A balance of deubiquitinating enzymes controls cell cycle entry

Claudine E. Mapa, Heather E. Arsenault, Michelle M. Conti, Kristin E. Poti, and Jennifer A. Benanti*
Department of Molecular, Cell and Cancer Biology, University of Massachusetts Medical School, Worcester, MA 01605

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 *ubp10*Δ cells. Similarly, deletion of *UBP14*, another DUB that promotes proteasomal activity, rescued the proliferation defect in *ubp10*Δ cells. Our results suggest that DUBs function through a complex genetic network in which their activities are coordinated to facilitate accurate cell cycle progression.**

Monitoring Editor
Mark J. Solomon
Yale University

Received: Jul 10, 2018
Revised: Sep 6, 2018
Accepted: Sep 7, 2018

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

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E18-07-0425) on September 12, 2018.

*Address correspondence to: Jennifer A. Benanti (Jennifer.Benanti@umassmed.edu).

Abbreviations used: DUB, deubiquitinating enzyme; UPS, ubiquitin-proteasome system.

interacting proteins that regulate their functions in vivo (Leggett *et al.*, 2002; Krogan *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 *ubp10*Δ cells and restored the stability of Ubp10 targets. Deletion of an alternate proteasome-regulatory DUB, *UBP14*, also rescued the proliferation defect in *ubp10*Δ 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 cell cycle defect, or broadly and nonspecifically affected ubiquitinated proteins. We overexpressed each DUB from a plasmid under the control of the galactose-inducible *GAL1* promoter. In agreement with previous reports, constitutive overexpression of no individual DUB 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 (CYCLoPs) 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.

DUB	Systematic name	Domains[a]	Localization[b]	Regulatory partners	References
Ubp1	YDL122W	USP	C, ER, CoP, CP, B		Chong *et al.*, 2015
Ubp2	YOR124C	USP	C	Rsp5	Chong *et al.*, 2015; Kee *et al.*, 2005
Ubp3	YER151C	USP	C	Bre5	Cohen *et al.*, 2003; Chong *et al.*, 2015
Ubp4, Doa4	YDR069C	USP, Rhod	C, E, M, SP	Bro1	Amerik *et al.*, 2006; Luhtala and Odorizzi, 2004; Chong *et al.*, 2015
Ubp5	YER144C	USP, Rhod	C, BN, N		Amerik *et al.*, 2006
Ubp6	YFR010W	USP, UBL	C, N, M, V	Proteasome	Chong *et al.*, 2015; Verma *et al.*, 2000
Ubp7	YIL156W	USP, Rhod	C, M		Chong *et al.*, 2015
Ubp8	YMR223W	USP, ZnF	N, M	SAGA	Chong *et al.*, 2015; Henry *et al.*, 2003
Ubp9	YER098W	USP	C		Chong *et al.*, 2015
Ubp10, Dot4	YNL186W	USP, IDR	N, No, M	Sir4, Dhr2, Utp22	Chong *et al.*, 2015; Kahana and Gottschling, 1999; Richardson *et al.*, 2012
Ubp11	YKR098C	USP			
Ubp12	YJL197W	USP, DUSP	C, M, V	Rad23, Cdc48	Chong *et al.*, 2015; Gödderz *et al.*, 2017
Ubp13	YBL067C	USP	C		Chong *et al.*, 2015
Ubp14	YBR058C	USP, ZnF	C, N, V, M		Chong *et al.*, 2015
Ubp15	YMR304W	USP, MATH	C, E, ER	Pex1/Pex6, Cdh1	Chong *et al.*, 2015; Debelyy *et al.*, 2011; Ostapenko *et al.*, 2015
Ubp16	YPL072W	USP	M, C, E		Chong *et al.*, 2015; Kinner and Kölling, 2003
Otu1	YFL044C	OTU, ZnF	C, N, V, M	Cdc48	Chong *et al.*, 2015; Rumpf and Jentsch, 2006
Otu2	YHL013C	OTU	C		Chong *et al.*, 2015
Rpn11	YFR004W	JAMM	N, V	Proteasome	Chong *et al.*, 2015; Verma *et al.*, 2000
Yuh1	YJR099W	UCH			
Miy1	YPL191C	MINDY	C		Chong *et al.*, 2015

[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.

TABLE 1: Summary of *Saccharomyces cerevisiae* DUBs.

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 SCF^{Cdc4} and SCF^{Grr1} 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

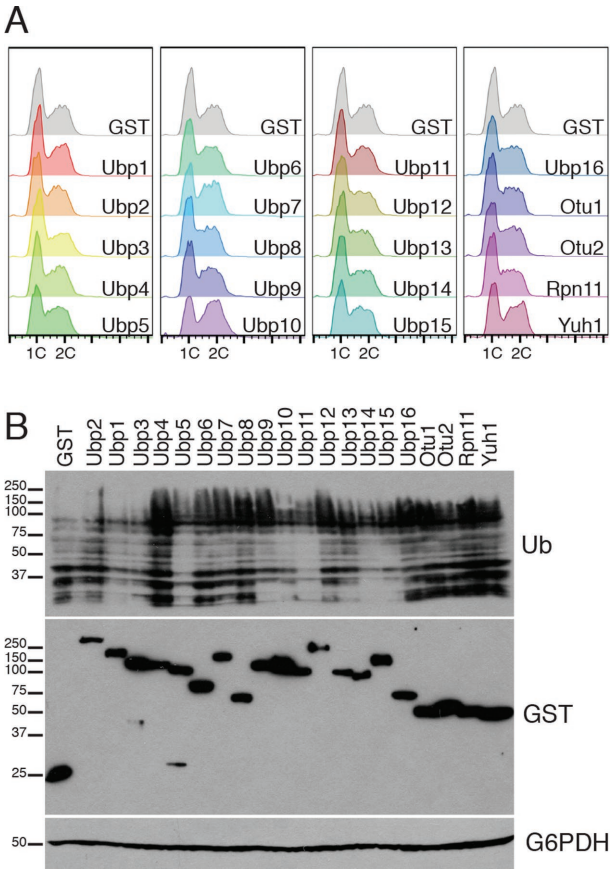

FIGURE 1: Acute overexpression of DUBs does not arrest the cell cycle. (A) Cell cycle analysis following DUB overexpression. Expression of GST-tagged DUBs was induced from the *GAL1* promoter for 4 h and DNA content quantified by flow cytometry. (B) Western blots for ubiquitin chains (Ub) and GST-DUB proteins following a 4-h induction. G6PDH is shown as a loading control.

assays were performed to examine the stability of these targets following overexpression of each of the five DUBs that regulated the most targets in our screen (Ubp5, Ubp6, Ubp7, Ubp10, and Yuh1), and GST as a control. Stabilization of substrates correlated well with up-regulation of target proteins by specific DUBs (Figure 3 and Supplemental Figure S3). Moreover, DUBs differentially stabilized target proteins. For example, the G1 cyclins Cln1 and Cln2 were stabilized most strongly by Ubp7. In contrast, the stabilities of Hst3 and Spo12 were not significantly affected by Ubp7 but were strongly stabilized by Ubp10. Thus, DUBs differentially stabilize proteasomal targets, which supports the conclusion that DUBs exhibit substrate specificity in vivo.

Ubp10 regulates the cell cycle

Ubp10 is a USP family DUB (Table 1 and Figure 4A) that has established roles in gene silencing, ribosome biogenesis, and recovery from DNA damage (Singer *et al.*, 1998; Kahana and Gottschling, 1999; Gallego-Sánchez *et al.*, 2012; Richardson *et al.*, 2012). Interestingly, Ubp10 up-regulated the greatest number of cell cycle proteins in our screen and we confirmed that 15 of 16 candidate targets were up-regulated by Ubp10 compared with GST (Figure 4B). Mutation of the catalytic cysteine residue of Ubp10 to serine was previously shown to eliminate its deubiquitinase activity (Kahana and Gottschling, 1999). We tested whether the deubiquitinase activity of Ubp10 is 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, *ubp10*Δ cells exhibited an ~15-min delay in initiating DNA replication when grown in rich medium (Figure 5, B 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 *ubp10*Δ cells. The expression of two proteins expressed in G2/M-phase, Hst3 and Spo12, was delayed ~15 min in *ubp10*Δ cells, in accordance with the requirement for Ubp10 to enter S phase on time. Although the expression of these proteins was delayed in *ubp10*Δ 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 *ubp10*Δ cells; however, peak expression levels of both proteins were decreased in the absence of Ubp10. In addition, Dbf4 was less stable in *ubp10*Δ 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 *ubp10*Δ 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 *ubp10*Δ cells contributes to the G1/S delay that we observed, arrest-release experiments were performed in wild-type and *ubp10*Δ cells that overexpressed Rpa190. Surprisingly, although overexpression of Rpa190 did increase the colony size of *ubp10*Δ 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 *ubp10*Δ 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.

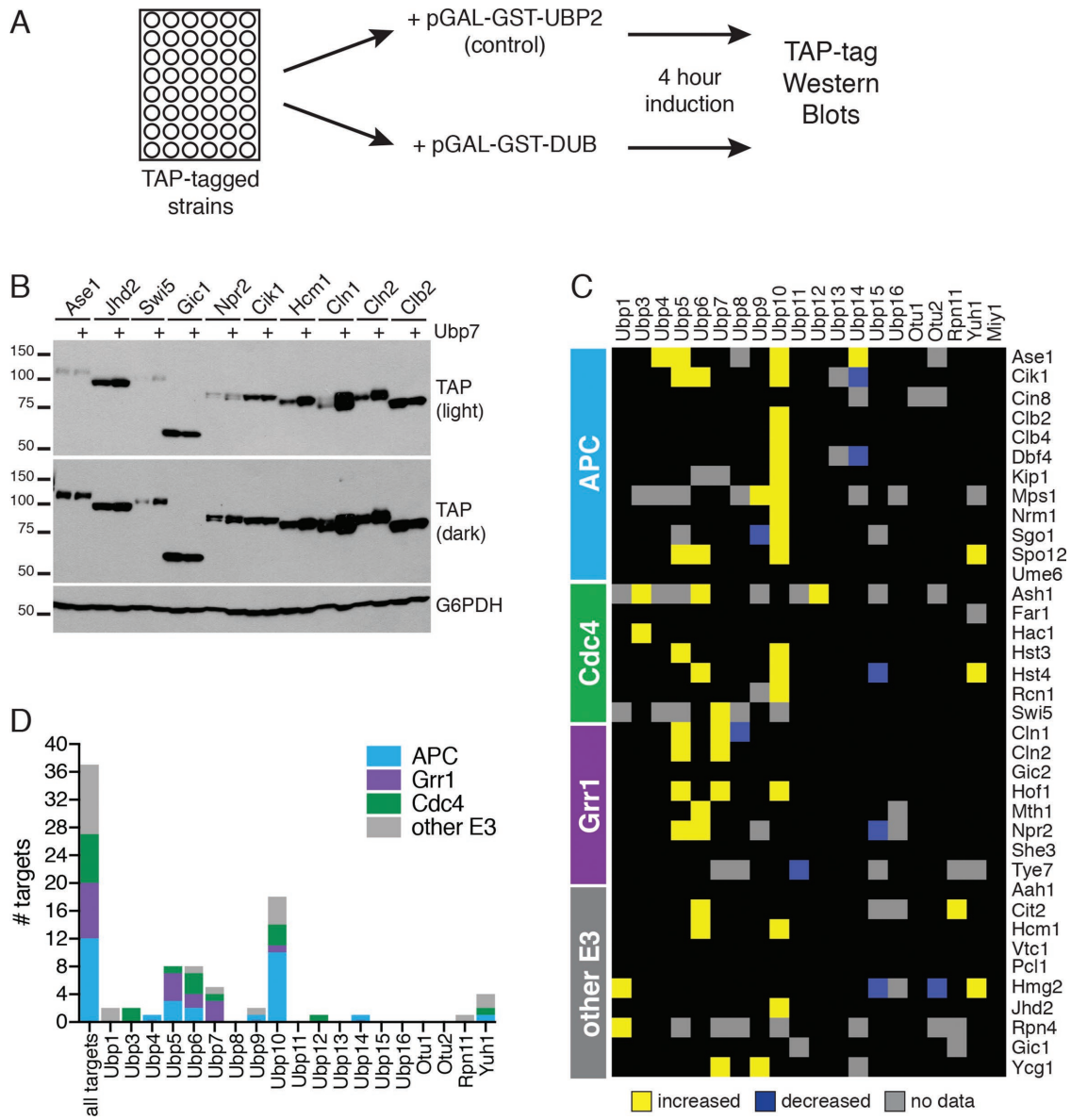

FIGURE 2: DUBs up-regulate specific subsets of cell cycle proteins. (A) Design of overexpression screen to identify DUBs that target specific cell cycle regulators for degradation. (B) Representative data from DUB overexpression screen. Western blots show levels of 10 TAP-tagged target proteins (light 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.

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 *ubp10*Δ 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 *ubp10*Δ cells. Notably, *ubp10*Δ 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

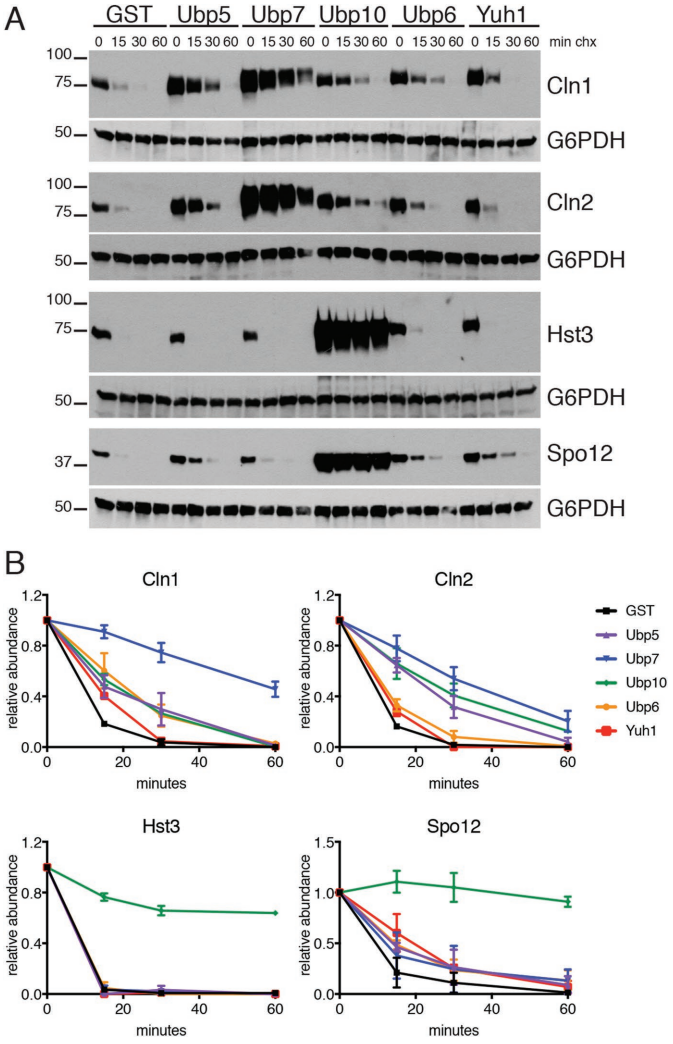

FIGURE 3: DUBs differentially stabilize substrates. (A) Cycloheximide-chase assays of the indicated targets following 4 h of overexpression of GST, Ubp5, Ubp7, Ubp10, Ubp6, or Yuh1. Western blots of TAP-tagged targets and G6PDH (loading control) are shown. (B) Quantitation of cycloheximide-chase assays from (A). Shown are the average of $n = 2$ experiments; errors bars represent the SEM.

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 *ubp10*Δ (Figure 7A). The average doubling time of *ubp10*Δ 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 *ubp6*Δ *ubp7*Δ *yuh1*Δ, 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 *ubp10*Δ strains. All strains that included both *ubp10*Δ and *ubp6*Δ had doubling times that are decreased significantly compared with the *ubp10*Δ 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 *ubp10*Δ cells by comparing the timing of S-phase entry in wild-type, *ubp6*Δ, *ubp10*Δ, and *ubp6*Δ *ubp10*Δ strains in G1 arrest-release experiments. Although deletion of *UBP6* had no effect on the cell cycle on its own, *ubp6*Δ in combination with *ubp10*Δ 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 *ubp10*Δ cells. To test this possibility, we assayed the half-life of Dbf4 and Rpa190 in *ubp6*Δ *ubp10*Δ strains. As previously observed, Dbf4 was degraded more quickly in *ubp10*Δ 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 *ubp6*Δ *ubp10*Δ cells compared with the *ubp10*Δ 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 *ubp6*Δ and *ubp6*Δ *ubp10*Δ 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 *ubp10*Δ 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 *ubp6*Δ 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 *ubp6*Δ cells. However, free ubiquitin levels were also reduced in *ubp10*Δ and *ubp6*Δ *ubp10*Δ 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

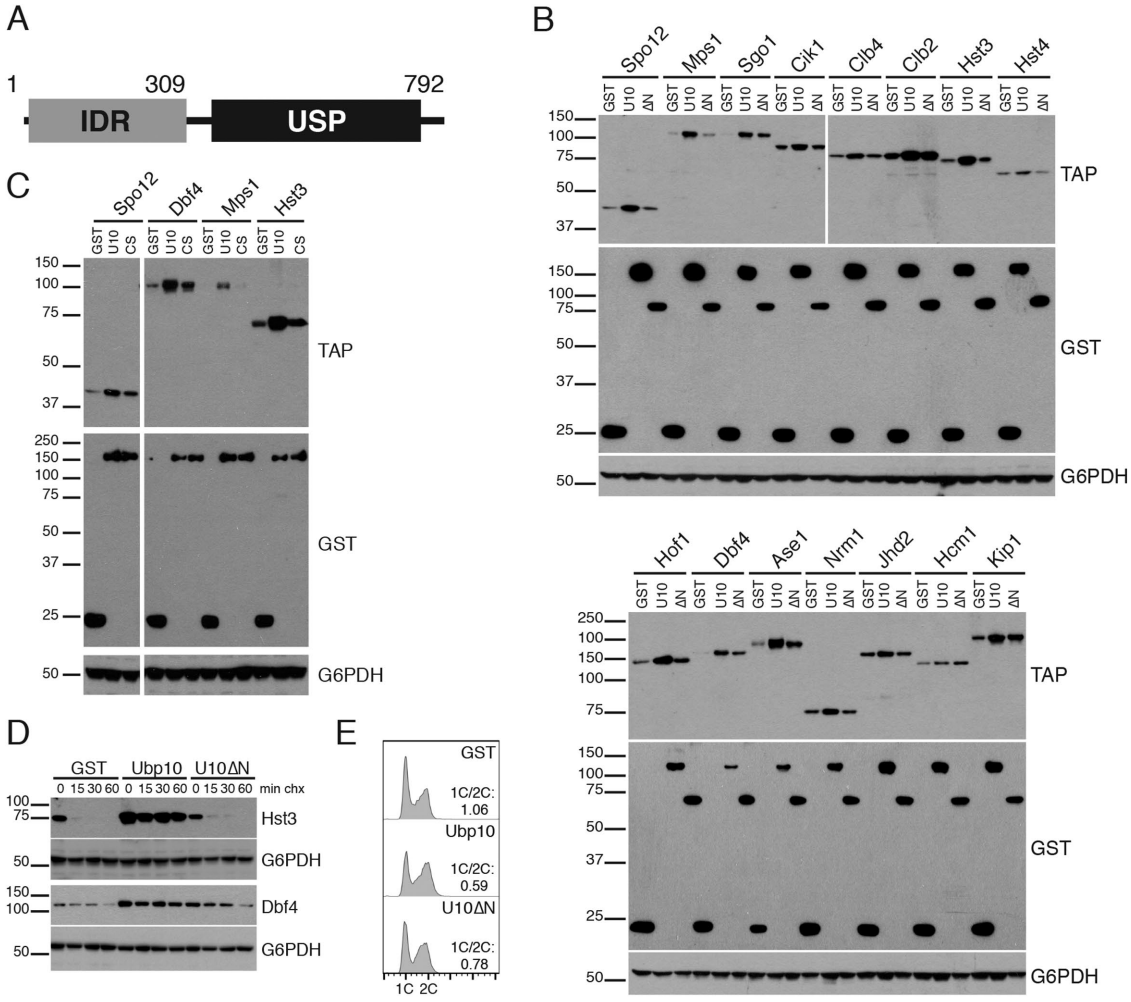

FIGURE 4: The catalytic activity and N-terminal IDR of Ubp10 contribute to target stabilization. (A) Diagram of domains in Ubp10. (B) Validation of Ubp10 candidate targets and analysis of the contribution of the IDR. Western blots showing levels of the indicated TAP-tagged candidate targets following 2-h induction of GST, Ubp10, or Ubp10Δ2-309 (ΔN) proteins with 0.5% galactose. GST blots show similar expression of GST and DUBs; G6PDH blots confirm equal loading. (C) Ubp10 catalytic function is important for stabilization of substrates. Western blot showing levels of the indicated TAP-tagged candidate targets following overexpression of GST, Ubp10, or Ubp10-C371S (CS) as in B. GST and G6PDH blots are shown as controls. (D) Cycloheximide-chase assays of Hst3 and Dbf4 following overexpression of GST, Ubp10, or Ubp10Δ2-309 (ΔN) as in B. Western blots of TAP-tagged Dbf4 and Hst3 are shown. G6PDH blot confirms equal loading. (E) FACS profiles showing DNA content of cells overexpressing GST, Ubp10, or Ubp10Δ2-309 (U10ΔN) as in B. 1C:2C ratio is shown to highlight the increased 2C population upon Ubp10 overexpression. Also see Supplemental Figure S4.

appear to be affected by *UBP6* deletion in the same way. N-end rule reporter substrates and the transcription factor Gcn4 are destabilized in *ubp6*Δ 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 *ubp6*Δ cells. Notably, none of these proteins was destabilized upon *UBP6* deletion. In addition to Dbf4, Spo12, Hst3, and Cik1 were more stable in *ubp6*Δ cells, whereas the stability of Cln2 was not changed (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 *ubp10*Δ cells by slowing the degradation of Ubp10 substrates.

If deletion of Ubp6 has a positive effect on *ubp10*Δ strains by impairing proteasomal function, then other mutants that have compromised proteasomal function should also rescue the defects in *ubp10*Δ 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 *ubp10*Δ 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

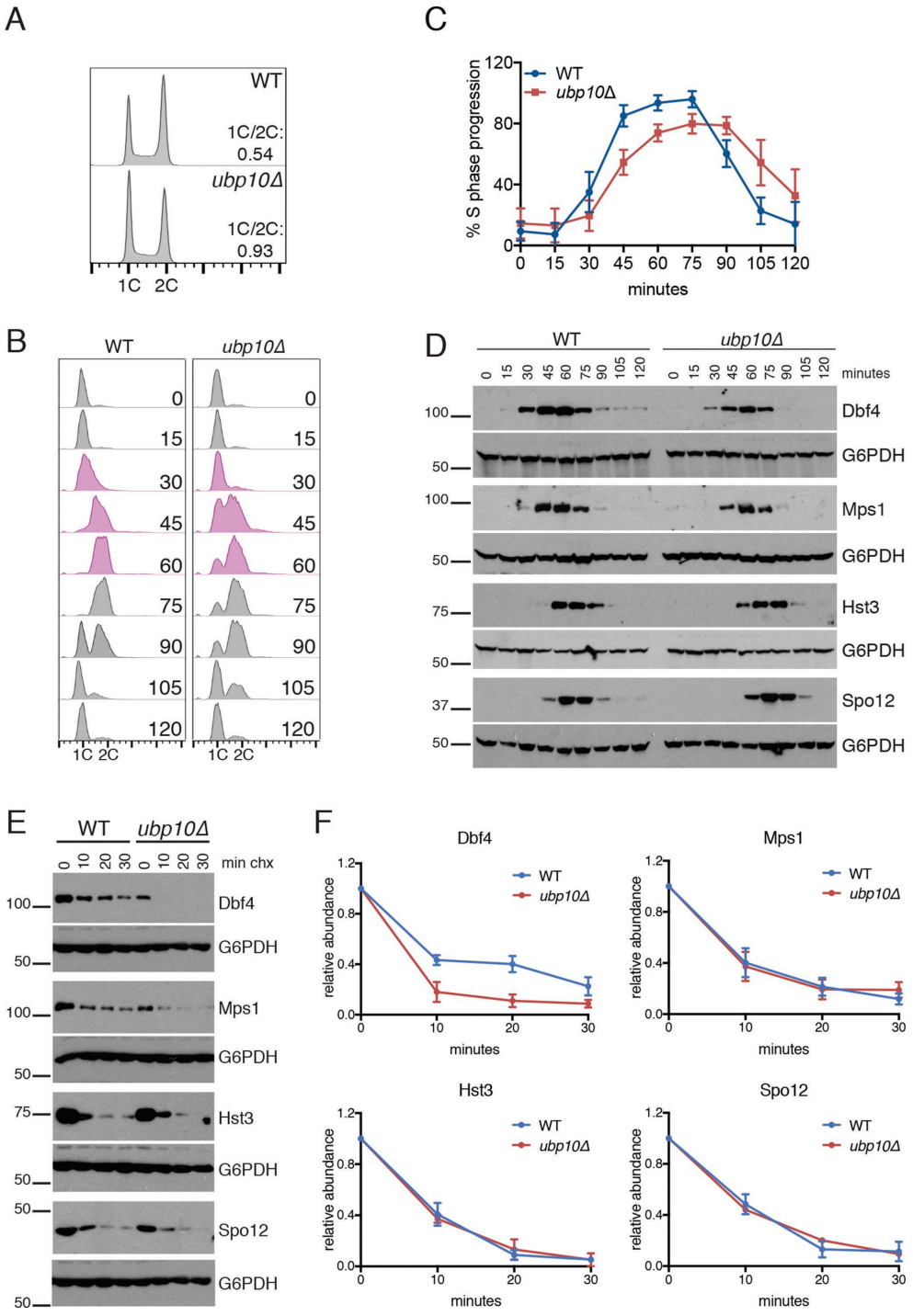

FIGURE 5: Ubp10 regulates entry into the cell cycle. (A) FACS profiles showing DNA content of asynchronous wild-type (WT) and *ubp10*Δ cells growing in rich medium. 1C:2C ratio is shown to highlight increased 1C population upon deletion of *UBP10*. (B) S phase is delayed in *ubp10*Δ cells. Wild-type (WT) and *ubp10*Δ 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 *ubp10*Δ strains with the four different TAP-tagged candidates shown in D. (D) Expression of Ubp10 candidate targets are reduced and/or delayed in *ubp10*Δ 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 *ubp10*Δ 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.

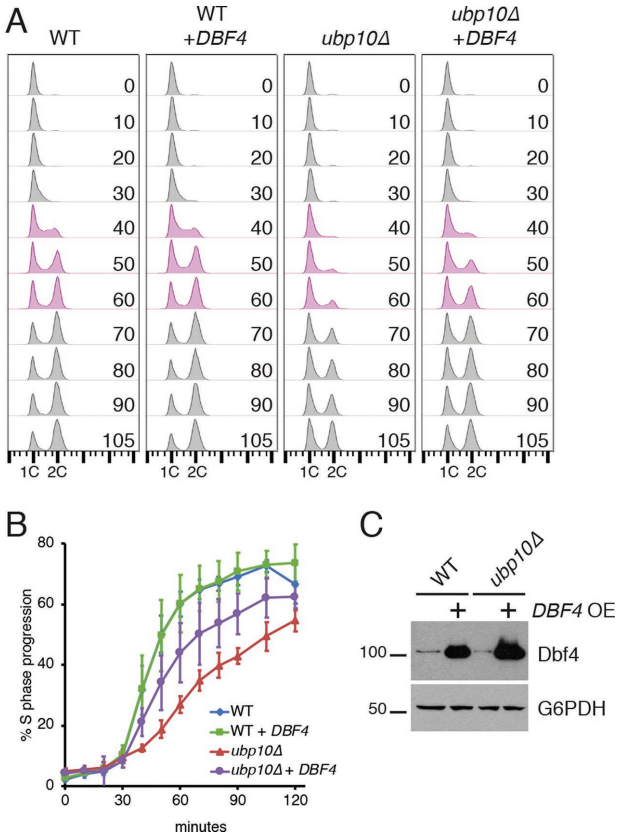

FIGURE 6: Dbf4 overexpression partially restores S-phase timing in *ubp10*Δ cells. (A) Strains expressing *DBF4* from its own promoter in a 2μ plasmid (*DBF4* OE) or an empty vector control were grown in synthetic medium lacking uracil, arrested in G1 with alpha factor for 3 h, and then released into medium without alpha factor. Nocodazole was added to the cultures after 30 min to arrest cells in the following mitosis. Samples were taken for FACS at 10–30-min intervals. A representative experiment is shown; time points that exhibit the greatest differences between strains are highlighted in purple. (B) Progression through S phase was calculated as described in *Materials and Methods*. Shown is an average of $n = 5$ experiments. Error bars represent standard deviations. (C) Western blot of TAP-tagged Dbf4 in asynchronous cells from A. G6PDH is shown as a loading control.

appropriate balance of DUB activities in the cell, since deletion of either *UBP6* or *UBP14* can counteract the negative effects of losing Ubp10.

DISCUSSION

Identifying the functions and substrates of DUBs has been challenging because the mechanisms by which most DUBs select and interact with their targets is not well understood. In yeast, single and double DUB deletion mutants display very minor phenotypes (Amerik and Hochstrasser, 2004; Costanzo *et al.*, 2016) and only modest changes in their proteome in normal growth conditions (Poulsen *et al.*, 2012; Isasa *et al.*, 2015). Moreover, many DUBs are capable of deubiquitinating the same model substrates in vitro (Schaefer and Morgan, 2011). These findings all suggest that there is redundancy among the 21 DUBs in yeast. Here, we explored this possibility using both overexpression and deletion approaches. Our overexpression screen enabled us to identify proteasomal substrates that can be targeted by each DUB, even if redundancies exist. The data from the screen show that more than half (12 of 21) of yeast DUBs are capable of up-regulating expression of one or more UPS targets in the cell. Notably, each of these 12 DUBs up-regulated a different subset of the 37 proteasomal substrates that were screened, demonstrating these DUBs exhibit specificity toward their targets in vivo.

In total, eight of 20 DUBs did not up-regulate of any of the 37 UPS targets in our screen. This result was expected because several DUBs are known to regulate nonproteasomal functions of ubiquitin. For instance, Ubp8 is a component of the SAGA complex and removes monoubiquitin from histone H2B (Henry *et al.*, 2003). In addition, Ubp16 is a membrane protein that associates with mitochondria (Kinner and Kölling, 2003). Another reason a DUB may not have regulated any UPS targets is if it needs to associate with other proteins to function. For example, Rpn11 is active only when it is incorporated into the proteasome (Verma *et al.*, 2002), and therefore proteins regulated by Rpn11 may not have been identified in our screen if the overexpressed protein is not proteasome-bound. Although we cannot draw conclusions about the eight DUBs that did not up-regulate any proteins in the screen, for each of the DUBs that did up-regulate a subset of proteasomal targets we can conclude that they are active upon overexpression. In addition, these DUBs must display substrate specificity, because no two DUBs up-regulated the same set of proteins.

In addition to the 20 yeast DUBs that fall into well-characterized DUB families, 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 same 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.*, 2008; Holt *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 *ubp10*Δ 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

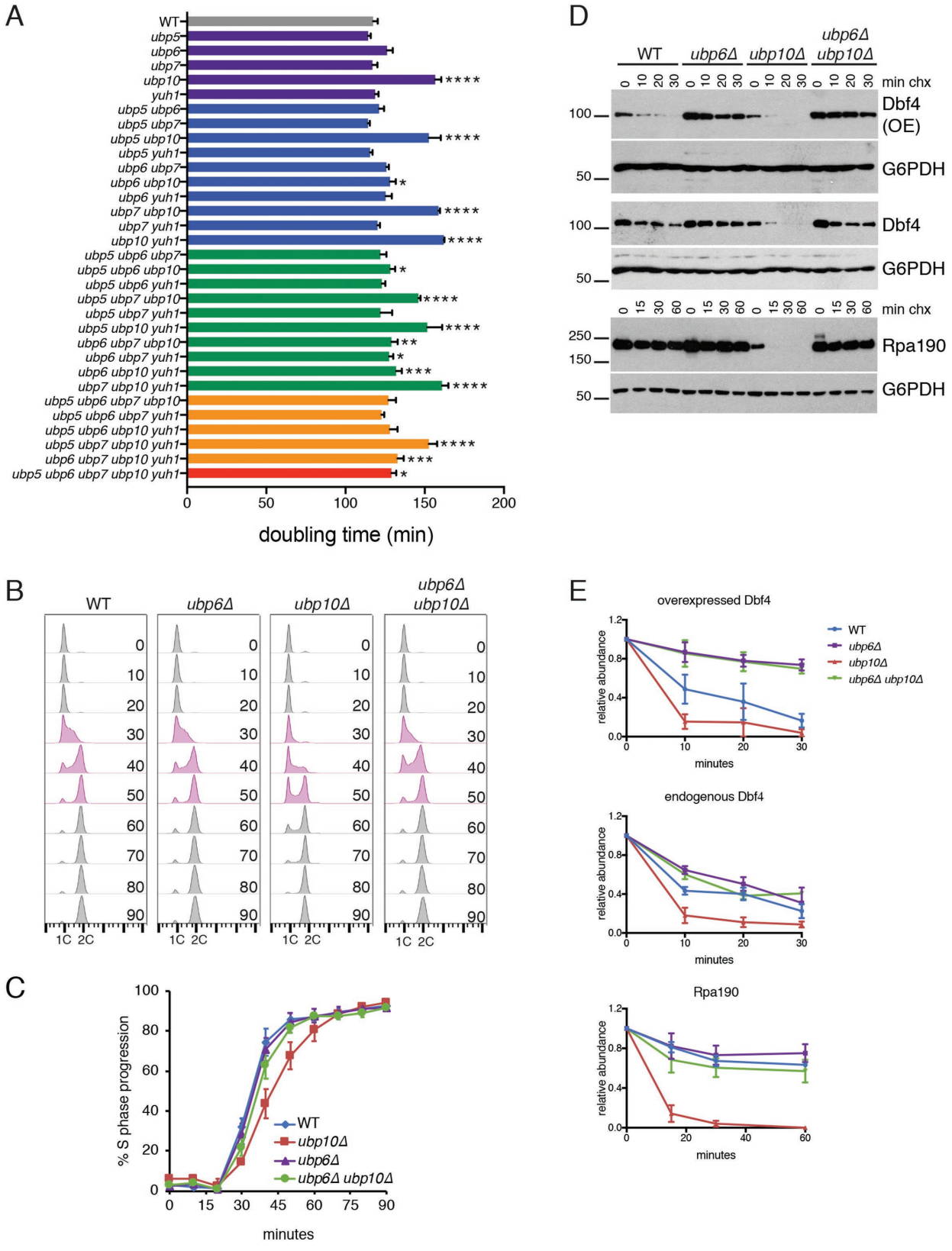

FIGURE 7: Deletion of *UBP6* restores cell cycle timing in *ubp10*Δ strains. (A) Doubling times of strains with deletions of the indicated DUBs. Colors represent the number of deletions: single mutants, purple; double mutants, blue; triple mutants, green; quadruple mutants, orange; quintuple mutant, 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,

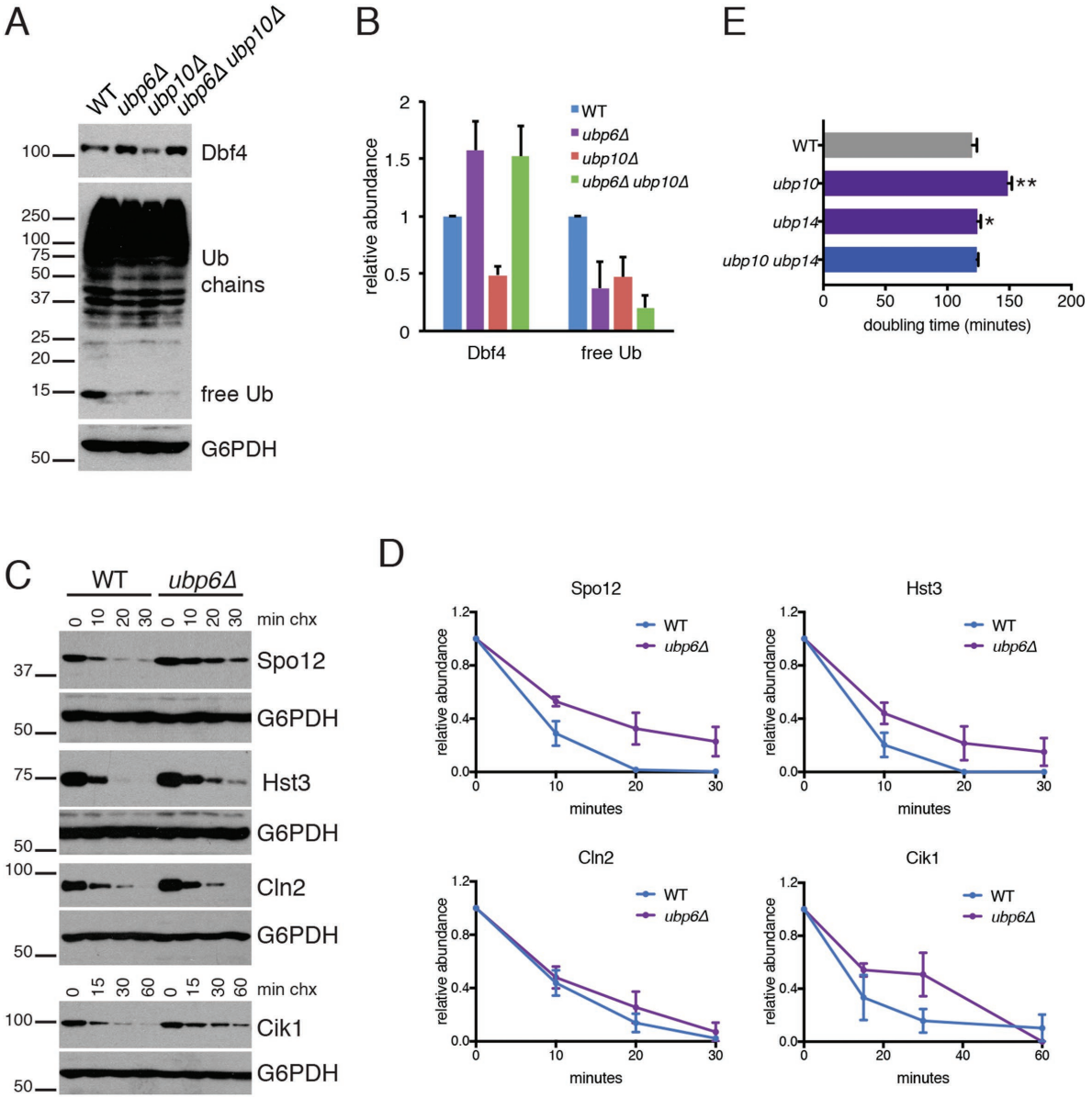

FIGURE 8: Deletion of DUBs that promote proteasome function rescue *ubp10*Δ phenotypes. (A) Representative Western blot of Dbf4-TAP and ubiquitin in asynchronous cells. G6PDH is shown as a loading control. (B) Quantitation of Western blots in part A. Dbf4-TAP and free ubiquitin levels were normalized 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 *ubp10*Δ 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 *ubp10*Δ), or $n = 4$ (endogenous Dbf4 in ubp6Δ and *ubp6*Δ *ubp10*Δ, Rpa190) experiments; error bars represent the SEM.

(Linghu *et al.*, 2002). However, we and others have found that *yuh1*Δ 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 *ubp10*Δ cells. However, a likely possibility is that the coordinate misregulation of several proteins in *ubp10*Δ 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 *ubp5*Δ *ubp6*Δ *ubp7*Δ *ubp10*Δ *yuh1*Δ 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 *ubp10*Δ 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 *ubp10*Δ 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, *ubp10*Δ 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 *ubp6*Δ 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 *ubp5*Δ *ubp6*Δ *ubp7*Δ *yuh1*Δ strain (YJB673) was crossed to a *ubp10*Δ 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 *URA3*-marked plasmid pYES2-GST (Kishi and Yamao, 1998). Previously described *UBP10* mutant genes (Richardson *et al.*, 2012) were also cloned into pYES2-GST. *RPA190-GFP* and *DBF4-TAP* sequences, along with their respective promoters, were amplified from genomic DNA from GFP (Huh *et al.*, 2003) and 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 FACS Calibur 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 independently 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.

ACKNOWLEDGMENTS

We thank Richard Gardner, Daniel Bolon, and Eduardo Torres for reagents and helpful discussions and Tom Fazzio and Marian Walhout for critical reading of the manuscript. This work was supported by funding from the Worcester Foundation for Biomedical Research and Grant no. R01GM115708 from the National Institutes of Health (NIH) to J.A.B. C.E.M. was supported in part by NIH training grant no. T32CA130807.

REFERENCES

Abdul Rehman SA, Kristariyanto YA, Choi S-Y, Nkosi PJ, Weidlich S, Labib K, Hofmann K, Kulathu Y (2016). MINDY-1 is a Member of an evolutionarily conserved and structurally distinct new family of deubiquitinating enzymes. Mol Cell 63, 146–155.

Albuquerque CP, Smolka MB, Payne SH, Bafna V, Eng J, Zhou H (2008). A multidimensional chromatography technology for in-depth phosphoproteome analysis. Mol Cell Proteomics 7, 1389–1396.

Amerik AY, Hochstrasser M (2004). Mechanism and function of deubiquitinating enzymes. Biochim Biophys Acta 1695, 189–207.

Amerik AY, Li SJ, Hochstrasser M (2000). Analysis of the deubiquitinating enzymes of the yeast *Saccharomyces cerevisiae*. Biol Chem 381, 981–992.

Amerik A, Sindhi N, Hochstrasser M (2006). A conserved late endosome-targeting signal required for Doa4 deubiquitylating enzyme function. J Cell Biol 175, 825–835.

Amerik AYU, Swaminathan S, Krantz BA, Wilkinson KD, Hochstrasser M (1997). In vivo disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome. EMBO J 16, 4826–4838.

Beckley JR, Chen J-S, Yang Y, Peng J, Gould KL (2015). A degenerate cohort of yeast membrane trafficking DUBs mediates cell polarity and survival. Mol Cell Proteomics 14, 3132–3141.

Benanti JA (2012). Coordination of cell growth and division by the ubiquitin-proteasome system. Semin Cell Dev Biol 23, 492–498.

Benanti JA, Cheung SK, Brady MC, Toczyski DP (2007). A proteomic screen reveals SCFGrr1 targets that regulate the glycolytic-gluconeogenic switch. Nat Cell Biol 9, 1184–1191.

Böhm S, Szakal B, Herken BW, Sullivan MR, Mihalevic MJ, Kabbinavar FF, Branzei D, Clark NL, Bernstein KA (2016). The budding yeast ubiquitin protease Ubp7 is a novel component involved in S phase progression. J Biol Chem 291, 4442–4452.

Chernova TA, Allen KD, Wesoloski LM, Shanks JR, Chernoff YO, Wilkinson KD (2003). Pleiotropic effects of Ubp6 loss on drug sensitivities and yeast prion are due to depletion of the free ubiquitin pool. J Biol Chem 278, 52102–52115.

Chong YT, Koh JLY, Friesen H, Duffy K, Cox MJ, Moses A, Moffat J, Boone C, Andrews BJ (2015). Yeast proteome dynamics from single cell imaging and automated analysis. Cell 161, 1413–1424.

Cohen M, Stutz F, Belgareh N, Haguenauer-Tsapis R, Dargemont C (2003). Ubp3 requires a cofactor, Bre5, to specifically de-ubiquitinate the COPII protein, Sec23. Nat Cell Biol 5, 661–667.

Costanzo M, VanderSluis B, Koch EN, Baryshnikova A, Pons C, Tan G, Wang W, Usaj M, Hanchard J, Lee SD, *et al.* (2016). A global genetic interaction network maps a wiring diagram of cellular function. Science 353, aaf1420.

Darling S, Fielding AB, Sabat-Pośpiech D, Prior IA, Coulson JM (2017). Regulation of the cell cycle and centrosome biology by deubiquitylases. Biochem Soc Trans BST20170087–12.

Debelyy MO, Platta HW, Saffian D, Hensel A, Thoms S, Meyer HE, Warscheid B, Girzalsky W, Erdmann R (2011). Ubp15p, a ubiquitin hydrolase associated with the peroxisomal export machinery. J Biol Chem 286, 28223–28234.

Elmore ZC, Beckley JR, Chen J-S, Gould KL (2014). Histone H2B ubiquitination promotes the function of the anaphase-promoting complex/cyclosome in *Schizosaccharomyces pombe*. G3 (Bethesda) 4, 1529–1538.

Ficarro SB, McCleland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM (2002). Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. Nat Biotechnol 20, 301–305.

Fragkos M, Ganier O, Coulombe P, Méchali M (2015). DNA replication origin activation in space and time. Nat Rev Mol Cell Biol 16, 360–374.

Gallego-Sánchez A, Andrés S, Conde F, San-Segundo PA, Bueno A (2012). Reversal of PCNA ubiquitylation by Ubp10 in *Saccharomyces cerevisiae*. PLoS Genet 8, e1002826.

Gardner RG, Nelson ZW, Gottschling DE (2005). Ubp10/Dot4p regulates the persistence of ubiquitinated histone H2B: distinct roles in telomeric silencing and general chromatin. Mol Cell Biol 25, 6123–6139.
Ghaemmaghami S, Huh W-K, Bower K, Howson RW, Belle A, Dephoure N, O'Shea EK, Weissman JS (2003). Global analysis of protein expression in yeast. Nature 425, 737–741.
Gödderz D, Giovannucci TA, Laláková J, Menéndez-Benito V, Dantuma NP (2017). The deubiquitylating enzyme Ubp12 regulates Rad23-dependent proteasomal degradation. J Cell Sci 130, 3336–3346.
Hanna J, Hathaway NA, Tone Y, Crosas B, Elsasser S, Kirkpatrick DS, Leggett DS, Gygi SP, King RW, Finley D (2006). Deubiquitinating enzyme Ubp6 functions noncatalytically to delay proteasomal degradation. Cell 127, 99–111.
Hanna J, Leggett DS, Finley D (2003). Ubiquitin depletion as a key mediator of toxicity by translational inhibitors. Mol Cell Biol 23, 9251–9261.
Harreman M, Taschner M, Sigurdsson S, Anindya R, Reid J, Somesh B, Kong SE, Banks CAS, Conaway RC, Conaway JW, *et al*. (2009). Distinct ubiquitin ligases act sequentially for RNA polymerase II polyubiquitylation. Proc Natl Acad Sci USA 106, 20705–20710.
Henry KW, Wyce A, Lo W-S, Duggan LJ, Emre NCT, Kao C-F, Pillus L, Shilatifard A, Osley MA, Berger SL (2003). Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. Genes Dev 17, 2648–2663.
Holt LJ, Tuch BB, Villén J, Johnson AD, Gygi SP, Morgan DO (2009). Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. Science 325, 1682–1686.
Huh W-K, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O'Shea EK (2003). Global analysis of protein localization in budding yeast. Nature 425, 686–691.
Isasa M, Rose CM, Elsasser S, Navarrete-Perea J, Paulo JA, Finley DJ, Gygi SP (2015). Multiplexed, proteome-wide protein expression profiling: yeast deubiquitylating enzyme knockout strains. J Proteome Res 14, 5306–5317.
Kahana A, Gottschling DE (1999). DOT4 links silencing and cell growth in *Saccharomyces cerevisiae*. Mol Cell Biol 19, 6608–6620.
Kee Y, Lyon N, Huibregtse JM (2005). The Rsp5 ubiquitin ligase is coupled to and antagonized by the Ubp2 deubiquitinating enzyme. EMBO J 24, 2414–2424.
Kee Y, Munoz W, Lyon N, Huibregtse JM (2006). The deubiquitinating enzyme Ubp2 modulates Rsp5-dependent Lys63-linked polyubiquitin conjugates in *Saccharomyces cerevisiae*. J Biol Chem 281, 36724–36731.
Kinner A, Kölling R (2003). The yeast deubiquitinating enzyme Ubp16 is anchored to the outer mitochondrial membrane. FEBS Lett 549, 135–140.
Kishi T, Yamao F (1998). An essential function of Grr1 for the degradation of Cln2 is to act as a binding core that links Cln2 to Skp1. J Cell Sci 111 (Pt 24), 3655–3661.
Kouranti I, McLean JR, Feoktistova A, Liang P, Johnson AE, Roberts-Galbraith RH, Gould KL (2010). A global census of fission yeast deubiquitinating enzyme localization and interaction networks reveals distinct compartmentalization profiles and overlapping functions in endocytosis and polarity. PLoS Biol 8, e1000471.
Krogan NJ, Cagney G, Yu H, Zhong G, Guo X, Ignatchenko A, Li J, Pu S, Datta N, Tikuisis AP, *et al*. (2006). Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. Nature 440, 637–643.
Landry BD, Doyle JP, Toczyski DP, Benanti JA (2012). F-box protein specificity for G1 cyclins is dictated by subcellular Llocalization. PLoS Genet 8, e1002851.
Landry BD, Mapa CE, Arsenault HE, Poti KE, Benanti JA (2014). Regulation of a transcription factor network by Cdk1 coordinates late cell cycle gene expression. EMBO J 33, 1044–1060.
Lee B-H, Lee MJ, Park S, Oh D-C, Elsasser S, Chen P-C, Gartner C, Dimova N, Hanna J, Gygi SP, *et al*. (2010). Enhancement of proteasome activity by a small-molecule inhibitor of USP14. Nature 467, 179–184.
Lee B-H, Lu Y, Prado MA, Shi Y, Tian G, Sun S, Elsasser S, Gygi SP, King RW, Finley D (2016). USP14 deubiquitinates proteasome-bound substrates that are ubiquitinated at multiple sites. Nature 532, 398–401.
Leggett DS, Hanna J, Borodovsky A, Crosas B, Schmidt M, Baker RT, Walz T, Ploegh H, Finley D (2002). Multiple associated proteins regulate proteasome structure and function. Mol Cell 10, 495–507.
Linghu B, Callis J, Goebl MG (2002). Rub1p processing by Yuh1p is required for wild-type levels of Rub1p conjugation to Cdc53p. Eukaryot Cell 1, 491–494.
Luhtala N, Odorizzi G (2004). Bro1 coordinates deubiquitination in the multivesicular body pathway by recruiting Doa4 to endosomes. J Cell Biol 166, 717–729.
Mocciaro A, Rape M (2012). Emerging regulatory mechanisms in ubiquitin-dependent cell cycle control. J Cell Sci 125, 255–263.
Morgan DO (2007). The Cell Cycle, London: New Science Press.
Ostapenko D, Burton JL, Solomon MJ (2015). The Ubp15 deubiquitinase promotes timely entry into S phase in *Saccharomyces cerevisiae*. Mol Biol Cell 26, 2205–2216.
Peth A, Besche HC, Goldberg AL (2009). Ubiquitinated proteins activate the proteasome by binding to Usp14/Ubp6, which causes 20S gate opening. Mol Cell 36, 794–804.
Poulsen JW, Madsen CT, Young C, Kelstrup CD, Grell HC, Henriksen P, Juhl-Jensen L, Nielsen ML (2012). Comprehensive profiling of proteome changes upon sequential deletion of deubiquitylating enzymes. J Proteomics 75, 3886–3897.
Reed BJ, Locke MN, Gardner RG (2015). A conserved deubiquitinating enzyme uses intrinsically disordered regions to scaffold multiple protein interaction sites. J Biol Chem 290, 20601–20612.
Richardson LA, Reed BJ, Charette JM, Freed EF, Fredrickson EK, Locke MN, Baserga SJ, Gardner RG (2012). A conserved deubiquitinating enzyme controls cell growth by regulating RNA polymerase I stability. Cell Rep 2, 372–385.
Rothstein R (1991). Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol 194, 281–301.
Rumpf S, Jentsch S (2006). Functional division of substrate processing cofactors of the ubiquitin-selective Cdc48 chaperone. Mol Cell 21, 261–269.
Schaefer JB, Morgan DO (2011). Protein-linked ubiquitin chain structure restricts activity of deubiquitinating enzymes. J Biol Chem 286, 45186–45196.
Schülein-Völk C, Wolf E, Zhu J, Xu W, Taranets L, Hellmann A, Jänicke LA, Diefenbacher ME, Behrens A, Eilers M, *et al*. (2014). Dual regulation of Fbw7 function and oncogenic transformation by Usp28. Cell Rep 9, 1099–1109.
Shi Y, Chen X, Elsasser S, Stocks BB, Tian G, Lee B-H, Shi Y, Zhang N, de Poot SAH, Tuebing F, *et al*. (2016). Rpn1 provides adjacent receptor sites for substrate binding and deubiquitination by the proteasome. Science 351, aad9421.
Singer MS, Kahana A, Wolf AJ, Meisinger LL, Peterson SE, Goggin C, Mahowald M, Gottschling DE (1998). Identification of high-copy disruptors of telomeric silencing in *Saccharomyces cerevisiae*. Genetics 150, 613–632.
Sopko R, Huang D, Preston N, Chua G, Papp B, Kafadar K, Snyder N, Oliver SG, Cyert M, Hughes TR, *et al*. (2006). Mapping pathways and phenotypes by systematic gene overexpression. Mol Cell 21, 319–330.
Sowa ME, Bennett EJ, Gygi SP, Harper JW (2009). Defining the human deubiquitinating enzyme interaction landscape. Cell 138, 389–403.
Sun X-X, He X, Yin L, Komada M, Sears RC, Dai M-S (2015). The nucleolar ubiquitin-specific protease USP36 deubiquitinates and stabilizes c-Myc. Proc Natl Acad Sci USA 112, 3734–3739.
Verma R, Aravind L, Oania R, McDonald WH, Yates JR, Koonin EV, Deshaies RJ (2002). Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. Science 298, 611–615.
Verma R, Chen S, Feldman R, Schieltz D, Yates J, Dohmen J, Deshaies RJ (2000). Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. Mol Biol Cell 11, 3425–3439.
Willis N, Rhind N (2009). Mus81, Rhp51(Rad51), and Rqh1 form an epistatic pathway required for the S-phase DNA damage checkpoint. Mol Biol Cell 20, 819–833.
Wu R, Haas W, Dephoure N, Huttlin EL, Zhai B, Sowa ME, Gygi SP (2011). A large-scale method to measure absolute protein phosphorylation stoichiometries. Nat Methods 8, 677–683.