Mcm10 Regulates the Turnover of DNA Polymerase alpha

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Abstract

Accurate and efficient duplication of the genome exactly once per cell cycle is crucial for prolonged health of an organism. Minichromosome maintenance (Mcm) 10 is an essential, highly conserved replication factor with multiple functions at the fork. Critical for Mcm10's role at the fork is its DNA binding activity. Utilizing the crystal structure of the conserved internal domain of *X. laevis* (x) Mcm10, we identified residues that, when mutated, significantly reduced xMcm10's ability to bind DNA *in vitro*. Importantly, the corresponding mutations in *S. cerevisiae* (sc) Mcm10 resulted in reduced viability after exposure to hydroxyurea, a drug that causes replication fork stalling. This suggests that the DNA binding activity of Mcm10 is important *in vivo* for fork stabilization during replication stress. In addition to its DNA binding activity, work from our laboratory has shown that Mcm10 regulates the stability of DNA polymerase (pol) alpha/primase, the only enzyme capable of *de novo* DNA synthesis, in yeast and humans. In the absence of Mcm10, Cdc17, the catalytic subunit of pol alpha, is rapidly degraded. We have determined that Cdc17 degradation is dependent on the proteasome via Ubc4 and Not4 and disrupting this degradation pathway results in elevated steady-state levels of Cdc17. Furthermore, Cdc17 shows synthetic dosage lethality with *not4Δ* cells. Importantly, overexpression of Cdc17 and Mcm10 causes slow growth, an increased mutation rate, and microsatellite-mediated gross DNA rearrangements, suggesting that regulated turnover of Cdc17 is crucial to maintain genome stability.

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List of Abbreviations

5-FOA	5-fluoroorotic acid
ACS	ARS consensus sequence
AP	Associated protein
APC	Anaphase promoting complex
ARS	Autonomously replicating sequence
AZ1	Antizyme-1
BRCA	Breast cancer gene
CAN	Canavanine
Ccr	Carbon catabolite repression
Cdc	Cell division cycle
Cdh	E-cadherin gene
Cdt	Cdc10 dependent transcript
Cdk	Cyclin dependent kinase
Clb	B-type cyclin
Cln	A-type cyclin
CMG	Cdc45-Mcm2-7-GINS
Cul	Cullin
Dbf	Dumbbell forming
DDK	Dbf4-dependent kinase
DDR	DNA damage response
Dia	Digs into agar
DMSO	Dimethyl sulfoxide
Dna	DNA synthesis defective
Dpb	DNA polymerase-binding
ds	double-strand
DSB	Double-strand break
E1	Ubiquitin activating enzyme
E2	Ubiqutin conjugating enzyme
E3	Ubiquitin ligase enzyme
Egd	Enhancer of Gal4 DNA binding
F-box	Originally identified in cyclin F
Fen	Flap endonuclease
G_1	Gap 1
G_2	Gap 2
GINS	Go, Ichi, Ni, San; Japanese for five, one, two, three
Grr	Glucose repression-resistant
HECT	Homologous to E6-AP carboxy terminus
HNPCC	Hereditary nonpolyposis colorectal cancer
HR	Homologous recombination

HU	Hydroxyurea
M	Mitosis
Mcm	Minichromosome maintenance
Mec	Mitosis entry checkpoint
MLH	MutL homolog
MMR	Mismatch repair
MMS	Methyl methanesulfonate sensitive
MSH	MutS homolog
NES	Nuclear export signal
NHEJ	Nonhomologous end joining
NMR	Nuclear magnetic resonance
Not	Negative regulator of transcription
nt	Nucleotide
OB	Oligonucleotide/oligosaccharide binding
ODC	L-ornithine decarboxylase
ORC	Origin recognition complex
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
Pdr	Pleiotropic drug resistance
Pol	DNA polymerase
PMS	Post-meiotic segregation
pre-RC	Pre-replicative complex
Pri	Primase
Prt	Proteolysis
Rad	Radiation sensitive
RFC	Replication factor C
RING	Really interesting new gene
ROS	Reactive oxygen species
RPA	Replication protein A
Rpn	Regulatory particle non-ATPase
Rrm	Recombination mutation
Rtt	Regulator of Ty1 transposition
S	DNA synthesis
SCF	Skip, cullin, F-box containing
SDL	Synthetic dosage lethality
sh	Short hairpin
si	Short interfering
Sld	Synthetically lethal with *dpb11-1*
ss	single-strand
Uba	Ubiquitin activating
Ubc	Ubiquitin conjugating

UPS	Ubiquitin-proteasome system
UV	Ultraviolet

Chapter 1

Introduction

Cell Cycle Control and DNA Replication

Cell Cycle Regulation. The cell cycle is a carefully regulated process that is divided into four distinct parts: G_1, S, G_2, and M phase. G_1 and G_2 phases are gap phases, the DNA is replicated during the DNA synthesis phase, and during mitosis cells segregate the duplicated DNA to opposite poles of the cell and undergo cytokinesis. Progression through the four phases of the cell cycle in yeast is controlled by a single cyclin dependent kinase, Cdk 1 (Hartwell *et al.*, 1973; Reed *et al.*, 1985). Several temporally regulated proteins known as cyclins bind to and differentially regulate Cdk1. There are nine cyclins in yeast, three G1 cyclins, cyclin (Cln) 1-3, also known as A-type cyclins, (Reed *et al.*, 1989; Richardson *et al.*, 1989), and six B-type cyclins (Clb) 1-6 (Surana *et al.*, 1991; Epstein and Cross, 1992; Schwob and Nasmyth, 1993). When bound to Cdk1, cyclins activate kinase activity and differentially regulate the specificity of Cdk1. The G_1 phase cyclins Cln1-3 are required for bud emergence, spindle pole body duplication, and the indirect activation of the B-type cyclins via promoting the degradation of the Clb-Cdk inhibitor, Sic1 (Fernandez-Sarabia *et al.*, 1992; Cvrckova and Nasmyth, 1993; Tyers, 1996; Verma *et al.*, 1997; Nash *et al.*, 2001). The B-type cyclins then advance the cell through S, G_2, and into M phase. More specifically, Clb5 and Clb6 are required for initiation of DNA replication, Clb3 and Clb4 are active during the S and G_2 phases, and Clb1 and Clb2 promote spindle formation, initiate mitosis, and inhibit mitotic exit and cell division (Fitch *et al.*, 1992; Richardson *et al.*, 1992; Schwob and Nasmyth, 1993; Surana *et al.*, 1993) (Figure 1). In order to exit M phase and complete cell division, Cdk1 activity must be inhibited. This inactivation is accomplished by the

anaphase promoting complex (APC), which becomes active during mitosis and inactivates Cdk1, allowing cells to exit mitosis and undergo cell division (King *et al.*, 1995; Sudakin *et al.*, 1995). As a general rule, those cyclins that are active during a given phase of the cell cycle need to be degraded before the cell can move on to the next phase of the cell cycle. Cln1 and Cln2 are degraded by a skip, cullin, F-box containing (SCF) E3 ligase complex that contains the F-box (originally identified in cyclin F)(Bai *et al.*, 1996) protein glucose repression-resistant (Grr) 1 (Barral *et al.*, 1995; Skowyra *et al.*, 1997). As an exception to the rule, Cln3 is not significantly cell cycle-regulated, but is an upstream regulator of Clb1 and Clb2 (Tyers *et al.*, 1993). Clb6 is the only B-type cyclin that is regulated by an SCF E3 ligase; it is degraded by SCF containing the F-box protein Cdc4 (Jackson *et al.*, 2006). The remaining B-type cyclins are degraded by the APC that is bound to either cell division cycle (Cdc) 20 or E-cadherin gene (Cdh) 1 (Peters, 2006).

DNA Replication Initiation. DNA replication is initiated throughout the eukaryotic genome at loci known as "replication origins". These origins are well-defined in budding yeast. They have autonomously replicating sequences (ARSs) that contain a ARS consensus sequence (ACS) known as an A element which is required for the sequence to be replicated. In addition, there are three elements - B1, B2 and B3 - that are also important for ARS function (Stinchcomb *et al.*, 1979; Chan and Tye, 1980; Kearsey, 1983, 1984; Rao *et al.*, 1994). In metazoa, origins of replications are much less defined by DNA sequence; they instead seem to be defined by chromosomal context and epigenetic factors in addition to primary sequence (Aladjem and Fanning, 2004; Mechali, 2010). Although origins of replication are defined differently between budding yeast and

metazoa with respect to their DNA sequences, they are nonetheless recognized and bound by the evolutionarily conserved protein complex known as the origin recognition complex (ORC) in all eukaryotes (Bell and Stillman, 1992; Gavin *et al.*, 1995; Rowles *et al.*, 1996; Pinto *et al.*, 1999). In G_1, ORC is required to recruit Cdc6 (Figure 2A), another evolutionarily conserved DNA replication factor that is required for DNA replication initiation (Hartwell, 1976; Cocker *et al.*, 1996). The ORC:Cdc6 complex then recruits Cdc10 dependent transcript (Cdt) 1 and the minichromosome maintenance (Mcm) 2-7 complex (Figure 2A)(Donovan *et al.*, 1997; Tanaka *et al.*, 1997; Maiorano *et al.*, 2000; Tanaka and Diffley, 2002). Once the Mcm2-7 complex has been loaded as a double hexamer, the assembled proteins are known as the pre-replicative complex, or pre-RC (Diffley *et al.*, 1995; Remus *et al.*, 2009) (Figure 2B). Mcm10, another evolutionarily conserved (Figure 5B), essential replication factor is then recruited to origin DNA (Merchant *et al.*, 1997; Izumi *et al.*, 2000; Wohlschlegel *et al.*, 2002b; Christensen and Tye, 2003; Ricke and Bielinsky, 2004) (Figure 2B). Mcm10 has been shown to be required for recruitment of Cdc45 to the pre-RC, the next step in replication initiation (Hopwood and Dalton, 1996; Aparicio *et al.*, 1997; Zou *et al.*, 1997; Saha *et al.*, 1998; Walter and Newport, 2000; Chou *et al.*, 2002; Wohlschlegel *et al.*, 2002a; Gregan *et al.*, 2003; Sawyer *et al.*, 2004b; Im *et al.*, 2009). However, others, including our laboratory, have shown that Mcm10 is not required for Cdc45 recruitment (Park *et al.*, 2008; Ricke and Bielinsky, unpublished data), making this a controversial issue (Figure 2B). Synthetically lethal with *dpb11-1* (Sld) 3 next binds to the pre-RC (Figure 2B), along with DNA polymerase-binding (Dpb) 11 and Sld2 (Araki *et al.*, 1995; Kamimura *et al.*,

1998; Araki, 2010). Dpb11 bridges Sld2 and Sld3 after they are phosphorylated by the Cdk1 (Zegerman and Diffley, 2007) (Figure 2C). The GINS (Go, Ichi, Ni, San; Japanese for five, one, two, three) complex is then recruited to the origin along with DNA polymerase (pol) ε (Kubota *et al.*, 2003; Takayama *et al.*, 2003; Muramatsu *et al.*, 2010) (Figure 2C). Finally, Cdk1 and dumbbell forming (Dbf) 4-dependent kinase DDK activities are responsible for the initiation of DNA replication with Sld2 and Sld3 being targeted by Cdk1 and Mcm2-7 being targeted by DDK (Lei *et al.*, 1997; Weinreich and Stillman, 1999; Zegerman and Diffley, 2007; Labib, 2010; Randell *et al.*, 2010). DNA replication is then initiated with the unwinding of DNA by the Cdc45-Mcm2-7-GINS (CMG) complex (Schwacha and Bell, 2001; Moyer *et al.*, 2006; Bochman and Schwacha, 2008), which allows access to the factors required for DNA synthesis (Figure 3).

Inhibition of Rereplication. It is important that a cell replicates its genome exactly once per cell cycle. Rereplication via deregulation of replication licensing factors can lead to genomic instability (Hook *et al.*, 2007; Teer and Dutta, 2008; Green *et al.*, 2010). For this reason, eukaryotic cells have evolved mechanisms to inhibit rereplication of their genome by inactivating the proteins required for pre-RC formation in S, G_2 and M phases, ensuring that pre-RC formation can only take place during G_1. All eukaryotes use multiple redundant pathways to prevent rereplication underscoring the importance of replicating the genome exactly once per cell cycle (Nguyen *et al.*, 2001; Li and Blow, 2005; Machida and Dutta, 2007). Yeast inhibit ORC via Cdk1-dependent phosphorylation (Nguyen *et al.*, 2001), whereas in humans, Orc1 is degraded in S phase (Machida *et al.*, 2005). After the Mcm2-7 complex has been loaded, Cdc6 is

phosphorylated by Cdk1 and this leads either to its degradation (yeast) or its export to the cytoplasm (mammals), making it impossible to load Mcm2-7 onto origins that have already fired (Petersen *et al.*, 1999; Calzada *et al.*, 2000; Delmolino *et al.*, 2001). Phosphorylation of Mcm2-7 during G_2 and M phases induces its export to the cytoplasm and inhibits Mcm2-7 chromatin association (Hendrickson *et al.*, 1996; Labib *et al.*, 1999; Nguyen *et al.*, 2000). Cdt1 is regulated very differently between yeast and metazoans. In yeast, Cdt1 is exported from the nucleus in a Cdk-dependent manner during S, G2, and early M phases (Tanaka and Diffley, 2002). In metazoa Cdt1 is regulated in multiple ways. Cdt1 is degraded independently by two different E3 ligase enzymes and it is also bound by an inhibitor, Geminin (Wohlschlegel *et al.*, 2000; Li *et al.*, 2003; Liu *et al.*, 2004; Arias and Walter, 2006; Jin *et al.*, 2006; Hook *et al.*, 2007). In yeast, Orc1 phosphorylation needs to be inhibited, Cdc6 needs to be stabilized and the Mcm2-7 complex needs to remain in the nucleus in order for re-replication to occur (Nguyen *et al.*, 2001). In metazoa, the mechanism to prevent re-replication is different, as it is dependent, for the most part, on the regulation of Cdt1. Depletion of Geminin, a protein that binds and inhibits Cdt1, and/or stabilization of Cdt1 appears to be sufficient for re-replication in most human cell types (Melixetian *et al.*, 2004; Zhu *et al.*, 2004; Jin *et al.*, 2006; Lovejoy *et al.*, 2006; Machida and Dutta, 2007).

DNA Replication Elongation and Termination. DNA synthesis proceeds bidirectionally whereby two replication forks are moving in opposite directions (Prescott and Kuempel, 1972). DNA is synthesized by three replicative DNA pols: α, δ and ε (Burgers, 2009). Since DNA is anti-parallel and DNA polymerases can only synthesize

DNA unidirectionally (5'-3'), synthesis occurs in a semi-discontinuous manner, with a leading strand and a lagging strand at each replication fork. Unwinding of the DNA exposes single-stranded (ss) DNA, which is immediately coated by a heterotrimeric protein complex, replication protein A (RPA) (Wold and Kelly, 1988). RPA, in conjunction with Mcm10, recruit pol α/primase to the DNA (Tanaka and Nasmyth, 1998; Zou and Stillman, 2000; Ricke and Bielinsky, 2004, 2006; Chattopadhyay and Bielinsky, 2007) (Figure 3). DNA primase initiates replication by synthesizing a short ~10-12 nucleotide (nt) RNA primer (Morris and Racine, 1978; Conaway and Lehman, 1982a, b). Pol α, the only enzyme capable of *de novo* DNA synthesis, subsequently adds 12-20 nt of DNA to the RNA primer, resulting in a short RNA/DNA primer (Denis and Bullock, 1993). Although pol α is required for initiation of DNA synthesis, it is a potentially mutagenic enzyme, as it lacks 3'-5' exonuclease (proofreading) activity, rendering it unable to repair incorrectly inserted bases (Morrison *et al.*, 1991). This is not trivial, as this initiation process happens approximately 6×10^4 times in a yeast cell and an astounding 2×10^7 times in a human cell, assuming an Okazaki fragment length of 200 bp, during every round of replication. That amounts to approximately 9.6×10^5 bases in yeast and 3.2×10^8 bases in humans, per cell, per round of replication, that are not proofread by pol α after insertion. Therefore, DNA synthesis by pol α is clearly an evolutionary force that needs to be tightly regulated. Bulk DNA synthesis, following synthesis of the short DNA primer, is handed off to pol ε on the leading strand and pol δ on the lagging strand (Waga *et al.*, 2001; Pursell *et al.*, 2007; Nick McElhinny *et al.*, 2008) (Figure 3). Both of these polymerases are more processive, aided by their

interaction with proliferating cell nuclear antigen (PCNA) (Eissenberg *et al.*, 1997), and contain intrinsic proofreading activity, which limits the number of mis-paired bases during DNA replication. (Nethanel and Kaufmann, 1990; Morrison *et al.*, 1991; Waga and Stillman, 1994; Garg *et al.*, 2004a; Pursell *et al.*, 2007; Nick McElhinny *et al.*, 2008). The precise mechanism of the polymerase switch is not known; however, there are clues as to what the mechanism may be. Aside from replication factor C (RFC), which loads PCNA onto DNA (Tsurimoto and Stillman, 1990; Podust *et al.*, 1995), Mcm10 also appears to play a role in this process. Mcm10 can be diubiquitinated and this modified form of Mcm10 interacts with PCNA (Das-Bradoo *et al.*, 2006). Importantly, the interaction between PCNA and diubiquitinated Mcm10 is essential in yeast, as mutants of Mcm10 and PCNA that disrupt the interaction are lethal (Das-Bradoo *et al.*, 2006). Interestingly, only the unmodified form of Mcm10 binds Cdc17, the catalytic subunit of pol α in yeast (Ricke and Bielinsky, 2004, 2006). The above data support a model whereby unmodified Mcm10 recruits and maintains pol α at the replication fork until the RNA/DNA primer has been synthesized. At this time Mcm10 becomes diubiquitinated, interacts with PCNA and likely releases Cdc17, since the diubiquitinated from of Mcm10 can no longer interact with Cdc17 (Das-Bradoo *et al.*, 2006). PCNA tethers pol δ to the lagging strand and pol ε to the leading strand template, thus completing the polymerase switch. Pol ε continuously synthesizes the leading strand, but the lagging strand is synthesized discontinuously, in approximately 200-bp sections called Okazaki fragments, by the repeated combined efforts of pol α/primase and DNA pol δ (Burgers, 2009). Each Okazaki fragment contains an RNA primer, synthesized by the primase component of pol

α/primase enzyme, that needs to be removed and replaced with DNA. This task is performed by flap endonuclease (Fen) 1 (Ishimi *et al.*, 1988; Harrington and Lieber, 1994; Waga *et al.*, 1994), DNA synthesis defective (Dna) 2 (Dumas *et al.*, 1982; Budd and Campbell, 1995) and pol δ. When pol δ reaches the RNA primer, it displaces the RNA as it synthesizes DNA, and thus creates an RNA flap (Figure 3). Most flaps are cleaved a few nucleotides at a time by Fen1 (Ayyagari *et al.*, 2003; Jin *et al.*, 2003; Garg *et al.*, 2004b). Some flaps, at a very low rate, escape cleaveage by Fen1 and are bound by RPA, which stimulates cleavage of the longer flap by Dna2 (Bae and Seo, 2000; Bae *et al.*, 2001). Therefore, Fen1 and Dna2 both cooperate to remove RNA flaps during Okazaki fragment maturation, but Fen1-mediated flap cleavage is the preferred pathway, as it has recently been shown that Fen1 activity is stimulated by the Dna2 "backup pathway" (Henry *et al.*, 2010). The resulting nick in the DNA after flap removal is sealed by DNA ligase I (Beard, 1972; Soderhall and Lindahl, 1973; Johnston, 1983; Waga *et al.*, 1994) (Figure 3). The process described above continues until the entire genome has been duplicated and replication is terminated. Replication termination is relatively well understood in *E. coli*, but not in eukaryotes. Briefly, *E. coli* chromosomes contain replication pause sites, called *Ter* sites, that are able to restrict replication termination to a terminus region, which is approximately opposite the origin of replication on the chromosome (Duggin *et al.*, 2008). Recently, the termination of eukaryotic replication has become clearer, as 71 *Ter* sites that contain replication pausing elements were identified in budding yeast (Fachinetti *et al.*, 2010). These same authors showed that these *Ter* sites were occupied by the Top2 DNA topoisomerase, the rDNA

recombination mutation (Rrm) 3 DNA helicase is involved in replication fork progression through *Ter* sites, and that both these enzymes are required for efficient replication termination and genome stability (Alver and Bielinsky, 2010; Fachinetti *et al.*, 2010).

Proteasomal Protein Degradation

Ubiquitin. Ubiquitin, an eight-kiloDalton (kD) protein that is ubiquitously expressed in eukaryotes was first identified in the thymus (Goldstein *et al.*, 1975). 30 years ago, it was identified as the factor required for ATP-dependent proteolysis in rabbit reticulocytes (Wilkinson *et al.*, 1980). Ubiquitin is expressed as either a polyubiquitin molecule that is subsequently cleaved into monomers (Ozkaynak *et al.*, 1984; Finley *et al.*, 1987) or as fusions of one ubiquitin molecule fused with a ribosomal protein (Finley *et al.*, 1989; Redman and Rechsteiner, 1989). The importance of the ubiquitin system for cells is underscored by the fact that it is essential for cell viability and by the major roles it plays in such diverse areas as cell cycle regulation, DNA repair, transcriptional regulation, protein synthesis, cellular stress response, endocytosis, and programmed cell death (Finley *et al.*, 1984; Finley *et al.*, 1987; Jentsch *et al.*, 1987; Goebl *et al.*, 1988; Finley *et al.*, 1989; Hochstrasser and Varshavsky, 1990; Schwartz *et al.*, 1990; Galan and Haguenauer-Tsapis, 1997; Terrell *et al.*, 1998). Again, speaking to the importance of ubiquitin for cell viability, it is critical that ubiquitin homeostasis be maintained within the cell. If the levels of free ubiquitin are too low, yeast cells become sensitive to heat, cadmium, and canavanine, a toxic amino acid analog (Papa and Hochstrasser, 1993; Swaminathan *et al.*, 1999; Chernova *et al.*, 2003). Interestingly, too much ubiquitin is

also detrimental to the yeast cells. Ubiquitin overexpression causes sensitivity to cadmium, arsenite, and paromycin (Chen and Piper, 1995). Moreover, ubiquitin overexpression exacerbates the slow growth phenotype of ubiquitin-proteasome system (UPS) mutants, possibly due to the build-up of ubiquitinated proteins (Kimura *et al.*, 2009). This is also likely important for mammals, since individual deletion of the two genes that encode polyubiquitin in mice, *Ubb* and *Ubc*, results in infertility and embryonic lethality, respectively (Ryu *et al.*, 2007; Ryu *et al.*, 2008).

Ubiquitin-Dependent Proteolysis. Much is now known about ubiquitin and ubiquitin-mediated proteasomal degradation and it is clear that this is the major pathway of regulated protein proteolysis in cells. Ubiquitin-mediated proteasomal degradation begins when a target protein meant for degradation becomes flagged by the addition of multiple molecules of ubiquitin. Modifying a protein with ubiquitin is a multi-step process involving several catalysts (Figure 6). First, ubiquitin is bound by an ubiquitin-activating (E1) enzyme via a thiol-ester bond to a cysteine residue within the E1 enzyme. Once bound, the ubiquitin molecule is considered "charged". Next, the ubiquitin molecule is transferred to an ubiquitin-conjugating (E2) enzyme, also via a thiol-ester bond. The target protein is then recruited to the E2 enzyme by an ubiquitin ligase (E3) enzyme, which provides the specificity for this pathway (Hershko *et al.*, 1983; Varshavsky, 2005). There are two types of E3 enzymes, really interesting new gene (RING) finger enzymes and homologous to E6-associated protein (AP) carboxy terminus (HECT) domain enzymes (Ardley and Robinson, 2005). RING finger E3 enzymes, the first characterized being proteolysis (PRT) 1 from Arabidopsis, act as a bridge between

the E2 enzyme and the target protein, with the E2 enzyme directly transferring the ubiquitin molecule to the target protein (Potuschak *et al.*, 1998; Joazeiro *et al.*, 1999; Lorick *et al.*, 1999; Yokouchi *et al.*, 1999). Alternatively, an E2 enzyme transfers the ubiquitin onto a HECT domain E3 enzyme, such as E6-AP, and this type of E3 enzyme subsequently transfers the ubiquitin to the target protein (Huibregtse *et al.*, 1995; Scheffner *et al.*, 1995; Schwarz *et al.*, 1998). In both cases the target protein becomes covalently modified with the ubiquitin molecule. In all cases the ubiquitin molecule is attached to the target protein via an isopeptide bond between the C-terminal glycine residue of ubiquitin and a lysine residue within the target protein (Goldknopf and Busch, 1977; Hochstrasser, 1996). Multiple ubiquitins are then added to the conjugated ubiquitin to form a chain of ubiquitin molecules on the target protein (Chau *et al.*, 1989; van Nocker and Vierstra, 1993). The polyubiquitinated protein is then recruited to the proteasome via ubiquitin receptors. Ubiquitin receptors can be proteasome subunits, such as regulatory particle non-ATPase (Rpn) 10, which remains anchored to the proteasome and binds polyubiquitin to recruit targeted substrates (Deveraux *et al.*, 1994; Kang *et al.*, 2007). Ubiquitin receptors can also act as "shuttling factors", as is the case with radiation sensitive (Rad) 23, which can only bind the proteasome when bound to polyubiquitin, potentially remotely capturing substrates targeted for degradation (Saeki *et al.*, 2002; Wang *et al.*, 2003). Furthermore, shuttling factors can bind E3 ligases and are only released once the substrate has been ubiquitinated allowing the substrate to be delivered to the proteasome (Kim *et al.*, 2004; Kaplun *et al.*, 2005; Richly *et al.*, 2005; Ivantsiv *et al.*, 2006). The 26S proteasome is a very large, multisubunit proteolytic machine. It

consists of a 20S catalytic core that is capped on both ends with 19S regulatory complexes, which control access to the catalytic core (Peters *et al.*, 1994; Groll *et al.*, 1997; Gorbea *et al.*, 1999). Once bound to the proteasome, the target protein is deubiquinated to sustain ubiquitin pools, unfolded to fit into the catalytic core, and degraded (Thrower *et al.*, 2000; Yao and Cohen, 2002; Finley, 2009).

Ubiquitin-Independent Proteolysis. Although the vast majority of degradation via the proteasome requires ubiquitination of the target protein, there are examples of proteins that are degraded by the proteasome independently of ubiquitination. The first and most well understood example of ubiquitin-independent proteasomal degradation to be described was L-ornithine decarboxylase (ODC) (Pegg, 2006). Antizyme-1 (AZ1) binds to ODC and increases its affinity for the proteasome, thus leading to the degradation of ODC while AZ1 is recycled (Glass and Gerner, 1987; Bercovich *et al.*, 1989). Therefore, in this instance AZ1 plays a functional role very similar to ubiquitin, even though it is not chemically similar to ubiquitin (Heller *et al.*, 1976; Kahana, 2009). Several other proteins have since been shown to be degraded via the proteasome independently of ubiquitin *in vivo*, such as p21 (Chen *et al.*, 2004), c-Fos (Bossis *et al.*, 2003), Fra-1 (Basbous *et al.*, 2007), p53 (Asher and Shaul, 2005), Rb (Kalejta and Shenk, 2003), HIFα (Kong *et al.*, 2006; Kong *et al.*, 2007) , thymidylate synthase (Pena *et al.*, 2006), and SRC-3/AIB1 (Li *et al.*, 2006). Whereas the exact mechanism of their degradation is not as well understood in some cases, it is assumed that ubiquitin is not required. These pathways were not rigorously investigated *in vitro*, as with ODC and AZ1. However, the proteins above were degraded either in *uba1* temperature sensitive

mutants that were not able to utilize ubiquitin at the restrictive temperature or when an ubiquitin mutant that had all seven lysines mutated to arginine – making chain formation impossible – was overexpressed, or in some cases both situations were shown. Degradation of these proteins was inhibited in the presence of proteasome inhibitors, however, suggesting that their degradation was indeed proteasome-dependent. Thus it seems that polyubiquitination is one mechanism for targeted proteasomal degradation, but not the only mechanism.

Genome Stability and Cancer

The genome of every organism aside from viruses holds all of the information required to construct and perpetuate that organism. For this reason, it is crucially important for cells to maintain the stability of their genome to promote normal growth and prolonged health. Unfortunately, the genome is under almost constant attack from both endogenous and exogenous sources that cause instability within the genome. Underscoring its importance, loss of genome stability (DNA rearrangements, mutations) is a major hallmark of all cancer cells and likely plays a causative role in tumorigenesis (Benson, 1961; Doll and Peto, 1981; Al-Tassan *et al.*, 2002; Cleaver, 2005; Gorgoulis *et al.*, 2005; Bartkova *et al.*, 2006).

Sources of DNA Damage. Endogenously, there are several sources of DNA damage, which result in ss and in some cases double-stranded (ds) DNA breaks (DSBs). Highly reactive oxygen-containing molecules with an unpaired electron, generally referred to as reactive oxygen species (ROS), are formed during normal cellular

metabolism (Fridovich, 1986; Nohl *et al.*, 2003). ROS can damage DNA by attacking bases and/or the phosphodiester backbone, causing ss and DSBs in the DNA (Kastan and Bartek, 2004; Ragu *et al.*, 2007). Our own enzymes can also be a source of DNA damage. For example, mutations can occur naturally through deamination of DNA bases at a rate of 3.5×10^{-8}/cell/day (Sargentini and Smith, 1985; Shen *et al.*, 1994), which can ultimately lead to the incorporation of an incorrect nucleotide opposite the deaminated base during the subsequent round of replication. If the incorrect nucleotide is not replaced with the correct one, the mutation will become permanent, or "fixed", during the next round of replication. Mutations in the DNA can also occur naturally during DNA replication when replicative pols incorporate incorrect nucleotides during DNA synthesis at a rate of 1×10^{-4} - 1×10^{-6} (Kunkel *et al.*, 1989; Morrison and Sugino, 1994; Shcherbakova *et al.*, 2003; Fortune *et al.*, 2005; McCulloch and Kunkel, 2008). The replication fork is also a source of DNA DSBs. When the replication fork encounters damaged DNA, it stalls and can potentially be processed into a DSB if it is not properly stabilized in a checkpoint-dependent manner (Lopes *et al.*, 2001; Tercero and Diffley, 2001; Branzei and Foiani, 2010).

There are also exogenous sources of DNA damage, including ultraviolet (UV) light, ionizing radiation, and xenochemicals. The latter are often metabolized into carcinogens such as those found in tobacco smoke (Hoffmann and Hoffmann, 1997; Belpomme *et al.*, 2007). UV light causes intrastrand crosslinking of adjacent thymine and cytosine bases (Setlow *et al.*, 1965a; Setlow *et al.*, 1965b). These crosslinked bases are not properly recognized by the DNA polymerase during replication and can lead to

the insertion of an incorrect nucleotide and fixed mutations as mentioned above. Ionizing radiation can produce ROS, damages many molecules in the cell, including DNA, and may cause up to 10% of all occupational cancers (Wakeford, 2004; Belpomme *et al.*, 2007). Finally, humans have produced hundreds of thousands of chemicals in the last 60 years, many of which are carcinogenic due to their ability to modify DNA bases. The production, use and disposal of these potential carcinogens are not properly regulated (Hogan *et al.*, 1981; Belpomme *et al.*, 2007). In conclusion, there are a multitude of ways that our DNA can be damaged every second of every day and it is crucial that our cells combat that damage.

Cellular Defenses Against Genomic Stress. Despite the overwhelming abundance of ways that DNA is damaged during normal cellular growth, the mutation rate of cells is very low (McCulloch and Kunkel, 2008). This apparent paradox is possible because cells are able to sense the presence of DNA damage (ss and ds breaks, mutations) and possess mechanisms to repair the damage (Ciccia and Elledge, 2010). When damage is sensed, checkpoints that halt the cell cycle are activated, so that the cell has time to repair the damage before moving on to the next phase of the cell cycle (Paulovich and Hartwell, 1995). As such, there are DNA damage checkpoints for G_1, intra-S, and G_2 phases of the cell cycle to ensure that the DNA is error free before proceeding with mitosis and cell division (Weinert and Hartwell, 1989; Kuerbitz *et al.*, 1992; Weinert, 1992; al-Khodairy *et al.*, 1994; Murakami and Okayama, 1995). Furthermore, there is a mitotic checkpoint that helps ensure that chromosomes are segregated properly (Gerring *et al.*, 1990; Li and Murray, 1991; Rowley *et al.*, 1992; Andreassen and Margolis, 1994; Poehlmann and

Roessner, 2010). If a checkpoint is activated, a process known as the DNA damage response (DDR) induces the expression of repair proteins to remove DNA lesions (Ciccia and Elledge, 2010). Cells have evolved different repair pathways to resolve different types of DNA damage. DSBs are repaired by nonhomologous end joining (NHEJ) during G1 phase and by homologous recombination (HR) during the S and G_2 phases of the cell cycle (Delacote and Lopez, 2008). NHEJ ligates the two ends of a DSB after processing them, making this process prone to errors (Wilson *et al.*, 1982; Roth *et al.*, 1985; Lieber *et al.*, 2003). In contrast, HR uses sequence from the sister chromatid as a template to repair a DSB, making this a mostly error-free process (Smithies *et al.*, 1971; Chlebowicz and Jachymczyk, 1979; Sung and Klein, 2006). In addition to DSBs, mutations in the DNA also need to be repaired by the cell. Mutations in the DNA are repaired either by the pol itself – as mentioned above, pols δ and ε contain intrinsic proofreading activity (Morrison and Sugino, 1994) – or by the mismatch repair (MMR) pathway, which can locate and remove the mutation and then insert the correct nucleotide (Wildenberg and Meselson, 1975; Bishop and Kolodner, 1986; Holmes *et al.*, 1990; Jiricny, 2006). Interestingly, it has recently been shown that MMR is more efficient in correcting errors introduced by pol α as compared to pol δ, which makes biological sense for the cell since pol α is the least accurate replicative polymerase (Nick McElhinny *et al.*, 2010). The replicative polymerases (α, δ, ε) cannot insert a nucleotide opposite a base that has been damaged or altered. Fortunately, cells have a different set of polymerases called translesion polymerases (ζ, η, κ, ι, Rev1), which are specialized to insert nucleotides opposite damaged bases, allowing these lesions to be bypassed and replication to resume

(Nelson *et al.*, 1996a, b; Johnson *et al.*, 1999; Zhang *et al.*, 2000a; Zhang *et al.*, 2000b). Translesion polymerases are recruited by mono-ubiquitinated PCNA to sites of damage via their ubiquitin binding domains (Hoege *et al.*, 2002; Stelter and Ulrich, 2003; Bienko *et al.*, 2005; Garg and Burgers, 2005; Zhuang *et al.*, 2008). The fidelity of the bypass synthesis depends on the lesion and polymerase used (Lehmann *et al.*, 2007), but a mutation is preferred by the cell to replication fork stalling, because, as mentioned above, replication fork stalling can lead to DSB formation, which is much more dangerous for the cell.

Genome Instability and Cancer. If genomic stability were important for cancer prevention, then the prediction would be that disruption of a pathway or pathways that maintain genome stability promotes tumorigenesis. Three lines of evidence support this notion: (1) Tumors can arise by spontaneous mutation. Cells are able to maintain a very low mutation rate (1×10^{-9}/round of replication) (McCulloch and Kunkel, 2008), but the rate is not zero; some mutations do persist. Loss-of-function mutations in tumor suppressor genes or gain-of-function mutations in protooncogenes can cause an increase in genome instability that is linked to cancer (Deng *et al.*, 1994; Eyfjord *et al.*, 1995; Taylor *et al.*, 1997; Halazonetis *et al.*, 2008). The resulting instability increases the likelihood of accumulating additional carcinogenic mutations that drive tumorigenesis. These mutations increase the likelihood of tumorigenesis. Indeed, sporadic cancers account for the large majority of all human cancers (Kastan and Bartek, 2004). (2) Heritable deficiencies that increase genome instability increase cancer susceptibility in afflicted individuals. This is well-documented; hereditary non-polyposis colorectal

cancer (HNPCC), Fanconi anemia, and Breast Cancer Gene 1 (BRCA1)-defiency are a few well-known examples (Sarna *et al.*, 1975; Lynch *et al.*, 1985; Futreal *et al.*, 1994; Steichen-Gersdorf *et al.*, 1994). HNPCC is characterized by the deficiency of at least one component in the mismatch repair pathway, leading to a much higher mutation rate and increased likelihood of cancer in affected individuals (Fishel *et al.*, 1993; Leach *et al.*, 1993). One of the major phenotypes of HNPCC is microsatellite instability (Ionov *et al.*, 1993; Boland and Goel, 2010). Microsatellites are short, repetitive DNA sequences that are found naturally throughout the genome (Tautz and Schlotterer, 1994). When the MMR pathway is deficient, as in HNPCC, microsatellites become unstable (becoming longer or shorter) (Aaltonen *et al.*, 1993), likely interfering with proper gene expression if the microsatellite is in the promoter or coding regions of a gene. Fanconi anemia is a disease in which cells are deficient for the repair of DNA interstrand cross-links by HR (Pace *et al.*, 2010), which causes an increase in chromosomal aberrations (Joenje and Patel, 2001). Individuals afflicted with this heritable disease show increased cancer susceptibility (Alter, 1996). Lastly, BRCA1 plays an important role in DNA repair by homologous recombination and its loss results in a dramatic increase in genome instability (Miki *et al.*, 1994; Brown *et al.*, 1995; Scully *et al.*, 1997; O'Donovan and Livingston, 2010). BRCA1 deficiency results in a breast cancer risk of 52% by age 70 in women (Milne *et al.*, 2008). (3) Forcing a decrease in replication fidelity increases tumorigenesis by increasing the rate of mutation. There is evidence from animal models that this is case. Mutations that render pol δ and ε exonuclease-deficient each increase the mutation rate in yeast cells 10- to 100-fold over wild type cells, which have a

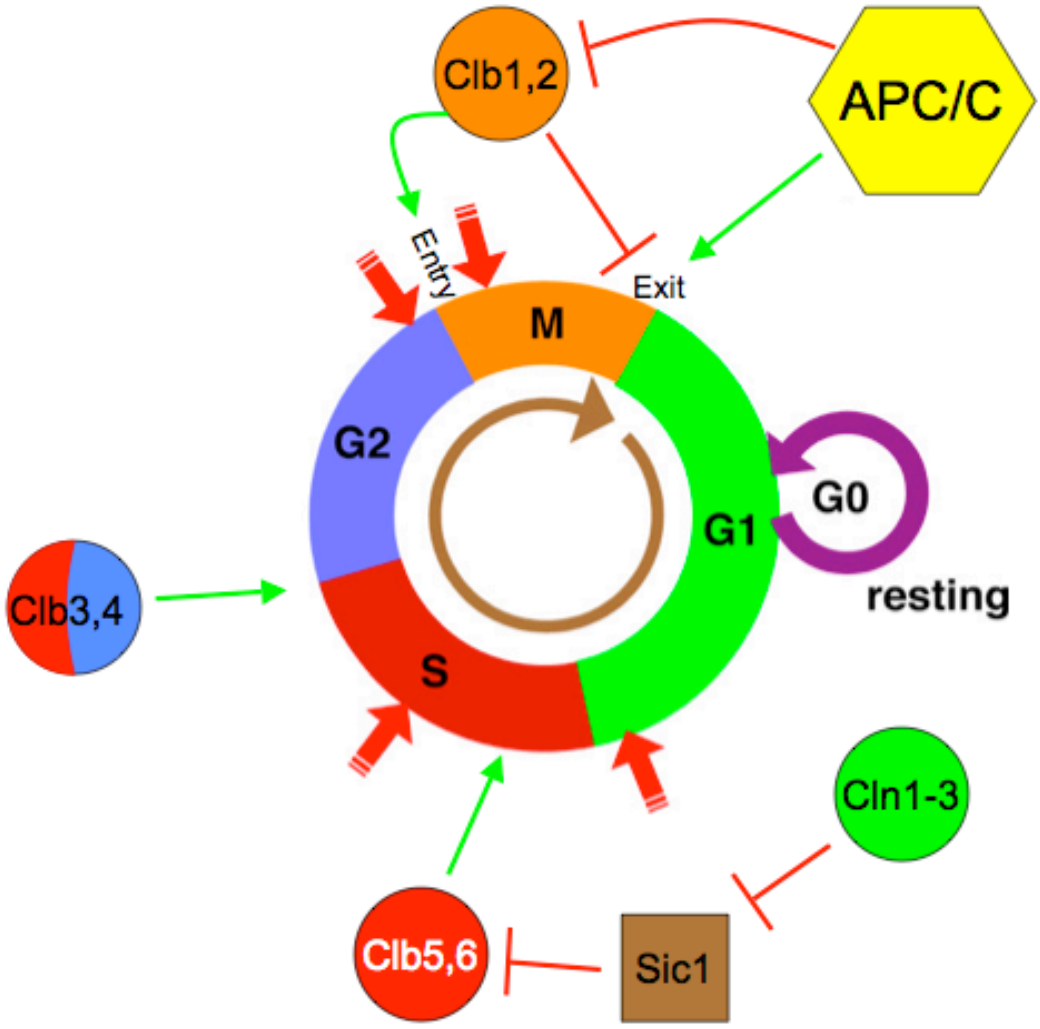
mutation rate of 1 x 10^{-8} (Morrison and Sugino, 1994). Mice homozygous for the corresponding mutation in murine DNA pol δ are viable, but develop lymphomas early in life and epithelial carcinomas later in life (Goldsby *et al.*, 2001; Goldsby *et al.*, 2002). Furthermore, mice homozygous for a mutation in the active site of pol δ that decreases nucleotide selection fidelity, but retains proofreading activity, were embryonic lethal (Venkatesan *et al.*, 2007). Importantly, the heterozygotes showed increased genomic instability and accelerated tumorigenesis (Venkatesan *et al.*, 2007). Finally, mice deficient for pol η or ι develop cancer after exposure to UV light and, although loss of pol ζ is embryonically lethal in mice, cells deficient for pol ζ show severe chromosome instability, underscoring the importance of the translesion bypass pathway (Dumstorf *et al.*, 2006; Lin *et al.*, 2006; Wittschieben *et al.*, 2006).

Rationale

Mcm10 is a highly conserved, essential DNA replication factor whose role is far from elucidated (Solomon *et al.*, 1992; Merchant *et al.*, 1997). Mcm10 depletion causes DNA damage and apoptosis in human cells (Chattopadhyay and Bielinsky, 2007), underscoring its importance to cellular health (Paulsen *et al.*, 2009). Although much has yet to be learned about Mcm10's function in the cell, there are some aspects of its role in DNA replication that are known. Mcm10 binds DNA (Homesley *et al.*, 2000) and travels with the replication fork (Ricke and Bielinsky, 2004), but it is not known whether Mcm10's DNA binding activity is required for efficient replication. The structure of the internal domain of Mcm10 has recently been solved and Figures 4 and 5 show that even though the C terminus of Mcm10 differs between species, the internal domain is highly conserved (Warren *et al.*, 2008). Our goal is to use this information to help learn more about the mechanism by which Mcm10 operates during replication. In addition to DNA, Mcm10 also binds to and regulates the stability of Cdc17, the catalytic subunit of pol α (Ricke and Bielinsky, 2004, 2006; Chattopadhyay and Bielinsky, 2007). Very little is known about the regulation of pol α, although some consequences of its misregulation have been documented. A *cdc17* mutant, *pol1-1*, that affects stability of the pol α/primase complex, causes genome instability (Lucchini *et al.*, 1988; Gutierrez and Wang, 2003). Furthermore, reducing the levels of wild type Cdc17 causes chromosomal translocations (Lemoine *et al.*, 2005). Even reduced activity of the wild type enzyme has a phenotype, as aphidicolin treatment, which inhibits the replicative polymerases, causes chromosomal breaks at fragile sites in human cells (Glover *et al.*, 1984). Since Mcm10

and Cdc17 are highly conserved in humans and the proper regulation of Cdc17 appears to be crucial to maintain genome integrity, we decided to investigate the regulated turnover of Cdc17 and the functional consequences of disrupting this pathway in budding yeast.

Figure 1



Cell cycle image modified from http://homepage.mac.com/enognog/checkpoint.htm

Figure 1. Cell cycle regulation by cyclins. The cell cycle consists of four phases (G_1, S, G_2, and M) and progression through these phases is regulated by the temporal expression of cyclin proteins that activate Cdk1 in yeast. Cln1-3 regulate activities during G_1 phase, including upregulation of Clb5 and Clb5 and inhibition of Sic1, an inhibitor of Clb-Cdks. Clb5 and Clb6 are required for entry into and intiation of S phase. Clb3 and Clb4 are active during the S and G_2 phases and help promote entry into mitosis. Clb1 and Clb2, the mitotic cyclins are required for entry into and progression through M phase, but inibit exit from mitosis. The APC negatively regulates Clb1 and Clb2 once the cell is ready to proceed through and exit mitosis, thus promoting mitotic exit and cell divison. The thick red arrows refer to the G_1/S, intra-S, G_2/M, and M phase checkpoints.

Figure 2

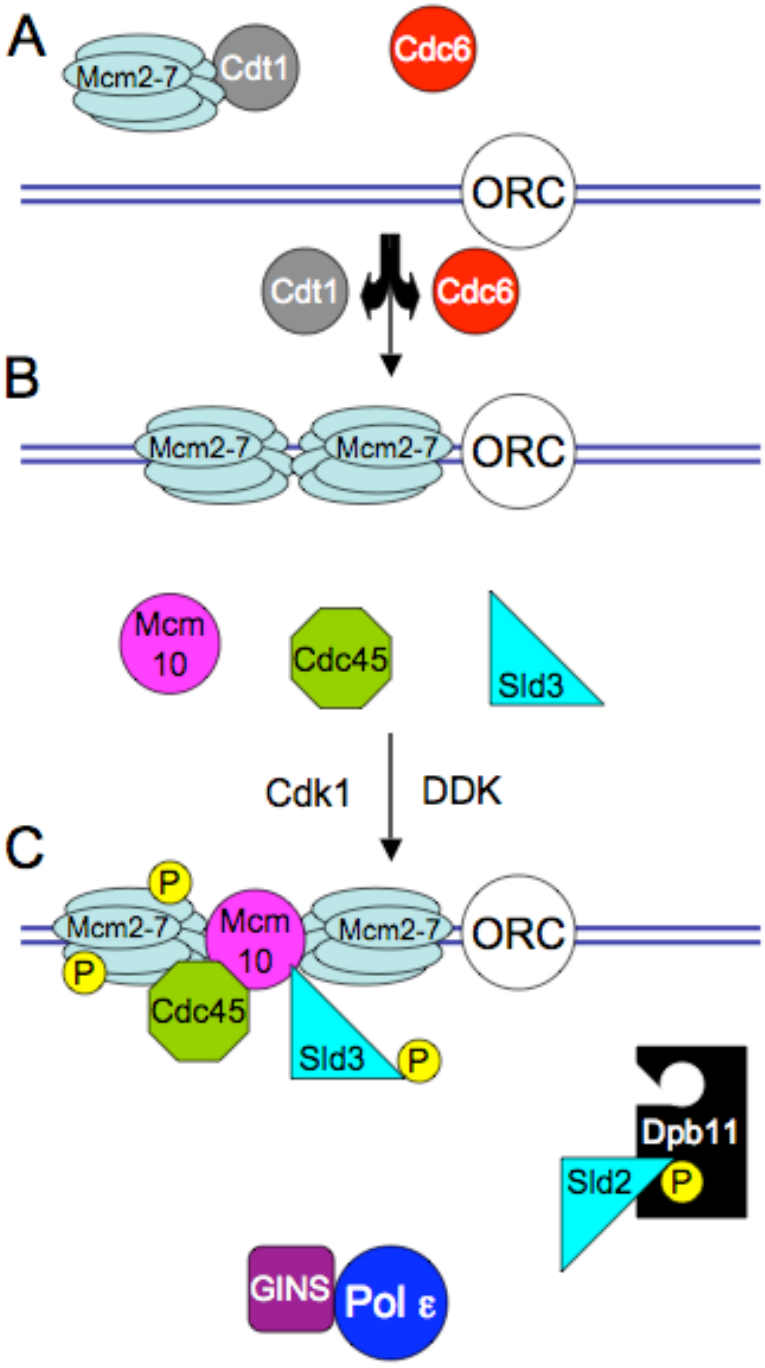

A
Mcm2-7
Cdt1
Cdc6
ORC
Cdt1
Cdc6
B
Mcm2-7
Mcm2-7
ORC
Mcm 10
Cdc45
Sld3
Cdk1
DDK
C
P
Mcm2-7
Mcm 10
Mcm2-7
ORC
P
Cdc45
Sld3
P
Dpb11
Sld2
P
GINS
Pol ε

Figure 2. DNA replication initiation in eukaryotes. The initiation of eukaryotic DNA replication is a multistep process requiring many proteins. (A) ORC recognizes and binds replication of origins and recruits Cdc6, which in turn recruits Cdt1 and the Mcm2-7 complex. (B) Mcm2-7 is loaded onto DNA forming the pre-replication complex (pre-RC); Cdc6 and Cdt1 are released from the DNA. Mcm10, Cdc45, and Sld3 are then recruited to the pre-RC. (C) Cdk1 activity is required for initiation and two of its targets are Sld2 and Sld3. Dpb11 acts as a bridge to bring phosphorylated Sld2 and Sld3 together. DDK is also required for initiation and phosphorylates the Mcm2-7 complex. Lastly, pol ε and the GINS are recruited to the origin, priming the cells for initation of DNA sythesis.

Figure 3

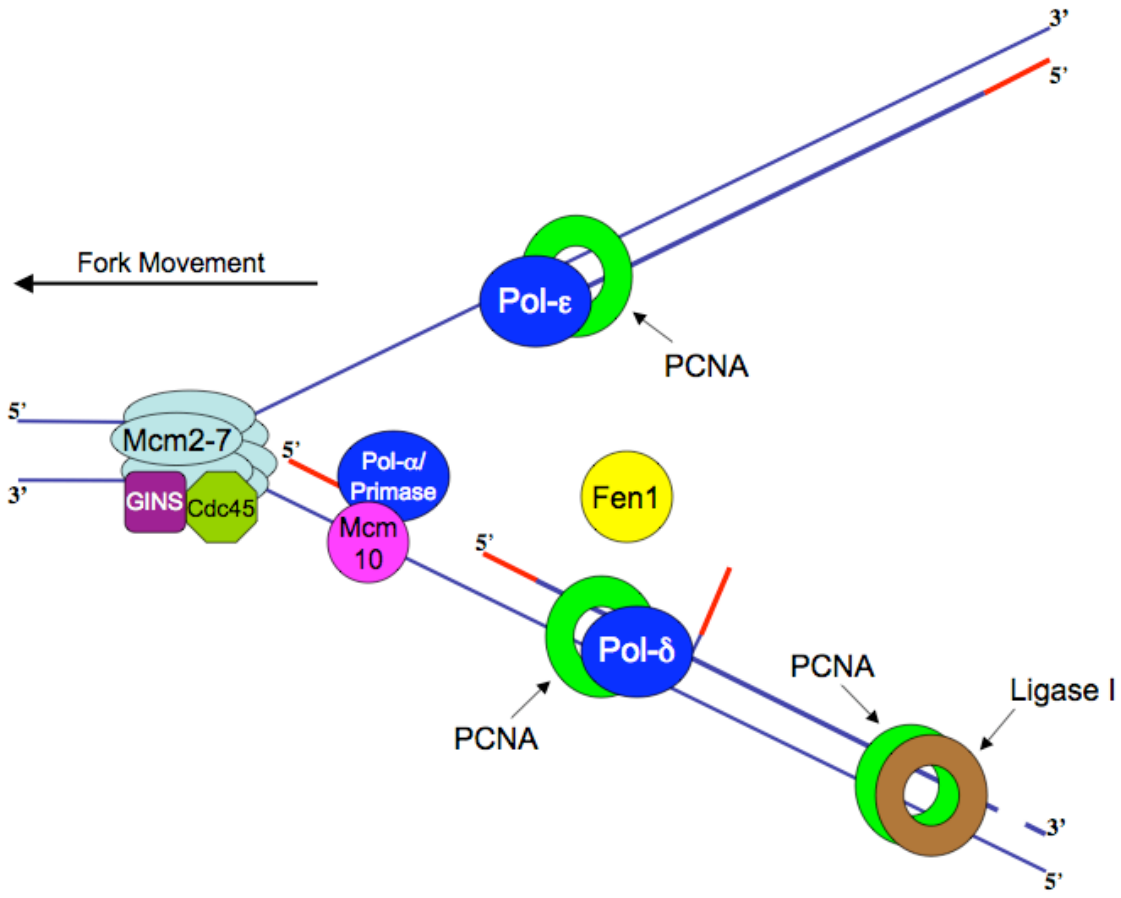

3'
5'
Fork Movement
Pol-ε
PCNA
5'
3'
Mcm2-7
GINS
Cdc45
5'
Pol-α/
Primase
Mcm
10
Fen1
5'
Pol-δ
PCNA
PCNA
Ligase I
3'
5'

Figure 3. Eukaryotic DNA replication elongation. DNA replication elongation is the process by which the cell makes exactly one copy of its genome. The DNA is unwound by the CMG helicase complex as the last step of replication initiation. Mcm10 then delivers pol α/primase to the replication fork where it synthizes a short RNA/DNA primer. Bulk DNA synthesis is then turned over to pol δ on the lagging strand and pol ε on the leading strand. Pol δ displaces the RNA primer, which is then removed by Fen1. The resulting nick in the DNA is sealed together by DNA Ligase I.

Figure 4

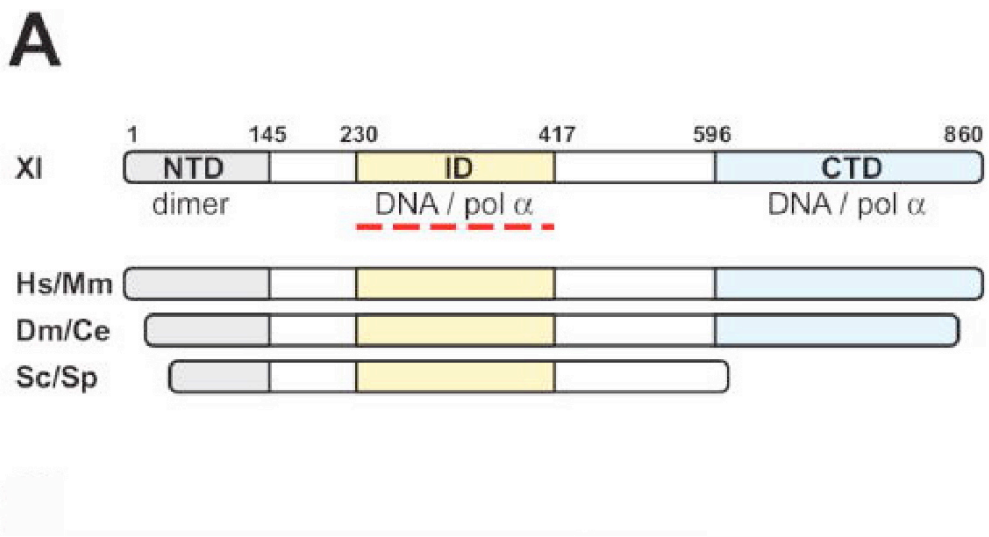

A
1
145
230
417
596
860
Xl
NTD
ID
CTD
dimer
DNA / pol α
DNA / pol α
Hs/Mm
Dm/Ce
Sc/Sp

Figure 4. The domains of Mcm10. (A) Mcm10 domain architecture. The three domains identified from *X. laevis* (Xl) Mcm10 are shown as colored bars and aligned with homologous regions of Mcm10 from *Homo sapiens* (Hs), *Mus musculus* (Mm), *Drosophila melanogaster* (Dm), *Caenorhabditis elegans* (Ce), *Saccharomyces cerevisiae* (Sc), and *Schizosaccharomyces pombe* (Sp). Data generated by Eric Warren (Warren *et al.*, 2008).

Figure 5

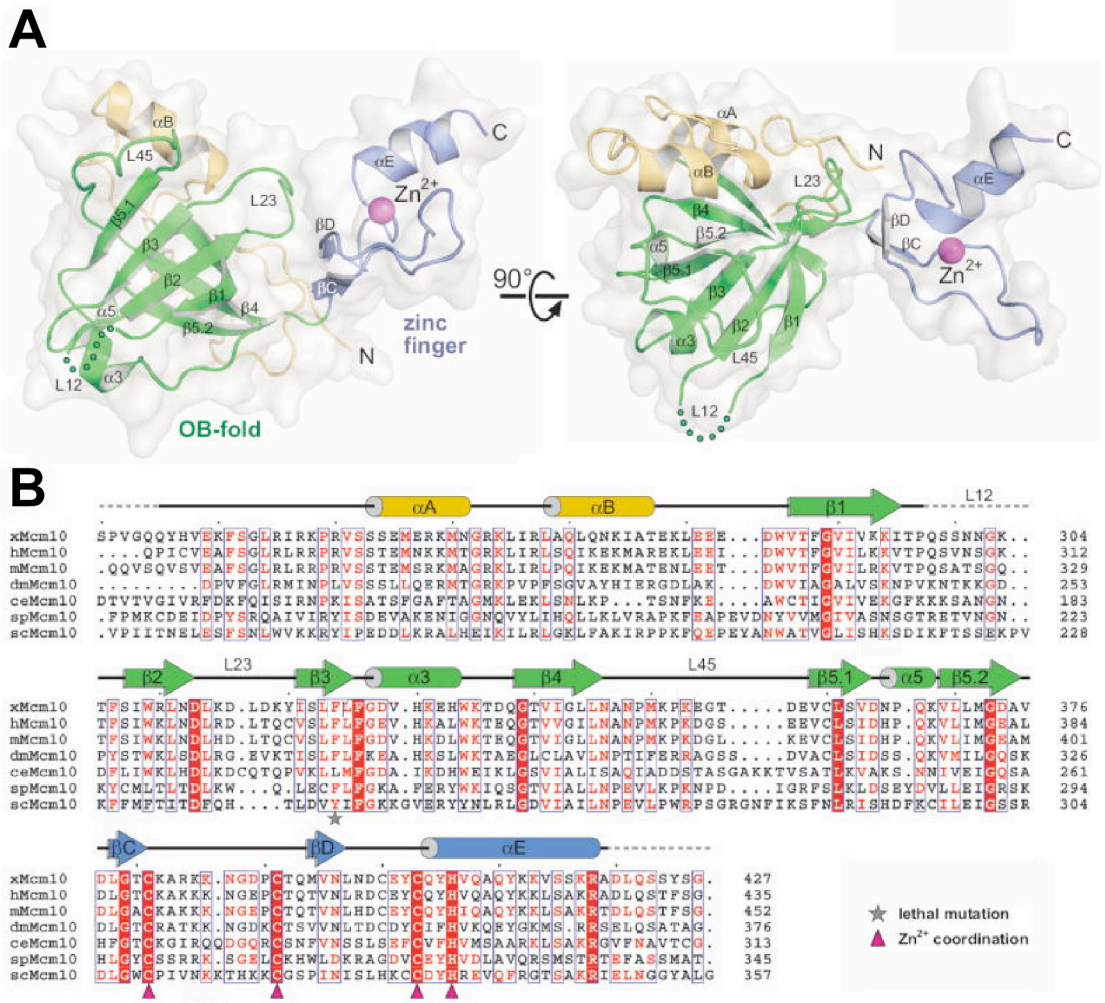

A
αB
L45
β5.1
L23
αE
Zn2+
C
βD
β3
β2
β1
β4
βC
α5
β5.2
zinc finger
N
L12
α3
OB-fold
90°
αA
αB
L23
N
C
αE
β4
β5.2
βD
α5
β5.1
βC
Zn2+
β3
β2
β1
α3
L45
L12
B
αA
αB
β1
L12
xMcm10 SPVGQQYHVEKFSGLRIRKPRVSSSEMERKMNGRKLIRLAQLQNKIATEKLEEE...DWVTFGVIVKKITPQSSNNGK.. 304
hMcm10 ....QPICVEAFSGLRLRRPRVSSTEMNKKMTGRKLIRLSQIKEKMAREKLEEI...DWVTFGVILKKVTPQSVNSGK.. 312
mMcm10 .QQVSQVSVEAFSGLRLRRPRVSSTEMSRKMAGRKLIRLPQIKEKMATENLEET...DWVTFGVILRKVTPQSATSGQ.. 329
dmMcm10 .........DPVFGLRMINPLVSSSLLQERMTGRKPVPFSGVAYHIERGDLAK....DWVIAGALVSKNPVKNTKKGD.. 253
ceMcm10 DTVTVGIVRFDKFQISIRNPKISATSFGAFTAGMKLEKLSNLKP...TSNFKE.....AWCTIGVIVEKGFKKKSANGN.. 183
spMcm10 .FPMKCDEIDPYSRQAIVIRYISDEVAKENIGGNQVYLIHQLLKLVRAPKFEAPEVDNYVVMGIVASNSGTRETVNGN.. 223
scMcm10 .VPIITNELESFSNLWVKKRYIPEDDLKRALHEIKILRLGKLFAKIRPPKFQEPEYANWATVGLISHKSDIKFTSSEKPV 228
β2
L23
β3
α3
β4
L45
β5.1
α5
β5.2
xMcm10 TFSIWRLNDLKD.LDKYISLFLFGDV.HKEHWKTDQGTVIGLLNANPMKPKEGT.....DEVCTSVDNP.QKVLLMGDAV 376
hMcm10 TFSIWKLNDLRD.LTQCVSLFLFGEV.HKALWKTEQGTVVGILNANPMKPKDGS.....EEVCTSIDHP.QKVLIMGEAL 384
mMcm10 TFSIWKLNDLHD.LTQCVSLFLFGDV.HKDLWKTEQGTVIGLLNANPMKPKDGL.....KEVCTSIDHP.QKVLIMGEAM 401
dmMcm10 PYSTWKLSDLRG.EVKTISLFLFKEA.HKSLWKTAEGLCLAVLNPTIFERRAGSS....DVACTSIDSS.QKVMILGQSK 326
ceMcm10 DFLIWKLHDLKDCQTQPVKLLMFGDA.IKDHWEIKLGSVIALISAQIADDSTASGAKKTVSATTKVAKS.NNIVEIGQSA 261
spMcm10 KYCMLTLTDLKW...Q.LECFLFGKA.FERYWKIQSGTVIALLNPEVLKPKNPD....IGRFSTKLDSEYDVLLEIGRSK 294
scMcm10 KFFMFTITDFQH....TLDVYIFGKKGVERYYNLRLGDVIAILNPEVLPWRPSGRGNFIKSFNTRISHDFKCILEIGSSR 304
βC
βD
αE
xMcm10 DLGTCKARKK.NGDPGTQMVNLNDCEYCQYHVQAQYKKVSSKRADLQSSYSG. 427
hMcm10 DLGTCKAKKK.NGEPGTQTVNLRDCEYCQYHVQAQYKKLSAKRADLQSTFSG. 435
mMcm10 DLGACKAKKK.NGEPGTQTVNLHDCEYCQYHIQAQYKKLSAKRTDLQSTFSG. 452
dmMcm10 DLGTCRATKK.NGDKGTSVVNLTDCDYGIFHVKQEYGKMS.RRSELQSATAG. 376
ceMcm10 HFGTCKGIRQQDGQRGSNFVNSSLSEFGVFHVMSAARKLSAKRGVFNAVTCG. 313
spMcm10 HLGYCSSRRK.SGELGKHWLDKRAGDVGEYHVDLAVQRSMSTRTEFASSMAT. 345
scMcm10 DLGWCPIVNKKTHKKGGSPINISLHKCGDYHHREVQFRGTSAKRIELNGGYALG 357
lethal mutation
Zn2+ coordination

Figure 5. Structure of the conserved internal domain of Mcm10 (A) The crystal structure of *X. laevis* Mcm10-ID (residues 230-427) is shown as a ribbon diagram with a white molecular surface. Two orthogonal views show the relative orientation of the OB-fold (green), zinc finger (blue ribbon, magenta Zn^{2+} sphere), and N-terminal α-helical/coil region (gold). (B) Sequence alignment of Mcm10-ID with schematic secondary structural elements colored as in (A). The gray star denotes a lethal mutation. Conserved Zn^{2+}-coordinating residues are marked with magenta triangles. Data generated by Eric Warren (Warren *et al.*, 2008).

Figure 6

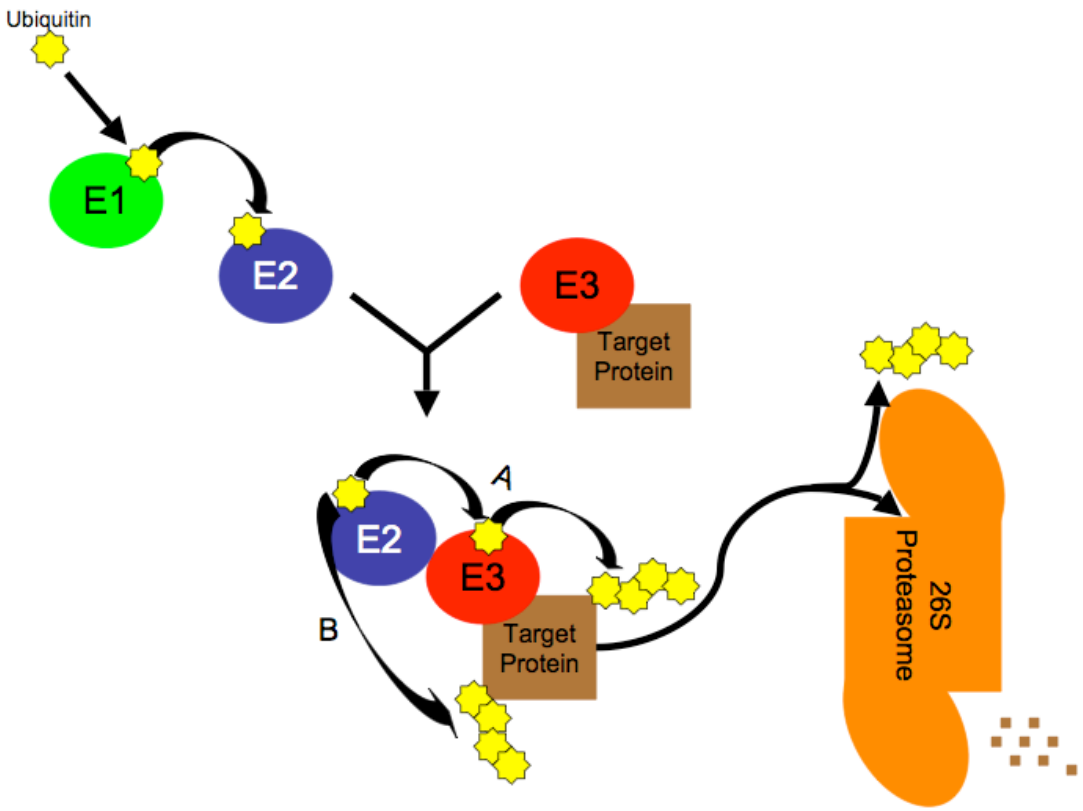

Ubiquitin
E1
E2
E3
Target Protein
A
E2
E3
B
Target Protein
26S Proteasome

Figure 6. Ubiquitin-mediated proteasomal degradation pathway. Ubiquitin-mediated proteasomal degradation is the major regulated proteolytic pathway in the cell. In this pathway, an ubiquitin molecule (yellow star) is first covalently attached to an E1 ubiquitin activating enzyme. The E1 enzyme then transfers the ubiquitin to an E2 ubiquitin conjugating enzyme. The E3 ubiquitin ligase enzyme, which provides the specificity for this pathway, binds the target protein to be degraded and brings it to the E2 enzyme. In the case of a HECT domain E3 ligase, the ubiquitin is transferred to the E3 ligase, which then transfers the ubiquitin to the target protein (A). For RING finger E3 ligases, the ubiquitin is transferred directly from the E2 to the target protein (B). The reaction is repeated, resulting in a polyubiquitinated target protein, which is then targeted for the proteasome. Once at the proteasome, the ubiquitin chain is removed from the target protein and the protein is subsequently unfolded and fed through the catalytic core of the proteasome, where it is degraded.

Chapter 2

Structural Basis for DNA Binding by Mcm10

Results

A Novel DNA Binding Platform

To expand our understanding of nucleic acid binding by Mcm10-ID (the highly conserved internal domain), ssDNA binding was investigated by monitoring perturbations in NMR chemical shifts as DNA was titrated into ^{15}N-enriched Mcm10-ID (Figure 1A). Sequence-specific resonance assignments were obtained using standard multidimensional triple-resonance experiments performed on ^{13}C, ^{15}N-enriched Mcm10-ID. The addition of ssDNA to Mcm10-ID resulted in a shift of a significant number but not all of the peaks in the 2D ^{15}N-^{1}H HSQC spectrum, which is consistent with a combination of effects from DNA binding and small conformational changes in the protein. Over the course of the titration, the peaks shifted continuously (fast exchange) with changes saturated at a 1:3 protein:DNA ratio (data not shown). These observations are consistent with the 3 μM binding affinity measured by fluorescence anisotropy (Robertson *et al.*, 2008). To determine the DNA binding site of Mcm10-ID, the residues exhibiting the most significant NMR chemical shift perturbations were mapped onto the crystal structure (Figure 1B). The largest shifts were observed for residues lining the β-barrel of the OB-fold and, surprisingly, the extended loop of the zinc finger. Very few perturbations were observed on the opposite face of the protein, demonstrating that DNA primarily contacts the common OB-fold/zinc loop surface.

A comparative structure search using the DALI server (Holm and Sander, 1993) revealed that the OB-fold in Mcm10-ID is most similar to those of the high-affinity ssDNA binding domains from the 70 kDa subunit of human RPA (RPA70AB) (Figures

2A and B). RPA70AB is composed of tandem OB-folds oriented with their ssDNA binding surfaces side-by-side, which allows eight nucleotides of ssDNA to traverse both binding pockets in a linear fashion (Figure 2A). Superposition of the Mcm10-ID and RPA70A OB-folds places the zinc finger in the same location as the second RPA70B OB-fold, suggesting that 8-10 nucleotides are needed to span the OB-fold/zinc loop surface. This correlates well with the length dependence of DNA binding to Mcm10-ID determined by fluorescence anisotropy, which showed that a 10-nucleotide oligomer was the shortest DNA that supported high affinity binding for ssDNA. Thus, the OB-fold and extended zinc loop in Mcm10-ID forms a molecular surface analogous to the DNA binding platform of the two RPA70AB OB-folds.

To validate that Mcm10 utilizes the entire OB-fold/zinc loop surface to engage DNA, mutational studies of Mcm10's DNA binding activities were performed. The assessment of mutants was based on DNA binding affinities for 25-mer ssDNA measured by a fluorescence anisotropy assay. A significant difference between Mcm10's putative DNA binding surface and that of RPA70AB is the cluster of basic residues (Lys293, Lys385, Lys386) on the zinc finger loop and in the cleft formed between the two motifs (Figure 2). Mutations in this electropositive region had a marked effect on Mcm10 binding to DNA. Most strikingly, a Lys385Glu/Lys386Glu double mutant on the extended zinc loop exhibited a 10-fold reduction in ssDNA binding affinity (Figures 3A and B). Lysine to glutamate substitutions along the zinc finger helix (Lys412Glu/Lys413Glu and Lys417Glu/Arg418Glu), by contrast, had a modest stimulatory effect on DNA binding. At the cleft between the OB-fold and zinc finger,

Lys293Ala reduced the affinity for ssDNA five-fold with respect to wild-type Mcm10-ID, and Tyr320Ala had a marginal but significant effect (Figure 3B).

On the OB-fold side, a cluster of three phenylalanine side chains (Phe306, Phe324, Phe326) and Lys353 are poised to interact with ssDNA in our model. Indeed, Phe324Ala on strand β3 and Lys353Ala in the L45 loop had a modest effect on DNA binding (Figure 3B). Substitution of any residue within the L12 loop, including Phe306, resulted in insoluble protein, which precluded analysis of L12 in our DNA binding assay. In RPA70AB, both OB-folds clamp the ssDNA between loops L12 and L45, and aromatic residues Phe238 (RPA70A) and Trp361 (RPA70B) at the C terminus of β3 form DNA base stacking interactions (Bochkarev *et al.*, 1997). Phe326 in xMcm10 is invariant among Mcm10 orthologs and superimposes with RPA Phe238 and Trp361 (Figures 2A and B). Surprisingly, substitution of Phe326 with alanine did not affect DNA binding to Mcm10-ID (Figure 3B). However, a single Phe238Ala mutation in RPA70A was also reported as not having a measurable effect on ssDNA binding, despite the observation of a direct contact to ssDNA in the crystal structure (Walther *et al.*, 1999). Thus, it is not possible to draw specific conclusions from the mutational data alone. The data do, however, reflect the redundancy in protein-DNA contacts along the extended binding site in Mcm10-ID. Taken together, the NMR and mutational data indicate that DNA spans the hydrophobic cleft of the OB-fold and the extended, positively charged loop of the variant zinc finger.

Functional Relevance of DNA Binding to Mcm10

To establish that the residues affecting DNA binding of xMcm10 in vitro have a role during DNA replication in vivo, we introduced the corresponding mutations into the endogenous *MCM10* locus of *S. cerevisiae* and tested for sensitivity to hydroxyurea (HU). Mid-log phase cultures were incubated in 0.2 M HU for 60, 120, or 180 min before they were diluted and plated in the absence of HU to determine the rate of recovery. Figure 4A shows that all mutants were expressed at levels similar to wild-type Mcm10. Under our test conditions, wild-type cells doubled in number during the course of the HU treatment, and mutations that showed no effect on DNA binding in vitro (Asn313Ala/Lys314Ala) behaved in the same manner. Those mutants exhibiting a modest decrease in DNA binding (His215Ala/Lys216Ala, corresponding to xMcm10 Lys293Ala) displayed a two-fold decline in survival (Figure 4B). Viability was more strongly compromised in the Phe230Ala/Phe231Ala mutant. Either of these two phenylalanines corresponds to Phe306 in xMcm10, which is implicated in DNA binding by our ssDNA docking model, but we were uable to test this directly because the protein was insoluble. Most strikingly, when Asn313 and Lys314 (corresponding to Lys385/386 in xMcm10) were changed to glutamic acid instead of alanine, survival was drastically reduced by more than seven-fold to about 30%, even after very short exposure to HU (Figure 4B). Taken together, these results extend our in vitro DNA binding studies and suggest that the corresponding residues in scMcm10 have an important role in DNA replication. Because neither Phe230/231 (located in the OB-fold) nor Asn313/Lys314 (located in the zinc finger loop) lies within the binding sites for pol α or PCNA (Das-

Bradoo *et al.*, 2006; Ricke and Bielinsky, 2006), it is likely that the HU sensitivity that we detected is directly due to a defect of scMcm10 in DNA binding.

Figure 1

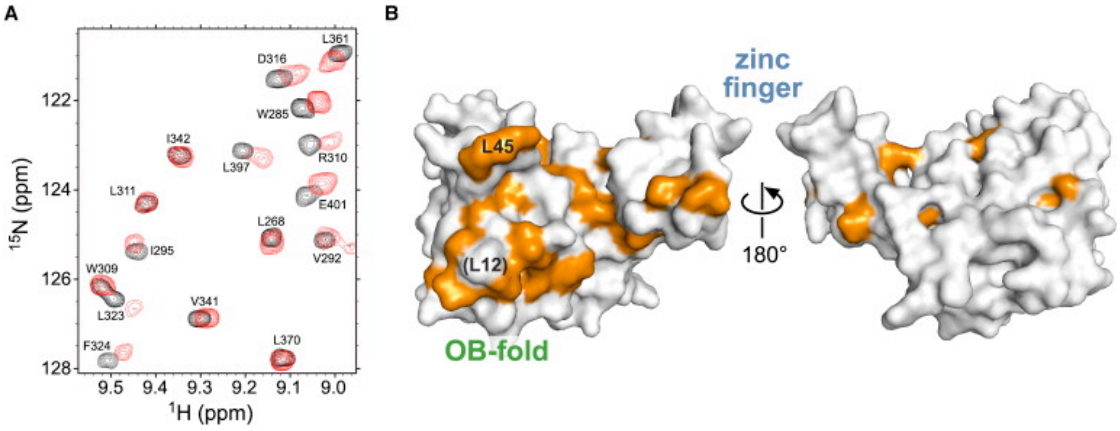
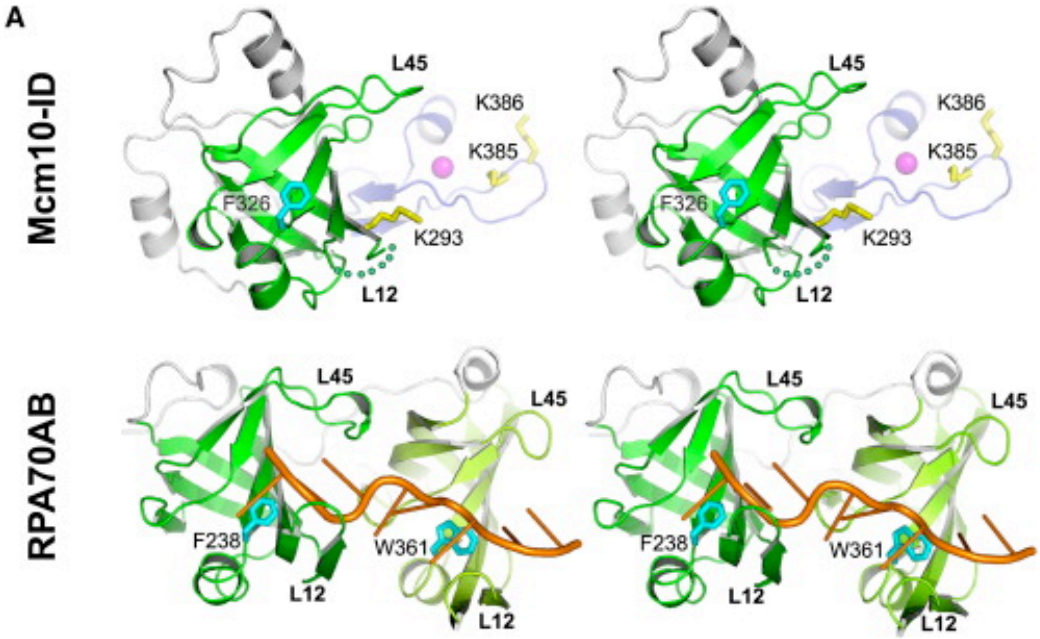

A
B
L361
D316
W285
I342
R310
L397
L311
E401
L268
I295
V292
W309
V341
L323
F324
L370
122
124
126
128
9.5
9.4
9.3
9.2
9.1
9.0
15N (ppm)
1H (ppm)
zinc
finger
L45
(L12)
180°
OB-fold

Figure 1. Mapping the Mcm10 DNA binding site. (A) NMR chemical shift perturbations in response to ssDNA binding to Mcm10. An overlay of a region of ^{15}N-^{1}H HSQC spectra of ^{15}N-enriched Mcm10 in the absence (black) and presence (red) of ssDNA (1:0.4 protein:DNA ratio) is shown. Peak assignments are labeled. (B) Surface representation of Mcm10-ID with all assigned residues showing significant chemical shift perturbation (>0.057 ppm) colored orange. Two orientations rotated 180˚ with respect to one another show that perturbations occur almost exclusively on one surface of the protein. Data generated by Eric Warren (Warren *et al.*, 2008).

Figure 2



B

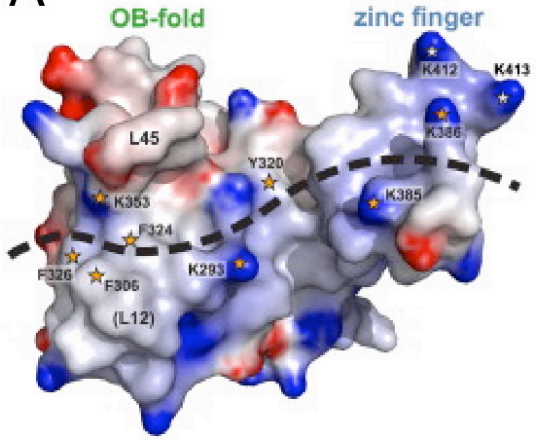

```
Mcm10   KLIRLAQL..QNKIATEKLEEEDWVTFGVIVKKITPQS.S.N....NGKTFSIWRLNDLKDLD  318
RPA70A  KVVPIASL..T.....PYQS..KWTIAARVTNKSQIRTWS.NSR.GEGKLFSL.ELVDES...  230
RPA70B  DFTGIDDLENK.....SKDS..LVDIIGICKSYEDATKITVRSNNREVAKRNIY.LMDTS..G  353
```


```
Mcm10   KYISLFLPGDVHKEHWK.TDQGTVIGLLNANPMKPK.EGTDE...VCLSVDNPQKVLLMGDA  375
RPA70A  GEIRATAFNEQVDKFFPLIEVNKVYYFSKGTLKIANKQFTAVKNDYEMTFNNETSVMPAED.  291
RPA70B  KVVTATLWGEDADKFDGSR..QPVLAIKGARVSDF.....G...GRSLSVLSSSTIIANPDI  405
```

Figure 2. The DNA binding surface of Mcm10. (A) Stereodiagram of crystal structures of Mcm10-ID and an RPA70AB/ssDNA complex. OB-folds are colored green, zinc finger blue with magenta Zn^{2+}, and ssDNA orange. Residues important for DNA binding to Mcm10 and RPA are rendered as yellow and cyan sticks, respectively. (B) Structure-based sequence alignment of OB-folds from Mcm10, RPA70A, and RPA70B. Residues shown by mutagenesis to affect in vitro DNA binding in Mcm10-ID are highlighted yellow, and conserved aromatic residues contacting ssDNA in RPA are highlighted cyan. DNA-binding residues identified from the RPA crystal structure are in boldface, and disordered residues in the Mcm10-ID crystal structure are gray. Data generated by Eric Warren (Warren *et al.*, 2008).

Figure 3

A



B

Mutant	K_d (μM)	Relative binding
WT	3.4 ± 0.5	(1.0)
K293A	17.8 ± 4.0	0.2
Y320A	4.8 ± 0.5	0.7
F324A	5.5 ± 1.9	0.6
F326A	3.1 ± 0.7	1.1
K353A	6.6 ± 1.3	0.5
K386E	27.3 ± 2.1	0.1
K385E / K386E	40.2 ± 8.6	0.1
K412E / K413E	1.7 ± 0.3	1.9
K417E / R418E	2.4 ± 0.4	1.4

Figure 3. Mutations that alter Mcm10 binding to DNA. (A) Mcm10-ID engages ssDNA at both the OB-fold and zinc loop surface. Mcm10-ID is shown as an electrostatic potential surface (blue, positive; red, negative). Residues implicated in DNA binding are highlighted with orange stars, and the positions of L12 and L45 loops known to contact ssDNA in other OB-folds are labeled. (B) ssDNA binding activity of Mcm10-ID mutants. The dissociation constants (K_d) for wild-type and mutant Mcm10-ID proteins for 25-mer ssDNA were determined in vitro using fluorescence anisotropy. Data generated by Eric Warren (Warren *et al.*, 2008).

Figure 4

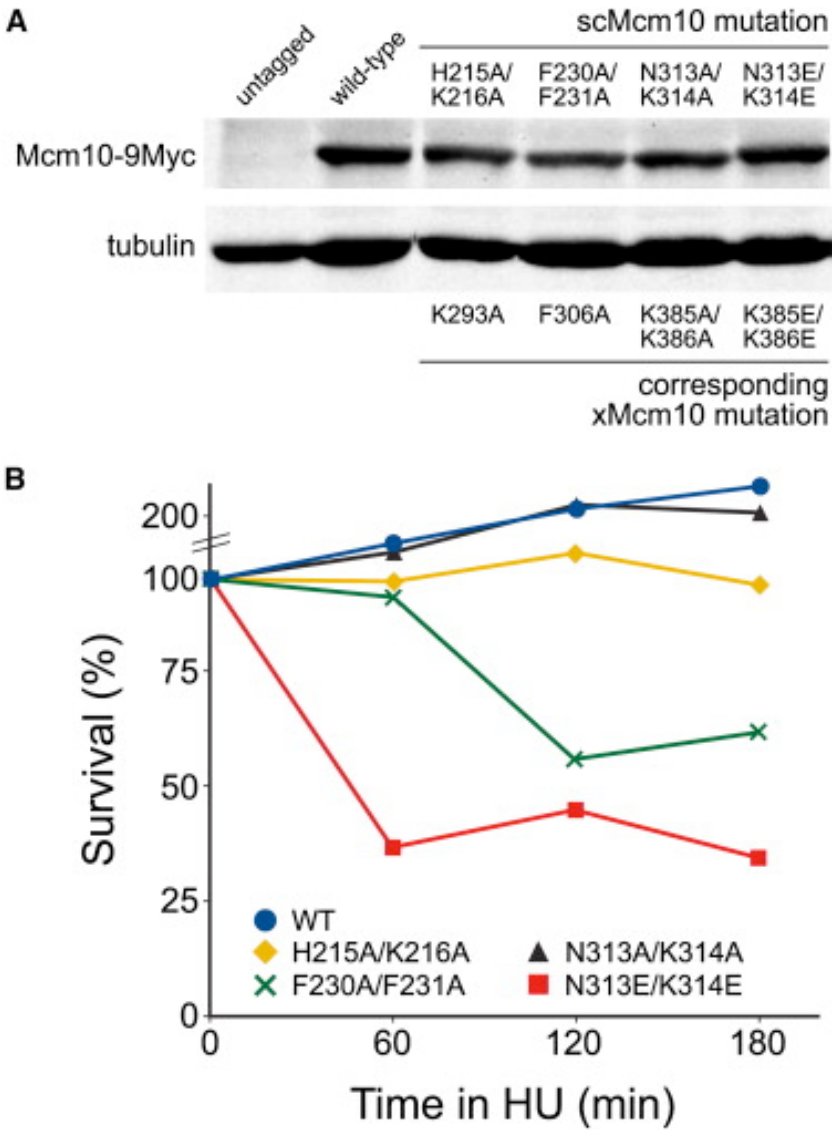

A
scMcm10 mutation
untagged
wild-type
H215A/ K216A
F230A/ F231A
N313A/ K314A
N313E/ K314E
Mcm10-9Myc
tubulin
K293A
F306A
K385A/ K386A
K385E/ K386E
corresponding xMcm10 mutation
B
Survival (%)
200
100
75
50
25
0
0
60
120
180
Time in HU (min)
WT
H215A/K216A
F230A/F231A
N313A/K314A
N313E/K314E

Figure 4. Mutations in the OB-fold and zinc finger domain of scMcm10 affect cell viability in hydroxyurea. (A) Total protein extracts prepared from mid-logarithmic phase cycling cells were analyzed by Western blot with anti-Myc and anti-α-tubulin antibodies. (B) Survival of wild-type, *mcm10-H215A/K216A*, *mcm10-F230A/F231A*, *mcm10-N313A/K314A*, and *mcm10-N313E/K314E* cells after treatment with 0.2 M hydroxyurea for 60, 120, or 180 min is shown in one representative experiment. Data generated by Justin Haworth.

Materials and Methods

Mcm10 Purification

Xenopus laevis Mcm10-ID (amino acids 230-427) was expressed and purified as previously reported (Robertson *et al.*, 2008). Briefly, Mcm10-ID was overexpressed as a N-terminal thioredoxin-His_6 fusion protein from a modified pET-32a expression vector (Novagen) in *Escherichia coli* BL21 (DE3) cells at 16˚C. For NMR experiments, protein was uniformly enriched with ^{13}C and ^{15}N by propagating cells in M9 minimal medium supplemented with 2 mg/ml $^{13}C_6$-glucose and/or 1 mg/ml $^{15}NH_4Cl$ (Cambridge Isotope Laboratories). Cells were harvested in 50 mM Tris (pH 7.5), 500 mM NaCl, and 10% glycerol, and lysed under pressure (25,000 psi) using an Avestin EmulsiFlex C3 homogenizer. Mcm10-ID was isolated using Ni-NTA affinity chromatography (Qiagen). Following cleavage of the affinity tag, the protein was further purified by ssDNA-cellulose (Sigma) and gel filtration chromatography using a Superdex 200 preparative column (GE Healthcare) equilibrated in 20 mM Tris (pH 7.5), 150 mM NaCl, and 5% glycerol.

X-Ray Crystallography

Mcm10-ID crystals were grown by hanging drop vapor diffusion from 100 mM Tris (pH 8.0), 100 mM KSCN, and 40% PEG 4000, and were flash frozen in mother liquor containing 10% glycerol prior to data collection. X-ray diffraction data were collected at beamline 22-ID at the Advanced Photon Source (Argonne, IL) and processed with HKL2000 (Otwinowski *et al.*, 1997). Mcm10-ID crystallized in space group $P2_1$ with three molecules in the asymmetric unit.

Experimental X-ray phases were obtained from a multiwavelength anomalous diffraction (MAD) experiment using a single crystal that was soaked for 5 hr at 25˚C in mother liquor supplemented with 1 mM $KAu(CN)_2$. Diffraction data were collected at 110 K at energies corresponding to the peak (1.0388 Å), inflection (1.0370 Å), and high-energy remote (1.0311 Å) settings for the gold L_{III} absorption edge. The positions of 10 gold atoms in the asymmetric unit were located by automated Patterson searching using SHELXD (Uson and Sheldrick, 1999), and initial phases to 3 Å were refined by solvent flattening using the SOLOMON option within autoSHARP (Vonrhein *et al.*, 2007). The model containing all three proteins in the asymmetric unit was built manually into the experimentally phased electron density using XtalView/Xfit (McRee, 1999). Electron density for residues 230-234 (N terminus), 420-427 (C terminus), and 298-304 (loop L12) were unobserved.

The model was refined against the native X-ray data (50-2.3 Å) with a maximum likelihood target for experimental phases as implemented in REFMAC 5.2 (Murshudov *et al.*, 1997). Improvements to the model were made by manual inspection of σ_A-weighted 2 mF_o-DF_c and mF_o-DF_c electron density maps, and they were judged successful by a decrease in R_{free} during refinement. Translation/liberation/screw-rotation (TLS) refinement in REFMAC was used to model anisotropic motion of each protein domain (three in total). Individual anisotropic B-factors were derived from the refined TLS parameters and held fixed during subsequent rounds of refinement, which resulted in a decrease in both R and R_{free} and a noticeable improvement in the electron density maps.

Analysis of the final structure using PROCHECK (Laskowski *et al.*, 1993) showed 91.5% and 8.3% of the total of 515 residues to be within the favored and allowed regions of the Ramachandran plot, respectively. Only one residue, located at the extreme N terminus, resided in the disallowed region.

NMR Spectroscopy

Spectra were acquired at 25˚C on Bruker DRX 500, 600, and 800 NMR spectrometers equipped with cryoprobes. Backbone resonance assignments were made using 3D triple resonance experiments acquired on the DRX 600: HNCA, HNCACB, CBCA(CO)NH, (H)C(CO)NH-TOCSY, and HNCO. Chemical shift perturbation data were collected by titrating unlabelled 15-mer oligonucleotide d(GGCGCATTGTCGCAA) into 250 μM ^{15}N-Mcm10-ID in 20 mM Tris-d_{11} (pH 7.0), 75 mM NaCl, and 5% D_2O. Gradient enhanced ^{15}N-^{1}H HSQC NMR spectra were recorded at protein/DNA ratios of 1:0, 1:0.5, 1:1, 1:3, and 1:5. The observation of chemical shift perturbations in the fast exchange limit (on the NMR time scale) enabled the peaks in the free protein and DNA complexes to be correlated. All spectra were processed and analyzed using Topspin v1.3 (Bruker, Billerca, MA) and Sparky v3.1 (University of California, San Francisco, San Francisco, CA).

Mutagenesis and In Vitro DNA Binding Assays

xMcm10 mutants were prepared using a Quik-Change Kit (Stratagene) and purified similarly to wild-type protein, except that the ssDNA-cellulose affinity step was replaced with an SP-sepharose (GE Healthcare) ion exchange step. DNA binding to Mcm10 mutants was measured by following an increase in fluorescence anisotropy as

protein was added to a fluorescently-labeled oligonucleotide d(ATGGTAGGCAACCATGTAGTAGTCA) containing a 6-carboxyfluorescein moiety at the 3'-end. For DNA length dependence measurements, 5-mer, 10-mer, and 15-mer oligonucleotides were derived from the 5'-end of the 25-mer sequence above. Protein was added over the concentration range of 0.1-50 μM to a solution containing 25 nM DNA in 20 mM Tris (pH 7.5), 100 mM NaCl, and 5% glycerol. Polarized fluorescence intensities using excitation and emission wavelengths of 495 and 515 nm, respectively, were measured for 30 s (1/s) and averaged. Anisotropy (r) was calculated using the equation $r = (I_{par} - I_{perp})/(I_{par} + 2I_{perp})$, where I_{par} and I_{perp} are the observed fluorescence intensities recorded through polarizers oriented parallel and perpendicular to the direction of vertically polarized light. Dissociation constants (K_d) were derived by fitting a simple two-state- binding model to data from three experiments using Kaleidagraph 3.5 (Synergy Software, Reading, PA).

Hydroxyurea Survival Assay

ScMcm10 mutant yeast strains were constructed by integrating pRS406-Mcm10-9Myc (ABy491), pRS406-Mcm10-9Myc-H215A/K216A (ABy492), pRS406-Mcm10-9Myc-F230A/F231A (ABy496), pRS406-Mcm10-9Myc-N313E/K314E (ABy503), and pRS406-Mcm10-9Myc-N313A/K314A (ABy525) into the endogenous *mcm10* locus of ABy014 (W303-1a). Total protein extracts were prepared from mid-logarithmic phase cycling yeast cultures ($OD_{600} = 0.6$) as previously described (Ricke and Bielinsky, 2006). Briefly, cells were pelleted, washed with 20% trichloroacetic acid and the cell wall was disrupted and the cells lysed by glass beads into 20% trichloroacetic acid. The resulting

lysate was pelleted by centrifugation at 3000 rpm for 10 min. The supernatant was removed and the pellet was resuspended in 2X Laemmli buffer (4% SDS, 20% glycerol, 120 mm Tris-Cl, pH 6.8, 200 mm dithiothreitol, 0.1% bromphenol blue). 1 M Tris was used to neutralize the lysates and the samples were boiled for 5 min. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed by Western blot with anti-Myc (9E11, LabVision Neomarkers) for Myc-tagged scMcm10 and anti-α-tubulin (MMS-407R, Covance). For the hydroxyurea survival assay, cells were grown to mid-logarithmic phase. All mutants tested had a doubling time comparable to the wild-type strain (data not shown). A 100 μl aliquot of cells was removed from each culture before adding 200 mM hydroxyurea; 100 μl aliquots were removed at timed intervals and diluted, and colony-forming units were scored for viability on YPD (rich medium) plates as described previously (Allen *et al.*, 1994). Percentage survival was determined relative to cells that were not exposed to hydroxyurea at the beginning of the experiment.

Chapter 3

The Degradation Pathway of Cdc17 and the Functional Consequences of Its Disruption

Results

Cdc17 Degradation is Proteasome-Dependent

Our laboratory has previously shown that Cdc17 is rapidly degraded when Mcm10 is depleted from yeast cells (Ricke and Bielinsky, 2004, 2006). The fact that this pathway is conserved in human cells (Chattopadhyay and Bielinsky, 2007) implies that it serves an important function. Thus, we were interested in elucidating the mechanism of the Cdc17 degradation pathway. Because Cdc17 was degraded very rapidly, we hypothesized that its turnover might be mediated by the proteasome. To test this hypothesis, we inhibited the proteasome and monitored Cdc17 levels in the absence of Mcm10 using the temperature sensitive *mcm10-1* mutant (Merchant *et al.*, 1997; Sawyer *et al.*, 2004a). We deleted *RPN4* and *PDR5* to sensitize cells to treatment with the proteasome inhibitor MG132 (Rock *et al.*, 1994; Fleming *et al.*, 2002). As expected, addition of MG132 at 30˚C had no effect (Figure 1). When cells were shifted to 37˚C in the presence of DMSO, Cdc17 was degraded. In contrast, when cells were shifted to 37˚C in the presence of MG132, Cdc17 was stabilized (Figure 1), suggesting that Cdc17 degradation is proteasome-dependent.

Ubc4 is Required for Cdc17 Degradation

We next wanted to identify the ubiquitin conjugating enzyme (E2) and the ubiquitin ligase (E3) responsible for Cdc17 degradation. There are only 11 E2 enzymes in yeast and only one, Cdc34, is essential (Hochstrasser, 1996). We therefore screened a collection of yeast strains that each lack a different nonessential E2 enzyme by overexpressing Cdc17. In addition, we overexpressed Cdc17 in strains lacking *MMS2*

(Hofmann and Pickart, 1999), an E2 enzyme variant, and *CUL3* and *RTT101*, both of which are components of separate E3 ligase complexes (Michel *et al.*, 2003). Because overexpressed Cdc17 is unstable (Muzi Falconi *et al.*, 1993), we reasoned that it would rapidly disappear in all strains except the one lacking the E2 enzyme required for its degradation. We overexpressed Cdc17 under the control of a galactose-inducible promoter from a high copy plasmid as described previously (Ricke and Bielinsky, 2006). To monitor Cdc17 stability, we grew cells in liquid medium to mid-log phase, induced Cdc17 expression by adding galactose, then added glucose to inhibit Cdc17 expression and observed Cdc17 protein levels by Western blot over the course of three hours. Cdc17 was indeed unstable when overexpressed in our strain background. The protein was nearly undetectable 3 hours after glucose addition (Figure 2). In most strains, overexpressed Cdc17 was degraded with kinetics very similar to those observed in wild-type cells with the notable exception of *ubc4Δ* mutants (Figure 2). Cdc17 was significantly stabilized in *ubc4Δ* cells compared to all other strains, including *ubc5Δ* mutants (Figure 2). We determined that Cdc17 overexpression levels in *ubc4Δ* mutants were approximately 10-fold higher than in wild-type cells by diluting our protein extracts (data not shown). Although Ubc4 and Ubc5 are 93% identical and largely functionally redundant, we attribute this discrepancy to the differential regulation of Ubc4 and Ubc5. *UBC5* mRNA is of low abundance in log phase cells (Seufert and Jentsch, 1990) and, although present at steady state levels similar to Ubc4, Ubc5 protein is much less stable (Panasenko *et al.*, 2009). We concluded from this data that Ubc4 functions as the E2

enzyme in the ubiquitin-mediated degradation of overexpressed Cdc17 in cycling yeast cells.

Because Ubc4 has been implicated in the degradation of misfolded proteins (Seufert and Jentsch, 1990), it was possible that overexpressed Cdc17 is aberrantly folded and the requirement of Ubc4 for the degradation of overexpressed Cdc17 is not physiologically relevant. Therefore, we determined if degradation of endogenous Cdc17 also required Ubc4. We deleted *UBC4* in *mcm10-1* mutants in which Cdc17 is tagged with three hemagglutinin (HA) epitopes and monitored Cdc17 stability in the absence of Mcm10. On Mcm10 depletion at 37˚C, Cdc17 was degraded in the presence of Ubc4, as expected (Figure 3, A and B; (Ricke and Bielinsky, 2004). Conversely, when Mcm10 was depleted from *mcm10-1 ubc4Δ* cells, Cdc17 remained stable (Figure 3, A and B). To ensure that Cdc17 stability was specific to *mcm10-1 ubc4Δ* cells, we monitored Cdc17 stability in *mcm10-1 ubc5Δ*, *mcm10-1 rad6Δ, mcm10-1 ubc13Δ*, and *mcm10-1 mms2Δ* double mutants. None of the other gene deletions had a significant effect (Figure 3, A and B). We observed a slight but reproducible mobility shift in Cdc17-3HA when it was degraded. Whether this shift was due to protein modification is unclear. Notably, this shift was absent in *ubc4Δ* cells (Figure 3A). We concluded that endogenous Cdc17 degradation following depletion of Mcm10 is Ubc4-dependent.

Not4 is the E3 Ligase Responsible for Cdc17 Destabilization

To identify the E3 ligase that cooperates with Ubc4 in Cdc17 destabilization, we took a candidate approach. Contrary to the small number of E2 enzymes in yeast, there are ~ 100 E3 ligases in budding yeast (Kostova *et al.*, 2007). Although it would have

been possible to screen 100 deletion strains, we took advantage of the fact that cells lacking Ubc4 along with Ubc5 have several characteristic phenotypes. For example, *ubc4Δ ubc5Δ* double mutants are resistant to heat shock and translational inhibitors (Seufert and Jentsch, 1990; Chuang and Madura, 2005). To identify candidate E3 ligases, we searched the literature for E3 ligase-deletion strains that phenocopied the heat shock resistance of *ubc4Δ ubc5Δ* cells. The E3 ligase Not4 fulfilled this criterion (Mulder *et al.*, 2007). Furthermore, Not4 binds Ubc4, has Ubc4-dependent ubiqutination activity *in vitro*, and associates with the proteasome (Panasenko *et al.*, 2006; Laribee *et al.*, 2007; Mulder *et al.*, 2007). Thus, we deleted *NOT4* in *mcm10-1* mutants and tested whether this gene is involved in Cdc17 degradation. When we shifted *mcm10-1 not4Δ* cells to the non-permissive temperature of 37˚C to deplete Mcm10, Cdc17 remained stable in the absence of Mcm10, to the same extent as in *mcm10-1 ubc4Δ* double mutants (Figure 4, A and B). To ensure that the observed Cdc17 stability was due to the E3 ligase activity of Not4 we constructed *not4-L35A*, which is unable to bind Ubc4 (Mulder *et al.*, 2007). We performed a temperature shift experiment as described above with *mcm10-1* mutants, *mcm10-1 not4Δ* double mutants, and *mcm10-1 not4Δ* double mutants supplemented with either pRS316-*NOT4* or pRS316-*not4-L35A*. Cdc17 degradation was largely rescued in cells in which *NOT4* was added back, but not in cells with *not4-L35A*, suggesting that Not4's E3 ligase activity was indeed required for the destabilization of Cdc17 (Figure 5, A and B). The incomplete rescue of Cdc17 degradation was likely due to decreased expression of Not4 protein from the plasmid compared to endogenous Not4 levels (Figure 5C, compare middle lane to left lane).

Because Cdc17 was stabilized in *mcm10-1* cells in the presence of MG132 (Figure 1), we attempted to detect ubiquitinated Cdc17. Unfortunately, we were unable to detect ubiquitin-protein conjugates, although we performed immunoprecipitation experiments under both native and denaturing conditions (data not shown). To address whether ubiquitination was necessary for Cdc17 destabilization, we overexpressed synthetic wild-type or mutant ubiquitin in *mcm10-1 pdr5Δ rpn4Δ* cells. Clearly, expression of the G75,76A ubiquitin mutant (Das-Bradoo *et al.*, 2010), which is incompetent for isopeptide bond formation blocked Cdc17 degradation, whereas wild-type ubiquitin had no effect (Figure 6). Based on the results presented in Figure 4, we argue that Ubc4 acts in concert with the E3 ligase Not4 to destabilize Cdc17 in the absence of Mcm10. However, it remains unclear if Cdc17 is a direct target of ubiquitination.

Cdc17 Degradation Occurs in the Nucleus

Because Ubc4 and Not4 localize to the cytoplasm as well as the nucleus, we addressed where in the cell Cdc17 is degraded. Although Cdc17 is localized to the nucleus, it is possible that the protein is translocated to the cytoplasm before degradation. To determine whether Cdc17 is degraded in the nucleus or the cytoplasm, we fused a strong nuclear export signal (NES) (Wen *et al.*, 1995) along with 3 HA epitopes to the C-terminus of Ubc4 to restrict its localization to the cytoplasm. We confirmed that Ubc4-NES-3HA retained its function by testing the allele in a heat shock assay (Seufert and Jentsch, 1990). As mentioned above, *ubc4Δ ubc5Δ* cells are resistant to heat shock. If Ubc4-NES-3HA is not a functional enzyme, then *UBC4-NES-3HA ubc5Δ* double mutants

should be resistant to heat shock. However, when we tested this particular strain, it was not resistant to heat shock, suggesting that Ubc4-NES-3HA retained E2 enzyme function (Figure 7A). We also confirmed that Ubc4-NES-3HA was primarily cytoplasmic by fusing GFP to its C-terminus and observing its cellular localization by fluorescence microscopy (Figure 7B). Having verified that Ubc4-NES-3HA is a functional cytoplasmic enzyme, we then determined whether Cdc17 is degraded in the nucleus or the cytoplasm. If Cdc17 degradation occurs in the nucleus, then Cdc17 should remain stable in *mcm10-1 UBC4-NES-3HA* double mutants. Conversely, if Cdc17 is degraded in the cytoplasm, then Cdc17 should be degraded after Mcm10 depletion from *mcm10-1 UBC4-NES-3HA* cells. Our experiments showed that the former was the case, although Cdc17 was not as stable in *UBC4-NES-3HA* cells as in *ubc4Δ* mutants (Figure 8, A and B). We attribute this difference in stability to the fact that a small portion of *mcm10-1 UBC4-NES-3HA* cells may not have efficiently excluded Ubc4 from the nucleus. Thus, the cells with Ubc4 in the nucleus were able to degrade Cdc17 and this resulted in overall lower levels of Cdc17 in the entire population of cells compared to *ubc4Δ* mutants. These data strongly suggested that Cdc17 is degraded primarily in the nucleus in an Ubc4- and Not4-dependent manner.

In the light of the fact that Not4 has been strongly implicated in transcriptional regulation and has thus far not been linked to the turnover of replication factors, we wondered whether Cdc17 transcript levels were affected in the various mutants that we analyzed in this study. To this end, we isolated total RNA and performed semi-

quantitative RT-PCR. We did not observe any significant changes in Cdc17 transcript levels (Figure 9), arguing that Ubc4/Not4 do not affect Cdc17 transcription.

TABLE 1

List of yeast strains used in this study

Strain name	Relevant genotype	Source
Strains derived from W303-1a (*MATa ura3-1 ade 2-1 his3-11,-15 leu2-3,-112 can1-100 trp1-1*)		
ABy013	*mcm10-1, CDC17::3HA-TRP1*	Ricke and Bielinsky, 2004
ABy342	*mcm10-1, ubc4::LEU2, CDC17::3HA-TRP1*	This Study
ABy455	*mcm10-1, UBC4::NES-3HA-URA3, CDC17::3HA-TRP1*	This Study
ABy529	*mcm10-1, not4::LEU2, CDC17::3HA-TRP1*	This Study
ABy363	*mcm10-1, ubc5::LEU2, CDC17::3HA-TRP1*	This Study
ABy359	*mcm10-1, rad6::LEU2, CDC17::3HA-TRP1*	This Study
ABy360	*mcm10-1, ubc13::LEU2, CDC17::3HA-TRP1*	This Study
ABy361	*mcm10-1, mms2::LEU2, CDC17::3HA-TRP1*	This Study
ABy781	*mcm10-1, CDC17::3HA-TRP1,* pRS316	This Study
ABy762	*mcm10-1, not4::LEU2, CDC17::3HA-TRP1*, pRS316	This Study
ABy738	*mcm10-1, not4::LEU2, CDC17::3HA-TRP1*, pRS316-*NOT4*	This Study
ABy739	*mcm10-1, not4::LEU2, CDC17::3HA-TRP1*, pRS316-*not4-L35A*	This Study
ABy596	*mcm10-1, CDC17::3HA-TRP1, NOT4::3HA-URA3*	This Study
ABy740	*mcm10-1, not4::LEU2, CDC17::3HA-TRP1*, pRS316-*NOT4-3HA*	This Study
ABy741	*mcm10-1, not4::LEU2, CDC17::3HA-TRP1*, pRS316-*not4-L35A-3HA*	This Study
ABy448	*CDC17::3HA-TRP1*	This Study
ABy605	*mcm10-1, UBC4::GFP-HIS3*	This Study
ABy660	*mcm10-1, UBC4::NES-GFP-HIS3*	This Study
ABy527	*mcm10-1, pdr5::LEU2, rpn4::HIS3, CDC17::2HA-URA3*	This Study
ABy661	*mcm10-1, pdr5::LEU2, rpn4::HIS3, CDC17::2HA-URA3*, YEp105	This Study
ABy1051	*mcm10-1, pdr5::LEU2, rpn4::HIS3, CDC17::2HA-URA3*, YEp105-G75,76A	This Study
Strains derived from S288C (*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0*)		
ABy150	*MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0*	Mortimer and Johnston, 1986
ABy152	*ubc4::KANMX*	This Study
ABy574	*UBC4::NES-3HA-URA3*	This Study
ABy577	*ubc5::LEU2, UBC4::NES-3HA-URA3*	This Study
GAP510	*ubc4::HIS3, ubc5::LEU2*	Trotter *et. al.*, 2001
ABy258	pRS426gal-*CDC17-2HA*	This Study
ABy259	*rad6::KANMX*, pRS426gal-*CDC17-2HA*	This Study
ABy260	*ubc4::KANMX*, pRS426gal-*CDC17-2HA*	This Study
ABy261	*ubc5::KANMX*, pRS426gal-*CDC17-2HA*	This Study
ABy262	*ubc7::KANMX*, pRS426gal-*CDC17-2HA*	This Study
ABy263	*ubc8::KANMX*, pRS426gal-*CDC17-2HA*	This Study
ABy264	*ubc10::KANMX,* pRS426gal-*CDC17-2HA*	This Study
ABy265	*ubc13::KANMX,* pRS426gal-*CDC17-2HA*	This Study

ABy266	*mms2::KANMX,* pRS426gal-*CDC17-2HA*	This Study
ABy267	*rtt101::KANMX,* pRS426gal-*CDC17-2HA*	This Study
ABy268	*cul3::KANMX,* pRS426gal-*CDC17-2HA*	This Study

TABLE 2

The *Saccharomyces cerevisiae* E2 ubiquitin-conjugating enzymes

Protein	kDa	Functions
Ubc1	24	Essential in the absence of Ubc4 and Ubc5. Important for growth from spores, endocytosis of membrane proteins
Ubc2/Rad6	20	DNA repair, induced mutagenesis, sporulation, repression of retrotransposition, N-end rule pathway
Ubc3/Cdc34*	34	Essential for viability, G1-S cell cycle progression, DNA replication, spindle pole body separation, degradation of p40 (Sic1), Gcn4, and Clns
Ubc4	16	[Ubc4 and Ubc5 are 92% identical] Degradation of abnormal proteins, sporulation,
Ubc5	16	resistance to stress conditions, degradation of MAT"2 and ubiquitin-fusion proteins, endocytosis of membrane proteins
Ubc6/Doa2	28	Degradation of MATα2 and certain membrane proteins (localizes to ER/nuclear envelope)
Ubc7	18	Involved in the ER-associated protein degradation pathway
Ubc8	25	Negatively regulates gluconeogenesis by mediating the glucose-induced ubiquitination of fructose-1,6-bisphosphatase[1]
Ubc9*	18	SUMO-conjugating enzyme[2]; essential for viability, G2-M cell cycle progression,
degradation		of Clb2 and Clb5 cyclins
Ubc10/Pas2	21	Peroxisome biogenesis and peroxisomal matrix protein import
Ubc11	17	Ubiquitin-conjugating enzyme most similar in sequence to Xenopus ubiquitin-conjugating enzyme E2-C, but not a true functional homolog of this E2; unlike E2-C, not required for the degradation of mitotic cyclin Clb2[3]
Ubc12	21	Mediates the conjugation of Rub1p, a ubiquitin-like protein, to other proteins[4]
Ubc13	18	Error-free DNA postreplication repair pathway[5]
Mms2	15	E2 variant involved in error-free postreplication DNA repair; forms a heteromeric complex with Ubc13p[6]

*Gene is essential for viability

Modified from Hochstrasser, *Annu. Rev. Genet.* 1996; [1]Schule T, *EMBO J* 2000; [2]Johnson ES and Blobel G, *J Biol Chem* 1997; [3]Townsley FI and Ruderman JV, *Yeast* 1998; [4]Liakopoulos D, *EMBO J* 1998; [5]Brusky J, *Curr Genet* 2000; [6]Hofmann RM and Pickart CM, *Cell* 1999

Figure 1

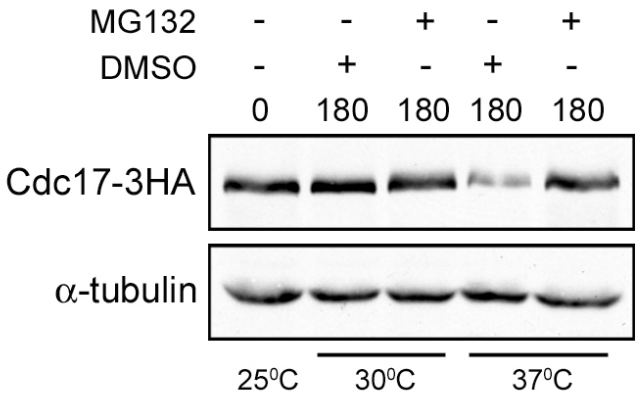

MG132 - - + - +
DMSO - + - + -
0 180 180 180 180
Cdc17-3HA
α-tubulin
25⁰C
30⁰C
37⁰C

Figure 1. Cdc17 degradation is proteasome-dependent. ABy527 (*mcm10-1, pdr5Δ, rpn4Δ*) cells were grown in rich liquid medium at 25˚C to midlog phase and split in four. MG132 was added to two of the cultures, DMSO was added to the other two, and cultures were shifted either to 30˚C or 37˚C for 180 minutes. Cdc17-3HA and α-tubulin were analyzed by Western blot. Data generated by Justin Haworth.

Figure 2

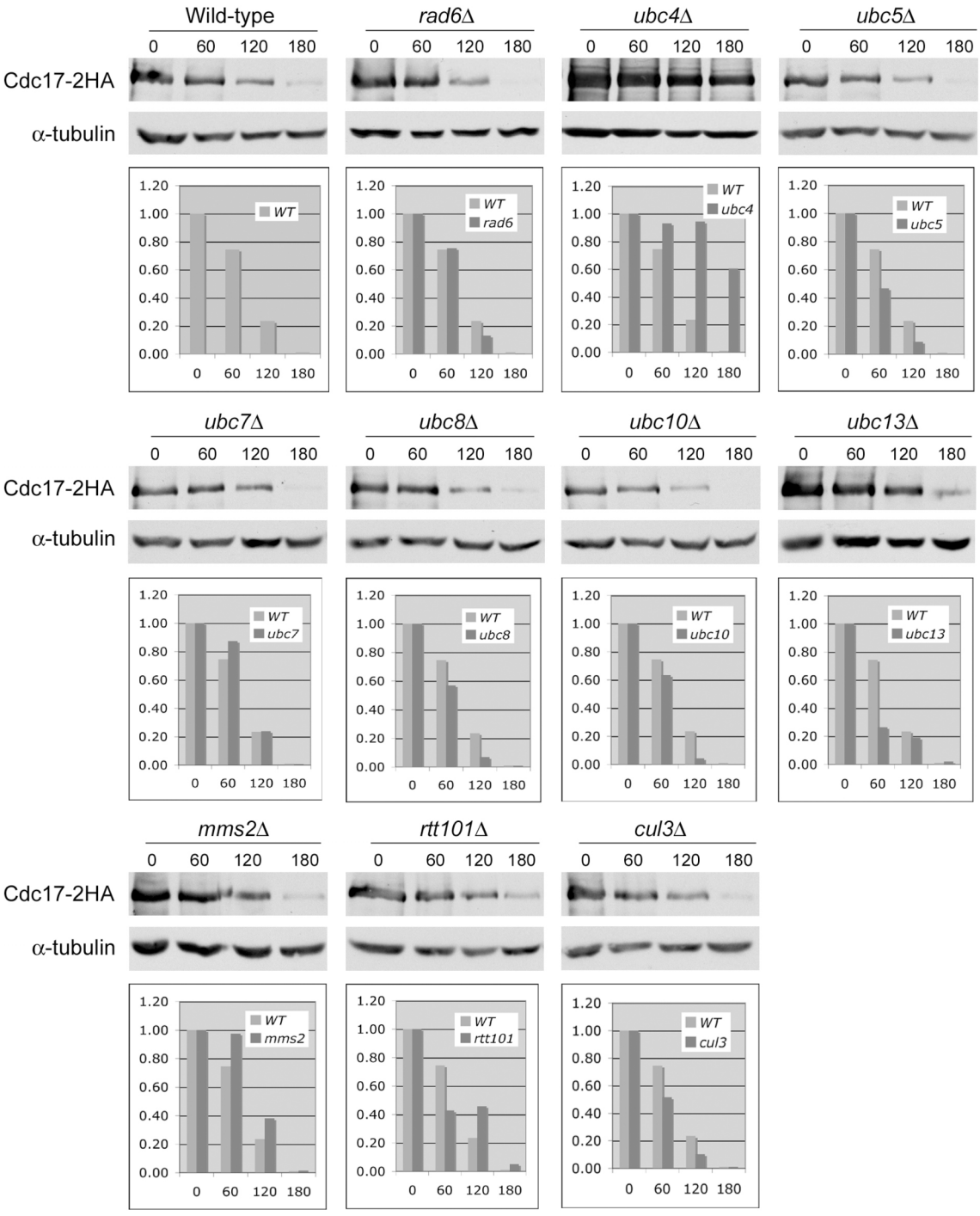

Wild-type
rad6Δ
ubc4Δ
ubc5Δ
ubc7Δ
ubc8Δ
ubc10Δ
ubc13Δ
mms2Δ
rtt101Δ
cul3Δ
0 60 120 180
Cdc17-2HA
α-tubulin
1.20
1.00
0.80
0.60
0.40
0.20
0.00
WT
rad6
ubc4
ubc5
ubc7
ubc8
ubc10
ubc13
mms2
rtt101
cul3

Figure 2. Overexpressed Cdc17 is stable in *ubc4Δ* cells. Strains were grown overnight in minimal medium with 2% raffinose. Cdc17-2HA was overexpressed from a galactose promoter in the presence of 2% galactose for 2 h. Cells were pelleted, resuspended in YPD, and samples were taken at 0, 60, 120, and 180 min after addition of glucose. Cdc17-2HA and α-tubulin, which served as a loading control, were analyzed by Western blot. The graphs show the quantification of Cdc17 relative to α-tubulin at each time point in each mutant strain relative to wild-type. The wild-type data in each graph is from the same experiment. Data generated by Justin Haworth.

Figure 3

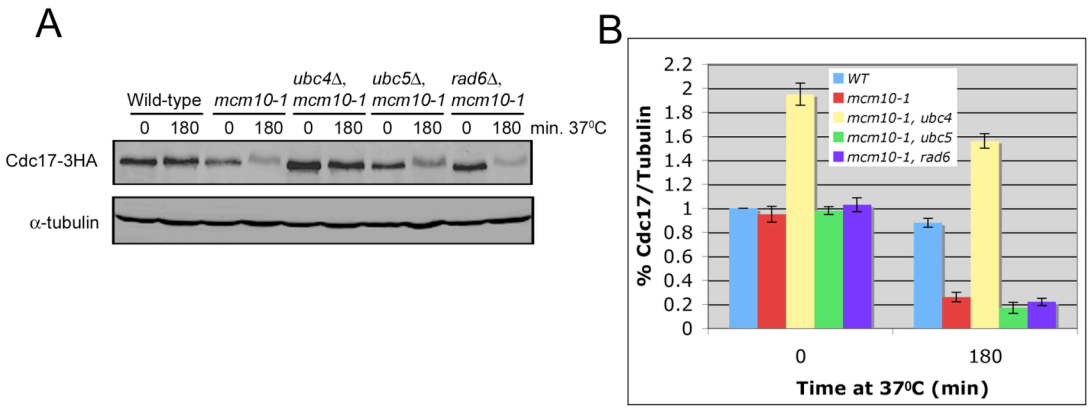

A
Wild-type
mcm10-1
ubc4Δ, mcm10-1
ubc5Δ, mcm10-1
rad6Δ, mcm10-1
0 180 0 180 0 180 0 180 0 180 min. 37°C
Cdc17-3HA
α-tubulin
B
% Cdc17/Tubulin
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
mcm10-1
mcm10-1, ubc4
mcm10-1, ubc5
mcm10-1, rad6
0
180
Time at 37°C (min)

Figure 3. Ubc4 is the E2 enzyme required for endogenous Cdc17 degradation in the absence of Mcm10. (A) Asynchronous cultures of ABy448 (W303-1a), ABy013 (*mcm10-1*), ABy342 (*mcm10-1*, *ubc4*Δ), ABy363 (*mcm10-1*, *ubc5*Δ) and ABy359 (*mcm10-1, rad6*Δ) grown at 25°C were shifted to 37°C for 180 min. Cdc17-3HA and α-tubulin were analyzed by Western blot. (B) The graph shows Cdc17/tubulin ratios at each time point for each strain relative to wild-type at time 0 (average of 3 separate experiments, bars represent mean +/- standard deviation). Data generated by Justin Haworth.

Figure 4

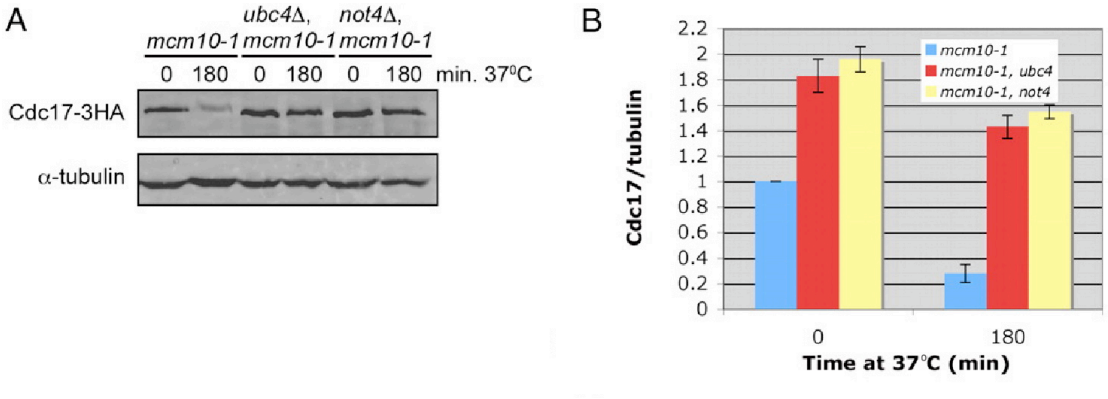

A
ubc4Δ,
not4Δ,
mcm10-1
mcm10-1
mcm10-1
0 180 0 180 0 180 min. 37°C
Cdc17-3HA
α-tubulin
B
Cdc17/tubulin
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
mcm10-1
mcm10-1, ubc4
mcm10-1, not4
0
180
Time at 37°C (min)

Figure 4. Not4 is the E3 ligase required for endogenous Cdc17 degradation in the absence of Mcm10. (A) Asynchronous cultures of ABy013 (*mcm10-1*), ABy342 (*mcm10-1*, *ubc4*Δ) and ABy529 (*mcm10-1, not4Δ*) grown at 25°C were shifted to 37°C for 180 min. Cdc17-3HA and α-tubulin were analyzed by Western blot. (B) The graph shows Cdc17/tubulin ratios at each time point for each strain relative to *mcm10-1* at time 0 (average of 3 separate experiments, bars represent mean +/- standard deviation). Data generated by Justin Haworth.

Figure 5

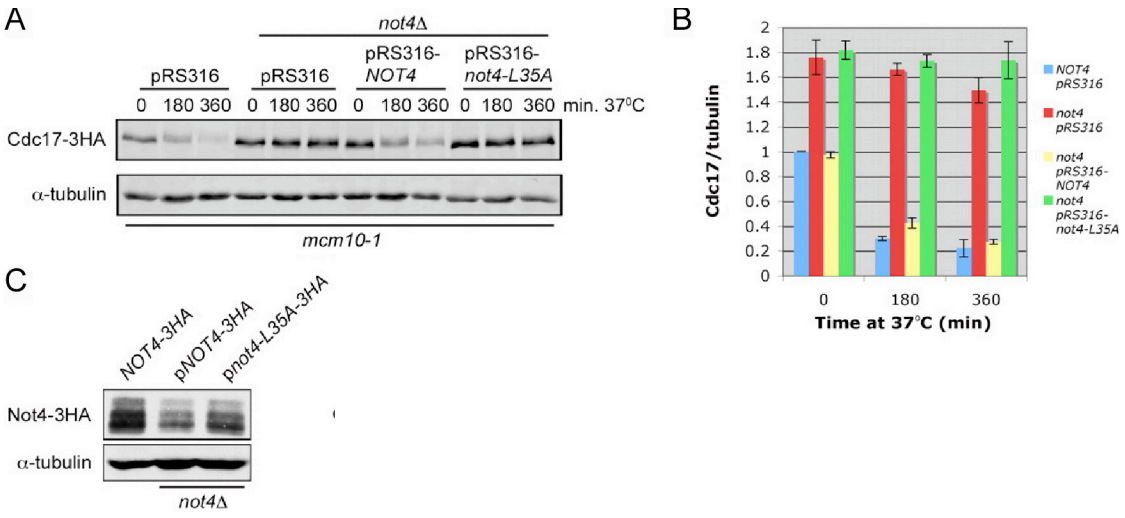

A
not4Δ
pRS316
pRS316
pRS316-NOT4
pRS316-not4-L35A
0 180 360 0 180 360 0 180 360 0 180 360 min. 37°C
Cdc17-3HA
α-tubulin
mcm10-1
B
Cdc17/tubulin
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
180
360
Time at 37°C (min)
NOT4 pRS316
not4 pRS316
not4 pRS316-NOT4
not4 pRS316-not4-L35A
C
NOT4-3HA
pNOT4-3HA
pnot4-L35A-3HA
Not4-3HA
α-tubulin
not4Δ

Figure 5. Cdc17 degradation depends on Not4's interaction with Ubc4. (A) Asynchronous cultures of ABy781 (*mcm10-1*, pRS316), ABy762 (*mcm10-1*, *not4*Δ, pRS316), ABy738 (*mcm10-1, not4Δ*, pRS316-*NOT4*) and ABy739 (*mcm10-1, not4Δ,* pRS316-*not4-L35A*) grown at 25°C were shifted to 37°C for 180 or 360 min. Cdc17-3HA and α-tubulin were analyzed by Western blot. (B) The graph shows Cdc17/tubulin ratios at each time point for each strain relative to ABy781 at time 0 (average of 3 separate experiments, bars represent mean +/- standard deviation). (C) Asynchronous cultures of ABy596 (*mcm10-1, NOT4-3HA*), ABy740 (*mcm10-1, not4Δ,* pRS316-*NOT4-3HA*) and ABy741 (*mcm10-1, not4Δ,* pRS316-*not4-L35A-3HA*) were grown in SC-ura liquid medium at 25˚C to midlog phase. Not4-3HA and α-tubulin were analyzed by Western blot. Data generated by Justin Haworth.

Figure 6

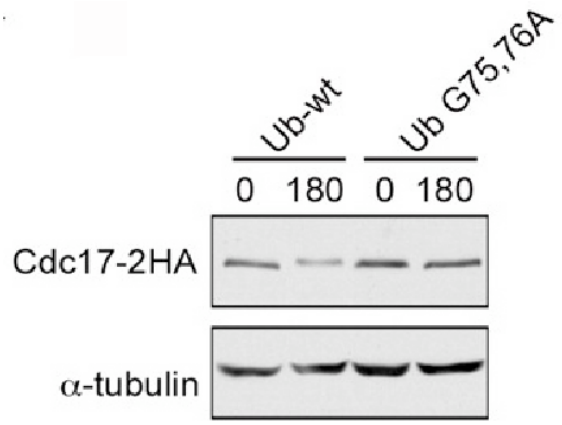


Figure 6. Ubiquitination is required for Cdc17 degradation. ABy661 (*mcm10-1, pdr5Δ, rpn4Δ*, YEp105) and ABy1051 (*mcm10-1, pdr5Δ, rpn4Δ*, YEp105-G75,76A) were grown in rich medium to OD_{600} = 0.6 at 25˚C and shifted to 37˚C for 3 hours. Total protein was precipitated with trichloroacetic acid and analyzed by Western blot using anti-HA and anti-tubulin antibodies. Data generated by Justin Haworth.

Figure 7

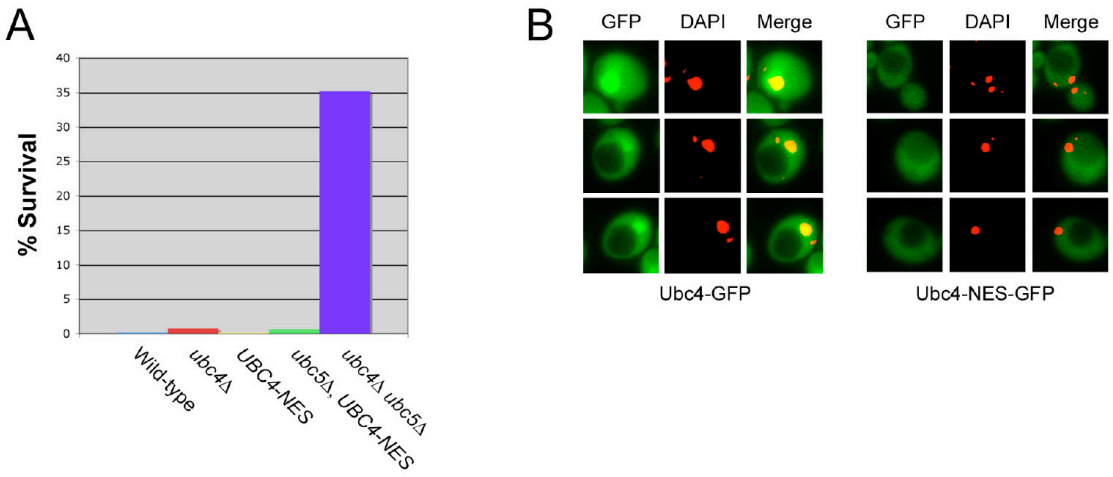

A
% Survival
40
35
30
25
20
15
10
5
0
Wild-type
ubc4Δ
UBC4-NES
ubc5Δ, UBC4-NES
ubc4Δ ubc5Δ
B
GFP
DAPI
Merge
GFP
DAPI
Merge
Ubc4-GFP
Ubc4-NES-GFP

Figure 7. Ubc4-NES is functional and cytoplasmic. (A) ABy150 (S288C), ABy152 (*ubc4Δ*), ABy574 (*UBC4-NES-3HA*), ABy577 (*ubc5Δ, UBC4-NES-3HA*) and GAP510 (*ubc4Δ, ubc5Δ*) were heat shocked as described in the *Materials and Methods*, aliquots were grown on YPD plates for 3 d at 30˚C, and colonies were counted to determine the percentage of cells that were viable following heat shock. (B) ABy605 (*mcm10-1, UBC4-GFP*) and ABy660 (*mcm10-1, UBC4-NES-GFP*) were examined using fluorescence microscopy. Merge represents GFP and DAPI. Yellow dots represent colocalization of Ubc4-GFP and DAPI-stained nuclei. Red dots represent DAPI-stained nuclei in which Ubc4-GFP is not present. Black circles represent vacuoles. Data generated by Justin Haworth.

Figure 8

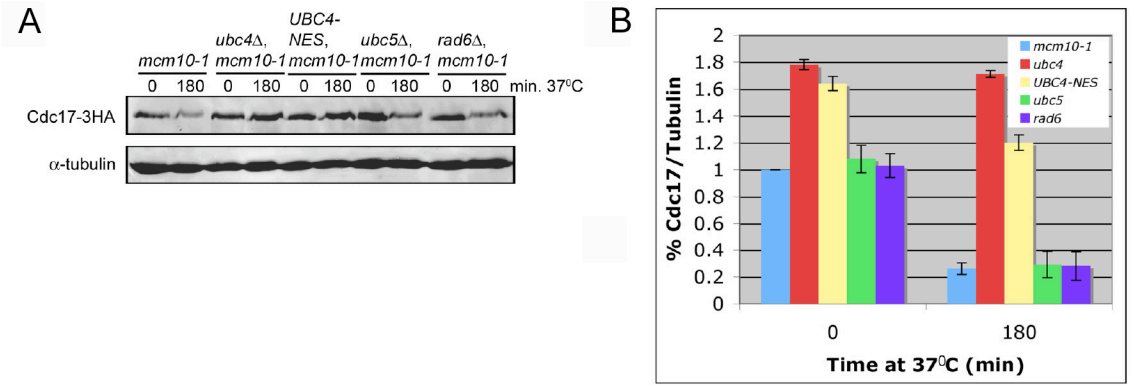

A
mcm10-1
ubc4Δ, mcm10-1
UBC4-NES, mcm10-1
ubc5Δ, mcm10-1
rad6Δ, mcm10-1
0 180 0 180 0 180 0 180 0 180 min. 37°C
Cdc17-3HA
α-tubulin
B
% Cdc17/Tubulin
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
180
Time at 37°C (min)
mcm10-1
ubc4
UBC4-NES
ubc5
rad6

Figure 8. Cdc17 is degraded primarily in the nucleus. (A) Asynchronous cultures of ABy013 (*mcm10-1*), ABy342 (*mcm10-1*, *ubc4*Δ), ABy455 (*mcm10-1, UBC4-NES-3HA*), ABy363 (*mcm10-1*, *ubc5*Δ) and ABy359 (*mcm10-1, rad6*Δ) grown at 25°C were shifted to 37°C for 180 min. Cdc17-3HA and α-tubulin were analyzed by Western blot. (B) The graph shows Cdc17/tubulin ratios at each time point for each strain relative to *mcm10-1* at time 0 (average of 3 separate experiments, bars represent mean +/- standard deviation). Data generated by Justin Haworth.

Figure 9

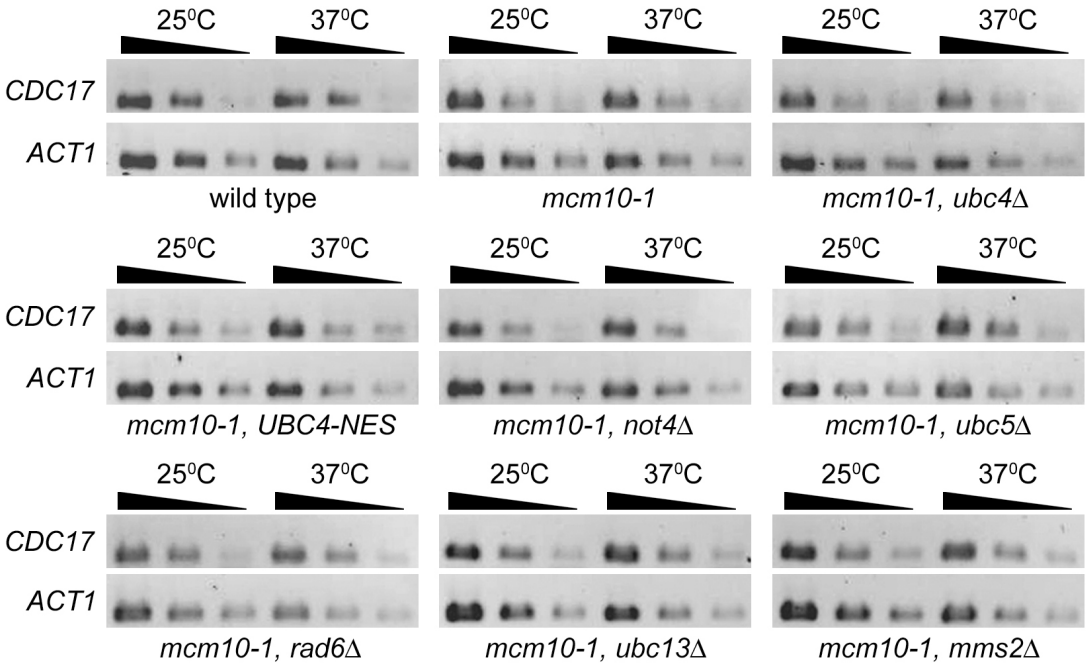

25⁰C
37⁰C
CDC17
ACT1
wild type
mcm10-1
mcm10-1, ubc4Δ
mcm10-1, UBC4-NES
mcm10-1, not4Δ
mcm10-1, ubc5Δ
mcm10-1, rad6Δ
mcm10-1, ubc13Δ
mcm10-1, mms2Δ

Figure 9. *CDC17* mRNA levels are unchanged in *not4Δ* mutants. ABy448 (W303-1a), ABy013 (*mcm10-1*), ABy342 (*mcm10-1, ubc4Δ*), ABy455 (*mcm10-1, UBC4-NES*), ABy529 (*mcm10-1, not4Δ*), ABy363 (*mcm10-1, ubc5Δ*), ABy359 (*mcm10-1, rad6Δ*), ABy360 (*mcm10-1, ubc13Δ*) and ABy361 (*mcm10-1, mms2Δ*) were grown in rich medium to midlog phase at 25˚C and shifted to 37˚C for 180 min. Total RNA was extracted from cells taken just before the shift to 37˚C and after 180 min at 37˚C. Semiquantitative RT-PCR was performed on each sample using 30, 6, and 3 ng total RNA with primers specific for either *CDC17* or *ACT1*. Data generated by Justin Haworth.

Materials and Methods

Strains and Plasmids

Strains are isogenic derivatives of either W303-1a or S288C. All strains carrying gene deletions were constructed using a PCR-based gene disruption method (Brachmann *et al.*, 1998). The genotypes of the yeast strains utilized in this study are listed in Table 1. All plasmids were constructed using standard molecular cloning techniques. pRS316-*NOT4* was constructed by cloning the entire *NOT4* coding sequence as well as 497 base pairs of promoter sequence into pRS316. Site-directed mutagenesis was used to generate pRS316-*not4-L35A* from pRS316-*NOT4*. The 3HA tag was added by PCR. Strains ABy661 and ABy1051 were constructed by transforming plasmid YEp105, which harbors a synthetic Myc-tagged ubiquitin gene (Ellison and Hochstrasser, 1991), and YEp105-G75,76A (Das-Bradoo *et al.*, 2010) into ABy527, respectively.

Protein Preparation and Western Blot Analysis

Total protein extracts were prepared as described previously by trichloroacetic acid extraction (Ricke and Bielinsky, 2006). Briefly, cells were pelleted, washed with 20% trichloroacetic acid and the cell wall was disrupted and the cells lysed by glass beads into 20% trichloroacetic acid. The resulting lysate was pelleted by centrifugation at 3000 rpm for 10 min. The supernatant was removed and the pellet was resuspended in 2X Laemmli buffer (4% SDS, 20% glycerol, 120 mm Tris-Cl, pH 6.8, 200 mm dithiothreitol, 0.1% bromphenol blue). 1 M Tris was used to neutralize the lysates and the samples were boiled for 5 min. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed by Western blot with anti-HA (16B12; Covance)

for HA-tagged proteins and anti-α-tubulin (B512; Covance). Alexa Fluor 680 (Invitrogen) secondary antibody was used to visualize proteins with Odyssey v3.0 software on an Odyssey scanner from LI-COR Biosciences.

Heat Shock Survival Assay

This assay was performed as described previously (Seufert and Jentsch, 1990). Briefly, cells were grown in YPD liquid medium to OD_{600} = 1.0. Before and after incubation at 52˚C for 5 min, appropriate aliquots were spread on YPD plates and incubated at 30˚C for 3 d. Colonies were counted and data presented as percentage of colonies formed after heat shock.

Microscopy

For nuclei staining, DAPI stain (10 μg/ml, Sigma, D1380) was added to cultures for 30 min, washed twice with sterile water, air-dried on a microscope slide, and visualized using a Zeiss AxioplanII microscope with a DIC Alpha Plan-Fluar 100x/1.45 objective and DAPI filter. Ubc4-GFP and Ubc4-NES-GFP were visualized using a GFP filter. All images were captured using an Axiocam HRm camera and Axiovision software 4.0.

RNA Extraction and RT-PCR

Total RNA extraction was performed with the Qiagen RNeasy kit according to the manufacturer's protocol. Total RNA was digested with RNase-free DNase (Ambion). Digested RNA (30, 6, and 3 ng) was subjected to a one-step RT-PCR reaction using the Superscript III One-Step RT-PCR kit (Invitrogen). The PCR cycle used was as follows: 52˚C for 30 min followed by 94˚C for 2 min and 30 cycles of 94˚C for 15 s, 55˚C for 30

s, and 68˚C for 30 s. *CDC17* was amplified with primer 5' *CDC17*-3454 (5'-GCTCTAAGAATGCGTAAGGCTGGTAG-3') and primer 3' *CDC17*-3712 (5'-CAAACGCACCACGTTGAAGCTATC-3') and *ACT1* was amplified with primer 5' *ACT1*-511 (5'- GTTACCCAATTGAACACGGTATTGTC-3') and primer 3' *ACT1*-836 (5'- CAAAATGGCGTGAGGTAGAGAGAAAC-3').

Chapter 4

Phenotypes Resulting from Cdc17 Overexpression

Results

Cdc17 Levels are Elevated in the Presence of Mcm10 in *ubc4*Δ and *not4*Δ Cells

Cdc17 is transcribed and translated at the G_1/S transition of every cell cycle (Wahl *et al.*, 1988), yet steady-state levels of Cdc17 remain unchanged throughout the cell cycle (Muzi Falconi *et al.*, 1993). It is possible that Cdc17 levels remain stable via degradation of any excess Cdc17 by Ubc4 and Not4. Because Mcm10 stabilizes Cdc17 (Ricke and Bielinsky, 2004, 2006; Chattopadhyay and Bielinsky, 2007), we asked whether steady-state levels of the catalytic subunit of pol-α were elevated in the presence of Mcm10-1 when Cdc17 degradation was disrupted. We grew asynchronous cultures of *ubc4Δ, UBC4-NES, not4Δ, ubc5Δ, rad6Δ, ubc13Δ,* and *mms2Δ* cells (all in the *mcm10-1* background) to midlog phase and analyzed the steady-state levels of Cdc17 by Western blot. The protein accumulated approximately two-fold in *ubc4Δ, UBC4-NES,* and *not4Δ* cells (Figure 1, A and B), whereas no significant increase was observed in *ubc5Δ, rad6Δ, ubc13Δ,* or *mms2Δ* cells. We observed similar trends in *ubc4Δ, not4Δ* and *ubc5Δ* mutants that carried the wild-type gene for *MCM10*, although the effect was most pronounced in *not4Δ* cells (Figure 1C). We concluded from these data that cells keep Cdc17 levels tightly regulated not only through Ubc4- and Not4-mediated degradation, but also by limiting the amount of Mcm10 that is available to bind and stabilize Cdc17.

Nuclear Ubc4 is Important for Replication Stress Tolerance

Because cells keep Cdc17 tightly regulated, we hypothesized that elevation of Cdc17 steady-state levels may have functional consequences during DNA replication. It has been shown previously that *not4Δ* cells are extremely sensitive to hydroxyurea (HU),

which correlates with Not4's role in the transcriptional upregulation of ribonucleotide reductase (Mulder *et al.*, 2005). Cells lacking Ubc4 have been shown to be moderately sensitive to HU, but the reason for this sensitivity was not explained (Mulder *et al.*, 2007), and we were curious as to whether it pertains to the nuclear function of Ubc4. Thus, we subjected *UBC4-NES-3HA* cells to HU treatment to determine if Ubc4's nuclear localization is important for replication stress tolerance. Interestingly, *UBC4-NES-3HA* cells behaved identically to *ubc4Δ* cells (Figure 2A). Whereas *ubc4Δ* mutants were moderately sensitive to HU, *not4Δ* cells were highly sensitive to the drug, as seen previously (Figure 2A). Cells lacking Ubc5 were no more sensitive to HU than wild-type cells. These results suggested that Ubc4 has a function in the nucleus that is important for replication stress tolerance.

To address whether Cdc17 plays a role in the observed HU sensitivity, we repeated the experiment in *mcm10-1* mutants. Because the *mcm10-1* mutation affects Cdc17 stability at 30˚C (Ricke and Bielinsky, 2006), we predicted that the HU sensitivity should be alleviated in the double mutants, despite the fact that the *mcm10-1* strain displays a higher HU sensitivity than wild-type cells (Figure 2A). This was indeed the case. Surprisingly, even the severe HU sensitivity of *not4Δ* mutants was suppressed by *mcm10-1* (Figure 2A). This suppression went hand-in-hand with reduced Cdc17 levels in *mcm10-1 not4Δ* double mutants (Figure 2B; we estimate a 2.5- to fivefold reduction compared with *not4Δ*). Therefore, the level of Cdc17 expression correlates well with the degree of HU sensitivity in *not4Δ* cells although we cannot exclude the possibility that

mcm10-1 ameliorates effects of replication stress in *not4Δ* mutants in a Cdc17-independent manner.

Overexpression of Cdc17 and Mcm10 Causes Slow Growth

We hypothesized that there may be other defects caused by elevated Cdc17 levels that occurred at low frequency, such that they would not be readily detected. To test this hypothesis we overexpressed Cdc17, along with its binding partner Mcm10, to exacerbate the effect of disrupting Cdc17 degradation. We overexpressed Cdc17 and Mcm10 from plasmids under the control of a galactose promoter (Figure 3A). To our surprise, 10-fold overexpression of Cdc17 and Mcm10 resulted in a significant growth defect in the absence of any exogenous stress (Figure 3B). According to our estimate, growth was inhibited approximately 25-fold. Importantly, cells carrying the empty vector controls did not display any growth phenotype (Figure 3B). Overexpression of Cdc17 alone did not affect growth, likely because Mcm10 was not present at high enough levels to recruit excess Cdc17 to chromatin where it functions during DNA replication (Ricke and Bielinsky, 2006). Similarly, overexpression of Mcm10 alone had little effect, suggesting that the primary reason for the proliferation inhibition was the excess of Cdc17. It was possible that the growth defect we observed upon co-overexpression of Cdc17 and Mcm10 was due to sequestration of the rest of the pol-α/primase complex. To address this possibility, we overexpressed the remaining subunits of pol-α/primase, Pol12, Pri1, and Pri2, together with Mcm10 and Cdc17 (Figure 93A). However, this did not rescue the observed phenotype (Figure 3B). From these data we concluded that careful regulation of Cdc17 is required for cells to undergo normal proliferation.

Overexpression of Cdc17 and Mcm10 Induces Genome Instability

As mentioned earlier, Cdc17 lacks proofreading activity, making it a potentially mutagenic enzyme (Morrison *et al.*, 1991). We therefore reasoned that higher levels of Cdc17 and Mcm10 might cause an elevated mutation rate in cells. To test this idea, we overexpressed either Cdc17 alone, Mcm10 alone, or Cdc17 with Mcm10 in wild-type cells and monitored the ability of these cells to acquire resistance to canavanine, a toxic amino acid analog (Whelan *et al.*, 1979). Individual colonies of each strain were grown in liquid medium containing galactose (to induce protein overexpression) to saturation and then plated on solid medium containing canavanine. A dilution of each culture was also plated on rich solid medium for a viable cell count. The numbers of canavinine-resistant (Can^R) colonies were counted after 3-4 days growth at 30˚C. Cells overexpressing both Cdc17 and Mcm10 showed a fivefold increase in mutation rate, whereas cells overexpressing Cdc17 or Mcm10 alone showed no significant increase in mutation rate (Figure 4, A and B). Cells containing overexpression vectors for Cdc17 and Mcm10 grown in glucose served as a negative control. We also tested *ubc4Δ* and *not4Δ* mutants and they displayed a 1.6- and 2.5-fold increase in Can^R colonies (Figure 4A).

Different pol-α mutants have also been shown to induce microsatellite instability in budding yeast (Gutierrez and Wang, 2003). We hypothesized that elevated levels of Cdc17 and Mcm10 might have the same effect because they interfere with normal proliferation (Figure 3B). To test this hypothesis, we used a plasmid-based assay to measure microsatellite instability (Henderson and Petes, 1992; Gutierrez and Wang,

2003). In this assay, a plasmid (pSH44) containing an in-frame insertion of a poly (GT) tract upstream of *URA3* was transformed into cells that contained either empty vector, vectors with galactose-inducible *CDC17* or *MCM10* alone, or vectors with galactose-inducible *CDC17* and *MCM10*, rendering these cells Ura^+ (Henderson and Petes, 1992). Alterations in the poly (GT) tract that change the reading frame result in Ura^- cells and these cells can be selected for on 5-fluoroorotic acid (5-FOA)-containing medium (Boeke *et al.*, 1984). We found that cells overexpressing both Cdc17 and Mcm10 disrupted the *URA3* gene at an ~ 20-fold higher rate than cells that carried empty vector controls (Figure 5A). Conversely, cells overexpressing Cdc17 alone showed no increase in mutation rate, whereas cells overexpressing Mcm10 alone showed a 4-fold increase in mutation rate (Figure 5A). To verify that 5-FOA resistant (FOA^R) cells carried mutations in the microsatellite tract we attempted to recover the plasmids of 30 colonies. Only 13 of these gave rise to ampicillin resistant bacteria colonies. DNA sequencing revealed that 5/13 had an intact poly (GT) tract, whereas 8/13, or 60 %, had lost the microsatellite tract, indicating that the plasmid DNA was rearranged. To confirm this finding, we cleaved the plasmids with HindIII, which should yield a 3.6- and 4.3 kb fragment (Gutierrez and Wang, 2003). All plasmids that had lost the poly (GT) tract displayed DNA rearrangements (Figure 6B). To further ascertain that the rearrangements were dependent on the microsatellite tract, we compared the number of FOA^R colonies between pSH44 and pRS316-*TRP1*, which is devoid of a poly (GT) tract. Whereas the former exhibited a 20-fold change, we only observed a fivefold increase in mutation rate for the latter in cells that cooverexpressed Cdc17 and Mcm10 (Figure 5B). Therefore, the majority of

plasmid rearrangements must have been triggered by replication through the microsatellite tract. Together, these findings demonstrate that disregulation of Cdc17 and Mcm10 can induce microsatellite-mediated DNA recombination.

Overexpression of Cdc17 results in synthetic dosage lethality with *not4Δ*

Thus far, all of our functional assays used the cooverexpression of Cdc17 and Mcm10. However, if cells are defective for Cdc17 degradation, the protein should accumulate and ultimately interfere with cell viability or fitness independently of cooverexpression of Mcm10. To test this prediction, we overexpressed either Cdc17 or Mcm10 in *ubc4Δ*, *ubc5Δ*, *ubc4Δ ubc5Δ*, and *not4Δ* mutants. Because *ubc4Δ ubc5Δ* double and *not4Δ* single mutants grow more slowly than the other strains, we evaluated cell growth after 3 and 5 d, respectively. Figure 7A shows that Cdc17 and Mcm10 were induced at similar levels in all strains after 1 hour in galactose-containing medium. As predicted, *ubc4Δ* mutants exhibited a significant growth defect when Cdc17, but not Mcm10, was overexpressed (Figure 7B). The effect was even more pronounced in *ubc4Δ ubc5Δ* double and *not4Δ* single mutants, which displayed SDL with *CDC17* overexpression. In the case of *ubc4Δ ubc5Δ* double mutants, this was also true for *MCM10* (Figure 7B). When we examined Cdc17 expression levels after overnight culture in galactose-containing medium, *ubc4Δ* and *not4Δ* cells had accumulated much higher levels of Cdc17 than the wild-type strain (Figure 8). The amount of Cdc17 protein detected in the different strains correlated well with the observed growth defect. These results are consistent with our claim that Ubc4 and Not4 regulate the degradation of Cdc17 either directly or indirectly.

TABLE 1

List of yeast strains used in this study

Strain name	Relevant genotype	Source
Strains derived from W303-1a (*MATa ura3-1 ade 2-1 his3-11,-15 leu2-3,-112 can1-100 trp1-1*)		
ABy013	*mcm10-1, CDC17::3HA-TRP1*	Ricke and Bielinsky, 2004
ABy342	*mcm10-1, ubc4::LEU2, CDC17::3HA-TRP1*	This Study
ABy455	*mcm10-1, UBC4::NES-3HA-URA3, CDC17::3HA-TRP1*	This Study
ABy529	*mcm10-1, not4::LEU2, CDC17::3HA-TRP1*	This Study
ABy363	*mcm10-1, ubc5::LEU2, CDC17::3HA-TRP1*	This Study
ABy359	*mcm10-1, rad6::LEU2, CDC17::3HA-TRP1*	This Study
ABy360	*mcm10-1, ubc13::LEU2, CDC17::3HA-TRP1*	This Study
ABy361	*mcm10-1, mms2::LEU2*, *CDC17::3HA-TRP1*	This Study
ABy448	*CDC17::3HA-TRP1*	This Study
ABy607	*ubc4::LEU2, CDC17::3HA-TRP1*	This Study
ABy597	*not4::LEU2, CDC17::3HA-TRP1*	This Study
ABy662	*UBC4::NES-3HA-URA3, CDC17::3HA-TRP1*	This Study
ABy764	*ubc5::LEU2, CDC17::3HA-TRP1*	This Study
ABy640	pSH44, pRS423gal, pRS425gal	This Study
ABy641	pSH44, pRS423gal, pRS425gal-*CDC17-2HA*	This Study
ABy642	pSH44, pRS423gal-*MCM10-3HA*, pRS425gal	This Study
ABy643	pSH44, pRS423gal-*MCM10-3HA*, pRS425gal-*CDC17-2HA*	This Study
ABy695	pRS423gal, pRS424gal, pRS425gal, pRS426gal	This Study
ABy694	pRS423gal, pRS424gal, pRS425gal, pRS426gal-*CDC17-2HA*	This Study
ABy696	pRS423gal-*MCM10-3HA*, pRS424gal, pRS425gal, pRS426gal	This Study
ABy705	pRS423gal-*MCM10-3HA*, pRS424gal, pRS425gal, pRS426gal-*CDC17-2HA*	This Study
ABy732	pRS423gal-*MCM10-3HA*/*POL12-3HA*, pRS424gal-*PRI1-3HA*/*PRI2-3HA*, pRS425gal, pRS426gal-*CDC17-3HA*	This Study
ABy962	pRS423gal	This Study
ABy963	pRS423gal-*CDC17-2HA*	This Study
ABy964	pRS423gal-*MCM10-3HA*	This Study
ABy965	*ubc4::LEU2*, pRS423gal	This Study
ABy966	*ubc4::LEU2*, pRS423gal-*CDC17-2HA*	This Study
ABy967	*ubc4::LEU2*, pRS423gal-*MCM10-3HA*	This Study
ABy968	*ubc5::LEU2*, pRS423gal	This Study
ABy969	*ubc5::LEU2*, pRS423gal-*CDC17-2HA*	This Study
ABy970	*ubc5::LEU2*, pRS423gal-*MCM10-3HA*	This Study
ABy971	*ubc4::LEU2, ubc5::TRP1*, pRS423gal	This Study
ABy972	*ubc4::LEU2, ubc5::TRP1*, pRS423gal-*CDC17-2HA*	This Study
ABy973	*ubc4::LEU2, ubc5::TRP1*, pRS423gal-*MCM10-3HA*	This Study
ABy974	*not4::LEU2*, pRS423gal	This Study

ABy975	*not4::LEU2*, pRS423gal-*CDC17-2HA*	This Study
ABy976	*not4::LEU2*, pRS423gal-*MCM10-3HA*	This Study
ABy977	pRS316-*TRP1*, pRS423gal, pRS425gal	This Study
ABy978	pRS316-*TRP1*, pRS423gal, pRS425gal-*CDC17-2HA*	This Study
ABy979	pRS316-*TRP1*, pRS423gal-*MCM10-3HA*, pRS425gal	This Study
ABy980	pRS316-*TRP1*, pRS423gal-*MCM10-3HA*, pRS425gal-*CDC17-2HA*	This Study
ABy831	*ubc4::LEU2*, *CDC17::3HA-TRP1,* pRS426gal-*CDC17-2HA*	This Study
ABy837	*not4::LEU2*, *CDC17::3HA-TRP1,* pRS426gal-*CDC17-2HA*	This Study
ABy981	*CDC17::3HA-TRP1,* pRS426gal-*CDC17-2HA*	This Study
Strains derived from S288C (*MATa his∆1 leu2∆0 met15∆0 ura3∆0*)		
ABy575	*not4::LEU2*	This Study
ABy574	*UBC4::NES-3HA-URA3*	This Study
ABy563	pRS426gal-*CDC17-2HA*	This Study
ABy564	pRS423gal-*MCM10-3HA*	This Study
ABy565	pRS423gal-*MCM10-3HA*, pRS426gal-*CDC17-2HA*	This Study

Figure 1

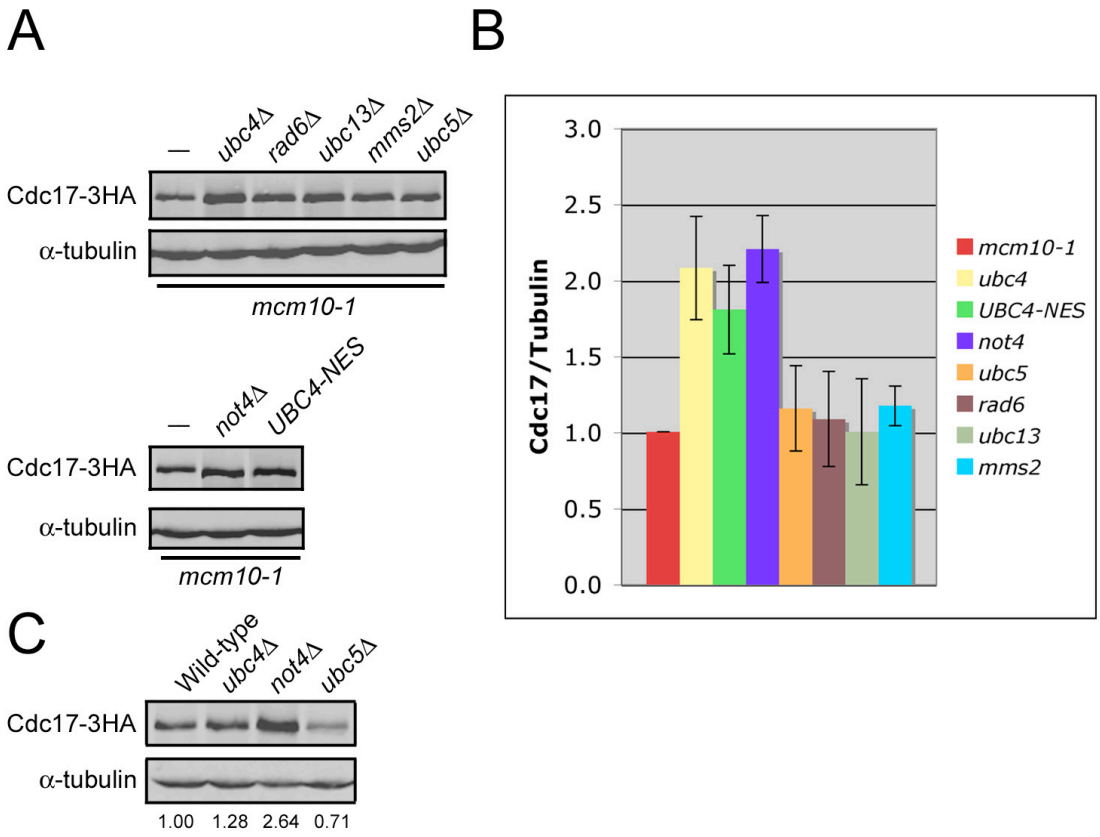
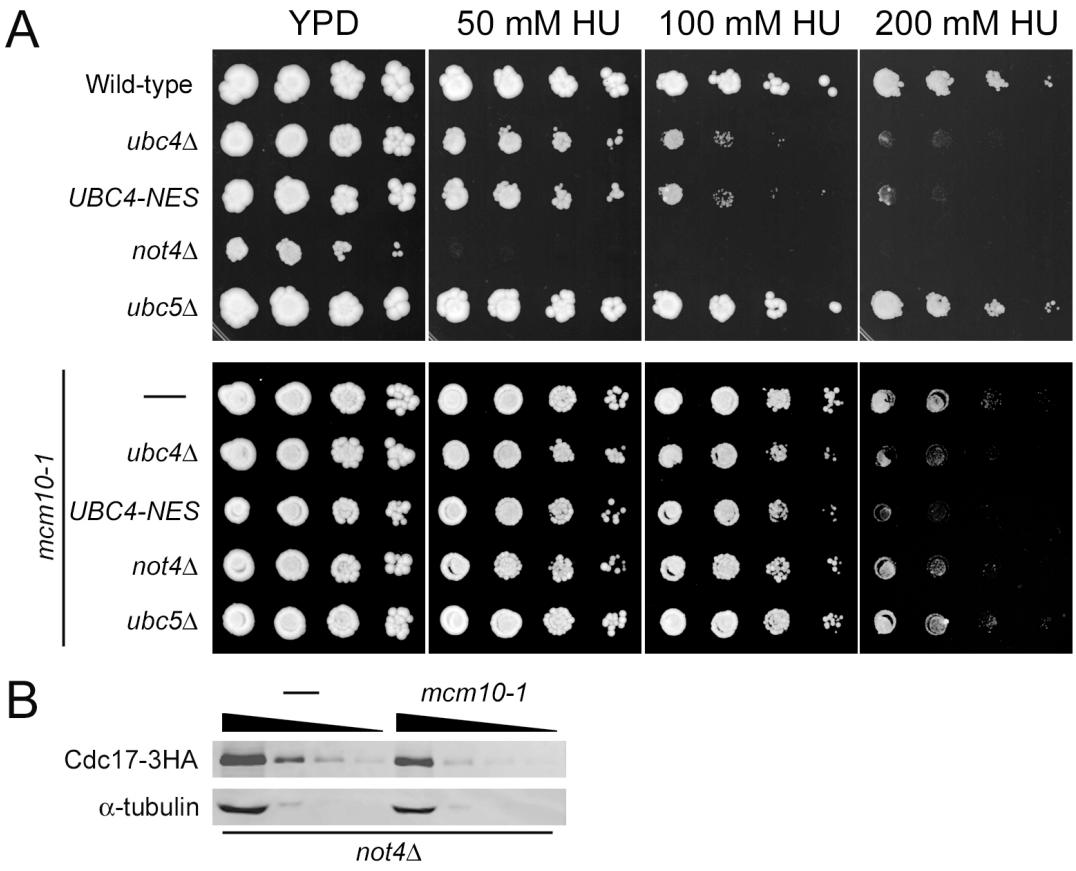

A
—
ubc4Δ
rad6Δ
ubc13Δ
mms2Δ
ubc5Δ
Cdc17-3HA
α-tubulin
mcm10-1
—
not4Δ
UBC4-NES
Cdc17-3HA
α-tubulin
mcm10-1
B
Cdc17/Tubulin
3.0
2.5
2.0
1.5
1.0
0.5
0.0
mcm10-1
ubc4
UBC4-NES
not4
ubc5
rad6
ubc13
mms2
C
Wild-type
ubc4Δ
not4Δ
ubc5Δ
Cdc17-3HA
α-tubulin
1.00
1.28
2.64
0.71

Figure 1. Cdc17 levels are elevated twofold in the presence of Mcm10 in *ubc4Δ*, *UBC4-NES-3HA*, and *not4Δ* cells. (A) Asynchronous cultures of ABy013 (*mcm10-1*), ABy342 (*mcm10-1*, *ubc4*Δ), ABy455 (*mcm10-1, UBC4-NES-3HA*), ABy363 (*mcm10-1*, *ubc5*Δ), ABy359 (*mcm10-1, rad6*Δ), ABy360 (*mcm10-1, ubc13*Δ), ABy361 (*mcm10-1, mms2*Δ) and ABy529 (*mcm10-1, not4*Δ) were grown at 25°C to midlog phase. Cdc17-3HA and α-tubulin were analyzed by Western blot. (B) The graph shows the fold change in Cdc17 in each strain relative to *mcm10-1* (average of 3 separate experiments, bars represent mean +/- standard deviation). (C) Asynchronous cultures of ABy448 (W303-1a), ABy607 (*ubc4Δ*), ABy597 (*not4*Δ), and ABy764 (*ubc5Δ*) were grown at 25°C to midlog phase. Cdc17-3HA and α-tubulin were analyzed by Western blot. The values represent the fold change in Cdc17 levels relative to wild-type. Data generated by Justin Haworth.

Figure 2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YPD
50 mM HU
100 mM HU
200 mM HU
Wild-type
ubc4Δ
UBC4-NES
not4Δ
ubc5Δ
mcm10-1
—
ubc4Δ
UBC4-NES
not4Δ
ubc5Δ
B
—
mcm10-1
Cdc17-3HA
α-tubulin
not4Δ

Figure 2. Ubc4 is required in the nucleus for tolerance to hydroxyurea. (A) Successive 10-fold dilutions of ABy448 (W303-1a), ABy607 (*ubc4Δ*), ABy662 (*UBC4-NES-3HA*), ABy597 (*not4Δ*), ABy764 (*ubc5Δ*), ABy013 (*mcm10-1*), ABy342 (*mcm10-1, ubc4Δ*), ABy455 (*mcm10-1, UBC4-NES-3HA*), ABy529 (*mcm10-1, not4Δ*) and ABy363 (*mcm10-1, ubc5Δ*) were grown for 3 d at 30˚C on YPD plates and YPD containing 50 mM, 100 mM, and 200 mM HU. (B) Asynchronous cultures of ABy597 (*not4Δ*) and ABy529 (*mcm10-1, not4Δ*) were grown to midlog phase. Cdc17-3HA and α-tubulin were analyzed by Western blot in undiluted extracts and extracts diluted 10-fold, 25-fold and 50-fold. Data generated by Justin Haworth.

Figure 3

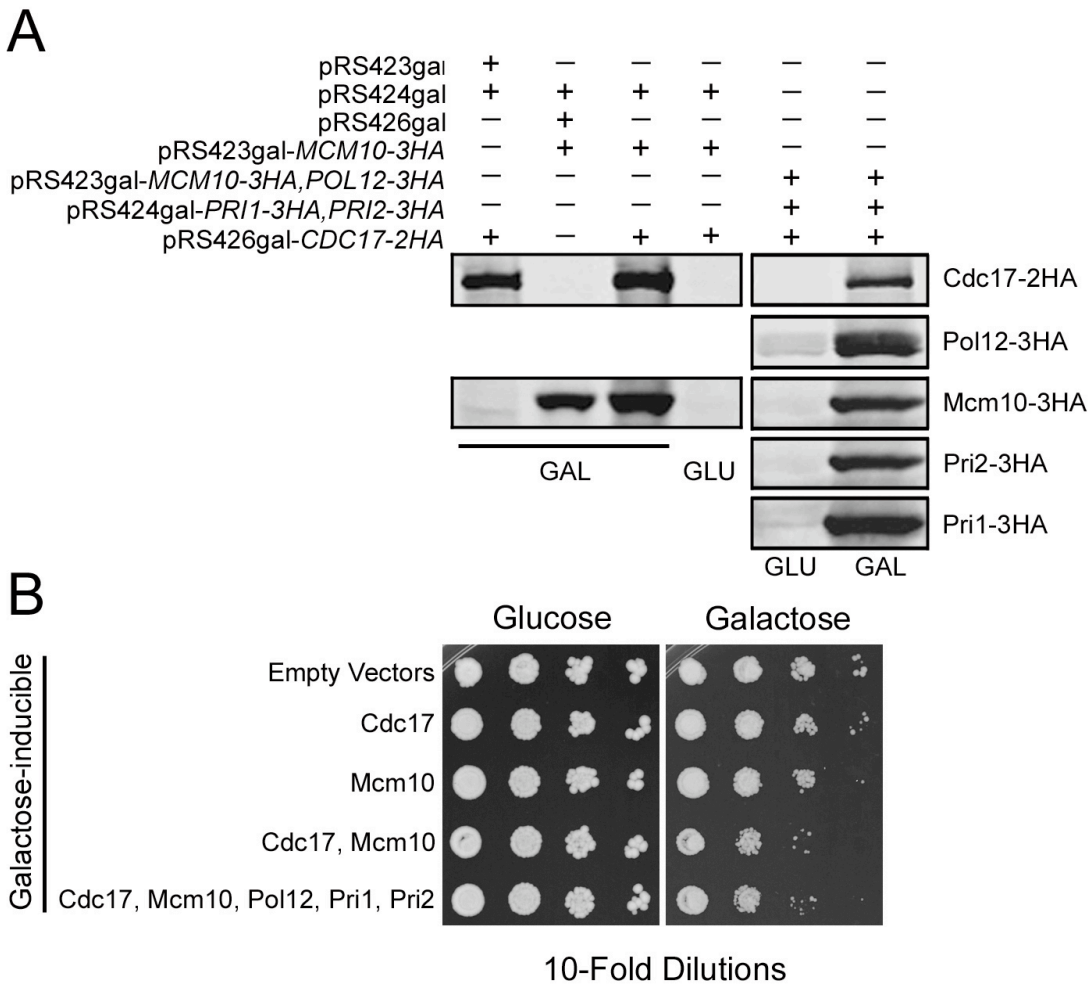
A
pRS423gal
pRS424gal
pRS426gal
pRS423gal-MCM10-3HA
pRS423gal-MCM10-3HA,POL12-3HA
pRS424gal-PRI1-3HA,PRI2-3HA
pRS426gal-CDC17-2HA
Cdc17-2HA
Pol12-3HA
Mcm10-3HA
Pri2-3HA
Pri1-3HA
GAL
GLU
GLU
GAL
B
Glucose
Galactose
Galactose-inducible
Empty Vectors
Cdc17
Mcm10
Cdc17, Mcm10
Cdc17, Mcm10, Pol12, Pri1, Pri2
10-Fold Dilutions

Figure 3. Overexpression of Cdc17 and Mcm10 causes slow growth. (A) Strains containing the indicated plasmids were grown in media containing 2% raffinose to midlog phase, either 2% galactose (GAL) or 2% glucose (GLU) was added, and cells were grown for an additional 2 h. Cdc17-2HA, Mcm10-3HA, Pol12-3HA, Pri1-3HA, and Pri2-3HA were analyzed by Western blot. (B) Successive 10-fold dilutions of ABy695 (empty vectors), ABy694 (*GAL-CDC17-2HA*), ABy696 (*GAL-MCM10-3HA*), ABy705 (*GAL-CDC17-2HA*, *GAL-MCM10-3HA*) and ABy732 (*GAL-CDC17-2HA, GAL-MCM10-3HA, GAL-POL12-3HA, GAL-PRI1-3HA, GAL-PRI2-3HA*) were grown for 3 d at 30˚C on SC-his-trp-ura with 2% glucose plates and SC-his-trp-ura with 2% galactose plates. Data generated by Justin Haworth.

Figure 4

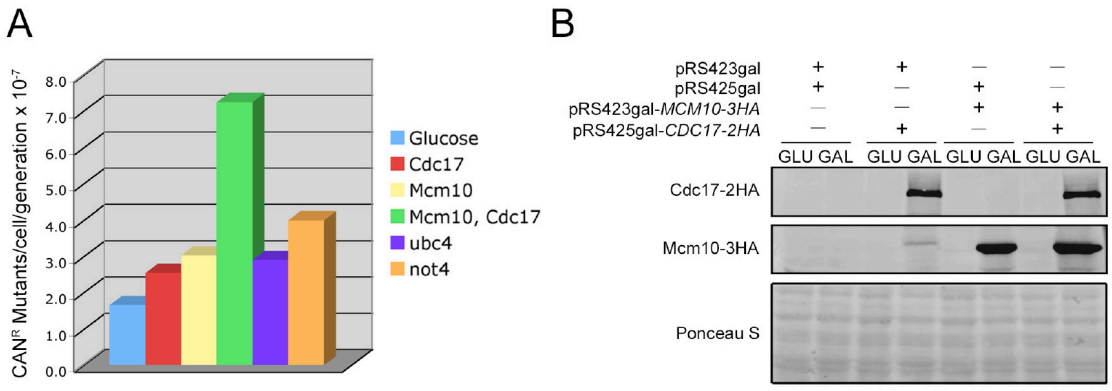
A
CAN^R Mutants/cell/generation x 10^-7
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Glucose
Cdc17
Mcm10
Mcm10, Cdc17
ubc4
not4
B
pRS423gal
pRS425gal
pRS423gal-MCM10-3HA
pRS425gal-CDC17-2HA
GLU GAL GLU GAL GLU GAL GLU GAL
Cdc17-2HA
Mcm10-3HA
Ponceau S

Figure 4. Overexpression of Cdc17 and Mcm10 increases the cellular mutation rate. (A) Seven individual colonies of ABy563 (pRS426gal-*CDC17-2HA*), ABy564 (pRS423gal-*MCM10-3HA*) and ABy565 (pRS423gal-*MCM10-3HA*, pRS426gal-*CDC17-2HA*) were grown to saturation in the presence of 2% galactose, and seven colonies of ABy565 were grown to saturation in the presence of 2% glucose. Cells were grown on -arg plates containing canavanine (60 mg/L) to select for mutants and on YPD plates for a viable cell count. Colonies were counted after 2-3 d at 30˚C. (B) Strains containing the indicated plasmids were grown in media containing 2% raffinose to midlog phase, either 2% galactose (GAL) or 2% glucose (GLU) was added, and cells were grown for an additional 2 h. Cdc17-2HA and Mcm10-3HA were analyzed by Western blot. Data generated by Justin Haworth.

Figure 5

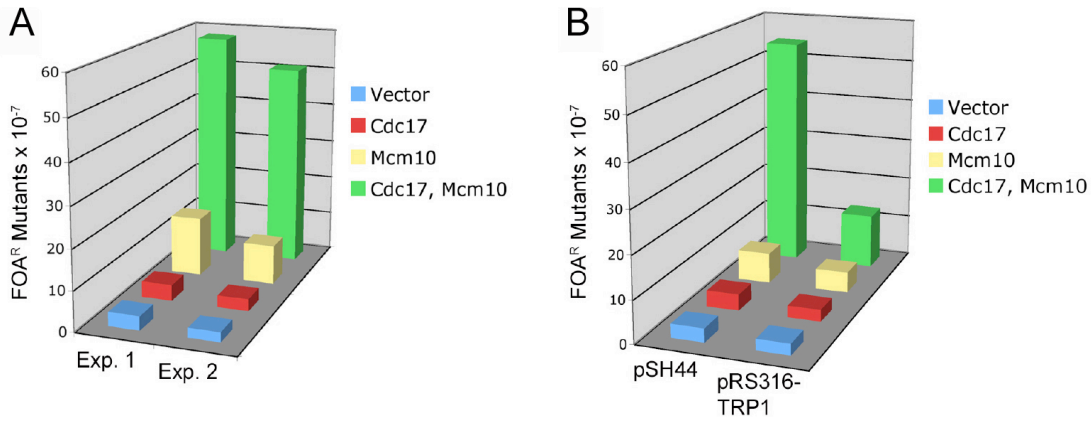
A
FOA^R Mutants x 10^-7
60
50
40
30
20
10
0
Exp. 1
Exp. 2
Vector
Cdc17
Mcm10
Cdc17, Mcm10
B
FOA^R Mutants x 10^-7
60
50
40
30
20
10
0
pSH44
pRS316-TRP1
Vector
Cdc17
Mcm10
Cdc17, Mcm10

Figure 5. Overexpression of Cdc17 and Mcm10 induces microsatellite instability. (A) Seven individual colonies each from ABy640, ABy641, ABy642 and ABy643 were grown in SC-his-trp-leu with 2% galactose to saturation at 30˚C. From each culture, 100 cells were plated on SC-trp with 2% glucose medium and 10^7 cells were plated on SC-trp with 2% glucose and 1 mg/ml 5-FOA medium. Plates were incubated for 5 d at 30˚C and colonies were counted. (B) Same as in A, but with the addition of strains ABy977, ABy978, ABy979 and ABy980, which contain pRS316-*TRP1* in place of pSH44. Data generated by Justin Haworth.

Figure 6

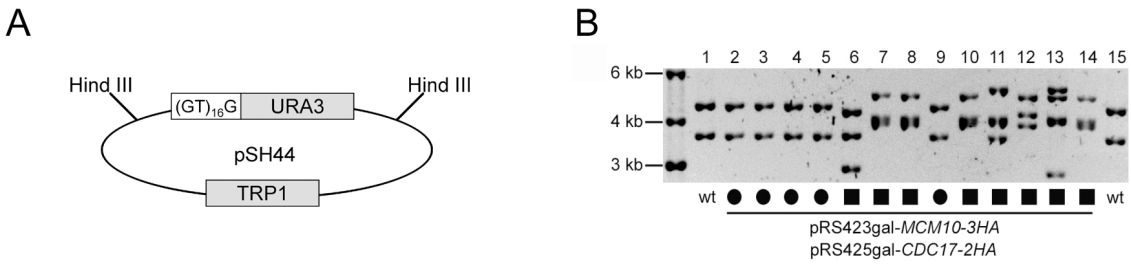
A
Hind III
(GT)16G
URA3
Hind III
pSH44
TRP1
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
6 kb
4 kb
3 kb
wt
wt
pRS423gal-MCM10-3HA
pRS425gal-CDC17-2HA

Figure 6. Overexpression of Cdc17 and Mcm10 induces gross DNA rearrangements. (A) Plasmid map of pSH44. (B) pSH44 was isolated from 5-FOA-resistant clones and digested with HindIII, which produces a 4.3-kb fragment (backbone) and a 3.6-kb fragment [poly(GT)-*URA3* coding sequence] in the wild type plasmid. Digested plasmids showed either no detectable size change (circles) or complex rearrangements (squares). Data generated by Justin Haworth.

Figure 7

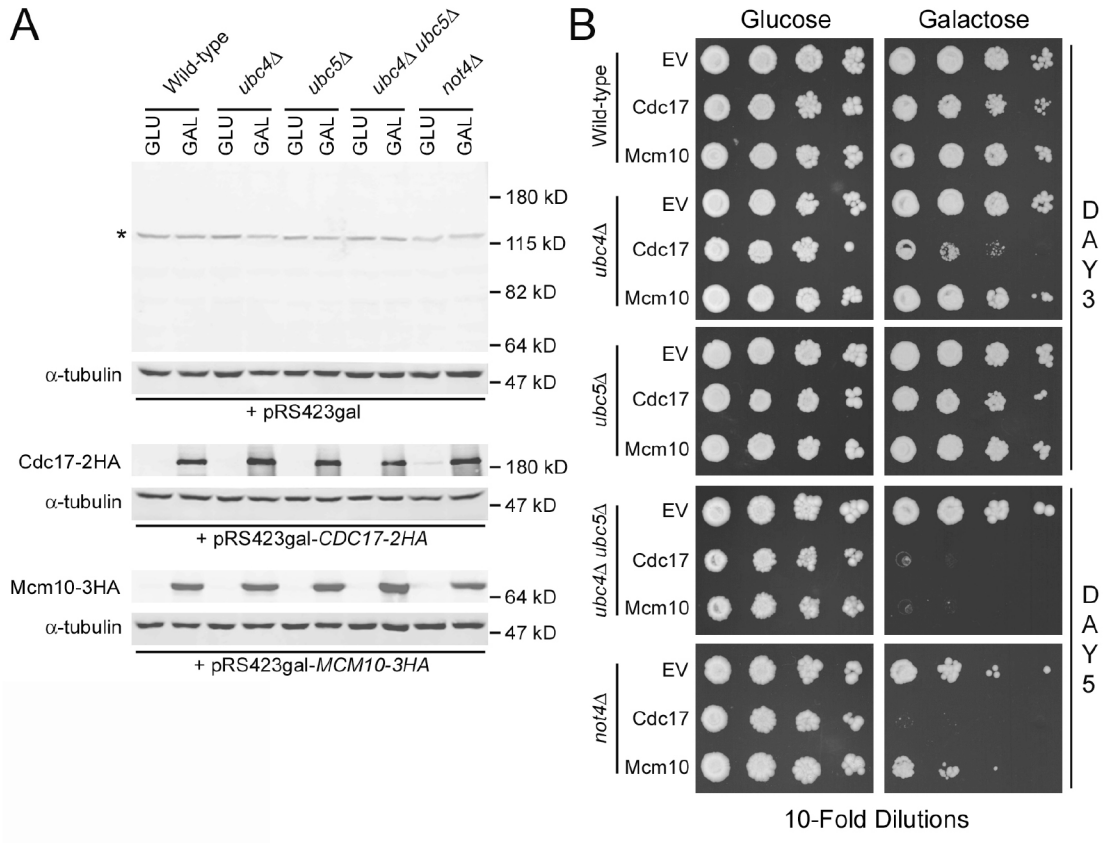
A
Wild-type
ubc4Δ
ubc5Δ
ubc4Δ ubc5Δ
not4Δ
GLU
GAL
180 kD
115 kD
82 kD
64 kD
47 kD
*
α-tubulin
+ pRS423gal
Cdc17-2HA
+ pRS423gal-CDC17-2HA
Mcm10-3HA
+ pRS423gal-MCM10-3HA
B
Glucose
Galactose
EV
Cdc17
Mcm10
DAY3
DAY5
10-Fold Dilutions

Figure 7. Overexpression of Cdc17 in *ubc4Δubc5Δ* and *not4Δ* mutants causes synthetic dosage lethality. (A) ABy962 (pRS423gal), ABy963 (*GAL-CDC17-2HA*), ABy964 (*GAL-MCM10-3HA*), ABy965 (*ubc4Δ*, pRS423gal), ABy966 (*ubc4Δ*, *GAL-CDC17-2HA*), ABy967 (*ubc4Δ*, *GAL-MCM10-3HA*), ABy968 (*ubc5Δ*, pRS423gal), ABy969 (*ubc5Δ*, *GAL-CDC17-2HA*), ABy970 (*ubc5Δ*, *GAL-MCM10-3HA*), ABy971 (*ubc4Δubc5Δ*, pRS423gal), ABy972 (*ubc4Δubc5Δ*, *GAL-CDC17-2HA*), ABy973 (*ubc4Δubc5Δ*, *GAL-MCM10-3HA*), ABy974 (*not4Δ*, pRS423gal), ABy975 (*not4Δ*, *GAL-CDC17-2HA*) and ABy976 (*not4Δ*, *GAL-MCM10-3HA*) were grown in media containing 2% raffinose to midlog phase, either 2% galactose (GAL) or 2% glucose (GLU) was added, and cells were grown for an additional hr. Cdc17-2HA, Mcm10-3HA and α-tubulin were analyzed by Western blot. The asterisk indicates a non-specific band recognized by the HA antibody. (B) Successive 10-fold dilutions of the strains in A were grown for the indicated time at 30˚C on SC-his-trp-ura with 2% glucose plates and SC-his-trp-ura with 2% galactose plates. Data generated by Justin Haworth.

Figure 8

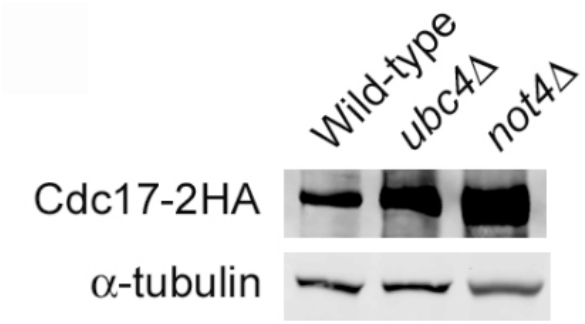

Figure 8. Accumulation of Cdc17 in *not4Δ* mutants. ABy981 (*CDC17-3HA*, pRS426gal-*CDC17-2HA*), ABy831 (*ubc4Δ*, *CDC17-3HA*, pRS426gal-*CDC17-2HA*) and ABy837 (*not4Δ, CDC17-3HA*, pRS426gal-*CDC17-2HA*) were grown overnight in SC-ura medium to mid-log phase. Cdc17 and α-tubulin were analyzed by Western blot. Data generated by Justin Haworth.

Materials and Methods

Strains and Plasmids

Strains are isogenic derivatives of either W303-1a or S288C. All strains carrying gene deletions were constructed using a PCR-based gene disruption method (Brachmann *et al.*, 1998). The genotypes of the yeast strains utilized in this study are listed in Table 1. All plasmids were constructed using standard molecular cloning techniques.

Protein Preparation and Western Blot Analysis

Total protein extracts were prepared as described previously by trichloroacetic acid extraction (Ricke and Bielinsky, 2006). Briefly, cells were pelleted, washed with 20% trichloroacetic acid and the cell wall was disrupted and the cells lysed by glass beads into 20% trichloroacetic acid. The resulting lysate was pelleted by centrifugation at 3000 rpm for 10 min. The supernatant was removed and the pellet was resuspended in 2X Laemmli buffer (4% SDS, 20% glycerol, 120 mm Tris-Cl, pH 6.8, 200 mm dithiothreitol, 0.1% bromphenol blue). 1 M Tris was used to neutralize the lysates and the samples were boiled for 5 min. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed by Western blot with anti-HA (16B12; Covance) for HA-tagged proteins and anti-α-tubulin (B512; Covance). Alexa Fluor 680 (Invitrogen) secondary antibody was used to visualize proteins with Odyssey v3.0 software on an Odyssey scanner from LI-COR Biosciences.

Protein Overexpression

Strains carrying plasmids for exogenous overexpression of Cdc17, Mcm10, Pol12, Pri1, and Pri2 were grown overnight in minimal medium with 2% raffinose.

Proteins were overexpressed from a galactose promoter in the presence of 2% galactose or repressed in the presence of 2% glucose. Total protein was extracted using the trichloroacetic acid extraction protocol. Cdc17-2HA, Mcm10-3HA, Pol12-3HA, Pri1-3HA, Pri2-3HA and α-tubulin were detected by Western blot using anti-HA (16B12) for HA-tagged proteins and anti-α-tubulin (B512).

Microsatellite Instability Assay

Assay was performed as described previously (Gutierrez and Wang, 2003) except for the following. Seven individual colonies from each strain tested were grown in liquid SC-his-trp-leu minimal medium with 2% galactose to saturation. From each culture, 10^7 cells were spread on SC-trp plates containing 1 mg/ml 5-FOA and 100 cells were spread on SC-trp plates for a viable cell count. Plates were incubated at 30˚C for 4-5 d and FOA^R colonies were counted. The frequency of FOA^R clones was calculated by dividing the number of FOA^R colonies by the number of viable cells. To assess whether microsatellites were altered, pSH44 was recovered from FOA^R clones and the microsatellite region was sequenced. For clones in which the microsatellite was unaltered, pSH44 was digested with *Hin*dIII to detect gross sequence changes. Sequencing of these plasmids indicated that all of them had lost the microsatellite region, which was replaced with *LEU2* sequence that likely recombined with the upstream *LEU2* promoter sequence that immediately precedes the microsatellite tract in pSH44 (Henderson and Petes, 1992). We constructed a control plasmid for this assay that had the same auxotrophic markers as pSH44, but lacked a microsatellite tract. To this end, *TRP1* was inserted into the multicloning site of pRS316 with *Kpn*I and *Sac*I.

Chapter 5

Discussion and Future Studies

Discussion

The aim of this research was to gain further insight into two of the known functions of the highly conserved replication factor, Mcm10: Mcm10's DNA binding activity and its ability to stabilize the catalytic subunit of pol α. By utilizing the crystal structure of the conserved internal domain of Mcm10, we were able to map its DNA binding surface. Mutational analysis of residues determined to be important for DNA binding by xMcm10 revealed that the DNA binding activity of scMcm10 is crucial for maintaining replication fork stability during replication stress. With respect to Mcm10's ability to stabilize Cdc17, we were able to determine that Cdc17 degradation in the absence of Mcm10 is dependent on Ubc4 and Not4. Furthermore, disruption of the degradation pathway of Cdc17 revealed that regular turnover of Cdc17 is required to prevent increased steady-state levels of Cdc17. Illustrating the importance of tightly regulating Cdc17 levels, cooverexpression of Cdc17 and Mcm10 resulted in slow growth, an increased mutation rate and microsatellite-induced recombination. This research highlights the importance of Mcm10's function in the cell: its DNA binding activity is important for replication fork stability during replication stress and its regulation of Cdc17 levels prevents genome instability.

Insights into Mcm10's role during DNA replication revealed by its structure

The DNA binding surface of Mcm10. The crystal structure of the conserved internal domain of xMcm10 revealed a novel arrangement of its OB-fold and zinc finger domains. The zinc finger domain of Mcm10 is on the opposite face of its OB-fold (near L23) compared to the zinc finger domains of other DNA binding proteins such as RPA70

(Bochkarev *et al.*, 1997), which reside within or adjacent to the L12 loop (Ch. 1, Figure 5A). The significance of Mcm10's novel OB-fold and zinc finger arrangement is not known, but it is possible that it is required for Mcm10's ability to bind both ss and dsDNA, which has been shown *in vitro* (Robertson *et al.*, 2008). The fact that mutations that reduce xMcm10's DNA binding activity *in vitro* result in hydroxyurea sensitivity when made in scMcm10 suggests that these residues are indeed important for DNA binding *in vivo*. Furthermore, the hydroxyurea sensitivity of the scMcm10 DNA binding mutants is evidence that the DNA binding activity of Mcm10 is important for replication fork stability during replication stress. This is a novel finding that has possible implications for using Mcm10 as a drug target in cancer cells, which I discuss in detail in the "Future Studies" section below.

The structure of Mcm10 helps elucidate Mcm10's role at the replication fork. Mcm10 physically interacts with DNA, pol α and PCNA all within the conserved internal domain (Das-Bradoo *et al.*, 2006; Ricke and Bielinsky, 2006; Robertson *et al.*, 2008). Presumably, it needs to bind both DNA and pol α simultaneously at the replication fork (Ricke and Bielinsky, 2004). Asn268 within scMcm10 is required to stabilize pol α (Ricke and Bielinsky, 2006) and this residue (xMcm10 Asn346) is surface exposed on the structure, which is consistent with its role in binding and stabilizing pol α (Ricke and Bielinsky, 2004). Importantly, Asn268 lies outside of the DNA binding surface of Mcm10, suggesting that Mcm10 can indeed bind DNA and pol α simultaneously. Mcm10 also interacts with PCNA, but only when Mcm10 is diubiquitinated (Das-Bradoo *et al.*, 2006). Tyr245 within Mcm10 is required for the interaction between

diubiquitinated Mcm10 and PCNA (Das-Bradoo *et al.*, 2006). Interestingly, mutation of the corresponding residue (Phe324Ala) in unmodified xMcm10 resulted in slightly reduced DNA binding, suggesting that this residue contributes to DNA binding. It is therefore possible that upon diubiquitination Mcm10 undergoes a conformational change that alters its interaction with DNA and allows PCNA to bind, which has been suggested previously (Das-Bradoo *et al.*, 2006). Identification of the ubiquitination sites on Mcm10 would allow for further investigation into the proposed conformational change.

The Pol α Degradation Pathway and its Significance

Our work has demonstrated that Cdc17 turnover is dependent on Ubc4 and Not4. This was unexpected because Not4 was originally isolated as a transcriptional regulator (Collart and Struhl, 1994). Not4 has also been shown to possess E3 ubiquitin ligase activity *in vitro* (Panasenko *et al.*, 2006; Mulder *et al.*, 2007). It is part of the nine-subunit Ccr4-Not complex that regulates trimethylation of histone H3 lysine 4 (Chen *et al.*, 2001; Laribee *et al.*, 2007). Importantly, we show that Cdc17 is not modulated at the transcript level (Ch 1; Figure 9), but rather through ubiquitin-mediated proteasomal degradation (Ch 1; Figures 1, 4, 5 and 6). This degradation is likely triggered when Mcm10 is ubiquitinated and no longer interacts with pol-α (Das-Bradoo *et al.*, 2006) or the replisome becomes unstable (Lee *et al.*, 2010). Our results suggest that yeast cells stabilize only enough Cdc17 to efficiently initiate replication. We propose that increased Cdc17 protein levels above a defined molecular threshold are detrimental to the cell and are therefore rapidly eliminated.

Cdc17 degradation is Not4-dependent. We have identified Cdc17 as a novel target of Not4-mediated degradation. Recently, Jhd2 (JmjC domain-containing histone demethylase) was reported to be a direct target of Not4 (Mersman *et al.*, 2009). In addition, Not4 may have a role in the degradation of Egd2, which is part of the ribosome-associated EGD (enhancer of Gal4 DNA binding) complex (Panasenko *et al.*, 2006). However, none of these proteins has a role in DNA replication. Cdc17 is stable in the absence of Mcm10 in *not4Δ* cells (Ch 3; Figure 4A) and accumulates in the presence of Mcm10 when *NOT4* is deleted (Ch 4; Figure 1C). We take this as evidence that Not4 is required for regular turnover of Cdc17. This is supported by the observation that Cdc17 destabilization is clearly dependent on ubiquitin conjugation (Ch 3; Figure 6), although it remains elusive whether Cdc17 is a direct target of ubiquitination. In this context, it is worthwhile to note that precedence exists for ubiquitin-independent proteasomal degradation (Schrader *et al.*, 2009). Also, both Ubc4 and Not4 can directly bind the proteasome (Chuang and Madura, 2005; Laribee *et al.*, 2007), making it feasible that Cdc17 is not ubiquitinated itself because it may be brought directly to the proteasome by Ubc4 and/or Not4. In addition, we excluded that accumulation of Cdc17 in *mcm10-1 not4Δ* cells (Ch 3; Figure 4A) was due to a failure to degrade Mcm10-1 protein at the non-permissive temperature (data not shown).

Cdc17 shows synthetic dosage lethality with not4Δ cells. Another striking observation supporting the claim that Not4 regulates Cdc17 turnover in conjunction with Ubc4 is provided by the SDL tests (Ch 4; Figure 7). SDL screens have been used to identify potential targets for protein kinases and might also be useful in revealing

potential targets for E3 ligases (Makhnevych *et al.*, 2009; Zou *et al.*, 2009). One question such a screen could address is whether Cdc17 is the only replication factor regulated by Not4 or if Not4 controls the turnover of other replication proteins as well. Mcm10 does not appear to be a potential target as its overexpression had little effect on the viability of *not4Δ* cells (Ch 4; Figure 7B). Nevertheless, a mutation in *MCM10* almost completely alleviated the severe HU-sensitivity of *not4Δ* cells (Ch 4; Figure 2). Because *mcm10-1* is HU-sensitive by itself, one might have expected that it exacerbates the drug sensitivity of the *not4Δ* strain. We believe that these results are most easily explained by the destabilizing effect that *mcm10-1* has on Cdc17 (Ricke and Bielinsky, 2006; Lee *et al.*, 2010). PCNA overexpression, however, was synthetically sick in *not4Δ* cells (Haworth, unpublished result), suggesting that Cdc17 may not be the only replication factor regulated by Not4. Even if Not4 does not regulate the stability of PCNA, this result implies that *not4Δ* mutants have an intrinsic replication fork defect that is exacerbated by excess PCNA, further suggesting that Not4 plays a role in maintaining efficient S phase progression. The noncatalytic p12 subunit of human pol δ is degraded in response to DNA damage (Zhang *et al.*, 2007) and human PCNA is degraded via the proteasome in the absence of phosphorylation at Tyr211 (Wang *et al.*, 2006). The degradation of p12 and PCNA inhibit S phase progression (Wang *et al.*, 2006; Zhang *et al.*, 2007), whereas our studies here demonstrate that degradation of Cdc17 is required for the efficient S phase progression, underscoring the conceptual novelty of this Cdc17 degradation pathway. It will be important to determine whether turnover of other replication elongation factors is required for efficient S phase progression, or whether this is specific

to pol α because aside from these examples, very little is known about regulation of the turnover of replication elongation factors. SDL screens, such as the one proposed above with *not4Δ* cells, could therefore be very informative for the regulation of replication factor turnover.

Proteasomal degradation at the replication fork. The data presented here raise the possibility that ubiquitin-mediated degradation of Cdc17 occurs at or in close proximity of the replication fork. There have been other hints that the ubiquitination machinery operates at the replication fork, but direct evidence is lacking to date. For example, Dia2, an F-box protein, has been shown to bind origins of replication and promote genome stability, but no degradation target of Dia2 has been identified (Blake *et al.*, 2006; Koepp *et al.*, 2006; Mimura *et al.*, 2009; Kile and Koepp, 2010). In addition, it was recently shown that ubiquitination of Spt16, a subunit of the FACT chromatin remodeling complex, is important for FACT recruitment to DNA replication origins and binding to the MCM2-7 complex, but does not affect FACT's role in transcription (Han *et al.*, 2010).

We propose that Ubc4 and Not4 may have a role in degrading Cdc17 to ensure efficient polymerase switching (pol-ε on the leading strand (Pursell *et al.*, 2007) and pol-δ on the lagging strand (Nick McElhinny *et al.*, 2008)). In support of this model, we demonstrated earlier that ubiquitinated Mcm10 binds PCNA, the processivity factor for pol-δ and pol-ε, but not pol-α (Das-Bradoo *et al.*, 2006). Therefore, it is possible that after pol-α synthesizes a short DNA primer, Mcm10 becomes ubiquitinated and interacts with PCNA, causing Cdc17 to be released from Mcm10. Once released, Cdc17 might

then be targeted for proteasomal degradation (Ch 5; Figure 1). We stress that a direct interaction between Cdc17 and Not4/Ubc4 is strictly hypothetical at present. Regardless of whether the interaction with Cdc17 is direct or indirect, Not4/Ubc4 could ensure that Cdc17, a potentially mutagenic enzyme, is not loaded back onto the DNA to further extend the RNA/DNA primer. The fivefold elevated mutation rate of cells overexpressing Cdc17 and Mcm10 (Ch 4; Figure 4A) is consistent with a mechanism by which Mcm10 "redelivers" pol-α to chromatin, thus interfering with the switch from pol-α to pol-δ (Ch 5; Figure 1). As a consequence, pol-α would further extend the RNA/DNA primer, which could account for the increased frequency of mutations (Ch 4; Figure 4A). Tight regulation of Mcm10 by ubiquitination is likely a prerequisite for regular turnover of Cdc17. Therefore, Mcm10 has a dual role, not only stabilizing but also providing timely degradation to inhibit the accumulation of Cdc17.

C*ooverexpression of Cdc17 and Mcm10 causes genome instability.* Inhibition of replicative pol activity induces chromosomal breaks, even at drug doses that are low enough to not affect the proliferation rate of cells (Glover *et al.*, 1984). In addition, a mutant of Cdc17, *pol1-1*, increases mutation frequency and microsatellite instability in budding yeast (Gutierrez and Wang, 2003). Furthermore, low levels of pol-α cause chromosomal translocations in budding yeast, even in the presence of a functional S phase checkpoint (Lemoine *et al.*, 2005). All of these observations demonstrate the effects of a decline in pol-α function. In contrast, we have shown here that increasing steady-state levels of wild-type pol-α causes genomic instability as well. In a plasmid-based *in vivo* assay (Figure 2), cooverexpression of Cdc17 and Mcm10 resulted in a

significant increase in microsatellite-mediated recombination (Ch 4; Figures 5 and 6). These events almost certainly require DNA breakage, and we speculate that breaks occur via improper progression of lagging strand synthesis. In this context it is interesting to note that a recent study in *Arabidopsis* suggests that the failure to properly switch from pol-α to pol-δ might trigger recombination events at replication forks (Schuermann *et al.*, 2009).

Pol α degradation and cancer. DNA rearrangements have been linked to many different types of cancer (Frohling and Dohner, 2008; Nambiar *et al.*, 2008). We hypothesize that microsatellite-induced instability could arise through increased levels of pol-α and its binding partner, Mcm10. In support of this hypothesis, p180, the catalytic subunit of pol-α in humans, and Mcm10 are upregulated in glioma cells (Sun *et al.*, 2006). Intriguingly, UbcH5, the human holomog of yeast Ubc4/5, is downregulated in these cells (Sun *et al.*, 2006). Furthermore, p180 and Mcm10 levels are increased, whereas CNOT4 levels, the human homolog of yeast Not4, are decreased in human male germ cell tumors (Korkola *et al.*, 2006). These data indicate that the degradation pathway identified here may also be important in human cells. More specifically, because the levels of p180, Mcm10, UbcH5, and CNOT4 are misregulated in cancer cells, this pathway may be important for cancer prevention and perhaps serve as a novel target for cancer therapy.

Future Studies

The structure and function of Mcm10

Screen for an inhibitor of Mcm10 DNA binding. Cancer cells need to proliferate, and Mcm10 is essential for cell proliferation. Importantly, drastic reduction of Mcm10 is known to cause a large amount of DNA damage, which leads to apoptosis (Chattopadhyay and Bielinsky, 2007; Paulsen *et al.*, 2009). Since Mcm10's ability to bind DNA is crucial for maintaining replication fork integrity, inhibition of DNA binding could mimic a reduction of the Mcm10, thereby causing DNA damage and eventually apoptosis. If this is the case, then the DNA binding activity of Mcm10 would be a prime candidate drug for targeting in cancer therapy. The elucidation of the molecular surface required for this DNA binding activity by Mcm10 should greatly facilitate the identification of a drug that can inhibit Mcm10's DNA binding activity. Two approaches could be taken to identify a drug since the structure and binding surface are known. One would be to use a molecular modeling program that identifies potential drugs *in silico* that would be good candidates to test functionally. Another approach would be to do a brute force drug screen using a high throughput DNA binding assay. One example of this would be a fluorescence resonance energy transfer (FRET)-based DNA binding assay where Mcm10 would be tagged with GFP and the DNA substrate would be tagged with Rhodamine so that if DNA binding occurs a FRET reaction takes place and can be measured (Aoki *et al.*, 2006). One could then screen a drug library looking for reduced FRET, which would indicate that DNA binding has been abolished. Drug hits could then

be tested in tumor cell lines to determine if they can inhibit proliferation and/or induce apoptosis.

Regulation of pol α

Cdc17 degradation pathway. Recently, it was shown that Cdc17 is stabilized in a checkpoint-dependent manner via the Mec1 checkpoint kinase in a *mcm10-1* suppressor strain that has reduced Mcm2-7 helicase function due to a G400D missense mutation in the *MCM2* gene (Lee *et al.*, 2010). The authors hypothesize that the checkpoint promotes the stability of Cdc17 in order to maintain replication fork integrity because Cdc17 is degraded in a *mec1* mutant that is deficient for checkpoint activation. In order to test if Not4 is required for the degradation of Cdc17 under these conditions, Cdc17 stabililty could be assessed in a *mcm10-1*, *mcm2-G400D*, *mec1*, *not4Δ* mutant. If Cdc17 was stable in this mutant at the restrictive temperature that would indicate that Not4 is indeed required for Cdc17 degradation in the presence of replication stress and the lack of a functional checkpoint. Conversely, if Cdc17 remains unstable in the *mcm10-1*, *mcm2-G400D*, *mec1*, *not4Δ* mutant that would suggest that there is another, Not4-independent, Cdc17 degradation pathway, which would be very intriguing. One could then perform a screen for stability of Cdc17 at the restrictive temperature in the *mcm10-1*, *mcm2-G400D*, *mec1*, *not4Δ* mutant background to search for the factors required for the novel Cdc17 degradation pathway.

It has recently been shown that Not4 is post-translationally modified through phosphorylation (Lau *et al.*, 2010). Several phosphorylation sites were identified by

mass spectrometry and mutation of these sites to alanine (*not4S/T5A*), which almost completely eliminated the slower migrating forms of Not4, resulted in sensitivity to cellular stresses similar to, but not as severe as, *not4Δ* mutants (Lau *et al.*, 2010). This indicates that phosphorylation of Not4 is important for some of its functions. Cdc17 stability, however, was not investigated in this study and it would be interesting to know whether Cdc17 can be degraded in the absence of Mcm10 in the *not4S/T5A* mutant background. If Cdc17 can be degraded in the *not4S/T5A* mutant background that would imply that phosphorylation of Not4 is not required for its role in the Cdc17 degradation pathway. If Not4 phosphorylation is required for Cdc17 degradation, then Cdc17 should be stable in the absence of Mcm10 in the *not4S/T5A* mutant background. Either experimental outcome would be informative. In addition, it would also be important to determine if Not4 phosphorylation is required to suppress the growth defect, increased mutation rate, and microsatellite-induced recombination phenotypes caused by cooverexpression of Cdc17 and Mcm10. This could be determined by repeating these assays in the *not4S/T5A* mutant background.

Is Cdc17 ubiquitinated in vitro? Although we have presented evidence here that Cdc17 is ubiquitinated in an Ubc4- and Not4-dependent manner, we were unable to detect ubiquitinated Cdc17 *in vivo*. It may be that Cdc17 is indeed ubiquitinated, but this ubiquitinated species is very short-lived and therefore technically difficult to observe. Alternatively, since both Ubc4 and Not4 are able to interact with the proteasome (Chuang and Madura, 2005; Laribee *et al.*, 2007), it is possible that Cdc17 itself does not need to be ubiquitinated prior to proteasomal degradation. In order to determine if Not4 is

capable of ubiquitinating Cdc17, we could perform an *in vitro* ubiquitination assay with purified Cdc17, Not4, Ubc4, Uba1 and ubiquitin, since it has been shown previously that Not4 has E3 ligase activity with Ubc4 *in vitro* (Mulder *et al.*, 2007). If Cdc17 is able to be ubiquitinated in a Not4-dependent manner *in vitro* that would suggest that ubiquitinated Cdc17 is too short-lived to observe *in vivo*. Alternatively, if Not4 is not able to ubiquitinate Cdc17 *in vitro* that would be evidence that Cdc17 is not ubiquitinated prior to its degradation via the proteasome.

Are Ubc4 and Not4 at the replication fork? It would be worthwhile to investigate whether Ubc4 and/or Not4 are present at replication origins and replication forks. Since not only the entire yeast genome sequence is known, but also the time at which specific sequences are replicated, it is possible to monitor whether Ubc4 and Not4 are bound to replication origins and the regions flanking replication origins during replication elongation. Our laboratory has performed this type of analysis previously using ChIP with Mcm10 at origins of replication that fire early in S phase, showing that Mcm10 travels with the replication fork (Ricke and Bielinsky, 2004). Therefore, Mcm10 could be used as a positive control for the Ubc4 and Not4 ChIP studies. Primers specific for a nonorigin region that is not replicated early in S phase would be used as a negative control for the ChIP experiments. If Ubc4 and/or Not4 were present at the same regions of the DNA as Mcm10 during S phase, and not present at nonorigin regions upon completion of S phase, that would strongly suggest that they travel with the replication fork and that Cdc17 is degraded at or near the replication fork.

Microsatellite-induced recombination caused by Cdc17 and Mcm10 overexpession. Since overexpression of Cdc17 and Mcm10 resulted in microsatellite-mediated recombination, it would be informative to perform the plasmid-based microsatellite instability assay in a *rad52Δ* mutant background in which homologous recombination is defective (Paques and Haber, 1999). If the observed phenotype is exacerbated compared to *RAD52* cells that would imply that homologous recombination has a protective effect and limits genomic instability.

The *pol1-1* mutant mentioned above, which reduces the stability of the pol α/primase complex (Lucchini *et al.*, 1988), showed genetic interactions with members of the mismatch repair pathway (Gutierrez and Wang, 2003). There are two heterotetrameric protein complexes involved in the yeast mismatch repair system. One contains MutS homolog (*MSH) 2*, *MSH6*, post-meiotic segregation (*PMS) 1*, and MutL homolog (*MLH) 1* gene products and is responsible for the repair of single-base mismatches and small loops of DNA (Johnson *et al.*, 1996). The other complex is identical except that *MSH3* replaces *MSH6* and the resulting complex repairs small loops up to eight bases (Sia *et al.*, 1997). The *pol1-1* microsatellite instability phenotype was enhanced in both *msh6Δ* and *msh3Δ* mutant backgrounds, suggesting that mismatch repair is the major pathway involved in correcting mistakes made in *pol1-1* mutants (Gutierrez and Wang, 2003). It would be interesting to investigate whether cooverxpression of Cdc17 and Mcm10 in the same mutant backgrounds would also result in an enhanced mutation rate. If that were the case, it would suggest that having excess

pol α at the replication fork creates the same requirement for mismatch repair as *pol1-1* mutants.

Degradation pathway of p180 in human cells. Although it is intriguing that p180, UbcH5, and CNOT4 are all misregulated in certain cancer types, their relationship is, at this point, only correlative. To help determine if the pol α degradation pathway is relevant in cancer cells, it will be crucial to investigate whether this pathway is conserved in human cells. Since Cdc17, Ubc4, and Not4 are all conserved in human cells - p180, UbcH5, and CNOT4, respectively – it should be possible to determine if CNOT4 and UbcH5 are required for the degradation of p180 in human cells. Our laboratory has a HCT116 cell line that is heterozygous for MCM10 in which one allele of MCM10 has been inactivated by gene targeting. MCM10 and p180 levels are 50% of wild type levels in this cell line, again demonstrating that MCM10 is required to stabilize p180 in human cells. There are several human homologs of yeast Ubc4 (of which UbcH5 is one), but only one human homolog of yeast Not4, which is CNOT4. Therefore, since CNOT4 is the only human homolog of yeast Not4, experiments targeting CNOT4 for depletion would likely be more straightforward, knocking down one protein rather than several variants of UbcH5. Small interfering (si) RNA knockdown of CNOT4 in these $MCM10^{+/-}$ cells followed by monitoring p180 levels would allow us to determine if p180 levels are regulated by CNOT4 in the short term of a transient knockdown. This experiment could also be performed in $MCM10^{+/+}$ cells, but we feel that detecting an increase in p180 levels in cells with reduced p180 may be more straightforward. An increase in p180 levels compared to $MCM10^{+/-}$ cells treated with a scrambled siRNA

would indicate that CNOT4 is indeed involved in p180 degradation. Alternatively, if no change in p180 levels is observed over the course of the transient knockdown of CNOT4, this could either mean that CNOT4 is not involved in p180 degradation or that a longer time course is needed to observe a significant increase in p180 levels. To differentiate between these interpretations, we could monitor p180 levels over a longer time course in $MCM10^{+/-}$ cells stably transfected with either a CNOT-specific short hairpin (sh) RNA or a scrambled shRNA. If p180 levels remain unchanged over a long time course, it would be reasonable to conclude that CNOT4 is not required for p180 degradation. Alternatively, if p180 stability is regulated by CNOT4, it will then be of interest to determine if human cells share similar phenotypes to yeast cells when the pol α degradation pathway is disrupted. More specifically, the proliferation rate, mutation rate, and sensitivity to hydroxyurea should be examined in CNOT4-depleted, $MCM10^{+/-}$ cells.

p180 overexpression in human cells. In addition to investigating the p180 degradation pathway, it will be important to determine if human cells are sensitive to overexpression of p180, as we have shown that yeast cells are sensitive to Cdc17 overexpression. In wild type yeast cells, Cdc17 overexpression resulted in phenotypes only when cooverexpressed with Mcm10. However, human cells are more sensitive to changes in MCM10 expression levels than are yeast. For example, $MCM10^{+/-}$ cells, which have approximately 50% of wild type MCM10 protein levels, proliferate more slowly than wild type cells (Chattopadhyay, unpublished result). In comparison, yeast cells show no growth defect in *mcm10-1* mutants that have only 10% of wild type Mcm10 protein levels at the permissive temperature (Haworth and Bielinsky,

unpublished result). Therefore, it is possible that human cells are also more sensitive than yeast cells to overexpression of the catalytic subunit of pol α. To test this hypothesis, p180 could be overexpressed in human cells and subsequently the growth and mutation rates can be analyzed. If p180 overexpression alone does not show any change in growth and mutation rates, then MCM10 could be cooverexpressed, as it had to be in yeast cells to observe a significant phenotype because Mcm10 is required for proper regulation of pol α at the replication fork. Since overexpression of Cdc17 showed synthetic dosage lethality with *not4Δ* mutants, it would also be worthwhile to overexpress p180 in CNOT4-depleted human cells and determine if this results in an inability to proliferate. The results of these experiments in human cells will be invaluable regardless of their outcome. They will either identify the p180 degradation pathway as a legitimate potential target for cancer therapy or show that this pathway is not conserved in human cells, thereby opening the door to investigation of how pol α stability is regulated in human cells.

Figure 1

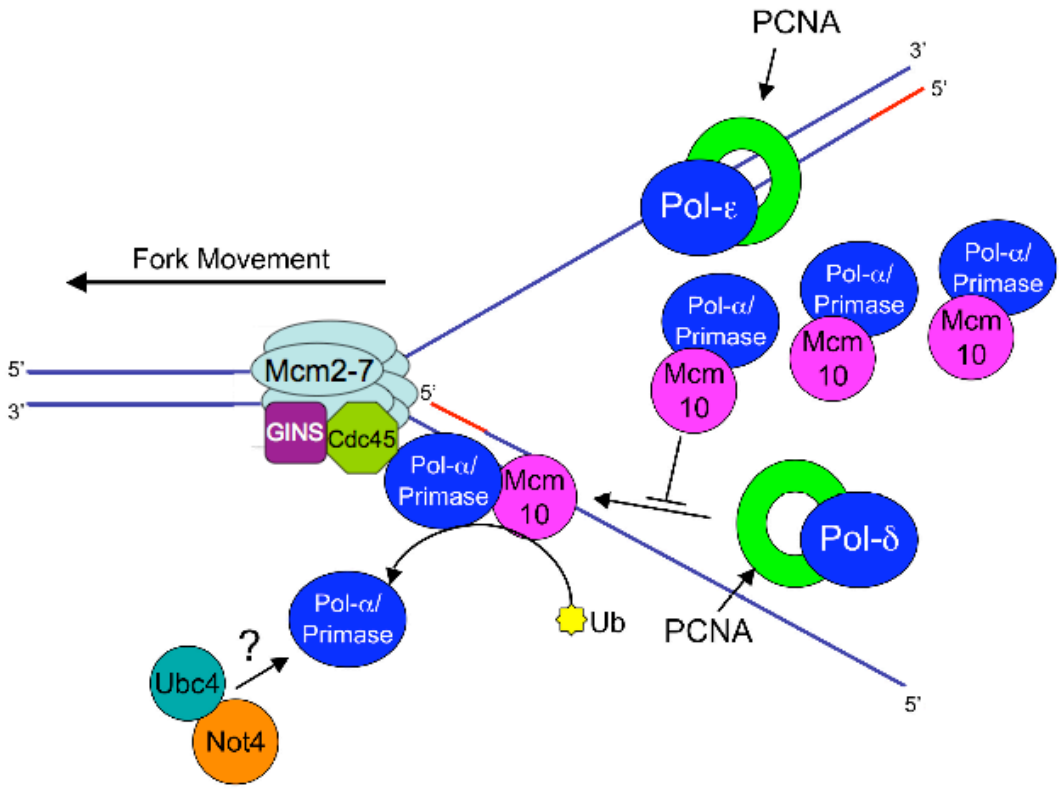
PCNA
3'
5'
Pol-ε
Fork Movement
Pol-α/
Primase
Mcm
10
5'
3'
Mcm2-7
GINS
Cdc45
5'
Pol-α/
Primase
Mcm
10
Pol-δ
Pol-α/
Primase
Ub
PCNA
?
Ubc4
Not4
5'

Figure 1. Overexpression of Mcm10 and Cdc17 may hinder polymerase switching. After pol-α extends the RNA primer (red line) by 12-20 nucleotides, Mcm10 is ubiquitinated at two distinct lysines (yellow star; (Das-Bradoo *et al.*, 2006)). PCNA is recruited to the lagging strand, and a polymerase switch occurs between pol-α and pol-δ, after which pol-α is released from ubiquitinated Mcm10 and recognized by Ubc4 and Not4. The question mark denotes the fact that it is currently unclear whether the interaction is direct or indirect. Accumulation of Cdc17 and Mcm10 interferes with the switch to pol-δ, allowing pol-α to synthesize more DNA than in wild-type cells.

Figure 2

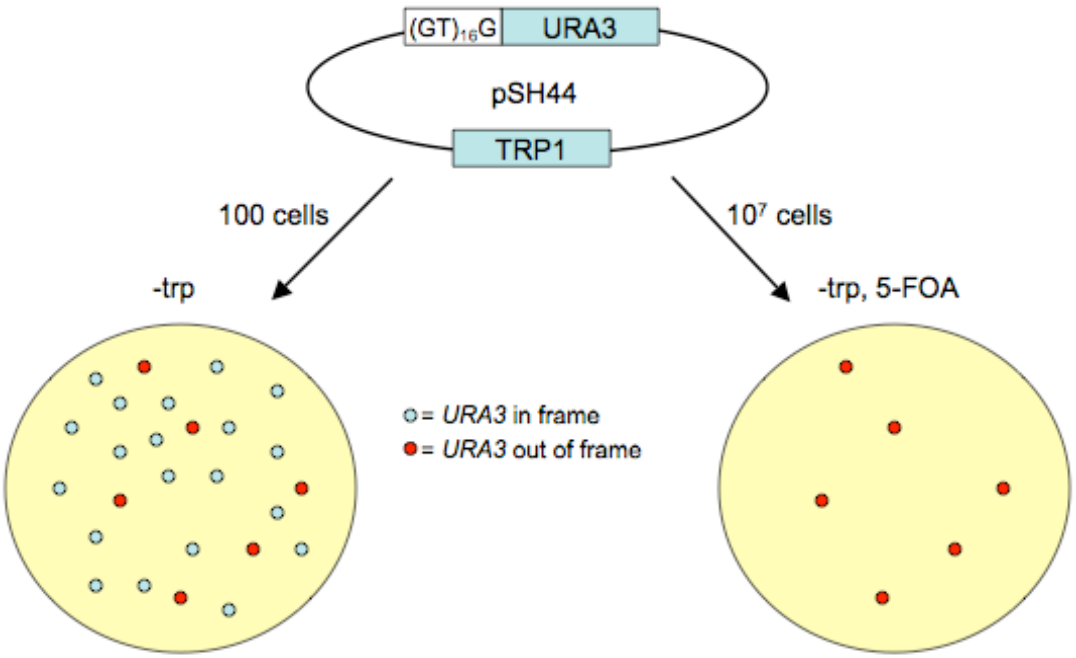
(GT)16G
URA3
pSH44
TRP1
100 cells
10^7 cells
-trp
-trp, 5-FOA
= URA3 in frame
= URA3 out of frame

Figure 2. Plasmid-based microsatellite instability assay. This assay takes advantage of the fact that fluoroorotic acid (5-FOA) is toxic to cells that express Ura3. pSH44 has 16 GT dinucleotide repeats plus one addition G in front of the *URA3* gene, which keeps URA3 in frame and able to be expressed, sensitizing cells with an intact microsatellite to 5-FOA (blue circles). Cells carrying a copy of pSH44 with a change in the length of the microsatellite (either contraction or expansion) that puts *URA3* out of frame results in a lack of *URA3* expression, rendering these cells resistant to 5-FOA (red circles). Thus, growth on media containing 5-FOA can be used as a readout for microsatellite instability in this assay. Importantly, growth on 5-FOA is likely an underestimate of microsatellite instability since some contractions and expansions of the microsatellite will not put *URA3* out of frame.

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