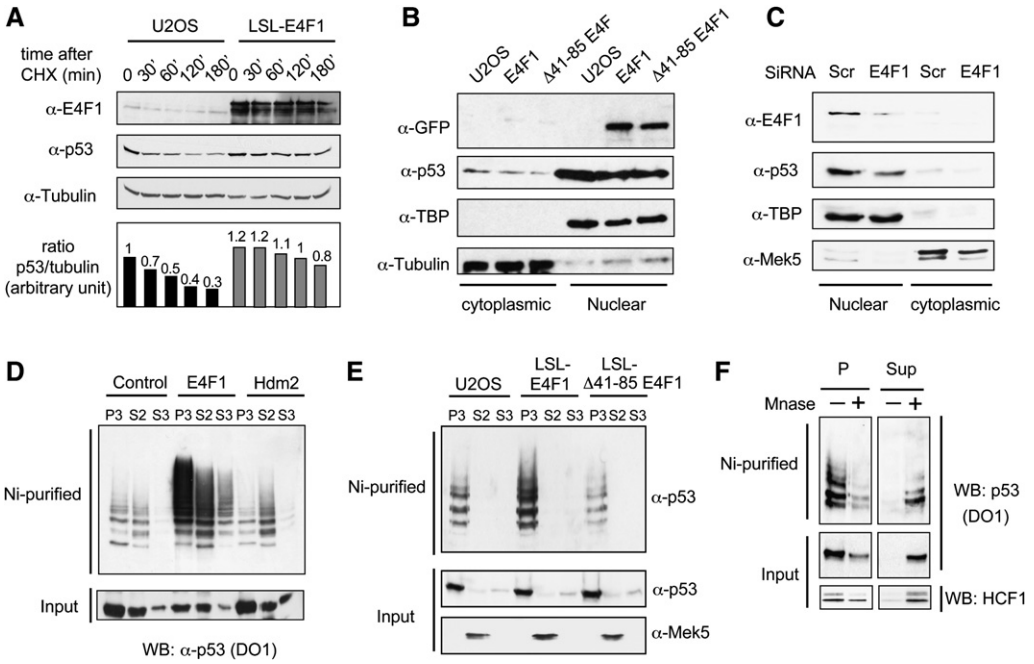

Figure 4. E4F1-Mediated Ubiquitylation of p53 Results in Neither p53 Destabilization nor Nuclear Export

(A) E4F1 overexpression results in a moderate stabilization of endogenous p53 protein. Half-life of p53 protein was measured in cycloheximide- (CHX) treated parental or LSL-E4F1 5 days after Cre-transduction. Protein extracts, prepared at indicated time points after CHX addition, were analyzed by western blotting with anti-E4F1, -p53 (DO1), and -tubulin Abs (upper three panels). (Lower panel) Quantification of the ratio of p53/tubulin (mean values of two independent experiments).

(B) The nuclear/cytoplasmic distribution of p53 does not change upon ectopic E4F1 expression. Western blot analysis of nuclear and cytoplasmic extracts prepared from Cre-infected parental, LSL-E4F1, and LSLΔ41–85E4F1 cells with anti-p53 (D01), -GFP, -TBP, or -tubulin Abs.

(C) The nuclear/cytoplasmic distribution of p53 does not change upon depletion of endogenous E4F1. Western blot analysis of nuclear and cytoplasmic extracts, prepared from U2OS cells treated for 72 hr with either control scrambled SiRNA (Scr) or E4F1 SiRNAs, and probed with anti-E4F1, -p53 (DO1), -TBP, or -Mek5 Abs.

(D) E4F1-, but not Hdm2-, stimulated Ub-p53 forms localize in chromatin-enriched fraction. U2OS cells were transiently cotransfected with 6XHis-Ub and p53 together with either E4F1, Hdm2, or control expression vectors. Cell lysates were fractionated into fractions S2 (soluble cytosolic and some soluble nuclear components), S3 (soluble nuclear components), and P3 (insoluble chromatin and nuclear matrix components). His-Ub-conjugated forms of p53 (ectopic + endogenous) present in each fraction were purified and analyzed as described in Figure 1F. Cells transfected with Hdm2 were treated for 2 hr with the proteasome inhibitor MG132 prior to the biochemical fractionation.

(E) Endogenous Ub-p53 forms stimulated by E4F1 localize in the chromatin-enriched fraction. Western blot analysis of Ub-conjugated proteins present in fractions P3, S2, and S3 prepared from parental U2OS, LSL-E4F1, or LSLΔ41–85E4F1 cells 5 days after Cre-transduction. His-Ub-conjugated forms of endogenous p53 present in each fraction were purified and analyzed as described in Figure 1F. Mek5 was used to assess the quality of the biochemical fractionation.

(F) Digestion of chromatin by Micrococcal Nuclease (Mnase) releases the E4F1-stimulated Ub-p53 forms from the chromatin-enriched fraction P3. P3 fractions prepared from LSL-E4F1 cells were treated with Mnase (+) or with mock enzymatic buffer (−). Digested suspensions were centrifuged and His-Ub-conjugated p53 forms were purified from both the supernatant (sup) and the pellet (P) as described in Figure 1F. Ni^{+}-purified His-Ub-conjugated proteins (Ni-purified) and a fraction of corresponding input extracts were probed for the presence of p53 and HCF1 (a control protein tightly associated with chromatin) by immunoblotting using anti-p53 (DO1) and -HCF1 Abs.

these results (Figures 5A, 5B, and data not shown). Importantly, *p21*^{Waf1} and *Cyclin G1* inductions were not detected in LSLΔ41–85E4F1 cells (Figure 5A) or LSL-E4F1 cells stably expressing short hairpin RNAs (ShRNAs) against p53 ((LSL-E4F1)Δp53; Figure 5B). These results suggested that the E3 activity of E4F1 activates a subset of the p53 transcriptional program. Consistently, chromatin immunoprecipitation (ChIP) assays showed enhanced recruitment of p53 on the *p21*, but not the *NOXA*, promoter region, upon E4F1 ectopic expression (Figure 6E).

The induction of this p53 transcriptional program led to the accumulation of E4F1-GFP-positive cells in the G1 phase of the cell cycle (Figure 5C), and the gradual loss of E4F1-GFP expressing cells in the total cell population (Figure 5D). Importantly, Annexin-V staining of LSL-E4F1 cells indicated that the E4F1-induced growth defect was not the consequence of enhanced cell death (Figure 6A). Notably, this growth defect was partly rescued in cells stably expressing ShRNAs directed against *p53* and *p21*^{WAF1}(Figures 5C and 5D), confirming the role of the p53 pathway in this process. A similar growth defect was not observed in LSLΔ41–85E4F1 cells, indicating that it depends on the E3 activity of E4F1. Moreover, while cooperation to induce cell cycle arrest was obtained

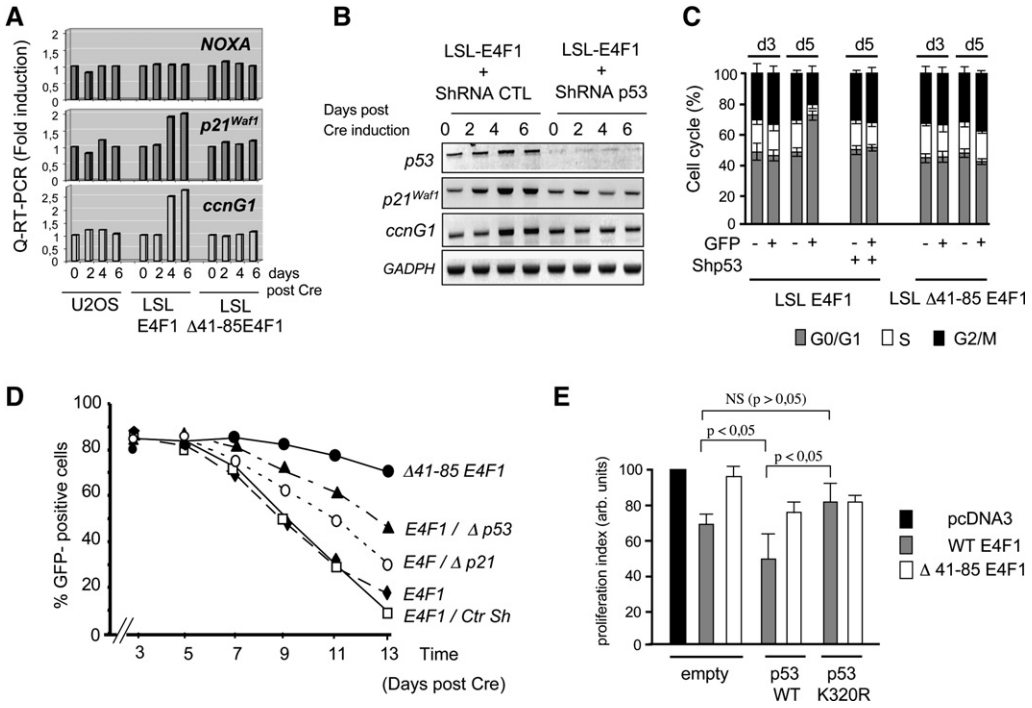

Figure 5. E4F1 Induces a p53-Dependent Growth Arrest Transcriptional Program that Requires Its E3 Ligase Activity

(A) E4F1, but not the E3 ligase mutant Δ41–85E4F1, stimulates expression of a subset of p53 target genes. Quantitative RT-PCR (QRT-PCR) analysis of *p21*^{Waf1}, *cyclin G1,* and *NOXA* gene expression in parental, LSL-E4F1, and LSLΔ41–85E4F1 cells at indicated time points following Cre- transduction. The data show the mean values obtained by QRT-PCR in three independent experiments.

(B) E4F1-mediated activation of *p21*^{Waf1} and *cyclin G1* genes is p53-dependent. Semiquantitative RT-PCR analysis of *p21* and *cyclin G1* mRNAs in Cre-transduced LSL-E4F1 cells stably expressing either a control ShRNA (CTL) or a ShRNA designed to knock down human p53 (ShRNA p53).

(C) E4F1-GFP expression results in a p53-dependent and E3 ligase-dependent growth arrest with cells accumulating in G0/G1. LSL-E4F1, LSLΔ41–85E4F1, and LSL-E4F1 stably expressing p53 ShRNA were harvested at the indicated time points after Cre-infection, and DNA content was analyzed by propidium iodide (PI) staining/FACScan analysis. PI profiles of E4F1-GFP-positive (+) and -negative (−) cells from the same cell population were analyzed. Data represents the mean ± SD of three independent experiments.

(D) E4F1-mediated growth defects are p53-dependent and require its Ub E3 ligase activity. Percentage of GFP-positive cells present in each cell population at the indicated time points after Cre-treatment of LSL-E4F1-GFP (E4F1) or LSLΔ41–85E4F1-GFP (Δ41–85) cells. Similar experiments were performed on LSL-E4F1-GFP cells stably expressing ShRNAs directed against *p53* (E4F1/Δp53), *p21*^{Waf1} (E4F1/Δp21), or control genes. Data represent the mean of three independent experiments.

(E) Lysine 320 of p53 is involved in E4F1-mediated cell cycle arrest. U2OS cells were transduced with control, WT p53, or K320R mutant p53 retroviruses (harboring also a CMV-GFP cassette) and transiently transfected for 24 hr with E4F1, Δ41–85E4F1, or empty control expression constructs. Cells were then incubated for 20 hr with BrdU, and both GFP-positive and E4F1-positive cells were assessed for BrdU uptake by immunofluorescence. Histograms represent the percentage of BrdU positive cells relative to control cells (control retrovirus + empty expression construct) set at 100 in arbitrary units. Statistical analyses were performed using ANOVA and Student-Newman-Keuls multiple comparisons tests. Data represents the mean ± SD of three independent experiments. NS, not significant.

in cells transiently overexpressing E4F1 and p53, this was observed neither with the ΔE3-E4F1 mutant nor with p53 mutated on its E4F1-ubiquitylation site (K320R-p53) (Figure 5E).

Altogether, these data suggest that E4F1-mediated ubiquitylation of p53 on K320 promotes the transcriptional activation of a subset of p53 target genes involved in cell cycle arrest.

E4F1 Exhibits p53-Dependent Antiapoptotic Activities

We next investigated whether E4F1 could also modulate the cellular response to p53-dependent apoptotic stimuli, such as UVC irradiation or p14^{ARF} overexpression. Annexin-V staining of UVC-irradiated LSL-E4F1 and LSLΔ41–85E4F1 cells revealed that cells overexpressing WT E4F1 were less prone to undergo UVC-induced apoptosis than parental cells or cells expressing the E3 deletion mutant (Figure 6A). Conversely, treatment of U2OS cells with E4F1 SiRNAs resulted in increased spontaneous cell death as well as potentiation of UVC- and p14^{ARF}-induced apoptosis (Figures 6B and 6C, respectively). Although incompletely so, this cell death induced by E4F1 depletion was rescued in cells stably expressing ShRNA directed against p53 (Figures 6B and 6C).

Consistent with the notion that E4F1 interferes with proapoptotic functions of p53, we also observed that E4F1 overexpression decreased the UVC-dependent

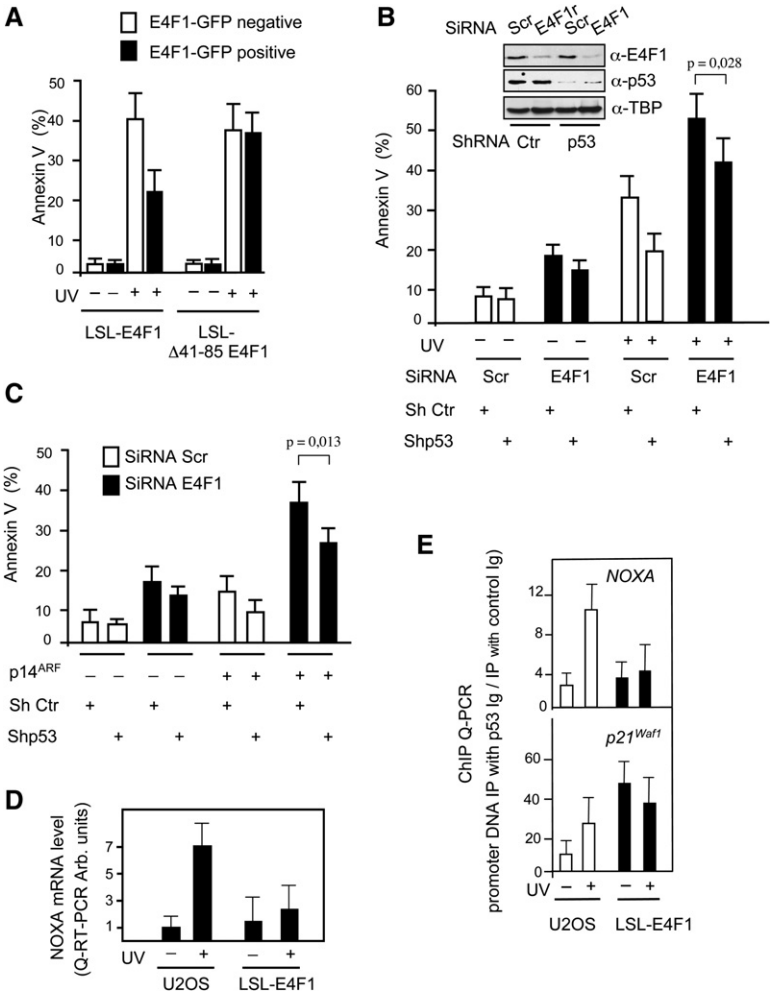

Figure 6. E4F1 Exhibits p53-Dependent Antiapoptotic Activities

(A) E4F1 ectopic expression protects cells from UVC-induced cell death. Five days after Cre-infection, LSL-E4F1 and LSLΔ41–85E4F1 cells were exposed to UVC (15 J/m^2) and analyzed, 36 hr later, for Annexin-V staining by FACSscan. Infection conditions were set to ensure that 50% of the cells express the E4F1-GFP transgene. Histograms represent the percentage of Annexin-V-labeled cells among GFP-positive and -negative cells from the same dish. Data are the mean ± SD of six independent experiments.

(B) E4F1 depletion sensitizes U2OS cells to UVC-induced cell death. This effect is only partly p53-dependent. U2OS cells stably expressing either control-ShRNAs (Sh Ctr) or p53-ShRNAs (Sh p53) were transiently transfected for 3 days with control scrambled SiRNA (Scr, open box) or E4F1-SiRNA (E4F1, black box). Cells were then exposed to UVC (15 J/m^2) and analyzed, 24 hr later, for Annexin-V staining by FACScan. The data represent the mean ± SD of three independent experiments. Statistical analysis was performed using a paired t test. E4F1 and p53 depletions were assessed by immunoblotting (upper panels).

(C) E4F1 depletion sensitizes U2OS cells to cell death induced by overexpression of p14^{ARF}. Annexin-V-positive cells were determined by FACScan after treatment of U2OS cells with either control SiRNA (Scr, open box) or E4F1-SiRNA (black box) and transduction with either empty (−) or p14^{ARF} (+) retroviruses. These experiments were performed in parallel in U2OS cells stably expressing p53 (Shp53) or control (Sh Ctr) ShRNAs. Data are the mean ± SD of three independent experiments. Statistical analysis was performed using a paired t test.

(D) E4F1 ectopic expression decreases the UVC-induced activation of the proapoptotic gene *NOXA*. Five days after Cre-transduction, parental and LSL-E4F1 cells were mock-treated or UVC-irradiated (15 J/m^2). Six hours after irradiation, *NOXA* mRNA expression was assessed by QRT-PCR. Data shown are the mean values ± SD of two independent experiments.

(E) Chromatin immunoprecipitations assays (ChIP) showing that E4F1 stimulates the recruitment of endogenous p53 at the *p21* promoter, but not at the *NOXA* promoter, region. Five days after Cre-transduction, parental and LSL-E4F1 cells were mock-treated or UVC-irradiated (15 J/m^2). Six hours later, similar amounts of formaldehyde-cross-linked chromatin samples were immunoprecipitated with anti-p53 (DO7) or control Ig, and precipitates were analyzed by quantitative PCR for the presence of human *p21^{Waf1}* and *NOXA* promoter fragments (centered around the major p53 sites). Histograms represent the ratio between the fraction of input promoter DNA (*p21* or *NOXA*) immunoprecipitated by the anti-p53 antibody and DNA immunoprecipitated by the control Ig. Histograms represent the mean values ± SD of three independent experiments.

induction of the proapoptotic p53 target gene *NOXA* (Q-RT-PCR, upper panel, Figure 6D) and the coincidental recruitment of p53 on the *NOXA* promoter region (Figure 6E). In these UV-irradiated E4F1-overexpressing cells, p53 recruitment on the *p21* promoter remained high (Figure 6E), a situation which coincided with the maintenance of a high level of p53 ubiquitylation (Figure S6C).

Taken together, these data strongly argue that E4F1-mediated ubiquitylation causes p53 to trigger activation of a transcriptional program involved in cell cycle arrest and antagonizes p53 ability to induce apoptosis.

E4F1-Stimulated Ubiquitylation Antagonizes p53 Acetylation by PCAF

The lysines of p53 targeted by E4F1 include K320, a residue previously shown to be acetylated by the acetyltransferase PCAF in response to various cellular stressors leading to apoptosis (Liu et al., 1999), as shown here in U2OS irradiated by UVC (Figures 7A and 7B and Figure S6B). We hypothesized that PCAF and E4F1 mediate competitive posttranslational modifications of p53 on K320. Such competition between acetylation and ubiquitylation has previously been reported on other p53 lysines

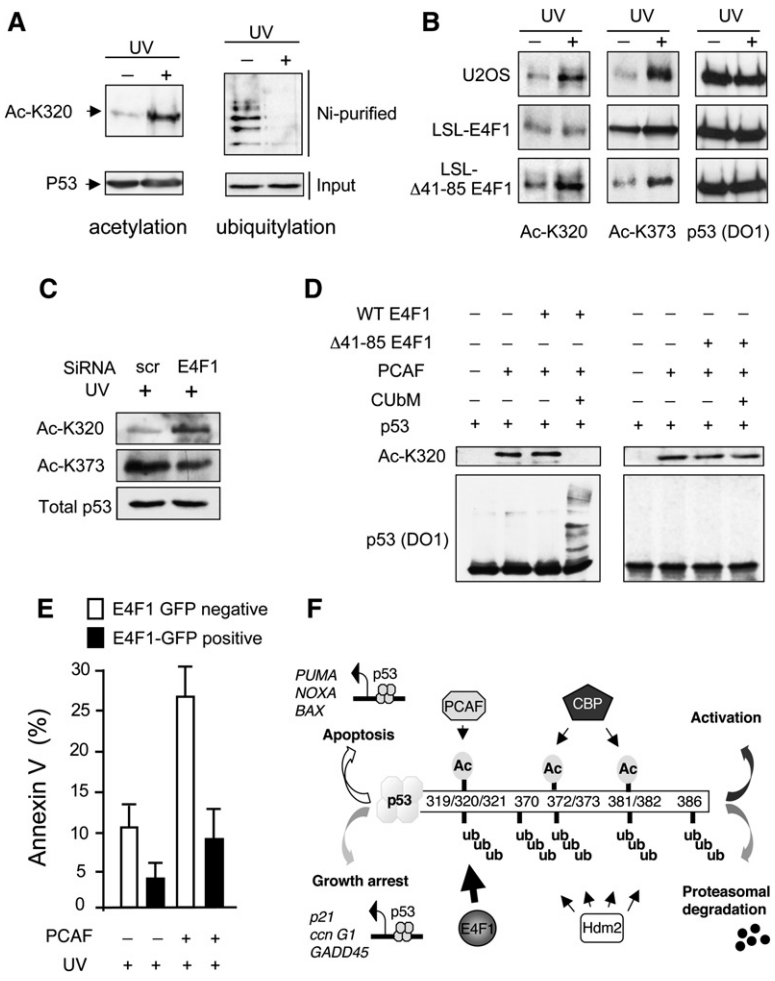

Figure 7. E4F1-Mediated Ubiquitylation of p53 Antagonizes the UVC-Stimulated and PCAF-Mediated Acetylation of K320

(A) Acetylation of K320 inversely correlates with ubiquitylation of p53 in UV-irradiated U2OS cells. U2OS cells were UVC-irradiated (15 J/m^2) (+) or mock treated (–). Four hours later, endogenous p53 was immunoprecipitated (DO7) and precipitates were probed by immunoblotting with an antibody recognizing either the K320-acetylated form of p53 (upper left panel) or all forms of p53 (FL393) (lower left panel). In parallel, ubiquitylation levels of endogenous p53 were assessed as described in Figure 1F (right panels). Neither cells nor cellular extracts were treated with proteasome inhibitors.

(B) E4F1 ectopic expression decreases UVC-induced acetylation of endogenous p53 on K320. Four hours after UVC irradiation (15 J/m^2) (+) or mock-treatment (–) of parental U2OS, LSL-E4F1, and LSLΔ41–85E4F1 cells, the PCAF-mediated acetylation on K320 and CBP/p300-mediated acetylation on K373 of endogenous p53 were assessed by IP-western experiments as described in Figure 7A. Immunoblottings were performed with antibodies recognizing either the K320- or K373-acetylated forms of p53.

(C) E4F1 depletion enhances UVC-induced acetylation of p53 on K320. Four hours after UVC irradiation, acetylation levels of K320 and K373 of endogenous p53 were assessed, as in Figure 7B, in U2OS cells treated for three days with control scrambled (scr) or E4F1 SiRNAs.

(D) E4F1-mediated ubiquitylation of recombinant p53 directly competes for PCAF-induced acetylation on residue K320 of p53 in vitro. Combinations of recombinant PCAF, p53, and WT or Δ41–85 E4F1 proteins were incubated with acetylation mix under standard conditions and then immunoblotted with Abs recognizing either the K320-acetylated form of p53 (upper panels) or all forms of p53 (DO1) (lower panels). E4F1-mediated ubiquitylation reactions were performed as described in Figure 1 prior to the acetylation reaction. CUbm, ubiquitylation mix: E1, E2, Ub, ATP.

(E) E4F1 expression protects cells from the sensitizing effects of PCAF to UVC-induced cell death. LSL-E4F1 cells were transiently transfected with empty (–) or PCAF expression vectors and exposed to UVC (15 J/m^2). Six hours after irradiation, E4F1-GFP-positive and GFP-negative cells were analyzed for Annexin-V staining by FACScan. Data are the mean ± SD of two independent experiments.

(F) Schematic representation of posttranslational modifications occurring at p53 lysine clusters K319–K321 and K370/372/373/381/382/386. Both clusters are subjected to mutually exclusive posttranslational modifications performed by acetyl-transferases (PCAF and CBP/p300, respectively) and Ub E3 ligases (E4F1 and Hdm2, respectively). Based on our results, we propose that these modifications have different output signals for p53 effector functions. Competition between PCAF-mediated acetylation and E4F1-mediated ubiquitylation at K320 modulates p53 ability to induce either growth arrest or apoptosis.

(including K373) that are targeted by p300/CBP and Hdm2.

Consistent with this model, the UVC-stimulated acetylation of K320 (Figure 7A) coincided with a decrease in the basal level of p53 ubiquitylation (mainly ascribable to E4F1 activity in those experimental conditions as shown in Figure 1F). Conversely, p53 acetylation on K320, but not on K373, was compromised in cells overexpressing E4F1 (Figure 7B) and potentiated upon SiRNA-mediated depletion of endogenous E4F1 (Figure 7C). Finally, in vitro K320 acetylation of recombinant p53 by recombinant PCAF was abolished when p53 was first ubiquitylated in vitro by E4F1 (Figure 7D). In contrast, in vitro acetylation of K373 mediated by p300 remained unaffected by E4F1 (Figure S5A). Importantly, E4F1 did not perturb acetylation of purified Histone H3, another PCAF substrate (Figure S5B), indicating that the decreased acetylation of p53 on K320 was not the indirect consequence of an alteration of PCAF acetyltransferase activity.

Of note, the in vivo competition between ubiquitylation and acetylation on K320 was not observed upon ionizing irradiation of U2OS cells (Figures S6B and S6C), an experimental condition which induces DNA double-strand breaks and p53 activation without inducing cell death in this cell line (Allan and Fried, 1999).

Collectively, these data indicate that E4F1 and PCAF mediate mutually exclusive posttranslational modifications of p53, a mechanism that might explain the control of E4F1 over p53-dependent induction of cell death (Figure 7E).

DISCUSSION

E4F1 was originally identified as an E1A-activated cellular transcription factor that directly associates with several important cellular regulators, including p53. Here, we show that E4F1 is also a bona fide Ub E3 ligase for p53, although it contains none of the typical domains previously described in other Ub E3 ligases. Instead, a region of E4F1 (aa 30–80) required for its Ub E3 activity presents sequence similarities with IR domains of the SUMO E3 ligase RanBP2. Despite these similarities, full-length E4F1 was unable to promote in vitro conjugation of SUMO 1 or, like Hdm2, of Nedd8 on p53. At this stage, we can not formally rule out the possibility that E4F1 can also function as a SUMO E3 in vivo under specific conditions or toward other substrates. However, it is tempting to speculate that E4F1 and RanBP2 E3 domains originate from a common ancestor, but have evolved to favor Ub or SUMO conjugation, a situation already observed with RING-type E3 domains.

E4F1-mediated oligo-ubiquitylation occurs in the lysine cluster K319–K321 of p53. These acceptor lysines are distinct from those targeted by Hdm2, and consistently, their ubiquitylation is Hdm2-independent. In striking contrast with previously described p53-Ub E3, we found that E4F1 overexpression neither changes the p53 nuclear/cytoplasmic ratio nor destabilizes p53. Accordingly, these E4F1-mediated p53-Ub conjugates were readily observed in cellular extracts without addition of proteasome inhibitors, reinforcing the notion that this modification does not target p53 to degradation. This was unexpected, since E4F1 predominantly favors Ub chain extension via K48, a branching type generally associated with proteasomal degradation. Nevertheless, there is evidence that the ability of K48 polyubiquitylated proteins to physically interact with the 26S proteasome relies on the length of the Ub chain (Thrower et al., 2000). Of note, E4F1-modified p53 Ub-conjugated ladders generated in our assays are shorter than those observed with Hdm2 (Figure 3E).

In striking contrast with Ub-p53 forms stimulated by Hdm2, which are mainly cytosoluble and targeted to the proteasome, we found that E4F1-stimulated Ub-p53 forms are tightly associated with chromatin, suggesting that they could be involved in transcription. Consistent with this notion, stimulation of the E4F1-mediated ubiquitylation of p53 coincides with the selective activation of p53 responsive genes involved in growth arrest, including *p21* and *cyclin G1*, but not of those involved in apoptosis. Accordingly, ChIP assays indicate that E4F1 stimulates the recruitment of p53 on the *p21* promoter, but not on its proapoptotic target-gene, *NOXA*. Interestingly, similar scenarios were recently reported about the ubiquitylation of the transcription factors Met4 and c-Myc, which promotes their recruitment to some, but not all, of their target genes (Adhikary et al., 2005; Flick et al., 2004).

Importantly, this E4F1-mediated activation of p53 is unlikely to be an indirect consequence of E4F1-induced DNA damage, since E4F1-overexpressing cells do not exhibit specific phospho-Ser139 γ-H2AX staining (Figure S6D) or enhanced phosphorylation of p53 on Ser15 (Figure S6E).

In addition, our data suggest that E4F1 exhibits antiapoptotic properties that depend on its E3 activity and, at least in part, on p53. This activity unlikely results from alterations of upstream signaling pathways that activate p53 in response to UVC-irradiation, since phosphorylation of p53 on Ser15 occurred at similar levels in control and E4F1-overexpressing cells after UVC irradiation (Figure S6E). Endogenous E4F1 seems to exert a similar cytoprotective function, since SiRNA-mediated depletion of E4F1 in U2OS cells resulted in spontaneous cell death and in increased apoptosis in response to UVC and $p14^{ARF}$ overexpression. However, p53 depletion incompletely rescues this cell death induced by E4F1 depletion, suggesting that the impact of E4F1 on survival extends beyond its effects on p53. Of note, gene expression profiling of LSL-E4F1 cells also identified several p53-independent survival genes whose expression was activated upon E4F1 expression, among which are members of the BCL and IGF families (E.J., L.L., and C.S., unpublished data).

How does E4F1-stimulated ubiquitylation modulate the p53 transcriptional activity? Hypotheses similar to the "histone code" have been advanced for the panoply of p53 posttranslational modifications, where multisite and competing modifications are important for coordinating qualitative and quantitative aspects of p53 functions in vivo (Yang, 2005). Consistent with the model that different modifications of p53 create gridlock at its C-terminal lysine residues, with one modification excluding others (Chuikov et al., 2004; Saito et al., 2003; Sakaguchi et al., 1998), we found that E4F1-mediated ubiquitylation competes for acetylation of K320 by PCAF, but leaves intact its capacity to be acetylated by CBP/p300. In contrast to the plethora of information regarding the regulation of p53 by CBP/p300, the roles of PCAF in p53-dependent stress-induced cell death/growth arrest remain unclear and controversial (Sakaguchi et al., 1998, Di Stefano et al., 2005, Knights et al., 2006). In U2OS cells, we found that p53 is rapidly acetylated on K320 and K373 after UVC irradiation at a dose that induces a strong apoptotic response. In contrast, this K320 acetylation was barely detectable upon exposure to ionizing radiation, which activates p53 but does not induce cell death in this cell line.

This might explain why E4F1-mediated ubiquitylation does not perturb the G2 cell cycle arrest induced by double-stranded DNA breaks (Figure S6F), a phenomenon that correlates with acetylation of p53 by CBP (K373), but not PCAF (K320). Moreover, we found that PCAF overexpression sensitizes cells to UVC-induced apoptosis. Collectively, these data strongly support a role for PCAF-stimulated acetylation of p53 on K320 in apoptosis, at least in U2OS cells. Consistent with this model, we propose that E4F1 antagonizes the PCAF-mediated acetylation of p53 and ability to trigger an apoptotic program in response to UVC. This E4F1-p53-PCAF model also provides new directions for exploring the poorly understood capacity of p53 to choose between a cell cycle arrest or apoptotic transcriptional program (Oren, 2003; Vousden and Lu, 2002). Based on this model, it could be interesting to reanalyze naturally occurring p53 mutants that exhibit different abilities to induce cell cycle arrest or apoptosis (Friedlander et al., 1996; Rowan et al., 1996; Ryan and Vousden, 1998) for their capacity to be either preferentially acetylated by PCAF or ubiquitylated by E4F1. Notably, p53 mutations/deletions near the lysine cluster 319–320 have already been shown to affect its capacity to induce apoptosis, but not growth arrest (Kong et al., 2001).

While the competition between PCAF and E4F1 provides an explanation for how E4F1 antagonizes the expression of proapoptotic genes, it does not address the molecular mechanism by which E4F1-stimulated K48 oligo-ubiquitylation promotes the activation of p53-responsive genes involved in growth arrest. This might occur through the mild stabilization of p53 that is detected in cells overexpressing E4F1. However, the degree of this stabilization appears to be too moderate to explain by itself the robust p53-dependent cell cycle arrest observed in these cells. It seems more likely that multiple mechanisms are involved in a way reminiscent of the acetylation of p53 at K382 that enhances DNA binding (Gu and Roeder, 1997; Luo et al., 2004), blocks ubiquitylation by Hdm2 (Nakamura et al., 2000), and specifically interacts with the bromodomain of CBP (Barlev et al., 2001; Espinosa and Emerson, 2001; Prives and Manley, 2001). Thus, it is possible that the K320 ubiquitylation of p53 also enhances physical interactions with transcriptional regulators such as other transcription factors, coactivators, or components of the basal transcriptional machinery.

In conclusion, our data show that E4F1 is a bona fide Ub E3 ligase for the p53 tumor suppressor both in vitro and in vivo. To our knowledge, this is the first example of p53-Ub forms that are associated with chromatin and are not linked to p53 inactivation. E4F1 plays a unique role in the p53 pathway as a novel component of the molecular switch that directs p53 toward cell cycle arrest or apoptosis. As such, it will be of considerable interest to identify pathways/signals that trigger the physiological activation of E4F1 Ub E3 activity, and to evaluate its implication in tumorigenesis. Interestingly, it was recently unraveled that there exists a physical and functional link between Bmi1 and E4F1 in hematopoietic stem cells (Chagraoui et al., 2006). Given the key role of Bmi1 in the regulation of the ARF-p53 pathway, it will be of great interest to address the role of Bmi1 on E4F1-mediated ubiquitylation of p53 in vivo.

EXPERIMENTAL PROCEDURES

In Vivo Ubiquitylation Assay

5.10^6 U2OS cells were transfected using Fugene (Roche) with 5 μg of 6XHis-tagged Ub and 2.5 μg of p53 expression constructs. Cells were lysed under denaturing conditions 24 hr after transfection. Briefly, cells were resuspended in 10 ml of denaturing buffer (6 M guanidinium-HCl, 0.5% Triton, 10 mM imidazole, 20 mM Tris-HCl [pH 8], 0.5 mM DTT, and 0.5 mM Iodoacetamide). His–Ub conjugated proteins were purified by nickel chromatography (Ni-NTA Agarose; Qiagen). Nickel-bound ubiquitylated proteins were subjected to western blot analysis using anti-p53 Ab (DO1). Note that levels of ubiquitylation were assessed on an equal amount of p53 proteins, as shown by western blotting of an unpurified fraction of the same extracts.

Generation of LSL-E4F1 Inducible Cell Lines

The WT full-length human E4F1 cDNA was cloned into pEGFP-N1 plasmid (Clontech). The Δ41–85 deletion of E4F1 was generated by PCR using the following oligonucleotides (5′ GCCTTGGCCCCCAG CGCCCCTCCGGAGGCC3′ and 5′ GGCCTCCGGAGGGGCGCTGG GGGCCAAGGC3′). After introduction of a transcriptional STOP cassette flanked by Lox-P sequences (Jackson et al., 2001), both constructs were stably transfected into U2OS cells. Puromycin-resistant clones were checked for Cre-inducible expression of the E4F1-GFP proteins by FACSscan analysis. Several independent clones expressing comparable levels of WT and Δ41–85 E4F1 protein were used.

Small-Scale Chromatin Fractionation Assay

4.10^7 U2OS cells transfected with a His-tagged Ub expression construct were collected for preparation of subcellular fractions as previously described (Wysocka et al., 2001). Ten percent of these fractions were used as input extracts and for Mnase treatment. Ninety percent were lysed in highly denaturing guanidinium buffer for purification of His-Ub conjugated proteins by nickel chromatography.

The list of antibodies, constructs, recombinant proteins, and oligonucleotides used in this manuscript, as well as detailed procedures for in vitro assays (ubiquitylation, acetylation, neddylation, sumoylation), FACSscan analysis, immunoprecipitation experiments, analysis of p53 stability, ChIP experiments, and gene expression assays are available in the Supplemental Data.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.cell.com/cgi/content/full/127/4/775/DC1/.

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