

Proteasome-Mediated Processing of Def1, a Critical Step in the Cellular Response to Transcription Stress

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SUMMARY

DNA damage triggers polyubiquitylation and degradation of the largest subunit of RNA polymerase II (RNAPII), a “mechanism of last resort” employed during transcription stress. In yeast, this process is dependent on Def1 through a previously unresolved mechanism. Here, we report that Def1 becomes activated through ubiquitylation- and proteasome-dependent processing. Def1 processing results in the removal of a domain promoting cytoplasmic localization, resulting in nuclear accumulation of the clipped protein. Nuclear Def1 then binds RNAPII, utilizing a ubiquitin-binding domain to recruit the Elongin-Cullin E3 ligase complex via a ubiquitin-homology domain in the Ela1 protein. This facilitates polyubiquitylation of Rpb1, triggering its proteasome-mediated degradation. Together, these results outline the multistep mechanism of Rpb1 polyubiquitylation triggered by transcription stress and uncover the key role played by Def1 as a facilitator of Elongin-Cullin ubiquitin ligase function.

INTRODUCTION

Substantial research effort is presently focused on understanding the cellular processes maintaining genome integrity and allowing faithful replication after DNA damage (Branzei and Foiani, 2010). Although such processes are of utmost importance for the long-term survival and fitness of cells and organisms, the key immediate response of cells suffering genotoxic insult is arguably to maintain gene expression. Indeed, without continued transcription, cells cannot proceed through the cell cycle, and even nondividing cells will perish. Like DNA replica-

tion, transcription is severely affected by DNA damage, with various DNA lesions resulting in RNAPII stalling, pausing, arrest, and/or backtracking (hereafter collectively referred to as transcription stress). It is therefore not surprising that cells have evolved a number of mechanisms to ensure that transcription can rapidly resume upon DNA damage (Svejstrup, 2010). One important mechanism is transcription-coupled nucleotide excision repair (TC-NER), which removes transcription-blocking lesions so that RNAPII can continue (Gaillard and Aguilera, 2013). In budding yeast, TC-NER is dependent on Rad26, the homolog of human Cockayne syndrome B (van Gool et al., 1994). Intriguingly, Rad26 interacts with another protein, Def1 (Woudstra et al., 2002). The phenotypes of cells lacking *DEF1* indicate a role for this factor in the DNA damage response, but Def1 is not involved in repair. Instead, it is required for a “mechanism of last resort.” During this alternative process, the largest subunit of RNAPII, Rpb1, becomes ubiquitylated and degraded, which results in disassembly of the large RNAPII complex and allows the lesion to be dealt with by other means (Wilson et al., 2013). Although it was originally identified as a response to DNA damage (Bregman et al., 1996; Beaudenon et al., 1999), it is now known that Rpb1 ubiquitylation and degradation occurs under a number of conditions that result in transcription stress (Hobson et al., 2012; Somesh et al., 2005; Sigurdsson et al., 2010). Obviously, Rpb1 ubiquitylation must be tightly regulated to specifically target the small subset of elongating polymerases that cannot otherwise be salvaged, as any unnecessary Rpb1 degradation will severely affect general gene expression and cell survival. Results obtained over the last decade have provided insight into the mechanisms by which Rpb1 is ubiquitylated and degraded (reviewed in Wilson et al., 2013), but although it is required for Rpb1 ubiquitylation, the precise role of Def1 has remained elusive.

Degradation of Rpb1 occurs by the addition of lysine 48-linked polyubiquitin chains, disassembly of the chromatin-associated RNAPII elongation complex, and proteasomal

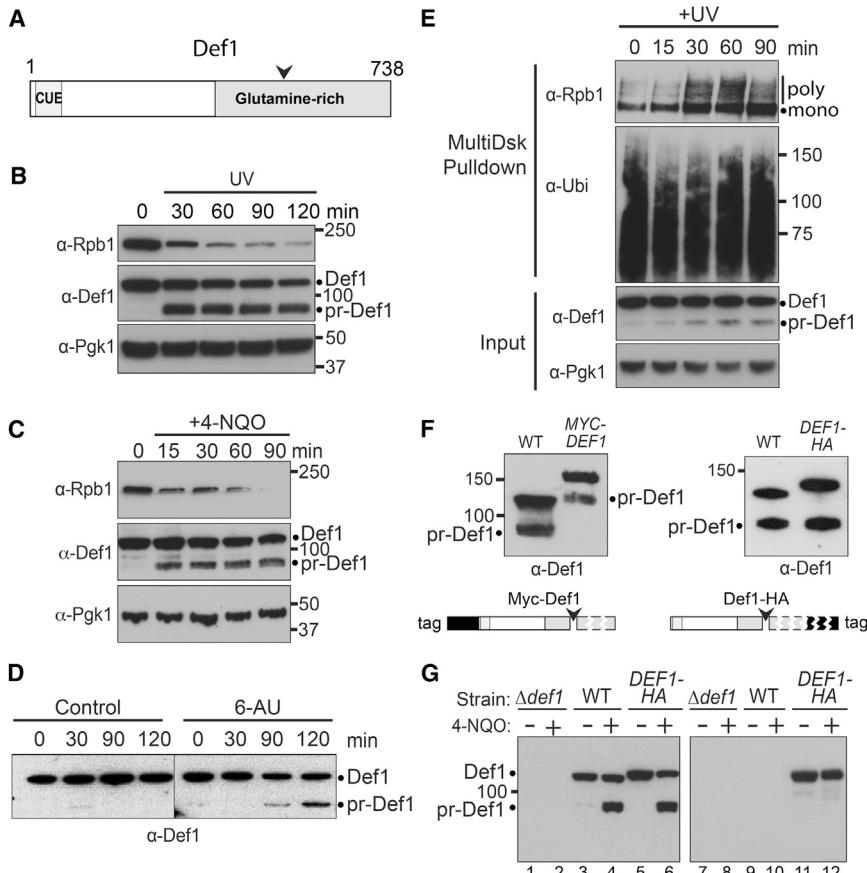


Figure 1. Def1 Is Processed in Response to Transcription Stress

(A) Schematic representation of Def1 indicating the CUE domain, area of processing (arrow), and glutamine rich region.

(B) Western blot of cell extracts at the indicated times after UV irradiation, using antibodies against Rpb1, Def1, and Pgk1 (loading control).

(C) As in (B), but after incubation with 4-nitroquinoline 1-oxide (4-NQO).

(D) Def1 processing induced by 6-azauracil (6-AU). Superfluous lanes between lanes 4 and 5 were removed.

(E) As in (B), but ubiquitylated proteins isolated using MultiDsk pull-down (Wilson et al., 2012).

(F) Western blot probed using anti-Def1 antibody, showing WT, or N-terminally 9xMyc-tagged Def1 (left), or WT, or C-terminally 6xHA-tagged Def1 (right), after incubation with 4-NQO for 1 hr.

(G) Western blots of identical samples from *Δdef1*, WT, or *DEF1-3xHA*, probed with anti-Def1 antibodies (left panel), or anti-HA antibodies (right).

See also Figure S1 and Tables S1 and S2.

degradation (Wilson et al., 2013). Notably, ubiquitylation of Rpb1 is a two-step process, involving distinct ubiquitin ligases (E3s) (Harreman et al., 2009). Briefly, stalled RNAPII in budding yeast is targeted by a HECT domain E3, Rsp5 (Beaudenon et al., 1999), which cooperates with Uba1 (E1, ubiquitin-activating enzyme) and Ubc5 (E2, ubiquitin-conjugating enzyme) to add a single ubiquitin moiety, probably at more than one site on Rpb1 (Somesh et al., 2007; Harreman et al., 2009). A second E3 ligase, a complex containing the Elc1, Ela1, Cul3, and Rbx1 proteins (“Elongin-Cullin complex”), then takes over and adds lysine 48-linked ubiquitin chains to the premonoubiquitylated Rpb1 (Harreman et al., 2009; Ribar et al., 2006, 2007). Following polyubiquitylation, a ubiquitin-specific ATPase, Cdc48, then delivers Rpb1 from the RNAPII elongation complex to the proteasome (Verma et al., 2011). The mechanism of Rpb1 ubiquitylation is highly conserved, with the process in mammals being catalyzed by NEED4 and the Elongin ABC-Cullin 5 complex, homologs of the budding yeast E3 proteins (Huibregtse et al., 1997; Anindya et al., 2007; Yasukawa et al., 2008; Harreman et al., 2009).

As mentioned above, polyubiquitylation and degradation of Rpb1 also requires the Def1 protein, both *in vivo* (Woudstra et al., 2002) and *in vitro* (Reid and Svejstrup, 2004). Def1 is an unusual protein, consisting largely of domains of low complexity, with a predicted N-terminal CUE (ubiquitin-binding)

domain as the only notable feature (Ponting, 2002) (Figure 1A). In this study, we show that ubiquitylation and degradation of Rpb1 encompasses an unusually wide variety of ubiquitin-related mechanisms centered on the Def1 protein. Together, these mechanisms facilitate nuclear accumulation of a proteasome-processed version of Def1, which then acts as a bridging factor between RNAPII and the Elongin-Cullin complex, triggering Rpb1 polyubiquitylation.

RESULTS

Def1 Is Processed in Response to DNA Damage and Other Transcription Stress

Under conditions that generate transcription-impeding DNA damage, such as UV irradiation and treatment with 4-nitroquinoline 1-oxide (4-NQO), we noticed the appearance of a faster migrating protein, hereafter called processed Def1 (pr-Def1), which specifically cross-reacted with a Def1 antibody (Figures 1B, 1C, and 1G, left, and Figure S1A available online). The faster migrating Def1 form was also observed upon treatment with the transcription elongation inhibitor, 6-azauracil (6-AU) (Figure 1D), suggesting that it is generated in response to transcription stress, rather than as a damage response per se. pr-Def1 formation correlated with polyubiquitylation (Figure 1E) and degradation (Figures 1B and 1C) of Rpb1.

Three distinct processes might theoretically explain the generation of pr-Def1. First, it might be caused by changes in the production of *DEF1* mRNA (e.g., alternative start- or stop-site usage or alternative splicing). Second, it might be caused by changes in mRNA translation. Third, it might occur at a posttranslational stage, via partial proteolysis. No significant changes in the

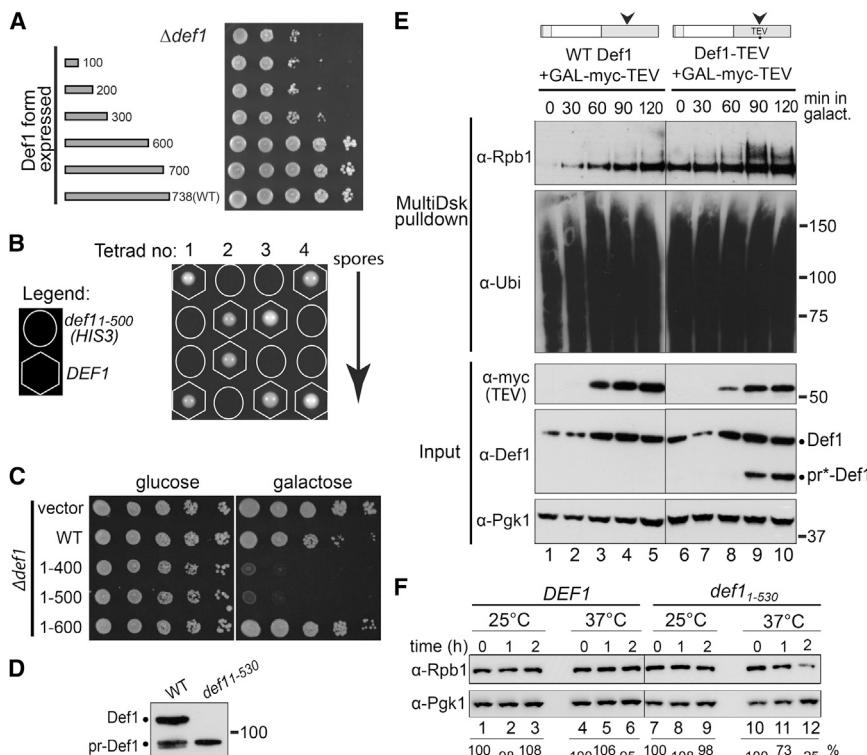


Figure 2. Processing of Def1 Produces an Activated Form

(A) Dilution growth series of cells expressing Def1 fragments.

(B) A ratio of 2:2 segregation of spores from four different tetrads from a heterozygous $\text{DEF1}/\text{def1-1500}:\text{HIS3}$ diploid, with spore genotype indicated. Viable HIS3 colonies were never observed.

(C) Dilution series of Δdef1 yeast cells (with GAL-driven plasmid indicated on left), grown on glucose or galactose. Overexpression of WT DEF1 from a 2 μm plasmid is slightly detrimental to Δdef1 cells.

(D) Western blot showing Def1 from 4-NQO-treated WT cells, and def1-1530 , respectively.

(E) Western blot of extract from cells expressing Myc-tagged TEV protease, as well as WT Def1, or Def1 containing a TEV protease cleavage site. Blots of ubiquitylated proteins shown in two upper panels, with extract blots shown below. Lanes 1 and 2 were underloaded, giving the false impression that growth in galactose results in higher Rpb1 monoubiquitylation. Superfluous lanes between lanes 5 and 6 were removed.

(F) Western blots of extracts from WT and def1-530 cells grown at permissive (25°C) or restrictive temperature (37°C). Rpb1 signal was quantitated relative to the Pgk1 control, and the values at time 0 set to 100; other values are expressed relative to that. Superfluous lanes between lanes 6 and 7 were removed.

See also Figure S2.

production of DEF1 mRNA were detectable in response to DNA damage (Figure S1B), and pr-Def1 was still generated in response to DNA damage in cells that no longer produced new Def1 protein (Figure S1C). This indicates that Def1 processing is a posttranslational event, a conclusion that was further supported by all subsequent experiments.

To investigate whether it is the N or C terminus of Def1 that is missing from pr-Def1, yeast strains expressing tagged versions of Def1 were generated. When N-terminally tagged Def1 was detected using the polyclonal anti-Def1 antibody, the processed version was larger than wild-type (WT) pr-Def1 (Figure 1F, left), whereas no mobility shift of pr-Def1 was observed when a HA-tag was positioned at the (degraded) C terminus (Figure 1F, right). Indeed, Def1, but not pr-Def1, could be detected with anti-HA antibody (Figure 1G, compare lanes 6 and 12). Together, these experiments demonstrate that pr-Def1 lacks the C-terminal region of full-length Def1. This short fragment has persistently evaded detection, suggesting that it is degraded during processing, or immediately thereafter.

Processing of Def1 Produces an Activated Form

If pr-Def1 plays a causative role in the response to transcription stress, it might be expected that artificial generation of pr-Def1-like protein species would result in Def1 activation and possibly lead to Rpb1 ubiquitylation and degradation. To address this possibility, the endogenous DEF1 gene was shortened by genomic recombination. Small C-terminal deletions (generating Def1-1600 and Def1-1700) had little or no effect on cell growth, whereas large deletions (generating Def1-100 to Def1-300)

resulted in a slow-growth phenotype, similar to that of a complete DEF1 deletion (Figure 2A). Strikingly, haploid strains expressing Def1-1400 and Def1-1500 (that would theoretically encode protein fragments of a size similar to that of pr-Def1) could not be generated despite repeated attempts, but a diploid strain expressing both WT Def1 and Def1-1500 was viable. However, upon sporulation and tetrad dissection of this diploid into haploid spores, only two of four spores were viable; these contained the WT version of DEF1 (Figure 2B), indicating that the Def1-1500 fragment is toxic. Def1 self-associates (Figure S2A) and might be either a dimer or a multimeric protein. This could explain why the expression of genetically truncated versions of Def1 is not lethal when a WT copy of DEF1 is also present. In apparent agreement with this idea, other proteolytically processed proteins have been reported to be dimeric (Rape and Jentsch, 2002).

Expression of Def1-1400 and Def1-1500 proteins from an inducible promoter also inhibited cell growth, whereas expression of WT Def1 and Def1-1600 had little or no effect (Figure 2C, galactose). Together, these data indicate that expression of genetically generated, shorter forms of Def1 that mimic pr-Def1 is strongly detrimental to the cell.

Repeated attempts to map the site of damage-induced Def1 processing by mass spectrometric analysis proved unsuccessful. As an alternative approach to better define processing, we therefore used genetic recombination to generate C-terminal DEF1 deletions in the equivalent of ten amino acid intervals between Def1 residues 500 and 600. DEF1 genes expressing proteins between 500 and 520 amino acids in length were lethal,

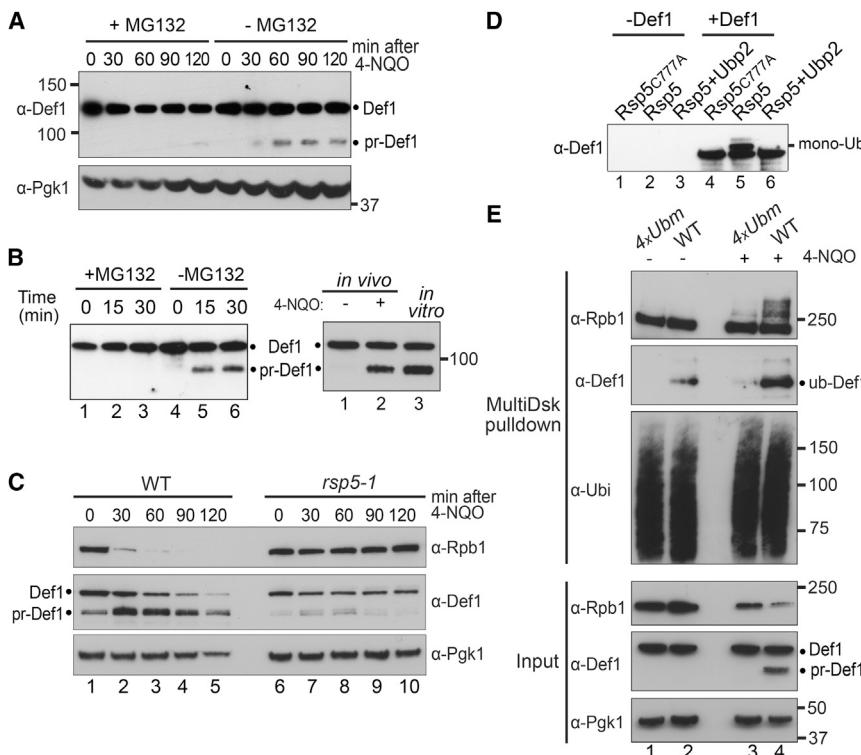


Figure 3. Proteasome- and Ubiquitin-Dependent Def1 Processing

(A) Western blot of extracts from cells treated with (or without) MG132 prior to incubation with 4-NQO for the indicated times.

(B) Left: Def1 processing reconstituted using pure Def1 and 26S proteasome (see Figure S3A), and its sensitivity to MG132. Right: western blot comparing pr-Def1 with Def1 processed by pure proteasome.

(C) Western blot of extract from WT and *rsp5-1* cells grown at 37°C for 2 hr before addition of 4-NQO for the indicated times. Lanes 4 and 5 are underloaded, giving the false impression that Def1 disappears at those time points. Normal processing was observed in *rsp5-1* at the permissive temperature (data not shown).

(D) Western blots of Def1 ubiquitylation, reconstituted using highly purified proteins (Def1, ubiquitin, Uba1, Ubc5, Rsp5, and Ubp2; see Figure S3A).

(E) Western blots of ubiquitylated proteins (upper three panels) and extracts (input; lower three panels) from UV-irradiated WT and Def1 ubiquitylation site mutant (4xUbm) cells, respectively. See also Figure S3.

ably because TEV-mediated Def1 nicking does not induce other pathways that are activated by transcription stress.

Next, the temperature-sensitive *def1₁₋₅₃₀* strain was used to further investigate the effect of Def1 processing. We surmised that because *def1₁₋₅₃₀* is slow growing at 37°C, the restrictive temperature likely activates this version of Def1. Overall protein levels and that of the P-gk1 control protein remained largely unchanged at 37°C. In contrast, Rpb1 protein levels decreased with time in *def1₁₋₅₃₀*, but not WT cells (Figure 2F, compare lanes 11 and 12 with 5 and 6), again suggesting that the processed version of Def1 triggers RNAPII ubiquitylation and degradation.

Def1 Is Processed in a Ubiquitin- and Proteasome-Dependent Manner

Ubiquitin- and proteasome-dependent protein processing, whereby a part of a protein is degraded to release a biologically active fragment, has been described for other proteins (reviewed in Rape and Jentsch, 2002). In agreement with a similar role for the proteasome in the processing of Def1, treatment of yeast cells that are sensitized to proteasome inhibitor (Collins et al., 2010) with MG132 inhibited the formation of pr-Def1 (Figure 3A).

In order to test whether the proteasome can directly process Def1, we reconstituted proteasomal processing in vitro using Def1 and 26S proteasome purified to virtual homogeneity (Figure S3A). Strikingly, in this simple assay correct proteasome-dependent Def1 processing was observed, with full-length Def1 processed to the shortened version (Figure 3B, lanes 5 and 6). As expected, this was greatly reduced in the presence of proteasome inhibitor (lanes 2 and 3). Significantly, pr-Def1 was not proteolyzed further: smaller proteolysis products were not observed, and the overall Def1 protein amount remained constant. Moreover, the size of processed Def1 generated by

whereas those generating Def1 proteins of 530 amino acids or more were viable (Figure S2B). Interestingly, a strain expressing Def1₁₋₅₃₀ (*def1₁₋₅₃₀*) was temperature-sensitive (Figure S2C). This version of the Def1 protein migrated slightly slower than pr-Def1 (Figure 2D), suggesting that the natural processing site lies somewhere in the region prior to amino acid 530. We attempted to disrupt Def1 processing by making internal deletions in this region, but these merely resulted in shifting the site to another position (Figure S2D). This suggests that Def1 processing does not occur at a specific, short amino acid motif, as has also been found for other processed proteins (Piwnko and Jentsch, 2006).

To investigate whether Def1 clipping in itself can trigger RNAPII ubiquitylation even in the absence of transcription stress, we now constructed a yeast strain expressing Def1 with a tobacco etch virus (TEV) protease cleavage site inserted between amino acids 522 and 523, around the predicted region of normal processing. Such modification had no obvious effect on *DEF1* function (Figure S2E). Upon induction of GAL-regulated Myc-TEV protease (Figure 2E, third panel from bottom), a fraction of Def1 was indeed cleaved, but only in the strain with the inserted TEV site (pr*-Def1; Figure 2E, second panel from bottom). Importantly, a fraction of Rpb1 reproducibly became polyubiquitylated concurrently with the emergence of pr*-Def1 (Figure 2E, top, compare lanes 9 and 10 with 4 and 5, respectively). These results indicate that Def1 processing can in itself trigger detectable Rpb1 polyubiquitylation, independently of DNA damage. Not surprisingly, the effect of TEV-induced cleavage was less pronounced than after DNA damage and did not result in appreciable degradation of the Rpb1 protein, presum-

purified proteasome in vitro was similar to that of pr-Def1 generated by transcription stress in vivo (Figure 3B, right panel, compare lanes 2 and 3).

Not all proteasome-mediated protein degradation requires ubiquitylation of the target protein (Jariel-Encontre et al., 2008). However, in other reported cases of proteasomal protein processing, ubiquitylation of the target protein has been implicated, at least in vivo (Palombella et al., 1994; Hoppe et al., 2000; Pan et al., 2006). Moreover, monoubiquitylation of NF- κ B p105 is sufficient for its proteasomal processing (Kravtsova-Ivantsiv et al., 2009), and we recently found that Def1 becomes monoubiquitylated in response to DNA damage (Wilson et al., 2012). The ubiquitin ligase Rsp5 binds and monoubiquitylates Rpb1 (Huibregtse et al., 1997; Harreman et al., 2009). Furthermore, this E3 has been implicated in proteasomal processing of other yeast proteins (Hoppe et al., 2000). We therefore investigated whether Rsp5 might be involved in Def1 ubiquitylation and proteasomal processing. Indeed, in a strain carrying a temperature-sensitive *rsp5* allele, Def1 failed to be efficiently processed in response to 4-NQO at the restrictive temperature (Figure 3C, middle panel on right), whereas Def1 processing and accompanying Rpb1 degradation was observed in the WT control strain (Figure 3C, left). The remaining proteasomal processing of Def1 observed in this experiment might be explained by residual Rsp5 activity at the restrictive temperature, or by ubiquitylation merely playing a stimulatory role in Def1 processing.

To ascertain whether Rsp5 can directly ubiquitylate Def1, we reconstituted the process with highly purified proteins. Def1 ubiquitylation was indeed observed upon incubation with Uba1 (E1), Ubc5 (E2), and ubiquitin, but only when active Rsp5 was included (Figure 3D, lane 5). Ubiquitylation was not observed when the active site of Rsp5 was mutated (lane 4), or in the presence of ubiquitin protease Ubp2 (lane 6), which associates with Rsp5 in vivo (Kee et al., 2005).

Using Def1 that had been ubiquitylated in vitro, two pairs of adjacent, potential ubiquitylation sites were now identified by mass spectrometry. Simultaneous mutation of these four sites (K281R, K288R, K328R, K329R; creating 4xUbm) resulted in strongly reduced Def1 ubiquitylation and perturbed Rpb1 polyubiquitylation, (Figure 3E, upper panels, compare lanes 3 and 4) and also affected Def1 processing and Rpb1 degradation (Figure 3E, lower panels), in response to DNA damage. Interestingly, 4xUbm did not always completely abrogate Def1 processing (see Figure S3B). This suggests that other potential ubiquitylation sites still remain available, or that proteasomal processing of Def1 is only accelerated by ubiquitylation, but not absolutely dependent on it, in apparent agreement with the results obtained in *rsp5-1* (Figure 3C). Furthermore, ubiquitylation of Def1 was not absolutely required for proteasomal processing in the reconstituted in vitro system, although it could accelerate the process (Figure S3C).

Taken together, these results strongly indicate that processing of Def1 involves Rsp5-mediated ubiquitylation and partial degradation by the proteasome.

pr-Def1 Accumulates in the Nucleus

The experiments above indicate that proteasomal processing activates Def1, but how pr-Def1 promotes polyubiquitylation of

Rpb1 remained unclear. We began to characterize this connection by examining the cellular localization of Def1. Previous studies indicated that Def1 is cytoplasmic (Huh et al., 2003; Tkach et al., 2012), despite being required for Rpb1 polyubiquitylation in chromatin (Woudstra et al., 2002; Verma et al., 2011). However, the earlier localization studies relied on a C-terminal GFP-tag on Def1, which would not visualize pr-Def1. By using an N-terminal GFP-tag on Def1 instead, we ensured that both forms of Def1 could be detected. Without UV treatment, GFP-Def1 was cytoplasmic, as expected (Figure 4A, panels c and f). After treating yeast cells with UV light, however, the GFP signal spread across the entire cell, including the DAPI-stained nucleus (Figure 4A, panels i and l). In contrast, C-terminally tagged Def1-GFP did not show relocalization after treatment with UV (Figures 4B and S4A).

Def1 was also GFP-tagged in the MG132-sensitized strain (Collins et al., 2010). When these cells were treated with proteasome inhibitor to block pr-Def1 formation, Def1 remained in the cytosol even after DNA damage (compare Figure 4C, panel f, with 4A, panel i), further indicating that only pr-Def1 accumulates in the nucleus.

It was now investigated whether altering steady-state subcellular localization might suppress the previously described toxicity of GAL-expressed Def1_{1–500}. Indeed, even though Def1_{1–500} was overexpressed, appending a prototypical leucine-rich nuclear export signal (NES) (Gerace, 1995) to its N terminus reproducibly decreased toxicity (Figure 4D), suggesting that the detrimental effect of Def1_{1–500} expression is at least partly due to nuclear localization.

We next examined whether the C-terminal region of Def1 might harbor a domain, which directly or indirectly leads to cytoplasmic localization by either inhibiting nuclear import or promoting nuclear export (Figure 4E). When a GFP control protein containing both a nuclear localization signal (NLS) and an NES was expressed, the fusion-protein was detected in the whole cell (Figure 4E, panels a–c) (Taura et al., 1998). In contrast, if the GFP construct contained an NLS, but lacked the NES and instead carried a C-terminal control domain (β -galactosidase), it was exclusively nuclear (Figure 4E, panels g–i) (Lee et al., 1996). Importantly, when this control domain was replaced with the Def1 C terminus (amino acids 500–738), the resulting GFP fusion protein was found in both the nucleus and the cytoplasm (Figure 4E, panels m–o), showing that the C terminus of Def1 promotes cytoplasmic localization.

Although the best-characterized mechanism of nuclear export occurs via NESs recognized by the Crm1 receptor (Hutten and Kehlenbach, 2007), 13 additional members of the karyopherin family of nuclear transport receptors are found in *Saccharomyces cerevisiae* (Ström and Weis, 2001). For the vast majority of these receptors, the motif targeting the cargo for transport has not been defined. However, export from the nucleus is typically dependent on components of the nuclear pore, such as Rat7 (Nup159) (Brykailo et al., 2007 and references therein). The Def1 C-terminal domain does not contain a classical NES motif, and as expected, Def1 indeed remained cytoplasmic when Crm1 was inhibited (Figure S4B). Importantly, however, the C-terminal domain of Def1 failed to bring about cytoplasmic localization and thus accumulated in the nucleus in the nuclear

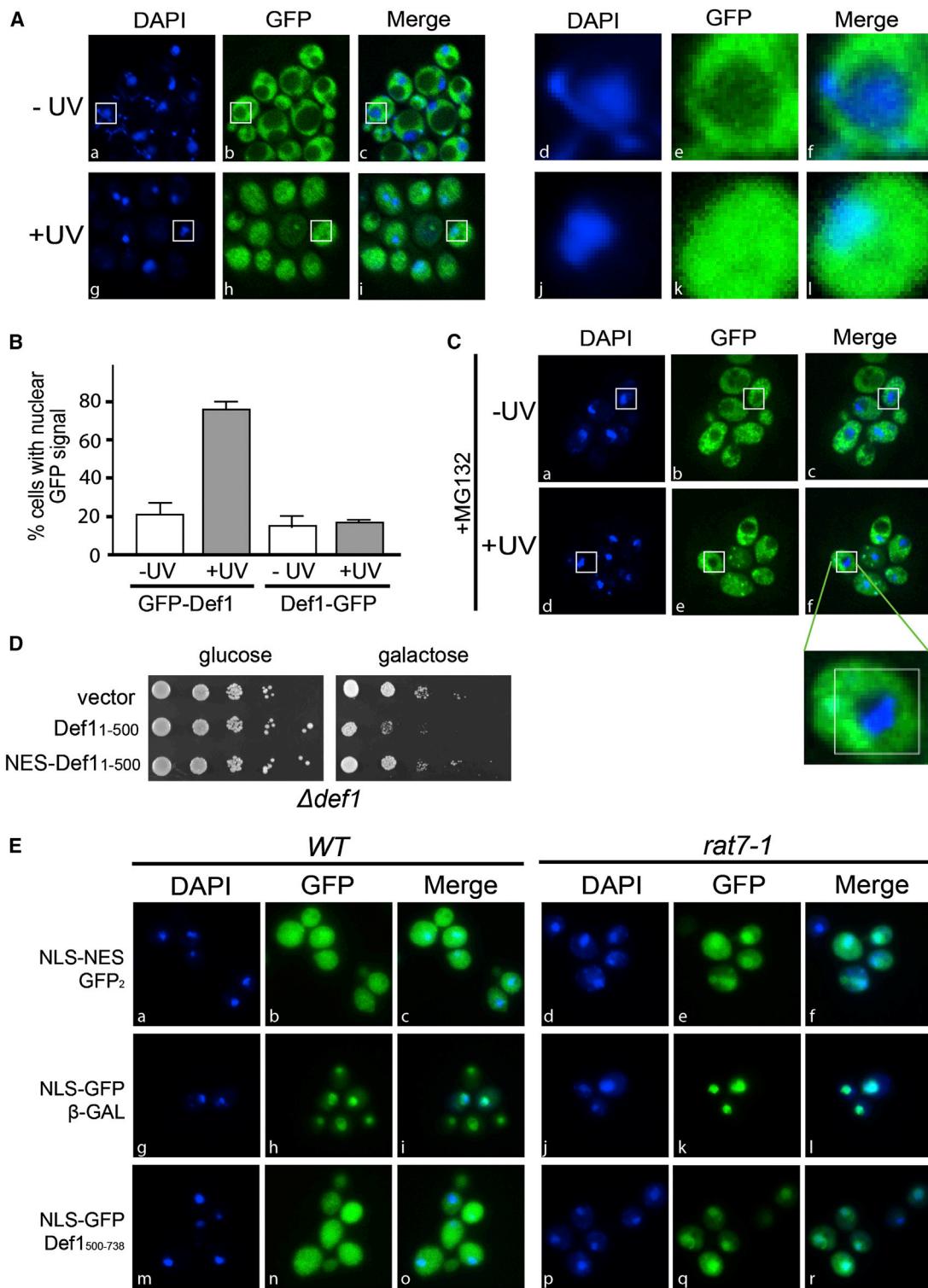


Figure 4. pr-Def1 Accumulates in the Nucleus

(A) Subcellular localization of N-terminally GFP-tagged Def1 after UV irradiation. Nucleus is marked by DAPI staining. Left: a typical field of cells. Right: enlargement of the yeast cell indicated by white box in panel on left. The number and size of vacuoles differed from experiment to experiment, but it had no influence on the nuclear accumulation observed.

(B) Quantification of damage-induced, nuclear accumulation of N-terminally tagged GFP-Def1 and C-terminally tagged Def1-GFP, respectively. Error bars indicate SD of three biological replicates, counting 200–300 cells for each condition.

(legend continued on next page)

pore mutant *rat7-1* (Figure 4E, panels p–r), opening the possibility that this Def1 domain works by promoting export from the nucleus rather than by inhibiting nuclear import.

Together, these data indicate that Def1 is normally efficiently exported from the nucleus so that it is mainly cytoplasmic at steady state. Upon DNA damage or other transcriptional stress, however, a region directing cytoplasmic localization is removed by proteolysis, allowing pr-Def1 to accumulate in the nucleus, facilitating Rpb1 ubiquitylation/degradation.

The CUE Domain Is Critical for Def1 Function

The data above indicate that transcription stress induces proteasomal Def1 processing, leading to pr-Def1 accumulation in the nucleus. However, the role of Def1 in Rpb1 ubiquitylation inside the nucleus remained to be established. We began by examining the role of the N-terminal CUE domain (Ponting, 2002), predicted to bind ubiquitin (Shih et al., 2003). Indeed, Def1's CUE domain bound to ubiquitin in vitro and when four key CUE residues were mutated (Shih et al., 2003) (Figure 5A, schematic) it reduced such binding (Figure S5A; see also Figure 6D). Strikingly, although expression of Def1_{1–500} was highly toxic, CUE domain mutation effectively suppressed this effect (Figure 5A). CUE mutation also abrogated DNA damage-induced Rpb1 polyubiquitylation (Figure 5B, upper panel; compare lanes 4 and 8). This was not due to a lack of Def1 activation: CUE mutation had no effect on Def1 processing (Figure 5B, middle panel) or on its accumulation in the nucleus (Figure S5B).

Together, these results indicate that the ubiquitin-binding CUE domain is critical to the nuclear function of pr-Def1.

pr-Def1 Associates with Ela1-Elc1 Complex via Its CUE Domain

Rpb1 is monoubiquitylated by Rsp5 in a Def1-independent manner and then polyubiquitylated by the Elongin-Cullin complex in a Def1-dependent fashion (Woudstra et al., 2002; Harriman et al., 2009). Consequently, the data presented above might be explained if Def1 helps mediate the interaction of monoubiquitylated polymerase with the Elongin-Cullin complex. In this model, Def1 would use its CUE domain to bind monoubiquitylated RNAPII, the Elongin-Cullin complex, or both.

We first performed coimmunoprecipitation experiments using extracts from UV-irradiated yeast cells. RNAPII and the Ela1 subunit of the Elongin-Cullin complex both coimmunoprecipitated with Myc-Def1 (Figure 5C, lane 5). Somewhat unexpectedly, however, mutation of the Def1 CUE domain had little or no effect on the interaction with RNAPII, but resulted in a reproducible decrease in the interaction with Ela1 (compare lanes 5 and 9).

To further analyze the intriguing interaction between Def1 and Elongin-Cullin complex, the same factors were now immunoprecipitated from a strain expressing a Myc-tagged version of the Elc1 protein. Myc-Elc1 coprecipitated Def1, but only if Def1 had a functional CUE domain (Figure 5D, compare lanes 3 and

7). Notably, Myc-Elc1 also failed to coprecipitate RNAPII unless Def1 had a functional CUE domain, indicating that Def1 is required to bridge the interaction of Elongin-Cullin with RNAPII.

Ela1 and Elc1 form a heterodimer, akin to the F-box/Skp1 substrate adaptor of other Cullin ligases (Koth et al., 2000), which we now produced in *Escherichia coli*. To test the interaction of this dimer with Def1, pure recombinant Def1_{1–500}, mimicking the active pr-Def1 fragment, was immobilized on beads. A similar amount of the corresponding CUE mutant was also immobilized (Figure 5E). Remarkably, although the recombinant, purified Ela1-Elc1 complex was not ubiquitylated, it associated with immobilized Def1_{1–500}, but not when the CUE domain was mutated (Figure 5E, compare lanes 6 and 7).

To further investigate whether Def1 bridges or stabilizes an interaction between RNAPII and the Elongin-Cullin complex, we used purified RNAPII, Def1, and recombinant Ela1-Elc1 complex in tripartite binding experiments (Figure 5F). In these experiments, RNAPII was immobilized on beads. Both Def1_{1–500} and Def1_{1–500/CUEm} bound to such beads, but showed only background binding to the empty control beads (Figure 5F, compare lanes 6 and 8 with 5 and 7, respectively). In contrast, Ela1-Elc1 complex bound poorly to RNAPII (Figure 5F, compare lanes 9 and 10). However, when Def1 was included in the binding reaction, a strong association of Ela1-Elc1 complex with RNAPII was detected (Figure 5F, compare lanes 10 and 12). Importantly, the ability of Def1 to bridge the interaction between RNAPII and Ela1-Elc1 was dependent on the CUE domain: even though Def1_{1–500/CUEm} associated with RNAPII-containing beads as efficiently as its WT counterpart, it had little or no stimulating effect on Ela1-Elc1 binding (Figure 5F, compare lanes 12 and 14 with lane 10). Interestingly, addition of Ela1-Elc1 also clearly increased Def1-RNAPII interaction (Figure 5F, compare Def1 binding in lane 12 with that in 6, 8, and 14), thus contributing to a more stable RNAPII/Def1/Ela1-Elc1 ternary complex.

A Functionally Important Ubiquitin-Homology Domain in the Ela1 Protein

Recombinant Ela1-Elc1 complex interacts directly with Def1 in a CUE (ubiquitin-binding domain [UBD]-dependent manner, suggesting that, per definition, Ela1 or Elc1 must contain a domain that resembles ubiquitin, e.g., a ubiquitin homology domain (UbH). Indeed, Ela1-Elc1 complex was also capable of associating with other purified UBD proteins or domains, such as Dsk2, RAP80, and Ataxin-3 (Dikic et al., 2009) (Figure 6A, compare lanes 4–6 with lanes 2 and 3). Interestingly, however, only Def1 retrieved Ela1-Elc1 from crude yeast extracts, but did not bind ubiquitylated proteins. Conversely, Dsk2, Rap80, and Ataxin-3 bound ubiquitylated proteins, but did not bind Ela1-Elc1 under the same conditions (Figure S6A). This indicates that Def1's CUE domain specifically interacts with Ela1-Elc1 through the putative UbH, but does not generally bind ubiquitylated proteins.

(C) Localization of GFP-Def1 in cells incubated with MG132 for 1 hr prior to UV irradiation. Image below shows enlargement of yeast cell indicated by white box.
(D) Dilution series of *Δdef1* cells (carrying the GAL-driven CEN plasmid indicated on the left) grown on glucose or galactose.

(E) Localization of GFP in fixed cells expressing NLS-NES-GFP₂ (panels a–f), NLS-GFP-β-galactosidase (panels g–l), or NLS-GFP-Def1_{500–738} (panels m–r), in WT cells (left), or *rat7-1* cells (right).

See also Figure S4.

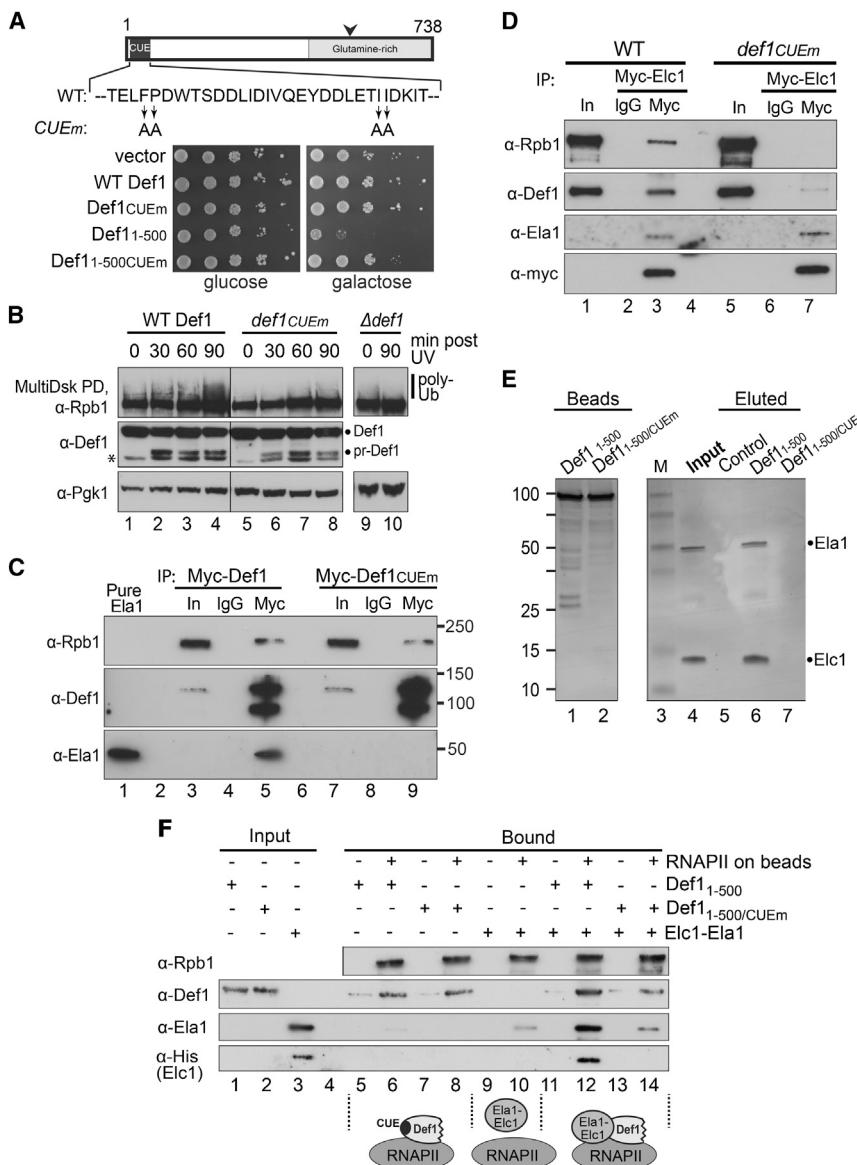


Figure 5. The CUE Domain of Def1 Is Essential for Rpb1 Polyubiquitylation

(A) Top: schematic of Def1 showing four key mutated residues of the CUE domain. Bottom: dilution series of Δ def1 cells (carrying the GAL-driven CEN plasmid indicated on the left), grown on glucose or galactose.

(B) Western blot of extracts from WT, $\text{def1}_{\text{CUEm}}$, or Δ def1 cells, UV-irradiated at time 0. Ubiquitylated Rpb1 isolated by MultiDsk pull-down. Asterisk denotes an additional band occasionally observed with the anti-Def1 antibody. Superfluous lanes between lanes 4 and 5 were removed.

(C) Western blot of Myc-Def1 and Myc-Def1_{CUEm}, immunoprecipitated from extracts prepared 1 hr after UV irradiation. Recombinant Ela1 is loaded as an antibody specificity control (lane 1).

(D) Western blot of Myc-Elc1 immunoprecipitated from chromatin-enriched extracts from WT or Def1_{CUEm} cells, prepared 1 hr after UV irradiation.

(E) Binding of recombinant Ela1-Elc1 heterodimer to immobilized, recombinant Def1₁₋₅₀₀ or Def1_{1-500/CUEm} (see also Figure S3A). Proteins were separated by SDS-PAGE and stained with silver. (F) Western blot analyzing binding of recombinant Ela1-Elc1 heterodimer to immobilized RNAPII (or empty control beads), in the absence or presence of recombinant Def1₁₋₅₀₀, or Def1_{1-500/CUEm}. Schematics of results are shown below the relevant lanes.

See also Figure S5.

sufficient for association with purified Def1₁₋₅₀₀ (Figure 6D, compare lane 5 with lanes 2 and 3).

These data prompted us to perform an alignment of ubiquitin with the Ela1 C-terminal domain required for Def1 binding (Figure S6B), which further supported the finding that Ela1 contains a UbH. Ela1₁₋₂₅₀ also interacted with Elc1 inside cells (Figure S6C) but failed to support damage-induced ubiquitylation (Figure 6E) and degradation of Rpb1, similar to a strain lacking

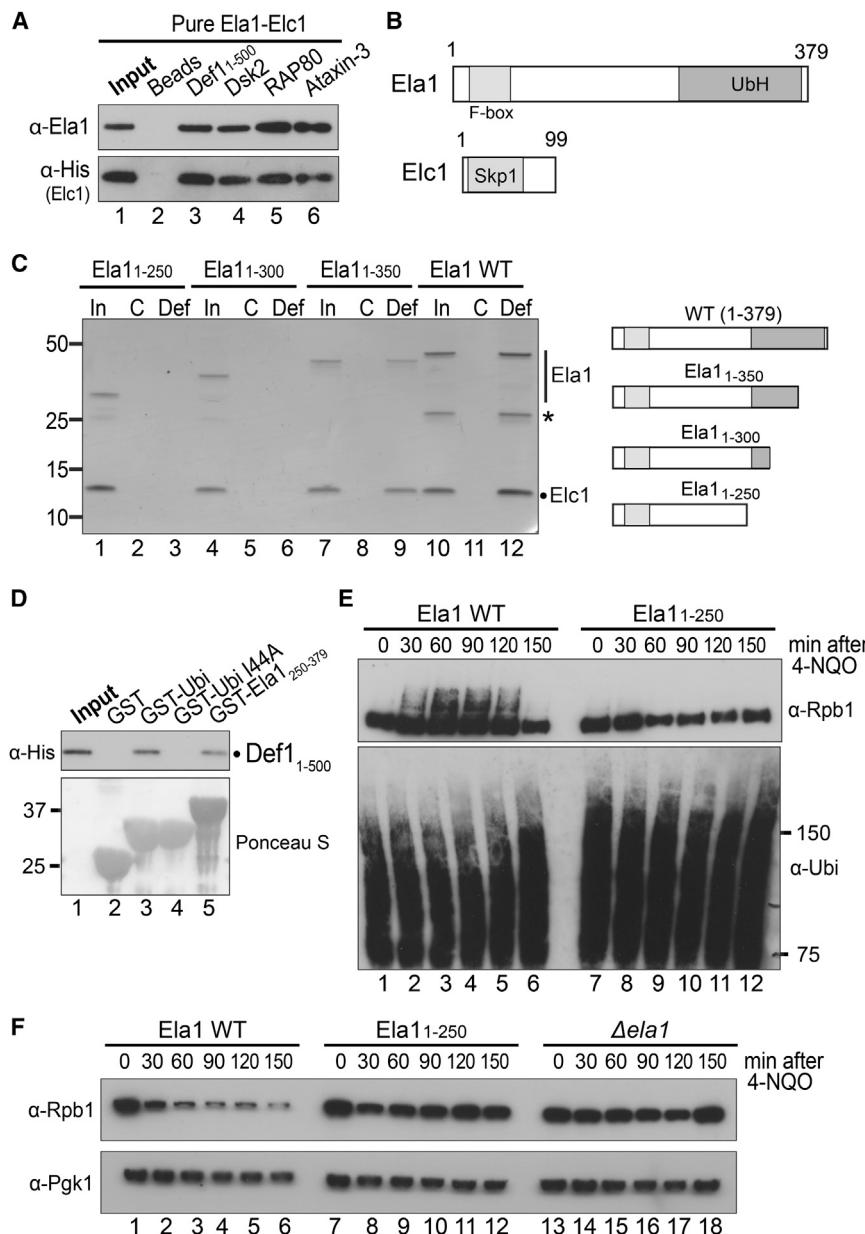
ELA1 altogether (Figure 6F, compare lanes 1–6 with 7–12 and 13–18, respectively), indicating that the UbH is important for cellular Ela1 function.

Together, these results indicate that Def1 bridges the interaction between RNAPII and the Elongin-Cullin ubiquitin ligase complex via an interaction between its CUE domain and a UbH in the Ela1 subunit of the Ela1-Elc1 adaptor complex.

DISCUSSION

The process leading to disassembly of RNAPII elongation complexes in response to transcription stress is remarkable by encompassing so many disparate ubiquitin-based events. For example, Def1 is partially processed, but not entirely degraded, via a ubiquitin-directed, proteasome-dependent mechanism.

Given that Elc1 is a very small, Skp1-like polypeptide (Figure 6B, lower), it seemed unlikely to contain a UbH. In contrast, Ela1 is significantly larger (upper), and the function of its C-terminal domain was unclear, prompting us to investigate this region. Constructs encoding different C-terminally truncated Ela1 forms (Figure 6C, schematic on right) were coexpressed with Elc1 in bacteria, and the resulting complexes purified and tested for their ability to bind Def1 immobilized on beads (Figure 6C, left). As expected from previous work (Koth et al., 2000), the different Ela1 forms were all capable of forming a complex with Elc1 (Figure 6C, lanes 1, 4, 7, and 10). However, Ela1-Elc1 complexes that only contained the first 250 or 300 amino acids of Ela1, respectively, were unable to bind Def1 beads (Figure 6C, compare lanes 3 and 6 with lanes 9 and 12). Moreover, the Ela1 C-terminal region was not only required, but also



Def1 also contains a ubiquitin-binding domain (UBD), but this domain does not have a ubiquitin-moiety as its primary target. Rather, it binds to a ubiquitin homology (UbH) domain in the Ela1 subunit of the yeast Elongin adaptor complex. This allows recruitment of the Elongin-Cullin E3 complex to RNAPII, which in turn results in Rpb1 becoming polyubiquitylated and degraded by the proteasome.

Proteasomal Processing and Activation of Def1

Several general principles of proteasome-mediated protein processing have emerged through the study of mammalian NF-κB proteins and their yeast homologs, Mga2 and Spt23 (Rape and Jentsch, 2002). For example, proteasomal processing

Figure 6. The CUE Domain of Def1 Binds Ela1-Elc1

(A) Western blot to detect binding of recombinant Ela1-Elc1 heterodimer to immobilized, recombinant Def₁₋₅₀₀ and Dsk2, or the ubiquitin-binding domains from Rap80 and Ataxin-3. See also Figures S3A and S6A.

(B) Schematic of Ela1 and Elc1, indicating relevant domains.

(C) Binding of recombinant Ela1-Elc1 heterodimers (Ela1 form schematically depicted on the right) to immobilized, recombinant Def₁₋₅₀₀ (Def), or control beads (C). Protein gel stained with silver. Asterisk indicates a contaminating protein from the purification of full-length Ela1-Elc1 complex.

(D) Western blot showing binding of purified, recombinant Def₁₋₅₀₀ to immobilized GST-fusion proteins, including GST-Ela1₂₅₀₋₃₇₉. Ponceau S stain of membrane shown as a loading control for GST-fusion proteins.

(E) MultiDsk pull-down, comparing damage-induced Rpb1 ubiquitylation in WT Ela1 (Ela1 WT) and cells lacking Ela1's UbH (ela1₁₋₂₅₀).

(F) As in (E), but comparing total Rpb1 levels after DNA damage.

typically involves ubiquitylation of the target protein, although not necessarily polyubiquitin chains: monoubiquitylation can be sufficient to elicit or speed up the reaction (Rape et al., 2001; Kravtsova-Ivansiv et al., 2009). Similar results are reported here: proteasomal Def1 processing is greatly stimulated by, but does not absolutely require, monoubiquitylation. Remarkably, the reaction can even be reconstituted with purified proteins: incubation of highly purified Def1 with highly purified proteasome results in correct processing. This suggests that all signals for this intriguing process may be intrinsic to the factors involved, e.g., that it does not require another protease to perform the initial “protein nicking,” or accessory factors to help stop the proteasome from completely degrading the target. The fully defined Def1 processing system should prove helpful in further defining the underlying mechanisms.

At the cellular level, our data are consistent with a model, in which Def1 constantly shuttles in and out of the nucleus, but is predominantly found in the cytoplasm at steady state; this is dependent on the C-terminal region of the protein. Def1 processing removes this region, allowing nuclear accumulation. As far as we know, Def1 represents the first example of a protein in which nuclear accumulation occurs as a result of regulated, proteolytic removal of a domain that directs cytoplasmic transport. We note that studies on proteins such as Huntingtin and Ataxin-7 have indicated that polyglutamine domain expansion

observed in disease-causing versions of these proteins affects subcellular localization (Nucifora et al., 2001; Chan et al., 2011). Further studies are needed to understand the details of Def1 subcellular transport, but it is an intriguing possibility that the extremely glutamine-rich regions in the C-terminal half of the protein also somehow regulate nucleocytoplasmic transport in the case of Def1.

Ubiquitylation and degradation of Rpb1 is used as a last-resort response to a variety of conditions, frequent or infrequent, that result in transcription stress (Bregman et al., 1996; Somesh et al., 2005; Sigurdsson et al., 2010; Hobson et al., 2012). Given that the C-terminal domain of Def1 promotes cytoplasmic localization, it is possible that Def1 normally shuttles in and out of the nucleus so that at least a small amount of Def1 is present in the nucleus at all times. In this scenario, the predominant cytoplasmic location of Def1 ensures that it is normally limiting for Rpb1 polyubiquitylation, helping to restrict Rpb1 degradation to a last resort solution to normal transcription elongation problems. Upon DNA damage or other genome-wide transcription stress, however, the protein is allowed to temporarily accumulate in the nucleus, so that RNAPII can be rapidly cleared and transcription restarted. Def1 ubiquitylation by Rsp5, as well as its processing by the proteasome, might occur in the nucleus, possibly even triggered at the stalled polymerase, but this remains to be determined.

Interestingly, Def1 is involved in a number of other, apparently unrelated cellular processes (Chen et al., 2005; Jordan et al., 2007; Suzuki et al., 2011), opening the possibility that other pathways might also rely on Def1 for their proper activation or inactivation in response to DNA damage and other stress. We note that the Def1 pathway has interesting parallels with the bacterial SOS response, as well as with metazoan apoptosis. In the bacterial SOS response, DNA damage results in the RecA-dependent autocatalytic clipping of the LexA repressor protein, resulting in upregulation of repair genes and allowing mutation-prone survival in the face of severe DNA damage (Schlacher and Goodman, 2007). In mammalian cells, severe DNA damage can elicit apoptosis by triggering the protease (caspase)-dependent cleavage and inactivation of ICAD/DFF45 to allow the nuclease CAD to enter the nucleus and fragment the DNA (Nagata, 2000). Proteolytic processing-dependent release of protein fragments, whose activity needs to be restricted to a severe cellular crisis situation, may thus have been utilized several times during evolution.

The Multiple Steps of Rpb1 Ubiquitylation and Degradation

Rpb1 ubiquitylation and degradation occurs via a complex, multistep mechanism (Wilson et al., 2013). In the initial step, Rpb1 is monoubiquitylated by Rsp5, before another E3, the Elongin-Cullin complex, takes over to perform polyubiquitylation (Figure 7, steps 1 and 4, respectively). Our data show how Def1 fits into this complex scheme: it is activated by Rsp5-mediated ubiquitylation, which allows its proteasomal processing and nuclear accumulation (step 2), so that Def1 can bind RNAPII and help recruit the Elongin-Cullin complex, promoting efficient polyubiquitylation (steps 3 and 4). Intriguingly, Def1 contains a CUE domain (UBD), shown here to be essential for this function. Interestingly, this domain is not used to bind the monoubiquity-

lated polymerase, but rather to recruit the Elongin-Cullin complex. Indeed, we found that the Ela1 subunit of the Elongin adaptor complex harbors a UbH. In humans, the related Elongin A complex comprises three subunits, Elongin A, B, and C (Aso et al., 1995). Budding yeast only has homologs of Elongin A (Ela1) and Elongin C (Elc1) (Koth et al., 2000). Remarkably, the small human Elongin B protein, which lacks a yeast homolog, contains a UbH (Garrett et al., 1995). Given that yeast Ela1 harbors a UbH in its C terminus (that is not conserved in mammalian Elongin A), it is an obvious possibility that sequences encoding this domain have become separated onto a separate gene, encoding Elongin B, during evolution. Our data provide evidence for an important role for this UbH in protein ubiquitylation. More specifically, we show that Elongin's UbH plays a role in mediating target recognition, rather than, for example, mediating proteasome delivery as has previously been hypothesized (Welchman et al., 2005). We note that, as is true for all proteins containing a ubiquitin-binding domain or a ubiquitin-homology domain (Hofmann, 2009), binding specificity must occur through combination with other recognition domains and motifs in Def1 and the Elongin-Cullin complex.

The Elongin complex is akin to the Skp1/F-box proteins of other Cullin-ligases (Koth et al., 2000). These proteins are substrate-specific adaptors, which mediate correct target recognition. Ela1/Elc1 complex is unusual in that this adaptor does not actually recognize its target protein, RNAPII, but rather the bridging factor Def1, which then connects it to the target (Figure 7B). The APC/C complex, a Cullin-like ligase, employs distinct substrate-specificity factors (e.g., Cdc20 and Cdh1), but these are integral components of the complex in specific phases of the cell cycle (Bassermann et al., 2013). In contrast, Def1, to the best of our knowledge, represents the first example of a discrete bridging factor being required for target recognition by a Cullin E3 ligase. Whether substrate-specific bridging factors are required for the diverse functions of Elongin-Cullin complex(es), and for Cullin-type E3 ligases in general, is an intriguing possibility that deserves further investigation.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids

Tables S1 and S2 describe *S. cerevisiae* strains and plasmids. Standard techniques were employed for their construction.

In Vivo Techniques

Whole-cell extracts were prepared by alkaline extraction (Kushnirov, 2000) or via bead-beating, with ubiquitylated proteins being isolated using MultiDsk resin (Wilson et al., 2012). A list of antibodies used in this study can be found in Table S3.

In Vitro Assays

Def1 ubiquitylation assays were performed essentially as described for RNAPII ubiquitylation (Somesh et al., 2005). Binding experiments were performed largely as described (Anindya et al., 2010). Reconstituted proteasome assays were performed with purified yeast proteasome (10 nM) and Def1 (1.5 μM) in reactions with and without MG132 (10 μM).

Fluorescence microscopy was carried out on live cells or in formaldehyde-fixed cells (Figures 4E and S4B). Deltavision microscopy was used to visualize yeast using an X100 UplanSApo 1.40 NA oil objective lens on an Olympus inverted microscope (IX71).

For further details, please see the Extended Experimental Procedures.

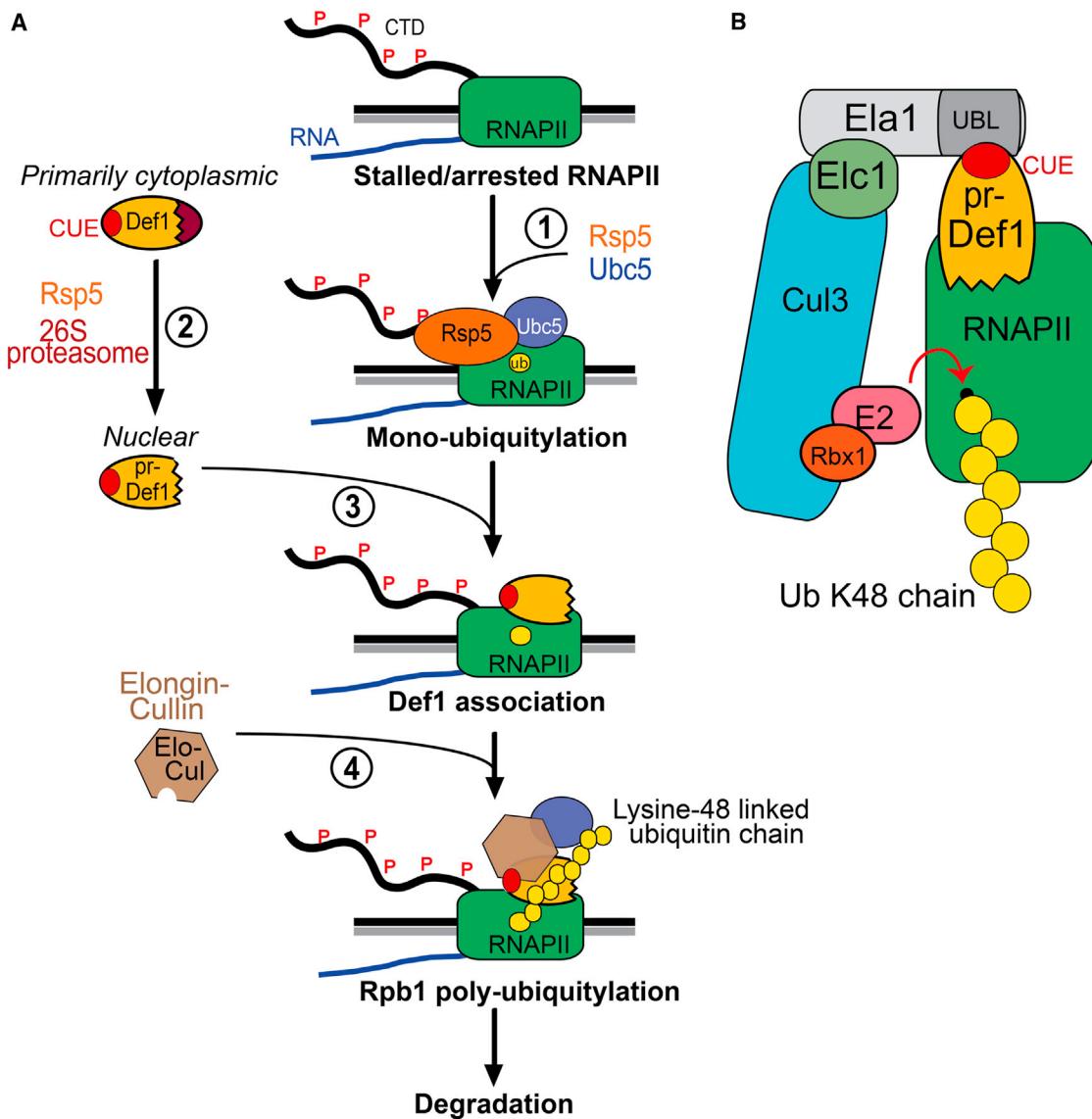


Figure 7. Model for Def1 Function

(A) Model for Def1-dependent polyubiquitylation of Rpb1. (1) Rsp5 monoubiquitylates Rpb1. (2) Concurrently, Rsp5 ubiquitylates Def1, resulting in proteasomal processing and nuclear accumulation. (3) Nuclear pr-Def1 binds RNAPII, (4) recruiting Elongin-Cullin complex to carry out Rpb1 polyubiquitylation. See text for details. (B) Schematic depicting the bridging function of Def1. Ela1-Elc1 complex may also have weak Def1-independent interactions with RNAPII, as suggested by the findings of Figure 5F.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2013.07.028>.

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