Saccharomyces cerevisiae ubiquitin-like protein Rub1 conjugates to cullin proteins Rtt101 and Cul3 *in vivo*

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In *Saccharomyces cerevisiae*, the ubiquitin-like protein Rub1p (related to ubiquitin 1 protein) covalently attaches to the cullin protein Cdc53p (cell division cycle 53 protein), a subunit of a class of ubiquitin E3 ligases named SCF (Skp1–Cdc53–F-box protein) complex. We identified Rtt101p (regulator of Ty transposition 101 protein, where Ty stands for transposon of yeast), initially found during a screen for proteins to confer retrotransposition suppression, and Cul3p (cullin 3 protein), a protein encoded by the previously uncharacterized open reading frame YGR003w, as two new *in vivo* targets for Rub1p conjugation. These proteins show significant identity with Cdc53p and, therefore, are cullin proteins. Modification of Cul3p is eliminated by deletion of the Rub1p pathway through disruption of either *RUB1* or its activating enzyme *ENR2*/*ULA1*. The same disruptions in the Rub pathway decreased the percentage of total Rtt101p that is modified from approx. 60 to 30 %. This suggests that Rtt101p has an additional *RUB1*- and *ENR2*-independent modification. All modified forms of Rtt101p and Cul3p were lost when a single lysine residue in a conserved region near the C-terminus was replaced by an arginine residue. These results suggest that this lysine residue is the site of Rub1p-dependent and -independent modifications in Rtt101p and of Rub1p-dependent modification in Cul3p. An *rtt101Δ* strain was hypersensitive to thiabendazole, isopropyl (*N*-3-chlorophenyl) carbamate and methyl methanesulphonate, but *rub1Δ* strains were not. Whereas *rtt101Δ* strains exhibited a 14-fold increase in Ty1 transposition, isogenic *rub1Δ* strains did not show statistically significant increases. Rtt101K791Rp, which cannot be modified, complemented for Rtt101p function in a transposition assay. Altogether, these results suggest that neither the *RUB1*-dependent nor the *RUB1*-independent form of Rtt101p is required for Rtt101p function. The identification of additional Rub1p targets in *S. cerevisiae* suggests an expanded role for Rub in this organism.

Key words: cullin, Cul3, post-translational modification, Rtt101 [regulator of Ty (transposon of yeast) transposition 101], Rub1 (related to ubiquitin 1), ubiquitin-like protein.

INTRODUCTION

Ubiquitin is a globular, 76-amino-acid protein found free or covalently attached to proteins. Attachment of ubiquitin to proteins is a regulated process requiring multiple enzymes (reviewed recently in [1]). The first step is catalysed by the ubiquitin-activating enzyme E1 (encoded by *UBA1* in budding yeast), forming a ubiquitin adenylate at the C-terminus of ubiquitin using ATP. Ubiquitin then forms an *S*-ester bond between its C-terminus and an internal cysteine residue of E1. From E1, ubiquitin is transferred to an internal cysteine residue of a second enzyme called E2 or ubiquitin-conjugating enzyme. The *S*-ester cascade includes a third protein or protein complex, called E3 or ubiquitin ligase. E3s bring together activated ubiquitin and the substrate protein to transfer ubiquitin typically to an ε-amino group of a lysine residue in the substrate. The substrate specificity of the ubiquitin conjugation pathway is provided by E2 and E3. When linked via Lys-48 within ubiquitin, ubiquitin chains attached to substrate proteins allow recognition and degradation of the substrate by the proteosome.

Recently, a number of analogous ubiquitin-like conjugation pathways have been described. The ubiquitin-like protein mouse NEDD8 (neuronal precursor cell expressed developmentally down-regulated-8) [2] and its orthologues, *Saccharomyces cerevisiae* Rub1p (related to ubiquitin 1 protein) [3] and *Arabidopsis thaliana* RUBs [4], have been identified and their conjugation pathways have been characterized. The Rub/Nedd8-activating enzyme was first discovered by the identification of a protein in *Arabidopsis* involved in hormone signalling, namely AXR1 (auxin resistant-1), which has high homology with the N-terminus of ubiquitin E1 [5]. Subsequent work in *S. cerevisiae* led to the characterization of the heterodimeric Rub1p-activating enzyme composed of the subunits Uba3p and Enr2p/Ula1p (E1 N-terminal region 2/ubiquitin-like activating 1 protein) [6,7]. A single protein, Ubc12p, is the Rub1p/Nedd8-conjugating enzyme [7,8]. The genes encoding Rub/Nedd8 or its conjugating enzymes are essential in *S. pombe* [9], *Caenorhabditis elegans* [10], *Drosophila melanogaster* [11] and *Mus musculus* [12]. All these organisms showed cell-cycle abnormalities, leading to death when null alleles were created or the mRNA was decreased by RNA interference. In addition, the *Arabidopsis axr1-12* null mutant showed insensitivity to the phytohormone auxin, which is involved in cell division, elongation and differentiation in plants [13]. These results point to an integral role for the Rub/Nedd8 pathway in these organisms.

The first proteins identified as targets for Rub1p conjugation were Cdc53p (cell division cycle 53 protein) *in vivo* in *S. cerevisiae* [6,7] and hCUL4a *in vitro* [14], two related proteins of the

Abbreviations used: Cdc53p, cell division cycle 53 protein; Enr2p/Ula1p, E1 N-terminal region 2/ubiquitin-like activating 1 protein; HA, haemagglutinin; ICPC, isopropyl (*N*-3-chlorophenyl) carbamate; IκBα, inhibitor of nuclear factor κBα; MMS, methyl methanesulphonate; ORF, open reading frame; RIG, retrotransposition indicator gene; Ty, transposon of yeast; SCF, Skp1–Cdc53–F-box protein complex; SUMO-1, small ubiquitin-related modifier-1.

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cullin family. At least one cullin protein in every organism studied is a subunit of a class of ubiquitin E3 ligases called SCF (Skp1–Cdc53–F-box protein complex), named after the first three components identified in yeast, Skp1, Cdc53p (or cullin) and F-box protein. In addition, the RING domain-containing proteins Hrt1 (also known as Roc1 and Rbx1) and Sgt1 were found as additional components of SCF complexes. Since there are multiple F-box proteins, SCF complexes promote the ubiquitination of a large number of diverse proteins [15].

In an effort to identify the biochemical function of Nedd8/Rub proteins, *in vitro* studies of mammalian SCF activity have identified that the Rub/Nedd8 pathway is required for maximal SCF activity. Podust et al. [16] isolated the components required for robust *in vitro* ubiquitination of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ from rabbit reticulocyte lysate and identified them to be Nedd8 and the Nedd8 conjugation pathway. To ubiquitinate the E2 protein, CDC34 and its endogenous target IκBα (inhibitor of nuclear factor κBα), the $SCF^{\beta TrCP}$ complex required Nedd8-modified CUL1 purified from HeLa cells or the Nedd8 conjugation enzymes [17]. Modification of CUL1 by Nedd8 increases the v_{max} value of the complex without changing the K_m value for its substrate [18], probably by facilitating recruitment of the E2-containing *S*-ester-linked ubiquitin [19]. Collectively, these results demonstrate that the cullin subunit requires Nedd8 conjugation for maximal SCF activity *in vitro* and presumably *in vivo* as well.

In *S. cerevisiae*, genetic interactions of *RUB1* with genes encoding components of the SCF complex were identified, suggesting a function related to the SCF activity [6,7]. However, in stark contrast with the situation in mammals, *D. melanogaster*, *C. elegans* and *S. pombe*, the Rub1 pathway is not essential in *S. cerevisiae*. Even more remarkable, no gross defects are seen in *rub1Δ* and *enr2Δ/ula1Δ* strains, whereas the SCF subunit genes *SKP1*, *CDC53* and *HRT1* are essential. Thus, despite the demonstrated role of Rub/Nedd8 in SCF function in mammals and *S. pombe*, and the importance of the *S. cerevisiae* SCF, the role of Rub1p conjugation to Cdc53p in *S. cerevisiae* SCF complexes remains unknown. For this reason, we sought to identify additional *in vivo* Rub1p targets to help elucidate Rub1p function in budding yeast. Two other cullin family members were identified as Rub1p targets in the present study.

EXPERIMENTAL

Yeast strains

The following strains are direct descendents of W303-1B (*MATα ade2-1*, *leu2-3,112*, *his3-11*, *trp1-1*, *ura3-1*, GAL^+) [20], with modifications indicated in the parentheses: *rub1Δ* (*rub1Δ::TRP1*, *rub1Δ::HIS3* or *rub1Δ::URA3*), *3xHA:RUB1* (*rub1::3xHA:RUB1*), *3xHA:RUB1 rtt101Δ* (*rub1::3xHA:RUB1*, *rtt101Δ::URA3* or *rtt101Δ::TRP1*). *enr2Δ* and *ENR2* are descendents from a cross between *rub1Δ::HIS3* (see above) and *skp1-12 enr2Δ* (*MATa skp1-12*, *enr2Δ::TRP1*, *ade2*, *his3*, *leu2*, *trp1*, *ura3*) [6]. rub1*Δ cdc53Δ*::*HIS3* is a descendent of a cross between *rub1Δ::HIS3* and NMY53*Δ* (*MATa cdc53::HIS3*, *his3*, *leu2*, *trp1*, *ura3*, pE3a) [6]. The pE3a plasmid (*CDC53 URA3*) was replaced with either pMT1857 (*CDC53-C-MYC6 TRP1 CEN*), pMT1858 (*CDC53-Δ 757-815-C-MYC6 TRP1 CEN*) or pMT1859 (*CDC53-Δ 794-815 C-MYC6 TRP1 CEN*). The truncated Cdc53p proteins are referred to as Cdc53*Δ* 757p and Cdc53*Δ* 794p in the present study. *3xHA:RUB1 rtt101Δ cdc53Δ::HIS3* pMT2235 is a descendent of a cross between *3xHA:RUB1 rtt101Δ* and *rub1Δ cdc53Δ::HIS3* pE3a. The plasmid pE3a was replaced by pMT2235 (*CDC53-K760R-C-MYC6 LEU2 CEN*). The *RUB1*, *RTT101* and *CUL3* loci were disrupted using a PCR-based technique [21] and disruption was confirmed by amplification of the loci using PCR with primers annealing outside the replaced region. Replacement of the *RUB1*-coding sequence by a *3xHA:RUB1*-coding sequence was achieved by a two-step replacement procedure. The *RUB1* gene was first replaced by the *URA3*-selective marker as described above. The *URA3* marker was then replaced with a *3xHA:RUB1*-coding region by transforming a PCR product containing *3xHA:RUB1* ORF (open reading frame) flanked by regions having homology with the 5′ and 3′ regions of the *RUB1* loci. All transformations were performed using a modified lithium acetate procedure [22].

Strain BY4742 (*MATα*, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0* [21]) and *rub1Δ kanMX4* and *rtt101Δ kanMX4* derivatives were obtained from Research Genetics (Huntsville, AL, U.S.A.). Strain JC3212 contains a chromosomal Ty1*his3AI[Δ1]*-3114 element (where Ty1 stands for transposon of yeast 1) integrated into strain BY4742 by the induction of transposition from plasmid pGTy1*his3AI[Δ1]*, performed as described previously [23]. An *rtt101Δ kanMX4* Ty1*his3AI[Δ1]*-3114 derivative was made by crossing JC3212 to an *rtt101Δ kanMX4* derivative of the congenic strain BY4741 (Research Genetics).

The *rtt101* strains JC2410, JC2412 and JC2440 contain an mTn3-*lacZ/LEU2* insertion 82 or 84 bp upstream or 438 bp downstream of the beginning of the *RTT101* ORF respectively, in strain JC2326 [*MATΔ-ura3*, cir0, *ura3*-167, *leu2::hisG*, *his3Δ200*, Ty1*his3AI*-270, Ty1*NEO*-588, Ty1 (*tyb1::lacZ*)-146]. Construction of these strains is described in [24].

Plasmid construction

The coding sequences of *RTT101*, *RUB1* and *CUL3* were amplified by PCR, cloned and placed downstream of three copies of the coding sequence for the HA (haemagglutinin) epitope in a pYES2-based plasmid (Invitrogen, Carlsbad, CA, U.S.A.). The *RTT101K791R* or *Cul3K688R* versions of the *RTT101* and *CUL3* plasmids were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). All sequences were verified by DNA sequencing.

Protein extraction and Western-blot analysis

Yeast cells were grown in 50 ml selective synthetic complete + galactose media at 30 °C until late exponential-phase growth (A_{600} 5–10). Yeast cells were collected by centrifugation, fast-frozen in liquid nitrogen, thawed and resuspended in 800 μl of STE (100 mM NaCl/10 mM Tris, pH 8.0/1 mM EDTA), 1 mM PMSF, 5 mM *N*-ethylmaleimide and protease inhibitors (Mini-Protease Inhibitors Complete; Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.). Glass beads (500 μl) were added and the tubes were shaken for three 15 min bursts using a multishaker at 4 °C. Cells were spun down for 10 min at 4 °C (20 000 ***g***). Supernatants were transferred to new microcentrifuge tubes and the total protein was quantified by the bicinchoninic acid method according to the manufacturer's instructions (Sigma).

SDS/PAGE was performed using the Laemmli buffer system and Western-blot analysis was performed as described previously using mouse anti-HA antibodies (12CA5) and mouse anti-Myc antibodies obtained from Roche Molecular Biochemicals [25]. The primary and secondary dilutions were 1:1000 in Blotto solution for the Luminol developing method or 1:2000 in Blotto solution for ECL+ developing method (Amersham Biosciences, Piscataway, NJ, U.S.A.). Western blots were visualized and quantified using the Storm 680 PhosphorImager and ImageQuant 4.0 software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

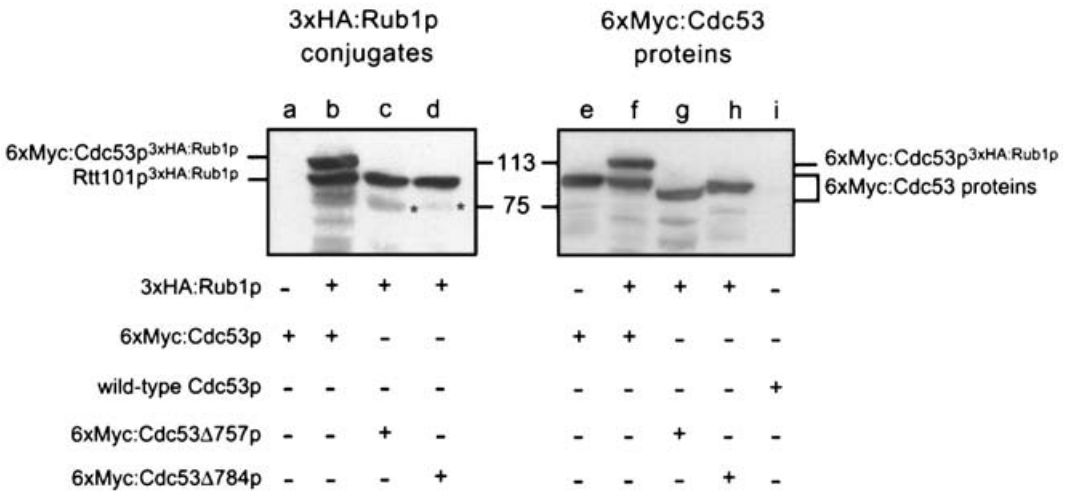

Figure 1 Two Rub1p conjugates are independent of Cdc53p

Western-blot analysis using anti-HA (lanes a–d) or anti-Myc (lanes e–i) antibodies on protein extracts from *rub1Δ* (lanes a, e and i) or *rub1Δ* expressing 3xHA:Rub1p (lanes b–d and f–h) strains and expressing one of the following Cdc53 proteins: full-length 6xMyc:Cdc53p (lanes a, b, e and f) or one of two C-terminal truncations starting at the indicated amino acid 6xMyc:Cdc53Δ757p (lanes c and g), 6xMyc:Cdc53Δ794p (lanes d and h) or non-epitope-tagged Cdc53p (lane i). Asterisks in lanes c and d indicate a second lower-abundance Cdc53p-independent 3xHA:Rub1p conjugate. +, Expression of the protein indicated on the left.

Ty1 retrotransposition and phenotypic assays

A quantitative assay for retrotransposition of Ty1 was performed in strain BY4742 and derivatives after the introduction of a Ty1 element marked with the RIG (retrotransposition indicator gene) *his3AI[Δ1]*, upstream of the *HIS4* locus using the *URA3*-based integrating plasmid pJC573 [24]. Retrotransposition of the Ty1*his3AI[Δ1]* element results in the formation of a His^+ prototroph. Saturated cultures of each strain grown in YPD media, as described in [29], at 30 °C were diluted 1:1000 in YPD broth and grown to saturation at 20 °C. A 2 μl of aliquot of each diluted culture was plated on ScD-Ura medium, and 400 μl (strain BY4742) or 100 μl (*rtt101Δ* and *rub1Δ* derivatives) of aliquots were plated on ScD-Ura–His medium to determine the fraction of Ura^+ cells that became His^+ at 20 °C.

Strain W303-1B and derivatives were transformed with the plasmid pJC562, which contains a Ty1 element marked with the RIG *kanMX-AI* in the *URA3* 2μ vector, Yep24. The *kanMX-AI* RIG contains an artificial intron [26] in antisense orientation at nt 385 of the *kan* ORF and the *Ptef* promoter from the *kanMX* cassette in plasmid pUG6 [27]. Retrotransposition of Ty1*kanMX-AI* results in resistance to G418. Saturated cultures of each strain grown in ScD-Ura broth at 30 °C were diluted 1:1000 in YPD broth and grown to saturation at 20 °C. The fraction of Ura^+ cells that became $G418^R$ at 20 °C was determined as above, except that SE-Ura + 200 μg/ml G418 plates, prepared as described by Cheng et al. [28], were used to score transposition events.

Strain JC3212 and an *rtt101Δ* derivative, each containing Ty1*his3AI[Δ1]*-3114, were transformed with a pYES2 vector (Invitrogen) alone or with pYES2 harbouring *3xHA:RTT101* or *3xHA:RTT101K791R*. Transformants were grown as patches on ScD-Ura agar for 2 days at 30 °C. Expression from the plasmid constructs was induced by replicating cells to rich medium containing galactose (YPGalactose) and incubating for 5 days at 20 °C. Cells were then replicated to YPD medium for 1 day at 20 °C to promote transposition. His^+ prototrophs, which arose by transposition in cells that retained a *URA3*-marked plasmid, were scored by replicating to ScD-Ura–His agar. His^+ Ura^+ colonies were photographed after 3 days of growth at 30 °C.

Stock solutions of ICPC [isopropyl (*N*-3-chlorophenyl) carbamate; Sigma] and thiabendazole (Sigma) were made in DMSO and appropriate amounts were added in each plate before solidification. To test for growth on media containing different chemicals, strains were grown overnight in YPD media at 30 °C and diluted to A_{600} 0.1 and 0.01 (for thiabendazole and ICPC test) or A_{600} 0.8 and serial dilutions [for MMS (methyl methanesulphonate]. Equal volumes were spotted on YPD plates containing 100 μg/ml thiabendazole, 1 mM ICPC, 0.01 % MMS or DMSO alone. In the tests for growth on thiabendazole- or ICPC-containing media, equal volumes of cells were also spotted on a synthetic medium lacking tryptophan (ScD-Trp) and a synthetic medium lacking leucine (ScD-Leu) to verify the genotype. Plates were incubated at 30 °C for 4 days before photographing.

RESULTS

Additional conjugates of Rub1p are present independent of Cdc53p

We previously identified Cdc53p as a target for the covalent addition of Rub1p using a plasmid-based Rub1p expression construct with a single HA epitope at the N-terminus [6]. To improve detection, the Rub1p ORF was modified to express Rub1p with three HA epitopes (3xHA:Rub1p). The 3xHA:Rub1p expression plasmid was introduced into an *rub1Δ*, *cdc53Δ* strain expressing 6xMyc:Cdc53p from a second plasmid [30]. This allowed us to track the migration of both Cdc53p and its Rub1p-modified form(s) and other Rub1p conjugates in the same yeast. In protein extracts from this strain, two abundant HA-immunoreactive proteins were visualized, along with a number of lower abundance bands when compared with an isogenic strain not expressing 3xHA:Rub1p (Figure 1, cf. lanes a and b). The 3xHA:Rub1p-containing Cdc53p species was identified by immunoblotting the same extracts with anti-Myc antibody to visualize 6xMyc:Cdc53p. Two anti-Myc reactive species were present (Figure 1, lane f) that are not visualized when non-epitope-tagged Cdc53p is expressed (Figure 1, lane i). The slower migrating 6xMyc:Cdc53p band co-migrated with the slowest migrating 3xHA:Rub1p

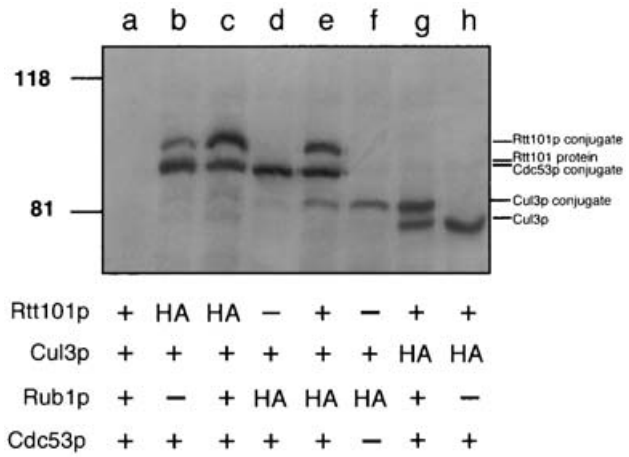

Figure 2 Previously uncharacterized Rub1p conjugates co-migrate with the slower migrating forms of Cul3p and Rtt101p and are completely dependent on *RUB1* for Cul3p and partially dependent on *RUB1* for Rtt101p

Western-blot analysis with anti-HA antibodies of protein extracts from the following yeast strains: expressing Rtt101p from a plasmid in an *RUB1*, *RTT101*, *CDC53*, *CUL3* strain (lane a), 3xHA:Rtt101p from a plasmid in *CDC53*, *CUL3 rub1Δ* or *RUB1* strain (lane b or c respectively), chromosomal 3xHA:Rub1p in *rtt101Δ*, *CDC53*, *CUL3* (lane d), chromosomal 3xHA:Rub1 in *RTT101*, *CDC53*, *CUL3* (lane e) or chromosomal 3xHA:Rub1 in rtt101Δ *cdc53::HIS3 CDC53K760R*, *CUL3* (lane f), or 3xHA:Cul3p in *RUB1* or *rub1Δ* strain (lane g or h respectively). A + or – indicates the expression or absence respectively of the chromosomally encoded untagged version. HA indicates expression of a 3xHA-epitope-tagged version, either on a plasmid (for 3xHA:Rtt101p and 3xHA:Cul3p) or at its endogenous chromosomal location (for 3xHA:Rub1p).

species and was lost in *rub1Δ* (Figure 1, lane e), clearly identifying them as 6xMyc:Cdc53p$^{3xHA:Rub1p}$.

The second major HA-immunoreactive band as well as others of lower abundance (Figure 1, lane b) could be other forms of Rub1p-modified Cdc53p, produced either *in vivo* or *in vitro*, or novel Rub1p conjugates. To distinguish between these possibilities, an approach was sought to eliminate the presence of all Cdc53p^{Rub1p} species. Since Cdc53p is essential, strains were constructed that express one of two C-terminal truncations of Cdc53p as the sole source of Cdc53p, namely 6xMyc:Cdc53Δ 757p or 6xMyc:Cdc53Δ 794p, which are lacking the last 48 or 21 amino acids respectively. Neither of these proteins is conjugated to Rub1p *in vivo*, although both are capable of complementing a *CDC53* deficiency (A. Willems and M. Tyers, personal communication). We verified that in strains expressing 3xHA:Rub1p, these proteins are present as a single species *in vivo* (Figure 1, lanes g and h respectively) migrating slightly faster than full-length unmodified 6xMyc:Cdc53p. When the same extracts were immunoblotted to identify 3xHA:Rub1p-containing proteins, the faster migrating, abundant HA-immunoreactive band identified previously (Figure 1, lane b) was not altered in either migration rate or abundance (Figure 1, cf. lane b with lanes c and d). This result demonstrates that the faster migrating 3xHA:Rub1p conjugate does not contain Cdc53p. An additional faint 3xHA-immunoreactive band was visible (marked with an asterisk in Figure 1, lanes c and d), suggesting the presence of a third 3xHA:Rub1p conjugate. The relative abundance of this conjugate varied (cf. Figure 1, lanes c and d; see also Figure 2, lanes d and e). Altogether, these results indicate the presence of one or possibly two additional Rub1p conjugates distinct from the previously described Cdc53p^{Rub1p}.

It is possible that the abundant novel conjugate was owing to Rub1p overexpression on a plasmid under the *GAL1* promoter. To exclude this possibility, the *RUB1* gene was replaced in the genome with a sequence encoding 3xHA:Rub1p under its own promoter in a strain expressing non-epitope-tagged Cdc53p. Three conjugates were readily visible when protein extracts were separated by SDS/PAGE optimized for the separation of high-molecular-mass proteins and the resulting protein blot subjected to Western-blot analysis using anti-HA antibodies (Figure 2, lane e). This result demonstrates that the presence of multiple Rub1p conjugates is not an artifact of 3xHA:Rub1p overexpression.

The calculated masses of these conjugates (Figure 2, lane e) were approx. 83, 100 and 110 kDa. The 100 kDa conjugate is close to the predicted mass of Cdc53p$^{3xHA:Rub1p}$ [6,7]. In this case, the Cdc53p$^{3xHA:Rub1p}$ is not the slowest migrating conjugate, since Cdc53p lacks the 6xMyc epitope tag. Possible candidates for the other Rub1p conjugates are other members of the cullin family in *S. cerevisiae*. There are two ORFs in the genome having significant identity with Cdc53p [31]. One ORF, YJL047c, encodes Rtt101p (regulator of Ty transposition 101 protein) [24], which has 21 % identity in the C-terminal 400 amino acids with the corresponding region of Cdc53p. The predicted mass of unmodified Rtt101p was 99 kDa, which suggested that the novel conjugate at 110 kDa could be Rtt101p$^{3xHA:Rub1p}$. The other Cdc53p-related protein is an uncharacterized ORF, namely YGR003w. YGR003w encodes a protein with a predicted mass of 86 kDa and 22 % overall identity with Cdc53p, which we named Cul3p (cullin 3 protein). The smaller mass of the faint Rub1p conjugate suggested that it could be Cul3p$^{3xHA:Rub1p}$.

To identify Rtt101p as an Rub1p target, *RTT101* was disrupted in the strain expressing 3xHA:Rub1p and the conjugation pattern in protein extracts was determined by Western-blot analysis. In an *rtt101Δ* strain, the 110 kDa conjugate was not present (Figure 2, lane d), whereas Cdc53p$^{3xHA:Rub1p}$ and the fastest migrating conjugate remained, demonstrating dependence of the 110 kDa conjugate on Rtt101p and suggesting that Rtt101p is modified by Rub1p *in vivo*. To test whether Rtt101p itself was the target for Rub1p conjugation and the Rub1p conjugate did not require *RTT101*, 3xHA:Rtt101p was expressed in a wild-type strain. Western-blot analysis revealed the presence of two forms of Rtt101p differing in mass consistent with the addition of one Rub1p (Figure 2, lane c). When compared with extracts expressing 3xHA:Rub1p with wild-type (non-epitope-tagged) Rtt101p, Cdc53p and Cul3p (Figure 2, lane e), the highest molecular-mass species of 3xHA:Rtt101p co-migrated with highest molecular-mass 3xHA:Rub1p conjugate, identifying them as 3xHA:Rtt101p^{Rub1p} and Rtt101p$^{3xHA:Rub1p}$ respectively.

To identify whether Cul3p was a Rub1p target, we constructed a strain lacking the Rub1p-modifiable forms of the first two Rub1p conjugates. *RTT101* was disrupted and *CDC53* was replaced by *CDC53K760R*, containing a mutation that complements *cdc53*, but prevents the attachment of Rub1p (A. Willems and M. Tyers, personal communication). This strain also expressed HA-tagged Rub1p from a chromosomal copy. Western-blot analysis using anti-HA antibodies was used to compare the 3xHA:Rub1p conjugation pattern in protein extracts from this strain with the isogenic strain with wild-type *RTT101* and *CDC53* (Figure 2, lanes f and e respectively). Three distinct HA-immunoreactive bands are present in the strain where both Rtt101p and Cdc53p can be modified by Rub1p (lane e). Only the fastest migrating band remained in the strain deleted for *RTT101* and expressing Cdc53pK760R (lane f), consistent with the identification of a third and novel Rub1p conjugate.

The migration rate of this novel conjugate is close to the predicted mass of Cul3p$^{3xHA:RUB1p}$. If Cul3p is modified by Rub1p, then it should be found in two different forms after SDS/PAGE in a similar manner as observed for Rtt101p and Cdc53p. A triple

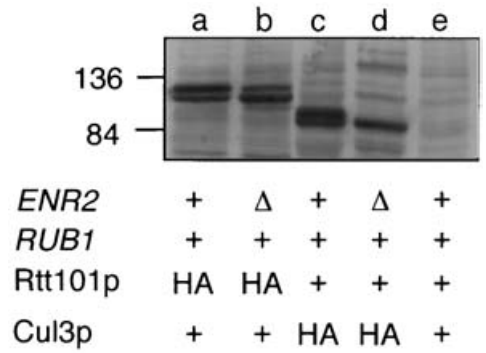

Figure 3 The slower migrating form of Cul3p and a portion of the slower migrating form of Rtt101p are dependent on *ENR2*

Western-blot analysis using anti-HA antibodies on protein extracts from strains expressing either 3xHA:Rtt101p (lanes a and b), 3xHA:Cul3p (lanes c and d) in either an *ENR2* (lanes a, c and e) or an *enr2Δ* (lanes b and d) background. Other symbols are the same as in Figure 2.

HA-epitope-tagged version of Cul3p was expressed in both the *RUB1* strain and the *rub1Δ* strain. Western-blot analysis revealed the presence of two forms of Cul3p in protein extracts from an *RUB1* strain (Figure 2, lane g). In contrast, only the faster migrating form of Cul3p was present in protein extracts from an *rub1Δ* strain (Figure 2, lane h), consistent with the fact that the slower migrating form was Rub1p-modified Cul3p. The slower migrating form of 3xHA:Cul3p (lane g) co-migrated with the fastest migrating 3xHA:Rub1p conjugate in the 3xHA:Rub1p strain expressing wild-type Rtt101p and Cdc53p (lane e), and co-migrated with the only band in the *rtt101Δ*/Cdc53pK760R/3xHA:Rub1p strain (lane f). Altogether, these results demonstrate that Cul3p exists in both a non- and Rub1p-modified form *in vivo*.

Rtt101p exists in a modified form that co-migrates with Rtt101p^{Rub1p}, but is Rub1p- and Enr2p-independent

If the slower migrating form of Rtt101p is modified solely by Rub1p, it should be dependent on *RUB1* as observed for Cul3p. 3HA:Rtt101p was expressed in isogenic *rub1Δ* and *RUB1* strains and anti-HA pattern was determined by Western-blot analysis. In contrast with the results observed for Cul3p, a slower migrating Rtt101p form remained in the *rub1Δ* strain (Figure 2, lane b). However, there was a reproducible decrease in its relative abundance compared with an *RUB1* strain (Figure 2, cf. lanes b and c). Quantification of the fraction of modified 3xHA:Rtt101p revealed that in the *RUB1* strain, the modified form was 60% of the total 3xHA:Rtt101p, whereas in the *rub1Δ* strain, the modified form was only 30% of the total 3xHA:Rtt101p. 3xHA:Rtt101p expressed in an *rtt101Δ* background also showed a similar decrease in the slower migrating form in *rub1Δ* relative to its isogenic *RUB1* strain (results not shown). These results suggest that the 110 kDa band represents two modified forms of Rtt101p, only one of which is Rub1p-dependent.

Enr2p/Ula1p is one subunit of the heterodimeric E1-like activating enzyme catalysing the first step in attachment of Rub1p to Cdc53p. To determine if the modified forms of Rtt101p and Cul3p require Enr/Ula1, 3xHA-tagged Rtt101p or Cul3p was expressed in isogenic *ENR2* and *enr2Δ* strains and protein extracts were analysed by Western-blot analysis (Figure 3). 3xHA:Cul3p^{Rub1p} was lost in the *enr2Δ* strain (Figure 3, cf. lanes c and d), demonstrating its dependence on *ENR2*. In contrast, and as seen previously in *RUB1* versus *rub1Δ* strains, a modified form of 3xHA:Rtt101p remained in the *enr2Δ* strain (Figure 3, cf. lanes a and b). Quantification of the bands revealed the same approximate change in the abundance of modified 3xHA:Rtt101p as observed in *RUB1* versus *rub1Δ* strains (results not shown). These experiments suggest that the Rtt101p modification by Rub1p requires Enr2p and the Rub1p-independent modification does not require Enr2p.

Cul3p^{Rub1p} and both Rub1p-dependent and -independent forms of Rtt101p require a conserved lysine residue at their C-termini

Both Rtt101p and Cul3p contain a lysine residue surrounded by an amino acid sequence conserved among cullin family members, although the conservation in this region of Rtt101p is considerably weaker (Figure 4A). To determine whether this lysine residue is required for Rub1p modification, the lysine codon was mutated to encode arginine in both Rtt101p and Cul3p. 3xHA:Rtt101K791Rp was expressed in both RUB1 and rub1Δ backgrounds and its modification state(s) were determined by Western-blot analysis using anti-HA antibodies (Figure 4B). Expression of Rtt101K791Rp in either *RUB1* or *rub1Δ* strain resulted in the loss of all the modified Rtt101p (Figure 4B, cf. lanes a and d with lanes b and e). Therefore both the *RUB1*-dependent and -independent modified forms require Lys-791 of Rtt101p. The modification state of 3xHA:Cul3K688Rp expressed *in vivo* was also determined, and it was found that only the unmodified form of Cul3p is present when the conserved lysine residue is substituted by an arginine residue (Figure 4C). These results indicate that Rtt101p Lys-791 and Cul3p Lys-688 are required for the detection of modified forms and they are probably the site of Rub1p attachment.

A disruption in *RUB1* is not phenotypically identical with an *rtt101Δ* strain

Two different studies have identified phenotypes for *rtt101Δ* strains. *RTT101* was first characterized in a screen for mutants that increase Ty1 transposition rates [24]. Systematic surveys for chemicals that affect cell growth showed that disruption of *RTT101* resulted in increased sensitivity to MMS [32,33], thiabendazole, azaserine and ICPC [34]. Thiabendazole is a microtubule-depolymerizing drug [35], azaserine is an inhibitor of purine biosynthesis and a glutamine antagonist [36,37], and ICPC is a mitotic poison [34]. Although the molecular basis for these sensitivities is not understood, if Rub1p attachment is required for Rtt101p activity, then disruption of *RUB1* should result in a strain with similar phenotype as a strain disrupted in *RTT101*. Tetrads segregating for *rtt101Δ* and *rub1Δ* were tested for sensitivity to ICPC, thiabendazole and MMS. Spotting experiments revealed that *rub1Δ* strains were not hypersensitive to either ICPC or thiabendazole, whereas *rtt101Δ* strains were, as reported previously (Figure 5). *rub1Δ* strains, in contrast with *rtt101Δ* strains, were also not hypersensitive to MMS (Figure 6).

We also tested if *RUB1* was required for *RTT101* function in suppressing Ty1 transposition. The Ty1 transposition rates in an *rub1Δ* strain, *rtt101Δ* strain and the isogenic wild-type strains were determined for two genetic backgrounds (Table 1). Consistent with previously published results indicating that Rtt101p is a regulator of Ty1 transposition [24], *rtt101Δ* mutant strains exhibited a 14-fold increase in Ty1 transposition frequency when compared with the corresponding wild-type strains. In contrast, the small increase in transposition detected in *rub1Δ* mutant strains was 8.8–10-fold less compared with the *rtt101Δ* mutant strains and was not statistically different from wild-type strains (Table 1).

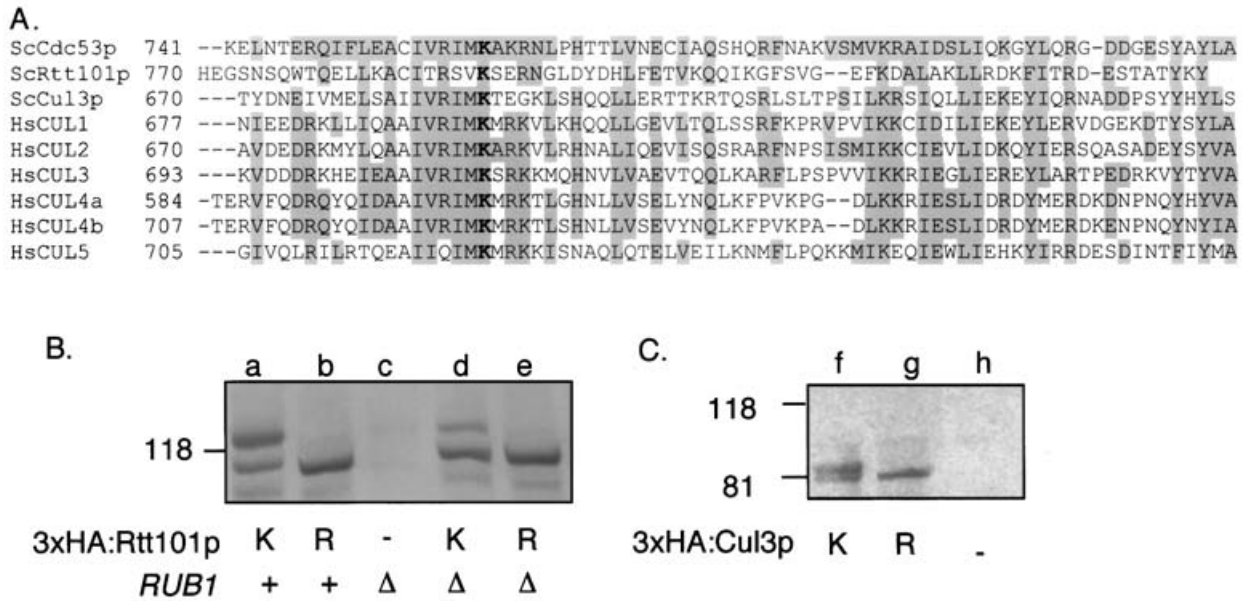

Figure 4 Rtt101p and Cul3p contain a lysine residue conserved among cullins that is required for Rub1p modification

(**A**) Alignment of the C-terminal region of *S. cerevisiae* proteins Cdc53p, Cul3p (YDR003w) and Rtt101p and human proteins CUL1, CUL2, CUL3, CUL4a, CUL4b and CUL5 using ClustalW. Shaded regions represent residues highly conserved between Cdc53p and the protein (with K = R, I = V = L and D = E). Bold letters represent conserved lysine residue and site of attachment of Rub1p/NEDD8. (**B**) Western-blot analysis using anti-HA antibodies of protein extracts from yeast expressing either 3xHA:Rtt101p (lanes a and d), 3xHA:Rtt101K791Rp (lanes b and d) or Rtt101p (lane c) in either an *RUB1* (lanes a and b) or *rub1Δ* (lanes c–e) background. (**C**) Western-blot analysis of protein extracts from yeast expressing either 3xHACul3p (lane f), 3xHACul3K688Rp (lane g) or non-tagged Cul3p (lane h).

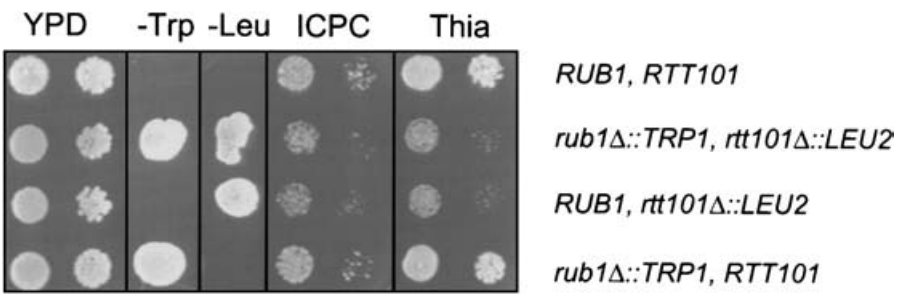

Figure 5 *rub1Δ* strains are not sensitive to thiabendazole or ICPC

Growth assays were performed by growing each strain overnight in YPD broth at 30 °C and then diluting to A_{600} 0.1 and 0.01. Equal volumes of cells were spotted on either a YPD medium with added DMSO (YPD), a synthetic medium lacking tryptophan (-Trp), synthetic medium lacking leucine (-Leu), YPD with 1 mM ICPC (ICPC) or YPD with 100 μg/ml thiabendazole (Thia). ScD-Trp and ScD-Leu select for disruption in *RUB1* or *RTT101* respectively.

Although it appears that Rub1p is not required for Rtt101p function, it remains possible that the Rub1-independent modification of Rtt101p is required. Therefore the ability of Rtt101K791Rp, which is not modified *in vivo*, to complement an *rtt101Δ* strain was tested. Expression of Rtt101K791Rp complemented the hypertransposition phenotype of an *rtt101Δ* mutant as evidenced by suppression of His^+ prototrophs (Figure 7). Interestingly, overexpression of both Rtt101p and Rtt101K791Rp suppressed transposition further in *RTT101* strains (Figure 7). This result indicates that neither lysine modification is required for Rtt101p function in this assay.

DISCUSSION

Despite its high degree of amino acid and structural identity with ubiquitin [4,38], the conjugation pattern of Rub1p/Nedd8 is very distinct from ubiquitin. A typical ubiquitin conjugation pattern visualized by SDS/PAGE is a smear, which may include thousands of proteins, including single and poly-ubiquitinated forms. In contrast, the Rub1p/Nedd8 conjugation pattern is simpler, displaying a number of distinct substrates [7]. We identified Rtt101p and Cul3p as targets of Rub1p conjugation in *S. cerevisiae*. These two cullin-like proteins are related to the previously described Rub1p target, Cdc53p. The existence of a yeast strain lacking all three known Rub1p conjugation targets, either by deletion of the gene encoding the proteins or by expression of proteins lacking the lysine residue required for attachment, would help to determine whether additional, less abundant Rub1p conjugates await discovery. Despite multiple attempts, we were unable to construct a strain with deficiencies in both Rtt101p and Cul3p when expressing a non-modifiable form of Cdc53p. The reasons for this remain unclear.

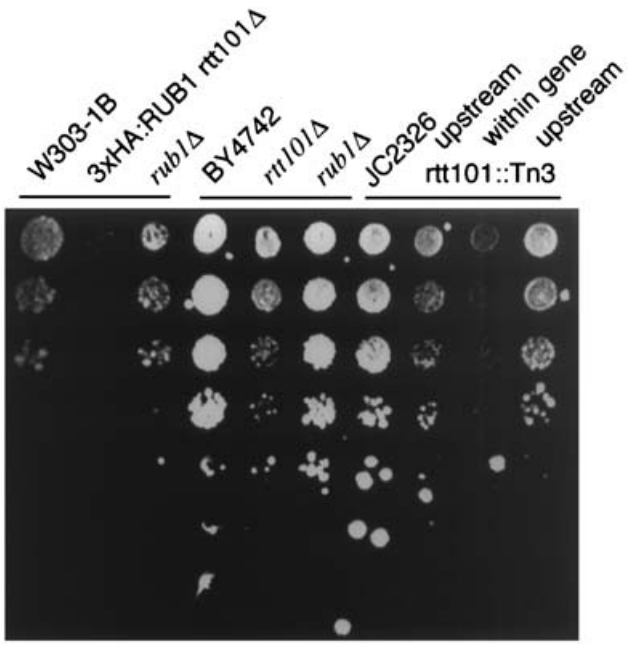

Figure 6 *rtt101Δ* strains are sensitive to MMS, whereas *rub1Δ* strains are not

Growth assays were performed by growing each strain overnight in YPD broth at 30 °C and then diluting to A_{600} 0.8. Samples (3 μl of each culture) and 10-fold serial dilutions were spotted on to YPD agar containing 0.01 % MMS. Each column indicates independent mutations generated as described in the Experimental section, with the corresponding wild-type strains, W303-1b, BY4742 and JC2326 shown on the right in each group. The Tn3 insertions in *RTT101* (last three columns) are strains JC2410, JC2440 and JC2412 (from left to right) whose exact Tn3 insertion sites are described in the Experimental section.

Table 1 Frequency of marked Ty1 elements in *rtt101Δ* and *rub1Δ* strains

Experiment	Strain background	Marked Ty1 element	Relevant genotype	Frequency of Ty1 mobility ($\times 10^{-7}$)*	Relative Ty1 mobility frequency†
I	BY4742	Ty1*his3AI[Δ1]*	wt	4.9±1.5	1
			rtt101Δ	68.0±16	14
			rub1Δ	6.7±1.2	1.4
II	W303-1B	Ty1*kanMX-AI*	wt	68±51	1
			rtt101Δ	968±344	14
			rub1Δ	110±36	1.6

* Number of His^+ Ura^+ (BY4742 background) or $G418^R$ Ura^+ (W303-1B background) prototrophs divided by the total number of Ura^+ cells. Each value is the average frequency ± S.D. calculated from 3–6 independent cultures grown at 20 °C.

† Frequency of mobility of the marked Ty1 element relative to that of the wild-type background strain.

The identity of the conjugates found in the present study are consistent with studies in other organisms where multiple members of the cullin family are targets for Rub/Nedd8 conjugation. NEDD8 conjugates to all members of the human cullin family *in vitro* [39]. *In vivo* NEDD8-modified forms of hCUL1 [18,40], hCUL2 [40,41] and hCUL3 [42] have been identified. In fission yeast, *S.p.*NEDD8 conjugates *in vivo* to two cullin proteins Pcu1 and Pcu4 [9]. The functions of several cullins, but not all, have been described. hCUL1 is a subunit of multiple SCFs, whereas hCUL2 is a part of the SCF-like complex called the von Hippel–Lindau complex [15]. These complexes facilitate transfer of ubiquitin from its *S*-ester-linked form on E2 to an amide linkage on a target protein. On the basis of the crystal structure of hCUL1, Zheng et al. [43] postulated that hCUL3 and its putative orthologues, including *S. cerevisiae* Cul3p, share residues that map to a putative N-terminal surface at a position analogous to the Skp1-binding site on hCUL1 orthologues. This suggests that Cul3-like proteins share an interaction with a currently unidentified protein. hCUL3 plays an undefined role in the ubiquitination of free cyclin E, and is required for free cyclin E proteolysis [42]. For the yeast cullins, Rtt101p and Cul3p, the only experimental result in support of their role in SCF-like complexes is that they interact with the SCF subunit Hrt1p/Rbx1p/Roc1p in a yeast two-hybrid experiment [44].

Rub/Nedd8 attachment to cullin family members has been correlated with increased interaction of an *S*-ester-linked E2 with the E3 ligase, resulting in an activation of ubiquitination activity *in vitro* in mammalian cell lines [17] and *in vivo* in *S. pombe* [9]. Since Rub/Nedd8 and its activating and conjugating enzymes are essential proteins in these organisms [9,12], it is probable that Rub/Nedd8 is required for sufficient SCF activity *in vivo*. In contrast, *RUB1* is not essential in *S. cerevisiae* and, therefore, is not probably required for SCF activity, which is necessary for cell-cycle progression. However, the observed genetic interactions of *rub1Δ* with temperature-sensitive alleles of SCF components suggest a subtle role for Rub1p in SCF function that remains undefined.

Identifying additional Rub1p targets in *S. cerevisiae* and learning about the role of Rub1p modification in their activity could potentially explain why Rub1p has been evolutionarily retained in this organism where its contribution to Cdc53p modification has not been clear. Our experiments found two additional Rub1p conjugates, suggesting that conjugation to these proteins may have a function. Whereas Rtt101p clearly plays a major role in regulating Ty1 transposition frequency, there is no equivalent role for Rub1p in this process. In addition, the Rub1p-independent form is not required, since Rtt101K791Rp is capable of suppressing transposition. Less is known about Cul3p. A *cul3* disruption strain is viable and an *rub1*/*cul3* double disruption does not differ from the *cul3* strain, suggesting no genetic interactions (results not shown). Additionally, expression studies have provided little insight into its function. *CUL3* expression does not change in a cell-cycle-dependent manner [45] during sporulation [46] or in response to exposure to MMS [47]. Given this limited understanding of Cul3p function, we cannot predict the effect of attachment of Rub1p to Cul3p. A better understanding of the biochemical activities of Cul3p and Rtt101p are required before the function of Rub1p attachment to these proteins can be elucidated.

Surprisingly, 3xHA:Rtt101p was found in two forms both in *rub1Δ* and *enr2Δ* strains. The *RUB1*- and *ENR2*-independent Rtt101p-modified forms co-migrated with Rub1p-modified Rtt101p and required the same lysine residue as did the *RUB1*- and *ENR2*-dependent modification. From these results, we hypothesize that Rtt101p is modified covalently at the same lysine residue by another protein having the same or nearly identical mass as Rub1p. If so, Rtt101p presents the first example of a protein being the target of both the Rub1p conjugation system and another protein-tagging system. However, we cannot formally exclude the presence of a non-protein modification or a modification at another site that requires Lys-791. On the basis of the differences in the ratio of modified to unmodified Rtt101p between *rub1Δ* and *RUB1* strains, the form modified independent of Rub1p represents a substantial fraction of the modified form. However, currently, the Rub1p-independent form can only be visualized in the absence of Rub1p or its conjugating enzyme. Hence, it remains possible that the Rub1p-independent modification will only occur in the absence of Rub1p modification. Identification of the nature

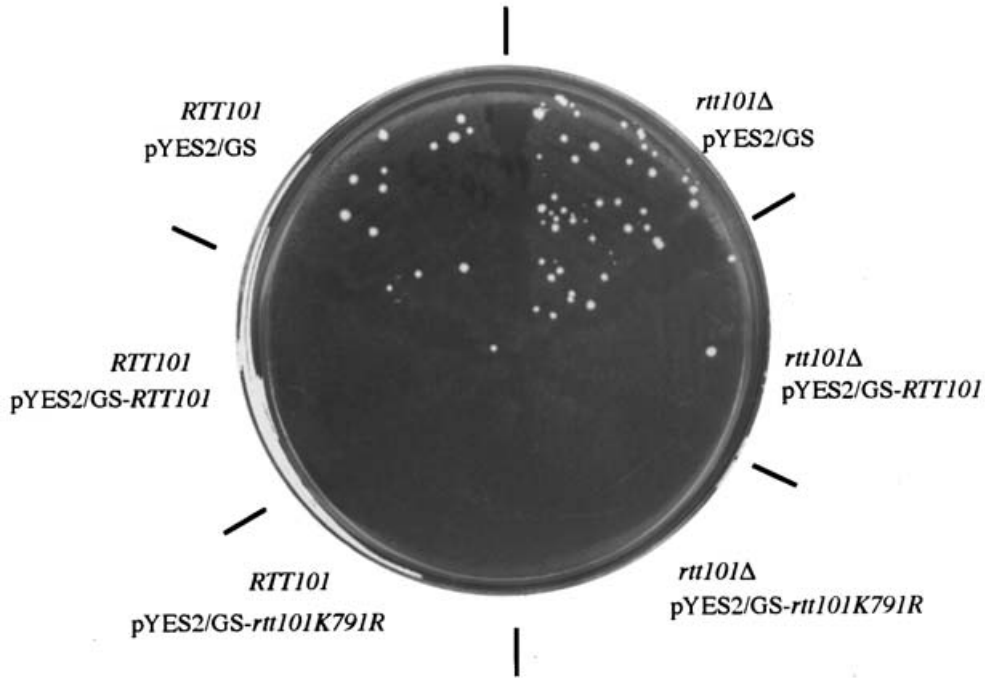

Figure 7 Modification of Rtt101p is not required for suppression of Ty1 transposition

An assay for His^+ prototrophs that arise as a consequence of Ty1 transposition was performed in strains expressing a plasmid vector (pYES2) or a vector plus insert (either *RTT101* or *RTT101K791R*). Expression from the *GAL1* promoter on each plasmid was induced by growth on a rich medium containing galactose. Transposition events in cells that retained the plasmid throughout induction were detected as colonies growing on a ScD-Ura–His plate. A plate showing one representative transformant of each plasmid in an *RTT101* strain and one of each in an isogenic *rtt101Δ* strain is shown here.

of the additional modification will help identify the circumstances under which these two modifications of Rtt101p are present.

Dual modification of a protein by Rub1p/Nedd8 and a second protein has not been described previously. However, modification of the mammalian inhibitor of the Rel family of transcription factors, IκBα, at the same site by either ubiquitin or the ubiquitin-like protein SUMO-1 (small ubiquitin-related modifier-1) has been observed. Lys-21 and Lys-22 in IκBα are sites of ubiquitination that result in degradation by the proteasome (reviewed in [48]). Desterro et al. [49] found that Lys-21 and Lys-22 are also sites of SUMO-1 attachment. Addition of SUMO-1 results in the protection of Lys-21/Lys-22 from ubiquitination and, therefore, from degradation. Thus, targeting of identical residues by multiple modification is similar to that seen in the present study for Rtt101p. However, the function for Rub1-dependent and -independent modification requires data on the biochemical function of this cullin-like protein.

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