Cell, Vol. 102, 67–76, July 7, 2000, Copyright ©2000 by Cell Press

# Cell Cycle–Regulated Modification of the Ribosome by a Variant Multiubiquitin Chain

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## Summary

**Ubiquitin is ligated to L28, a component of the large ribosomal subunit, to form the most abundant ubiquitin–protein conjugate in *S. cerevisiae*. The human ortholog of L28 is also ubiquitinated, indicating that this modification is highly conserved in evolution. During S phase of the yeast cell cycle, L28 is strongly ubiquitinated, while reduced levels of L28 ubiquitination are observed in $G_1$ cells. L28 ubiquitination is inhibited by a Lys63 to Arg substitution in ubiquitin, indicating that L28 is modified by a variant, Lys63-linked multiubiquitin chain. The *K63R* mutant of ubiquitin displays defects in ribosomal function in vivo and in vitro, including a dramatic sensitivity to translational inhibitors. L28, like other ribosomal proteins, is metabolically stable. Therefore, these data suggest a regulatory role for multiubiquitin chains that is reversible and does not function to target the acceptor protein for degradation.**

## Introduction

Ubiquitination is involved in a large number of biological processes, including cell cycle control, DNA repair, inflammation, morphogenesis, transcriptional control, and antigen presentation (Hochstrasser, 1996; Hershko and Ciechanover, 1998; Peters et al., 1998). The known role of ubiquitin in these processes is to serve as a molecular tag to target proteins for degradation. Cytosolic proteins are targeted for degradation by the proteasome (Hochstrasser, 1996; Hershko and Ciechanover, 1998; Peters et al., 1998), whereas ubiquitination can target cell-surface proteins for endocytosis and subsequent degradation in the lysosome (Hicke, 1997).

Formation of ubiquitin–protein conjugates involves ligation of the carboxy terminus of ubiquitin to amino groups of the target protein (Hershko and Ciechanover, 1998). Ubiquitination is catalyzed by a multienzyme cascade. In an initial, ATP-dependent reaction, a high energy thioester bond is formed between the carboxy terminus of ubiquitin and the active site cysteine of a ubiquitin activating enzyme, or E1. Subsequently, ubiquitin is transferred to the active site cysteine of a ubiquitin conjugating enzyme (E2). Ubiquitin may be then directly ligated to the substrate protein or ligation may be mediated by a ubiquitin protein ligase (E3) that confers substrate specificity (Hershko and Ciechanover, 1998; Laney and Hochstrasser, 1999). An additional factor, E4, assists in the elongation of some multiubiquitin chains (Koegl et al., 1999). The proteasome preferentially degrades multiubiquitinated substrates in which the carboxy terminus of one ubiquitin is ligated to lysine 48 of the previously attached ubiquitin (Chau et al., 1989; Pickart, 1997; Thrower et al., 2000).

Ubiquitin has six other lysine residues, and we and others have shown that many of these residues can also be used in the formation of multiubiquitin chains (Arnason et al. 1994; Spence et al., 1995; Johnson et al., 1995; Baboshina and Haas, 1996; Galan and Haguenauer-Tsapis, 1996; Springael et al., 1999, Hofmann and Pickart, 1999; Mastrandrea et al., 1999; for review, see Dubiel and Gordon, 1999). In particular, we constructed yeast strains in which each lysine in ubiquitin was individually substituted with arginine. One of these mutants, *ubiK63R*, was sensitive to ultraviolet light and ethyl methane sulfonate, and defective for UV-induced mutagenesis (Spence et al., 1995). The structure of K63-linked ubiquitin chains should differ significantly from that of K48-linked chains and therefore these two types of chains are likely to perform distinct functions. K63-linked chains can be synthesized by a heterodimer of Ubc13, a ubiquitin conjugating enzyme, and Mms2, a ubiquitin conjugating enzyme variant (UEV; Hofmann and Pickart, 1999). UEVs are homologous to E2s but lack the active site cysteine (Sancho et al., 1998; Xiao et al., 1998; Hofmann and Pickart, 1999). The DNA damage sensitivity of the *ubiK63R* strain can be accounted for by Ubc13/Mms2-dependent formation of K63-linked multiubiquitin chains, but the relevant acceptor protein remains to be identified. Two substrates for K63-linked chains have been identified as the plasma membrane permeases Fur4 (Galan and Haguenauer-Tsapis, 1996) and Gap1 (Springael et al., 1999). *ubiK63R* mutants are deficient in the endocytosis and lysosomal degradation of these proteins.

We have found that the *ubiK63R* mutation resulted in the disappearance of the ubiquitinated forms of a low molecular mass, basic protein (Spence et al., 1995). In this paper, we identify this substrate as ribosomal protein L28. We show that L28 is ubiquitinated in intact ribosomes and that heavily ubiquitinated ribosomes are active in translation in vitro. Moreover, the *ubiK63R* mutant displays defects in translation, including a hypersensitivity to translational inhibitors and polysome instability. The ubiquitination of L28 is cell cycle dependent in that it is low in $G_0$ and $G_1$ and high in S phase. Since L28 is a stable protein (Gorenstein and Warner, 1976, 1977; Warner et al., 1985), these data suggest that a variant multiubiquitin chain may function in a reversible, nonproteolytic manner.

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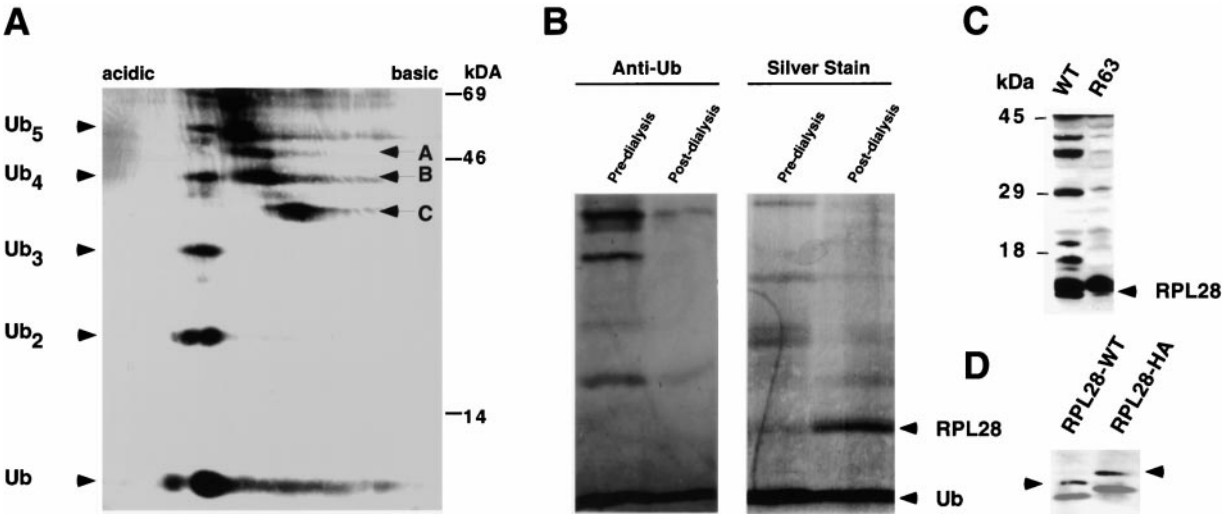


Figure 1. Identification of L28 as a Major Substrate for Ubiquitination

(A) Two-dimensional IEF/SDS-PAGE analysis of affinity-purified total cellular ubiquitin conjugates. JSY171 cells, expressing 6His-*myc*-ubiquitin, were UV irradiated and lysed in the presence of 8 M urea. The clarified lysate was subjected to chromatography on Ni-NTA resin, and the eluate analyzed by IEF/SDS-PAGE, followed by immunoblotting with anti-ubiquitin antibody. The pH range of the IEF gel was 3–10 (see Spence et al., 1995). Spots A, B, and C correspond to progressively multiubiquitinated forms of L28, as shown below. Ub, free ubiquitin; Ub2-Ub4, free ubiquitin chains. The mobility of electrophoretic markers is shown at right, with molecular masses given in kDa.

(B) Disassembly of affinity-purified ubiquitin conjugates releases a single major protein, identified as L28 by Edman degradation. The Ni-NTA column eluate was dialyzed against a neutral buffer to remove urea. After resolving the dialysate by SDS-PAGE, parallel gels were either silver stained to reveal total proteins or immunoblotted with anti-ubiquitin antibody.

(C) Immunoblot analysis of extracts from wild-type (SUB280) and *ubiK63R* (SUB413) cells, prepared according to Tarun and Sachs (1995). The filter was probed with an antibody to L28. An arrow indicates unmodified L28. The mobility of electrophoretic markers is shown at left, with molecular masses given in kDa.

(D) Epitope tagging of L28 confirms that electrophoretically retarded species reacting with the anti-L28 antibody contain L28 protein. Arrows indicate presumptive monoubiquitinated forms of wild-type and tagged L28.

## Results

### Identification of L28 as a Substrate for K63-Linked Chain Formation

Formation of the most abundant ubiquitin–protein conjugate in yeast is abolished in the *ubiK63R* mutant (Spence et al., 1995). To identify the substrate component of this conjugate, a yeast strain was constructed which expresses ubiquitin with an N-terminal extension of six histidines followed by a *myc* epitope (see Beers and Callis, 1993). Ubiquitin conjugates were isolated on a nickel-NTA column and eluted at low pH in the presence of 8 M urea. Immunoblots of two-dimensional gels of the eluate, as probed by anti-ubiquitin antibodies, revealed a vertical series of free ubiquitin chains and a diagonal series of spots representing multiply ubiquitinated forms of a basic ubiquitin–protein conjugate (Figure 1A). Upon dialysis to remove the urea, the ubiquitin conjugates were disassembled, presumably by a copurifying ubiquitin-specific isopeptidase, releasing a single detectable protein that was not recognized by antibodies to ubiquitin (Figure 1B). The N-terminal sequence of this 16–19 kDa protein was determined to be PSRFTKTRKHRGHVSAGKGRIIKHRKH, which matches the mature N terminus of ribosomal protein L28 at 26 of 27 residues (Kaufer et al., 1983). (In alternative nomenclatures, this protein is referred to as rp44, L29, and as the product of the *CYH2* gene [Mager et al., 1997]).

To confirm the ubiquitination of L28, we prepared antibodies to this protein. Immunoblots performed with these antibodies revealed a ladder of modified forms of L28, which were, as expected, eliminated in the *ubiK63R* strain (Figure 1C). In addition, epitope tagging of L28 results in an equivalent size shift in both the unmodified and apparent monoubiquitinated form, indicating that components of both bands are derived from the same gene (Figure 1D).

### Ubiquitinated L28 Is Associated with 80S Ribosomes and Polysomes

Ribosomal proteins, once assembled into ribosomes, are stable, but those that have not been incorporated into ribosomes are rapidly degraded, most likely through the ubiquitin pathway (Gorenstein and Warner, 1976, 1977; Warner et al., 1985; Maicas et al., 1988). To test whether this phenomenon can explain the ubiquitination of L28, we determined whether L28 is ubiquitinated when present in ribosomes. Cell extracts were prepared from exponentially growing wild-type and *ubiK63R* cells and analyzed by sucrose gradient sedimentation. Proteins in each fraction of the gradient were resolved by SDS-PAGE and immunoblotted. In immunoblots of wild-type extracts visualized with antibody to L28, a ladder of slower-migrating forms was observed (Figure 2A, bands A–C) in addition to unmodified L28. The modified forms of L28 displayed a complex sedimentation pattern, with peak intensities corresponding to those of monosomes and polysomes. In contrast, only a single band, corresponding to unmodified L28, was detected in immunoblots of *ubiK63R* cells (Figure 2B). These data indicate

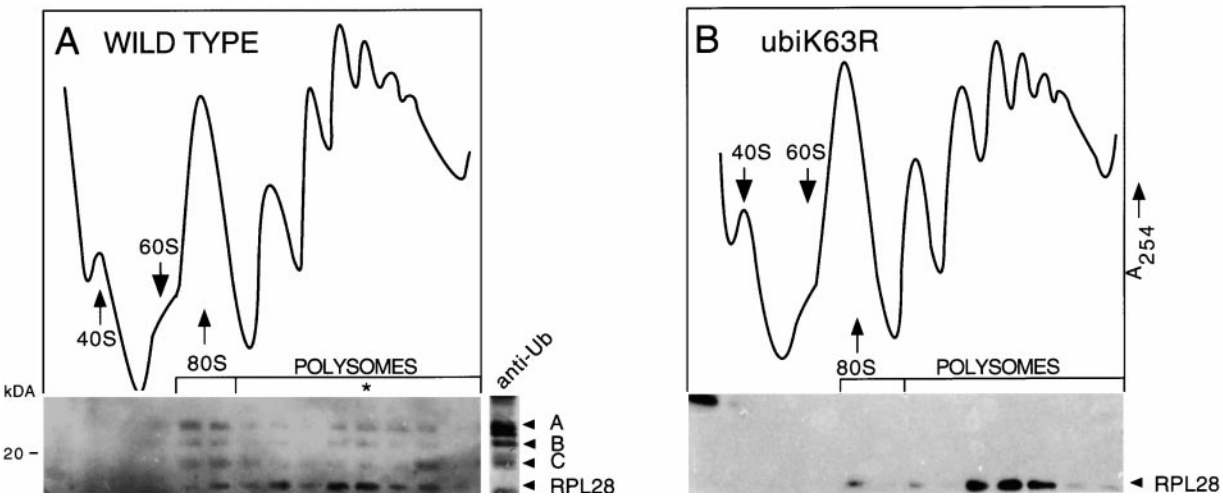


Figure 2. Ribosome-Associated L28 Is Modified by a Variant Multiubiquitin Chain

Polysomes from cycloheximide-treated cells were analyzed on sucrose gradients, followed by SDS-PAGE and immunoblotting. Gradient profiles are shown with the top of the gradient to the left and are aligned with the blots. The blots were probed with antibodies to L28. (A), wild-type extract; (B), *ubiK63R* extract. The side panel to the right of (A) shows the pattern of ubiquitin-reactive bands observed on the same blot (the side panel corresponds to the lane marked with an asterisk). These data indicate that bands A–C represent ubiquitinated forms of L28. The band between A and B that is detected using the anti-ubiquitin but not anti-L28 antibody is likely to represent a free multiubiquitin chain (data not shown). Some variation in L28 amounts from fraction to fraction resulted from incomplete recovery of L28 during concentration of gradient fractions for electrophoresis.

that L28 can be modified by a variant multiubiquitin chain in actively translating ribosomes.

**Ubiquitination of the Human Homolog of L28**

If the ubiquitination of L28 were functionally significant, it would be expected to be conserved in evolution. Although ribosomes have been subjected to extensive biochemical analysis, ubiquitination of the ribosome has not previously been reported for any species. We found that the antibody to yeast L28, which is raised against evolutionarily conserved amino acid sequences, detected a ribosomal protein in extracts from human cells (see below). To test whether this protein was the human counterpart of yeast L28, we expressed the orthologous human gene (RPL27a) in *E. coli*. Recombinant L27a was detected by the antibody and comigrated electrophoretically with the major form of the ribosomal protein recognized by the antibody (not shown).

To test for posttranslational modification of L27a, cytoplasmic extracts from human 293 cells were resolved by sucrose gradient analysis, and the fractions subjected to immunoblotting with the antibody to yeast L28 (Figure 3A). A complex set of high molecular weight bands was found to cosediment with ribosomal protein L27a, suggesting that the human homolog of L28 is posttranslationally modified. When the same blot was reprobed with antibodies to ubiquitin, many bands were observed, most of which could be aligned with the modified forms of L27a (Figure 3B). To confirm the presence of L27a within these conjugates, a FLAG epitope was appended to the C terminus of the protein. FLAG-L27a was expressed in 293T cells, and cytoplasmic extracts from these cells were fractionated on sucrose gradients. Figure 3C shows an immunoblot analysis of an 80S monosome containing fraction from the gradient. The results indicate that ribosome associated FLAG-L27a is posttranslationally modified, apparently by ubiquitination, insofar as most of the modified forms of FLAG-L27a can be aligned with anti-ubiquitin bands from the same blot.

To confirm that the modified forms of L27a contain ubiquitin, as opposed to a cross-reacting, ubiquitin-like protein, 293T cells were transfected with a plasmid expressing 6His-*myc*-tagged ubiquitin (Ward et al., 1994). Total cellular ubiquitin–protein conjugates were affinity purified from these cells using a Ni-NTA column. L27a containing species of greater than 40 kDa were retained on this column (Figure 3D). These high molecular weight forms of L27a are shown in Figure 3A to be ribosomally associated. Modified forms of L27a that migrate in the 25–35 kDa region of the gel were not efficiently purified on Ni-NTA columns, possibly because retention of modified L27a on the column is enhanced in the presence of multiple 6His-*myc*-ubiquitin molecules. In summary, these experiments indicate that the capacity to be modified by multiubiquitination when present within mature ribosomes is common to L28 orthologs from yeast to humans.

**The *ubiK63R* Mutant Is Hypersensitive to Translational Inhibitors**

Because L28 is ubiquitinated on 80S ribosomes and polysomes, *ubiK63R* and wild-type yeast cells were tested for their sensitivity to translational inhibitors. This phenotype has often been used to reveal underlying defects in translation (Masurekar et al., 1981; Nelson et al., 1992). L28 has been localized to the peptidyl transferase center of the ribosome (Xiang and Lee, 1989). Accordingly, *ubiK63R* mutants were found to be extremely sensitive to the transpeptidylation inhibitors anisomycin and trichodermin (Figure 4A and data not shown). The target of these inhibitors is not L28 but

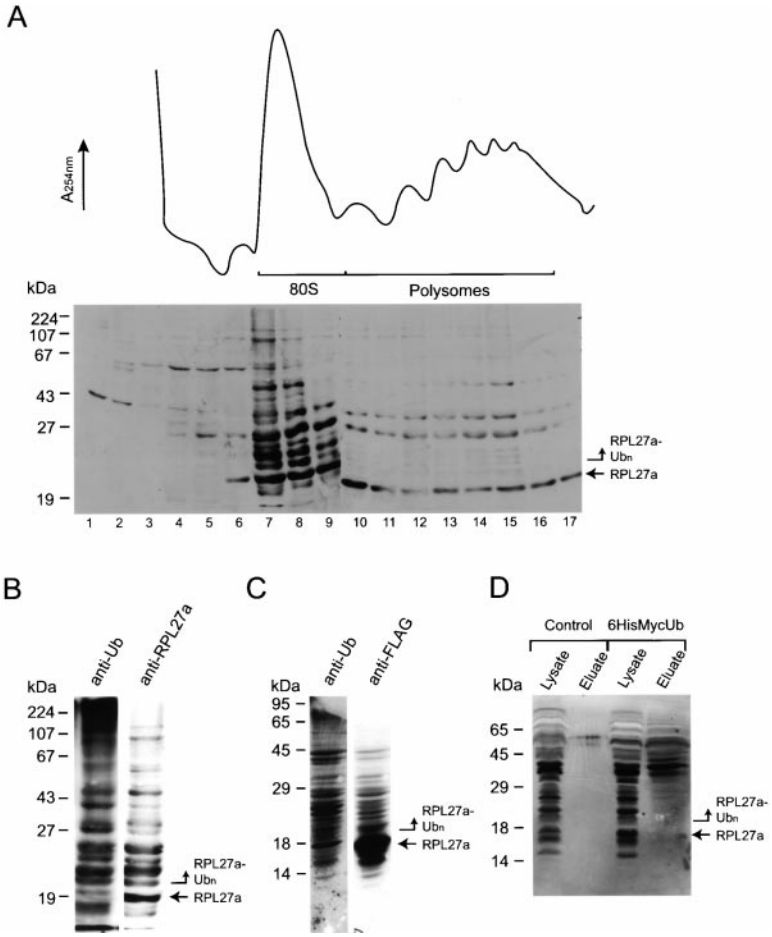


Figure 3. Ribosome-Associated L29 Is Modified by a Multiubiquitin Chain in Human Cells

(A) Cytoplasmic extract from 293 cells was subjected to sucrose gradient analysis, followed by SDS-PAGE and immunoblotting with antibody to yeast L28. Molecular mass standards are shown at left. The A254 profile shown at top is aligned with the blot.

(B) Lane 7 from (A), probed with antibodies to either ubiquitin or yeast L28, as indicated. A close correspondence between ubiquitin containing and RPL27a containing bands is apparent. However, these data do not exclude the possibility that ribosomal proteins other than RPL27a are also ubiquitinated.

(C) Extracts were prepared from 293T cells expressing FLAG-tagged L27a, and fractionated by sucrose gradient analysis. A sucrose gradient fraction corresponding to the 80S peak was immunoblotted and sequentially probed with antibodies to the FLAG epitope and to ubiquitin. The major bands seen with anti-FLAG antibody were not observed in the absence of FLAG-RPL27a expression (data not shown).

(D) 293T cells were transfected with a plasmid expressing either 6His-*myc*-ubiquitin or a control vector that does not express ubiquitin. Cytoplasmic extracts were fractionated using Ni-NTA resin. Proteins eluted from the column by imidazole were resolved by SDS-PAGE, immunoblotted, and probed with antibody to yeast L28. The effectiveness of Ni-NTA fractionation was confirmed by reprobing the blot with anti-*myc* (data not shown).

rather the 25S rRNA, as well as L3, another component of the peptidyl transferase center (Jimenez et al., 1975; Fried and Warner, 1981; Sweeney et al., 1991; Rodriguez-Fonseca et al., 1995). The mutants are moderately sensitive to rapamycin and geneticin. rRNA molecules were present at wild-type levels in the mutants and appeared to be properly processed (data not shown), suggesting that the sensitivity of *ubiK63R* mutants to translational inhibitors does not reflect a defect in ribosome biogenesis. The sensitivity to translational inhibitors is

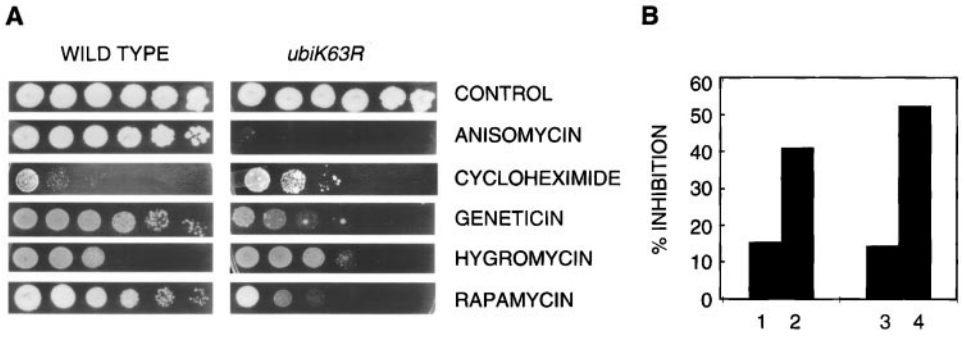


Figure 4. Hypersensitivity of *ubiK63R* Mutants to Antibiotics That Inhibit Translation

(A) *ubiK63R* (SUB413) and wild-type (SUB280) cells were grown to exponential phase. Serial 5-fold dilutions were spotted onto YPD plates containing the indicated antibiotics. After incubation for 3 to 8 days, the plates were photographed. See Experimental Procedures for details.

(B) The *ubiK63R* mutation enhances the sensitivity of protein synthesis to anisomycin. Wild-type cells (lanes 1 and 3) and *ubiK63R* mutants (lanes 2 and 4) were grown to exponential phase in methionine-free synthetic medium. Anisomycin was added at 25 μg/ml, followed 15 min later by [$^{35}$S]methionine. After 5 min of labeling, total protein synthesis was measured as described (Ciechanover et al., 1984). In samples 3 and 4, cells were arrested in S phase by incubation in the presence of 200 mM hydroxyurea for 4 hr prior to the addition of anisomycin. The percent inhibition of protein synthesis is shown for each sample.

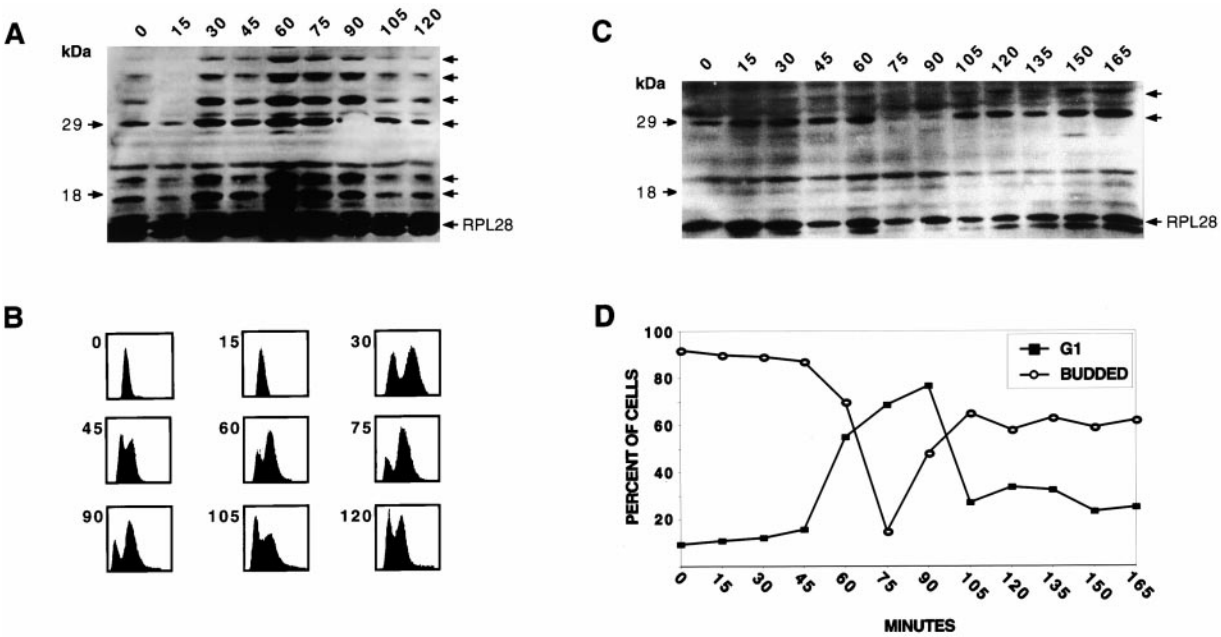


Figure 5. The Multiubiquitination of L28 Is Cell Cycle Regulated

(A and B) Daub15 cells were arrested in $G_1$ with $\alpha$ factor. At 15 min time intervals after removal of $\alpha$ factor, the cells were lysed and aliquots were taken and processed for SDS-PAGE/immunoblotting (A) and FACS analysis (B). The blot was probed with antibodies to L28. The molecular masses of electrophoretic markers are given in kDa at the left. Unmodified L28 and ubiquitin-L28 conjugates are indicated at right.

(C and D) SUB280 cells were arrested in M phase with nocodazole. At 15 min intervals following release from arrest, aliquots were taken for immunoblot analysis with anti-L28 antibodies (C), FACS analysis (D), and budding index determination (D).

specific insofar as *ubiK63R* mutants do not exhibit a broad spectrum drug sensitivity; they are slightly more resistant to hygromycin and cycloheximide (Figure 4A), and there is no differential sensitivity to paromomycin or neomycin (data not shown). In addition, substitutions of other residues of ubiquitin (K6, K11, K27, K29, and K33), many of which engender a strong growth defect (Spence et al., 1995), produced no sensitivity to anisomycin (data not shown). Thus, anisomycin sensitivity is not simply the result of attenuated ubiquitin function, but rather it is a specific phenotype of the *K63R* mutation, presumably owing to the mutant's inability to form alternate multiubiquitin chains.

To clarify the mechanism of growth suppression by anisomycin in the *ubiK63R* mutant, the level of inhibition of protein synthesis was quantitated following exposure to the drug. Translation was repressed approximately 3-fold more in the mutant cells than in the wild type (Figure 4B), indicating that the influence of K63 chains on drug sensitivity reflects a direct alteration of ribosome function. The strength of this effect was enhanced when cells were arrested in S phase with hydroxyurea, as compared to exponentially growing cells (Figure 4B). This might reflect higher chain levels seen in S phase (Figure 5) and in hydroxyurea arrested cells (data not shown).

### L28 Ubiquitination Is Cell Cycle Regulated

By inhibiting the Tor kinases, rapamycin arrests the cell cycle of yeast in $G_1$ (Barbet et al., 1996). Thus, the sensitivity of *ubiK63R* mutants to rapamycin suggested that L28 ubiquitination may be cell cycle regulated. Yeast cells were therefore synchronized by $\alpha$ factor arrest, released from the cell cycle block, and, at 15 min intervals, aliquots were taken and analyzed by immunoblotting and flow cytometry. During the arrest period, as well as the remainder of $G_1$, L28 conjugate levels were low. Following the onset of S phase, L28 was strongly ubiquitinated (Figures 5A and 5B). The levels of L28 ubiquitination were reduced when $\alpha$ factor arrested cells exited mitosis and reentered $G_1$ (Figures 5A and 5B). Similar results were obtained when yeast cells were released from a nocodazole-induced mitotic block (Figures 5C and 5D). In this case, L28 ubiquitination was high during the block and fell once cells progressed into $G_1$, and the budding index reached its lowest value. Thus, inhibition of cell cycle progression does not itself result in reduced L28 chain levels, but rather the reduced L28 ubiquitination observed during the $\alpha$ factor arrest reflects the cell cycle phase of the block. With the onset of S phase and the formation of new buds, L28 ubiquitination fully recovered (Figures 5C and 5D).

### Ubiquitinated Ribosomes Are Active in Translation In Vitro

The presence of a multiubiquitin chain at the peptidyl transferase center might be expected to interfere with the elongation step of protein synthesis. To test whether the translational efficiency of ribosomes is reduced by ubiquitination, extracts were prepared from cells arrested in $G_1$ with $\alpha$ factor, or in S phase with hydroxyurea (Wang et al., 1997). Immunoblotting confirmed that, in extracts from $\alpha$ factor arrested cells, L28 was primarily

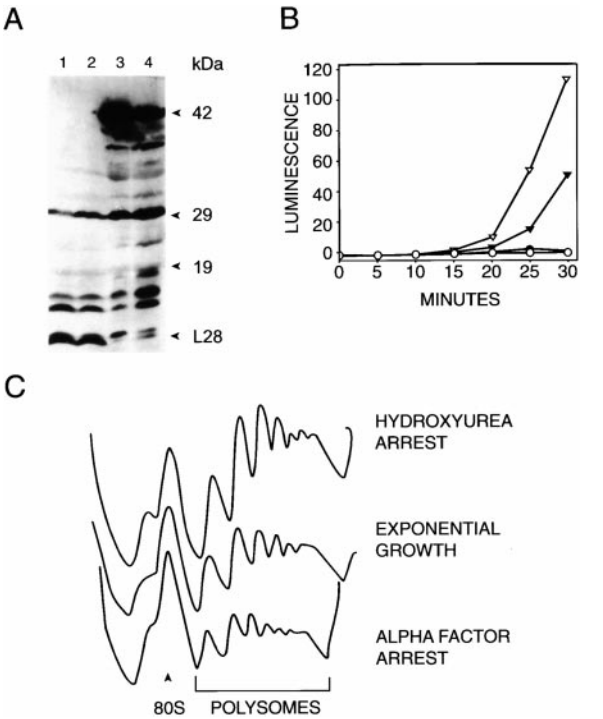


Figure 6. In Vitro Translation of Luciferase mRNA in Cell Extracts in Which L28 Is Multiubiquitinated

(A) Immunoblot of Daub15 cell extracts used for in vitro translation, probed with antibody to L28. Extracts were subjected to immunoblotting following Sephadex G25 chromatography and immediately prior to in vitro translation (see Experimental Procedures). L28 is heavily ubiquitinated in exponentially growing cell extracts and extracts from hydroxyurea arrested cells. Sources of extracts were: lane 1, cells were harvested 20 min after release from $\alpha$ factor arrest; lane 2, $\alpha$ factor arrested cells; lane 3, exponentially growing cells; and lane 4, hydroxyurea arrested cells.

(B) In vitro translation of capped, polyadenylated luciferase mRNA by the extracts shown in panel (A), as determined by the measurement of luminescence over time. Sources of extracts were: open circles, $\alpha$ factor arrested cells; closed circles, $\alpha$ factor arrest, 20 min postrelease; closed triangles, exponentially growing cells; open triangles, hydroxyurea arrested cells.

(C) Polysome profiles from $\alpha$ factor arrested cells, exponentially growing cells, and hydroxyurea arrested cells as analyzed on 20% to 47% sucrose gradients. A254 readings are shown, with sedimentation from left to right. The ratio of polysomes to 80S is reduced in $\alpha$ factor arrested cells. Control experiments confirmed that incorporation of radiolabeled methinione into protein in vivo is similarly reduced in $\alpha$ factor arrested cells.

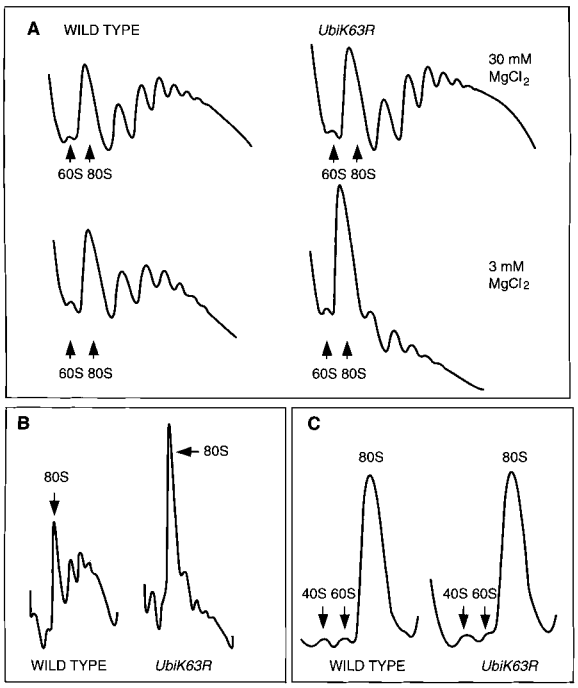


Figure 7. Polysomes from *ubiK63R* Cells Are Unstable In Vitro

(A) *ubiK63R* polysomes are hypersensitive to low magnesium concentrations. Wild-type (SUB280) and *ubiK63R* (SUB413) cells were treated with 50 $\mu$g/ml cycloheximide for 20 min, and lysed in a buffer containing 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and either 3 mM or 30 mM $MgCl_2$. Clarified lysates were loaded onto 20% to 47% sucrose gradients prepared with the same buffer and with the same magnesium concentration as the lysate.

(B) As (A), except that wild-type and *ubiK63R* cells were treated with 10 $\mu$g/ml cycloheximide for 20 min prior to polysome preparation as described above. (30 mM $MgCl_2$ was used in the lysis and sucrose gradient buffers, which yields polysome profiles equivalent to those at 12 mM $MgCl_2$ [data not shown]).

(C) Cells were treated with 15 mM sodium azide for 20 min prior to harvesting, and lysed with 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 3 mM $MgCl_2$. Lysates were loaded onto 15% to 35% sucrose gradients prepared with the same buffer. In all gradients, A254 readings are shown, and sedimentation is from left to right.

unmodified, with a subpopulation of mono- and diubiquitinated forms; whereas approximately 95% of the L28 was ubiquitinated in extracts from hydroxyurea treated cells (Figure 6A). Translation of capped, polyadenylated luciferase mRNA was low in extracts from $\alpha$ factor arrested cells, intermediate in exponentially growing cell extracts, and highest in extracts from hydroxyurea treated cells in which L28 was heavily ubiquitinated (Figure 6B). Polysome profiles showed a similar pattern; the highest levels were observed in extracts from hydroxyurea treated cells, and lowest in $\alpha$ factor arrested cells (Figure 6C). In summary, the most efficient translation was observed in extracts in which $>$95% of ribosomes were ubiquitinated. These experiments indicate that ubiquitination at the peptidyl transferase center does not inhibit translation, and may have a stimulatory effect. Consistent with the latter possibility, translation rates were reduced by 3- to 4-fold in the *ubiK63R* mutant (data not shown). The relative reduction in rate was independent of capping and polyadenylation (data not shown).

### *ubiK63R* Polysomes Are Unstable in Reduced $MgCl_2$

The sensitivity of *ubiK63R* cells to translational inhibitors suggested the existence of a biochemical defect in polysomes from *ubiK63R* mutants. To directly test for such a defect, the stability of *ubiK63R* polysomes was analyzed by sucrose gradient sedimentation under various conditions. An important requirement for polysome stability is $MgCl_2$, which is necessary for the association of mRNA and tRNA with the ribosome (Wintermeyer et al., 1990). At 30 mM $MgCl_2$, polysome profiles from wild-type and mutant cells were comparable (Figure 7A). However, when polysomes from the mutant cells were

analyzed in the presence of the reduced $MgCl_2$ concentration of 3 mM, they dissociated into 80S ribosomes to a greater extent than those from wild-type cells (Figure 7A). Similarly, polysomes from *ubiK63R* cells were stabilized to a much lower extent than wild-type polysomes by a reduced cycloheximide concentration of 10 μg/ml (Figure 7B). This result is consistent with the partial resistance to cycloheximide that is conferred by the *ubiK63R* mutation.

Because $MgCl_2$ is also required for the structural integrity of monosomes and ribosomal subunits, we analyzed the effect of reduced $MgCl_2$ concentration on the stability of free 80S ribosomes. Runoff ribosomes were generated by treating cells with sodium azide prior to lysis. The extent of dissociation of 80S ribosomes into 40S and 60S subunits in 3 mM $MgCl_2$ was comparable in mutant and wild-type extracts (Figure 7C). This result suggests that the in vitro instability observed with polysomes from *ubiK63R* mutants is due to dissociation of the ribosome from the mRNA rather than a defect in either 60S subunit stability or the association of the 40S and 60S subunits. These data also show that the translational defect in *ubiK63R* mutants does not arise from an imbalance between the levels of 40S and 60S subunits.

## Discussion

The importance of ubiquitination in targeting proteins for degradation is well established. In this work, we show that ubiquitination can facilitate protein synthesis in addition to protein degradation. This alternative, nonproteolytic role for ubiquitination involves reversible modification of the target protein by a variant multiubiquitin chain. In vivo data demonstrate that K63-linked multiubiquitin chains are required for proper functioning of the translational apparatus; the *ubiK63R* mutant is hypersensitive to the translational inhibitors anisomycin and trichodermin due to a direct effect on the rate of protein synthesis, and polysomes from this strain are less stable, at least in vitro, than those of wild type.

Consistent with these observations, we have found that ribosomes are abundantly ubiquitinated. This modification occurs on ribosomal protein L28, is dependent on Lys63 of ubiquitin, and is cell cycle regulated. The functional significance of L28 ubiquitination is suggested by its broad evolutionary conservation and by the observation that the translational inhibitors to which *ubiK63R* mutant are most sensitive are those that target the peptidyl transferase center, which contains L28. Interestingly, L28 ubiquitination is induced when cells are treated with inhibitors of transpeptidylation such as anisomycin and trichodermin (J. S., unpublished data). Since cell survival is compromised in the absence of such ubiquitination, upregulation of L28 ubiquitination may potentially participate in an adaptive response under conditions of compromised translation. Interestingly, such conditions are associated with degradation of a high fraction of newly synthesized proteins (Schubert et al., 2000).

The location of L28 at the peptidyl transferase center and the sensitivity of *ubiK63R* mutants to certain transpeptidylation inhibitors suggest that L28-ubiquitin chains may influence the elongation phase of protein synthesis. While the mechanism of this effect remains to be identified, it is striking that the in vitro properties of polysomes are substantially altered by the *ubiK63R* mutation. The instability of *ubiK63R* polysomes in reduced $MgCl_2$ suggests that L28 ubiquitination may result in an enhanced association of ribosomes with mRNA transcripts. The instability of *ubiK63R* polysomes in low cycloheximide is also of interest because the target of cycloheximide, by genetic criteria, is L28 (Kaufer et al., 1983). Cycloheximide is routinely used to stabilize polysomes for analysis on sucrose gradients.

The present data, together with previous studies of the *ubiK63R* mutant, indicate that K63-linked multiubiquitin chains play multiple regulatory roles in *S. cerevisiae*. Although entirely proficient in proteasome-mediated protein degradation of test substrates, these mutants are deficient in DNA repair (Spence et al., 1995; Hofmann and Pickart, 1999), endocytic targeting (Galan and Haguenauer-Tsapis, 1996; Springael et al., 1999), mitochondrial inheritance (Fisk and Yaffe, 1999), and mRNA translation. Thus, the pleiotropic *ubiK63R* phenotype is remarkably different from what one would predict based on ubiquitin's canonical role as a signal for proteasomal degradation. We suggest that this divergence reflects functional differentiation of multiubiquitin chains based on their linkage sites.

One pathway for K63 chain formation in both yeast and mammals involves a heterodimeric Ubc13/Mms2 ubiquitin-conjugating complex (Hofmann and Pickart, 1999). Mutations in the corresponding genes confer DNA repair defects that are comparable to those of the *ubiK63R* mutant, and epistasis experiments indicate that these mutants and the *ubiK63R* mutant are defective in the same pathway (Hofmann and Pickart, 1999). This provides strong support for the view that the pleiotropic defects of the *ubiK63R* mutant reflect an inability to form K63-linked multiubiquitin chains. However, preliminary studies suggest that the Ubc13/Mms2 complex is not responsible for the formation of K63 chains bound to L28, insofar as *ubc13* and *mms2* mutants are not sensitive to translational inhibitors (R. G. and D. F., unpublished data). Consistent with this view, we previously showed that the ubiquitination of a protein identified in this report as L28 requires the ubiquitin-conjugating enzyme Ubc4 (Spence et al., 1995). It will be interesting to determine how many pathways for K63 chain formation exist, and what mechanisms govern their regulation by cell cycle phase, translation, and apparently, DNA damage.

K48-linked multiubiquitin chains are essential for viability in yeast (Finley et al., 1994), and are required for the degradation of many proteins in vivo and in vitro (Pickart, 1997; Peters et al., 1998). Several variant chain linkage sites in ubiquitin have been observed, including K6, K11, K29, and K63. Most of these studies have employed artificial substrates, and only in the case of K63 chains have endogenous targets and physiological functions been identified. Interestingly, however, degradation of ubiquitin-Pro-β-galactosidase is inhibited when the N-terminal ubiquitin of the fusion protein carries a K29R substitution (Johnson et al., 1995). The Ufd2 protein, which is specifically required to convert oligoubiquitinated Ub-Pro-β-galactosidase to a long-chain

form (Koegl et al., 1999), might be involved in switching the specificity of chain linkages from K29 to K48, thus allowing extended chains to be formed.

As the first cell cycle dependent modification of the ribosome to be identified, L28 ubiquitination may provide a means of communication between the cell cycle and protein synthesis machinery. The regulation of L28 ubiquitination is novel in that modification levels are low during $G_1$ and elevated during S, $G_2$, and M phases of the cell cycle, whereas cyclin ubiquitination exhibits a nearly complementary profile of activation during M phase and early $G_1$, and repression during S and $G_2$ (King et al., 1996; Koepp et al., 1999). The ubiquitination of L28 differs more generally from previously described cell cycle–dependent ubiquitination events in that it does not serve to target the substrate for degradation. A nonproteolytic role for ubiquitin was previously described in which the cotranslation of ubiquitin and specific ribosomal proteins from the natural gene fusions *UBI1-UBI3* facilitates ribosome biogenesis (Finley et al., 1989). The present results suggest that multiubiquitination can also serve a nonproteolytic role, and that a variant form of multiubiquitin chain can regulate protein function through a reversible mechanism. A number of proteins, such as histones H2A and H2B, are modified by ubiquitination (primarily monoubiquitination) but not degraded. Consistent with our data, ubiquitination of histones, which is nonproductive in terms of degradation, may nonetheless function in regulation (Robzyk et al., 2000).

The ubiquitin-proteasome pathway has assumed many key roles in the regulation of cell cycle progression, apparently because the chemically irreversible nature of proteolysis makes it a fail-safe mechanism to drive the exclusively forward progression of the cell cycle (King et al., 1996; Koepp et al., 1999). However, given the existence of a large number of deubiquitinating enzymes, the ubiquitination step itself can be rapidly reversible. This reversibility has been viewed primarily as a means to regulate recognition of the target protein by the proteasome. Our data suggest a different model in which, for some proteins, ubiquitination may produce a reversibly altered functional state. The interplay between reversible and irreversible steps may endow the ubiquitin pathway with a capacity to serve diverse regulatory functions.

## Experimental Procedures

### Yeast Strains and Media

Strain SUB280 (*MAT*a *lys2–810 leu2–3,-112 ura3–52 his3-Δ200 trp1–1[am] ubi1-Δ1::TRP1 ubi2-Δ2::ura3 ubi3-Δub-2 ubi4-Δ2::LEU2* [pUB39] [pUB100]), is referred to for simplicity as wild type; it expresses wild-type ubiquitin, at an approximately normal level (Finley et al., 1994), from a single synthetic gene, and it is comparable to wild type in growth rate and other properties. pUB39 expresses ubiquitin and pUB100, the tail of Ubi1 (Finley et al., 1994). SUB413 is isogenic to SUB280 except for a *K63R* mutation in the ubiquitin sequence (Spence et al., 1995). JSY171 was constructed by the introduction of a high copy number *URA3*-marked plasmid (pUB221) encoding 6His-*myc*-ubiquitin under the control of the *CUP1* promoter into SUB280. The sequence of yeast 6His-*myc*-ubiquitin was identical to that of wild-type ubiquitin except for an N-terminal extension of MEIHHHHHHAGEQKLISEEDLG. The resident plasmid (pUB39) was evicted using α-aminoadipic acid (Spence et al., 1995). In all experiments, yeast cells were grown at 30°C. YPD medium consisted of 2% peptone, 1% yeast extract, and 2% dextrose. Synthetic media were prepared as described (Finley et al., 1994).

### Purification of L28-Ubiquitin Conjugates for Sequencing

Three liters of JSY171 were grown to exponential phase in YPD, harvested, washed in $H_2O$, resuspended in $H_2O$, and UV irradiated. The cells were then harvested, resuspended in 8 M urea, 0.1 M $NaH_2PO_4$, and 10 mM Tris-HCl (pH 8.0), and lysed by the glass bead method. The lysate was clarified by centrifugation and loaded onto a $Ni^{2+}$-NTA column (Qiagen). The column was washed and ubiquitin conjugates were eluted at pH 5.5 and in the presence of 8 M urea according to manufacturer's instructions. The eluate was dialyzed against 10 mM $NaPO_4$, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and concentrated by ultrafiltration. Proteins were separated on a 10% to 20% polyacrylamide-SDS gradient gel and blotted onto PVDF membrane. Procedures for SDS gel electrophoresis (as well as IEF) were as described (Spence et al., 1995). The blot was stained with 0.1% Ponceau S in 1% acetic acid, and the major band that was not detectable by immunoblotting with ubiquitin antibodies in a parallel lane was excised and processed for sequencing. Amino-terminal sequencing was performed by the Harvard Biopolymers facility.

### Antibody Preparation and Epitope Tagging

Antibodies were prepared against the L28-derived peptides KTRKLRGHVS, GRIGKHRKHPGGRG, and AGGMHHHRINMDKYH by alternate injections of keyhole limpet hemacyanin-conjugated peptides and peptides adsorbed to aluminum hydroxide as described (Harlow and Lane, 1988). For immunoblotting, antibodies were purified using peptide affinity columns. Antibody specificity was confirmed by immunoblotting extracts from yeast strains HF250-14C and HF251-5C. These strains express wild-type L28 and HA-tagged L28, respectively (H. M. Fried, personal communication). Anti-ubiquitin-conjugate antibodies were from either Affiniti Research Products or Art Haas (Haas and Bright, 1985).

RPL27a was tagged with the FLAG epitope by appending the sequence DYKDDDK directly to its C terminus. The FLAG-RPL27a coding region was cloned downstream from the CMV promoter in the plasmid pRGB4 (a gift from R. Kopito, see Ward et al., 1994) to create pGD125. FLAG-RPL27a is competent for incorporation into ribosomes.

### Sucrose Gradient Analysis of Extracts from Yeast and Human Cells

Polysomes were prepared essentially as described (Sagliocco et al., 1996). Yeast cells were grown to exponential phase in YPD and treated with 100 μg/ml cycloheximide for 15 to 20 min. 25 $OD_{600}$ of cells were harvested, washed in lysis buffer (100 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl [pH 7.5]), resuspended in 500 μl of lysis buffer and lysed using the glass bead method. 25 $OD_{260}$ of the clarified lysate was loaded onto 20% to 47% sucrose gradients prepared in 100 mM NaCl, 30 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.5). Polysome gradients were routinely run at 188,000 × g for 4 hr at 4°C. To increase resolution in the monosome region (Figure 7C), a 15% to 30% gradient was used (centrifuged for 17 hr at 54,000 × g). Gradients were fractionated using either a Buchler Auto Densi-Flow IIC or a Brandel gradient fractioner. Proteins from each fraction were precipitated by adding an equal volume of 40% TCA. The pellet was washed with acetone, dried, and resuspended in 8 M urea and 2% NP40. Electrophoresis on 12% polyacrylamide-SDS gels and immunoblotting were performed as described (Spence et al., 1995). For the recovery of ubiquitin conjugates from JS171 cell extracts, TCA-precipitated gradient fractions were resuspended in 8 M urea, 2% NP40, and 10 mM Tris and loaded onto TALON resin (Clontech). Conjugates were eluted by 10 mM EDTA and loaded directly onto a 12% polyacrylamide-SDS gel after the addition of 2× SDS sample buffer. Immunoblotting with antibody to L28 was performed as described (Spence et al., 1995).

Cytoplasmic extracts of 293T cells (ATCC Number 45504) were prepared in a lysis buffer consisting of 10 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, and 20 mM iodoacetamide. (No translational inhibitors were used in the case of 293T cells.) After 30 min of incubation on

ice, the cells were lysed by passage through a 27 1/2 gauge needle five times. The extract was clarified by centrifugation at 2000 × g for 5 min prior to gradient analysis. Clarified extracts from 293T cells were sedimented on 20% to 47% sucrose gradients prepared as described above and centrifuged for 17 hr at 54,000 × g.

293 cells were treated with 50 μg/ml cycloheximide for 25 min, then washed with cycloheximide containing PBS, and lysed in 100 mM NaCl, 30 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5), 50 μg/ml cycloheximide, 5 U/ml RNAsin by passage through a 27 1/2 gauge needle ten times. Clarified extracts were loaded onto a 15% to 50% sucrose gradient in the same buffer without cycloheximide or RNAsin.

**Affinity Chromatography of Ubiquitin-L27a Conjugates**

pCW7, which expresses 6His-*myc*-ubiquitin from the CMV promoter (Ward et al., 1994), and pRGB4, a control vector, were transfected in 293T cells. Cells were suspended in 8 M urea, 0.1 mM $NaH_2PO_4$, and 10 mM Tris-HCl (pH 8.0) and lysed by repeated passage through a 25 gauge needle. Extracts were clarified by centrifugation and the supernatants mixed with Ni-NTA-agarose for 1 hr with agitation. The agarose beads were transferred to mini-columns and washed with 20 column volumes of lysis buffer. Ubiquitin conjugates were eluted with 100 mM imidazole-NaOH (pH 7.4) and analyzed by SDS-PAGE, followed by immunoblotting.

**Antibiotic Sensitivity**

In plate assays, antibiotics were used at the following concentrations: 20 μg/ml anisomycin, 30 ng/ml rapamycin, 60 μg/ml geneticin, 100 μg/ml hygromycin, 10 μg/ml cycloheximide, and 20 μg/ml trichodermin. To measure protein synthesis (Ciechanover et al., 1984), [$^{35}$S]methionine-labeled cell extracts were spotted onto 3MM paper filters (Whatman) that had been presoaked in 10% (w/v) TCA. The filters were washed in cold 10% TCA, immersed for 5 min in 10% TCA at 90°C, washed twice in 10% cold TCA, washed twice in 95% EtOH, and dried. Radioactivity was determined using a scintillation counter. Nonspecific background was subtracted from all readings as described (Ciechanover et al., 1984).

**Cell Cycle Synchronization**

Because of the difficulty in obtaining homogenous α factor arrested populations in the genetic background of the *ubiK63R* mutant, cultures of 15Daub (*MAT*a *ade1 his2 leu2–3,-112 trp1 ura3*Δ*ns bar1*Δ; Mondesert and Reed, 1996) were used for this purpose. α factor was used at 100 ng/ml in YPD to arrest 15Daub cells; >95% of the cells displayed schmoo morphology prior to release from arrest. Nocodazole was used at 15 μg/ml with 1% DMSO in YPD to arrest SUB280. Cells were released from 3 hr cell cycle arrests by 3 washes with YEP (2% peptone, 1% yeast extract) followed by resuspension in YPD. In the case of nocodazole arrest, 1% DMSO was included in the YEP wash buffer. At 15 min intervals, aliquots were taken for analysis by FACS and immunoblotting. Budding index was determined microscopically. For immunoblotting, 1 $OD_{600}$ of cells was harvested, washed once with $H_2O$, and resuspended in 8 M urea, 10 mM $Na_2HPO_4$, and 1 mM Tris-HCl (pH 8.0). Cells were lysed using glass beads for 1 min, and the lysate was frozen on dry ice. Samples were later clarified by centrifugation, and an equal volume of 2× SDS sample buffer was added; electrophoresis and immunoblotting were performed as described (Spence et al., 1995). FACS analysis was carried out essentially as described (Hutter and Eipel, 1979).

**In Vitro Translation**

All procedures were essentially as described by Tarun and Sachs (1995) with some modifications. Two to three liters of 15Daub were grown to exponential phase in YPD. Alternatively, the cells were arrested for 3 hr, either in $G_1$ by the addition of 100 ng/ml α factor or in S phase by the addition of 150 mM hydroxyurea. Cells were lysed and the lysate was clarified and fractionated on a Sephadex G25 column as described (Tarun and Sachs, 1995). Fractions with an $OD_{260}$ of 0.9 or higher were pooled and frozen in aliquots at −70°C. Luciferase RNA transcripts were prepared from BamH1-linearized T3LUCpA DNA (a gift from Alan Sachs) with a RiboMAX large scale RNA kit (PROMEGA). Capped mRNA was synthesized using the cap analogue 7-methyl-GpppG (New England Biolabs). RNA concentration was determined by $OD_{260}$ and RNA integrity was confirmed by gel electrophoresis. In vitro translation was performed as described (Tarun and Sachs, 1995). Luciferase activity was measured by adding 10 μl of reaction mix to 50 μl of Luciferase Assay Reagent (PROMEGA) and recording the activity immediately for 1 min in a Beckman LS6000SC scintillation counter.

**Acknowledgments**

We would particularly like to thank Corinne John for carrying out the drug-sensitivity tests, Chris Larsen for generous help in constructing figures, and Curt Wittenberg and Karin Flick for assistance in cell cycle–related experiments. We also thank Steve Reed, Alan Sachs, and Ron Kopito for yeast strains and plasmids; Howard Fried for the generous gifts of trichodermin, strains, and advice; Art Haas for anti-ubiquitin antibodies; and, for comments on the manuscript, Cecile Pickart, Marion Schmidt, Alan Sachs, and Robin Reed. Supported by NIH grants GM12702 (to F. S.) and GM43601 (to D. F.), and DOE grant FG03-86ER60429 (to M. K). G. D. was supported by a fellowship from the Deutsche Forschungsgemeinschaft.

Received July 15, 1999; revised May 2, 2000.

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