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Damage-Induced Ubiquitylation of Human RNA Polymerase II by the Ubiquitin Ligase Nedd4, but Not Cockayne Syndrome Proteins or BRCA1

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SUMMARY

UV-induced RNA polymerase II (RNAPII) ubiquitylation and degradation are important DNA damage responses, conserved from yeast to man. However, the identity of the human enzymes that mediate these responses has been unclear. Previously, Cockayne syndrome proteins and BRCA1 were implicated in the process. Surprisingly, using a recently developed assay system, we found that these factors are not directly involved in RNAPII ubiquitylation. The defects in RNAPII ubiquitylation observed in CS cells are caused by an indirect mechanism: these cells shut down transcription in response to DNA damage, effectively depleting the substrate for ubiquitylation, namely elongating RNAPII. Instead, we identified Nedd4 as an E3 that associates with and ubiquitylates RNAPII in response to UV-induced DNA damage in human cells. Nedd4-dependent RNAPII ubiquitylation could also be reconstituted with highly purified proteins. Together, our results indicate that transcriptional arrest at DNA lesions triggers Nedd4 recruitment and RNAPII ubiquitylation.

INTRODUCTION

Eukaryotic cells make use of multiple, highly conserved pathways to contend with UV-induced DNA damage. One important response mechanism is transcription-coupled repair (TCR), during which DNA lesions in the transcribed strand of an active gene are repaired much faster than in the genome overall (Hanawalt, 2001; Svejstrup, 2002; Laine and Egly, 2006). Patients suffering from the severe human disorder Cockayne syndrome (CS) have defective TCR due to mutations in either the CSA or the CSB gene. In response to DNA damage, RNAPII becomes ubiquitylated and degraded. Because cell lines lacking CSA or CSB were found to be defective for RNAPII ubiquitylation, it was proposed that TCR might involve RNAPII ubiquitylation/degradation (Bregman et al., 1996; Ratner et al., 1998). However, experiments in yeast have indicated that degradation of RNAPII is not required for TCR but is rather an alternative to it and that, in the absence of both pathways, cells become extraordinarily sensitive to DNA damage (Woudstra et al., 2002; Somesh et al., 2005, 2007; Lommel et al., 2000). Together, the evidence accumulated to date in yeast supports a model in which RNAPII progression is obstructed by DNA damage. Damage-stalled RNAPII then first triggers TCR, or—as a last resort—the polymerase becomes ubiquitylated and degraded if repair of the lesion by TCR is not possible. The damage may then be removed by other, slower repair pathways (Svejstrup, 2002, 2007). Whether a similar mechanism operates in human cells has remained unclear.

CSB encodes a Swi/Snf-like DNA-dependent ATPase (Troelstra et al., 1992; Selby and Sancar, 1997; Citterio et al., 2000), whereas CSA is part of a multisubunit ubiquitin ligase complex, containing Cullin 4A, Roc1, and DDB1 (Groisman et al., 2003). The association of CSA with an ubiquitin ligase activity (E3) might potentially explain the observed requirement for this protein in RNAPII ubiquitylation, and a current model indeed incorporates the CSA complex as the E3 in this modification pathway (Laine and Egly, 2006). However, the E3 for RNAPII ubiquitylation in yeast is the *h*omologous to the *E*6-AP *c*arboxyl *t*erminus (HECT) ubiquitin ligase Rsp5 (Beaudenon et al., 1999; Reid and Svejstrup, 2004; Somesh et al., 2005), which has several human homologs (Ingham et al., 2004). Moreover, it has also been suggested that BRCA1 is involved in damage-induced RNAPII ubiquitylation in human cells (Starita et al., 2005; Kleiman et al., 2005).

We investigated the role of CSA, CSB, BRCA1, and several Rsp5 homologs in ubiquitylation and degradation of RNA polymerase II in human cells. Here we show that neither CSA nor CSB or BRCA1 is directly involved in RNAPII ubiquitylation and provide evidence to explain previous results pointing to a role for CSA and CSB in this pathway. We also find that Nedd4—but not five other Rsp5 homologs—is involved in ubiquitylation of human RNAPII, in vitro and in vivo.

RESULTS

Experiments in yeast have shown that transcriptional arrest results in ubiquitylation of RNAPII (Somesh et al.,

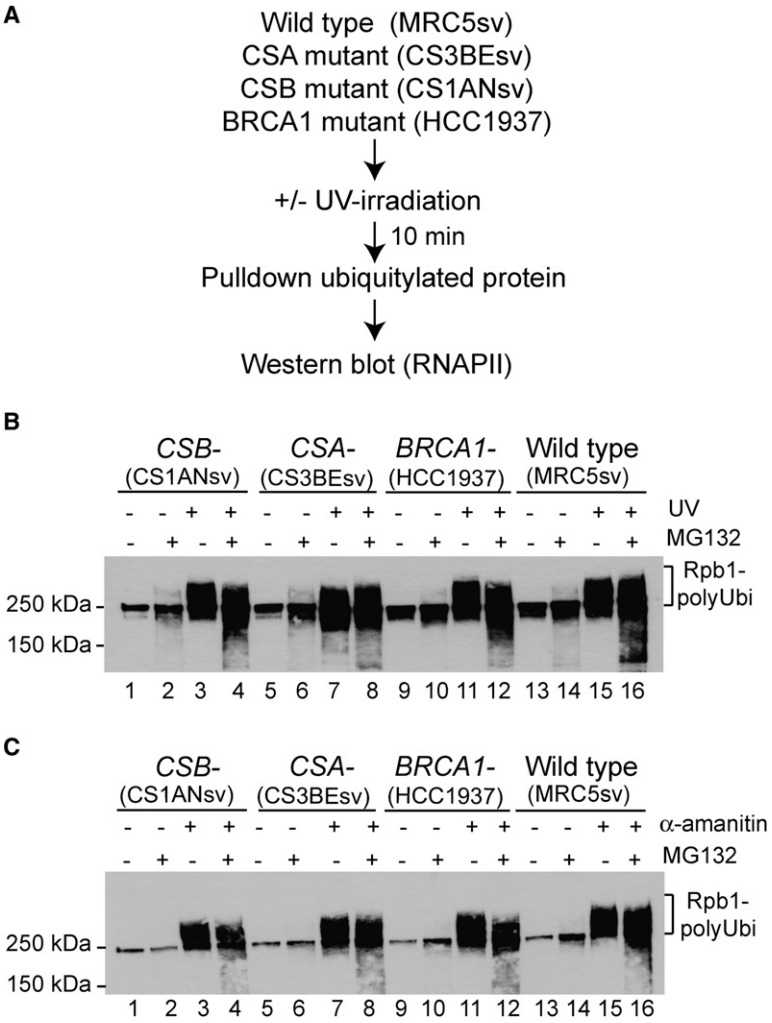

Figure 1. UV Irradiation and α-Amanitin-Induced Transcriptional Arrest Result in Ubiquitylation of RNAPII in Human Cells In Vivo

(A) Outline of the experimental procedure.

(B) Ubiquitylated proteins, isolated by Dsk2p-affinity chromatography, from extracts of wild-type, BRCA1 mutant (*BRCA1*−), CSA mutant (*CSA*−), or CSB mutant (*CSB*−) cells irradiated, (+) or not irradiated (−), with UV light were immunoblotted and probed with anti-Rpb1 antibody (4H8).

(C) As in (B), but extract treated (+), or not treated (−), with α-amanitin. MG132, proteasome inhibitor.

2005). In human cells, UV irradiation is known to cause ubiquitylation of the largest subunit (Rpb1) of RNAPII (Bregman et al., 1996). To examine whether ubiquitylation also occurs as a direct result of RNAPII transcriptional arrest in higher cells, we examined the effect of α-amanitin. Binding of α-amanitin to RNAPII permits nucleotide entry to the active site and one round of RNA synthesis but prevents the translocation of DNA and RNA needed to empty the site for the next round of synthesis (Bushnell et al., 2002). It is therefore an extremely specific inhibitor of transcript elongation. To isolate ubiquitylated proteins from crude extracts in these experiments, we took advantage of the properties of the yeast Dsk2 protein, which has a UBA domain that binds strongly to mono- and polyubiquitylated proteins (Funakoshi et al., 2002). MRC5 fibroblast cells were exposed to UV irradiation or treated with α-amanitin, and sonicated whole-cell extracts were incubated with immobilized GST-Dsk2p to affinity purify ubiquitylated proteins. These were then immunoblotted with Rpb1-specific antibody to detect ubiquitylated RNAPII (Figure 1A). In agreement with earlier reports (Bregman et al., 1996; Ratner et al., 1998; Luo et al., 2001), UV irradiation resulted in significant enrichment of slowly migrating (polyubiquitylated) Rpb1 after incubation with the Dsk2-affinity resin (Figure 1B, lane 15). This slowly migrating Rpb1 was indeed polyubiquitylated, because incubation of resin-bound proteins with ubiquitin protease led to disappearance of these forms and significant dissociation of the polymerase (Figure S1 in the Supplemental Data available with this article online). Western blots probed with phosphorylation-specific (H5 and H14) antibody indicated that the ubiquitylated polymerase was also phosphorylated (data not shown), as previously reported (Bregman et al., 1996).

Interestingly, α-amanitin treatment induced an RNAPII ubiqutylation pattern that was virtually indistinguishable from that triggered by UV-irradiation (Figure 1C, lane 15), indicating that damage-independent transcriptional arrest indeed also triggers RNAPII ubiquitylation in human cells. Others have previously reported that α-amanitin treatment of murine and human fibroblasts results in the disappearance of Rpb1 (Clark et al., 1991; Nguyen et al.,

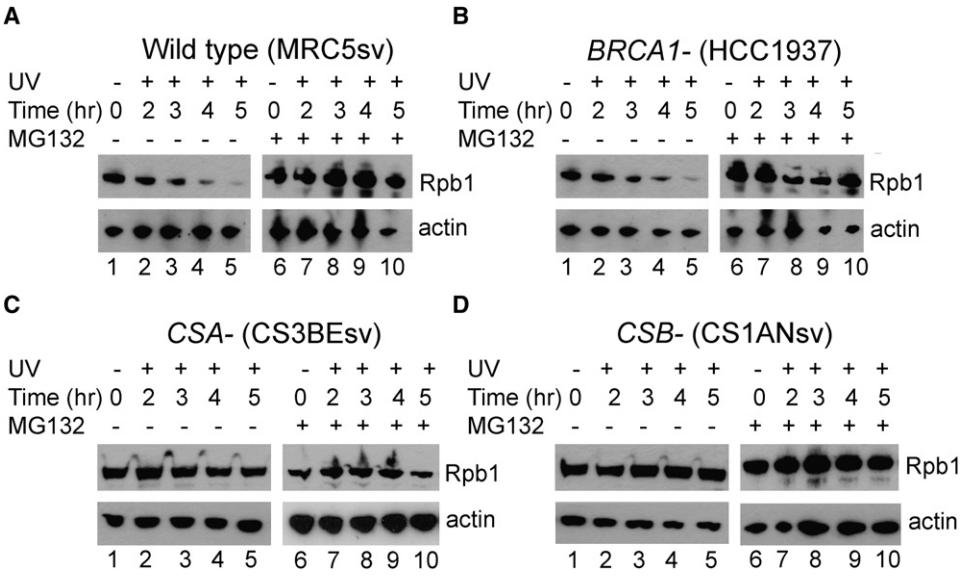

Figure 2. UV-Dependent Degradation of RNAPII

The amount of RNAPII in wild-type (A), BRCA1 mutant (B), CSA mutant (C), and CSB mutant cells (D) at different times after UV irradiation (30 J/m^2) in the presence (+) or absence (−) of proteasome inhibitor MG132 was investigated by western blotting, probing with anti-Rpb1 antibodies. β-actin (actin) was used as loading control.

1996). Our results indicate that this occurs via ubiquitylation (Figure 1C) and degradation by the proteasome (Figure S2).

CS Proteins and BRCA1 Are Dispensable for RNAPII Ubiquitylation

Although UV-induced RNAPII ubiquitylation was first reported more than 10 years ago (Bregman et al., 1996), the identity of the factor responsible has remained unclear. We tested the involvement of CSA, CSB, and BRCA1 with the new assay system. For this purpose, we used the CSA mutant cell line CS3BEsv and the CSB mutant cell line CS1ANsv (Cleaver et al., 1999). Breast cancer cell line HCC1937, in which ubiquitylation of known BRCA1 targets is abolished (Tomlinson et al., 1998), was used to examine the role of BRCA1. Surprisingly, when wild-type, CSB, CSA, and BRCA1 mutant cells were exposed to UV irradiation, a comparable amount of ubiquitylated RNAPII was observed (Figure 1B, compare lanes 1 and 3, 5 and 7, and 9 and 11 with 13 and 15). This result indicates that CS proteins and BRCA1 are not required for damage-induced ubiquitylation of RNAPII. Moreover, α-amanitin also induced RNAPII ubiquitylation normally in the mutant cells (Figure 1C, compare lanes 1 and 3, 5 and 7, and 9 and 11 with 13 and 15), suggesting that DNA damage-independent RNAPII ubiquitylation occurs in the absence of functional CS proteins and BRCA1 as well.

We next compared DNA damage-dependent degradation of Rpb1 in wild-type and mutant cells. The different cell lines were subjected to UV irradiation in the presence of the protein synthesis inhibitor cycloheximide. Whole-cell extracts were prepared at different time points after irradiation, and these were subjected to immunoblot analysis. As shown in Figures 2A and 2B (lanes 1–5), the level of Rpb1 (but not actin) decreased similarly over time in wild-type and BRCA1 mutant cells, indicating that BRCA1 is not required for damage-induced degradation of Rpb1. As expected, the Rpb1 decrease was due to proteasomal degradation, because inclusion of the proteasome inhibitor MG132 stabilized it (Figures 2A and 2B, lanes 6–10). Interestingly, in apparent contradiction of our previous ubiquitylation experiments, degradation was clearly inhibited in CSA and CSB mutant cells. These cells were severely defective for degradation of RNAPII in response to UV irradiation (Figures 2C and 2D).

To investigate this conundrum further, the level of ubiquitylated RNAPII at different times after UV irradiation in wild-type and CS cells was compared. As shown in Figure 3A, RNAPII was ubiquitylated immediately after UV irradiation, and the ubiquitylation level slowly decreased over 4 hr but was clearly detectable even after 3 hr in wild-type cells. Please note that only a small fraction of cellular RNAPII is ubiquitylated at any given time. Upon ubiquitylation, RNAPII is rapidly degraded. Thus, it is not the same polymerases that are ubiquitylated after, for example, 10 and 180 min.

The ubiquitylation profile was markedly different from WT in CS cells (Figures 3B and 3C). Here, ubiquitylation occurred normally immediately after UV (compare lanes 2, 3, and 4 in Figures 3A–3C) but disappeared rapidly and was undetectable already after 80 min (compare lanes 5–8 in Figures 3A–3C). The profile of RNAPII ubiquitylation was also tested in mutant cells rescued by expression of the relevant CS protein and was found to be similar to

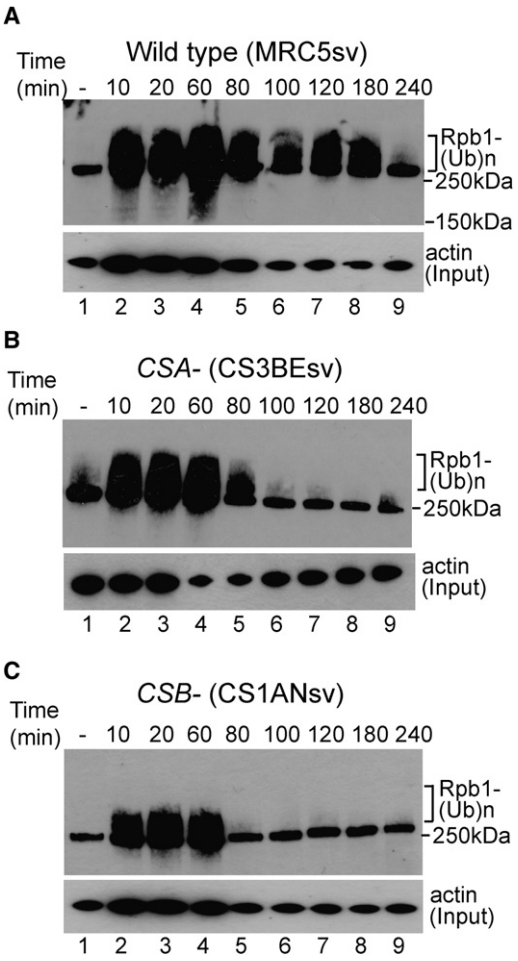

Figure 3. Time Course of UV-Dependent Ubiquitylation of RNAPII

Upper panels, western blots of ubiquitylated RNAPII, isolated by Dsk2p-affinity chromatography, from extracts of wild-type (MRC5sv) (A), CSA mutant (CS3BEsv) (B), or CSB mutant (CS1ANsv) (C) irradiated (+), or not irradiated (–), with UV (30 J/m^2) and probed with anti-Rpb1 antibody at the indicated times after UV irradiation. Lower panels, actin control for amount of extract loaded onto DSK2 resin.

that of wild-type cells (data not shown), indicating that, as expected, the effect was due to the CS proteins.

These results suggest that, although RNAPII undergoes UV-induced ubiquitylation both in wild-type and CS cells, ubiquitylation of RNAPII continues over several hours in wild-type cells, whereas it quickly ceases in CSA and CSB mutant cells. As expected, the disappearance of ubiquitylated Rpb1 was due to its proteasomal degradation, as the level of ubiquitylated polymerase remained constant in the presence of proteasome inhibitor (data not shown).

Ubiquitylation of RNAPII Requires Transcription

The results described above might best be explained if the effect in CS cells were indirect. In this connection, it is important to note that UV irradiation causes global inhibition of transcriptional initiation in mammalian cells (Mayne and Lehmann, 1982; Rockx et al., 2000). However, although transcription inhibition is partial and temporary in wild-type cells (recovering to normal levels within the first few hours), transcription remains suppressed for more than 18 hr in CS cells, and the extent of inhibition is also much more dramatic in these cells (Rockx et al., 2000; Proietti-De-Santis et al., 2006; Figure S3). Moreover, RNAPII ubiquitylation is directly triggered by arrest of elongating polymerase (Figure 1C; Somesh et al., 2005; Lee et al., 2002). These observations could potentially explain why RNAPII ubiquitylation is similar in wild-type and CS cells immediately after UV irradiation (where the level of transcription is similar), whereas there is much less RNAPII ubiquitylation at later time points in CS cells because transcription in these cells ceases. In wild-type cells, there is continuous transcription (although temporarily at lower levels) and therefore arrest of polymerase at unrepaired DNA lesions, resulting in unbroken cycles of ubiquitylation and degradation at DNA lesions, reducing RNAPII levels over the first hours after irradiation in these cells.

In order to test this hypothesis, we sought to artificially suppress transcription initiation in wild-type cells, thereby mimicking one specific effect of CS mutation after DNA damage. Others have previously shown that significant inhibition of transcription can be achieved by RNAi-mediated knockdown of general initiation factor TFIIB, if a dominant-negative TFIIB mutant (which is not targeted by RNAi) is simultaneously expressed (Elsby et al., 2006). We established TFIIB "activity knockdown" in HEK293 cells that was similar to that obtained previously (Figure 4A; see Elsby et al. [2006] for details). When these cells were UV irradiated, much less ubiquitylated RNAPII was detected immediately after UV irradiation, and—as previously observed in CS cells—the ubiquitylated polymerase persisted for a shorter time than in wild-type cells (Figure 4B, compare lanes 2–4 with 6–8). This indicates that active transcription is required for RNAPII ubiquitylation, and supports the model that elongating polymerase is the target of the ubiquitylation machinery.

In order to further investigate whether transcription inhibition causes the defect in RNAPII degradation observed in CS cells, wild-type and CSB mutant cells were treated with α-amanitin (for 2 hr) to induce ubiquitylation of arrested RNAPII 3 hr after subjecting the cells to UV irradiation. In controls, in the absence of UV irradiation, α-amanitin treatment resulted in ubiquitylation in both wild-type and CSB mutant cells (Figures 4C and 4D, lane 8), in agreement with our previous results (Figure 1B). In wild-type cells, α-amanitin also elicited RNAPII ubiquitylation when added 3 hr after UV irradiation, showing that efficient transcription occurred at this point so that RNAPII was present on genes to be targeted for ubiquitylation by the drug. In striking contrast, no α-amanitin-induced ubiquitylation occurred after UV irradiation in CSB mutant cells (compare lane 6 in Figures 4C and 4D). Similar results were obtained in CSA mutant cells (data not shown). When wild-type CSB was ectopically expressed in CSB

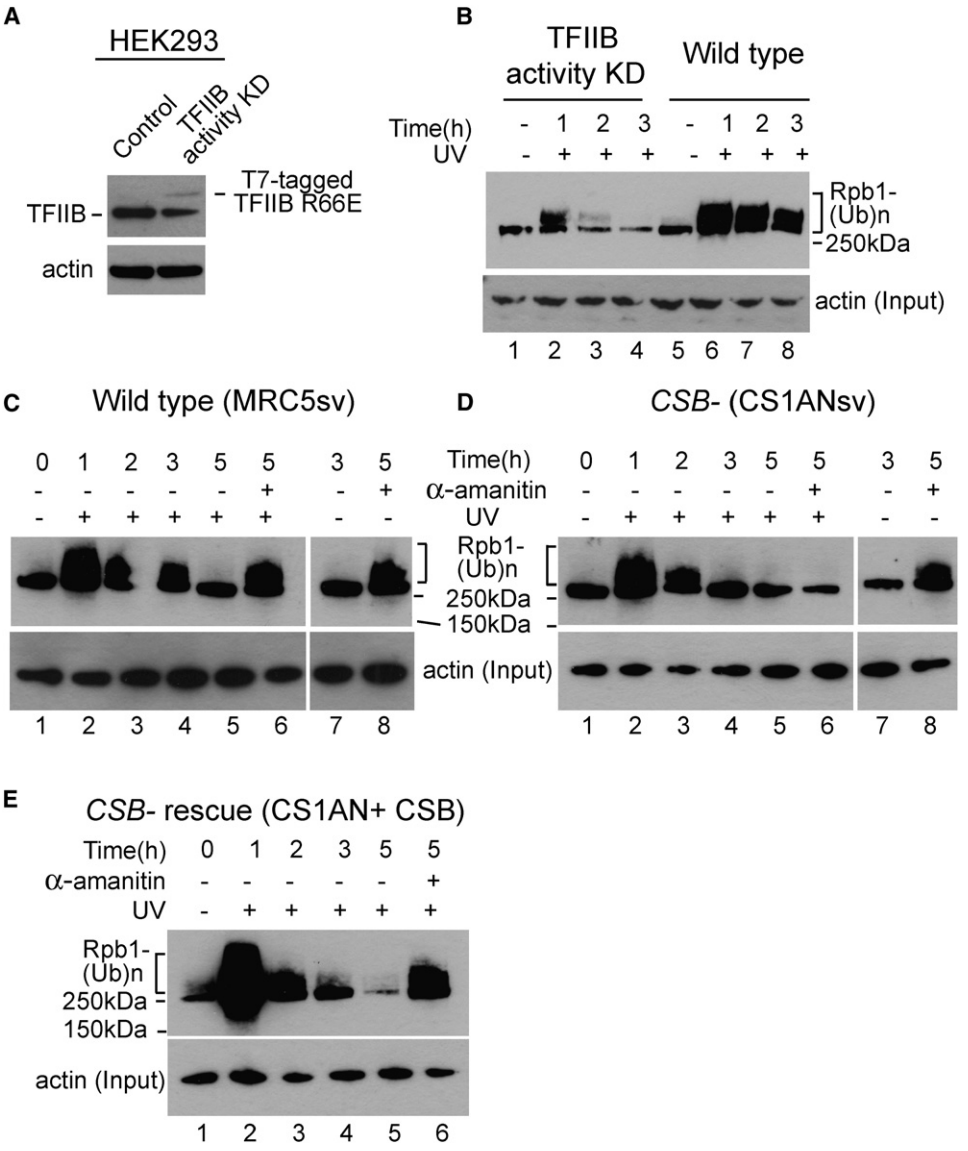

Figure 4. Effect of Transcription Initiation Inhibition on UV-Dependent Ubiquitylation of RNAPII

(A) TFIIB knockdown in HEK293 cells. Cells were cotransfected with plasmid expressing mutant TFIIB and a TFIIB siRNA expressing plasmid (Elsby et al., 2006). Ninety-six hours after transfection, whole-cell extracts were analyzed by immunoblotting with anti-TFIIB antibody. β-actin (actin) was used as loading control. Please note that, as observed and discussed by Elsby et al., TFIIB can only be knocked down to a certain extent, necessitating coexpression of a dominant-negative TFIIB mutant to achieve efficient inhibition of transcription.

(B) Effect of TFIIB activity knockdown on ubiquitylation of RNAPII. Upper panel, western blot of ubiquitylated RNAPII isolated 10 min after irradiation by Dsk2-affinity chromatography from extracts of HEK293 cells, transiently transfected either with specific anti-TFIIB plasmids (TFIIB KD) or control plasmids (wild-type), treated (+) or not treated (−) with UV (30 J/m^2), and probed with anti-Rpb1 antibody to detect RNAPII at different times after irradiation. Lower panel, actin control for equal loading of extract onto DSK2 resin.

(C–E) Effect of α-amanitin treatment on RNAPII ubiquitylation in UV-irradiated cells. Wild-type (MRC5sv) (C), CSB mutant (CS1ANsv) (D), and rescued CSB (CS1ANsv+tagged CSB) cells (E) were UV irradiated (30 J/m^2) and allowed to recover for 3 hr before treatment with (or without) α-amanitin for 2 hr. Ubiquitylated proteins were isolated by Dsk2-affinity chromatography and immunoblotted with anti-Rpb1 antibody (upper panels). In control experiment (small panels in [C] and [D]), nonirradiated cells were α-amanitin treated as a control. Lower panels, actin control for amount of extract loaded onto DSK2 resin. Time (h) indicates time after irradiation.

mutant cells, ubiquitylation of RNAPII was restored (Figure 4E, lane 6), indicating that, as expected, the defect observed in CSB mutant cells was indeed due to the absence of the CSB protein.

These data provide additional evidence for the conclusion that the defect in damage-induced RNAPII ubiquitylation and degradation in CS cells is indirect: it is caused by the UV-induced shutdown of transcription in these cells.

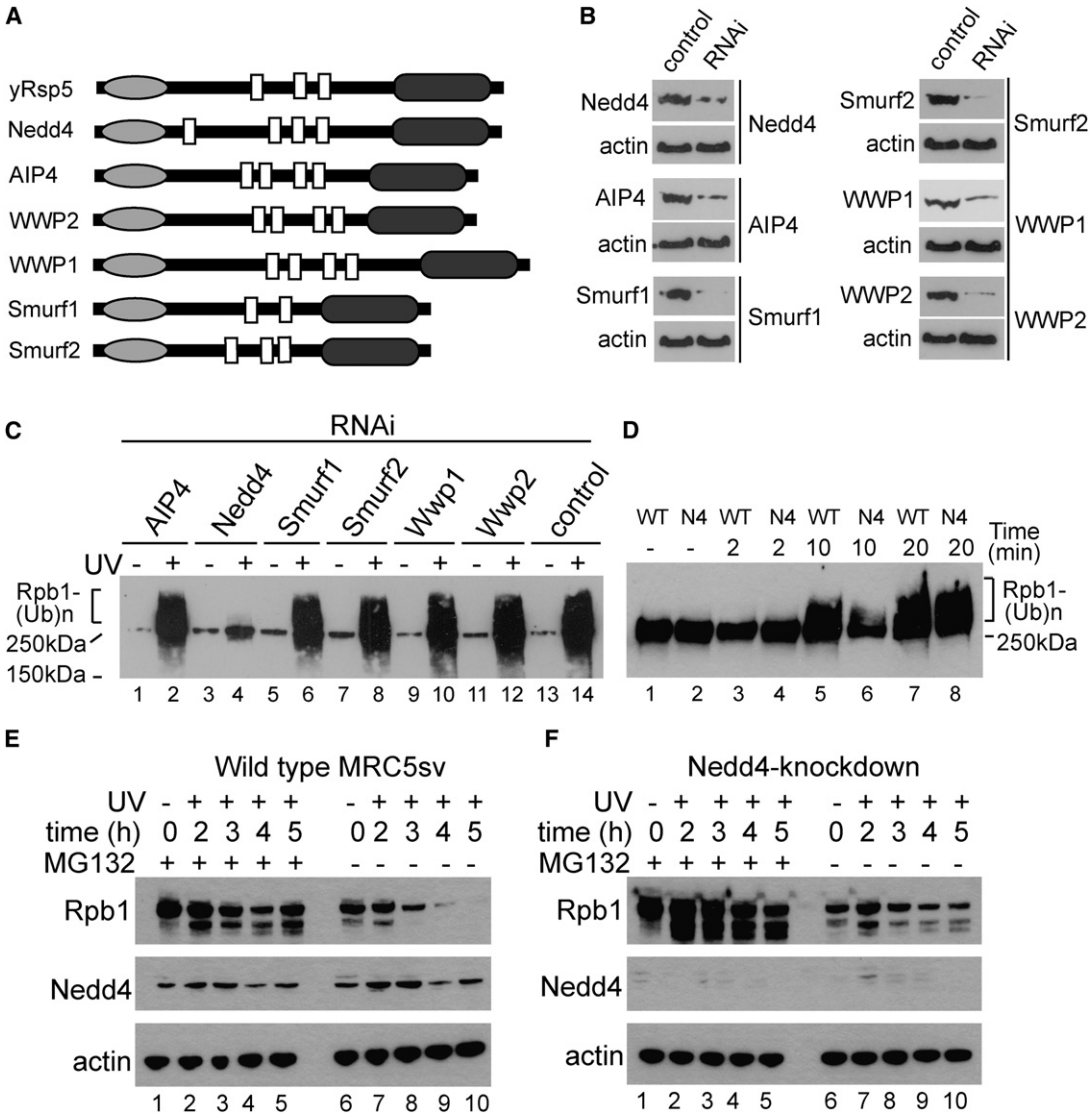

Figure 5. Knockdown of Rsp5 Homologs by RNAi

(A) Human Rsp5 homologs. The positions of the C2 domain (light gray sphere), WW domain (white rectangles), and the HECT domain (dark gray spheres) are shown (Ingham et al., 2004).

(B) Effect of siRNA knockdown on endogenous levels of Rsp5 homologs. HEK293 cells were transfected with the indicated relevant siRNA. Level of endogenous protein was analyzed in whole-cell extract by immunoblotting with the antibodies indicated on the left.

(C) Effect of RNAi-mediated knockdown on ubiquitylation of RNAPII. Western blot of ubiquitylated protein isolated by Dsk2-affinity chromatography 10 min after irradiation (30 J/m^2) from extracts of the HEK293 cells transfected with siRNA, irradiated (+) or not irradiated (−) with UV, and probed with anti-Rpb1 antibody.

(D) Western blot of ubiquitylated RNAPII in extracts from wild-type and stable Nedd4 knockdown cells (N4) at different times after UV irradiation, prepared as in (C).

(E and F) Degradation of RNAPII in wild-type (E) and stable Nedd4 knockdown cells (F) after UV irradiation (30 J/m^2) in the presence (+) or absence (−) of the proteasome inhibitor MG132 was investigated by western blotting, probing with anti-Rpb1 antibody. Nedd4 levels were detected by anti-Nedd4 antibody.

Nedd4 Promotes Ubiquitylation and Degradation of RNAPII In Vivo

The data presented above indicate that neither BRCA1 nor CSA or CSB is directly involved in ubiquitylation and degradation of RNAPII. In *Saccharomyces cerevisiae*, the E3 for RNAPII ubiquitylation is Rsp5 (Beaudenon et al., 1999; Somesh et al., 2007; Reid and Svejstrup, 2004). There are six obvious sequence homologs of Rsp5 in human cells with similar size and domain organization: AIP4, Nedd4, Smurf1, Smurf2, WWP1, and WWP2 (Figure 5A

and Ingham et al., 2004). Among these, Nedd4 has the highest homology to Rsp5 (52% identity, 69% similarity).

In order to investigate if any of the Rsp5 homologs are involved in ubiquitylation of RNAPII in vivo, the homologs were knocked down individually by using siRNAs. The effectiveness of RNAi was first assessed by the use of antibodies specific for the individual proteins. Significant RNAi-dependent reductions in protein levels were achieved in all cases (Figure 5B). We next established that RNAi expression did not affect overall cell morphology or cell-growth rates during the course of the experiments (data not shown). These knockdown cells were then UV irradiated, and total ubiquitylated protein was isolated and immunoblotted to detect ubiquitylated RNAPII (Figure 5C). Several independent experiments showed that UV-induced ubiquitylation was reproducibly affected only by Nedd4 siRNA (Figure 5C, compare lane 4 with lane 14 [control] and other knockdowns; data not shown). This indicates that Nedd4 is required for UV-induced ubiquitylation of RNAPII in vivo. The residual RNAPII ubiquitylation activity observed in Nedd4 siRNA cells might either be due to the remaining Nedd4 protein (typically 10%–20% of normal levels) or to a compensating activity of one of the other Rsp5 homologs. To investigate if the function of Nedd4 overlaps with any of the other homologs, the RNAPII ubiquitylation assay was also carried out in cells in which the level of both Nedd4 and one other homolog was reduced by RNAi. None of these combinations reproducibly reduced RNAPII ubiquitylation beyond what was observed with Nedd4 siRNA alone (data not shown), supporting the conclusion that Nedd4 is a major E3 for UV-induced RNAPII ubiquitylation in human cells.

To further investigate the role of Nedd4 in ubiquitylation of RNAPII in vivo, a stable Nedd4 knockdown MRC5 fibroblast cell line was generated, which constitutively reduced the level of Nedd4 significantly (~5-fold; Figure 5F). We reckoned that, in the absence of complete Nedd4 removal, reductions in RNAPII ubiquitylation would only be easily detected immediately after DNA damage but that, because RNAPII ubiquitylation is short lived and proteolysis appears to be limiting for RNAPII turnover during extensive DNA damage (Somesh et al., 2007), the remaining Nedd4 catalytic activity would quickly establish apparently normal levels of modification at steady state. A delay in the ubiquitylation of individual RNAPII molecules in this cell line might then result in a concomitant delay in overall UV-induced RNAPII degradation. Dramatically reduced UV-dependent ubiquitylation was indeed observed in the stable Nedd4 knockdown cells compared to wild-type cells immediately after UV irradiation, but after 20 min, the level of ubiquitylation in the two cell lines was already similar, as expected (Figure 5D, compare lanes 5 and 6, and 7 and 8).

We now investigated the effect of stable Nedd4 knockdown on UV-induced degradation of Rpb1. Wild-type and Nedd4 knockdown cells were subjected to UV irradiation, and Rpb1 levels were checked by immunoblotting. Significantly, the level of Rpb1 consistently decreased less dramatically in the knockdown cells (Figures 5E and 5F, compare lanes 6–10, and data not shown), indicating that Nedd4 is indeed required for UV-induced Rpb1 degradation. Treatment of cells with proteasome inhibitor prevented degradation (Figures 5E and 5F, lanes 1–5).

Taken together, these results indicate that Nedd4, the human homolog of budding yeast Rsp5, is required for normal ubiquitylation and degradation of Rpb1 in response to UV-induced DNA damage in vivo.

Ubiquitylation of RNAPII by Nedd4 In Vitro

We next sought to establish a highly purified ubiquitylation system for human RNAPII to confirm and extend the in vivo findings by investigating whether Nedd4 can directly ubiquitylate the polymerase. In order to reconstitute RNAPII ubiquitylation with purified proteins, the six Rsp5 homologs were cloned, expressed in *E. coli*, and purified (Figure 6A). HECT domain E3 ligases form ubiquitin-thioester chains on themselves in the presence of E1 and E2 enzyme (Wang et al., 1999), and by using this assay, we found that the recombinant E3s were indeed all catalytically active (Figure 6B). To investigate whether they could support human RNAPII ubiquitylation in vitro, highly purified human RNAPII was used as substrate, with human Uba1 and UbcH7 (or UbcH5C) involved as E1 and E2, respectively (Figure 6C, lanes 7–10). In this highly purified, reconstituted human RNAPII ubiquitylation system (see Figure S4 for purity of these proteins), Nedd4 (and Smurf1), but none of the other Rsp5 homologs, supported robust ubiquitylation of RNAPII (Figure 6C, lanes 2 and 3).

The data obtained with highly purified proteins provide biochemical support for the concept that Nedd4 directly ubiquitylates RNAPII in vivo.

Nedd4 Is Recruited to Chromatin-Associated RNAPII after UV-Induced DNA Damage

If Nedd4 were directly involved in RNAPII ubiquitylation as indicated above, it might be expected that it interacts with transcribing RNAPII in a manner that is affected by DNA damage inside cells. The form of RNAPII engaged in active transcription is associated with chromatin, but it can be solubilized by nuclease digestion (Figure 7A). RNAPII from chromatin was immunoprecipitated, and the association of the six Rsp5 homologs was tested by western blotting (Figures 7B–7G). Nedd4, but none of the other Rsp5 homologs, coimmunoprecipitated with RNAPII from chromatin, and importantly, more of this protein was associated with the polymerase after UV irradiation (Figures 7B–7G, compare lanes 1 and 2 with 5 and 6). Together, these data indicate that Nedd4 associates with RNAPII in chromatin, enabling its ubiquitylation upon UV-damage-induced transcriptional arrest.

DISCUSSION

Considerable effort has been invested in understanding the transcription and repair responses elicited by UV-induced DNA damage in human cells. However, a detailed

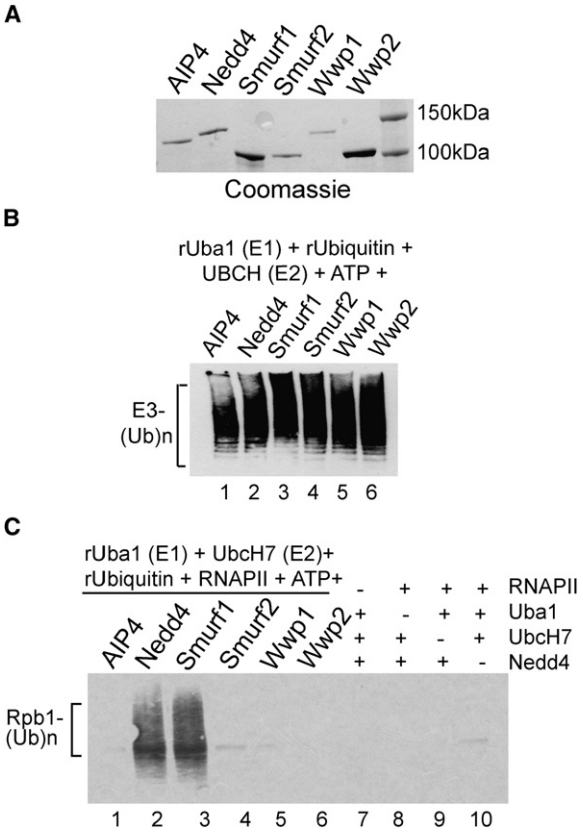

Figure 6. Reconstitution of Human RNAPII Ubiquitylation with Purified Proteins

(A) Recombinant E3 ligases employed in conjunction with Uba1, UbcH7, and human RNAPII (Figure S4) to reconstitute human RNAPII ubiquitylation with myc-tagged ubiquitin.

(B) Activity of the different E3 ligases, in the presence of E1 and E2.

(C) RNAPII ubiquitylation in vitro detected by probing western blots with 9E11 antibody (Somesh et al., 2005). When E1 (Uba1), E2 (UbcH7), E3 (Nedd4), or RNAPII was omitted, no RNAPII ubiquitylation was observed (lanes 7–10). For simplicity, only RNAPII ubiquitylation using UbcH7 is shown. However, AIP4, Smurf2, WWP1, and WWP2 also failed to ubiquitylate RNAPII with UbcH5C (data not shown). A larger concentration of E3 was required to detect self-ubiquitylation (B), explaining the absence of background signal during RNAPII ubiquitylation (C).

understanding of important issues such as the mechanism and factors governing RNAPII ubiquitylation/degradation and the role of CS proteins in this damage response has been lacking. Here, we addressed these issues and reached several conclusions. First, ubiquitylation/degradation of RNAPII in human cells is not a damage response per se, but rather a consequence of RNAPII progression being blocked by lesions during transcript elongation. So, if there is no transcription, there is little or no RNAPII ubiquitylation. Second, neither CSA, CSB, nor BRCA1 plays a direct role in RNAPII ubiquitylation, in contrast to what had been suggested by previous studies. The effect of CS mutation on RNAPII ubiquitylation is indirect and is brought about via the dramatic decrease in general transcription after DNA damage in these cells. Third, the homolog of yeast Rsp5, Nedd4, is required for RNAPII ubiquitylation in human cells, whereas we failed to find evidence for other Rsp5 homologs playing a role. As expected from this finding, Nedd4 associates with RNAPII in chromatin and this interaction is increased after DNA damage.

Blocks to Transcription Elicit RNAPII Ubiquitylation and Degradation

When RNAPII ubiquitylation was first reported more than a decade ago (Bregman et al., 1996), it was thought of as a specific response to UV-induced DNA damage. Later experiments in vitro, and in vivo (in yeast), have suggested that ubiquitylation is more likely to be a response to RNAPII arrest during transcript elongation (Lee et al., 2002; Somesh et al., 2005, 2007; Svejstrup, 2007). Such arrest would, of course, be much more frequent in the presence of transcription-blocking DNA lesions, providing a likely explanation for the dramatic increase in RNAPII ubiquitylation observed upon UV irradiation. The results attained in human cells reported here corroborate this hypothesis. First, RNAi-mediated knockdown of TFIIB activity (which decreases the level of actively transcribing RNAPII on genes) dramatically decreases UV-induced RNAPII ubiquitylation, showing that UV-induced DNA lesions—or UV-induced DNA damage signaling pathways—are not in themselves enough to trigger RNAPII ubiquitylation. Second, RNAPII ubiquitylation is triggered not only by UV irradiation but also by α-amanitin. This drug specifically targets the elongating form of RNAPII, inhibiting the translocation step of RNA synthesis. In combination with previous results, our results in human cells thus strongly support the idea that blocks to transcript elongation are the actual trigger for RNAPII ubiquitylation.

Neither CS Proteins nor BRCA1 Is Involved in RNAPII Ubiquitylation

A surprising outcome of this study was the finding that neither CSA nor CSB or BRCA1 is involved in RNAPII ubiquitylation. A number of studies on these factors had previously suggested that they were either required for, or somehow directly involved in, RNAPII ubiquitylation (Kleiman et al., 2005; Starita et al., 2005; Ratner et al., 1998; McKay et al., 2001; Bregman et al., 1996). The conclusions reached in previous studies were often based primarily on experiments in which weak smearing above the band representing Rpb1 was taken to represent ubiquitylation, and often only at single time points after UV irradiation. Because of our failure to obtain consistent, dependable results in the different cell lines we were studying by using this measure, we developed a simple, more reliable system to study the kinetics of protein ubiquitylation. This system is based on the enrichment of ubiquitylated proteins via their binding to an UBA domain (GST-Dsk2 resin). The numerous experiments we have performed with the protocols described here failed to support the idea that BRCA1 or CS proteins play a direct role in RNAPII ubiquitylation.

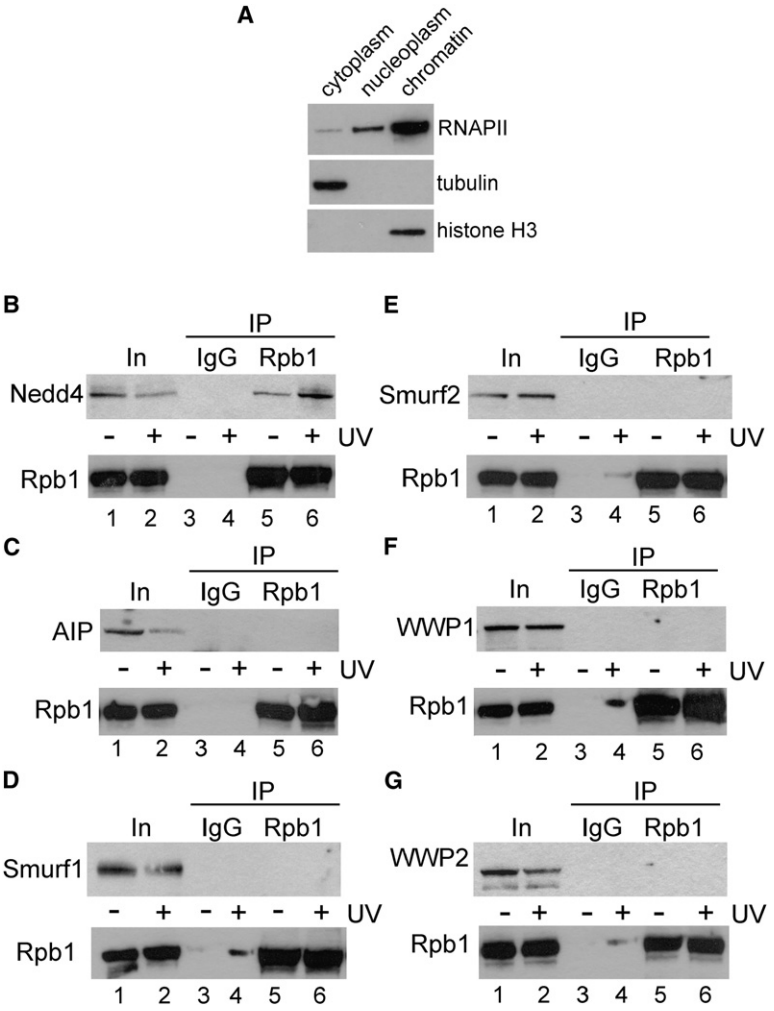

Figure 7. UV-Induced Recruitment of Nedd4 to Chromatin-Associated RNAPII

(A) Subcellular fractionation of proteins into cytoplasm, (chromatin-free) nucleoplasm, and chromatin. Western blots of RNAPII, tubulin (cytoplasmic marker), and histone H3 (chromatin-marker) are shown.

(B) Western blots showing Nedd4 coimmunoprecipitation with RNAPII before and after UV irradiation (30 J/m^2) (lanes 5 and 6). Control IP experiments, performed with IgG beads, are shown in lanes 3 and 4. Benzonase (which degrades both DNA and RNA) was used to release chromatin-associated proteins prior to and during immunoprecipitation, ensuring that the interaction was not indirect via nucleotides.

(C–G) As in (B), but with the other Rsp5 homologs.

We were particularly intrigued by the finding that CS cell lines had normal RNAPII ubiquitylation immediately after UV irradiation but apparently failed to degrade RNAPII. This led us to discover that the effect of CS mutation on RNAPII ubiquitylation and degradation is indirect: these mutant cell lines shut down transcription in response to DNA damage and therefore do not contain the substrate for RNAPII ubiquitylation, namely the transcribing (elongating) form. As mentioned above, we mimicked the shutdown of transcription observed in cells lacking CS proteins by depleting TFIIB activity in otherwise normal cells (Elsby et al., 2006). As a consequence, UV-induced RNAPII ubiquitylation was dramatically reduced. Moreover, we took advantage of the specificity of α-amanitin for the elongating form of RNAPII and its ability to induce RNAPII ubiquitylation in much the same way as UV-induced DNA damage. In wild-type cells where transcription recovers quickly, α-amanitin induced substantial RNAPII ubiquitylation a few hours after UV irradiation. In CS cells, however, it did not. Together, these results indicate that the failure of CS cells to correctly ubiquitylate and degrade RNAPII is due to their failure to restart transcription after DNA damage. These findings have important consequences for the assessment of data obtained on RNAPII ubiquitylation in mutants or under conditions that affect general transcription levels. Indeed, the possibility that BRCA1 might under certain circumstances affect RNAPII ubiquitylation indirectly, for example through an involvement with (Scully et al., 1997), or effect on, the general transcription machinery, merits future investigation.

In a broader perspective, the data presented here indicate that the basic mechanisms underlying TCR and RNAPII ubiquitylation are conserved between yeast and man. Our results thus effectively dispel the idea that CS cells are defective for RNAPII ubiquitylation and that this underlies their failure to support transcription-coupled repair. In support of this conclusion, Nedd4 knockdown cells are not sensitive to UV irradiation (Figure S5), in contrast to CS cells, which are defective for TCR (Venema et al., 1990). In humans as in yeast, TCR and RNAPII ubiquitylation are thus alternative pathways in the response to DNA damage and RNAPII transcriptional arrest (Svejstrup, 2007).

Nedd4 Is Required for RNAPII Ubiquitylation

In yeast, Rsp5 is required for RNAPII ubiquitylation upon DNA damage and transcriptional arrest in vivo (Beaudenon et al., 1999; Somesh et al., 2005) and in vitro (Somesh et al., 2005, 2007; Reid and Svejstrup, 2004). Rsp5 has several human homologs, among which Nedd4 is the most likely functional homolog based on sequence comparison and characteristics. For example, although Nedd4 is primarily a cytoplasmic factor (it carries a nuclear export signal), it is also found in the nucleus, where its activity regulates the stability of, for example, human *p*roline-*r*ich *t*ranscript, *b*rain-expressed (hPRTB) protein (Hamilton et al., 2001). Our experiments show that Nedd4 is also required for RNAPII ubiquitylation in the chromatin of human cells. This E3 supports ubiquitylation of human RNAPII in a highly purified, reconstituted ubiquitylation system (with Uba1 and either UbcH5C or UbcH7), and Nedd4 RNAi knockdown affects both ubiquitylation and degradation of RNAPII in vivo. In agreement with the idea that Nedd4 targets elongating RNAPII, ubiquitylation of this polymerase form was indeed more efficient in the highly purified ubiquitylation system (Figure S6), as previously reported for the yeast system (Somesh et al., 2005). Interestingly, other Rsp5/Nedd4 homologs did not appear to have the same capability to ubiquitylate RNAPII. Although Smurf1 supported RNAPII ubiquitylation in vitro, RNAi knockdown of this protein (or any of the other Rsp5/Nedd4 homologs) failed to reproducibly affect RNAPII ubiquitylation in vivo, even when combined with Nedd4 knockdown. Smurf1 also did not coimmunoprecipitate with RNAPII from chromatin. However, although our evidence points to Nedd4 as the main Rsp5 homolog required for damage-induced RNAPII ubiquitylation, we cannot from our negative results conclude that other homologs do not target RNAPII, especially in the absence of complete knockdown of the tested proteins. Moreover, other homologs might conceivably ubiquitylate RNAPII in response to other cellular signals than DNA damage or in specific cell types. It is also worth noting that Nedd4 itself is found in two isoforms (Nedd4-1 and -2), differing by the presence in Nedd4-2 of an extra ~250 amino acids. The biochemical reconstitution experiments described here were performed with Nedd4-1, as were the in vivo experiment testing Nedd4's RNAPII interaction. However, the RNAi knockdown constructs were directed against sequences found in both proteins. We therefore cannot exclude the possibility that Nedd4-2 is also capable of supporting RNAPII ubiquitylation.

Taken together, the data presented in this report support the idea that RNAPII transcriptional arrest at DNA lesions in human cells results in the recruitment of Nedd4 and thereby RNAPII ubiquitylation, eventually leading to degradation by the proteasome.

It is interesting to note that Nedd4 is a potential proto-oncogene responsible for regulating the stability of the PTEN tumor suppressor and that Nedd4 overexpression is correlated with an increased cancer risk in mice (Trotman et al., 2007; Wang et al., 2007). These findings underscore the potential importance of understanding the consequences of Nedd4 deregulation for RNAPII stability after DNA damage. Future experiments should thus address whether changes in Nedd4 activity affect the general damage response and thereby potentially the sensitivity of certain cancer cell types to DNA damaging agents used as antineoplastic drugs.

EXPERIMENTAL PROCEDURES

Plasmids

Open reading frames encoding human AIP4, Nedd4, Smurf1, Smurf2, WWP1, and budding yeast Dsk2p were cloned into the pGex vector (Amersham), in-frame with GST. His-tagged WWP2 was subcloned into pET21 (Novagen). Human ubiquitin was cloned into pET21 with an amino-terminal his-myc tag. For transient expression in human cell, myc-tagged AIP4, -Nedd4, -Smurf1, -Smurf2, -WWP1, and -WWP2 were cloned into pIRES-puro (Clontech).

Proteins

GST-Uba1 and the human UbcH5C and UbcH7 (E2) proteins were purchased from Boston Biochemical. GST-tagged proteins (AIP4, Nedd4, Smurf1, Smurf2, and WWP1) were expressed in *E. coli* and purified on glutathione Sepharose by standard techniques. His-tagged WWP2 and human his-myc-tagged ubiquitin were expressed in *E. coli* and purified by Ni-NTA agarose affinity chromatography. See Figure S4 for details.

Cell Culture and Knockdown of Nedd4 Homologs by RNAi

Cells were grown in monolayer in DMEM containing 10% fetal bovine serum (GIBCO) in a 5% CO_2/95% air atmosphere at 37°C.

Validated Stealth RNAi pools were obtained from Invitrogen. The siRNA sequences specific for human Smurf1, Smurf2, AIP4, WWP1, and WWP2 mRNAs corresponded to nucleotides 1163–1187, 1294–1318 (Smurf1), 611–635, 932–956 (Smurf2), 1371–1395, 2342–2366, 2425–2449 (AIP4), 192–216, 1124–1148, 1766–1790 (WWP1), 360–384, 1241–1265, 1978–2012 (WWP2), and 2155–2179, 2649–2673, and 555–579 (Nedd4) of the respective coding regions. The Stealth siRNAs were transfected into HEK293 cells as per the manufacturer's instructions. To determine knockdown efficiency, extracts were prepared 60 hr after transfection, blotted, and probed with specific antibody against AIP4 (Santa Cruz), Nedd4 (Abcam), Smurf1 (Abgent), Smurf2 (Abcam), WWP1 (Abcam), and WWP2 (Santa Cruz). As negative control, stealth RNAi negative control (Invitrogen) was used.

For measuring DNA damage-induced ubiquitylation in different knockdown cells, HEK293 cells were grown in 6 cm tissue culture dishes and transfected with specific stealth RNAi. Sixty hours after transfection, cells were irradiated with UV at 30 J/m^2 using a germicidal lamp. After incubation in fresh media for 10 min at 37°C, whole-cell extracts were prepared and used directly for immunoblotting or pull-down experiment.

For stable knockdown of Nedd4, a specific, double-stranded, short hairpin oligo that targets Nedd4 (5′-AATGGCAACATTCAACTGCAA-3′) was cloned into pSilencer2-U6-puro small interfering RNA vector (Ambion) and transfected into HEK293 cells with Lipofectamine 2000 (invitrogen). After 24 hr of transfection, cells were transferred to, and grown on, 0.5 μg/ml puromycin for 14 days. Clones were pooled and passaged for an additional 6 weeks and analyzed for endogenous Nedd4 level by western blot using Nedd4 antibody.

In Vivo Ubiquitylation and Degradation

For DNA damage-induced ubiquitylation/degradation experiments, fibroblasts were grown and irradiated as above. UV doses from 5 to 30 J/m^2 were used, but because only damage-arrested RNAPII is ubiquitylated, smaller doses resulted in less RNAPII ubiquitylation per cell

being detectable. Therefore, 30 J/m^2 was used for the experiments shown. α-amanitin treatment (10 μg/ml; Calbiochem) was for 2 hr at 37°C. Where indicated, proteasome inhibitor MG132 (5 μM; Sigma) or protein synthesis inhibitor cycloheximide (25 μg/ml; Sigma) was added 1 hr before treatment. For whole-cell extract preparation, cells were lysed in TENT buffer (20 mM Tris-HCl [pH 8.0], 2 mM EDTA, 150 mM NaCl, and 1% Triton X-100) containing protease inhibitor cocktail, sonicated briefly, precleared by centrifugation at 20,000 × g for 10 min at 4°C, and used directly for immunoblotting or pull-down experiment.

Isolation of ubiquitylated proteins took advantage of the properties of budding yeast Dsk2p. Briefly, GST-Dsk2 protein was expressed in *E. coli* and purified on/bound to glutathione Sepharose 4B. Cell extracts in TENT buffer were incubated with this resin (or GST alone as control) at 4°C overnight. After washing with PBS, ubiquitylated proteins were eluted from the beads with 50 mM Tris-HCl (pH 8.2), 10 mM reduced glutathione. The samples were fractionated on a 4%–12% SDS-PAGE gel (Bio-Rad) and blotted and probed with an Rpb1-specific mAb (4H8, which recognizes both phosphorylated and nonphosphorylated RNAPII, was used). Where required, extract input was adjusted so that comparable amounts from different cells lines or time points were loaded onto the Dsk2 resin.

In Vitro Ubiquitylation

For in vitro ubiquitylation of RNAPII, 300 ng RNAPII was incubated with 25 nM rUba1, 25 nM rGST-UbcH7 (or rGST-UbcH5C), 25 nM rGST-Nedd4 (or other E3), 2 μg rHis-myc-ubiquitin, and 2 mM ATP in ubiquitylation buffer (25 mM Tris-Cl [pH 8.0], 125 mM NaCl, 2 mM MgCl2, and 50 μM DTT). Reactions were incubated at 30°C for 90 min before adding SDS-PAGE loading buffer. Samples were fractionated by 4%–12% SDS-PAGE (Bio-Rad) and blotted and probed with anti-myc antibody (9E11). In vitro (self) ubiquitylation of Nedd4 homologs was done as above, but 100 nM E1, E2, and E3 was used. Reactions were stopped by adding SDS-PAGE loading buffer without reducing agent and were analyzed by western blotting as above.

Cellular Fractionation, Isolation of Chromatin-Associated Proteins, and Coimmunoprecipitation Experiments

Cells were lysed with cytoplasmic lysis buffer (10 mM Tris-HCl [pH 7.9], 0.34 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT, 0.5% NP-40, and protease inhibitors [Otero et al., 1999]), and nuclei pelleted by centrifugation at 3500 × g for 15 min. Nuclei were washed with cytoplasmic lysis buffer without NP-40, then lysed with nuclear lysis buffer (20 mM HEPES [pH 7.9], 3 mM EDTA, 10% glycerol, 150 mM potassium acetate, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, and protease inhibitors). The nucleoplasmic fraction was cleared by centrifugation at 15,000 × g for 30 min. The chromatin-enriched pellet was resuspended in nuclease incubation buffer (150 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 150 mM KOAc, and protease inhibitors), and DNA and RNA in the suspension were digested with 25 U/μl Benzonase (Novagen). The sample was cleared by centrifugation at 20,000 × g for 30 min, and the supernatant collected.

Coimmunoprecipitation was done overnight at 4°C with the solubilized chromatin fraction. Immunocomplexes were washed extensively with IP wash buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% NP-40, and 1 mM EDTA), boiled in SDS-PAGE sample buffer, resolved in 4%–12% Bis-Tris gradient polyacrylamide gels (Bio-Rad), and analyzed by western blotting.

Supplemental Data

Supplemental Data include six figures and can be found with this article online at http://www.molecule.org/cgi/content/full/28/3/386/DC1/.

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