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The TFIIH subunit Tfb3 regulates cullin neddylation

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Summary

Cullin proteins are scaffolds for the assembly of multi-subunit 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 did it require 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 as well as 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 multi-subunit 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

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Supplemental Information
Supplemental Information includes Supplemental Experimentation Procedures, 4 Figures, and 2 Tables that can be found with this article online.

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 towards the Nedd8-conjugating enzyme Ubc12 and mediates cullin nedylation *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 homologues 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 orthologue Cdc53 (Kurz et al., 2008) and orients Ubc12 to promote Hrt1-mediated Cdc53 neddylation (Scott et al., 2010). Moreover, the human Dcn1 homologue 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 791 (K791) contributes to Rtt101 function, we assayed the resistance of yeast mutants to CPT and MMS (Fig. 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 *rub1Δ* cells was comparable to 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 (Fig. 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 homologue Ubc5 (Fig. 1B; Suppl. Information, Fig. S1), 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) (Fig. 1C). Conjugation of 9Myc-Ubq to Rtt101 was observed in wild type cells, although to a lesser extent than in *rub1Δ* cells (Fig. 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 *rub1*Δ and *ubc4*Δ cells are resistant to CPT (Fig. 1D), *rub1*Δ *ubc4*Δ 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 *rub1*Δ *ubc4*Δ *rtt101(K791R)* triple 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. Indeed, while Dcn1 is required for efficient neddylation of Cdc53 (Kurz et al., 2005) (Fig. 2A), we observed that neither neddylation nor ubiquitylation of Rtt101 were altered in *dcn1Δ* cells (Fig. 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 (Fig. 2B). First, we mutated a conserved isoleucine (I57A) located in the RING interface with E2s. Corresponding mutations in Hrt1 orthologues 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 Cln2 (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 (Fig. 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 A51Δ loop mutant, I57A and C81Y RING mutants strongly impaired the turnover of Cdc4, resulting in its accumulation (Fig. 2C, Suppl. Information Fig. S2). Similar results were observed with Cln2 (Suppl. Information Fig. 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 (Fig. 2D). In contrast, the catalytically active A51Δ mutant was barely able to modify Rtt101, and Cdc53 neddylation was reduced (Fig. 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 are 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 non-essential yeast genes (see Suppl. Information online). 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* (Fig. 3A). Likewise, Rtt101

neddylation *in vitro* was strongly impaired in protein extracts prepared from *tfb3(C16Y)* mutant cells (Fig. 3B). While neddylation of Cdc53 was only slightly diminished in *tfb3(C16Y)* cells (Fig. 3A), neddylation of Cul3 required both Dcn1 and intact Tfb3, both *in vivo* and *in vitro* (Fig. 3D and 3E). Interestingly, residual modification of Cul3 was detectable in both *dcn1*Δ and *tfb3(C16Y)* strains but not in the double mutant (Fig. 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*Δ strain was not dependent on Tfb3 (Suppl. Information, Fig. 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 (Fig. 3C), indicating that, like Rtt101, Tfb3 contributes to the cellular resistance against DNA-replication stress. Following 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, Rbp1 degradation was compromised in cells harboring non-neddylatable Cul3 (Cul3(K688R)) as well as in *rub1*Δ cells (Fig. 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 (Fig. 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 TFIIH, a well characterized complex involved in transcription initiation and nucleotide excision repair (NER) (Mydlikova et al., 2010). To determine whether these functions of TFIIH are involved in Rtt101 regulation, we examined Rtt101 modification in various TFIIH mutants with impaired transcription or NER activities. For instance, *ccl1-ts4*, *kin28-ts*, *rad3-14* and *ssl2-ts* mutants severely reduce general transcription activity (Guzder et al., 1994; Lee and Lis, 1998; Qiu et al., 1993; Valay et al., 1996; Valay et al., 1995), while *tfb1-1*, *tfb5*Δ or *rad2*Δ 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 (Fig. 4A and Suppl. Information Fig. S4A. Furthermore, previously published microarray experiments showed that general transcription is not impaired in *tfb3(C16Y)* cells (Jona et al., 2002). Together this indicates 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 *RRI1*, the catalytic subunit of the CSN, nor deletion of *LAG2* rescued Rtt101 modification in *tfb3(C16Y)* cells (Fig. 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 (Suppl. Information Fig S4B), excluding the possibility that *tfb3(C16Y)* cells contain a *trans*-inhibitor of Rtt101 neddylation. We conclude that Tfb3 directly or indirectly activates the Rtt101 neddylation and ubiquitylation.

The fact that Cdc53 neddylation was almost normal in *tfb3(C16Y)* cells (see Fig. 3A, 4B and Suppl. Information, Fig. S3) indicates that Tfb3 functions downstream of Ubc12. Indeed, Ubc12 was correctly expressed and efficiently charged with Rub1 in *tfb3(C16Y)* cells (Fig. 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 (Fig. 4D). In contrast, the RING domain of Ssl1 -another TFIIH subunit previously reported to function as a ubiquitin E3 (Takagi et al., 2005)- was preferentially precipitated by Ubc4 (Fig. 4D). To determine whether Tfb3 also physically interacts with the Rtt101/Hrt1 complex, we performed GST pull-down experiments. As shown in Fig. 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 (Suppl. Information Fig. S4C). Together, these results suggest that the RING-finger protein Tfb3 regulates Rtt101 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.

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 TFIIH subunits that affect Rtt101 modification, it is unlikely that activation of Rtt101 and Cul3 neddylation is linked to TFIIH function in transcription initiation or NER. However, we currently do not know whether TFIIH 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 Suppl. Information Table S1. They were grown at 30°C and manipulated using standard techniques. For the spotting experiments (Fig. 1 and 3) photographs of the plates were taken after an incubation of ~60h at 30°C. A list of all plasmids used in this study is provided in Suppl. Information 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 (20mM Hepes pH 7.5, 100mM NaCl, 10mM $MgCl_2$) to a final protein concentration of 25μg/μL. Neddylation of cullins present in the extract was started by addition of 1μM recombinant Rub1 and 2mM ATP. The reactions were incubated at 30°C for 15min to 1h, stopped with 2× Laemmli loading buffer, and boiled for 5min at 95°C.

Interaction of E2 conjugating enzymes with RING domain containing proteins

Plasmids expressing GST, GST-Ubc12, GST-Ubc4 or GST-Rad6 were co-transformed 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 using the auto-induction method. After an overnight incubation at 25°C, cells were harvested, resuspended in 125mM Tris pH 7.5, 50mM NaCl, 5mM mercaptoethanol and 2mM 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 1h at room temperature. The beads were then washed extensively in a buffer containing 50mM Tris pH 7.5, 200mM NaCl, 0.1% NP40 and 5mM mercaptoethanol, resuspended in sample buffer, and processed for immunoblotting.

GST-binding assays of Tfb3 from yeast extracts

GST, GST-Hrt1 and GST-Hrt1 co-expressed with the Rtt101 C-terminal domain were produced in *E. coli* BL21(DE3)-RIL cells using the auto-induction method. After an overnight incubation at 18°C, cells were harvested, resuspended in lysis buffer (50 mM Tris pH 7.5, 200mM NaCl, 10mM mercaptoethanol, and 2mM PMSF) and lysed by sonication. The extracts were clarified and incubated with glutathione beads for 1h at room temperature. The beads were washed extensively with lysis buffer and resuspended in 50mM Tris pH 7.5, 150mM NaCl, 0.5% Triton X100, and 5mM mercaptoethanol. A fraction of the beads was then used to pull-down Protein A-Tfb3 from a yeast protein extract. Approximately 10^8 cells expressing Tfb3-TAP were harvested, resuspended in 300μL of yeast lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 0.5% Triton X100, 10mM mercaptoethanol, and a protease inhibitor cocktail (Complete EDTA-free, Roche)), and lysed with a FastPrep® instrument (QBiogene) in the presence of 1mL of 0.5mm glass beads (20s, speed 4.5). After 1h rotation at room temperature, the glutathione beads were extensively washed with the yeast lysis buffer, resuspended in sample buffer, and further processed for immunoblotting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Ubiquitylation and neddylation of Rtt101 activate its function *in vivo*
- Rtt101 modifications neither require Dcn1 nor fully active Hrt1
- Tfb3 regulates the modifications and activities of Rtt101 and Cul3

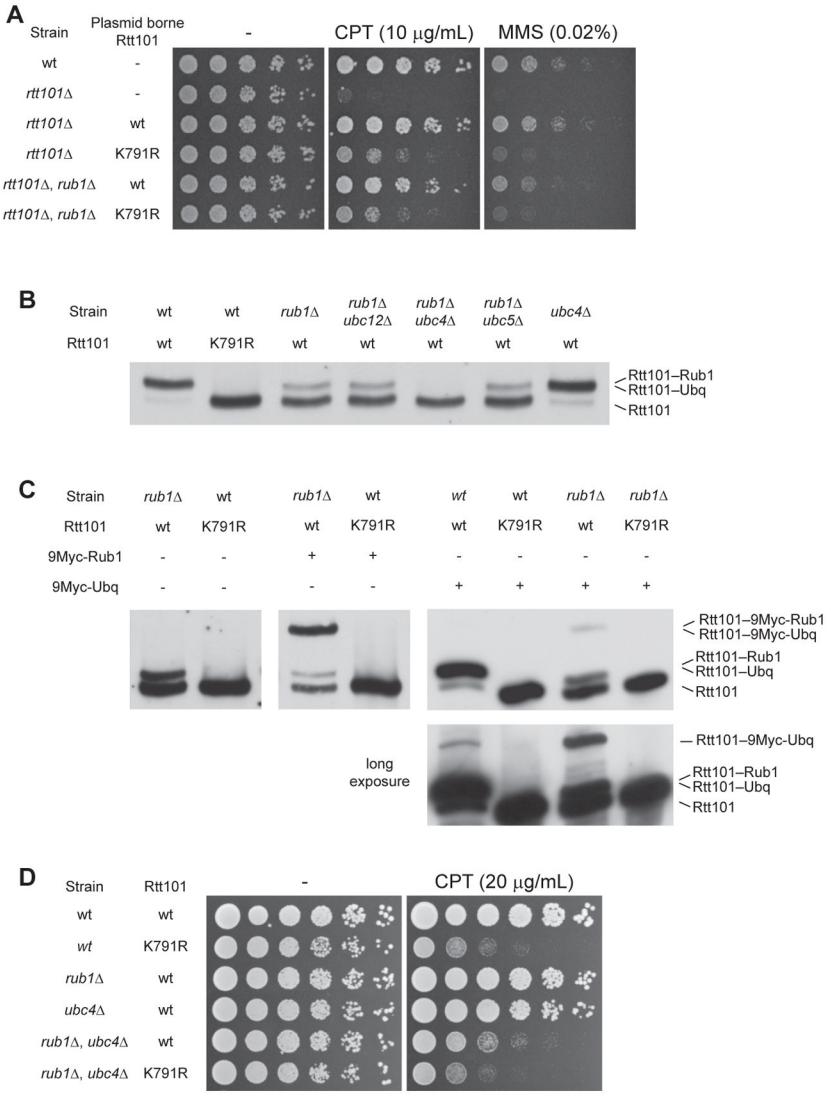

Figure 1.

Neddylation 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 Suppl. Information online.

(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 Suppl. Information, 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 20μg/mL CPT.

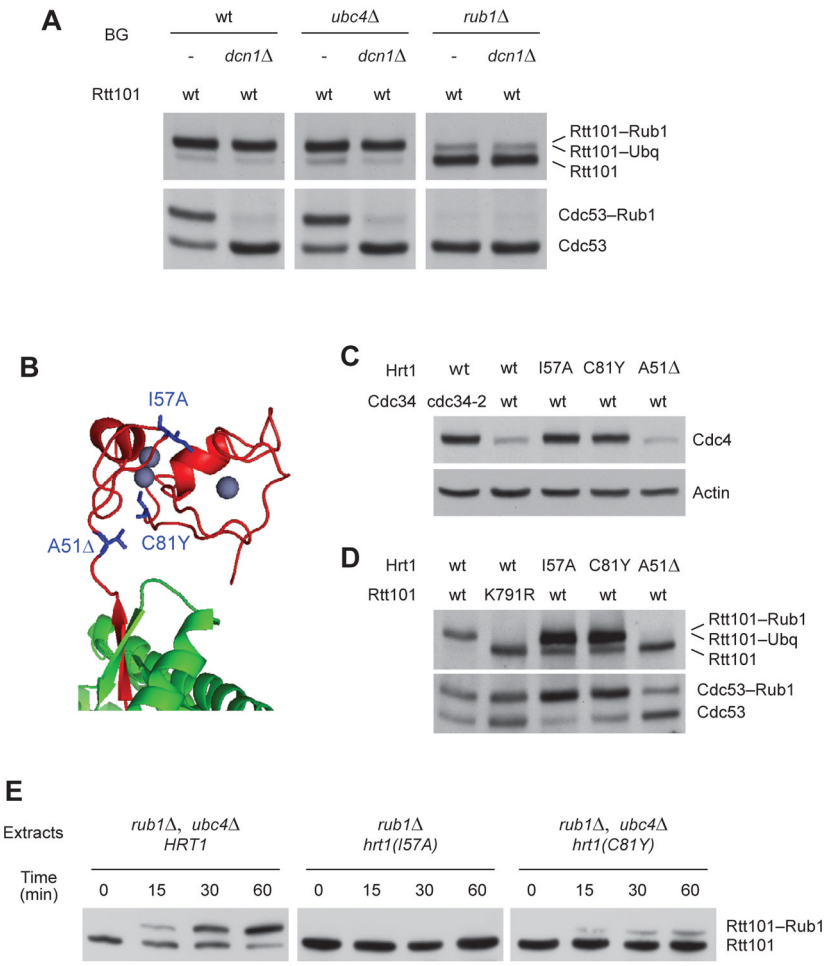

Figure 2.

Rtt101 modification in *DCN1* and *HRT1* mutant cells

(A) Rtt101 neddylation and ubiquitylation is not impaired in *dcn1*Δ 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/pdb3dqv/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 Suppl. Information, Fig. 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.

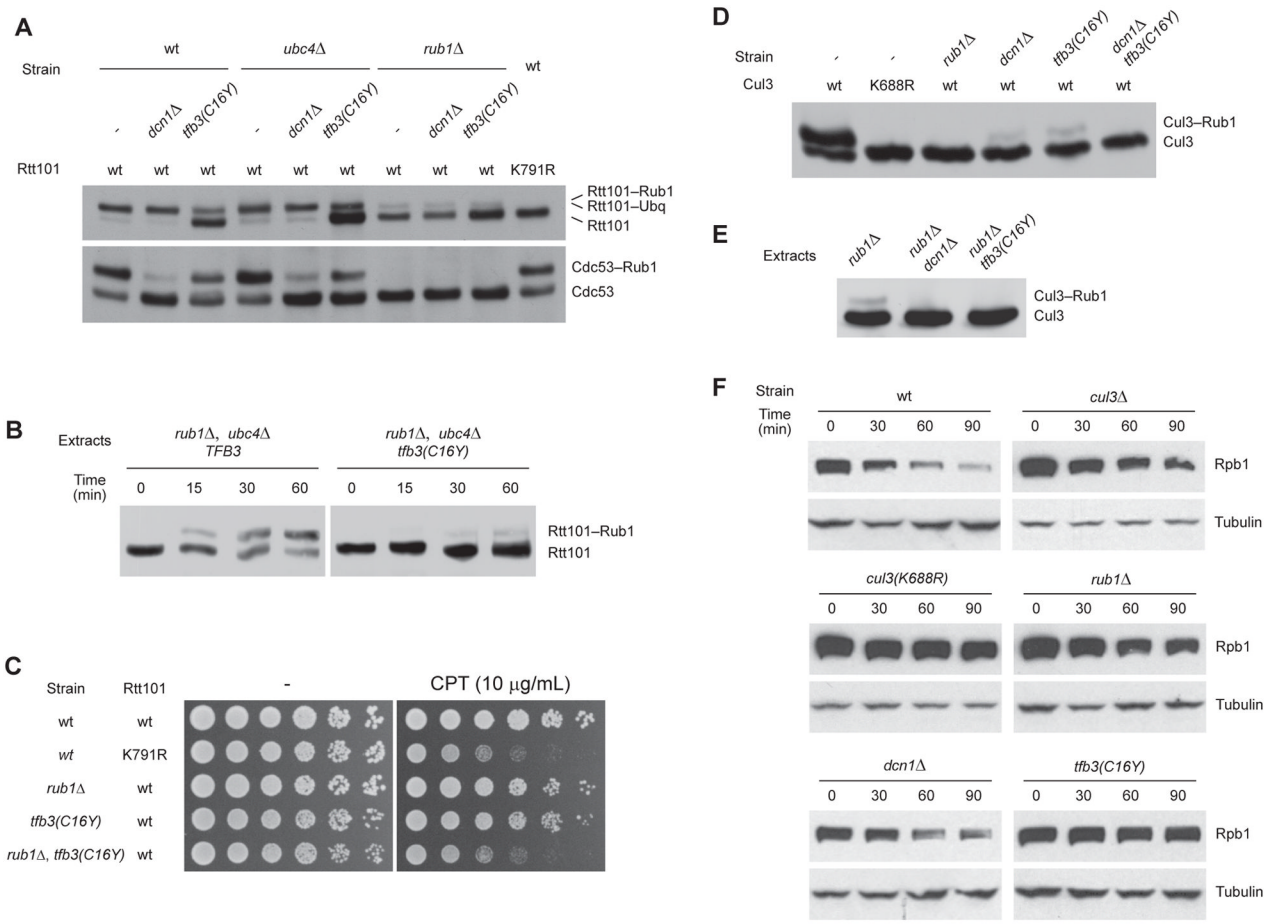

Figure 3.

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 Suppl. Information, Fig. 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 10μg/mL CPT.

(D) Cul3 neddylation is impaired in *dcn1*Δ 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 1h after addition of recombinant Rub1 and ATP.

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

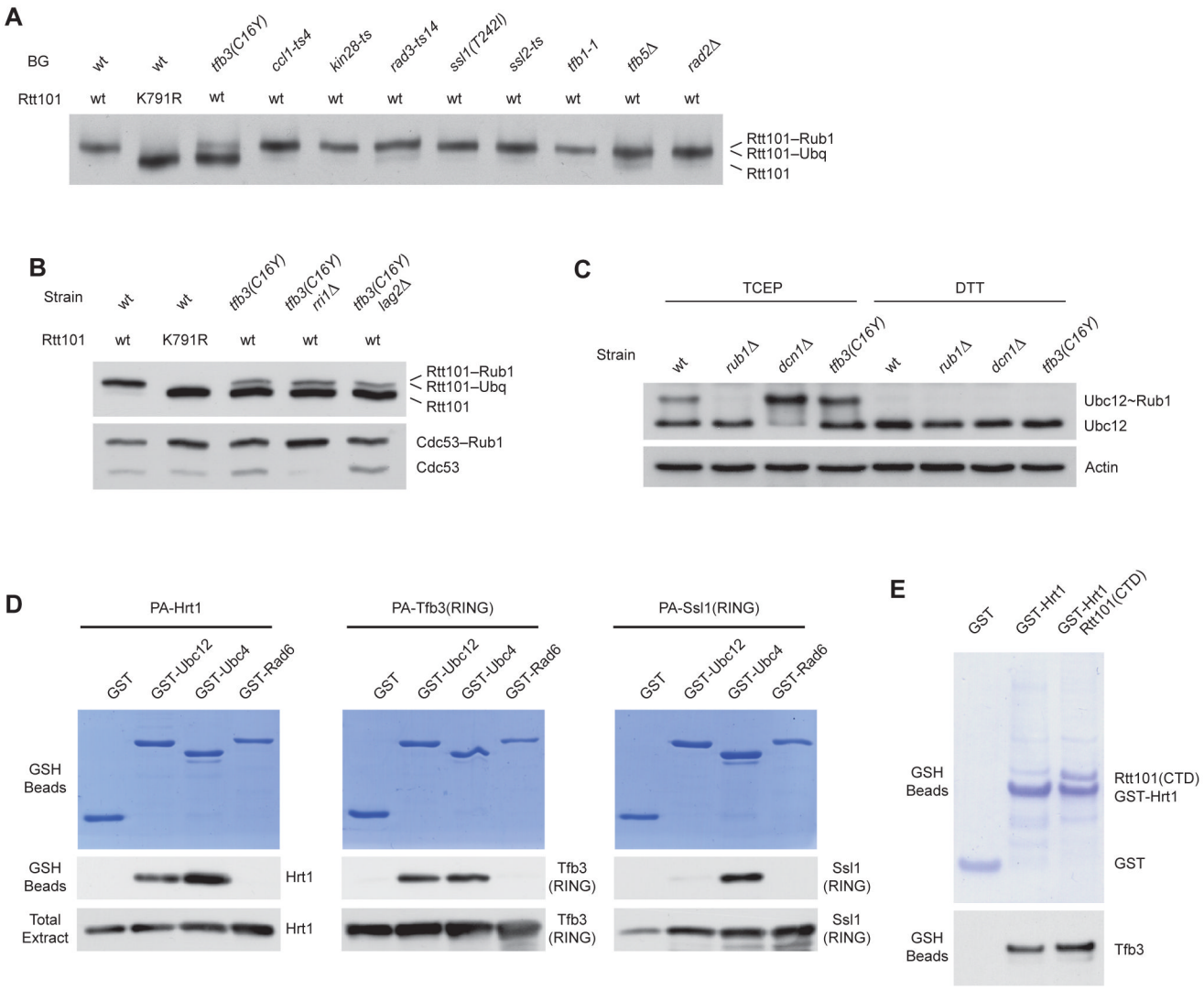

Figure 4.

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 Suppl. Information, Fig. 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 (upper panel) and immunoblotting (middle panel). An

aliquot of the extracts prior to precipitation was included for control (total extract, bottom panel).

(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 (upper panel) and immunoblotting (bottom panel).