Quantitative Proteomic Analysis of Chromatin Reveals that Ctf18 Acts in the DNA Replication Checkpoint

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Yeast cells lacking Ctf18, the major subunit of an alternative Replication Factor C complex, have multiple problems with genome stability. To understand the in vivo function of the Ctf18 complex, we analyzed chromatin composition in a ctf18Δ mutant using the quantitative proteomic technique of stable isotope labeling by amino acids in cell culture. Three hundred and seven of the 491 reported chromosomal proteins were quantitated. The most marked abnormalities occurred when cells were challenged with the replication inhibitor hydroxyurea. Compared with wild type, hydroxyurea-treated ctf18Δ cells exhibited increased chromatin association of replisome progression complex components including Cdc45, Ctf4, and GINS complex subunits, the polymerase processivity clamp PCNA and the single-stranded DNA-binding complex RPA. Chromatin composition abnormalities observed in ctf18Δ cells were very similar to those of an mrc1Δ mutant, which is defective in the activating the Rad53 checkpoint kinase in response to DNA replication stress. We found that ctf18A cells are also defective in Rad53 activation, revealing that the Ctf18 complex is required for engagement of the DNA replication checkpoint. Inappropriate initiation of replication at late origins, because of loss of the checkpoint, probably causes the elevated level of chromatin-bound replisome proteins in the ctf18Δ mutant. The role of Ctf18 in checkpoint activation is not shared by all Replication Factor C-like complexes, because proteomic analysis revealed that cells lacking Elg1 (the major subunit of a different Replication Factor C-like complex) display a different spectrum of chromatin abnormalities. Identification of Ctf18 as a checkpoint protein highlights the usefulness of chromatin proteomic analysis for understanding the in vivo function of proteins that mediate chromatin transactions. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.005561, 1–14, 2011.
Because its pleiotropic effects suggest that various chromosome maintenance pathways could be affected in the ctf18Δ mutant, we took a proteomic strategy to investigate chromatin abnormalities. Possible targets of regulation by Ctf18-RLC include proteins involved in the processes of DNA replication, the replication stress response, or establishment of sister chromatid cohesion. Central to all these processes is the replisome, the multiprotein complex that replicates the DNA, as described below. Replisome assembly begins during G1 phase, with the formation of prereplication complexes by loading of heterohexameric Mcm2–7 complexes at origin sites (18). Replication initiation then involves recruitment by Mcm2–7 hexamers of Cdc45 and the GINS complex (containing the four subunits Sld5, Psf1, Psf2, and Psf3), leading to assembly of the Cdc45-MCM-GINS complex. Cdc45-MCM-GINS is central to replisome function, probably forming the activated replicative helicase that unwinds the DNA, enabling its replication on the leading strand by DNA polymerase ε and on the lagging strand by DNA polymerase δ (along with its processivity factor PCNA and the DNA polymerase α priming complex) (18–20). During the replication process, exposed single-stranded DNA is coated by the single-stranded binding protein RPA (containing subunits Rfa1, Rfa2, and Rfa3) to stabilize and protect it.

A recent biochemical study revealed that the Cdc45-MCM-GINS is the central component of the so-called “replisome progression complex” (RPC), which contains additional factors including Ctf4 (21). Ctf4 appears to link Cdc45-MCM-GINS to Polymerase α (22, 23), and is required for the establishment of cohesion (12), the mechanism through which duplicated sister chromatids are held together until anaphase. Sister chromatids are held together by the ring-shaped cohesin complex, which contains subunits Smc1, Smc3, Scc1, and Scc3 (24). Establishment of cohesion appears to be closely coupled to replication through RPC-mediated events, and yeast cells lacking RPC proteins such as Ctf4 display cohesion defects (25).

A further component of the RPC is the checkpoint mediator protein Mrcl, essential for cells to respond correctly to replication stress, such as fork blockage events induced by the DNA replication inhibitor HU (21, 26, 27). In response to replication stress, eukaryotic cells activate a DNA replication checkpoint pathway that suppresses new replication initiation events at unfired origins, stabilizes replication forks, induces transcription of DNA stress response genes, and delays cell cycle progression (28). The current model suggests that a checkpoint kinase Mec1, the budding yeast homolog of human ATR, is recruited to replication block sites and phosphorylates target proteins. One of the targets is Mrc1, which provides an activation platform for the checkpoint kinase Rad53 to initiate the checkpoint response (27). mrc1Δ cells demonstrate a significant delay in Rad53 activation in response to a replication block (26). In wild-type cells HU treatment causes suppression of further replication initiation events, but HU-treated mrc1Δ cells inappropriately initiate replication at late origins, because of failure of DNA replication checkpoint activation (26, 29).

To investigate the in vivo function of Ctf18-RLC, we analyzed the differences in chromatin composition between wild-type and ctf18Δ cells. We took advantage of recent advances in quantitative protein mass spectrometry, which provide the tools to enable the analysis of entire chromatin composition in an unbiased way. Here, we have applied stable isotope labeling by amino acids in cell culture (SILAC)-based quantitative proteomics (30) for comparison of chromatin components from wild type and ctf18Δ _Saccharomyces cerevisiae_. Budding yeast provides an ideal organism for developing this work because of its small, relatively well-characterized proteome. This novel approach has provided us with a large-scale view of changes in chromatin composition in a ctf18Δ mutant, and revealed that the Ctf18-RLC complex acts in the same pathway as Mrc1 to mediate the DNA replication checkpoint.

**EXPERIMENTAL PROCEDURES**

_Yeast Strains—_ _S. cerevisiae_ strains used are listed in supplemental Table S1. SHY201 was generated by sporulation of the diploid strain BY4743 and selection of a MATa lysine auxotroph, followed by disruption of _ARG4_. To construct TKY1, a _ctf18Δ::kanMX4_ construct was PCR-amplified from the relevant EUROSCARF gene deletion strain and transformed into SHY201. TKY18, TKY130, and TKY131 were constructed in the same way, using _elg1Δ::kanMX4_ and _rad9Δ::kanMX4_ fragments. A strain carrying a deletion of the entire _MRC1_ gene was created by PCR-based one-step gene replacement using pFA6a-kanMX6 as a template (31). Myc-, FLAG-, and HA-tagging was carried out using standard PCR-based gene insertion methods (31). Disrupted and tagged alleles were confirmed by PCR. Primer sequences are available on request.

_SILAC Labeling—_ For lysine and arginine double labeling, _lys2Δ_ arg4Δ strains TKY1, TKY18, or TKY111 were grown for at least ten generations in “heavy” medium, which is synthetic yeast medium containing: 6.9 g/lL yeast nitrogen base without amino acids (FORME-DIUM), 1.85 g/lL amino acid dropout mixture without arginine and lysine (Kaiser formulation, FORMEDIUM), 2% glucose, 15 mg/lL [5-^13^C]l-arginine and 30 mg/lL [5-^15^N]l-lysine. SHY201 cells were grown in “light” medium, containing 15 mg/lL l-arginine and 30 mg/lL l-lysine. For lysine single labeling, TKY1 or SHY201 cells were grown for ten generations in the same synthetic medium but containing 15 mg/lL l-arginine and either 30 mg/lL [5-^13^C]l-lysine or 30 mg/lL [5-^15^N]l-lysine.

_Cell Synchronization and Release—_ Cells were grown in heavy or light media at 30 °C to early log phase (3–10^6^ cells/ml), and synchronized by treating with 5 μg/ml α-factor for 2.5 h at 30 °C. For release into HU, cells were spun down, washed once in heavy or light medium, re-suspended in HU-containing heavy or light medium, and incubated with shaking for 1.5 h at 30 °C. To release cells from α-factor arrest into normal S phase, cells were synchronized in the same way and resuspended in heavy or light media lacking HU. The cultures were then incubated with shaking at 30 °C and harvested at a mid-S phase time point, as determined by flow cytometry analysis. Flow cytometry was carried out using SYTOX Green nucleic acid stain (Invitrogen Molecular Probes) as previously described (32).

_Preparation of Chromatin-enriched Fraction—_ Chromatin-enriched fractions were prepared according to Sheu et al. (33), modified to incorporate a nuclear isolation procedure (34). Approximately 4 × 10^6_ cells (~1 × 10^7_ cells/ml) were harvested and resuspended in 10 ml of
prespheroplasting buffer (100 mm PIPES/KOH, pH 9.4, 10 mm dithio- treitol (DTT), 0.1% sodium azide) then incubated for 10 min at room temperature, followed by incubation in 10 ml of spheroplasting buffer (50 mm KH2PO4/K2HPO4, pH 7.4, 0.6 mM Sorbitol, 10 mM DTT) containing 200 μg/ml Zymolyase-100T and 5% Glusulase at 37 °C for 30 min with occasional mixing. Spheroplasts were washed with 5 ml of 20 mM -glycerophosphate, 1 mM phenyl-methylsulfonyl fluoride (PMSF), Protease inhibitor tablets (EDTA free, Roche)) and resuspended in 5 ml of ice-cold wash buffer. The suspension was overlaid onto 5 ml of 7.5% Ficoll-Sorbitol cushion buffer (7.5% Ficoll, 20 mM KH2PO4/K2HPO4, pH 6.5, 0.6 mM Sorbitol, 1 mM MgCl2, 1 mM DTT, 20 mM -glycerophosphate, 1 mM PMSF, Protease inhibitor tablets) and the spheroplasts were spun through the cushion buffer at 5000 rpm for 5 min to remove proteases derived from Zymolyase-100T. The pelletted spheroplasts were resuspended in 200 μl of ice-cold wash buffer and dropped into 14 ml of 18% Ficoll buffer (18% Ficoll, 20 mM KH2PO4/K2HPO4, pH 6.5, 1 mM MgCl2, 1 mM DTT, 20 mM -glycerophosphate, 1 mM PMSF, Protease inhibitor tablets, 0.01% Nonidet P-40) with stirring. At this stage, it was confirmed microscopically that the cytoplasmic membranes were lysed, but that nuclei and vacuoles (often attached together) were intact. The suspension was subjected to 10 strokes with a loose-fitting pestle in a Potter-Elvehjem homogenizer (which releases nuclei from vacuoles and improves recovery of nuclei). Unbroken cells were removed by two low-speed spins (5000 × g for 5 min at 4 °C). Nuclei were then pelleted by a high-speed spin (16,100 × g for 20 min) and the cytoplasmic fraction removed. After washing nuclei in ice-cold wash buffer, the nuclei were resuspended in 200 μl of Extraction Buffer EB (50 mm HEPES/KOH, pH 7.5, 100 mm KCl, 2.5 mM MgCl2, 0.1 mM ZnSO4, 2 mM NaF, 0.5 mm spermidine, 1 mM DTT, 20 mM -glycerophosphate, 1 mM PMSF, Protease inhibitor tablets) and lysed by addition of Triton X-100 to 0.25%, followed by incubation on ice for 10 min. The lysate was overlaid on 500 μl of EBX-S buffer (EB buffer, 30% sucrose, 0.25% Triton X-100), and spun at 12,000 rpm for 10 min at 4 °C. The top layer (nucleoplasmic fraction) was removed and the chromatin pellet was washed in 1 ml of EBX buffer (EB buffer, 0.25% Triton X-100) and spun at 10,000 rpm for 2 min at 4 °C. The chromatin pellet was resuspended in 40 μl of 1.5× Tris-Glycine SDS Sample Buffer and incubated for 2 min at 85 °C, followed by spinning at 10,000 rpm for 30 s before loading on a Novex 8–16% Tris-Glycine Gel (Invitrogen). Whole-cell extract was prepared by lysing the washed spheroplasts in EBX buffer. Protein concentration of whole cell extract was measured using Qubit Fluorometer and Quant-IT Protein Assay Kit (Invitrogen). Protein concentration of chromatin fraction was calculated by comparing intensities of protein bands of chromatin fraction with those of whole cell extract on SYPRO Ruby (Bio-Rad) stained gel. S phase chromatin was prepared in the same way (see above) with the following adjustments: sodium azide was added directly to the culture to a final concentration of 0.1%, and the culture was immediately chilled on ice for 10 min. After harvesting, the cells were resuspended and incubated in prespheroplasting buffer for 10 min on ice (instead of at room temperature). 0.1% sodium azide was included in the spheroplasting buffer.

Wild-type and ctf18Δ chromatin were prepared separately and then mixed, to avoid complications arising from differences of these strains in cell size and susceptibility to spheroplasting enzymes.

**Mass Spectrometry and Data Analysis**—Equal amounts (60 μg each) of proteins from chromatin-enriched fractions (differentially-labeled with isotopes) were mixed and size-fractionated by one-dimensional SDS-PAGE (Novex 8–16% Tris-Glycine Gel, Invitrogen). Proteins were visualized by colloidal Coomassie staining (Colloidal Blue Staining Kit, Invitrogen), and the entire protein gel lane was excised and cut into 12 slices. The gel slices were destained in dH2O and 20 mM NH4HCO3. Each gel slice was subjected to in-gel digestion with trypsin (Trypsin Gold, Promega) for lysine- and arginine-labeled proteins or Lys-C (Lysyl Endopeptidase, Wako) for lysine-labeled proteins (35). The resulting peptides were extracted and analyzed in automated LC-MS/MS as described previously (36). Mass spectrometry analysis was performed using a nanoflow high performance liquid chromatography system connected to a linear ion trap-orbitrap hybrid mass spectrometer (linear trap quadrupole-Orbitrap XL or Velos, Thermo Fisher Scientific Inc) via a nanoelectrospray ion source (Proxeon Biosystems) (36).

Quantitation was performed using the program MaxQuant (version 1.0.12.31. or 1.0.13.13) (37). The derived peak list generated by Quant.exe (in the first part of MaxQuant) was searched using the Mascot search engine (Matrix Sciences, version 2.2.2) for peptide identifications against the yeast GenBank database (released May 2006), containing 11,168 S. cerevisiae protein sequences with the addition of 175 commonly observed contaminants and all the reversed sequences. The initial mass tolerance was set to 7 ppm, and tandem MS (MS/MS) mass tolerance was 0.5 Da. The enzyme was specified as trypsin or Lys-C, with a maximum of two missed cleavages. Carbamidomethylation of cysteine was searched as a fixed modification, whereas N-acetyl protein and oxidation of methionine were searched as variable modifications. As discussed in Cox et al. (37), peptides and proteins were accepted in reverse order of their PEP (probability of false hit) scores whereas the number of forward database identifications remained 100-fold higher than the number of reverse database identifications (i.e. until reverse identifications exceeded 1% of those accepted), thus resulting in a false discovery rate of 1%. Because the use of MaxQuant software has significantly improved reliability and accuracy of peptide quantitation and assignment to proteins (37), proteins were considered to be identified if represented by at least one unique peptide, and were considered quantified if they had at least one quantified SILAC pair. The data quality (in particular, the number of unique peptides and number of quantification events for each protein) was however an important additional parameter when considering the confidence to be given to specific results. Taking account of the data quality, we indicate the names of a selected number of proteins that were quantitated by at least two peptides in Figs. 3A, 4B, 6D, 7A, and 7C.

**Categorization of Proteins**—Protein categorization annotations are generally according to the Gene Ontology Cellular Component in the Saccharomyces Genome Database. Where Gene Ontology Cellular Component makes multiple assignments for a particular gene product, it was assigned to the category appearing first in the following list: Chromosome, Nucleolus, Nucleoplasm, Cytoplasm, Other. For example, a protein with a Gene Ontology Cellular Component annotation “chromosomal, nuclear, and cytoplasmic” was assigned to the category “chromosome” (and not to “nucleoplasm” or “cytoplasm”). Potential proteins encoded by dubious open reading frames were included in the category “Other.” Some probable chromatin proteins (e.g. transcription, chromatin remodelling and repair proteins, such as RNA polymerase components, SWI/SNF, SAGA, and RSC subunits, “Rad” proteins, Mec1, and Tel1) were manually re-allocated from “nucleoplasm” to “chromosome” categories.

**Quantification of Proteins in Chromatin-enriched Fractions by Western Blotting**—Whole cell extracts and chromatin-enriched fractions were prepared from HU-arrested cultures of epitope-tagged strains as described above. Samples were electrophoresed on NuPAGE 4–12% Bis-Tris gels (Invitrogen) gels and blotted on PVDF membrane (Hybond-P, GE Healthcare). Antibodies used in detection of epitope-tagged proteins were mouse anti-HA (HA.11, Covance Research Products, Princeton, NJ) and mouse anti-Myc (ab32, Abcam, Cambridge, MA). Antibodies used in detection of Mcm2, Adh1, and histone H3 were goat anti-Mcm2 (sc-6680, Santa Cruz, Santa
RESULTS

Workflow for Quantitative Analysis of Chromatin Components in Wild-type and ctf18Δ Cells—To understand the in vivo function of Ctf18-RLC, we used SILAC-based comparative proteomics (30) to compare chromatin composition in wild-type and ctf18Δ cells (Fig. 1A). ctf18Δ cells were grown in “heavy” media, i.e., containing 13C/15N-substituted arginine and/or lysine; complete labeling of cellular proteins was facilitated by the use of lysine and arginine auxotrophic mutants (lys2Δ arg4Δ). Wild-type cells were grown in “light”, i.e., unlabeled (12C/14N) media. Both wild-type and ctf18Δ cultures were synchronized during DNA replication by release from G1 phase arrest into medium containing the replication inhibitor HU. Chromatin proteins were prepared from wild-type and ctf18Δ cells separately (see Fig. 2A), mixed and then size-fractionated using SDS-PAGE, digested, and subjected to LC-MS/MS analysis, allowing calculation of heavy/light ratios for the peptides and proteins identified. Strains used are TKY1 and SHY201. B, Specimen MS spectrum for a PCNA peptide, showing increased loading of PCNA on chromatin in the ctf18Δ strain.

Immunoprecipitation—Approximately 2 × 10⁸ spheroplasts were resuspended in 600 μl of lysis buffer (50 mM HEPES/KOH, pH 7.5, 150 mM NaCl, 3 mM MgCl₂, 10% glycerol, 0.2% Nonidet P-40, 10 mM NaF, 20 mM β-glycerophosphate) containing protease inhibitors (1× Complete (Roche), 1% Protease Inhibitor Mixture (Sigma P8215)). Lysates were treated with 200 units of DNase I (Roche) at 4 °C for 30 min and then centrifuged to produce cleared whole cell extract. This extract was incubated with 2 μl of anti-FLAG M2 antibody (Sigma) conjugated with 20 μl of Dynabeads Protein G (Dynal) at 4 °C for 2 h. Samples were washed four times with lysis buffer, and then resuspended in SDS sample buffer. To examine Mcm4 binding for Cdc45 and Pol1, EBX buffer (as used for chromatin fractionation) or low salt buffer (50 mM HEPES/KOH, pH 7.5, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, 10% glycerol, 0.1% Nonidet P-40, 2 mM NaF, 2 mM β-glycerophosphate, 1× Complete (Roche), 1% Protease Inhibitor Mixture (Sigma P8215)) was used instead of lysis buffer. Cdc45 protein was detected using sheep polyclonal anti-Cdc45 antibody (kindly gifted from Dr. Karim Labib).

Two-dimensional Gel—Genomic DNA was prepared as described (38, 59). DNA fragments digested using EcoRI were separated by neutral/neutral two-dimensional agarose gel electrophoresis (40) and transferred to Neutral membrane (Qbiogene, Illkirch, France) by capillary blotting. EcoRI fragments containing ARS305 and ARS1413 were detected using suitable 32P-labeled probes.

Rad53 Phosphorylation—SHY201, TKY1, TKY18, TKY111, TKY130, or TKY131 cells were arrested in G1 phase using α-factor, and released into S phase in the presence or absence of 200 μM HU at 25 °C. Cells were sampled at indicated time points, washed twice with water and incubated in 0.1 M NaOH for 5 min at room temperature. The cells were spun down and resuspended in SDS sample buffer before SDS-PAGE electrophoresis and Western blotting as described above. Rad53 protein was detected using a goat polyclonal anti-Rad53 antibody (sc-6748, Santa Cruz Biotechnology).

Quantitative Proteomic Analysis of ctf18Δ Mutant Chromatin

Cruz, CA), rabbit anti-Adh1 (ab34680, Abcam), and rabbit anti-histone H3 (ab46765, Abcam). Secondary antibodies were alkaline phosphatase (AP)-conjugated anti-mouse IgG (sc-2008, Santa Cruz), AP-conjugated anti-rabbit IgG (S3731, Promega, Madison, WI), and AP-conjugated anti-goat IgG (sc-2022, Santa Cruz). The detection substrate was ECF Western blotting reagent (GE Healthcare). Chemiluminescent signals were scanned by FLA-3000 (excitation, 473 nm; filter, OS80, FUJIFILM) and quantified using Image Gauge V4.21 software. To compare the amounts of proteins in chromatin fractions from wild type and mutant (see Figs. 3, 4, and 7), a standard plot was drawn based on analysis of a dilution series of chromatin from wild type (WT) (e.g., supplemental Fig. S3), and values for experimental samples were measured by placement on that plot. Values were adjusted for variations in loading based on histone H3 signal. To compare chromatin-bound PCNA in wild type and elig1Δ cells by Western blot, chromatin fractions were prepared as described above and PCNA was detected using mouse monoclonal anti-PCNA antibody (ab70472, Abcam).

Immunoprecipitation—Approximately 2 × 10⁸ spheroplasts were resuspended in 600 μl of lysis buffer (50 mM HEPES/KOH, pH 7.5, 150 mM NaCl, 3 mM MgCl₂, 10% glycerol, 0.2% Nonidet P-40, 10 mM NaF, 20 mM β-glycerophosphate) containing protease inhibitors (1× Complete (Roche), 1% Protease Inhibitor Mixture (Sigma P8215)). Lysates were treated with 200 units of DNase I (Roche) at 4 °C for 30 min and then centrifuged to produce cleared whole cell extract. This extract was incubated with 2 μl of anti-FLAG M2 antibody (Sigma) conjugated with 20 μl of Dynabeads Protein G (Dynal) at 4 °C for 2 h. Samples were washed four times with lysis buffer, and then resuspended in SDS sample buffer. To examine Mcm4 binding for Cdc45 and Pol1, EBX buffer (as used for chromatin fractionation) or low salt buffer (50 mM HEPES/KOH, pH 7.5, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, 10% glycerol, 0.1% Nonidet P-40, 2 mM NaF, 2 mM β-glycerophosphate, 1× Complete (Roche), 1% Protease Inhibitor Mixture (Sigma P8215)) was used instead of lysis buffer. Cdc45 protein was detected using sheep polyclonal anti-Cdc45 antibody (kindly gifted from Dr. Karim Labib).

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loading on chromatin is altered in ctf18Δ, the change is reflected in the abundance of heavy-labeled peptides. A specimen MS spectrum for a PCNA peptide is shown in Fig. 1B. This heavy and light peptide pair (SILAC peptide pair) indicates an ~2-fold increase in PCNA in the ctf18Δ chromatin preparation, compared with wild-type. Using MaxQuant software (37), protein ratios are calculated as the median of all SILAC peptide ratios for each protein identified, including a normalization step adjusting for any inequality in protein loading in the two samples.

Validation of Chromatin Preparation—When we used a published chromatin enrichment protocol (33), we found that only 44% of proteins quantitated were nuclear or chromosomal and the remainder were cytoplasmic or other contaminants of the chromatin preparation (data not shown). To refine the analysis, we incorporated a nuclear isolation procedure (adapted from Young et al. (34)) into the protocol (Fig. 2A and supplemental Fig. S1). A chromatin-enriched fraction prepared by this modified procedure was analyzed by SDS-PAGE and SYPRO Ruby staining (Fig. 2B). Western blotting demonstrated that chromatin proteins (the MCM complex subunit Mcm2 and histone H3) were efficiently recovered, whereas the cytoplasmic protein Adh1 was undetectable in the chromatin-enriched fraction (Fig. 2C). With this modified procedure, 56% of proteins identified by mass spectrometry were nuclear or chromosomal, covering 63% (307 out of 491) of chromosomal proteins (Fig. 2D). As a category, chromosomal and nucleolar proteins were recovered and identified in the highest proportions. Many contaminating cytoplasmic proteins were nevertheless quantitated, as expected because our chromatin enrichment procedure does not constitute a full chromatin purification. Because our purpose was to measure, rather than to identify, chromatin proteins, the contaminating cytoplasmic proteins do not interfere with quantification of chromatin components. Thus, we had established a suitable methodology for analyzing chromatin composition using SILAC quantitative proteomics.

Chromatin Binding of DNA Replication Proteins (PCNA, RPA, GINS, and Ctf4), the Checkpoint Kinase Mec1, and Cohesin are Increased in HU-treated ctf18Δ Cells—To emphasize consistent changes within related groups of proteins, we plot graphs showing both heavy/light ratios and relative peptide abundance (i.e. MS peptide intensity) (37, 41). Log2 ratios of the 307 identified chromosomal proteins are plotted against summed MS peptide intensities for each protein (Fig. 3A). Validating the approach, “heavy” peptides derived from Ctf18 protein were not identified, and the protein showing the largest decrease on chromatin in the ctf18Δ mutant was the Dcc1 subunit of Ctf18-RLC (Fig. 3A). The Ctf8 subunit of Ctf18-RLC was not identified in this experiment. The list of chromosomal proteins quantitated is shown in supplemental Table S2. One striking abnormality observed in HU-treated ctf18Δ cells was a two- to fourfold increase in many replisome factors—including PCNA, single-strand DNA-binding proteins Rfa1, Rfa2, and Rfa3, GINS complex proteins Psf1, Psf2, and Psf3, the Ctf4 connector protein that binds GINS and Pol α, and the checkpoint kinase Mec1. We also observed a modest, but reproducible, increase in chromatin loading of the cohesin complex components Smc1, Smc3, and Scc3, and the cohesin establishment factor Pds5 (Fig. 3A). We observed a slight decrease in the telomeric chromatin component Rap1, and Pol α-prime subunits Pol12 and Pri2 (Fig. 3A). Because Rap1 binds telomere repeat sequences, a reduction in chromatin-bound Rap1 is consistent with the shortened telomeres.
observed in ctf18Δ cells (14). Fig. 3B shows a color-coded schematic representation of the changes we observed. We repeated this experiment with minor modifications and observed similar chromatin abnormalities (Supplemental Fig. S2 and Table S3).

To confirm the changes observed, we epitope-tagged specimen proteins and performed Western blotting to examine their representation in chromatin preparations from HU-treated wild-type and ctf18Δ cells. Overall expression levels of Rfa1–3HA, Ctf4–13Myc, PCNA-3Myc, and Psf2–13Myc were similar in wild-type and ctf18Δ/H9004, as assessed by analysis of whole cell extracts (Fig. 3C). However, these proteins were increased, when compared with wild-type, in chromatin-enriched fractions prepared from HU-treated ctf18Δ (Fig. 3C and Fig. 3D).
The levels of increase closely reflect the changes observed by SILAC mass spectrometry (Fig. 3E). In summary, we successfully used SILAC quantitative proteomics to detect abnormalities in ctf18Δ chromatin, observing the most significant change to be increased loading of various replisome components.

Chromatin Loading of Replisome Components did not Increase in the ctf18Δ Mutant in Normal S Phase—ctf18Δ cells show cohesion and telomere defects in normal growth (12, 13), so we tested for chromatin abnormalities in ctf18Δ cells in an unchallenged S phase (“normal” S phase). We used SILAC to compare chromatin isolated from wild-type and ctf18Δ cultures progressing synchronously through normal DNA replication. For these experiments, unlabeled wild-type and labeled ctf18Δ cultures were released from G1 arrest and sampled at a mid-S phase time point (Fig. 4A). Chromatin was then prepared for SILAC-based chromatin profiling as described above. In general, we observed only slight abnormalities in ctf18Δ chromatin composition, and the changes observed on HU treatment were not apparent in normal S phase (Fig. 4B and supplemental Table S4). The levels of cohesin loading appeared very similar to wild-type (supplemental Fig. S4). Chromatin loading of DNA replication proteins (such as RPA components Rfa2 and Rfa3, GINS, and Ctf4) was in general not substantially changed in ctf18Δ when compared with wild type. We did observe a slight increase in loading of Rfa1, and a slight decrease in chromatin loading of the DNA polymerase catalytic subunit Pol3 (Fig. 4B). Loading on chromatin of PCNA was also slightly decreased in ctf18Δ during normal S phase (Fig. 4B, 4C and 4D), in contrast to its behavior in HU-treated ctf18Δ cells where PCNA loading was increased. We repeated this experiment and observed similar changes (supplemental Fig. S5 and Table S5). Taken the results in Figs. 3 and 4 together, we conclude that the increased loading of replisome components onto chromatin in ctf18Δ cells occurs in response to HU, and is not observed in ctf18Δ cells undergoing normal S phase.

Excess Active Helicase Complex is Present in HU-Treated ctf18Δ Cells—GINS and Ctf4 are subunits recruited to the MCM helicase during RPC formation (21). The increased GINS
and Ctf4 observed in the chromatin fraction of HU-treated ctf18Δ cells (Fig. 3) suggested an increase in the proportion of GINS and Ctf4 complexed with MCM (suggesting a larger number of RFCs). To test this possibility, we immunoprecipitated Mcm4–3Flag and compared the amount of Ctf4–13Myc or the GINS subunit Psf2–13Myc that co-immunoprecipitated in wild-type and ctf18Δ cells (Figs. 5A and 5B). We found that the amounts of Ctf4 and Psf2 complexed with Mcm4 were noticeably increased in HU-treated ctf18Δ cells (Figs. 5A, 5B, and 5D).

Next, we examined whether Cdc45 showed increased association with Mcm4. Cdc45 forms another central subunit of the physical connector between the replicative helicase complex and Ctf4 observed in the chromatin fraction of HU-treated ctf18Δ cells. Cells were synchronized in G1 phase (G1) using α-factor, and then released in the presence of 200 mM HU for 90 min (HU). Mcm4–3Flag was immunoprecipitated (Mcm4 IP), followed by analysis of co-precipitated Psf2–13Myc (A), Ctf4–13Myc (B), or Cdc45 and Pol1–6HA (C: upper panels). Immunoblotting detection used anti-Flag, anti-Myc, anti-Cdc45 or anti-HA antibodies. Immunoprecipitation was also performed under low salt conditions (50 mM potassium acetate) (C: lower panels). Strains used are TKY52, TKY78, TKY59, and TKY85. D, Histogram shows ctf18Δ/WT ratios of Mcm4 binding for Psf2–13Myc, Ctf4–13Myc, Cdc45, and Pol1–6HA. Values were normalized based on the amount of Mcm4–3Flag precipitated in each experiment.

Ctf4 binds to GINS and DNA Pol α and is thought to form a physical connector with the replicative helicase complex Cdc45-MCM-GINS and the DNA Pol α-primase complex (22, 23). Although Ctf4 association with chromatin increased in HU-treated ctf18Δ cells, the SILAC analysis suggested that chromatin association of Pol α-primase subunits, such as Pol1, Pol12 and Pri2, actually decreased (Fig. 3A and supplemental Fig. S4), potentially suggesting an imbalance in replisome components. We used co-IP to examine whether the amount of Pol1 binding to the helicase complex in HU-treated ctf18Δ cells is really reduced. Consistent with the SILAC data, slightly less Pol1 appeared to associate with Mcm4 in HU-treated ctf18Δ when pull-down was carried out under the same buffer conditions as for SILAC chromatin fractionation (Fig. 5C, upper three panels), However, the signal from co-immunoprecipitated Pol1 was weak and close to background level. To improve Pol1 signal strength, we performed immunoprecipitation under lowered salt conditions (50 mM potassium acetate) as described previously (22). With this adjustment, Pol1 signal strength was increased and it was clear that in HU-treated ctf18Δ cells, the amount of Pol1 associated with Mcm4 is elevated (Fig. 5C, bottom and 5D). This observation suggests that in HU-treated ctf18Δ cells the amount of active, replisome-associated Pol1 (and Pol α-primase) is in fact increased, like other RPC components. It appears however that particular characteristics of the Pol α-primase complex preclude identification of this increase by SILAC (see Discussion).

Ctf18 is Required for Full Activation of Rad53 in Response to HU—The hundreds of replication origins in the S. cerevisiae genome initiate replication sequentially according to a temporal program (42). In wild-type cells exposed to HU, initiation at late origins is inhibited by the DNA replication checkpoint pathway. In mutant cells deficient in the DNA replication checkpoint (e.g. mrc1Δ), late origins are activated (26, 29). One possibility is that the excess active helicase complex present in HU-treated ctf18Δ cells is caused by inappropriate initiation and RPC formation at late replication origins, because of a defective DNA replication checkpoint. To test this idea, we examined whether late origin ARS1413 fires in HU-treated ctf18Δ cells using two-dimensional gel analysis. No replication intermediates were detected at ARS1413 in HU-treated wild-type cells, but a clear bubble arc was detected in HU-treated ctf18Δ (Fig. 6A), indicative of initiation at ARS1413 and suggestive of a defect in the replication checkpoint. An even more intense bubble arc at ARS1413 in mrc1Δ cells may reflect differing kinetics in ctf18Δ and mrc1Δ strains of activating the DNA damage checkpoint, which also affects late origins (see below).

HU exposure causes hyperphosphorylation of the checkpoint kinase Rad53 and stimulation of its kinase activity (43). We compared the phosphorylation kinetics of Rad53 in HU-treated wild-type and ctf18Δ cells (Fig. 6B). We found that Rad53 phosphorylation was delayed in HU-treated ctf18Δ cells, similar to the delay in mrc1Δ cells (compare 30 min time points). We conclude that Ctf18 is required for timely activa-

Quantitative Proteomic Analysis of ctf18 Mutant Chromatin

Fig. 5. Increased association of GINS, Ctf4 and Cdc45 with MCM in HU-treated ctf18Δ, when compared with HU-treated wild-type cells. Cells were synchronized in G1 phase (G1) using α-factor, and then released in the presence of 200 mM HU for 90 min (HU). Mcm4–3Flag was immunoprecipitated (Mcm4 IP), followed by analysis of co-precipitated Psf2–13Myc (A), Ctf4–13Myc (B), or Cdc45 and Pol1–6HA (C: upper panels). Immunoblotting detection used anti-Flag, anti-Myc, anti-Cdc45 or anti-HA antibodies. Immunoprecipitation was also performed under low salt conditions (50 mM potassium acetate) (C: lower panels). Strains used are TKY52, TKY78, TKY59, and TKY85. D, Histogram shows ctf18Δ/WT ratios of Mcm4 binding for Psf2–13Myc, Ctf4–13Myc, Cdc45, and Pol1–6HA. Values were normalized based on the amount of Mcm4–3Flag precipitated in each experiment.
tion of Rad53 and efficient DNA replication checkpoint engagement in response to HU. In the absence of the Mrc1-mediated replication checkpoint, HU treatment causes accumulating DNA damage, with the result that Rad53 becomes phosphorylated through the Rad9-dependent DNA damage checkpoint pathway (26). To test whether the somewhat delayed Rad53 activation in HU-treated ctf18Δ cells depends on the Rad9 pathway, we constructed a ctf18Δ rad9Δ double mutant and tested phosphorylation kinetics of Rad53. On HU treatment, phosphorylated forms of Rad53 accumulate in rad9Δ cells, but are hardly detected in the ctf18Δ rad9Δ double mutant in HU (Fig. 6C). These results suggest that Ctf18, like Mrc1, is required to activate the DNA replication checkpoint pathway. In the absence of Ctf18, Rad53 activation occurs only through the Rad9-dependent checkpoint pathway, and probably reflects accumulating DNA damage.

We conclude that the excess active helicase present in HU-treated ctf18Δ cells results from inappropriate initiation at
late replication origins because of a defect in the DNA replication checkpoint.

Alteration of Chromatin Composition in HU-treated ctf18Δ Cells is Similar to that in HU-treated Cells Lacking a Checkpoint Mediator Mrc1—We used SILAC quantitative proteomics to examine the chromatin of HU-treated mrc1Δ cells, to test whether the chromatin abnormalities observed in ctf18Δ cells are similar to those of another checkpoint-deficient mutant. We found increased chromatin association of replication proteins including PCNA, Cdc45, GINS components, Ctf4, and RPA subunits in HU-treated mrc1Δ cells (Fig. 6D and Supplemental Table S6), very similar to the changes observed for the ctf18Δ mutant exposed to HU. The similarity in chromatin profile between HU-treated ctf18Δ and mrc1Δ included a slight increase in chromatin association of the cohesin complex (Fig. 6E). The close resemblance of chromatin profiles of HU-treated mrc1Δ and ctf18Δ mutants supports the suggestion that Ctf18 acts in the same pathway as Mrc1 in the cellular response to HU, affecting chromatin through the DNA replication checkpoint.

HU Treatment of Cells Lacking the Elg1-RLC Reveals a Spectrum of Chromatin Abnormalities that Differs from ctf18Δ—The function of Elg1-RLC, another alternative RFC complex, is not well understood. To investigate the function of Elg1-RLC, we used SILAC to compare chromatin fractions from HU-treated wild-type and elg1Δ cells. There was no significant increase in chromatin association of Cdc45, GINS, Ctf4, RPA, Mec1 and the cohesin complex subunits Scc1 and Scc3 in HU-treated elg1Δ cells (Fig. 7A and supplemental Table S7). This chromatin profile differs sharply from the ctf18Δ mutant and suggests that Elg1 is not essential for activation of DNA replication checkpoint. Interestingly, we observed that chromatin association of PCNA and the flap endonuclease Rad27 (a yeast FEN1 homolog) was substantially increased in HU-treated elg1Δ cells (Figs. 7A and 7B). Rad27 is believed to act in Okazaki fragment processing and maturation. Chromatin association of PCNA and Rad27 was also abnormally high during normal S phase in elg1Δ cells (Figs. 7C and 7D and supplemental Table S8). Increased chromatin loading of PCNA in elg1Δ is consistent with recently published data (44). Finally, we confirmed that the DNA replication checkpoint is intact in elg1Δ cells, by demonstrating that late origin ARS1413 is repressed and Rad53 phosphorylation occurs normally in HU-treated elg1Δ cells (Fig. 7E and 7F). Elg1 is therefore not required for the DNA replication checkpoint, and that the chromatin composition abnormalities seen in elg1Δ presumably result from a different defect. Despite certain phenotypic similarities (7, 8), Ctf18-RLC and Elg1-RLC therefore appear to have distinct in vivo functions in DNA metabolism.

DISCUSSION
To investigate the role of Ctf18-RLC in S phase, we used SILAC-based proteomics to analyze chromatin composition. About 63% of known chromatin proteins were identified and quantified (Fig. 2D). We found significant and reproducible changes in chromatin composition in the ctf18Δ mutant, which were confirmed by Western blot analysis (Fig. 3). The results demonstrate that our quantitative proteomic approach is useful to obtain a large-scale view of changes in chromatin composition.

Ctf18 is Required for DNA Replication Checkpoint Activation—SILAC proteomic analysis revealed increases in chromatin-bound RPC components such as GINS, Cdc45, and Ctf4 in HU-treated ctf18Δ cells, suggesting the presence of excess active DNA helicase complex (Fig. 3). Immunoprecipitation assays confirmed that the amount of Mcm4-bound GINS, Cdc45, and Ctf4 (Fig. 5) was increased, suggesting increased RPC formation and the presence of a larger number of active replisomes. Because this effect was observed only when replication was challenged by HU and not during normal S phase (Figs. 3 and 4), we suspected that the apparent increase in active replisomes might reflect inappropriate late origin initiation because of defective DNA replication checkpoint activation. We found that a late origin is derepressed in HU-treated ctf18Δ cells (Fig. 6A) and phosphorylation of the checkpoint kinase Rad53 is delayed (Fig. 6B), showing that Ctf18 is required for normal activation of the replication checkpoint. The close resemblance of chromatin composition profiles from HU-treated ctf18Δ cells and mrc1Δ cells (Fig. 6E) supports the idea that most of the abnormalities observed in HU-treated ctf18Δ chromatin are caused by defective DNA replication checkpoint activation. Using a different approach Crabbé et al. (45) also recently showed that Ctf18-RLC is required for the DNA replication checkpoint. Our results are moreover consistent with previous data suggesting that the Ctf18-RLC subunits Dcc1 and Ctf8 are required for the DNA replication checkpoint pathway (46), and with the suggestion that lack of human Ctf18-RLC alters the dynamics of replication (47).

The Rad53 phosphorylation that does still occur in the ctf18Δ mutant on HU treatment depends on the Rad9-mediated DNA damage checkpoint. The delay to Rad53 activation in the absence of Ctf18 probably provides a time window allowing initiation at late origins. The fairly slight retardation of Rad53 phosphorylation probably explains why earlier studies of the ctf18Δ mutant (which used less rigorous sampling protocols) did not detect a checkpoint defect (5, 48).

Chromatin Profile Abnormalities of HU-treated ctf18Δ Cells Could Result From a Defective DNA Replication Checkpoint—ctf18Δ cells show defects in sister chromatid cohesion (12). Cohesin loading on chromatin appeared normal in ctf18Δ cells in an unchallenged S phase. Unexpectedly however, we observed slightly increased loading of the cohesin complex on chromatin in HU-treated ctf18Δ cells (Fig. 3 and supplemental Fig. S4). Extra cohesin is therefore loaded onto chromatin when replication is blocked in ctf18Δ cells, when compared with wild-type. In addition to the normal quota of
Quantitative Proteomic Analysis of *ctf18* Mutant Chromatin

**A**

HU-arrested

![Graph showing protein intensities for HU-arrested cells.]

**B**

WCE Ch

- PCNA
- Mcm2
- Adh1
- Histone H3

**C**

Normal S

![Graph showing protein intensities for normal S phase.]

**D**

WT *elg1Δ*

- Async
- α-factor arrested
- mid-S phase

**E**

ARS305 (early origin) ARS1413 (late origin)

**F**

WT *elg1Δ*

| Time (min) | HU | Normal S |
|-----------|----|----------|
| 0         | ![Image](image1) | ![Image](image2) |
| 20        | ![Image](image3) | ![Image](image4) |
| 30        | ![Image](image5) | ![Image](image6) |
| 40        | ![Image](image7) | ![Image](image8) |
| 50        | ![Image](image9) | ![Image](image10) |

**Graphs and images**

1. HU-arrested cell analysis showing changes in protein intensities.
2. Western blot analysis for PCNA, Mcm2, Adh1, and Histone H3 in WCE and chromatin (Ch) extracts.
3. Normal S phase analysis showing protein intensities.
4. Flow cytometry data for WT and *elg1Δ* cells in asynchronous (Async), α-factor arrested, and mid-S phase.
5. DNA replication origin analysis for early and late origins.
6. Western blot analysis for Rad53-P protein levels in HU-arrested and normal S phase conditions.
cohesin loaded in late G1 phase, extra cohesin can be loaded onto chromatin during G2/M phase to reinforce cohesion at sites of DNA damage (49). We suspect that the additional cohesin observed on chromatin in HU-challenged ctf18Δ cells reflects a cellular response to abnormal levels of DNA damage, which may result from the formation of extended tracts of single-stranded DNA in HU-treated ctf18Δ cells because of the replication checkpoint activation defect. Regions of single-stranded DNA have been observed at HU-blocked replication forks in checkpoint-deficient mutants (50). Increased chromatin loading of the repair protein Rad52 in HU-treated ctf18Δ and mrc1Δ cells (supplemental Fig. S2 and Fig. 6D) supports the idea that these mutants accumulate DNA damage when challenged with HU, as does their accumulation of H2A(X) phosphorylation (45). HU-treated mrc1Δ cells display an increase of chromatin-bound cohesin complex similar to that of ctf18Δ (Fig. 6E), consistent with its resulting from the DNA replication checkpoint defect.

The checkpoint kinase Mec1-Ddc2, homolog of human ATR-ATRIP, is recruited to RPA-coated single-stranded DNA in response to HU or DNA damage (28). We therefore suspect that increased chromatin loading of Mec1 is probably because of increased RPA loading in HU-arrested ctf18Δ (Fig. 3), caused by single-stranded DNA accumulating at HU-blocked replication forks in this checkpoint-deficient mutant. The reduction in chromatin-bound Plm2 we observed when either ctf18Δ or mrc1Δ is HU-treated probably also reflects the checkpoint defect. Plm2 is a putative transcription factor either blocked replication forks in this checkpoint-deficient mutant. H2A(X) phosphorylation (45). HU-treated mrc1Δ cells display an increase of chromatin-bound cohesin complex similar to that of ctf18Δ (Fig. 6E), consistent with its resulting from the DNA replication checkpoint defect.

What is the Molecular Activity of the Ctf18-RLC?—In vitro, the Ctf18-RLC can load PCNA onto DNA and also unload it from DNA (9–11). Increased complex formation on chromatin in HU-treated ctf18Δ cells (Fig. 3) might be taken to suggest that Ctf18-RLC unloads PCNA from DNA in vivo. However, because chromatin-associated PCNA also increased in HU-treated mrc1Δ cells (Fig. 6), it is likely that the increase in chromatin-bound PCNA in HU-treated ctf18Δ cells results mainly from loading of PCNA at late origins, possibly mediated by RFC. In contrast to our SILAC results, ChIP-microarray and ChIP-qPCR studies reported decreased PCNA in HU-treated ctf18Δ cells (16). This apparent inconsistency may reflect different quantification methods. In ChIP-microarray and ChIP-qPCR datasets it is difficult to distinguish between PCNA destabilization at replication forks, and replisomes that are themselves abnormally spread out (perhaps as a result of defective checkpoint activation).

During normal S phase in ctf18Δ cells, we did observe a decrease in PCNA loading on chromatin (Fig. 4). This result suggests that Ctf18 may load PCNA during normal S phase, possibly at specific genomic sites. Further experiments will however be needed to test this idea, because reduced PCNA loading could equally be an indirect effect of the ctf18Δ mutation.

It is unclear how the Ctf18-RLC mediates DNA replication checkpoint activation. Because it binds DNA polymerase ε (53, 54), one possibility is that Ctf18-RLC coordinates the replisome components to allow checkpoint activation by RPA and Mec1. Alternatively, the Ctf18-RLC might load or unload a modified form of PCNA required for checkpoint activation.

Distinct Functions of Ctf18-RLC and Elg1-RLC—In contrast to the ctf18Δ mutant, yeast cells lacking Elg1 showed no increase in GINS, Cdc45 and Ctf4 on chromatin, and displayed normal Rad53 activation on HU treatment (Fig. 7). Ctf18-RLC and Elg1-RLC therefore appear to have distinct roles in maintaining genome stability.

Association of PCNA with chromatin was increased in elg1Δ cells both during normal S phase and when replication forks are challenged by HU addition (Fig. 7). This increased chromatin association of PCNA is unlikely to result from inappropriate late origin initiation, because there was no matching increase in Cdc45-MCM-GINS complex formation. The accumulation of PCNA in elg1Δ could potentially result from failure of PCNA unloading by Elg1-RLC (although so far there has been no in vitro demonstration of PCNA unloading by Elg1-RLC). Increased loading of the flap endonuclease Rad27 in elg1Δ cells may suggest involvement of Elg1 in PCNA transactions during Okazaki fragment maturation, a possibility requiring further investigation.

Limitations of SILAC-based Chromatin Profiling—Using SILAC analysis of HU-treated ctf18Δ chromatin we observed changes consistent with increased Cdc45-MCM-GINS-Ctf4 complex formation; however, the amount of Pol α-primase in the chromatin fraction appeared slightly decreased (Fig. 3).
Further investigation of the behavior of Pol α-prime using co-IP experiments, including adjustment of assay conditions, suggested that interaction between the helicase complex and the Pol α subunit is actually increased in HU-treated ctf18Δ cells (Fig. 5C), in a way that resembles the increased association of other RPC components with helicase. Increased Pol α-prime loading was not detected in our chromatin fractionation partly because of salt sensitivity of its interaction with the replisome (Fig. 5D), and partly because background levels of Pol α-prime binding to chromatin reduce the proportional increase observed on genuine replisome formation. Similarly, only small increases were observed in the chromatin association of certain other RPC subunits (e.g. Tof1, Spt16, Pob3, and Top1) in HU-treated ctf18Δ cells (supplemental Fig. S4), probably because the proportion of these proteins bound non-specifically to chromatin obscures genuine increases in their replisome association (i.e. where background association of a protein with chromatin is high, biologically meaningful changes can appear marginal). These cases highlight the importance of considering the limitations of the chromatin enrichment procedure, and the implications for interpreting results.

Conclusion and prospects—Chromatin profiling using SILAC-based proteomics represents the first method to obtain a large-scale view of changes in chromatin composition. This method is particularly useful in highlighting the significance of relatively small changes that nonetheless occur consistently among specific groups of proteins, and in this way has revealed that Ctf18 acts in the DNA replication checkpoint. Our chromatin profiling approach will be very useful to investigate changes in chromatin composition that occur in other mutant cells and in response to drugs.

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