A balance of deubiquitinating enzymes controls cell cycle entry

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ABSTRACT

Protein degradation during the cell cycle is controlled by the opposing activities of ubiquitin ligases and deubiquitinating enzymes (DUBs). Although the functions of ubiquitin ligases in the cell cycle have been studied extensively, the roles of DUBs in this process are less well understood. Here, we used an overexpression screen to examine the specificities of each of the 21 DUBs in budding yeast for 37 cell cycle–regulated proteins. We find that DUBs up-regulate specific subsets of proteins, with five DUBs regulating the greatest number of targets. Overexpression of Ubp10 had the largest effect, stabilizing 15 targets and delaying cells in mitosis. Importantly, UBP10 deletion decreased the stability of the cell cycle regulator Dbf4, delayed the G1/S transition, and slowed proliferation. Remarkably, deletion of UBP10 together with deletion of four additional DUBs restored proliferation to near–wild-type levels. Among this group, deletion of the proteasome-associated DUB Ubp6 alone reversed the G1/S delay and restored the stability of Ubp10 targets in ubp10Δ cells. Similarly, deletion of UBP14, another DUB that promotes proteasomal activity, rescued the proliferation defect in ubp10Δ cells. Our results suggest that DUBs function through a complex genetic network in which their activities are coordinated to facilitate accurate cell cycle progression.

INTRODUCTION

Progression through the eukaryotic cell cycle is controlled by the periodic expression of regulatory proteins that are expressed precisely at the times their functions are needed (Morgan, 2007). This pattern of cyclical protein expression is dependent on the ubiquitin-proteasome system (UPS), which is the primary mechanism of regulated protein degradation. Within the UPS, E3 ubiquitin ligases recognize specific protein targets and attach chains of ubiquitin to direct those proteins to the proteasome for destruction. The actions of E3s can be opposed by deubiquitinating enzymes (DUBs) that remove ubiquitin chains.

Although many E3s have established roles in targeting cell cycle–regulatory proteins for degradation (Benanti, 2012; Mocciaro and Rape, 2012), the roles of DUBs in cell cycle control are just beginning to be understood. Some DUBs appear to affect the cell cycle indirectly. For example, in fission yeast Ubp8 indirectly antagonizes the function of the essential mitotic-regulatory E3, the anaphase promoting complex (APC; Elmore et al., 2014). In mammalian cells, multiple DUBs have been identified that impact the cell cycle through deubiquitination of individual cell cycle regulators (Darling et al., 2017). In budding yeast, only a few DUBs of the 21 total DUBs have been found to have cell cycle–regulatory roles. Ubp15 has been shown to control the cell cycle directly by deubiquitinating the B-type cyclin Clb5 and promoting S-phase entry (Ostapenko et al., 2015). Two other DUBs, Ubp7 and Ubp10, are implicated in cell cycle control following DNA damage. Cells lacking UBP7 are sensitive to replication stress; however, the substrate(s) responsible for this role of Ubp7 is not known (Böhm et al., 2016). Ubp10 also plays a role in the DNA damage response, by removing ubiquitin from PCNA after DNA repair to promote recovery from S-phase arrest (Gallego-Sánchez et al., 2012). Beyond these few examples, the roles of DUBs in regulation of the yeast proteome during the cell cycle remain to be identified.

One reason identification of DUB substrates has been challenging is that several lines of evidence suggest that DUBs may have overlapping or redundant functions. This possibility is supported by in vitro studies. Although DUBs form distinct complexes with
interacting proteins that regulate their functions in vivo (Leggett et al., 2002; Krogh et al., 2006; Sowa et al., 2009; Kouranti et al., 2010; Richardson et al., 2012), assays of different DUB complexes purified from budding yeast demonstrate that most have some ability to deubiquitinate the same ubiquitinated proteins in vitro (Schaefer and Morgan, 2011). Redundancy presents a technical hurdle for identifying DUB substrates using loss-of-function approaches, because mutations in multiple DUBs are necessary to prevent the ubiquitination of target proteins. For example, simultaneous deletion of five DUBs is required to disrupt ubiquitin-regulated membrane trafficking in fission yeast (Kouranti et al., 2010; Beckley et al., 2015). To circumvent these issues, proteomics of ubiquitinated proteins has been used to identify specific DUB substrates involved in membrane trafficking (Beckley et al., 2015). However, this approach has not yet been applied to DUB substrates whose ubiquitination leads to their degradation by the UPS. In general, UPS substrates that are rapidly degraded are expressed at low levels, making them difficult to detect by proteomic approaches. Indeed, quantitative whole cell proteomics of budding yeast strains with deletions of individual DUBs have not detected many UPS targets (Poulsen et al., 2012; Isasa et al., 2015). Therefore, additional approaches to identify DUB targets are necessary.

Here we take an alternative approach to identify substrates by testing whether elevated levels of individual DUBs can stabilize cell cycle proteins in vivo. We determined which of the 21 yeast DUBs, upon overexpression, can stabilize each of 37 cell cycle–regulatory proteins that are degraded by the UPS. We find that overexpression of the majority of DUBs leads to an increase in the levels of one or more cell cycle–regulatory proteins. Overexpression of Ubp10 increased the levels of 15 cell cycle proteins tested (40%), suggesting it plays a central role in regulating the cell cycle proteome. Significantly, either overexpression or deletion of UBP10 impaired cell cycle progression, demonstrating that precisely tuned levels of Ubp10 are critical for normal proliferation. We further showed that deletion of the proteasome-associated DUB Ubp6 rescued the cell cycle defects of ubp10Δ cells and restored the stability of Ubp10 targets. Deletion of an alternate proteasome-regulatory DUB, UBP14, also rescued the proliferation defect in ubp10Δ cells, suggesting that partial proteasome inhibition can counteract the accelerated degradation of proteins that occurs in the absence of Ubp10. These studies uncover new roles for these DUBs in cell cycle control and demonstrate the coordinated activities of an interconnected network of DUBs is necessary for accurate progression through the cell cycle.

RESULTS
A gain-of-function screen to examine DUB specificity
Because evidence suggests that DUBs act redundantly (Kouranti et al., 2010; Schaefer and Morgan, 2011), potentially masking the effects of mutations in individual DUBs, we sought to design an overexpression/gain-of-function screen to identify DUBs that can regulate cell cycle protein levels. Budding yeast express 21 DUBs that can be classified into five families based on their catalytic domains (Table 1). We first tested whether overexpression of any of these 21 DUBs resulted in a permanent growth arrest (Sopko et al., 2006; Gallego-Sánchez et al., 2012); however, seven strains overexpressing individual DUBs (UBP1, UBP3, UBP10, UBP11, UBP12, UBP14, UBP15) exhibited reduced growth after induction (Supplemental Figure S1A). We next examined the consequences of a 4-h DUB induction, which was the amount of time it took to induce maximum expression of DUBs from the GAL1 promoter (Supplemental Figure S1B). Importantly, no cell cycle arrest was observed following overexpression of any DUB for 4 h (Figure 1A). In addition, there was no evident decrease in long ubiquitin chains, which might be observed if a particular DUB could nonspecifically target all ubiquitinated proteins in the cell (Figure 1B). Based on these results, a 4-h induction time was selected to perform the screen for the stabilization of any of the selected proteins upon DUB overexpression.

To identify DUBs that can regulate the degradation of specific cell cycle proteins, we tested a matrix of 777 pairs and asked whether overexpression of each of the 21 DUBs could up-regulate any of 37 TAP-tagged cell cycle proteins (Figure 2A). The 37 target proteins that were selected fit three criteria: 1) the target has been shown to be up-regulated upon inactivation of an E3 or inhibition of the proteasome, 2) expression of the target is cell cycle regulated, and 3) TAP-tagged alleles are included in a previously constructed TAP-tag strain collection (Supplemental Data S1; Ghaemmaghami et al., 2003). Pilot experiments indicated that overexpression of Ubp2 did not significantly increase levels of any cell cycle protein compared with overexpression of glutathione S-transferase (GST) alone (Supplemental Figure S2A and Supplemental Data S2), so Ubp2 was used as a negative control for the screen. To perform the screen, expression of either the control (Ubp2) or the test DUB was induced in each TAP-tagged strain for 4 h. Western blotting was performed with anti-TAP–tag antibodies to quantify cell cycle proteins and G6PDH was used as a loading control (Figure 2B). Proteins that changed at least twofold in two replicates of the screen were considered high-confidence changes (Supplemental Figure S2 and Supplemental Data S2).

Among the 777 DUB-target pairs tested, 50 increases (6.9% of all pairs) and nine decreases (1.2%) in protein levels were identified (Figure 2C). Targets (27 of 37) were up-regulated by overexpression of at least one DUB, and overexpression of 12 of 20 DUBs resulted in the up-regulation of at least one target protein. The fact that more proteins exhibited increased levels than decreased levels is consistent with the prediction that overexpression of DUBs leads to ubiquitin chain removal, stabilization, and increased levels of their targets. Another notable result is that each DUB regulated a different subset of cell cycle proteins, demonstrating that DUBs exhibit specificity for subsets of ubiquitinated proteins in vivo, even in an overexpression setting.

How do DUBs achieve substrate specificity? One simple explanation might be that DUBs and ubiquitinated proteins need only to be colocalized in the same subcellular compartment to facilitate their interaction. To address this possibility, the localization of all targets and DUBs was examined in the collection of yeast cells and localization patterns (CYCLOPaS) database (Chong et al., 2015), which reports high-confidence localization data for the majority of yeast proteins. With the exception of two DUBs whose localizations are unknown (Ubp11 and Yuh1), all DUBs localize to the nucleus, the cytoplasm, or both (Table 1). Among the target proteins, 31 of 37 are localized to some extent in both the nucleus and the cytoplasm, which would make these proteins accessible to all DUBs tested (Supplemental Data S3). Of the six targets that are not reported to be nuclear or cytoplasmic, two (Hst3 and Swi5) are inferred to be nuclear based on their established functions, one (Hmg2) is membrane localized, and the localization of three is not known. However, the extent of overlap in localization patterns between the majority of targets and DUBs argues that localization cannot explain most DUB specificity observed in the screen.
Several DUBs have been found to interact with E3 ubiquitin ligases in cells (Kee et al., 2005; Rumpf and Jentsch, 2006; Sowa et al., 2009), and in some instances an E3 has been shown to function as an adaptor to recruit the DUB to its substrates (Kee et al., 2006; Harreman et al., 2009; Schülein-Völk et al., 2014; Sun et al., 2015). Because the identities of the E3s that ubiquitinate most of the cell cycle proteins in our screen are known, we examined whether any DUB regulated all substrates of a given E3, which would suggest this type of recruitment mechanism. We found that Ubp10 up-regulated most of the APC substrates in our screen, whereas Ubp5 and Ubp7 up-regulated many SCFCdc4 and SCFCentr substrates (Figure 2D). However, each of these DUBs also up-regulated targets ubiquitinated by other E3s, and no DUB regulated all substrates of any particular E3, suggesting that additional mechanisms contribute to DUB-substrate specificity.

Although DUBs are predicted to stabilize targets by removing ubiquitin chains and blocking their degradation, it is possible that up-regulation may be indirect, for instance if a transcriptional activator of cell cycle proteins is stabilized by a DUB. To determine whether DUBs regulate the stability of candidate target proteins, eight targets were investigated further, to determine whether DUB overexpression impaired their degradation. Cycloheximide-chase

### TABLE 1: Summary of Saccharomyces cerevisiae DUBs.

<table>
<thead>
<tr>
<th>DUB</th>
<th>Systematic name</th>
<th>Domains(^a)</th>
<th>Localization(^b)</th>
<th>Regulatory partners</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubp1</td>
<td>YDL122W</td>
<td>USP</td>
<td>C, ER, CoP, CP, B</td>
<td>Rsp5</td>
<td>Chong et al., 2015</td>
</tr>
<tr>
<td>Ubp2</td>
<td>YOR124C</td>
<td>USP</td>
<td>C</td>
<td></td>
<td>Chong et al., 2015; Kee et al., 2005</td>
</tr>
<tr>
<td>Ubp3</td>
<td>YER151C</td>
<td>USP</td>
<td>C</td>
<td>Bre5</td>
<td>Cohen et al., 2003; Chong et al., 2015</td>
</tr>
<tr>
<td>Ubp4, Doa4</td>
<td>YDR069C</td>
<td>USP, Rhod</td>
<td>C, E, M, SP</td>
<td>Bro1</td>
<td>Amerik et al., 2006; Luhtala and Odorizzi, 2004; Chong et al., 2015</td>
</tr>
<tr>
<td>Ubp5</td>
<td>YER144C</td>
<td>USP, Rhod</td>
<td>C, BN, N</td>
<td></td>
<td>Amerik et al., 2006</td>
</tr>
<tr>
<td>Ubp6</td>
<td>YFR010W</td>
<td>USP, UBL</td>
<td>C, N, M, V</td>
<td>Proteasome</td>
<td>Chong et al., 2015; Verma et al., 2000</td>
</tr>
<tr>
<td>Ubp7</td>
<td>YIL156W</td>
<td>USP, Rhod</td>
<td>C, M</td>
<td></td>
<td>Chong et al., 2015</td>
</tr>
<tr>
<td>Ubp8</td>
<td>YMR223W</td>
<td>USP, ZnF</td>
<td>N, M</td>
<td>SAGA</td>
<td>Chong et al., 2015; Henry et al., 2003</td>
</tr>
<tr>
<td>Ubp9</td>
<td>YER098W</td>
<td>USP</td>
<td>C</td>
<td></td>
<td>Chong et al., 2015</td>
</tr>
<tr>
<td>Ubp10, Dot4</td>
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<td>USP, IDR</td>
<td>N, No, M</td>
<td>Sir4, Dhr2, Utp22</td>
<td>Chong et al., 2015; Kahana and Gottschling, 1999; Richardson et al., 2012</td>
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<td></td>
<td></td>
<td></td>
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<td>USP, DUSP</td>
<td>C, M, V</td>
<td>Rad23, Cdc48</td>
<td>Chong et al., 2015; Gödderz et al., 2017</td>
</tr>
<tr>
<td>Ubp13</td>
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<td>USP</td>
<td>C</td>
<td></td>
<td>Chong et al., 2015</td>
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<tr>
<td>Ubp14</td>
<td>YBR058C</td>
<td>USP, ZnF</td>
<td>C, N, V, M</td>
<td></td>
<td>Chong et al., 2015</td>
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<tr>
<td>Ubp15</td>
<td>YMR304W</td>
<td>USP, MATH</td>
<td>C, E, ER</td>
<td>Pex1/Pex6, Cdh1</td>
<td>Chong et al., 2015; Debelyv et al., 2011; Ostapenko et al., 2015</td>
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<tr>
<td>Otu1</td>
<td>YFL044C</td>
<td>OTU, ZnF</td>
<td>C, N, V, M</td>
<td>Cdc48</td>
<td>Chong et al., 2015; Rumpf and Jentsch, 2006</td>
</tr>
<tr>
<td>Otu2</td>
<td>YHL013C</td>
<td>OTU</td>
<td>C</td>
<td></td>
<td>Chong et al., 2015</td>
</tr>
<tr>
<td>Rpn11</td>
<td>YFR004W</td>
<td>JAMM</td>
<td>N, V</td>
<td>Proteasome</td>
<td>Chong et al., 2015; Verma et al., 2000</td>
</tr>
<tr>
<td>Yuh1</td>
<td>YJR099W</td>
<td>UCH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miy1</td>
<td>YPL191C</td>
<td>MINDY</td>
<td>C</td>
<td></td>
<td>Chong et al., 2015</td>
</tr>
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\(^a\)DUB catalytic domains: USP, ubiquitin-specific protease; UCH, ubiquitin C-terminal hydrolase; OTU, ovarian tumor; JAMM, JAB1/MPN/Mov34 metalloenzyme; MINDY, motif interacting with Ub-containing novel DUB family. DUB accessory domains: Rhod, rhodanese-like; UBL, ubiquitin-like; ZnF, zinc finger; IDR, intrinsically disordered region; DUSP, domain in ubiquitin-specific proteases; MATH, Merpin and traf homology domain.

\(^b\)C, cytoplasm; N, nucleus; No, nucleolus; ER, endoplasmic reticulum; CoP, cortical patches; CP, cell periphery; B, bud; BN, bud-neck; E, endosome; M, mitochondria; SP, spindle pole; V, vacuole.
We tested whether the deubiquitinase activity of Ubp10 was necessary for its ability to stabilize its targets, and for all four targets that were tested, the catalytic activity of Ubp10 contributed to their up-regulation (Figure 4C). Ubp10 has a unique N-terminus containing an intrinsically disordered region (IDR) that is required for its interaction with several established binding partners (Reed et al., 2015). To determine whether this domain is also required to stabilize the targets that we identified, a mutant of Ubp10 that lacks the IDR (Ubp10ΔN) was tested in an overexpression assay. Although Ubp10 and Ubp10ΔN were not induced to equal levels in all experiments, stabilization of nine of 15 targets was reproducibly dependent on the N-terminal IDR, whereas five others showed intermediate effects and one was unaffected (Figure 4B). To confirm that changes in protein levels reflected changes in protein stability, the half-life of one target whose up-regulation was completely dependent upon the IDR (Hst3), and one target that showed intermediate regulation by Ubp10ΔN (Dbf4) were assayed (Figure 4D). For both targets, stabilization by Ubp10ΔN correlated with the extent of up-regulation that was observed. These data strongly suggest that Ubp10 is recruited to its targets via its N-terminal IDR to remove ubiquitin and stabilize these proteins.

The fact that Ubp10 regulated expression of 40% of cell cycle proteins tested suggested that it controls progression through the cell cycle. Consistent with this possibility, asynchronous populations of cells overexpressing Ubp10 showed a reduced fraction of cells in G1 and an increased fraction of mitotic cells, with Ubp10ΔN overexpression having an intermediate effect (Figure 4E, Supplemental Figure S4). Moreover, deletion of Ubp10 had the opposite effect, resulting in an increased fraction of G1 cells in an asynchronous population (Figure 5A). These data suggest that Ubp10 regulates entry into S phase. To test this, cells were arrested in G1, released, and DNA content was monitored at 15-min intervals. Compared to wild-type cells, ubp10Δ cells exhibited an ∼15-min delay in initiating DNA replication when grown in rich medium (Figure 5B and C). We next examined the levels of four representative target proteins that are stabilized by Ubp10 overexpression, to determine whether their expression was altered in ubp10Δ cells. The expression of two proteins expressed in G2/M-phase, Hst3 and Spo12, was delayed ∼15 min in ubp10Δ cells, in accordance with the requirement for Ubp10 to enter S phase on time. Although the expression of these proteins was delayed in ubp10Δ cells, the peak levels of each protein were comparable to peak levels in wild-type cells (Figure 5D). In contrast, Dbf4 and Mps1 protein levels increased with similar timing in wild-type and ubp10Δ cells; however, peak expression levels of both proteins were decreased in the absence of Ubp10. In addition, Dbf4 was less stable in ubp10Δ cells, whereas Mps1, Spo12, and Hst3 half-lives were relatively unchanged (Figure 5, E and F). These results suggest that Dbf4 is deubiquitinated and stabilized by Ubp10 during an unperturbed cell cycle.

A previous study found that the slow growth phenotype of ubp10Δ cells growing on solid medium could be reversed by overexpression of Rpa190, a Ubp10 target that regulates ribosome biogenesis (Richardson et al., 2012). To determine whether reduced Rpa190 expression in ubp10Δ cells contributes to the G1/S delay that we observed, arrest-release experiments were performed in wild-type and ubp10Δ cells that overexpressed Rpa190. Surprisingly, although overexpression of Rpa190 did increase the colony size of ubp10Δ strains growing on plates (Supplemental Figure S5, A and B), it did not restore the timing of DNA replication following release from a G1 arrest to wild type (Supplemental Figure S5C). Therefore, other pathways must be altered in ubp10Δ cells that result in delayed DNA replication.

Among the candidate Ubp10 targets identified in our screen, the target most likely to impact DNA replication timing is Dbf4.
Dbf4 is the activating subunit of the kinase Cdc7, which together phosphorylate subunits of the MCM helicase to initiate DNA replication (Fragkos et al., 2015). We found that Dbf4 was less stable and expressed at lower levels in ubp10Δ cells (Figure 5, D–F), which could be the cause of the delay in DNA replication. To test this possibility, Dbf4 was overexpressed from a plasmid in ubp10Δ cells. Notably, ubp10Δ cells showed a greater delay in G1/S entry when growing in synthetic medium compared with rich medium (Figures 5B and 6A), and this delay was partially reversed upon Dbf4 overexpression (Figure 6). This result supports the possibility that stabilization of Dbf4 by Ubp10 is important for timely cell cycle progression. However, because Dbf4 overexpression only partially restored the timing of S-phase entry, the deubiquitination of additional proteins by Ubp10 must also contribute to its cell cycle–regulatory role.

Genetic analysis of the DUB network
The modest phenotypes that have been reported for most DUB deletion strains suggest that there may be redundancies within the DUB network. However, most pairwise DUB deletions that have been examined do not exhibit negative genetic interactions, which would be expected if two DUBs redundantly regulate a critical

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**Figure 2:** DUBs up-regulate specific subsets of cell cycle proteins. (A) Design of overexpression screen to identify DUBs that target specific cell cycle regulators for degradation. (B) Representative data from DUB overexpression screen. Western blots show levels of 10 TAP-tagged target proteins (light and dark exposures are shown) and G6PDH following 4 h of induction of a control (Ubp2) or Ubp7. (C) Summary heat map of DUB overexpression screen. DUBs are in columns, targets in rows. Targets are grouped by their corresponding E3 ubiquitin ligase (left). Yellow indicates the target increased at least twofold in two replicates of the screen, blue indicates the target decreased at least twofold in two replicates. Gray indicates no data. All primary data are reported in Supplemental Data S2. (D) Comparison of the number of targets up-regulated by each DUB. Bars are color-coded to group targets by their regulatory E3.
process (Costanzo et al., 2016). To explore potential higher order redundancies, we focused on the five DUBs that were able to stabilize the greatest number of cell cycle proteins upon overexpression (Ubp5, Ubp6, Ubp7, Ubp10, and Yuh1), and constructed strains carrying all possible combinations of these deletions. All strains were viable, including a strain with all five DUBs deleted, indicating that these DUBs are not redundantly required for any essential process.

To assay for potential combinatorial effects on proliferation, the doubling times of strains with each of the 32 genotypes (all possible combinations of deletions in the five selected DUBs) were measured. Among the single mutant strains, the only strain with a significant proliferation defect was ubp10Δ (Figure 7A). The average doubling time of ubp10Δ strains was 157 min in rich medium, compared with 118 min for wild-type strains. Almost all combinations of deletions that included the remaining four DUBs, including all single deletions, had doubling times that were not significantly different from wild type (Figure 7A). The one exception was ubp6Δ ubp7Δ yuh1Δ, which displayed a relatively small increase in doubling time. These results indicate that there are no strong negative genetic interactions among these five DUBs, indicating a lack of redundancy at least for cell cycle progression. Strikingly, however, we did discover a novel positive genetic interaction: deletion of UBP6 rescued the proliferation defect observed in ubp10Δ strains. All strains that included both ubp10Δ and ubp6Δ had doubling times that are decreased significantly compared with the ubp10Δ single mutant (Figure 7A and Supplemental Table S1).

Because deletion of UBP10 slows proliferation by delaying entry into the cell cycle (Figure 5, B and C), we tested whether deletion of UBP6 reversed the G1/S delay in ubp10Δ cells by comparing the timing of S-phase entry in wild-type, ubp6Δ, ubp10Δ, and ubp6Δ ubp10Δ strains in G1 arrest-release experiments. Although deletion of UBP6 had no effect on the cell cycle on its own, ubp6Δ in combination with ubp10Δ restored the timing of DNA replication to wild type (Figure 7, B and C). This result suggests that Ubp6 activity may counteract Ubp10 function in cells and that the balance of their activities is important for cell cycle timing.

Ubp6 is one of two DUBs that associate with the proteasome, where it removes ubiquitin from proteasomal substrates and protects ubiquitin from degradation (Leggett et al., 2002; Hanna et al., 2003). Several studies have shown that Ubp6 inhibits the proteasome and that cells lacking Ubp6 display increased proteasomal activity (Hanna et al., 2006; Lee et al., 2010). In contrast, another report showed that loss of Ubp6 impairs proteasomal function by preventing maximal opening of the gate to the substrate translocation channel (Peth et al., 2009). Although the precise effect of Ubp6 on the proteasome remains unclear, these studies raise the possibility that altered proteasomal activity upon UBP6 deletion might reverse the accelerated degradation of Ubp10 substrates in ubp10Δ cells. To test this possibility, we assayed the half-life of Dbf4 and Rpa190 in ubp6Δ ubp10Δ strains. As previously observed, Dbf4 was degraded more quickly in ubp10Δ cells compared with wild type (Figures 5, E and F, and 7, D and E). In addition, Rpa190 was stable in wild-type cells, but was destabilized in the absence of Ubp10 (Figure 7, D and E), consistent with a previous report (Richardson et al., 2012). Interestingly, the levels and stabilities of both proteins were restored to wild type in ubp6Δ ubp10Δ cells compared with the ubp10Δ single mutant. The effect of UBP6 deletion on Dbf4 that was overexpressed from a plasmid was greater than its effect on endogenous Dbf4, and overexpressed Dbf4 was significantly stabilized in both ubp6Δ and ubp6Δ ubp10Δ cells compared with wild type (Figure 7, D and E). These data demonstrate that deletion of UBP6 impairs degradation of these substrates by the UPS and reverses the accelerated degradation that occurs in ubp10Δ cells.

Loss of UBP6 results in accelerated degradation of ubiquitin itself, and as a result, decreases the pool of free ubiquitin in the cell that is available to be conjugated to substrates (Amerik et al., 2000; Leggett et al., 2002; Chernova et al., 2003; Hanna et al., 2003). We therefore considered the possibility that Ubp10 substrates may be stabilized in ubp6Δ cells because ubiquitin levels are reduced. To explore this possibility, we quantified free ubiquitin levels in DUB deletion strains. Consistent with previous studies, there was less free ubiquitin in ubp6Δ cells. However, free ubiquitin levels were also reduced in ubp10Δ and ubp6Δ ubp10Δ strains, and there was no correlation between increased levels of Dbf4 and decreased levels of ubiquitin (Figure 8, A and B). This result suggests that ubiquitin levels are not limiting for Dbf4 degradation, and that Ubp6 does not affect the stability of Ubp10 substrates by reducing the amount of available ubiquitin in the cell.

Previous studies have also found that UBP6 deletion alters the stability of proteasome substrates. However, not all substrates...
appear to be affected by UBP6 deletion in the same way. N-end rule reporter substrates and the transcription factor Gcn4 are destabilized in ubp6Δ strains (Hanna et al., 2006), whereas the model proteasomal substrates Ub-Pro-β-gal and Ub-Leu-β-gal are more stable upon UBP6 deletion (Leggett et al., 2002). Together with our findings on Dbf4 and Rpa190, these results suggest that not all proteasomal substrates are affected by UBP6 deletion in the same way.

To determine whether Ubp6 regulates the stability of additional endogenous UPS substrates, we examined another four UPS targets to determine whether their stabilities were altered in ubp6Δ cells. Notably, none of these proteins was destabilized upon UBP6 deletion (Figure 8, C and D). These results are consistent with the model that Ubp6 is required for the efficient degradation of many proteasomal substrates in vivo and that UBP6 deletion rescues cell cycle defects in ubp10Δ cells by slowing the degradation of Ubp10 substrates.

If deletion of Ubp6 has a positive effect on ubp10Δ strains by impairing proteasomal function, then other mutants that have compromised proteasomal function should also rescue the defects in ubp10Δ strains. To test this possibility, we combined deletion of an alternate DUB, UBP14, with UBP10 deletion. Ubp14 degrades free ubiquitin chains in the cell (Amerik et al., 1997). Because free ubiquitin chains inhibit the proteasome, UBP14 deletion causes an accumulation of free ubiquitin chains and impairs degradation of proteasomal substrates. As predicted by our model, deletion of UBP14 reduced the doubling time of ubp10Δ mutants to a near wild-type level (Figure 8E). This result suggests that Ubp14 and Ubp6 act similarly to promote degradation of UPS substrates in vivo. Moreover, it highlights the importance of maintaining the
FIGURE 5: Ubp10 regulates entry into the cell cycle. (A) FACS profiles showing DNA content of asynchronous wild-type (WT) and ubp10Δ cells growing in rich medium. 1C:2C ratio is shown to highlight increased 1C population upon deletion of UBP10. (B) S phase is delayed in ubp10Δ cells. Wild-type (WT) and ubp10Δ cells growing in rich medium were arrested in G1 with alpha factor and released into the cell cycle. Additional alpha factor was added back after 30 min to arrest cells in the subsequent G1 phase. Representative FACS plots are shown; S-phase time points are highlighted in purple. (C) Progression through S phase was calculated as described in Materials and Methods. An average of n = 8 experiments is shown. Error bars represent standard deviations. The eight replicates include two experiments each performed in WT and ubp10Δ strains with the four different TAP-tagged candidates shown in D. (D) Expression of Ubp10 candidate targets are reduced and/or delayed in ubp10Δ cells. Strains expressing TAP-tagged candidate targets were followed over the cell cycle, as in B. TAP and G6PDH Western blots are shown. (E) Cycloheximide-chase assays showing the half-life of candidate targets in WT and ubp10Δ cells. Western blots for TAP-tagged targets and G6PDH are shown. (F) Quantitation of cycloheximide-chase assays from E. Shown are the average of n = 8 (Dbf4), n = 5 (Mps1), or n = 3 (Spo12, Hst3) experiments; errors bars represent the SEM.
show that more than half (12 of 21) of yeast DUBs are capable of up-regulating the same model substrates in vitro (Schaefer and Morgan, 2011). These findings all suggest that there is redundancy among the DUBs, a recent study identified two additional yeast proteins belonging to a newly discovered DUB family that exhibits specificity for K48-linked ubiquitin chains (Abdul Rehman et al., 2016). We tested one of these proteins, Miy1, in our screen and found that it did not alter expression of any of the UPS targets tested. The homologous enzyme Miy2 was not screened because it does not display any activity toward ubiquitin chains in vitro (Abdul Rehman et al., 2016).

Our data show that many DUBs can recognize specific substrates in vivo; however, we cannot rule out the possibility that some of the DUB-target interactions we identified do not normally occur when the DUB is expressed at endogenous levels. The best way to confirm that a candidate target is an endogenous substrate of a particular DUB is to show that the target is also less stable when that particular DUB is deleted. However, in some instances we did not observe target destabilization in DUB delete cells (Figure 5, E and F). There could be several reasons for this result. One possibility is that there is redundancy and that more than one DUB may need to be deleted for a target to be destabilized. Alternatively, the DUB in question may not be active during the context of an unperturbed cell cycle. Out of 21 DUBs, 18 have been found to be phosphorylated in proteome-wide screens (Ficarro et al., 2002; Albuquerque et al., 2009; Wu et al., 2011), which suggests that their activities are subject to regulation. If future studies identify particular environmental conditions or states when specific DUBs are active, it will be interesting to reexamine many of these substrates to determine whether they are regulated by those DUBs in those contexts. Although we cannot rule out that overexpression may drive interactions with some substrates in our screen, we have shown that one candidate target of Ubp10, Dbf4, is less stable in ubp10Δ cells (Figure 5, E and F) and that the established protein interaction domain of Ubp10 and its catalytic activity contribute to Dbf4 stabilization by Ubp10 (Figure 4, B and C). Therefore, many candidate targets identified here are likely to be endogenous substrates of the identified DUBs in some context.

Our data set also identified DUBs with potential cell cycle–regulatory roles. Among the DUBs that regulated several targets in our screen, Yuh1 is a good candidate to regulate the cell cycle because it is required for Rub1 modification of the SCF subunit Cdc53.
FIGURE 7: Deletion of UBP6 restores cell cycle timing in ubp10Δ strains. (A) Doubling times of strains with deletions of the indicated DUBs. Colors represent the number of deletions: single mutants, purple; double mutants, blue; triple mutants, green; quadruple mutants, orange; quintuple mutant, red. Shown are average doubling times for \( n = 2-6 \) independently derived strains of each genotype. Error bars represent SD. Asterisks indicate strains that are significantly different from wild type (WT) as measured by one-way ANOVA (*, \( p < 0.05 \); **, \( p < 0.005 \); ***, \( p < 0.0005 \); ****, \( p < 0.0001 \)). All data are included in Supplemental Table S1. (B) G1 arrest-release of strains with the indicated genotypes.
FIGURE 8: Deletion of DUBs that promote proteasome function rescue ubp10Δ phenotypes. (A) Representative Western blot of Dbf4-TAP and ubiquitin in asynchronous cells. G6PDH is shown as a loading control. (B) Quantitation of Western blots in part A. Dbf4-TAP and free ubiquitin levels were normalized to G6PDH. Shown is an average of $n = 3$ experiments; error bars represent SD. (C) Cycloheximide-chase assay of TAP-tagged UPS targets in wild-type (WT) and ubp10Δ cells as indicated. TAP and G6PDH blots are shown. (D) Quantitation of cycloheximide-chase assays from C. Shown is an average of $n = 3$ experiments; error bars represent the SEM. (E) Doubling time of strains with deletions in the indicated DUBs. Shown is the average of $n = 4$ replicates; error bars represent SD. Asterisks indicate strains that are significantly different from wild type (WT) as measured by one-way ANOVA (*, $p < 0.05$; **, $p < 0.005$).

Growing in rich medium. Cells were arrested in G1 with alpha factor and released into medium without alpha factor. Nocodazole was added to the medium 30 min after release to arrest cells in the following mitosis. Representative FACS plots are shown; time points that exhibit the greatest differences between strains are highlighted in purple. (C) Progression through S phase was calculated as described in Materials and Methods. Averages of $n = 3$ biological replicates are shown. Error bars represent SD. (D) Cycloheximide-chase assays of Dbf4-TAP and Rpa190-TAP in the indicated strains. In the top panels Dbf4 was overexpressed from a 2μ plasmid (as in Figure 6); the middle and lower panels represent Dbf4 and Rpa190 expressed from their genomic loci. TAP and G6PDH (loading control) Western blots are shown. (E) Quantitation of cycloheximide-chase assays from A. Shown are average of $n = 3$ (Dbf4 OE), $n = 8$ (endogenous Dbf4 in WT and ubp10Δ), or $n = 4$ (endogenous Dbf4 in ubp6Δ and ubp6Δ ubp10Δ, Rpa190) experiments; error bars represent the SEM.
(Linghu et al., 2002). However, we and others have found that yuh1Δ cells proliferate as well as wild-type cells (Figure 7A; Amerik et al., 2000). This suggests that another enzyme can compensate for Yuh1 loss, or that blocking Rub1 modification of Cdc53 does not impair SCF activity enough to impact the cell cycle.

The DUB that had the greatest effect on the cell cycle was Ubp10. Notably, both overexpression and deletion of Ubp10 resulted in delays in different phases of the cell cycle. Previous studies have linked Ubp10 to regulation of diverse cellular processes through removal of both monoubiquitin and polyubiquitin chains. Ubp10 regulates nonproteasomal roles of ubiquitin by removing monoubiquitin from both PCNA and histone H2B (Gardner et al., 2005; Gallego-Sánchez et al., 2012). This role in H2B deubiquitination is partially redundant with Ubp8 and regulates telomeric silencing (Singer et al., 1998; Kahana and Gottschling, 1999). Ubp10 can also impact protein stability by removing polyubiquitin chains from proteasomal substrates. For example, Ubp10 deubiquitinates and stabilizes the largest subunit of RNA polymerase I, Rpa190 (Richardson et al., 2012). Stabilization of Rpa190 by Ubp10 is critical for maximal rRNA synthesis and ribosome biogenesis.

Our data suggest that Ubp10 also controls the stabilities of many cell cycle proteins and regulates the timing of the G1/S transition (Figures 4 and 5). Although our results suggest that stabilization of Dbf4 by Ubp10 contributes to proper S-phase timing (Figure 6), other functions of Ubp10 must also be involved. This delay does not appear to be the result of reduced Rpa190 expression (Supplemental Figure 5), and it is also unlikely that other established Ubp10 targets are involved in this delay. The silencing defect resulting from increased H2B ubiquitination is not expected to affect the cell cycle, because other mutations that cause silencing defects do not alter cell cycle distribution (Richardson et al., 2012). Regulation of PCNA by Ubp10 is also unlikely to impact the cell cycle, because PCNA is not ubiquitinated during an unperturbed S phase in budding yeast, and it is only regulated by Ubp10 following exposure to DNA damage (Gallego-Sánchez et al., 2012). Additional, unidentified Ubp10 substrates may contribute to the delay in S-phase entry in ubp10Δ cells. However, a likely possibility is that the coordinate misregulation of several proteins in ubp10Δ cells results in the G1/S delay.

With the exception of Ubp10, no cell cycle changes were observed upon overexpression of any additional DUBs, and previous studies suggest DUB deletion strains proliferate at rates similar to wild type (Amerik and Hochstrasser, 2004; Poulsen et al., 2012). For this reason, we attempted to uncover additional redundancies in the network by carrying out a comprehensive genetic analysis of the five DUBs that our screen implicated in cell cycle regulation. The expectation was that if more than one of these DUBs redundantly regulates a critical cell cycle protein, combinations of those deletions might be lethal or slow proliferation. Remarkably, no strong negative genetic interactions were identified among these five DUBs, and even the ubp5Δ ubp6Δ ubp7Δ ubp10Δ yuh1Δ strain proliferated nearly as well as a wild-type strain. It is possible that strains with combinations of DUB deletions have fitness defects in alternate environmental states. Future studies may uncover context-dependent redundancies between DUBs.

The surprising finding that our genetic analysis uncovered was that deletion of UBP6 rescued the proliferation defect and cell cycle delay in ubp10Δ cells (Figure 7, A–C). UBP6 deletion also restored the stabilities of Ubp10 targets Dbf4 and Rpa190 in the absence of Ubp10 (Figure 7, D and E). This rescue is most likely an indirect effect that is a result of the central role of Ubp6 in regulating proteasomal function. Ubp6 associates with the proteasome base and removes ubiquitin chains from substrates before they are translocated into the proteasomal channel (Leggett et al., 2002; Lee et al., 2016; Shi et al., 2016). Our results suggest that this deubiquitination by Ubp6 is important for efficient degradation of Ubp10 substrates, because they are stabilized in its absence. This is a surprising result because previous studies suggest that cells lacking Ubp6 have increased proteasomal activity (Hanna et al., 2006; Lee et al., 2010). However, not all studies agree (Peth et al., 2009) and we find that several endogenous UPS substrates are stabilized in the absence of Ubp6 (Figure 8, C and D). Moreover, deletion of a second DUB that promotes proteasome function (Ubp14) also reversed the proliferation defect in ubp10Δ cells (Figure 8E). Together these findings suggest Ubp6 is normally required for efficient degradation of many UPS substrates.

The mechanism by which Ubp6 promotes degradation of proteasomal substrates is unclear. One possibility is that deletion of UBP6 reduces the amount of free ubiquitin in the cell, limiting the amount of ubiquitin available for substrate degradation. However, ubp10Δ cells also have reduced free ubiquitin (Figure 8A), and Dbf4 and Rpa190 degradation is accelerated in these cells (Figure 7, D and E), which suggests that ubiquitin levels are not limiting for their degradation. Moreover, not all proteasomal substrates are stabilized in ubp6Δ cells (Figure 8, C and D). Therefore, Ubp6 does not affect all proteasomal substrates equally. A recent report found that Ubp6 (and its human homologue Usp14) deubiquitinates only substrates that have more than one ubiquitin chain (Lee et al., 2016), raising the possibility that the Ubp6 deubiquitinates only substrates with particular ubiquitin chain configurations. In the future, it will be of interest to elucidate how Ubp6 affects degradation of some substrates and not others. These experiments will also shed light on the cellular consequences of disrupting the balance of DUB activities.

**MATERIALS AND METHODS**

**Yeast strains and plasmids**

All yeast strains are in the S288c background and were grown in rich (YM-1) or synthetic (C) medium at 30°C (Benanti et al., 2007). Strains from the TAP-tag collection were used for the DUB overexpression screen (Ghaemmaghami et al., 2003). To generate the panel of DUB deletion strains used in Figure 6, DUB genes were deleted using standard methods (Rothstein, 1991). Subsequently, a ubp5Δ ubp6Δ ubp7Δ yuh1Δ strain (YJB673) was crossed to a ubp10Δ strain (YCM313) and all possible combinations of genotypes were recovered. A complete list of strains is in Supplemental Table S2.

DUB overexpression plasmids were obtained from the GST-tagged collection (Sopko et al., 2006) and sequenced to verify the correct insert. Any GST-DUB plasmids that contained an incorrect sequence were reconstructed by cloning the gene from genomic DNA into the similar DBF4-TAP sequence, along with their respective promoters, were amplified from genomic DNA from GFP (Huh et al., 2012) were also cloned into pYES2-GST. GST-DUB overexpression plasmids were transformed into 37 TAP-tag strains, respectively, and cloned into pRS426. A complete list of plasmids is in Supplemental Table S3.

**DUB overexpression screen**

GST-DUB overexpression plasmids were transformed into 37 TAP-tag strains expressing the tagged target proteins of interest. For the screen, strains were grown to mid-log phase in C medium lacking uracil (C-Ura) containing 2% raffinose in deep-well 96-well plates. To induce overexpression of DUBs, galactose was added to the
medium at a final concentration of 2% and cells were incubated for 4 h at 30°C. Cells were pelleted in 96-well plates, lysed using a trichloroacetic acid (TCA) lysis protocol, and Western blots performed as described below. For each test DUB, the control (Ubp2) was induced in a matched set of strains and lysed side by side. All DUBs were screened twice.

Multiple exposures of all Western blots were collected. Quantification was performed using ImageStudioLite software (Li-Cor Biosciences) on the lightest exposure in which a given protein was detected in both control and DUB samples. All TAP-signals were normalized to the loading control G6PDH and log2 fold change values were calculated. Any protein that could not be detected in one or both samples in a given experiment was not included in the data set, because it was not possible to calculate an accurate fold change value. Proteins were considered up- or down-regulated if levels changed at least twofold in both replicates of the screen. All fold change values are reported in Supplemental Data S2.

**Western blotting**
For the DUB screen and all experiments except for those in Figure 4, equivalent optical densities of cells were collected and lysed using a previously described TCA lysis protocol (Landry et al., 2014). For experiments in Figure 4, cells were lysed by bead beating in sample buffer (Landry et al., 2012). Western blotting was performed with antibodies against TAP (CAB1001; ThermoFisher), GST (clone 4C10; BioLegend), ubiquitin (clone P4D1), GFP (clone JL8; Clontech), Cdc28 (sc-6709; Santa Cruz Biotechnology), and G6PDH (A9521; Sigma). Where indicated, Western blots were normalized using ImageStudioLite software, as described above.

**Flow cytometry**
Cells were fixed in 70% ethanol and stained with Sytox green (Invitrogen) as previously described (Landry et al., 2012). Data were collected on a Becton Dickinson FACSCalibur or a Millipore Guava easyCyte HT. Data were analyzed using FlowJo software (FlowJo, LLC). In arrest-release experiments, S-phase progression was calculated from the mean DNA content of the population, as previously described (Willis and Rhind, 2009).

**Cycloheximide-chase assays**
Cells were grown to mid-log phase, and 50 μg/ml cycloheximide was added to inhibit protein synthesis. Samples were collected at the indicated time points and analyzed by Western blotting.

**Doubling time assays**
To calculate doubling times of DUB deletion strains, saturated overnight cultures were diluted to an optical density of 0.2 in rich medium containing 2% dextrose in 96-well plates. Population growth was monitored using a Tecan Infinite PRO microplate reader at 30°C with continuous shaking, measuring the optical densities every 20 min. Doubling times were calculated using GraphPad Prism software. For each genotype the doubling times of two to six independent derived strains were measured and statistical significance calculated using one-way analysis of variance (ANOVA). All data are included in Supplemental Table S1.

**Serial dilution assays**
Fivefold dilutions of strains with the indicated genotypes were plated on C-Ura plates containing 2% dextrose. Plates were imaged after 24–72 h of incubation.

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