Rtt101 and Mms1 in budding yeast form a CUL4^{DDB1}-like ubiquitin ligase that promotes replication through damaged DNA

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In budding yeast the cullin Rtt101 promotes replication fork progression through natural pause sites and areas of DNA damage, but its relevant subunits and molecular mechanism remain poorly understood. Here, we show that in budding yeast Mms1 and Mms22 are functional subunits of an Rtt101-based ubiquitin ligase that associates with the conjugating-enzyme Cdc34. Replication forks in *mms1Δ*, *mms22Δ* and *rtt101Δ* cells are sensitive to collisions with drug-induced DNA lesions, but not to transient pausing induced by nucleotide depletion. Interaction studies and sequence analysis have shown that Mms1 resembles human DDB1, suggesting that Rtt101^{Mms1} is the budding yeast counterpart of the mammalian CUL4^{DDB1} ubiquitin ligase family. Rtt101 interacts in an Mms1-dependent manner with the putative substrate-specific adaptors Mms22 and Crt10, the latter being a regulator of expression of ribonucleotide reductase. Taken together, our data suggest that the Rtt101^{Mms1} ubiquitin ligase complex might be required to reorganize replication forks that encounter DNA lesions.

Keywords: ubiquitin; Rtt101; Cul4; Mms1; DDB1

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INTRODUCTION

The S phase of the cell cycle is a period of great vulnerability for the maintenance of genomic integrity. In particular, events that impede the correct progression of the replication fork can induce the formation of abnormal DNA structures that might contribute to genome instability and, consequently, to carcinogenesis. Eukaryotic cells have therefore developed complex mechanisms to protect their genome during replication (Tourrière & Pasero, 2007). In budding yeast, the cullin Rtt101 assembles a multi-subunit ubiquitin ligase that is required for accurate replication through natural pause sites and damaged templates (Luke *et al*, 2006). Interestingly, Rtt101 is recruited to chromatin in a process that requires the histone H3 acetyltransferase Rtt109 (Collins *et al*, 2007; Roberts *et al*, 2008), which, together with the poorly characterized proteins Mms1 and Mms22, seems to regulate sister chromatid exchanges induced by replisome blockage (Duro *et al*, 2008). However, little is known about the function and the composition of the Rtt101-based ubiquitin ligase at stalled forks.

Cullins are a family of proteins that act as scaffolds for the assembly of multisubunit ubiquitin ligases. They are characterized by a conserved carboxyl-terminal domain that interacts tightly with the RING finger protein RBX1/ROC1/Hrt1, which recruits a ubiquitin-conjugating enzyme. The amino terminus of cullins is more divergent and mediates the association of each cullin with distinct families of substrate-specific adaptor proteins. Although most cullins interact with adaptor or linker proteins that contain a characteristic BTB fold domain (Pintard *et al*, 2004), human CUL4A binds to the β-propeller B (BPB) domain of the DNA-damage-binding protein 1 (DDB1; Li *et al*, 2006). Recently, a family of DDB1- and CUL4-associated factors (DCAFs) has been described, which contain WD40 repeats and function as substrate-specific adaptors in CUL4-based ubiquitin ligases (Jin *et al*, 2006; Lee & Zhou, 2007). Interestingly, CUL4^{DDB1} complexes seem to regulate several DNA transactions that participate in the maintenance of genome integrity such as the control of replication

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licensing and nucleotide excision repair (Higa & Zhang, 2007; O'Connell & Harper, 2007).

The budding yeast *Saccharomyces cerevisiae* encodes three cullin proteins: Cdc53, Cul3 and Rtt101. Although Cdc53 and Cul3 are orthologues of mammalian CUL1 and CUL3, respectively, careful bioinformatics analysis failed to place Rtt101 in a known cullin subfamily. Through genetic screening, DNA combing and biochemical analysis, we identified Mms1 as a homologue of mammalian DDB1. Furthermore, Mms22 and Crt10 interact with Rtt101 in an Mms1-dependent manner, suggesting that they could function as substrate-specific adaptors for the Rtt101-based ubiquitin ligase, and might target proteins involved in replication through damaged templates and expression of ribonucleotide reductase (RNR).

RESULTS

Mms1, Mms22 and Cdc34 function with Rtt101

To identify new components of the Rtt101 pathway, we screened the Euroscarf deletion collection for mutants with sensitivity to the topoisomerase I poison camptothecin (CPT). The identified mutants were analysed further by spotting serial dilutions onto YPD plates containing the DNA-methylating agent methyl methanesulphonate (MMS) or the RNR inhibitor hydroxyurea. Cells lacking *MMS1*, *MMS22* and *RTT101* showed a drug-sensitivity pattern similar to *rtt101Δ* cells (Fig 1A; data not shown), suggesting that they might function as regulators or subunits of the Rtt101-based ubiquitin ligase. In contrast to *rtt101Δ rtt107Δ* double mutants, *rtt101Δ mms1Δ* and *rtt101Δ mms22Δ* cells did not show increased sensitivity to MMS compared with each single mutant (Fig 1A). Furthermore, a genetic interaction network showed that *MMS1*, *MMS22* and *RTT101* most likely function in the same pathway *in vivo* (Fig 1B; Blake et al, 2006; Pan et al, 2006; Collins et al, 2007).

To establish which ubiquitin-conjugating enzyme (E2) functions with Rtt101, we first screened all E2 gene deletions for sensitivity to various genotoxins. Although several E2s were required to protect cells against MMS (supplementary Fig 1A online), only *cdc34-2* and possibly *rad6Δ* cells were sensitive to both CPT and MMS (Fig 1C; supplementary Fig 1B online). To test whether Cdc34 or Rad6 is able to bind to Rtt101, we performed *in vitro* binding experiments using Cdc34, Rad6 and, for control, Ubc4 purified as glutathione S-transferase (GST) fusion proteins from *Escherichia coli*. Haemagglutinin-tagged Rtt101 expressed in yeast extracts was specifically retained on GST-Cdc34 beads, but did not associate with GST alone, GST-Ubc4 or GST-Rad6 (Fig 1D). These functional and biochemical data suggest that similar to Cdc53, the Rtt101-based ligase functions with the ubiquitin-conjugating enzyme Cdc34.

Mms1 and Mms22 promote replication in MMS

Consistent with previous studies (Luke et al, 2006; Duro et al, 2008), we found that *rtt101Δ*, *mms1Δ* and *mms22Δ* cells were competent for non-homologous end joining and homologous recombination at a homothallic switching endonuclease (HO)-induced double strand break (DSB; supplementary Fig 2A; online). To test whether, similar to Rtt101, Mms1 and Mms22 are required to promote fork progression through replication-impeding obstacles, we analysed completion of DNA replication by pulsed-field electrophoresis (PFGE) after releasing synchronized cells from an MMS- or hydroxyurea-induced block. Although

wild-type cells efficiently resumed DNA replication after MMS removal, completion of DNA replication in *mms1Δ* and *mms22Δ* cells was severely delayed (supplementary Fig 2B,C online). This effect was comparable to *rtt101Δ* cells and similarly affected all tested chromosomes (supplementary Fig 2B,C,D online).

To confirm whether this delay is indeed due to replication fork defects, we monitored the recovery of individual forks after MMS treatment by DNA combing. Consistent with PFGE analysis, we detected a 3- to 4-fold increase in unreplicated gaps in *rtt101Δ*, *mms1Δ* and *mms22Δ* cells relative to wild type (Fig 2A,B). These gaps correspond to small regions (around 30 kb) devoid of backup replication origins, which are flanked by irreversibly arrested forks and induce a prolonged activation of the Rad53 checkpoint kinase (Fig 2C). By contrast, and similar to Rtt101, Mms1 and Mms22 were not required to complete replication after fork pausing induced by hydroxyurea (Fig 2C; data not shown). Taken together, these data indicate that Rtt101, Mms1 and Mms22 promote completion of DNA replication after fork arrest induced by DNA damage.

Ubiquitination of the replication clamp proliferating-cell nuclear antigen (PCNA) at Lys 164 by the Rad6–Rad18 complex is an important mechanism to protect replication forks encountering DNA lesions (Moldovan et al, 2007). Therefore, we tested whether the Rtt101-based ubiquitin ligase could participate in PCNA ubiquitylation. In contrast to *rad18Δ* controls, PCNA was efficiently ubiquitinated in MMS-treated wild-type, *rtt101Δ* and *mms1Δ* cells (Fig 2D), showing that the Rtt101 complex is not involved in PCNA modification.

Mms1 is similar to human DDB1

To test whether Mms1 interacts physically with Rtt101 *in vivo*, we expressed protein A-tagged Mms1 in cells containing calmodulin-binding peptide (CBP)-9Myc-tagged Rtt101. Mms1 co-precipitated with Rtt101 (Fig 3A; Suter et al, 2007), but was not present in control purifications prepared from cell extracts containing protein A-tagged Cdc53 (data not shown). This interaction was not altered in *mms22Δ* cells (Fig 3A), suggesting that Mms22 is not required to assemble or stabilize the Mms1–Rtt101 complex. Bioinformatic analysis of Mms1 showed sequence homology to the human CUL4-associated protein DDB1 (supplementary Fig 3A online). Although overall sequence identity was less than 10%, localized regions were conserved in terms of sequence and predicted secondary structure (supplementary Fig 3A online). Closer inspection of these regions predicted that Mms1 probably adopts a DDB1-like β-propeller structure, with a conserved substrate interaction domain on blade 7 within the BPA domain (Fig 3B). Furthermore, the BPB domain of DDB1, which mediates its interaction with CUL4 (Li et al, 2006), is most likely fully intact within Mms1 (Fig 3B; supplementary Fig 3B online).

DDB1 is known to interact with the N-terminal domain of the CUL4-type cullins. Indeed, Rtt101 deleted for its 50 (ΔN50) or 100 (ΔN100) N-terminal residues failed to restore growth of *rtt101Δ* cells on plates containing 0.015% MMS, although the mutant proteins were expressed at comparable levels (Fig 3C; data not shown). Furthermore, significantly reduced amounts of Mms1 co-precipitated with Rtt101-ΔN50 compared with wild-type controls (Fig 3A), indicating that the N-terminal domain of Rtt101 is required for interacting with Mms1.

To analyse the relationships of DDB1 and MMS1 homologues, we constructed a dendrogram using the neighbour-joining

Fig 1 | *RTT101* interacts with *MMS1*, *MMS22* and *CDC34*. (A) The indicated yeast deletion mutants were grown overnight to saturation in YPD medium at 30 °C and serially diluted onto YPD plates either in the presence (right panel) or absence (left panel) of 0.0025% MMS, and incubated for a further 2 days at 30 °C. (B) *MMS22*, *MMS1* and *RTT101* interact genetically with many of the same genes involved in DNA replication and homologous recombination. Green, synthetic lethality; grey, synthetic growth defect. (C) *rtt101Δ* and *cdc34-2* mutants were grown overnight in YPD medium at 25 °C, and serial dilutions were spotted onto YPD media containing, as indicated, 0.01% MMS or 10 μM CPT. Cells were grown for 2 days at 32.5 °C. (D) Bacterially expressed GST, GST-Rad6, GST-Ubc4 and GST-Cdc34 were immobilized onto glutathione beads and incubated with yeast extracts containing HA-Rtt101. Bound HA-Rtt101 was detected by Western blotting (upper panel), whereas the purified GST fusion proteins were visualized by Ponceau S staining (lower panel, the band corresponding to full-length GST-Cdc34 is marked with an asterisk). CPT, camptothecin; GST, glutathione S-transferase; HA, haemagglutinin; MMS, methyl methanesulphonate; YPD, yeast extract, peptone, dextrose; WT, wild type.

algorithm (Fig 3D). Four clades can be inferred from the resulting tree, which are formed by DDB1, RSE1, CFT1 and MMS1 homologues, respectively. Apparently, the MMS1 and the DDB1 homologues do not belong to a common clade. Furthermore, although *S. cerevisiae* encodes a single MMS1-like protein, both *Schizosaccharomyces pombe* and *Neurospora crassa* have an MMS1-like protein and an authentic DDB1. By contrast, the MMS1-like protein is not present in human cells, where only DDB1 exists. These results indicate that MMS1 is a distant homologue of human DDB1.

Mms22 and Crt10 interact with Rtt101 via Mms1

Human DDB1 bridges the interaction with cullin 4 and the DCAF substrate-specific adaptors. To identify putative substrate-specific adaptors of the Rtt101 complex, we prepared extracts from strains expressing tandem affinity purification (TAP)-tagged Mms1 and Rtt101, and for control Cdc53. After TAP purifications, co-purifying proteins were analysed by mass spectrometry. Interestingly, Crt10, a regulator of RNR gene transcription (Fu & Xiao, 2006), co-purified with both Rtt101 and Mms1, but not with Cdc53 (Fig 4A; data not shown). The interaction between Crt10 and Rtt101 was dependent

on Mms1 (Fig 4A). By contrast, Crt10 efficiently interacted with Mms1 in the absence of Rtt101 (data not shown), and Crt10 failed to co-precipitate with Rtt101-ΔN50, which is defective for binding to Mms1 (Fig 4A). Interestingly, Mms22 was also recovered in Rtt101 TAP purifications (data not shown), suggesting that Mms22 might also be a subunit of the Rtt101 complex. Indeed, similar to Crt10, the interaction between Mms22 and Rtt101 was dependent on Mms1 and required an intact N-terminal domain of Rtt101 (Fig 4B). However, Crt10 interacted with Rtt101 in the absence of MMS, whereas the interaction between Mms22 and Rtt101 was stimulated after MMS treatment (Fig 4B), suggesting that Rtt101 might form two distinct complexes with separate functions. Taken together, we conclude that Mms1 bridges the interaction between Crt10 and Mms22 with Rtt101 (Fig 4C).

DISCUSSION

Rtt101^{Mms1} is the CUL4^{DDB1} equivalent

Several lines of evidence suggest that Mms1 is the *S. cerevisiae* equivalent of mammalian DDB1. First, Mms1 binds to the N-terminal domain of Rtt101, which is required for its function *in vivo*. Second, bioinformatic analysis showed that Mms1 and

Fig 2 | *mms1Δ*, *mms22Δ* and *rtt101Δ* cells are unable to replicate past MMS-induced DNA damage. (A) Wild-type, *rtt101Δ*, *mms1Δ* and *mms22Δ* cells were arrested in G₁ with α -factor and released into S phase in the presence of 0.033% MMS and 400 μ g/ml BrdU. After 60 min, the cells were resuspended in a fresh medium containing BrdU. The persistence of unreplicated gaps along individual chromosomes was monitored by DNA combing 130 min after release from MMS. Representative DNA fibres are shown, with arrowheads pointing to unreplicated gaps. Green: BrdU; red: DNA. Scale bar, 50 kb. (B) The frequency of unreplicated gaps in wild-type, *rtt101Δ*, *mms1Δ* and *mms22Δ* cells was quantified 130 min after release from MMS. (C) The indicated yeast strains expressing endogenously tagged Rad53-TAP were grown to exponential phase at 30 °C in YPD (0–) after which MMS was added for 2 h (0.02% final concentration). MMS was then quenched with sodium thiosulphate and the cells were released into pre-warmed YPD media (0+). Whole-cell protein extracts prepared at the indicated time points were probed for Rad53-TAP by Western blotting with the PAP antibody (Sigma, Buchs, Switzerland). (D) The indicated yeast mutants expressing 6 $\times$ HIS-tagged PCNA were grown and exposed to MMS as outlined in (C). PCNA was precipitated in the presence of 6 M guanidine HCl to preserve sensitive post-translational modifications, and ubiquitylated PCNA was detected using mouse monoclonal antibodies directed against ubiquitin (Cell Signaling, Allschwil, Switzerland). BrdU, 5-bromodeoxyuridine; HU, hydroxyurea; MMS, methyl methanesulphonate; PAP, peroxidase anti-peroxidase; PCNA, proliferating-cell nuclear antigen; YPD, yeast extract, peptone, dextrose; WT, wild type.

DDB1 share sequence similarity in the BPB and BPA domains, which in DDB1 are known to interact with CUL4 and the substrate-specific adaptors, respectively. Finally, Mms1 acts as a linker that bridges the interaction between Rtt101 and Mms22 or Crt10, which are thus likely to function as substrate-specific adaptors analogous to mammalian DCAFs. Taken together, these results support the idea that the Rtt101^{Mms1} complex belongs to the CUL4^{DDB1} subfamily of cullin-based ubiquitin ligases. Similar to Rtt101 in budding yeast, mammalian CUL4A has been shown to participate directly in the maintenance of genome integrity. For example, CUL4A mediates the ubiquitination and degradation of the replication-licensing factor CDT1, which is an important regulatory mechanism to prevent re-replication in metazoans (Higa & Zhang, 2007; O'Connell & Harper, 2007). CUL4A also controls nucleotide excision repair in response to ultraviolet-induced DNA damage (Higa & Zhang, 2007; O'Connell & Harper, 2007). By analogy with the function of Rtt101 in budding yeast,

our results now suggest that CUL4^{DDB1} complexes might also promote DNA replication through damaged templates.

Rtt101 might regulate nucleotide synthesis

Previously, Crt10 has been implicated in the transcriptional regulation of RNR and is upregulated in response to DNA damage (Fu & Xiao, 2006). RNR catalyses the rate-limiting step in the production of deoxynucleotides, and its activity is tightly controlled to provide appropriate amounts of deoxynucleotides for DNA replication and repair (Nordlund & Reichard, 2006). In fission yeast, CUL4 and DDB1 homologues regulate RNR activity by controlling the proteolysis of the RNR inhibitor Spd1 at the onset of S phase and following DNA damage (Bondar *et al*, 2004; Liu *et al*, 2005). Our observation that Crt10 might function as a substrate-specific adaptor for the Rtt101-based ubiquitin ligase therefore raises the possibility that Rtt101 and Mms1 might similarly regulate the activity of RNR in budding yeast.

Fig 3 | Mms1 belongs to the DDB1 family of cullin 4 adaptors. (A) Extracts prepared from wild-type (–), *crt10Δ* and *mms22Δ* cells expressing protein A-tagged Mms1 and either full-length (WT) or amino-terminally truncated (ΔN50) CBP-9Myc-Rtt101 were incubated with calmodulin beads, and the total extract (upper panels) and eluate (lower panels) were probed for the presence of bound Rtt101 and Mms1. (B) Three-dimensional representation of the DDB1 structure adapted from Li *et al* (2006); PDB accession 2b5m. The three β-propeller domains are labelled as BPA, BPB and BPC. Each propeller is characterized by seven repeats of antiparallel four-stranded β-sheets and can be assigned to distinct functions. BPB has been described to be a cullin-binding domain, whereas the BPA and BPC domains seem to bind to substrates. Stretches found to be conserved between Mms1 and DDB1 homologues are coloured in orange. (C) Full-length and the indicated N-terminal Rtt101 truncation mutants Δ50 and Δ100 were expressed as the only Rtt101 copy from *GAL1,10* promoter. An empty vector (Vc) acted as a negative control. (D) Dendrogram of the DDB1/Mms1 homology using the neighbour-joining algorithm. For the tree construction, only reliable and gap-free alignment columns were used. Species names: Ag, *Anopheles gambiae*; At, *Arabidopsis thaliana*; Br, *Brachydanio rerio*; Ca, *Candida albicans*; Dm, *Drosophila melanogaster*; Eg, *Eremothecium gossypii*; En, *Emicella nidulans*; Hs, *Homo sapiens*; Kw, *Kluyveromyces waltii*; Mm, *Mus musculus*; Nc, *Neurospora crassa*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Xt, *Xenopus tropicalis*. The principal subfamilies are indicated at the right border of the figure. BPA, β-propeller A; BPB, β-propeller B; BPC, β-propeller C; DDB1, DNA-damage-binding protein 1; PDB, Protein Data Bank.

Rtt101^{Mms1/Mms22} at stalled replication forks

Similar to Rtt101 (Luke *et al*, 2006), Mms1 and Mms22 promote replication through damaged templates. Indeed, recent evidence indicates that Mms22 and Mms1 promote S-phase-specific sister chromatid recombination, a function that also involves the unconventional acetyltransferase Rtt109 and the histone chaperone Asf1 (Duro *et al*, 2008). Interestingly, genetic evidence indicates that Rtt101, Mms1 and Mms22 function downstream from Rtt109 (Collins *et al*, 2007), and that Rtt109-dependent acetylation is required to recruit Rtt101 to chromatin in the presence of DNA damage (Roberts *et al*, 2008). Taken together, these data indicate that the Rtt101^{Mms1/Mms22} complex might promote recombinational repair at stalled replication forks. The BRCA1 C terminus (BRCT) repeat-containing protein Rtt107 also interacts with Rtt101 (Rouse, 2004; Roberts *et al*, 2008) and functions at stalled replication forks (Rouse, 2004). However, Rtt107 is not required for recombination at stalled forks

(Duro *et al*, 2008), and we have shown that deletion of *RTT101* and *RTT107* has additive sensitivities to MMS. Therefore, Rtt107 is unlikely to be involved directly in the ubiquitin ligase function of Rtt101 at stalled replication forks.

Finally, our data provide significant mechanistic insights into the function of the Rtt101^{Mms1/Mms22} complex. First, the interaction between Mms22 and Rtt101 is regulated by DNA damage, suggesting that the active ubiquitin ligase is assembled on stalling of the replication fork. It will be informative to investigate further how replication fork stalling triggers the assembly of the Rtt101^{Mms1/Mms22} complex and whether it depends on the Rtt109 acetyltransferase. Second, the analogy with CUL4-based ubiquitin ligases suggests that Mms22 might function as a substrate-specific adaptor. This is an important step towards the identification of Rtt101 ubiquitination substrate(s) relevant for its function in maintaining genome stability during replication.

Fig 4 | Mms1 bridges the interaction of Mms22 and Crt10 with Rtt101. (A,B) Extracts prepared from wild-type (–) and *mms1Δ* cells expressing, as indicated, either full-length (WT) or N-terminally truncated (ΔN50) CBP-9Myc-Rtt101 together with protein A-tagged Crt10 (A) or Mms22 (B) were incubated with calmodulin beads. The total extract (upper panels) and eluates (lower panels) were then probed for the presence of bound Rtt101, Crt10 and Mms22. In (B), the extracts were prepared from cells treated for 20 min with or without 0.1% MMS. Note that the interaction of Rtt101 with Mms22 is regulated by DNA damage. (C) The DDB1-like protein Mms1 interacts with the N-terminal domain of Rtt101 and allows the recruitment of the putative substrate-specific adaptors Crt10 and Mms22. The Rtt101^{Mms1/Crt10} complex might regulate the activity of RNR, whereas the Rtt101^{Mms1/Mms22} complex promotes replication through DNA lesions, most likely through ubiquitination of an unknown target protein. DDB1, DNA-damage-binding protein 1; MMS, methyl methanesulphonate; RNR, ribonucleotide reductase.

METHODS

Yeast strains and plasmid construction. All yeast strains were derivatives of the Euroscarf BY4741 background (*MATa*, *ura3Δ0*, *leu2Δ0*, *his3Δ0* and *met15Δ0*). All DNA manipulations were performed according to standard protocols; construct details are available on request.

Affinity precipitations. Yeast strains were transformed with centromeric plasmids expressing either CBP-9Myc-Rtt101 or CBP-9Myc-Rtt101-ΔN50 in combination with protein A-tagged Mms1, Crt10 or Mms22 expressed from the *ADH* promoter. Yeast pellets prepared from 10 ml exponentially growing cultures, which were treated as indicated with 0.1% MMS for 20 min, were resuspended in 300 μl extraction buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM CaCl₂, 0.5% Triton, 10 mM β-mercaptoethanol, 1 × antiproteases (Complete EDTA-free; Roche, Basel, Switzerland) and lysed with 1.25 mm glass beads in a FastPrep bead beater (QBiogene, Basel, Switzerland; 20 s beating at 4 m/s). Total extracts were diluted to 1 ml in extraction buffer, incubated for 1 h at 4 °C with 10 μl calmodulin agarose resin (Stratagene, Basel, Switzerland) and washed six times with 1 ml extraction buffer; bound proteins were eluted with 10 μl elution buffer (20 mM Tris pH 8.0, 1 M NaCl, 100 mM EDTA). Total extracts and eluates were separated on 6% Tris–tricine acrylamide gels and blotted on polyvinylidene fluoride membranes. Protein A-tagged Mms1, Crt10 and Mms22 were visualized with rabbit PAP (peroxidase anti-peroxidase) complexes (Sigma, Buchs, Switzerland; P1291), and Rtt101 with a rabbit anti-Myc (Gramsch, Schwabhausen, Germany).

GST pull downs. Yeast cells expressing HA₃-Rtt101 were lysed in immunoprecipitation buffer (20 mM Tris pH 7.5, 100 mM NaCl,

5 mM MgCl₂, 0.2% NP-40, 5% glycerol, 0.5 mM dithiothreitol, 1 mM NaF, 20 mM β-glycerophosphate, 1 × antiproteases; Roche) with glass beads, and the extracts were incubated for 1 h at 4 °C with purified GST or GST-tagged Cdc34, Ubc4 or Rad6. GST-tagged proteins were then purified using GST- beads and washed extensively with immunoprecipitation buffer, and bound proteins were eluted with sample buffer. The elutes were separated on SDS–polyacrylamide gel electrophoresis, immunoblotted with haemagglutinin antibodies, and the membrane was stained with Ponceau S to visualize bound GST proteins.

Sequence alignments and dendrogram. Sequence database searches were performed with a non-redundant data set calculated from current releases of UniProt, TrEMBL and GenPept. Several alignments were calculated by T-Coffee, using excised domains instead of the complete sequences. Generalized profile construction and searches were performed using the pftools package, version 2.1 (available at <ftp://ftp.isrec.isb-sib.ch/sib-isrec/pftools/>), and the BLOSUM45 substitution matrix and default penalties of 2.1 for gap opening and 0.2 for gap extension. Statistical significance of profile matches was calculated from the analysis of the score distribution of a randomized database. Only sequence matches found with a probability of $P < 0.01$ were included in the following rounds of iterative profile refinement. Dendrogram analysis was carried out using the ‘neighbour-joining’ algorithm.

MMS recovery and DNA combing experiments. Cells were arrested in G₁ with α-factor and released into S phase in the presence of 0.033% MMS (Sigma) and 400 μg/ml BrdU (Sigma). After 60 min, the cells were washed with sodium thiosulphate (Sigma) and resuspended in fresh medium. The cells were collected every 15 min and the electrophoretic mobility of chromosomes,

which is indicative of completion of DNA replication, was monitored by PFGE using a Gene Navigator system (GE Healthcare, Glattbrugg, Switzerland). PFGE gels were stained with ethidium bromide (Sigma) and transferred on Hybond-XL membranes (GE Healthcare) to analyse specific chromosomes by Southern blot or on nitrocellulose membranes (GE Healthcare) to analyse BrdU incorporation with an anti-BrdU antibody (clone BU1/75; AbCys, Paris, France). Chromosome mobility was quantified with a Typhoon Trio+ (GE Healthcare).

DNA combing was performed as described previously (Luke et al, 2006), following the detailed protocol published at <http://www.epigenome-noe.net/researchtools/protocol.php?protid=36>. For each strain, 50–100 Mb of genomic DNA was analysed.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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