

The cohesin acetyltransferase Eco1 coordinates rDNA replication and transcription

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Abstract

Eco1 is the acetyltransferase that establishes sister-chromatid cohesion during DNA replication. A budding yeast strain with an *eco1* mutation that genocopies Roberts syndrome has reduced ribosomal DNA (rDNA) transcription and a transcriptional signature of starvation. We show that deleting *FOB1*—a gene that encodes a replication fork-blocking protein specific for the rDNA region—rescues rRNA production and partially rescues transcription genome-wide. Further studies show that deletion of *FOB1* corrects the genome-wide replication defects, nucleolar structure, and rDNA segregation that occur in the *eco1* mutant. Our study highlights that the presence of cohesin at the rDNA locus has a central role in controlling global DNA replication and gene expression.

Keywords cohesin; Eco1; DNA replication

Subject Categories Cell Cycle; DNA Replication, Repair & Recombination; Transcription

DOI 10.1002/embr.201337974 | Received 10 September 2013 | Revised 11 February 2014 | Accepted 12 February 2014 | Published online 14 March 2014

EMBO Reports (2014) 15: 609–617

Introduction

The evolutionarily conserved cohesin complex contributes to chromosome function in many ways. Cohesin contributes to the processes of chromosome segregation, DNA replication, chromosome condensation, and DNA damage repair. Cohesin mutations reduce ribosomal DNA (rDNA) transcription and translation in both budding yeast and human cells [1]. Cohesion also promotes nucleolar structure and function in both budding yeast and human cells [2, 3]. Roberts syndrome (RBS) is a human disease caused by mutation of *ESCO2*, a homolog of the yeast cohesin acetyltransferase *Eco1* gene [4]. Mutations in cohesin are also associated with Cornelia de Lange syndrome (CdLS) and myeloid neoplasms. These diseases are caused by changes in gene expression, rather than aneuploidy. However, the mechanisms by which the cohesin complex influences the transcriptome are unclear.

Cohesin binds to the approximately 150 highly transcribed tandem repeats that make up the budding yeast rDNA locus [5]. In fact, cohesin binds to the rDNA regions in every eukaryotic genome in which binding has been examined. Replication is a challenge for this highly transcribed region. Fob1 controls rDNA replication in budding yeast, allowing it to occur only in the direction of transcription. The replication fork barrier (RFB) provided by Fob1 ensures that the replication apparatus does not disrupt transcription of the 35S gene [6, 7]. Human rDNA repeats contain a similar RFB. DNA replication forks move more slowly in human *ESCO2* mutant cells [8]. Moreover, the heterochromatic repulsion observed at centromeres and nucleolar organizing centers in RBS cells suggests that these regions might have cohesion defects due to difficulty with replication [4]. The cohesin complex binds adjacent to the RFB in the rDNA [5] and is important for replication fork restart [9]. These observations indicate an intimate connection between cohesin function and DNA replication, and a special role for cohesin at the rDNA.

In this study, we observed many defects in DNA replication in an *eco1* mutant. Defects in replication, rRNA production, and genome-wide transcription were partially rescued by deleting *FOB1*. While replication defects have been reported in other cohesin mutants [8, 10–13], it has not been appreciated that replication defects may interfere with transcription of the rDNA region. We propose that replication defects associated with mutations in cohesin greatly influence gene expression.

Results and Discussion

***FOB1* deletion partially rescues the genome-wide expression pattern in an *eco1* mutant**

We asked how deletion of *FOB1* would affect the phenotypes associated with the *eco1-W216G* mutation (*eco1*) that causes decreased acetyltransferase activity in RBS [14, 15]. Gcn4 is a transcriptional activator that is translated when translational activity is poor [16]. We employed a Gcn4-lacZ reporter as an indicator for ribosome function. The *eco1* strain shows a fourfold increase in β -galactosidase

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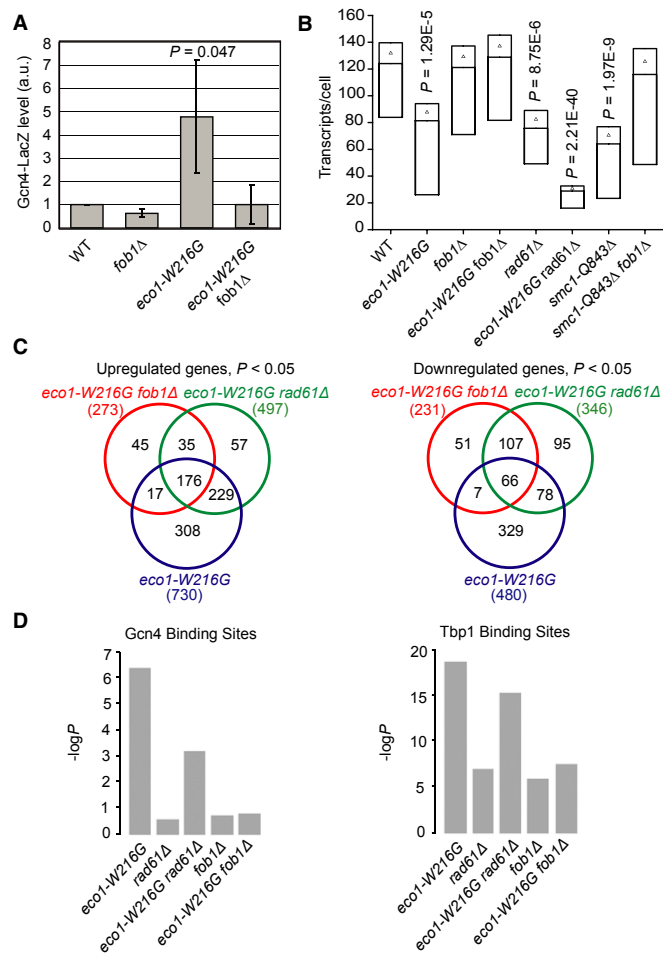


Figure 1. *FOB1* deletion partially rescued differential gene expression in the *eco1* mutant.

- A** β -galactosidase activity for each strain was measured in triplicate. All values were normalized to the level of the WT strain and are shown in arbitrary units (a.u.). The P -values were calculated by Student's t -test, comparing mutant to WT.
- B** FISH was used to measure transcription in strains with a unique sequence inserted into one rDNA repeat and the indicated mutation. For each strain, at least three independent cultures were monitored and at least 300 cells per culture were quantified. In the plot shown, the dot is the average, the box is the 95% confidence standard error, and the horizontal line within the box is the median. The P -values were calculated by Student's t -test, comparing mutant to WT. The data for WT and *eco1-W216G* strains were first published in Bose *et al* (2012) [1].
- C** Venn diagrams of upregulated genes and downregulated genes with $P < 0.05$ in the indicated strains are shown.
- D** Genes with Gcn4- or Tbp1-binding sites in their promoters were assessed as a group in each data set by a gene set enrichment test. The resulting P -values are shown as $-\log_{10}(P\text{-value})$. The P -value is calculated by a hypergeometric test using the number of differentially expressed genes with the binding site versus the number of genes in the genome with the site.

activity compared to a WT strain [1]. When we deleted the *FOB1* gene in the *eco1* mutant background, β -galactosidase levels were reduced (Fig 1A), suggesting that *FOB1* deletion rescued the poor translational activity in the *eco1* strain. Moreover, when the Fob1 protein was over-expressed, the β -galactosidase activity in the *eco1*

mutant increased further (Supplementary Fig S1), indicating further impaired translational activity.

Ribosome function depends on rRNAs transcribed from the rDNA locus. We speculated that deleting *FOB1* rescued ribosome function in the *eco1* mutant by rescuing rDNA transcription. We used FISH to detect transcription of a single ribosomal repeat [17]. As previously observed, the rRNA transcript level in the *eco1* strain was half that in a WT strain [1]. However, deleting *FOB1* in the *eco1* strain restored rRNA transcripts to WT levels (Fig 1B). For comparison, we measured rRNA transcripts in an *eco1 rad61Δ* double mutant strain. *RAD61* negatively regulates cohesion establishment and deleting it rescues the temperature sensitivity of the *eco1* strain, but not the elevated expression from the Gcn4-lacZ reporter [1]. While *fob1Δ* is expected to have an rDNA-specific effect, *rad61Δ* should produce a more general effect on cohesin. In contrast to *fob1Δ*, *rad61Δ* did not rescue rRNA transcription in the *eco1* strain.

Eco1 has other targets in addition to the subunits of the cohesin complex [18, 19]. To exclude the possibility that *fob1Δ* might rescue rDNA transcription through a different mechanism, we measured the rRNA level in an *smc1-Q843Δ fob1Δ* double mutant. Smc1 is a subunit of the cohesin complex. The mutation is a single amino acid deletion associated with CdLS [1]. The level of rRNA in the *smc1-Q843Δ* strain was also rescued by *fob1Δ* (Fig 1B), suggesting that *fob1Δ* rescues rDNA transcription through a cohesin-related mechanism.

To assess the effect of *fob1Δ* on genome-wide gene expression in the *eco1* strain, we performed microarray analysis of RNA from the following strains: (1) *eco1*, (2) *eco1 fob1Δ*, (3) *eco1 rad61Δ*, (4) *fob1Δ*, (5) *rad61Δ*, and (6) WT. Differentially expressed genes were selected based on a fold change between mutant and WT of at least 1.4-fold and an adjusted P -value < 0.05 . The number of differentially expressed genes was less in the *eco1 fob1Δ* strain (504) than in the *eco1* strain (1210) (Supplementary Fig S2). The *eco1 fob1Δ* strain also had fewer differentially expressed genes than the *eco1 rad61Δ* strain (843, Fig 1C, Supplementary Fig S2). Since genes containing binding sites for the sequence-specific transcriptional activators Gcn4 and Tbp1 are differentially expressed in the *eco1* strain [1], we asked whether these targets were less differentially expressed in the double mutant strains. The number of differentially expressed genes with these sites was decreased in the *eco1 fob1Δ* strain compared to the *eco1* and the *eco1 rad61Δ* strain (Fig 1D). Collectively, these experiments suggest that differential gene expression in the *eco1* strain may be due in part to reduced levels of rRNA. Restoration of rRNA levels significantly rescues the transcriptional profile of the mutant.

***FOB1* deletion rescues DNA replication defects associated with the *eco1* mutation**

Given the RFB function of Fob1 at the rDNA, we speculated that *fob1Δ* would rescue rRNA levels in the *eco1* mutant by its effect on DNA replication. To examine DNA replication, we measured cell cycle progression by cytometry analysis. Cells were synchronized in G1 by α -factor treatment and then released at 33°C to pass through S phase. 33°C is a permissive temperature for growth, but the *eco1-W216G* mutation is lethal at 37°C, so we reasoned 33°C might accentuate any phenotype (Supplementary Fig S3). A shift in DNA content was observed at 20 min in the *eco1* mutant, indicating

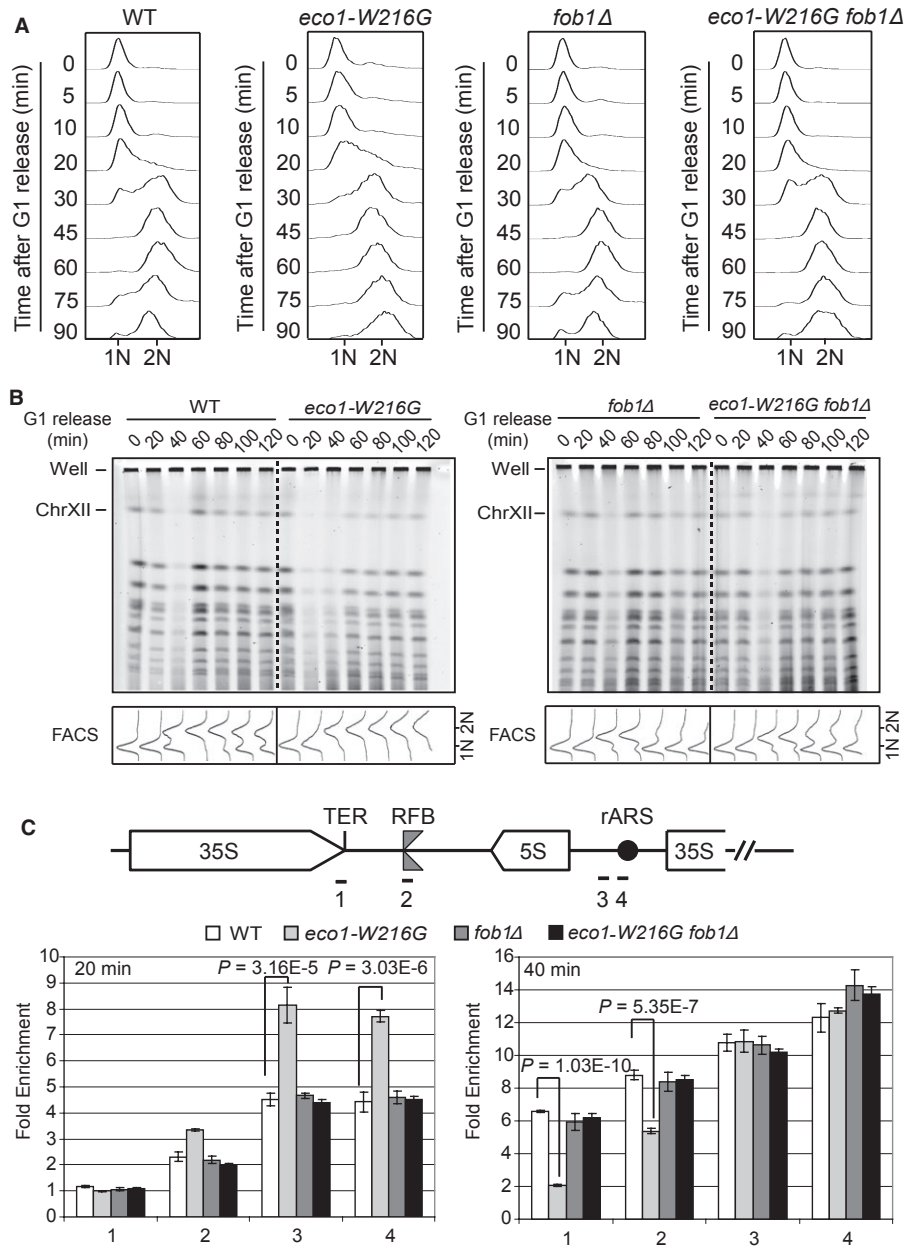


Figure 2. *FOB1* deletion rescues DNA replication defects in the *eco1* mutant.

A Each strain was synchronized in G1 using α -factor at 30°C, released at 33°C and samples were collected at the indicated time points for analysis of DNA content by cytometry.

B Each strain was synchronized in G1 using α -factor at 30°C, released at 33°C, and another dose of α -factor was added at 60 min to avoid a second round of DNA replication. DNA samples were collected for PFGE at the indicated time points.

C BrdU labeling was carried out in cells synchronized and released as described in the Supplementary Methods. Following ChIP with anti-BrdU antibody, the DNA eluates were used as a template for qPCR with the four primer pairs indicated at the rDNA. The region around the rARS (primer pairs 3 and 4) has more BrdU incorporation at the 20-min time point in the *eco1* mutant, but the double mutant is similar to WT. The regions most distant from the rARS, when replication is unidirectional (primer pairs 1 and 2), are under-replicated in the *eco1* mutant compared to WT or the double mutant at 40 min. Bars indicate the average value, and error bars indicate the standard deviation. Two independent biological replicates were performed with two technical replicates each. *P*-values were calculated by Student's *t*-test.

earlier progression to S phase than in a WT strain (Fig 2A). However, both WT and *eco1* strains complete the shift to 2N at approximately the same time, suggesting that the *eco1* strain takes longer to complete replication than WT. To assess the effect of

fob1Δ on cell cycle progression in the *eco1* strain, we measured cell cycle progression in *fob1Δ* and *eco1 fob1Δ* strains. The double mutant did not initiate S phase earlier, suggesting that *FOB1* deletion rescued the replication defect (Fig 2A).

We next examined DNA replication in cells synchronized with α -factor using pulsed field gel electrophoresis (PFGE). In PFGE, chromosomes cannot migrate into the gel while undergoing replication due to replication intermediates. DNA samples were collected at the indicated times following release from G1. Consistent with the cytometry data, less chromosome migration was detectable at 20 min in the *eco1* strain compared to a WT strain (Fig 2B). This result confirmed that DNA replication initiated earlier in the *eco1* strain, and further demonstrated that all chromosomes were affected. The *eco1 fob1 Δ* strain did not initiate DNA replication early (Fig 2B), suggesting that *fob1 Δ* rescued DNA replication. Therefore, deletion of the rDNA-specific factor *FOB1* appeared to rescue a genome-wide replication defect in the *eco1* mutant.

While Fob1 has fork-blocking activity, it also regulates recombination and copy number at the rDNA. Eco1 plays a role in DNA damage repair and recombination [15, 20, 21]. However, the *eco1* mutation does not affect recombination or copy number at the rDNA locus [1, 22], nor does it have a synthetic growth phenotype with lower copy number of rDNA (Supplementary Fig S3), suggesting that *fob1 Δ* is unlikely to rescue recombination or copy number issues. In addition, deletion of *FOB1* alone does not alter the frequency of origin firing in the rDNA or the fraction of active rDNA genes [23]. Therefore, *fob1 Δ* may rescue the DNA replication defect in the *eco1* mutant by allowing bidirectional replication at the rDNA, thereby promoting the completion of rDNA replication. Because rDNA replication and transcription do not occur simultaneously, completion of replication may facilitate efficient transcription of the locus. Deletion of *FOB1* has also been shown to relieve replication stress in the *smc6-9* mutant at the rDNA locus [24], suggesting a shared role for SMC complexes in regulating rDNA replication.

To further address how *FOB1* deletion rescues replication of the rDNA locus, we measured replication using BrdU labeling followed by ChIP/qPCR [25]. Cells were arrested in G1 with α -factor and then released into medium with BrdU. BrdU incorporation was detected using ChIP followed by qPCR. The detection primers were chosen to measure replication at the rARS (primer pairs 3 and 4), or the most distant point from the rARS (primer pairs 1 and 2) when replication is unidirectional. The enrichment for rARS sequences in the *eco1* mutant strain was higher than in the WT strain at 20 min, demonstrating that the rDNA begins replication earlier (Fig 2C). However, at the 40-min time point, the *eco1* strain had poor replication of the rARS distal sequences compared to either WT or the *eco1 fob1 Δ* double mutant, strongly suggesting that replication at the rDNA region is incomplete in the single mutant but more complete in the double mutant. A replication fork travels an average of 20 kb in budding yeast, but the average distance is closer to 50 kb at the rDNA, making these replication forks some of the longest in the genome [26, 27]. Although these ARSs fire early, the replication of the region continues throughout S phase [28]. The observed defects in replication are consistent with the hypothesis that prolonged replication of the rDNA interferes with its transcription in the *eco1* mutant strain.

Eco1 regulates origin firing activity

To further address origin firing, we investigated the association of the replication initiation factor Cdc45 with the rARS in WT and *eco1*

mutant cells using ChIP [29, 30]. To measure the kinetics of Cdc45 binding, we released yeast from G1 arrest at 16°C to slow down the replication process. The level of Cdc45 binding to the rDNA origin of replication (rARS) in the *eco1* mutant peaked at 90 min, earlier than the peak at 105 min observed in WT cells (Fig 3A), further confirming that the rARS fires earlier in the *eco1* mutant than in WT.

To study how the *eco1* mutation affects replication genome-wide, we measured DNA content by deep sequencing of genomic DNA in WT and *eco1* cells [31, 32]. Samples of genomic DNA were collected at 0, 20, and 40 min following release from G1 arrest. The origin firing pattern was different between WT and *eco1* strains at 20 min (Fig 3B, Supplementary Figs S4 and S5). More early origins fire in the WT strain than in the *eco1* mutant strain, but late origins fire about equally well in the two strains at 20 min, indicating that the origin firing sequence is disrupted in the *eco1* mutant. Origin firing in the *eco1* mutant also occurred at non-ARS sites as well as mapped ARS sites (Fig 3B, Supplementary Figs S4 and S5), but replication from any single site was generally less pronounced in the *eco1* mutant than in the WT. This might be due to the titration of the replication factors by the firing of many additional sites. Replication factors can be limiting for replication progression [33]. Because our previous experiments suggested slow DNA replication in the *eco1* mutant, we measured the completeness of DNA replication genome-wide at late S phase. Replication was less complete in the *eco1* mutant at 40 min (Supplementary Fig S6).

To confirm the origin firing defect in the *eco1* mutant, we measured origin activity by transforming WT and *eco1* mutant strains with plasmids containing (1) no ARS sequence, (2) rARS sequence, or (3) ARS1 sequence [34]. ARS1 is a well-studied highly efficient early ARS located on chromosome IV. We used these plasmids to assess the ability of these three sequences to promote autonomous plasmid maintenance, likely reflecting the efficiency of firing of the ARS in the genomic context. In the genome, each rDNA repeat contains the rARS sequence. However, in a given cell cycle, approximately 1 in 5 of these rARSs will fire [27]. We observed more transformants for the rARS-containing plasmid in the *eco1* background compared to WT, using the same amount of plasmid DNA (Fig 3C), suggesting more firing of this ARS in the mutant, consistent with the BrdU labeling experiment. An increase in rARS firing could contribute to less transcription of 35S in the context of the genomic locus. The ARS1-containing plasmid in the *eco1* strain had fewer transformants, consistent with the result derived from sequencing that ARS1 fires less efficiently in the *eco1* mutant than in WT (Supplementary Fig S5). Interestingly, the no ARS plasmid was replicated with low efficiency in the mutant (Fig 3C), which could reflect the origin fidelity defect observed in genome-wide sequencing. The above results suggest that Eco1 regulates origin firing.

Cohesin is reported to be enriched at replication origins and to spatially organize replication factories [11]. Cohesin could directly regulate origin firing at ARS sites. Another possibility is that mutations in cohesin alter the dNTP pool [10]. Increases in the nucleotide pool can modulate origin choice and interorigin spacing [35, 36]. In a genome-wide proteomic study of the *eco1* strain, we found evidence supporting the latter possibility. Many proteins involved in dNTP synthesis were present at higher levels in the *eco1* mutant, which could increase the dNTP pool (Supplementary Fig S7). The gene expression profile of the *eco1* mutant strain is very similar to starvation [1], such that the expression of many genes involved in purine,

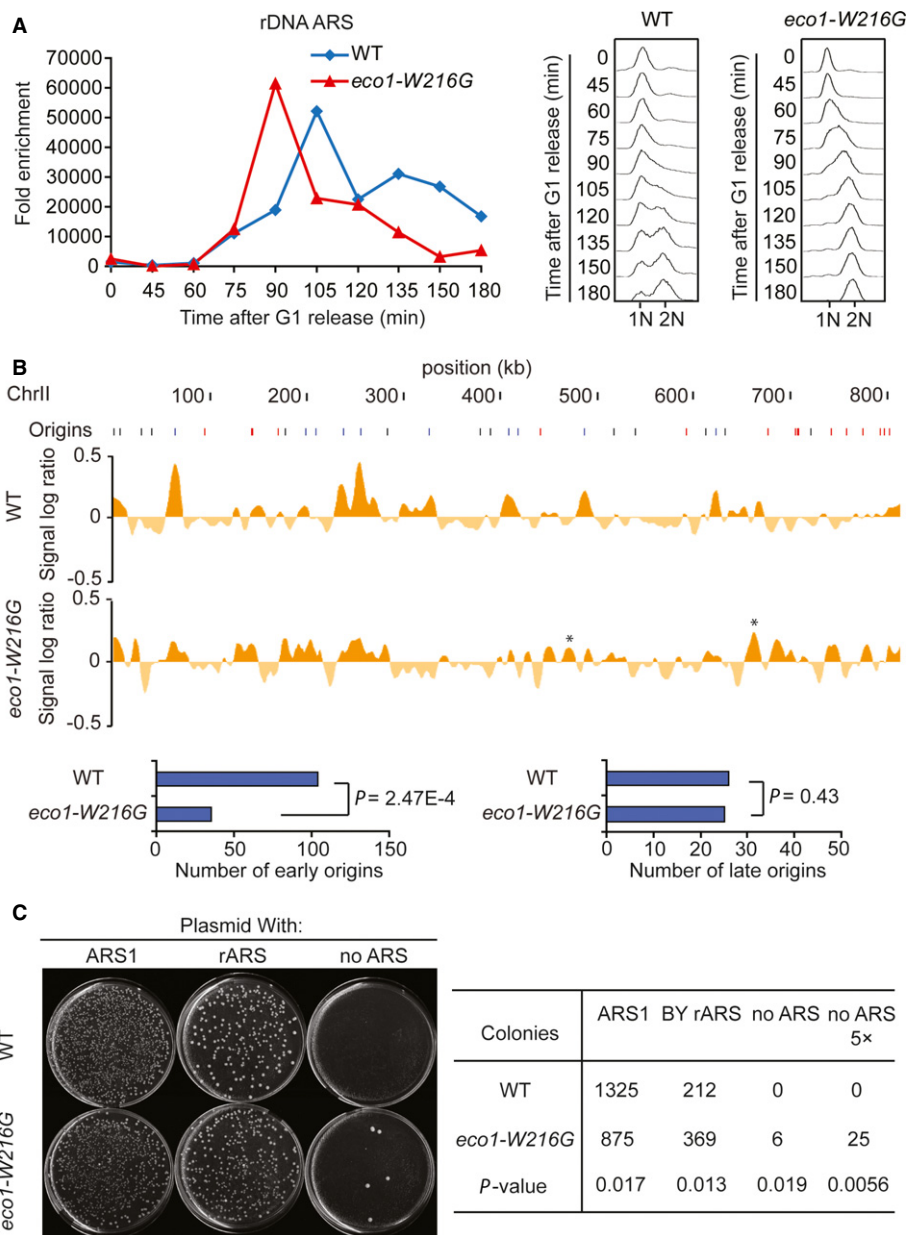


Figure 3. The *eco1* mutation disrupted replication origin activity.

A ChIP of Cdc45-FLAG was performed with anti-Flag antibody and analyzed by qPCR using primers specific for the rDNA ARS. WT and *eco1* strains with Cdc45-Flag were synchronized in G1 using α -factor at 30°C, released at 16°C, and samples were collected at the indicated time points.

B Strains were cultured as in Fig 2A. Genomic DNA was collected at 0, 20, and 40 min and sequenced. The signal intensities relative to a G1 phase strain are shown along ChrII of *S. cerevisiae*. Early and late origins along ChrII are indicated using blue and red color, respectively. Origins shown in black indicate the ARS is either inactive or replication timing data is not available. The asterisks indicate replication at non-ARS sites. The lower panel shows the numbers of early and late origins fired in the indicated strains. The number of fired origins was calculated by counting the peaks on all chromosomes using a 5-kb window centered by origin. We observed similar patterns of origin firing in biological replicates. The *P*-values were calculated by Student's *t*-test, comparing mutant to WT.

C DNA origin activity in WT and *eco1* strains was measured using plasmids. Strains transformed with the indicated plasmid were replica-plated to YPD plates with G418 after a day of growth on YPD medium to assess the efficiency of origin firing. The number of colonies is shown to the right. The *P*-values were calculated as in (B).

pyrimidine, and amino acid biosynthetic processes is misregulated. However, this signature is not present in the *eco1 fob1Δ* strain (Supplementary Figs S2 and S7). The misregulation of metabolic processes could cause too many regions to fire, which could

subsequently lead to the depletion of nucleotide pools and replication factors such that replication forks cannot proceed with optimal speed [37]. Therefore, cohesin may influence origin usage, firing fidelity, and timing in part through its effect on gene expression.

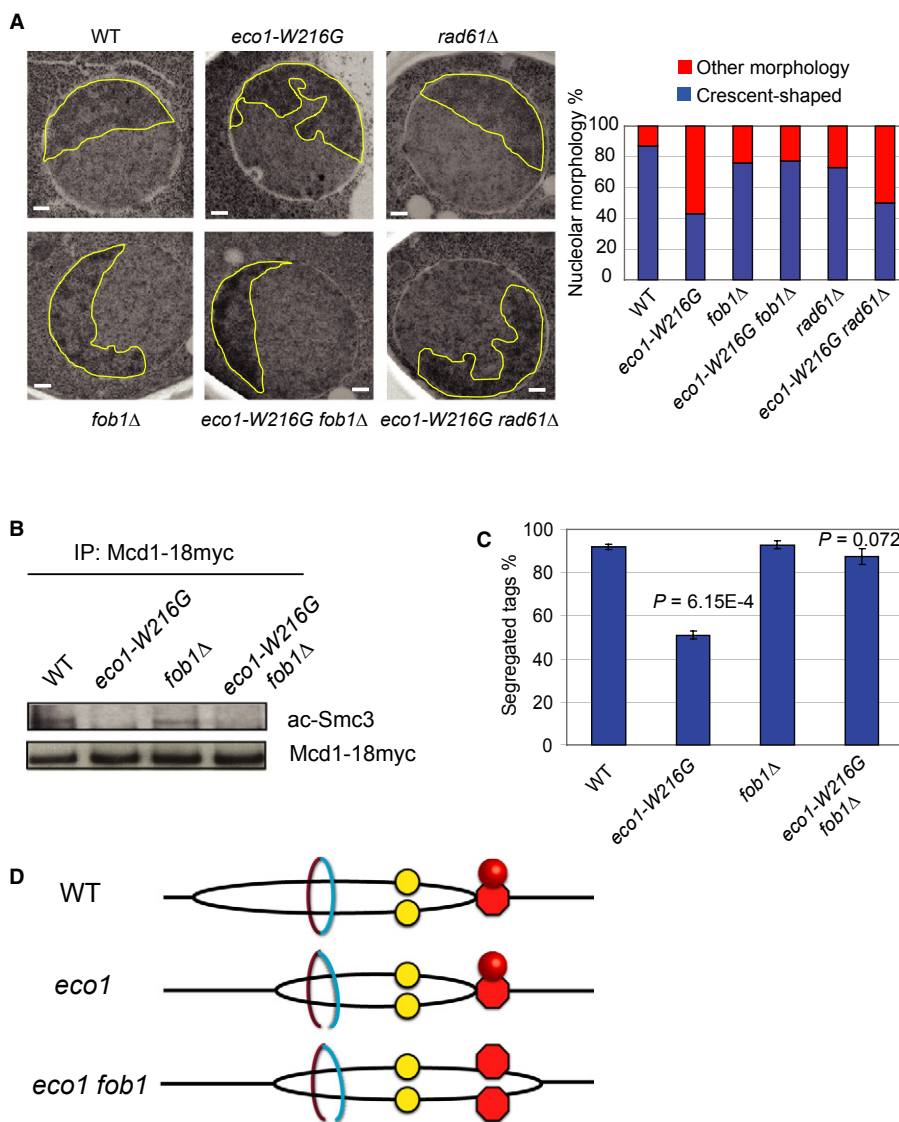


Figure 4. *FOB1* deletion rescues nucleolar structure and chromosome segregation in the *eco1* mutant.

- A Nucleolar morphology of WT, *eco1*, *fob1Δ*, and *eco1 fob1Δ* strains was examined by EM. The nucleolus is the electron-dense area. At least 25 nucleoli were scored per strain. The scale bar represents 0.2 μm. The data for WT and *eco1-W216G* strains were first published in Harris et al [2].
- B *FOB1* deletion does not rescue cohesin acetylation in the *eco1* mutant. The cohesin complex was immunoprecipitated with α-Myc affinity gel and then analyzed by western blotting with α-acetyl-lysine antibody for Smc3 acetylation level (upper panel). The same immunoprecipitated samples were blotted for anti-Myc antibody as a loading control (lower panel).
- C Segregation of the rDNA in WT, *eco1*, *fob1Δ*, and *eco1 fob1Δ* strains was measured 80 min after the release from G1. All binucleated cells were counted to determine segregation. Error bars indicate standard deviation from three independent experiments. At least 150 cells per strain were counted per experiment. The *P*-values were calculated by Student's *t*-test, comparing mutant to WT.
- D A model for how deletion of *FOB1* (red ball) rescues replication at the rDNA locus in the *eco1* mutant by allowing replication from the rARS (yellow) to pass through the replication fork block (red stop sign). Cohesin is shown as a red/blue ring.

***FOB1* deletion rescues nucleolar morphology and chromosome segregation defects associated with the *eco1* mutation**

Electron microscopy shows the budding yeast nucleolus, home of the rDNA repeats, as a single dense crescent-shaped structure abutting the nuclear envelop in a WT strain. However, in the *eco1* strain, the nucleolus is irregularly shaped (Fig 4A). To assess the effect of *fob1Δ* on nucleolar morphology, we analyzed nucleoli in *fob1Δ* and

eco1 fob1Δ strains. *FOB1* deletion rescued the irregular nucleolar morphology in the *eco1* strain (Fig 4A). In contrast to *fob1Δ*, *rad61Δ* had less of a rescue effect for nucleolar structure. The lack of rescue with *rad61Δ* correlates with the lack of rescue for rDNA transcription and the global transcriptional profile.

Because cohesin establishment is coupled to DNA replication [12, 38, 39], we wondered whether *fob1Δ* restored nucleolar morphology by improving the levels of acetylated cohesin. We

measured the acetylation of K112 and K113 of Smc3, the lysines targeted by Eco1 for replication-coupled cohesion [38, 39]. *fob1Δ* did not rescue acetylation (Fig 4B), suggesting that the recovery of nucleolar morphology in the double mutant is more likely due to the rescue of the replication and transcription of the rDNA locus.

Replication stress could induce chromosome segregation defects and genome instability [40, 41]. To study rDNA segregation, we used tetR-YFP to detect tetO repeats inserted in the telomere proximal end of the rDNA [24]. We observed that in the *eco1* strain, approximately 50% of spots did not segregate correctly at 80 min after release from G1 (Fig 4C). This is consistent with the finding that cohesin mutation-induced replication defects lead to segregation defects in mice [42]. In contrast to the delay in separation of the rDNA, we did not observe a delay in centromere segregation (Supplementary Fig S8), suggesting that the rDNA region is specifically delayed in the *eco1* mutant.

Next, we addressed whether the rDNA segregation delay in the *eco1* strain could be rescued by relieving incomplete replication via *fob1Δ*. We observed that in the *eco1 fob1Δ* double mutant strain, the rDNA segregated with normal timing. This suggests that the replication defect induced by the *eco1* mutation could cause the rDNA segregation delay.

Figure 4(D) shows a model summarizing the rDNA replication phenotypes for the *eco1* and *eco1 fob1Δ* mutants. Replication stress has been reported to cause sister-chromatid bridging, especially at fragile loci such as the rDNA [40]. The rDNA locus could play a “sensor” role for cellular functions. Our study suggests that cohesin affects gene expression and DNA replication genome-wide via control of these same processes at the rDNA region. We speculate that the replication defects associated with cohesin mutations interfere with the transcription of rDNA, leading to transcriptional and translational defects that contribute to human disease.

Materials and Methods

Yeast strains and cell synchronization

Yeast strains and primers used in this study are listed in Supplementary Table S1. Exponentially growing cells were arrested in G1 phase by the addition of α -factor (1.5×10^{-8} M final) for 2 h. To release cells from α -factor arrest, cells were spun down and washed twice in media containing 0.1 mg/ml Protease (Sigma, P-6911).

Data access

All deep sequencing and Affymetrix microarray data have been submitted to the NCBI Gene Expression Omnibus (GEO accession number GSE54743). All primary data associated with this manuscript can be found at <http://odr.stowers.org/websimr/datasetview/632/0/>.

Cytometry and microscopy

Cytometry analysis was performed to analyze cell cycle phase on cells fixed in 70% ethanol. Cells were washed with FACS buffer (50 mM sodium citrate), treated with RNase, stained with propidium iodide (4 μ g/ml final), and analyzed by using a MACSQuant analyzer (Miltenyi Biotech). Fluorescence signal was observed using

a Zeiss Axiovert 200M microscope (63 \times objective, NA = 1.40). Image acquisition and analysis was performed with Axiovision (Carl Zeiss).

Pulse-field gel electrophoresis (PFGE)

PFGE was carried out as previously described [43].

β -Galactosidase assay

Yeast cells were grown overnight at 30°C in SD-ura and then diluted to OD₆₀₀ = 0.2 in YPD+CSM. Cells were allowed to grow for two generations and were collected. Protein extracts were made by bead beating. β -galactosidase activity was measured following standardized protocols, using ONPG (o-nitrophenyl- β -D-galactopyranoside) as the substrate.

Gene expression analysis

Gene expression analysis was carried out using Affymetrix Yeast Genome 2.0 microarrays and following the protocol as previously described [1].

FISH

FISH experiments were carried out following the protocol as previously described [1].

Supplementary information for this article is available online: <http://embor.embopress.org>

Acknowledgments

We thank Sue Jaspersen and Jingjing Chen for assistance and helpful suggestions, Ivan Liachko for advice on the analysis of genome-wide replication data, A. Bedalov and A. Hinnebusch for plasmids, and the Aragon, Pasero, Grunstein, Petes, Kobayashi, and van Oudenaarden laboratories for strains. We thank SIMR for funding.

Author contributions

SL and JLG wrote the paper. GH, LF, CS, and SL conducted data analysis. SL, JLG, KKL, BH, BX, AS, and TB carried out the experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

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