Yeast UBL-UBA proteins have partially redundant functions in cell cycle control
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Abstract

**Background:** Proteins containing ubiquitin-like (UBL) and ubiquitin associated (UBA) domains have been suggested to shuttle ubiquitinated substrates to the proteasome for degradation. There are three UBL-UBA containing proteins in budding yeast: Ddi1, Dsk2 and Rad23, which have been demonstrated to play regulatory roles in targeting ubiquitinated substrates to the proteasome for degradation. An involvement of these proteins in cell cycle related events has also been reported. We tested whether these three proteins act redundantly in the cell cycle.

**Results:** Here we show that the UBL-UBA proteins are partially redundant for cell cycle related roles. RAD23 is redundant with DDI1 and DSK2, but DDI1 and DSK2 are not redundant with each other and the triple deletion shows a synthetic effect, suggesting the existence of at least two roles for RAD23 in cell cycle control. The rad23Δddi1Δdsk2Δ triple deletion strain delays both in G2/M-phase and in mid-anaphase at high temperatures with duplicated spindle pole bodies. Cell cycle progression in the triple deletion strain can only be partially rescued by a rad23 allele lacking the c-terminal UBA domain, suggesting that RAD23 requires its c-terminal UBA domain for full function. In addition to their ability to bind ubiquitin and the proteasome, the UBL-UBA proteins also share the ability to homodimerize. Rad23 and Dsk2 dimerization requires their UBL and/or UBA domains whereas Ddi1 dimerization does not. Here we show that Ddi1 homodimerization is necessary for its cell cycle related functions.

**Conclusion:** The three yeast UBL-UBA proteins have partially redundant roles required for progression through mitosis.

**Background**

The ubiquitin-proteasome pathway is a complex protein degradation system that is conserved from yeast to mammals and plays an important role in many processes such as cell cycle control, endocytosis and DNA repair [1-5]. In Saccharomyces cerevisiae Rad23, Ddi1 and Dsk2 are the three UBL-UBA proteins, which are hypothesized to shuttle ubiquitinated substrates to the proteasome for degradation [6-11] due to their ability to interact with the proteasome through their UBL domains [12-15] as well as with ubiquitin and polyubiquitinated substrates through their UBA domains [7,8,16-18]. Consistent with the shut-
tling hypothesis, downregulation of human RAD23 (hHR23) using siRNA induces accumulation of p53 which is known to be continuously degraded by the ubiquitin-proteasome pathway [19]. In addition, yeast Rad23 is sufficient for docking of ubiquitin conjugates to proteasomes isolated from a strain carrying a mutation in the ubiquitin-interacting-motif (UIM) of the proteasome subunit Rpn10 [20], which is thought to act as a receptor that binds ubiquitinated substrates. Furthermore, proteasomes isolated from a rad23Δdsk2Δ strain are defective in their association with endogenous ubiquitin conjugates and this defect is stronger than the one observed in rpn10 mutants that are unable to bind ubiquitin, suggesting that the UBL-UBA proteins are more active than Rpn10 in delivery of ubiquitinated substrates to the proteasome [20].

Interestingly, none of the UBL-UBA proteins are essential for viability. Single deletion of RAD23 or DSK2 induces partial stabilization of a model degradation-substrate whereas the rad23Δdsk2Δ double deletion completely stabilizes the substrate [7] and accumulates endogenous polyubiquitinated proteins at high temperatures [21], suggesting that Rad23 and Dsk2 function redundantly in ubiquitin-mediated protein degradation. In addition to these genetic interactions, UBL-UBA proteins have been shown to physically interact, forming both homodimers and heterodimers [7,22]. Dsk2 and Rad23 homodimerization occurs through their UBL and/or UBA domains [17,23,24], whereas Ddi1 homodimerization requires neither of these domains [17]. Although, in vivo, heterodimerization of UBL-UBA proteins is likely to also occur via bridging molecules [22], direct interactions between the UBL and UBA domains promoting heterodimer formation have been demonstrated [22,23]. Rad23 homo and heterodimerization of UBL-UBA family members has been suggested to play a role in regulating their interactions with other components of the ubiquitin-proteasome pathway and to lead to the formation of multimeric complexes with polyubiquitin chains [23,25]. Such interactions could increase the targeting efficiency of ubiquitinated substrates for degradation.

One of the cellular processes that relies on ubiquitin-proteasome dependent proteolysis is cell cycle progression. Cell division is a complex process; it requires that a series of steps are fulfilled in a specific and unidirectional order. When cell cycle regulation fails genetic instability and aneuploidy often arise, which are hallmarks of and might initiate cancers [26,27]. A long list of cell cycle regulators are known to be degraded by the ubiquitin-proteasome pathway [2]. Several pieces of evidence suggest a role for UBL-UBA containing proteins in cell cycle control. In budding yeast, overexpression of Dsk2 is toxic, inducing accumulation of ubiquitinated substrates [12] and arresting the cells in mitosis with abnormal nuclear position and short bipolar spindles [28]. Ddi1 is involved in the degradation of an SCF component, the F-box protein Ufo1, involved in the G1/S transition [29], as well as one of its targets the Ho endonuclease [30]. Overexpression of DDI1 or RAD23 suppresses the temperature sensitive phenotype of a PDS1 mutant allele (pds1–128) [31]. Combined deletion of the RAD23 and DDI1 C-terminal UBA, but not the single deletions, results in premature loss of cohesion and spindle elongation in the presence of hydroxyurea (HU), which is known to activate the S-phase checkpoint [31]. In addition, the dsk2Δrad23Δ double deletion, but not the single deletions, is reported to be defective in Spindle Pole Body (SPB) duplication at high temperature, inducing the formation of monopolar spindles and subsequently arresting the cells in mitosis [28]. These results suggest redundant roles for the UBL-UBA proteins in cell cycle events.

Here we demonstrate that deleting RAD23 in combination with either DDI1 or DSK2 induces cell cycle delays in the G2/M-phase and anaphase at high temperatures, indicating that DDI1 and DSK2 are redundant with RAD23. In addition, we provide evidence that DDI1 and DSK2 are not functionally redundant and therefore that RAD23 has at least two cell cycle-related functions. This hypothesis is supported by the synthetic effect observed in the triple deletion, confirming that RAD23-DDI1 redundancy is different from that of RAD23-DSK2 (i.e. the triple deletion shows an additive phenotype). Surprisingly however, the arrest is not due to failure in SPB duplication.

**Results**

**RAD23, DDI1 and DSK2 have partially redundant roles in cell cycle progression**

Possible roles for Rad23 and Ddi1 in the cell cycle have been suggested by their ability to rescue the temperature sensitivity of pds1–128 [31], while Rad23 and Dsk2 have been reported to have roles in SPB duplication and Dsk2 overexpression leads to mitotic arrest [28]. To test whether the three yeast UBL-UBA proteins have a redundant role in cell cycle progression we took a genetic approach, obtaining strains deleted for each one of the UBL-UBA genes (Table 1) as well as all the double deletion combinations and the triple deletion, and asked whether any redundancy in terms of functions in cell cycle progression could be observed.

Although none of the single deletions have temperature sensitive phenotypes, the rad23Δdsk2Δ double deletion has been reported to accumulate ubiquitinated substrates at 37°C [21], as well as have a temperature sensitivity phenotype at 35°C [28]. Partially consistent with these results, we observed that rad23Δ ddi1Δ or dsk2Δ singly deleted strains as well as rad23Δ ddi1Δ and ddi1Δdsk2Δ
Table 1: Yeast strains used in this study

|     | Strain Description                                      |
|-----|--------------------------------------------------------|
| L167| MATalpha pds1–128                                      |
| L1494| pds1–128 GAL-ddi1::UBA [HIS]                           |
| L1493| pds1–128 GAL-Ddi1 [HIS]                                |
| 1671| MATa bar1::Δ GPTUB1::ura3 ARG4                         |
| 2073| MATalpha pds1–128 GAL-ddi1::A407L                      |
| 2074| MATalpha pds1–128 GAL-ddi1::L426A                      |
| L153| MATalpha pds1–128 arg4 Gal gal-ddi1::ΔUBA2 [HIS]       |
| L156| MATalpha pds1–128 arg4 Gal gal-ddi1::ΔUBA2 [HIS]       |
| L150| MATalpha pds1–128 arg4 Gal gal-ddi1 [LEU]              |
| L169| MATa bar1::Δ spc42::GFP [TRP]                          |
| L182| MATa bar1::Δ dsk2::KAN spc42::GFP [TRP]                |
| L185| MATa bar1::Δ rad23::KAN dsk2::KAN spc42::GFP [TRP]     |
| L187| MATa bar1::Δ ddi1::KAN dsk2::KAN spc42::GFP [TRP]      |
| L189| MATa bar1::Δ rad23::KAN spc42::GFP [TRP]               |
| L196| MATa bar1::Δ ddi1::KAN rad23::KAN spc42::GFP [TRP]     |
| L201| MATa bar1::Δ ddi1::KAN rad23::KAN dsk2::KAN TUB1::GFP [URA] |
| L218| MATa bar1::Δ ddi1::KAN dsk2::KAN rad23::KAN spc42::GFP [TRP] |
| L221| MATa bar1::Δ ddi1::KAN spc42::GFP [TRP]                |
| L263| MATa bar1::Δ ddi1::KAN dsk2::KAN TUB1::GFP::URA3 RAD23::UBA2-MYC [TRP] |

The c-terminal UBA domain of Rad23 is necessary for its cell cycle functions

Although both UBA domains of Rad23 have been shown to bind ubiquitin both in yeast [17] and in humans (hHR23A) [32], there is a specific requirement for its c-terminal UBA domain (UBA2) in mediating cell cycle arrest after binding to the HIV-1 Vpr protein [33]. Therefore we tested whether UBA2 is required for the cell cycle function of Rad23 by examining cell cycle progression in a strain in which the sole UBL-UBA protein is rad23ΔUBA2 (Figure 2). A recent study suggested that the C-terminal UBA domain of Rad23 acts as a stabilization domain [34], preventing proteasome-dependent degradation of Rad23. It was therefore important to determine if rad23ΔUBA2 was stable in our strains. As shown in Figure 2A, the protein levels of rad23ΔUBA2 and Rad23 were identical, whether these genes were expressed from their endogenous promoters or exogenously from the GAL1 promoter. This was also the case when we compared the protein levels of Ddi1 and ddi1ΔUBA (Figure 2A). After confirming that the wild type and mutant proteins were present in identical amounts in our strains, we spotted serial dilutions of the corresponding strains onto rich medium and grew at a range of temperatures for several days (Figure 2B). A partial recovery of the temperature sensitivity of the rad23Δddi1Δdsk2Δ triple deletion strain was observed after introducing rad23ΔUBA2, but full recovery was observed only when the full-length RAD23 gene was introduced (Figure 2B). These data suggest that, although both UBA-motifs can interact with ubiquitin [17], full Rad23 activity requires both UBA domains.

To explore this result in more detail, we examined the kinetics of cell cycle progression in strains that contained...
Deletion of DDI1, DSK2 and RAD23 has a synthetic effect on cell cycle progression at high temperatures. (A) Cells were grown to mid-log phase in liquid YEPD at 30°C then shifted to 37°C for 0, 4 and 6 hrs. Cell cycle distribution was determined by bud morphology: cells in G1 are unbudded (yellow), S-phase cells with small buds (blue) and G2/M cells with large buds (red). Large buds (dumbbells) are defined as cells where the bud is as big as the mother cell. In parallel, samples were taken for FACScan analysis of DNA content (lower panel).
Cell cycle arrest in cells lacking DDI1, DSK2 and the c-terminal UBA (UBA2) domain of Rad23. (A) Western blots showing relative protein levels of Rad23, Ddi1 and mutant forms in yeast extracts. Left two panels show endogenous levels of these proteins and the right panel shows levels produced after expression of constructs from the GAL1 promoter. In each case, the wild type and mutant version were tagged identically at their C-termini – [MYC]6x for Rad23 and [HIS]6x for Ddi1. PSTAIRE is a loading control. Strains expressed (from left to right) – Left panel: RAD23 [MYC]6x, DDI1 [HIS]6x, RAD23ΔUBA2 [MYC]6x, DDI1ΔUBA [HIS]6x; Middle Panel: no tag control, RAD23 [MYC]6x and DDI1 [HIS]6x, RAD23ΔUBA2 [MYC]6x and DDI1ΔUBA [HIS]6x, RAD23 [MYC]6x and DDI1 [HIS]6x, RAD23ΔUBA2 [MYC]6x and DDI1ΔUBA [HIS]6x; Right Panel: no tag control, GAL1-RAD23 [MYC]6x, GAL1-DDI1 [HIS]6x, GAL1-DDI1ΔUBA [HIS]6x. Upper band of Ddi1 is a phospho-
rylated species (data not shown). (B) Synthetic effect of UBL-UBA mutants on temperature sensitivity. The indicated strains were grown to mid-log phase and serial dilutions were spotted onto YEPD plates and incubated at the indicated temperatures for 48 hrs. Levels of Rad23 and Rad23ΔUBA2 protein expression were determined as shown in A. (C) Kinetics of cell cycle progression in wild type, ddi1Δ dsk2Δ rad23Δ and ddi1Δ dsk2Δ rad23ΔUBA2 cells. (ddi1Δ dsk2Δ strains behaved identically to the wild type control; Figure 1 and data not shown.) Cells were arrested in G1 at 30°C with alpha-factor and released in rich medium at 37°C. Cell cycle progression was monitored by bud morphology: unbudded cells (G1, yellow line), cells with small buds (S-phase, blue line) and cells with large buds (G2/M, red line).
either no UBL-UBA proteins (rad23Δdsk1Δrad23Δ or rad23ΔUBA2 (dsk1Δdsk2Δrad23ΔUBA2) as the sole UBL-UBA protein (Figure 2C). After release from a G1 arrest induced by alpha-factor treatment at the non-permissive temperature of 37°C, re-entry into the cell cycle and budding occurred with the same kinetics in these strains and in a wild type control. G2/M accumulation (red line) in the wild type strain peaked at 120 minutes and decreased to 28% 140 minutes after release as the cells entered the next cell cycle. Initiation of a second cell cycle was also indicated by the presence of a second wave of small-budded cells (blue line) appearing at 130 minutes. In contrast, the triple deletion strain entered G2/M with the same kinetics as wild type, but remained there even after 140 minutes (75% in G2/M). Meanwhile, ddi1Δ dsk2Δ  rad23ΔUBA2 cells entered G2/M with the same kinetics as both the wild type and the triple deletion strains, but accumulated in G2/M, albeit to a lesser extent than the triple deletion (60% at 140 minutes). This finding suggests that rad23ΔUBA2 partially alleviates the delay and confirms that complete function of Rad23 requires the full-length protein and that the internal UBA domain (UBA1) can only partially alleviate the G2/M delay.

**G2/M arrest is not due to a failure in SPB duplication**

Since defects in SPB duplication leading to G2/M arrest have been reported for strains lacking RAD23 and DSK2 [28], we asked if there is a SPB duplication defect in the triple deletion strain. To visualize SPBs in live cells we used strains expressing SPC42::GFP (Table 1). Wild type or rad23Δddi1Δdsk2Δ triple deletion strains expressing SPC42::GFP were arrested in G1 using alpha-factor and released into fresh medium at the restrictive temperature. Samples were taken every 10 minutes, visualized (Figure 3A–B) and scored according to SPB number (Figure 3C). Surprisingly, the triple deletion strain separated SPBs normally (Figure 3A–C). The kinetics of SPB separation in the triple deletion were indistinguishable from that of the wild type strain (Figure 3D), except that the triple deleted cells arrested as large budded cells with two SPBs (Figure 3E, D). In the wild type strain, SPB separation initiated at ~40 minutes after release from G1 at the restrictive temperature (Figure 3C, yellow line) and peaked at ~80 minutes. In accordance, the population of cells with a single SPB signal (Figure 3C, blue line) was depleted within the same timeframe. After 100 minutes a second wave of cells with a single SPB signal peaked, followed by a second wave of SPB separation as cells enter another cell cycle. A mixed population of un budded cells with single SPB signals and re-budded cells with newly separated SPBs could be observed 110 minutes after the alpha-factor release (Figure 3A, arrow head), consistent with cells entering the next cell cycle. ddi1Δdsk2Δrad23Δ cells separated their SPBs with the same kinetics as wild type cells, but then delayed or arrested with large buds and two SPBs (Figure 3C, red line). A very similar phenotype was seen in dsk2Δrad23Δ mutants (data not shown). Interestingly, the arrest observed in the dsk2Δrad23Δ and the triple deleted cells was not homogeneous, since some of the cells arrested as dumbbells with well separated SPBs, resembling anaphase cells (Figure 3B, right cells, and Figure 3D), while the rest of the arrested cells possessed SPBs that were separated by a distance typical of G2 cells (Figure 3B, arrow).

DAPI staining of DNA in these arrested cells confirmed that some cells were arrested in a G2-like stage with a single nucleus (Figure 3E, left two panels), while other cells were arrested in early (3rd panel) or late anaphase (right-most panel). Arrest in G2 or anaphase could have been due to defects in spindle assembly. Therefore, to further explore these results and examine in more detail the dynamics of spindle assembly in the triple deletion, we constructed strains harboring a TUB1::GFP gene (Table 1).

In agreement with the results observed in the SPC42::GFP strains, wild type cells and ddi1Δdsk2Δrad23Δ cells assembled their spindles with the same timing, relative to bud emergence (Figure 3F).

In a final series of experiments, we performed time courses in which cells were released from a nocodazole arrest at the restrictive temperature of 37°C (Figure 3G). The percentage of wild type cells with G2-SPBs (Figure 3G, top panel, green line) at the time wild type arrested their spindles (Figure 3G, lower panel, blue line). In contrast, the triple deleted cells showed a delay in anaphase onset, as judged by the distance between the SPBs, and ~30% remained in G2 (Figure 3G, top panel, green line) at the time wild type G2-cells had been completely depleted. Similar data were obtained in strains expressing TUB1::GFP. Within about 10 minutes after release of the nocodazole block, almost all large budded cells had assembled G2 spindles (Figure 3G, lower panel). Wild type cells initiated spindle elongation ~20 minutes after release from nocodazole and by 40 minutes most of the cells had completed anaphase (Figure 3G, lower panel, blue line). In contrast, some ddi1Δdsk2Δrad23Δ cells remained in G2, as judged by their short (1–4 μM) spindles, even 90 minutes after the release (Figure 3G, lower panel, green line). Together, these results suggest ddi1Δdsk2Δrad23Δ cells delay cell cycle progression at two distinct points when grown at high temperatures: a fraction of the triple deleted cells delay before anaphase with duplicated SPBs but short spindles, whereas other cells delay in a mid-anaphase or late-anaphase state with partly-elongated or fully-elongated spindles. This suggests that combined deletion of RAD23, DDI1 and DSK2 affects spindle dynamics and cell cycle progression, but not SPB duplication.
Figure 3

SPB duplication and spindle function. (A) Photomicrograph of wild type SPC42:GFP cells from time course in C, 110 minutes after alpha-factor release. Arrowhead shows rebudded cell that has already undergone a second SPB duplication. (B) Photomicrograph of rad23Δdsk2Δddi1ΔSPC42:GFP cells from time course in C, 110 minutes after alpha factor release. Arrow shows cell with G2-like short inter-SPB distance. (C) SPB duplication kinetics. Wild type (WT) and rad23Δdsk2Δddi1Δ (3Δ) cells were arrested with alpha factor and released into rich medium at 37°C. Samples were taken every 10 minutes and SPBs in each cell were counted. (D) Photomicrographs showing SPBs (SPC42:GFP) in wild type, rad23Δdsk2Δ and rad23Δdsk2Δddi1Δ cells at 90 minutes after release from G1 arrest at the restrictive temperature. (E) Photomicrographs showing DIC, SPC42:GFP signals, and DAPI staining of DNA in rad23Δdsk2Δddi1Δ cells at 180 minutes after release from G1 arrest at the restrictive temperature of 37°C. Left two panels show G2-like cells (52% of the large budded cells) with a single nucleus and SPBs typically separated by less than 4 μm. Right two panels show anaphase cells with divided nuclei (44% of the large budded cells) and SPBs typically separated by > 4 μm. The remaining large budded cells had stretched single nuclei. (F) Timing of bud emergence and spindle assembly after release from alpha-factor induced G1 arrest in wild type, rad23Δdsk2Δ, and rad23Δdsk2Δddi1Δ cells. Photomicrograph shows rad23Δdsk2Δddi1ΔTUB1:GFP cells at 37°C with both G2 and anaphase spindles. (G) Nocodazole release experiment in rad23Δdsk2Δddi1ΔSPC42:GFP cells. Cells were arrested with alpha factor, released into medium containing nocodazole and grown at 30°C for 2 hours, then washed and released into medium containing alpha factor to prevent re-budding upon progression to G1. Samples were taken every 10 minutes and SPBs with short G2-like inter-SPB distance (1–4 μm, inset micrograph) were counted (upper panel). This protocol was repeated in rad23Δdsk2Δddi1ΔTUB1:GFP cells (lower panel). Samples were taken every 10 minutes and short G2 spindles (inset micrograph) were counted.
**Ddi1 homodimerization is necessary for pds1–128 rescue**

In addition to their abilities to bind ubiquitin and the proteasome, Dsk2, Ddi1 and Rad23 share the ability to form homodimers [22,24]. The importance of dimerization for the function of these proteins is not known however. Dsk2 and Rad23 homodimerization involves their UBL and/or UBA domains [17,23,24], which makes it difficult to obtain mutants that disrupt homodimerization but remain capable of binding to ubiquitin and the proteasome. Ddi1, on the other hand does not require either the UBA or the UBL domains for homodimerization [22].

Therefore we tested whether homodimerization is important for the role of Ddi1 in one specific cell cycle related event; stabilization of Pds1–128. Firstly we more precisely defined the domain important for Ddi1 homodimerization to a region encompassing residues 184 to 285 (Figure 4C) and asked whether this mutant could rescue the temperature sensitivity of this strain (Figure 4D). When overexpressed, neither ddi1Δ184–285, nor ddi1 alleles that disrupt UBA-ubiquitin interaction (ddi1ΔUBA or ddi1-L426A [17]) rescued the temperature sensitivity of the pds1–128 strain but overexpression of wild type DDI1 or ddi1-A407L (a ddi1-UBA mutant that still interacts with ubiquitin) did rescue the temperature sensitivity of pds1–128. The protein levels of wild type Ddi1 and the mutant alleles were the same (Figure 2A). Therefore, these results suggest that both homodimerization and ubiquitin interaction are required for rescue of pds1–128 by overproduced Ddi1.

**Discussion**

The UBL-UBA proteins Ddi1, Dsk2 and Rad23 are conserved from yeast to humans but are not essential for viability in yeast, suggesting that they might perform overlapping functions. We investigated this possibility by removing all three UBL-UBA proteins to find that they are essential for viability and cell cycle progression at high temperatures. Interestingly, these analyses did not provide evidence for a functional overlap between Ddi1 and Dsk2, but rather indicate that Rad23 has redundant functions with each of Ddi1 and Dsk2. A strain deleted for all three of these genes exhibited a synthetic effect, suggesting that RAD23 has at least two independent roles that are required for viability at high temperatures; one of them shared with DDI1 and the other one with DSK2. Interestingly, these redundancies do not seem to be a product of evolutionary divergence of duplicated genes, since neither RAD23, DSK2 or DDI1 are elements in the duplication blocks that have been described to have arisen after the yeast genome duplication event. Furthermore, they lie next to elements from different duplication blocks: block 28 for RAD23, block 46 for DSK2 and block 13 for DDI1 [35], which further suggests that they are functionally related, but not evolutionarily related.

Since these proteins have been shown to be involved in cell cycle related phenomena [28,31], we examined cell cycle progression at the restrictive temperature and found that ddi1Δdsk2Δrad23Δ cells arrest or delay in G2/M and anaphase. Surprisingly, the arrest observed in the triple deletion was not due to a failure in SPB duplication. We were also not able to observe SPB duplication defects in the rad23Δdsk2Δ double deletion strains at 37°C (data not shown), as had been previously reported [28]. We currently do not know the reason for this contradictory result, but it could be due to differences in the genetic background between the BF264-15 Daub (this study) and S288c strains [28].

Interestingly, when we examined the kinetics of spindle elongation (Figure 3) we found that the arrest observed in the triple deletion strain was not homogeneous. There were two distinct populations: cells that arrested with G2-like spindles and had a single nucleus, and cells that arrested with partly or fully elongated spindles and divided nuclei. One possibility is that the ddi1Δdsk2Δrad23Δ cells progressively accumulate ubiquitinated substrates that somehow interfere with degradation of other cell cycle regulatory proteins. The existence of two arrest points might therefore be a reflection of two cell cycle stages at which proteasome function is required: some of the mutant cells may have accumulated enough substrates to enforce arrest at the earlier time point whereas other cells might be able to reach the latter cell cycle stage. However, the specific reasons why subpopulations of the triple deleted cells arrest at the earlier or later time point remain to be determined.

The ability of UBL-UBA proteins to both hetero and homodimerize, as well as to bind to polyubiquitin in tandem [23] suggests the possibility that UBL-UBA dimerization regulates binding of these proteins to ubiquitinated substrates, possibly by bringing more than one UBL-UBA protein in close proximity to the ubiquitin chain [23,36]. Ddi1 is the only UBL-UBA protein in which homodimerization does not involve the UBL or UBA domains, allowing the study of the role of dimerization without affecting its interaction with ubiquitin or the proteasome. We have shown that disruption of Ddi1 homodimerization affects
Ddi1's ability to rescue the pds1–128 temperature sensitivity, suggesting that Ddi1 homodimerization is necessary for its role in Pds1–128 stabilization. Ddi1 dimerization does not involve the cystein residues at positions 276 and 300 (Figure 4A, blue) since a C276S C300S double mutant is still able to interact with wild type Ddi1 (data not shown). Interestingly, it has been suggested that Pds1–128 stabilization by Ddi1 might be due to de-ubiquitination of Pds1–128 through the aspartyl-protease domain of Ddi1 (underlined in Figure 4A)[37]. Our results suggest that this could be possible. First, the ddi1Δ184–285 allele lacks half of the aspartyl-protease domain of Ddi1 (Figure 4B). A Yeast two hybrid assay reveals that residues 184–285 are important for homodimerization of Ddi1 but not for its interaction with Rad23 (Figure 4C). Production of blue color in x-gal containing medium signals for a positive interaction between two proteins [17]. ddi1Δ184–285 interaction with DDI1 is highly reduced but ddi1Δ184–285 interaction with RAD23 is similar to that of wild type DDI1. (C) Western blots showing levels of different Ddi1 variants. Cells were grown on YEPR overnight, diluted 1:10 and expression of DDI1 constructs was induced from the GAL1 promoter by Galactose addition. These strains corresponded to the pds1–128 DDI1 plus: GAL1:ddi1ΔUBA GAL1:DDI1 GAL1:ddi1Δ184–285 GAL1:ddi1Δ184–285 plus: GAL1:ddi1ΔUBA GAL1:DDI1 GAL1:ddi1Δ184–285 GAL1:ddi1Δ184–285 plus: GAL1:ddi1A407L GAL1:ddi1L426A GAL1:DDI1 GAL1:ddi1L426A GAL1:ddi1A407L (D) Rescue of pds1–128 temperature sensitivity. Serial dilutions of the indicated strains (corresponding to those tested in D) were spotted onto YEPD or YEPG plates and incubated for 24 hr at the indicated temperatures.
domain. Second, the aspartyl protease domain requires dimerization to become active, and the ddi1Δ184–285 allele does not dimerize, suggesting that the predicted Ddi1 aspartyl-protease activity is not functional in ddi1Δ184–285 and could hypothetically be involved in pds1–128 rescue.

**Conclusion**
Redundancy of function among the budding yeast UBL-UBA proteins can be predicted based on their common abilities to bind ubiquitin and the proteasome. We have shown that the yeast UBL-UBA proteins have partially redundant functions, Rad23 being redundant with both Ddi1 and Dsk2. Surprisingly Ddi1 and Dsk2 do not share redundant functions but a synthetic phenotype is observed in the triple deletion strain in which cells become delayed in both G2 and anaphase.

**Materials and methods**

**Strains and Plasmids**
Strains are derivatives of BF264-15Daub [38], unless otherwise noted. Standard genetic procedures were used [39]. Spindles were visualized by expressing a GFP:TUB1 construct [40,41] integrated at the URA3 locus. SPBs were visualized by expressing a GFP-SPC42 construct [28]. Cultures were grown on YEPD at 30°C unless otherwise stated. Micrographs were acquired with a Zeiss Axioplan2 Microscope, using an alpha-Plan-FLUAR 100X/1,45 oil objective, an AxioCam HRm camera and Axiovision software.

The pb42AD-ddi1Δ184–285 vector was obtained by Clal digestion of the pb42AD-DDI1 two-hybrid vector [17,31] followed by self-ligation. pYIPG2-DDI1 and pYIG2-ddi1Δ184–285 were obtained by PCR from the respective pb42AD-vectors. The PCR fragments were cloned into a pCR2.1-TOPO vector (Invitrogen), cut out with BamHI and cloned into the pYIPG2 vector. These constructs were subsequently integrated at the LEU2 locus. pb42AD-DDI1-C276S-C300S was constructed using the QuickChange site directed mutagenesis kit (Stratagene) using the following mutagenesis primers: DDIC300S-1 5'CTGAAAAGGCATTGGCTAGTGTGGACTTAAAGGAAAAC3', DDIC300S-2 5'GTITTTCCTTTAAATGGCTAGTGGACITAAAGGAAAAC3', DDIC276S-1 5'GGTTTTCCTTTAAGTCCACACTAGCCAAATGCCITTTTCA
G3', DDIC276S-2 5'AAAATAGAAACACAATATATTCCAAGCAGTTTACCCTCITAGATACGT3' and DDIC276S-2 5'CAGTATCTAAGACGGTAAAACTGGGTAATATATTG
TGTTTCTATTTT3'.

DSK2 was disrupted by homologous recombination using a fragment obtained by PCR from the FA6 cassette using the primers: dsk2kan-5' 5'-ATAAGACCGATCAAAGACCCGAATCATCTTAGCAC-
GATACAGCTGAAGCTTGCACGCT-3' and dsk2kan-3' (5'-TAGGTAAAAGATATATAGTTGCGGCATCTA-GACGTTTATGCAATAGCCTGATC-3').

**Time courses**
Yeast strains were grown in rich YEPD medium containing extra adenine overnight and diluted 1:20 before the experiment. For G1-release, diluted cultures were incubated for ~2 hours in the presence of 0.2 μg/mL alpha-factor (Sigma), then washed twice and released in fresh medium at 37°C. For nocodazole release, an alpha-factor arrest was performed first and cells were released into fresh medium at 37°C containing 15 μg/mL Nocodazole for 11/2 hours. Cells were washed twice with pre-warmed sterile water and released into pre-warmed rich medium containing 0.4 μg/mL alpha-factor to stop the cells from entering the next cell cycle. Alpha-factor was also added to the nocodazole-arrest medium 1/2 hour before releasing. At least 100 cells were counted per timepoint.

**Western blots**
Whole cell lysates were separated by SDS-PAGE (10% acrylamide w/v), transferred to an Immobilon PVDF membrane and probed using rabbit polyclonal anti-Ddi1 1:5000, or rabbit polyclonal anti-Rad23 1:5000 [22].

**List of Abbreviations**
GFP – Green Fluorescent Protein
SPB – Spindle Pole Body
UBL – Ubiquitin Like
YEPD – Yeast Peptone Dextrose

**Competing interests**
The author(s) declare that they have no competing interests.

**Authors’ contributions**
Experiments performed by LADM, YK. Experiment design by KJW and DJC.

**Acknowledgements**
The authors would like to thank B. Bertoalet, S. Haase, S. Reed, G. Mondevert and R. Wright for strains, plasmids and advice. J. Berman, JF Giménez-Abián, C. Andrews, S. Erickson, A. Vas and the University of Minnesota yeast community for helpful comments and discussions, and B. Meier for excellent technical assistance. This work was funded by NIH grant CA099033 (DJC). LADM was supported by DOD grant B043245.

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