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## Proteasomal Regulation of the Mutagenic Translesion DNA Polymerase, *Saccharomyces cerevisiae* Rev1

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### Abstract

Translesion DNA synthesis (TLS) functions as a tolerance mechanism for DNA damage at a potentially mutagenic cost. Three TLS polymerases (Pols) function to bypass DNA damage in *Saccharomyces cerevisiae*: Rev1, Pol ζ, a heterodimer of the Rev3 and Rev7 proteins, and Pol η (Rad30). Our lab has shown that *S. cerevisiae* Rev1 protein levels are under striking cell cycle regulation, being ~50-fold higher during G2/M than during G1 and much of S phase (Waters and Walker, 2006). *REV1* transcript levels only vary ~3-fold in a similar cell cycle pattern, suggesting a posttranscriptional mechanism controls protein levels. Here, we show that the *S. cerevisiae* Rev1 protein is unstable during both the G1 and G2/M phases of the cell cycle, however the protein's half-life is shorter in G1 arrested cells than in G2/M arrested cells, indicating that the rate of proteolysis strongly contributes to Rev1's cell cycle regulation. In the presence of the proteasome inhibitor, MG132, the steady-state levels and half-life of Rev1 increase during G1 and G2/M. Through the use of a viable proteasome mutant, we confirm that the levels of Rev1 protein are dependent on proteasome-mediated degradation. The accumulation of higher migrating forms of Rev1 under certain conditions shows that the degradation of Rev1 is possibly directed through the addition of a polyubiquitination signal or another modification. These results support a model that proteasomal degradation acts as a regulatory system of mutagenic TLS mediated by Rev1.

### Keywords

Rev1; translesion synthesis; DNA damage tolerance; protein degradation

## 1. Introduction

Cells constantly face the challenge of maintaining genomic integrity as a result of DNA damage arising from endogenous and exogenous sources. To prevent the negative consequences of DNA damage, the cell is equipped with DNA repair and tolerance mechanisms. DNA repair restores the original state of the DNA. DNA damage tolerance, however, allows DNA lesions to remain in the genome even during replication.

When the cell employs translesion DNA synthesis (TLS) to tolerate DNA damage, specialized DNA polymerases with members from all domains of life [1] catalyze

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replication opposite lesions that normally prevent the replicative DNA polymerases' activity. Most TLS polymerases belong to the Y-family of DNA polymerases, which are better able to accommodate bulky DNA lesions because the active sites are less sterically constrained than those of the high-fidelity, replicative polymerases [2]. Given this structural property of TLS polymerases and their lack of any proofreading activity, TLS polymerases can exhibit high error rates. TLS across from lesions can be relatively error-free or quite error-prone depending on the lesion and polymerase involved [3–5]. Following bypass, the DNA repair pathways can later remove the DNA lesion, which remains in the DNA.

There are three known TLS polymerases in *S. cerevisiae*: Rev1 and Pol  $\eta$  (Rad30) of the Y-family and the B-family member, Pol  $\zeta$ , a heterodimer of Rev3 and Rev7. All three are highly conserved among eukaryotes. The *REV1*, *REV3*, and *REV7* genes were discovered in screens for reversionless mutants in yeast (a phenotype indicating loss of a mutagenic activity) [6,7]. The *rev1* $\Delta$  mutant phenotypes include an increased sensitivity to certain DNA damaging agents and a decreased damage-induced mutation frequency, indicating Rev1's instrumental role in DNA damage resistance and mutagenesis [8].

Rev1's DNA polymerase activity exhibits unique properties that include specificity for a template G and a preference for inserting dCMPs as a consequence of pairing the incoming dNTP with one of its own residues instead of with a template base [9–11]. Despite this clear and evolutionarily conserved catalytic activity (Wiltout and Walker, submitted), the non-catalytic functions of Rev1 appear to be more critical for DNA damage tolerance and mutagenesis *in vivo* based on known mutant phenotypes. In *S. cerevisiae*, the *rev1-1* (G193R) mutant of the BRCT domain leads to almost null phenotypes *in vivo*, but the mutant protein retains about 60% of the catalytic activity *in vitro* [12]. Additionally, the ubiquitin binding motif (UBM2) [13–15] and the conserved region of Rev1's C-terminus that interacts with other TLS polymerases [16–20] are critical for cellular survival and mutagenesis after DNA damage [reviewed in [21]]. Therefore, beyond its DNA polymerase function, Rev1 serves to regulate the other TLS polymerases through protein-protein interactions or direct interaction with the DNA.

The mutagenic nature of Rev1 indicates that the activity must be tightly regulated. The conservation of Rev1 in higher eukaryotes suggests that the evolutionary benefits outweigh the risks of its potentially mutagenic activity, although it is possible that all of Rev1's *in vivo* functions are not known.

Not surprisingly, disrupting the normal protein levels of TLS polymerases has negative consequences. In *S. cerevisiae*, ectopic overexpression of Pol  $\zeta$ 's Rev3 and Rev7 proteins leads to a greater sensitivity to UV radiation and an increase in UV-induced mutation frequency [22]. In one study in mammalian cells, a 2 to 4.5-fold overexpression of the TLS DNA Pol  $\kappa$  interferes with replication fork progression in CHO cell lines [23]. In another report one study, overexpression of human REV1 in ovarian carcinoma cells demonstrates the potential danger of misregulated Rev1 levels [24]. Therefore, understanding how the regulation of *REV1* properly balances survival and mutagenesis in the cell is crucial.

Currently, limited data exists regarding the regulation of *REV1* gene expression. Unlike some other genes encoding DNA repair proteins, *S. cerevisiae REV1* transcription is not inducible by DNA damage or heat shock [25]. *REV1* transcript levels are, however, upregulated during sporulation in *S. cerevisiae* [26–28]. At the protein level, previous work from our lab has shown that Rev1 is under striking cell cycle control with protein levels peaking during G2/M rather than S phase when the bulk of replication occurs [29]. Despite the approximately 50-fold change at the protein level, *REV1* transcript levels only increase 3-fold during G2/M relative to G1. Interestingly, Rev1 is phosphorylated in a similar cell

cycle-dependent manner, demonstrating another potential method of regulation [30]. The molecular means controlling the unexpected cell cycle regulation of Rev1, however, are not yet fully understood.

More recent studies support the hypothesis that cell cycle regulation of Rev1 is functionally important. For example, the action of Rev1 and Pol  $\zeta$  is key for bypass of ultraviolet-induced DNA damage during and after S phase of the cell cycle and can occur separately from bulk genomic replication [31]. In another study, the use of G2-specific promoters to express Rev3 and Rad30 complemented the deletion of the TLS polymerases with respect to survival and mutagenesis phenotypes in response to specific types of DNA damage [32]; a G2-specific promoter was not used to express Rev1 in this study.

Several genetic studies indicate that TLS may be subject to regulation by the proteasome. These studies took advantage of the *ump1 $\Delta$*  strain, which is a viable mutant of a gene encoding a maturation factor for the 20S catalytic core of the 26S proteasome [33]. The spontaneous and UV-induced mutator phenotype of the *ump1 $\Delta$*  strain is dependent on the TLS polymerase gene, *REV3*, which is generally placed in the same genetic pathway as *REVI* [34,35]. The *ump1 $\Delta$*  strain is hypermutable, whereas *rev3 $\Delta$*  and *ump1 $\Delta$  rev3 $\Delta$*  strains are hypomutable, suggesting that Ump1 may act as a negative regulator of Rev3 activity, possibly through Rev1's interaction with Pol  $\zeta$ . The authors of this study, however, did not examine *REVI*'s genetic interactions with *UMPI*. In an *ump1* strain, short-lived proteins are stabilized and ubiquitin-protein conjugates accumulate [33]. Therefore, we hypothesized the involvement of proteasomal degradation in TLS regulation as a means for control of this potentially mutagenic process.

Selective protein turnover through ubiquitination and subsequent proteasomal degradation represents an essential regulatory mechanism in eukaryotic cells. The irreversibility of protein degradation ensures both spatial and temporal control and eliminates improper reactivation of the protein. The attachment of monoubiquitin or polyubiquitin chains to specific proteins is critical for a variety of cellular processes from DNA repair and replication to gene silencing, in addition to protein degradation [36,37].

Here, we studied the role that proteasomal degradation has in regulating the mutagenic TLS polymerase Rev1, the levels of which are cell cycle regulated. We show that Rev1 is a moderately short-lived protein throughout the cell cycle but is degraded more rapidly during G1 than during G2/M. Our data indicate that Rev1 undergoes proteasome-mediated degradation during both G1 and G2/M arrests that is potentially targeted through a polyubiquitin modification. Overall, these results indicate that proteasomal degradation serves as an efficient and irreversible mechanism of regulating the potentially mutagenic effects of Rev1's action.

## 2. Material and methods

### 2.1. Yeast strains

A strain list for this study is described in Table 1. All strains are derivatives of the W1588-4C (*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5*) [38] parent strain. The Rev1 protein was tagged at its native locus with a C-terminal TEV-ProA-His<sub>7</sub> epitope tag (marked with *HIS3*) using pYM10 [39], similar to that previously described [17,29]. *UMPI* and *ERG6* (also called *ISE1*) were each separately deleted via a one-step replacement, amplifying the *ump1::kanMX4* or *erg6::kanMX4* cassette from the deletion library and transforming the product into the appropriate strain background [40]. The *BAR1* gene was disrupted by a one-step gene replacement using digested pZV77 to aid in arresting cells with  $\alpha$  factor (gift from S. Bell). The multicopy vector, pMRT7 (pCK322), contains the

*P<sub>CUP1</sub>-myc-UBI* expression cassette and the *URA3* marker [41] (gift from C. Kaiser). All cassettes and plasmids were introduced through a standard lithium acetate protocol [42]. Oligonucleotide sequences that were used in strain construction are available up request.

## 2.2. Cell cycle arrest

Cells were grown in YEPD media at 30°C with the exception of *ump1Δ* strains that were grown at 25°C. When the culture reached an OD of 0.5, the cells were split into two cultures for arrest, one G1 arrested with  $\alpha$  factor (50 ng/ml) and the other G2/M arrested with nocodazole (15μg/ml). Cells were treated for 3 to 4 hours prior to starting the assays.

## 2.3. Immunoblot

Protein extracts were made using a trichloroacetic acid (TCA) procedure similar to that published [39]. TCA precipitations were run on 7.5% SDS-PAGE gels (Lonza), and the immunoprecipitation samples were run on NuPAGE 3–8% tris-acetate gels (Invitrogen) before being transferred to polyvinylidene difluoride membranes (PVDF, Immobilon-P; Millipore). PVDF membranes were probed with rabbit peroxidase-anti-peroxidase soluble complex (PAP, Sigma) for ProA-tagged proteins and anti-3-phosphoglycerate kinase (yeast), mouse IgG, monoclonal antibody (anti-PGK, Molecular Probes) with mouse secondary for the Pgk1 control.

## 2.4. Flow cytometry

Cells were prepped as in [43] and analyzed on a Becton Dickinson FACSCalibur flow cytometer.

## 2.5. Cycloheximide chase assay

G1 or G2/M arrested cells were treated with cycloheximide (Sigma) (50 μg/ml) after the full 3 to 4 hours for arrest. For logarithmic growing cells, cultures were grown to an equivalent O.D. of 0.7, and then cycloheximide was added at 50 μg/ml to start the time course. At specific time points, cells were collected for flow cytometry (0.5 ml) or TCA precipitations (1.5 ml). Cells for TCA precipitations were immediately spun down, frozen in liquid nitrogen, and stored at –20°C.

## 2.6. Proteasome inhibitor assay

Cultures were treated with MG132 (Z-Leu-Leu-Leu-al, 50 μM, Sigma) for G1 and G2/M arrested cells. All experiments involving MG132 were completed in an *erg6Δ (ise1)* strain background to allow for MG132 permeability [44]. The cells were collected as described in Section 2.5.

## 2.7. Immunoprecipitation

Lysis and immunoprecipitations were carried out as described [16] with the following modifications. The immunoprecipitated strains were subcultured into 500 ml of SC media lacking uracil (for selection of pMRT7) or histidine (for the strain lacking the plasmid) and grown to an OD of 0.7 at 25°C. Copper sulfate (0.5 μM) was added to induce the expression of Myc-tagged ubiquitin. Cells were harvested in 50 ml tubes, washed in cold water, transferred to and pelleted in 2 ml screw cap tubes, and stored in lysis buffer at –80°C until the remaining steps of the lysis and immunoprecipitation protocol were completed.

### 3. Results

#### 3.1. *S. cerevisiae* Rev1 is unstable during the G1 and G2/M phases of the cell cycle

Given Rev1's profound cell cycle regulation, we wanted to know to what extent protein degradation contributes to the significant drop in Rev1 levels during the G1 stage of the cell cycle. Proteolysis influences the cell cycle regulation of many proteins in *S. cerevisiae* [45]. To monitor Rev1 protein stability, we inhibited translation by adding cycloheximide to arrested cells, collected samples at subsequent time points, and visualized ProA-tagged Rev1 by immunoblot. *REV1* was expressed under its native promoter at the endogenous locus, and the protein produced had a tag at its C-terminus that does not affect Rev1's contribution to survival and mutagenesis after UV damage [29]. Cells were arrested during the G1 stage of the cell cycle with  $\alpha$  factor and during the G2/M stages with nocodazole. Interestingly, Rev1 is unstable during both G1 when protein levels are the lowest and during G2/M when protein levels are highest (Fig. 1A and B). The flow cytometry data indicates that the cells remain in the arrested state even after the addition of cycloheximide (1N DNA content for G1 arrested cells, 2N DNA content for G2/M arrested cells) (Fig. 1C and D). Our measurements indicate that the half-life of Rev1 during a G1 arrest is 18 minutes and 32 minutes during a G2/M arrest (Fig. 1E and F) as determined by densitometry measurements and half-life calculations performed similarly to that described by Belle *et al.* [46]. Since Rev1 protein is degraded faster during G1 arrest when protein levels are the lowest than during G2/M arrest when protein levels are the highest, these results suggest that protein degradation acts as a necessary component of Rev1's striking cell cycle regulation. The slower degradation during G2/M, when Rev1 levels are highest, may be a means to limit Rev1 levels throughout the cell cycle.

#### 3.2. Inhibition of the proteasome causes an increase in Rev1 protein levels

After learning that Rev1 protein is unstable throughout the cell cycle with faster degradation in G1 than in G2/M, we asked whether this property was dependent on proteasomal degradation. We used the proteasome inhibitor, MG132, and assessed Rev1 protein levels following treatment. All experiments involving MG132 were performed in an *erg6* background to allow the drug to enter the cells. The steady-state level of Rev1 protein increased in the presence of the proteasome inhibitor for both G1 and G2/M arrested cells indicating that the proteasome function is associated with Rev1's degradation (Fig. 2A and B). Flow cytometry analysis confirmed that the *erg6* $\Delta$  cells arrest normally in the absence or presence of proteasome inhibitor (data not shown, also see Fig. 2E and F).

#### 3.3. The proteasome is responsible for Rev1's relatively short half-life

To monitor the effect that the disruption of proteasome function has on the half-life of Rev1 protein, G1 and G2/M arrested cells were pre-incubated with MG132 for 30 minutes. The time course began with the addition of cycloheximide. The half-life of Rev1 during G1 or G2/M arrest is longer in the presence of proteasome inhibitor than when translation is inhibited in its absence (Fig. 2C and D). The flow cytometry data does not show any abnormalities for the arrests (Fig. 2E and F). As seen in another report [47], MG132 does not completely prevent degradation of Rev1 in this cycloheximide-chase assay.

#### 3.4. Rev1 steady-state levels increase when proteasome function is defective

To further support our proteasome inhibitor results with a genetic approach, we utilized one of the viable mutants associated with the assembly of the proteasome (*ump1*), which lacks the gene encoding a maturation factor for the 20 S proteasome. In cells that have been arrested by  $\alpha$  factor or nocodazole, the steady-state levels of Rev1 are significantly greater in the *ump1* $\Delta$  cells than in wild type cells during G1 and are moderately increased during G2/

M (Fig. 3A and B at time points 0) similar to the effect observed after the addition of the proteasome inhibitor drug. These experiments were carried out at 25°C instead of 30°C to avoid problems with the temperature sensitivity of an *ump1Δ* strain. Rev1 levels are much greater in the *ump1Δ* background during the G1 arrest. In fact, Rev1 is not even visible in the blot of the wild type strain in the exposure selected to illustrate Rev1 levels in the *ump1Δ* mutant (Fig. 3A). A similar large increase in Rev1 levels was not observed after the addition of nocodazole, and the half-life of Rev1 in the *ump1Δ* strain during nocodazole arrest does not seem to significantly differ from the estimated half-life in wild type from Fig. 1F (Fig. 3B). However, this discrepancy appears to arise from an effect of the deletion of *UMP1* on the cellular response to nocodazole. The flow cytometry reveals that the *ump1Δ* cells arrest normally with  $\alpha$  factor but fail to properly arrest in nocodazole (Fig. 3C and D). This is most likely due to the pleiotropic nature of phenotypes as a result of deleting *UMP1*. Since more cells accumulate with 1N DNA content (when Rev1 levels are the lowest) in *ump1Δ* cells treated with nocodazole than with a wild type strain, the Rev1 protein levels shown in Fig. 3B are an underestimate of the levels actually present during G2/M. These results indicate that the proteasome is involved in the degradation of Rev1 and are consistent with the data obtained using MG132. The differences seen between the proteasome inhibitor and proteasome assembly mutant experiments can be attributed to the pleiotropic effects of deleting *UMP1* and only partial loss of proteasome function in both cases.

### 3.5. Higher migrating forms of Rev1 indicate targeting of the protein to the proteasome

After observing that the disruption of proteasome function affected Rev1 protein levels, we assessed whether Rev1 is modified to be targeted to the proteasome. In general, the attachment of a polyubiquitin chain of at least four Lys48-linked ubiquitins will target proteins for degradation by the 26S proteasome [48]. To detect higher migrating forms of Rev1 and subsequently test if these forms represent polyubiquitinated Rev1, we immunoprecipitated ProA-tagged Rev1 in an *ump1* strain background (Fig. 3E, lane 2). This strain also included myc-tagged ubiquitin under a copper-inducible promoter. We compared this immunoprecipitation to one of ProA-tagged Rev1 in an *UMP1* strain lacking myc-tagged ubiquitin (Fig. 3E, lane 1) and to another immunoprecipitation of non-tagged Rev1 in an *ump1* strain with myc-tagged ubiquitin (Fig. 3E, lane 3). When probing for ProA-tagged Rev1, a significant smear appears above the Rev1 band for the immunoprecipitation done in the presence of myc-tagged ubiquitin in an *ump1* background (Fig. 3E, lane 2). No Rev1 is detected when the protein lacks the ProA tag (Fig. 3E, lane 3).

The blot for myc-tagged ubiquitin with anti-myc after immunoprecipitation showed no bands and for anti-ubiquitin did not show any distinct bands or smears corresponding to Rev1's migration or higher that were specific to the ProA-tagged Rev1 immunoprecipitations (data not shown). In both cases, this could be due to Rev1 protein levels still being very low even after the immunoprecipitation, given that they are only detectable with by immunoblot of the ProA tag and not silver stain. The anti-myc or anti-ubiquitin may not be sensitive enough to detect the small fraction of Rev1 that is modified. Also, the anti-ubiquitin blot is not ideal, since the immunoblot had a very high background despite the fact that we had immunoprecipitated the protein of interest.

The higher migrating smear in lane 2 (Fig. 3E) implies that a modified form of Rev1 exists and is more easily detected in the *ump1Δ* strain background. Since the deletion of *ump1* is known to cause an accumulation of ubiquitin-conjugated proteins [33], it seems likely that the smear in lane 2 (Fig. 3E) represents polyubiquitinated Rev1. However, the very low levels of the Rev1 present in cells made these experiments technically challenging and thus we cannot conclusively rule out the possibility that the Rev1 modification associated with its degradation results from another protein modification.

## 4. Discussion

Since Rev1 protein levels fluctuate ~50-fold as cells progress through the cell cycle and transcript levels only undergo a ~3-fold change [29], we hypothesized that proteolysis contributes to the cell cycle regulation of Rev1. Indeed, we find that Rev1 is degraded during both G1 and G2/M in a manner that is dependent on the proteasome function, with the half-life during G1 being shorter than during G2/M. Faster degradation of Rev1 by the proteasome during G1 than during G2/M seems to be a major mechanism responsible for Rev1's striking cell cycle regulation. Aside from the transcriptional and proteasomal mechanisms of regulation, it is possible that some other posttranscriptional mechanism could also contribute to the significant increase of Rev1 protein levels during G2/M.

Degradation by the proteasome serves as an excellent mechanism to ensure the proper timing and positioning of a protein for action. Proteolysis eliminates the protein in an effective and irreversible way to prevent action and can destroy aberrant proteins. For potentially mutagenic TLS polymerases, ensuring that these polymerases do not interfere with the replicative DNA polymerases and only function when needed is critical to avoid widespread mutagenesis. These results demonstrate that *S. cerevisiae* uses proteasomal degradation to keep Rev1 protein levels low in general or low at specific times in the cell cycle. Similarly, TLS proteins are regulated by degradation in *E. coli*. For example, UmuC, the catalytic subunit of Pol V, undergoes proteolysis by the Lon protease [49].

In higher eukaryotes, more data is emerging that other DNA polymerases involved in base excision repair and capable of TLS, Pols  $\lambda$  and  $\beta$ , are targeted for proteasome-mediated degradation [50,51]. Interestingly, phosphorylation of Pol  $\lambda$  stabilizes the protein during late S and the G2/M stages of the cell cycle. These are the same cell cycle stages that Rev1 protein levels are the highest and phosphorylated in *S. cerevisiae* [30]. Future work will be required to know if Rev1 degradation is modulated by phosphorylation or if Rev1 is targeted for degradation through another protein modification.

A few examples of ubiquitin-independent proteasomal degradation exist such as Spe1 degradation mediated by the interaction with Oaz1 in *S. cerevisiae* [52]. If not polyubiquitination, the higher migrating form of Rev1 could represent another protein interacting with Rev1 or phosphorylation of specific residue(s) or the addition of alternative protein modifications. If the higher migrating form of Rev1 is due to polyubiquitination, then the attachment of polyubiquitin on Rev1 will involve an E2 and E3 ubiquitin ligase. The timing of the anaphase-promoting complex/cyclosome (APC/C) activity coincides with the lowest levels of Rev1 occurring during G1. The APC/C, a multisubunit ubiquitin-protein ligase, controls cell cycle progression by targeting key proteins for 26S proteasome degradation during late mitosis and G1 [45]. As shown here though, Rev1 is degraded throughout the cell cycle and therefore may not be a substrate for classical ubiquitin ligases associated with cell cycle regulation.

Our initial discovery that Rev1 protein levels peak during the G2/M phase of the cell cycle had seemed inconsistent with this translesion DNA polymerase functioning mainly during S phase. Furthermore, Lopes *et al.* [53] observed that in UV-irradiated *S. cerevisiae*, small ssDNA gaps accumulate along replicated duplexes that likely result from the repriming of DNA synthesis downstream of UV lesions on both leading and lagging strands. Their observation that TLS can help to counteract the accumulation of these gaps without affecting fork progression led them to suggest that the bulk of TLS takes behind replication forks and thus contributes postreplicatively to restore the integrity of replicated duplexes [53]. More recent studies have lent further support to the concept that TLS polymerases act post bulk genomic replication and during G2/M (in addition to during S phase) for full DNA damage

tolerance [31,32,54-56]. It seems likely that the control of Rev1 levels through cycle cycle-dependent proteosomal degradation is an important way of limiting the action of Rev1/3/7-dependent mutagenic TLS more to G2/M. Such a strategy can reduce the amount of mutagenesis occurring after DNA damage by delaying some portion of mutagenic TLS until after high fidelity repair and more accurate damage tolerance mechanisms have had a chance to act [29]. This would be especially important for cells undergoing replication and experiencing large amounts of DNA damage. Under these circumstances, DNA repair might not have enough time to completely remove all of the lesions before replication takes place, thereby leading to postreplicational gaps opposite lesions in G2/M.

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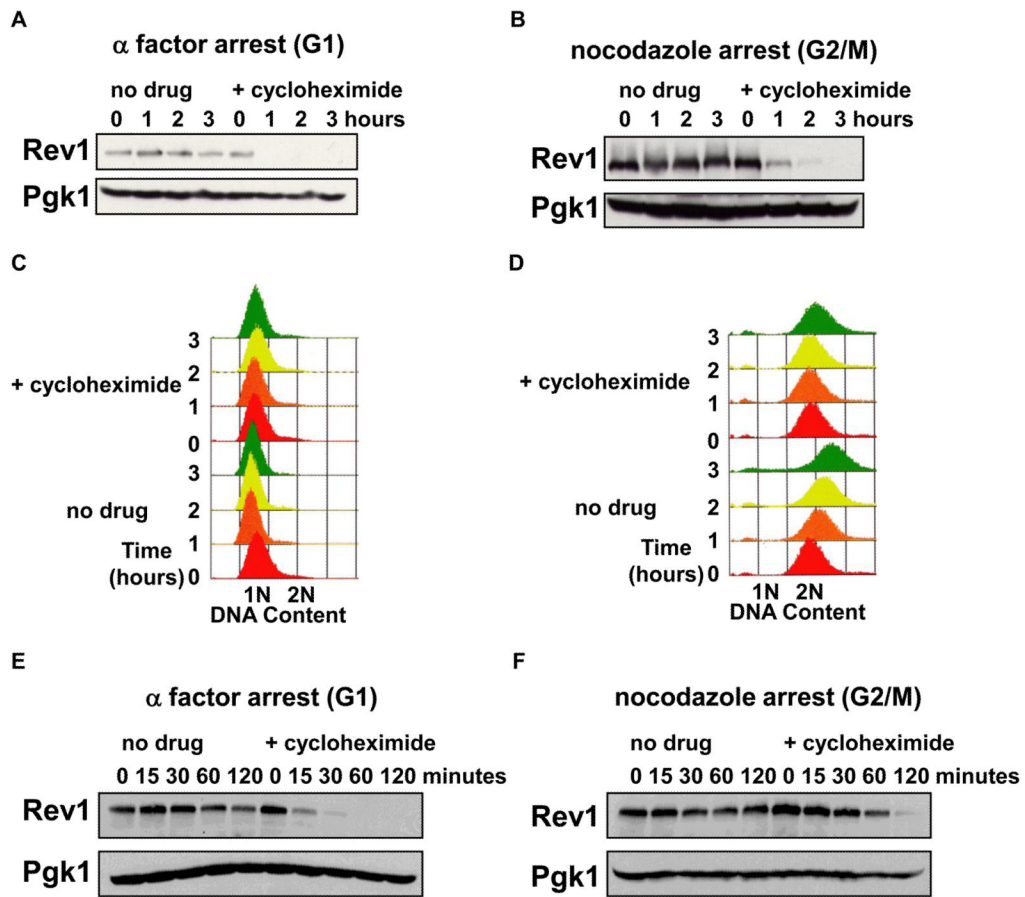
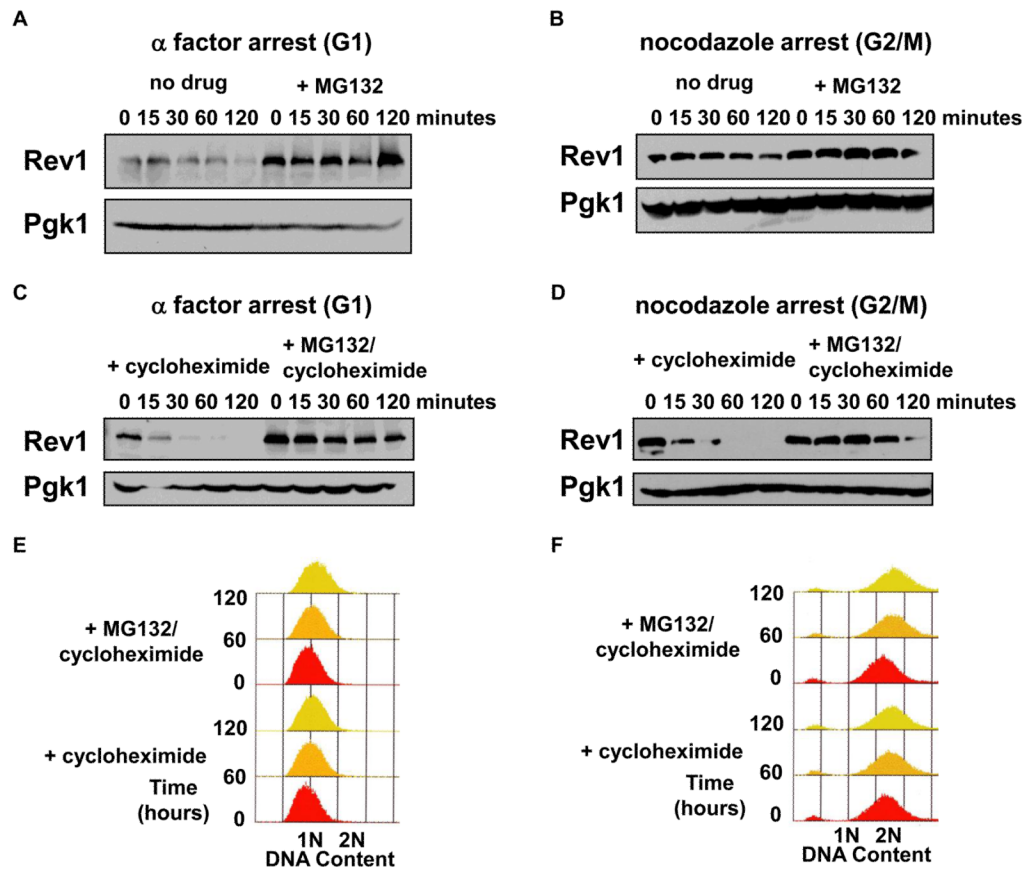


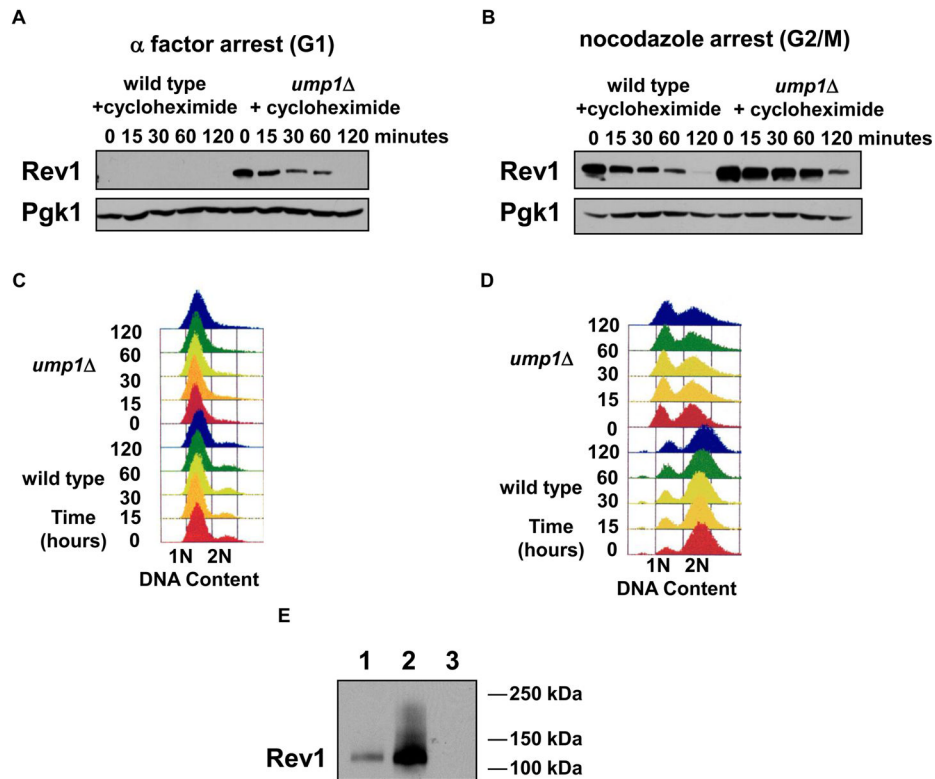
Fig. 1.

Rev1 protein experiences turnover during the G1 and G2/M phases of the cell cycle. (A) Rev1 levels decrease during G1 arrest after cycloheximide treatment. The cells from the Rev1-ProA strain were arrested with  $\alpha$  factor at 30°C, and then split into a cycloheximide treated culture and a non-treated culture before time points were collected each hour. Immunoblots are probed with PAP for the ProA-tagged Rev1 and with anti-Pgk1 for the Pgk1 loading control. (B) Rev1 levels decrease during G2/M arrest after cycloheximide treatment. The assay was performed as in (A), except that the arrest was done with nocodazole. (C) Rev1-ProA cells stay arrested after cycloheximide treatment with 1N DNA content. Flow cytometry data is shown for  $\alpha$  factor-arrested cells with and without cycloheximide. (D) Rev1-ProA cells remain in G2/M arrest after the addition of cycloheximide. Flow cytometry data is given for nocodazole-arrested cells in the presence and absence of cycloheximide. (E) The half-life of Rev1 in G1 arrested cells is less than in G2/M arrested cells. The half-life was estimated to be 18 minutes. The assay for the immunoblot was carried out as in (A), except that time points were taken at smaller intervals. (F) The half-life of Rev1 in G2/M arrested cells is greater than in G1 arrested cells. The half-life is estimated to be 32 minutes. The assay was completed as in (E), except that cells were arrested with nocodazole.



**Fig. 2.**

Normal proteasome function regulates Rev1 protein levels. (A) Rev1 protein levels significantly increase in the presence of the proteasome inhibitor, MG132, in G1 arrested cells. Cells were arrested with  $\alpha$  factor at 30°C, and then divided into a MG132 treated and non-treated cultures before time points were taken. The strain background is *erg6Δ*. The immunoblot was probed with PAP for ProA-tagged Rev1 and anti-Pgk1 for the Pgk1 loading control. (B) Rev1 protein levels are also greater after MG132 treatment in G2/M arrested cells. The assay was carried out as in (A), apart from the arrest being done with nocodazole. (C) Rev1 protein levels are stabilized when the proteasome is inhibited during G1. After a pre-incubation of  $\alpha$  factor-arrested cells with MG132 at 30°C, cycloheximide was added to start the time course. The strain background is *erg6Δ*. The immunoblot shows Pro-tagged Rev1 and the loading control, Pgk1. (D) The half-life of Rev1 during G2/M is also lengthened in the presence of MG132. The assay was performed as in (C), but cells were arrested with nocodazole. (E) Cells maintain 1N DNA content after MG132 and cycloheximide treatment. Flow cytometry data is shown for select time points in the presence of cycloheximide alone or cycloheximide and MG132. (F) MG132 does not affect the nocodazole arrest. Flow cytometry data is depicted as in (E), apart from the cells being in a G2/M arrest.



**Fig. 3.** The steady-state levels of Rev1 are greater in a proteasome-defective strain background, and a higher migrating form of Rev1 accumulates. (A) Rev1 protein levels increase in the *ump1Δ* background during G1 arrest. Cells were arrested with  $\alpha$  factor at 25°C, and then treated with cycloheximide to start the time course using the Rev1-ProA or Rev1-ProA *ump1Δ* strains. The immunoblot shows ProA-tagged Rev1 and Pgk1 as a loading control. (B) Rev1 protein levels are greater in the *ump1Δ* background during G2/M arrest. The assay was completed as in (A), except that the cells were arrested with nocodazole. (C) The *ump1Δ* strain background does not change the ability of cells to arrest with 1N DNA content. Flow cytometry is shown for the Rev1-ProA or Rev1-ProA *ump1Δ* strains during  $\alpha$  factor arrest. (D) The *ump1Δ* cells accumulate more with 1N DNA content during nocodazole arrest than the wild type background. Flow cytometry shows the DNA content for G2/M arrested cells of the Rev1-ProA or Rev1-ProA *ump1Δ* strains. (E) A higher migrating form of Rev1 exists when *UMP1* is deleted and myc-tagged ubiquitin is overexpressed. Strains are Rev1-ProA (lane 1), Rev1-ProA *ump1Δ* + pMRT7 (lane 2), and W1588-4C *ump1Δ* + pMRT7 (lane 3). The immunoblot for the Rev1-ProA immunoprecipitation samples were probed with PAP for ProA-tagged Rev1.

**Table 1**

Yeast strains used in this study.

Strain	Relevant Genotype	Source
YLW70	W1588-4C <i>bar1Δ::LEU2</i>	[16]
Rev1-ProA	W1588-4C <i>bar1Δ::LEU2 REV1-TEV-ProA-7HIS</i>	[29]
Rev1-ProA <i>ump1Δ</i>	same as Rev1-ProA but <i>ump1Δ::kanMX4</i>	This study
Rev1-ProA <i>erg6Δ</i>	same as Rev1-ProA but <i>erg6Δ::kanMX4</i>	This study
W1588-4C <i>ump1Δ</i> + pMRT7	W1588-4C <i>bar1Δ::LEU2 ump1Δ::kanMX4</i> p <i>P<sub>CUP1</sub>-myc-UBI</i>	This study
Rev1-ProA <i>ump1Δ</i> + pMRT7	Same as Rev1-ProA <i>ump1Δ</i> with p <i>P<sub>CUP1</sub>-myc-UBI</i>	This study

All strains are derivatives of W1588-4C (*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5*) [38].