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Mapping ubiquitination sites of *S. cerevisiae* Mcm10

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ABSTRACT

Minichromosome maintenance protein (Mcm) 10 is a part of the eukaryotic replication machinery and highly conserved throughout evolution. As a multivalent DNA scaffold, Mcm10 coordinates the action of proteins that are indispensable for lagging strand synthesis, such as the replication clamp, proliferating cell nuclear antigen (PCNA). The binding between Mcm10 and PCNA serves an essential function during DNA elongation and is mediated by the ubiquitination of Mcm10. Here we map lysine 372 as the primary attachment site for ubiquitin on *S. cerevisiae* Mcm10. Moreover, we identify five additional lysines that can be ubiquitinated. Mutation of lysine 372 to arginine ablates ubiquitination of overexpressed protein and causes sensitivity to the replication inhibitor hydroxyurea in cells that are S-phase checkpoint compromised. Together, these findings reveal the high selectivity of the ubiquitination machinery that targets Mcm10 and that ubiquitination has a role in suppressing replication stress.

1. Introduction

Minichromosome maintenance protein (Mcm) 10 is an essential replication factor required for origin unwinding and DNA synthesis initiation. Multiple independent studies have identified Mcm10 as a major protector of genome integrity [1,2], especially at common fragile sites [3]. Mcm10 binds to replication origins at the end of G1 phase after the Mcm2-7 core helicase has been loaded onto chromatin as an inactive double-hexamer [4–6]. Mcm10 directly interacts with Mcm2-7 dimers and facilitates the remodeling into monomers [7,8]. This process is likely driven by Mcm10's DNA binding activity [9]. The change in Mcm2-7 conformation and the assembly of the mature replicative helicase, the Cdc45:Mcm2-7:GINS (CMG) complex, occur in early S phase through the coordinated action of cell cycle regulated kinases [10,11]. For the duration of DNA replication Mcm10 stays attached to Mcm2-7 and serves as a DNA binding scaffold that links the CMG complex to the polymerization machinery [4]. Other interaction partners at replication forks include the chromosome transmission fidelity protein 4 (Ctf4), DNA polymerase-α (pol-α), the replication clamp, proliferating cell nuclear antigen (PCNA), and the 9-1-1 checkpoint clamp [2]. Mcm10 is required to maintain some of these key factors on chromatin [12]. It is therefore not surprising that partial loss of Mcm10 triggers replication stress in both *S.cerevisiae* and human tissue culture cells. Under those conditions, replication forks move slowly and elicit responses triggered by the accumulation of single-stranded (ss) DNA, a byproduct of fork stalling [13]. In budding yeast, this is counteracted by the activity of a small ubiquitin-like modifier (SUMO) targeted ubiquitin ligase, the action of which facilitates mitotic progression in the presence of incompletely replicated chromosomes [14].

Ubiquitination also regulates the binding between Mcm10 and PCNA, which is dependent on the cell cycle and a PCNA interacting peptide (PIP) box that is buried in the central domain of Mcm10 [15]. Curiously, the PIP box is part of a highly conserved oligonucleotide/-saccharide binding (OB) fold. These β-barrel motifs are common in RNA and DNA binding proteins and form a cleft that allows for the direct interaction with the nucleic acid backbone [16]. Perpendicular to the PIP box, which resides on the third β-sheet of the OB-fold, is a hydrophobic patch, the Hsp10-like domain that has been implicated in the binding of pol-α [9]. Due to the spatial arrangement of the PIP box and the Hsp10-like motifs, the simultaneous interaction of Mcm10 with pol-α and PCNA seems highly unlikely. Indeed, co-immunoprecipitation experiments revealed that Mcm10 has to be ubiquitinated in order to interact with PCNA, whereas pol-α physically associates only with the unmodified form of Mcm10 [15]. A previous study had suggested that two lysines of Mcm10 are mono-ubiquitinated [15]. Here, we identify lysine (K) 372 as the primary site and map several alternative sites for ubiquitin attachment on Mcm10 in *S. cerevisiae*.

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2. Materials and methods

2.1. Strains and plasmids

Yeast strains used in this study are isogenic derivatives of W303. To overexpress wild type Mcm10 or Mcm10 mutants, cells were transformed with galactose inducible plasmids expressing histidine (His) and hemagglutinin (HA) tagged Mcm10. A copper inducible ubiquitin plasmid (YEp105) was transformed to overexpress ubiquitin [15]. To express the *MCM10* transgene under the control of its endogenous promoter, the coding sequence and 372 bp of upstream promoter sequence were cloned into pRS316 [17]. To express the C-terminally tagged 3HA- and His_8-Mcm10 from the endogenous locus, pRS406 integration plasmids were used, which contained the 3′ half of the *MCM10* gene with the respective epitope tags. pRS406 constructs were linearized and transformed into the desired strains. Point mutations were introduced by a QuikChange Lightning Site Directed Mutagenesis Kit (Agilent Technologies). All strains used in this study were verified by DNA sequencing of genomic DNA and are listed in Table S1.

2.2. Protein overexpression and nickel affinity purification

Ubiquitin expression was induced with 100 μM Copper from the beginning of log phase (OD=0.2) to mid-log phase (OD=0.6) [18]. 2% galactose was added to induce Mcm10 expression at mid-log phase and cells were grown for 3 h. Cells were harvested and lysed with 1.85 M NaOH and 7.5% β-mercaptoethanol. Total protein was precipitated with 55% percent trichloroacetic acid (TCA) and resuspended in buffer A (8 M urea, 300 mM NaCl, 0.5% NP-40, 50 mM Na_2HPO_4, and 50 mM Tris). Lysate was bound to Ni-NTA beads (QIAGEN) at a ratio of 100 μl slurry per 1 mg protein. After overnight binding, three separate washes were applied with buffer A. The first wash was carried out with 10 bead volumes of buffer A' (pH=8). The second wash was applied with 10 bead volumes of buffer A' (pH=6.3). The third wash was performed with 10 bead volumes of buffer A' (pH=6.3) to which 10 mM imidazole was added. Protein was eluted with 5 bead volumes of buffer B (8 M urea, 200 mM NaCl, 2% SDS, 50 mM Na_2HPO_4, 10 mM EDTA, 50 mM Tris pH=4.3). Eluates were concentrated to 500 μl-1 mL with Amicon filter units and fractionated on SDS-PAGE.

2.3. In-gel trypsin digestion

Comassie Brilliant Blue stained bands of interest were cut into 1 × 1 mm pieces and destained with destaining solution (25 mM ammonium bicarbonate, 50% acetonitrile) for 1 h. Samples were then treated with reducing buffer (25 mM ammonium bicarbonate, 50 mM (Tris [2-carboxyethyl] phosphine) for 1 h, and alkylation buffer (25 mM ammonium bicarbonate, 100 mM iodoacetamide) for 1 h. Alkylated gel pieces were washed with 25 mM ammonium bicarbonate and digested with 10 μg/μl trypsin overnight at 37 °C. Bands of interest were extracted by 1.67% formic acid and 66.7% acetonitrile, lyophilized and sent to the Center for Mass Spectrometry and Proteomics at the University of Minnesota for mass spectrometry analysis.

2.4. Mass spectrometry analysis

The peptide mixtures were desalted using the Stage Tip protocol [19] and approximately 1 μg was injected into a capillary liquid chromatography (LC) column online with a mass spectrometer. We analyzed the samples on an Orbitrap XL system with LC-tandem mass spectrometry (LC-MS/MS) with the following MS acquisition specifications: data dependent acquisition (DDA) was performed on the top 6 most intense ions from MS1 scans, ion trap automatic gain control setting was 3000 and the dynamic exclusion time period was 20 s. We analyzed two samples from a replicate experiment on an Orbitrap Velos system as described previously [20] with the following exceptions to the MS acquisition method: DDA on the top 10 most intense peaks detected in MS1 mode with CID (collision induced dissociation) activation at 35% normalized collision energy and 30 msec activation time, automatic gain control at 1 × 10E4 and 100 msec maximum injection time; dynamic exclusion duration was 30 s.

2.5. Data base searching

We used two data base search programs for tandem mass spectral interpretation. MSconvert (http://proteowizard.sourceforge.net/) was used for the conversion of .RAW files to mzXML files. mzXML files were converted to DTA files with an in-house tool (https://github.com/jmchilton/tint/). We searched the spectra with Sequest v27 rev12 (Thermo Fisher, San Jose, CA) [21] against the NCBI RefSeq yeast protein database from 2011 concatenated with a protein contaminants database in a combined forward and reversed fashion for false discovery rate calculations (13388 total entries). Search parameters were: precursor mass tolerance 0.1 Da, product ion mass tolerance 0.8 Da, fixed modification carbamidomethyl cysteine, variable modifications methionine oxidation (15.9949), lysine ubiquitination (114.0429) and 2 missed cleaves per peptide. Criteria for protein identification were as follows:

Scaffold (version Scaffold_4.3.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95% probability by the Peptide Prophet algorithm [22]. Protein identifications were accepted if they could be established at greater than 95% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm [23]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. We also used PEAKS® Studio software [24] v 7.0 (Bioinformatics Solutions, Inc, Waterloo, ON CA) for interpretation of tandem mass spectra and protein inference. We used the following parameters in PEAKS®: merge spectra within 15 ppm precursor mass tolerance and 0.2 min retention time, correct precursors between charge states 2 and 9, filter quality setting > 0.5; de novo precursor mass error 20 ppm and fragment mass error 0.8 Da, trypsin specificity, fixed modification carbamidomethyl cysteine, variable modification oxidation of methionine, 3 maximum variable modifications; database search precursor mass error 50 ppm and fragment mass error 0.8 Da, enzyme and standard modification settings identical to de novo parameters, maximum 3 missed cleave sites, maximum 4 variable modifications per peptide, NCBI RefSeq yeast database (6/18/13) combined with contaminant proteins (http://www.thegpm.org/cRAP/index.html) and FDR estimation enabled; PTM module parameters included: pyro-glutamic acid (−17.0265), N and Q deamidation (0.9840), M dioxidation (31.9898), K ubiquitination as GG (114.0429). Only peptides were considered that were identified more than once, unless noted otherwise.

2.6. Protein preparation and immunoblotting

Protein extract was prepared from asynchronous yeast cultures. Total protein was obtained by trichloroacetic acid (TCA) precipitation, fractionated by SDS-PAGE and subsequently transferred onto nitrocellulose membrane. HA- or His-tagged Mcm10 was visualized using a horseradish peroxidase (HRP)-conjugated anti-HA (Roche 3F10). LexA tagged Mcm10 was detected with an anti-LexA antibody (Abcam, ab14553).