Within the ubiquitin degradation pathway, the canonical signal is a lysine 48-linked polyubiquitin chain that is assembled upon an internal lysine residue of a substrate protein. Once constructed, this ubiquitin chain becomes the principle signal for recognition and target degradation by the 26S proteasome. The mechanism by which polyubiquitin chains are assembled on a substrate protein, however, has yet to be clearly defined. In an in vitro model system, purified E2-ubiquitin thiolester was unable to catalyze the formation of polyubiquitin chains in the absence of the ubiquitin-activating enzyme E1. Mutagenesis of key residues within the E1 active site revealed that its conserved catalytic cysteine residue is essential for the formation of these chains. Moreover, inactivation of the E2 active site had no effect on the ability of E1 to catalyze ubiquitin chain formation. These findings strongly suggest E1 is responsible for not only the activation of ubiquitin but also for the direct catalytic extension of a lysine 48-linked polyubiquitin chain.

The ubiquitin (Ub)5 proteolytic system is a universal physiological regulator within the eukaryotic cell, and its dysregulation is associated with a host of disease states. In the Ub proteolytic pathway, a canonical Lys48-linked polyUb chain is built upon an internal lysine residue of a protein destined for destruction (1). Once assembled, this chain is quickly recognized by the 26S proteasome resulting in the rapid degradation of the substrate (2–4). The traditional conceptualization of polyUb chain assembly begins with the ATP-dependent activation of free Ub, by the Ub-activating enzyme (E1) in a two-step process that involves: 1) the adenylation of the C terminus of Ub through the hydrolysis of ATP and 2) the transfer of this Ub to a conserved cysteine residue. Following its activation, Ub is passed from the conserved E1 active site cysteine residue to a catalytic cysteine residue on a ubiquitin-conjugating enzyme (E2). The E2, in concert with a protein, or protein complex known as a ubiquitin protein ligase or E3, binds a target, and then modifies it by the addition of a Ub molecule on an exposed lysine residue (5–7). It is presently thought that, following the initial identification and conjugation of a substrate with Ub, the assembly of the polyUb chain occurs through a mechanism involving this same E2/E3 complex (5, 7–9). While a number of studies have attempted to elucidate the precise mechanism of polyUb chain assembly, none have clearly established that either E2 or E3, in the absence of E1, is capable of catalyzing the formation of polyUb chains sufficient in length for recognition by the 26S proteasome. Here we describe and characterize a novel model system with which to study the underlying mechanism of Lys48-linked polyUb chain extension. Importantly, this system demonstrates for the first time that not only is E1 essential for the initial ATP-dependent activation of Ub, it is also capable of the catalytic extension of the polyUb chain on a mono-ubiquitinated substrate.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Strains—Escherichia coli** expression plasmids for Ub and Ubc1Δ450 have been previously described (10). D. Finley generously provided the Leu2 yeast expression vector, pUBA1 containing the 3’ His6-tagged UBA1 (YKL210W), and the ΔUBA1 Saccharomyces cerevisiae strain PJ325. Uba1 was expressed in S. cerevisiae MHY-501 (MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1, gal2) (11). The *E. coli* strain MC1061 was used for PCR mutagenesis and plasmid biosynthesis (12).

**E1 Mutagenesis**—E1atp was produced through a glycerine to valine substitution at amino acid position 446 by substitution of G-T at base 1337 (GGT to GTT) within the UBA1 gene YKL210W. E1ala was produced in a similar manner through a cysteine to alanine substitution at position 600, by altering bases 1788–1800 from TGT to TGT. E1ala was produced in a similar manner through a cysteine to alanine substitution at position 600, by altering bases 1788–1800 from TGT to TGT. The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.
**Protein Expression and Purification**—His$_6$-tagged E1 was expressed and purified from MHY-501 cells grown in S.D. (−leu) at 30 °C. E1 expression was induced from the CUP promoter by the addition of 100 mM CuSO$_4$, followed by 12 h of growth at 30 °C. Cells were pelleted and re-suspended in 50 mM Tris, pH 7.5, 10 mM MgCl$_2$, 1 mM sorbitol, 1 mM DTT, and spheroplasts were formed by the addition of 1 mg of zymolyase (Seikagaka Corp). The spheroplasts were pelleted and re-suspended in buffer A (80 mM Na$_2$HP0$_4$, 93 mM NaH$_2$PO$_4$, 4 mM NaCl, pH 7.4). To ensure lysis, spheroplasts were vortexed with a 1:5 volume of acid washed glass beads (Biospec). The lysate was clarified by high speed centrifugation at 40,000 rpm, and the supernatant filtered using a 0.45-μm syringe tip filter (Millipore). His$_6$-E1 was isolated by passing the clarified lysate over a 1-ml HiTrap chelating column (Amersham Biosciences) charged with 100 mM NiSO$_4$. The column was washed and E1 eluted from the column with buffer A containing 500 mM imidazole. Finally, E1 was passed over a Hi Load Superdex 75 16/60 FPLC column (Amersham Biosciences) equilibrated with buffer B (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA). Expression and purification of recombinant Ubc1$_{Δ450}$ and [³⁵S]Ub have been previously described (13, 14).

**In Vitro Ubiquitination Reactions**—All ubiquitination assays were carried out in ubiquitination buffer consisting of 10 mM HEPES (pH 7.5), 5 mM MgCl$_2$, 40 mM NaCl, 20 μg/ml protease inhibitors (antipain, aprotinin, chymostatin, leupeptin, pepstatin A), 180 mg/ml phenylmethylsulfonyl fluoride, 0.6 units/ml inorganic pyrophosphatase (Sigma Aldrich), and 5 mM ATP (unless otherwise stated).

**Sizing Columns**—Reactions where only the E1-dependent activation of Ub was followed contained 10 or 100 nM E1, 100 nM E2, and 100 nM [³⁵S]Ub. E2-K-Ub or 100 nM [³⁵S]Ub were incubated at 30 °C for 60 min and immediately loaded on a Superdex 75 HR 10/30 gel filtration column (Amersham Biosciences). Reaction products were eluted with buffer B and 0.5-ml fractions collected. The total counts per minute (cpm) of incorporated [³⁵S]Ub was determined by scintillation counting on a Beckman LS-6800 Liquid Scintillation counter.

**SDS-PAGE Gels**—PolyUb chain building reactions contained 10 or 100 nM E1, 100 nM E2, and either 100 nM [³⁵S]Ub or 100 nM E2~[³⁵S]Ub thiolester or 100 nM E2~[³⁵S]Ub conjugate and were incubated at 30 °C for 1–8 h and were subsequently stopped by the addition of 10% trichloroacetic acid, followed by centrifugation. Protein pellets were resuspended in SDS loading buffer containing DTT and boiled for 10 min and were then applied to a 10% SDS-polyacrylamide gel. Samples were then visualized by autoradiography using a Fuji Film BAS2000 phosphorimager.

**Purification and Stability of the E2-S~Ub Thiolester**—E2-S~Ub thiolester was purified in a reaction containing 1.2 mM E2, 1.2 mM [³⁵S]Ub, and 8 nM yeast E1 (15). This reaction was incubated at 30 °C for 5 h and immediately passed over a Superdex 75 16/30 gel exclusion column that had been equilibrated with buffer B containing 50 mg/ml bovine serum albumin. Peak fractions were analyzed for E1-S~[³⁵S]Ub thiolester as described previously (17).

**Formation and Ethyleneimine Treatment of Conjugate Thiolester**—The conjugate thiolester was formed by incubating 165 nM E2-K-Ub$_{C48}$ conjugate in the presence of 165 nM [³⁵S]Ub, 10 nM yeast E1, and ATP. Following a 3-h incubation at 30 °C reactions were passed over a Superdex column equilibrated with buffer B containing 50 mg/ml bovine serum albumin, and the stable [³⁵S]Ub~S-E2-K-Ub$_{C48}$ conjugate thiolester was isolated. The [³⁵S]Ub~S-E2-K-Ub$_{C48}$ conjugate thiolester was then inactivated by treatment with ethyleneimine as described previously (16).

**Formation of and Purification of the E2-K-Ub and Ub$_{C48}$ Conjugates**—Mono-ubiquitinated E2-K-Ub conjugate was purified in a way similar to that for the E2-S~Ub thiolester (17), with the following alterations: unlabeled Ub was used and reactions were incubated at 30 °C for 16 h rather than 5 h. DTT was then added to a final concentration of 100 mM, to eliminate any remaining E2~S~Ub thiolester. The reaction was incubated for an additional hour in the presence of DTT and then passed over a Superdex 75 16/30 gel exclusion column that was equilibrated with buffer B containing 50 mg/ml bovine serum albumin. SDS-PAGE Coomassie staining of mono-conjugate peak fractions revealed that the E2-K-Ub and E2-K-Ub$_{C48}$ conjugates had been purified to homogeneity.

**Uba1 Back-transfer Reactions**—The back-transfer of activated Ub onto the active site cysteine residue within E1 was performed in ubiquitination buffer lacking ATP (18). Purified E2-S~[³⁵S]Ub thiolester (100 nM) and 100 nM wt E1 or E$_{atp}$ were incubated at 30 °C for 30 min and immediately loaded onto a Superdex 75 HR 10/30 gel filtration column that had been equilibrated with buffer B containing 50 mg/ml bovine serum albumin. Peak fractions were analyzed for E1-S~Ub thiolester as described previously (17).

**Iodoacetamide Treatment of E2-K-Ub Mono-conjugate**—Inactivation of the active site cysteine residue with iodoacetamide has been described previously for several E2s (19–22). Approximately 200 mg of the E2-K-Ub or E2-K-Ub$_{C48}$ conjugates were incubated with a 1000× molar excess of iodoacetamide at 30 °C for 60 min. This was then followed by the addition of a 2× molar excess of DTT and dialysis against 4 liters of buffer B for 12 h at 4 °C.

**RESULTS**

We have previously described the formation of Lys$_4$-linked polyUb chains using a three-protein in vitro system containing recombinant versions of yeast E1, Ub, and the core catalytic domain of the E2, Ubc1 (Ubc1$_{Δ450}$) (10). Time course analysis of a complete ubiquitination reaction demonstrates completion of chain assembly after ~8 h (Fig. 1A). This system represents a powerful and easily manipulated model of polyUb chain assembly occurring at a similar rate to that previously described for long Ub chains on a covalently-linked target, and does not require the participation of ancillary proteins such as E3s, E4s or a separate substrate (23).
In a complete ubiquitination reaction, containing E1, E2, Ub, and ATP, the chain is initiated by the relatively slow intramolecular transfer of Ub from the active site of the E2–Ub thiolester to a nearby lysine (Lys93). We observed that rate of polyUb chain assembly exhibited by the complete reaction is similar at early time points to the rate of spontaneous E2–K–Ub conjugate synthesis observed in the E2–S–Ub thiolester reaction (Fig. 1B). This similarity suggested that it is the formation of the E2–K–Ub conjugate that defines the rate-limiting step in the assembly of Lys93–linked polyUb chains. When the total amount of E2–K–Ub conjugates was followed for reactions containing only the 270 nM purified E2–S–Ub thiolester and 27 nM E1 in the absence of any exogenous ATP, the total amount of E2–linked Ub was deconvoluted into the mono-conjugate (open circles) or the total sum of all the E2–Ub, (n–1) conjugates (open triangles). D, the total amount of Ub incorporated into each individual E2–Ub conjugate was determined at time points corresponding to 1 h (open squares), 2 h (open triangles), and 4 h (open circles).

Considering this model system within the context of the currently accepted mechanism for polyUb chain assembly, the E2–S–Ub thiolester should be the sole biochemical intermediate responsible for extension of the Ub chain. The inherent ability of the E2–S–Ub thiolester to produce the E2–K–Ub conjugate spontaneously suggested that its purification alone should be sufficient to produce polyUb chains. However, our previous observation that purified E2–S–Ub thiolester remained stable over the course of hours suggested that it alone was not sufficient to support chain assembly (Fig. 2, lane 2) (15).

If the E2 catalytic cysteine plays a direct role in chain assembly then there are three possible routes by which this may occur (Supplemental Fig. S1): 1) an intermolecular transfer where Ub is passed from the E2–S–Ub thiolester to the Lys93–linked Ub molecule of the E2–K–Ub conjugate, or, 2) an intermolecular transfer of Ub from the E2–S–Ub thiolester to the Ub–S–E2–K–Ub thiolester-conjugate, or, 3) an intramolecular transfer where Ub is passed from the active site of the Ub–S–E2–K–Ub thiolester-conjugate to its own Lys93–linked Ub. While this last alternative is formally possible, following the addition of the first Ub to the growing chain subsequent addition clearly becomes topologically improbable. We therefore considered only the first two hypotheses involving the intermolecular transfer of Ub to the growing chain as likely.

Testing these hypotheses first required the purification of the two E2 thiolester species (Supplemental Fig. S2). Purification of the E2–S–Ub thiolester was straightforward, as we had previously determined that it remains stable over the course of several hours (15). Purification of the Ub–S–E2–K–Ub thiolester-conjugate initially proved problematic because purified E2–K–Ub mono-conjugate is a potent precursor for chain assembly in the presence of E1, leaving no evidence of the con-
jugate-thiolester intermediate. High yields of purified conjugate-thiolester could only be achieved when Lys<sup>48</sup> of the conjugated Ub molecule was replaced with Cys<sup>48</sup> to create a dead-end substrate, blocking subsequent chain assembly. Here, the role of Cys<sup>48</sup> was 2-fold: 1) As a chain terminator, it resulted in sufficient yields of the conjugate-thiolester to facilitate its purification, 2) its chemical modification with ethyleneimine created a functional Lys<sup>48</sup> mimic (S-aminoethylcysteine) fully capable of participating in chain extension (4, 7, 16).

In Fig. 2, a complete reaction containing E1, E2, Ub, and ATP illustrates the rapid synthesis of Lys<sup>48</sup>-linked Ub chains onto the E2-K-Ub mono-conjugate (lane 1). Having already determined that the formation of the E2-K-Ub conjugate was rate-limiting, both the E2-K~Ub thiolester and E2-K-Ub conjugate were incubated at equimolar concentrations (lane 6). The absence of polyUb chains from this reaction illustrated that the intermolecular transfer of Ub from E2-S~Ub thiolester is not the mechanism by which polyUb chains are assembled. In the case of purified Ub~S-E2-K-Ub thiolester-conjugate (lane 8), chemical conversion of Cys<sup>48</sup> to a functional lysine mimic also did not result in the appearance of polyUb chains (lane 9). Notably, the addition of E1 to either reaction stimulated chain assembly despite the absence of both ATP and free Ub (lanes 3, 7, and 10). It is therefore clear that neither the E2-S~Ub thiolester nor Ub~S-E2-K-Ub thiolester mono-conjugate are capable of catalyzing polyUb chain assembly in the absence of E1.

The previous experiments demonstrate that in this model E1 plays either a facilitative role, acting as a scaffold for E2 interactions, or a direct catalytic role in polyUb chain assembly. To study this question, we created three active site substitutions: C600A-Uba1 (E1<sub>ala</sub>) and C600S-Uba1 (E1<sub>ser</sub>) at the active site study this question, we created three active site substitutions: C600A-Uba1 (E1<sub>ala</sub>) and C600S-Uba1 (E1<sub>ser</sub>) at the active site. To investigate the theoretical possibility that the cysteine-to-alanine substitution may have altered a critical binding interface between E1 and E2 with the more conservative substitution of cysteine-to-serine at the same position (E1<sub>ser</sub>). We observed an identical absence of polyUb chain assembly (Fig. 3). These observations additionally demonstrate that the E1 molecule plays no facilitative role in an E2-S~Ub thiolester-dependent chain assembly mechanism. The E1<sub>ser</sub> reaction on the other hand, synthesized chains at a level comparable to that of wt E1 (lane 2).

We investigated the theoretical possibility that the cysteine-to-alanine substitution may have altered a critical binding interface between E1 and E2 with the more conservative substitution of cysteine-to-serine at the same position (E1<sub>ser</sub>). We observed an identical absence of polyUb chain assembly (Fig. 4).

Analysis of the E1/E2 interaction has always been complicated by the fact that it is transient and not observable <i>in vitro</i>. However, we feel that a conservative point substitution within the active site of E1 will not substantially alter binding affinities between these proteins. In fact, within the Nedd8-activating E1 homolog Uba3, residues located within the major E2 binding cleft do not include residues near the active site (31). Furthermore, a structure of the Ubc12/Uba3 complex has also demonstrated that there is no direct interaction between the E1 active site region and E2 (32). Taken together, the results of Figs. 1–4 reinforce the notions that within this simple system, E1 does not simply function as an inert scaffold for the homotypic interaction of E2s, and that the E1 active site cysteine is less essential
for the formation of the E2-S~Ub thiolester than it is for the catalysis of polyUb chain assembly.

Having demonstrated that the E2-thiolester plays no direct catalytic role in Ub chain assembly, we then sought to clarify the enzymatic contribution of E1 itself. In order to accomplish this, we first needed to generate an E2-K-Ub mono-conjugate species that could act solely as a substrate for chain assembly without any potential for catalytic contribution. Iodoacetamide is a highly efficient and specific alkylator of cysteine residues and has been used in numerous previous studies to selectively inactivate components of the Ub activation cascade (19, 20, 22). We therefore purified the E2-K-Ub mono-conjugate and chemically inactivated its active site cysteine residue with iodoacetamide to preclude the possibility of its catalytic involvement in subsequent steps. The extent of inactivation was judged to be complete based on the inability of its identically treated E2-K-UbC48 counterpart to form the conjugate thiolester in the presence of Ub, E1 and ATP (Fig. 5A). Active and inactive forms of E2-K-UbK48 were then combined with purified E1-S/H11011 Ub thiolester (prepared using the back-transfer reaction already described) in the presence of the non-hydrolyzable ATP analogue AMP-PNP to prevent the subsequent reactivation of free Ub. Additionally, we found the use of AMP-PNP stabilized E1-S~Ub thiolester, preventing Ub hydrolysis during transfer (Fig. 4A, top panel). Notably, the level of chain assembly in each of these reactions was comparable regardless of the functional state of the E2 active site cysteine (Fig. 5B). Furthermore, inactivation of the E2 active site cysteine transformed the E2 mono-conjugate into a more preferred substrate for polyUb chain assembly presumably by inactivation of the alternative Ub transfer site. The average chain length of iodoacetamide-treated E2 was observed to be greater than that of the untreated E2 (Fig. 5C).
DISCUSSION

The fundamental biochemistry of ubiquitination is of great interest to the scientific community, and has been the subject of intense investigation over the past two decades. The central problem of this system remains the mechanism by which polyUb chains are assembled on a substrate protein prior to its degradation. The formation of polyUb chains involves the coordinated assembly and polymerization of Ub proteins into a distinct molecular architecture. This differs greatly from other forms of post-translational modification, such as phosphorylation, in the sense that nonspecific protein interactions will not result in the artificial formation of these conserved structures in vitro.

The interdependent nature of target selection and chain assembly in the ubiquitin system coupled with the exceedingly large numbers of enzymes involved has made it difficult to determine the precise role that each component plays in vivo. Here we characterize a novel and powerful in vitro system that allows for the study of only the essential enzymatic components of ubiquitin chain assembly.

The necessity for precise target selection within the ubiquitin proteolytic system has given rise to a large family of both E2 and E3 proteins. The extent of variability is best illustrated by the observation that a subset of protein targets are recognized and efficiently ubiquitinated by only an E2 (18, 33, 34) while others require the presence of both an E2 and E3 (35, 36). According to the currently accepted model of polyUb chain formation, these E2s and E3s are responsible for the catalytic assembly of Ub chains on substrates. This model has gained widespread acceptance despite a paucity of direct biochemical evidence demonstrating this to be the case. The solution of the crystal structure of the SCF Ub ligase complex revealed a 50–60 Å gap between the distal substrate-binding component of the complex and the active site cysteine of the E2 (37). This observation makes it very difficult to rationalize an E2/E3-only model of polyUb chain assembly, as the gap presents two major conceptual challenges. First, a 50-Å distance between the target lysine and active site cysteine of E2 appears too great for the efficient transfer of Ub. Second, if this model is correct, it is unclear how this mechanism could ultimately result in the formation of Ub chains because as the chain is extended, the configuration and distance of the target changes with respect to the fixed E2-S—Ub thiolester. A great deal of effort has been spent in recent years trying to find the appropriate “fit” that reconciles the structural evidence with the biochemical evidence. Most recently, Petroski et al. (7) hypothesized a diffusion-driven mechanism for substrate ubiquitination, but it is important to note that reactions in this study, as well as all studies examining chain assembly, contain active E1. As such, any catalytic contribution from E1 cannot simply be discounted.

In the canonical mechanism of polyUb chain assembly, E1 supplies the downstream components of the enzymatic cascade with activated Ub while playing no direct role in substrate recognition or polyUb chain assembly. The inability of E1 alone to identify and ubiquitinate substrate proteins in a variety of experimental systems has supported this narrow functional classification (18, 19, 34, 38). The capacity of E1 to activate and transfer Ub to the active site of an E2 follows a common mechanistic theme shared with other synthetic enzymes such as the amino acid tRNA synthetases and acetyl-CoA synthetase. E1, however, differs from these enzymes at a key step of the transfer mechanism. With these and other synthetases, a carboxyl group is first activated as an adenylate followed by its direct transfer to an autonomous molecular moiety in a single enzymatic step. By comparison, the transfer of activated Ub to the E1 cysteine introduces an intermediate reaction step. Even E1 evolutionarily related prokaryotic homologue MoeB (Supplemental Fig. S4) is known to transfer an adenylated form of the Ub-like MoeD directly to elemental sulfur coordinated within the NifS-like sulfur transferase (39). This two-step activation of Ub by E1 is a biochemical curiosity that has been the subject of recent conjecture and previously rationalized as a detour around a potential topological barrier during the transfer of the Ub-like protein, NEDD8, from its E1 to E2 (40, 41). The fact however that the Ub adenylate can be transferred directly from the E1 ATP-binding site to E2 (this work) illustrates that although the E1 cysteine is critical to cell viability, it is not essential for Ub transfer. Alternatively, here we provide strong evidence that the E1 active site cysteine has a separate and specific function, as a direct catalytic participant in the assembly of polyUb chains (Fig. 6).

The conclusions of this study have been consolidated in the mechanistically simplified experiment presented in Fig. 5. Here we demonstrate that polyUb chain extension does not require the active site cysteine of E2, and occurs instead through the direct transfer of Ub-thiolester from the active site cysteine of E1 to the terminal Lys48 of the growing chain. We also note that because the E1-S—Ub thiolester is not regenerated in these reactions, chain growth must proceed by a nonprocessive mechanism involving a new E1 molecule for each round of Ub addition.

One caveat with this study is that E2 serves as both enzyme and substrate within this model system, which is different from situations in vivo where a separate substrate is targeted. We cannot therefore exclude the possibility that the E2 is participating in the recognition and formation of polyUb chains in a non-catalytic role. Additionally, it is possible that multiple mechanisms for polyUb chain assembly coexist within a single cell. A mechanistic model using a single enzyme for the conserved catalytic steps, such as assembly of the Lys48-linked polyUb chain, with diversity-related functions, such as target selection and polymerization of alternate linkage chains, occur-
PolyUb Chain Catalysis by E1

...ring through the catalytic activity of a large structurally varied family of enzymes, has intuitive logic. For instance, it is well known that some cellular targets are mono-ubiquitinated at one or multiple sites (33, 43), while others have short Ub chains (33) and still others are conjugated with Ub chains of alternate linkages (44, 45). All of these enzymatic pathways may collaborate to produce a combinatorial diversity of function based on both availability and compartmentalization of the constituent enzymatic components.

In summary, we have presented evidence that illustrates a mechanism for polyUb chain assembly which proceeds in four discrete steps: activation of Ub by E1, transfer of Ub from E1 to the E2 active site cysteine, substrate mono-ubiquitination, and lastly chain extension on the substrate by E1. The dual role of E1 in Ub activation and Lys48-linked polyUb chain assembly, therefore, consolidates within a single polypeptide two functions that are both essential and universal to Ub-dependent protein turnover. Significantly, these activities constitute the initial targeting of a substrate protein.

While the precise mechanism by which polyUb chain extension occurs remains to be clearly defined and the physiological relevance of this result remains to be determined, this is the first evidence to demonstrate that E1 directly catalyzes Lys48-linked polyUb chain assembly.

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REFERENCES

1. Chau, V., Tobias, J. W., Bachmair, A., Marriot, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989) *Science* 243, 1576–1583
2. Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (1994) *J. Biol. Chem.* 269, 7059–7061
3. Seufert, W., and Jentsch, S. (1992) *EMBO J.* 11, 3077–3080
4. Gregory, I., Poosch, M. S., Cousins, G., and Chau, V. (1990) *J. Biol. Chem.* 265, 8354–8357
5. Chen, Z., and Pickart, C. M. (1990) *J. Biol. Chem.* 265, 21835–21842
6. Scheffner, M., Nuber, U., and Huijbregts, J. M. (1995) *Nature* 373, 81–83
7. Petrofski, M. D., and Deshaies, R. J. (2005) *Cell* 123, 1107–1120
8. Van, N., S., and Vierstra, R. D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 10297–10301
9. Schulman, B. A., Carrano, A. C., Jeffrey, P. D., Bowen, Z., Kinnucan, E. R., Finnin, M. S., Elledge, S. J., Harper, J. W., Pagano, M., and Pavletich, N. P. (2000) *Nature* 408, 381–386
10. Hodgins, R., Gwozd, C., Arnason, T., Cummings, M., and Ellison, M. J. (1996) *J. Biol. Chem.* 271, 28766–28771
11. Papa, F. R., and Hochstrasser, M. (1993) *Nature* 366, 313–319
12. Cassadan, M. J., and Cohen, S. N. (1980) *J. Mol. Biol.* 138, 179–207
13. Hodgins, R. R., Ellison, K. S., and Ellison, M. J. (1992) *J. Biol. Chem.* 267, 8807–8812
14. Ptak, C., Prendergast, J. A., Hodgins, R., Kay, C. M., Chau, V., and Ellison, M. J. (1994) *J. Biol. Chem.* 269, 26539–26545
15. Hamilton, K. S., Ellison, M. J., Barber, K. R., Williams, R. S., Huzil, J. T., McKenna, S., Ptak, C., Glover, M., and Shaw, G. S. (2001) *Structure (Camb)* 9, 897–904
16. Piotrowski, J., Beal, R., Hoffman, L., Wilkinson, K. D., Cohen, R. E., and Pickart, C. M. (1997) *J. Biol. Chem.* 272, 23712–23721
17. Hatfield, P. M., Callis, J., and Vierstra, R. D. (1990) *J. Biol. Chem.* 265, 15813–15817
18. Haas, A. L., and Bright, P. M. (1988) *J. Biol. Chem.* 263, 13258–13267
19. Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983) *J. Biol. Chem.* 258, 8206–8214
20. Haldeman, M. T., Xia, G., Kasperek, E. M., and Pickart, C. M. (1997) *Biochemistry* 36, 10526–10537
21. Klemperer, N. S., Berleth, E. S., and Pickart, C. M. (1989) *Biochemistry* 28, 6053–6054
22. Mastrandrea, L. D., Kasperek, E. M., Niles, E. G., and Pickart, C. M. (1998) *Biochemistry* 37, 9784–9792
23. Staschuck, M. (2007) *Int. J. Biochem. Cell Biol.* 39, 319–326
24. McGrath, J. P., Jentsch, S., and Varshavsky, A. (1991) *EMBO J.* 10, 227–236
25. Hatfield, P. M., and Vierstra, R. D. (1992) *J. Biol. Chem.* 267, 14799–14803
26. Dohmen, R. I., Steppeyn, R., McGrath, J. P., Forrova, H., Kolarov, J., Gofnau, A., and Varshavsky, A. (1995) *J. Biol. Chem.* 270, 18099–18109
27. Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M. D., Klonsky, D. J., Ohsumi, M., and Ohsumi, Y. (1998) *Nature* 395, 395–398
28. Johnson, E. S., Schwienhorst, I., Dohmen, R. J., and Blobel, G. (1997) *EMBO J.* 16, 5509–5519
29. Liakopoulos, D., Doenges, G., Matuschewski, K., and Jentsch, S. (1998) *EMBO J.* 17, 2208–2214
30. Walker, J. E., Eberle, A., Gay, N. J., Runswick, M. J., and Saraste, M. (1982) *Biochem. Soc. Trans.* 10, 203–206
31. Huang, D. T., Miller, D. W., Mathew, R., Roussel, M. F., and Schulman, B. A. (2004) *Struct. Mol. Biol.* 11, 927–935
32. Huang, D. T., Paydar, A., Zhung, M., Waddele M. B., Holton J. M., and Schulman, B. A. (2005) *Mol. Cell* 17, 341–350
33. Pickart, C. M., and Vella, A. T. (1988) *J. Biol. Chem.* 263, 15076–15082
34. Haas, A., Reback, P. M., Pratt, G., and Rechsteiner, M. (1990) *J. Biol. Chem.* 265, 21664–21669
35. van Nocker, S., and Vierstra, R. D. (1993) *J. Biol. Chem.* 268, 24766–24773
36. Sung, P., Berleth, E., Pickart, C., Prakash, S., and Prakash, L. (1991) *EMBO J.* 10, 2187–2193
37. Zheng, N., Schulman, B. A., Song, L., Miller, J. I., Jeffrey, P. D., Wang, P., Chu, C., Koepf, D. M., Elledge, S. J., Pagano, M., Conaway, R. C., Conaway, J. W., Harper, J. W., and Pavletich, N. P. (2002) *Nature* 416, 703–709
38. Hershko, A., Leshinsky, E., Ganoth, D., and Heller, H. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 1619–1623
39. Leimkuhler, S., and Rajagopalan, K. V. (2001) *J. Biol. Chem.* 276, 22024–22031
40. VanDemark, A. P., and Hill, C. P. (2003) *Nat. Struct. Biol.* 10, 244–246
41. Walden, H., Podgorski, M. S., and Schulman, B. A. (2003) *Nature* 422, 330–334
42. Mastrandrea, L. D., You, J., Niles, E. G., and Pickart, C. M. (1999) *J. Biol. Chem.* 274, 27299–27306
43. Terrell, J., Shih, S., Dunn, R., and Hicke, L. A. (1998) *Mol Cell* 1, 193–202
44. Arnason, T., and Ellison, M. J. (1994) *Mol. Cell. Biol.* 14, 7876–7883
45. Hofmann, R. M., and Pickart, C. M. (1999) *Cell* 96, 645–653