Replisome Function During Replicative Stress Is Modulated by Histone H3 Lysine 56 Acetylation Through Ctf4

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ABSTRACT Histone H3 lysine 56 acetylation in *Saccharomyces cerevisiae* is required for the maintenance of genome stability under normal conditions and upon DNA replication stress. Here we show that in the absence of H3 lysine 56 acetylation replisome components become deleterious when replication forks collapse at natural replication block sites. This lethality is not a direct consequence of chromatin assembly defects during replication fork progression. Rather, our genetic analyses suggest that in the presence of replicative stress H3 lysine 56 acetylation uncouples the Cdc45–Mcm2-7–GINS DNA helicase complex and DNA polymerases through the replisome component Ctf4. In addition, we discovered that the N-terminal domain of Ctf4, necessary for the interaction of Ctf4 with Mms22, an adaptor protein of the Rtt101–Mms1 E3 ubiquitin ligase, is required for the function of the H3 lysine 56 acetylation pathway, suggesting that replicative stress promotes the interaction between Ctf4 and Mms22. Taken together, our results indicate that Ctf4 is an essential member of the H3 lysine 56 acetylation pathway and provide novel mechanistic insights into understanding the role of H3 lysine 56 acetylation in maintaining genome stability upon replication stress.

KEYWORDS Ctf4; H3K56 acetylation; Mms22; replicative stress; replisome

THE eukaryotic replisome consists of polymerases and an essential DNA helicase that are linked by a number of factors assembled during the initiation of chromosome replication. Progression of the replication fork depends on the activity of the replisome progression complex (RPC). This complex is uniquely present during S phase (Gambus *et al.* 2006) and remains associated with the replication fork until completion of DNA replication. In *Saccharomyces cerevisiae*, the RPC is made up of Mcm10, Mrc1, Tof1, Csm3, Ctf4, Top1, FACT (Spt16 and Pob3), and the CMG complex comprising Cdc45, Mcm2-7 (MCM), and the *go ichi ni san* (GINS) complex. The CMG constitutes the core replicative helicase responsible for the movement and activities of the replication fork (Pacek *et al.* 2006; Bochman and Schwacha 2009).

The link between helicase and polymerases is a crucial determinant for the regulation of the replisome. The leading-strand DNA polymerase- ϵ was recently shown to be integrated into the replisome via an interaction with the GINS complex (Sengupta *et al.* 2013). Furthermore, the DNA polymerase- α -primase complex, which initiates DNA synthesis at replication origins and continues to prime Okazaki fragments at the fork, remains associated with the RPC via the Ctf4 trimer, which simultaneously interacts with the GINS complex (Gambus *et al.* 2009; Tanaka *et al.* 2009; Gosnell and Christensen 2011; Simon *et al.* 2014).

Cells have evolved different mechanisms to maintain genome integrity under the conditions threatening replication progression (Jossen and Bermejo 2013; Leman and Noguchi 2013). The S-phase checkpoint mediated by *MRC1* was initially characterized as a pathway activated by fork stalling and able to both stabilize the replisome and delay cell cycle progression (Elledge 1996; Sancar *et al.* 2004; Labib and De Piccoli 2011).

It was further shown that, during DNA replication stress, lack of either *MRC1* or *CTF4* leads to uncoupling between the replicative polymerases and RPC, as well as a dissociation of replisome components (Bando *et al.* 2009; Tanaka *et al.* 2009;

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Mimura *et al.* 2010). Unlike *MRC1*, *CTF4* is not required for S-phase checkpoint activation.

Ctf4 was initially identified in *S. cerevisiae* as a chromosome transmission fidelity factor required for the maintenance of genome stability and sister-chromatid cohesion (Spencer *et al.* 1990; Jawad and Paoli 2002; Gambus *et al.* 2006; Lengronne *et al.* 2006). *CTF4* is not essential for budding yeast viability (Miles and Formosa 1992), but its deletion greatly sensitizes cells to DNA replication drugs (Ogiwara *et al.* 2007). Mechanistically, *Ctf4* is required for coordination between DNA unwinding and synthesis, and it also stabilizes polymerase- α at the replication forks (Gambus *et al.* 2009; Tanaka *et al.* 2009; Mimura *et al.* 2010). Among various partners, *Ctf4* interacts with an F-box protein *Dia2* involved in the regulation of DNA replication (Mimura *et al.* 2009) and with *Mms22*, an adaptor protein of the Cul4(Ddb1)-like E3 ubiquitin ligase complex (Gambus *et al.* 2009; Mimura *et al.* 2009, 2010). The latter also includes *Mms1* and cullin *Rtt101*, both crucial for maintaining replisome integrity in hydroxyurea and therefore for efficient recovery from replication stress (Luke *et al.* 2006; Duro *et al.* 2008; Zaidi *et al.* 2008; Gambus *et al.* 2009; Mimura *et al.* 2010; Vaisica *et al.* 2011).

The *Rrm3* helicase travels with the replication fork and facilitates the progression of replication forks through non-histone protein–DNA complexes throughout the genome (Azvolinsky *et al.* 2009; Fachinetti *et al.* 2010). In the absence of *RRM3*, chromosome breaks occur at discrete fork pause sites at specific genomic locations (Ivessa *et al.* 2003). A number of studies indicate that the DNA breaks generated in *rrm3* Δ cells affect cell viability in the absence of the so-called “H3K56 acetylation pathway” that comprises *ASF1*, *RTT109*, *RTT101*, *MMS1*, and *MMS22* (Tong *et al.* 2004; Luke *et al.* 2006; Pan *et al.* 2006; Collins *et al.* 2007; Duro *et al.* 2008; Roberts *et al.* 2008; Zaidi *et al.* 2008; Costanzo *et al.* 2010; Koh *et al.* 2010; Mimura *et al.* 2010).

In *S. cerevisiae*, H3K56 localizes at the DNA entry and exit points of a nucleosome (Masumoto *et al.* 2005; Ozdemir *et al.* 2005; Xu *et al.* 2005). H3K56 is transiently acetylated during the S phase of the cell cycle and after DNA damage and is rapidly de-acetylated by the action of the sirtuins *Hst3* and *Hst4*, when cells enter the transition between G2 and M phases and after DNA repair (Masumoto *et al.* 2005; Xu *et al.* 2005). *Asf1* binds to all newly synthesized H3 and presents the H3–H4 dimer to the *Rtt109* lysine acetyltransferase for H3K56 acetylation (H3K56ac). Following acetylation of H3K56, ubiquitylation of H3 and H4 by the *Rtt101–Mms1–Mms22* E3 ligase complex weakens the *Asf1*–H3–H4 interaction (Han *et al.* 2013) and facilitates the transfer of H3–H4 to other histone chaperones complexes, thereby coordinating nucleosome formation as well as stable progression of the replication fork (Li *et al.* 2008; Clemente-Ruiz *et al.* 2011; Han *et al.* 2013). In addition to its well-characterized function in replication-coupled chromatin assembly, H3K56ac is also required for a number of other processes such as transcription, DNA repair-coupled chromatin assembly, deactivation of the DNA damage checkpoint, and repair of DNA lesions that occur during DNA replication

(Chen *et al.* 2008; Endo *et al.* 2010; Wurtele *et al.* 2011; Tanaka *et al.* 2012; Haber *et al.* 2013; Muñoz-Galván *et al.* 2013). Yet, despite its multiple roles, the mechanism by which the H3K56ac pathway sustains viability under replication stress and its targets remain unknown.

Here, we show that the H3K56ac pathway is essential for the viability of cells lacking *RRM3*. Strikingly, we discovered that, in cells devoid of *RRM3*, *CTF4* mediates a deleterious effect in the absence of H3K56ac. This finding poises *Ctf4* as a potential target of the H3K56ac pathway. Genetic analysis of the negative effect of *CTF4* revealed that it is related to the interaction of *Ctf4* with the GINS complex and DNA polymerase- α . Consistently, we found that destabilization of the catalytic subunit of polymerase- α rescues the viability of *rrm3* Δ cells in the absence of H3K56ac. Finally, our data strongly suggest that this effect is dependent upon an interaction between *Ctf4* and *Mms22*. Similarly to *ctf4* Δ , deletion of *MRC1* induces uncoupling between helicase and polymerase (Tanaka *et al.* 2009; Mimura *et al.* 2010; Vaisica *et al.* 2011). In accord with this notion, we found that the replication function of *MRC1* is also strongly deleterious for cells experiencing constitutive replicative damages in the absence of a functional H3K56ac pathway.

Materials and Methods

Strain construction

All strains used in this study are listed in [Supporting Information, Table S1](#). Null mutations were obtained after polymerase chain reaction amplification of a disruption cassette as described previously (Corda *et al.* 2005).

Cell cycle analysis

For synchronous cell cultures, yeast cells were grown at 25° or 30° in yeast extract peptone dextrose (YPD) to OD₆₀₀ = 0.6 and then arrested in G1 by the addition of 15 μ g/ml of α -factor (GENEPEP SA). After 2 hr, α -factor was removed to allow cells to progress synchronously through the cell cycle either in the presence or absence of 40 μ M camptothecin (CPT). Samples were taken every 10 min for fluorescence-activated cell sorting (FACS), Ctf4-Myc chromatin-binding assay, and H3K56ac measurement.

Protein chromatin-binding assay

The assays were performed as described previously (Liang and Stillman 1997). Briefly, cells were harvested and treated with sodium azide at the indicated time and spheroplasted by incubating them first in 3 ml of prespheroplasting buffer [100 mM PIPES Piperazine-1,4-bis(2-ethanesulfonic acid) (pH 9.4), 10 mM dithiothreitol (DTT)] for 10 min at room temperature and then in 2 ml of spheroplasting buffer [50 mM KH₂PO₄/K₂HPO₄ (pH 7.5), 0.6 M sorbitol, 10 mM DTT] containing 80 μ l of 1 mg/ml of oxalyticase (Sigma) at 30° for 25 min with occasional shaking. Spheroplasts were washed with 1 ml of cold wash buffer [100 mM KCl, 50 mM HEPES–KOH

(pH 7.5), 2.5 mM MgCl₂, and 0.4 M sorbitol], pelleted at 4000 × g for 1 min at 4°, and resuspended in 100 µl of extraction buffer (EB) [100 mM KCl, 50 mM HEPES–KOH (pH 7.5), 2.5 mM MgCl₂, 50 mM NaF, 5 mM Na₄P₂O₇, 0.1 mM NaVO₃] containing protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 20 µg/ml of leupeptin, 2 µg/ml of pepstatin, 2 mM benzamidinium HCl, and 0.2 mg/ml of bacitracin]. The suspension was split into two tubes. Spheroplasts were lysed by adding Triton X-100–0.25% and incubating on ice for 5 min with gentle mixing. The lysate was underlayered with 50% vol of 30% sucrose and spun at 12,000 × g for 10 min at 4°. Supernatants correspond to the soluble fractions. Pellets were washed with 25% EB containing 0.25% Triton X-100, spun again at 10,000 × g for 5 min at 4°, and resuspended in Laemmli buffer. Proteins were transferred onto a nitrocellulose membrane. Ctf4-Myc was detected with the 9E10 anti-Myc monoclonal antibody (Santa Cruz). We detected H3K56ac by using H3K56ac antibody (Active Motif).

Mass spectrometry

Cell culture and cross-link: Cells were synchronized as described above. At time 0 min (G1 phase) and 20 min (S phase), cells were treated with 1% formaldehyde (Sigma-Aldrich) at room temperature for 15 min. Formaldehyde was quenched at room temperature with 0.125 M glycine for 5 min. Finally, cells were washed three times with 1× cold TBS, frozen in liquid nitrogen, and stored at –80°. Cell cycle progression was monitored by FACS.

Isolation of Ctf4-green fluorescent protein for spectrometry analysis from yeast cell extracts: Purification of the Ctf4-green fluorescent protein (Ctf4-GFP) was performed as previously described (De Piccoli *et al.* 2012) with minor modifications. Pellets were resuspended in lysis buffer (mass/vol) containing 50 mM HEPES (pH 7.9), 140 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) supplemented with a protease inhibitor cocktail (Roche) and 1 mM PMSF, 0.1 mM NaVO₃, 2 mM NaF, and 1 mM DTT. Cells were disrupted using a Fast Prep machine (MP Biomedicals, three runs of 30 sec at maximum speed). After addition of 0.25 vol of extraction buffer (50 mM HEPES pH 7.9, 140 mM NaCl, 1 mM EDTA, 5% Triton-X100, 0.5% sodium deoxycholate), the samples were sonicated in a bioruptor (Diagenode), and insoluble material was removed by centrifugation at 16,000 × g for 30 min. The cell extracts were then incubated with anti-GFP-coated magnetic beads (Meek *et al.* 2012) for 2 hr before washing three times with each buffer 1 [50 mM HEPES (pH 7.9), 500 mM NaCl, 1 mM EDTA, 1% Triton-X100, 0.1% Na deoxycholate] and buffer 2 [10 mM Tris–HCl (pH 8), 250 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA]. The immunoprecipitated protein samples were incubated for 10 min at 65° with elution buffer [50 mM Tris–HCl (pH 8), 10 mM EDTA, 1% SDS] before addition of Laemmli buffer and incubation at 95° for 30 min.

Sample preparation: Pulldown protein extracts were loaded on NuPAGE 4–12% Bis–Tris acrylamide gels in MOPS buffer

according to the manufacturer's instructions (Invitrogen, Life Technologies). Electrophoresis was stopped as soon as proteins were stacked as a single band. Protein-containing bands were stained with Thermo Scientific Imperial Blue, cut from the gel, and, following reduction and iodoacetamide alkylation, digested with high-sequencing grade trypsin (Promega, Madison, WI). Extracted peptides were concentrated before mass spectrometry (MS) analysis. MS analyses were carried out by liquid chromatography–tandem mass spectrometry using a LTQ-Velos-Orbitrap (Thermo Fisher Scientific) connected to a nanoLC Ultimate 3000 rapid separation liquid chromatography system (Dionex, Sunnyvale, CA). Five microliters corresponding to 20% of each sample was injected in triplicate into the system. After preconcentration and washing of the sample on a Dionex Acclaim PepMap 100 column (C18, 2 cm × 100 µm i.d. 100-Å pore size, 5-µm particle size), peptides were separated on a Dionex Acclaim PepMap RSLC column (C18, 15 cm × 75 µm i.d., 100-Å, 2-µm particle size) at a flow rate of 300 nL/min with a two-step linear gradient (4–20% acetonitrile/H₂O; 0.1% formic acid for 90 min and 20–45% acetonitrile/H₂O; 0.1% formic acid for 30 min). For peptide ionization in the nanospray source, spray voltage was set at 1.4 kV and the capillary temperature at 275°. All samples were measured in a data-dependent acquisition mode. Each analysis was preceded by a blank MS run to monitor system background. The peptide masses were measured in a survey full scan [scan range 300–1700 m/z, with 30-K full width at half maximum resolution at m/z = 400, target automatic gain control value of 1.00 × 10⁶ and maximum injection time of 500 ms]. In parallel to the high-resolution full scan in the Orbitrap, the data-dependent collision-induced dissociation scans of the 10 most intense precursor ions were fragmented and measured in the linear ion trap (normalized collision energy of 35%, activation time of 10 ms, target automatic gain control value of 1.00 × 10⁴, maximum injection time of 100 ms, isolation window of 2 Da). Parent masses obtained in the Orbitrap analyzer were automatically calibrated on the 445.120025 ion used as lock mass. The fragment ion masses were measured in the linear ion trap to have a maximum sensitivity and the maximum amount of MS/MS data. Dynamic exclusion was implemented with a repeat count of 1 and exclusion duration of 30 sec.

Data analysis: Raw files generated from mass spectrometry analysis were processed with Proteome Discoverer 3.1 (Thermo Fisher Scientific). This software was used to search data via an in-house Mascot server (version 2.4.1; Matrix Science Inc., London) against the *S. cerevisiae* database subset (7802 sequences) of the SwissProt database (version 2014-06). For the database search, the following settings were used: a maximum of two miscleavages, oxidation as a variable modification of methionine, carbamido-methylation as a fixed modification of cysteine, and trypsin as the enzyme. A peptide mass tolerance of 6 ppm and a fragment mass tolerance of 0.8 Da were allowed. Only peptides with a high-stringency Mascot score were used for protein identification. A peptide false discovery rate of <1% was used. The precursor ion area detector from Proteome

Figure 1 Asf1 interaction with histone H3 is crucial for viability of yeast *rrm3Δ* cells. (A) Deletion of *ASF1* is lethal in *rrm3Δ* cells. Tetrad dissection of the diploid strain *asf1Δ/ASF1 rrm3Δ/RRM3*. In this and subsequent figures, the four spores from a given tetrad are in a vertical line on a YPD plate. Four representative tetrads are shown after 3 days (left) and after 5 days (right) at 30°. Squares indicate the *rrm3Δ* single mutants. Circles indicate the *asf1Δ* single mutants. Assuming that 2:2 segregation of the marker allows one to identify *asf1Δ rrm3Δ* double mutants (indicated by dashed circles). (B) Wild-type and *asf1Δ rrm3Δ* cells (from microcolonies shown in A, right) analyzed by differential interference contrast and 4',6-diamidino-2-phenylindole staining. Almost all *asf1Δ rrm3Δ* cells analyzed have no distinct nucleus compared to wild-type cells. (C) Effects of Asf1 interactions on the viability of *rrm3Δ* cells. Tetrad analysis of the meiotic progeny of *asf1Δ/ASF1 rrm3Δ/RRM3* diploid cells expressing *asf1-D37R E39R* (i) or *asf1-V94R* (ii) mutated forms of *ASF1* from plasmid pRS314. The presence of *asf1Δ rrm3Δ* spores is indicated by dashed circles. *asf1Δ rrm3Δ* spores expressing *asf1-D37R E39R* (i) or *asf1-V94R* (ii) are indicated by circles. A plus sign (+) indicates spores carrying the plasmid. wt, A1, R3, and A1R3 indicate wild-type, *asf1Δ*, *rrm3Δ*, and *asf1Δ rrm3Δ* spores, respectively. (iii) Tetrad analysis of the meiotic progeny of *rad53-ALRR/RAD53 rrm3Δ/RRM3* diploid cells. Circles indicate the *rad53-ALRR* mutant. Dashed circles indicate the *rad53-ALRR rrm3Δ* double mutant. wt, R3, ALRR, and ALRR R3 indicate wild-type, *rrm3Δ*, *rad53-ALRR*, and *rad53-ALRR rrm3Δ* spores, respectively.

Discoverer 3.1 was used for relative quantitation of proteins (sum of the area of the three more intense precursor ions used for protein identification). Protein lists obtained from cells with or without Ctf4-GFP were compared to discriminate between those proteins that specifically interact with Ctf4 from contaminant proteins.

Results

Loss of chaperone function of Asf1 causes *rrm3Δ* lethality

As previously suggested by genetic screens (Pan *et al.* 2006; Collins *et al.* 2007; Fiedler *et al.* 2009; Costanzo *et al.* 2010), we observed that *RRM3* disruption strongly affects the growth of *asf1Δ* cells (see below). To characterize the genetic interaction between *ASF1* and *RRM3*, one allele of *ASF1* was deleted in an *RRM3/rrm3Δ* diploid. Dissection of meiotic tetrads shows that *asf1Δ rrm3Δ* spores do not form visible colonies after 3 days at 30° (Figure 1A, left). This synthetic lethality between *asf1Δ* and *rrm3Δ* cannot be attributed to spore germination defects because *asf1Δ rrm3Δ* spores formed visible microcolonies after 5 days at 30° (Figure 1A, right). Microscopic analysis

indicated that *asf1Δ rrm3Δ* cells displayed aberrant morphologies, and that for almost all cells, the lethality can be attributed to death during mitosis (Figure 1B).

The histone chaperone *Asf1* fulfils various chromatin-related functions in both replication-coupled and replication-independent fashion and through physical interactions with multiple partners (Mousson *et al.* 2007). *Asf1* participates in the assembly and disassembly of chromatin through its H3 histone chaperone activity, in transcriptional silencing via an interaction with *Hir1* and in several aspects of the cellular response to genotoxic stress through an interaction with H3 or *Rad53* (Hu 2001; Sharp *et al.* 2001; Sutton *et al.* 2001; Celic *et al.* 2006; Recht *et al.* 2006; Takahata *et al.* 2009; Jiao *et al.* 2012; Burgess *et al.* 2014).

To understand which function of *Asf1* is crucial for viability of *rrm3Δ* cells, we tested various separation-of-function mutants of *Asf1*. Plasmids expressing *asf1* mutant proteins were introduced individually into an *asf1Δ/ASF1 rrm3Δ/RRM3* diploid strain. After sporulation of diploids, 100 tetrads were dissected and the genotypes of the viable spores were determined. To assess the replication-independent chromatin assembly function of *Asf1*, we first analyzed the double mutation *D37R E39R* (*asf1-DE*) that abolishes *Asf1-Hir1* interaction

but not histone H3 binding (Mousson *et al.* 2005). The HIR complex (formed by *Hir1*, *Hir2*, *Hir3*, and *Hpc2*) binds to *Asf1* and promotes replication-independent chromatin assembly (Green *et al.* 2005). We found that the *asf1-DE* mutant protein expressed in the *asf1Δ rrm3Δ* cells complemented the lethality of the double mutant (Figure 1C, i). In the complementary experiment we observed that the *HIR2* deletion did not affect *rrm3Δ* viability (Figure S1). Given that *HIR2* is required for the integrity of the HIR complex and, consequently, for histone deposition activity of *Asf1*/HIR (Green *et al.* 2005; Silva *et al.* 2012), these observations demonstrate that the replication-independent chromatin assembly function of *Asf1* is not required for *rrm3Δ* cell viability. We next evaluated the contribution of the single-residue substitution V94R, important for histone H3 interaction (Mousson *et al.* 2005; Jiao *et al.* 2012). We found that expression of the *asf1-V94R* allele was not able to complement *asf1Δ rrm3Δ* lethality (Figure 1C, ii). The checkpoint kinase *Mec1* and the *Ddc1-Mec3-Rad17* sliding clamp regulate the interaction between *Asf1* and *Rad53* (Hu 2001; Sharp *et al.* 2001; Burgess *et al.* 2014). Since the *asf1-V94R* mutation also affects *Asf1-Rad53* interaction (Jiao *et al.* 2012; Dennehey *et al.* 2013), we tested the viability of the *Rad53-A806R-L808R* (*rad53-ALRR*) mutant, which is strongly affected in its interaction with *Asf1* (Jiao *et al.* 2012) in the *rrm3Δ* background. We found that the *rad53-ALRR rrm3Δ* double mutant is viable (Figure 1C, iii). These data suggest that the crucial function of *ASF1* required for *rrm3Δ* cells viability is related to its histone chaperone function.

H3K56 acetylation is necessary for viability of *rrm3Δ* cells

Cells expressing the *asf1-V94R* mutant that cannot bind to histone H3-H4 (Mousson *et al.* 2005) lose H3K56ac (Recht *et al.* 2006), an important mark of all newly synthesized histone H3's preceding the histone fold domain (Masumoto *et al.* 2005; Celic *et al.* 2006; Han *et al.* 2007b). H3K56ac is catalyzed by the histone acetyl transferase (HAT) *Rtt109* upon presentation of the H3-H4 heterodimer by the histone chaperone *Asf1* (Schneider *et al.* 2006; Driscoll *et al.* 2007; Han *et al.* 2007a; Tsubota *et al.* 2007; Dahlin *et al.* 2014). To ascertain the contribution of H3K56ac to *rrm3Δ* viability, we analyzed the consequences of deleting *RTT109* in *rrm3Δ* cells. We found that similarly to *ASF1*, *RTT109* is required in the absence of *RRM3* (Figure S2). Because *RTT109* also acetylates other H3 and H4 lysines (Fillingham *et al.* 2008; Abshiru *et al.* 2013) and has functions independent of its H3K56 HAT activity (Roberts *et al.* 2008), we crossed a strain expressing *H3K56R* from a centromeric plasmid as the sole source of histone H3 to an *rrm3Δ* strain and analyzed the spores after sporulation of the diploid. Results obtained from the dissection of 160 tetrads are presented in Table 1. We were unable to recover any *rrm3Δ* spores expressing wild-type H4 and the *H3K56R* mutant as the sole source of histone H3, showing that H3K56ac is vital in *rrm3Δ* cells (Figure 2A and Table 1).

The *Sir2*-related *Hst3* and *Hst4* histone deacetylases regulate histone H3K56ac both during the normal cell cycle and

Table 1 Histone H3-H4 dependence on *rrm3Δ* viability

Histone genes	<i>RRM3</i>	<i>rrm3Δ</i>
<i>HHT1-HHF1 HHT2-HHF2</i>	58	44
<i>HHT1-HHF1 hht2Δ-hhf2Δ</i>	24	48
<i>hht1Δ-hhf1Δ HHT2-HHF2</i>	20	41
<i>hht1Δ-hhf1Δ hht2Δ-hhf2Δ</i>	0	0
<i>hht1Δ-hhf1Δ hht2Δ-hhf2Δ+hht1-K56R-HHF1</i>	20	0
<i>HHT1-HHF1 hht2Δ-hhf2Δ+hht1-K56R-HHF1</i>	37	27
<i>hht1Δ-hhf1Δ HHT2-HHF2+hht1-K56R-HHF1</i>	37	37
<i>HHT1-HHF1 HHT2-HHF2+hht1-K56R-HHF1</i>	75	31

A total of 160 tetrads from diploids for *hht1Δ-hhf1Δ/HHT1-HHF1 hht2Δ-hhf2Δ/HHT2-HHF2 rrm3Δ/RRM3* expressing *HHF1* and *hht1-K56R* from a centromeric plasmid were dissected, and the genotype of the viable spores was determined. The number of viable spores carrying each deletion and/or plasmid is indicated. Among the *rrm3Δ* spores carrying the *hht1-K56R* allele, only spores also expressing the wild-type *HHT1* allele are viable.

after DNA damage. Consequently, loss of *HST3* and *HST4* results in the constitutive hyperacetylation of H3K56 (Masumoto *et al.* 2005; Celic *et al.* 2006; Maas *et al.* 2006; Miller *et al.* 2006; Yang *et al.* 2008; Delgoshiaie *et al.* 2014). *SIR2* is also required to deacetylate H3K56 at specific heterochromatic sites (F. Xu *et al.* 2007). To further examine the impact of H3K56ac in the absence of *RRM3*, we deleted one allele of *RRM3*, *HST3*, *HST4*, and *SIR2* in a diploid strain. By analyzing the spores derived from this diploid, we found that both the *hst3Δ hst4Δ rrm3Δ* triple and *sir2Δ hst3Δ hst4Δ rrm3Δ* quadruple mutants were viable (Figure 2B). Thus, in contrast to the absence of H3K56ac, constitutive H3K56ac is not deleterious in the absence of *RRM3*.

H3K56ac-dependent coordination between nucleosome assembly and stability of advancing replication forks is not required for viability in the absence of *RRM3*

We have shown that HIR-dependent replication-independent chromatin assembly is not required for the viability of *rrm3Δ* cells. We then asked if the replication-coupled chromatin assembly function of H3K56ac is important for *rrm3Δ* cell viability. H3K56ac facilitates replication-coupled chromatin assembly by increasing the association of new histone molecules with CAF-1 and *Rtt106* (Li *et al.* 2008; Han *et al.* 2013). This pathway coordinates nucleosome assembly and stability of the advancing replication forks but is not required for H3K56ac-mediated protection against replicative DNA-damaging agents by DNA repair/tolerance mechanisms (Clemente-Ruiz *et al.* 2011). Consequently, during replication, similar defects arise in the *asf1Δ cac1Δ rtt106Δ* and the *cac1Δ rtt106Δ* mutants (Clemente-Ruiz *et al.* 2011; Prado and Clemente-Ruiz 2012). Thus, we analyzed the viability of *rrm3Δ cac1Δ rtt106Δ* cells by looking at the meiotic progeny of a diploid strain heterozygous for *RRM3*, *CAC1*, and *RTT106* deletions. We found that simultaneous deletion of *CAC1* and *RTT106* in the *rrm3Δ* causes slow growth, but unlike *asf1Δ*, is viable (Figure 3, A and B). This result indicates that a defective DNA repair/tolerance mechanism, rather than an alteration of the replication-coupled chromatin assembly *per se*, causes lethality of *rrm3Δ* cells in the absence of H3K56ac.

A *hht1Δ-hhf1Δ/HHT1-HHF1 hht2Δ-hhf2Δ/HHT2-HHF2 rrm3Δ/RRM3*
+ *phht1-K56R-HHF1*

Figure 2 Hyperacetylation and hypoacetylation of lysine 56 of histone H3 affect *rrm3Δ* cells differently. (A) H3K56R mutation is lethal in *rrm3Δ* cells. Tetrads from diploids for *hht1Δ-hhf1Δ/HHT1-HHF1 hht2Δ-hhf2Δ/HHT2-HHF2 rrm3Δ/RRM3* expressing *HHF1* and *hht1-K56R* from a centromeric plasmid were dissected and analyzed for the presence of auxotrophic markers. Dashed circle indicates *rrm3Δ* spore expressing *H3K56R* as sole source of histone H3. (B) *rrm3Δ* cells are viable with constitutively acetylated H3K56. The *hst3Δ/HST3 hst4Δ/HST4 rrm3Δ/RRM3 sir2Δ/SIR2* diploid strain was dissected. The presence of *rrm3Δ hst3Δ hst4Δ* and *rrm3Δ sir2Δ hst3Δ hst4Δ* mutants is indicated by a circle and by dashed circles, respectively.

B *hst3Δ/HST3 hst4Δ/HST4 rrm3Δ/RRM3 sir2Δ/SIR2*

***CTF4* is harmful upon DNA damage in absence of a functional H3K56ac pathway**

Both *RTT107*, which supposedly functions in the same genetic pathway as *RTT101*, *MMS1*, and *MMS22* to maintain genome stability, and *CTF4* interact with the *Rtt101-Mms1* complex through the adaptor protein *Mms22* (Pan *et al.* 2006; Collins *et al.* 2007; Gambus *et al.* 2009; Mimura *et al.* 2010). We have analyzed the consequences of *RTT101*, *MMS1*, *MMS22*, *RTT107*, and *CTF4* deletions in *rrm3Δ* cells and found that all double mutants are lethal with the only exception of *ctf4Δ rrm3Δ* mutant (Figure S3). Based on the fact that *ctf4Δ* suppresses some negative phenotypes associated with constitutive H3K56ac (Celic *et al.* 2008) and on our observations, we hypothesized that *Ctf4* may be a target of the H3K56ac pathway. To test this hypothesis, we evaluated the effects of deleting *CTF4* when the *RRM3* deletion was combined with mutations affecting different steps of the H3K56ac pathway. The results presented in Figure 4 show that, despite its negative effect in the single *rrm3Δ* mutant, the inactivation of *CTF4* restores the growth of each of the double mutants (*asf1Δ rrm3Δ*, *rtt109Δ rrm3Δ*, *rtt101Δ rrm3Δ*, *mms1Δ rrm3Δ*, and *mms22Δ rrm3Δ*). To ascertain whether the effect of *ctf4Δ* is linked to H3K56ac, we further examined the impact of the *CTF4* deletion in the *rrm3Δ H3K56R* double mutant. As expected, we found that *ctf4Δ* restored the viability of *rrm3Δ* cells expressing H3K56R as the sole source of H3 (Figure S4). Strikingly, the growth of each triple mutant seemed similar to the one of the *rrm3Δ ctf4Δ* mutant with one exception: *ctf4Δ mms22Δ rrm3Δ* (Figure 4). The slower growth of the *ctf4Δ mms22Δ rrm3Δ* strain may be explained by the multiple roles of *Mms22* in the response to DNA damage (Wurtele *et al.* 2011). Moreover, we have found that *ctf4Δ* is not able to modify the viability of *rtt107Δ rrm3Δ* cells (Figure S5), indicating that *CTF4* and *RTT107* act in distinct pathways to deal with replicative damage and suggesting that *RTT107* does not belong to the H3K56ac pathway.

We next investigated the deleterious effect of *CTF4* when cells affected in the H3K56ac pathway were treated with the methylating agent methyl methanesulfonate (MMS). Deletion of *CTF4* increased the viability of *asf1Δ*, *rtt109Δ*, *mms1Δ*, and *mms22Δ* single mutants. These results corroborate our findings obtained in the settings of the *RRM3* deletion and further support the notion that H3K56ac modulates replisome function during replicative stress through *CTF4* (Figure 5). The only exception is the double mutant *ctf4Δ rtt101Δ*, which is more sensitive compared to the *rtt101Δ* single mutant. A previous report showed that, in addition to its function in the H3K56ac pathway, *RTT101* exerts an *MMS1*- and *MMS22*-independent function during replication through its interaction with histone chaperones (Han *et al.* 2010). Interestingly, the *ctf4Δ rtt101Δ* double mutant is less sensitive to MMS as compared to *ctf4Δ*, suggesting that the *CTF4* deletion is beneficial in the presence of MMS in the *rtt101Δ* mutant.

***Ctf4*-mediated uncoupling of DNA polymerase- α and GINS is crucial for the viability of *rrm3Δ* cells experiencing DNA damage**

Ctf4 couples the CMG helicase to polymerase- α in the replisome by interacting simultaneously with both polymerase- α and the GINS complex (Gambus *et al.* 2009; Tanaka *et al.* 2009; Simon *et al.* 2014). To determine the importance of the *Ctf4*-bridging function, we analyzed a *Ctf4*-(1-383) truncated mutant (*Ctf4*-NT) that is unable to bind the GINS and polymerase- α together with the *Ctf4*-(1-383) mutant (*Ctf4*- Δ NT) that has kept its ability to bind GINS and polymerase- α (Gambus *et al.* 2009). The *ctf4*-NT construct restores the viability of *asf1Δ rrm3Δ* (Figure 6A), as does the complete absence of *CTF4*. In contrast, analysis of >100 tetrads did not yield a single *ctf4*- Δ NT *asf1Δ rrm3Δ* triple mutant (Figure 6B). Similar results were observed when the double mutants *ctf4*-NT *asf1Δ* and *ctf4*- Δ NT *asf1Δ* were exposed to CPT (Figure 6C). Based on these results we suggest that the

A

asf1Δ/ASF1 rrm3Δ/RRM3 cac1Δ/CAC1 rtt106Δ/RTT106

B

Figure 3 A Cac1/Rtt106-independent function of H3K56ac is required for viability of *rrm3Δ* cells. (A) Defective Cac1/Rtt106-dependent chromatin assembly does not cause lethality in the absence of *RRM3*. Tetrads from the *rtt106Δ/RTT106 cac1Δ/CAC1 asf1Δ/ASF1 rrm3Δ/RRM3* diploid strain were dissected. Diamonds indicate *cac1Δ rtt106Δ* mutants. Hexagon indicates the *rtt106Δ rrm3Δ* mutant. Square indicates the *cac1Δ rrm3Δ* mutant. Circle indicates the *cac1Δ rtt106Δ rrm3Δ* mutant. Dashed circles indicate *asf1Δ rrm3Δ* mutants. Triangle indicates *asf1Δ cac1Δ* mutant. (B) Effects of *cac1Δ* and *rtt106Δ* on viability of *rrm3Δ* cells. Yeast strains of indicated genotypes were streaked onto YPD plates and grown at 30° for 3 days.

bridging function of *Ctf4* is deleterious in the absence of H3K56ac following DNA damage.

To confirm that the restoration of the viability of the *asf1Δ rrm3Δ* mutant by the *CTF4* deletion is associated with the uncoupling of the helicase and polymerase- α , we used the thermosensitive (ts) mutant *cdc17-1* encoding the catalytic subunit of the DNA polymerase- α . In agreement with our hypothesis, we found that the *cdc17-1* mutation rescues *asf1Δ rrm3Δ* cell viability at the semirestrictive temperature (30°), but not at the permissive temperature (25°) (Figure 6D).

By analyzing the *Ctf4* truncations, we observed that the *ctf4-ΔNT* allele was lethal by itself when combined with *rrm3Δ* (Figure 6B). Based on the results from K. Labib's and T. Kamura's laboratories showing that the N-terminal region of *Ctf4* interacts with *Mms22* (Morohashi *et al.* 2009; Mimura *et al.* 2010) and because the double *ctf4Δ rrm3Δ* was viable, we hypothesized that the lethality of the *ctf4-ΔNT rrm3Δ* mutant could be due to the inability of *Ctf4-ΔNT* to interact with *Mms22* and consequently to modulate *Ctf4* interaction with GINS and polymerase- α . If this hypothesis is valid, we expect that the *Ctf4-Mms22* interaction functions in the H3K56ac pathway and that a defect in this interaction phenocopies a lack of H3K56ac. In such a case, the viability of *hst3Δ hst4Δ rrm3Δ* should be also compromised by *ctf4-ΔNT* despite the constitutive hyperacetylation of H3K56. We have shown that the hyperacetylation of H3K56 resulting from the simultaneous deletion of *HST3* and *HST4* is not lethal in *rrm3Δ* cells (Figure 2B). We now show that the viability of *hst3Δ hst4Δ rrm3Δ* is compromised by *ctf4-ΔNT* (Figure S6). These observations reinforce the idea that the *ctf4-ΔNT* mutant is insensitive to H3K56ac and strongly suggest that the role of

Ctf4 in the H3K56ac pathway relies on its interaction with *Mms22*. These results indicate that the key function of *Ctf4* in coupling DNA polymerase- α to the CMG helicase in wild-type cells becomes deleterious in *rrm3Δ* cells lacking H3K56ac. Taken together, these genetic analyses suggest that one function of H3K56ac could be to modulate the replisome in the presence of DNA damage, probably through an interaction between *Ctf4* and *Mms22*.

Ctf4 association to chromatin is not notably affected in *rrm3Δ* cells

To further examine the relationships between *CTF4* and H3K56ac, we analyzed the chromatin-bound *Ctf4* together with H3K56ac (see *Materials and Methods*). Consistent with published data (Masumoto *et al.* 2005; Wang *et al.* 2010), we observed that *Ctf4* was bound to chromatin mainly during S phase and dissociated in late S or G2 phase (Figure 7A). Interestingly, chromatin dissociation of *Ctf4* occurred concomitantly with the increase in H3K56ac (Figure 7A). In the absence of *RRM3*, H3K56ac levels were not significantly affected, but the timing of *Ctf4* dissociation from chromatin was delayed (Figure 7B). We assumed that the persistence of *Ctf4* association to chromatin in the *rrm3Δ* mutant probably reflected the fact that *rrm3Δ* cells took longer to traverse from late S phase into the next cell cycle, as a consequence of the accumulation of DNA lesions during S phase in the absence of *RRM3*. In agreement with this, we found that *Ctf4* persists in chromatin also in the presence of CPT (Figure 7C). These results indicate that the uncoupling observed upon DNA damage is not mediated by *Ctf4* degradation. Finally, we analyzed the global level of *Ctf4* in the wild-type control strain *CTF4-MYC* and in

Figure 4 *CTF4* deletion suppresses *rrm3Δ* lethality in different genetic contexts affecting the H3K56ac pathway. (A) *CTF4* deletion rescues *asf1Δ rrm3Δ* lethality. Tetrads from diploids heterozygous for *ctf4Δ*, *rrm3Δ*, and *asf1Δ* were dissected and analyzed after 3 days at 30°. Circles indicate *ctf4Δ asf1Δ rrm3Δ* mutants. Dashed circle indicates *asf1Δ rrm3Δ* mutants. In A–E, diamonds indicate *ctf4Δ rrm3Δ* mutants. (B) *CTF4* deletion rescues *rtt109Δ rrm3Δ* lethality. Tetrads from diploids heterozygous for *ctf4Δ*, *rrm3Δ*, and *rtt109Δ* were dissected and analyzed as in A. Circles indicate *ctf4Δ rtt109Δ rrm3Δ* mutants. Dashed circles indicate *rtt109Δ rrm3Δ* mutants. (C) *CTF4* deletion rescues *rtt101Δ rrm3Δ* lethality. Tetrads from diploids heterozygous for *ctf4Δ*, *rrm3Δ*, and *rtt101Δ* were dissected and analyzed as in A. Circle indicates *ctf4Δ rtt101Δ rrm3Δ* mutant. Dashed circles indicate *rtt101Δ rrm3Δ* mutants. (D) *CTF4* deletion rescues *mms1Δ rrm3Δ* lethality. Tetrads from diploids heterozygous for *ctf4Δ*, *rrm3Δ*, and *mms1Δ* were dissected and analyzed as in A. Circle indicates *ctf4Δ mms1Δ rrm3Δ* mutants. Dashed circles indicate *mms1Δ rrm3Δ* mutants. (E) *CTF4* deletion partially rescues *mms22Δ rrm3Δ* lethality. Tetrads from diploids heterozygous for *ctf4Δ*, *rrm3Δ*, and *mms22Δ* were dissected and analyzed as in A. Circles indicate *ctf4Δ mms22Δ rrm3Δ* mutants. Dashed circles indicate *mms22Δ rrm3Δ* mutants.

H3K56R CTF4-MYC mutant cells. Consistent with our genetic analysis and with the fact that *CTF4* function is detrimental to yeast cells lacking H3K56ac (Pan *et al.* 2006), we repeatedly observed that the amount of Ctf4 was reduced in the *H3K56R* mutant (Figure 7D), suggesting that one adaptation of *H3K56R* cells is to reduce their levels of Ctf4 to promote their growth.

Interaction between Mms22 and the replisome is promoted in *rrm3Δ* cells

Next, we examined whether the interaction between the replisome and Mms22 is modulated by *RRM3* deletion using a quantitative mass spectrometry approach. Mass spectrometry analyses conducted in wild-type and *rrm3Δ* cells after immunoprecipitation of Ctf4-GFP protein during S phase allowed us to identify Mms22, Mcm2-7 helicase, the catalytic subunit of DNA polymerase- α (Cdc17), and other components of the replisome-progressing complex and factors that will be described elsewhere (Figure 8A and Figure S7). Consistent with

our previous finding that Ctf4 is not degraded in *rrm3Δ* cells (Figure 7), we found similar Ctf4 levels in either the presence or absence of *RRM3*. Interestingly, despite lower Mms22 levels in *rrm3Δ* cells (Figure 8B, right), we repeatedly observed, after immunoprecipitation of Ctf4-GFP, an enrichment of Mms22 without any increase of Ctf4, Mcm2-7, or Cdc17 levels in the absence of *RRM3* (Figure 8A). These results, showing a specific enrichment of Mms22 at forks in the absence of *RRM3*, are consistent with our genetic observations obtained with the Ctf4 mutants. They reinforce our conclusion that the interaction between Ctf4 and Mms22 is regulated during replicative damage and is crucial in dealing with replicative stress.

Replisome destabilization allows cells lacking a functional H3K56ac pathway to survive under replicative stress

To better understand the role of H3K56ac at forks, we extended our analysis to another crucial component of the

Figure 5 In presence of MMS, *CTF4* becomes harmful for cells affected in the H3K56 acetylation pathway. Fivefold serial dilutions of exponentially growing cells were spotted onto a YPD plate or 0.015% MMS plate and incubated at 30° for 3 days.

RPC. In an unperturbed S phase, *Mrc1* senses and regulates replisome integrity by interacting with multiple components of the replisome, such as *Tof1/Csm3* and CMG complexes, *Dia2*, *Ctf4*, and the DNA polymerase- ϵ (Kanemaki *et al.* 2003; Katou *et al.* 2003; Nedelcheva *et al.* 2005; H. Xu *et al.* 2007; Lou *et al.* 2008; Komata *et al.* 2009; Mimura *et al.* 2009; Morohashi *et al.* 2009; Naylor *et al.* 2009; Uzunova *et al.* 2014). Interestingly, as shown for *ctf4* Δ cells, replication forks progress more slowly in *mrc1* Δ cells (Szyjka *et al.* 2005; Tourrière *et al.* 2005; Hodgson *et al.* 2007). Considering our findings with respect to *CTF4*, and the results of previous genetic screens (Collins *et al.* 2007; Fiedler *et al.* 2009; Haber *et al.* 2013), it is possible that the replicative function of *MRC1* could also be deleterious in the presence of DNA damage, when H3K56ac is compromised. To test this possibility, because *mrc1* Δ is synthetic lethal with *rrm3* Δ (Naylor *et al.* 2009), we first examined the effects of the *MRC1* deletion in *asf1* Δ cells subjected to replicative stress. We evaluated the viability of the *asf1* Δ *mrc1* Δ cells in the presence of DNA damage induced by CPT and MMS. We observed that deleting *MRC1* strongly suppressed the CPT and MMS sensitivity of *asf1* Δ cells without affecting *asf1* Δ thermo-sensitivity (Figure 9A). In addition to its role in replication, *MRC1* is also required for checkpoint activation upon DNA replication stress. To further determine which function of *MRC1* is harmful to *asf1* Δ cells in the presence of DNA replication stress, we tested two separation-of-function mutants of *MRC1*. We found that the *mrc1-C14* mutant that is compromised for its replication function (Naylor *et al.* 2009) behaved as *mrc1* Δ , whereas the checkpoint defective *mrc1-AQ* mutant had no effect (Figure 9B). We obtained similar results with *mms1* Δ cells (Figure S8). These results point out

the importance of the *MRC1* replication function in the sensitivity to DNA replication stress of cells deficient for the H3K56ac pathway.

Discussion

In this study, we first show that, in the absence of *ASF1*, the *rrm3* Δ growth defect is a direct consequence of the lack of H3K56ac. Our detailed genetic analyses indicate that, upon DNA replication stress, the lack of H3K56ac mainly affects DNA repair and/or DNA damage tolerance mechanisms implicated in the response to replicative DNA damage.

Both *ASF1* and *RTT109* are required for H3K56R and function together with the E3 ubiquitin ligase complex *Rtt101-Mms1-Mms22* (which is itself dispensable for H3K56ac) in the H3K56ac pathway (Collins *et al.* 2007). We have discovered that inactivating *CTF4* restores the viability of *asf1* Δ *rrm3* Δ , *rtt109* Δ *rrm3* Δ , *mms1* Δ *rrm3* Δ , and *mms22* Δ *rrm3* Δ mutants. Our results show that the loss of *CTF4* allows the cell to survive when the fork encounters obstacles in the absence of a functional H3K56ac pathway. According to the *Ctf4* role in coupling the MCM helicase to polymerase- α during normal replication (Gambus *et al.* 2009; Tanaka *et al.* 2009), we propose that the regulation of this function is crucial in the presence of replicative damages. The importance of uncoupling the helicase from polymerase- α was further strengthened by our observations showing that the *ctf4-NT* mutation that affects *Ctf4* interactions with GINS and polymerase- α (Gambus *et al.* 2009), and consequently its coupling ability between the helicase and polymerase, also restores the viability of *asf1* Δ cells upon replicative damage in contrast to the *ctf4- Δ NT* mutation that preserves the *Ctf4* coupling function (Gambus *et al.* 2009). Similarly, we have shown that affecting the stability of the large subunit of the DNA polymerase- α also restores the viability of *asf1* Δ *rrm3* Δ cells devoid of H3K56ac (see below). We therefore concluded that in the presence of replicative stress a coordinated progression of helicase and DNA polymerase- α is harmful in the absence of a functional H3K56ac pathway. We propose that one consequence of H3K56ac upon DNA replication stress would be to modulate replisome integrity by weakening the coupling between the MCM helicase and polymerase- α through *Ctf4*.

We also point out the importance of the *MRC1* replication function in the sensitivity to replication stress of cells deficient for the H3K56ac pathway. Indeed, during normal replication, a *Ctf4* trimer interacts with multiple replisome components including *Mrc1* and polymerase- ϵ (Simon *et al.* 2014). Moreover, loss of *Ctf4* affects the association of both leading and lagging replication proteins with the fork (Tanaka *et al.* 2009), suggesting that *Ctf4* plays a role in the coordinated progression of the MCM helicase with both leading- and lagging-strand synthesis. Therefore, we propose that, in response to DNA replication stress, *Ctf4* could modulate the coordinated progression of MCM helicase with both lagging- and

Figure 6 Uncoupling of MCM helicase and DNA polymerase- α favors cell viability during replicative stress in the absence of H3K56 acetylation. (A) The inability of Ctf4 to bind GINS and DNA polymerase- α restores the viability of the *asf1Δ rrm3Δ* mutant. Tetrad dissection from the *ctf4-NT/CTF4 asf1Δ/ASF1 rrm3Δ/RRM3* diploid strain. Diamond, circle, and dashed circle indicate *ctf4-NT rrm3Δ*, *ctf4-NT asf1Δ rrm3Δ*, and *asf1Δ rrm3Δ* mutants, respectively. (B) The ability of Ctf4 to bind GINS and DNA polymerase- α is lethal in the *asf1Δ rrm3Δ* mutant. Diamond, circles, and dashed circle indicate *ctf4-ΔNT rrm3Δ*, *ctf4-ΔNT asf1Δ rrm3Δ*, and *asf1Δ rrm3Δ* mutants, respectively. (C) Sensitivity to CPT of the combination of the mutants *ctf4Δ*, *ctf4-NT*, and *ctf4-ΔNT* with *asf1Δ*. Fivefold serials dilutions of exponentially growing cells were spotted onto YPD and 4 μ M CPT plates and incubated at 30° for 3 days. (D) Affecting the stability of the catalytic subunit of the DNA polymerase- α (Cdc17) restores the viability of *asf1Δ rrm3Δ* mutant. Tetrad dissection from *cdc17-1/cdc17-1 asf1Δ/ASF1 rrm3Δ/RRM3* diploid strain. Circles indicate the *asf1Δ rrm3Δ cdc17-1* mutants.

leading-strand polymerases in a H3K56ac-dependent way. Our findings showing that *CTF4* negatively affects the growth of *rrm3Δ* cells in the absence of a functional H3K56ac pathway (Figure 4) is consistent with the idea that Ctf4 is the major, if not only, target of the H3K56ac pathway during DNA replication stress.

Among the *ctf4Δ*, *ctf4-NT*, and *ctf4-ΔNT* mutants we found that only *ctf4-ΔNT*, which retains the ability to bind GINS and DNA polymerase- α , is synthetic-lethal with *rrm3Δ* cells despite the presence of normal levels of H3K56ac. This negative genetic interaction indicates that deficient replication fork progression arising in *rrm3Δ* cells requires a *CTF4*-

dependent regulation of the replisome function. Ctf4 has been shown to interact with the Rtt101-Mms1-Mms22 complex through an interaction with Mms22 (Mimura *et al.* 2010). Since Ctf4 needs its N-terminal portion to interact with Mms22 (Gambus *et al.* 2009; Mimura *et al.* 2010), we speculate that the inability of Ctf4- Δ NT to interact with Mms22 affects the H3K56ac-dependent uncoupling between helicase and polymerase, required to cope with DNA replication stress, and causes *rrm3Δ* lethality. In the absence of Ctf4 or its C-terminal part, this interaction is probably no longer required for *rrm3Δ* cells viability because of the uncoupling between MCM helicase and polymerase- α . Taken together,

Figure 7 Ctf4 chromatin association is not affected in presence of replicative damages. (A) Cell cycle chromatin association of Ctf4. Ctf4-Myc cells were synchronized in G1 with α -factor and released into fresh medium at 25°. Samples were collected every 10 min, crude chromatin was prepared and analyzed by Western blot with 9E10 antibody for Ctf4-Myc (upper) and H3K56ac antibody for H3K56 acetylation (lower) using the same blot. Cell cycle progression was followed by FACS analysis (right). (B) Cell cycle chromatin association of Ctf4 in *rrm3* Δ cells. Ctf4-Myc *rrm3* Δ cells were treated and analyzed as in A. (C) Cell cycle chromatin association of Ctf4 in the presence of CPT. Ctf4-Myc cells were synchronized in G1 with α -factor and released in a new cell cycle at 25° in the presence of 40 μ M of CPT. Samples were collected, prepared, and analyzed as in A. (D) The level of Ctf4 is reduced in *hht1-K56R* cells. *ctf4-myc* and *ctf4-myc hht1 Δ -hhf1 Δ hht2 Δ -hhf2 Δ* +phht1-K56R-HHF1 cells were synchronized in G1 with α -factor and released in a fresh medium at 30°. Samples were collected every 10 min and analyzed by Western blot with 9E10 antibody for Ctf4-Myc detection (upper). Anti-Rfa1 antibody was used as a loading control. Cell cycle progression was followed by FACS analysis (right).

these results reinforce the notion that *CTF4* belongs to the H3K56ac pathway.

How could Ctf4 action be regulated by the H3K56ac pathway in the presence of DNA replicative damage? Post-translational modifications of replisome components play a key role in regulating fork progression (Zech and Dalgaard 2014). A recent study has shown that *Schizosaccharomyces pombe* cells elicit a program to degrade replisome upon DNA replication stress through the action of the ubiquitin–proteasome system SCF^{Pof3}, a homolog of the budding yeast SCF^{Dia2} ubiquitin–proteasome system (Roseaulin *et al.* 2013a,b). Because Ctf4 physically interacts with Dia2, which drives CMG helicase and replisome disassembly at the end of DNA replication (Maric *et al.* 2014; Moreno *et al.* 2014) and is ubiquitylated by SCF^{Dia2} (Ho *et al.* 2002; Collins *et al.* 2007; Mimura *et al.* 2009; Morohashi

et al. 2009), one possibility could be that Ctf4 is targeted by Dia2 in response to replicative stress in a way dependent on H3K56ac. However, because the *ctf4- Δ NT* mutation that preserves the Ctf4 interaction with Dia2 (Mimura *et al.* 2009; Morohashi *et al.* 2009) does not restore the viability of *asf1 Δ rrm3 Δ* cells and is lethal in the *rrm3 Δ* mutant (in contrast to the *ctf4-NT* mutation that loses the Ctf4–Dia2 interaction), we exclude the possibility that Ctf4 action could be regulated during replicative stress by H3K56ac through an action mediated by the SCF^{Dia2}.

Mms22 is recruited to chromatin at stalled replication forks (Dovey *et al.* 2009; Ben-Aroya *et al.* 2010; Vaisica *et al.* 2011). An attractive hypothesis would be that Ctf4 is a substrate of the Rtt101–Mms1–Mms22 complex and is further degraded at stalled replication forks under replicative stress. However, we failed to see any decrease in Ctf4 level in the

A

Figure 8 Replicative stress induced by the absence of *RRM3* increases Mms22 association with the replisome. (A) Pull-down protein extracts were loaded on Bis-Tris acrylamide gels in MOPS buffer and stained as a single band before trypsin digestion followed by mass spectrometry analysis. (Left) Mass spectroscopy data obtained after immunoprecipitation of Ctf4-GFP during S phase for Mms22, the catalytic subunit of the DNA polymerase- α (Cdc17), and MCM helicase. Spectral counts show the total number of identified peptide sequences for the indicated protein in each sample (*RRM3* CTF4-GFP, *rrm3Δ* CTF4-GFP, and control CTF4). (Right) Relative quantitation of Mms22 protein compared to Ctf4 protein measured by the ratio of the sum of the areas of the three more intense precursor ions used for each protein identification. The averages of several independent experiments are shown. (B) The level of Mms22 is decreased in *rrm3Δ* cells. Protein extracts from *mms22Δ* + *pG16adh-TAP-MMS22* and *mms22Δ rrm3Δ* + *pG16adh-TAP-MMS22* strains were prepared from S-phase-synchronized cells and analyzed by Western blot with a protein-A antibody (right). Total proteins on the membrane were stained with Ponceau S as a loading control (left).

B

presence of replicative damage (Figure 7, A and B; Figure 8A). Although we cannot exclude that Ctf4 degradation could occur locally and transiently, we presume that Ctf4 is not degraded. We infer that, if the Rtt101-Mms1-Mms22 complex ubiquitylates Ctf4, this ubiquitylation mediates a certain function of Ctf4, rather than Ctf4 degradation. Another possibility could be that Ctf4 is released from forks at damaged chromatin. However, we also failed to observe a significant difference in the amount of Ctf4 associated with bulk chromatin in the absence or presence of replicative damages, suggesting that the H3K56ac pathway did not affect Ctf4 association in chromatin globally. Thus, putative ubiquitylation of Ctf4 would neither affect Ctf4 stability nor Ctf4 chromatin association but would regulate Ctf4 action at the replisome. Interestingly, we found that a H3K56R mutant known to contain intrinsic DNA damage (Hyland *et al.* 2005; Masumoto *et al.* 2005; Pan *et al.* 2006; Recht *et al.* 2006; Celic *et al.* 2008; Wurtele *et al.* 2011) exhibited a lower level of Ctf4, suggesting that cells lacking H3K56ac increased their fitness by reducing the amount of Ctf4 and thereby the Ctf4 action.

Upon DNA replication stress, H3K56ac is maintained at high levels behind the replication fork in a checkpoint-dependent

manner (Masumoto *et al.* 2005; Maas *et al.* 2006). The accumulation of H3K56ac at forks is thought to increase DNA accessibility (Masumoto *et al.* 2005; Driscoll *et al.* 2007; Yang *et al.* 2008) and to create a unique chromatin environment allowing cells to deal with replicative stress. Cells with constitutively high levels of H3K56ac are extremely sensitive to subtle perturbations in DNA replication, and deletion of CTF4 partially suppresses some of their phenotypes (Celic *et al.* 2008). We found that *rrm3Δ* cells, known to accumulate DNA replication stress at specific regions throughout the genome, were viable in this context, confirming that the chromatin environment induced by acetylation of H3K56 behind replication forks is crucial. Because the viability of *ctf4-ΔNT rrm3Δ hst3Δ hst4Δ* cells experiencing constitutively high levels of H3K56ac is compromised, we assume that an interaction between Mms22 and Ctf4 promotes H3K56ac-, Rtt101-Mms1-Mms22-, and Ctf4-dependent molecular events required to rescue replication fork damages during replicative stress. This hypothesis is clearly reinforced by our findings showing that *RRM3* deletion leads to an increased enrichment of Mms22. In good agreement with the fact that Mms22 is recruited to chromatin in a DNA damage-, *RTT109*-, and *RTT101*-dependent manner (Dovey *et al.*

Figure 9 The replicative function of *MRC1* is deleterious in *asf1Δ* cells experiencing replicative damages. (A) Effects of *mrc1Δ* on viability of the *asf1Δ* cells. Tenfold serial dilutions of exponentially growing cells were spotted onto YPD plates incubated at 30° or 38°, onto 5-μM CPT, and 0.01%-MMS plates incubated at 30° for 3 days. (B) Effects associated with the replicative and checkpoint functions of *MRC1* on viability of the *asf1Δ* cells. Tenfold serial dilutions of exponentially growing cells were spotted onto YPD, 5-μM CPT, and 0.005%-MMS plates and incubated at 30° for 3 days.

2009; Ben-Aroya *et al.* 2010; Vaisica *et al.* 2011) and, in the light of recent work showing that the *Rtt101–Mms1–Mms22* complex binds H3K56ac-H4 preferentially over unmodified H3-H4 (Han *et al.* 2013), we foresee that H3K56ac signals DNA damage and directly recruits the *Rtt101–Mms1–Mms22* complex behind the fork to ubiquitylate H3K56 acetylated histones, *Ctf4*, and various substrates leading to an increased DNA accessibility at damage sites and improved DNA repair. Alternatively, *Ctf4* could also recruit the *Rtt101–Mms1* at the DNA damage fork through its interaction with *Mms22*, and its function at the replisome could be subsequently regulated by the complex. Consistent with the deleterious effect of *CTF4* in *rrm3Δ* cells lacking H3K56ac, the coupling function of *Ctf4*, and its ability to act as a platform for multivalent interactions (Simon *et al.* 2014), we propose that upon DNA replication stress *Ctf4* acts downstream of H3K56ac and the *Rtt101–Mms1–Mms22* complex to modulate the integrity of the replisome by affecting both lagging and leading strands at the forks through an interaction with *Mms22* (Figure 10).

DDB1, which binds CUL4A in humans to form the CUL4A^{DDB1} E3 ubiquitin ligase, shares sequence homology with *Mms1* (Lee and Zhou 2007; Jackson and Xiong 2009; Havens and Walter 2011). The CUL4A^{DDB1} complex ubiquitylates various proteins in response to DNA damage (Nouspikel 2011), regulates both the replication-coupled and replication-independent nucleosome assembly (Han *et al.* 2013), and is

critical for new histone H3.3 accumulation at sites of DNA damage (Adam *et al.* 2013). H3K56ac is conserved in humans and seems to also be regulated in a DNA damage-dependent way (Das *et al.* 2009; Tjeertes *et al.* 2009; Yuan *et al.* 2009). Increased levels of H3K56ac have been observed in cancer cells in a manner that is proportional to tumor grade (Das *et al.* 2009). In mammals, H3K56 is acetylated by Gcn5 (Tjeertes *et al.* 2009; Burgess and Zhang 2010). Interestingly, the *Ctf4* human homolog And-1 interacts with H3 and Gcn5, impairs the interaction between Gcn5 and an E3 ligase complex, and modulates the H3K56ac level (Li *et al.* 2011, 2012). Similarly to *Ctf4*, And-1 also plays an important role in bridging between CMG helicase and DNA polymerases, suggesting that And-1 coordinates DNA unwinding and polymerase activities in humans (Zhu *et al.* 2007; Aze *et al.* 2013; Kang *et al.* 2013). Our results raise the possibility that the H3K56ac pathway is conserved between yeast and humans and that And-1 regulates the human replisome in an H3K56ac-dependent manner through the action of the CUL4A^{DDB1} complex, which is thought to increase DNA accessibility at damage sites (Nouspikel 2011). An attractive possibility that needs to be tested in yeast would be that *Ctf4* directly interacts with histones and/or histone chaperones behind the fork and, by sensing the nature and the level of histone modifications, together with the *Rtt101–Mms1–Mms22* complex, regulates replisome integrity and function in response to replicative stress.

Figure 10 H3K56 acetylation prevents genomic instability by affecting Ctf4 function. During S phase, in an unperturbed cell cycle (left), Asf1 functions cooperatively with Rtt109 to acetylate new histone H3 at lysine 56 (solid circles). The Rtt101-Mms1-Mms22 complex binds and ubiquitylates new H3K56ac histones (shaded circles) and promotes an efficient progression of the replication fork and nucleosome assembly by favoring H3-H4 transfer from Asf1 to other histone chaperones. At the end of S-phase, Hst3 and Hst4 deacetylases remove H3K56ac. How ubiquitylation is removed is still unknown. In response to DNA-damaging agents and in *rrm3Δ* cells (right), replication fork progression is affected, leading to checkpoint activation and subsequently to transcriptional repression of *HST3* and *HST4* and degradation of Hst3 and Hst4, allowing H3K56ac to persist. The unique chromatin environment created by H3K56ac accumulation behind the fork triggers a crucial interaction between Ctf4 and the Rtt101-Mms1-Mms22 complex through the N-terminal domain of Ctf4. This interaction modulates replisome structure by uncoupling MCM helicase and DNA polymerases and increases genome accessibility at replication defective forks, thus preserving genome integrity. It is currently unclear whether the interaction between Ctf4 and the Rtt101-Mms1-Mms22 complex is required to ubiquitylate Ctf4 itself and modulate its function

or to recruit the Rtt101-Mms1-Mms22 complex to the site of DNA damage to ubiquitylate other replisome components. The function of the H3K56ac pathway is totally abolished in the absence of the N-terminal domain of Ctf4 required for its interaction with Mms22, positioning Ctf4 in the H3K56ac pathway.

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Supporting Information

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Replisome Function During Replicative Stress Is Modulated by Histone H3 Lysine 56 Acetylation Through Ctf4

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Figure S1 The HIR complex is not required for the viability in absence of *RRM3*. Tetrad analysis of the meiotic progeny of the *hir2Δ/HIR2 rrm3Δ/RRM3 asf1Δ/ASF1* diploid strain. Four representative tetrads are shown after 5 days at 30° on YPD plate. Square indicates the *hir2Δ rrm3Δ* double mutant. Circle indicates the *hir2Δ asf1Δ* double mutant. Assuming 2:2 segregation of the marker allows one to identify *asf1Δ rrm3Δ* double mutants (indicated by dashed circles) and *hir2Δ rrm3Δ asf1Δ* triple mutants (indicated by dashed squares).

Figure S2 The Rtt109 histone acetyl transferase is required for the viability in absence of *RRM3*. Tetrad analysis of the meiotic progeny of the *rtt109Δ/RTT109 rrm3Δ/RRM3* diploid strain was dissected and subsequently incubated for 5 days at 30° on YPD plate. Squares indicate *rrm3Δ* mutants. Circles indicate *rtt109Δ* mutants. Dashed circles indicate *rtt109Δ rrm3Δ* mutants.

Figure S3 *RRM3* is required for cell viability in different genetic contexts. (A) *RRM3* deletion strongly affects *rtt101Δ* mutant viability. Tetrads from diploids heterozygous for *rrm3Δ* and for *rtt101Δ* were dissected and analyzed after 3 days at 30°. Squares indicate *rrm3Δ* mutants. Circles indicate *rtt101Δ* mutants. Dashed circles indicate *rrm3Δ rtt101Δ* mutants. (B) *RRM3* deletion strongly affects *mms1Δ* mutant viability. Tetrads from diploids heterozygous for *rrm3Δ* and for *mms1Δ* were dissected and analyzed as in A. Squares indicate *rrm3Δ* mutants. Circles indicate *mms1Δ* mutants. Dashed circles indicate *mms1Δ rrm3Δ* mutants. (C) *RRM3* deletion strongly affects *mms22Δ* mutant viability. Tetrads from diploids heterozygous for *rrm3Δ* and for *mms22Δ* were dissected and analyzed as in A. Squares indicate *rrm3Δ* mutants. Circles indicate *mms22Δ* mutants. Dashed circles indicate *mms22Δ rrm3Δ* mutants. (D) *RRM3* deletion strongly affects *rtt107Δ* mutant viability. Tetrads from diploids heterozygous for *rrm3Δ* and for *rtt107Δ* were dissected and analyzed as in A. Squares indicate *rrm3Δ* mutants. Circles indicate *rtt107Δ* mutants. Dashed circles indicate *rtt107Δ rrm3Δ* mutants. (E) *RRM3* deletion affects *ctf4Δ* mutant viability. Tetrads from diploids heterozygous for *rrm3Δ* and for *ctf4Δ* were dissected and analyzed as in A. Squares indicate *rrm3Δ* mutants. Circles indicate *ctf4Δ* mutants. Dashed circles indicate *ctf4Δ rrm3Δ* mutants.

hht1Δ-hhf1Δ/HHT1-HHF1 hht2Δ-hhf2Δ/HHT2-HHF2 + phht1-K56R-HHF1
ctf4Δ/CTF4 rrm3Δ/RRM3

○ *ctf4Δ rrm3Δ hht1Δ-hhf1Δ hht2Δ-hhf2Δ*
+ *phht1-K56R-HHF1*

■ *rrm3Δ hht1Δ-hhf1Δ hht2Δ-hhf2Δ*
+ *phht1-K56R-HHF1*

Figure S4 *CTF4* is deleterious in *hht1-K56R rrm3Δ* cells. Tetrads from diploids for *hht1Δ-hhf1Δ/HHT1-HHF1 hht2Δ-hhf2Δ/HHT2-HHF2 rrm3Δ/RRM3* expressing *hht1-K56R* and *HHF1* from a centromeric plasmid were dissected and analyzed. Dashed circle indicates *rrm3Δ* spore expressing *H3K56R* as sole source of histone H3. Circle indicates *ctf4Δ rrm3Δ* spore expressing *hht1-K56R* as sole source of histone H3. Three representative tetrads are shown after 3 days at 30°.

Figure S5 H3K56 acetylation and *RTT107* function in separate pathways upon DNA replication stress. Tetrad analysis of the meiotic progeny of the *rtt107Δ/RTT107 rrm3Δ/RRM3 ctf4Δ/CTF4* diploid strain. *rtt107Δ rrm3Δ* growth is not rescued by *CTF4* deletion. Circles indicate *rtt107Δ ctf4Δ rrm3Δ* mutants. Dashed circles indicate *rtt107Δ rrm3Δ* mutants. Four tetrads are shown after 3 days at 30°.

Figure S6 The N-terminal part of *CTF4* is required for the viability of *rrm3Δ* cells in presence of permanent H3K56 acetylation. Tetrads from the diploid strain *ctf4-ΔNT/CTF4 rrm3Δ/RRM3 hst3Δ/HST3 hst4Δ/HST4* were dissected and analyzed for the presence of auxotrophic markers. Diamond indicates *rrm3Δ ctf4-ΔNT* mutant. Dashed circle indicates *hst3Δ hst4Δ rrm3Δ ctf4-ΔNT* mutant. The presence of *hst3Δ hst4Δ rrm3Δ* and *hst3Δ hst4Δ ctf4-ΔNT* mutants is indicated by circles and square, respectively.

MS analysis of IPs from S cell extracts

Identified Proteins	Spectral counts		
	<i>RRM3</i> (<i>CTF4-GFP</i>)	<i>rrm3Δ</i> (<i>CTF4-GFP</i>)	<i>Control</i> (<i>CTF4</i>)
Ctf4	283	299	0
Mms22	29	39	0
Cdc17	36	35	0
Pol12	7	0	0
Pol2	9	6	0
Dpb2	4	5	0
Mcm2	8	8	0
Mcm3	6	2	0
Mcm4	13	9	0
Mcm5	8	5	0
Mcm6	12	7	0
Mcm7	6	0	0
Cdc45	7	0	0
PCNA	12	4	0
Rfc2	6	6	0
Rfc3	10	10	0
Rfc4	2	0	0
Pri1	5	0	0
Pri2	6	0	0
Rfa1	40	33	0
Rfa2	7	4	0

Figure S7 Summary of DNA replication proteins identified by mass spectrometry. The name of the replication proteins identified after immunoprecipitation of Ctf4-GFP during S-phase as well as the number of peptides identified for each protein are listed.

Figure S8 The replication function of *MRC1* is harmful in *mms1 Δ* cells upon DNA replication stress. Effects associated with the replicative and checkpoint functions of *MRC1* on viability of *mms1 Δ* cells. Tenfold serial dilutions of exponentially growing cells were spotted onto YPD, 5 μ M CPT, and 0.01% MMS plates and incubated at 30° for 3 days.

TABLE S1 Strains used in this study (All based on W303-1a)

CMY1562	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1 rad53-ALRR::URA3::rad53Δ::HIS3</i> (Jiao <i>et al.</i> 2012)
HMY140	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1 hht1-hhf1Δ::LEU2 hht2-hhf2Δ::kanMX3 trp1::HHT1 K56R-HHF1::TRP1</i> (Masumoto <i>et al.</i> 2005)
M354	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1 cdc17-1</i> (Pappas <i>et al.</i> 2004)
PP397	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 RAD5 trp1-1 ura3-1 mrc1::HIS5::mrc1-AQ::LEU2</i> (Tourriere <i>et al.</i> 2005)
Y2544	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-11 mrc1-C14-myc13::kanMX</i> (Naylor <i>et al.</i> 2009)
YAG172-1	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1 ctf4ΔNT-9MYC::TRP1; kanMX</i> (Gambus <i>et al.</i> 2009)
YAG190-1	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1 9MYC-ctf4NT::TRP1; kanMX</i> (Gambus <i>et al.</i> 2009)
YL1103	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1 CTF4-9MYC::HIS3</i> (Wang <i>et al.</i> 2010)
YL1104	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 CTF4-GFP:: kanMX</i> (Wang <i>et al.</i> 2010)
YVC200	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1 YFP-SML1 rrm3Δ::natMX</i>
YVC201	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1 YFP-SML1 rrm3Δ::natMX</i>
YVC202	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1 YFP-SML1 asf1Δ::kanMX6</i>
YVC203	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 YFP-SML1/YFP-SML1 rrm3Δ::natMX/RRM3 asf1Δ::kanMX6/ASF1</i>
YVC204	<i>CMY1562 X YVC201 rad53-ALRR::URA3::rad53Δ::HIS3/RAD53 rrm3Δ::natMX/RRM3</i>
YVC205	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 hir2Δ::TRP1/HIR2 rrm3Δ::natMX/RRM3</i>
YVC206	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 rtt109Δ::LEU2/RTT109 rrm3Δ::natMX/RRM3</i>

YVC207	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 hht1-hhf1Δ::LEU2/HHT1-HHF1 hht2-hhf2Δ::kanMX3/HHT2-HHF2 trp1::HHT1 K56R-HHF1::TRP1 rrm3Δ::natMX/RRM3</i>
YVC208	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 hst3Δ::HIS5/HST3 hst4Δ::natMX/HST4 sir2Δ::TRP1/SIR2 rrm3Δ::natMX/RRM3</i>
YVC209	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 cac1Δ::hphMX/CAC1 rtt106Δ::LEU2/RTT106 rrm3Δ::natMX/RRM3</i>
YVC210	Spore wild-type arising from the dissection of YVC209
YVC211	Spore <i>asf1Δ::kanMX6</i> arising from the dissection of YVC209
YVC212	Spore <i>rrm3Δ::natMX</i> arising from the dissection of YVC209
YVC213	Spore <i>cac1Δ::hphMX</i> arising from the dissection of YVC209
YVC214	Spore <i>rtt106Δ::LEU2</i> arising from the dissection of YVC209
YVC215	Spore <i>asf1Δ::kanMX6 cac1Δ::hphMX</i> arising from the dissection of YVC209
YVC216	Spore <i>asf1Δ::kanMX6 rtt106Δ::LEU2</i> arising from the dissection of YVC209
YVC217	Spore <i>rrm3Δ::natMX cac1Δ::hphMX</i> arising from the dissection of YVC209
YVC218	Spore <i>rrm3Δ::natMX rtt106Δ::LEU2</i> arising from the dissection of YVC209
YVC219	Spore <i>cac1Δ::hphMX rtt106Δ::LEU2</i> arising from the dissection of YVC209
YVC220	Spore <i>rrm3Δ::natMX cac1Δ::hphMX rtt106Δ::LEU2</i> arising from the dissection of YVC209
YVC221	Spore <i>asf1Δ::kanMX6 cac1Δ::hphMX rtt106Δ::LEU2</i> arising from the dissection of YVC209
YVC222	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 rtt101Δ::URA3/RTT101 rrm3Δ::natMX/RRM3</i>
YVC223	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 mms1Δ::TRP1/MMS1 rrm3Δ::natMX/RRM3</i>
YVC224	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 mms22Δ::TRP1/MMS22 rrm3Δ::natMX/RRM3</i>
YVC225	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 rtt107Δ::LEU2/RTT107 rrm3Δ::natMX/RRM3</i>

YVC226	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 ctg4Δ::TRP1/CTF4 rrm3Δ::natMX/RRM3</i>
YVC227	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 asf1Δ::kanMX6/ASF1 ctg4Δ::TRP1/CTF4 rrm3Δ::natMX/RRM3</i>
YVC228	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 rtt109Δ::TRP1/RTT109 ctg4Δ::TRP1/CTF4 rrm3Δ::natMX/RRM3</i>
YVC229	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 rtt101Δ::URA3/RTT101 ctg4Δ::TRP1/CTF4 rrm3Δ::natMX/RRM3</i>
YVC230	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 mms1Δ::TRP1/MMS1 ctg4Δ::TRP1/CTF4 rrm3Δ::natMX/RRM3</i>
YVC231	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 mms22Δ::TRP1/MMS22 ctg4Δ::TRP1/CTF4 rrm3Δ::natMX/RRM3</i>
YVC232	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 rtt107Δ::LEU2/RTT107 ctg4Δ::TRP1/CTF4 rrm3Δ::natMX/RRM3</i>
YVC233	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 ctg4Δ::TRP1/CTF4 hht1-hhf1Δ::LEU2/HHT1-HHF1 hht2-hhf2Δ::kanMX3/HHT2-HHF2 trp1::HHT1 K56R-HHF1::TRP1 rrm3Δ::natMX/RRM3</i>
YVC234	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 ctg4ΔNT-9MYC::TRP1; kanMX/CTF4 asf1Δ::kanMX6/ASF1 rrm3Δ::natMX/RRM3</i>
YVC235	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 9MYC-ctg4NT::TRP1; kanMX/CTF4 asf1Δ::kanMX6/ASF1 rrm3Δ::natMX/RRM3</i>
YVC236	MATa <i>ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1 cdc17-1 asf1Δ::kanMX6</i>
YVC237	MAT α <i>ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1 cdc17-1 rrm3Δ::natMX</i>
YVC238	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 cdc17-1/cdc17-1 rrm3Δ::natMX/RRM3 asf1Δ::kanMX6/ASF1</i>

YVC239	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 hst3Δ::HIS5/HST3 hst4Δ::natMX/HST4 ctf4ΔNT-9MYC::TRP1; kanMX/CTF4 rrm3Δ::natMX/RRM3</i>
YVC240	MATa <i>ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1 CTF4-9MYC::HIS3 rrm3Δ::natMX</i>
YVC241	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 mrc1Δ::TRP1/MRC1 rrm3Δ::natMX/RRM3 asf1Δ::kanMX6/ASF1</i>
YVC261	MATa <i>ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1 hht1-hhf1Δ::LEU2 hht2-hhf2Δ::kanMX3 trp1::HHT1 K56R-HHF1::TRP1 CTF4-9MYC::HIS3</i>
YVC242	Spore <i>mrc1Δ::TRP1</i> arising from the dissection of YVC241
YVC243	Spore <i>mrc1Δ::TRP1 asf1Δ::kanMX6</i> arising from the dissection of YVC241
YVC244	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 RAD5/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 mrc1::HIS5::mrc1-AQ::LEU2/MRC1 asf1Δ::kanMX6/ASF1 rrm3Δ::natMX/RRM3</i>
YVC245	Spore <i>mrc1::HIS5::mrc1-AQ::LEU2</i> arising from the dissection of YVC244
YVC246	Spore <i>mrc1::HIS5::mrc1-AQ::LEU2 asf1Δ::kanMX6</i> arising from the dissection of YVC244
YVC247	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 mrc1-C14-myc13::kanMX/MRC1 csm3Δ::URA3/CSM3 asf1Δ::kanMX6/ASF1 rrm3Δ::natMX/RRM3</i>
YVC248	Spore <i>mrc1-C14-myc13::kanMX</i> arising from the dissection of YVC247
YVC249	Spore <i>mrc1-C14-myc13::kanMX asf1Δ::kanMX6</i> arising from the dissection of YVC247
YVC250	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 mrc1Δ::TRP1/MRC1 mms1Δ::TRP1/MMS1</i>
YVC251	Spore <i>mms1Δ::TRP1</i> arising from the dissection of YVC250
YVC252	Spore <i>mrc1Δ::TRP1 mms1Δ::TRP1</i> arising from the dissection of YVC250
YVC253	MATa/MAT α <i>ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 RAD5/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 mrc1::HIS5::mrc1-AQ::LEU2/MRC1 mms1Δ::TRP1/MMS1</i>

YVC254	Spore <i>mrc1::HIS5::mrc1-AQ::LEU2 mms1Δ::TRP1/MMS1</i> arising from the dissection of YVC253 YVC255 <i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 mrc1-C14-MYC13::kanMX/MRC1 mms1Δ::TRP1/MMS1</i>
YVC255	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 rad5-535/rad5-535 trp1-1/trp1-1 ura3-1/ura3-1 mrc1-C14-MYC13::kanMX/MRC1 mms1Δ::TRP1/MMS1</i>
YVC256	Spore <i>mrc1-C14-MYC13::kanMX mms1Δ::TRP1</i> arising from the dissection of YVC255
YVC261	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 ura3-1 mms22Δ::TRP1 + pGR0383</i>
YVC262	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 ura3-1 CTF4-GFP::kanMX mms22Δ::TRP1 + pGR0383</i>
YVC263	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 ura3-1 CTF4-GFP::kanMX rrm3Δ::natMX mms22Δ::TRP1 + pGR0383</i>
YVC270	Spore wild-type arising from the dissection of YVC227
YVC271	Spore <i>ctf4Δ::TRP1</i> arising from the dissection of YVC227
YVC272	Spore <i>asf1Δ::kanMX6</i> arising from the dissection of YVC227
YVC273	Spore <i>ctf4Δ::TRP1 asf1Δ::kanMX6</i> arising from the dissection of YVC227
YVC274	Spore <i>rtt109Δ::TRP1</i> arising from the dissection of YVC228
YVC275	Spore <i>rtt109Δ::TRP1 ctf4Δ::TRP1</i> arising from the dissection of YVC228
YVC276	Spore <i>rtt101Δ::URA3</i> arising from the dissection of YVC229
YVC277	Spore <i>rtt101Δ::URA3 ctf4Δ::TRP1</i> arising from the dissection of YVC229
YVC278	Spore <i>mms1Δ::TRP1</i> arising from the dissection of YVC230
YVC279	Spore <i>mms1Δ::TRP1 ctf4Δ::TRP1</i> arising from the dissection of YVC230
YVC280	Spore <i>mms22Δ::TRP1</i> arising from the dissection of YVC231
YVC281	Spore <i>mms22Δ::TRP1 ctf4Δ::TRP1</i> arising from the dissection of YVC231
YVC285	Spore wild-type arising from the dissection of YVC234
YVC286	Spore <i>ctf4Δ::TRP1</i> arising from the dissection of YVC227
YVC287	Spore <i>asf1Δ::kanMX6</i> arising from the dissection of YVC234
YVC288	Spore <i>ctf4Δ::TRP1 asf1Δ::kanMX6</i> arising from the dissection of YVC227
YVC289	Spore <i>9MYC-ctf4NT::TRP1; kanMX</i> arising from the dissection of YVC235
YVC290	Spore <i>9MYC-ctf4NT::TRP1; kanMX asf1Δ::kanMX6</i> arising from the dissection of YVC235

YVC291 Spore *ctf4*Δ*NT-9MYC::TRP1*; *kanM asf1*Δ::*kanMX6* arising from the dissection of YVC234

YVC292 Spore *ctf4*Δ*NT-9MYC::TRP1*; *kanMX asf1*Δ::*kanMX6* arising from the dissection of YVC234

Plasmids used in this study

pRS314-*asf1-V94R* (Carl Mann)

pRS314-*asf1-D37R E39R* (Carl Mann)

pGR0383 pG16adh-PATEV-MMS22 (Zaidi *et al.* 2008)