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Mammalian Elongin A complex mediates DNA-damage-induced ubiquitylation and degradation of Rpb1

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The Elongin complex stimulates the rate of transcription elongation by RNA polymerase II (pol II) by suppressing transient pausing of the pol II at many sites along the DNA. Elongin is composed of a transcriptionally active A subunit and two small regulatory B and C subunits, which can form an isolable Elongin BC subcomplex. Here, we have shown that both the ubiquitylation and proteasomal degradation of the largest subunit of pol II (Rpb1) following UV-irradiation are significantly suppressed in Elongin A-deficient cells; however, in both cases suppression is rescued by transfection of wild-type Elongin A. Moreover, we have demonstrated that the Elongin A–Elongin BC complex is capable of assembling with the Cul5/Rbx2 module, and that this hetero-pentamer complex efficiently ubiquitylates Rpb1 *in vitro*. Mechanistic studies indicate that colocalization of Elongin A and Cul5 in cells and the interaction of Elongin A with the Ser5-phosphorylated form of Rpb1 are strongly enhanced following UV-irradiation. Taken together, our results suggest that mammalian Elongin A is directly involved in ubiquitylation and degradation of Rpb1 following DNA damage.

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Introduction

Eukaryotic mRNA synthesis by RNA polymerase II (pol II) is regulated by the concerted action of a set of transcription factors that control the activity of pol II during the initiation and elongation stages of transcription. At least six general transcription factors have been identified in eukaryotic cells and found to promote the selective binding of pol II to promoters and to support a basal level of transcription (Roeder, 1996). In addition, a diverse collection of elongation factors that promote efficient elongation of transcripts by pol II *in vitro* have also been identified (Conaway *et al*, 2000; Shilatifard *et al*, 2003; Sims *et al*, 2004). These factors fall into two broad functional classes based on their ability either to reactivate arrested pol II or to suppress the transient pausing of pol II. The first class is composed of members of the SII family (Wind and Reines, 2000; Shilatifard *et al*, 2003; Sims *et al*, 2004). The second class comprises a collection of elongation factors, including TFIIF (Price *et al*, 1989), Elongin (Bradsher *et al*, 1993; Aso *et al*, 1995), ELL (Shilatifard *et al*, 1996) and CSB (Selby and Sancar, 1997), which increase the overall rate of mRNA chain elongation by decreasing the frequency and/or duration of transient pausing by pol II at sites along the DNA template.

Elongin was identified as a heterotrimer composed of A, B, and C subunits of ~770, 118, and 112 amino acids, respectively (Bradsher *et al*, 1993; Garrett *et al*, 1994, 1995; Aso *et al*, 1995). Elongin A is the transcriptionally active subunit, whereas Elongins B and C are positive regulatory subunits that can form an isolable Elongin BC subcomplex (Aso *et al*, 1995, 1996; Duan *et al*, 1995). Two additional Elongin A family members, Elongin A2 and Elongin A3, have been identified in mammalian cells (Aso *et al*, 2000; Yamazaki *et al*, 2002). All three Elongin A proteins function similarly to stimulate the overall rate of elongation by pol II *in vitro* and have been shown to bind to the Elongin BC complex through a 10-amino acid degenerate sequence motif referred to as the BC-box with consensus [(T,S,P)LXXX(C,A,S)XXX(V,I,L)]. Recently, Elongin A was found to belong to a larger family of Elongin BC-box proteins that can all be linked through Elongins B and C to a heterodimeric module composed of Cullin family proteins Cul2 or Cul5 and the RING finger proteins Rbx1 or Rbx2 to form multisubunit complexes that can function as E3 ubiquitin ligases (Kamura *et al*, 2001). The best characterized of these Elongin BC-based ubiquitin ligases is the von Hippel-Lindau (VHL) tumour suppressor complex, where the VHL protein serves as a substrate recognition subunit that recruits target proteins for ubiquitylation and the Cullin/Rbx module functions to activate ubiquitylation of target proteins by an E2 ubiquitin-conjugating enzyme. Although it remained unknown whether Elongin A actually functions as a substrate recognition subunit of ubiquitin ligases and, if so, what its substrates might be, it has been speculated that one function of Elongin A may be to recruit a Cullin/Rbx module directly to transcription elongation complexes to target ubiquitylation of pol II or other components of the transcription apparatus (Shilatifard *et al*, 2003). Indeed, Prakash and co-workers have recently reported that yeast homologues of mammalian Elongin A

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and Elongin C (Ela1 and Elc1, respectively) appeared to be required for ubiquitylation and subsequent degradation of the largest subunit of pol II (Rpb1) in DNA-damaged yeast cells (Ribar *et al*, 2007).

In this study, we have investigated the role of Elongin A in ubiquitylation and degradation of Rpb1 in mammalian cells. Using recombinant proteins expressed in insect cells, we have shown that Elongin A is capable of assembling with the Cul5/Rbx2 module through Elongins B and C, and that this heteropentamer complex efficiently ubiquitylates Rpb1 *in vitro*. Moreover, degradation of Rpb1 following UV-irradiation is significantly inhibited by the reduced expression of Elongin A and Cul5. Taken together, our results suggest that mammalian Elongin A is directly involved in ubiquitylation and degradation of Rpb1, both *in vitro* and *in vivo*.

Results

Stability of hyperphosphorylated Rpb1 following DNA damage is increased in Elongin A-deficient cells

It has been shown that DNA damage causes phosphorylation of a subpopulation of pol II, followed by ubiquitylation and subsequent degradation by the proteasome (Ratner *et al*, 1998; Luo *et al*, 2001; McKay *et al*, 2001). To examine whether Elongin A is involved in ubiquitin-mediated degradation of pol II, the protein levels of Rpb1 after DNA damage were compared in Elongin $A^{+/+}$ and Elongin $A^{-/-}$ ES cells. As shown in Figure 1A, the level of the hypophosphorylated form of Rpb1 (pol IIA) detected with 8WG16 decreased dramatically in Elongin $A^{+/+}$ ES cells following treatment with UV or cisplatin. In contrast, the pol IIA levels were substantially less reduced in Elongin $A^{-/-}$ ES cells. To confirm that the observed diminution of pol IIA in Elongin $A^{+/+}$ ES cells after DNA damage is due to phosphorylation of its C-terminal domain (CTD) and conversion into the hyperphosphorylated form (pol IIO), Elongin $A^{+/+}$ and Elongin $A^{-/-}$ ES cells were UV-irradiated and allowed to recover for the indicated periods of time in the presence or absence of the CTD-kinase inhibitors H7 and DRB. As shown in Figure 1B, the UV-induced decline of pol IIA in Elongin $A^{+/+}$ ES cells was indeed prevented by either of these CTD-kinase inhibitors. An appropriate interpretation of these results therefore is that DNA-damage-induced ubiquitylation and subsequent degradation of pol IIO prevent its being recycled (through dephosphorylation) to pol IIA, so that the steady-state level of pol IIA diminishes in Elongin $A^{+/+}$ ES cells. In Elongin $A^{-/-}$ ES cells, however, the ubiquitin-mediated degradation of Rpb1 is possibly impaired so that the steady-state level of pol IIA is less significantly altered compared with Elongin $A^{+/+}$ ES cells.

To further evaluate the effect of Elongin A on the degradation of Rpb1, the rate of decay of Rpb1 in the presence of cycloheximide was measured in Elongin $A^{+/+}$, Elongin $A^{-/-}$ ES cells, and Elongin $A^{-/-}$ ES cells transfected with wild-type Elongin A (Elongin $A^{-/-}$ + Elongin A ES cells). As shown in western blots with N20, which recognizes both pol IIA and pol IIO, the decrease of the level of pol IIO following UV-irradiation was significantly delayed in Elongin $A^{-/-}$ ES cells compared with Elongin $A^{+/+}$ ES cells; however, the delay observed in Elongin $A^{-/-}$ ES cells was overcome by the introduction of wild-type Elongin A (Figure 1C). In addition, the diminution of pol IIO in Elongin A-expressing cells is

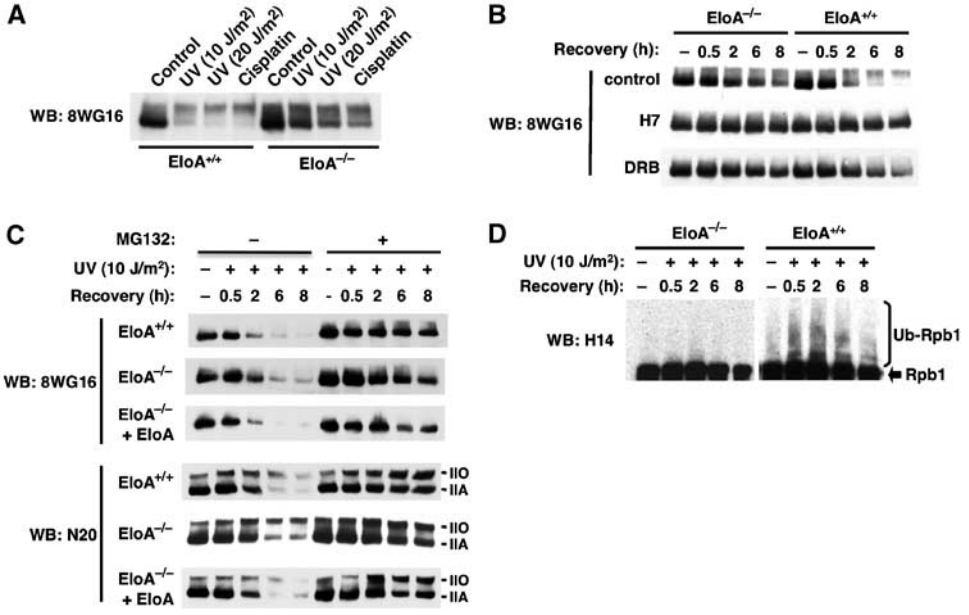

Figure 1 Stability of Rpb1 following DNA damage is increased in Elongin $A^{-/-}$ ES cells. (**A**) Elongin $A^{+/+}$ and Elongin $A^{-/-}$ ES cells were treated with UV-irradiation (10 or 20 J/m^2) or cisplatin (20 μg/ml) and recovered for 8 h in the presence of the protein synthesis inhibitor cycloheximide. Total cell lysates were prepared, separated by 6% SDS–PAGE, and immunoblotted with 8WG16 antibody. (**B**) Elongin $A^{+/+}$ and Elongin $A^{-/-}$ ES cells were treated with UV-irradiation (10 J/m^2) and recovered for the indicated period of time in the presence of cycloheximide with or without the CTD-kinase inhibitors H7 and DRB. Total cell lysates were prepared, separated by 6% SDS–PAGE, and immunoblotted with 8WG16 antibody. (**C**) Elongin $A^{+/+}$, Elongin $A^{-/-}$ ES cells, and Elongin $A^{-/-}$ ES cells transfected with Elongin A were treated with UV-irradiation (10 J/m^2) and recovered for the indicated period of time in the presence of cycloheximide with or without the proteasomal inhibitor MG132. Total cell lysates were prepared, separated by 6% SDS–PAGE, and immunoblotted with 8WG16 or N20 antibody. (**D**) Elongin $A^{+/+}$ and Elongin $A^{-/-}$ ES cells were treated with UV-irradiation (10 J/m^2) and recovered for the indicated period of time in the presence of MG132. Total cell lysates were prepared, separated by 6% SDS–PAGE, and immunoblotted with H14 antibody.

prevented by the proteasomal inhibitor MG132, indicating that the loss of pol IIO results from proteasomal degradation. Furthermore, UV-irradiation clearly stimulated the formation of a high molecular mass smear characteristic of ubiquitin-conjugates in Elongin $A^{+/+}$ ES cells, but failed to induce its formation in Elongin $A^{-/-}$ ES cells (Figure 1D). Taken together, our results suggest that mammalian Elongin A functions in targeting pol IIO for degradation through the ubiquitin-mediated proteolysis pathway in response to DNA damage.

The Elongin A–Elongin BC complex assembles with a Cul5/Rbx2 module to reconstitute a ubiquitin ligase of Rpb1

VHL and other Elongin BC-box proteins can assemble with the Elongin BC complex and a heterodimeric module composed of a member of the Cullin protein family and RING finger protein Rbx1 or Rbx2 to form multiprotein ubiquitin ligase complexes (Kamura *et al*, 1999, 2000, 2001, 2004). In mammalian cells the Cullin protein family includes at least five members, referred to as Cul1, Cul2, Cul3, Cul4, and Cul5 (Kipreos *et al*, 1996). Each of these Cullin proteins has been shown to assemble with either Rbx1 or Rbx2 to reconstitute a module that is capable of activating ubiquitylation by E2 ubiquitin-conjugating enzymes (Ohta *et al*, 1999; Kamura *et al*, 2001, 2004).

To confirm that the Elongin A–Elongin BC complex can assemble into similar complexes, Sf9 insect cells were infected with combinations of baculoviruses encoding 3 × Flag–Elongin A, Elongins B and C, 3 × myc–Rbx2, myc–Rbx1, and individual Cullin proteins containing N-terminal HA epitope tags. When immunoprecipitations were performed with anti-HA and anti-Flag antibodies, Elongin A, Elongins B and C, and Rbx2 could be specifically coimmunoprecipitated with HA–Cul5, but not with HA–Cul1, HA–Cul2, HA–Cul3, or HA–Cul4 (Figure 2A). Rbx2 was reproducibly present in all the anti-Flag immunoprecipitates. It is most likely that Rbx2 is linked to Elongin A through

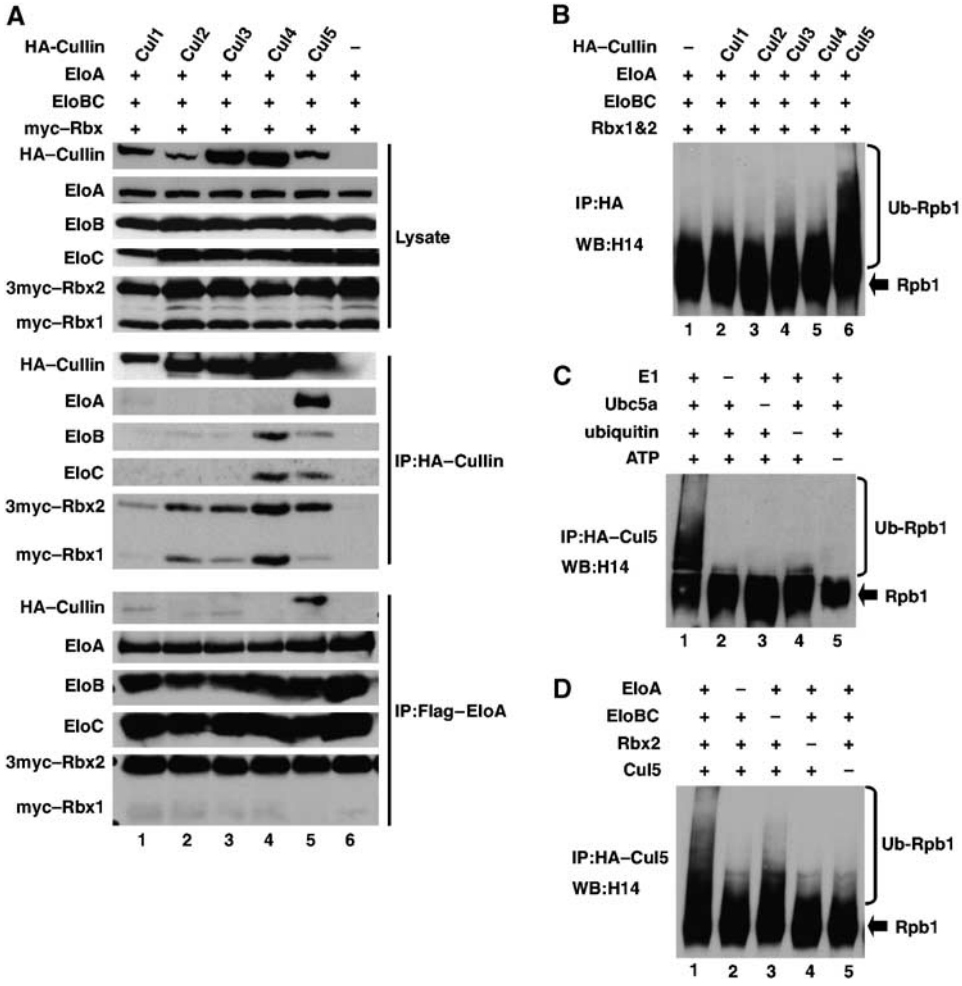

Figure 2 The Elongin A–Elongin BC complex assembles with a Cul5/Rbx2 module to reconstitute a ubiquitin ligase of Rpb1 *in vitro*. (**A**) Sf9 cells were coinfected with the baculoviruses indicated in the figure. Anti-HA and anti-Flag immunoprecipitations were performed as described under Materials and methods. Total cell lysates and anti-HA and anti-Flag immunoprecipitates were separated by SDS–PAGE and immunoblotted with the indicated antibodies. (**B**, **D**) Lysates from Sf9 cells infected with the indicated baculoviruses were immunoprecipitated with anti-HA antibody and were subjected to *in vitro* ubiquitylation assay as described under Materials and methods. Samples were analyzed on 6% SDS–PAGE and processed for western blotting with H14 antibody. (**C**) Lysates from Sf9 cells containing Elongin A, HA-Cul5, Rbxs 1 and 2, and Elongins B and C were immunoprecipitated with anti-HA antibody and were subjected to *in vitro* ubiquitylation assay as described under Materials and methods, with omission of E1 (lane 2), hUbc5a (lane 3), ubiquitin (lane 4), or ATP (lane 5) from reaction mixtures. Samples were analyzed on 6% SDS–PAGE and processed for western blotting with H14 antibody.

endogenous insect cell Cul5, although we cannot rule out the possibility that Rbx2 is bound directly to Elongin A in these assays.

To determine whether the Elongin A–Elongin BC–Cul5–Rbx2 complex possesses ubiquitin ligase activity for Rpb1, anti-HA immunoprecipitates from Sf9 cell lysates were assayed for their abilities to activate Rpb1 ubiquitylation in the presence of ATP, ubiquitin, E1, the E2 ubiquitin-conjugating enzyme hUbc5a, and purified pol II. As shown in Figure 2B, maximal activation of Rpb1 ubiquitylation was observed when reaction mixtures contained complexes purified from cells that were overexpressing Elongin A, Elongins B and C, Rbx proteins, and Cul5, but not other members of the Cullin family. Activation of Rpb1 ubiquitylation by the Elongin A complex was strongly dependent on ATP, ubiquitin, E1, and the E2 ubiquitin-conjugating enzyme hUbc5a (Figure 2C). To investigate the contributions of individual subunits of the Elongin A complex to Rpb1 ubiquitylation, Sf9 cells were coinfected with various combinations of baculoviruses encoding 3 × Flag–Elongin A, Elongins B and C, 3 × myc–Rbx2, and HA–Cul5. Anti-HA immunoprecipitates were then assayed for their abilities to activate Rpb1 ubiquitylation in the presence of ATP, ubiquitin, E1, hUbc5a, and purified pol II. As shown in Figure 2D, maximal activation of Rpb1 ubiquitylation was observed in the presence of Elongin A-containing complexes purified from cells overexpressing all five subunits of the Elongin A complex. A low level of Rpb1 ubiquitylation was observed when reaction mixtures contained complexes purified from cells that were not overexpressing Elongins B and C, most likely because of the presence of a substoichiometric amount of contaminating endogenous insect cell Elongins B and C in immunoprecipitated Elongin A complexes. These results, thus, demonstrate that the Elongin A–Elongin BC complex is capable of assembling with a Cul5/Rbx2 module to reconstitute a ubiquitin ligase that targets Rpb1.

Localization of Elongin A regions important for the assembly and the Rpb1-ubiquitylation activity of the Elongin A ubiquitin ligase

It has been reported recently that, in addition to the BC-box, mammalian Elongin A contains sequences similar to the canonical Cul5-box consensus ϕxxLPϕPxxϕxx[Y/F][L/I], immediately downstream of the BC-box (Kamura *et al*, 2004; Mahrour *et al*, 2008) (Figure 3A). To investigate whether these BC-box and potential Cul5-box sequences of Elongin A are required for the assembly and the Rpb1 ubiquitylation activity of the Elongin A ubiquitin ligase, we compared the activities of wild-type Elongin A and three Elongin A mutants. An Elongin A double point mutant Elongin A[L550P;C554F] contains BC-box mutations that were shown previously to disrupt binding of the BC-box family members to the Elongin BC complex (Aso *et al*, 1996; Kamura *et al*, 1998). Internal deletion mutants Elongin A[Δ(549–558)] and Elongin A[Δ(568–588)] lack the BC-box and Cul5-box sequences, respectively.

Sf9 cells were coinfected with various combinations of baculoviruses encoding Elongins B and C, 3 × myc–Rbx2, myc–Rbx1, HA–Cul5, and 3 × Flag-tagged Elongin A, Elongin A[L550P;C554F], Elongin A[Δ(549–558)], or Elongin A[Δ(568–588)]. Complexes were immunoprecipitated from cell lysates with anti-HA or anti-Flag antibody and analyzed (i) for the presence of individual subunits of the Elongin A complex by immunoblotting and (ii) for their abilities to activate Rpb1 ubiquitylation in the presence of ATP, ubiquitin, E1, the E2 ubiquitin-conjugating enzyme hUbc5a, and purified pol II. As shown in Figure 3B, Elongin A mutants, Elongin A[L550P;C554F] and Elongin A[Δ(549–558)] were severely, and Elongin A[Δ(568–588)] was less severely, impaired in their abilities to bind to Elongins B and C. Consistent with the previous observation that entry of Cullins into the BC-box protein complexes depends on the presence of Elongins B and C (Kamura *et al*, 2000, 2001), Cul5 was not as efficiently coimmunoprecipitated with Elongin A[L550P;C554F], Elongin A[Δ(549–558)], or Elongin A[Δ(568–588)] as wild-type Elongin A. Furthermore, anti-HA immunoprecipitates purified from insect cells expressing Elongin A mutants, Elongin A[L550P;C554F], Elongin A[Δ(549–558)], or Elongin A[Δ(568–588)] failed to activate Rpb1 ubiquitylation above the low background level observed in the absence of Elongin A (Figure 3C).

In addition, we examined the requirement of the N-terminal SII similarity region and the elongation activation domain of Elongin A for activating Rpb1 ubiquitylation. As shown in Figure 3D, anti-HA immunoprecipitate purified from insect cells expressing Elongin A[121–773], which lacks the region similar to SII, exhibited a level of activity comparable with that of full-length Elongin A, suggesting that the N terminus of Elongin A is dispensable for activating Rpb1 ubiquitylation. The relative Rpb1 ubiquitylation activities of wild-type Elongin A and a series of N-terminal, C-terminal, and internal deletion mutants of Elongin A were then compared (Figure 3E). Anti-HA immunoprecipitates purified from insect cells expressing Elongin A deletion mutants lacking sequences between residues 521 and 680, which have been shown to be most critical for inducible activation of Elongin A transcriptional activity by Elongin BC (Aso *et al*, 1996), failed to activate Rpb1 ubiquitylation above the background level observed in the absence of Elongin A (Figure 3F). These results, therefore, demonstrate (i) that both the BC-box and Cul5-box sequences of Elongin A are essential for the proper assembly and Rpb1 ubiquitylation activity of the Elongin A ubiquitin ligase, and (ii) that the Elongin A region required for Rpb1 ubiquitylation is almost identical to the elongation activation domain of Elongin A.

Elongin A colocalizes with Cul5 in cell nuclei after UV-irradiation

We have previously shown that Elongin A is predominantly localized to the cell nucleus (Yasukawa *et al*, 2007). Although the distribution of Cul5 has been controversial, recent reports suggest that it migrates between the nucleus and cytoplasm depending on the cell cycle (Querido *et al*, 2001; Ceremuga *et al*, 2003; Burnatowska-Hledin *et al*, 2004). It is therefore important to determine whether Elongin A and Cul5 colocalize in cells after UV-irradiation. HeLa cells were UV-irradiated and fixed after the indicated period of time, and then localization of Elongin A, Cul5, and also Cul2 as a control was analyzed by laser confocal microscopy. Consistent with the previous results, Elongin A predominantly localized to the nucleus. Cul5 also primarily localized to the nucleus, as did Cul2. Before UV-irradiation, neither Cul5 nor Cul2 showed significant colocalization with Elongin A. However, 30–40 min after UV-irradiation, Cul5 (Figure 4A), but not

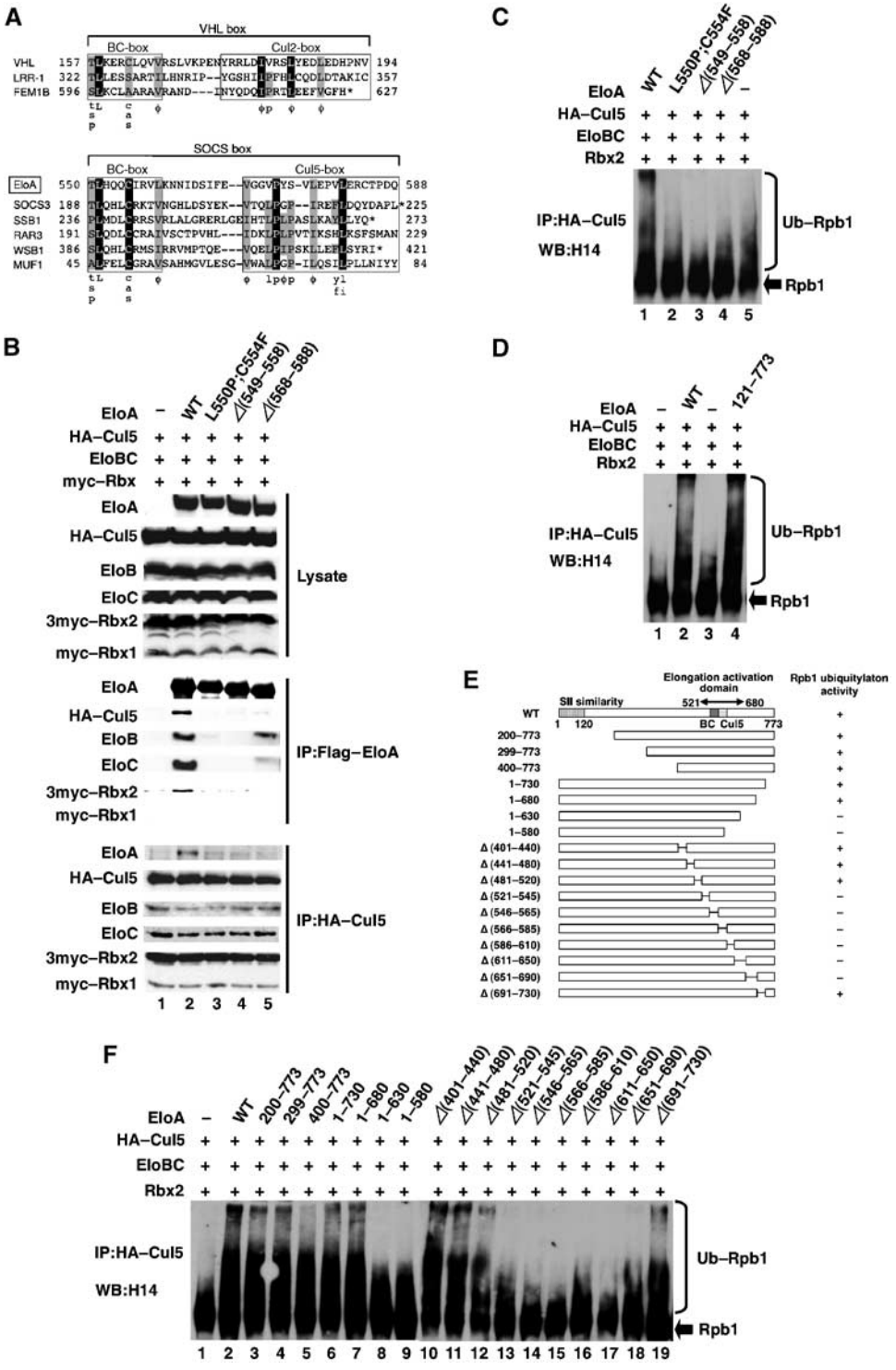

Figure 3 Localization of Elongin A regions required for the assembly and the Rpb1 ubiquitylation activity of the Elongin A ubiquitin ligase complex. (**A**) Alignment of amino acid sequences of VHL-box and SOCS-box proteins. The BC-box, Cul2-box, and Cul5-box are indicated (boxed). Identical (black) and similar (grey) amino acids are shaded. Asterisks indicate C termini. Sequences are of the human proteins. Sequences of the SOCS-box of Elongin A are perfectly conserved between human and rat, and correspond to amino acids 549–587 in rat Elongin A. The canonical BC-box and Cul5-box sequences and conserved Cul2-box residues are indicated below each alignment. (**B**) Sf9 cells were coinfected with the baculoviruses indicated in the figure. Anti-Flag and Anti-HA immunoprecipitations were performed as described under Materials and methods. Total cell lysates and anti-Flag and anti-HA immunoprecipitates were separated by SDS–PAGE and immunoblotted with the indicated antibodies. (**C**, **D**, **F**) Lysates from Sf9 cells infected with the indicated baculoviruses were immunoprecipitated with anti-HA antibody and were assayed for their ability to activate Rpb1 ubiquitylation as described under Materials and methods. Samples were analyzed on 6% SDS–PAGE and processed for western blotting with H14 antibody. (**E**) N-terminal, C-terminal, and internal deletion mutants of Elongin A analyzed in this study. On the right, the results of the assay shown in (F) are shown.

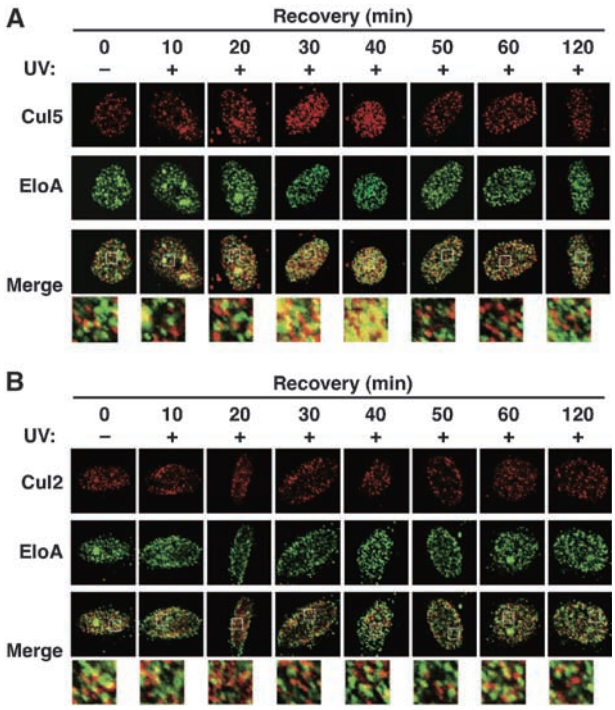

Figure 4 Intracellular colocalization of Elongin A and Cul5 after UV-irradiation. (**A**, **B**) HeLa cells treated with UV-irradiation (10 J/m^2) were incubated for the indicated period of time, then were fixed and analyzed by laser confocal microscopy. In panel A, Cul5 was detected using anti-Cul5 rabbit polyclonal antibody and Alexa 594 anti-rabbit IgG antibody (Top). Elongin A was detected using anti-Elongin A mouse monoclonal antibody and Alexa 488 anti-mouse IgG antibody (Middle). In panel B, Cul2 was detected using anti-Cul2 mouse monoclonal antibody and Alexa 488 anti-mouse IgG antibody (Top). Elongin A was detected using anti-Elongin A rabbit polyclonal antibody and Alexa 594 anti-rabbit IgG antibody (Middle). Merged images are displayed (Bottom). The box indicates the area that is enlarged. Images were pseudocoloured using Adobe Photoshop so that Elongin A is shown in green and Cul5 or Cul2 is shown in red. The areas of overlap appear as yellow.

Cul2 (Figure 4B), showed extensive colocalization with Elongin A in the nucleus. The transient nature of the colocalization of these two proteins suggests that the Elongin A–Elongin BC complex associates with a Cul5/Rbx2 module only temporarily in cells after UV-irradiation.

Reduced expression of Elongin A and Cul5 inhibits the degradation of Ser5-phosphorylated Rpb1 following DNA damage

To confirm the role of Elongin A and Cul5 in DNA-damage-induced degradation of pol IIO, we knocked down Elongin A or Cul5 in mouse embryonic fibroblasts (MEFs) or HeLa cells, respectively, by RNA interference (RNAi)-mediated gene silencing. Two independent sequences for each molecule were used as targets of RNAi. Cells infected with a retroviral vector encoding either Elongin A (EloA-1 or EloA-2) or Cul5 (Cul5-1 or Cul5-2) shRNAs specific for Elongin A or Cul5 mRNAs exhibited a significant decrease in the abundance of Elongin A or Cul5, respectively, compared with that apparent in cells infected with a control vector for EGFP shRNA (Figure 5A). Expression of EGFP shRNA did not affect Rpb1 degradation. The reduction in the level of pol IIO and Ser5-phosphorylated Rpb1 detected with H14 following UV-irradiation, however, was significantly inhibited by expression of Elongin A or Cul5 shRNAs (Figure 5B). The stability of Ser2-phosphorylated Rpb1 detected with H5, however, was not significantly increased by these shRNAs. These results suggest that both Elongin A and Cul5, indeed, function in mediating preferential degradation of the Ser5-phosphorylated form of Rpb1 in response to DNA damage *in vivo*.

Wild-type Elongin A, but not Elongin A[L550P;C554F], rescues delayed degradation of Rpb1 in Elongin A-deficient cells

To confirm that the ubiquitylation mediated by Elongin A will result in a rapid turnover of pol IIO through proteasomal degradation *in vivo*, either wild-type Elongin A or Elongin A[L550P;C554F], which was unable to support pol II ubiquitylation *in vitro*, was transfected into Elongin $A^{-/-}$ ES cells, and the rate of decay of Ser5-phosphorylated Rpb1 following UV-irradiation in the presence of cycloheximide was measured in these cells. As shown in western blots with H14, the observed delay in the degradation of Rpb1 in Elongin $A^{-/-}$ ES cells was almost unchanged by the introduction of Elongin A[L550P;C554F], whereas the rate of decay of Rpb1 in Elongin $A^{-/-}$ ES cells was significantly increased by the introduction of wild-type Elongin A (Figure 6). In addition, the degradation of Rpb1 induced by expression of wild-type Elongin A was prevented by the proteasomal inhibitor MG132. These results demonstrate that Ser5-phosphorylated Rpb1 undergoes proteasome-dependent degradation in an Elongin A-dependent manner *in vivo*, suggesting that Rpb1 is a target of Elongin A ubiquitin ligase for ubiquitylation and rapid degradation by the proteasome.

UV-irradiation enhances the interaction of Elongin A with the Ser5-phosphorylated form of Rpb1

The effect of the Elongin A complex on Rpb1 ubiquitylation would most likely be achieved through a direct interaction between Elongin A and pol II. Such interactions might be constitutive, but also could be induced by DNA damage. To clarify these possibilities, Elongin $A^{-/-}$ ES cells transfected with Flag-tagged Elongin A were UV-irradiated, and cell extracts prepared at different times during recovery in the presence of MG132 were immunoprecipitated with anti-Flag antibody. The presence of Rpb1 in these immunoprecipitates was examined by immunoblotting (Figure 7). A low but clearly detectable level of the Ser5-phosphorylated form of Rpb1 coimmunoprecipitated with Elongin A in the absence of UV-irradiation (Figure 7, lane 1). However, a dramatic increase in the level of Ser5-phosphorylated Rpb1 associated with Elongin A was observed 30 min after UV-irradiation (Figure 7, lane 2). As cells recovered after UV-irradiation, detectable interaction between Elongin A and Ser5-phosphorylated Rpb1 also decreased (Figure 7, compare lanes 2, 3, and 4), whereas neither unphosphorylated nor Ser2-phosphorylated forms of Rpb1 were detectable in those immunoprecipitates. These results, therefore, suggest that the primary target for Elongin A ubiquitin ligase may be the Ser5-phosphorylated form of Rpb1 and that they interact in a way that is strongly enhanced by UV-irradiation.

Discussion

In this report, we provide evidence (i) that both the ubiquitylation and proteasomal degradation of Rpb1 following UV-

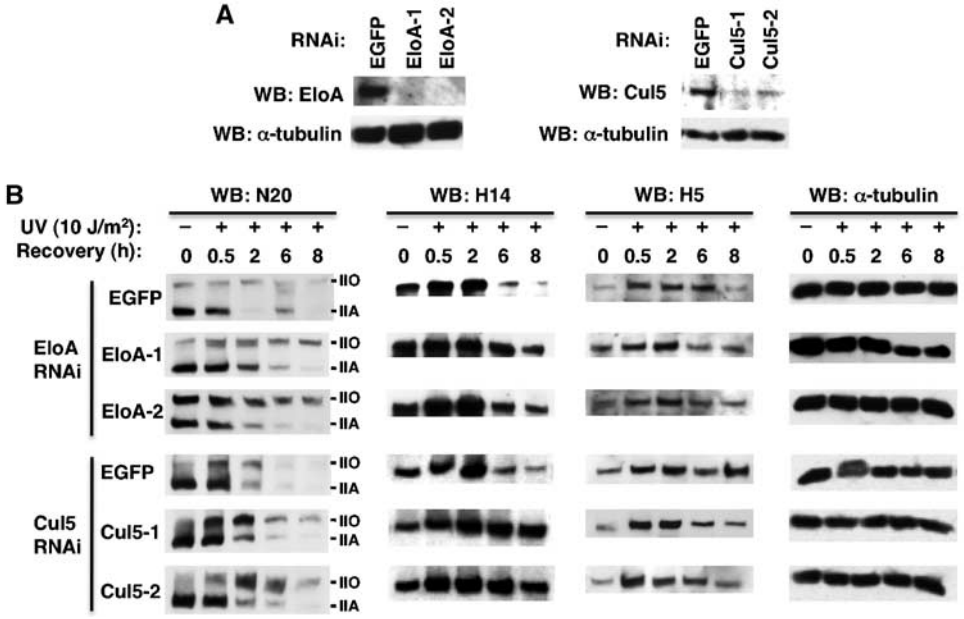

Figure 5 Effect of RNAi knockdown of Elongin A and Cul5 on the UV-induced degradation of Rpb1. (**A**, **B**) MEFs or HeLa cells were infected with a retroviral vector encoding either Elongin A (EloA-1 or EloA-2), Cul5 (Cul5-1 or Cul5-2), or EGFP (control) shRNAs. Lysates of the cells were subjected to western blot analysis with anti-Elongin A, anti-Cul5, N20, H14, H5, or anti-α-tubulin antibody (control).

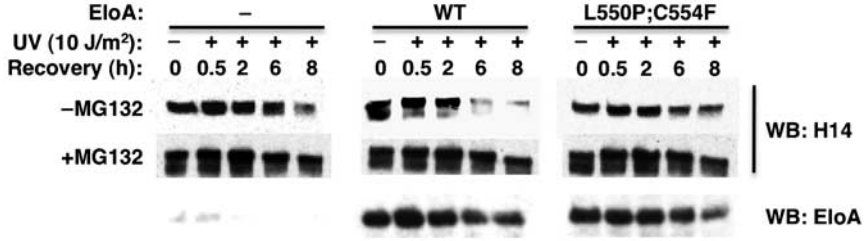

Figure 6 Wild-type Elongin A, but not Elongin A[L550P;C554F], rescues delayed degradation of Rpb1 in Elongin A-deficient cells. Elongin $A^{-/-}$ ES cells transfected with either pCAG-IPG empty vector, pCAG-IPG-Flag-Elongin A, or pCAG-IPG-Flag-Elongin A[L550P;C554F] were treated with UV-irradiation (10 J/m^2) and recovered for the indicated period of time in the presence of cycloheximide with or without MG132. Total cell lysates were prepared, separated by SDS–PAGE, and immunoblotted with H14 antibody. Expression of wild-type Elongin A and Elongin A[L550P;C554F] in the absence of MG132 was detected by anti-Flag antibody.

irradiation are suppressed in Elongin A-deficient mammalian cells, but are rescued by transfection of Elongin A, (ii) that Elongin A is capable of assembling with a Cul5/Rbx2 module through Elongin BC and that this heteropentamer complex efficiently ubiquitylates Rpb1 *in vitro*, (iii) that UV-irradiation enhances not only the colocalization of Elongin A and Cul5 in cell nuclei, but also the interaction of Elongin A with the Ser5-phosphorylated form of Rpb1, and (iv) that reduced expression of Elongin A and Cul5 inhibits the degradation of Ser5-phosphorylated Rpb1 following UV-irradiation. Taken together, our results suggest that at least one function of mammalian Elongin A may be to act as a substrate recognition subunit of a ubiquitin ligase that is directly involved in ubiquitylation and degradation of Ser5-phosphorylated Rpb1.

Mammalian Elongin A complex is one of the ubiquitin ligases that target pol II

Using an elegant assay, Svejstrup and co-workers have recently shown that Nedd4, one of the human homologues of yeast Rsp5, acts as a ubiquitin ligase for human Rpb1, *in vitro* and *in vivo* (Anindya *et al*, 2007). Their conclusion that Nedd4 is a major ubiquitin ligase of Rpb1 in mammalian cells was drawn from the findings (i) that CSA, CSB, and BRCA1, which have been thought to be the key players for Rpb1 ubiquitylation in mammals (Ljungman and Lane, 2004; Lainé and Egly, 2006; Svejstrup, 2007), turned out not to be directly involved in this process, and (ii) that UV-induced ubiquitylation of Rpb1 was significantly inhibited by RNAi-mediated knockdown of Nedd4. In their time course experiment, however, significantly reduced UV-dependent ubiquitylation was indeed observed in the Nedd4-knockdown cells compared with wild-type cells immediately after UV-irradiation, but after 20 min, the levels of ubiquitylation in the two cell lines were almost comparable (Anindya *et al*, 2007). This result suggests the possibility that Nedd4 is not the only factor responsible for Rpb1 ubiquitylation in mammalian cells and that factors other than Nedd4 might exert their function with delayed time intervals after UV-irradiation. Our finding that maximal enhancement of the intracellular colocalization of Elongin A and Cul5 was observed 30–40 min after UV-irradiation (Figure 4A) is in good agreement with this possibility. In addition, the primary target for ubiquityla-

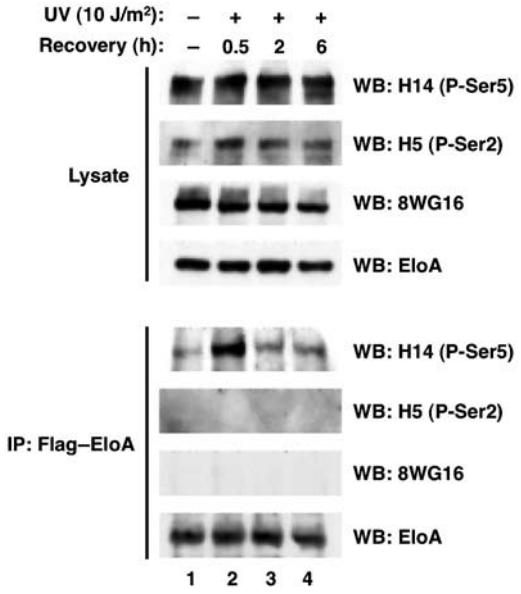

Figure 7 UV-irradiation enhances the interaction of Elongin A with the Ser5-phosphorylated form, but not with the Ser2-phosphorylated or the unphosphorylated form, of Rpb1. Elongin $A^{-/-}$ ES cells transfected with Flag-tagged Elongin A were treated with UV-irradiation (10 J/m^2) and recovered for the indicated period of time in the presence of MG132. Total cell lysates were prepared, immunoprecipitated with anti-Flag antibody, and subjected to 6% SDS–PAGE and western blot analysis with the indicated antibodies.

tion by Rsp5 is known to be the processively transcribing Ser2-phosphorylated pol II, which predominates distal to the promoters (Komarnitsky *et al*, 2000; Somesh *et al*, 2005), whereas that of Elongin A ubiquitin ligase appears to be Ser5-phosphorylated pol II, which predominates proximal to the promoters and is therefore thought to be an early elongation complex (Komarnitsky *et al*, 2000). Moreover, it was recently shown that the VHL protein complex, which contains Elongins B and C and a Cul2/Rbx1 module, is also induced to bind to the Ser5-phosphorylated pol II following UV-irradiation and targets it for ubiquitylation in human cells (Kuznetsova *et al*, 2003). Thus, it is possible that Elongin A and VHL complexes might have an overlapping function in pol II ubiquitylation and that these complexes and Nedd4 may act in a complementary manner to survey transcribing pol II throughout the genome.

Mechanism of activation of the Elongin A ubiquitin ligase

What is the mechanism of activation of the Elongin A ubiquitin ligase by DNA damage? We cannot rule out the possibility that the Elongin A–Elongin BC complex and Elongin A ubiquitin ligase are constitutively present as separate complexes in cells. However, evidence suggests that many fully assembled SCF ubiquitin ligase complexes are highly unstable in cells (Zhou and Howley, 1998; Galan and Peter, 1999; Wirbelauer *et al*, 2000). Consistent with this, we and others have not been able to isolate a complex containing both wild-type Elongin A and Cul5 by immunoprecipitation from mammalian cell extracts (Kamura *et al*, 2004; Mahrour *et al*, 2008; and data not shown). In addition, the result of our immunofluorescence analysis shows that Elongin A and Cul5 colocalize in cells in a way that is significantly enhanced by UV-irradiation. The transient nature of the colocalization suggests that the Elongin A ubiquitin ligase complex would be present only temporarily in cells. It is, thus, tempting to speculate that DNA damage may trigger the recruitment of the Cul5/Rbx2 module to the Elongin A–Elongin BC complex to assemble an Elongin A ubiquitin ligase, thereby enabling transcribing pol II to be targeted for ubiquitylation, although the exact manner in which this occurs remains unknown.

Role of pol II ubiquitylation by the Elongin A ubiquitin ligase

Although ubiquitylation of pol II was originally uncovered as a response to DNA damage (Bregman *et al*, 1996; Ratner *et al*, 1998), it turned out to be rather a frequent event during transcription and also occurs in the absence of DNA damage; indeed, pol II ubiquitylation is now thought to be a general response to arrested elongation complexes (Lee *et al*, 2002; Somesh *et al*, 2005, 2007; Svejstrup, 2007). Evidence suggests that the Elongin A–Elongin BC complex has a function as a transcription elongation factor that decreases the frequency and/or duration of the transient pausing by pol II (Bradsher *et al*, 1993; Aso *et al*, 1995). We suggest that once Elongin A–Elongin BC encounters an arrested pol II, a Cul5/Rbx2 module may be recruited to assemble an Elongin A ubiquitin ligase, which then exerts its function to remove arrested pol II from the DNA by ubiquitylation and proteasome-mediated degradation, thereby enabling the next pol II to transcribe the gene. If the obstacle is a DNA lesion, this will also allow the DNA repair machinery access to the damage. Our results, therefore, suggest that mammalian Elongin A contributes to increasing the overall efficiency of transcript elongation in cells by at least two mechanisms—(i) by preventing transient pausing of pol II as a transcription elongation factor and (ii) by removing arrested pol II by ubiquitylation and degradation as a component of an E3 ubiquitin ligase.

Materials and methods

Cell culture

Mouse Elongin A containing an N-terminal Flag tag was amplified from pcDNA3.1-Flag-Elongin A (Tamura *et al*, 2003) by PCR with primers containing *Bam*HI and *Not*I restriction sites and subcloned into pCAG-IPG vector (provided by Dr H Niwa). Elongin $A^{-/-}$ ES cells were generated from CCE ES cells as described earlier (Yamazaki *et al*, 2003). Elongin $A^{+/+}$ and Elongin $A^{-/-}$ + Elongin A ES cell clones were generated by transfections of pCAG-IPG empty vector into CCE ES cells and pCAG-IPG-Flag-Elongin A into Elongin $A^{-/-}$ ES cells, respectively. ES cells were cultured on gelatin-coated dishes in the absence of feeder cells in DMEM (Sigma) supplemented with 20% fetal bovine serum (FBS), glutamine, non-essential amino acids, antibiotics, 100 μM β-mercaptoethanol and recombinant LIF, as described earlier (Yamazaki *et al*, 2003). HeLa cells were maintained in DMEM containing 10% FBS. Cells were collected and lysed in ice-cold buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM DTT, 0.5% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, and the Complete Protease Inhibitor Cocktail (Roche Applied Science), and centrifuged at 10 000 *g* for 20 min at 4°C. For UV-irradiation studies, cells were washed with phosphate-buffered saline (PBS), irradiated with UV light (254 nm; UVP Inc.) at the indicated doses, and grown in fresh medium for various times. Cisplatin treatment (20 μg/ml) was for 3 h at 37°C. Where indicated, 50 μM H7, 50 μM DRB, 5 μM MG132 or 25 μg/ml cycloheximide was added to cells 1 h before UV or cisplatin treatment.

Expression of recombinant proteins in Escherichia coli

Human Ubc5a (hUbc5a) with an N-terminal 6-histidine tag and a C-terminal FLAG tag was expressed in *E. coli* strain BL21(DE3) and purified by Ni^{2+}–agarose chromatography (Kamura *et al*, 2000). After dialysis against 40 mM Hepes–NaOH (pH 7.9), 60 mM potassium acetate, 2 mM DTT, 5 mM $MgCl_2$, 0.5 mM EDTA, and 10% (vol/vol) glycerol, proteins were stored at −80°C.

Expression of recombinant proteins in Sf9 insect cells

Rat wild-type Elongin A and a series of N-terminal, C-terminal, and internal deletion mutants and a double point mutant of Elongin A, containing 3 × Flag tags at their C-termini were subcloned into pBacPAK-His1. Human Cul5 containing an N-terminal HA tag was subcloned into pBacPAK-His2. Mouse Rbx2 containing an N-terminal 3 × myc tag was subcloned into pBacPAK8. Recombinant baculoviruses were generated with the BacPAK baculovirus expression system (Clontech). Baculoviruses encoding rat Elongin B, rat Elongin C, mouse Rbx1 containing an N-terminal myc tag, human Cul1, Cul2, and Cul3, and mouse Cul4 containing N-terminal HA tags were described earlier (Kamura *et al*, 1999, 2001; Aso *et al*, 2000). Baculovirus encoding *Arapidopsis thaliana* E1 with an N-terminal 6-histidine tag was provided by Dr W Krek.

Sf9 cells were cultured at 27°C in Grace's insect medium (Gibco) with 10% FBS and kanamycin (100 μg/ml) and infected with the recombinant baculoviruses indicated in the figures. Seventy-two hours after infection, cells were collected and lysed in ice-cold buffer containing 40 mM Hepes–NaOH (pH 7.9), 150 mM NaCl, 1 mM DTT, 0.5% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, and the Complete Protease Inhibitor Cocktail, and centrifuged at 10 000 *g* for 20 min at 4°C.

Immunoprecipitation and western blotting

Anti-myc (9E10) and anti-HA (12CA5) monoclonal antibodies (Roche Applied Science), anti-Flag (M2) monoclonal antibody (Sigma), anti-RNA polymerase II monoclonal antibodies (8WG16, H14, and H5; Covance) and polyclonal antibody (N20; Santa Cruz), anti-Cul5 antibody (Santa Cruz), anti-Elongin A antibody (Yasukawa *et al*, 2007), anti-Elongin B antibody (Garrett *et al*, 1995), and anti-Elongin C monoclonal antibody (Transduction Laboratories) were used in immunoprecipitations and immunoblotting. Lysates of ES cells or baculovirus-infected Sf9 cells were incubated with the antibodies indicated in the figures for 1 h at 4°C and then with protein A-Sepharose (GE Healthcare) for 1 h, or incubated with anti-FLAG M2-conjugated agarose beads (Sigma) for 2 h at 4°C. Beads were washed four times in buffer containing 40 mM Hepes–NaOH (pH 7.9), 150 mM NaCl, 1 mM DTT, 0.5% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, and once in buffer containing 40 mM Hepes–NaOH (pH 7.9), 150 mM NaCl, 1 mM DTT, and 10% (vol/vol) glycerol. Immunoprecipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS–PAGE), transferred to a polyvinylidene difluoride membrane (Millipore), and analyzed by immunoblotting with the antibodies indicated in the figures. Immunoblots were visualized with either Western Lightning (Perkin Elmer) or SuperSignal West Femto chemiluminescent reagent (Pierce) according to the manufacturer's instructions.

Assay of ubiquitylation in vitro

RNA polymerase II was purified as described from rat liver nuclear extracts (Conaway and Conaway, 1990). To assay immunoprecipitated Elongin A complexes for their ability to activate Rpb1 ubiquitylation, Sf9 cells infected with the baculoviruses indicated in the figures were lysed as described above. After centrifugation at 10 000 *g* for 20 min at 4°C, the supernatants were immunoprecipitated with 2 μg of anti-HA (12CA5) antibody and 10 μl of protein A-Sepharose. The beads were mixed with ∼100 ng of E1, ∼100 ng of hUbc5a, 5 μg of bovine ubiquitin (Sigma), 0.5 μg of ubiquitin aldehyde (Boston Biochem), and an aliquot of purified RNA polymerase II in a 20-μl reaction containing 20 mM Tris–HCl (pH 7.9), 2 mM DTT, 5 mM $MgCl_2$, 1.5 mM ATP, 10 mM creatine phosphate, 10 μg of creatine phosphokinase, and 5 mM NaF. Reaction mixtures were incubated for 30 min at 30°C. Reaction products were subjected to 6% SDS–PAGE and analyzed by western blotting with H14 antibody.

Immunofluorescence studies

HeLa cells grown in 4-well chamber slides were UV-irradiated (10 J/m^2) and incubated for the time indicated in the figures. Cells were washed in ice-cold PBS and fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. Cells were permeabilized in PBS containing 0.5% Triton X-100 for 20 min at room temperature and blocked in PBS containing 3% BSA for 1 h at room temperature. Cells were incubated with primary antibodies at appropriate dilutions for 2 h at room temperature, followed by incubation with appropriate secondary antibodies at room temperature for 2 h. To examine colocalization of Elongin A and Cul5, Elongin A was detected using anti-Elongin A mouse monoclonal antibody and Alexa Fluor 488-conjugated anti-mouse IgG antibody (Molecular Probes), whereas Cul5 was detected using anti-Cul5 rabbit polyclonal antibody (Santa Cruz) and Alexa Fluor 594-conjugated anti-rabbit IgG antibody (Molecular Probes). To examine colocalization of Elongin A and Cul2, Elongin A was detected using anti-Elongin A rabbit polyclonal antibody and Alexa Fluor 594-conjugated anti-rabbit IgG antibody, whereas Cul2 was detected using anti-Cul2 mouse monoclonal antibody (Transduction Laboratories) and Alexa Fluor 488-conjugated anti-mouse IgG antibody. For staining DNA, cells were treated with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) from Sigma. Slides were mounted in SlowFade (Molecular Probes), and examined under an Olympus FV300 confocal laser-scanning microscope. Multiple images containing several cells were collected and representative cells are shown. All images were digitally processed for presentation using Adobe Photoshop.

Isolation of MEFs

Primary MEFs were isolated from 13.5-day-postcoitum wild-type embryos and cultured as described earlier (Miyata *et al*, 2007). Only nonsenescent MEFs (passages 1–3) were used for experiments.

RNAi

The mouse U6 gene promoter, followed by DNA corresponding to an shRNA sequence was subcloned into the *Not*I and *Xho*I sites of pMX–puro II, yielding pMX–puro II-U6/siRNA, as described earlier (Kamura *et al*, 2004). The DNA for the shRNA encoded a 21-nucleotide hairpin sequence specific to the mRNA target, with a loop sequence (-TTCAAGAGA-) separating the two complementary domains, and contained a tract of five T nucleotides to terminate transcription. The hairpin sequences specific for mouse Elongin A (EloA-1, EloA-2), for human Cul5 (Cul5-1, Cul5-2), and for EGFP (Clontech) mRNAs corresponded to nucleotides 1115–1135 (EloA-1), 2081–2101 (EloA-2), 1637–1657 (Cul5-1), 2202–2222 (Cul5-2), and 126–146 (EGFP) of the respective coding regions. The resulting vectors were used to transfect Plat E cells and thereby to generate recombinant retroviruses. HeLa cells stably expressing mouse ecotropic retrovirus receptor or MEFs were infected with the recombinant retroviruses. After selection, in a medium containing puromycin (10 μg/ml), cells were UV-irradiated (10 J/m^2) and incubated for the time indicated in the figure, and then harvested and subjected immunoblotting.

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