Introduction

In order for DNA replication to occur, the DNA duplex strands need to be separated by a replicative helicase [1]. Cellular replicative helicases tend to be hexameric rings that bind DNA within their central channels [2,3]. The ring shape is thought to maintain association with DNA thus enhancing the processivity of the helicase [4], and may be important for DNA unwinding by potentially excluding one strand from the central channel [5,6]. Regardless of the exact mechanism for DNA unwinding, the helicase must use nucleotide binding and hydrolysis to translocate along the bound DNA.

X-ray structures of homo-hexameric replicative helicases that are members of the AAA+ family, including the superfamily 3 (SF3) helicase from bovine papillomavirus (E1) and mini-chromosome maintenance (MCM) from archaeal species, provide insight into how DNA translocation is achieved [7–10]. Notably, a β hairpin from each subunit projects into the central channel of the helicase. The structure of the E1 hexameric helicase with single-stranded DNA in its central channel identifies residues at the tip of the hairpin that contact the sugar phosphate backbone; in particular a lysine side-chain forms a salt-bridge with the DNA backbone [11]. ATP binding and hydrolysis are thought to drive conformational changes, leading to a sweeping motion of the β hairpins that moves DNA through the central channel [9]. Later structures of archaeal MCM proteins demonstrated the existence of the β hairpins with a lysine residue near the tip [7,10]. These hairpins are referred to as the pre-sensor 1 (PS1) hairpins due to their position adjacent to the sensor 1 motif of the AAA+ domain as shown for the Sulfolobus solfataricus (Sso) MCM (Figure 1).

In eukaryotic cells, the replicative helicase is comprised of six paralogous proteins of the AAA+ family, termed Mcm2-7. Each of the six subunits is essential for DNA replication in cells from yeast to mammals [13,14]. The requirement for six distinct subunits may reflect the greater need for control of DNA replication and hence cell proliferation in eukaryotic cells compared to other systems. Indeed, the Mcm2-7 subunits are differentially targeted by protein kinases for control of cell proliferation [15–25], and have distinct roles in the activity of the intact complex [26]. In this regard, ATP sites found within each of the Mcm subunits are formed at the interface of neighboring subunits, and contribute differently to the overall ATPase activity of the complex [26–28]. Not all of the ATP sites are essential for DNA unwinding, even though the ATP sites are essential for viability [28–31]. Models for DNA unwinding by the homo-hexameric helicases suggest each subunit makes an identical contribution. This is not the case for Mcm2-7 as suggested by the distinct sequences of the components.

The pre-sensor 1 (PS1) hairpin is found in ring-shaped helicases of the AAA+ family (ATPases associated with a variety of cellular activities) and is implicated in DNA translocation during DNA unwinding of archaean mini-chromosome maintenance (MCM) and superfamily 3 viral replicative helicases. To determine whether the PS1 hairpin is required for the function of the eukaryotic replicative helicase, Mcm2-7 (also comprised of AAA+ proteins), we mutated the conserved lysine residue in the putative PS1 hairpin motif in each of the Saccharomyces cerevisiae Mcm2-7 subunits to alanine. Interestingly, only the PS1 hairpin of Mcm3 was essential for viability. While mutation of the PS1 hairpin in the remaining MCM subunits resulted in minimal phenotypes, with the exception of Mcm7 which showed slow growth under all conditions examined, the viable alleles were synthetic lethal with each other. Reconstituted Mcm2-7 containing Mcm3 with the PS1 mutation (Mcm3K499A) had severely decreased helicase activity. The lack of helicase activity provides a probable explanation for the inviability of the mcm3K499A strain. The ATPase activity of Mcm2-7K499A was similar to the wild type complex, but its interaction with single-stranded DNA in an electrophoretic mobility shift assay and its associations in cells were subtly altered. Together, these findings indicate that the PS1 hairpins in the Mcm2-7 subunits have important and distinct functions, most evident by the essential nature of the Mcm3 PS1 hairpin in DNA unwinding.

Abstract

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were constructed in a two-step process. The promoter region was amplified for each of the MCM genes. MCM2, MD89/MD90, and MD90/MD91 were used to amplify MD81/MD82, MD83/MD84, MD85/MD86, MD87/MD88, MD89/MD90, and MD90/MD91 were amplified by PCR using a UTR primer and a start primer, then inserted into YCplac111 using the restriction sites in Table 1. The remaining coding region was amplified by PCR using oligonucleotides and cloned into YIplac211 (pMD241). pMD241 was digested with SmaI and a BpiI linker inserted to generate pMD260. The remaining coding sequence of MCM7 was inserted from a pET expression plasmid [27] using Sacl and BglII to give pMD261. To generate mcm3K499A (pMD391; MD411/M412), mcm5K506A (pMD441; MD34/435), mcm6K665A (pMD413/M414), mcm5K499R (MD50) and mcm3K499A (MD612/M613), mcm3K499A (MD558; MD611/M662), and mcm3K499A (MD559; MD663/M664) YCplac111, the Stratagene QuickChange site-directed mutagenesis kit was used with the indicated primer pairs. Isolated clones were sequenced.

To clone MCM3 (pMD562) and mcm3K499A (pMD563) into YEpplac101, a SphI-SacI fragment of pMD255 and pMD300 was ligated into the same sites of YEpplac101. A fragment of SphI-Sacl from pMD306 was also ligated to the same sites in YIplac211 to generate mcm3K499A-YIplac211 (pMD561). For cloning mcm4K658A into YIplac211, we first amplified mcm4K658A by PCR using oligonucleotides MD85, MD274, and mcm4K658A-YCplac111 as templates. This product was cloned into YIplac211 using PstI and BamHI to give pMD439. For mcm5K499A, a BpiI site was introduced into YIplac211 at the Smal site. mcm5K499A was introduced into this plasmid from mcm5K499A-YCplac111 as a SphI-BpiI fragment. pMD440 was constructed by inserting a SphI-BamHI fragment of mcm6K665A-YCplac111 into YIplac211. To generate MCM3 and mcm3K499A myc8 N-terminally tagged expression plasmids, MCM3 and mcm3K499A were amplified by PCR using

and the different ATPase activity of subunit pairs [26,27]. However, the exact contribution each subunit makes to the DNA unwinding by Mcm2-7 is currently unknown.

Here, we have mutated the conserved lysine residue in the PS1 hairpin of each of the S. cerevisiae Mcm2-7 subunits to alanine and examined the effect of the mutations. Interestingly, only the PS1 hairpin of Mcm3 is essential for viability. Mutation of the PS1 hairpin in Mcm7 resulted in growth related phenotypes, and strains with pairwise mutations in the remaining PS1 hairpins displayed synthetic slow or lethal interactions. Consistent with the observed loss of viability, Mcm2-7 complexes containing Mcm3 bearing the PS1 mutation (Mcm3K499A) show decreased DNA unwinding in vitro. The Mcm3K499A-containing Mcm2-7 has reduced binding to single-stranded DNA in an electrophoretic mobility shift assay, and analysis of Mcm3K499A in yeast cell extracts revealed differences in its molecular associations. Together our results indicate the importance of the PS1 hairpins in Mcm2-7 function, and identify an essential function of Mcm3.

Materials and Methods

Plasmids

Oligonucleotides used to construct plasmids are listed in Table 1. For plasmid shuffling of the MCM genes, two plasmids for each wild-type gene were constructed (plasmids are listed in Table 2). The first set (pMD264, 245, 244, 227 238 and 228, representing wild-type gene were constructed (plasmids are listed in Table 2). For plasmid shuffling of the MCM genes, two plasmids for each

Figure 1. Structure of the Mcm proteins. Organization of the Mcm proteins in both the linear protein sequence (top) and in the folded proteins, based on the crystal structure of the Solfolobus solfataricus Mcm protein (PDB-ID 3F9W) [7]. The Mcm proteins are members of the AAA+ family of ATPases. The ATPase active sites are formed at the interface between two subunits. The Walker A (red), Walker B (magenta), and Sensor-1 (green) motifs are contributed by one subunit; the Arginine Finger (orange) and Sensor-2 (cyan) motifs are contributed by a second subunit (reviewed in 13). The Pre-Sensor 1 motif (PS1; blue) harbors a conserved lysyl residue at the turn between the two β-strands, and is not directly involved in ATP hydrolysis; this lysyl residue is the subject of the current work. For clarity, the PS2 motif is not indicated on the 3-dimensional structure.
oligonucleotides MD84, MD556, and cloned using NotI and SacI into a derivative of YCplac111 where the DED1 promoter drives expression of a mycN-terminally tagged protein [33]. The pET24a-mcm3K499A was cloned by cutting mcm3K499A-YCplac111 with NdeI and SacI and ligating into the same sites of pET24a.

To construct MCM3 and mcm3K499A Flag3-tagged expression plasmids, a GAL10 promoter containing YCplac111 plasmid (pMD407) was linearized with NdeI and SacI, and ligated into the same sites of pET24a. Oligonucleotides MD659/MD660 were annealed and ligated into linearized pMD407. MCM3 and mcm3K499A isolated from pMD502 and pMD503 were then inserted as NotI-SacI fragments to give pMD560 and pMD554.

Plasmid shuffling

Diploid heterozygous strains containing a KanMX deletion of a mcm gene were obtained from Open Biosystems. The mcm2::his3 disruption strain (MDY54) was a derivative of YMD33 [31]. Each of these was transformed with the relevant MCM-YCplac33 plasmid and sporulated to give MDY16, 17, 40, 41, 70, and 100. mcm deletion haploid strains containing their corresponding MCM-YCplac33 were transformed with a mcmKA-YCplac111 or MCM-YCplac111. The transformed strains were grown in YPD, then plated on 5-FOA-containing media to select for cells that lost the MCM-YCplac33 [34].
Table 2. Plasmids used in this study.

| Plasmid | Description            |
|--------|------------------------|
| pMD264 | MCM2-YCplac33          |
| pMD245 | MCM3-YCplac33          |
| pMD244 | MCM4-YCplac33          |
| pMD227 | MCM5-YCplac33          |
| pMD238 | MCM6-YCplac33          |
| pMD228 | MCM7-YCplac33          |
| pMD229 | MCM3 5′UTR-YCplac111   |
| pMD230 | MCM4 5′UTR-YCplac111   |
| pMD232 | MCM5 5′UTR-YCplac111   |
| pMD240 | MCM6 5′UTR-YCplac111   |
| pMD241 | MCM7 5′UTR-YCplac111   |
| pMD266 | MCM2-YCplac33-pL9U9     |
| pMD235 | MCM3-YCplac111         |
| pMD242 | MCM4 5′UTR-YCplac111   |
| pMD379 | MCM4 YCplac111         |
| pMD237 | MCM5 5′UTR-YCplac111   |
| pMD378 | MCM5-YCplac111         |
| pMD239 | MCM6-YCplac111         |
| pMD260 | MCM7 5′UTR-YCplac111   |
| pMD261 | MCM7-YCplac111         |
| pMD307 | mcm2K665A-YCplac111    |
| pMD386 | mcm3K499A-YCplac111    |
| pMD391 | mcm4K658A-YCplac111    |
| pMD411 | mcm5K506A-YCplac111    |
| pMD358 | mcm6K665A-YCplac111    |
| pMD308 | mcm7K550A-YCplac111    |
| pMD438 | mcm4K658A-Ylap211      |
| pMD439 | mcm5K506A-Ylap211      |
| pMD440 | mcm6K665A-Ylap211      |
| pMD346 | mcm7K550A-Ylap211      |
| pMD466 | mcm3K499A-pET24a       |
| pMD501 | mcm3K499A-YCplac111    |
| pMD558 | mcm3K550A-YCplac111    |
| pMD559 | mcm3K550A-YCplac111    |
| pMD502 | DDE1-myc3′-MCM3-YCplac111 |
| pMD503 | DDE1-myc3′-mcm3K499A-YCplac111 |
| pMD407 | GAL10-YCplac111        |
| pMD554 | GAL10-Flag3′-mcm3K499A-YCplac111 |
| pMD560 | GAL10-Flag3′-MCM3-YCplac111 |
| pMD561 | mcm3K499A-Ylap211      |
| pMD562 | MCM3-YEplac181         |
| pMD563 | mcm3K499A-YEplac181    |

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Yeast strains

All yeast strains are listed in Table 3. Two-step gene replacement was used to integrate PS1 hairpin mutation into the yeast genome [35]: mcm2K665A (MDY225 and 226), MCM3/mcm3K499A (MDY411), mcm7K550A (MDY220), mcm2K665A (MDY221 and 222), mcm6K665A (MDY255 and 254). Each mutation incorporated a unique replacement was used to integrate PS1 hairpin mutation into the yeast genome [35]: mcm2K665A (MDY225 and 226), MCM3/mcm3K499A (MDY411), mcm7K550A (MDY220), mcm2K665A (MDY221 and 222), mcm6K665A (MDY255 and 254). Each mutation incorporated a unique

restriction site (Table 1) for identification. Ylpal211 plasmids were linearized with MsdI (mcm3), AgeI (mcm6), BspEI (mcm3), MloI (mcm6), or BamHI (mcm7) and transformed into BY4743. URA3 positive colonies were grown in YPD liquid media, and then selected on 5-FOA-containing media. PCR amplification of the MCM loci was performed and restriction mapping used to confirm integration of mcm3K499A. The heterozygous diploid strains were sporulated and haploid mcm3K499A mutants isolated.

Imaging yeast overexpression of MCM3 and mcm3K499A

BY4741 transformed with YCplac111-GAL10-MCM3 or YCplac111-GAL10-mcm3K499A was grown in minimal media lacking leucine supplemented with 2% galactose overnight. The overnight cultures were diluted to 10^6 cells/mL with minimal media lacking leucine supplemented with 2% galactose. After two hours cells were imaged under bright field using a Nikon Eclipse Ti microscope. Measurements were taken using NIS Elements Imaging Software.

Proteins

The recombinant Mcm subunits were purified from Escherichia coli and reconstituted into Mcm2-7 as described [27].

Mcm3K499A purification

The mcm3K499A pET24a plasmid was transformed into BL21 DE3 Codon+. Twelve liters of transformed cells were grown in LB media with 100 mg/L of ampicillin, and 25 mg/L of chloramphenicol to a density of A600 = 0.6. Cells were cooled to 15°C and iodoacetamide β-D-thiogalactopyranoside added to a final concentration of 1 mM. Cells were incubated at 15°C for 20 hours prior to collecting the cell pellet. The cell pellet was resuspended in 250 mL of Buffer H (20 mM HEPES, pH 7.5, 2 mM DTT, 10% v/v glycerol, and 0.1 mM EDTA) and lysed at 15000 psi in an Emulsiflex-C3 high pressure homogenizer. Debris was pelleted by centrifugation at 15000 g for 25 minutes and the supernatant decanted. Ammonium sulfate was added to the supernatant (0.25 g/mL) with stirring at 4°C. Ammonium sulfate precipitate was collected by centrifugation, and resuspended with 150 mL of Buffer H. The solution was dialyzed overnight at 4°C in 4 L of Buffer H with stirring and then loaded onto a Fast flow Q Sepharose column equilibrated with Buffer H and washed with 7 column volumes of Buffer H containing 50 mM NaCl. Mcm3K499A was eluted over 10 column volumes in a gradient of 0–500 mM NaCl in Buffer H. Fractions containing Mcm3K499A were collected and dialyzed overnight in 750 mL of Buffer H at 4°C. A single-stranded (ss) DNA-Sepharose column was made by coupling boiled and sonicated salmon sperm DNA to cyanogen bromide-activated Sepharose 4B (GE Life Sciences). The dialyzed solution was loaded onto the ssDNA-Sepharose column equilibrated with Buffer H and washed with 10 column volumes and protein eluted with 10 column volumes of a 0–500 mM NaCl gradient in Buffer H. The fractions containing Mcm3K499A were pooled and ammonium sulfate added (0.3 g/mL) with stirring at 4°C. The precipitate was collected by centrifugation, resuspended and dialyzed against 400 mL of Buffer A (20 mM Tris–HCl, pH 7.5, 2 mM DTT, 10% v/v glycerol, and 0.1 mM EDTA). The solution was loaded onto a MonoQ (GE Life Sciences) column equilibrated with Buffer A containing 50 mM NaCl and the protein eluted over 20 column volumes of a 0-500 mM NaCl gradient in Buffer A. Fractions containing Mcm3K499A were pooled and dialyzed in one liter of Buffer H with stirring overnight at 4°C. The dialyzed solution was loaded onto a MonoS (GE Life Sciences) column equilibrated with Buffer H containing 50 mM NaCl, and the protein eluted over 20 column volumes of a
Western blotting and frozen at −80°C.

**Western blotting**

Western blotting was performed using polyvinylidene difluoride membranes and anti-myc (Sigma-Aldrich) as described by Mutiu et al [36].

**Biochemical assays**

DNA unwinding and ATPase assays were performed essentially as described by Stead et al [25] with the exception that intact complex was used. ATP hydrolysis was assayed using thin-layer chromatography. Each 15-μl reaction contained 1 mM [γ-32P]ATP (20 mCi/mmol; Perkin Elmer Life Sciences), 20 mM Tris–HCl (pH 7.5), 10 mM magnesium acetate, and 2 mM DTT, and 200 nM Mcm2-7. At the indicated times, 2 μl of each reaction was removed and quenched with 2 μl of 50 mM EDTA (pH 8). One microliter was spotted onto a polyethylenglycol cellulose sheet (EM Science), developed in 0.6 M potassium phosphate (pH 3.4), dried, exposed to a PhosphorStorage screen, and scanned with a Storm 860 scanner (GE Healthcare). DNA unwinding measurements were performed with a DNA substrate containing 30 nucleotides of duplex, with 60 nucleotides of single-stranded DNA on one strand and a 5' biotin on the other strand. Each reaction (6 μl) contained 20 mM Tris–HCl (pH 7.5), 10 mM magnesium acetate, 100 μM EDTA, 5 mM DTT, 5 mM ATP, 67 nM streptavidin, 1 nM substrate with 100 nM,
Figure 2. Growth of strains bearing the PS1 hairpin alleles. (A) Alignment of the PS1 hairpin and Sensor 1 in SsoMcm with S. cerevisiae Mcm2-7 using T-Coffee. (B) Plasmid shuffling of Mcm PS1 hairpin mutations. The wild-type Mcm gene (MCM), the Mcm gene with PS1 hairpin mutation (mcmK499A), or the empty LEU2-CEN plasmid (Vector) were transformed into a haploid yeast strain deleted for the genomic copy of the corresponding Mcm gene and containing a copy of the gene on a URA3-CEN plasmid. Transformed yeast were grown overnight at 30°C in YPD media, serially diluted, and then spotted onto a YPD plate or a plate containing 5-FOA. (C) The diploid strain MDY411 (MCM3/mcm3K499A) was sporulated and tetrads dissected. The dissection plates (YPD) were incubated at 30°C. (D) MDY16 (mcm3::KanMX YEpplac33 MCM3 URA3) was transformed with 2 micron plasmid YEplac181 (2-µm Vector), pMD562 (2-µm MCM3), or pMD563 (2-µm mcm3K499A). Transformants were grown overnight at 30°C in YPD media, serially diluted, and then spotted onto a YPD plate or a plate containing 5-FOA.

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Figure 3. Effect of temperature and genotoxic agents on PS1 hairpin mutants. (A) Cultures of yeast strains BY4741 (wild-type), MDY225 (mcm2K633A), MDY220 (mcm4K658A), MDY222 (mcm5K506A), MDY226 (mcm6K665A), and MDY254 (mcm7K550A) were grown overnight in YPD at 30°C, serially diluted 10-fold, spotted onto YPD plates and incubated at either 16°C, 30°C or 37°C. (B) Cultures of yeast strains BY4741 (wild-type), MDY225 (mcm2K633A), MDY220 (mcm4K658A), MDY222 (mcm5K506A), MDY226 (mcm6K665A), and MDY254 (mcm7K550A) were grown in YPD, and 10-fold serial dilutions spotted onto YPD and YPD containing either 0.03% methyl methanesulfonate (MMS) or 200 mM hydroxyurea (HU). Plates were incubated at 30°C. A mec2-1 [51] strain, known to be sensitive to genotoxic stress, was also spotted on the plates.

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200 nM or 400 nM Mcm2-7. Samples were analyzed by native PAGE using an 8% gel in Tris–borate–EDTA buffer.

DNA binding assay

The single-stranded DNA affinity chromatography was performed with a 200 μL single-stranded DNA Sepharose column (see above Mcm3K499A purification). Five micrograms of Mcm2-7 complex were applied to the column in buffer H containing 5 mM ATP and 50 mM NaCl and eluted with buffer containing 5 mM ATP and either 50 mM, 100 mM, 200 mM, 300 mM, 400 mM, or 500 mM NaCl. Each elution was performed twice with one column volume. 24 μL of each fraction was separated by SDS-PAGE (6%). The polyacrylamide gels were stained with colloidal blue stain, and then washed with deionized water to destain the gels for imaging. The destained gels were then silver stained according to protocol provided in Pierce Silver Stain Kit (Thermo Scientific) to detect protein in the column fractions.

The electrophoretic mobility shift assay was adapted from Stead et al. [25]. Briefly, Mcm2-7 and Mcm2-7 3K499A complexes were incubated with 1 nM of 59-32P-labeled oligonucleotide (ATGTCCTAGCAAGCCAGAATTCGGCAGCGTC-(T)60) at 37°C in buffer containing 20 mM Tris–HCl (pH 7.5), 10 mM magnesium acetate, 100 μM EDTA, 5 mM DTT, and 5 mM ATP for 10 minutes. One microgram of anti-Mcm7 antibody (Santa Cruz Biotech) was added to another set of Mcm2-7 samples prior to incubation with radiolabeled oligonucleotide to disrupt Mcm2-7 binding. Four microliters of 12.5% glycerol was added to each reaction and then resolved in a 5% native (Tris–borate–EDTA) polyacrylamide gel (19:1 acrylamide:bis–acrylamide; BioShop Canada) containing 5% glycerol, 0.1% NP-40 and 10 mM Mg(CH3COO)2 at 30 mA for 2.5 hours. The gel was dried, and exposed to film.

Gel filtration chromatography

Proteins extracts were prepared cryogenically as described by Saleh et al. [37]. Five mg of yeast extract prepared in 50 mM sodium phosphate pH 7.0, 150 mM NaCl was loaded at a flow rate of 0.3 mL/min onto a 24 mL FPLC Superose 6HR10/30 column (Amersham Pharmacia Biotech.). Protein from 10 μL aliquots of 250 μL fractions for wild type protein and 10 μL for Mcm3K499A were resolved by SDS-PAGE and proteins detected by western blotting.

Modeling of S. cerevisiae Mcm2-7

Individual Mcm2 through Mcm7 subunits were modeled based on the 4.35-Å resolution structure of Solfolobus solfataricus Mcm (SsoMcm; PDB ID 3F9V; [7]). This was done using a multiple sequence alignment incorporating SsoMcm residues 9 to 603 and residues 204 to 849 of Mcm2; 22 to 744 of Mcm3; 188 to 837 of Mcm4; 25 to 692 of Mcm5; 109 to 839 of Mcm6; and 15 to 728 of Mcm7.

Table 4. Synthetic lethal crosses of mcm PS1 alleles.

| Cross                        | Spore colonies examined | Viable with two mutations | P - value |
|------------------------------|-------------------------|---------------------------|-----------|
| mcm2K633A x mcm4K658A       | 24                      | 0                         | 0.002     |
| mcm2K633A x mcm5K506A       | 21                      | 0                         | 0.007     |
| mcm2K633A x mcm6K665A       | 18                      | 0                         | 0.007     |
| mcm2K633A x mcm7K550A       | 19                      | 0                         | 0.007     |
| mcm4K658A x mcm5K506A       | 24                      | 6                         | 0.161     |
| mcm4K658A x mcm6K665A       | 16                      | 0                         | 0.018     |
| mcm4K658A x mcm7K550A       | 15                      | 0                         | 0.018     |
| mcm5K506A x mcm6K665A       | 19                      | 0                         | 0.007     |
| mcm5K506A x mcm7K550A       | 21                      | 0                         | 0.007     |
| mcm6K665A x mcm7K550A       | 24                      | 0                         | 0.002     |

1 A random spore analysis was performed by isolating individual spore colonies from tetrads.

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The comparative modeling protocol of Rosetta was used to thread the sequences onto the SsoMcm structure, build loop regions and additional domains in the Mcm2 through Mcm7 subunits that were not present in SsoMcm, and refine the overall structure of the subunits [38,39]. A model for the Mcm2-7 hexamer was then assembled by superimposing the N-terminal domains of the Mcm2 through Mcm7 subunits on the N-terminal domains of Methanobacterium thermoautotrophicum hexamer (PDB ID 1LTL; [40]). Molecular graphics were generated using PyMOL (Version 1.5.0.5, Schroédinger, LLC) and electrostatic surface calculations were carried out using PDB2PQR [41] and APBS [42].

Results and Discussion

Effects of PS1 mutations in Mcm2-7 on yeast growth

Each of the Mcm subunits contains a pre-sensor 1 (PS1) hairpin adjacent to the sensor 1 motif of the AAA+ domain (Figure 1). To determine whether the PS1 hairpin motifs are important for the function of Mcm2-7, the replicative helicase in eukaryotic cells, we mutated the conserved lysine residue to alanine in each of the Mcm subunits (Figure 2A). The mutant genes, encoded on LEU2-containing centromeric plasmids, or empty plasmid (vector), were transformed into MDY16 (mcm3Δ YCplac33-MCM3). The transformed yeast were grown overnight at 30°C in liquid YPD, serially diluted, and spotted onto YPD, synthetic complete lacking leucine, and YPD containing 5-FOA. (B) The MCM3 and mcm3K499R plasmid-shuffled strains were grown overnight at 30°C, serially diluted and spotted on YPD and YPD containing 0.03% MMS, 200 mM hydroxyurea (HU), or 20 mM caffeine. The mec2-1 strain was subjected to the same growth assay as a positive control for genotoxic stress [51]. (C) The MCM3 and mcm3K499R plasmid-shuffled strains were grown overnight at 30°C, serially diluted, spotted on YPD, and grown at 16°C, 30°C or 37°C.

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Figure 5. Characterization of mcm3K499R, mcm3K499N and mcm3K499Q alleles. (A) The MCM3, mcm3K499R, mcm3K499N and mcm3K499Q genes encoded on LEU2-containing centromeric plasmids, or empty plasmid (vector), were transformed into MDY16 (mcm3Δ YCplac33-MCM3). The transformed yeast were grown overnight at 30°C in liquid YPD, serially diluted, and spotted onto YPD, synthetic complete lacking leucine, and YPD containing 5-FOA. (B) The MCM3 and mcm3K499R plasmid-shuffled strains were grown overnight at 30°C, serially diluted, and spotted on YPD and YPD containing 0.03% MMS, 200 mM hydroxyurea (HU), or 20 mM caffeine. The mec2-1 strain was subjected to the same growth assay as a positive control for genotoxic stress [51]. (C) The MCM3 and mcm3K499R plasmid-shuffled strains were grown overnight at 30°C, serially diluted, spotted on YPD, and grown at 16°C, 30°C or 37°C.

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Mcm7. The comparative modeling protocol of Rosetta was used to thread the sequences onto the SsoMcm structure, build loop regions and additional domains in the Mcm2 through Mcm7 subunits that were not present in SsoMcm, and refine the overall structure of the subunits [38,39]. A model for the Mcm2-7 hexamer was then assembled by superimposing the N-terminal domains of the Mcm2 through Mcm7 subunits on the N-terminal domains of Methanobacterium thermoautotrophicum hexamer (PDB ID 1LTL; [40]). Molecular graphics were generated using PyMOL (Version 1.5.0.5, Schrödinger, LLC) and electrostatic surface calculations were carried out using PDB2PQR [41] and APBS [42].

Results and Discussion

Effects of PS1 mutations in Mcm2-7 on yeast growth

Each of the Mcm subunits contains a pre-sensor 1 (PS1) hairpin adjacent to the sensor 1 motif of the AAA+ domain (Figure 1). To determine whether the PS1 hairpin motifs are important for the function of Mcm2-7, the replicative helicase in eukaryotic cells, we mutated the conserved lysine residue to alanine in each of the Mcm subunits (Figure 2A). The mutant genes, encoded on LEU2-containing centromeric plasmids, or empty plasmid (vector), were transformed into MDY16 (mcm3Δ YCplac33-MCM3). The transformed yeast were grown overnight at 30°C in liquid YPD, serially diluted, and spotted onto YPD, synthetic complete lacking leucine, and YPD containing 5-FOA. (B) The MCM3 and mcm3K499R plasmid-shuffled strains were grown overnight at 30°C, serially diluted, and spotted on YPD and YPD containing 0.03% MMS, 200 mM hydroxyurea (HU), or 20 mM caffeine. The mec2-1 strain was subjected to the same growth assay as a positive control for genotoxic stress [51]. (C) The MCM3 and mcm3K499R plasmid-shuffled strains were grown overnight at 30°C, serially diluted, spotted on YPD, and grown at 16°C, 30°C or 37°C.

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Figure 5. Characterization of mcm3K499R, mcm3K499N and mcm3K499Q alleles. (A) The MCM3, mcm3K499R, mcm3K499N and mcm3K499Q genes encoded on LEU2-containing centromeric plasmids, or empty plasmid (vector), were transformed into MDY16 (mcm3Δ YCplac33-MCM3). The transformed yeast were grown overnight at 30°C in liquid YPD, serially diluted, and spotted onto YPD, synthetic complete lacking leucine, and YPD containing 5-FOA. (B) The MCM3 and mcm3K499R plasmid-shuffled strains were grown overnight at 30°C, serially diluted, and spotted on YPD and YPD containing 0.03% MMS, 200 mM hydroxyurea (HU), or 20 mM caffeine. The mec2-1 strain was subjected to the same growth assay as a positive control for genotoxic stress [51]. (C) The MCM3 and mcm3K499R plasmid-shuffled strains were grown overnight at 30°C, serially diluted, spotted on YPD, and grown at 16°C, 30°C or 37°C.

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The PS1 Hairpins in Mcm2-7
To further characterize the effects of the PS1 mutations, we examined the growth of viable strains bearing the mutations at different temperatures (Figure 3A). For these experiments the PS1 hairpin mutations were integrated into the genome. At each of the temperatures, the relative growth of \textit{mcm7K550A} was reduced. The \textit{mcm2K633A} containing strain grew somewhat more slowly at 16°C.

Mutations in the Mcm subunits often result in sensitivity to genotoxic agents. Therefore, we examined the growth of each of the strains with a PS1 mutation on media containing the ribonucleotide reductase inhibitor hydroxyurea, or the DNA-damaging agent methyl methanesulfonate (MMS) (Figure 3B). Consistent with its slow growth at different temperatures, strains containing \textit{mcm7K550A} grew slowly on both agents, with a slight sensitivity to MMS noted. Similarly strains bearing the other PS1 mutations were not sensitive to hydroxyurea or MMS.

Our plasmid shuffling experiments indicate that a single mutation of the conserved PS1 lysine (K499) residue in Mcm3 results in loss of viability. In contrast, the homo-hexameric \textit{S. solfataricus} Mcm (SsoMcm) accommodates several subunits with disruptions in catalytic elements and still maintains significant helicase activity [44]. Therefore, we investigated the effect of mutating two different PS1 hairpins in the Mcm2-7 complex. We mated the haploid strains containing individual PS1 hairpin mutations to produce all the possible pair-wise combinations. After sporulating the heterozygous strains, we screened the spore colonies for viable double mutants. Only \textit{mcm4K658A} and \textit{mcm5K506A} were viable (Table 4). These grew more slowly than wild-type or strains containing either \textit{mcm4K658A} or \textit{mcm3K499A} and were more sensitive to hydroxyurea and MMS (Figure 4).

The lysine residue on the PS1 hairpin is predicted to make an electrostatic interaction with the sugar phosphate backbone of DNA to facilitate translocation of DNA and unwinding [8]. We converted the lysine of the PS1 hairpin of Mcm3 to arginine, glutamine, or asparagine to determine whether the charge is important for function. Of the three alleles examined by plasmid shuffling, only \textit{mcm3K499R} supported viability (Figure 5A). The strain containing this allele displayed no overt growth defects when plated on 200 mM hydroxyurea, 0.03% MMS, and 20 mM caffeine (Figure 5B). Additionally, the rate of growth was the same as wild type at 16°C, 30°C and 37°C (Figure 5C). This suggests that the positive charge at residue 499 of Mcm3 is essential for function.

To begin to investigate how Mcm3 K499A disrupts function, we addressed whether its overexpression would have a dominant negative effect. A plasmid expressing \textit{mcm3K499A} or \textit{MCM3} from a \textit{GAL10} promoter was transformed into BY4741 (\textit{MCM3}), and serial dilutions plated onto media containing glucose, raffinose, or galactose. In the presence of glucose or raffinose, where the \textit{GAL10} promoter is transcriptionally repressed or not induced respectively, there was no effect on growth, whereas in galactose-containing media induction of \textit{mcm3K499A} expression resulted in a slow growth phenotype (Figure 6A). In addition, there was an increase of approximately three-fold in cell diameter for the \textit{GAL10-mcm3K499A} transformed strain compared to \textit{GAL10-MCM3} transformed strain when grown in galactose-containing media (Figure 6B and 6C). Based on these observations, overexpression of \textit{mcm3K499A} in the context of a wild type background leads to a dominant negative effect that is likely associated with a cell cycle defect.

The Mcm3 PS1 hairpin is required for DNA unwinding

To determine the biochemical effects of Mcm3K499A on the activity of the complex, we reconstituted it into Mcm2-7 to yield Mcm2-7K499A. Each of the subunits, including Mcm3K499A was
expressed as a recombinant protein in E. coli, and checked for the absence of contaminating nuclease or ATPase activity. Individual Mcm subunits were mixed in equal molar ratios to reconstitute the hexameric complex, the final step of the reconstitution being elution from a gel filtration column. Mcm2-7 3K499A eluted at a volume corresponding to the MCM hexamer, similar to wild-type Mcm2-7 (600 kDa; Figure 7A). We examined the DNA unwinding of wild type and mutant Mcm complexes using a radiolabeled synthetic fork substrate where DNA unwinding is measured as the amount of single stranded DNA liberated from the duplex substrate. At a concentration of 200 nM the wild-type complex converted 1.5 fmoles of substrate to single-stranded DNA in 10 minutes (Figures 7B and 7C). By contrast, the 200 nM concentration of Mcm2-7 3K499A unwound 0.1 fmol of the fork substrate. Therefore Mcm2-7 3K499A has a ∼15-fold reduction in helicase activity, indicating that the Mcm3 PS1 hairpin is critical for DNA unwinding.

The loss of helicase activity in Mcm2-7 3K499A may be due to a role for the Mcm3 PS1 hairpin in the ATPase activity of the complex. Interestingly, of the isolated dimer pairs, the pair of Mcm3 and Mcm7 has the highest ATPase activity, approaching that of the intact Mcm2-7 hexamer [25]. ATP hydrolysis was measured for intact wild-type Mcm2-7 and Mcm2-7 3K499A complexes. As shown in Figure 8A, the ATP hydrolysis rate for Mcm2-7 3K499A was not significantly different from the wild-type Mcm2-7. We next addressed whether Mcm2-7 3K499A is capable of single-stranded DNA binding. Mutant and wild-type complexes were chromatographed on a single-stranded Sepharose affinity...
Figure 8. ATPase activity and DNA binding by Mcm2-73K499A. (A) The reconstituted Mcm2-7 and Mcm2-73K499A complexes were tested for ATPase activity by incubating with \([\gamma^{32}P]\)ATP and measuring the radioactivity of free P, produced at the time points indicated. (B) Elution profile of Mcm2-7 and Mcm2-73K499A from ssDNA-Sepharose. Five micrograms of Mcm2-7 (upper panel), Mcm2-73K499A (middle panel), and the peptidyl prolyl isomerase Pin-1 (lower panel) were chromatographed on a 200 μL single-stranded DNA Sepharose column in the presence of 5 mM ATP. At each step the column was washed twice with 200 μL of buffer containing 50 mM, 100 mM, 200 mM, 300 mM, 400 mM, or 500 mM NaCl. Twenty-four microliters of each fraction was separated by SDS-PAGE (6%) and stained with colloidal blue to detect the load (left) and then silver stain to detect the fractions (right). (C) An electrophoretic mobility shift assay of DNA binding by Mcm2-7 (lanes 3-6) and Mcm2-73K499A (lanes 11–14). \(^{5}\text{P}\) radiolabeled ATGTCTAGCAAGCCAGAATTCGGCAGCGTC(T)\(_n\) was incubated with increasing concentrations (50, 100, 200, or 400 nM) of the Mcm hexameric complexes and separated in a 5% native polyacrylamide gel. Lanes 7-10 included a pre-incubation of the wild-type complex with an anti-Mcm7 antibody. Lanes 1 and 2 show the position of the DNA in the absence of protein, and in the presence of the anti-Mcm7 antibody, respectively. doi:10.1371/journal.pone.0082177.g008
column in the presence of ATP, and eluted with increasing salt concentration. As shown in Figure 8B, wild type Mcm2-7 eluted from this column primarily in the 200 and 300 mM NaCl wash fractions (upper panel). The elution profile for Mcm2-7K499A closely resembled that of the wild type complex (middle panel) indicating that the Mcm2-7K499A is capable of binding single stranded DNA. Lack of binding by the peptidyl prolyl isomerase Pin-1 [lower panel], a relatively basic protein with a pI of 9.4, indicated that the binding by the Mcm complexes was specific for DNA and not simply due to charge interactions. We next used an electrophoretic mobility shift assay in an attempt to detect more subtle differences in DNA binding. A 5’ radiolabeled oligonucleotide of 90 bases was used as the substrate. As shown in Figure 8C (lanes 3–6) increasing concentrations of wild-type Mcm2-7 depleted the substrate band and resulted in the appearance of a discrete band of reduced mobility. To confirm that the band of slower mobility was a Mcm2-7-DNA complex, Mcm7 antibody was pre-incubated with Mcm2-7 prior to addition of radiolabeled oligonucleotide (Figure 8C lanes 7–10). In the presence of the antibody the band of slower mobility was diminished, indicating that it contained Mcm2-7. When the DNA binding activity of Mcm2-7K499A was assayed (lanes 11–14), the amount of Mcm2-7K499A-DNA complex was reduced suggesting that Mcm2-7K499A is less able to bind the 90 base single-stranded DNA or that the binding is unstable under the electrophoresis conditions.

The loss of helicase activity is the most pronounced functional effect of the Mcm3 hairpin mutation, and may explain the inviability of the mcm3K499A strain. To investigate the effects of the Mcm3 hairpin mutation in cells, we analyzed myc3-Mcm3K499A expressed in yeast to determine if its ability to associate with other components required for replication differs from the wild type protein. Whole cell extracts containing mcm3K499A were prepared from cells grown to mid-log phase in YPD media and analyzed by gel filtration chromatography on a Superose 6 column. As shown in Figure 9, wild type Mcm3 elutes from the Superose 6 column in two peaks. The first peak is broad and corresponds to complexes with a molecular mass greater than 2 MDa. We suspect that this may represent the Mcm2-7 complex associated with chromatin. The second peak elutes in the molecular mass range from 150 to 350 kDa. This likely represents Mcm3 in association with other molecules, and perhaps an equilibrium between subcomplexes of Mcms. The elution profile of mcm3K499A resembled that of the wild type in that it eluted as two peaks, but with significant differences for the high- and low- molecular weight complexes. In the high molecular weight complex, Mcm3 appears as a single band that migrates with a mass of 135 kDa, while in the mcm3K499A strain, Mcm3 appears as a 135 kDa band but there are also two prominent bands at approximately 150 kDa and 175 kDa. These were not detected in the wild-type Mcm3 cells, even after prolonged exposure of the film. The reduced mobility forms of Mcm3 are likely the result of protein modification, but the nature of this modification is unclear. The second difference between wild-type and mcm3K499A cells is that the smaller complex from wild-type cells elutes at a peak at 200 kDa (fraction 34 on the profile, Figure 9), which was shifted to approximately 260 kDa in the mcm3K499A strain (fraction 32). Since the purified Mcm2-7K499A hexamer dissociates and behaves similarly to the wild-type complex in vitro, these results suggest that in cells the altered activities of the Mcm2-7K499A complex result in changes in its molecular associations.

Figure 9. Analysis of myc3-tagged Mcm3 and Mcm3K499A by gel filtration. Extracts were prepared from yeast strains MDY405 (myc3-Mcm3) and MDY406 (myc3-Mcm3K499A). Five mg of protein was separated on a Superose 6HR10/30 column. Protein from 10 μL aliquots of 250 μL fractions for Mcm3 and 20 μL for Mcm3K499A were resolved by SDS-PAGE and myc-tagged protein detected by western blotting with anti-myc antibody. Fraction numbers are indicated below (L, load). The migration of molecular mass standards on the gel is shown on the left. The fractions corresponding to the peak elution of mass standards from the Superose 6 column is shown above. doi:10.1371/journal.pone.0082177.g009
although the degree to which it obstructs the central channel will depend on its structure, which has not been modeled. In addition to the funnel shape, the surface charge of the central channel exhibits a systematic change: the large opening formed by the C-terminal domains carries a negative surface charge, while the surface formed by the more constricted N-terminal domains is positively charged (Figure 10B).

The second important observation from the Mcm2-7 model is that the PS1 hairpins of all Mcm subunits are somewhat recessed and do not project into the interior of the central channel. Together with the reduced stability of DNA binding by Mcm2-7<sup>K542A</sup>, these observations lead us to propose that the PS1 hairpin may be a component of an exit channel that directs one strand of incoming duplex DNA through the side of the Mcm2-7 hexamer. A structural model of the SsoMCM hexamer suggests that side channels are formed at the interface of subunits and run from the central channel to the outside of the ring [7]; the side channels are wide enough to accommodate single-stranded DNA. Similar channels are seen in electron micrographs of eukaryotic Mcm2-7 [47] and are present in our Mcm2-7 model (Figure 10C). The extrusion of single-stranded DNA through a side channel of the Mcm2-7 complex is a possible explanation for reduced helicase activity upon mutation of the Mcm3 PS1 hairpin. Of note, the channel incorporating the Mcm3 PS1 hairpin is formed at the interface between Mcm3 and Mcm7 (Figure 10C), which is critically important for Mcm2-7 function. For example, when the various Mcm subunits are expressed independently to generate dimeric species, it is the isolated Mcm3/7 dimer that has the highest ATPase activity, almost as high as the ATPase of the intact Mcm2-7 hexamer [26,27]. Furthermore, expression of Mcm7 with mutations in its Walker A or Walker B motif, or expression of Mcm3 with an R542A mutation in its “arginine finger” lead to a strong dominant-lethal phenotype [26]. Taken together, the shape and charge features of the Mcm2-7 “funnel”, along with the functional importance of catalytic components found in the Mcm3/7 interface, are consistent with either double-stranded DNA or single-stranded DNA entering Mcm2-7 at the larger C-terminal end. If double-stranded DNA enters the channel, it may be destabilized due to the negative surface charge of the channel interior: one of the separated strands would be actively extruded through the Mcm3/7 interface, while the other strand could exit through the positively charged N-terminal end of the Mcm2-7 hexamer, or through another side channel. In a second possible scenario, single-stranded DNA would enter the Mcm2-7 channel and would be actively extruded through the Mcm3/7 interface, while the other strand would be sterically excluded from entering the channel.

The importance of the PS1 hairpins in Mcm function is most apparent from the loss of viability when PS1 of Mcm3 was mutated. We demonstrate that the Mcm3 PS1 hairpin participates in DNA unwinding by Mcm2-7, and based upon our in vitro experiments suggest that it may do so by altering the interaction of the complex with single-stranded DNA. This result is similar to findings with SsoMCM and the SF3 viral replicative helicases where the PS1 hairpin is essential for helicase activity [8,9,48]. We also note that the interactions of Mcm3<sup>K499A</sup> are altered in cells as demonstrated by changes in its elution from a Superose 6 column. Whether these changes are due to or the cause of the defects in cellular function of the protein is unclear.

A key finding of our study is that of the six PS1 hairpins in the heterohexameric Mcm2-7 complex, only the PS1 hairpin of Mcm3 is essential. This strongly suggests that it has a unique role in Mcm2-7 function. The finding that the PS1 hairpin of Mcm3 is essential for viability is somewhat surprising since Mcm3 has been proposed to act principally in the regulation of the other Mcm2-7 subunits rather than have a direct role in DNA unwinding [49,50]. Although not essential, the Mcm2, Mcm4, Mcm5, Mcm6 and Mcm7 PS1 hairpins are important for function as lysine to alanine mutations in any two subunits leads to inviability or slow growth.

Figure 10. Model of Mcm2-7. (A) Two views of the Mcm2-7 hexamer: on the left, looking through the central channel from the C-terminal end, and on the right, rotated 90° about a horizontal axis. The N- and C-terminal domains of the subunits are indicated. Subunits 3 and 7 are highlighted in magenta and blue, respectively, and the side chains of the PS1 lysyl residues are shown as space-filling models in red. ADP modeled onto the Walker A motifs is shown as a yellow space-filling model. (B) The hexamer is split into two halves - Mcm7/3/5 and Mcm2/6/4 - to show the electrostatic surface of the Mcm2-7 central channel; red indicates a negative surface potential, and blue a positive surface potential. The outer diameter of the hexamer is approximately 120 Å, while the inner diameter decreases from approximately 40 Å in the region formed by the C-terminal domains, to 20 Å or less in the region formed by the N-terminal domains. The N-terminal region will be further constricted by a 70-residue insertion in Mcm6, which is expected to occupy the region indicated with a green circle. A yellow square on the Mcm7/3/5 trimer indicates the potential side exit tunnel in the Mcm3/7 interface, which is shown in greater detail in Panel C. (C) Stereodiagram of the potential exit channel in the Mcm3/7 interface, illustrating the position of the Mcm3 PS1 hairpin (purple) and essential K499 residue (red) relative to ADP bound to Mcm7 and the motifs associated with ATP binding and hydrolysis: the Walker A (WA, red), Walker B (WB, magenta), and Sensor 1 (S1, green) of Mcm7, and the Arginine Finger (orange) and Sensor 2 (S2, cyan) of Mcm3.

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The finding that the mcm4K658A mcm5K506A double mutation strain was viable, in contrast to the lack of viability of other pairwise combinations is also another clear indication that each subunit contributes differently to the function of Mcm2-7.

Supporting Information

Figure S1 Growth of mcm3K958A and mcm7K958A plasmid shuffled yeast strains. Harploid yeast strains deleted for MCM3 or MCM7 and bearing MCM3 or MCM7 on a UR3-CEN plasmid were transformed with LEU2-LEU2 plasmids containing either MCM3, mcm3K958A, MCM7, mcm7K958A or the empty LEU2-CEN plasmid (Vector). The transformed yeast were grown overnight at 30 °C in YPD media, serially diluted, and then spotted onto a YPD plate or a plate containing 5-FOA. The plates or MCM3 LEU2-CEN was viable, in contrast to the lack of viability of other pairwise beads, and 10, 20 or 40 μg of total protein separated by SDS-PAGE. Blots of these gels were probed with anti-Mcm2, (Santa Cruz Biotech) or anti-myc (Sigma-Aldrich) antibody to assess the level of Mcm subunit. We note that for Mcm3 detection the plasmids were transformed into BY4741 and thus contain wild-type Mcm3.

(TIF)

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Author Contributions

Conceived and designed the experiments: MJD CJB BSS SKWL. Performed the experiments: MJD CJB BSS SKWL. Analyzed the data: MJD CJB BSS SKWL. Contributed reagents/materials/analysis tools: MJD CJB BSS SKWL. Wrote the paper: MJD CJB BSS SKWL.

Supporting Information

Figure S2 Expression levels of Mcm 2 and 3. Yeast strains MDY70 (MCM2), MDY71 (mcm2K633A), MDY405 (DED1-myc2-MCM3) and MDY406 (DED1-myc2-mcm3K958A) were grown to mid-log phase; yeast extracts were prepared by grinding with glass beads, and 10, 20 or 40 μg of total protein separated by SDS-PAGE. Blots of these gels were probed with anti-Mcm2, (Santa Cruz Biotech) or anti-myc (Sigma-Aldrich) antibody to assess the level of Mcm subunit. We note that for Mcm3 detection the plasmids were transformed into BY4741 and thus contain wild-type Mcm3.

(TIF)

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