Ubc4 and Not4 Regulate Steady-State Levels of DNA Polymerase-α to Promote Efficient and Accurate DNA Replication

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INTRODUCTION

The accurate duplication of the genome is crucial for the prolonged health of eukaryotic organisms. Inaccurate DNA replication and/or replication of any portion of the genome more than once can result in genomic instability, which is a consistently observed hallmark of cancer cells (Vaziri et al., 2003; Venkatesan et al., 2007). It is crucial, therefore, to understand the entire process of DNA replication. The initiation of DNA replication requires the coordinated recruitment of several proteins. Prereplicative complexes (Difffley et al., 1994), including the core helicase Mcm2–7 (Bochman and Schwacha, 2008), form at origins of replication, are converted to pre-initiation complexes (Zou and Stillman, 1998), and DNA is subsequently unwound (reviewed in Bell and Dutta, 2002). Once the DNA is unwound, the accuracy of DNA replication depends in large part upon DNA polymerases (pol)-α, -δ, and -ε, all of which coordinate to synthesize the nascent copy of DNA during replication in eukaryotes (Burgers, 2009). Highlighting the importance of accurate DNA replication, 43% of mice homozygous for a proofreading-deficient allele of pol-δ develop cancer, primarily lymphoma, but also squamous-cell carcinoma (Goldsbey et al., 2001). Interestingly, pol-α, the only enzyme that can synthesize DNA de novo, naturally lacks proofreading activity (Morrison et al., 1991). Unlike pol-δ, pol-α is foremost a replicative — and not a repair — polymerase (Wu et al., 2001; Wang et al., 2004). The lack of proofreading activity likely reflects one mechanism by which nature enforces evolution. However, this raises the question of how humans use a potentially mutagenic polymerase responsible for the initiation of more than 30 million Okazaki fragments without regularly developing cancer. Fortunately, pol-α incorporates only 12–20 nucleotides before turning over bulk DNA synthesis to either pol-δ (lagging strand) or pol-ε (leading strand), both of which are more processive and contain proofreading activity (Nethanel and Kaufmann, 1990; Morrison et al., 1991; Waga and Stillman, 1994; Garg et al., 2004; Pursell et al., 2007; Nick McElhinny et al., 2008).

The fact that pol-α is limited to synthesizing only 12–20 nucleotides of DNA implies a very tight regulation of this enzyme. Our laboratory and others have shown previously that minichromosome maintenance (Mcm) 10 interacts with the catalytic subunit of pol-α in budding and fission yeast and higher eukaryotes (Fien et al., 2001; Wang et al., 1998; Pursell et al., 2007). Mcm10 is an essential DNA replication factor that is conserved from yeast to humans (Merchant et al., 1997; Aves et al., 1998; Homesley et al., 2000;}

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Abbreviations used: Mcm, Minichromosome maintenance; pol-α, DNA polymerase-α; PCNA, proliferating cell nuclear antigen; Ubc, ubiquitin conjugating enzyme.

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Izumi et al., 2000). It associates with replication origins before replication initiates and is required for the stable association of pol-α with chromatin (Ricke and Bielinsky, 2004). In addition to interacting with the catalytic subunit of pol-α, Mcm10 also binds to proliferating cell nuclear antigen (PCNA), the processivity clamp for both DNA pol-δ and -ε. The interaction between Mcm10 and PCNA is essential in budding yeast and depends on diubiquitination of Mcm10 (Das-Bradoo et al., 2006). At the same time, this modification appears to inhibit the interaction between Mcm10 and pol-α (Das-Bradoo et al., 2006). These data suggest that Mcm10 plays an integral role in the switch from pol-α to pol-δ and pol-ε. As mentioned above, Mcm10 does not only interact with Cdc17 it also regulates its stability (Ricke and Bielinsky, 2004), and this function is evolutionarily conserved (Chattopadhyay and Bielinsky, 2007). The catalytic subunit of pol-α is rapidly degraded after depletion of Mcm10 from yeast and human cells (Ricke and Bielinsky, 2004; Chattopadhyay and Bielinsky, 2007). However, the pathway responsible for this degradation is not known.

The ubiquitin-mediated degradation system is a major proteolytic pathway in cells. In this system, ubiquitin, an 8-kDa peptide, is transferred from an ubiquitin activating (E1) enzyme to an ubiquitin conjugating (E2) enzyme, then to an ubiquitin ligase (E3) and finally, to the target protein (Hershko et al., 1983). This process is repeated several times to polyubiquitinate the protein and this serves as a signal for degradation by the proteasome, a large, multisubunit proteolytic machine (Varshavsky, 2005). The fact that Cdc17 is rapidly degraded in the absence of Mcm10 suggests that it might be a target of proteasomal degradation.

Here, we first sought to elucidate the mechanism of Cdc17 degradation in the absence of Mcm10. We found that Cdc17 was degraded via the proteasome and we identified ubiquitin conjugating enzyme (Ubc) 4 and negative regulator of transcription (Not) 4 as the E2 and E3 enzymes required for the destabilization of Cdc17. Although we were unable to detect ubiquitin conjugates of Cdc17, protein turnover clearly depended on ubiquitination. Moreover, overexpression of Cdc17 resulted in slow growth of ubc4Δ mutants and revealed synthetic dosage lethality (SDL) with a NOT4 deletion. Lastly, cooverexpression of Cdc17 and Mcm10 exhibited a similar slow growth phenotype and a significant increase in microsatellite-mediated DNA rearrangements. Our results suggest that Cdc17 levels are carefully regulated in the cell through Ubc4- and Not4-dependent destabilization counterbalanced by Mcm10-dependent stabilization. We propose that tipping this balance in favor of increasing Cdc17 and Mcm10 levels causes genomic instability.

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 (Buchman et al., 1996). The genotypes of the yeast strains used 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 Ably661 and Ably1051 were constructed by transforming plasmid YEp105, which harbors a synthetic Myc-tagged ubiquitin gene (Ellison and Hochstrasser, 1991), and YEp105-GFP76A (Das-Bradoo et al., 2010) into Ably527, respectively.

Protein Preparation and Western Blot Analysis

Total protein extracts were prepared as described previously by trichloroacetic acid extraction (Ricke and Bielinsky, 2006). Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed by Western blot with anti-HA (16B12; Covance, Princeton, NJ) for HA-tagged proteins and anti-γ-tubulin (B512; Covance). Alexa Fluor 680 (Invitrogen, Carlsbad, CA) secondary antibody was used to visualize proteins with Odyssey v3.0 software on an Odyssey scanner from LI-COR Biosciences (Lincoln, NE).

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).

Heat Shock Survival Assay

This assay was performed as described previously (Seufert and Jentsch, 1990). Briefly, cells were grown in YPD liquid medium to OD600 = 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.

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, 107 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 FOAΔ colonies were counted. The frequency of FOAΔ colonies was calculated by dividing the number of FOAΔ colonies by the number of viable cells. To assess whether microsatellites were altered, pSH44 was recovered from FOAΔ clones and the microsatellite region was sequenced. For clones in which the microsatellite was unaltered, pSH44 was digested with HindIII 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 sequence 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 KpnI and SacI.

Microscopy

For nuclei staining, DAPI stain (10 μg/ml, Sigma, St. Louis, MO, D1380) was added to cultures for 30 min, washed twice with sterile water, air-dried on a microscope slide, and visualized using a Zeiss Axioplan microscope with a DIC Alpha Plan-Fluar ×100/1.45 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, Austin, TX). 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 60°C for 30 s. CDC17 was amplified with primer 5′ CDC17-3712 (5′-GCTCTAAGATCTGGAAGGCTGCTAG-3′) and primer 3′ CDC17-3712 (5′-AAACCTCACCCGCGTTGAAAGCTATC-3′) and ACTI was amplified with primer 5′ ACTI-511 (5′-GTACACCAATGGACACCG-GTATGTC-3′) and primer 3′ ACTI-836 (5′-CAGAAAGCGCTGGGAGA- GAAAC-3′).

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.

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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.

| Strain name | Relevant genotype | Source |
|-------------|-------------------|--------|
| ABY6013     | mcml-1, CDC17::3HA-TRP1 | Ricke and Bielinsky, 2004 |
| ABY342      | mcml-1, ubc4::LEU2, CDC17::3HA-TRP1 | This study |
| ABY455      | mcml-1, UBC4::NES-3HA-URA3, CDC17::3HA-TRP1 | This study |
| ABY529      | mcml-1, not4::LEU2, CDC17::3HA-TRP1 | This study |
| ABY363      | mcml-1, ubc5::LEU2, CDC17::3HA-TRP1 | This study |
| ABY359      | mcml-1, rad6::LEU2, CDC17::3HA-TRP1 | This study |
| ABY360      | mcml-1, ubc13::LEU2, CDC17::3HA-TRP1 | This study |
| ABY361      | mcml-1, mms2::LEU2, CDC17::3HA-TRP1 | This study |
| ABY781      | mcml-1, CDC17::3HA-TRP1, pRS316 | This study |
| ABY762      | mcml-1, CDC17::3HA-TRP1, pRS316 | This study |
| ABY738      | mcml-1, not4::LEU2, CDC17::3HA-TRP1, pRS316-Not4 | This study |
| ABY739      | mcml-1, not4::LEU2, CDC17::3HA-TRP1, pRS316-not4-L35A | This study |
| ABY796      | mcml-1, CDC17::3HA-TRP1, NOT4::3HA-URA3 | This study |
| ABY740      | mcml-1, not4::LEU2, CDC17::3HA-TRP1, pRS316-Not4-HA | This study |
| ABY741      | mcml-1, not4::LEU2, CDC17::3HA-TRP1, pRS316-Not4-L35A-HA | This study |
| ABY448      | CDC17::3HA-TRP1 | This study |
| ABY407      | 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 |
| ABY674      | ubc5::LEU2, CDC17::3HA-TRP1 | This study |
| ABY605      | mcml-1, UBC4::GFP-HIS3 | This study |
| ABY660      | mcml-1, UBC4::NES-GFP-HIS3 | This study |
| ABY527      | mcml-1, pdr5::LEU2, pmk4::HIS3, CDC17::2HA-URA3 | This study |
| ABY661      | mcml-1, pdr5::LEU2, pmk4::HIS3, CDC17::2HA-URA3, YEp105 | This study |
| ABY1051     | mcml-1, pdr5::LEU2, pmk4::HIS3, CDC17::2HA-URA3, YEp105-G75,76A | 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 |
| ABY645      | pRS423gal, pRS424gal, pRS425gal, pRS426gal | This study |
| ABY649      | pRS423gal, pRS424gal, pRS425gal-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, POL1-2-3HA, pRS424gal-PR1-3HA, pRS425gal | This study |
| ABY862      | pRS423gal | This study |
| ABY863      | pRS423gal-CDC17-2HA | This study |
| ABY864      | pRS423gal-MCM10-3HA | This study |
| ABY865      | ubc4::LEU2, pRS423gal | This study |
| ABY866      | ubc4::LEU2, pRS423gal-CDC17-2HA | This study |
| ABY867      | ubc4::LEU2, pRS423gal-MCM10-3HA | This study |
| ABY868      | ubc5::LEU2, pRS423gal | This study |
| ABY869      | 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 |
| ABY974      | ubc4::LEU2, ubc5::TRP1, pRS423gal-MCM10-3HA | This study |
| ABY975      | not4::LEU2, pRS423gal | This study |
| ABY976      | not4::LEU2, pRS423gal-CDC17-2HA | 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 |

Continued
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, and monitored Cdc17 stability in the absence of Mcm10. On MCM10-3HA mutants (Figure 2), Cdc17 was significantly stabilized in ubc4Δ cells compared with all other strains, including ubc5Δ mutants (Figure 2). We determined that Cdc17 overexpression levels in ubc4Δ mutants were ~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 whether degradation of endogenous Cdc17 also required Ubc4. We deleted UBC4 in mcm10Δ-1 mutants in which Cdc17 is tagged with three hemagglutinin (HA) epitopes and monitored Cdc17 stability in the absence of Mcm10. On MCM10-1 deletion 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 mcm10Δ-1 ubc4Δ cells, Cdc17 remained stable (Figure 3, A and B). To ensure that Cdc17 stability was specific to mcm10Δ-1 ubc4Δ cells, we monitored Cdc17 stability in mcm10Δ-1 ubc5Δ, mcm10Δ-1 rad6Δ, mcm10Δ-1 ubc13Δ, and

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**Table 1. Continued**

| Strain name | Relevant genotype | Source |
|-------------|------------------|--------|
| ABy150      | MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 | Mortimer and Johnston, 1986 |
| ABy152      | ubc4::KANMX      | This study |
| ABy157      | hos4::LEU2       | This study |
| ABy1574     | UBC4::NEM-3HA-URA3 | This study |
| ABy1577     | ubc5::LEU2, UBC4::NEM-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 |
| ABy269      | pRS426gal-CDC17-2HA | This study |
| ABy264      | pRS423gal-MCM10-3HA | This study |
| ABy265      | pRS423gal-MCM10-3HA, pRS426gal-CDC17-2HA | This study |

**Figure 1.** Cdc17 degradation is proteasome-dependent. ABy527 (mcm10-1, pdr5Δ, rpm4Δ) cells were grown in rich liquid medium at 25°C to mid-log 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 min. Cdc17–3HA and α-tubulin were analyzed by Western blot.
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 are from the same experiment.
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 the absence of Mcm10. (A) Asynchronous cultures of ABy448 (W303-1a), ABy013 (mcm10-1), ABy342 (mcm10-1, ubc4Δ), ABy363 (mcm10-1, ubc5Δ), and ABy399 (mcm10-1, rad6Δ) 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 ± SD).

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 rpm4Δ 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 4F). 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 whether 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 (Seufert and Jentsch, 1990). 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 5A). 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 5B). Having verified that Ubc4-NES-3HA is a functional cytoplasmic enzyme, we then determined whether Cdc17 was degraded in the nucleus or the cytoplasm. If Cdc17 degradation occurred in the nucleus, then Cdc17 should have remained stable in mcm10-1 UBC4-NES-3HA ubc5Δ double mutants. Conversely, if Cdc17 was degraded in the cytoplasm, then Cdc17 should have been 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 5C, C and D). 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 nu-
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 with aubc4Δ 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 analyzed Cdc17 transcript levels (Figure 6), arguing that Ubc4/Not4 do not affect Cdc17 transcription.

**Cdc17 Levels Are Elevated in the Presence of Mcm10 in aubc4Δ and not4Δ Cells**

Cdc17 is transcribed and translated at the G1/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 aubc4Δ, 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 mcm10-1, not4Δ, ubc5Δ, ubc13Δ, or mms2Δ cells. We observed similar trends in aubc4Δ, not4Δ and ubc5Δ mutants that carried the wild-type gene for MCM10, although the effect was most pronounced in not4Δ cells (Figure 7C). 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 whether Ubc4’s nuclear localization is important for replication stress tolerance. Interestingly,

Figure 5. Cdc17 is degraded primarily in the nucleus. (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. (C) Asynchronous cultures of ABy013 (mcm10-1), ABy342 (mcm10-1, ubc4Δ), ABy555 (mcm10-1, UBC4-NES-3HA), ABy363 (mcm10-1, ubc5Δ), and ABy359 (mcm10-1, rad6Δ) grown at 25°C were shifted to 37°C for 180 min. Cdc17–3HA and α-tubulin were analyzed by Western blot. (D) 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 ± SD).

Figure 6. CDC17 mRNA levels are unchanged in not4Δ mutants. ABy48 (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.
UBC4-NES-3HA cells behaved identically to ubc4Δ cells (Figure 8A). Whereas ubc4Δ mutants were moderately sensitive to HU, not4Δ cells were highly sensitive to the drug, as seen previously (Figure 8A). 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 8A). This was indeed the case. Surprisingly, even the severe HU sensitivity of not4Δ mutants was suppressed by mcm10-1 (Figure 8A). This suppression went hand-in-hand with reduced Cdc17 levels in mcm10-1 not4Δ double mutants (Figure 8B; 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 9A). To our surprise, 10-fold overexpression of Cdc17 and Mcm10 resulted in a significant growth defect in the absence of any exogenous stress (Figure 9B). According to our estimate, growth was inhibited 25-fold. Importantly, cells carrying the empty vector controls did not display any growth phenotype (Figure 9B). 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 cooverexpression 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 9A). However, this did not rescue the observed pheno-
type (Figure 9B). 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 galactose (to induce protein overexpression) or glucose (to repress protein expression) to 30°C. Cells overexpressing Cdc17 alone showed no increase in mutation rate, whereas cells overexpressing Mcm10 alone showed a fourfold increase in mutation rate (Figure 9B). To further ascertain that the rearrangements were dependent on the microsatellite tract, we compared the number of FOA-resistant colonies between pSH44 and pRS316-TRP1, which is devoid of a (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 overexpressed Cdc17 and Mcm10 (Figure 10D). Therefore, the majority of plasmid rearrangements must have been triggered by replication through the microsatellite tract. Together, these findings demonstrate that deregulation 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 not4Δ, 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 11A shows that Cdc17 and Mcm10 were induced at similar levels in all strains after 1 h in galactose-containing medium, whereas cells overexpressing Mcm10 alone exhibited a significant growth defect when Cdc17, but not Mcm10, was overexpressed (Figure 11B). 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 11B). 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 11C). The amount of Cdc17 protein detected in the different strains correlated well with the observed growth defect.

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**Figure 9.** 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.

**Figure 10.** Overexpression of Cdc17 and Mcm10 disrupts the URA3 gene at an ~20-fold higher rate than that of cells that carried empty vector controls (Figure 9, B and C). Conversely, cells overexpressing Cdc17 alone showed no increase in mutation rate, whereas cells overexpressing Mcm10 alone showed a fourfold increase in mutation rate (Figure 9, B and C). To verify that 5-FOA resistant (FOAr) 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 pol (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 10D). To further ascertain that the rearrangements were dependent on the microsatellite tract, we compared the number of FOAR colonies between pSH44 and pRS316-TRP1, which is devoid of a (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 overexpressed Cdc17 and Mcm10 (Figure 10E). Therefore, the majority of plasmid rearrangements must have been triggered by replication through the microsatellite tract. Together, these findings demonstrate that deregulation of Cdc17 and Mcm10 can induce microsatellite-mediated DNA recombination.
These results are consistent with our claim that Ubc4 and Not4 regulate the degradation of Cdc17 either directly or indirectly.

**DISCUSSION**

In this study, we demonstrate 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 (Figure 6) but rather through ubiquitin-mediated proteasomal degradation (Figures 1 and 4). 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.

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 (Figure 4A) and accumulates in the presence of Mcm10 when NOT4 is deleted (Figure 7C). We take this as evidence that Not4 is required for regular turnover of Cdc17. This is supported by the obser-
vation that Cdc17 destabilization is clearly dependent on ubiquitin conjugation (Figure 4F), 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). In addition, we excluded that accumulation of Cdc17 in mcm10-1 not4Δ/H9004 cells (Figure 4A) was due to a failure to degrade Mcm10-1 protein at the nonpermissive temperature (data not shown). Another striking observation supporting the claim that Not4 regulates Cdc17 turnover in conjunction with Ubc4 is provided by the SDL tests (Figure 11). SDL screens have been used to identify potential targets for protein kinases (Maknhevych et al., 2009; Zou et al., 2009) and might also be useful in revealing potential targets for E3 ligases. 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/H9004 cells (Figure 11B). Nevertheless, a mutation in MCM10 almost completely alleviated the severe HU-sensitivity of not4Δ cells (Figure 8). Because mcm10-1 is

Figure 12. Overexpression of Mcm10 and Cdc17 may hinder polymerase switching. After pol-ε extends the RNA primer (red line) by 12–20 nucleotides, Mcm10 is ubiquitinated at two distinct lysines (yellow star; (Das-Bra-ndo et al., 2006). PCNA is recruited to the lagging strand, and a polymerase switch occurs between pol-ε and pol-δ, after which pol-ε 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-ε to synthesize more DNA than in wild-type cells.

**Figure 11.** Overexpression of Cdc17 in ubc4Δ-ubc5Δ 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), and ABY972 (ubc4Δubc5Δ, GAL-CDC17-2HA) 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 nonspecific 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. (C) ABY981 (CDC17-3HA, pRS426gal-CDC17-2HA), ABY983 (ubc4Δ, CDC17–3HA, pRS426gal-CDC17-2HA), and ABY983 (not4Δ, CDC17–3HA, pRS426gal-CDC17-2HA), and ABY987 (not4Δ, GAL-MCM10-3HA) were grown overnight in SC-ura medium to midlog phase. Cdc17 and α-tubulin were analyzed by Western blot.
HU-sensitive by itself, one might have expected that it exacerbates the drug sensitivity of the not4 strain. We believe that these results are most easily explained by the destabilizing effect that mcm10-1 has on Cdc17 (Rickie and Bielinsky, 2006; Lee et al., 2010).

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).

We propose that Ubc4 and Not4 may have a role in degrading Cdc17 to ensure efficient polymerase switching (pol-α 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 (Figure 12). 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 (Figure 10A) is consistent with a mechanism by which Mcm10 “redelivers” pol-α to chromatin, thus interfering with the switch from pol-α to pol-δ (Figure 12). As a consequence, pol-α would further extend the RNA/DNA primer, which could account for the increased frequency of mutations (Figure 10A). 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.

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 investigations 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, cooverexpression of Cdc17 and Mcm10 resulted in a significant increase in microsatellite-mediated recombination (Figure 10, C–E). 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).

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 up-regulated in glioma cells (Sun et al., 2006). Intriguingly, UbcH5, the human homolog of yeast Ubc4/5, is down-regulated in these cells. 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.

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