Rtt109 Prevents Hyper-Amplification of Ribosomal RNA Genes through Histone Modification in Budding Yeast

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Abstract

The genes encoding ribosomal RNA are the most abundant in the eukaryotic genome. They reside in tandem repetitive clusters, in some cases totaling hundreds of copies. Due to their repetitive structure, ribosomal RNA genes (rDNA) are easily lost by recombination events within the repeated cluster. We previously identified a unique gene amplification system driven by unequal sister-chromatid recombination during DNA replication. The system compensates for such copy number losses, thus maintaining proper copy number. Here, through a genome-wide screen for genes regulating rDNA copy number, we found that the rtt109 mutant exhibited a hyper-amplification phenotype (~3 times greater than the wild-type level). RTT109 encodes an acetyl transferase that acetylates lysine 56 of histone H3 and which functions in replication-coupled nucleosome assembly. Relative to unequal sister-chromatid recombination-based amplification (~1 copy/cell division), the rate of the hyper-amplification in the rtt109 mutant was extremely high (>100 copies/cell division). Cohesin dissociation that promotes unequal sister-chromatid recombination was not observed in this mutant. During hyper-amplification, production level of extra-chromosomal rDNA circles (ERC) by intra-chromosomal recombination in the rDNA was reduced. Interestingly, during amplification, a plasmid containing an rDNA unit integrated into the rDNA as a tandem array. These results support the idea that tandem DNA arrays are produced and incorporated through rolling-circle-type replication. We propose that, in the rtt109 mutant, rDNA hyper-amplification is caused by uncontrolled rolling-circle-type replication.

Introduction

The ribosome is an abundant macromolecular protein-RNA complex that translates mRNA into protein. In Saccharomyces cerevisiae, ribosomal proteins (RP) account for approximately 50% of total protein and ribosomal RNA (rRNA) represents approximately 80% of total RNA [1]. While the amount of RP increases during translation from mRNA, the amount of rRNA is dependent on transcription level. To meet this huge biosynthetic demand for rRNA in eukaryotic cells, the rRNA genes are present in hundreds of copies and are transcribed by a highly efficient RNA polymerase, RNA pol I [1]. We recently reported that about half of the rDNA genes are located on chromosome XII (Figure 1A) [3]. One unit of rDNA is 9.1 kb and is composed of a region encoding a pre-35S rRNA, a small 5S rRNA and two intergenic spacer (IGS). The IGS region encodes the key elements involved in the amplification system that maintains rDNA gene copy number [4]. During S phase, progress of the DNA replication fork is blocked at the replication fork barrier sequence (RFB) through the action of the fork-blocking protein, Fob1 [5]. This inhibition induces DNA double-strand breaks (DSBs) and subsequent repair by unequal sister-chromatid recombination which increases rDNA copy number through re-replication of neighbor copies [6]. Moreover, this amplification is promoted by RNA pol II dependent transcription from E-pro, a non-coding bi-directional promoter near the RFB sequence [7]. Transcription from E-pro is proposed to clear cohesin proteins from nearby, which in turn may promote unequal sister-chromatid recombination, because cohesin proteins suppress unequal sister-chromatid recombination [7]. In a wild type strain with ~150 rDNA copies, E-pro transcription is repressed by a histone deacetylase, Sir2. Once rDNA copy number is reduced, Sir2 repression is relieved and amplification by unequal sister-chromatid recombination is enhanced. This amplification gradually increases copy number at a rate of ~ one per cell division until a maximum of about 150 copies is reached [3].

To elucidate the mechanism by which rDNA copy number is regulated, we sought and found mutants in which copy number increased abnormally by screening a genome-wide deletion library consisting of ~4,800 mutants (OpenBiosystems). Among these mutants, the rtt109A strain exhibited a prominent phenotype in...
Author Summary

Gene amplification is one of the major strategies used by cells to increase the abundance of gene products. We have been studying amplification of the ribosomal RNA genes cluster, also known as rDNA (ribosomal DNA), in yeast and found that unequal sister-chromatid recombination increased copy number following accidental deleterious recombination events among the repeats. This amplification is highly regulated and ceases when the copy number reaches ~150. We isolated mutants, including rtt109, which have abnormally high copy numbers of rDNA. RTT109 encodes an acetyl transferase that affects chromatin structure. In the mutant, rolling circle-type amplification that is observed in the early developmental stage in amphibians, oogenesis occurred. We speculate that RTT109 plays a key role in regulating the mode of rDNA amplification. Variation in gene copy number (amplification) has been widely observed in a variety of organisms, contributing to both beneficial adaptation and pathology (e.g., cancer). Our results shed new light on molecular mechanisms of gene amplification.

which rDNA copy number is ~400 in contrast to ~150 in the wild type. RTT109 encodes an acetyltransferase that is stimulated by the histone chaperone Asf1 [8-11] to acylate histone H3 on residue K56. The acetylated histones are incorporated into newly-synthesized DNA to promote replication-coupled nucleosome assembly by chromatin assembly factor 1 (CAF-1) and histone chaperone Rtt106 [12]. It is known that the defect in acetylation affects DNA damage sensitivity, stability of the replication fork, and recombination between sister-chromatids [8,13-16]. Recently, Housley and Tollervey also independently found this phenotype in the asf1 and rtt109 mutants [17].

We analyzed the relationship between histone acetylation and rDNA expansion in depth. We found that mutation of K56 of histone H3 also induced “hyper-amplification” of rDNA. Moreover, the histone deacetylase double mutant, hist3 hist4, had an increased rDNA copy number as well. Interestingly, the rate of the hyper-amplification in the hist3 hist4 mutant was much faster (~100 copies per cell division), than the ~1 copy per cell division in wild-type. During hyper-amplification, extra chromosomal rDNA circles (ERC) generated from the rDNA by Fob1-dependent recombination did not accumulate. Instead, integration of plasmids with rDNA sequences was detected. They replicated sequentially and repeatedly during the integration step. These observations suggest that intra-chromosomal recombinational repair of Fob1-driven DSB that produces ERC does not occur. Instead, the data suggest that break-induced replication (BIR) takes place between the DSB end and ERC, resulting in rolling circle replication and hyper-amplification. We conclude that Rtt109 prevents such unusual replication and facilitates completion of recombinational repair with a sister-chromatid after a DNA double-stranded break occurs at the blocking site.

Results

Identification of mutants with high rDNA copy number
To elucidate the mechanism which regulates rDNA copy number, we measured the size of rDNA in the ~4,800 mutants that comprise the yeast deletion library (S288C genetic background) by pulsed-field gel electrophoresis (CHEF, Figure S1). We identified eight mutants with higher-than-usual copy numbers (~150), and presumed that they were defective in copy number regulation (Table 1). The rtt109 mutant was among those with the highest rDNA copy number. Chromosome XII of the rtt109 mutant is bigger than the biggest size maker (3.13 Mb) and sharper than other chromosome XIs (lane 6 in Figure S1). This indicates that the chromosome XII is too big to be analyzed under these electrophoretic conditions and failed to migrate.

To confirm the “hyper-copy” rDNA phenotype in the rtt109 mutant, we deleted RTT109 in a different genetic background (W303) and tested the size of chromosome XII in this mutant by CHEF. As shown in Figure 2A, chromosome XII in the rtt109 strain was much longer than in the wild type strain. Moreover, we measured rDNA copy number in the rtt109 mutant based on signal intensities of Southern blots after BglII digestion (Figure 2B). The copy number was ~2.5 times greater (~400 copies) than in the wild type strain (~150 copies, Figure 2B).

To determine whether hyper-amplification of the rDNA was a direct result of the deletion of RTT109, we transformed the rtt109 mutant with plasmid-encoded RTT109 and monitored the size of chromosome XII by CHEF. Chromosome XII remained longer up to ~24 generations following the transformation (Figure 2C, lane 6–10), but decreased in size to that observed in the wild-type

Figure 1. Structure of rDNA region in budding yeast. rARS and RFB are the replication origin and blocking site, respectively. IGS 1 and 2 are intergenic spacers. E-pro is a bidirectional promoter for noncoding transcription. Arrows indicate the direction of transcription of the 5S and 35S rRNA genes. The fork-blocking protein, Fob1p binds to the replication fork blocking site (RFB) in the IGS1, and inhibits the replication fork passing from the left in the figure. Sir2 represses transcription from E-pro.

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Acetylation and deacetylation of histone H3K56 inhibit hyper-amplification

Rtt109 acetylates the K56 residue of newly-synthesized histone H3 and plays an important role in chromatin remodeling during DNA replication, repair, and recombination [8], [12], [14], [15]. Acetylation of K56 no longer occurs in the rtt109 mutant [9–10]. To determine whether the acetylation activity is related to the hyper-amplification of rDNA, we measured rDNA copy number in the histone H3 mutants, H3K56R (non-acetylated) and H3K56Q (acetylated mimic). As expected, the H3K56R mutant had a longer chromosome XII similar to the rtt109 mutant (Figure 3A, lane 2), indicating that the hyper-amplification of rDNA occurred by inhibition of acetylation of the histone H3K56 residue. However, unexpectedly, the H3K56Q mutant also had a longer chromosome XII (Figure 3A, lane 3), although the rDNA copy number did not reach the level observed in the histone deacetylase mutant. Moreover, in the W303 genetic background, the double H3K56 histone deacetylase (HDAC) mutant, hst3 hst4, in which almost all H3 K56 residues were deacetylated, exhibited the hyper-amplification phenotype as well (Figure 3B) [18]. These results indicate that deacetylation of K56 on histone H3 on the sister chromatin also prevents hyper-amplification and maintains proper copy number.

Table 1. List of mutants with higher-than-usual rDNA copy numbers.

| Gene name | Description from Saccharomyces Genome Database |
|-----------|-----------------------------------------------|
| POP2 | RNase of the DEDD superfamily, subunit of the Ccr4-Not complex that mediates 3' to 5' mRNA deadenylation |
| CCR4 | Component of the CCR4-NOT transcriptional complex, which is involved in regulation of gene expression; component of the major cytoplasmic deacetylase, which is involved in mRNA poly(A) tail shortening |
| IME4 | Probable mRNA N6-adenosine methyltransferase required for entry into meiosis; transcribed in diploid cells; haploids repress IME4 transcription via production of antisense IME4 transcripts; antisense transcription is repressed in diploids |
| RTT109 | Histone acetyltransferase critical for cell survival in the presence of DNA damage during S phase; acetylates H3-K56 and H3-K9; involved in non-homologous end joining and in regulation of Ty1 transposition; interacts physically with Vps75p |
| MMS22 | Subunit of an E3 ubiquitin ligase complex involved in replication repair; stabilizes protein components of the replication fork, such as the fork-pausing complex and leading strand polymerase, preventing fork collapse and promoting efficient recovery during replication stress; required for accurate meiotic chromosome segregation |
| CTF4 | Chromatin-associated protein, required for sister chromatid cohesion; interacts with DNA polymerase alpha (Pol1p) and may link DNA synthesis to sister chromatid cohesion |
| RRN10 | Protein involved in promoting high level transcription of rDNA, subunit of UAF (upstream activation factor) for RNA polymerase I |
| DHH1 | Cytoplasmic DEAD/HH-box helicase, stimulates mRNA decapping, coordinates distinct steps in mRNA function and decay, interacts with both the decapping and deadenylase complexes, may have a role in mRNA export and translation |

We measured the size of rDNA in the ~4,800 mutants from the yeast deletion library (Open Biosystems) by pulsed-field gel electrophoresis. The listed 8 mutants had extremely high rDNA copy numbers. The descriptions are taken from the Saccharomyces Genome Database (http://www.yeastgenome.org/) [46]. doi:10.1371/journal.pgen.1003410.t001

Hyper-amplification is accelerated by Fob1

We have previously shown that rDNA amplification from low copy number to wild type levels is dependent on the replication fork blocking activity of Fob1 and subsequent recombination [3]. To determine whether hyper-amplification starting from wild type copy number levels also requires this blocking activity, we deleted RTT109 in the fob1 mutant. As shown in Figure 4A (right, lanes 9–10), hyper-amplification in the double mutant was not observed. However, after more than 50 generations, some rtt109 fob1 clones had a longer chromosome XII than the single fob1 mutant, although they were still shorter than chromosome XII in the rtt109 single mutant (Figure S2, lanes 15–16). To confirm the contribution of Fob1 to the hyper-amplification phenotype, we monitored amplification rate in the rtt109 fob1 GAL-FOB1 double mutant over a time course after induction of FOB1 transcription. Amplification was observed 6 hrs post-induction (Figure 4B, asterisks). Because the doubling time of the mutant we measured was 3.4 hrs, the hyper-amplification phenotype was detectable within ~2 generations. On the other hand, in cells grown on glucose where FOB1 was not induced, chromosome XII appeared slightly smeared in the gel, but the longer chromosome XII variant was not detected even after 32 hrs (Figure 4B). Taken together, these observations indicate that Fob1 plays an essential role in the hyper-amplification phenotype. We also tested whether hyper-amplification occurred in mutants belonging to the RTT109 epistasis group. A histone chaperone, Asf1, functions with Rtt109 to acylate histone H3K56 [19], [20]. In an asf1 mutant, the rDNA was also hyper-amplified, although the length of chromosome XII was much shorter than in the rtt109 mutant (Figure 4A, lane 4–5). Deletion of the other histone chaperone Vps75/Rtt109 complex that acetylates H3 N-terminal residues did not promote a hyper-amplification phenotype (Figure 4A, lane 6–7) [21–23]. A similar result was reported by Houseley & Tollervey (2011) [17]. In the double fob1 asf1 mutant, amplification was substantially repressed, while in the vps75 mutant, no hyper-amplification was observed (Figure 4A, lane 11–14). This is consistent with a previous report that showed that an Rtt109-Asf1, but not an Rtt109-Vps75 complex, acetylated the histone H3/H4 dimer, which is a key event required for genome stability [9].

The rtt109 mutation does not affect replication fork stability, non-coding transcription, or cohesin association in the intergenic region

Because histone H3K56 acetylation is important for replisome integrity at a stalled replication fork under replication stress [9], [10], we monitored stability of a replication fork arrested at the RFB site in the rtt109 mutant, in which FOB1 is induced by galactose as used in Figure 4B, by two-dimensional gel electrophoresis during hyper-amplification. In both the wild type and rtt109 mutant, the RFB spot on the Y-arc appeared only when
**Figure 2. Hyper-amplification of rDNA is induced by deletion of RTT109.** A) Chromosome XII with rDNA in the wild type and rtt109 mutant; DNA was analyzed by pulsed-field gel electrophoresis (CHEF), and stained with EtBr (left) and hybridized with rDNA (right). Lanes 1 and 2 are the wild type strains and lanes 3–7 are the rtt109 mutant in the W303 genetic background. Chromosome XII indicated with the asterisk is longer after hyper-amplification. B) Quantitation of rDNA copy number in the wild type (lane 1) and rtt109 mutant (lanes 2 and 3) by Southern hybridization. The upper panel shows the rDNA band after BglII digestion. MCM2 is the internal control used for normalization. The lower panel indicates quantification of the signal intensity of each band detected by Southern blotting with rDNA and MCM2 probes, respectively. Values are means of three independent experiments and bars are S.D. values. C, D) Rtt109 restored wild type levels of rDNA copies in the rtt109 mutant. Chromosome XII was analyzed by CHEF analysis followed by Southern hybridization with an rDNA probe in the rtt109 mutant at –24 generations (C) and at –90 generations (D) after introduction of the empty vector (C: lanes 1–5, D: lanes 1–4, 9–12) or plasmid-encoded RTT109 (C: lanes 6–10, D: 5–8, 13–16). Mutants were constructed in the W303 and S288c genetic backgrounds.

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FOB1 was induced (cells grown on galactose). Because the signal intensities were similar (Figure 5A), the fork stability at the RFB appears not to be affected by RTT109 function. In addition, the amount of recombination intermediates was not increased in the rtt109 mutant (Figure 5A).

Non-coding transcription from the IGS region (E-pro) is up-regulated during amplification from low copy number rDNA [7]. The transcription reduces association of the cohesin complex, resulting in unequal sister-chromatid recombination [7]. Therefore, we tested whether transcription increased in the rtt109 mutant. By northern analysis, the RNA increased in the rtt109 mutant (Figure 5B). However, high level E-pro expression was not observed in the rtt109 fob1 double mutant (Figure 5B) in which rDNA copy number is maintained at wild type levels (Figure 4A, lanes 9–10). This suggests that the increased rDNA copy number causes the high level of E-pro transcription rather than deletion of RTT109. To address this possibility, we measured the level of E-pro in the wild type strain with a higher rDNA copy number constructed by introduction of a plasmid-encoded RTT109 into the rtt109 mutant shown in Figure 2C. As the result (Figure S3), the strain with the longer rDNA had a high level of E-pro expression as well as the rtt109 mutant. Therefore, we conclude that Rtt109 is not involved in repression of E-pro transcription, but that the abnormally increased rDNA copy number leads to a failure to repress transcription. Moreover, we examined the effect of deletion of RTT109 on the association of cohesin with the IGS region of rDNA by chromatin immunoprecipitation (ChiP) assay. To exclude the indirect effect of increased rDNA copy number, we compared cohesin (Mcd1) association level between the rtt109 mutant and the wild type in a FOB1 defective background. As shown in Figure 5D, we confirmed cohesin association in the IGS in the mutant. Because the association represses unequal sister-chromatid recombination [7], the hyper-amplification in the rtt109 mutant is most likely not induced by the recombination.

**Rolling circle amplification occurs during hyper-amplification**

It appears to be difficult for break-induced unequal sister-chromatid amplification at the standard level to achieve the high rate of amplification observed in the rtt109 mutant (>100 copies/cell division). As mentioned above, we detected cohesin association that repressed the frequency of unequal sister-chromatid recombination. Rather, rolling circle type amplification appears to occur in such a manner that a large increase in copy number occurs in just a few cell divisions. In fact, the rolling circle structure was observed in the fraction remaining in the agarose gel plug subjected to exclusion.

**Figure 3. Dysfunction of histone H3K56 acetylation and deacetylation induces hyper-amplification of rDNA copy number.** A) Chromosome XII in mutants with amino acid substitutions in histone H3: K56 to R and Q analyzed by CHEF. The left panel shows an EtBr-stained gel and the right panel shows hybridization with an rDNA probe. Lane 1: no substitution (H3K56), lane 2: arginine substitution for lysine (H3K56R), lane 3: glutamine substitution for lysine (H3K56Q). B) Chromosome XII in the hst3 hst4 double mutant analyzed by CHEF. Lane 1 is the wild type strain as control, and lanes 2–5 are four independent clones of the hst3 hst4 double mutant strain grown at 23°C.

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to pulsed-field gel electrophoresis (Figure 6). To initiate rolling circle replication, the broken end at the RFB must recombine with the intra-sister-chromatid (Figure 7B) or with popped-out circular molecules (ERC, extra chromosomal rDNA circle, Figure 7C). In the former case, the recombination usually produces an ERC [4]. Therefore, we monitored ERC formation during hyper-amplification by 2D gel electrophoresis in which the non-linear DNA molecules are separated from genomic DNA. In the wild type control, ERCs were detected in a Fob1-dependent manner as molecules are separated from genomic DNA. In the wild type cation by 2D gel electrophoresis in which the non-linear DNA molecules (ERC, extra chromosomal rDNA circle, Figure 7C). In the former case, the recombination usually produces an ERC [4]. Therefore, we monitored ERC formation during hyper-amplification by 2D gel electrophoresis in which the non-linear DNA molecules are separated from genomic DNA. In the wild type control, ERCs were detected in a Fob1-dependent manner as molecules are separated from genomic DNA. In the wild type cation by 2D gel electrophoresis in which

**Figure 4. Fob1 promotes hyper-amplification in rtt109 mutant.** A) FOB1 dependency of hyper-amplification in rtt109 and related mutants.

\[ \text{RTT109} \rightarrow \text{rtt109} \rightarrow \text{asf1} \rightarrow \text{vps75} \]

B) Time course analysis of hyper-amplification in the rtt109 mutant. Times after galactose-mediated FOB1 induction or glucose-mediated FOB1 repression are indicated. Asterisks show the positions of hyper-amplified chromosome XIII containing rDNA. Details are provided in Materials and Methods.

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![Image](58x537)

The ribosomal RNA gene is the most abundant and unstable gene in cells. Abundance is maintained in spite of the instability through several mechanisms. Gene amplification after deleterious recombination is one. We found that modification of a histone represses rolling circle replication and maintains wild type rDNA copy number.
Study of the relationship between histone modification, as a phenotype of the sir2 mutant, and rDNA stability was initiated by Gottlieb and Esposito (1989) [29]. They found that rDNA recombination was greatly enhanced in a sir2 mutant. Fritze et al. (1997) found that the mutation affected chromatin structure and speculated that it prevented recombination (instability) in the repeat [30]. Later, Sir2 was identified as a histone deacetylase [31]. Subsequently, we found that such alterations of chromatin structure repressed the noncoding promoter (E-pro) whose transcription reduces cohesion association with rDNA, and induces recombination between unequal sister-chromatids, because cohesin proteins hold sister-chromatids together and suppress unequal sister-chromatid recombination [7]. Therefore, one putative mechanism for rDNA maintenance by histone modification is through E-pro regulation. As reported here, however, deletion of RTT109 alone did not activate E-pro expression when copy number was normal (Figure 5B). Instead, as the result of the hyper-amplification, E-pro transcription was

Figure 5. Replication fork stability at RFB, non-coding RNA from IGS and cohesin association in rtt109 mutant. A) 2D gel electrophoresis to detect replication fork blocking activity at the RFB site with or without Fob1 induction. The scheme is shown in the right side of the figure. Ri is recombination intermediates. The wild type (RTT109) and rtt109 mutant with YCplac22-GAL-FOB1 in the fob1 defective background were incubated in the medium with raffinose or galactose for 2D gel electrophoresis. See Materials and Methods for details. B, C) E-pro transcription in the rtt109 and rtt109 fob1 double mutants. Transcripts from E-pro were detected by northern analysis with the probe positioned on the IGS1 region described in (C) [7]. RNA from two clones of each strain were prepared and analyzed. The 18S rRNA is a loading control. D) ChIP assay for cohesin association to the IGS. The wild type (fob1 background) and rtt109 mutant (fob1 background) both of which carry FLAG-tagged MCD1 were used for the ChIP assay. Four regions within rDNA (C1 to C4 shown in C) were analyzed by PCR. (Left panel) The PCR products from serially-diluted (2-fold) samples were separated in a 2.5% agarose gel and stained with ethidium bromide. (Right panel) Quantification of PCR products. Values of immunoprecipitated DNA (IP) were normalized to total DNA (WCE) and C1 (WT). doi:10.1371/journal.pgen.1003410.g005

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elevated in the rtt109 mutant. This may be because the copy number is too high to be silenced transcriptionally. It is known that the shorter rDNA also induces a high level of E-pro and induces rDNA amplification [7]. In this case, the activation can be explained by self-regulation of SIR2 as follows. When rDNA copy number is reduced, a large amount of Sir2 is released from the rDNA and may silence other parts of the genome. In fact, Michell et al. reported that telomere silencing is enhanced in a low rDNA copy strain (2005) [32]. They also showed that SIR2 itself is a target for repression. Therefore, we propose that autoregulated less SIR2 expression induces more E-pro transcription to amplify rDNA in a low rDNA copy strain. As copy number increases, more Sir2 is consumed by interaction with rDNA and repression of SIR2 is reduced. When copy number reaches wild type levels, SIR2 expression is maximal which represses E-pro resulting in a halt to further amplification. Therefore, in the case of hyper-amplified rDNA, Sir2 levels may be too low to interact with all rDNA copies resulting in a net partial loss of E-pro repression.

Hyper-amplification was observed in the mms22 mutant which has been shown to interact genetically with RTT109 (Table 1) [33]. In both rtt109 and mms22 mutants, cells become more sensitive to DNA damage during S phase than in wild type. Both Rtt109 and Mms22 are also involved in regulation of homologous recombination induced by replication inhibition [14]–[16], [34]. Our results show that the hyper amplification is accelerated by replication fork-blocking activity in the RFB. Therefore, one explanation is that the blocked fork induces DNA double stranded breaks and that recombinational repair of the broken sister-chromatid can be modulated by these proteins through histone modification. However, as shown in our 2D gels, no significant abnormality was observed in amount of recombination intermediates in the rtt109 mutant (Figure 5A), e.g., the early steps of recombination (DSB, homologous annealing, strand invasion and formation of Holliday structure). This suggests that Rtt109 and Mms22 function in later steps of the recombination reaction. For example, after formation of the Holliday structure, the donor strand may not separate from the template strand, or, alternatively, replication, such as break-induced replication (BIR) may occur [35]. In case of repetitive sequences such as rDNA, this BIR initiates rolling circle replication which generates an enormous increase in copy number during the cell cycle (Figure 7B).

Figure 6. Images of rolling circle structure by atomic force microscopy. DNA was isolated from the well of the CHEF gel for the rtt109 mutant (12 hr, Figure 4B). By atomic force microscopy, the rolling circle structure with a similar length (8–11 kb) as a unit of rDNA was observed. We observed ~1% DNA fragments with loop in the rtt109 mutant. In contrast, in the wild type, such molecules were detected at a frequency of ~0.1%.

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The pattern of integration of the IGS plasmid into the chromosomal region indicates that rolling circle replication occurs in the *rtt109* mutant (Figure 8D). However, integration of the ERC-like plasmid does not seem to be the sole or main contribution to rDNA amplification. The total signal intensities of both an integrated ERC-like plasmid into chromosome XII [Figure 8C, Chr XII(hyp)] and intermediates of on-going rolling circle replication in the well were ~15 times greater than that of single copy *URA3* on Chromosome V (Figure 8C; lanes 7–8 in the right panel, Chr. V). Of course, because both the ERC-like plasmid and native ERC become templates for rolling circle replication, the actual extent of replication is expected to be greater. Intra-chromosomal recombination may also contribute to promote an additional pathway of rolling circle replication. As shown in Figure 7B, the broken end selects the intra-sister chromatid as a recombination template, instead of ERC, and converts into a Break-Induced Replication (BIR) event. Thus, we think that such template switch from sister-chromatid to ERC (results in ERC integration) and BIR conversion (results in less ERC production) reduce Fob1-induced ERC level during hyper-amplification in the *rtt109* mutant (Figure 8A and 8B).

Our rolling circle replication model is supported by other reports concerning Rtt109 function. Wurtele et al. (2012) showed that the histone H3K56R mutation reduced marker loss rate in rDNA [36]. As we showed in Figure 2A, the mutation induced rDNA hyper-amplification which should have reduced loss of rDNA copies. Another study reported that mutated *RTT109* reduced the frequency of unequal recombination between sister chromatids in non-rDNA regions [16]. This is consistent with our observation that cohesin associates with the IGS region of rDNA. This suggests that rDNA amplification by unequal sister-chromatid recombination (Figure 7A) does not seem elevated in the mutant. Houseley and Tollervey (2011) recently reported that hyper-amplification occurred in an *asf1* mutant and proposed a model based on unequal sister chromatid interaction [17]. Because the rate of amplification is much lower in the *asf1* mutant than in the *rtt109* mutant, their model appears plausible. But in the case of the *rtt109* mutant, models other than one based on rolling circle replication would not seem compatible with such a high amplification rate. In addition to the *rtt109* mutant and the histone H3K56R mutant, we found that the double deletion mutant of histone deacetylase that acts on the K56 residue of histone H3, Hst3 Hst4, and the H3K56Q mutant all exhibited a hyper-amplification phenotype. These mutations also increased sensitivity to DNA damage relative to wild type cells [18]. Either an excess or an absence of acetylation on H3K56 of sister chromatids during recombination at the replication fork blocking site leads to rolling-circle amplification of rDNA. While we currently lack a satisfying explanation, the downstream event of the unusual H3K56 modification is important for the completion of replication.
Figure 8. Less ERC is generated and multimeric ERC integrates into rDNA during hyper-amplification. A) 2D gel electrophoresis to detect ERC before (growth on raffinose) and after (growth on galactose) FOB1 induction. The wild type (RTT109) and rtt109 mutant with YCplac22-GAL-FOB1 in the fob1 defective background were incubated as in the experiment to monitor hyper-amplification and were sampled for DNA at time zero (Raffinose) and 9 hr after galactose-mediated FOB1 induction (Galactose). The scheme is shown in the right side of the figure. B) Quantification of ERC before and after FOB1 induction. Quantification is described in Materials and Methods. C) Integration of plasmid with rDNA into chromosome XII during hyper-amplification; EtBr staining (left) and hybridization with URA3 as probe (right). Asterisks indicate the positions of the plasmid with and without rDNA (See Figure S4 to confirm the band with asterisks derived from the plasmid). The arrowhead indicates the position of the hyper-amplified band of chromosome XII [ChrXII(hyp)]. The rtt109 mutant with an empty vector (lanes 1–4) or with a plasmid containing an rDNA unit (ERC-like plasmid, lanes 5–8) were incubated after FOB1 induction. As the control, the wild type strain with the ERC-like plasmid was incubated (lanes 9–10, RTT109). At the indicated time after Fob1 induction, DNA was prepared for CHEF analysis. Southern hybridization was performed with a URA3 probe. D) 2D gel electrophoresis to detect the repeats amplified by rolling circle replication. (Upper) A scheme for rolling circle amplification with the IGS plasmid. The amplification forms a cluster of plasmid sequences in the rDNA. Copy number of a cluster is estimated by BglII digestion. In this case, five copies were amplified. (Lower) Plasmid sequences were detected by a specific probe (amp). The scheme is shown on the right side of the figure. The numbers below the linear position are copy numbers generated by rolling circle amplification.
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Figure 9. Hyper-amplification of rRNA genes is not a result of transcriptional compensation. A) Nascent rRNA transcription in the rtt109 mutant. Pre-35S (25S) rRNA and ACT1 were detected in the fob1 and rtt109 fob1 double mutants by northern blotting. (Left) EtBr-stained gel. The bands are mature rRNAs. (Right) Hybridization with pre-35S rRNA-specific oligo probe. ACT1 was detected with ACT1 specific probe. B) Quantitation of nascent rRNA transcripts. The pre-35S (25S) rRNA was normalized to ACT1. Values relative to the wild type strain. The probe hybridizes with the ETS region of pre-35S rRNA. Therefore, nascent pre-35S and just-processed 25S rRNA with the ETS sequence were detected. Both bands were measured as nascent rRNA transcripts. Values are means of three independent experiments and bars are S.D. values. As for the rtt109 fob1 double mutants, two sets of experiments were performed. C) The ratio of active (transcribed) to silent (non-transcribed) copies of rtt109 mutant in the psoralen assay. Genomic DNA from psoralen-treated cells was purified and digested with EcoRI. After gel migration, two bands ("s" and "f") were detected by Southern hybridization with an rDNA probe. "s" and "f" indicate slow and fast moving bands that correspond with active and inactive copies of rDNA, respectively.
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of recombination at the RFB in order to inhibit BIR-based rolling circle replication.

Rolling circle replication is known to play important roles in cell physiology. The most well known example may be rDNA amplification during amphibian oogenesis [37]. In this stage, extra-chromosomal rolling circle amplification increases copy number up to 2-3 orders of magnitude resulting in a huge increase in rRNAs (and ribosomes) needed for early development [38], [39]. This mechanism of replication is not limited to rDNA but occurs elsewhere in the genome and in some cases, helps cells adapt to various stresses (for review, see [40]). In cancer cells, gene amplification is frequently observed and may be mediated in part by rolling circle replication [41]. Abnormal histone modification has also been reported in malignant tissue [42] consistent with the possibility that gene amplification plays a role in accelerating tumorigenesis.

Materials and Methods

Yeast strains, plasmids, growth conditions for hyper amplification

Yeast strains used in this study were derived from NOY408-1b in the W303 genetic background and from strain S288c (OpenBiosystems) and are listed in Table S1. The strains were grown at 30°C in YPD medium except for the hsd3 hsd4 double mutant that was cultured at 23°C. To monitor rDNA hyper-amplification, the rtt109 mutant with the plasmid containing galactose-inducible FOB1 (YCplac22-Gal-FOB1) was precultured in synthetic complete medium minus tryptophan with raffinose (SC-trp-raf). The strain was then transferred to SC-trp with galactose instead of raffinose (at time zero) and harvested at the indicated times (0, 6, 12, 18, 24, 36 hr) to prepare genomic DNA in agarose plugs [43]. As a control, the RTT109 strain was monitored in parallel. Raffinose (1/10 volume of a 10X raffinose stock) and galactose were added just before use. Yeast strains with amino acid substitutions at histone K56 were generated by plasmid shuffling [44].

Genome-wide screening for yeast mutants with high rDNA copy number

Genomic DNA was purified from ~4,800 yeast mutants from the genome-wide deletion library (OpenBiosystems) as described [43]. They were then subjected to pulsed-field gel electrophoresis as described below. Gels were stained with ethidium bromide (EtBr). The mutant strains with a longer chromosome XII than the 3.13 Mb size marker (H. wingei) were classified as hyper-amplified rDNA mutants (Table 1). We used a wide gel with a long comb (45 cm) and a 3.13 Mb size marker (EtBr). The mutant strains with a longer chromosome XII than the 3.13 Mb size marker were then subjected to pulsed-field gel electrophoresis in TAE at 1 V/cm for 20 hours. After excising each lane above 5 kb from the 1D gel, 2D gel electrophoresis was performed in 1.0% SeaKem LE Agarose gel (Lonza) with EtBr at 6 V/cm for 5 hours at 4°C. The gel was blotted and part of the membrane containing DNAs longer than 7 kb was hybridized with an rDNA-specific probe. The other part of the membrane containing DNAs shorter than 7 kb was probed with URA1. The ERC signal was normalized to the URA1 signal in each blot. The relative amounts of ERC were determined from three independent experiments as shown in Figure 8B. To detect rolling circle amplification (Figure 8D) by 2D gel electrophoresis, cells with the IGS plasmid, YEplac195 containing the IGS region of rDNA were used. DNA was isolated from cells 48 hrs after release into SC–trp +Gal. Conditions used for 2D gel electrophoresis and induction of hyper-amplification were similar to those described above, except that DNA was digested with BglII before electrophoresis. BglII does not cut the plasmid but cuts the chromosomal rDNA, such that only plasmids integrated on the chromosome form linear structures following BglII digestion. The plasmid sequence was detected using a specific probe (amp).

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as described [6].

Atomic force microscopy (AFM)

DNA fraction remaining in the agarose gel plug after pulsed-field gel electrophoresis was extracted by crushing. AFM observation was performed as described [45]. During the sample preparation, DNA was sheared into smaller fragments (~50 kb). Among these fragments (~1,500), we looked for the circle with the proper size (8–11 kb). Imaging was carried out with a Digidal Instrument MultiMode AFM in the tapping mode. Image J (NIH) was used for measurement of DNA length.

Supporting Information

Figure S1 An example of the pulsed-field gel electrophoresis screen for strains in which rDNA copy number increased. This is a collection of representative mutants in which rDNA stability was reduced. The gel is stained with EtBr. The largest bands are chromosome XII with rDNA in each lane. WT is the wild type strain. Others are mutants from the deletion library. Lane 6 is the rtt109 mutant. M is a size marker from H. wingei.

Figure S2 Fob1 is required for efficient hyper-amplification. The size of chromosome XII in the fob1 mutant was monitored for multiple generations after deletion of RTT109, fob1A is a control harboring wild type RTT109. Three independent colonies were picked and streaked on YPD to yield single colonies. DNA were then prepared from each and subjected to CHEF analysis. SCI1 is chromosome XII after a single colony isolation (lanes 1–3, 11–13) while SCI3 is a clone obtained after three consecutive single colony isolations (lanes 4–6, 14–16). SCI5 is a clone obtained after five consecutive single colony isolations (lanes 7–9, 17–19).
**Figure S3** E-pro expression in the rtt109 mutant before and after complementation with the wild type allele. E-pro expression in the rtt109 mutant following complementation with Rtt109. Transcripts of E-pro were detected by northern analysis in the wild type, rtt109 mutant and rtt109 mutant transformed with YCp-RTT109. Two clones were analyzed per strain. (EPS)

**Figure S4** Confirmation of extra-plasmid-derived bands. EtBr staining (left) and hybridization with URA3 (right). Data are similar to those shown in Figure 8C except for Figure S3 in the rtt109 mutant following complementation with Rtt109. Two clones were analyzed per strain.

This suggests that the other bands are derived from the plasmid carrying URA3 and may be replication intermediates of plasmid monomers and multimers.

(EPS)

**Table S1** Yeast strains and plasmids used in this study. (XLS)

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**Author Contributions**

Conceived and designed the experiments: SI TK. Performed the experiments: SI KS TK. Analyzed the data: SI TK. Contributed reagents/materials/analysis tools: SI KS TK. Wrote the paper: SI TK.

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