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Investigation

Genome stability is guarded by yeast Rtt105 through multiple mechanisms

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Abstract

Ty1 mobile DNA element is the most abundant and mutagenic retrotransposon present in the genome of the budding yeast *Saccharomyces cerevisiae*. Protein regulator of Ty1 transposition 105 (Rtt105) associates with large subunit of RPA and facilitates its loading onto a single-stranded DNA at replication forks. Here, we dissect the role of *RTT105* in the maintenance of genome stability under normal conditions and upon various replication stresses through multiple genetic analyses. *RTT105* is essential for viability in cells experiencing replication problems and in cells lacking functional S-phase checkpoints and DNA repair pathways involving homologous recombination. Our genetic analyses also indicate that *RTT105* is crucial when cohesion is affected and is required for the establishment of normal heterochromatic structures. Moreover, *RTT105* plays a role in telomere maintenance as its function is important for the telomere elongation phenotype resulting from the Est1 tethering to telomeres. Genetic analyses indicate that *rtt105Δ* affects the growth of several *rfa1* mutants but does not aggravate their telomere length defects. Analysis of the phenotypes of *rtt105Δ* cells expressing NLS-Rfa1 fusion protein reveals that *RTT105* safeguards genome stability through its role in RPA nuclear import but also by directly affecting RPA function in genome stability maintenance during replication.

Keywords: Rtt105; replication; chromatin; cohesion; telomere

Introduction

The Ty1 element of the budding yeast *Saccharomyces cerevisiae* is the best-studied LTR-retrotransposon among the five species of retrotransposable elements present in budding yeast (Voytas and Boeke 1992; Curcio *et al.* 2015). The stability of Ty1-related sequences, which are the most repetitive components of the *Saccharomyces cerevisiae* genome is modulated by host factors. These factors influence retrotransposition either by promoting efficient Ty1 retromobility (retromobility host factors, *RHF* genes) or by maintaining transpositional dormancy (restrictors of Ty1 transposition, *RTT* genes) (Curcio *et al.* 2007; Nyswaner *et al.* 2008; Dakshinamurthy *et al.* 2010).

RTT genes are involved in different aspects of host–genome maintenance and the product of these genes function essentially during S phase of the cell cycle (Scholes *et al.* 2001). For example, *RTT106* encodes a histone chaperone involved with CAF-1 in replication coupled chromatin assembly and in the integrity of advancing replication forks (Li *et al.* 2008; Clemente-Ruiz *et al.* 2011; Han *et al.* 2013). Rtt109 is a histone acetyl transferase that modifies lysine 56 of histone H3 (Driscoll *et al.* 2007). Rtt109 is required, with Rtt106, Rtt101, and Mms1 (Rtt108), during normal replication, and to modulate replisome function during replicative stress to promote cell survival in the presence of DNA damages (Han *et al.* 2013; Luciano *et al.* 2015). On the other hand, Rtt102 is a component of both SWI/SNF and RSC chromatin remodeling complexes involved in DNA replication stress response (Schubert *et al.* 2013) while Rtt103 associates with sites of DNA breaks and functions in the DNA damage response (Srividya *et al.* 2012). Rrm3 (Rtt104) travels with the fork and helps the replication fork traverse protein–DNA complexes (Azvolinsky *et al.* 2006). Elg1 (Rtt110) is a subunit of an alternative replication factor C complex important for DNA damage recovery during replication, involved in cohesion, and in telomere maintenance (Kanellis *et al.* 2003; Smolikov *et al.* 2004; Parnas *et al.* 2009). Rtt107 is implicated in DNA repair during S phase and recruits Smc5/6 to double-stranded breaks (DSB) (Leung *et al.* 2011).

A genome-wide analysis indicated that *RTT105* exhibited genetic interaction with genes involved in genome maintenance (Collins *et al.* 2007). Immunoprecipitation and mass spectroscopy revealed that Rtt105 co-purifies with the three subunits of RPA and Kap95, the primary karyopherin responsible for RPA import (Li *et al.* 2018, 2019). The Rtt105/RPA interaction is required for the association of RPA with Kap95 and peaks in S phase (Li *et al.* 2018). Rtt105 was proposed to form an alternative adapter for RPA nuclear import, modulating RPA level in nucleus, and to assist RPA in adopting a more extended conformation to contact ssDNA (Li *et al.* 2018). Of note, Rtt105 is not present in the final RPA–ssDNA complex, revealing that the functions of Rtt105 are highly analogous to histone chaperones in regulating histone

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behaviors (Li *et al.* 2018). As a consequence, *rtt105Δ* cells die in the presence of hypomorph alleles of *ORC2* or *POL3*, are sensitive to genotoxic agents such as HU, CPT, MMS, and bleomycin, and present a mild delay of S phase progression during DNA replication (Li *et al.* 2018). Importantly, while the binding level of RPA is strongly reduced in the absence of *RTT105*, no dramatic decrease of global DNA synthesis level under unperturbed replication forks is detected. These observations suggest that Rtt105 is more important for regulating RPA binding at perturbed replication forks where more and longer ssDNA intermediates are generated (Sogo *et al.* 2002). RPA is the main single-stranded DNA-binding protein involved in multiple processes including replication, transcription, recombination, checkpoints, telomere maintenance, elimination of G-rich DNA secondary structures, and DNA repair. To advance our understanding of the relationship between Rtt105 and RPA, it is crucial to determine the role of Rtt105 in the multiple functions of RPA and if these functions are directly affected through Rtt105 ability to chaperone RPA.

In this study, we further investigate the importance of *RTT105* in replication and in various replication-coupled mechanisms in which RPA plays a critical role. We show that *RTT105* is essential for the viability of cells when replisome progression or S-phase checkpoint is affected. Consistent with these results, homologous recombination (HR), but not nonhomologous end joining (NHEJ) is required to sustain the growth of *rtt105Δ* mutant. We further report multiple genetic interactions between *RTT105*, and genes involved in chromatin structure formation and in cohesion establishment. We also reveal that *RTT105* is required for telomere elongation by telomerase likely at a step independent of telomerase recruitment but related to its function as RPA chaperon. Finally, our data show that *RTT105* affects DNA metabolism and genome stability not only via its role in RPA nuclear import and indicate that *RTT105* exerts a crucial role during replication.

Materials and methods

Strain construction

All strains used in this study are listed in Supplementary Table S1. To construct the pRS316-*NLS-RFA1* plasmid allowing the expression of *NLS-RFA1*, a first PCR was used to amplify a fragment containing the promoter of *RFA1* fused to an NLS (encoding PKKKRKV). A second PCR was performed to amplify the full *RFA1* coding sequence. The primers were designed in order to generate overlapping sequences between the two PCR products. A third PCR combining the two previous PCR products as templates produced a fragment coding for *NLS-RFA1* under the control of *RFA1* promoter. This fragment was gel-purified, digested with *Bam*HI-HF and *Hind*III-HF (BioLabs), and cloned into pRS316. The resulting pRS316-*NLS-RFA1* vector was confirmed by DNA sequencing.

Fluorescence microscopy

All microscopy analyses were performed in liquid (SC synthetic media) using a Nikon Eclipse Ti microscope with a 100× objective. Images were collected using a Neo sCMOS camera (Andor). Exposure time was DIC: 100 ms; CFP: 500 ms. Images were analyzed using ImageJ on 2D-maximum projections from 11-Z-stacks spaced 0.5μ each. Cells were prepared by growing at 30°C.

Telomere length and cell senescence analyses

Telomere length analysis and cell senescence assays were performed as described (Simon *et al.* 2021). Telomere lengths were determined with Quantity one software (Bio Rad) using a semi-log plot generated from the distance migrated on the same agarose gel by DNA fragments in the Eurogentec SmartLadder. Average lengths ± standard deviations are reported in Table 1. Liquid senescence assays were performed starting with the spore products of *est1Δ/EST1 rtt105Δ/RTT105* diploid strain. The diploid strain has been propagated for at least 50 population doublings (PDs) on YPD plates to ensure homogeneous telomere length before sporulation. The senescence assay was performed as described by Simon *et al.* (2021).

Table 1 Telomere length analysis of *rtt105Δ*, *yku80Δ*, and *rtt105Δ yku80Δ* mutants

Mutant	Telomere length relative to WT (bp)
rtt 105Δ	−106 ± 14
yku80Δ	−148 ± 13
rtt105Δ yku80Δ	−167 ± 7

Values based on $n = 7$.

Analysis of CLB2-rfa1 protein level

Yeast cells were grown at 30°C in YPD to an OD_{600} = 0.8 and arrested in S-phase by adding 200 mM HU (Sigma) for 2 h. HU was removed to allow cells to progress synchronously through the cell cycle in the presence of 25 μg/ml nocodazole. Samples were taken at the indicated time point for FACS analyses to monitor the progression of the cell cycle and for protein extraction. Cells were then lysed by bead beating in the presence of 20% TCA. The pellets were recovered by centrifugation and incubated with 1× Laemmli buffer at 95°C for 5 min to recover proteins. Subsequently, proteins were separated on 10% poly-acrylamide gel (Life Technologies) followed by Western blotting with anti-Rfa1 antibody (Agrisera).

Protein chromatin-binding assay

Asynchronous cells were harvested and incubated in 3 ml of prespheroplasting buffer (100 mM PIPES (pH 9.6), 10 mM dithiothreitol (DTT) for 10 min at 30°C. After centrifugation, cells were resuspended in 2 ml of spheroplasting buffer (50 mM KH_2PO_4/K_2HPO_4 (pH 7.5), 0.6 M Sorbitol, 10 mM DTT, 0.5 mM PMSF) containing 10 μl of 10 mg/ml of Zymolase (AMSBIO) and incubated at 30°C for 25 min with gentle shaking. Spheroplasts were washed with 1 ml of cold Diffley buffer (20 mM PIPES (pH 6.8, 150 m KOAc, 2 mM $MgOAc_2$, and 0.4 M Sorbitol) containing 1 mM PMSF and protease inhibitors, pelleted at 3000 rpm for 5 min at 4°C, resuspended in 600 μl of Diffley buffer containing 1% Triton X-100 and incubated on ice for 5 min with gentle mixing. Finally, lyzed nuclei were centrifugated at 13.000 rpm for 15 min at 4°C and the pellets corresponding to the chromatin-associated proteins fraction were resuspended in 1× Laemmli buffer.

Data availability

All data and method required to confirm the conclusions of this work are within the manuscript: Supplementary Table S1: Strains used in this study. Supplementary Figure S1: *RTT105* is required for normal cell growth. Figure S2: *RTT105* exhibits genetic interactions with S-phase checkpoint components. Supplementary Figure S3: *RTT105* is important for cells affected in the replication-dependent nucleosome assembly process. Supplementary Figure S4: *RTT105* is important for cells affected in the replication-dependent nucleosome assembly process but not in replication-independent nucleosome assembly. Supplementary Figure S5: *RTT105* inactivation exacerbates the telomeric defects arising in the absence of *YKU* and *EST1* genes. Supplementary

Figure S6: *RTT105* inactivation affects senescence and survivor formation in *est1Δ* cells. Supplementary Figure S7: Exogenous expression of *rtt105Δ155-208* mutant failed to rescue the growth in *rtt105Δ* cells. Supplementary Figure S8: Cell cycle regulation of CLB2-rfa1. Supplementary Figure S9: Bringing Rfa1 into the nucleus rescues the viability of *rfa1Δ* and *rfa1-D228Y rtt105Δ* cells but not the growth defect and HU sensitivity in *rtt105Δ* cells. Supplementary Figure S10: Bringing Rfa1 into the nucleus rescues the growth of *rtt105Δ* cells affected in S-phase checkpoint, cohesion, or repair.

Supplementary material is available at figshare DOI: https://doi.org/10.25386/genetics.13373021.

Results

The *rtt105Δ* mutant grows slowly at 25°C and is not sensitive to replication stress induced by the absence of PIF1 family helicases

One allele of *RTT105* was disrupted in a wild-type diploid yeast strain. After spore dissection, we noticed that *rtt105Δ* spores exhibited a slow-growth defect at 30°C (Supplementary Figure S1, upper panel, left). This slow growth phenotype was amplified at 25°C without affecting spore viability (Supplementary Figure S1, upper panel, right). We obtained similar results by deleting *RTT105* directly in haploid cells (Supplementary Figure S1, lower panels). *rtt105Δ* cells were previously reported to be highly sensitive to DNA-damaging agents (Li *et al.* 2018). During replication, forks encounter natural impediments throughout the genome (Gadaleta and Noguchi 2017). We assessed the importance of *RTT105* in the absence of Pif1 and Rrm3 that assist the replisome in the replication of difficult to replicate genomic regions. Both proteins perform overlapping and distinct roles in replication, repair, telomere length maintenance, and cohesion (reviewed in Muellner and Schmidt 2020). We found that *RTT105* was dispensable for the growth of *pif1Δ* cells (Figure 1A, left) suggesting that Rtt105 and Pif1 work in the same pathway to counteract DNA damages arising from G4 DNA secondary structures (Maestroni *et al.* 2020). Surprisingly, we observed that *RTT105* was also dispensable for *rrm3Δ* growth (Figure 1A, right). Indeed, in cells lacking Rrm3, chronic stalling of forks at protein–DNA barriers is associated with increased DNA damage and checkpoint activation (Ivessa *et al.* 2003; Azvolinsky *et al.* 2006; Schmidt and Kolodner 2006). These results indicate that *RTT105* functions with specific type, and/or with a certain level of DNA damage.

RTT105 exhibits synthetic genetic interactions with genes encoding replisome components

The *rtt105Δ* mutation is synthetic lethal when combined with mutations in either the origin recognition complex (ORC) or polymerase δ (Li *et al.* 2018). Having shown that the replisome component Rrm3 was not crucial for *rtt105Δ* cells, we investigated the importance of *RTT105* for replication fork progression that depends on the replisome progression complex (RPC) (Gambus *et al.* 2006). The RPC consists of Mcm2–Mcm7 proteins, Mcm10, the go ichi ni san (GINS) complex, Cdc45, the trimeric complex of regulatory factors comprising Tof1, Csm3, and the checkpoint mediator Mrc1, Ctf4, Top1, and the histone chaperon FACT (Spt16 and Pob3). In agreement with the fact that *RTT105* plays a role during replication (Li *et al.* 2018), we found that combining *rtt105Δ* with the thermosensitive *mcm2-1* helicase mutant resulted in a marked slow growth phenotype (Figure 1B, left). We further found that *rtt105Δ cdc17-1* double mutant was dead at 30°C (Figure 1B, right), indicating that *RTT105* is important in cells experiencing lagging strand-induced replicative stress. Along the same line, cells lacking *RTT105* were inviable in the absence of Mrc1 that promotes replisome progression at the leading strand (Yeeles *et al.* 2017) (Figure 1C, left). In addition to its role in replication, *MRC1* is also required for checkpoint activation after DNA replicative stress. We found that the *mrc1-C14* mutant that is compromised for its replication function but proficient for its checkpoint function (Naylor *et al.* 2009) was synthetically lethal or very slow growing with *rtt105Δ* (Figure 1C, middle). Finally, because Mrc1 together with Tof1/Csm3 also forms a complex required for fork protection (Calzada *et al.* 2005; Tourrière *et al.* 2005; Bando *et al.* 2009; Eickhoff *et al.* 2019), we analyzed the contribution of *TOF1/CSM3* to *rtt105Δ* viability. Deleting *TOF1* also caused synthetic lethality with *rtt105Δ* (Figure 1C, right) suggesting that the stability of the replication fork is strongly affected in absence of *RTT105*. Taken together, these genetic interactions show that *RTT105* becomes crucial when replisome integrity is affected.

The S-phase checkpoint pathway is required for *rtt105Δ* viability

We next evaluated whether loss of *RTT105* caused a synthetic interaction with mutations in the S-phase checkpoint. The S-phase checkpoint is divided into two branches: the DNA replication checkpoint (DRC), which is specific to S phase, and is mediated by *MRC1*, the RFC-*CTF18* complex checkpoint mediator, and other fork components, and the DNA-damage checkpoint (DDC) which operates throughout the cell cycle and depends on the checkpoint mediator Rad9 (reviewed in Pardo *et al.* 2017). Both branches are activated by the sensor kinase Mec1 and converge on the effector kinase Rad53. We found that Mec1 and Rad53 are required for cell viability in the absence of *RTT105* (Supplementary Figure S2, center).

Then, we evaluated the importance of the DRC in *rtt105Δ* by testing the *ctf18Δ* mutant, which is compromised for its DRC function. We found that *ctf18Δ* strongly affects *rtt105Δ* cell growth (Supplementary Figure S2, left). To assess the contribution of the DDC in *rtt105Δ* cells, we deleted *RAD9* and found that DDC inactivation also strongly affects the viability of the *rtt105Δ* mutant (Supplementary Figure S2, right). These data indicate that in *rtt105Δ* cells both DRC and DDC are important to sustain their growth. These results support the notion that *rtt105Δ* cells exhibit replicative defects and associated DNA damages.

Homologous recombination is required for *rtt105Δ* viability in contrast to nonhomologous end joining

It has been recently shown that *rtt105Δ* cells exhibit a synthetic sick phenotype with mutations in key genes involved in HR and nonhomologous end joining suggesting that *RTT105* have important role in these repair processes (Li *et al.* 2018).

The MRX complex (Mre11-Rad50-Xrs2) and Sae2 function together to initiate end resection, an essential early step in homology-dependent repair of DSB (Longhese *et al.* 2010; Seeber *et al.* 2016; Gnügge and Symington 2017). Our results showed that *rtt105Δ* cells lacking either one of the MRX component or Sae2 were unable to grow or grew very poorly indicating that initial steps of HR are required to sustain the viability of *rtt105Δ* cells (Figure 2, A and B). Of note, this synthetic lethality could not be attributed to spore germination defects since *mre11Δ rtt105Δ* spores formed micro-colonies after 7 days at 30°C. To show that

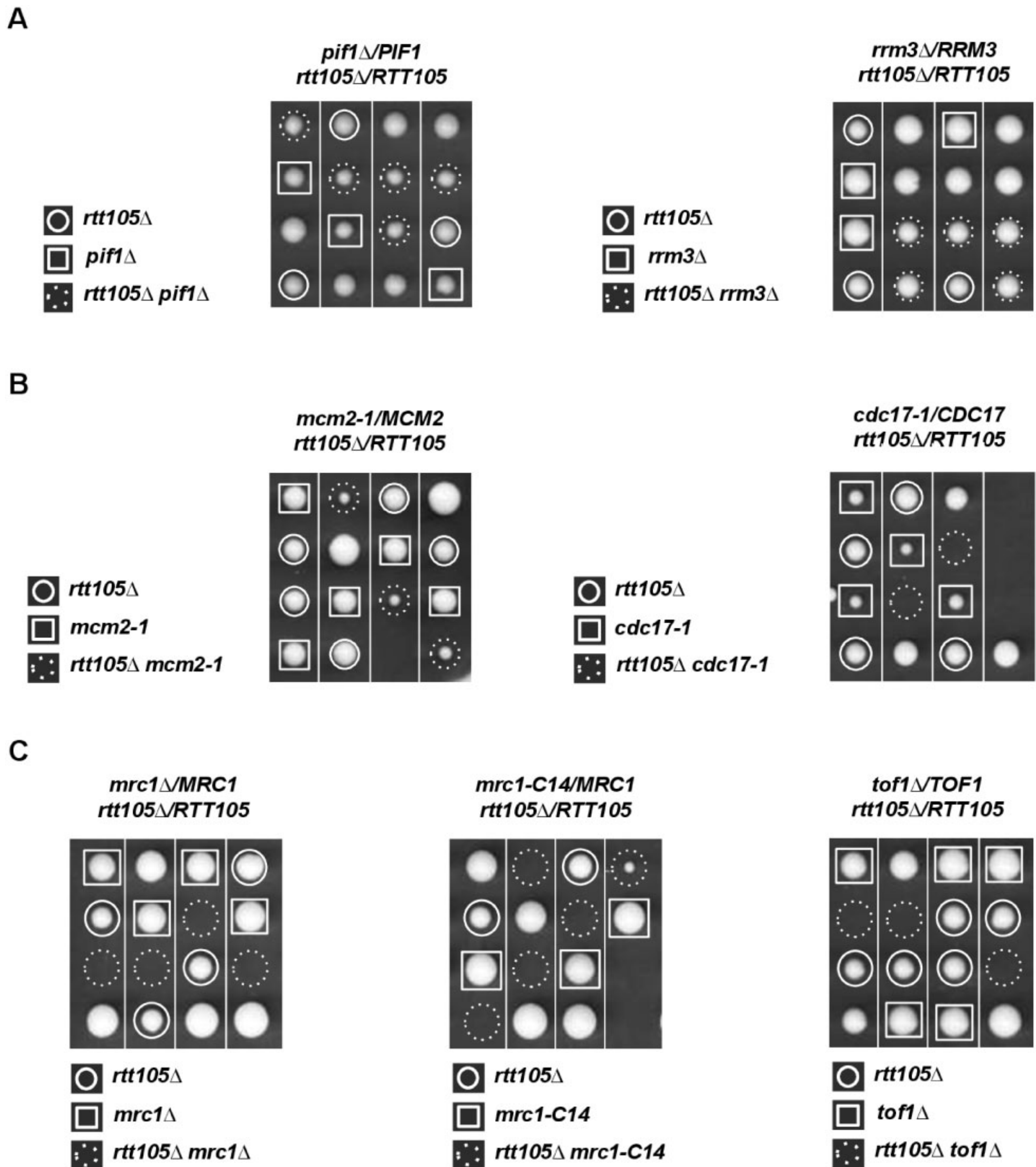

Figure 1 *RTT105* inactivation results in cell lethality in different genetic contexts affecting replisome progression. (A) Members of the PIF1 DNA helicase family are not required for viability of yeast *rtt105Δ* cells. Tetrad dissection of the diploid strains *pif1Δ/PIF1 rtt105Δ/RTT105* and *rrm3Δ/RRM3 rtt105Δ/RTT105*. In this and subsequent figures, the spores from a given tetrad are in vertical line in a YPD plate. Four representative tetrads are shown after 3 days at 30°C. (B) Genetic interaction of *mcm2-1* and *cdc17-1* with *RTT105*. The diploid strains *mcm2-1/MCM2 rtt105Δ/RTT105* (left) and *cdc17-1/CDC17 rtt105Δ/RTT105* (right) were sporulated and dissected. (C) *RTT105* is required for viability in the absence of the replicative function of *MRC1* or the Tof1-Csm3 complex. Left, tetrads from diploids heterozygous for *mrc1Δ*, and for *rtt105Δ* were dissected and analyzed as in (A). Center, tetrads from *mrc1-C14*, and *rtt105Δ* heterozygous diploids were dissected and analyzed. Right, tetrads from diploids heterozygous for *tof1Δ*, and for *rtt105Δ* were dissected and analyzed.

rtt105Δ-induced DNA damage was actually repaired by HR, we monitored nuclear localization of Rfa1-CFP that forms fluorescence foci representing DNA repair centres of multiple DSBs (Lisby *et al.* 2004). Despite the fact that deleting *RTT105* gave a diffuse signal and reduced the level of RPA associated to the fork (Li *et al.* 2018, 2019) we found that *rtt105Δ* exhibited a very high frequency of spontaneous Rfa1-CFP foci compared to WT (67% *vs* 4%, respectively) (Figure 2C). We noted that some Rfa1-CFP foci appeared brighter (27%), likely reflecting abnormally long region of RPA-bound single-stranded DNA.

We next investigated the requirement of the nonhomologous end-joining repair pathway for *rtt105Δ* viability. We found that

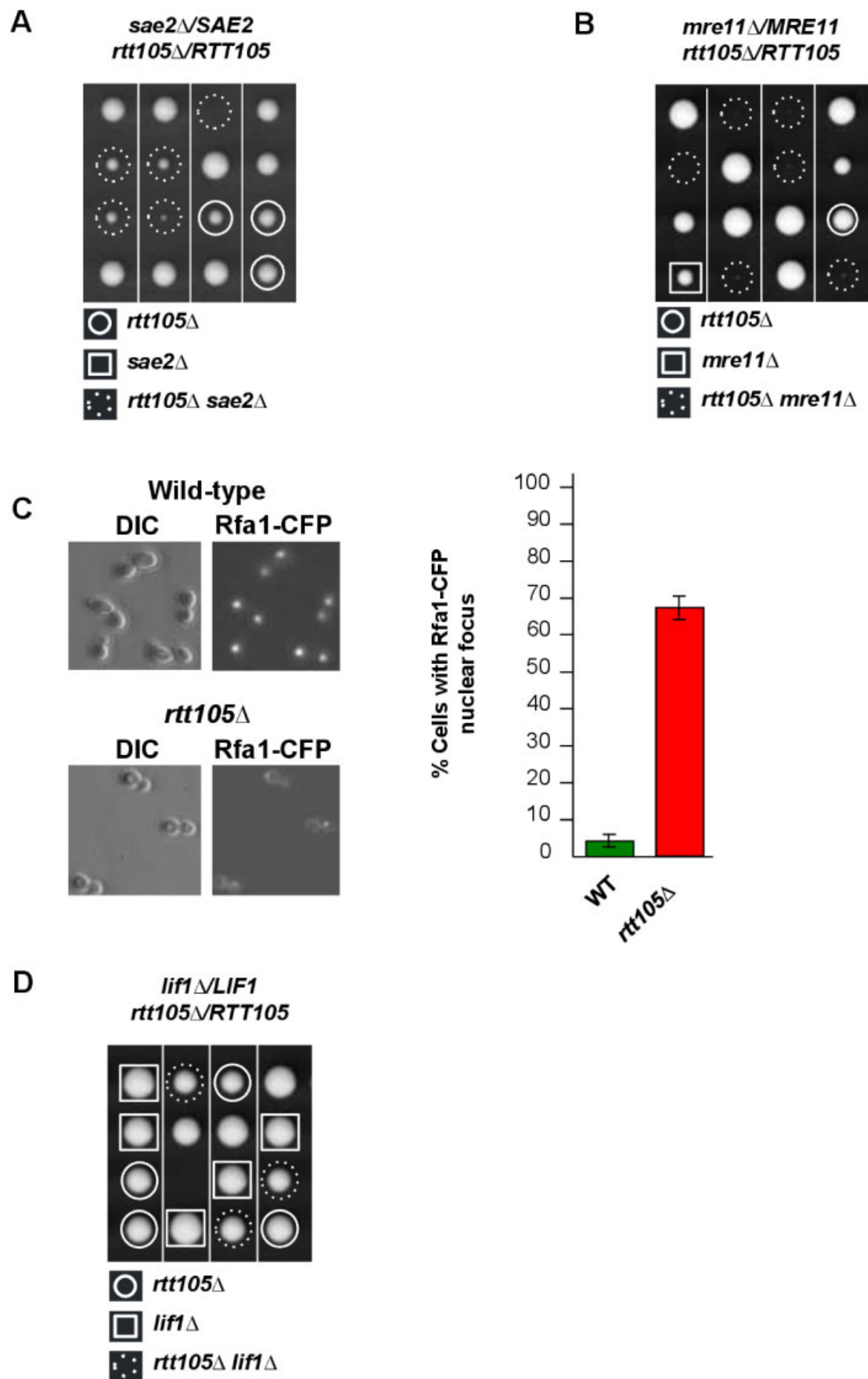

Figure 2 *RTT105* shows genetic interactions with components involved in DNA repair by homologous recombination. (A, B) *sae2Δ/SAE2 rtt105Δ/RTT105* and *mre11Δ/MRE11 rtt105Δ/RTT105* diploid strains were dissected and the resulting spores were incubated at 30°C for 3 days. (C) Left, Rfa1 foci are detected in *rtt105Δ* cells. Wild-type and *rtt105Δ* cells encoding Rfa1-CFP were analyzed with differential interference contrast (DIC) (left) and with florescence microscopy (right). Right, numbers indicate the percentage of cells that contained Rfa1-CFP foci. Rfa1 foci were analyzed in asynchronously growing cells. At least 200 cells were analyzed for each strain. (D) Genetic interaction of *LIF1*, with *RTT105*. *lif1Δ/LIF1 rtt105Δ/RTT105* were dissected. Tetrads were grown at 30°C for 3 days.

RTT105 was dispensable for the growth of *lif1Δ* cells (Figure 2D). This result indicates that NHEJ *per se* is not important for *rtt105Δ* growth.

Collectively, these data strongly support the notion that the absence of *RTT105* induces DSB and/or ssDNA gaps that form as a result of DNA replication perturbation and are repaired by HR but not by NHEJ (see *Discussion*).

Cohesion defects create a requirement for *RTT105*

Considering that many replisome components are part of sister chromatid cohesion pathways (Xu *et al.* 2007) and that replication fork passage is intimately linked to the establishment of the sister chromatid cohesion, we further investigated the importance of *RTT105* in cells experiencing cohesion defects. We focused on *SCC1* (also known as *MCD1*), which encodes one of the essential

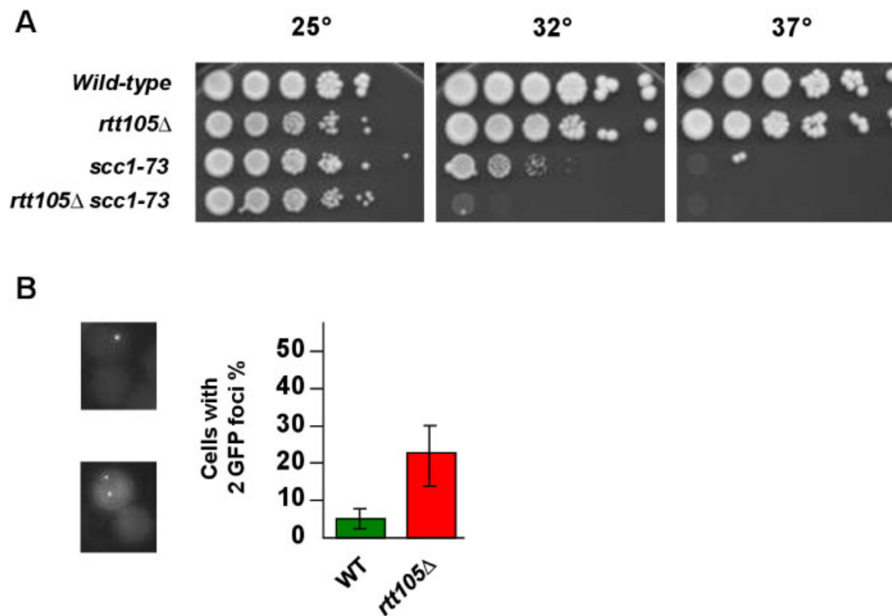

Figure 3 *RTT105* is important for cells affected in sister chromatid cohesion. (A) Tenfold serial dilutions of exponentially growing cells with the indicated mutations were spotted onto YPD plates and incubated at 25°C, 32°C, or 37°C for 3 days. (B) Cohesion is affected in *rtt105Δ* cells. Sister chromatid cohesion was analyzed by monitoring the tagged centromere of chromosome III. Over 150 cells were counted for each experiment. The results represent the average of three independent experiments.

subunits of the cohesin complex. We found that the *ts* allele *scc1-73* which displays increased cohesion loss when shifted to the semi-restrictive temperature (32°C) (Michaelis *et al.* 1997) is lethal in the absence of *RTT105* at this temperature (Figure 3A). We directly evaluated the cohesion defects in a *rtt105Δ* mutant using strains bearing Lac operator repeats integrated at a site near the centromere of chromosome III (Figure 3B). We found that *rtt105Δ* cells exhibited failure in cohesion (around 23%). Our results reveal that *RTT105* is important to sustain the viability of cells with cohesion defects suggesting that *RTT105* contributes to efficient sister chromatid cohesion.

RTT105 genetically interacts with genes encoding histone chaperones and histone H3–H4 lysine mutants with defects in nucleosome assembly during replication

Cohesion and replication-coupled nucleosome assembly have been functionally linked (Zhang *et al.* 2017a, 2017b). H3K56ac is an important mark required for chromatin assembly (Chen and Tyler 2008; Li *et al.* 2008). This mark found in all newly synthesized histone H3 and deposited behind replication forks in S-phase is dependent on *ASF1* and catalyzed by *RTT109* (Driscoll *et al.* 2007; Han *et al.* 2007a, 2007b; Tsubota *et al.* 2007). To ascertain the importance of *RTT105* in cells unable to acetylate H3K56, we analyzed the consequences of deleting either *ASF1* or *RTT109*. We found that *rtt105Δ* cells showed significant growth defects when combined with *asf1Δ* and *rtt109Δ* (Supplementary Figure S3A). Consistent with this result, the substitution *K56R* in H3 also impaired the growth of *rtt105Δ* cells (Supplementary Figure S3B, compare green and red circles). We next analyzed the importance of other post-translational modifications on newly synthesized histones known to regulate the replication-coupled nucleosome assembly (Ai and Parthun 2004; Li *et al.* 2009). We observed that *H3K9,14,18,23,27R* and *H4K5,8,12R* mutations significantly reduced *rtt105Δ* growth (Supplementary Figure S4A). On the contrary, deletion of *HIR1* or *HIR2* which are involved in replication-independent nucleosome assembly (Green *et al.* 2005) caused no apparent effect on *rtt105Δ* growth (Supplementary Figure S4B). Taken together, these data support the idea that *RTT105* is functionally linked to nucleosome assembly during replication.

RTT105 is required for heterochromatin silencing and genetically interacts with *SIR* complex

We tested if *RTT105* is involved in gene silencing at the three heterochromatin-like loci in *S. cerevisiae*. We first ask whether *RTT105* is required for telomeric position effect (TPE) using cells containing the reporter gene *URA3* integrated at the left arm of telomere VII. We found that TPE was impaired in the *rtt105Δ* strain (Figure 4A). To determine whether silencing defects also occur at *HMR* in *rtt105Δ* cells, we used a color assay utilizing an *ADE2* reporter in which the *ADE2* expression is regulated by the *HMR* silencer (Sussel *et al.* 1993). As expected, we obtained pink colonies with the wild-type strain while *rtt105Δ* cells grew as white colonies indicating that silencing of *ADE2* at *HMR* was lost (Figure 4B). We finally investigated if *rtt105Δ* cells show defects in rDNA silencing by using a reporter strain containing a *URA3* gene inserted as a single copy at the rDNA locus (Chang and Winston 2011). When plated on minimal medium without uracil, WT cells showed slow growth whereas *rtt105Δ* cells (as well as *sir2Δ* cells used as control) were able to grow reflecting defects in rDNA silencing (Figure 4C).

Subtelomeric regions, rDNA array, and the cryptic mating-type loci are all transcriptionally silenced by SIR proteins (reviewed in Gartenberg and Smith 2016). We assess the impact of heterochromatin disruption on growth of *rtt105Δ* cells by deleting SIR proteins. Strikingly, we uncover that deleting both *SIR2* and *SIR4* rescued the *rtt105Δ* growth defect.

Taken together, these results show that *RTT105* is required for efficient silencing at the heterochromatic loci and suggest that the impaired localization of Sir proteins in the *rtt105Δ* contributes to its slow growth.

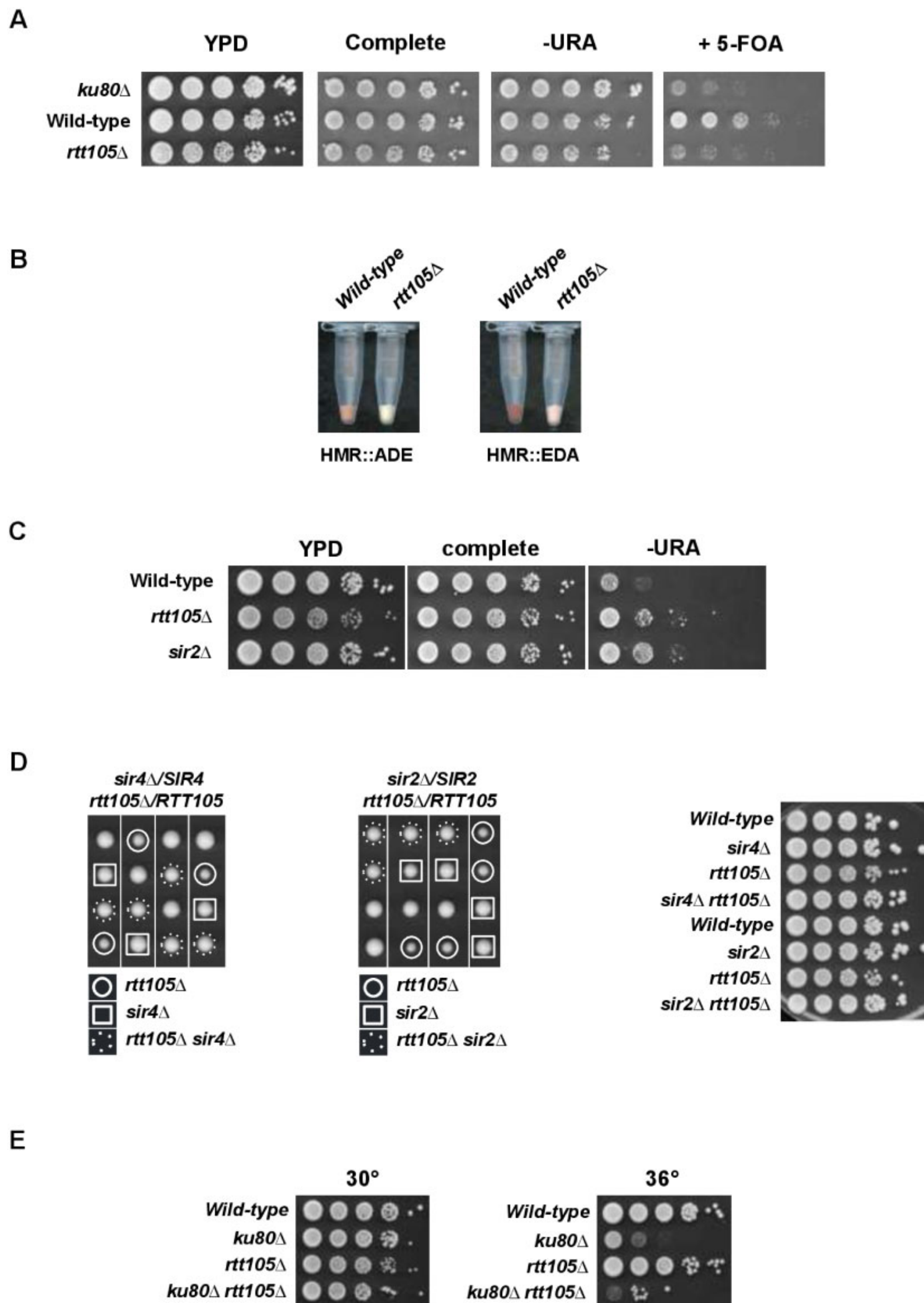

Figure 4 *RTT105* is required for gene silencing and interacts genetically with SIR and YKU complexes. (A) Deletion of *RTT105* reduces silencing of a telomere-proximal *URA3* gene. Tenfold serial dilutions of exponentially growing cells were spotted onto YPD, complete, SD-URA, and 5-FOA plates and incubated at 30°C for 2 days. *yku80***Δ** mutant was used as positive control. The absence of growth reveals a non-silenced state of the *URA3* gene. (B) Deletion of *RTT105* reduces *ADE2* silencing at *HMR*. Strains containing the *HMR*::*ADE2* allele or the *HMR*::*2EDA* allele (Sussel *et al.* 1993) were grown overnight in YPD liquid medium without additional adenine. A dark red/pink colony color indicates silencing of *ADE2*. A white/slightly pink colony color indicates a nonsilenced state of *ADE2*. *2EDA* indicates that the *ADE2* gene has been placed in a promotor-distal orientation with respect to the E-silencer (Sussel *et al.* 1993). (C) Deletion of *RTT105* gene reduces silencing at rDNA. Tenfold serial dilutions of exponentially growing cells were spotted onto YPD, complete, and SD-URA plates and incubated at 30°C for 2 days. All the different strains carry an *mURA3* reporter in a single copy within the rDNA (Chang and Winston 2011). Growth on medium without uracil (-URA) assesses the degree of reporter silencing. *sir2***Δ** mutant was used as positive control. The absence of growth reveals a silenced state for the *URA3* gene. (D) Deletion of *SIR* genes rescues *rtt105***Δ** growth defect. Tetrad dissection of *sir4***Δ**/*SIR4 rtt105***Δ**/*RTT105* (left) and *sir2***Δ**/*SIR2 rtt105***Δ**/*RTT105* (center) diploid strains. Right, effects of *sir2***Δ** and *sir4***Δ** on viability of the *rtt105***Δ** cells. Tenfold serial dilutions of exponentially growing cells were spotted onto YPD plates and incubated at 30°C for 3 days. (E) *rtt105***Δ** *yku80***Δ** mutant is not viable at 36°C. Tenfold serial dilutions of exponentially growing cells were spotted onto YPD plates and incubated at 30°C or 36°C for 3 days.

Disruption of *RTT05* affects telomere maintenance

We analyzed the *ts* phenotype of the *yku80*Δ which reflects telomere structure defects in the *rtt105*Δ (Gravel *et al.* 1998; Polotnianka *et al.* 1998). At 30°C, a permissive temperature for *ku80*Δ mutant, the double mutant *rtt105*Δ *ku80*Δ grew normally while at 36°C (the restrictive temperature) the *rtt105*Δ deletion abolished the ability of *ku80*Δ mutant to form colonies (Figure 4E) suggesting that the telomere structure defect of the *ku80*Δ mutant was aggravated by inactivation of *RTT105*. Interestingly, we often but not systematically noted the emergence of colonies in the double mutant that could reflect possible activation of telomerase-independent maintenance of telomeres in *rtt105*Δ *yku80*Δ cells. Since overexpression of telomerase was reported to suppress *yku80*Δ *ts* phenotype (Nugent *et al.* 1998; Teo and Jackson 2001), we investigated if *rtt105*Δ affected telomere length. As shown in Figure 5A, loss of *RTT105* resulted in shortening of telomeres revealing that *RTT105* positively regulates telomere length. In *S. cerevisiae*, recruitment of telomerase to telomeres was reported to be mediated by two pathways involving yKu-TLC1/Sir4 and Est1/Cdc13 interactions (Hass and Zappulla 2015; Chen *et al.* 2018). In addition to its role in recruiting telomerase, *YKU* that associates with TLC1 regulates TLC1 nuclear retention and also telomerase subunit Est1 accumulation at telomeres (Stellwagen 2003; Fisher *et al.* 2004; Gallardo *et al.* 2008; Lemon *et al.* 2019). We examined the genetic interaction between *RTT105*, *YKU80*, and *SIR4* genes related to telomere length. As previously described (Longhese *et al.* 2000; Hass and Zappulla 2015; Chen *et al.* 2018), we found that *sir4*Δ slightly decreases telomere size, and that *yku80*Δ cells have short but stable telomere (Figure 5B). We discovered that deleting *RTT105* diminished telomere length of *sir4*Δ mutant (Figure 5B, right) and slightly reduced telomere shortening of *yku80*Δ cells (Figure 5B, left and Table 1). These results suggest that *RTT105* acts in a pathway different than the yKu-TLC1/Sir4 recruitment pathway. We also evaluated the telomere size of *yku80*Δ *rtt105*Δ emerging colonies arising at 36°C in the spot assays (refer to Figure 4E, right). When these *yku80*Δ *rtt105*Δ emerging colonies were grown in liquid cultures, their growing colonies exhibited amplification of the tandemly repeated Y' short and Y' long subtelomeric elements (Type I survivors) with the disappearance of X-only telomeres. These results suggested that deleting *RTT105* in *yku* cells abolished telomerase activity at nonpermissive temperature leading to the appearance of Type I survivors (Supplementary Figure S5A) (Lundblad and Blackburn, 1993; Fellerhoff *et al.* 2000). We next inspected if telomere lengthening occurred in *rtt105*Δ cells when telomerase recruitment was bypassed by a fusion between Est1 or Est2 and the DNA-binding domain of Cdc13 (Evans and Lundblad 1999). We transformed wild-type and *rtt105*Δ cells with a plasmid expressing the hybrid proteins (Est1-DBD_{Cdc13} or Est2-DBD_{Cdc13}). In wild-type cells, expression of the Est1-DBD_{Cdc13} or Est2-DBD_{Cdc13} protein caused elongated telomeres as previously reported (Evans and Lundblad 1999). In contrast, in all *rtt105*Δ clones ($n = 5$) that we analyzed, artificial tethering of Est1 or Est2 led only to a modest lengthening of telomere size (Figure 5C) suggesting that *RTT105* is required for telomerase action when telomerase is artificially tethered to telomeres by the Est1-DBD_{Cdc13} or Est2-DBD_{Cdc13} fusion proteins.

We next monitored the binding of Cdc13 and RPA at telomeres by performing ChIP experiments in WT and *rtt105*Δ cells. Telomere-ChIP experiments revealed that *RTT105* deletion did not decrease Cdc13 binding to telomeres (Figure 5D) strongly favoring the hypothesis that Rtt105 acts at step independent of telomerase recruitment by the Cdc13 pathway. We also found that the absence of Rtt105 caused a decrease in the amount of RPA associated with telomeres (Figure 5E). Taken together, these data indicate that the role of *RTT105* in telomere maintenance likely relies on RPA and is independent of the canonical telomerase recruitment pathways.

Afterward, we analyzed the impact of *RTT105* inactivation on replicative senescence by analyzing the senescence profiles of *est1*Δ cells in the absence of *RTT105*. As expected, the *est1*Δ single mutant showed a decrease in growth over generations. Analysis of *rtt105*Δ *est1*Δ spore colonies revealed that growth defect of the double mutant was more severe than the one of the single *est1*Δ mutant (Supplementary Figure S5B). As expected, we did not notice signs of senescence in *rtt105*Δ cells that can grow indefinitely. In agreement with these observations, we found that recombined telomeres appeared more quickly in *rtt105*Δ *est1*Δ double mutant compared to *est1*Δ single mutant (Supplementary Figure S5C). Our results show that the deletion of *RTT105* accelerates replicative senescence without altering the rate of telomere shortening. To confirm these results, we performed liquid senescence assays. Wild-type, *rtt105*Δ, *est1*Δ, and *rtt105*Δ *est1*Δ spores arising from *rtt105*Δ/*RTT105* *est1*Δ/*EST1* diploid strain with an *EST1*-expressing plasmid were isolated after micromanipulation on YPD plate and then propagated in liquid cultures for around 120 PDs via serial dilution every 24 h (Aguilera *et al.* 2020). As observed on YPD plates, we did not notice any sign of senescence in *rtt105*Δ cells (Figures 6 and Supplementary Figure S6A, green lines). As expected, proliferation of *est1*Δ mutant declined progressively until the cells reached crisis after about 70 PDs before formation of the survivors (black lines). Deleting *RTT105* in *est1*Δ cells increased the rate of senescence as indicated by the early appearance of the crisis that appeared after about 50 PDs (blue lines). Interestingly, we noticed that the double *rtt105*Δ *est1*Δ mutant stayed for prolonged time in crisis before appearance of survivors, which reflect defects in survivor formation (Figures 6 and Supplementary Figure S6A, blue lines). These results highlight the role of *RTT105* in telomere replication that is particularly manifested in the absence of telomerase activity (Simon *et al.* 2016).

Finally, to compare the telomere structure in *rtt105*Δ *est1*Δ and *est1*Δ telomeres during replicative senescence, telomere length was analyzed at different time points of the senescence kinetics by southern blot. As expected, at the later time points the liquid culture of *est1*Δ cells gave rise to long TG1-3 tracts, heterogeneous in length, corresponding to type II survivors (Lundblad and Blackburn, 1993). Using several independently isolated clones, we found that similarly to *est1*Δ clones, *rtt105*Δ *est1*Δ clones produced type II survivors (Supplementary Figure S6B).

Taken together these results unveils the importance of *RTT105* in telomere length maintenance according to a scenario in which *RTT105* favors the restart of stalled replication forks at eroded telomeres in cells lacking telomerase activity (Simon *et al.* 2016).

Genetic interactions between *RTT105* and RPA mutants

Because Rtt105 functions as an "RPA chaperone" (Li *et al.* 2018, 2019), we conducted epistasis analysis between *rtt105*Δ and *rfa1* mutants. We first analyzed the genetic interaction between *rtt105*Δ and the *rfa1-D228Y* mutation reported to decrease the affinity of Rfa1 complex to ssDNA (Smith and Rothstein 1999; Audry *et al.* 2015). We found that most *rfa1-D228Y rtt105*Δ cells did not form visible colonies after 3 days at 30°C or formed

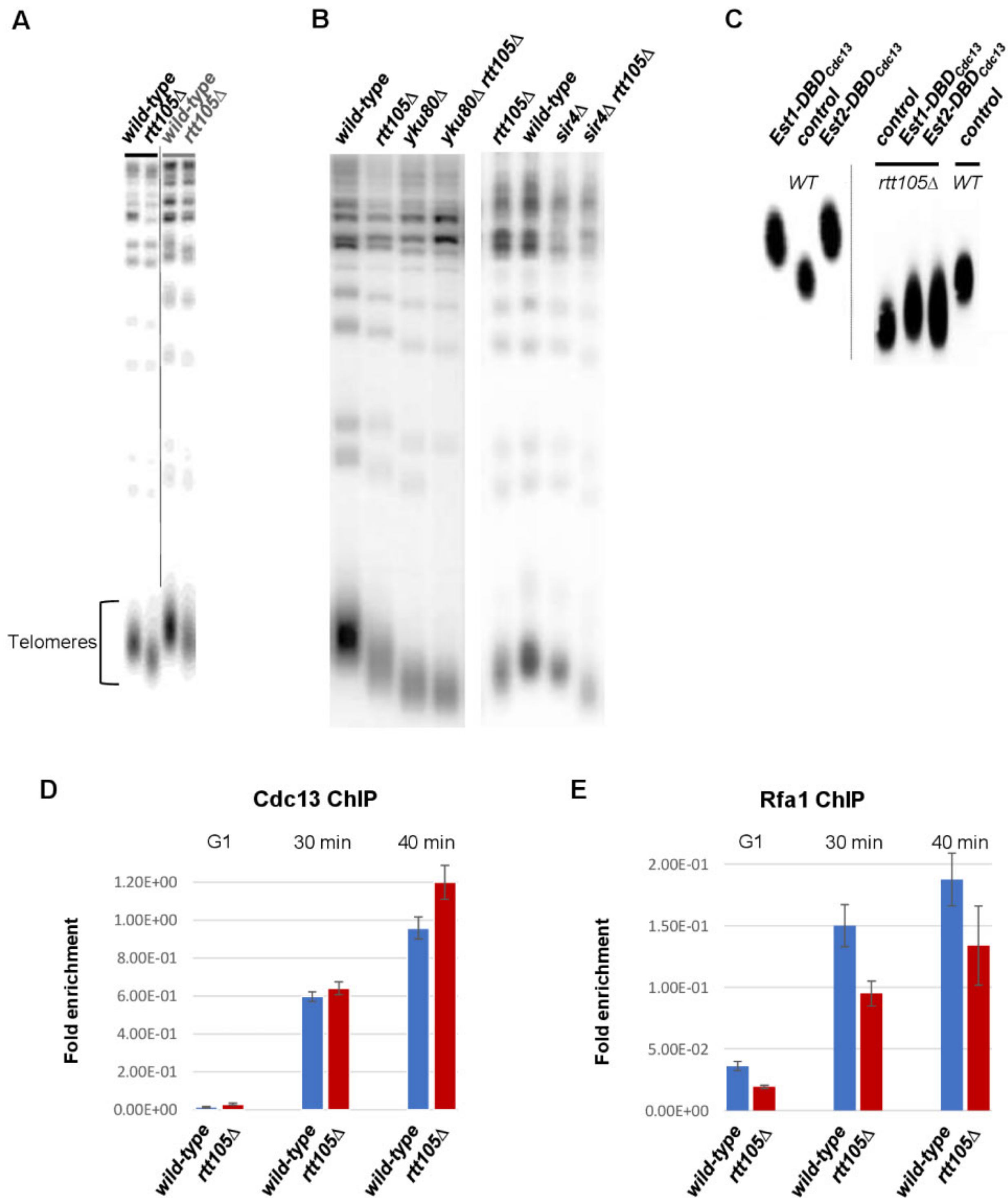

Figure 5 *RTT105* is required for telomere maintenance. (A) Telomere length of *rtt105*Δ cells measured by southern blotting. In this and subsequent figures, genomic DNA from each cell culture was digested by *Xho*I, separated on a 0.8% agarose gel, and hybridized to a poly(GT) telomere specific probe. Two different genetic backgrounds were used. Left (black), W303 background. Right (grey), LPY917 backgound. (B) Telomere length analysis of *rtt105*Δ in *yku80*Δ, and *sir4*Δ cells. (C) Tethering of Est1 and Est2 in *rtt105*Δ cells. Telomere length of wt and *rtt105*Δ strains transformed with either pVL1120 which directs the expression of Est1-DBD$_{CDC13}$ or pVL1107 which directs the expression of Est2-DBD$_{CDC13}$ (Evans and Lundblad 1999). Controls: wild-type and *rtt105*Δ strains were transformed with the empty plasmid. (D, E) Cdc13 binding at telomere is not reduced in *rtt105*Δ cells while binding of RPA is compromised. Cells were synchronized in G1-phase of the cell cycle using alpha-factor and subsequently released in YPD. ChIP experiments were performed at $t = 0$ min (G1), $t = 30$ min (S), and $t = 40$ min (late S) using an antibody either against the MYC-Tag of Cdc13-Myc (D) or against Rfa1 (Agrisera) (E). The immunoprecipitated DNA was quantified with real time PCR using primers amplifying the left arm of telomere XV (Tel-XV-L) (Bianchi and Shore 2007). Experiments were performed in triplicate.

microcolonies that grew extremely slowly (Figure 7A, left). This result suggests that decreasing the level of RPA is toxic for the cell when RPA ssDNA binding activity is compromised. We also noticed that replication-proficient but recombination-defective *rfa1-t11* (K45E) mutant (Vanoli et al. 2010) was very sick in the absence of *RTT105* (Figure 7A, right) confirming the necessity of

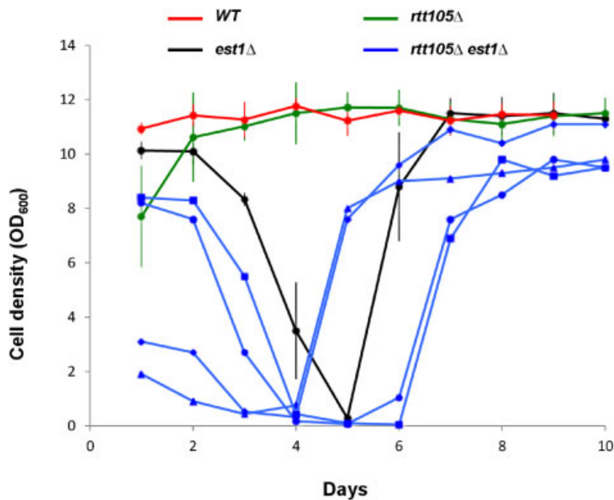

Figure 6 *RTT105* inactivation affects senescence and survivor formation in *est1*Δ. Mean senescence profiles of the WT, *rtt105*Δ, and *est1*Δ clones, and senescence profiles of the *rtt105*Δ *est1*Δ clones analyzed in the course of this study. At least three clones were analyzed in each case. Each clone was isolated by sporulation of a heterozygous diploid strain and subsequently propagated in liquid culture through daily serial dilutions. OD_{600} was measured every day to estimate the cell density reached in 24 h.

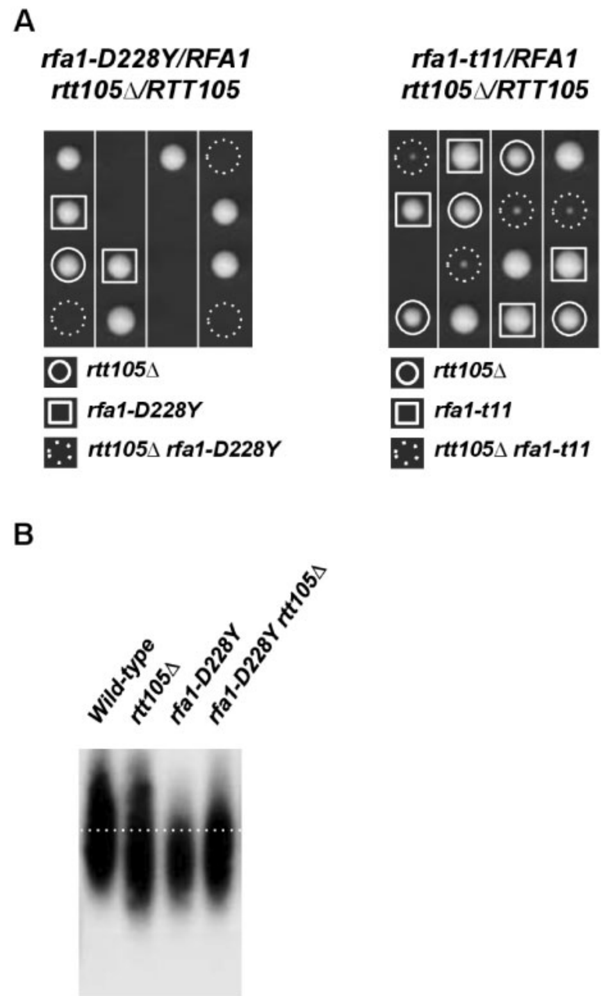

Figure 7 *rtt105*Δ displays genetic interactions with *rfa1* alleles. (A) Genetic interaction of *RFA1* with *RTT105*. *rfa1-D228Y/RFA1 rtt105*Δ/*RTT105* (left), and *rfa1-t11/RFA1 rtt105*Δ/*RTT105* (right) were dissected and incubated 30°C for 3 days. (B) Deleting *RTT105* does not increase the telomere length defect of *rfa1-D228Y* cells. Teloblots were performed as in Figure 5. The dashed line indicates wild-type telomere position.

recombinational process for normal growth in *rtt105*Δ cells. Since *rfa1-D228Y* allele was previously reported to shorten telomeres (Smith and Rothstein 2000; Luciano *et al.* 2012), we examined telomeres of *rfa1-D228Y rtt105*Δ cells. No synergistic reduction in telomere length occurred in the *rfa1-D228Y rtt105*Δ double mutant (Figure 7B) suggesting that the reduction in telomere size observed in *rtt105*Δ cells is related to RPA functions.

Reducing the level of RPA in S-phase phenocopies *rtt105*Δ

The question that arises from all the above results is whether these effects are indeed related to RPA functions. To answer this question, we analyzed if the *rtt105*Δ*155-208* mutant whose association to Rfa1 is compromised (Li *et al.* 2018) mimics the *rtt105*Δ mutation. After sporulating *rtt105*Δ/*RTT105* diploid strains containing a plasmid either expressing *RTT105* or the *rtt105*Δ*155-208* mutant, we observed that cells carrying the *rtt105*Δ*155-208* had similar phenotypes as *rtt105*Δ cells (Table 2, Supplementary Figure S7). This result suggests that the observed phenotypes described in the absence of *RTT105* are related to a lack of interaction between Rtt105 and Rfa1.

To confirm these observations, we examined if reducing the RPA levels specifically during S-phase gave rise to similar phenotype as those obtained in *rtt105*Δ cells. We took advantage of results indicating that restricting the expression of genes in G2/M phase can lead to potentially hypomorphic alleles in S phase (as a consequence of their reduced expression in S-phase). We used a diploid strain to swap the promoter of one allele of *RFA1* to create *CLB2-rfa1* allele in which *RFA1* is under the control of the mitotic Clb2 promoter and fused to 5′ region of *CLB2* encoding Clb2 degron (Hombauer *et al.* 2011). We found that *CLB2-rfa1* cells were viable and exhibited modest growth defect (Figure 8A). These results indicate that the amounts of CLB2-rfa1 persists in S-phase as shown in Supplementary Figure S8, and that N-terminal *CLB2* fusion *per se* was not sufficient to compromise vital S-phase function of *RFA1*. We performed chromatin immunoprecipitation (ChIP) to measure the level of RPA binding at the early origin ARS607 after replication fork stalling in *CLB2-rfa1* cells. ChIP-qPCR revealed that the association of Rfa1 subunit with replicating DNA (analyzed after HU treatment) was significantly reduced in *CLB2-rfa1* cells as shown for *rtt105*Δ (Li *et al.* 2018) but slightly higher than in *rtt105*Δ background (Figure 8B). We next examined the sensitivity of *CLB2-rfa1* cells in cells experiencing replicative stress. We found that similarly to *rtt105*Δ, *CLB2-rfa1* cells were sensitive to chronic exposure to HU but not to replicative damages arising in cells lacking *RRM3* helicase (Figure 8C). We further conducted an extensive genetic analysis with *CLB2-rfa1* (Table 3). We found that *orc5-1*, *cdc17-1*, *mrc1*Δ, *tof1*Δ, *ctf18*Δ, *rad53-K227A*, *rad52*Δ, *mre11*Δ, *sae2*Δ, *scc1-73*, *asf1*Δ, and *rtt109*Δ mutations all strongly affected the viability or growth of *CLB2-rfa1* cells in contrast to *lif1*Δ, and *sir4*Δ that did not (Table 3). Having shown that the deletion of *RTT105* reduced telomere length, we analyzed the average size of telomeres in *CLB2-rfa1* cells and found that *CLB2-rfa1* caused a shortening of telomere length (Figure 8D). Consistent with our ChIP-qPCR experiment showing more Rfa1 signal in *CLB2-rfa1* than in *rtt105*Δ mutant, we observed a smaller telomeres size reduction in *CLB2-rfa1* compared to *rtt105*Δ (Figure 8B). Altogether, these results show that *CLB2-rfa1* and

Table 2 Genetic dependence on *rtt105Δ155-208* viability. *rtt105Δ/RTT105* strains heterozygous for the indicated deletions expressing *rtt105Δ155-208* or *RTT105* from a centromeric plasmid were sporulated, dissected, and the genotype of the variable spores were determined

Mutant	Growth defect with *rtt105Δ* + *rtt105Δ155-208*	Growth defect with *rtt105Δ* + *RTT105*	Growth defect with *rtt105Δ* + Empty vector
orc5-1	Synthetic lethal	–	Synthetic lethal
mcm2-1	+++	–	+++
cdc17-1	Synthetic lethal	–	Synthetic lethal
mrc1Δ	Synthetic lethal	–	Synthetic lethal
tof1Δ	Synthetic lethal	–	Synthetic lethal
ctf18Δ	Synthetic lethal	–	Synthetic lethal
rad9Δ	+++	–	Synthetic lethal
rad53-K227A	+++	–	Synthetic lethal
rad52Δ	Synthetic lethal	–	Synthetic lethal
mre11Δ	Synthetic lethal	–	Synthetic lethal
sae2Δ	+++	–	+++
lif1Δ	–	–	–
*scc1-73**	Synthetic lethal	–	Synthetic lethal
asf1Δ	+++	–	+++
sir4Δ	–	–	–

A "–" represents no effect on growth over the individual single mutants. A "+" represents a synthetic effect on growth. More "+" indicate a more dramatic synthetic effect in comparison with other strain tested. All the analyses were conducted at 30°C excepted for those marked by a "*", which were conducted at 32°C.

rtt105Δ cells exhibit similar phenotypes and suggest that the observed phenotypes described in the absence of *RTT105* are due to lack of RPA binding at fork.

Targeting Rfa1 into the nucleus only partially rescues *rtt105Δ* phenotypes

To distinguish whether the defects in DNA metabolism that we reported in the absence of *RTT105* were the consequence of the RPA nuclear import defect or resulted from a loss of function of Rtt105 in genome stability maintenance, we expressed a fusion protein constituted by a nuclear localization signal (NLS, PKKKRKV) fused to the N-terminal part of the full-length Rfa1 sequence. We observed that Rfa1 levels in the nucleus and Rfa1-bound to chromatin were both increased in *rtt105Δ* cells expressing NLS-Rfa1 indicating that NLS fused to Rfa1 promoted its import (Supplementary Figure S9A). Moreover, NLS-Rfa1 rescued the growth of *rfa1Δ* cells and the lethality of *rfa1-D228Y rtt105Δ* mutant showing that the fusion protein is functional (Supplementary Figure S9B).

We further found that expressing NLS-Rfa1 failed to rescue the growth defect at 25°C and sensitivity to HU of *rtt105Δ* cells (Supplementary Figure S9C), suggesting that the impact on replication due to the absence of Rtt105 is not fully related to its role in importing RPA to the nucleus and to its role in mediating RPA binding to ssDNA. We further asked whether NLS-Rfa1 suppressed the genome stability defects displayed in *rtt105Δ* cells. We found that in the absence of *RTT105*, NLS-Rfa1 efficiently restored the viability of *ctf18Δ*, *scc1-73* and *rad52Δ* mutants, respectively required for activation of the replication checkpoint, cohesion and repair (Supplementary Figure S10). Likewise, we explored the growth of *rtt105Δ NLS-RFA1* cells devoid of *MRC1* or *TOF1*, two genes important to prevent chromosome fragility through their multiple functions during replication (Tourrière *et al.* 2005; Pardo *et al.* 2017; Puddu *et al.* 2017; Yeeles *et al.* 2017; Gellon *et al.* 2019). *mrc1Δ rtt105Δ* and *tof1Δ rtt105* expressing NLS-Rfa1 were able to form colonies after 3 days at 30°C (Figure 9, A and B) indicating that NLS-Rfa1 could restore viability in *mrc1Δ rtt105Δ* and *tof1Δ rtt105Δ* mutants. However, *mrc1Δ rtt105Δ* and *tof1Δ rtt105Δ* cells expressing NLS-Rfa1 exhibited severe growth defects (Figure 9, A and B) revealing that NLS-Rfa1 failed to rescue growth in the absence of *RTT105* and suggesting that Rtt105 functions in replication independently of its known RPA chaperone function. This result was consistent with the fact that NLS-Rfa1 does not rescue the HU sensitivity in *rtt105Δ* cells (Supplementary Figure S9C, bottom). Finally, we also evaluated the telomere size of *rtt105Δ* cells expressing NLS-Rfa1 and found that NLS-Rfa1 exogenous expression suppressed only partially telomere length defect in *rtt105Δ* (Figure 9C). Altogether these results show that bringing Rfa1 to the nucleus and on chromatin only partly rescued some of the phenotypes of *rtt105Δ* cells pointing out that the pleiotropic phenotypes arising in *rtt105Δ* cells are not exclusively due to the function of Rtt105 in chaperoning RPA during DNA metabolism. Collectively these results strongly suggest that in addition to its role in RPA nuclear import and in the regulation of RPA binding to DNA replication forks Rtt105 exerts a role in the maintenance of genome stability during S phase either by directly affecting RPA function and/or through an unknown function independent of RPA.

Discussion

Our extensive genetic analysis reveals that *RTT105* is important for replication and multiple vital co-replicational events by facilitating RPA nuclear localization and by supporting RPA function during replication.

We show that the weakening of coupling between MCM helicase and DNA polymerases is lethal in the absence of *RTT105*. In addition, we show neither Pif1 nor Rrm3, whose function is to assist fork progression across pausing sites, is essential for the growth of *rtt105Δ* cells despite the fact that these cells are sensitive to exogenous DNA-damaging agents. One explanation could be the number of damages arising in *pif1Δ* and *rrm3Δ* cells is less important than damages created by exogenous DNA damaging agents. The fact that *RRM3* deletion affects cell viability in the absence of genes involved in replication, checkpoint, or repair (Torres *et al.* 2004; Schmidt and Kolodner, 2006) rather suggests that *RTT105* function is required for cells undergoing specific replicative damages. Another possibility could be that replicative damages induced by either *rtt105Δ* or *rrm3Δ* are similar and therefore processed in the same way.

Our study reveals that both branches of S-Phase checkpoint, DRC, and DNA damage checkpoint are required for *rtt105Δ* viability. These interactions strengthen the notion that *RTT105* carries out an important function during DNA replication. Along the same line, Mre11 and Sae2 that initiate resection at stalled forks are critical in cells lacking *RTT105* (Mimitou and Symington 2008; Tittel-Elmer *et al.* 2009; Bentsen *et al.* 2013; Delamarre *et al.* 2020), confirming the importance of recombination in cells lacking *RTT105* (Li *et al.* 2018). The fact that *yku80Δ rtt105Δ* cells generated after the sporulation of the diploid *yku80Δ/YKU80 rtt105Δ/RTT105* exhibited growth defects (Li *et al.* 2018) could have suggested that NHEJ was required for the growth of *rtt105Δ* cells. Nevertheless, our discovery that *lif1Δ rtt105Δ* grew normally at 30°C indicates that NHEJ is not required for *rtt105Δ* fitness. Considering that NHEJ predominantly operates in G1 while Rtt105 acts in S-phase (Chiruvella *et al.* 2013), it is not so surprising that NHEJ is not required to sustain the growth of *rtt105Δ* cells. We also report in this work that deleting *RTT105* aggravates cohesion defect of *scc1-73* cells consistent with the fact that a

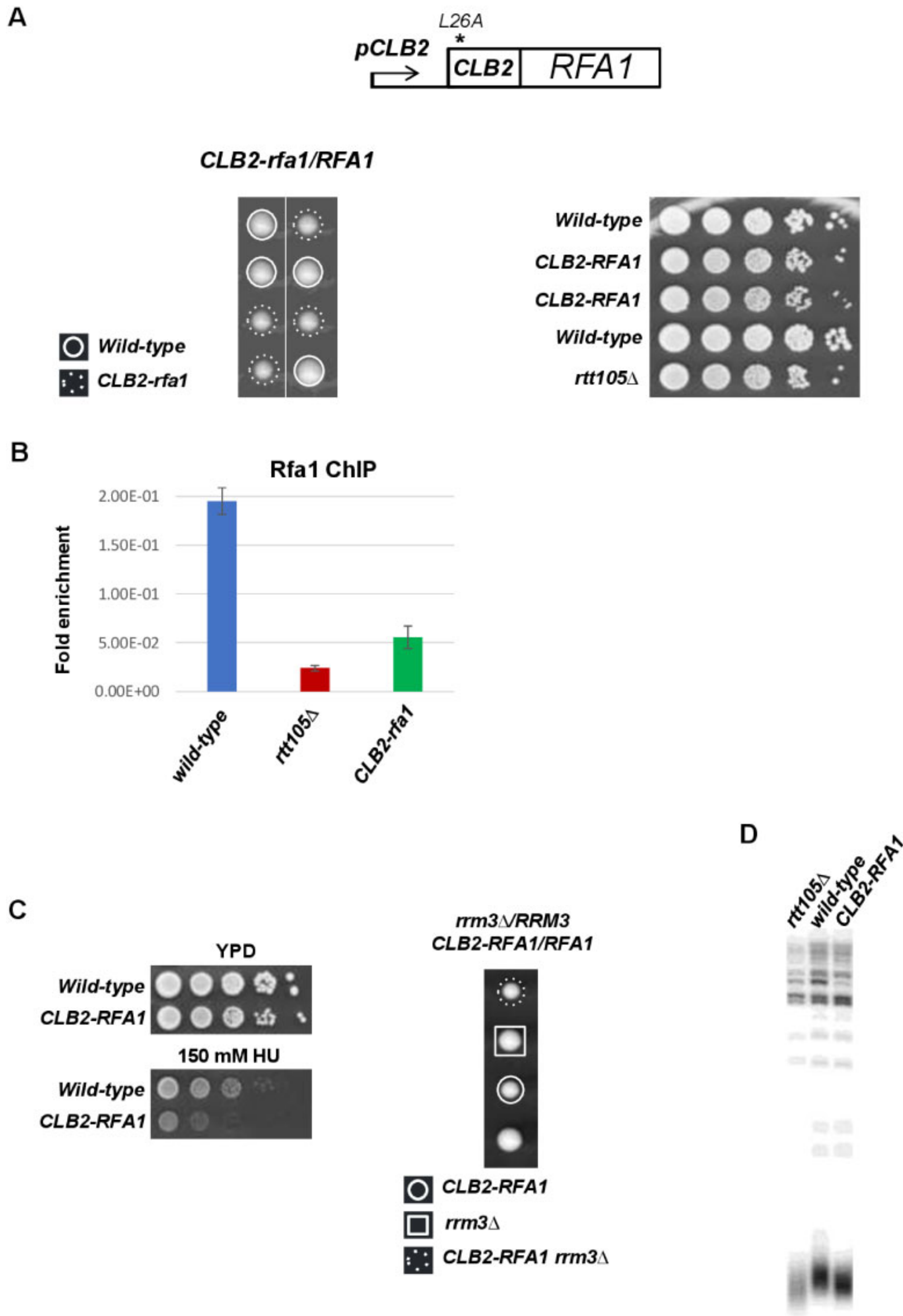

Figure 8 Phenotypes of *CLB2-rfa1* cells. (A) Top, schematic representation of the *CLB2-rfa1* construct used in this study. Down, *RTT105* is required for normal cell growth. Left, tetrad dissection of the diploid strain *CLB2-rfa1/RFA1*. Right, 10-fold serial dilutions of exponentially growing haploid cells were spotted. (B) Reduced binding of Rfa1 at fork in *CLB2-rfa1* cells. Asynchronous cells were blocked in S-phase with 200 mM HU. ChIP experiments were performed in triplicate using an antibody against Rfa1 (Agrisera) and the resulting DNA was quantified with real time PCR using primers amplifying ARS607 (CGTGCGGCAGTATAAGTTCA and GCAGGATCGACCTGACTCTT). (C) *CLB2-rfa1* mutant viability is affected by HU but not by *RRM3* inactivation. Left, 10-fold serial dilutions of exponentially growing cells were spotted onto YPD plate or 150 mM HU plate. Right, tetrad dissection of the diploid strain *CLB2-rfa1/RFA1 rrm3Δ/RRM3*. Plates were incubated at 30°C for 3 days. (D) Effect of *CLB2-rfa1* on telomere length measured by Southern blotting.

number of replication proteins play important roles in sister chromatid cohesion. However, because cohesion establishment factors localize to replication forks to promote fork restart (Lengronne *et al.* 2006; Gambus *et al.* 2009; Terret *et al.* 2009; Frattini *et al.* 2017) it is likely that repair of broken replication forks which arise in the absence of *RTT105* requires an intact cohesion (Klein *et al.* 1999; Sjögren and Nasmyth 2001).

Taken together, our genetic analyses suggest that the absence of *RTT105* affects replication and leads to the emergence of DSBs, which are subsequently repaired by HR.

Table 3 Genetic dependence on *CLB2-rfa1* viability. *CLB2-rfa1/RFA1* strains heterozygous for the indicated deletions were sporulated, dissected, and the genotype of the variable spores were determined

Mutant	Growth defect with *CLB2-rfa1*
orc5-1	Synthetic lethal
cdc17-1	Synthetic lethal
mrc1Δ	Synthetic lethal
tof1Δ	Synthetic lethal
ctf18Δ	Synthetic lethal
rad53-K227A	+++
rad52Δ	Synthetic lethal
mre11Δ	Synthetic lethal
sae2Δ	+++
lif1Δ	–
*scc1-73**	Synthetic lethal
asf1Δ	Synthetic lethal
sir4Δ	–

A "–" represents no effect on growth over the individual single mutants. A "+" represents a synthetic effect on growth. More "+" indicates a more dramatic synthetic effect in comparison with other strain tested. All the analyses were conducted at 30°C excepted for those marked by a "*," which were conducted at 32°C.

rtt105Δ mutation exhibits synthetic defects with mutations involved in the regulation of replication-coupled nucleosome assembly. On the contrary, deletion of the HIR complex, which is involved in replication-independent nucleosome assembly, did not induce a growth defect in *rtt105Δ* cells. These data highlight the importance of *RTT105* in a chromatin assembly process linked to replication, and ruled out a role for *RTT105* in replication-independent chromatin assembly. We further show that *RTT105* is required for efficient transcriptional silencing at the three heterochromatic regions that are transcriptionally silenced by the SIR proteins. Cells exhibiting defects in replication, in nucleosome assembly, and in sister chromatid cohesion have defective transcriptional silencing (Zhang *et al.* 2000; Sharp *et al.* 2001; Suter *et al.* 2004; Huang *et al.* 2007; Burgess *et al.* 2012). Because these processes are affected in *rtt105Δ* cells, it is possible that *RTT105* deletion affects silencing through its impact on these mechanisms. Curiously, we observed that deleting *SIR2* or *SIR4* improved the growth of *rtt105Δ* cells, which pinpoint Sir proteins contribution to *rtt105Δ* mutant fitness reduction. Deleting Sir2 and to a lesser extend Sir4 were reported to suppress *cdc6-4 ts* lethality (Pappas *et al.* 2004), and to target sensitive origins on chromosome III and VI (Crampton *et al.* 2008). Recently, it was shown that Sir2 inactivation rescued MCM loading at most euchromatic regions in the context of a *cdc6-4* mutant (Hoggard *et al.* 2018). These results therefore reinforce the notion that Rtt105 targets DNA replication.

We next showed that deleting *RTT105* reduces telomere length. Telomerase has two main recruitment pathways assisting and providing enzyme access to telomere. An essential pathway requires interaction of Est1 with Cdc13 (Evans and Lundblad 1999). The other pathway requires yKu80 and its binding to Sir4 (Peterson *et al.* 2001; Stellwagen 2003; Fisher *et al.* 2004; Hass and Zappulla 2015; Chen *et al.* 2018). Our genetic analysis between *RTT105* and *SIR4* suggests that *RTT105* promotes telomere lengthening independently of TLC1-Ku-Sir4 pathway. We further found that the telomere overelongation phenotype conferred by the artificial tethering of Est1 and Est2 via Est1-DBD_{Cdc13} or Est2-DBD_{Cdc13} fusion proteins was partially suppressed by *rtt105Δ*. Since deletion of *RTT105* does not impair Cdc13 binding at telomeres, this result suggests that the telomere shortening observed in the absence of *RTT105* is due to a decrease in telomerase activity independent of its recruitment. We also observed that in *est1Δ* cells absence of *RTT105* accelerates senescence without affecting the kinetics of telomere shortening suggesting that *RTT105* has a particular role in telomeres replication that are known to be prone to replication stress (Maestroni *et al.* 2017). We found that similarly to *est1Δ* cells, *est1Δ rtt105Δ* cells produced type II survivors but with a delayed kinetics suggesting that the absence of *RTT105* affects the appearance of survivors.

RPA protects and stabilizes ssDNA generated during DNA metabolism. Our genetic epistasis analysis between *rtt105Δ* mutant and *rfa1-t11* mutant revealed strong negative genetic interactions (respect to growth). Because *rfa1-t11* mutant is replication proficient but defective in recombination repair (Lee *et al.* 1998; Umezu *et al.* 1998; Kantake *et al.* 2003) we assume that the sickness of the *rfa1-t11 rtt105Δ* double mutant is due to the inability of *rfa1-t11* mutant to repair replicative damages provoked by the absence of *Rtt105*. Consistent with this hypothesis, we have shown that *rtt105Δ* displays also synthetic lethality phenotype with *mre11Δ*, which is itself functionally epistatic with *rfa1-t11* (for survival of replication fork stress or DSB recovery) (Seeber *et al.* 2016). Genetic epistasis analysis between *rtt105Δ* and *rfa1-D228Y* also pinpoints strong negative genetic interaction (respect to growth). Since RPA level bound to ssDNA and replication forks is reduced in *rfa1-D228Y* cells (Audry *et al.* 2015; Ruff *et al.* 2016), the synthetic lethality/sickness between *rfa1-D228Y* and *rtt105Δ* could be at least in part a consequence of the lower affinity of RPA for ssDNA. We also report the absence of a synergistic reduction in telomere length when the *rfa1-D228Y* mutant allele is combined with null mutation of *RTT105*. This result suggests that the negative effect on telomere length associated to *RTT105* deletion is related to RPA function at telomeres (Schramke *et al.* 2004; Luciano *et al.* 2012).

Genetic analyses that we have conducted with *rtt105Δ155-208* (Li *et al.* 2018) and *CLB2-rfa1* mutants in which both exhibit reduced level of RPA on ssDNA reveal that these two mutants phenocopy the *rtt105Δ* mutant. Because both alleles show genetic interactions similar to those of *rtt105Δ*, we assume that the pleiotropic effects observed in *rtt105Δ* cells are mostly related to the role of Rtt105 in chaperoning RPA a notion reinforced by recent observation that *RTT105* and *RPA* both play a role in removing G4 structures (Maestroni *et al.* 2020). The fact that *rtt105Δ155-208* phenocopies *rtt105Δ* but cannot rescue nuclear localization defect of Rfa1 (Li *et al.* 2018), could suggest that the role of Rtt105 in RPA nuclear import is the cause of the observed phenotypes. However, since Rtt105–Rfa1 interaction occurs predominantly in the nucleus and is also required to promote the binding of RPA to ssDNA (Li *et al.* 2018) one cannot distinguish whether the role of Rtt105 is related to defect in the nuclear localization of RPA or to a more direct effect on genome stability.

We observed that NLS-Rfa1 rescues *rtt105Δ* combined to either *scc1-73*, or *rad52Δ* suggesting that Rtt105 contributes to cohesion, and repair by chaperoning RPA. We propose that the lethality of *rad52Δ rtt105Δ* cells is a consequence of replicative damages leading to the emergence of DSBs, which cannot be repaired by HR in the absence of Rad52. Therefore, we consider that Rtt105 is not directly involved in Rad52-dependent HR repair pathways. NLS-Rfa1 also rescues the viability of *rfa1-D228Y rtt105Δ* double mutant, however the double mutant exhibits a slow growth. Given that *rfa1-D228Y* mutant is not functional for break-induced replication, which involves long ssDNA intermediates, but is largely functional for both intra-homologue gene conversion and single strand annealing (Ruff *et al.* 2016), we favor the idea that *rfa1-*

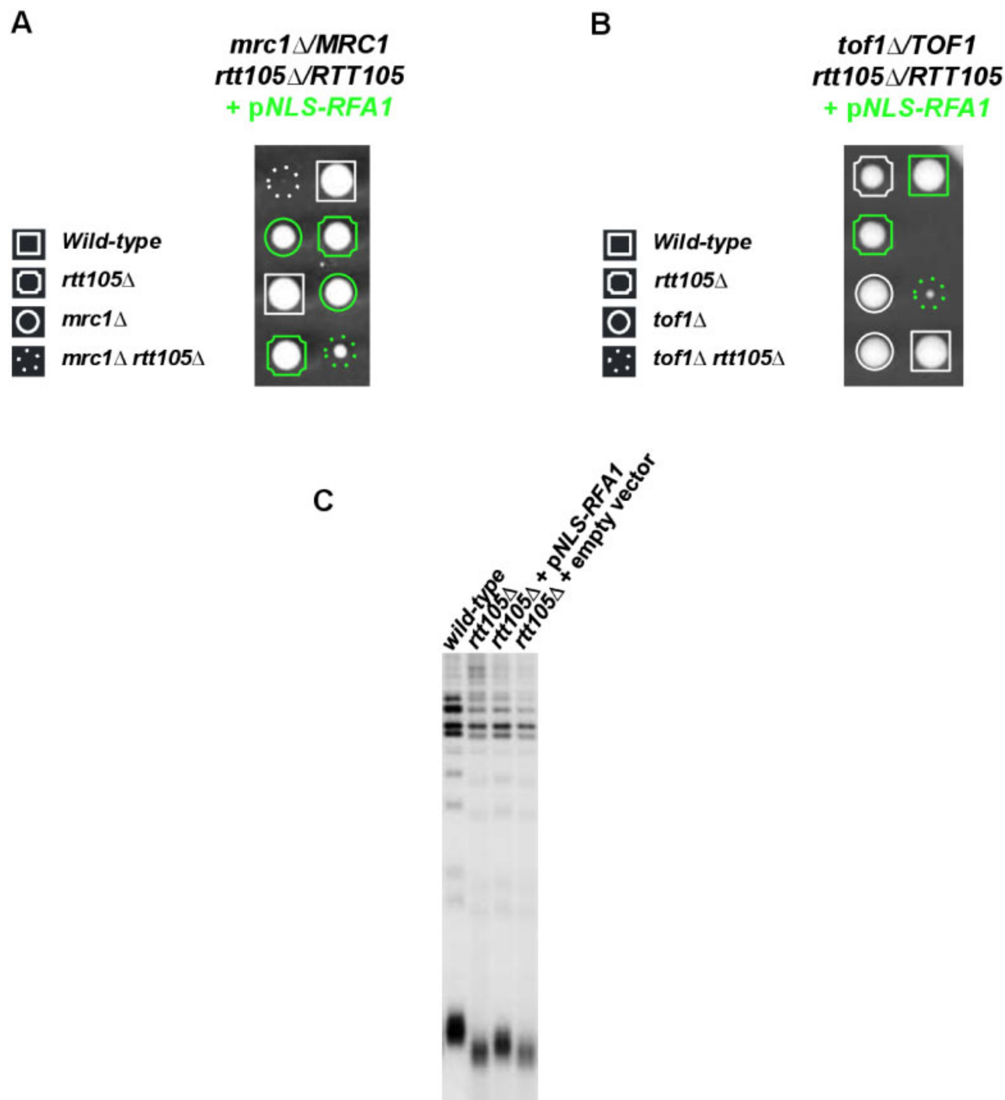

Figure 9 Bringing Rfa1 into the nucleus does not rescue the growth of *rtt105*Δ cells in absence of *MRC1* and *TOF1*. (A) Genetic interaction of *mrc1*Δ with *RTT105*. The diploid strains *mrc1*Δ/*MRC1 rtt105*Δ/*RTT105* expressing the fusion protein NLS-Rfa1 was sporulated and dissected. (B) Genetic interaction of *tof1*Δ with *RTT105*. The diploid strain *tof1*Δ/*TOF1 rtt105*Δ/*RTT105*, expressing the fusion protein NLS-Rfa1 was sporulated, and dissected. The green color indicates spores expressing the NLS-Rfa1 fusion protein. Plates were incubated at 30°C for 3 days. (C) Effect of NLS-Rfa1 expression in *rtt105*Δ cells on telomere length measured by Southern blotting.

D228Y mutant goes to the nucleus and that Rtt105 helps *rfa1-D228Y* cells to perform its essential function. Finally, we show that NLS-Rfa1 does not rescue the sensitivity to HU of *rtt105*Δ cells and only poorly rescues the growth of mutants affected in factors protecting stalled forks (*mrc1*Δ, *tof1*Δ, *ctf18*Δ). The fact that Ctf18 and Mrc1 act in separate pathways to maintain stability of repeat sequences (Gellon *et al.* 2011; Stokes *et al.* 2020) could explain the difference in RPA requirement observed between *ctf18*Δ *rtt105*Δ and *mrc1*Δ *rtt105*Δ cells. On their side, Mrc1 and Tof1 are both crucial for preventing fork breakage in the presence of secondary structures and are equally important for preventing instability at long repeat sequences (Gellon *et al.* 2019). It is tempting to speculate that as Mrc1 and Tof1, Rtt105 could have a role in fork stabilization when long ssDNA intermediates are generated and that this role is not related to RPA. This could explain the extreme instability that we have recently observed in presence of G4 structures in *rtt105*Δ cells (Maestroni *et al.* 2020).

In summary, our studies reveal that Rtt105 guards genome stability through multiple mechanisms. We have shown that in the absence of *RTT105* yeast cells require the two branches of the S phase checkpoint and HR to survive, indicating the presence of replicative defects. Furthermore, our detailed genetic analyses demonstrate that *RTT105* is important for several vital mechanisms intimately connected to replication fork progression, as sister chromatid cohesion and replication-dependent nucleosome assembly. We also point out the importance of *RTT105* in heterochromatin silencing and in telomere-length maintenance. This work reveals novel roles for *RTT105* during DNA metabolism and show that the pleiotropic effects of loss of *RTT105* are not only related to Rtt105's role in chaperoning Rfa1. Rtt105 may exert a crucial role in the maintenance of genome stability during replication by directly affecting RPA function and/or through an unknown function, independent of RPA.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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