The Ubiquitin–Proteasome System of *Saccharomyces cerevisiae*

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ABSTRACT Protein modifications provide cells with exquisite temporal and spatial control of protein function. Ubiquitin is among the most important modifiers, serving both to target hundreds of proteins for rapid degradation by the proteasome, and as a dynamic signaling agent that regulates the function of covalently bound proteins. The diverse effects of ubiquitylation reflect the assembly of structurally distinct ubiquitin chains on target proteins. The resulting ubiquitin code is interpreted by an extensive family of ubiquitin receptors. Here we review the components of this regulatory network and its effects throughout the cell.

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doi: 10.1534/genetics.112.140467
Manuscript received March 13, 2012; accepted for publication July 28, 2012
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THE modification of proteins by the covalent attachment of ubiquitin is a regulatory process whose influence is felt throughout the cell in all eukaryotes. Ubiquitylation targets proteins to the proteasome to be degraded, a process that dynamically sculpts the proteome, with hundreds of yeast proteins being rapidly and selectively degraded (Belle *et al.* 2006). However, many ubiquitin modifications act through nonproteolytic mechanisms, such as in DNA repair, chromatin dynamics, mRNA export, the extraction of proteins from multisubunit complexes, and the trafficking of membrane proteins. These differing fates of ubiquitylated proteins are controlled by the nature of the ubiquitin modification; a single ubiquitin is often insufficient to target the substrate to the proteasome, whereas substrates modified by a polyubiquitin chain can be preferentially targeted to the proteasome. Thus, the degree of processivity of a ubiquitin ligase is crucial in determining the consequences of the modification.

Ubiquitin is usually attached to protein lysine residues. Ubiquitin itself has seven lysines, all of which can be conjugated to a second ubiquitin molecule (Peng *et al.* 2003). This allows for the construction of topologically distinct polyubiquitin chains and a diversity of signaling modes beyond those associated with chain length. For example, Lys48-linked chains are critical for protein degradation, whereas Lys63-linked chains are used in DNA repair and the trafficking of membrane proteins. This flexibility in

signaling is fundamental to the ubiquitin system and may account for the pervasive influence of ubiquitin in cellular regulatory pathways. Ubiquitin receptors, many of which display specificity or preference for ubiquitin chain linkage type or length, play a key role in decoding the signals embedded in the structure of ubiquitin chains (Dikic *et al.* 2009).

The ubiquitin–proteasome and autophagy systems represent the principal modes of breakdown of intracellular proteins in eukaryotes. Autophagy, the hydrolysis of intracellular proteins within the vacuole (Nakatogawa *et al.* 2009), will be described in another article from this series. Autophagy is responsible for the selective breakdown of whole organelles, such as mitochondria and peroxisomes, as well as at least one large protein complex, the ribosome, but in *Saccharomyces cerevisiae*, autophagy is otherwise thought to be nonselective as compared to proteasomal degradation. Rapid protein breakdown within the cytoplasm and nuclei of eukaryotic cells, as exemplified by substrates such as cyclins, is generally mediated by the proteasome.

The paradigm of ubiquitylation, in which a small protein with a β-grasp fold covalently modifies other molecules via its C-terminal glycine, extends to several ubiquitin-like proteins (UBLs), each with a dedicated conjugation machinery: Smt3 (this modification is known as SUMOylation), Rub1 (NEDDylation), Urm1 (urmylation), and the autophagy factors Atg8 and Atg12 (Hochstrasser 2009; Inoue and Klionsky 2010). The target of conjugation is not always a protein; Atg8 is conjugated to phosphatidylethanolamine, thus converting it from a soluble to a membrane-bound protein, and Urm1 acts both as a protein modifier and as a sulfur carrier to support thiolation of tRNAs. Because of space limitations we will discuss these modification pathways below only as they relate to ubiquitylation itself. Space restrictions also prevent us from giving a complete description of the ubiquitin–proteasome system in yeast, and we apologize for any gaps in coverage.

We begin this review by describing the various components of the ubiquitin–proteasome system, including the conjugation cascade, the deubiquitylating enzymes, the proteasome, and the ubiquitin-selective chaperone Cdc48. The nature of substrate recognition in this pathway is then discussed, with special emphasis on the selective modification and degradation of defective proteins. Finally, we consider the many specialized functions of ubiquitylation in the nucleus, endomembrane system, and other subcellular sites.

Ubiquitin–Protein Conjugation

Ubiquitylation reaction

Ubiquitin is typically linked to substrates through an isopeptide bond between the ε-amino group of a substrate lysine residue and the carboxyl terminus of ubiquitin. Ubiquitin conjugation involves the E1–E2–E3 cascade of enzymes (Figure 1A). The reaction is initiated by the ubiquitin-activating enzyme E1 (Uba1), which forms a high-energy thioester bond with the main-chain carboxyl group of the terminal glycine residue of ubiquitin. This step consumes ATP in forming a ubiquitin–adenylate intermediate with subsequent release of AMP and pyrophosphate. Activated ubiquitin is transferred to one of the ubiquitin-conjugating enzymes (E2 or Ubc enzymes) by transesterification. Finally, E3 enzymes (ubiquitin ligases) catalyze the formation of isopeptide bonds between ε-amino groups of lysine residues in substrate proteins and the activated carboxyl group of ubiquitin (Deshaies and Joazeiro 2009; Varshavsky 2012). Through successive rounds of conjugation, polyubiquitin chains are synthesized. Lysines are by far the most common acceptor sites but ubiquitin ligation to the N-terminal amino group in higher eukaryotes (Bloom *et al.* 2003; Ben-Saadon *et al.* 2004; Kirisako *et al.* 2006; Rahighi *et al.* 2009; Tokunaga *et al.* 2009), as well as to serine, threonine, or cysteine in both yeast and mammals, has been observed (Cadwell and Coscoy 2005; Ravid and Hochstrasser 2007; Wang *et al.* 2007; Shimizu *et al.* 2010). The conjugation machinery shows hierarchical organization with one or two E1s (one in yeast), multiple E2s (11 in yeast) (Table 1), and a large family of E3s (60–100 in yeast) (Table 2). E3s mediate the exquisite selectivity of ubiquitylation by direct interaction with substrates.

Topology of ubiquitin conjugates

Monoubiquitylation describes the attachment of a single ubiquitin molecule to a substrate protein, whereas the attachment of more than one ubiquitin is referred to as polyubiquitylation or multiubiquitylation (Figure 1A). Polyubiquitylation represents the characteristic degradation signal, synthesized through isopeptide bond formation between lysine residues on substrate-anchored ubiquitin molecules and activated free-ubiquitin moieties. In contrast, multiubiquitylation is the attachment of multiple single ubiquitin molecules to several acceptor lysine residues in one protein. Marking proteins for degradation by the proteasome is the primary function of most polyubiquitin chains. In contrast, multi- or monoubiquitylation often, but not always (Dimova *et al.* 2012), mediates proteasome-independent functions such as protein binding, subcellular localization, intracellular trafficking, and modulation of activity (Hicke 2001; Kravtsova-Ivantsiv *et al.* 2009; Ziv *et al.* 2011).

Polyubiquitin chain assembly involves the formation of ubiquitin–ubiquitin conjugates, and any of the seven lysines of ubiquitin (K6, K11, K27, K29, K33, K48, and K63) can serve as an isopeptide bond acceptor in yeast and mammals (Figure 1B; Peng *et al.* 2003; Tagwerker *et al.* 2006; Meierhofer *et al.* 2008; Xu *et al.* 2009; Komander and Rape 2012). The resulting chains may define distinct signals, though all of them except K63-linked chains appear to mark proteins for degradation by the proteasome (Meierhofer *et al.* 2008; Xu *et al.* 2009; Kim *et al.* 2011). Depending on the substrate, some K63 chains might do so as well (Saeki *et al.* 2009b). Given that K48 and K63 chains have long been considered as canonical chain topologies, quantitation of the different polyubiquitin chains in yeast revealed a surprisingly

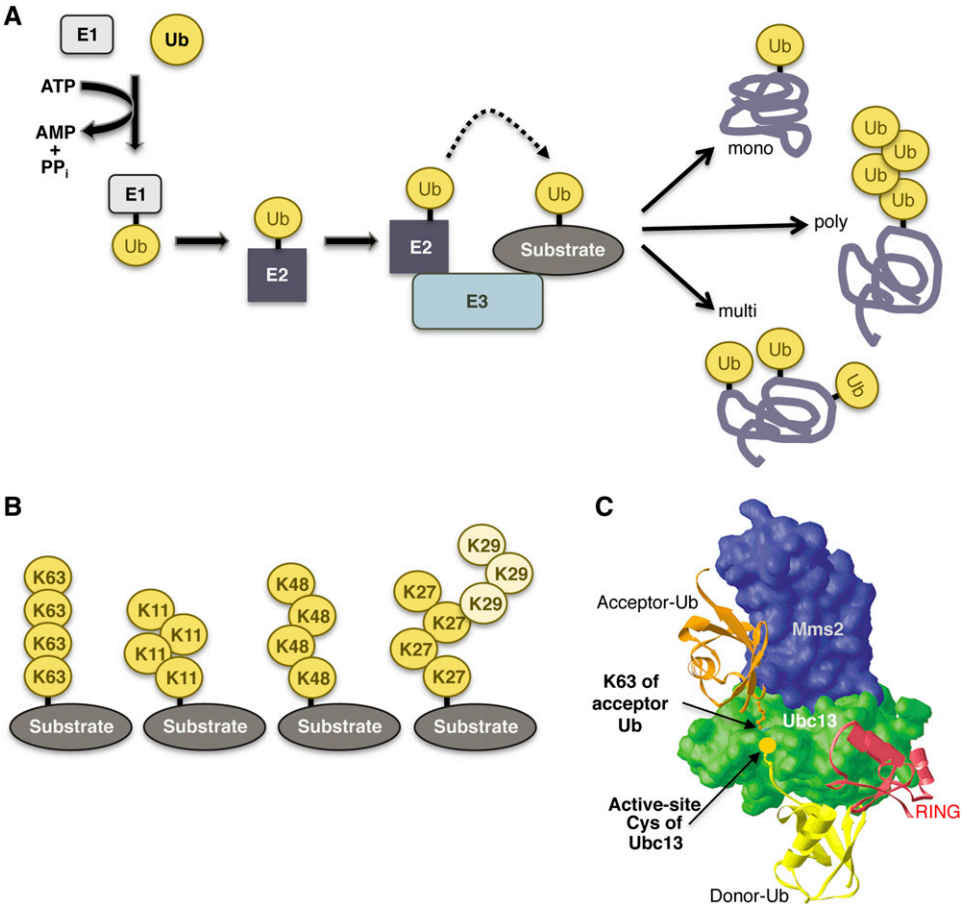

Figure 1 Protein ubiquitylation. (A) Ubiquitin is activated by E1 in an ATP-dependent step, transferred to the active site cysteine in an ubiquitin-conjugating enzyme (E2), and covalently attached to substrate proteins. Substrate selection depends on ubiquitin ligases (E3). Conjugation of a single ubiquitin molecule generates monoubiquitylated proteins. Repeated rounds of ubiquitin activation and conjugation lead to multi- or poly-ubiquitylated proteins. (B) Different polyubiquitin chain topologies can be synthesized depending on the specific lysine residue in ubiquitin used for chain formation. Three of the eight possible unbranched chain topologies (K6, K11, K27, K29, K33, K48, K63, and linear chains), and only one type of the possible forked polyubiquitin chains are shown. (C) Structural model for synthesis of K63-linked polyubiquitin chains by Ubc13/Mms2. Mms2 positions the acceptor ubiquitin with K63 in proximity to the active site cysteine of Ubc13. Figure adapted with permission from Macmillan Publishers Ltd: Chan, N. L., and C. P. Hill, 2001 Nat. Struct. Biol. **8:** 650–652.

high abundance of unconventional linkages. In unperturbed cells, ~29% of all ubiquitin–ubiquitin linkages are through K48, 16% through K63, and 28% through K11. The remaining topologies are less abundant, with K6 at 11%, K27 at 9%, and ~3% each for K29 and K33 (Xu *et al.* 2009).

The concept of specific signaling functions mediated through different ubiquitin chain topologies emerged from the engineering of yeast cells expressing K48R or K63R ubiquitin mutants to prevent formation of K48- or hypothetical K63-linked ubiquitin chains, respectively (Finley *et al.* 1994; Spence *et al.* 1995). K48-linked chains were found to target proteins for degradation and to be essential for viability, whereas K63-chains were dispensable during unstressed growth and did not affect degradation of proteasome substrates, but were required for the DNA damage response. The specific functions of other chain topologies

Table 1 Ubiquitin conjugating enzymes of *Saccharomyces cerevisiae*

UBC	Viable[a]	Biological processes and/or unique features
Ubc1	+	Vesicle biogenesis, ERAD, nuclear protein quality control, E2 for APC
Ubc2/Rad6	+	DNA repair, N-end rule, H2B monoubiquitylation
Ubc3/Cdc34	–	Cell cycle, E2 for SCF ligases
Ubc4	+	Protein quality control outside the nucleus, E2 for APC
Ubc5	+	Comparable to Ubc4 but expression is elevated in stationary phase
Ubc6	+	ERAD, has transmembrane region, can synthesize K11-chains *in vivo*
Ubc7	+	ERAD
Ubc8	+	Regulation of gluconeogenesis
Ubc9[b]	–	E2 for Smt3 (SUMO) conjugation
Ubc10/Pex4	+	Peroxisomal E2 important for peroxisome biogenesis
Ubc11	+	Cytoplasmic localization
Ubc12[b]	+	E2 for Rub1 (Nedd8) conjugation
Ubc13	+	DNA repair, dimerizes with Mms2 for synthesis of K63 chains

[a] In rich medium at 30°.
[b] E2s for conjugation of ubiquitin-like proteins.

Table 2 Ubiquitin ligases and components of *Saccharomyces cerevisiae*

E3	Viable	Biological processes and/or unique features[a]
HECT E3s		
Hul4	+	Unknown
Hul5	+	Cytoplasmic PQC[b], proteasome-associated protein, potential E4
Rsp5	–	Nedd4 family ligase, multiple functions: MVB sorting, endocytosis, transcription,
Tom1	+	mRNA export, degradation of excess histones
Ufd4	+	Ubiquitin fusion degradation pathway, N-end rule
Rsp5 adaptors		
Art1/Ldb19	+	Regulation of endocytosis, localized at plasma membrane
Art2/Ecm21	+	Regulation of endocytosis, localized at plasma membrane
Art3/Aly2	+	Control of nutrient-mediated intracellular sorting of GAP1
Art4/Rod1	+	Regulation of endocytosis, localized at plasma membrane
Art5	+	Regulation of endocytosis, localized at plasma membrane
Art6/Aly1	+	Regulation of endocytosis
Art7/Rog3	+	Regulation of endocytosis
Art8/Csr2	+	Regulation of endocytosis, regulates use of nonfermentable carbon sources
Art9/Rim8	+	Essential for anaerobic growth, PH response
Art10	+	Unknown function, cytoplasmic
Bsd2	+	Facilitates trafficking of metal transporters, localized at Golgi/endosome
Bul1	+	Post-Golgi endosomal sorting, temperature sensitive, functional homolog of Bul2
Bul2	+	Post-Golgi endosomal sorting, functional homolog of Bul1
Ear1	+	Cargo sorting at multivesicular bodies, localized at Golgi/endosome
Ssh4	+	Cargo sorting at multivesicular bodies, localized at Golgi/endosome
Tre1	+	Degradation of metal stransporter smf1, function is redundant with that of Tre2
Tre2	+	Degradation of metal stransporter smf1, function is redundant with that of Tre1
RING E3s[a]		
Asi1	+	SPS sensor signaling of amino acids, homologous to Asi3, transmembrane protein
Asi3	+	SPS sensor signaling of amino acids, homologous to Asi1, transmembrane protein
Asr1	+	RNA Pol II modification, alcohol stress response
Bre1	+	Histone H2B monoubiquitylation on K123
Cwc24	–	Pre-mRNA and snoRNA splicing
Dma1	+	Spindle positioning, orthologs of human Rnf8, redundant with Dma2
Dma2	+	Spindle positioning, orthologs of human Rnf8, redundant with Dma1
Doa10	–	ERAD-C, N-end rule ubiquitylation of acetylated proteins
Etp1	+	Required for growth in ethanol
Fap1	+	Response to rapamycin
Far1	+	G_1 cyclin dependent kinase inhibitor, pheromone response, putative E3
Hel2	+	Degradation of excess histone
Hrd1	+	ERAD-M, ERAD-L
Gid9/Fyv10	+	Degenerate ring domain, cooperates with RMD5 in ubiquitin ligation (see below)
Irc20	+	Unknown, localized to nucleus and mitochondria, has helicase domain
Mag2	+	Unknown function, cytoplasmic, homologous to human Rnf10
Nam7	+	Nonsense mediated mrna degradation, telomere maintenance
Not4	+	Subunit of Ccr4–Not complex, ubiquitylates NAC and histone demethylase Jhd2p
Pep3	+	Vacuolar protein sorting
Pep5	+	Vacuolar protein sorting
Pex2	+	Peroxisomal membrane E3, peroxisomal matrix protein import
Pex10	+	Peroxisomal membrane E3
Pex12	+	Peroxisomal membrane E3, required for peroxisome biogenesis
Pib1	+	Localized in endosomal and vacuolar membranes
Psh1	+	Cse4 ubiquitylation
Rad5	+	PCNA polyubiquitylation, postreplication repair
Rad16	+	Nucleotide excision repair
Rad18	+	PCNA-K164 monoubiquitylation, postreplication repair
Rkr1/Lnt1	+	Ubiquitylation of proteins translated from nonstop mRNAs
Rmd5	+	Gluconeogenesis, degradation of fructose-1,6-bisphosphatase
Rtc1	+	Unknown function
San1	+	Nuclear PQC
Slx5	+	SUMO-directed ligase, genotoxic stress response, forms STUbL together with Slx8
Slx8	+	SUMO-directed ligase, genotoxic stress response, forms STUbL together with Slx5
Snt2	+	Degradation of excess histone
Ssm4	+	mRNA stability, localized to ER/nuclear membrane
Ste5	+	Scaffold protein for MAPK cascade proteins

(continued)

Table 2, *continued*

E3	Viable	Biological processes and/or unique features[a]
Tfb3	−	Cul3 and Rtt101 neddylation, nucleotide excision repair
Tul1	+	Membrane protein sorting, localized to Golgi
Ubr1	+	N-recognin (N-end rule pathway), PQC
Ubr2	+	Rpn4 ubiquitylation, cytoplasmic PQC; Mub1 assists in recognition of Rpn4
Uls1	+	Degradation of SUMOylated proteins
Upf1	+	RING-related, nonsense-mediated decay of mRNA
YBR062C	+	Unknown function
U-box proteins		
Prp19	−	Splicing, U-box protein
Ufd2	+	Ubiquitin fusion degradation pathway, U-box protein, E4 activity, Cdc48 partner
RBR E3s		
Hel1	+	Degradation of excess histone, putative RING-in-between-RING ligase
Itt1	+	Putative RING-in-between-RING ligase
CRL core components		
Cdc53	−	Cullin 1, many functions including cell cycle
Cul3	+	Cullin 3, RNA Pol II ubiquitylation
Rtt101	+	Functional homolog of human cullin 4, DNA repair, rRNA decay
Skp1	−	SCF ligase component, many functions including cell cycle
Elc1	+	Elongin C, binds Cul3, RNA Pol II ubiquitylation
Mms1	+	Adaptor for Rtt101
Hrt1	−	Rbx1/Roc1, RING component of CRL ligases, many functions including cell cycle
F-box proteins		
Amn1	+	Mitotic exit network
Cdc4	−	Cell cycle, many other functions
Cos111	+	Unknown function, localizes to mitochondria
Ctf13	−	Subunit of centromere binding factor 3
Das1	+	Similarity to YDR131C, 6-azauracil sensitive
Dia2	+	Protection from DNA damage and replication stress, part of the RPC
Ela1	+	Elongin A, component of CRL3 ligase, RNA Pol II degradation
Grr1	+	G_1 cyclin degradation, regulates glucose repression
Hrt3	+	Unknown function
Mdm30	+	Mitochondrial fusion
Met30	−	Cell cycle, heavy metal stress response, sulfur compound homeostasis
Mfb1	+	Mitochondria morphology, mitochondria associated
Rav1	+	Component of RAVE complex, important for V-ATPase assembly
Rcy1	+	Recycling of internalized plasma membrane proteins
Roy1	+	Intracellular trafficking, inhibits Ypt52 GTPase activity
Saf1	+	Entry into quiescent phase
Skp2	+	Unknown function, homology to human Skp2
Ufo1	+	HO endonuclease degradation
YDR131C	+	Similarity to Das1
YLR224W	+	Unknown function
YDR306C	+	Unknown function
YLR352W	+	Unknown function
Substrate receptors of Cul3 and Rtt101 ligases		
Crt10	+	Substrate receptor for Rtt101 E3, ribonucleotide reductase gene expression
Elc1	+	Elongin C, component of CRL3 ligase, RNA Pol II degradation
Mms22	+	Substrate receptor for Rtt101 E3, DNA damage response
Rad7	+	Nucleotide excision repair, putative substrate receptor of CRL3
YDR132C	+	Unknown function, putative BTB domain protein
YIL001W	+	Unknown function, putative BTB domain protein
YLR108C	+	Unknown function, putative BTB domain protein
APC cyclosome core components		
Apc1	−	Cell cycle, largest APC/C subunit
Apc2	−	Cell cycle, cullin homology
Cdc27	−	Cell cycle
Apc4	−	Cell cycle
Apc5	−	Cell cycle
Cdc16	−	Cell cycle
Cdc23	−	Cell cycle
Apc9	+	Cell cycle
Doc1/Apc10	+	Cell cycle, coreceptor for D-box recognition

(continued)

Table 2, *continued*

E3	Viable	Biological processes and/or unique features[a]
Apc11	–	Cell cycle, RING-finger subunit of APC/C
Cdc26	+	Cell cycle
Swm1	+	Cell cycle
Mnd2	+	Meiosis
APC cyclosome substrate receptors		
Ama1	+	APC/C activator for meiosis
Cdc20	–	APC/C activator, degradation of Pds1 and other mitotic regulators
Cdh1	+	APC/C activator, degradation of mitotic cyclins

[a] Note that biochemical evidence for ubiquitin ligase activity has so far not been reported for many of these proteins. They are listed here because they contain RING (like) motifs, homology to F-box motifs, or other sequence features frequently associated with ubiquitin ligases. Several proteins that are possible E3s have been excluded from this list: Air1, Air2, Nse1, and Yvh1.
[b] Protein quality control.

are less clear. Interestingly, preventing K11 chain formation in yeast by K11R-ubiquitin replacement results in hypersensitivity to the ER-stress inducers DTT and tunicamycin, indicating that K11 chains are important for the endoplasmic-reticulum–associated degradation (ERAD) pathway (Xu *et al.* 2009).

Methods for the detection and quantification of ubiquitin conjugates have recently been reviewed (Kim *et al.* 2011; Laney and Hochstrasser 2011).

Ubiquitin-activating enzyme

In yeast, a single E1 enzyme is responsible for activation of ubiquitin. E1 is encoded by the essential *UBA1* gene (McGrath *et al.* 1991). Several temperature-sensitive *uba1* alleles exist; *uba1-206* is a tight mutant, and shows rapid depletion of ubiquitin conjugates at nonpermissive temperature as well as other phenotypes expected from a general block of ubiquitylation (Ghaboosi and Deshaies 2007).

Ubiquitin-conjugating enzymes

The first yeast E2 enzymes identified were Rad6/Ubc2 (Jentsch *et al.* 1987) and Cdc34/Ubc3 (Goebl *et al.* 1988). A total of 13 yeast *UBC* genes have been designated (Table 1), though further biochemical analyses revealed that Ubc9 and Ubc12 do not conjugate ubiquitin, but rather the ubiquitin-like proteins Smt3 (mammalian SUMO) and Rub1 (mammalian Nedd8), respectively (Johnson and Blobel 1997; Liakopoulos *et al.* 1998). Among the 11 genuine ubiquitin-conjugating enzymes only Cdc34/Ubc3 is essential for viability (Goebl *et al.* 1988). Temperature-sensitive *cdc34* mutants arrest at the G1-to S-phase transition of the cell cycle due to a defect in degradation of the cyclin-dependent kinase inhibitor Sic1 (Schwob *et al.* 1994). Cdc34 has many other substrates and most are selected by the Skp1–Cdc53–F-box (SCF) ubiquitin ligase family for which Cdc34 serves as the main, if not only, E2 enzyme (Petroski and Deshaies 2005). In addition, Cdc34 together with the ubiquitin ligase San1 functions in the nuclear protein quality control pathway (Gardner *et al.* 2005a; see below). Ubc1 is an alternative ubiquitin-conjugating factor for San1 (Gardner *et al.* 2005a).

Several other E2 enzymes are important for protein quality control pathways outside the nucleus. Ubc4 and Ubc5 are highly similar and function redundantly in conjugation of ubiquitin to abnormal proteins in the cytosol to induce their degradation by the proteasome (Seufert and Jentsch 1990). The double mutant is inviable in some genetic backgrounds (Panasenko *et al.* 2009; Stoll *et al.* 2011); in others it shows severe growth defects (Seufert and Jentsch 1990; Chen *et al.* 1993). Three E2 enzymes are involved in degradation of misfolded proteins from the endoplasmatic reticulum (ERAD pathway; see below): Ubc1, Ubc6, and Ubc7. Among these, only Ubc6 is directly anchored to the ER membrane by a C-terminal transmembrane region (Sommer and Jentsch 1993), whereas Ubc7 is recruited to the ER membrane and activated by ER-bound Cue1 (Biederer *et al.* 1997; Bazirgan and Hampton 2008). Ubc6 but not Ubc7 contributes significantly to total cellular protein modification with K11-linked polyubiquitin chains (Xu *et al.* 2009).

Multiple E2 enzymes can be involved in degradation of a single substrate, the MATα2 transcriptional regulator being a complex case in which four different UBCs have been implicated (Ubc4, Ubc5, Ubc6, and Ubc7) (Chen *et al.* 1993). However, many substrates may rely on a single E2. Ubiquitin-conjugating enzymes can also operate sequentially for efficient substrate polyubiquitylation, as demonstrated for Ubc1 and Ubc4 in polyubiquitylation of cell cycle regulators targeted by a ubiquitin ligase known as the anaphase promoting complex, or cyclosome (APC/C; Rodrigo-Brenni and Morgan 2007). Polyubiquitylation requires two distinct types of conjugation events: Attachment of the first ubiquitin to the substrate protein in an initial monoubiquitylation step, followed by cycles of ubiquitin chain elongation. In yeast, the rate-limiting monoubiquitylation step for APC/C substrates is catalyzed by Ubc4, whereas efficient ubiquitin chain synthesis requires Ubc1 (Rodrigo-Brenni and Morgan 2007). A C-terminal ubiquitin associated (UBA) domain that binds ubiquitin—a feature of Ubc1 not shared with any other yeast E2 (Merkley and Shaw 2004)—is required for optimal processivity of this reaction (Rodrigo-Brenni and Morgan 2007).

Some E2s are poised for synthesis of polyubiquitin chains. For example, heterodimeric E2s such as the yeast Ubc13/Mms2 complex synthesize polyubiquitin chains by transferring the thioester-bound donor ubiquitin from the catalytically active subunit (Ubc13) onto an acceptor ubiquitin that is noncovalently bound to a catalytically inactive UEV (ubiquitin E2 variant) binding partner (Mms2) (Hofmann and Pickart 1999; Eddins *et al.* 2006). Other E2s may transfer preassembled polyubiquitin chains onto substrates, as described for the mammalian Ube2g2 enzyme and its yeast ortholog Ubc7 (Li *et al.* 2007b; Ravid and Hochstrasser 2007). However, the same E2 can often catalyze both mono- and polyubiquitylation. For example, Rad6/Ubc2 catalyzes monoubiquitylation of the proliferating cell nuclear antigen PCNA (Hoege *et al.* 2002) and histone H2B (Robzyk *et al.* 2000), but forms polyubiquitin chains in the context of the N-end rule (Dohmen *et al.* 1991), a conserved pathway that relates protein stability to the identity of the amino terminal residue (Varshavsky 1992; Varshavsky 2011; Tasaki *et al.* 2012). Rad6/Ubc2 functions with different ubiquitin ligases in these pathways, and it appears that ligases and E2 enzymes, as well as the substrates themselves, can be determinants deciding between mono- or polyubiquitylation.

Ubiquitin-conjugating enzymes also help to define the linkage type during polyubiquitin chain synthesis. In particular, E2s dictate chain architecture when paired with **r**eally **i**nteresting **n**ew **g**ene (RING) domain ubiquitin ligases, whereas HECT (homologous to E6-AP carboxy terminus) domain E3s override any intrinsic chain topology preference of E2s. Synthesis of polyubiquitin chains with specific architectures by RING–E3/E2 pairs requires positioning of the E2 such that the linkage-defining lysine residue in the acceptor ubiquitin is proximal to the charged active site cysteine of the E2. The best-studied example in yeast is Ubc13, which synthesizes K63-linked chains (Figure 1C). In the Ubc13/Mms2 heterodimer, Mms2 positions the acceptor ubiquitin so that only K63 is allowed to approach the active site cysteine of Ubc13 (Eddins *et al.* 2006). A related mechanism was demonstrated for the mammalian ubiquitin-conjugating enzyme Ube2S, where a ubiquitin-binding region in the E2 orients the acceptor ubiquitin for K11-selective chain synthesis (Wickliffe *et al.* 2011).

The ubiquitin-conjugating enzymes are at the center of the E1–E2–E3 cascade. They interact with E1 and E3 but also ensure unidirectional handoff of ubiquitin from E1 to the substrate. E1 and E3 use a shared binding site on E2s, preventing recharging of E2s while bound to E3s and forcing their dissociation before the next round of conjugation (Eletr *et al.* 2005). Directionality of ubiquitin transfer is ensured by E1-dependent ATP hydrolysis as well as the different affinities of charged and uncharged E2s for E1 and E3. The ubiquitin-activating enzyme E1 binds uncharged E2s with higher affinity than the E2∼Ub leading to release of the loaded E2∼Ub (Hershko *et al.* 1983; Pickart and Rose 1985). Similarly, E3s have somewhat higher affinity for E2∼Ub than for the uncharged E2, facilitating processive ubiquitin chain synthesis (Siepmann *et al.* 2003; Saha and Deshaies 2008).

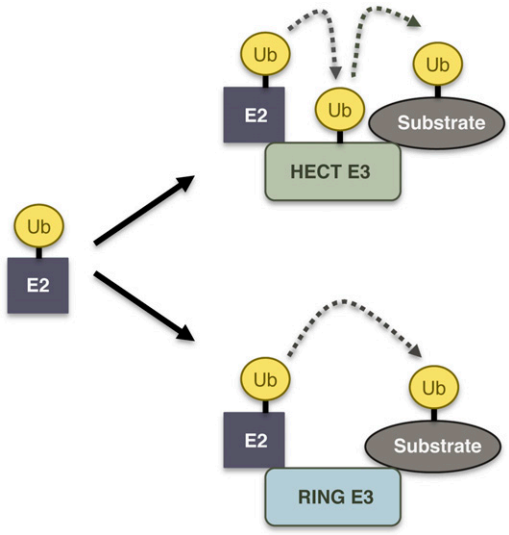

Figure 2 HECT and RING E3 ubiquitin ligases. Substrate ubiquitylation with HECT E3s involves an E3∼Ub thioester intermediate. Ubiquitin is transferred from the HECT E3 to the substrate. RING E3s typically do not form thioester intermediates but promote ubiquitin conjugation by bridging the interaction between E2 and substrate proteins. RING E3s also stimulate E2 activity. A subclass of RING-based ligases, the RING-in-between-RING (RBR) proteins, function like RING/HECT hybrids and form thioester intermediates. This mechanism remains to be confirmed for putative yeast RBR ligases.

Ubiquitin ligases

Ubiquitin ligases (E3s) form the largest group of proteins involved in ubiquitylation and they confer selectivity to the process. They bind E2s and substrate proteins to facilitate substrate-specific ubiquitylation. The first E3 identified was Ubr1, a mediator of the N-end rule pathway. Ubr1 binds protein substrates with different affinities based on their N-terminal amino acids (Bartel *et al.* 1990; Varshavsky 1992). Many other E3 enzymes were subsequently identified, all falling into two major classes: RING domain E3s (including the structurally related U-box domain E3s) and HECT domain E3s. Considering sequence features frequently associated with ubiquitin ligases, such as RING (like), F box, or HECT motifs, there are 60–100 putative E3s in yeast (Table 2). Most belong to the class of RING domain E3s, and only five HECT domain E3s are encoded in the yeast genome (Table 2). RING and HECT domain E3s follow distinct mechanisms to catalyze ubiquitylation (Figure 2). HECT domain E3s contain an active site cysteine within the HECT domain, which forms a thioester with ubiquitin received from an E2 prior to its transfer to the substrate (Scheffner *et al.* 1995). RING E3s do not form thioester intermediates; they instead facilitate ubiquitin transfer by positioning the charged E2∼Ub in proximity to the acceptor lysine in the substrate. In addition, RING domain ligases seem to activate E2s to facilitate ubiquitylation (Deshaies and Joazeiro 2009).

A subclass of RING domain E3s, the RING-in-between-RING (RBR) proteins, appear to function as RING/HECT

hybrids (Wenzel and Klevit 2012). They bind E2s with one RING domain and stimulate the transfer of ubiquitin onto a conserved cysteine residue in the other RING domain, forming an E3∼Ub thioester before conjugation to the substrate (Wenzel *et al.* 2011). Homology searches revealed two putative RBR ligases in yeast (Hel1 and Itt1) (Eisenhaber *et al.* 2007). Whether they indeed form E3∼Ub intermediates is unknown.

Functional interaction between RING and HECT domain E3s has been demonstrated for the N-end rule pathway (Hwang *et al.* 2010). The RING-type Ubr1 and HECT-type Ufd4 ligases form a complex to enhance processivity of substrate ubiquitylation. A similar role for the Ubr1/Ufd4 complex in the ubiquitin-fusion degradation pathway has also been suggested (Hwang *et al.* 2010). Interestingly, the Ubr1/Ufd4 complex may function as an E3/E4 pair. E4 enzymes—a small subgroup of ubiquitin ligases—select substrate proteins based on their having been previously ubiquitylated, and E4s function to extend these ubiquitin chains (Koegl *et al.* 1999).

HECT ubiquitin ligases: HECT domain E3s are named after their founding member E6AP, which ubiquitylates mammalian p53 in cells expressing the human papilloma virus protein E6. Yeast has five HECT E3s: Rsp5, Ufd4, Hul4, Hul5, and Tom1. The HECT domain is an ∼350-residue region consisting of the N-terminal lobe, which binds an E2, and the C-terminal lobe containing the active site cysteine, which forms a thioester intermediate with ubiquitin. N and C lobes are connected by a flexible hinge region (Huang *et al.* 1999). The five yeast HECT domain ubiquitin ligases function in diverse processes ranging from multivesicular body (MVB) sorting, endocytosis, histone degradation, and processing of ubiquitylated proteins (Hoppe *et al.* 2000; Shcherbik *et al.* 2003; Rape and Jentsch 2004; Crosas *et al.* 2006; Rotin and Kumar 2009; Singh *et al.* 2009).

The E3∼Ub thioester intermediate mediates E3-instructed ubiquitin chain assembly as demonstrated for Rsp5, which has been shown to dictate synthesis of K63-linked chains independently of the E2 enzymes used (Kim and Huibregtse 2009). Although the molecular mechanism is not known in detail, mutational studies suggest that the carboxy-terminal region of Rsp5 is involved in acceptor ubiquitin orientation to favor nucleophilic attack from lysine-63 in ubiquitin.

Rsp5 is the only yeast HECT E3 essential for viability in rich medium. Rsp5 is a particularly active E3 that mediates ubiquitylation of a large number of substrates and contributes to regulation of diverse biological pathways (Gupta *et al.* 2007; Rotin and Kumar 2009). Rsp5 is required for upregulation of expression of the fatty acid desaturase *OLE1* by the homologous transcription factors Spt23 and Mga2, and accordingly the lethality of *rsp5* mutants can be rescued by addition of oleic acid to the growth medium (Hoppe *et al.* 2000). While Spt23 and Mga2 are normally anchored in the ER membrane, Rsp5-mediated ubiquitylation induces proteasomal processing and release of transcriptional activation domains from these proteins (Hoppe *et al.* 2000; Shcherbik *et al.* 2003). Hul5, another HECT domain protein, is discussed below in the *Proteasome* section.

RING domain ubiquitin ligases: There are 44 yeast proteins containing RING domains and two proteins of the U-box family, which are structurally related to RING E3s but do not bind zinc (Ufd2 and Prp19). Although conclusive biochemical evidence for ubiquitin ligase activity is not available for all RING domain proteins, most of them probably have this activity. The globular RING domains bind E2 enzymes (Zheng *et al.* 2000) and appear to stimulate ubiquitin transfer by induction of subtle structural changes (Ozkan *et al.* 2005). Substrate recruitment, the central function of ubiquitin ligases, is achieved either by substrate binding domains within the same polypeptide chain as the RING domain (single subunit RING E3s) or by engaging specialized substrate receptors to form multisubunit RING E3s (Deshaies and Joazeiro 2009). Examples of the former are the N-recognin Ubr1 (Bartel *et al.* 1990); the ubiquitin ligase Bre1 that together with Rad6/Ubc2 catalyzes histone H2B ubiquitylation (Wood *et al.* 2003); the regulator of nuclear protein quality control San1 (Gardner *et al.* 2005a); Rkr1/Ltn1, which ensures degradation of potentially cytotoxic translation products produced from mRNAs that lack stop codons (Bengtson and Joazeiro 2010); and the two RING E3s, Rad18 and Rad5, which catalyze mono- and polyubiquitylation of PCNA, respectively (Hoege *et al.* 2002; see below). Prominent members of the multisubunit RING E3s are the APC/C (Pesin and Orr-Weaver 2008) and the largest group of ligases, the modular cullin-RING ligases (CRLs) (Petroski and Deshaies 2005; Zimmerman *et al.* 2010; Duda *et al.* 2011). Although one subunit (Apc2) of APC/C contains a cullin-like domain, the overall ligase architecture is very different from that of true CRLs. Detailed studies of other RING domain proteins (Table 2) may identify additional multisubunit E3s as has been shown for the seven-subunit Gid (glucose-induced degradation-deficient) E3, which controls the metabolic switch between glycolysis and gluconeogenesis (Santt *et al.* 2008; Menssen *et al.* 2012).

APC/C: APC/C is perhaps the most complex ubiquitin ligase. Its core is composed of 13 subunits (Apc1, Apc2, Cdc27, Apc4, Apc5, Cdc16, Cdc23, Apc9, Doc1, Apc11, Cdc26, Swm1, and Mnd2), with Apc11 being the RING domain component that binds Ubc1 and Ubc4, the two primary E2s functioning with yeast APC/C (McLean *et al.* 2011). The core APC/C associates with one of three activators that bind substrates and are crucial targets for APC/C regulation. Cdh1 and Cdc20 are activators controlling mitotic cell cycle progression and Ama1 recruits meiotic targets to APC/C (Visintin *et al.* 1997; Cooper *et al.* 2000).

Degradation of several important APC/C substrates ensures ordered progression through the steps of chromosome segregation. A cascade of mitotic events is unleashed by APC/C-mediated degradation of Pds1/securin to initiate

the metaphase-to-anaphase transition (Cohen-Fix *et al.* 1996; Yamamoto *et al.* 1996). Pds1 is an inhibitor of Esp1/separase, a protease that cleaves the cohesin Scc1 to allow sister chromatid separation (Ciosk *et al.* 1998; Uhlmann *et al.* 1999). Clb2 and other B-type cyclins are degraded by APC/C from anaphase until the end of the subsequent G_1 phase, which ensures a period of low cyclin-dependent kinase activity that is important for cytokinesis and the assembly of prereplication complexes (Irniger *et al.* 1995). Many other mitotic and meiotic regulators are APC/C substrates, and their degradation controls both normal mitotic processes and cell cycle checkpoint pathways (Pesin and Orr-Weaver 2008; McLean *et al.* 2011).

Tight regulation of Cdh1 and Cdc20 restricts APC/C activity to M phase and G_1 of the mitotic cell cycle (Pesin and Orr-Weaver 2008). G_2/M-phase–induced *CDC20* expression, APC/C phosphorylation-dependent binding of Cdc20 (Rudner and Murray 2000; Rudner *et al.* 2000), combined with active Cdc20 degradation during G_1 by APC/C^{Cdh1}, limit Cdc20 association with APC/C to M phase (Prinz *et al.* 1998; Foe *et al.* 2011). In contrast, Cdh1 levels are largely constant throughout the cell cycle, but binding to APC/C is prevented by Cdh1 phosphorylation during most of the cell cycle, except late M phase and G_1 (Zachariae *et al.* 1998).

APC/C substrates share distinct degradation motifs, the most common being the classic destruction box (D box) and the KEN box (Glotzer *et al.* 1991; Pfleger and Kirschner 2000). Although APC/C activators play a crucial role in D-box and KEN-box recognition, the core subunit Apc10/Doc1 serves as a coreceptor in D-box recognition (Carroll *et al.* 2005; Da Fonseca *et al.* 2011). Regulation occurs at the level of activator abundance, phosphorylation of activators and core components, as well as binding of the APC/C^{Cdc20} inhibitors Mad2 and Mad3 (McLean *et al.* 2011).

Cullin-RING ligases: CRLs form the largest group of ubiquitin ligases in all eukaryotes. A typical CRL ligase consists of four subunits: the RING protein Hrt1/Rbx1/Roc1, a cullin, a linker protein, and one of many alternative substrate receptors (Petroski and Deshaies 2005; Zimmerman *et al.* 2010; Duda *et al.* 2011). CRLs are assembled on a central scaffold subunit, the cullin, three of which are found in budding yeast (Cdc53, Cul3, and Rtt101). The C-terminal regions of cullins bind the small RING domain subunit Hrt1 (Kamura *et al.* 1999; Ohta *et al.* 1999; Seol *et al.* 1999), which in turn recruits and activates the E2 Cdc34. The N-terminal regions of cullins interact with substrate receptor subunits (F box, SOCS box, or DCAF proteins), usually through linker proteins (Skp1, Elc1, and Mms1) (Figure 3). Depending on the cullin, different classes of CRLs are formed. Cdc53 and Cul3 are orthologs of human Cul1 and Cul3, respectively. Rtt101 does not show significant homology to any particular vertebrate cullin but is functionally similar to human Cul4. The canonical CRLs, the SCF ligases, are assembled onto Cdc53/Cul1.

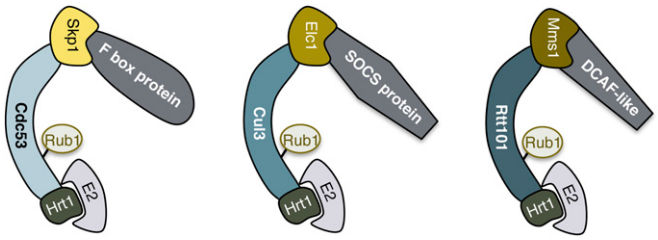

Figure 3 Cullin RING ligases (CRLs). A large class of multisubunit RING-based ligases is nucleated around cullins. Yeast has three classes of CRLs formed with the cullins Cdc53 (cullin 1), Cul3, and Rtt101 (functionally similar to human Cul4). The C-terminal regions of cullins bind the RING protein Hrt1/Rbx1/Roc1, and the N-terminal portions interact with specific adaptor proteins (Skp1, Elc1, and Mms1), which recruit substrate receptor proteins (F-box, SOCS-box, or DCAF proteins). Putative substrate receptors are listed in Table 3.

Proteins containing the F-box motif form substrate receptors of SCF ligases and recruit proteins with their C-terminal protein binding domains for ubiquitylation (Bai *et al.* 1996). Often substrate phosphorylation creates a binding surface that is recognized by the F-box subunit (Feldman *et al.* 1997; Skowyra *et al.* 1997). Yeast encodes 22 F-box proteins (Table 2), most of which form SCF ligases with distinct substrate specificities. Three F-box proteins (Cdc4, Met30, and Ctf13) are essential for viability in rich medium. Ctf13 likely does not form a conventional SCF E3, but is a structural component of the centromere binding complex CBF3 (Russell *et al.* 1999a). The best-studied yeast F-box proteins are Cdc4, Grr1, and Met30. The corresponding ligases SCF^{Cdc4}, SCF^{Grr1}, and SCF^{Met30} each control ubiquitylation of cell cycle regulators and proteins involved in nutrient signaling and may thus be key factors for integration of cell cycle progression and nutrient status.

SCF^{Cdc4} controls entry into S phase by degradation of the cyclin-dependent kinase inhibitor Sic1 (Feldman *et al.* 1997; Skowyra *et al.* 1997) and regulates the response to amino acid starvation through ubiquitylation and degradation of the transcription factor Gcn4 (Meimoun *et al.* 2000; Chi *et al.* 2001). SCF^{Cdc4}/Sic1 is probably the best-studied ligase/substrate pair, and much of our understanding about CRL function comes from biochemical characterization of Sic1 ubiquitylation. SCF^{Grr1} ubiquitylates the G_1 cyclins Cln1 and Cln2 to control their abundance (Barral *et al.* 1995; Seol *et al.* 1999; Skowyra *et al.* 1999). Consequently, SCF^{Grr1} is an important regulator of cyclin-dependent kinase activity during G_1. In addition, SCF^{Grr1} induces degradation of Mth1, which is critical for glucose sensing and adaptation to varying glucose concentrations (Flick *et al.* 2003). SCF^{Cdc4} and SCF^{Grr1} have many additional substrates and functions (Benanti *et al.* 2007; Skaar *et al.* 2009).

Fewer substrates are currently known for SCF^{Met30}, but their analyses have taught us about diversity and flexibility of ubiquitin signaling. SCF^{Met30} coordinates cell division with nutrient or heavy metal stress (Kaiser *et al.* 2006). One key substrate in this pathway is the transcription factor Met4, which is directly inactivated by modification with

Table 3 Deubiquitylating enzymes of *Saccharomyces cerevisiae*

DUB	Type	Localization/complex	Phenotype[a]
Ubp1	USP	Cytoplasmic, ER	Mild
Ubp2	USP	Ubp2/Rsp5/Rup1	Pleiotropic
Ubp3	USP	Ubp3/Bre5	Pleiotropic
Doa4/Ubp4	USP	Endosomal, Doa4/Bro1	Ub deficient, partial ts, can^s
Ubp5	USP	Bud neck	Assorted mild phenotpyes
Ubp6	USP	Proteasomal	Ub deficient; enhanced proteolysis, can^s
Ubp7	USP	Cytoplasmic	Increased prion formation
Ubp8	USP	Nuclear; SAGA	Sensitive to heat and γ-rays; partial ts
Ubp9	USP	Cytoplasmic	Mild
Ubp10	USP	Nuclear	Decreased silencing, partial cs, can^s
Ubp11	USP		Pleiotropically stress sensitive, can^s
Ubp12	USP		can^s
Ubp13	USP		Pleiotropically stress sensitive
Ubp14	USP		Elevated free ubiquitin chains, can^s
Ubp15	USP		Stress sensitive, partial ts, strong cs, can^s
Ubp16	USP	Mitochondrial	Can^s, slow growth on nonfermentable carbon
Rpn11	JAMM	Proteasomal	Essential (DUB activity not essential)
Otu1	OTU	Cdc48	Pleiotropically stress sensitive
Otu2	OTU	Ribosome associated (?)	Pleiotropically stress sensitive
Yuh1	UCH	Cytoplasmic	Acts preferentially on Rub1 (*vs.* ubiquitin)

[a] can^s, sensitive to amino acid analog canavanine; cs, cold-sensitive; ts, temperature-sensitive.

a K48-linked ubiquitin chain, but degradation is prevented because two ubiquitin binding motifs in Met4 shield the polyubiquitin chain from signaling degradation (Flick *et al.* 2006; Tyrrell *et al.* 2010). Although ubiquitylated Met4 is inactive as a transcription factor, it functions as a substrate receptor in the context of the extended SCF$^{Met30/Met4}$ ubiquitin ligase to trigger ubiquitylation and degradation of several Met4 binding factors, including Met32, which induces cell cycle arrest when stabilized (Ouni *et al.* 2010). The dual function of Met4 as transcription factor and ubiquitin ligase component allows it to coordinate cell cycle progression with response to nutrient or heavy metal stress.

An interesting aspect of CRL regulation is a ubiquitin-like modification found on cullins. Cullins are covalently modified on a conserved lysine residue in the C-terminal region by the ubiquitin-like protein Rub1, the yeast ortholog of metazoan Nedd8 (Lammer *et al.* 1998; Liakopoulos *et al.* 1998). Cullin modification with Nedd8 induces a major conformational change such that the E2-binding interface of the RING component Hrt1 extends out from the cullin surface, remaining tethered only by a flexible linker region. This not only allows the E2 to closely approach the substrate, but also provides the flexibility to adopt different conformations necessary for polyubiquitin chain synthesis (Duda *et al.* 2008). Rub1 modification is not essential for viability of budding yeast, but it is required for robust CRL activity and is essential in other organisms (Willems *et al.* 2004).

Deubiquitylation

Deubiquitylating enzymes catalyze the hydrolysis of the isopeptide bonds that link ubiquitin to its targets (Reyes-Turcu *et al.* 2009). Twenty deubiquitylating enzymes (DUBs) are found in yeast (Table 3), falling into four families: the Usp family, including 16 members; the Otu family, with two members; and the JAMM and Uch families, with one member each. Additional paralogs exist, with specificity for ubiquitin-like proteins such as Smt3 and Rub1. Yuh1, the lone Uch-type DUB in yeast, may serve primarily in the removal of Rub1 from target proteins, although capable of deubiquitylation as well (Linghu *et al.* 2002). Most DUBs are thiol proteases, the only exception being Rpn11, a zinc metalloprotease (Verma *et al.* 2002; Yao and Cohen 2002). The three-dimensional structures of several DUBs from yeast and other organisms are available (Johnston *et al.* 1999; Hu *et al.* 2005; Li *et al.* 2007a; Sato *et al.* 2008; Reyes-Turcu *et al.* 2009; Köhler *et al.* 2010).

The DUBs are highly diverse functionally, reflecting both their subcellular localization and their inherent substrate specificities. For example, Ubp8 is a component of the SAGA complex, a nuclear particle involved in chromatin remodeling (Henry *et al.* 2003). Ubp10 is also a specific regulator of nuclear processes such as the silencing of gene expression (see below). Other DUBs seem to function specifically on endosomes and multivesicular bodies, such as Doa4/Ubp4 (Luhtala and Odorizzi 2004; Amerik *et al.* 2006). One DUB, Ubp16, is thought to be an integral membrane protein and fractionates with mitochondria (Kinner and Kölling 2003). The enzymatic specificity of DUBs from yeast is only partially characterized (Amerik *et al.* 2000b; Schaefer and Morgan 2011). DUBs are presented with potential substrates that must number in the hundreds and possibly thousands, given the breadth of the ubiquitin pathway (Kim *et al.* 2011). Systematic identification of DUB substrates in yeast has not been attempted, and it is even unclear in general how rapidly ubiquitin modifications of protein substrates are reversed within cells.

DUB activity is required not only for the disassembly of ubiquitin–protein conjugates but also for biosynthetic

processing of the Ubi1–Ubi4 gene products, ubiquitin fusion proteins that are the sole source of ubiquitin in the cell. *UBI1*–*UBI3*, which supply most of the ubiquitin in growing, unstressed cultures, encode ubiquitin as N-terminal fusions to ribosomal proteins L40 and S31 (Finley *et al.* 1989). *UBI4*, the stress-responsive ubiquitin gene (Finley *et al.* 1987), has a series of six tandem repeats of the ubiquitin coding sequence (Ozkaynak *et al.* 1984). DUB activity is essential to release ubiquitin from these precursor forms, as their C termini are blocked. It is not known which DUBs are responsible for these cleavage events, but they exhibit fast reaction kinetics, as observed for artificial ubiquitin–β-galactosidase fusion proteins (Bachmair *et al.* 1986).

An important function of the DUBs is to recycle ubiquitin by recovering it from ubiquitin–protein conjugates before the target protein is degraded. Defects in this process give rise to reduced ubiquitin levels and pleiotropic stress sensitivities. The main DUBs responsible for recovering ubiquitin from conjugates that are en route to being degraded are Ubp6, Rpn11, and Doa4 (Swaminathan *et al.* 1999; Amerik *et al.* 2000b; Leggett *et al.* 2002; Hanna *et al.* 2003, 2007; Chernova *et al.* 2003; Kimura *et al.* 2009). Ubp6 and Rpn11 rescue ubiquitin from degradation by the proteasome, and Doa4 releases ubiquitin from membrane proteins that are about to be internalized within multivesicular bodies en route to the lysosome. Both Ubp6 and Rpn11 can release ubiquitin from proteasome substrates in the form of unanchored chains (Verma *et al.* 2002; Yao and Cohen 2002; Hanna *et al.* 2006). If not promptly disassembled, such chains can inhibit the proteasome by competing with ubiquitin–protein conjugates for access to proteasomal ubiquitin receptors. Ubp14 is dedicated to breaking down such unanchored chains (Amerik *et al.* 1997). Its specificity is achieved by recognition of the free C terminus of the proximal ubiquitin of the chain, leading to allosteric activation and cleavage of the isopeptide bond joining the proximal ubiquitin to the penultimate member of the chain (Reyes-Turcu *et al.* 2009). Doa4 can also disassemble free chains and plays a major role in this process upon heat shock (Kimura *et al.* 2009).

DUBs often function within protein complexes, and in such cases are typically activated by incorporation into the complex. For example, Ubp6 and Rpn11 are thought to be active only when associated with the proteasome (Leggett *et al.* 2002; Verma *et al.* 2002), Ubp3 is activated by Bre5 (Cohen *et al.* 2003), and Otu1 functions in association with Cdc48 (Rumpf and Jentsch 2006). A particularly elegant example is the activation of Ubp8 as a DUB when it is incorporated into the SAGA complex (Köhler *et al.* 2010). Additional modes of DUB regulation are exemplified by the transcriptional induction of the *UBP6* gene in response to reduced ubiquitin levels (Hanna *et al.* 2007); the inhibition of Doa4 by Rfu1, which is relieved upon heat shock (Kimura *et al.* 2009); and stimulation of Ubp3 activity by Hog1 kinase-dependent phosphorylation upon osmotic stress (Solé *et al.* 2011).

Some DUBs antagonize specific ubiquitin ligases. Ubp2 forms a complex with the ligase Rsp5, and deubiquitylates those proteins that Rsp5 modifies (Kee *et al.* 2005, 2006; Harreman *et al.* 2009). Other cases of DUB–ligase antagonism involve E4 enzymes. Thus, the E4 Ufd2 is antagonized by Otu1, with both residing on Cdc48 (Rumpf and Jentsch 2006), and the E4 Hul5 is antagonized by Ubp6, with both residing on the proteasome (Crosas *et al.* 2006). It would be interesting to understand why DUB–ligase pairs have evolved in these cases, since most ligases do not seem to be pitted against a specific DUB in this way.

Because of the abundance of DUBs in yeast, it is necessary to take precautions against postlysis deubiquitylation when assessing the role of ubiquitylation in any setting. DUBs that are thiol proteases are inactivated by the alkylating agent *N*-ethylmaleimide, but a zinc chelating agent such as *o*-phenanthroline is recommended in addition to neutralize the metalloprotease Rpn11 (Verma *et al.* 2002).

Proteasome

The proteasome has 33 distinct subunits (Table 4) and is the most complex protease known (Finley 2009). Its principal function is to degrade ubiquitin–protein conjugates. The proteasome is found in all eukaryotes and is highly conserved in evolution. Proteasomes are organized into two subassemblies, the 19S regulatory particle (RP) and the 20S core particle (CP). The RP recognizes substrates to be degraded, while the CP contains the proteolytic active sites. The proteolytic sites are sequestered within an interior space of the CP, ensuring that access to these sites is under strict control and nonspecific proteolysis is minimized (Figure 4). Substrates are routed from the RP to the CP through a narrow substrate translocation channel, which can exist in open and closed states (Figure 4). Globular proteins must be unfolded to traverse this channel. Unfolding is an active process mediated by the six distinct ATPases of the RP, Rpt1–Rpt6, which form a heteromeric ring complex (Figure 5A). Simple methods are available for testing whether an unstable protein is degraded in a proteasome-dependent manner (Fleming *et al.* 2002; Liu *et al.* 2007).

Core particle

The CP is a barrel-like structure composed of four stacked rings of subunits (Groll *et al.* 1997). The two outer rings are known as α rings, the two inner rings as β rings (Figure 4). CP components are generally referred to as α1–α7 and β1–β7 (Table 4). The proteolytic activity of the proteasome resides in the β ring; subunits β1, β2, and β5 are proteolytically active and are founding members of the threonine class of proteases. In each case, the active site nucleophile is the N-terminal α-amino group of the main chain. β1, β2, and β5 are synthesized as proenzymes and cleaved upon CP assembly to reveal a threonine residue at the new N terminus (Chen and Hochstrasser 1996; Arendt and Hochstrasser 1997; Groll *et al.* 1997). The specificities of the β1, β2, and

Table 4 Proteasome components and cofactors

Subcomplex or gene	Alias	Domains	Notes
CP			
Scl1	α1		
Pre8	α2		
Pre9	α3		Nonessential
Pre6	α4		
Pup2	α5		
Pre5	α6		
Pre10	α7		
Pre3	β1	Propeptide	Proteolytically active
Pup1	β2	Propeptide	Proteolytically active
Pup3	β3		
Pre1	β4		
Pre2	β5	Propeptide	Proteolytically active
Prs3	β6	Propeptide	
Pre4	β7	Propeptide	
RP base			
Rpt1		AAA, OB, CC	ATPase
Rpt2		AAA, OB, CC, HbYX	ATPase
Rpt3		AAA, OB, CC, HbYX	ATPase
Rpt4		AAA, OB, CC	ATPase
Rpt5		AAA, OB, CC, HbYX	ATPase
Rpt6		AAA, OB, CC	ATPase
Rpn1		TPR-like repeats	Apparent scaffold
Rpn2		TPR-like repeats	Apparent scaffold
Rpn13		PRU domain	Ub receptor, nonessential
Rpn10		VWA, UIM	Ub receptor, nonessential
RP lid			
Rpn3		PCI	
Rpn5		PCI	
Rpn6		PCI	
Rpn7		PCI	
Rpn8		MPN	
Rpn9		PCI	Nonessential
Rpn11		MPN	DUB activity
Rpn12		PCI	
Sem1			Nonessential
Associated proteins			
Ubp6		UBL and USP	DUB activity
Hul5		HECT	Ub ligase activity
Ufd4		HECT	Ub ligase activity
Ubc4		E2	E2 enzyme
Ecm29		HEAT	Possible chaperone
Blm10		HEAT	Opens CP gate
Rad23		UBL and UBA	Ub receptor
Dsk2		UBL and UBA	Ub receptor
Ddi1		UBL and UBA	Ub receptor

β5 active sites are trypsin-like, caspase-like, and chymotrypsin-like, in that they prefer basic, acidic, or hydrophobic residues, respectively, N-terminal to the scissile bond (Groll *et al.* 2005).

The α rings regulate substrate access into the inner chamber of the CP (Groll *et al.* 2000; Whitby *et al.* 2000; Bajorek *et al.* 2003). In the free form of the CP, the center of the α ring is occupied by N termini from all seven subunits, which converge into a defined but irregular structure that blocks substrate access to the chamber. Another important function of the α ring is to serve as a docking site for the RP and other regulators of the CP, such as Blm10. Both the RP and Blm10 activate the peptidase of the CP by shifting the α N termini away from the center of the α ring, and thus creating an opening for the passage of substrate (Finley 2009; Sadre-Bazzaz *et al.* 2010). The interfaces of the α subunits form seven pockets, which provide docking sites for the RP and Blm10 (Sadre-Bazzaz *et al.* 2010; Tian *et al.* 2011). The C termini of the Rpt proteins project into these pockets to stabilize the association between the RP and CP and drive opening of the CP channel (see below).

Regulatory Particle

Subunit organization of the regulatory particle: The spatial organization of the RP has been resolved in recent

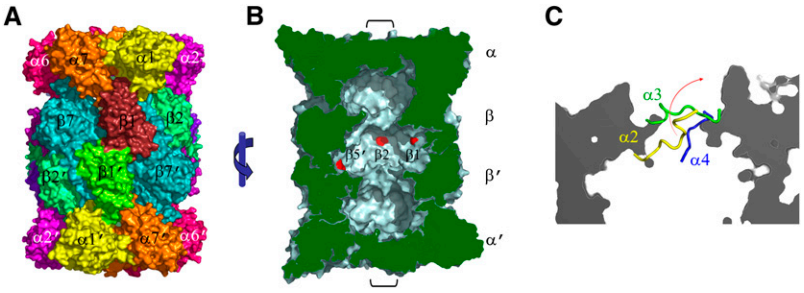

Figure 4 Proteasome core particle. (A) Space-filling exterior view of the CP, with subunits differentiated by color. Note the $\alpha_7\beta_7\beta_7\alpha_7$ organization. (B) Medial cut-away view of the CP, showing the interior cavity and active sites (red) sequestered within it. The substrate transloction channel is fully closed in the crystal structure of the free CP, but brackets indicate the approximate position of the channel in its open state. (C) Detail of the CP gate. The N-terminal tails of the α subunits, particularly α2, α3, and α4, as shown, block substrate access. The bodies of the α subunits are rendered in gray. Arrow indicates the movement of the tails that constitutes gate opening, a likely upward and outward migration (Förster *et al.* 2003). Images modified from Groll *et al.* 1997 and Tian *et al.* 2011, with permission.

electron microscopy studies (Lander *et al.* 2012; Lasker *et al.* 2012; Pathare *et al.* 2012; Sakata *et al.* 2012), as summarized in Figure 5. The RP is composed of the 10-subunit base and nine-subunit lid subassemblies (Table 4; Glickman *et al.* 1998; Finley 2009). The RP is anchored to the CP principally through the base, but the lid subunit Rpn6 also contacts the CP (Lander *et al.* 2012; Pathare *et al.* 2012). Dissociation of the RP into base and lid is observed upon purification of proteasomes from *rpn10Δ* mutants, or upon purification of wild-type proteasomes in the presence of high salt (Glickman *et al.* 1998; Saeki *et al.* 2000). Moreover, the base and lid are intermediates in RP assembly (see below). Thus, the base–lid dichotomy reflects the fundamental organization of the RP.

Unfolding of the protein substrate and its translocation into the CP are driven by ATP hydrolysis (Schrader *et al.* 2009; Sauer and Baker 2011; Smith *et al.* 2011a). The heterohexameric Rpt ring of the base represents the ancient core of the machinery that defines ATP-dependent proteases in all kingdoms of life (Figure 5A). The 13 additional components of the RP are peculiar to eukaryotes and seem designed in large part to recognize or process the ubiquitin component of the ubiquitin–protein conjugate, as discussed below. For example, two components of the base are ubiquitin receptors, and other components of the base, Rpn1 and Rpn2, are large subunits that serve as scaffolds (Figure 5C), allowing for the recruitment of a variety of factors, such as shuttling receptors (see below) with their cargo of ubiquitin–protein conjugates.

Substrate recognition: Two subunits of the RP, Rpn10, and Rpn13, bind ubiquitin chains. Rpn10 binds via its α-helical **U**biquitin-**I**nteracting **M**otif (UIM) element (Elsasser *et al.* 2004; Verma *et al.* 2004; Mayor *et al.* 2007), and Rpn13 via a pleckstrin homology (PH) domain known as the **P**leckstrin-like **R**eceptor for **U**biquitin (PRU) domain (Husnjak *et al.* 2008). Rpn10 and Rpn13 are situated on opposite sides of the substrate entry port, with Rpn13 more distant from the port due its apical position (Figure 5C; Lander *et al.* 2012; Sakata *et al.* 2012). Although not proximal to one another (Figure 5B), Rpn10 and Rpn13 might simultaneously engage the same ubiquitin chain, given adequate chain length. The UIM element of Rpn10 appears to contact the coiled-coil domain shared by Rpt4 and Rpt5 (Figure 5B). Rpt5 has been hypothesized to be a ubiquitin receptor based on cross-linking studies (Lam *et al.* 2002), though never shown to bind ubiquitin directly; and the proximity of its coiled-coil element to the UIM of Rpn10 (Lander *et al.*

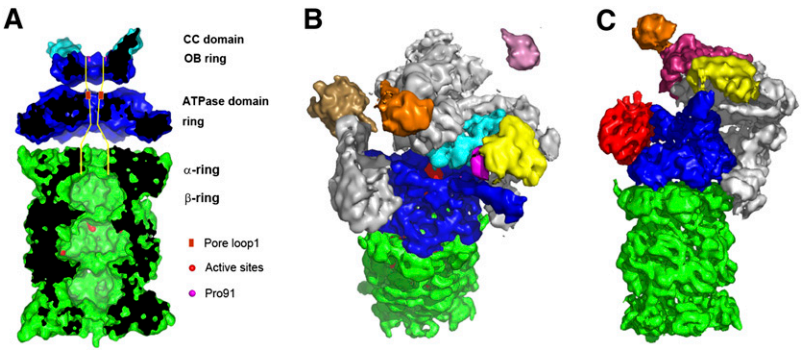

Figure 5 The proteasome holoenzyme. (A) Model of the Rpt ring of the proteasome in association with the yeast CP. Medial cut-away view, with the Rpt ring modeled from observations of the PAN ATPase from Archaea (adapted from Zhang *et al.* 2009b, with permission). The ATPase domain of the Rpt ring and the smaller OB domain above it both in blue. Coiled-coil elements (turquoise) emerge distally from the OB domain with their trajectory influenced by Pro91 (pink). The CP is in green, with proteolytic sites in red. Slice surfaces of the CP and Rpt ring are in black. The presumptive substrate translocation channel is demarcated with yellow lines: The entry port of the translocation channel is thought to be the OB ring, and substrates must migrate to the proteolytic active sites (red) to be hydrolyzed. The driving force for translocation is thought to be axial motions of the pore loops from the ATPase domain that line the translocation channel (gold rectangles). (B) Tilted view of the RP based on EM studies (Lander *et al.* 2012). The Rpt ring and CP are colored as in A. The DUB Rpn11 is in turquoise, with the presumptive substrate entry port directly beneath it (red-orange). The ubiquitin receptor Rpn13 is in orange. To its left is Ubp6 (approximate position), contacting Rpn1. To the right is Rpn10, with its **V**on **W**illebrand **A** (VWA) domain in yellow and its ubiquitin-binding UIM domain in red. All other RP subunits are in gray. Shown for comparison at upper right is free ubiquitin (pink). (C) Lateral view of the RP (derived from Lander *et al.* 2012). Highlighted are Rpn1 (red-orange), Rpn2 (pink), Rpn13 (orange), and Rpn10 (yellow). Lid subunits are in gray. B and C are from Tian *et al.* (2012), with permission.

2012) provides a plausible explanation of the cross-linking result. The ability of Rpn10 to recognize ubiquitin chains is regulated by its ubiquitylation; ubiquitin covalently linked to Rpn10 can fold back to occupy the UIM site (Isasa *et al.* 2010).

The RP also recognizes ubiquitin conjugates through a family of UBL–UBA proteins that serve as shuttling receptors: Rad23, Dsk2, and Ddi1 (Table 4; Schauber *et al.* 1998; Chen and Madura 2002; Elsasser *et al.* 2002; Rao and Sastry 2002; Saeki *et al.* 2002a; Elsasser and Finley 2005; Finley 2009; Rosenzweig *et al.* 2012). The N-terminal UBL (ubiquitin-like) domain in each shuttling receptor serves as a docking site for the proteasome, and the UBA domain (or domains) binds ubiquitin chains. Rpn1 and Rpn13 have been identified as receptor sites for UBL–UBA proteins (Elsasser *et al.* 2002; Saeki *et al.* 2002b; Husnjak *et al.* 2008; Peth *et al.* 2010; Gomez *et al.* 2011; Rosenzweig *et al.* 2012). Of the five proteasomal ubiquitin receptors described above, none is essential, and there is some degree of functional redundancy in addition to distinct roles. The biochemical basis of their functional differentiation remains largely unknown.

The shuttling receptors have divergent properties. Ddi1, for example, contains an aspartyl protease domain that is likely to be functional based on its crystal structure and on the identification of a defined phenotype in an active-site substitution mutant (Sirkis *et al.* 2006; White *et al.* 2011). Thus, the protease activity of Ddi1 could possibly provide an alternative to the proteasome as a means to attack ubiquitylated proteins. Dsk2 is distinguished by the existence of an extraproteasomal pool that is largely complexed to a free pool of Rpn10 (van Nocker *et al.* 1996; Matiuhin *et al.* 2008; Zhang *et al.* 2009a). In this complex, the UBL domain of Dsk2 binds the UIM element of Rpn10, which is the ubiquitin-binding element of Rpn10 (Zhang *et al.* 2009a). Interestingly, the UBL–UIM interaction can be displaced by a substrate-bound ubiquitin chain to form a ternary complex, that, with an unoccupied Dsk2 UBL domain, is activated for proteasome binding. Despite this interaction, Dsk2 does not bind proteasomes via Rpn10 (Elsasser *et al.* 2002; Matiuhin *et al.* 2008). Interestingly, a mammalian homolog of Dsk2 has been implicated in amyotrophic lateral sclerosis (Deng *et al.* 2011). As described below, Rad23 participates in the nucleotide excision repair (NER) pathway of DNA repair. Finally, mutated variants of the shuttling receptors have been studied as unique model substrates of the proteasome, although their wild-type forms are relatively stable (Heessen *et al.* 2005; Fishbain *et al.* 2011; Heinen *et al.* 2011; Sekiguchi *et al.* 2011)

Deubiquitylation at the proteasome: The lid is positioned for the most part laterally to the base, and its subunits extend like fingers to contact the base at many points (Figure 5C; Lander *et al.* 2012; Lasker *et al.* 2012). A key function of the lid is to deubiquitylate proteasome substrates, an activity mediated by its subunit Rpn11 (Maytal-Kivity *et al.* 2002; Verma *et al.* 2002; Yao and Cohen 2002). Rpn11, a metalloprotease, is thought to cleave at the substrate-proximal tip of the chain, thus removing the chain entirely. Rpn11 activity is typically dependent on ATP, though it is unlikely that Rpn11 is an ATPase. Rpn11 activity is likely coupled to ATP hydrolysis by Rpt proteins of the base, which is thought to translocate the substrate through the axial channel formed by the Rpt proteins. Presumably the substrate-attached chain is thereby moved toward the entry port of the channel, where it may encounter Rpn11. In agreement with this model, Rpn11 is found near the entry port of the substrate translocation channel (Figure 5B; Lander *et al.* 2012).

Remarkably, the lid is paralogous to two free complexes found in eukaryotic cells, eIF3 and the COP9 signalosome complex (Glickman *et al.* 1998). It appears that in the course of evolution the lid gave rise to the COP9 signalosome and eIF3. The COP9 signalosome is active in the removal of the ubiquitin-like protein Rub1 (see above) from covalent adducts to the cullin Cdc53 (Cope *et al.* 2002). Thus, the COP9 signalosome functions analogously to the lid, except that as it lost its association with the proteasome, its specificity was modified so that it cleaves a ubiquitin-like protein rather than ubiquitin.

Ubp6 is a second major proteasome-associated deubiquitylating enzyme (Verma *et al.* 2000; Leggett *et al.* 2002). *ubp6* null mutants are ubiquitin deficient (Amerik *et al.* 2000b; Leggett *et al.* 2002), due to elevated rates of ubiquitin turnover by the proteasome (Chernova *et al.* 2003; Hanna *et al.* 2003). Thus Ubp6 serves, like Rpn11, to protect ubiquitin from degradation by the proteasome by removing ubiquitin before it is translocated into the CP. However, Ubp6 does so quite differently from Rpn11. First, the position of Ubp6 is distant from the substrate entry port (Figure 5B; Lander *et al.* 2012) Unlike Rpn11, Ubp6 disassembles ubiquitin chains in an ATP-independent manner. Ubp6 serves to inhibit protein degradation by the proteasome, using two distinct mechanisms. Its deubiquitylating activity can shorten a chain before the substrate is productively engaged by the proteasome, leading to release of intact substrate. This has been shown most clearly with Ubp6's mammalian ortholog, Usp14 (Lee *et al.* 2010). Second, a catalytically inactive form of Usp14 can also inhibit protein degradation, through an unknown mechanism (Hanna *et al.* 2006). Finally, Ubp6 can influence gating of the substrate translocation channel (Peth *et al.* 2009). *ubp6* mutants show an exceptional ability to tolerate aneuploidy (Torres *et al.* 2010), owing apparently to enhanced quality-control protein degradation, perhaps reflecting enhanced proteasome activity.

Substrate deubiquitylation by the proteasome is antagonized by Hul5, a proteasome-associated ubiquitin ligase (Crosas *et al.* 2006). Numerous proteins are stabilized or degraded nonprocessively in *hul5* mutants, consistent with a generalized E4 activity of Hul5 (Crosas *et al.* 2006; Kohlmann *et al.* 2008; Aviram and Kornitzer 2010; Fang *et al.* 2011). The balance of Hul5 and Ubp6 activity can fine tune

proteasome activity to cellular conditions (Hanna *et al.* 2007; Fang *et al.* 2011; Park *et al.* 2011). In particular, Hul5 has been shown to be the major ubiquitin ligase targeting misfolded cytosolic proteins upon heat stress (Fang *et al.* 2011).

Initiation sites: Some proteins are resistant to protein degradation by the proteasome, even when modified by canonical ubiquitin chains. One potential explanation is that such proteins are inherently resistant to unfolding. However, this property does not correlate with the thermal melting profile of these proteins (Lee *et al.* 2001). Such proteins can be converted into favored substrates by appending short peptide segments to their N- or C termini (without perturbing their thermal melting profile). Unstructured peptide elements that are necessary for proteasome-mediated degradation (Prakash *et al.* 2004; Takeuchi *et al.* 2007; Schrader *et al.* 2009) are known as *initiation sites*. Such sites may be employed to dissociate specific subunits of a protein complex for selective degradation (Johnson *et al.* 1990; Verma *et al.* 2001; Prakash *et al.* 2009). Degradation is thought to proceed from an initiation site (Piwko and Jentsch 2006; Schrader *et al.* 2009), usually continuing to completion. In rare cases, degradation is interrupted and stable protein fragments escape from the proteasome, owing to the inability of the proteasome to effect complete substrate unfolding. As described below, this type of mechanism is used to activate certain transcription factors (Hoppe *et al.* 2000; Piwko and Jentsch 2006; Schrader *et al.* 2009).

Rpt ring: Crystallographic studies on the homohexameric **P**roteasome-**A**ctivating **N**ucleotidase (PAN) complex of Archaea, which is orthologous to the Rpt ring, have identified major structural features (Zhang *et al.* 2009b). A channel is formed at the center of the ring of ATPase domains, and within this channel are two "pore loops" that are likely to contact substrates (Figure 5A). When ATP is hydrolyzed, conformational changes of the ATPase domains are thought to move the pore loops along the axis of the channel, providing the driving force for substrate unfolding and translocation (Sauer and Baker 2011; Erales *et al.* 2012). The pore loops are expected to interact first with the initiation sites of the substrate, and then to track along the polypeptide as substrate translocation into the CP proceeds.

The Rpt proteins also contain oligonucleotide/oligosaccharide-binding (OB) domains (Zhang *et al.* 2009b), positioned on the N-terminal sides of the ATPase domains (Figure 5A). In the case of PAN, the OB domain self-assembles into a homohexameric ring complex (also known as the N ring). This ring is coaxial with the ATPase domain ring (Figure 5A). Most likely the OB ring serves as the substrate entry port of the proteasome, and the substrate's initiation site must thread through the central channel of the OB ring before coming into contact with the pore loops of the ATPase domain. Whether the OB ring engages substrates or provides a passive pore, one likely function of this ring is to impose a stringent criterion on the length of a functional initiation sequence. The presence of the OB domain may allow for eukaryotic proteins to have significant stretches of unstructured sequence without being readily degraded by the proteasome.

The OB domain of PAN forms a trimer of dimers. Each dimer is asymmetric in that the peptide bond at Pro91 is in the *trans* configuration in one subunit but in *cis* in its partner. Pro91 is positioned between the coiled-coil and OB domains, so this kink in the trajectory of the main chain allows for the α-helical elements emerging from partnered OB domains to coalesce into a coiled coil. This trimer of dimers arrangement is evidently replicated in the yeast proteasome (Zhang *et al.* 2009b), with "*cis*-Rpt's" alternating around the ring (Tomko *et al.* 2010).

Interface between the RP and CP: The Rpt proteins belong to the **A**TPases **A**ssociated with a variety of cellular **A**ctivities (AAA) family of ATPases. A distinguishing feature of the AAA family is the C domain, which is positioned at the perimeter of the ATPase domain. The C-terminal "tails" of the Rpt proteins are thought to be flexible, and some or all of the tails emerge from the C domains and insert into the α pockets of the CP. A motif at the end of the tail, the HbYX motif, is found on three of the six Rpt proteins, and these three Rpts—Rpt2, Rpt3, and Rpt5—are critical for CP gating (Smith *et al.* 2007). The Rpt tails have been mapped to the α pockets into which they insert by cross-linking (Tian *et al.* 2011). Surprisingly, the interface has an asymmetric character, with fixed contacts between the Rpt2, Rpt6, and Rpt3 tails and the α pockets into which they insert and on the other side of the ring, a less defined pocket specificity among the other tails (Tian *et al.* 2011). The CP–RP interface is stabilized not only by the insertion of Rpt tails into α pockets, but also presumably by an interaction between Rpn6 and α2 (Lander *et al.* 2012; Pathare *et al.* 2012).

Blm10 and ubiquitin-independent protein degradation by the proteasome

Not all proteasome substrates require modification by ubiquitin. One example is ornithine decarboxylase (ODC, encoded by *SPE1*), which catalyzes the committed step in polyamine biosynthesis, and is under intricate feedback control (Kurian *et al.* 2011). ODC is antagonized by ODC antizyme (Oaz1). When polyamine levels are high, Oaz1 is induced and binds to ODC. This exposes a peptide in ODC that can serve as an initiation site; ODC is then unfolded by the RP and degraded by the CP (Takeuchi *et al.* 2008).

In contrast to ODC, the proteasome's ubiquitin-independent substrates may typically be degraded without the participation of the RP. Other factors can replace the RP on the cylinder end of the CP, open the CP channel, and promote protein degradation (Finley 2009). The most conserved of these "CP activators" is Blm10, a 246-kDa HEAT-repeat protein (Schmidt *et al.* 2005). Approxiately 20% of proteasomes in yeast are hybrid RP–CP–Blm10 complexes (Schmidt *et al.*

2005). Blm10 binds to the cylinder end of the CP in the form of a turban and inserts its C-terminal HbYX element into the α5/α6 pocket to open the CP gate (Sadre-Bazzaz *et al.* 2010). An aperture in Blm10, though small, could provide access to the CP channel for an unfolded protein. Perhaps in this way, Blm10 promotes degradation of Sfp1, a transcriptional activator of ribosomal protein genes (Dange *et al.* 2011; Lopez *et al.* 2011). Blm10 also participates in assembly of the CP (Fehlker *et al.* 2003; Marques *et al.* 2007).

Proteasome activators such as Blm10 seem to lack both the capacity to recognize ubiquitin and to hydrolyze ATP. Their ability to promote protein degradation relies on opening of the CP channel, to provide access to substrate. They may preferentially catalyze the degradation of proteins that can bypass an ATP-dependent unfolding step, either because the substrate spontaneously unfolds at a high frequency or is constitutively unfolded (Dange *et al.* 2011).

Regulation of proteasome activity

The transcription factor Rpn4 recognizes consensus binding elements upstream of all genes encoding major proteasome components (Mannhaupt *et al.* 1999; Leggett *et al.* 2002). The protein is extremely unstable, being a substrate for the Ubr2 ligase (Wang *et al.* 2004; Ju *et al.* 2008), and Rpn4 is also degraded by the proteasome in a ubiquitin-independent pathway (Ju and Xie 2006; Ha *et al.* 2012). Consequently, when proteasome function is compromised, Rpn4 levels rise, leading to homeostatic restoration of proteasome activity (Xie and Varshavsky 2001; Metzger and Michaelis 2009; Wang *et al.* 2010). Under conditions of "proteasome stress," proteasomes also exhibit altered composition (Park *et al.* 2011). Chronic upregulation of proteasome activity by overexpression of Rpn4 leads to extended replicative lifespan in yeast (Kruegel *et al.* 2011; see also Chen *et al.* 2006).

Proteasome Assembly

CP assembly

An early step in CP assembly is formation of the seven-membered α ring. This ring is then used as a template for assembly of the β ring. The resulting structures, or "half-mers," are subsequently joined through β ring–β ring interactions to form the mature $\alpha_7\beta_7\beta_7\alpha_7$ CP. The proteolytic sites of the CP are held in an inactive state until the $\alpha_7\beta_7\beta_7\alpha_7$ complex is fully assembled, so that the proteolytic sites are never active unless sequestered from the cytoplasm. This pathway is ordered through the action of five dedicated assembly chaperones (Table 5) (Ramos *et al.* 1998; Le Tallec *et al.* 2007; Li *et al.* 2007c; reviewed by Kusmierczyk and Hochstrasser 2008).

The Pba1–Pba2 heterodimer binds the outer, RP-binding surface of the α ring, and the Pba3–Pba4 heterodimer the inner surface, which abuts the β ring in the mature particle. Interestingly, Pba1 and Pba2 contain HbYX motifs, suggesting that they may suppress premature Rpt tail insertion into nascent CP species (Kusmierczyk *et al.* 2011). The crystal structure of a Pba3–Pba4–α5 ternary complex indicates that these chaperones occlude interaction surfaces between the α and β rings (Yashiroda *et al.* 2008). However, the Pba3–Pba4 heterodimer has more complex effects on assembly, since its absence results in the substitution of α4 for α3 in a subset of proteasomes (Kusmierczyk *et al.* 2008).

Table 5 Assembly chaperones for the proteasome

CP chaperones	Domains/motifs	Ligands
Pba1	HbYX	An α pocket?
Pba2/Add66	HbYX	An α pocket?
Pba3/Irc25		α5
Pba4/Poc4		α5
Ump1		β5 propeptide
RP chaperones		
Nas2	PDZ	Rpt5 C domain
Nas6	Ankyrin repeats	Rpt3 C domain
Rpn14	WD40 repeats	Rpt6 C domain
Hsm3	Arm-like repeats	Rpt1 C domain

The three catalytically active β subunits, as well as two of the catalytically inactive subunits, are synthesized with N-terminal propeptides. Propeptide removal follows upon the joining of two half-mers, reflecting that formation of the interface between β rings is required for the proteolytic sites to acquire catalytic activity (Arendt and Hochstrasser 1997). Interestingly, the propeptide of β5 is essential for this subunit's incorporation into the CP (Chen and Hochstrasser 1996). The β5 propeptide also interacts physically with Ump1 (Heink *et al.* 2005), a chaperone that suppresses half-mer dimerization until the β ring is complete (Li *et al.* 2007c). The β ring is completed with the addition of the β7 subunit (Marques *et al.* 2007). This subunit has a C-terminal tail that reaches to the neighboring β ring and inserts into the interface between β1 and β2. As half-mers are joined, Ump1 is encapsulated in the nascent CP and degraded (Ramos *et al.* 1998).

RP assembly

The base and lid appear to have independent assembly pathways, and are joined to form the RP late in the pathway. Base assembly involves four dedicated and evolutionarily conserved chaperones, which are not found in mature proteasomes (Table 5) (Funakoshi *et al.* 2009; Kaneko *et al.* 2009; Le Tallec *et al.* 2009; Park *et al.* 2009; Roelofs *et al.* 2009; Saeki *et al.* 2009a). Each of these "RP chaperones" binds to the C domain of an Rpt protein, which constitutes a notable example of convergent evolution, because they have no sequence or structural homology.

The base is assembled from three precursor complexes or modules. Each module is defined by a pair of Rpt proteins, containing one *cis* and one *trans* subunit with respect to Pro91 (Rpt1–Rpt2, Rpt3–Rpt6, and Rpt4–Rpt5) (Funakoshi *et al.* 2009; Kaneko *et al.* 2009; Saeki *et al.* 2009a; Tomko *et al.* 2010). Thus, the slow steps in Rpt ring assembly are those involving the presumably weak interdimeric interfaces.

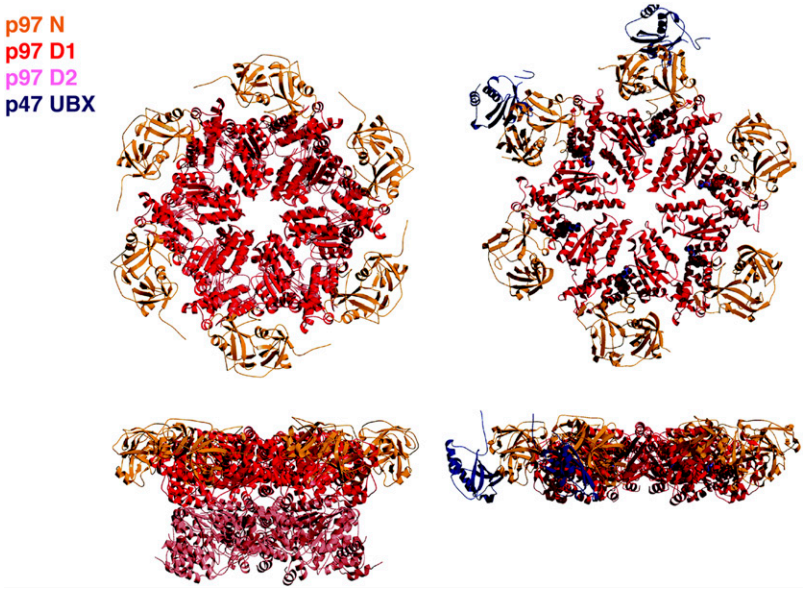

Figure 6 Structure of p97/Cdc48. Left: Ribbon representations of full-length p97. Top and side views are shown. The N, D1, and D2 domains are indicated in different colors. Right: Ribbon representations of p97 N and D1 domains interacting with p47. Top and side views, as at left. These images were reproduced with permission from Dreveny *et al.* (2004).

Each module contains at least one RP chaperone as well: the Rpt1–Rpt2 module is found with Hsm3 bound to Rpt1, whereas the Rpt4–Rpt5 module has Nas2 on Rpt5. The final module has both Nas6 and Rpn14 bound to Rpt3 and Rpt6, respectively.

The Rpt C-terminal tails appear to be critical for assembly; deletion of a single amino acid from the C terminus of either Rpt6 or Rpt4 leads to a dramatic defect in RP formation (Park *et al.* 2009). The Rpt proteins that have strong effects on gating—Rpt2, Rpt3, and Rpt5—have, by contrast, little effect on proteasome assembly. The assembly phenotypes of the *rpt6* and *rpt4* mutants suggest a role of the CP in RP assembly, and indeed, RP assembly is defective in several mutants whose primary defect is in CP assembly (Kusmierczyk *et al.* 2008; Park *et al.* 2011). Such CP mutants do not interfere nonspecifically with RP assembly but rather block an early step of Rpt ring formation, consistent with a templating model (Park *et al.* 2011)

How the chaperones promote proper base assembly remains to be solved. Interestingly, when bound to an Rpt C domain, some or all of the RP chaperones may project partly in the direction of the CP (Roelofs *et al.* 2009; Barrault *et al.* 2012). Chaperone binding is thus hypothesized to occlude contacts between the Rpt tail and its cognate α pocket, thus minimizing the formation of premature or incorrect RP–CP contacts (Roelofs *et al.* 2009). The RP chaperones Hsm3, Nas6, and Rpn14 do not bind the Rpt tail, only the proximal C domain (Roelofs *et al.* 2009; Takagi *et al.* 2012). Thus, they may occlude the tail by virtue of the proximity of the C domain to the tail. Consistent with this idea, Rpn14 remains stably associated with the proteasome when the C-terminal amino acid of Rpt6 is deleted (presumably leading to poor engagement of this tail), and Nas6 remains associated with proteasomes in the C-terminal mutant of Rpt3 (Park *et al.* 2009). Thus, negative regulation of the insertion of Rpt C termini into the α pockets of the CP may be a key mechanism of chaperone action. Nas2 appears to bind both the C domain and the tail itself, so it may well conform to the model (Lee *et al.* 2011). Some chaperones may positively regulate lateral interactions between ATPases as well, as indicated by recent work on Hsm3 (Barrault *et al.* 2012).

Whereas base assembly is guided by multipe chaperones, no chaperones have been identified for lid assembly. A landmark in lid assembly is the incorporation of Rpn12, the last subunit to join the complex (Fukunaga *et al.* 2010; Tomko and Hockstrasser 2011). The arrival of Rpn12 converts the nascent lid into a state competent to join with the base to form the RP. This property of Rpn12 is likely accounted for by direct contacts between this subunit and the base (Tomko and Hockstrasser 2011).

Cdc48 ATPase

An essential factor involved widely in ubiquitin-dependent processes is the chaperone Cdc48 (Meyer *et al.* 2012). This enzyme's ortholog in mammalian cells is p97 or valosin containing protein (VCP). Cdc48 belongs to the AAA family of ATPases (Halawani and Latterich 2006; Ye 2006; Jentsch and Rumpf 2007). It comprises two AAA ATPase domains, D1 and D2, and a terminal N domain (Figure 6). The chaperone assembles into cylindrical homohexamers that undergo nucleotide-dependent conformational changes, predominantly between the N and D1 domains (Pye *et al.* 2006). Genetic defects in the chaperone give rise to VCP disease (Ju and Weihl 2010), a progressive autosomal disorder associated with inclusion body myopathy, Paget disease of the bone, and frontotemporal dementia, accompanied by a marked accumulation of polyubiquitylated proteins.

The first link of Cdc48 to ubiquitin was found in a screen for stabilizing mutants in the N-end rule pathway (Ghislain *et al.* 1996). However, Cdc48 participates in diverse cellular processes such as cell cycle progression, homotypic

membrane fusion, DNA repair, and transcription factor processing. To support this wide range of functions, ancillary proteins regulate Cdc48 activity toward individual substrates in a spatially and temporally controlled manner (Schuberth and Buchberger 2008). The majority of ancillary factors are ubiquitin receptors that deliver ubiquitylated proteins to Cdc48, suggesting that it acts downstream of ubiquitylation and upstream of the proteasome. At least some Cdc48-associated proteins bind mutually exclusively to Cdc48 and thus define functionally distinct subcomplexes (Schuberth and Buchberger 2008). Interestingly, Cdc48 action may also lead to proteolysis in the vacuole. Cdc48 acts on substrates modified with the ubiquitin-like molecule Atg8 in the course of macroautophagy (Krick *et al.* 2010) and it plays a role in ribophagy under starvation conditions (Ossareh-Nazari *et al.* 2010).

Proteins that associate with Cdc48 are classified according to their Cdc48-binding domains. For example, regulatory cofactors containing ubiquitin regulatory X (UBX) domains or suppressor of high-copy PP1 (SHP) boxes bind to distinct regions within the N domain (Schuberth and Buchberger 2008). In contrast, several proteins harboring a peptide:N-glycanase/UBA or UBX-containing (PUB) or PLAP, Ufd3, and Lub1 (PUL) domain bind near the C terminus of Cdc48 (Madsen *et al.* 2009).

Cdc48's mechanism of action is not fully understood. Initial insight derived from analysis of the oleic acid (OLE) pathway, which controls the synthesis of unsaturated fatty acids in yeast, from the ERAD pathway, and from analysis of membrane fusion events. Transcription of *OLE1* is driven by Spt23 and its homolog Mga2. Spt23 is synthesized as an inactive precursor (p120), which is anchored in the membrane of the ER. In the absence of unsaturated fatty acids, p120 is ubiquitylated by the ubiquitin ligase Rsp5 and cleaved by the proteasome. The resulting p90 fragment lacks a transmembrane anchor and can thus drive transcription of *OLE1* (Hoppe *et al.* 2000). A complex of Cdc48 and the cofactors Ufd1 and Npl4 is involved in Spt23 activation. After activation, Spt23 exists in the ER membrane as a homodimer, only one subunit of which is processed. Cdc48–Ufd1–Npl4 binds and mobilizes the ubiquitylated and processed p90, separating it from the unprocessed Spt23. These data suggested that Cdc48 acts as a segregase to disassemble protein complexes (Rape *et al.* 2001; Jentsch and Rumpf 2007; Shcherbik and Haines 2007).

In support of a segregase function, Cdc48 is required to remove ubiquitylated Rpb1, the largest subunit of RNA Pol II, from chromatin. Rbp1 turnover is induced by UV (see below) and dependent on Cdc48, Ufd1, Npl4, Ubx4, and Ubx5 (Verma *et al.* 2011). In the absence of functional Cdc48, with Rpb1 degradation inhibited, ubiquitylated forms of Rbp1 are still delivered to the proteasome (Verma *et al.* 2011). These findings suggest that Cdc48 need not function strictly upstream of the proteasome but might act on proteasome-bound ubiquitin–protein conjugates. In some cases, however, the ATPases of the proteasome seem to be sufficient to extract a proteolytic target from a protein complex (see above). The mechanism whereby Cdc48 separates subunits of a protein complex from one another remains to be understood at the biochemical level. In particular, it is unresolved whether this activity involves threading of substrates through a central channel in Cdc48, in analogy to the mechanism of the proteasome.

In the turnover of ERAD substrates, Cdc48 acts subsequently to ubiquitylation but prior to the proteasome. The cofactors Ufd1 and Npl4 contain ubiquitin binding domains and participate in the export of polyubiquitylated proteins from the lumen and membrane of the ER (Bays *et al.* 2001b; Ye *et al.* 2001; Braun *et al.* 2002; Jarosch *et al.* 2002; Rabinovich *et al.* 2002). The integral membrane protein Ubx2 recruits Cdc48–Ufd1–Npl4 to the ER membrane and establishes its interaction with ubiquitin ligase complexes involved in ERAD (Neuber *et al.* 2005; Schuberth and Buchberger 2005). In membrane fusion processes, Cdc48 associates with the cofactor Shp1/Ubx1 (or, in mammals, with the homologous protein p47), to promote the homotypic fusion of membranes derived from the nucleus, the ER, and the Golgi apparatus (Hetzer *et al.* 2001).

Notably, some Cdc48 partner proteins modify the ubiquitin chains of bound substrates, thereby regulating the fate of these substrates. Thus, the antagonistic actions of the E4 enzyme Ufd2 and the DUB Otu1 determine the length of polyubiquitin chains on certain Cdc48 substrates, such as Spt23. According to a current model, Cdc48 accepts oligoubiquitylated substrates from ligases and then adjusts the length of a polyubiquitin chain prior to the substrate's dissociation from the Cdc48–Ufd1–Npl4 complex. The handoff of ubiquitylated proteins from Cdc48 to the proteasome is facilitated by the interaction of Ufd2 with the UBL domains of Rad23 and Dsk2. Once dissociated from Ufd2, these UBL domains are free to deliver substrate to the proteasome (Richly *et al.* 2005; Rumpf and Jentsch 2006; Hänzelmann *et al.* 2010; see also Kim *et al.* 2004).

Substrate Recognition in the Ubiquitin Pathway

Quality-control protein degradation

One of the major functions of the ubiquitin–proteasome system is the disposal of misfolded and damaged proteins. Cells are highly sensitive to such proteins (Geiler-Samerotte *et al.* 2011) and possess several mechanisms, in addition to the ubiquitin–proteasome system, to neutralize them (Liu *et al.* 2011). Misfolded proteins are often localized in subcellular compartments that may either reduce the toxicity of these proteins or promote efficient quality-control protein turnover (Kaganovich *et al.* 2008). Several E3s involved in protein quality control have been discovered. They include the nuclear quality control ligase San1 (Gardner *et al.* 2005a) and the endoplasmic-reticulum–associated degradation E3s Hrd1 and Doa10 (see below). The principal quality

control ligases in the cytoplasm appear to be Ubr1, Hul5, and the ribosome-bound ubiquitin ligase Rkr1/Ltn1 (Eisele and Wolf 2008; Bengtson and Joazeiro 2010; Heck *et al.* 2010; Nillegoda *et al.* 2010; Fang *et al.* 2011). In addition, Ubr2 may contribute to removal of misfolded cytopasmic proteins (Nillegoda *et al.* 2010).

How do these ubiquitin ligases recognize their substrates? San1 appears to bind directly to misfolded proteins through exposed hydrophobic patches, without the need for chaperones (Fredrickson *et al.* 2011; Rosenbaum *et al.* 2011). This interaction is mediated by multiple binding sites for substrates with different properties, embedded into intrinsically disordered regions in San1 (Rosenbaum and Gardner 2011; Rosenbaum *et al.* 2011). San1 ubiquitylates a range of mutant and thus presumably misfolded nuclear proteins, and deletion of the *SAN1* gene induces a cellular stress response (Gardner *et al.* 2005a). Remarkably, even some misfolded cytoplasmic proteins are subject to San1-dependent degradation after Hsp70-dependent import into the nucleus (Prasad *et al.* 2010), suggesting an important function of the nucleus in ubiquitin/proteasome-mediated protein quality control. In contrast to San1, Ubr1 is dependent on molecular chaperones in its quality control functions (Heck *et al.* 2010; Nillegoda *et al.* 2010). Ubr1 might bind molecular chaperones and employ them for substrate recognition as described for the mammalian cytoplasmic quality control ligase **C**arboxyl terminus of **H**sp70-**I**nteracting **P**rotein (CHIP) (McDonough and Patterson 2003). Alternatively, chaperones may promote substrate solubility or conformations that are directly recognized by Ubr1.

How Hul5 recognizes misfolded proteins is unknown. Hul5 is proposed to function as an E4 in the context of the proteasome (Crosas *et al.* 2006; see above), and it is thus possible that as yet unidentified E3s cooperate with Hul5 in substrate recognition and ubiquitylation. Consistent with this idea, *hul5*Δ mutants primarily affect poly- but not mono-ubiquitylation of misfolded proteins during heat stress (Fang *et al.* 2011).

The RING E3 Rkr1/Ltn1 is associated with ribosomes and ubiquitylates aberrant proteins arising from mRNAs that lack stop codons (Bengtson and Joazeiro 2010). Such non-stop mRNAs can result from errors in gene expression, and their poly(A) tails are translated into polylysine tracts. The positive charge of polylysine induces translational pausing due to strong electrostatic interaction with the negatively charged ribosome exit channel (Lu and Deutsch 2008). The resulting translationally paused or arrested nascent polypeptides seem to be targeted by Rkr1/Ltn1 (Bengtson and Joazeiro 2010). The precise mechanism is unknown, but translational pausing may transmit a conformational change to the surface of the ribosome that can be recognized by Rkr1/Ltn1.

Protein quality control in the endoplasmic reticulum

Proteins of the secretory pathway enter the ER through the Sec61 channel in an unfolded state and adopt their native conformation after clearing the channel. A protein quality-control system retains immature molecules in the ER until folding is completed. Terminally misfolded polypeptides are singled out by the ER protein quality-control system and routed to the cytoplasm for degradation by the ubiquitin–proteasome system. This highly conserved process, ERAD, promotes cellular homeostasis by preventing the accumulation and eventual aggregation of defective proteins within the secretory pathway (Hirsch *et al.* 2009; Buchberger *et al.* 2010; Smith *et al.* 2011b). Central to ERAD are membrane-bound ubiquitin ligases that are organized in multimeric protein complexes. They coordinate protein quality-control activities with cytoplasmic ubiquitylation, the action of the AAA–ATPase Cdc48, and the proteasome. The specificity of ERAD is primarily assured by substrate-recruitment factors that are integral components of ubiquitin ligase complexes. They selectively bind aberrant conformers and deliver them to downstream-acting factors (Meusser *et al.* 2005).

The ERAD pathway handles misfolded glycosylated and nonglycosylated proteins of the ER lumen, as well as membrane proteins, both single spanning and multispanning. The diversity of ERAD substrates is reflected in distinct pathways of ubiquitylation, as defined by individual E3 ligase complexes and their accessory factors: ERAD-C degrades proteins with defective cytosolic domains, ERAD-M targets lesions in transmembrane segments, and ERAD-L processes substrates with luminal defects (Vashist and Ng 2004). In yeast, two E3 ligase complexes, the HMG–CoA reductase degradation (HRD) ligase and Doa10, target this diverse pool of clients.

Ubiquitin ligase Doa10: Doa10 (**d**egradation **o**f **α**2) was identified in a screen for factors required for the degradation of the soluble transcriptional repressor Matα2 (Swanson *et al.* 2001) and subsequently shown to act as well on ERAD-C substrates. Doa10 features an unusual N-terminal RING-finger domain and 14 transmembrane segments. This ligase functions with the E2 enzymes Ubc6 and Ubc7. Ubc6, a C-terminally anchored membrane protein (Sommer and Jentsch 1993), is also a target of Doa10-dependent turnover (Walter *et al.* 2001). Ubc7 is recruited to the ER membrane via Cue1 (Biederer *et al.* 1997). If Cue1 is missing, Ubc7 is mislocalized to the cytoplasm and targeted for proteasome-mediated degradation by Ufd4-dependent ubiquitylation. This turnover of Ubc7 is most likely signaled by a polyubiquitin chain synthesized on the E2 active site cysteine (Ravid and Hochstrasser 2007). Doa10 also associates with Ubx2, which stabilizes the interaction of Doa10 with Cdc48 (Neuber *et al.* 2005; Schuberth and Buchberger 2005). Doa10 is not only found in the ER membrane but also in the inner nuclear membrane, where it targets nuclear substrates for degradation (Deng and Hochstrasser 2006). As no substrate-recruitment factors for Doa10 have been identified to date, the selection of targets is perhaps accomplished by the ligase itself. Although distinct short-lived client proteins are processed by the HRD ligase and Doa10, some overlapping functions

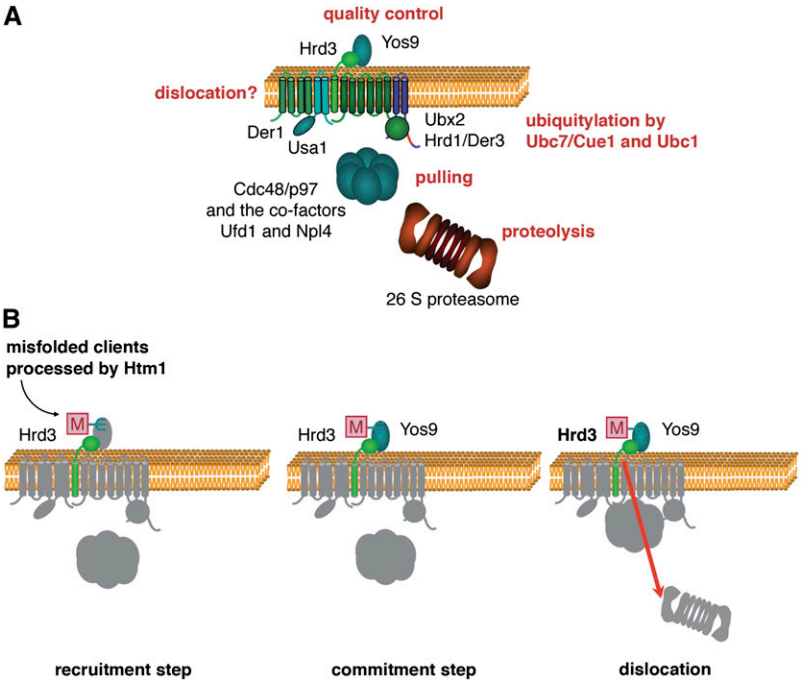

Figure 7 HRD ubiquitin ligase. (A) HRD ubiquitin ligase consists of six core subunits: Hrd1 exposes a RING-finger domain on the cytoplasmic surface of the ER membrane and acts together with the E2 enzymes Ubc7/Cue1 and Ubc1 (both not depicted). Hrd3 together with Yos9 forms the ER luminal domain of the ligase complex. Usa1 bridges Hrd1 with Der1. Ubx2 binds Hrd1 and also, via a UBX domain, Cdc48. The transmembrane organization of the ligase complex suggests that it connects ER-luminal quality-control functions, dislocation, ubiquitylation, and the generation of pulling forces with proteolysis by the proteasome. (B) Hypothetical model of how the ER-luminal domain of the HRD ligase selects ERAD substrates. The glycans of misfolded proteins are processed by Htm1 to generate the glycan signal $Man_7GlcNAc_2$. Hrd3 first binds the misfolded protein in a "recruitment step" (left). Then Yos9 controls the identity of the glycan signal in a "commitment step" (center). Only when both interactions are productive is the client protein dislocated into the cytoplasm for proteasomal digestion.

seem to exist: Double mutants of *doa10* and *hrd1* display enhanced cadmium sensitivity and show an activated unfolded protein response (Swanson *et al.* 2001; for further reading on the unfolded protein response (UPR), see Walter and Ron 2011).

HRD ubiquitin ligase: ERAD-L and ERAD-M substrates are targeted by the HRD ligase. Key elements of this ligase complex have been identified in two genetic screens. In one of the screens, an ERAD-L substrate was used (Knop *et al.* 1996)—a mutant version of the vacuolar enzyme carboxypeptidase Y, CPY* (Finger *et al.* 1993). A mutation that stabilized CPY* was found in *UBC7*, which was the first indication that misfolded proteins of the ER lumen are degraded by cytoplasmic pathways (Hiller *et al.* 1996). Since *UBC7* was also required for turnover of a mutant form of the translocation component Sec61 (*sec61-2*; Biederer *et al.* 1996) it became evident that ERAD-L and ERAD-M substrates can be degraded by the same cytoplasmic pathway. The other genetic screen was performed using an 3-hydroxy–3-methylglutaryl–CoA reductase (HMG-R) isozyme, Hmg2 (Hampton *et al.* 1996), which is not a *bona fide* misfolded protein. Instead, Hmg2 turnover is regulated through feedback control involving the mevalonate pathway. The two screens revealed overlapping genes (the "HRD" and the "DER" genes), indicating that Hmg2 is channeled into an ERAD pathway that also acts on misfolded proteins. Indeed, farnesol opens the conformation of Hmg2, which could make it accessible to the HRD ligase. Interestingly, the effect of farnesol requires an intact Hmg2 sterol-sensing domain (Shearer and Hampton 2005).

The HRD ligase complex is composed of at least six subunits and the requisite E2 enzymes (Figure 7A). The central component of this complex, Hrd1/Der3, comprises six transmembrane segments and ubiquitylates substrates at the cytoplasmic surface of the ER through its C-terminal RING-finger domain (Bordallo *et al.* 1998; Bays *et al.* 2001a). Similarly to Doa10, Hrd1 functions with Cue1-tethered Ubc7. Cue1 not only localizes with Ubc7 but also stimulates its enzymatic activity (Bazirgan and Hampton 2008). Ubx2, an additional component shared by Hrd1 and Doa10, connects Cdc48 to the HRD ligase pathway (Neuber *et al.* 2005; Schuberth and Buchberger 2005).

Another key element of the HRD ligase is Usa1, comprising two transmembrane helices and a UBA domain. Usa1 mediates the interaction of Hrd1 with the small membrane protein Der1, which spans the membrane four times and is selectively required for the breakdown of ERAD-L substrates (Knop *et al.* 1996; Carvalho *et al.* 2006). Additionally, Usa1 acts as a scaffold that binds Hrd1 and promotes its dimerization. This function of Usa1 is generally required for proteolysis of ERAD-L and ERAD-M substrates (Horn *et al.* 2009; Carvalho *et al.* 2010).

In vivo cross-linking studies have suggested that Hrd1 may bind ERAD-L substrates (Carvalho *et al.* 2010). However, Hrd1 carries only small loops facing the lumen of the ER. Therefore, it is likely that the luminal substrate-binding module of the HRD ligase is formed primarily by Hrd3 and Yos9 (Denic *et al.* 2006; Gauss *et al.* 2006a,b). Hrd3 is a type I transmembrane protein that exposes an ~80-kDa domain into the ER lumen. Yos9 interacts with the HRD ligase via Hrd3 and contains a mannose-6 phosphate receptor homology domain (MRH). The luminal Hrd3/Yos9 module links the ligase to the chaperone system of the ER by recruiting the Hsp70-type chaperone Kar2 to the E3 (Denic *et al.* 2006). Hrd3 binds misfolded CPY* irrespective of its glycan

modifications and also in absence of Yos9. Therefore, Hrd3 was proposed to be the primary receptor for misfolded proteins at the ligase complex (Gauss *et al.* 2006b). Yos9 specifically binds terminal α1,6-bonded mannose moieties on misfolded glycoproteins (Quan *et al.* 2008). These are generated by Mns1 and Htm1, which convert $Man_9GlcNAc_2$ into $Man_7GlcNAc_2$ (Clerc *et al.* 2009; Gauss *et al.* 2011). The binding characteristics of Hrd3 and Yos9 reflect the key features of degradation signals in ERAD substrates, one being misfolding of the client, probably recognized by hydrophobic interactions, and the other, a specific glycan signal, a $Man_7GlcNAc_2$ modification (Figure 7B). Since glycoproteins that are not processed by Mns1 and Htm1 are protected from degradation, these two mannosidases act as a timer that allows newly synthesized proteins to be distinguished from those that have failed to fold correctly (Jakob *et al.* 1998). While these data apply to glycan-modified ERAD-L model substrates, targeting of ERAD-M client proteins may differ. A mutational analysis of the Hrd1p membrane anchors indicated that the transmembrane segments may play a crucial role in detecting misfolding of ERAD-M substrates (Sato *et al.* 2009).

Although the Hrd1 and Doa10 ligases exhibit similar activities in ERAD, their topological organization is different. While it is likely that the domains of Doa10 involved in substrate selection and ubiquitin conjugation both reside in the cytoplasm, these domains are separated by the ER membrane in the case of the HRD ligase. Thus, at least ERAD-L clients have to be exported from the ER prior to ubiquitylation. This process, termed dislocation or retrotranslocation, most likely involves a proteinaceous channel in the ER membrane. It has been speculated that such a channel may be formed by the components of the HRD ligase itself (Hampton *et al.* 1996; Swanson *et al.* 2001; Horn *et al.* 2009). However, a function of the translocon in dislocation has also been proposed, based on a physical interaction between Hrd3 and Sec61 (Schafer and Wolf 2009). Moreover, an apparent interaction of CPY* with Sec61 is maintained until the misfolded protein is ubiquitylated on the cytoplasmic surface (Schafer and Wolf 2009). These findings support previous genetic data pointing to a function of Sec61 in ERAD (Plemper *et al.* 1997).

Degradation signals

Mechanisms of substrate selection by ubiquitin ligases are diverse and rely on a variety of degradation signals (*degrons*) (reviewed in Ravid and Hochstrasser 2008). Generally we can distinguish between signal-specific degrons on regulatory proteins and degrons controlled by protein folding and assembly. We briefly discussed the latter in the previous section, as they are key to protein quality-control pathways.

The first systematically studied and perhaps most surprising degrons are determined by the N-terminal amino acid residue of the substrate protein (Bachmair *et al.* 1986; Varshavsky 2011). The N-end rule ubiquitin ligase Ubr1 (Bartel *et al.* 1990) binds proteins with different affinities depending on the side chain of the first amino acid and thereby relates the protein's N terminus to protein stability (Table 6) (Choi *et al.* 2010). The specificity of Ubr1 is essentially complementary to that of methionine aminopeptidases, so that newly synthesized proteins will rarely present destabilizing residues; if the penultimate residue is destabilizing, methionine aminopeptidase will not remove the initiator methionine. Rather, destabilizing N-terminal residues are formed as a result of endoproteolytic cleavage by proteases such as separase (see below), or other post-translational events (Varshavsky 2011). For example, acidic N-terminal residues generated by endoproteases are not recognized by Ubr1, but are substrates for Ate1, an enzyme that ligates arginine to the substrate's N terminus. This allows for subsequent Ubr1-mediated recognition, ubiquitylation, and degradation. N-terminal glutamine and asparagine residues are funneled into the N-end rule pathway by the action of Nta1, an N-terminal amidase, whose reaction products are in turn substrates for Ate1 (Baker and Varshavsky 1995). These pathways mediate major regulatory events in many eukaryotes, such as the sensing of oxygen and nitric oxide levels (Licausi *et al.* 2011; Varshavsky 2011).

Table 6 N-end rule in *Saccharomyces cerevisiae*

Residue at N terminus	Half-life of X-ßgal
Arg	2 min
Lys	3 min
Phe	3 min
Leu	3 min
Trp	3 min
His	3 min
Asp	3 min
Asn	3 min
Tyr	10 min
Gln	10 min
Ile	30 min
Glu	30 min
Cys	>20 hr
Ala	>20 hr
Ser	>20 hr
Thr	>20 hr
Gly	>20 hr
Val	>20 hr
Pro	ND
Met	>20 hr

Adapted from Bachmair *et al.* (1986), with permission. ND, not done.

Acetylated N termini, which are found in most proteins, are not recognized by Ubr1. Instead, they present separate degrons recognized by the Doa10 ligase (Hwang *et al.* 2010). Many proteins are metabolically stable despite the presence of these targeting elements, presumably due to poor exposure of their N termini, suggesting a potential involvement of this pathway in recognition of misfolded proteins and quality control degradation (Hwang *et al.* 2010).

Many other regulated degradation pathways also use post-translational modifications to activate degrons. For

example, phosphorylation often generates a high-affinity interaction site for E3 recruitment. Such phosphodegrons are widely used by SCF ligases (Petroski and Deshaies 2005; Zimmerman *et al.* 2010; Duda *et al.* 2011). The cell cycle inhibitor Sic1 contains an array of phosphodegrons with relatively low affinities for SCF^{Cdc4}. This arrangement requires processive multiphosphorylation by the G1 and S-phase kinases Cln2/Cdc28 and Clb5/Cdc28 for efficient Sic1 degradation and transforms the graded kinase activity into a switch-like cell-cycle transition (Nash *et al.* 2001; Petroski and Deshaies 2003; Koivomagi *et al.* 2011).

Another interesting variety of degron formed by post-translational modification is recognized by the small ubiquitin-related modifier (SUMO)-targeted ubiquitin ligases (STUbLs) (Perry *et al.* 2008). These E3s contain SUMO interacting motifs that mediate binding to SUMOylated substrate proteins for ubiquitylation (see below).

Other degrons are less well defined and include short surface-exposed hydrophobic stretches such as the N-terminal degron in Matα2, which can be masked by heterodimerization with Mat**a**1 (Johnson *et al.* 1998).

Sites in target proteins that recruit E3s and allow ubiquitin conjugation constitute the canonical form of a degradation signal in the ubiquitin–proteasome system. These sites are remarkably varied, consistent with the multiplicity of ubiquitin ligases and their diverse substrate recognition modes. However, additional features to support proteasome-mediated degradation of the substrate are also critical, particularly an unfolded segment to serve as an initation site for the proteasome, as discussed above (Prakash *et al.* 2004).

Ubiquitylation of Membrane Proteins

As discussed earlier, membrane-associated ubiquitin ligases play key roles in the protein quality-control pathway of the ER. However, other membrane systems of the cell are also sites of abundant ubiquitylation, where it acts to direct protein sorting. On the one hand, ubiquitylation drives transport from the *trans*-Golgi and the plasma membrane. On the other, it helps to concentrate proteins in the MVB compartment. The MVB sorting step leads ultimately to proteolysis of the cargo—not in the proteasome, however, but in the vacuole (Lauwers *et al.* 2010).

Ubiquitin function in endocytosis

The abundance of receptors and transporters at the plasma membrane is regulated by endocytosis, often in a signal-dependent manner. Internalized proteins are transported to the endocytic compartment. From there, they are either recycled to the plasma membrane or packaged into multivesicular bodies for delivery to lysosomes. Ubiquitin serves as an important internalization signal for endocytosis. In some cases, ubiquitin seems to act redundantly with other signals.

A function of ubiquitin in protein sorting at the plasma membrane was suggested by the observation that ubiquitylated Ste6, the yeast pheromone transporter, accumulated at the plasma membrane when endocytosis is blocked (Kölling and Hollenberg 1994). Moreover, ubiquitylation was found to be necessary and sufficient for ligand-induced endocytosis of the pheromone receptor Ste2 (Hicke and Riezman 1996). Accordingly, the nitrogen permease inactivator Npi1 was identified as the HECT domain ligase Rsp5 (Hein *et al.* 1995; Huibregtse *et al.* 1995). A number of other plasma membrane proteins were subsequently shown to undergo ubiquitylation, including the permeases Fur4 and Gap1.

After Rsp5-dependent selection and modification of the cargo, it is most likely recognized by endocytic adaptors (Shih *et al.* 2002). Yeast endocytic adaptors Ent1 and Ent2 bind ubiquitylated cargo via UIM domains and localize to the plasma membrane by interacting with phosphatidylinositol-(4,5)-bisphosphate and clathrin. An additional endocytic scaffold protein, Ede1, an EH domain protein, contains a UBA domain and it may also contribute to cargo interaction and concentration (Dores *et al.* 2010).

Several nutrient permeases are ubiquitylated on multiple lysines by short K63 ubiquitin chains, consistent with the linkage specificity of Rsp5 (Kim and Huibregtse 2009). Though a single ubiquitin molecule is sufficient to promote endocytosis, multiple monoubiquitylation and short K63 chains accelerate the rate of endocytosis, to an extent that may depend on the substrate (Galan and Haguenauer-Tsapis 1997; Springael *et al.* 1999). Notably, the Jen1 transporter shows a strict requirement for K63 chains (Paiva *et al.* 2009).

Rsp5 contains WW domains, named for the presence of two highly conserved tryptophan residues, which directly interact with PPx(Y/F) motifs in substrate proteins. However, many cargo molecules, including several permeases, do not carry such a motif. In these cases, interaction of the cargo with Rsp5 is mediated by a family of adaptor proteins, such as the arrestin-related trafficking adaptors (ARTs). Unlike mammals, yeast does not have canonical arrestins, in that the yeast ART proteins lack adaptin and clathrin-binding sequences. Instead they interact with Rsp5 through a PxY sequence (Lin *et al.* 2008; Leon and Haguenauer-Tsapis 2009; Nikko and Pelham 2009). ART proteins (Art1–Art10) regulate the ubiquitylation of specific cargos at the plasma membrane in response to specific stimuli and may be part of a quality-control system that targets damaged and misfolded membrane proteins for degradation in the vacuole. This variety of adaptors explains how a single ubiquitin ligase can regulate endocytosis of many different proteins. Another level of regulation is provided by differential localization of the Rsp5 adaptors. For example, the adaptors Bul1 and Bul2 work both at the plasma membrane and the *trans*-Golgi (Nikko and Pelham 2009). The Art1–Art10 proteins (Lin *et al.* 2008; Nikko and Pelham 2009; MacGurn *et al.* 2011) function mainly at the plasma membrane, while the Rsp5 adaptor proteins Ear1, Ssh4, Bsd2, Tre1, and Tre2 are located mainly at endosomes (Liu *et al.* 1997; Stimpson *et al.* 2006; Leon *et al.* 2008).

Nutrient uptake is controlled by a regulatory loop that adjusts the level of amino acid transporters at the plasma membrane through Art1. Npr1 (nitrogen permease reactivator 1 kinase) phosphorylates residues near the N terminus of Art1 to inhibit its transport to the plasma membrane. Endocytosis of amino acid transporters is thereby suppressed. Npr1 itself is negatively regulated by the TORC1 kinase, which thus acts on ubiquitin-dependent endocytosis to fine tune the activity and composition of proteins of the plasma membrane (MacGurn *et al.* 2011).

Function of ubiquitin in the MVB pathway

Proteins of the late endosome destined for proteolysis are sorted into multivesicular bodies, which deliver cargo to the yeast vacuole for degradation (Katzmann *et al.* 2001; Henne *et al.* 2011). Formation of these vesicles involves invagination of membranes into the endosomal compartment. Mature MVBs subsequently fuse with the lysosome and release their contents. Crucial players in the MVB pathway are specific multisubunit **e**ndosomal **s**orting **c**omplexes **r**equired for **t**ransport (ESCRT): ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III, and the AAA ATPase Vps4 (part of the fifth ESCRT complex). These act sequentially (Katzmann *et al.* 2001; Babst *et al.* 2002a,b) in early cargo recruitment and concentration (ESCRT-0, -I, and -II) and later in cargo deubiquitylation and membrane sculpting (ESCRT-III and Vps4).

A prerequisite for selective sorting and concentration of membrane proteins into endosomal microdomains and, eventually MVBs, is the ubiquitylation of cargo proteins (Katzmann *et al.* 2001; Lauwers *et al.* 2010; Henne *et al.* 2011). ESCRT complexes have several distinct ubiquitin-binding motifs for cargo recognition. Subunits of the ESCRT-0 complex bind ubiquitin in several ways: Vps27 contains a VHS (**V**ps27 **H**rs **S**TAM) and two UIM domains in tandem, while the Hse1 subunit contains both a UIM and a VHS domain (Bilodeau *et al.* 2002). Thus, ESCRT-0 contains five ubiquitin-binding domains. However, it remains unclear whether this allows binding of several cargoes simultaneously or binding with high affinity to poly- or multiubiquitylated cargo (Ren and Hurley 2010). In addition to its ubiquitin-binding activity, ESCRT-0 may bind cargo through interactions with the clathrin vesicle machinery, suggesting microdomains in which clathrin lattices, ESCRT-0, and ubiquitylated cargo meet. ESCRT-I also contains ubiquitin-binding domains: the subunit Vps23 contains a UEV domain (Pornillos *et al.* 2002), and Mvb12 harbors a novel ubiquitin-binding domain (Shields *et al.* 2009). So far only one ubiquitin-binding domain has been identified in ESCRT-II, an **N**pl4 **Z**inc **F**inger (NZF) motif in Vps36. Also participating in ubiquitin-dependent sorting are the ubiquitin-binding adaptor proteins Gga1 and Gga2, although their exact roles in the pathway are not understood (Lauwers *et al.* 2009, 2010).

Though it was initially assumed that monoubiquitylation is sufficient to direct targets into the MVB pathway, it is now accepted that K63 ubiquitin chains are needed. Whether these ubiquitin moieties are added at the endosome or persist from endocytosis-associated ubiquitylation events at the plasma membrane is unresolved. In any case, this modification must be removed prior to packaging of cargo into vesicles to avoid depletion of ubiqutin by its uptake into the vacuole. In yeast, this step involves the DUB Doa4 (Amerik *et al.* 2000a). Doa4 is recruited into the ESCRT-III complex by the adaptor protein Bro1, which also stimulates the deubiquitylating activity of Doa4 (Luhtala and Odorizzi 2004; Richter *et al.* 2007).

In addition to ubiquitin binding, ESCRT components also serve as targets for ubiquitylation. For instance, Vps27 can be monoubiquitylated (Polo *et al.* 2002; Stringer and Piper 2011). Although the physiological function of this modification remains unknown, Hrs, the human homolog of Vps27, is inhibited by monoubiquitylation because an intramolecular interaction between the UIM and the ubiquitin modification prevents the binding of ubiquitylated cargo. Similar observations have been made for the endocytic adaptor Eps15, raising the possibility of a general role for monoubiquitylation in downregulation of these pathways (Hoeller *et al.* 2006).

Ubiquitylation and protein import into peroxisomes

Peroxisomes are small cytoplasmic vesicles housing ~50 enzymes that mediate β oxidation of fatty acids and other metabolic processes. Luminal proteins of the peroxisome are imported post-translationally by a complex, receptor-mediated process (Girzalsky *et al.* 2010). The import apparatus includes three RING ligases (Pex2, Pex10, and Pex12) that reside in the peroxisomal membrane, as well as as a cognate E2 (Ubc10/Pex4) (Williams *et al.* 2008; Platta *et al.* 2009). The principal role of ubiquitin in peroxisomes is apparently the monoubiquitylation of Pex5, a receptor for protein import into peroxisomes that delivers cargo to the peroxisome by cycling between the cytoplasm and the peroxisomal membrane (Carvalho *et al.* 2007; Platta *et al.* 2007; Grou *et al.* 2008). After cargo delivery, Pex5 must return to the cytoplasm for another round of import. This recycling step is dependent on Pex5 ubiquitylation. Other peroxisomal import receptors such as Pex18 and Pex20 are ubiquitylated and potentially follow a similar cycle. A heteromeric protein complex of the AAA family, composed of Pex1 and Pex6 monomers, mediates ATP-dependent extraction of Pex5 into the cytosol by an unknown mechanism (Platta *et al.* 2005). A hypothetical model accounting for these data are that the Pex1/Pex6 complex recognizes Pex5 via its ubiquitin modification, and functions analogously to Cdc48 in its extraction of membrane proteins from the ER in the ERAD-M pathway, as described above.

Nuclear Functions of the Ubiquitin System

Nuclear functions of ubiquitin fall into two general categories: on one hand, there is a nuclear form of the above-described protein quality-control pathways. On the other

hand, as described below, ubiquitylation contributes, both in its proteolytic and its noncanonical mode, to virtually all aspects of DNA metabolism (Ulrich 2002), such as DNA replication and repair (Bergink and Jentsch 2009; Ulrich and Walden 2010), gene expression and chromatin structure (Muratani and Tansey 2003; Shilatifard 2006), and chromosome dynamics and segregation (Pines 2006).

Coupling cell cycle progression to DNA replication and chromosome segregation

DNA replication and chromosome segregation are intimately coupled to cell cycle progression and hence subject to regulation by the ubiquitin system, mostly by means of proteolytic destruction of important regulators. These processes have been discussed in detail in excellent reviews (Nasmyth 1996; Pines 2006; Diffley 2010).

Replication initiation: At the G1-to-S transition, Sic1 degradation, initiated by SCF^{Cdc4}, allows the activation of the cyclin-dependent kinase (CDK) Cdc28 in complex with the S-phase–specific, B-type cyclins, Clb5 or Clb6, leading to phosphorylation and activation of the replication initiation factors Sld2 and Sld3 (Tanaka *et al.* 2007; Zegerman and Diffley 2007). In parallel, *SIC1* transcription is terminated by ubiquitylation and destruction of a transcriptional activator, Swi5, also mediated by SCF^{Cdc4} (Kishi *et al.* 2008).

There is also evidence for nondegradative contributions of ubiquitin to replication initiation. The cullin Rtt101 associates with early replication origins and ubiquitylates Spc16, a subunit of the **FA**cilitator of **C**hromatin **T**ransactions (FACT) complex, predominantly via K63-linked chains (Han *et al.* 2010). FACT is a histone chaperone with functions in transcription, DNA replication, and repair (Winkler and Luger 2011). Deletion of *RTT101* results in a weakening of the interactions between FACT and the replicative helicase (the Mcm2-7 hexamer), and a partial loss of both complexes from a subset of replication origins (Han *et al.* 2010). Whether or not ubiquitylation of Spc16 is responsible for this phenomenon has not been determined, but the activity of Rtt101 appears to impinge specifically on the replication-related functions of FACT.

Origin licensing: The mechanism that limits replication to a single round per cell cycle is called origin licensing. This restricts the activation of replication origins to S phase and prevents renewed firing until the next cell cycle. All organisms use multiple strategies to achieve this goal, including ubiquitin-mediated proteolysis of key regulatory factors (Diffley 2010). In budding yeast, the primary target is Cdc6, a component of the prereplicative complex (pre-RC). Cdc6 is phosphorylated in late G1 and S phase, which causes ubiquitylation by SCF^{Cdc4} and subsequent degradation (Drury *et al.* 1997). While in mammalian cells the pre-RC component Cdt1 is subject to proteolysis, its yeast homolog Tah11 is instead inactivated by export from the nucleus (Diffley 2010).

Chromosome segregation: One of the most important features of cell division is the even distribution of replicated chromosomes to the daughter cells, a process controlled by ubiquitin-dependent proteolysis (Pines 2006). During this cell cycle stage, the dominant E3 is the APC/C, which couples mitosis to cytokinesis and ensures correct chromosome segregation (Harper *et al.* 2002). Important substrates include cyclins; components of the spindle checkpoint that monitor the correct assembly of the mitotic spindle; and the securin protein, Pds1, an inhibitor of separase (Esp1) that initiates anaphase by cleaving the cohesin subunit Scc1, thus allowing sister chromatid separation (Nasmyth *et al.* 2000). The C-terminal proteolytic fragment of Scc1 is subject to proteasomal degradation by the N-end rule pathway, initiated by ubiquitylation via the RING-finger E3 Ubr1 with the E2 Rad6, and interference with this process causes chromosome loss (Rao *et al.* 2001; see also Buonomo *et al.* 2000).

Responses to replication stress

Mechanisms of replication fork protection: Whereas the coupling of replication initiation and origin licensing to the cell cycle mostly involves proteolytic functions of the ubiquitin system, the role of ubiquitylation in the course of replication appears more diverse. A number of ubiquitin ligases have been found to contribute to genome stability by protecting replication forks from stress, but their mechanisms of action are poorly understood.

The F-box protein Dia2 is a constitutive component of the replisome progression complex (RPC), tethered to the RPC components Mrc1 and Ctf4 by means of its N-terminal domain (Morohashi *et al.* 2009). Dia2 acts as a substrate adaptor for SCF^{Dia2}. Its association with replication forks appears to facilitate the replication of difficult templates and protects cells from DNA damage and replication stress (Mimura *et al.* 2009; Morohashi *et al.* 2009). Mrc1 and Ctf4 are ubiquitylated by SCF^{Dia2} and seem to be degraded, but it is unclear whether these substrates are functionally critical (Mimura *et al.* 2009). Dia2 itself is an unstable protein, and it is stabilized by replication stress (Kile and Koepp 2010).

The cullin Rtt101, in addition to its role in replication initiation, also contributes to protecting replication forks from collapse when they encounter DNA lesions (Luke *et al.* 2006). Thus, *rtt101Δ* mutants are sensitive to DNA-damaging agents and unable to recover from damage-induced fork stalling. In response to DNA damage, Rtt101 forms a ubiquitin ligase with the RING finger protein Hrt1, the linker protein Mms1, and the putative substrate adaptor Mms22 (Zaidi *et al.* 2008). Interestingly, Rtt101's function in replication fork protection appears unrelated to its action on the FACT complex, as the latter does not require the presence of Mms1 or Mms22 (Han *et al.* 2010). A second substrate adaptor, Crt10, is recruited to the Rtt101 cullin complex via Mms1 in a damage-independent manner and has been suggested to affect replication by regulating nucleotide levels (Fu and Xiao 2006; Zaidi *et al.* 2008).

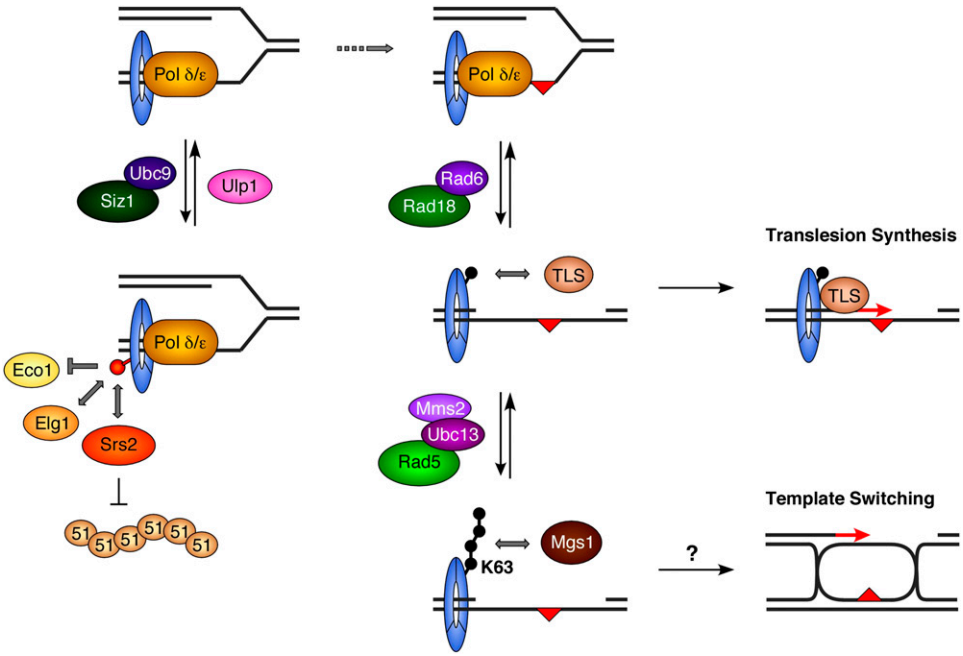

Figure 8 Modifications of the replication factor PCNA. During undisturbed replication, PCNA (blue ring shape) promotes processive DNA synthesis by replicative polymerases δ and ε (Pol δ/ε), and is modified by SUMO (red lollipop shape). The modification prevents binding of Eco1, but causes the recruitment of Elg1 and Srs2. Srs2 prevents the formation of the recombinogenic Rad51 filament (51), inhibiting unscheduled recombination at replication forks. Upon damage-induced replication fork stalling, PCNA is modified by mono- and polyubiquitin (black lollipop shapes) at postreplicative daughter-strand gaps. Monoubiquitylation recruits damage-tolerant DNA polymerases (TLS) for translesion synthesis, while K63 polyubiquitylation causes recruitment of Mgs1 and initiates damage bypass by template switching in an unknown manner. Conjugating enzymes, ligases, and DUBs are highlighted in shades of purple, green, and pink, respectively.

An interesting example of cross-talk between ubiquitin and the small ubiquitin-related modifier SUMO has emerged from the identification of a class of E3s, called STUbLs, which recognize SUMO-modified proteins as targets for ubiquitylation (Perry *et al.* 2008). In budding yeast, the RING-finger proteins Hex3/Slx5 and Slx8 have been implicated in preventing the accumulation of DNA damage during replication (Zhang *et al.* 2006). They form a heterodimer that promotes the ubiquitylation and degradation of highly sumoylated cellular proteins (Uzunova *et al.* 2007; Xie *et al.* 2007). The SUMO moieties are recognized by SUMO-interacting motifs within Hex3. It remains to be resolved whether the contribution of SUMO-dependent ubiquitylation to replication fork protection is attributable to the removal of specific sumoylated proteins for regulatory purposes or to preventing bulk accumulation of potentially toxic poly-SUMO conjugates.

Control of DNA damage bypass: An independent pathway for lesion processing during replication is called postreplication repair, DNA damage bypass, or DNA damage tolerance (Lawrence 1994). The process provides resistance to DNA-damaging agents, but is capable of generating genomic instability through damage-induced mutagenesis. It is initiated by ubiquitylation of the proliferating cell nuclear antigen (PCNA) Pol30 (Hoege *et al.* 2002), a homotrimeric sliding clamp that ensures processivity of the replicative polymerases and also acts as an interaction platform for a multitude of proteins involved in various aspects of DNA metabolism (Moldovan *et al.* 2007). The PCNA modification system (Figure 8) provides an example where mono- and polyubiquitylation at a single site elicit distinct cellular responses (Ulrich 2009), mediated by a range of ubiquitin receptors (Table 7).

Monoubiquitylation at a single conserved lysine, K164, mediated by the E2–E3 complex Rad6–Rad18 (Hoege *et al.* 2002), is a prerequisite for a process called translesion synthesis (Stelter and Ulrich 2003). This reaction involves a series of specialized DNA polymerases capable of using damaged DNA as a template for DNA synthesis (Waters *et al.* 2009). Although there is evidence for ubiquitin-independent translesion synthesis in vertebrates, the principle by which ubiquitylated PCNA activates damage-tolerant polymerases appears to be conserved: a series of ubiquitin-binding domains of the **U**biquitin-**B**inding **Z**inc Finger (UBZ) or **U**biquitin-**B**inding **M**otif (UBM) type, present in a subset of the polymerases (Table 7), affords enhanced affinity for the monoubiquitylated form of PCNA and thereby allows their recruitment and activation in response to DNA damage (Bienko *et al.* 2005). In budding yeast, this applies to polymerase η (encoded by *RAD30*), which mediates error-free translesion synthesis over UV-induced lesions, and Rev1, which in cooperation with polymerase ζ (encoded by *REV3* and *REV7*) is responsible for a large part of damage-induced mutagenesis (Garg and Burgers 2005; Guo *et al.* 2006; Parker *et al.* 2007).

The consequences of PCNA polyubiquitylation are less well defined. The modification is a prerequisite for an error-free pathway of template switching (Hoege *et al.* 2002), which mediates damage bypass by avoiding the use of damaged DNA as a replication template. PCNA polyubiquitylation involves the synthesis of a K63-linked chain by the E3 Rad5 in cooperation with the heterodimeric E2 Ubc13–Mms2 (Hoege *et al.* 2002; Parker and Ulrich 2009). The modification is likely to serve a nondegradative function (Zhao and Ulrich 2010) and enhances the affinity of an ATPase, Mgs1, for PCNA (Hishida *et al.* 2006; Saugar *et al.*

Table 7 Ubiquitin receptors in the DNA damage response

Protein	Domain	Function/significance	Pathway
Def1	CUE[a]	?	RNA polymerase II degradation
Mgs1	UBZ	Recruitment to mono- and polyubiquitylated PCNA	Postreplication repair
Mms2	UEV	Cooperation with Ubc13 in K63-chain synthesis	Postreplication repair
Pso2	UBZ[a]	?	Interstrand cross-link repair
Rad18	UBZ	?	Postreplication repair
Rad2	UBM[a]	?	Nucleotide excision repair
Rad23	UBA1	Preference for K63-linked chains	Nucleotide excision repair
	UBA2	Preference for K48-linked chains	
Rad30	UBZ	Recruitment to monoubiquitylated PCNA	Postreplication repair
Rev1	UBM1	Nonfunctional?	
	UBM2	Recruitment to monoubiquitylated PCNA	Postreplication repair

[a] Predicted by bioinformatics, but ubiquitin binding has not yet been demonstrated experimentally.

2012). However, the recruitment of this protein to sites of replication problems by means of its UBZ domain (Table 7) cannot fully explain the function of PCNA polyubiquitylation in damage bypass.

PCNA ubiquitylation is induced by DNA damage and replication stress, which involves the recruitment of Rad18 to stretches of single-stranded DNA covered by replication protein A (Davies *et al.* 2008). Yet, unlike the Dia2- and Rtt101-dependent mechanisms discussed above, damage bypass can be separated from bulk genome replication (Daigaku *et al.* 2010; Karras and Jentsch 2010), indicating that it operates on postreplicative daughter-strand gaps rather than directly at the fork.

The function of PCNA is further diversified by additional modifications: Attachment of the small ubiquitin-like modifier SUMO (Smt3 in yeast) occurs constitutively during replication and involves predominantly the same site that is targeted for damage-induced ubiquitylation, K164 (Hoege *et al.* 2002; Parker *et al.* 2008). This SUMOylation event prevents unscheduled recombination by recruiting an antirecombinogenic helicase, Srs2 (Papouli *et al.* 2005; Pfander *et al.* 2005). As with the recruitment of damage-tolerant polymerases by monoubiquitylated PCNA, Srs2 is targeted to SUMO-modified PCNA by means of tandem receptor motifs that independently recognize SUMO and PCNA (Armstrong *et al.* 2012). Under conditions of replication stress, Srs2 thus allows damage processing by ubiquitin-dependent bypass. At the same time, PCNA sumoylation enhances the affinity of an alternative clamp loader, Elg1, for PCNA (Parnas *et al.* 2010) and prevents the interaction of PCNA with an acetyltransferase important for the establishment of sister chromatid cohesion, Eco1 (Moldovan *et al.* 2006). In contrast to ubiquitylation, the functions of PCNA sumoylation are unlikely to be fully conserved in vertebrates, although an Srs2-related protein, bearing SUMO- and PCNA-interacting motifs, was recently identified in humans and shown to restrict unscheduled homologous recombination (Moldovan *et al.* 2012).

DNA repair

Among the DNA repair pathways, NER specializes in removing bulky lesions from double-stranded DNA by means of excising the damaged stretch and filling the resulting gap by DNA synthesis (Hoeijmakers 2001). The pathway operates in two distinct modes, depending on the way in which lesions are initially recognized. In global genome repair (GGR), Rad4 serves as the principal lesion recognition factor, in complex with its binding partner Rad23 (Figure 9A). In transcription-coupled repair (TCR), an RNA polymerase II stalled at a lesion initiates the events that lead to preferential repair of actively transcribed genes (Figure 9, B and C). Both branches are influenced by the ubiquitin–proteasome system.

Global genome repair: GGR is affected by the ubiquitin system in several ways (Reed and Gillette 2007; Dantuma *et al.* 2009). A cullin-based E3, containing the Cul3, Elc1, the SOCS box protein Rad7, and the RING-finger protein Rad16, mediates ubiquitylation of Rad4 (Gillette *et al.* 2006) (Figure 9). Although the ubiquitylated protein is degraded by the proteasome, its modification rather than its degradation was found to be important for repair (Gillette *et al.* 2006). Yet, the overall efficiency of GGR is highly dependent on Rad4 levels, which are controlled by Rad23 (Lommel *et al.* 2002; Ortolan *et al.* 2004). As a consequence, Rad4 is strongly depleted in *rad23Δ* mutants, and the resulting damage sensitivity can in part be compensated by boosting Rad4 abundance. Thus, contrary to its role as a ubiquitin receptor in proteasomal targeting, Rad23 appears to stabilize Rad4 rather than induce its degradation. This may be mediated simply by binding to Rad4 and thereby preventing its misfolding (Dantuma *et al.* 2009) or alternatively, by means of shielding its ubiquitylated form from proteasomal access (Ortolan *et al.* 2000). It has even been reported that *de novo* protein synthesis is required for the stabilizing effect of Rad23 on Rad4, suggesting a regulation via damage-induced transcription (Gillette *et al.* 2006). In addition, the N-terminal ubiquitin-like domain of Rad23, known for its function as a proteasome docking site (Elsasser *et al.* 2002), contributes to GGR, possibly by providing a link between the NER machinery and the ATPase activities of the 19S cap (Watkins *et al.* 1993; Schauber *et al.* 1998; Russell *et al.* 1999b). Rad23's UBA domains (Table 7), which as described above are critical for ubiquitin chain

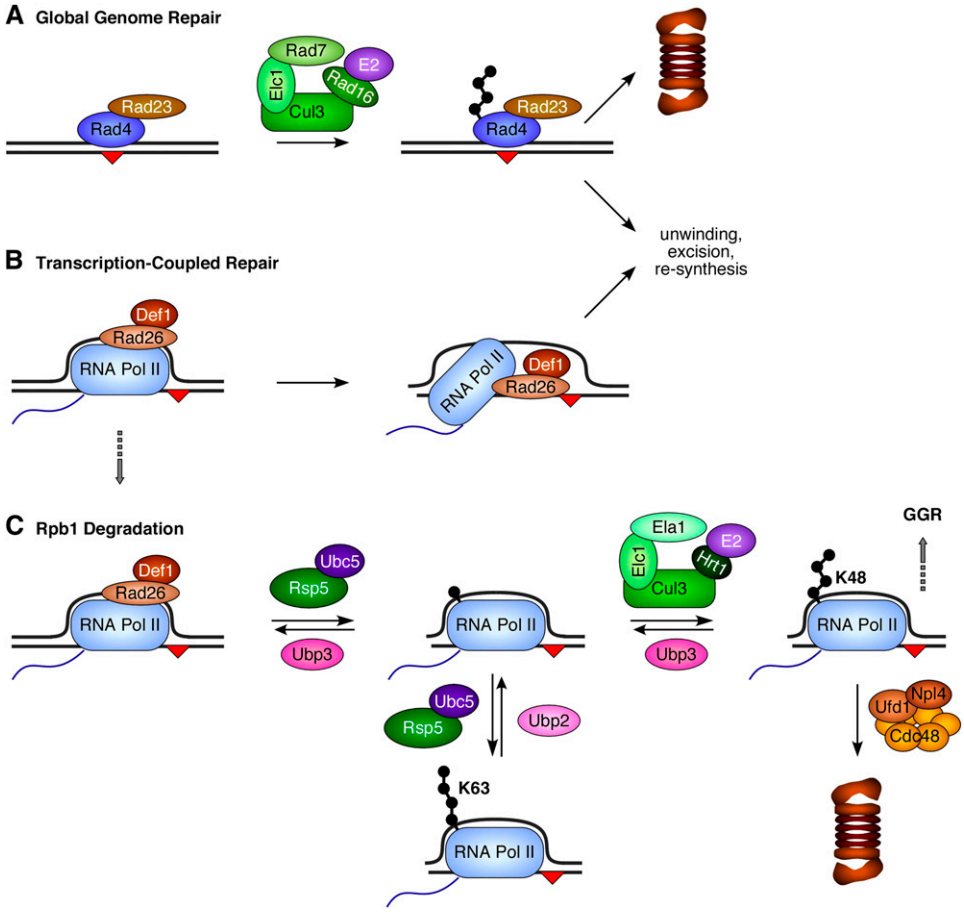

Figure 9 Ubiquitylation during nucleotide excision repair. (A) For global genome repair, lesions are recognized by Rad4 in complex with Rad23. Ubiquitylation of Rad4 is important for subsequent steps of repair. Ubiquitylated Rad4 is degraded by the proteasome. (B) Lesions on the transcribed strand of actively expressed genes are repaired by transcription-coupled repair, where RNA polymerase II (RNA Pol II) contributes to lesion recognition. Following removal of the enzyme by the action of Rad26, strand unwinding, excision of the lesion and resynthesis proceed as in global genome repair. (C) An irreversibly stalled RNA polymerase II is targeted for ubiquitylation and proteasomal degradation in a Def1-dependent manner. This frees the lesion and allows global genome repair. Conjugating enzymes, ligases, and DUBs are highlighted in shades of purple, green, and pink, respectively. Distinct polyubiquitin chain linkages are indicated as K48 or K63.

recognition by the proteasome, are not specifically required for NER (Bertolaet *et al.* 2001).

Transcription-coupled repair: The influence of ubiquitylation on TCR may be viewed as a solution to the problem of an irreversibly stalled RNA polymerase II (Svejstrup 2010) (Figure 9C). In this situation, the enzyme's large subunit, Rpb1, is ubiquitylated and degraded by the proteasome, which clears the transcription machinery from the site of damage and allows subsequent repair via GGR (Beaudenon *et al.* 1999; Woudstra *et al.* 2002). Degradation is dependent on the **C**oupling of **U**biquitin conjugation to **ER** degradation (CUE)-domain protein Def1 (Table 7), but whether this domain actually binds ubiquitin has not been determined. The E3 Rsp5 attaches either a single ubiquitin or a short K63-linked polyubiquitin chain to Rpb1, the latter of which may be trimmed by the DUB enzyme Ubp2 (Beaudenon *et al.* 1999; Harreman *et al.* 2009). A second E3, containing Cul3 in complex with Elc1, Ela1, and Hrt1, but not Rad7 and Rad16, has been implicated in Rpb1 modification as well (Ribar *et al.* 2006, 2007). This complex uses monoubiquitylated Rpb1 as a substrate for polyubiquitylation with a K48-linked chain (Harreman *et al.* 2009). Hence, Rpb1 polyubiquitylation, like PCNA modification, is characterized by the successive action of two E3s. As discussed above, degradation of polyubiquitylated Rpb1 requires the Cdc48–Ufd1–Npl4 complex (Verma *et al.* 2011), and the process might be balanced by Ubp3-mediated deconjugation (Kvint *et al.* 2008).

Regulation of gene expression and chromatin structure

Modulation of the transcription machinery: Tight control over gene expression is essential for adaptation to changes in a cell's environment. The ubiquitin–proteasome system contributes to this activity in many aspects (Muratani and Tansey 2003; Shilatifard 2006; Ouni *et al.* 2011; Geng *et al.* 2012). Among the most direct modes of influence is control over the levels of transcriptional regulators. Hence, many transcription factors are short-lived proteins, such as Gcn4, a target of SCF^{Cdc4} (Meimoun *et al.* 2000), and Gal4, whose abundance is limited by SCF^{Grr1} (Muratani *et al.* 2005). In many cases, including Gcn4, the transcriptional activation domain was found to overlap with the degradation signal (Salghetti *et al.* 2000). Ubiquitin-dependent proteolysis was thus found to be intimately coupled to the activity of natural and engineered transcription factors (Salghetti *et al.* 2001; Lipford *et al.* 2005; Wang *et al.* 2010). These observations led to the hypothesis that periodic promoter clearance is important for maximal activity. In the case of Gal4, two parallel degradation pathways have been described: the SCF^{Grr1}-dependent mode, which is independent of Gal4 activity and downregulates the protein in the absence of galactose, and a pathway

mediated by the F-box protein Dsg1, which applies to activated Gal4 (Muratani *et al.* 2005).

Ubiquitin-dependent activation of transcription factors does not need to involve complete degradation, but can also proceed by proteolytic processing. This was observed for the transcriptional activators Spt23 and Mga2, whose membrane-bound precursors are ubiquitylated by Rsp5, leading to their processing and relocalization into the nucleus (Hoppe *et al.* 2000; see above).

Finally, as discussed above, RNA polymerase II itself is subject to ubiquitylation and degradation upon transcription stalling. In addition, ubiquitylation of Rpb1 and Rpb2 by the RING-finger E3 Asr1 apparently causes an eviction of the polymerase subunits Rpb4 and Rpb7, leading to the enzyme's inactivation (Daulny *et al.* 2008). It remains to be seen, however, to what extent this strategy is used as a regulatory measure.

Several observations suggest possible nonproteolytic contributions of the proteasome to transcription. The RP, but not the CP, has been implicated in transcription elongation (Ferdous *et al.* 2001), and its ATPase subunits were found in association with active promoters (Gonzalez *et al.* 2002; Sulahian *et al.* 2006). Also, chromatin immunoprecipitation analyses have suggested limited overlap between RP and CP components on chromatin and have localized proteasome subunits to internal and 3′ regions of transcribed genes as well (Auld *et al.* 2006; Sikder *et al.* 2006). These data have resulted in a model postulating a nonproteolytic and chaperone-like activity of the proteasomal ATPases in transcription. In the context of transcription initiation, this has been linked to the recruitment and stimulation of the **S**pt-**A**da-**G**cn5 **A**cetyltransferase (SAGA) complex, a histone acetyltransferase (Lee *et al.* 2005). However, the physiological relevance of these findings is still debated (see for example Collins *et al.* 2009), and the mechanism by which the proteasome might act here remains to be elucidated.

Regulation of chromatin structure: The higher organization of genes into chromatin and their accessibility by the transcription machinery are crucial determinants of gene expression (Osley 2006; Shilatifard 2006). The first evidence for a contribution of the ubiquitin system to chromatin structure came from the identification of monoubiquitylated histone H2B (Robzyk *et al.* 2000). The modification is attached to K123 within the C-terminal tail of H2B by Rad6 in complex with the RING–E3 Bre1 (Wood *et al.* 2003), and is a prerequisite for the subsequent di- and trimethylation of histone H3 on K4 and K79 by the methyltransferases Set1 and Dot1, respectively (Dover *et al.* 2002; Ng *et al.* 2002; Sun and Allis 2002). The relationships between the levels of ubiquitylated H2B and methylated H3 are complex, and the mechanism by which one modification induces the other has not been fully explained; but the reaction appears to occur cotranscriptionally and is important for telomeric gene silencing. H2B ubiquitylation is also observed on the body of transcribed genes and has been associated with transcriptional initiation and elongation, but also repression (Henry *et al.* 2003; Kao *et al.* 2004; Xiao *et al.* 2005; Osley 2006). In a reconstituted system derived from mammalian cells, the effect of H2B monoubiquitylation on elongation by RNA polymerase II is due to a stimulation of the FACT complex, and a similar situation may apply in yeast (Pavri *et al.* 2006).

Interestingly, optimal transcription *in vivo* requires both ubiquitylation and subsequent deubiquitylation of H2B. Deconjugation is mediated by the two DUBs, Ubp8 and Ubp10 (Henry *et al.* 2003; Emre *et al.* 2005; Gardner *et al.* 2005b). Ubp8 acts as an integral component of the SAGA complex, and during the early steps of transcription. In contrast, Ubp10 works independently and influences mainly telomeric silencing, indicating nonredundant roles (Emre *et al.* 2005; Gardner *et al.* 2005b). A recent genome-wide analysis of ubiquitin and methylation marks on H2B revealed that the two DUBs affect different pools of cellular H2B (Schulze *et al.* 2011).

The cross-talk of histone ubiquitylation and methylation affects not only gene expression, but also genome maintenance, via an influence of H2B monoubiquitylation and subsequent H3 K79 methylation on the DNA damage checkpoint (Game and Chernikova 2009). In this context, the checkpoint mediator protein Rad9 recognizes K79-dimethylated H3 via Rad9's tudor domain and facilitates DNA repair by homologous recombination. As a consequence, *bre1* and *dot1* mutants are equally sensitive to agents that cause double-strand breaks (Game *et al.* 2006).

Processing of mRNAs: Gene expression can be regulated post-transcriptionally by modulating the maturation, export, or stability of mRNA, all of which are affected by the ubiquitin system. Maturation of pre-mRNAs is controlled by the splicing factor Prp19, an E3 of the U-box type, whose activity is essential for spliceosome function (Ohi *et al.* 2003). A possible substrate of Prp19 is the splicing factor Prp8, which is ubiquitylated *in vivo* (Bellare *et al.* 2008), but also contains a ubiquitin-binding domain of the Jab1/**M**pr1, **P**ad1, **N**-terminal (MPN) class that is essential for splicing (Bellare *et al.* 2006). Following splicing, mRNA is exported from the nucleus, and this process is guided by two E3s of the HECT family, Rsp5 and Tom1 (Duncan *et al.* 2000; Rodriguez *et al.* 2003). Rsp5 together with Ubc4 ubiquitylates the mRNA export factor Hpr1 in a transcription-dependent manner (Gwizdek *et al.* 2005). Ubiquitylated Hpr1 is targeted to the proteasome, but at the same time, the modification enhances interaction with the mRNA export receptor Mex67. This interaction is in part mediated by a UBA domain within Mex67, which was demonstrated to bind to polyubiquitin chains but also to interact with Hpr1 directly (Gwizdek *et al.* 2006). As a consequence, Mex67 stabilizes ubiquitylated Hpr1 by protecting it from proteasomal degradation. At the same time, it contributes to the recruitment of Hpr1 to actively transcribed genes, thus coordinating mRNA export with transcription (Gwizdek *et al.* 2006).

A relevant target of Tom1 appears to be Yra1, an adaptor protein linking mRNA to Mex67 (Iglesias *et al.* 2010). Mono-ubiquitylation and K48-diubiquitylation of Yra1 do not induce proteolysis, but promote dissociation from the Mex67–mRNP complex, which facilitates mRNA export. Finally, nonsense-mediated decay of mRNAs containing premature termination codons requires an RNA-dependent ATPase, Upf1, which also harbors a RING-related domain and displays E3 activity that is necessary for its function (Takahashi *et al.* 2008). However, the substrates and mechanism of this cytoplasmic pathway have not been elucidated.

Ccr4–Not complex: The multisubunit Ccr4–Not complex impinges on chromatin modification and transcription elongation, but also on RNA processing, export, translation, and stability (Collart and Panasenko 2011). Its Not4 subunit, a RING-finger protein, displays E3 activity (Albert *et al.* 2002), but it is unclear how many of the functions ascribed to the Ccr4–Not complex actually require this activity. Two targets have been identified: Jhd2, a histone H3K4 demethylase involved in regulating gene expression (Mersman *et al.* 2009), and the so-called "nascent associated polypeptide complex," a chaperone for nascent peptides at the ribosome that may be involved in protein quality control (Panasenko *et al.* 2006). Although Not4 cooperates with Ubc4 and/or Ubc5 in both cases, Jhd2 is polyubiquitylated and degraded, while the latter substrate undergoes monoubiquitylation. In addition, Not4 directly interacts with the proteasome and appears to contribute to its structural integrity (Panasenko and Collart 2011). It has been suggested that the complex acts as a general chaperone platform by means of associating with multiple interaction partners, but further research is clearly needed to uncover the basis of its multifunctionality.

Perspectives

For more than 25 years, the study of ubiquitylation in yeast has been a major driving force in the ubiquitin field, with countless original insights that have proven to be general across eukaryotes. Discoveries in this area have also fertilized many other aspects of cell biology, such as DNA repair and protein trafficking. Our understanding of ubiquitylation in yeast is more advanced than in other species but nonetheless far from mature.

In the coming years, this vast system will no doubt be charted more effectively through large-scale, mass-spectrometry–based proteomics efforts. The major goals of such studies will be to identify the set of all yeast proteins that undergo ubiquitylation; to identify the sites of ubiquitylation and the topologies of the ubiquitin chains at these sites, if any; to determine the set of yeast proteins that are substrates for the proteasome; and to match all substrates of the pathway to ubiquitin ligases, DUBs, and ubiquitin receptors that act on them. Such work should provide many fresh insights into the basic biology of yeast.

However, the type of pathway map that may emerge from studies of this kind will be limited. We will additionally need a better understanding of how the signaling information captured in the topology of ubiquitin chains is interpreted by ubiquitin receptors, and more generally deeper inroads must be made into the specificity, mechanisms, regulation, dynamics, and cell biology of the pathway. Vast networks of ubiquitin receptors, such as in the MVB and proteasome pathways, need to be deciphered. Ubiquitin chains are now studied as static entities, but they are likely to be very dynamic. It will be important, although challenging, to follow such key dynamics in the cell without perturbing pathway function.

Ackowledgments

We thank Geng Tian, Suzanne Elsasser, Soyeon Park, An Tyrrell, Karin Flick, Ingfei Chen, and especially Marion Schmidt for critical input and assistance with the figures. Work in our laboratories is supported by National Institutes of Health grants GM43601 and GM095526 (to D.F.), GM66164 and CA112560 (to P.K.); Deutsche Forschungsgemeinschaft grants SFB740, DIP, SPP1365 (to T.S.); and Cancer Research UK (H.D.U.)

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Communicating editor: T. Davis

Cell, Vol. 79, 233–244, October 21, 1994, Copyright © 1994 by Cell Press

The B-Type Cyclin Kinase Inhibitor p40^{SIC1} Controls the G1 to S Transition in S. cerevisiae

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Summary

When yeast cells reach a critical size, they initiate bud formation, spindle pole body duplication, and DNA replication almost simultaneously. All three events depend on activation of Cdc28 protein kinase by the G1 cyclins Cln1, -2, and -3. We show that DNA replication also requires activation of Cdc28 by B-type (Clb) cyclins. A sextuple *clb1-6* mutant arrests as multibudded G1 cells that resemble cells lacking the Cdc34 ubiquitin-conjugating enzyme. *cdc34* mutants cannot enter S phase because they fail to destroy p40^{SIC1}, which is a potent inhibitor of Clb but not Cln forms of the Cdc28 kinase. In wild-type cells, p40^{SIC1} protein appears at the end of mitosis and disappears shortly before S phase. Proteolysis of a cyclin-specific inhibitor of Cdc28 is therefore an essential aspect of the G1 to S phase transition.

Introduction

Sustained cell proliferation requires duplication of all cell constituents, followed by their segregation to daughter cells. Most constituents are synthesized continuously throughout the interdivision period, but chromosomes are duplicated during a discrete interval known as S phase. Cells ensure that chromosomes are duplicated with the same frequency as cells double their mass by controls that prevent S phase entry until cells have grown to a critical size (Killander and Zetterberg, 1965). Experiments involving the fusion of cells at different stages of the cell cycle suggested that S phase is triggered by an inducer that is produced in late G1 as cells reach the critical size and declines later in G2 (Rao and Johnson, 1970).

Candidates for such an inducer were first identified by genetic studies (Hartwell, 1974). DNA replication in the yeast Saccharomyces cerevisiae depends on activation of the Cdc28 protein kinase through its association with G1-specific cyclins called Cln1, -2, and -3 (Richardson et al., 1989). Cdc28 kinase activity associated with Cln1 or Cln2 rises in late G1 owing to the transcriptional activation of the *CLN1* and *-2* genes (Koch and Nasmyth, 1994). This process depends on Cln3 (L. Dirick, personal communication), which seems to be present throughout G1. Premature induction of *CLN1* and *-2* transcription, caused by ectopic expression or by raising Cln3 levels, triggers cells to enter S phase prematurely, indicating that the onset of *CLN1* and *-2* transcription is normally rate limiting for S phase entry. A similar logic may apply to the G1 to S phase transition in metazoan cells, where the appearance in late G1 of cyclin E transcripts may be important (Ohtsubo and Roberts, 1993; Knoblich et al., 1994).

When G1 yeast cells reach a critical size, they not only initiate DNA replication, but they also form buds and duplicate their spindle pole bodies, which are the first steps towards cytokinesis and chromosome segregation, respectively (Pringle and Hartwell, 1981). Cln cyclins are needed for all three events, and their function is therefore not specific to the induction of DNA synthesis. Three genes (*CDC4*, *-34*, and *-53*) are more specifically required for S phase entry. *CDC34* encodes a ubiquitin-conjugating enzyme (Goebl et al., 1988), and *CDC4* encodes a protein that contains copies of a motif found in β-transducins (Yochem and Byers, 1987). Mutants defective in these genes duplicate their spindle pole bodies and bud repeatedly, but they fail to enter S phase at the restrictive temperature. High levels of all three Cln–Cdc28 kinases accumulate in the mutants (Tyers et al., 1992), implying that Cln kinases, though rate limiting, cannot alone trigger DNA replication. The dependence of S phase entry on a ubiquitin-conjugating enzyme (Cdc34) suggests that the G1 to S phase transition needs the degradation of specific proteins.

In addition to Cln cyclins, Cdc28 associates with six B-type cyclins called Clb1-6 (Nasmyth, 1993). Clb1 and -2 proteins appear during G2, whereas Clb3 and -4 appear at the end of S phase (Grandin and Reed, 1993). Mutants lacking Clb1-4 enter S with normal kinetics but fail to form bipolar spindles (Amon et al., 1993). Transcripts from *CLB5* and *CLB6* appear in late G1 around the same time as those from *CLN1* and *-2* (Epstein and Cross, 1992; Schwob and Nasmyth, 1993). It is possible that a rise in Cdc28 kinase activity associated with Clb5 and Clb6 might be more directly involved in activating DNA replication than increases in Cln1 and -2 kinases because S phase, but not budding, is delayed by at least 30 min in *clb5 clb6* double mutants. There are two problems with this hypothesis: first, S phase is only delayed in the absence of Clb5 and -6, and second, premature *CLB5* expression from the *GAL1* promoter does not advance the onset of S (Schwob and Nasmyth, 1993).

We show here that DNA replication in *clb5 clb6* double mutants depends on the activity of the mitotic cyclins Clb1-4. Cells lacking all six Clbs fail entirely to enter S phase but continue budding like *cdc34* mutants, suggesting that *CDC34* might be needed for Clb kinase acitivation. Indeed, there is no Cdc28 kinase activity associated with either Clb2 or Clb5 in *cdc34*-arrested cells. A specific inhibitor of Clb–Cdc28 kinases, p40^{SIC1}, accumulates as cells exit from mitosis and normally disappears shortly before S phase. The inhibitory protein fails to disappear

†The first two authors contributed equally to this work.

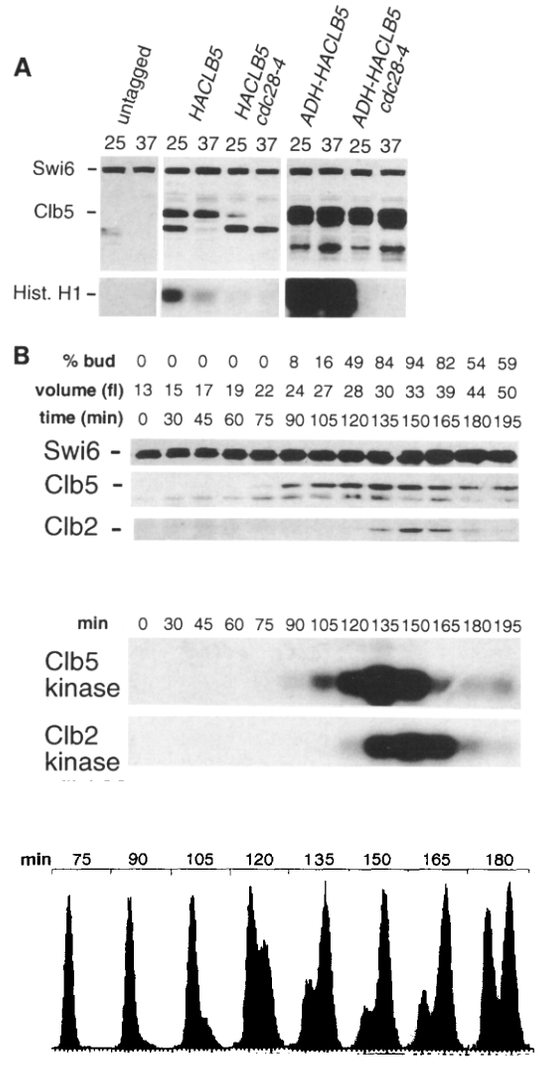

Figure 1. Clb5 Protein and Clb5-Associated Kinase Peak around S Phase

(A) Clb5-associated histone (Hist.) H1 kinase activity is *CDC28* dependent. Clb5 protein (top, in 50 μg of total protein) and Clb5-associated H1 kinase (bottom, 150 μg) in wild-type (untagged), K3662 (*HA6CLB5*), K3717 (*cdc28-4 HA6CLB5*), K3819 (*clb5::HIS3 ADH–HA6CLB5*), and K3913 (*cdc28-4 clb5::HIS3 ADH–HA6CLB5*) strains at permissive (25°C) or restrictive (37°C) temperature for 2.5 hr. Swi6, internal loading control.

(B) Pattern of Clb5 accumulation during the cell cycle. Early G1 cells (K3889, *CLB5HA3*) were isolated by elutriation and were released into YEPR medium at 30°C. (Top) Western blot (50 μg) is shown; the percentage of budded cells and modal cell volume (in femtoliters, fl) are indicated at the top. (Middle) H1 kinase activity immunoprecipitated with the 12CA5 antibody (for Clb5HA) or an anti-Clb2 antiserum. min, minutes. (Bottom) DNA content of the population measured by FACScan. The left and right peaks correspond to cells having 1N or 2N DNA content, respectively. Swi6, internal loading control; min, minutes.

at the equivalent stage of the cell cycle in *cdc34* mutants, and this is responsible for their failure to enter S phase. Premature expression of *CLB5* advances the initiation of S in mutants that lack p40. Thus, activation of Clb–Cdc28 kinases through proteolysis of $p40^{SIC1}$ could be a crucial step in the initiation of DNA replication in yeast.

Results

Clb5–Cdc28 Kinase Activity Peaks around S Phase

To investigate whether Clb5 binds to a protein kinase in vivo, we replaced the endogenous *CLB5* gene with an epitope-tagged version (*HACLB5*), the product of which can be detected on Western blots or immunoprecipitated using the 12CA5 monoclonal antibody (MAb). Clb5-associated histone H1 kinase activity is detected in immunoprecipitates of wild-type cells but is absent in the *cdc28-4* mutant (Figure 1A). However, very little HAClb5 protein accumulates in this mutant, presumably because *CLB5* transcription depends on *CDC28*. Cells expressing HAClb5 from the *ADH* promoter accumulate much higher levels of Clb5 protein in wild-type and mutant cells. This generates more histone H1 kinase activity in wild-type cells but still none in *cdc28-4* mutants, suggesting that this activity is due to Cdc28.

The levels of HAClb5 protein and its associated kinase activity were measured during the synchronous outgrowth of a culture of G1 daughter cells isolated by centrifugal elutriation (Figure 1B). Clb5 appeared as cells began to bud at 90 min, preceding the appearance of the G2-specific Clb2 cyclin. Clb5-associated kinase activity appeared as cells entered S phase. The absence of Clb5 in the starting population of G1 cells suggests that most if not all Clb5 protein made during late G1 and S phases is destroyed by the end of cell division. It is less clear from these data exactly when Clb5 protein is degraded. It seems to linger during G2, suggesting that it may be degraded at the end of the cell cycle. The distribution of Clb5–Cdc28 kinase activity during the cell cycle is consistent with this kinase having a role in the initiation of S phase.

B-Type Cyclins Are Required for DNA Replication

Any hypothesis assuming a role for Clb5 and -6 in the initiation of S phase must explain why S phase is merely delayed in *clb5 clb6* double mutants. To test whether DNA replication in the absence of Clb5 and -6 is dependent on Clb1-4, we constructed a strain in which *CLB1*, *-3*, *-4*, and -6 are deleted and which carries a temperature-sensitive allele of *CLB2* (Amon et al., 1993) and a version of *CLB5* that is under control of the *GAL1* promoter. This strain can only grow in medium containing galactose at 25°C. Unbudded G1 cells were isolated by centrifugal elutriation and were incubated at 37°C in medium lacking galactose. Few if any cells underwent DNA replication, even after 330 min (Figure 2A). At 150 min, when 35% of the cells had formed buds, an aliquot of the culture was incubated with galactose for 15 min and was then returned to medium lacking galactose. This short burst of *CLB5* expression caused all cells to replicate but did not allow them to undergo nuclear division or cytokinesis (Figure 2A). We conclude that DNA replication is totally dependent on *CLB* gene activity. Cln cyclins are therefore insufficient. Clb5 and Clb6 are most likely to perform this S phase–promoting function (owing to their early expression), but in their absence, one or more of the mitotic Clbs can substitute. Inactivation of Clb1-4 does not affect progression through

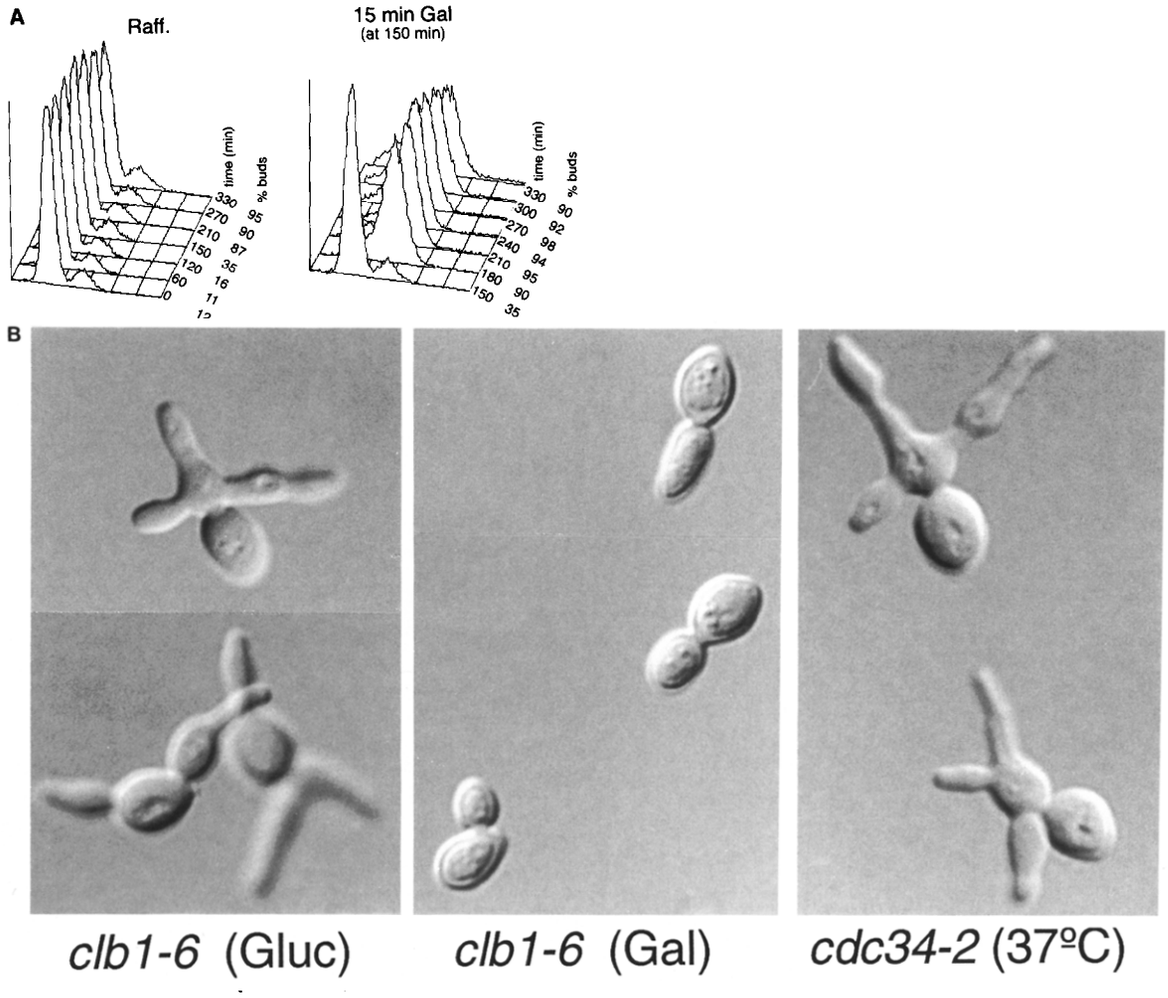

Figure 2. Phenotype of Cells Lacking the Clb1-6 Cyclins

clb1-6 mutant cells (K4057, *clb1Δ clb2^{ts}* clb3::TRP1 clb4::HIS3 clb5::GAL–CLB5/URA3 clb6::LEU2) were grown in YEPR medium containing 0.3% galactose (Gal) at 25°C to midlog phase. G1 cells were isolated by elutriation 1 hr after addition of glucose (2%, Gluc) and were then incubated in YEPR medium at 37°C; after 150 min, a fraction of the population was subjected to a 15 min pulse of 0.3% galactose, washed, and then returned to YEPR plus 2% glucose medium at 37°C.

(A) FACScan of cells lacking B-type cyclin activity (left) or transiently expressing Clb5 (right). Raff., raffinose.

(B) Photomicrographs of K4057-elutriated G1 cells kept for 4.5 hr in YEPD medium at 37°C (left) or for 4 hr in YEPRG medium at 37°C (middle); for comparison, *cdc34-2* cells (K4083, *cdc34-2*) were shifted for 5 hr at 37°C in YEPD (right).

S phase, suggesting that these mitotic cyclins are not normally needed for DNA replication (Amon et al., 1993). Finally, Clb5 alone can trigger what seems to be a complete S phase in the absence of Clb1, -2, -3, -4, and -6. It has been suggested that in some strains, Clb1-4 are needed to complete S phase (Richardson et al., 1992).

Despite being unable to replicate DNA, the sextuple *clb* mutant cells formed buds with normal kinetics (Figure 2A). However, bud growth was hyperpolarized, and most cells budded several times. The terminal phenotype of the sextuple *clb* mutant resembles that of *cdc4, cdc34*, and *cdc53* mutants, which also arrest in G1 with multiple buds (Figure 2B). Induction of Clb5 with galactose caused the buds to adopt a more normal shape and prevented rebudding. In agreement with the results of Lew and Reed (1993), we conclude that Clbs are needed to specify the pattern of bud growth and to prevent rebudding.

Posttranslational Control of Clb5 Kinase in Early G1

CLB5 transcripts are very rare in early G1 cells and only accumulate to high levels shortly before S phase. Premature expression of *CLB5* from the *GAL1* promoter does not advance the onset of DNA replication, suggesting either that Clb5 kinase is in fact insufficient to trigger S phase or that it is also regulated posttranscriptionally (Schwob and Nasmyth, 1993). For example, destruction box–mediated proteolysis of Clb2, which is initiated during mitosis, remains active during the subsequent G1 period until Cln cyclins are activated in late G1 (Amon et al., 1994). A similar phenomenon could affect accumulation of Clb5

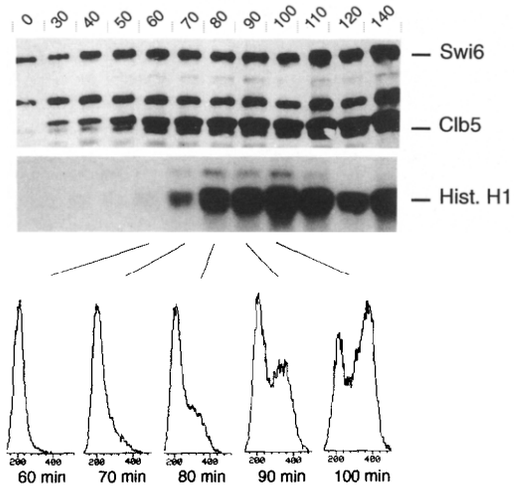

Figure 3. Posttranslational Regulation of the Clb5–Cdc28 Kinase

Elutriated early G1 cells from strain K3916 (*GAL–CLB5ΔDBHA3*) were incubated in YEPRG medium at 30°C for the indicated times. The procedures shown are Western blot (50 μg, top), histone (Hist.) H1 kinase assay (100 μg, middle), and DNA content (bottom). Swi6, internal loading control.

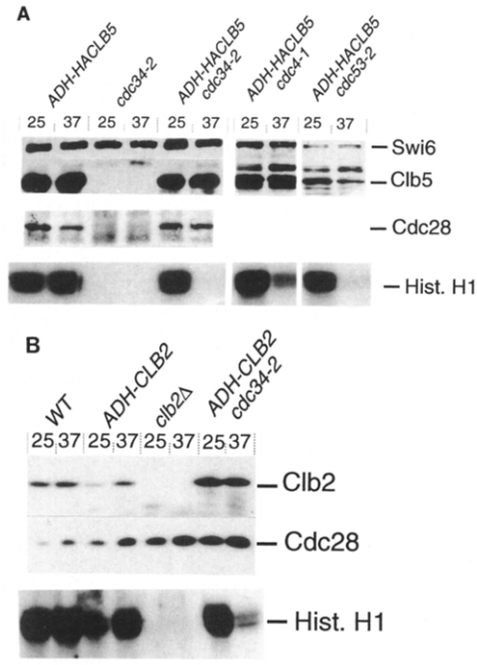

Figure 4. *cdc34*, *cdc4*, and *cdc53* Mutants Contain Little or No Clb5-Associated Kinase Activity

(A) Clb5 protein, coprecipitated Cdc28, and associated histone (Hist.) H1 kinase in *cdc4*, *-34*, and *-53* mutants. (Top) Western blot on extracts (30 μg) from K3819 (wild-type, *ADH–HA6CLB5*), K4083 (nontagged, *cdc34-2*), K3947 (*cdc34-2 clb5::HIS3 ADH–HA6CLB5*), K4024 (*cdc4-1 ADH–HA6CLB5*), or K4026 (*cdc53-2 ADH–HA6CLB5*) cells expressing Clb5 from the *ADH* promoter. (Middle) Western blot of 12CA5 immunoprecipates (from 400 μg extracts) probed with anti-Cdc28 antiserum (in the first six lanes only). (Bottom) Clb5-associated H1 kinase in 100 μg (or 40 μg for *cdc53-2*) extracts immunoprecipitated with 12CA5. Swi6, internal loading control.

(B) Clb2, Cdc28 proteins (Western blot, top), and associated histone H1 kinase (bottom) in K699 (wild type, WT), K3241 (*clb2::LEU2 TRP1::adh–CLB2*), K2902 (*cdc34-2 clb2::LEU2*), or K2925 (*cdc34-2 clb2::LEU2 TRP1::adh–CLB2*) cells at 25°C or 37°C.

protein in early G1 cells because it also contains destruction box–like sequences that contribute to its proteolysis in *cdc28* mutants arrested in G1 (S. Irniger, personal communication).

To address whether cell cycle–regulated transcription and proteolysis are alone responsible for preventing premature activation of the Clb5 kinase, we constructed a version of Clb5 that is driven by the *GAL1* promoter and lacks a putative destruction box (Clb5Δdb). Induction of Clb5Δdb in elutriated early G1 cells had no effect on the timing of DNA replication (see Figure 7B) even though much Clb5Δdb protein accumulated by 50 min, i.e., long before cells entered S phase (Figure 3). Interestingly, histone H1 kinase activity associated with Clb5Δdb did not appear until 70 min, at which time DNA replication commenced. Thus, Clb5 can accumulate in early G1 cells, at least when its destruction box is removed, but it cannot generate an active Cdc28 kinase. We conclude that Clb5–Cdc28 kinase is negatively regulated in early G1 by a posttranslational mechanism, which would explain why premature Clb5 expression fails to advance S phase.

Clb–Cdc28 Kinases Are Inactive in *cdc4*, *cdc34*, and *cdc53* Mutants

Because the phenotype of *cdc4*, *-34*, and *-53* mutants resembles that of cells lacking B-type cyclins (see Figure 2B), we investigated whether they might be defective in the activation of Clb kinases. When expressed from the *ADH* promoter, high levels of Clb5 protein accumulated in wild-type and mutant strains grown at 25°C or 37°C (Figure 4). Wild-type cells contained similar levels of Clb5-associated H1 kinase activity at both temperatures. Kinase activity was present at 25°C but absent at 37°C in *cdc34* and *cdc53* mutants and was strongly reduced in *cdc4* mutants. The lack of kinase activity in *cdc34* mutants is not due to a failure of Clb5 to interact with Cdc28 since the amount of Cdc28 protein coprecipitated with Clb5 was equal at both temperatures and similar to the amount found in wild type (Figure 4A). Similar results were obtained when Clb2-associated kinase activity was measured in wild-type and *cdc34* mutants expressing *CLB2* from the Schizosaccharomyces pombe *adh* promoter (Figure 4B). Despite the accumulation of Clb2 protein in the mutant cells at 37°C, the amount of kinase activity associated with this protein was much reduced compared with 25°C or with wild type at either temperature. The absence of Clb2–Cdc28 or Clb5–Cdc28 kinase activity in *cdc34* mutants contrasts with the high levels of kinase associated with Cln cyclins in these mutants (Tyers et al., 1992).

G1 Cells Contain an Inhibitor of the Clb5 Kinase

Because *CDC34* encodes a ubiquitin-conjugating enzyme thought to promote proteolysis (Goebl et al., 1988), we reasoned that the inactivity of Clb kinases in *cdc34* mutants might stem from their failure to destroy a Clb kinase–specific inhibitor protein. Such a protein might normally

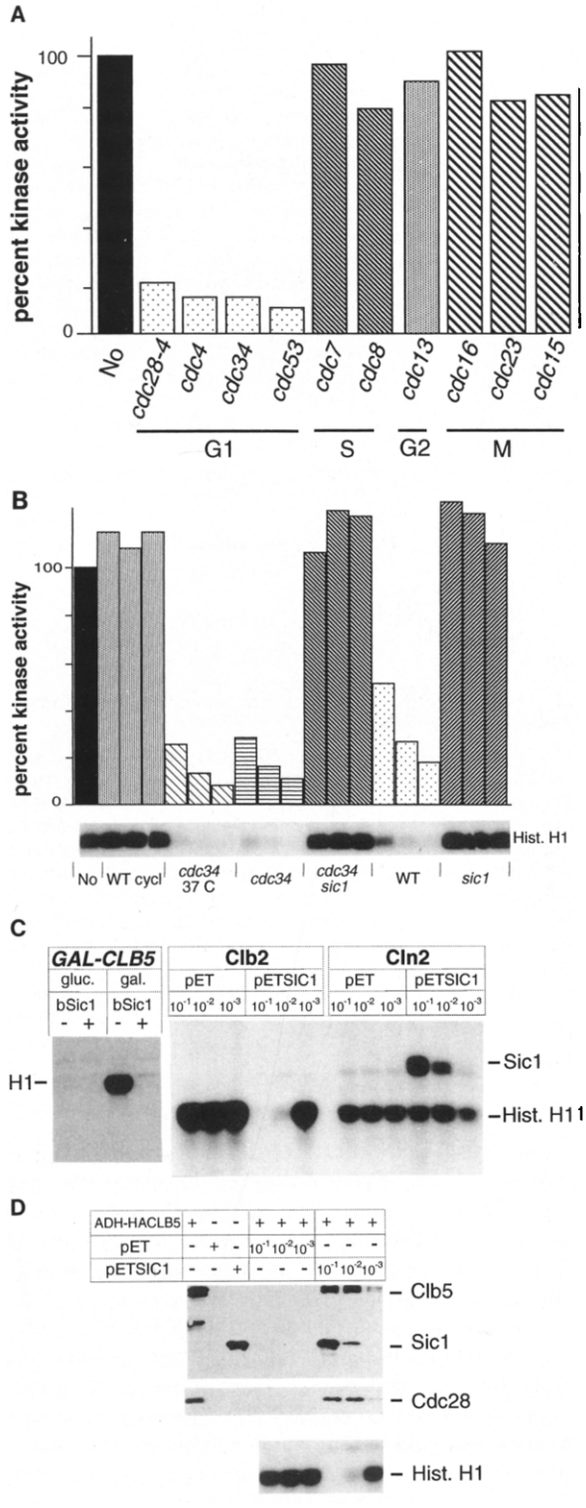

Figure 5. p40^{SIC1}-Mediated Inhibition of the Clb5–Cdc28 Kinase

(A) An inhibitor of the Clb5 kinase is present in G1 cells. Extracts (80 μg) from various *cdc* mutants arrested for 3 hr at 37°C were added to an extract (40 μg) containing tagged Clb5 (K3819, *ADH–HA6CLB5*), and their ability to inhibit the Clb5-associated kinase (leftmost bar, no extract added) was tested by measuring the H1 kinase activity of 12CA5 immunoprecipitates. The following *cdc* strains were used. K1990 (*cdc28-4*), K2018 (*cdc4-1*), K4083 (*cdc34-2*), K1838 (*cdc53-2*), K2032 (*cdc7-1*), K2537 (*cdc8- 1*), K2035 (*cdc13-1*), K2530 (*cdc16-1*), K2532 (*cdc23-1*), and K1994 (*cdc15-2*).

be present in early G1 cells and prevent the premature activation of Clb5 kinase. To test this idea, we mixed crude extracts containing high levels of a hemagglutinin-tagged (HA-tagged) Clb5 protein from cycling cells with extracts from cells (lacking any HA tag) at various stages of the cell cycle. The tagged Clb5 protein was then immunoprecipitated, and histone H1 kinase activity was measured (Figures 5A and 5B). A 2-fold excess of extract from cycling cells added to the HAClb5 extract had no effect on the activity of Clb5. Extracts from cells arrested in S phase by *cdc7* or *cdc8*, in G2 by *cdc13*, in metaphase by *cdc16* or *cdc23*, or in anaphase/telophase by *cdc15* mutations also failed to inhibit the kinase. In contrast, extracts from G1 cells, obtained either by centrifugal elutriation or by incubating *cdc28*, *cdc4*, *cdc34*, and *cdc53* mutants at the restrictive temperature, caused a 5-fold reduction in the Clb5 kinase activity. We found a similar but less drastic inhibition of a tagged Clb2 kinase (data not shown). These data suggest that G1 cells, but not those from other cell cycle stages, contain an inhibitor of Clb–Cdc28 kinases, which could be responsible for the lack of Clb5 kinase in the *cdc34* class of mutants and in early G1 cells expressing Clb5 protein. They also affect the interpretation of kinase measurements performed on imperfectly synchronous cultures since mixing G1 cells with S phase cells could mask the Clb5 kinase activity of the latter.

p40^{SIC1} Binds to and Inhibits the Clb5–Cdc28 Kinase

The Clb kinase inhibitory activity cannot be due to the Far1 protein implicated in Cln kinase inhibition by pheromones because diploid cells, which also arrest in G1 when *CDC34*

(B) The inhibitory activity depends on *SIC1*. Increasing amounts (20, 40, or 80 μg) of extracts from the following strains were added to 20 μg of K3819 (*ADH–HA6CLB5*) extracts (and kinase activity was measured): K699 (cycling wild-type [WT cycl]), K4083 (*cdc34-2* 37°C, arrested for 2.5 hr at 37°C) or (*cdc34-2*, elutriated G1 cells), K4163 (*cdc34-2 sic1::HIS3*), K3916 (*GAL–CLB5ΔDBHA3*, grown on raffinose), and K4137 (*sic1::HIS3 GAL–CLB5ΔDBHA3*, grown on raffinose) cells. No, no extract added.

(C) Purified Sic1 inhibits the Clb5- and Clb2-associated kinases but not the Cln2 kinase in vitro. (Left) Sic1 purified from E. coli (bSic1, 1 μl of 125 μg/ml) was added (plus sign) or not added (minus sign) to 12CA5 immunoprecipitates from strain MDMy649 (*sic1::HIS3 GAL–CLB5HA3*) grown in the presence (gal) or absence (gluc) of galactose. (Right) HisHASic1 (pETSIC1, 1 mg/ml) was added (1 μl of 10- [10^{-1}], 100- [10^{-2}], or 1000-fold [10^{-3}-fold] dilution) to yeast extracts from K3962 (*CLB2HA3*, 200 μg) or K4492 (*GAL–CLN2HA3*, 500 μg). After 30 min incubation at 4°C, Clb2 or Cln2, Sic1, and their associated proteins were immunoprecipated with the 12CA5 antibody, and H1 kinase activity was measured. In control lanes (pET), E. coli extracts from cells containing the empty vector were added.

(D) p40^{SIC1} associates with Clb5–Cdc28 and inhibits its kinase activity. Purified HisHASic1 protein (pETSIC1) was added (24 μl of a 10- [10^{-1}], 100- [10^{-2}], or 1000-fold [10^{-3}-fold] dilution of a 1 mg/ml solution) to an *ADH–HA6CLB5* extract (400 μg, total volume of 100 μl). Sic1-associated proteins were isolated on Ni^{2+}/NTA beads and were analyzed by Western blotting using 12CA5 (for Sic1 and Clb5, top) or anti-Cdc28 (middle) antibodies; as a negative control, an extract was prepared from E. coli containing the empty vector (pET). The same HisHASic1 (3 μl of 10-, 100-, and 1000-fold dilution) was tested for inhibition of the Clb5–Cdc28 kinase (50 μg of *ADH–HACLB5* extract) after immunoprecipitation with the 12CA5 MAb (bottom). The three leftmost lanes are control Western blots and immunoprecipitations.

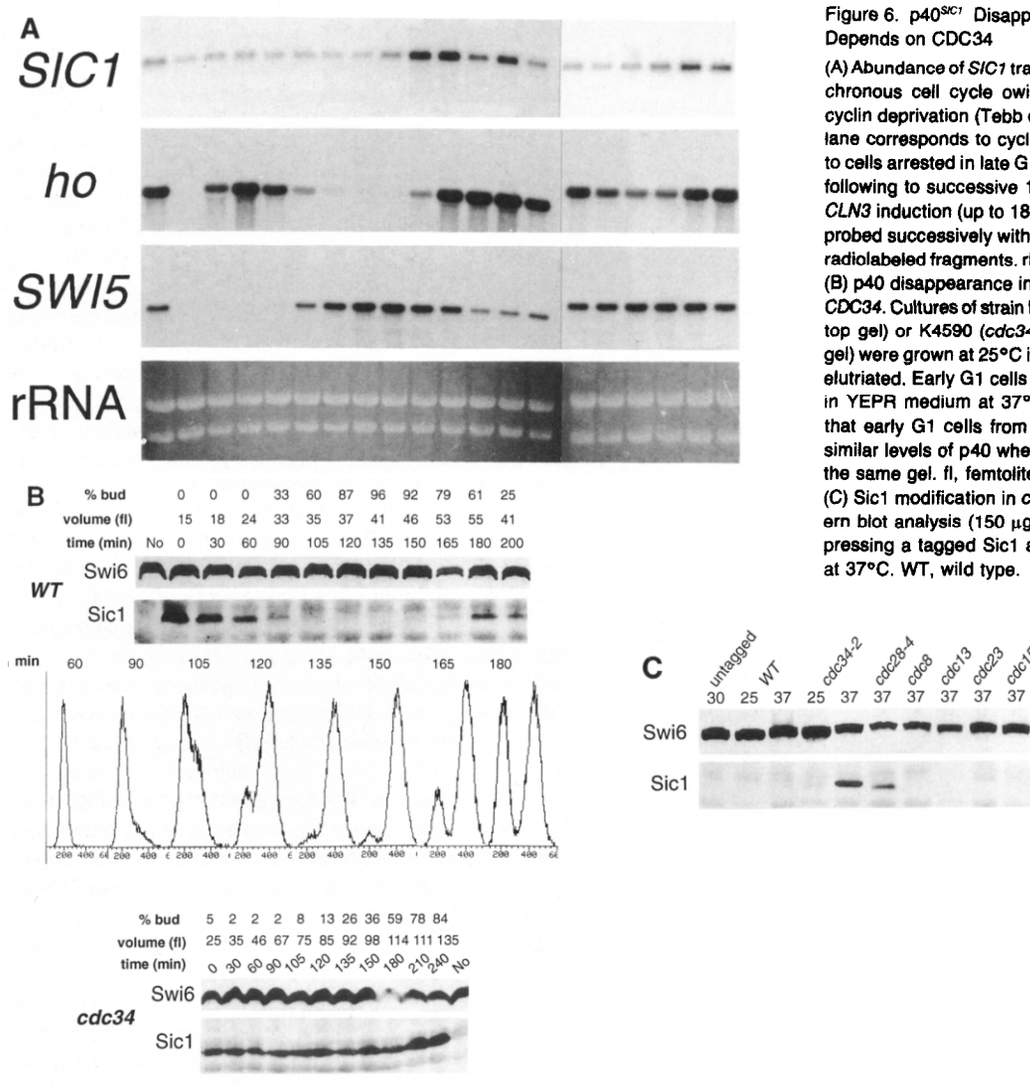

Figure 6. p40^{SIC1} Disappearance in Late G1 Depends on CDC34

(A) Abundance of *SIC1* transcripts during a synchronous cell cycle owing to temporary G1 cyclin deprivation (Tebb et al., 1993). The first lane corresponds to cycling cells, the second to cells arrested in late G1 (t = 0 min), and the following to successive 10 min intervals after *CLN3* induction (up to 180 min). The filter was probed successively with *SIC1*, *HO*, and *SWI5* radiolabeled fragments. rRNA, loading control.

(B) p40 disappearance in late G1 depends on *CDC34*. Cultures of strain K4677 (WT, *SIC1HA1*; top gel) or K4590 (*cdc34-2 SIC1HA1*; bottom gel) were grown at 25°C in YEPR medium and elutriated. Early G1 cells were then incubated in YEPR medium at 37°C (t = 0 min). Note that early G1 cells from both strains contain similar levels of p40 when run and probed on the same gel. fl, femtoliters; No, no tag.

(C) Sic1 modification in *cdc34* mutants. Western blot analysis (150 μg) of *cdc* mutants expressing a tagged Sic1 and arrested for 3 hr at 37°C. WT, wild type.

is mutated (D. Knapp, personal communication), do not transcribe *FAR1* (Chang and Herskowitz, 1990). Another candidate is p40, which was first described as a tightly bound substrate of Cdc28 (Reed et al., 1985) and was later found to be a potent inhibitor of the kinase (Mendenhall, 1993). p40 is encoded by the *SIC1* gene (Nugroho and Mendenhall, 1994) and is present in G1 cells that contain the Clb5 kinase inhibitor. Deletion of *SIC1* is not lethal, which enabled us to test whether the inhibitory activity of wild-type G1 or *cdc34* cells depends on p40. Figure 5B shows that the inhibition exerted by both types of extract is abolished in *sic1* mutants. Thus, p40^{SIC1} alone could be responsible for the Clb5 kinase inhibitory activity present in G1 and *cdc34* extracts.

To test whether p40^{SIC1} directly inhibits the Clb5 kinase, we added p40 purified from Escherichia coli to 12CA5 immunoprecipitates from cells expressing an HA-tagged Clb5. Figure 5C shows that p40 is a potent inhibitor of the Clb5-associated kinase. To address the specificity of inhibition by p40^{SIC1}, we purified from E. coli (on Ni^{2+}/NTA beads, which bind histidine residues) a version of p40 containing six histidines and a single HA tag at its N-terminus (HisHASic1), and we added varying amounts to crude yeast extracts containing HA-tagged Clb2 or Cln2 cyclins. The Cdk complexes were immunoprecipitated with the 12CA5 MAb, and H1 kinase activity was measured. Clb2-associated kinase was inhibited by HisHASic1 but Cln2 kinase was not. Moreover, p40 was a good substrate for the Cln2 kinase (Figure 5C). This experiment suffers from the criticism that the inhibitor and cyclin were coprecipitated, which could either enhance or diminish the ability of HisHASic1 to act as an inhibitor. The data nevertheless suggest that p40 may be a specific inhibitor of Clb but not of Cln-associated Cdc28 kinases. This is consistent with the behavior of *cdc34* mutants, which both bud and express genes regulated by the SBF and MBF transcription factors at the restrictive temperature (Amon et al., 1993). Both events depend on Cln cyclins, which must therefore be active in mutant cells in vivo despite the presence of the p40 inhibitor.

To determine whether p40^{SIC1} forms complexes with Clb5, Cdc28, or both, we incubated the HisHASic1 protein (pETSIC1) with yeast extracts containing an HA-tagged Clb5 and pulled down p40 using Ni^{2+}/NTA beads. Western analysis performed on the beads revealed the presence of p40^{SIC1}, Clb5, and Cdc28 proteins (Figure 5D). None of these proteins were found when the same experiment was repeated using extracts from E. coli cells that do not express p40 (pET). In the same extracts, Clb5-associated H1 kinase activity measured on 12CA5 immunoprecipitates was inhibited by HisHASic1 in a dose-dependent fashion and not by control E. coli extracts. These data, along with those from Figure 4A showing that Clb5–Cdc28 complexes are inactive in *cdc34* mutants, suggest that p40 inhibits the Clb5–Cdc28 kinase by forming ternary complexes with Clb5 and Cdc28.

Disappearance of p40 at the G1 to S Boundary Depends on *CDC34*

SIC1-dependent inhibitory activity is high in G1 cells but undetectable in S, G2, and M phase cells. To determine how this pattern is generated, we analyzed the level of *SIC1* transcripts and protein during the cell cycle. Yeast cells were arrested in late G1 by Cln cyclin deficiency and were induced to reenter the cell cycle synchronously by expressing *CLN3* from the *GAL1-10* promoter (Figure 6A). *SIC1* transcripts were present at all stages of the cell cycle, but their abundance increased 3- to 4-fold as cells underwent division, shortly after the peak of *SWI5* transcription in G2.

To detect p40^{SIC1}, we replaced the endogenous *SIC1* gene by a copy fused at its extreme C-terminus to sequences encoding a single HA epitope. This gene fusion was fully functional in vivo. Figure 6B shows the level of p40 during the synchronous outgrowth of G1 daughter cells isolated by centrifugal elutriation. p40 levels were high during early G1 but started to drop 30 min before initiation of S phase, were absent during S and G2 phases, and reappeared as cells underwent division. When the experiment was repeated using an otherwise congenic *cdc34* mutant strain, cells budded at a larger cell size and ultimately formed multiple elongated buds but failed to enter S phase. p40 levels were initially similar to those seen in wild-type but remained high as the mutant cells budded and for 2 hr thereafter. Thus, the drop in p40^{SIC1} levels in late G1 in wild type depends on *CDC34*.

We also analyzed the accumulation of p40 after shifting asynchronous cultures of wild-type and various *cdc* mutant cells from 25°C to 37°C (Figure 6C). Very little protein is found in wild-type cultures, presumably because they contain few early G1 cells. Furthermore, little or no p40 accumulates in *cdc8*, *cdc13*, *cdc23*, and *cdc15* mutants during their S, G2, or M phase arrests. In contrast, p40^{SIC1} accumulates to high levels in *cdc28* and *cdc34* mutants at 37°C. The protein migrated more rapidly in *cdc28* mutants, suggesting that it might be phosphorylated by Cdc28 in *cdc34* mutants. The greater abundance of p40 in *cdc34* cells (at 37°C) is not due to a corresponding increase in the level of *SIC1* transcripts (data not shown).

Effects of Deleting *SIC1* on S Phase Entry

We next addressed whether accumulation of p40 in *cdc34* mutants is alone responsible for their failure to enter S phase. We tested whether deleting *SIC1* would allow *cdc34* cells to undergo DNA replication. We isolated G1 *cdc34* cells and congenic *cdc34 sic1* cells by elutriation, and we incubated both at 37°C. The *cdc34* single mutant cells failed to replicate even after 6 hr, whereas *cdc34 sic1* double mutant cells replicated by 2 hr (Figure 7A) at the same size (44 fl) as congenic *cdc34* cells grown at permissive temperature. These cells underwent what appears to be a complete round of DNA replication but largely failed to undergo nuclear division and accumulated as large budded cells with a single nucleus (data not shown). Thus, deletion of *SIC1* allowed *cdc34* mutants not only to enter S phase but also to depolarize their bud growth, and it prevented them from rebudding. It did not suppress the lethality of the *cdc34-2* mutation, suggesting that *CDC34* may have an essential function in addition to promoting p40 degradation. Deletion of *SIC1* altered the phenotype of *cdc4* and *cdc53* mutants in a similar fashion, causing them to arrest with 2N DNA content and a single round bud (data not shown).

We also investigated whether p40 is responsible for preventing premature Clb5 kinase activation and DNA replication in early G1 cells when *CLB5* is expressed from the *GAL1* promoter. Addition of galactose to elutriated *GAL–CLB5ΔDBHA* cells did not advance S phase (Figure 7B). In contrast, galactose advanced entry into S phase by 25 min when the experiment was repeated with a congenic *sic1* deletion strain (Figure 7C). *SIC1* cells entered S with a volume of 26 fl with or without galactose. In contrast, galactose lowered the size at which *sic1* cells entered S phase to 22 fl. Thus, inactivation of the *SIC1* gene enabled early G1 cells that express Clb5Δdb ectopically to enter S phase at a smaller size than normal, i.e., to undergo premature DNA replication. When we repeated the same experiment in minimal medium to obtain more G1 cells, we found that deletion of *SIC1* allowed Clb5 kinase to become active in early G1 cells with the same kinetics as Clb5 protein accumulation (Figure 7D; compare with Figure 3). DNA replication was triggered in most cells by 50 min in this experiment (data not shown), by which time only modest amounts of Clb5 protein had accumulated (i.e., to a level comparable to that found in wild type during late G1).

In the absence of ectopic *CLB5* expression, *sic1* cells entered S before they budded. The two events are simultaneous in wild-type cells (Figures 7B and 7C). However, since the cell size at which DNA replication began is similar in mutant and wild type, we cannot say whether S phase is advanced or whether budding is delayed in the mutant. *SIC1* might be only one of several factors that prevents premature DNA replication in wild-type cells. We also noticed that *sic1* cells take longer than wild type to complete S phase. In wild type, the distribution of DNA contents becomes bimodal very soon after S phase entry, whereas in the mutant, it drifts more slowly to the 2N position as a single peak.

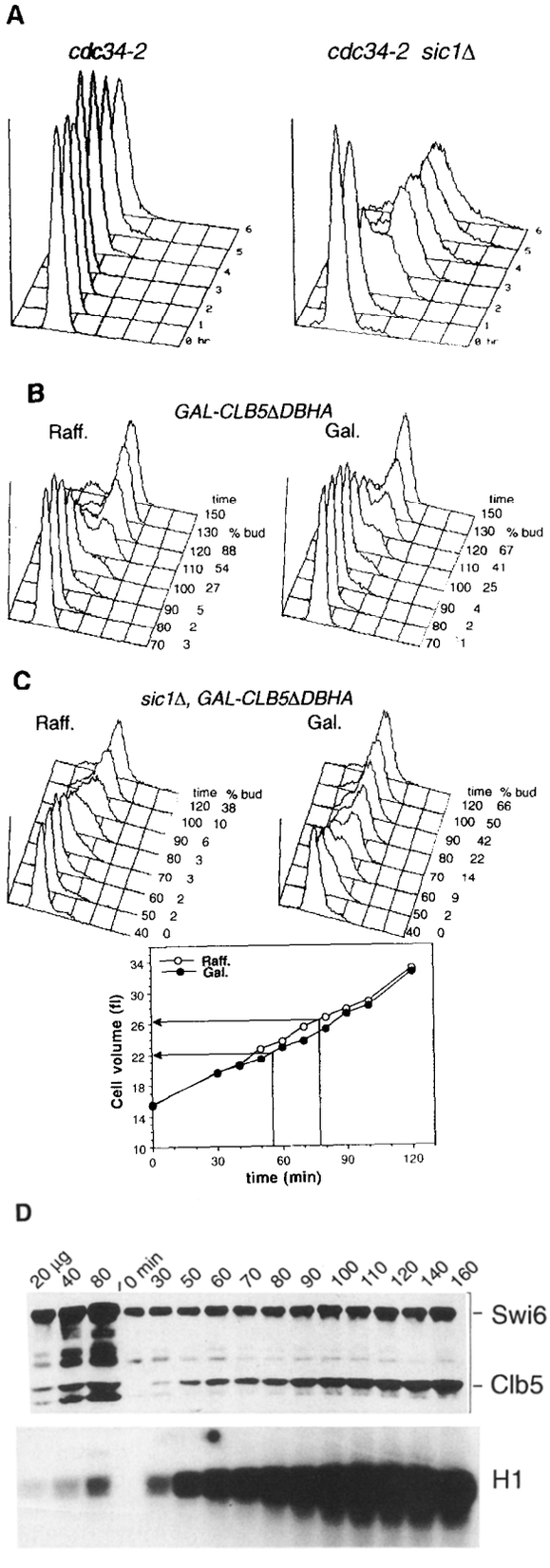

Figure 7. Role of *SIC1* In Vivo

(A) *SIC1* prevents DNA replication in *cdc34* mutants. *cdc34-2* (K4083, left) or *cdc34-2 sic1* (K4163, right) cells were presynchronized in stationary phase on plates and were elutriated. Early G1 cells were then incubated in YEPD medium at 37°C, and DNA content was measured at the indicated times (in hours) after the temperature shift.

(B) Ectopic expression of Clb5Δdb does not cause premature DNA replication. Early G1 cells from strain K3916 (*GAL–CLB5ΔDBHA3*) were incubated in YEPR medium lacking galactose (raffinose, Raff, is present) or containing 2% galactose (Gal). Time was measured in minutes.

(C) Premature DNA replication in *sic1* cells expressing Clb5Δdb. Early G1 cells from strain K4137 (*sic1::HIS3 GAL–CLB5ΔDBHA3*) congenic to K3916 were incubated in YEPR or YEPRG medium at 30°C at t = 0 min. DNA content (FACScan, top) and modal cell volume (bottom) were measured; peak cell volume at which about 30% of the cells in both cultures have replicated is indicated by an arrow. Raff., raffinose; fl, femtoliters; and Gal., galactose.

(D) Clb5 kinase activation parallels Clb5 protein accumulation in *sic1* mutants. A culture from strain K4137 growing in minimal medium plus 2% raffinose was elutriated and early G1 cells put back in minimal medium containing raffinose and galactose at 30°C. (Top) Clb5 Western blot (20 μg); (bottom) Clb5-associated H1 kinase activity. The three leftmost lanes contain 20, 40 and 80 μg of protein from an asynchronously growing *CLB5HA3* (K3889) culture. Swi6, internal loading control.

Discussion

Clb Kinases Are Necessary for Initiation of DNA Replication

Activation of the Cdc28 protein kinase by the G1-specific Cln1, -2, and -3 cyclins is required for budding, DNA replication, and spindle pole body duplication. Three additional genes, *CDC4*, *CDC34*, and *CDC53*, are more specifically required for S phase entry. *CDC34* encodes a ubiquitin-conjugating enzyme (Goebl et al., 1988), suggesting that degradation of certain proteins might be important for initiating DNA replication.

The Cdc28 kinase is needed for both DNA replication and mitosis, and it was thought that these events were triggered by the successive activation of Cdc28 by G1-specific (Cln1, -2, -3) and G2-specific cyclins (Clb1, -2, -3, -4) (Nasmyth, 1993). This tidy picture was, however, jolted by the discovery of two novel B-type cyclins, Clb5 and Clb6, which are synthesized in late G1 along with Cln1 and -2 and which are needed for punctual DNA replication (Epstein and Cross, 1992; Schwob and Nasmyth, 1993). It was therefore suggested that the immediate trigger for S phase might not be activation of Clns but rather the activation of Clb5 and -6. There existed two problems with this hypothesis: S phase is merely delayed in a *clb5 clb6* double mutant, and it fails to be advanced upon ectopic expression of *CLB5*. We have solved both problems by showing that Clb1-4 can substitute for Clb5 and Clb6 and that posttranslational regulation of the Clb5–Cdc28 kinase prevents premature DNA replication in G1.

Mutants lacking all six Clb activities form buds but fail altogether to enter S phase, despite having active Clns. Whereas Clns cannot trigger S phase in the absence of Clbs, ectopic expression of either Clb5 (Schwob and Nasmyth, 1993) or Clb2Δdb (Amon et al., 1994) can trigger S phase in the absence of Clns. Thus, Clb–Cdc28 kinases and not Cln–Cdc28 kinases may be the true S phase inducers. We presume that Clb5 and Clb6 fulfill this function

in wild-type cells and that, in their absence, S phase is merely delayed until Clb1-4 accumulate to sufficient levels.

Destruction of p40^{SIC1} Is Needed for Clb Kinase Activation and S Phase Entry

Three different mechanisms prevent Clb kinases from becoming active in early G1. First, *CLB* genes are not transcribed until late G1 (*CLB5* and *CLB6*), S (*CLB3* and *CLB4*), or G2 (*CLB1* and *CLB2*) phases (Koch and Nasmyth, 1994). Second, Clb cyclins are degraded as cells exit mitosis and, at least in the case of Clb2, remain unstable during the following pre-START G1 period (Amon et al., 1994). We have now discovered a third mechanism: Clb–Cdc28 kinases are kept inactive by an inhibitory protein, p40^{SIC1} (Mendenhall, 1993), that accumulates as cells exit from mitosis and only disappears shortly before S phase by a process that depends on the Cdc34 ubiquitin-conjugating enzyme. We have shown that both Clb2 and Clb5 proteins associate with Cdc28 in *cdc34* mutants, but they fail to activate the kinase probably owing to the formation of ternary complexes with p40^{SIC1} (Figure 4). The same may happen to Clb1, -3, -4, and -6, which would explain why *cdc34* cells cannot enter S phase.

The phenotypes of *cdc4*, *cdc34*, and *cdc53* mutants suggest that the inactivation of p40 in late G1 is an essential step for the initiation of DNA replication in yeast. *cdc34* daughter cells are born with amounts of p40 (Figure 6) and *SIC1*-dependent inhibitory activity (Figure 5) that are comparable to the amounts in newly borne wild-type cells, but neither p40 nor its inhibitory activity disappears in *cdc34* cells when they would be expected to enter S phase. Crucially, the persistence of p40^{SIC1} is largely if not solely responsible for the lack of DNA replication in *cdc34* cells because deletion of the *SIC1* gene allows them to enter S phase. Sufficient p40 exists in wild-type early G1 cells to prevent premature activation of Clb5 kinase and advancement of S phase (Figure 7) when *CLB5* is expressed ectopically. Destruction of this protein is therefore likely to be essential for the G1 to S transition in wild-type yeast cells. Our data suggest that *CDC4* and *CDC53* also participate in the destruction of p40.

The abundance of *SIC1* transcripts is not much altered in *cdc34* mutants, implying that the persistence of p40 is due either to increased translation or to a lack of proteolysis. The latter seems more likely given that Cdc34 is a ubiquitin-conjugating enzyme. *cdc34* mutants may also be defective in the degradation of the G1 cyclin proteins Cln2 and Cln3 (Tyers et al., 1993; D. Finley and R. Deshaies, personal communication). The lack of Cln cyclin degradation in *cdc34* mutants might not be relevant to their G1 arrest because deletion of *SIC1* alone allows *cdc34* cells to undergo DNA replication and because hyperactivation of Clns advances rather than delays S phase.

Regulation of p40^{SIC1} Levels

A key question is this: what triggers disappearance of the p40^{SIC1} inhibitor during late G1? We found that prompt activation of the Clb5 kinase is critically dependent upon Clns. In cells lacking Clns and expressing Clb5Δdb from the *GAL* promoter, Clb5 kinase becomes active 105 min later than in cells expressing Cln2 from the *MET3* promoter (data not shown). We do not yet know whether this delay depends on *SIC1*. Thus, Cln cyclins may play an important part in the activation of Clb5 kinase during late G1 in wild-type cells not only by promoting Clb5 synthesis but also by relieving inhibition of Clb5–Cdc28 complexes by p40^{SIC1}. p40 is a substrate for the Cln2 kinase in vitro (Figure 5C). Furthermore, the p40 protein that accumulates in *cdc28* mutants migrates more rapidly than the protein isolated from *cdc34* mutants (Figure 6C), which possess active Cln kinases. If this difference were due to phosphorylation, it would indicate that p40 might be an in vivo substrate of Cln kinases. Deletion of *CLN1* and *CLN2* delays S phase but not the transcription of *CLB5* (L. Dirick, personal communication), suggesting that the Cln1 and -2 kinases may be needed to trigger p40 degradation.

SIC1 is transcribed throughout the cell cycle with a peak around cell division (Figure 6A), but p40 disappears at the G1 to S transition and does not reappear until cell division (Figure 6B). It is not known what turns p40 degradation off and thereby triggers accumulation of p40 at the end of mitosis. It occurs around the same time as the onset of Clb2 proteolysis and may contribute to the inactivation of Clb kinases, which is thought to be necessary for exit from mitosis. The reappearance of p40 could explain why cells can exit from mitosis in the presence of moderate amounts of a nondestructible Clb2 protein (Amon et al., 1994). In this regard, it is interesting that the *SIC1* gene has also been isolated as a multicopy suppressor of *dbf2* mutants, which arrest in late anaphase (Donovan et al., 1994).

Functions of p40^{SIC1}

Cells lacking p40 are viable but have an increased frequency of broken and lost chromosomes. Daughter cells seem particularly afflicted and tend to arrest at a point late in the cell cycle (Nugroho and Mendenhall, 1994). It is currently unclear which aspect of p40^{SIC1} function makes it important for nuclear division. p40 helps to prevent premature entry into S, and it may thereby prevent errors in DNA replication. p40 also seems important for the rapid completion of S phase. The slow DNA replication of *sic1* mutants is similar to that of *clb5* mutants. p40 might help to direct Clb kinases to appropriate sites within the nucleus or to ensure that they are activated synchronously or only when Clbs have accumulated to high levels. Thus, p40 may actually help Clb kinases to function even though it inhibits them.

If p40 proteolysis were normally dependent on the prior activation of Clns, then p40^{SIC1} could also have an important role in ensuring that Clb kinases do not become active before Cln kinases. The latter are particularly important for bud formation, and premature activation of certain Clbs would interfere with this process (Amon et al., 1994). This might explain why budding is delayed relative to DNA replication in *sic1* mutants. Another event that could be jeopardized by premature activation of Clb kinases is spindle pole body duplication. This early step of spindle formation may depend on Cln kinases since it

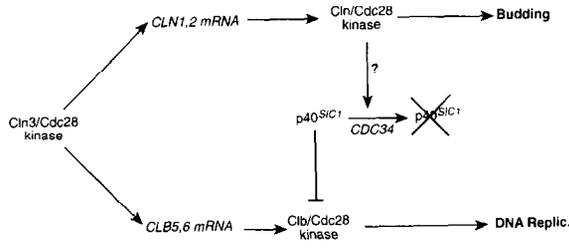

Figure 8. Model for the G1 to S Transition in Yeast

The Cln3–Cdc28 kinase activates *CLN1* and *-2* and *CLB5* and *-6* transcription when cells reach a critical size. The kinases associated with Cln1 and -2 are active, while the Clb–Cdc28 complexes are kept in an inactive state by the specific inhibitor $p40^{SIC1}$. The Clb kinases become active and cells enter S phase only when p40 is degraded. p40 proteolysis, necessary for S phase entry, might be triggered by the Cln kinase and depends on the Cdc34 ubiquitin-conjugating enzyme.

takes place in *cdc4* and *-34* mutants that lack Clb kinase activity. Faulty spindle pole body duplication could conceivably contribute to the increased chromosome loss and mitotic failure of *sic1* mutants.

The Role of Other Cyclin Kinase Inhibitors

There is a human *CDC34* homolog that complements the yeast mutant (Plon et al., 1993). Therefore, destruction of a p40 homolog might also be required for S phase entry in mammalian cells. Several cyclin kinase inhibitors have already been identified in mammalian cells (Pines, 1994), but so far none bears sequence similarity with p40.

There are striking similarities between the behavior of Sic1 and Far1 proteins in yeast. *FAR1* is needed for G1 arrest by pheromones and is thought to act by inhibiting Cln kinases (Chang and Herskowitz, 1990). It too disappears at the G1 to S phase transition in a *CDC34*-dependent manner and reappears at the end of mitosis (McKinney et al., 1993). Far1 is induced to associate with Cln cyclins upon pheromone treatment, but it is not yet clear whether it inhibits the Cdc28 kinase (Peter et al., 1993; Tyers and Futcher, 1993). If so, its spectrum of inhibition may differ from that of p40. Far1 might be expected to inhibit preferentially Cln kinases, whereas $p40^{SIC1}$ seems to inhibit preferentially Clbs. Far1, like the mammalian Cdk inhibitors p21 (El-Deiry et al., 1993; Dulić et al., 1994) and p27 (Polyak et al., 1994) are thought to mediate cell cycle arrest in response to external signals. $p40^{SIC1}$, on the other hand, seems to be part of an "intrinsic" regulatory network that ensures the orderly activation of cyclin-dependent kinases during unrestrained cell cycles. The Rum1 protein, which delays entry into S phase in S. pombe, may perform a function similar to $p40^{SIC1}$ (Moreno and Nurse, 1994).

Regulating S and M

Our current understanding of the events leading to initiation of S phase is outlined in Figure 8. When cells grow to a critical size, Cln3–Cdc28 kinase activates *CLN1* and *-2* and *CLB5* and *-6* transcription. Cln1 and -2 proteins accumulate and form active Cdc28 kinase. All three Cln–Cdc28 kinases are now active, but they cannot trigger S phase, which awaits activation of Clb–Cdc28 kinases upon p40 degradation. Phosphorylation of p40 by Cln1–Cdc28 and Cln2–Cdc28 kinases might trigger its degradation via the ubiquitin-conjugating enzyme Cdc34. It is currently unclear whether synthesis, protein stabilization, or p40 destruction limits the rate of Clb kinase activation in normal cells. These three processes could occur concurrently, and it is striking that all may depend on Cln–Cdc28 kinases. According to this model, Cln kinases control the kinetics of S phase entry, even though they are not the direct trigger for DNA replication.

Our results indicate that proteolysis is a key mechanism for regulating the G1 to S transition in yeast. It is likely that degradation of mitotic cyclins is required for exit from mitosis (Murray et al., 1989; Surana et al., 1993), and it is possible that degradation of other proteins might be required for sister chromatid segregation (Holloway et al., 1993). Why might cell cycle–regulated proteolysis be such a common theme? Because of its irreversibility, proteolysis may be an ideal mechanism for ensuring the procession of cell cycle events.

The discovery that B-type cyclins are inducers of S and M phases poses a question as to how cells determine the correct order of these two events. Any one of the six Clbs may be able to promote S phase. Clb5 seems most effective, but the occurrence of S phase, albeit slowly, in *clb3 clb4 clb5 clb6* quadruple mutants suggests that Clb1 and Clb2 can perform the same function, which is consistent with the induction of DNA replication by Clb2Δdb in triple *cln* mutants (Amon et al., 1994). It is therefore conceivable, although heretical, that the simultaneous activation of one or more B-type cyclins (possibly Clb2 alone if it is sufficiently expressed) would be able to promote an orderly succession of chromosome duplication and segregation in yeast. Successive waves of different types of cyclin-dependent kinases might not after all be needed for ordering these two key cell cycle events. This could be relevant to the mechanism by which rereplication during G2 is avoided. Why would a kinase that is able to promote both S and M not promote a second S phase in G2 cells, and what then determines the timing of mitosis?

Experimental Procedures

Strains, Media, and Reagents

All yeast strains were derivatives of or were at least backcrossed three times to W303 (*HMLa, HMRa*, ho ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 ssd1). Cells were grown in YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) supplemented as indicated with 2% glucose (YEPD), raffinose (YEPR), or galactose (YEPG), unless otherwise stated. Minimal medium is yeast nitrogen base (0.8%) supplemented with amino acids, adenine, uracil, and sugar. Secondary antibodies were purchased from Amersham, and proteins were detected by an enhanced chemiluminescence system according to the manufacturer.

Plasmid Constructions and Genetic Manipulations

For tagging Clb5 at the N- or C-terminus, a NotI cassette containing three tandem repeats of the HA epitope (*HA3*; Tyers et al., 1992) was inserted after the initiation codon or before the termination codon of *CLB5*. The resulting plasmids, C2598 (*HA6CLB5*) and C2667 (*CLB5HA3*), were transplaced to the *CLB5* locus of K3095 (*clb5::URA3*) by transformation and 5-FOA selection. A *CLB5HA3* version lacking the nine amino acids (56–64) of the destruction box (Glotzer et al.,

1991) was generated by PCR (C2668), cloned under control of the *GAL1* promoter to generate *GAL-CLB5ΔDBHA3* (C2670), and then integrated at the *URA3* locus of K699 to generate K3916.

For making the sextuple *clb* mutant, *CLB6* was first disrupted by *LEU2* (Schwob and Nasmyth, 1993) in the *clb1-4* strain (K3080; Amon et al., 1993) to generate K3671. A truncated *CLB5* gene driven by the *GAL1* promoter in a *URA3*-based integrative vector was constructed by deleting a 1.0 kb ClaI fragment that removes the last 64 residues of Clb5 (C2711). After linearization (with BspEI), this plasmid was integrated at the *CLB5* locus of K3671. Ura$^+$ transformants were selected on raffinose plus 0.3% galactose plates lacking uracil at 25°C and were then scored for glucose lethality at 25°C. *SIC1* disruptions are described in Nugroho and Mendenhall (1994). Sic1 was tagged at its N-terminus by inserting a triple HA cassette in a unique NotI site generated after the initiation codon (*HA3SIC1*, C2762). To tag Sic1 at the C-terminus, a fragment carrying a single HA epitope was inserted before the stop codon of *SIC1* to generate C2801. This plasmid was then cut with EcoRI and XbaI and was transplaced at the *SIC1* locus.

Cell Synchronization

Centrifugal elutriations were performed as described previously (Schwob and Nasmyth, 1993). For the experiment in Figure 7A, elutriation was preceded by stationary phase presynchronization on YEPD plates (4 days at 25°C).

Western Blot Analysis and Histone H1 Kinase Assay

Western blot analysis was performed as described in Surana et al. (1993). After transferring the proteins to Immobilon P membranes (Millipore), proteins were detected using an enhanced chemiluminescence detection system (ECL, Amersham). Dilutions of the antibodies were 1:100 for the 12CA5 MAb, 1:2000 for Clb2 antiserum, 1:2000 for Cdc28, and 1:40,000 to 1:200,000 for Swi6. For detection of Sic1HA1, the signal was amplified using a rabbit anti-mouse antibody (1:2500 or 1:5000, Jackson Immunoresearch). In experiments in which both Western blotting and H1 kinase assay were performed, the extracts prepared for the kinase assay were also used for Western blotting. Histone H1 kinase assays were performed as described previously (Surana et al., 1993).

Purification of Sic1 from E. coli

Purification of native Sic1 from E. coli (bSic1) will be described elsewhere. To produce the double-tagged HisHASic1 protein, a *HA1SIC1* fragment from C2762 was cloned into the pETKH1 vector (pET11d derivative; Studier et al., 1990). Induction results in the production of a fusion protein carrying six histidine residues and a single HA epitope at the N-terminus of Sic1 (pETSIC1, C2841). The pETSIC1 plasmid or the parental vector (pET) were transformed into BL21::DE3, and the fusion proteins were purified on Ni^{2+}/NTA beads as described (Qia *expressionist* Handbook, Qiagen). The beads were washed three times with NP-40 lysis buffer (Amon et al., 1993) and twice with the same buffer containing 10 mM imidazole; bound proteins were eluted with 250 mM imidazole. The eluted solutions (referred to as pET and pETSIC1) were adjusted to 1 mg/ml (in 35% glycerol) and were stored at −80°C.

Mixing and Kinase Inhibition Assay

Crude cell extracts from a strain overexpressing HA6Clb5 (K3819) were mixed with extracts from untagged wild-type cells, various *cdc* mutants, elutriated G1 cells, or E. coli purified p40^{SIC1}. After 30 min incubation at 4°C, Clb5 was immunoprecipitated with the 12CA5 MAb (1:10 dilution), and histone H1 kinase activity was determined. Inhibition by p40^{SIC1} was performed in NP-40 lysis buffer with 2 mM $MgCl_2$ and without DTT (Amon et al., 1993). To show complex formation between p40^{SIC1}, Clb5, and Cdc28, cell extracts containing HA6Clb5 (from K3819) were mixed with pET or pETSIC1 extracts and were incubated for 30 min at 4°C. Ni^{2+}/NTA beads were then added, and the suspension was incubated for 1 hr at 4°C. Beads were collected by centrifugation and washed twice with NP-40 buffer and twice with NP-40 buffer containing 10 mM imidazole. Beads were boiled in SDS loading buffer, and the supernatant was used for Western blot analysis.

Other Techniques

Flow cytometric DNA quantitation was determined according to Epstein and Cross (1992) on a Becton-Dickinson FACScan. Cell size analysis was performed on a CASY1 Cell Counter Plus Analyzer (Model TTC; Schärfe System). Cells were fixed for 2 hr in 3.8% formaldehyde and 0.1 M potassium phosphate (pH 6.4). About 20,000 cells were counted, and peak cell volume (in femtoliters) was measured in duplicate.

Acknowledgments

We would especially like to acknowledge the contributions made by Uttam Surana and Angelika Amon, who first noticed that Clb2 protein fails to activate Cdc28 in *cdc34* mutants. We would also like to thank Christian Dahmann, whose data is presented in Figure 2A, and Angelika Amon for the data presented in Figure 4B. We are grateful to Gotthold Schaffner, Robert Kurzbauer, Ivan Botto, Sissy Aigner for DNA sequencing and oligonucleotides synthesis, and Hannes Tkadletz for photographs. We thank Christian Koch and Christian Dahmann for comments on the manuscript as well as Bruce Futcher, Kim Arndt, and Stefan Jentsch for plasmids, strains, and antibodies. T. B. was supported by the Jubileumsfondprojekt 4637 of the Austrian National Bank and by Fond zur Wissenshaftlichen Förderung grant P09979/MOB. M. D. M. acknowledges the American Cancer Society (grant CB-70B) for funding.

Received May 31, 1994; revised August 11, 1994.

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