

# The TFIIH Subunit Tfb3 Regulates Cullin Neddylolation

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DOI 10.1016/j.molcel.2011.05.032

## SUMMARY

Cullin proteins are scaffolds for the assembly of multisubunit ubiquitin ligases, which ubiquitylate a large number of proteins involved in widely varying cellular functions. Multiple mechanisms cooperate to regulate cullin activity, including neddylation of their C-terminal domain. Interestingly, we found that the yeast Cul4-type cullin Rtt101 is not only neddylated but also ubiquitylated, and both modifications promote Rtt101 function in vivo. Surprisingly, proper modification of Rtt101 neither correlated with catalytic activity of the RING domain of Hrt1 nor required the Nedd8 ligase Dcn1. Instead, ubiquitylation of Rtt101 was dependent on the ubiquitin-conjugating enzyme Ubc4, while efficient neddylation involves the RING domain protein Tfb3, a subunit of the transcription factor TFIIH. Tfb3 also controls Cul3 neddylation and activity in vivo, and physically interacts with Ubc4 and the Nedd8-conjugating enzyme Ubc12 and the Hrt1/Rtt101 complex. Together, these results suggest that the conserved RING domain protein Tfb3 controls activation of a subset of cullins.

## INTRODUCTION

Nedd8 (Rub1 in yeast) is the closest relative to ubiquitin and functions as a posttranslational protein modifier (Rabut and Peter, 2008). The best-characterized Nedd8 substrates are cullins, a family of proteins that serve as scaffolds for the assembly of multisubunit ubiquitin ligases (E3s). Cullin-based E3s interact with various substrate specificity factors to regulate diverse cellular processes including cell-cycle progression, genome integrity, and signal transduction (Petroski and Deshaies, 2005; Pintard et al., 2004). Neddylation of cullins occurs on a conserved lysine residue in their C-terminal domain (CTD), which triggers a structural reorganization that not only initiates the assembly of E3 complexes but also activates ubiquitin-transfer to the

bound substrate (Duda et al., 2008; Saha and Deshaies, 2008; Yamoah et al., 2008).

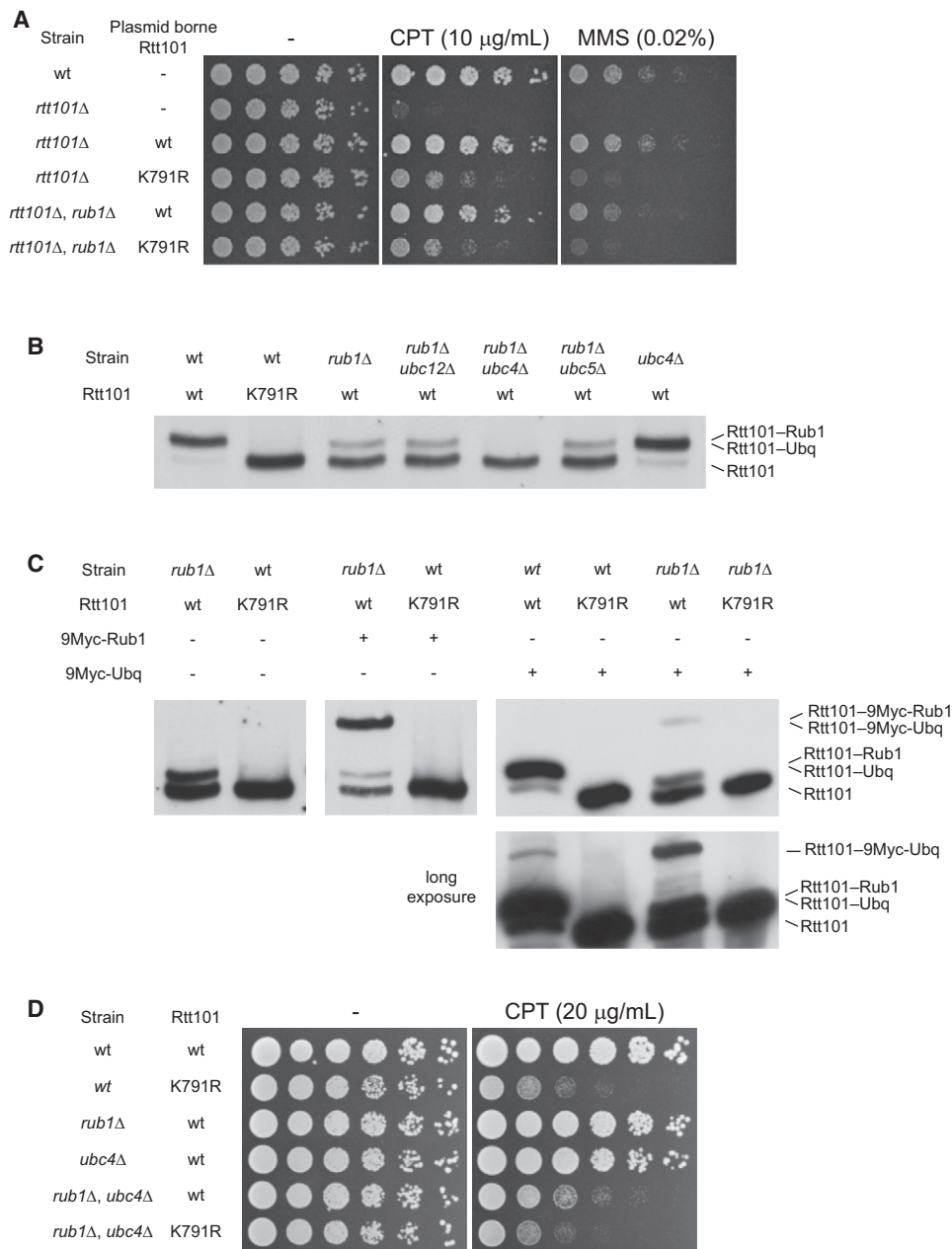
Owing to the importance of neddylation in the control of cullin activity, it is critical to understand how this modification is regulated. The conserved CTD of cullins tightly interacts with a RING domain protein termed Hrt1 or Rbx1, which in turn recruits ubiquitin-loaded conjugating enzymes (E2s) and constitutes the catalytic core of cullin-based E3s (Seol et al., 1999; Skowyra et al., 1999). Hrt1/Rbx1 also functions as an E3 toward the Nedd8-conjugating enzyme Ubc12 and mediates cullin neddylation in vitro (Scott et al., 2010, and references therein). However, Hrt1/Rbx1 is not sufficient to regulate cullin neddylation in vivo. For instance, the yeast Dcn1 protein and its homologs are required for efficient neddylation of various cullins in different organisms (Kim et al., 2008; Kurz et al., 2005). Dcn1 directly interacts with the CTD of the Cul1 ortholog Cdc53 (Kurz et al., 2008) and orients Ubc12 to promote Hrt1-mediated Cdc53 neddylation (Scott et al., 2010). Moreover, the human Dcn1 homolog DCNL3 recruits Cul3 to the plasma membrane and stimulates its neddylation (Meyer-Schaller et al., 2009), suggesting that Dcn1 family proteins promote cullin neddylation and activity at specific cellular localizations or in response to specific signals.

Here, we further characterized the mechanisms regulating cullin neddylation. Unlike Cdc53, we found that the yeast Cul4-type cullin Rtt101 is not only neddylated but also ubiquitylated in vivo on the same lysine residue. Surprisingly, these modifications do not require Dcn1 or fully functional Hrt1, but depend on the RING domain of Tfb3, a conserved subunit of the TFIIH complex involved in transcription initiation and nucleotide excision repair. In addition to Rtt101, Tfb3 also regulates neddylation and activity of Cul3, but not Cdc53. These results indicate that multiple pathways cooperate to regulate the activity of distinct cullins.

## RESULTS

### Neddylation and Ubiquitylation Activate Rtt101

Rtt101 function is required for yeast survival upon DNA-replication stress induced by genotoxic drugs such as camptothecin (CPT, an inhibitor of topoisomerase 1) or methyl methanesulfonate (MMS, a DNA methylating agent) (Luke et al., 2006). To dissect how neddylation of its conserved C-terminal lysine



**Figure 1. Neddylaton and Ubiquitylation of Lysine 791 Activate Rtt101**

(A) Rtt101 lysine 791 but not neddylation is required for yeast resistance to replication stress. Equivalent amounts of the indicated yeast strains were spotted in 5-fold serial dilutions onto YPD plates supplemented as indicated with MMS or CPT. More details about the yeast strains used in each figure panel can be found in the [Supplemental Experimental Procedures](#).

(B) The Rub1-independent modification of Rtt101 depends on Ubc4. Immunoblot of total extracts prepared from yeast strains with either wild-type (wt) or the K791R mutant of tagged Rtt101 expressed from the endogenous promoter. See also [Figure S1](#).

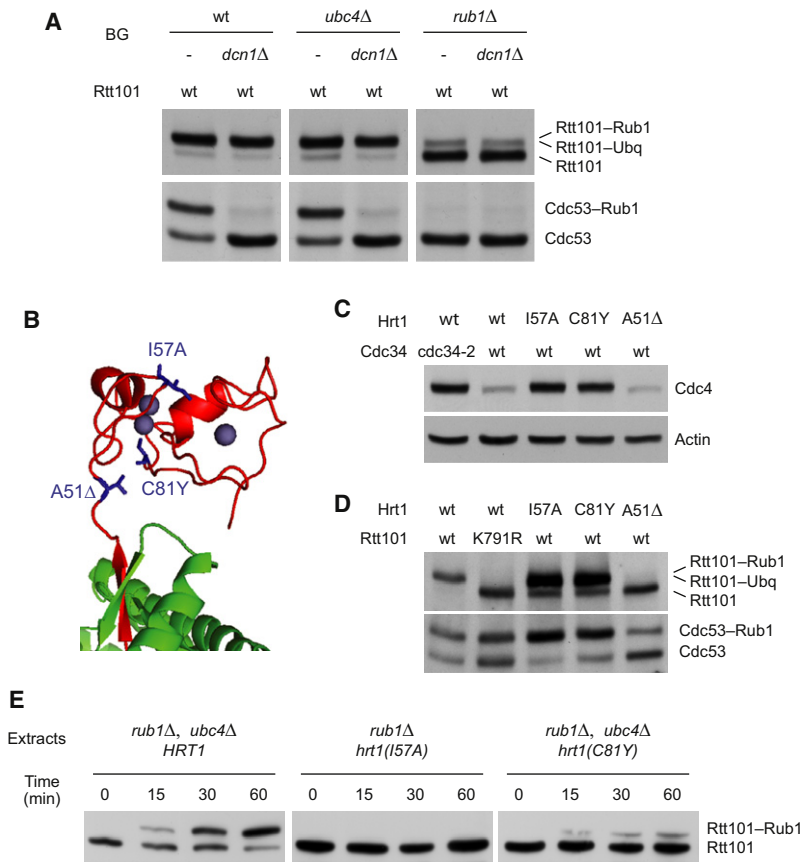
(C) Rtt101 is ubiquitylated in a K791-dependent manner. Immunoblot showing the modification of Rtt101 in cells expressing 9Myc-tagged ubiquitin or 9Myc-tagged Rub1.

(D) Neddylation and ubiquitylation redundantly activate Rtt101. Equivalent amounts of the indicated yeast strains were spotted in 5-fold serial dilutions onto YPD plates, supplemented with 20  $\mu$ g/ml CPT.

See also [Figure S1](#) and [Tables S1](#) and [S2](#).

791 (K791) contributes to Rtt101 function, we assayed the resistance of yeast mutants to CPT and MMS ([Figure 1A](#)). In contrast to wild-type, a Rtt101 mutant in which K791 was changed to an

arginine (K791R) was only partially able to complement the phenotype of *RTT101* inactivation. Surprisingly however, the CPT and MMS sensitivity of *rub1* $\Delta$  cells was comparable to



**Figure 2. Rtt101 Modification in *DCN1* and *HRT1* Mutant Cells**

(A) Rtt101 neddylation and ubiquitylation is not impaired in *dcn1Δ* cells. Immunoblots showing the modification of Rtt101 and Cdc53 in the indicated yeast strains.

(B) Position of I57A, C81Y and A51Δ mutations depicted on the structure of Rbx1 (red) in complex with neddylated Cul5 CTD (green) (DOI:10.2210/pdb3dq/pdb).

(C) Substrate ubiquitylation is impaired in *hrt1(C81Y)* and *hrt1(I57A)* cells. Immunoblot showing the steady state-level of Cdc4 in the indicated yeast strains. Actin was used to control equal loading. See also Figure S2.

(D) Rtt101 and Cdc53 modification is not impaired in *hrt1(C81Y)* and *hrt1(I57A)* cells. Immunoblot showing the modification of Rtt101 and Cdc53 in the indicated yeast strains. Actin was used to control equal loading.

(E) Kinetics of Rtt101 neddylation in vitro. Immunoblots showing Rtt101 modification in crude extracts after addition of recombinant Rub1 and ATP. See also Figure S2 and Tables S1 and S2.

mutants was comparable to Rtt101(K791R)-expressing cells. These results indicate that either neddylation or ubiquitylation of Rtt101 can activate its function in vivo.

**Modification of Cdc53 and Rtt101 In Vivo Do Not Require Fully Active Hrt1**

The observation that Rtt101 can be ubiquitylated in vivo suggests that the modification of this cullin involves specific mechanisms.

wild-type controls, indicating that K791, but not neddylation, is required for proper Rtt101 function. Indeed, while Rtt101(K791R) appeared as a single band by western blot, two forms of Rtt101 could readily be detected even in the absence of Rub1 or Ubc12 (Figure 1B), implying that Rtt101 is not only neddylated but also subject to another modification which similarly depends on K791 (Laplaza et al., 2004). Interestingly, the second modification of Rtt101 is drastically (but not fully) reduced in strains deleted for the ubiquitin-E2 Ubc4, but not its closest homolog Ubc5 (Figure 1B and Figure S1 available online), suggesting that Rtt101 may also be ubiquitylated in vivo. To determine whether ubiquitin can indeed be conjugated to K791, we used plasmids expressing 9Myc-tagged ubiquitin (9Myc-Ubq) or control 9Myc-Rub1. Both constructs were conjugated to wild-type Rtt101 but not to Rtt101(K791R) (Figure 1C). Conjugation of 9Myc-Ubq to Rtt101 was observed in wild-type cells, although to a lesser extent than in *rub1Δ* cells (Figure 1C), indicating that Rtt101 ubiquitylation occurs even in the presence of Rub1. Altogether, these results imply that Rtt101-K791 can be specifically ubiquitylated in a Ubc4-dependent manner.

To determine whether ubiquitylation activates Rtt101, we assayed the involvement of Ubc4 in DNA-replication stress. While both *rub1Δ* and *ubc4Δ* cells are resistant to CPT (Figure 1D), *rub1Δ ubc4Δ* double mutants were almost as sensitive to replication stress as Rtt101(K791R)-expressing cells. This sensitivity phenotype is most likely due to the loss of Rtt101 modification as the CPT sensitivity of *rub1Δ ubc4Δ rtt101(K791R)* triple

Indeed, while Dcn1 is required for efficient neddylation of Cdc53 (Kurz et al., 2005) (Figure 2A), we observed that neither neddylation nor ubiquitylation of Rtt101 were altered in *dcn1Δ* cells (Figure 2A). As Dcn1 is required to position Hrt1 to promote Cdc53 neddylation (Scott et al., 2010), we next tested whether Hrt1 is required for Rtt101 modification in vivo. As *HRT1* is essential, we used three distinct point mutants of Hrt1 that differentially affect its activity (Figure 2B). First, we mutated a conserved isoleucine (I57A) located in the RING interface with E2s. Corresponding mutations in Hrt1 orthologs considerably reduce neddylation of cullins in vitro (Huang et al., 2009). Second, we mutated a conserved cysteine (C81Y) that coordinates one of the zinc ions that stabilize the RING fold, thereby greatly reducing the turnover of several Cdc53 substrates such as Cin2 (Blondel et al., 2000). Third, we deleted an alanine (A51Δ) that lies within a loop at the junction between the RING domain of Hrt1 and the beta strand that anchors Hrt1 to cullin CTD. While this mutation should not affect the interaction between the Hrt1 RING domain and E2s, it alters the conformation of the cullin/Hrt1 dimer. The corresponding mutation in human Rbx1 severely inhibits in vitro neddylation of Cul1 CTD, but does not affect in vitro ubiquitylation of model substrates (Duda et al., 2008). Consistent with published observations, the efficiency of Rtt101 neddylation in vitro in crude protein extracts prepared from *hrt1(I57A)* and *hrt1(C81Y)* strains was strongly reduced (Figure 2E). We also examined the effect of these mutations on Cdc4, an F-box protein that interacts with

Cdc53 and is constitutively ubiquitylated and degraded throughout the cell cycle (Galan and Peter, 1999). In contrast to the A51 $\Delta$  loop mutant, I57A and C81Y RING mutants strongly impaired the turnover of Cdc4, resulting in its accumulation (Figure 2C and Figure S2). Similar results were observed with Cln2 (Figure S2), confirming that the I57A and C81Y mutations strongly impair Hrt1 catalytic activity. Surprisingly however, Rtt101 and Cdc53 accumulated in their modified forms in these mutants (Figure 2D). In contrast, the catalytically active A51 $\Delta$  mutant was barely able to modify Rtt101, and Cdc53 neddylation was reduced (Figure 2D). Thus, while Hrt1 is required for cullin neddylation in vitro, the steady-state level of Rtt101 and Cdc53 neddylation in intact cells is not correlated with Hrt1 catalytic activity. This implies that additional mechanisms must exist to ensure efficient cullin neddylation in vivo.

### Rtt101 and Cul3 Modifications Are Regulated by the RING Domain Protein Tfb3

As neither Dcn1 nor Hrt1 catalytic activity is sufficient to control Rtt101 modification in vivo, we decided to search for other regulator(s) of Rtt101 ubiquitylation and/or neddylation. A genetic screen did not identify such factors in a collection of 4308 nonessential yeast genes (see the Supplemental Experimental Procedures). We therefore examined whether essential genes with characteristic E3 features would regulate the modifications of Rtt101. Interestingly, we found that a mutation in the RING domain of Tfb3 (C16Y) significantly reduced Rtt101 neddylation and, to a lesser extent, ubiquitylation in vivo (Figure 3A). Likewise, Rtt101 neddylation in vitro was strongly impaired in protein extracts prepared from *tfb3(C16Y)* mutant cells (Figure 3B). While neddylation of Cdc53 was only slightly diminished in *tfb3(C16Y)* cells (Figure 3A), neddylation of Cul3 required both Dcn1 and intact Tfb3, both in vivo and in vitro (Figures 3D and 3E). Interestingly, residual modification of Cul3 was detectable in both *dcn1 $\Delta$*  and *tfb3(C16Y)* strains but not in the double mutant (Figure 3D). In contrast, the residual modification of Rtt101 in the *tfb3(C16Y)* strain was not due to Dcn1 and, conversely, the residual neddylation of Cdc53 in the *dcn1 $\Delta$*  strain was not dependent on Tfb3 (Figure S3). Taken together, these results demonstrate that Tfb3 is a regulator of cullin neddylation and imply that different cullins are activated by distinct mechanisms in vivo.

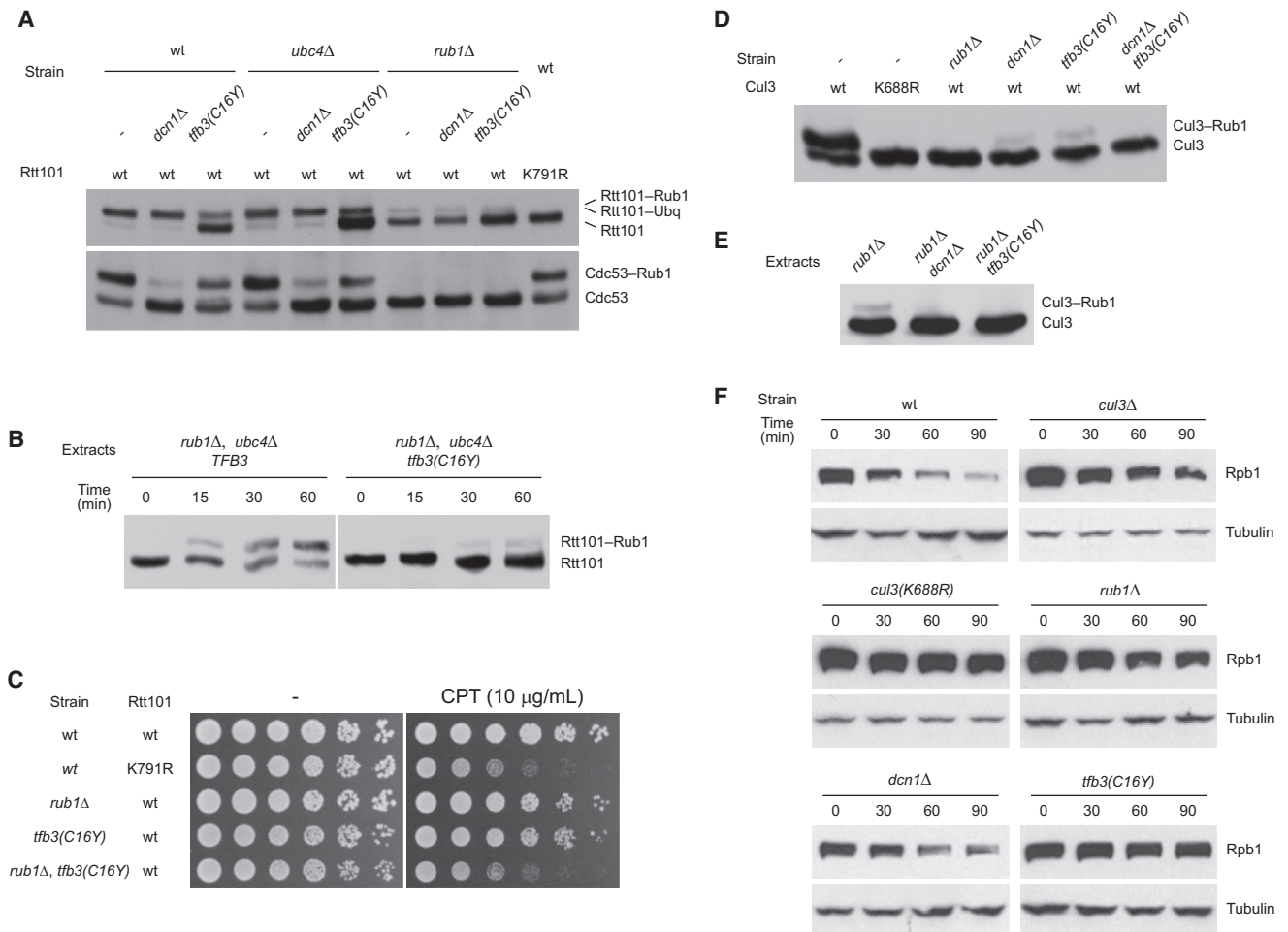
We next asked whether Tfb3 is functionally linked to Rtt101 and Cul3. Interestingly, deletion of *RUB1* strongly sensitized *tfb3(C16Y)* cells to CPT (Figure 3C), indicating that, like Rtt101, Tfb3 contributes to the cellular resistance against DNA-replication stress. After ultraviolet (UV) damage, Cul3 ubiquitylates the RNA polymerase II subunit Rpb1, which triggers its degradation by 26S proteasomes (Verma et al., 2011, and references therein). As expected, Rpb1 degradation was compromised in cells harboring nonneddylatable Cul3 [Cul3(K688R)] as well as in *rub1 $\Delta$*  cells (Figure 3F), demonstrating that Cul3 neddylation stimulates Rpb1 ubiquitylation. Interestingly, Rpb1 degradation upon UV damage was strongly impaired in *tfb3(C16Y)* cells, while deletion of *DCN1* had little or no effect (Figure 3F). Together, these results suggest that Tfb3 promotes Rtt101 neddylation to regulate DNA-replication stress and that Tfb3 is needed for Cul3 neddylation, which promotes Rpb1 degradation upon UV damage.

### Tfb3 Probably Regulates the Conjugation Step of Cullin Neddylation

Tfb3 is part of TFIIF, a well characterized complex involved in transcription initiation and nucleotide excision repair (NER) (Mylkova et al., 2010). To determine whether these functions of TFIIF are involved in Rtt101 regulation, we examined Rtt101 modification in various TFIIF mutants with impaired transcription or NER activities. For instance, *ccl1-ts4*, *kin28-ts*, *rad3-14*, and *ss2-ts* mutants severely reduce general transcription activity (Guzder et al., 1994; Lee and Lis, 1998; Qiu et al., 1993; Valay et al., 1995, 1996), while *tfb1-1*, *tfb5 $\Delta$*  or *rad2 $\Delta$*  mutants exhibit NER defects (Habraken et al., 1996; Matsui et al., 1995; Ranish et al., 2004). However, none of these mutations affected the total level of K791 modification or ubiquitylation (Figure 4A and Figure S4A). Furthermore, previously published microarray experiments showed that general transcription is not impaired in *tfb3(C16Y)* cells (Jona et al., 2002). Together, these data indicate that the defect of Rtt101 modification in *tfb3(C16Y)* cells is unlikely to be caused by general transcription or NER defects.

Loss of Rtt101 neddylation in *tfb3(C16Y)* cells could be due to decreased activity of neddylation activator(s) or due to increased activity of neddylation inhibitor(s). Currently, the only known inhibitors of cullin neddylation in yeast are the COP9 signalosome (CSN), a protein complex that catalyzes cullin deneddylation (Cope et al., 2002), and Lag2, an inhibitor of Cdc53 that blocks access to its neddylation site (Liu et al., 2009; Siergiejuk et al., 2009). If one of these factors was activated in *tfb3(C16Y)* cells, its inactivation should restore Rtt101 neddylation. However, neither deletion of *RR11*, the catalytic subunit of the CSN, nor deletion of *LAG2* rescued Rtt101 modification in *tfb3(C16Y)* cells (Figure 4B). Furthermore, in vitro neddylation of tagged Rtt101 was not significantly inhibited by a 4-fold excess of protein extract prepared from *tfb3(C16Y)* cells (Figure S4B), excluding the possibility that *tfb3(C16Y)* cells contain a *trans*-inhibitor of Rtt101 neddylation. We conclude that Tfb3 directly or indirectly activates Rtt101 neddylation and ubiquitylation.

The fact that Cdc53 neddylation was almost normal in *tfb3(C16Y)* cells (see Figures 3A and 4B and Figure S3) indicates that Tfb3 functions downstream of Ubc12. Indeed, Ubc12 was correctly expressed and efficiently charged with Rub1 in *tfb3(C16Y)* cells (Figure 4C). To examine whether the RING domain of Tfb3 could function as a Rub1 or ubiquitin-E3, we probed its interaction with E2s using recombinant proteins. Both GST-tagged Ubc12 and Ubc4, but not Rad6, were able to bind Hrt1 and the RING domain of Tfb3 (Figure 4D). In contrast, the RING domain of Ssl1—another TFIIF subunit previously reported to function as a ubiquitin-E3 (Takagi et al., 2005)—was preferentially precipitated by Ubc4 (Figure 4D). To determine whether Tfb3 also physically interacts with the Rtt101/Hrt1 complex, we performed GST pull-down experiments. As shown in Figure 4E, recombinant GST-Hrt1, either alone or in complex with the Rtt101 CTD, was able to specifically retain tagged Tfb3 in yeast extracts. Interestingly, a 4-fold excess of protein extract containing wild-type Tfb3 was not able to significantly rescue neddylation of tagged Rtt101 from *tfb3(C16Y)* extracts (Figure S4C). Together, these results suggest that the RING-finger protein Tfb3 regulates Rtt101



**Figure 3. Tfb3 Controls Cul3 and Rtt101 Modification and Contributes to Their Function**

(A) Rtt101 modification is impaired in *tfb3(C16Y)* cells. Immunoblots showing the modification of Rtt101 and Cdc53 in the indicated yeast strains. See also Figure S3.

(B) Kinetics of Rtt101 neddylation in *tfb3(C16Y)* extracts. Immunoblots showing Rtt101 modification in crude extracts after addition of recombinant Rub1 and ATP.

(C) Tfb3 contributes to the cellular resistance to replication stress. Equivalent amounts of the indicated yeast strains were spotted in 5-fold serial dilutions onto YPD plates, supplemented with 10 μg/ml CPT.

(D) Cul3 neddylation is impaired in *dcn1Δ* and *tfb3(C16Y)* cells. Immunoblot showing Cul3 neddylation in the indicated yeast strains.

(E) Cul3 neddylation in vitro. Immunoblot showing Cul3 modification in crude extracts 1 hr after addition of recombinant Rub1 and ATP.

(F) Tfb3 is needed for Rpb1 degradation after UV irradiation. Immunoblots showing Rpb1 levels after UV irradiation in the indicated yeast strains. Tubulin was used to control equal loading.

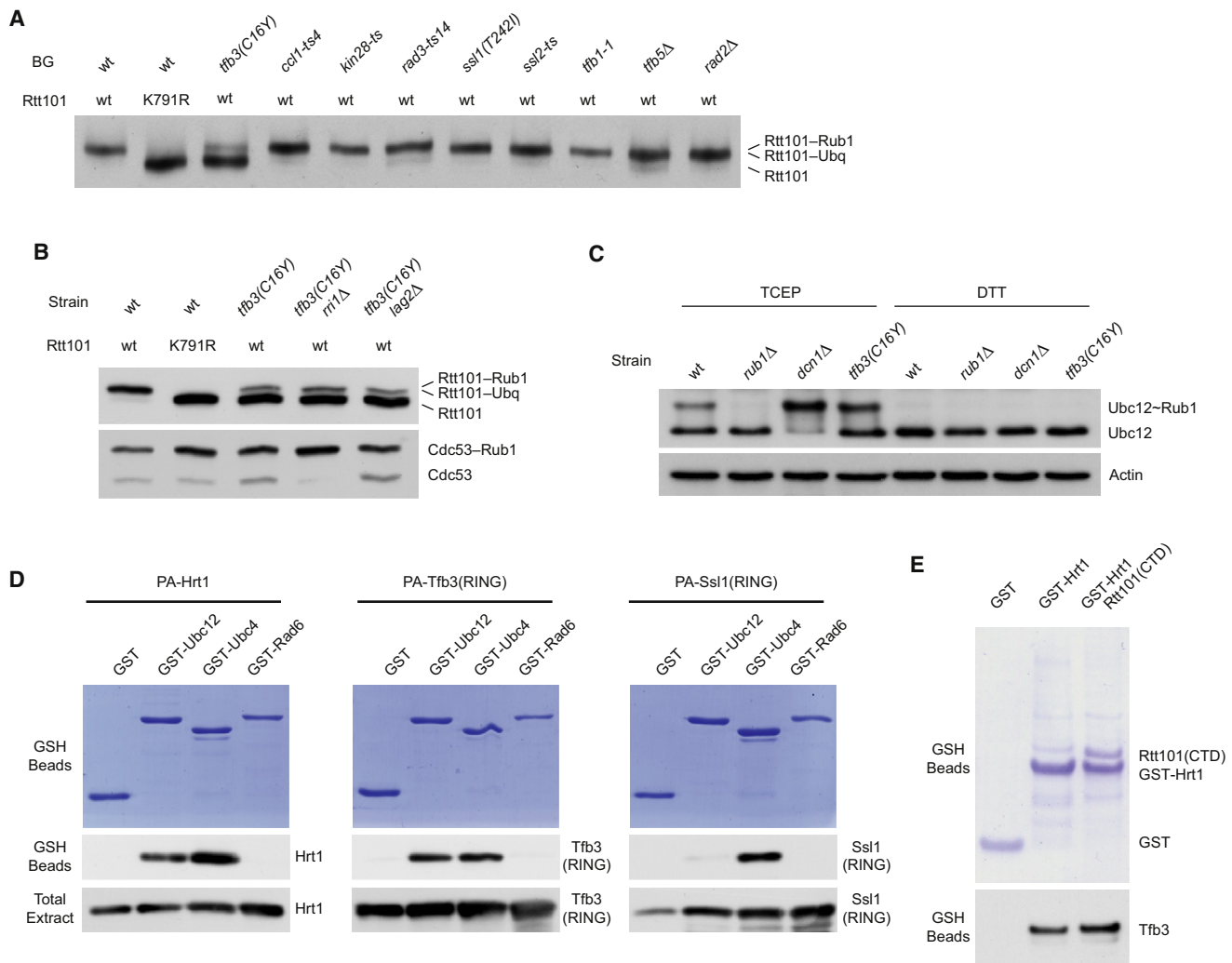
See also Figure S3 and Tables S1 and S2.

modification in “cis” and may promote Rtt101 neddylation and ubiquitylation through the interaction of its RING domain with E2 conjugating enzymes.

## DISCUSSION

Our finding that ubiquitylation of lysine 791 activates the yeast cullin Rtt101 in vivo reveals an unexpected mode of cullin regulation. As ubiquitylation of this residue is functionally redundant with its neddylation, both modifications may share a common activation mechanism. The recently solved structure of the neddylated CTD of Cul5 demonstrated that neddylation triggers

a conformational switch that favors substrate ubiquitylation (Duda et al., 2008; Saha and Deshaies, 2008; Yamoah et al., 2008). In this structure, Cul5 interacts with several residues of Nedd8 that are conserved in ubiquitin (Duda et al., 2008), including Leu8 and Ile44, that are important to promote Cul1 activity in vitro (Sakata et al., 2007). It is therefore likely that ubiquitylation of Rtt101 triggers a similar conformational switch as neddylation, and ubiquitylation of Cul1 can indeed activate substrate ubiquitylation in vitro (Duda et al., 2008). It will therefore be important to determine whether cullins other than Rtt101 can also be regulated by ubiquitylation in vivo and whether cullin ubiquitylation and neddylation can be independently regulated.



**Figure 4. Tfb3 Functions Downstream of Ubc12 and Interacts with Ubc12, Ubc4, and the Rtt101/Hrt1 Complex**

(A) Mutations in TFIIH subunits other than Tfb3 do not impair Rtt101 modification. Immunoblots showing the modification of Rtt101 in the indicated yeast strains. See also Figure S4A.

(B) Rri1 and Lag2 do not inhibit Rtt101 modification in *tfb3(C16Y)* cells. Immunoblots showing the modification of Rtt101 and Cdc53 in the indicated yeast strains.

(C) Ubc12 is properly charged with Rub1 in *tfb3(C16Y)* cells. Immunoblots of DTT- or TCEP-treated total extracts showing the amount of Rub1 charged Ubc12 (Ubc12~Rub1) in the indicated yeast strains. Contrary to TCEP, DTT hydrolyses the thioester bond of Ubc12 charged with Rub1. Actin was used to control equal loading.

(D) The RING domain of Tfb3 interacts with Ubc12 and Ubc4. Protein A-tagged Hrt1 (PA-Hrt1), and the RING-domains of Tfb3 [PA-Tfb3(RING)] or Ssl1 [PA-Ssl1(RING)] were expressed in *E. coli* together with GST, GST-Ubc12, GST-Ubc4, or GST-Rad6. Protein extracts prepared from these cells were incubated with glutathione beads (GSH beads) to precipitate GST-tagged E2 as well their binding partners. Proteins bound to GSH beads were visualized by Coomassie staining (top) and immunoblotting (middle). An aliquot of the extracts prior to precipitation was included for control (total extract, bottom).

(E) Hrt1 and the Rtt101/Hrt1 complex physically interact with Tfb3. Recombinant GST, GST-Hrt1 and a complex of GST-Hrt1 and the Rtt101 CTD was immobilized on glutathione beads (GSH-beads), and incubated with extracts prepared from yeast cells expressing protein A-tagged Tfb3. Proteins bound to GSH beads were detected by Coomassie staining (top) and immunoblotting (bottom).

See also Figure S4 and Tables S1 and S2.

Since Hrt1/Rbx1 neddylates cullins in vitro, it is generally assumed that this protein also governs cullin neddylation in vivo. Surprisingly, our results show that catalytic mutants of Hrt1 greatly impair cullin neddylation in vitro but not in vivo. To explain this discrepancy, we propose that Hrt1-dependent neddylation observed in vitro may reflect a basal activity of Hrt1, while intact cells require additional factors such as Dcn1

and Tfb3 to ensure efficient neddylation of specific cullins. Indeed, Tfb3 is needed for efficient neddylation of Cul3 and for neddylation and ubiquitylation of Rtt101, two yeast cullins regulating DNA-associated processes. Tfb3 is an essential subunit of the TFIIH complex, which mediates transcription initiation and is also involved in nucleotide excision repair (NER) (Mydlikova et al., 2010). Because we have not found

other mutations in TFIH subunits that affect Rtt101 modification, it is unlikely that activation of Rtt101 and Cul3 neddylation is linked to TFIH function in transcription initiation or NER. However, we currently do not know whether TFIH integrity is needed for modifying Rtt101 and Cul3 in vivo. As Tfb3 functions downstream of Ubc12 and contains a RING domain that is characteristic of E3s, it is tempting to speculate that Tfb3 itself may function as an E3 for a subset of cullins. In agreement with this model, we observed that Tfb3 interacts with Ubc12 and Ubc4 and also binds Rtt101 in complex with Hrt1. Alternatively, Tfb3 may promote cullin modification like Dcn1 by stimulating Hrt1-mediated cullin neddylation. Finally, it is possible that Tfb3 does not act at the modification step per se, but may rather alter the cullin conformation to a “neddylatable” state. Consistent with this mechanism, we were unable to rescue Rtt101 neddylation in extracts prepared from *tfb3(C16Y)* cells with wild-type Tfb3.

Irrespective of the precise molecular mechanism of Tfb3 function, our data demonstrate that Tfb3 and Dcn1 differentially regulate cullin modification. While efficient Rtt101 modification only requires Tfb3, Cul3 neddylation requires both Tfb3 and Dcn1, and Cdc53 neddylation primarily depends on Dcn1. Interestingly, all three cullins maintained low level of modification in either *DCN1* or *TFB3* mutated cells. The remaining modification of Rtt101 in the *tfb3(C16Y)* strain may be due to residual activity of Tfb3 in this strain or it may reflect the existence of additional activation mechanisms.

In conclusion, our findings imply that cells use distinct pathways to activate cullins by covalent modification of their C terminus. As each cullin regulates different cellular processes, this separation allows activating a specific subset of cullin complexes in response to different signals. For example, it is tempting to speculate that DNA/chromatin-associated functions of cullins may be controlled by a Tfb3-dependent neddylation mechanism. Additional mechanisms that affect particular cullin complexes include sequestration by the assembly inhibitor CAND1/Lag2, and stable binding of some members to the CSN complex (Olma et al., 2009). Together with regulated binding of substrates and substrate-specific adaptor modules, these mechanisms collectively orchestrate the spatial and temporal ubiquitylation of substrates.

## EXPERIMENTAL PROCEDURES

### Yeast Strains and Plasmids

All yeast strains used in this study are haploid derivatives of either BY4743 (ATCC 201390) or W303, and are listed in Table S1. They were grown at 30°C and manipulated via standard techniques. For the spotting experiments (Figures 1 and 3) photographs of the plates were taken after an incubation of ~60 hr at 30°C. A list of all plasmids used in this study is provided in Table S2.

### Cullin Neddylation in Crude Yeast Protein Extracts

Concentrated yeast protein extracts were prepared as described (Kong and Svejstrup, 2002). These extracts were then diluted in neddylation buffer (20 mM HEPES [pH 7.5], 100 mM NaCl, 10 mM MgCl<sub>2</sub>) to a final protein concentration of 25 μg/μl. Neddylation of cullins present in the extract was started by addition of 1 μM recombinant Rub1 and 2 mM ATP. The reactions were incubated at 30°C for 15 min to 1 hr, stopped with 2× Laemmli loading buffer, and boiled for 5 min at 95°C.

### Interaction of E2 Conjugating Enzymes with RING Domain-Containing Proteins

Plasmids expressing GST, GST-Ubc12, GST-Ubc4, or GST-Rad6 were cotransformed with plasmids expressing Protein A (PA)-tagged Hrt1, PA-Tfb3(RING), or PA-Ssl1(RING) into *E. coli* BL21(DE3)-RIL cells. Proteins were expressed via the autoinduction method. After an overnight incubation at 25°C, cells were harvested, resuspended in 125 mM Tris (pH 7.5), 50 mM NaCl, 5 mM mercaptoethanol, and 2 mM PMSF, and lysed by sonication. The extracts were clarified and diluted to contain similar concentration of GST-tagged E2 conjugating enzymes. Diluted extracts were then incubated with glutathione beads for 1 hr at room temperature. The beads were then washed extensively in a buffer containing 50 mM Tris (pH 7.5), 200 mM NaCl, 0.1% NP40 and 5 mM mercaptoethanol, resuspended in sample buffer, and processed for immunoblotting.

### GST-Binding Assays of Tfb3 from Yeast Extracts

GST, GST-Hrt1, and GST-Hrt1 coexpressed with the Rtt101 C-terminal domain were produced in *E. coli* BL21(DE3)-RIL cells via the autoinduction method. After an overnight incubation at 18°C, cells were harvested, resuspended in lysis buffer (50 mM Tris [pH 7.5], 200 mM NaCl, 10 mM mercaptoethanol, and 2 mM PMSF), and lysed by sonication. The extracts were clarified and incubated with glutathione beads for 1 hr at room temperature. The beads were washed extensively with lysis buffer and resuspended in 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, and 5 mM mercaptoethanol. A fraction of the beads was then used to pull down Protein A-Tfb3 from a yeast protein extract. Approximately 10<sup>8</sup> cells expressing Tfb3-TAP were harvested, resuspended in 300 μl yeast lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% Triton X-100, 10 mM mercaptoethanol, and a protease inhibitor cocktail [Complete EDTA-free, Roche]), and lysed with a FastPrep instrument (QBiogene) in the presence of 1 ml of 0.5 mm glass beads (20 s, speed 4.5). After 1 hr rotation at room temperature, the glutathione beads were extensively washed with the yeast lysis buffer, resuspended in sample buffer, and further processed for immunoblotting.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimentation Procedures, four figures, and two tables and can be found with this article online at doi:10.1016/j.molcel.2011.05.032.

## ACKNOWLEDGMENTS

We thank C. Rupp, C. Zbinden and R. Oania for technical assistance, and B. Schulman and members of the Peter laboratory for helpful discussions. G. Rabut was recipient of a Human Frontier Science Program Long-Term fellowship and is currently supported by INSERM and Région Bretagne. Work in the Deshaies and Peter laboratories is supported by the Howard Hughes Medical Institute and by research grants from Oncosuisse, the Swiss National Science Foundation, and the ETH Zurich, respectively.

Received: December 1, 2010

Revised: April 4, 2011

Accepted: May 16, 2011

Published: August 4, 2011

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