Life is Degrading:
SCF Ubiquitin Ligases--Their Components and Functions

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"People who make positive choices are winners, and people
who make negative choices are losers.
I despise losers."

-- Arnold Schwarzenegger

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Abstract

The cell cycle is defined by a series of phases demarcated by biochemically imposed transitions. In the budding yeast S. cerevisiae, initial genetic experiments indicated that the G1-S phase transition requires the activities of the essential *CDC4*, *CDC34*, *CDC53*, and *SKP1* gene products as well as an active G1 cyclin/Cdc28 kinase. These proteins promote entry into S phase by enabling the destruction of the S phase cyclin/Cdc28 inhibitor Sic1. That the ubiquitin proteolytic pathway plays a specific role in the elimination of Sic1 was suggested by the previous identification of Cdc34 as a ubiquitin conjugating, or E2, enzyme. However, the roles of the other gene products in eliminating Sic1 were not understood. We showed that Cdc4, Cdc53, and Skp1 assemble into a ubiquitin ligase complex named SCF^{Cdc4}. When combined with purified forms of Cdc34, the Uba1 ubiquitin-activating enzyme, and ubiquitin, SCF^{Cdc4} catalyzes the in vitro ubiquitination of Sic1 molecules that were first phosphorylated by active cyclin/Cdc28. SCF^{Cdc4} has emerged as a paradigm for elucidating the activities of highly evolutionarily conserved SCF complexes throughout eukaryotes. Within SCF, the F-box containing protein Cdc4 and its homologs act as receptors for phosphorylated substrates and are linked to the rest of SCF via F-box mediated binding to Skp1. Skp1 also interacts with Cdc53 or a related cullin family member. Cdc53 plus a newly identified SCF component, the essential RING-H2 protein Hrt1, form a highly conserved core ubiquitin ligase machine; their closest budding yeast homologs are present in the other known cell cycle ubiquitin ligase, the Anaphase Promoting Complex (APC). Cdc53/Hrt1 function by a novel mechanism to stimulate E2 enzyme activity, possibly by promoting an allosteric activation of E2 enzyme. The growing list of SCF components--including over 400 currently identified F-box proteins--and their substrates highlight the deep evolutionary conservation of SCF complexes and the diverse array of cellular processes that they control.

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Chapter 1. Introduction

Introduction to the Cell Cycle and Sic1

Every cell faces two fundamental challenges during its life. First, it must faithfully duplicate its DNA, and then assemble a mitotic spindle and accurately segregate sister chromatids into what will subsequently become two daughter cells. To accomplish these distinct events, the cell divides its lifetime into phases: DNA is duplicated in S phase and partitioned by the mitotic spindle to opposite poles of the cell during mitosis, or M phase; intervening periods before and after S phase are called G1 and G2, respectively. To define, generate, and execute these mutually exclusive states, cells employ a cell cycle "engine" driven at its core by a kinase called a cyclin–dependent kinase that phosphorylates essential targets required for cell cycle progression. As their name implies, cyclin-dependent kinases (CDKs) are activated by a binding partner, called a cyclin. First characterized in the early 1980's (Evans et al., 1983) cyclins are inherently short-lived proteins expressed in related families or groups at various stages of the cell cycle. Alternating waves of expression of S- and M-phase specific cyclins bind to CDKs and promote the requisite events of S (e.g,. replication origin firing) and M phases (e.g., initiating the release and separation of sister chromatids).

The molecular mechanism of transitions between phases of the cell cycle is a fundamental area of investigation. CDKs drive the cell cycle and are themselves governed by positive factors --e.g., cyclin binding, activating phosphorylations—and, surprisingly, negative factors-- inhibitory phosphorylations and binding of CDK inhibitory molecules. These negative factors generate steep barriers to cell cycle transitions thereby ensuring that the checklist of duties required in one phase is completed before permitting the next phase to begin.

In contrast with the complicated and redundant molecular regulatory networks that are the hallmark of mammalian cells, the budding yeast Saccharomyces cerevisiae affords a

relatively simplified and easily manipulated system to study the cell cycle and the regulation of CDK activity. In budding yeast, the commitment of a newborn cell to initiate S phase requires the presence of at least one S phase cyclin or Clb as they are collectively known. Two S phase cyclins, Clb5 and Clb6, are present in G1 and complexed with the single essential yeast CDK, Cdc28. However, during G1 these complexes are maintained in an inactive state by the binding of a third subunit named Sic1. Sic1 was isolated biochemically by purifying Cdc28 from yeast cells and examining associated proteins. A 40 kD associated protein termed Sic1 was found to be a stoichiometric inhibitor of Clb/Cdc28 complexes (Mendenhall, 1993; Nugroho and Mendenhall, 1994). Simultaneously, the *SIC1* gene was cloned as a multicopy suppressor of a temperature sensitive *dbf2*^{ts} mutant that caused a mitotic arrest phenotype (Donovan et al., 1994). Sequencing and preliminary characterization of *SIC1* suggested that it is not essential for viability, and cells lacking Sic1 enter S phase with approximately normal kinetics, but *sic1*Δ cells exhibit diminished viability, increased chromosome loss rates, and tend to accumulate in G2/M phase (Nugroho and Mendenhall, 1994).

Genetics of the G1-S Transition and Sic1

How does Sic1 coordinate proper cell cycle control? Genetic screens initiated nearly two decades ago gave the first tantalizing hints at a mechanism underlying the G1-S transition. Pringle and Hartwell (1981) performed a large-scale screen for yeast mutants that arrested at a given point in the cell cycle, thus displaying a cell division cycle or *cdc* phenotype. Two mutants that arrested in late G1– *cdc4*^{ts} and *cdc34*^{ts}—were identified and the wild-type alleles subsequently cloned by complementation of the mutant phenotypes. Another *cdc* mutant, *cdc53*, was identified in a similar screen for mutants exhibiting a *cdc34*-like phenotype (Goebl et al., 1988; Peterson et al., 1984). A fourth gene, *SKP1*,

was identified in a screen for genes that, when overexpressed, suppressed the *cdc4*^{ts} phenotype (Bai et al., 1996)). The Skp1 protein was also identified in human cells as a subunit of unkown function of a cyclin A/CDK2 complex (Zhang et al., 1995) and a component of the CBF3 centromere binding complex (Connelly and Hieter, 1996). In addition to these *CDC* genes, at least one functional G1 cyclin is required to traverse G1 phase. Cells lacking all three G1 cyclins (Cln1, 2, or 3) arrest as small unbudded cells at an early point in G1 termed START, prior to the *cdc4//4/53* arrest point (see Nasmyth and Hunt, 1993 for review). This arrest reflects a requirement for G1 cyclins to drive the cell cycle as well as to initiate key G1-specific events, such as spindle pole body duplication and bud formation. Finally, completing the exit from G1 requires the activity of at least one of the S phase/mitotic cyclin proteins, (Clb1-6) (Schwob et al., 1994).

Early genetic experiments suggested a role for the *CDC4/34/53* gene products in the G1-S transition. Cells carrying temperature-sensitive alleles of *cdc4*, *cdc34*, *cdc53*, or certain alleles of *skp1* arrest at the restrictive temperature with a common multi-budded ("weenie-buds") cell morphology and unreplicated DNA. Cells doubly mutant for these *cdc* genes exhibit synthetic lethality, while overexpression of *CDC4* complements a *cdc53*^{ts} mutation and vice versa (Johnson, 1991), suggesting a functional interaction between these gene products. Preliminary biochemical evidence that Cdc4, Cdc53 and Cdc34 could interact in vivo substantiated these initial genetic observations (Mathias et al., 1996; Willems et al., 1996). These genetic and physical interaction experiments suggested that these CDC genes function in a common pathway or complex, sometimes referred to for convenience as the "Cdc34 pathway" (Deshaies, 1995).

A fundamental advance in our understanding of the G1-S transition came from work by the Nasmyth lab relating cyclins, the *CDC4/34/53* genes, and Sic1. Schwob and co-workers (1994) showed that in wild-type cells, Sic1 appears at the end of mitosis and persists until mid G1 phase when it is rapidly degraded after cells execute START. In *cdc4*, *cdc34*, or *cdc53* mutant cells, however, Sic1 persists and cells remain arrested in late

G1 with low Clb/Cdc28 but high G1 cyclin (Cln)/Cdc28 activity. Since Sic1 is an inhibitor of Clb/Cdc28 complexes, maintaining high levels of Sic1 should block entry into S phase. In fact, simultaneously deleting Sic1 in a *cdc4*, *cdc34*, or *cdc53* background suppresses the *cdc* mutant G1 arrest phenotype, suggesting that Sic1 is a critical G1 target of the Cdc34 pathway. Intriguingly, *cdc34* (or *cdc4*) *sic1*Δ double mutants complete S phase but subsequently arrest in G2, suggesting the existence of other essential targets of the Cdc34 pathway (Schwob et al., 1994).

The Ubiquitin System

The aforementioned genetic studies suggested a role for the Cdc34 pathway in the elimination of Sic1. However, neither these studies nor the sequences of *CDC4*, *CDC53*, or *SKP1* yielded any significant clue as to the possible function of Cdc34 pathway members. Only the previous identification of Cdc34 as a component of the ubiquitin system (Goebl et al., 1988) provided any insight into the possible role of these *CDC* gene products in eliminating Sic1 and thereby regulating the G1-S transition.

Utilizing the covalent attachment of ubiquitin onto a target protein as a marker for its destruction, the ubiquitin proteolytic pathway is responsible for the proteolysis of most intracellular proteins (Hochstrasser, 1996). The ubiquitin pathway is described in detail below; briefly, ubiquitin is first activated by a ubiquitin-activating enzyme and then transferred to a ubiquitin conjugating enzyme. Ubiquitin is then covalently conjugated to substrate, often with the help of a ubiquitin ligase activity.

That the ubiquitin system plays a critical role in regulating the cell cycle was reinforced by several key observations in the 1980s. First, workers in the Varshavsky lab demonstrated that a temperature-sensitive mammalian cell line exhibiting cell cycle progression defects was shown to contain a mutant form of ubiquitin-activating enzyme (Finley et al., 1984) providing the first hint at a physiological role for the ubiquitin system.

The identification of *CDC34* as encoding a ubiquitin conjugating enzyme essential for budding yeast cell cycle progression was the first specific example of ubiquitination regulating the cell cycle. Complementing these efforts, experiments utilizing frog egg extracts demonstrated that the ubiquitin-dependent proteolysis of a mitotic cyclin (cyclin B) was necessary for exit from mitosis (Glotzer et al., 1991). This result led to hypothesis that an essential mechanism of cell cycle transitions was the phase-specific elimination of cyclins to clear the way for the next wave of expression and activity. The subsequent discovery of negative regulators as a key means of cell control aded a new layer of complexity to this view.

Initially regarded as merely a system for eliminating aberrant or damaged proteins, the results mentioned above set the stage for a groundswell of interest in the ubiquitin system--its components, regulation, and substrates. In hindsight, it is not surprising that ubiquitination should play such a critical role in cellular regulation; in fact, it is ideally suited to do so. The efficiency of the ubiquitin system make it an excellent means to control cellular decisions; unlike a potentially reversible modification like phosphorylation, proteolysis is inherently irreversible, affording the possibility of rapidly altering protein levels and thus the cellular biochemical state. It can also be a highly selective process, eliminating one subunit of a multicomponent complex--for example, destroying Sic1 while leaving Clb/Cdc28 intact. The temporal restriction of Sic1 destruction to the latter portion of G1 suggests that ubiquitin-mediated proteolysis can be exquisitely regulated. Potentially either the substrate or a component of the ubiquitin machinery must be activated before becoming active; examples of both forms of regulation are now known (see below). First, we consider in more detail the components of the ubiquitin pathway.

Ubiquitin. A scant 76 amino acids in size, ubiquitin (Ub) is one of the most highly conserved proteins in eukaryotes, from yeast to humans. Ubiquitin was initially described in 1975 (Goldstein et al., 1975) as a molecule attached to histone 2A via an isopeptide bond

between the carboxyl group of ubiquitin and the epsilon-amino group of a lysine residue in the histone (Goldknopf and Busch, 1977). That ubiquitin plays an important role in protein degradation was suggested by experiments identifying ubiquitin as APF, the ATP-dependent proteolysis factor required for protein degradation in vitro using a fractionated rabbit reticulocyte extract (Ciechanover et al., 1978; Wilkinson et al., 1980). These initial observations and subsequent in vitro experiments led to the conclusion that the attachment of ubiquitin to a protein serves as a marker for that protein's rapid destruction. In contrast to the monoubiquitination observed on histones, marking a protein for proteolysis entailed engineering a long linear chain of ubiquitin onto the target. The initial covalent attachment of a ubiquitin molecule serves as a 'landing pad', with lysines (typically Lys48) within a ubiquitin serving as the linkage site for subsequent molecules. Repeating this reaction generates long chains of ubiquitin molecules whose existence prefaces the immediate destruction of the target by the 26S proteasome.

Ubiquitin-activating enzyme (E1). Ubiquitin's journey from cytosol to attachment onto a substrate requires participation of at least three different activities, defined initially by Hershko and co-workers utilizing biochemical fractionation of cell extracts (Hershko et al., 1983). As a first step, ubiquitin is activated by an adenylation reaction executed by the ubiquitin-activating enzyme, or E1. Once activated, ubiquitin is covalently attached to a reactive cysteine residue that forms the active site. Unlike the stable, isopeptide attachment of ubiquitin to substrate, ubiquitin is bound to E1 via a labile, high-energy thiolester bond sensitive to attack from free thiols (Ciechanover et al., 1982). Most eukaryotes contain a single E1 enzyme; in budding yeast, E1 activity is supplied by Uba1, a 114 kDa protein required for viability. Uba1 (and E1 enzymes in general) are not thought to be subject to post-translational modifications governing their activity or abundance.

Ubiquitin conjugating enzyme (E2). Having formed a thiolester bond with a ubiquitin molecule, E1 enzyme unloads its cargo onto the active site of a member of a second class of enzyme, collectively termed ubiquitin conjugating, or E2, enzymes, of which Cdc34 is a member. There are currently over 30 known E2s, including 14 in yeast, although two of these are known to be involved in delivering ubiquitin-like molecules (UBLs) such as Rub1. E2 enzymes have been shown to play roles in diverse processes, including cell cycle progression, DNA repair, removal of misfolded proteins, heavy metal toxicity resistance and organelle biogenesis, but only Cdc34 and Ubc9 in budding yeast are absolutely essential for cell viability. All E2 enzymes share a common (>35% identity) core domain, the 16 kD UBC domain (Jentsch, 1992). A reactive cysteine lies in the middle of this domain and forms thiolesters with ubiquitin transferred from E1. In addition to this UBC domain, some E2s possess either N- or C-terminal extensions. Experiments from several labs suggest that these extensions mediate interactions between E2 enzymes and substrate or ubiquitin ligases (see below) (Kolman et al., 1992; Madura et al., 1993). Cdc34 is a 295 amino acid (34 kD) protein and is unique among the E2s in that it contains a fairly lengthy C-terminal extension comprising over half its total length. Initial domain swapping experiments demonstrated the C-terminal tail can confer Cdc34's essential cell cycle function onto a heterologous E2 enzyme, Rad6, that is not normally required for cell cycle functions (Kolman et al., 1992; Silver et al., 1992). Specifically, the 40 residues adjoining Cdc34's N-terminal UBC domain are required to bind E3 components (Mathias et al., 1998) and may promote the oligomerization of Cdc34 (Ptak et al., 1994).

Interestingly, Cdc34 exhibits another unique property: in the presence of E1 enzyme, ATP, and ubiquitin, Cdc34 can covalently attach ubiquitin onto itself, both in vivo and in vitro (Banerjee et al., 1993; Goebl et al., 1994). Ubiquitin is attached to one of four Lys residues located at the extreme C-terminus of Cdc34. However, these residues (and, in fact, the C-terminal 86 amino acids of Cdc34) are dispensable for Cdc34 function. Furthermore, Cdc34 is a constitutively stable, abundant protein throughout the cell cycle,

suggesting that the ability of Cdc34 to autoubiquitinate is not essential for its function or regulation. Instead, it may represent a side reaction as a Cdc34 molecule loaded with a thiolesterified ubiquitin deposits its cargo onto a convenient local acceptor lysine in its own tail in the absence of a bona fide substrate.

Ubiquitin ligases (E3). Once E2 has acquired a thiolesterified ubiquitin, its mission is to attach its cargo onto a target substrate. Some E2s may be able to execute this step alone, at least in vitro (Haas et al., 1991). However, most E2s probably require the assistance of a third component of the ubiquitin pathway, called a ubiquitin ligase or E3, functionally defined as an activity necessary for the transfer of ubiquitin from E2 to substrate. E3s can bind both E2 and substrate and are therefore the critical means in regulating substrate recognition and ubiquitin transfer. While occupying a position of supreme importance, E3s have historically been the least characterized components of the ubiquitin pathway, due in large part to their limiting cellular protein abundance and a lack of sequence homology between E3 families. Prior to the work described in Chapter 2, only two distinct families of E3s were known: Ubr1 and its mammalian homolog E3α, which mediate the N-end Rule and the HECT-domain family of E3s, typified by E6-AP.

The N-end Rule states that the half life of certain proteins is determined by the identity of that protein's N-terminal residue (Bachmair et al., 1986; Varshavsky, 1992; Varshavsky, 1996). A variant of the N-end Rule has been shown to exist in all organisms examined so far; in budding yeast, the 225 kD ubiquitin ligase Ubr1 mediates the N-end Rule. Ubr1 is not essential but has been shown to play an important role in mediating dipeptide uptake (Byrd et al., 1998). Ubr1 can bind its cognate E2, Ubc2 (also called Rad6) as well as potential N-end Rule substrates, thereby targeting them for ubiquitination and destruction (Bartel et al., 1990; Madura et al., 1993). While the physical basis for interactions between Ubr1, Ubc2 and other enzymes specific to the N-end Rule pathway

has been intensely investigated, the reaction mechanism underlying the transfer of ubiquitin from E2 to substrate is not well understood.

In contrast, the mechanistic details of ubiquitin transfer by the HECT-domain family of ubiquitin ligases are better understood. Investigations of the activated destruction of the human tumor suppressor p53 showed that a cellular protein, E6-AP, can collaborate with the E6 protein of human papillomavirus to form an E3 (Scheffner et al., 1993; Scheffner et al., 1995). The E6-AP protein contains an active site cysteine residue required for ubiquitin transfer to p53; in fact, the cysteine transiently forms a thiolester bond with ubiquitin, an obligate step in the E1-E2-E3 "bucket brigade"(Scheffner et al., 1995). Mutating this cysteine by site-directed mutagenesis yields an inactive, dominant-negative form of E6-AP; likewise, chemical inactivation by treatment with the alkylating agent N-ethyl maleimide (NEM) abolishes ligase activity (S. Schwarz, personal communication). This reactive cysteine lies in a domain that has since been identified in several proteins and has been named the HECT domain. A HECT domain containing protein from yeast, Rsp5, has been shown to act by an identical thiolester-containing transient intermediate state (Wang et al., 1999). However, whether all E3s, particularly non-HECT domain ligases, operate by a similar mechanism remains a subject for further investigation.

The 26S Proteasome. Once a target protein has been conjugated with multiubiquitin chains, it becomes a target for proteolytic destruction by a large, ATP-dependent, multisubunit complex called the 26S proteasome. The proteasome consists of a central, 20S core proteolytic region and two 19S caps or regulatory particles. The 20S particle is composed of two sets of rings, alpha and beta, each containing 7 subunits and stacked together to form a hollow cylinder, as confirmed by the recent elucidation of the three-dimensional structure by X-ray crystallography (Groll et al., 1997). The inside surface of the 20S core exhibits highly potent trypsin, chymotrypsin, and peptidyl-glutamyl-peptide proteolytic activities (Hochstrasser, 1996; Jentsch and Schlenker, 1995). Flanking either

end of the tube formed by the 20S core, the 19S caps contain at least 17 different proteins, including six ATPase subunits that may be involved in unfolding of incoming substrates (Rubin et al., 1998; Rubin et al., 1997). The cap is thought to act generally as a regulatory hinge or trapdoor for the 20S core, specifically permitting entry of multiubiquitinated proteins (Groll et al., 1997). It may achieve this specificity through 19S subunits that bind to multiubiquitinated proteins—for example, the yeast protein Mcb1 and its human homolog S5a (Deveraux et al., 1994; van Nocker et al., 1996). However, yeast MCB1 is not an essential gene, suggesting that a possible functional redundancy resides in other cap subunits. The proteasome also associates with ubiquitin hydrolases (or isopeptidases), enzymes that selectively cleave between ubiquitin moieties thereby releasing ubiquitin monomers. These isopeptidases play an important role in disassembling ubiquitin chains from proteins in the process of being degraded; failure to do so may constipate the proteasome and could be responsible for the slow-growth phenotype observed in yeast cells mutant in DOA4, a proteasome-associated isopeptidase (Papa and Hochstrasser, 1993).

Biochemical Reconstitution of the G1-S Transition

As mentioned previously, the identification of Cdc34 as an essential E2 enzyme posited a role for the ubiquitin system in regulating cell cycle progression (Goebl et al., 1988). However, while genetic experiments and preliminary protein-protein interaction studies hinted at a role for Cdc4, Cdc53, and Skp1 in a Cdc34-dependent pathway for the elimination of Sic1, the specific roles and functions of these proteins were unclear.

By contrast, genetic studies provided significantly more insight into the role of G1 cyclins (Cln)/Cdc28 complexes in promoting the transition through G1 phase. Cells deleted for all three *CLN* genes are inviable but are rescued by simultaneous deletion of

SIC1 (Schneider et al., 1996; Tyers, 1996). Furthermore, cells released from a *cdc34*^{ts} arrest require Cln/Cdc28 activity to initiate S phase (Schneider et al., 1996). Taken together, these data implicate Sic1 as the essential G1 target of Cln/Cdc28. A careful biochemical analysis of Sic1 phosphorylation revealed that Cln/Cdc28 can phosphorylate up to 9 sites on Sic1 in vivo (Verma et al., 1997b). Four of these sites, located in the N-terminal 160 amino acids of Sic1, are essential; mutating all of them produces a Sic1 that cannot be ubiquitinated in vitro and is stable in vivo, causing a G1 arrest phenotype when overexpressed. Once phosphorylated, Sic1 can be ubiquitinated in vitro by fractionated yeast cell extracts lacking CDK activity, demonstrating that phosphorylation is an essential prerequisite for Sic1's subsequent ubiquitination (Verma et al., 1997b). This requirement to phosphorylate by G1 cyclin provides an elegant means of ensuring that S phase is not initiated until Cln/Cdc28 activity is sufficiently high to ensure execution of START and other G1 functions like spindle pole body duplication.

Although insights into Cdc34 and Cln/Cdc28 activities shed light onto the mechanism underlying the G1-S transition, the role of Cdc4, Cdc53, and Skp1 remained unclear. Do they participate directly in Sic1 elimination by functioning as a ubiquitin ligase or do they act indirectly, for example, by activating a 26S proteasome subunit or modulating the activity of an as-yet undiscovered E3? Are those gene products the only ones required to drive the G1-S transition? To answer these questions we established an in vitro assay system to define the minimal set of proteins that are sufficient and necessary to catalyze the G1-S transition by ubiquitinating Sic1. Initially capitalizing on an established fractionated yeast extract system (Deshaies et al., 1995) we showed a direct requirement for Cdc34 and Cdc4 to ubiquitinate phosphorylated Sic1 (Verma et al., 1997a). Subsequent efforts used proteins highly purified from budding yeast, E. coli, and baculovirus-infected insect cells to demonstrate that a set of proteins—ubiquitin, E1, Cdc34, Cdc4, Cdc53, and Skp1—was competent to ubiquitinate phosphorylated Sic1. Importantly, Skp1-Cdc53-F-

box protein (i.e., Cdc4; see below) form a complex, SCF, that acts as a ubiquitin ligase (Feldman et al., 1997; Skowyra et al., 1997)(see Chapter 2).

SCF Has a Fourth Subunit

To investigate SCF components and their possible modes of regulation in vivo, we constructed an "epitope-tagged" form of Cdc53 that could be efficiently immunoprecipitated from yeast cells. A gene encoding this tagged form of Cdc53 was introduced into yeast cells and the expressed protein captured by immunoprecipitation. Associated co-purifying proteins, presumably SCF components or substrates, were resolved by SDS-PAGE and analyzed by mass spectrometry. As detailed in Chapter 3, this protocol revealed the existence of a fourth subunit of SCF, named Hrt1 (Seol et al., 1999). Similar biochemical purifications and two-hybrid experiments carried out by other researchers established that Hrt1 is a conserved member of SCF in rabbit, rat, and human cells, where it has been named Rbx1 or Roc1 (Kamura et al., 1999; Ohta et al., 1999; Tan et al., 1999). In budding yeast, *HRT1* is essential and cells carrying a temperature-sensitive allele of *HRT1* arrest with a *cdc4/34/53/skp1*-like *cdc* phenotype. Hrt1 contains a zinc ion-binding RING-H2 domain that is required for it to associate with Cdc53/cullin proteins. Hrt1 can also bind Cdc4 and Cdc34 but not Skp1. Intriguingly, the closest budding yeast paralog of Hrt1 is Apc11, a component of the other major cell-cycle ubiquitin ligase, the Anaphase Promoting Complex (APC). Furthermore, the Cdc53 subunit of SCF shares homology with another subunit of the APC, Apc2 (Zachariae et al., 1998). We used the autoubiquitination of Cdc34 as an assay to monitor E3 activity in the absence of substrate binding. These data, presented in Chapter 3, argue strongly that Cdc53 plus Hrt1 form a conserved core ubiquitin ligase that act by a unique mechanism to stimulate E2 enzyme activity, thereby promoting substrate ubiquitination.

The experiments described in Chapter 2 marked the beginning of a groundswell of interest in SCF and related ubiquitin ligases—particularly in elucidating the composition of SCF, the function, and mechanism of these components, and the diverse cellular roles played by SCF ubiquitin ligases. A tremendous amount of research on SCF has been reported in the two intervening years between publication of the work described in Chapters 2 and 3. From these efforts, a few important themes have emerged. SCF is a highly evolutionarily conserved machine and its components are being discovered in a broad range of organisms. The architecture of SCF—a core ubiquitin ligase attached to a potentially varied repertoire of F-box containing receptor modules—enables it to recognize a broad array of potential substrates and thereby participate in an broad array of cellular functions. Some key results from analyses of SCF and its components are summarized below.

Architecture of SCF Components

Cdc4 and the F-box. The experiments described in Chapter 2 revealed that the Cdc4 subunit of SCF is the receptor for substrate, binding phosphorylated (and not unphosphorylated) Sic1. Analysis of the *CDC4* coding sequence revealed two domains of interest: a C-terminal region of seven tandem WD-40 domains and a domain called the F-box. The WD-40 domain mediates the interaction between Sic1 and Cdc4, since a complete WD-40 domain is required to bind Sic1 (Skowyra et al., 1997) and the WD-40 regions alone are sufficient to interact with Sic1 (C. Correll, personal communication). In addition to Sic1, all currently known substrates of Cdc4—Far1 (Henchoz et al., 1997), Gcn4 (Y. Chi, personal communication), and Cdc6 (Drury et al., 1997)(J. Campbell, personal communication)—must be phosphorylated before binding to Cdc4, suggesting that the WD-40 domain functions as a phosphopeptide receptor. The known substrates of

Cdc4 share no obvious sequence homology or structural features, although a detailed analysis of the binding motifs present in substrates awaits further investigation. How might Cdc4 bind such a broad repertoire of substrates? Based on the X-ray crystallographic structure of the Gβ subunit of heterotrimeric G proteins, the WD-40 repeats are predicted to fold into a β-propeller structure (Lambright et al., 1996; Sondek et al., 1996; Wall et al., 1995). This structure provides a large surface area capable of potentially supporting a broad range of diverse protein-protein interactions. Most likely, it contains a phosphate-binding pocket; additional substrate sequence or structural requirement are still not known.

Cdc4 interacts with the rest of SCF via its F-box, a region of approximately 45 amino acids necessary for binding Skp1. First identified in human cyclin F, computer database searches have revealed the existence of many F-box proteins in a range of species (Bai et al., 1996; Patton et al., 1998b). While systematic mutational analysis on the F box has not yet been carried out, an intact F-box is required to bind Skp1. While the F-box is necessary, it may not be sufficient to bind Skp1, especially in vivo. For example, mutating residues in the C-terminus of the F-box protein Skp2 disrupts Skp1 binding, even though these residues are distal to the F-box (Lisztwan et al., 1998). A fragment of Cdc4 containing the N-terminus (with F-box) but lacking the WD-40 repeats binds poorly to Skp1 (Zhou and Howley, 1998). Thus, regions outside the F-box may play a role in interacting with Skp1 in some F-box proteins.

Skp1. The highly evolutionarily conserved molecule Skp1 juxtaposes the F-box and its captive substrate with the ubiquitin-delivering machinery. Budding yeast Skp1 is 163 amino acids in size, and its highly conserved human ortholog, while 30 residues shorter, can complement the defect of *skp1*^{ts} yeast mutants. Domain analysis suggest that the first 91 amino acids of Skp1 can bind Cdc53-like proteins (Michel and Xiong, 1998) while an

overlapping region is responsible for binding the F-box (Ng et al., 1998). This binding is likely to be direct since Skp1 can bind the human F-box protein Skp2 or the Cdc53-related protein Cul1 when all proteins are produced in E. coli (Lyapina et al., 1998; Yam et al., 1999). Furthermore, Skp1 produced in E. coli is fully functional in in vitro ubiquitination assays, suggesting that no eukaryotic-specific post-translational modifications are required for activity. However, as suggested above, Skp1—F-box interactions may be regulated by additional domains; for example, in vivo the Grr1-Skp1 interaction is positively regulated by the presence of glucose, although the mechanistic details of this regulation are not yet well defined (Li and Johnston, 1997).

Cdc53 and cullins. Cdc53 is an 814-amino acid protein that forms a core component of the SCF ubiquitin ligase. Mutational analyses and binding studies have identified three major subdomains with Cdc53: an N-terminal domain of approximately 219 amino acids capable of binding Skp1, an internal domain capable of binding Cdc34 and Hrt1, and a C-terminal region of unknown function. The extreme C-terminus is highly conserved with other Cdc53 homologs and contains the site of attachment for the ubiquitin-like molecule Rub1 (Lammer et al., 1998; Liakopoulos et al., 1998; Patton et al., 1998a). However, this domain is not required for Cdc53 function in vitro or in vivo (Feldman et al., 1997; Lammer et al., 1998; Patton et al., 1998a). Portions of Cdc53, including the central Cdc34- and Hrt1-binding domain share homology with a C. elegans protein, cullin-1 (Cul-1)(Kipreos et al., 1996). Additional analyses revealed a domain shared between Cdc53 and the APC subunit Apc2, subsequently named the cullin homology (CH) domain (Yu et al., 1998; Zachariae et al., 1998). Cullins have since been identified in a range of organisms including fungi, rats, rabbits, and humans (Kipreos et al., 1996; Kominami et al., 1998; Yu et al., 1998). The CH domain probably comprises an E2 enzyme-RING-H2-cullin interaction domain. N-terminally deleted forms of Cdc53 that cannot bind Skp1 can still bind Cdc34 and mammalian Hrt1, and a *cdc53* mutant (*cdc53-1*) with a lesion in the

CH domain specifically disrupts Cdc53-Cdc34 interactions while still retaining the ability to bind Skp1 (Ohta et al., 1999; Patton et al., 1998a). Like Cdc34, Hrt1 and its mammalian homologs also bind Cdc53/cullins via the CH domain and have been shown to enhance the E2-cullin binding interaction (Ohta et al., 1999; Skowyra et al., 1999).

Hrt1 and RING-H2 domain proteins. Hrt1 is the newest known member and also the smallest, weighing in at a mere 121 amino acids, over half of which (residues 54-121) comprise the zinc ion-binding RING-H2 domain (Suarin et al., 1996). Its size is deceptive, however, as Hrt1 can bind Cdc53/cullins, Cdc34/E2 enzymes, and Cdc4/F-box proteins (Kamura et al., 1999; Seol et al., 1999; Skowyra et al., 1999). Database searches indicate a wide potential array of Hrt1 homologs across all eukaryotes. However, these homology searches identify the common RING-H2 domain, and it is not yet conclusively demonstrated what fraction of these proteins do indeed function as ubiquitin ligase subunits. The APC contains a RING-H2 protein, Apc11, that can bind the cullin homology subunit Apc2 (Zachariae et al., 1998). Furthermore, the human Hrt1 ortholog, Rbx1/Roc1, interacts with all known cullins as demonstrated by two-hybrid assays and direct immunoprecipitations (Ohta et al., 1999). The binding of Roc1 to cullin is extremely strong, resisting disruption even by the denaturing detergent SDS (Ohta et al., 1999). These data suggest that a common structural feature of cullins is to recruit RING-H2 proteins as well as E2 enzymes. The tight interaction between Hrt1 and cullin, the small size of Hrt1, and the high homologies between Hrt1 relatives explain why initial in vitro reconstitution assays (Chapter 2) did not indicate a requirement for Hrt1 (Feldman et al., 1997). Most likely, a source of Hrt1 activity co-purified with Cdc53 expressed in insect cells and replaced bona fide Hrt1, permitting the ubiquitination of Sic1 (but not Cln2) (see Chapter 3).

The Cdc53/Hrt1 Ubiquitin Ligase Operates by a Novel Mechanism

Several key pieces of evidence implicate Cdc53 plus Hrt1 as the core ubiquitin ligase machine. First, Cdc53/Hrt1 potently stimulate the autoubiquitination activity of the E2 enzyme Cdc34 (Seol et al., 1999) (see Chapter 3). Cdc53 associates tightly with Hrt1, as do their nearest cousins in yeast, the ubiquitin ligase APC subunits Apc2 and Apc11 respectively. Significantly, Cdc53/Hrt1 (as representatives of cullins plus RING-H2 protein-based ligases) appear to function by a novel mechanism, in comparison with the HECT-domain family of molecules typified by E6-AP. As discussed previously, E6-AP contains an essential active cysteine residue and forms a transient thiolester with ubiquitin, an obligate step in the transfer of ubiquitin from E2 to substrate. Data presented in Chapter 3 (Seol et al., 1999) and other reports strongly argue that SCF operates by a different mechanism. First, there are no essential cysteine residues present in either Skp1 or Cdc53; every cysteine can be eliminated without effect (Patton et al., 1998a). Hrt1 has 9 cysteines, but 5 of these are predicted to participate in the RING-H2 metal coordination domain. Mutating these cysteines inactivates Hrt1 but still permits cullin binding (Kamura et al., 1999; Ohta et al., 1999). The authors of these papers argue that these results predict an essential, thiolester-generating role for these cysteines, but they do not inspect the ability of these mutants to independently interact with other SCF components or E2 enzyme, the likely cause of the defect. In contrast, three other highly conserved cysteines in Hrt1 that are not part of the RING-H2 domain are not essential for viability (Kamura et al., 1999; Skowyra et al., 1999). Second, SCF activity and Cdc53/Hrt1 activity in particular are resistant to treatment with high levels of the cysteine-alkylating agent N-ethyl maleimide (NEM), in contrast with the exquisite sensitivity of E1 and E2 to NEM treatment. Third, polycations such as polylysine can promote the autoubiquitination of Cdc34 and in the case of Gcn4, can serve as an E3, replacing SCF complexes to promote ubiquitination (Seol et al., 1999).

Cdc53/Hrt1 are though to act by (1) stabilizing the reactive intermediate formed during ubiquitin transfer, and (2) stimulating the activity of the E2 enzyme by causing a conformational change. Transfer of ubiquitin from a reactive thiolester bond to stable isopeptide linkage is predicted to occur via a reactive oxyanion intermediate. A cluster of positive charges provided by SCF may stabilize the reactive intermediate and facilitate ubiquitin transfer; a tantalizing cluster of six lysine residues is found in the N-terminal portion of Hrt1, perhaps filling the role of positive charge providers. In addition, Cdc34 likely undergoes an activating conformational change as a result of binding SCF. In support of this, a mutant form of Cdc34 that lacks its normal autoubiquitination acceptor lysines is able to autoubiquitinate in the presence of Cdc53/Hrt1, utilizing heretofore cryptic lysines. An allosteric activation mechanism would ensure that only those Cdc34 molecules properly bound to SCF would be functional and thereby restrain the large cellular pool of Cdc34 in excess of SCF. Interestingly, a growing list of known or potential ubiquitin ligases contains a RING-H2 domain, including Ubr1 (Kwon et al., 1998), Hrd1/Der3 (Bordallo et al., 1998; Hampton et al., 1996), SINA (Hu and Fearon, 1999), Rad18 (Bailly et al., 1997), and Mdm2 (Honda et al., 1997). Whether these function analagously to Hrt1 remains a subject of investigation.

The F-box Hypothesis

The initial biochemical characterization of SCF focused on defining the components required to ubiquitinate Sic1. Simultaneously, a parallel effort focused on the in vitro ubiquitination of the G1 cyclin Cln2. Cln2 was already known to be degraded in a Cdc34-dependent manner (Deshaies et al., 1995) and this degradation is preceded by its autophosphorylation carried out by active Cln/Cdc28 complexes (Lanker et al., 1996). The requirement for phosphorylation makes Clns inherently unstable. In contrast to Sic1

whose stability depends on Cdc4, initial genetic experiments suggested that Cln stability depends on the products of the *GRR1* gene, previously known to be involved in glucose repression (Barral et al., 1995). Skowyra et al. (1997) first demonstrated that an SCF complex containing Grr1 in lieu of Cdc4 could bind Cln; the in vitro reconstitution of Cln ubiquitination followed the discovery of Hrt1 (Seol et al., 1999; Skowyra et al., 1999). The activity imparted by Grr1 is substrate specific: SCF complexes containing Grr1 cannot bind or ubiquitinate Sic1 and SCF complexes containing Cdc4 cannot recognize Cln2 (Skowyra et al., 1997). Just as Cdc4 as multiple substrates, Grr1 also mediates the stability of two proteins involved in initiating polarized bud growth, Gic1 and Gic2 (Jacquenoud et al., 1998).

Although largely dissimilar over their entire lengths, Cdc4 and Grr1 do share one common region: the F box. Since both Cln and Sic1 ubiquitination require Skp1, Cdc53, Hrt1, and Cdc34, it was proposed that Skp1 serves as an adapter, linking a core ubiquitin ligase (Cdc53 plus Hrt1) to F-box proteins that act as interchangeable receptor modules responsible for recruiting specific substrates (Bai et al., 1996). This idea has been called the "F-box hypothesis". Since the F-box protein is the inherently variable part of SCF, the nomenclature of SCF designates the identity of the F-box unit (e.g., SCF^{Cdc4}, SCF^{Grr1}). The F-box hypothesis predicts that the F-box protein contains a protein-protein interaction domain capable of recruiting substrates. As discussed previously, the WD-40 domain of Cdc4 fulfills this role (Neer et al., 1994). Grr1 lacks WD-40 domains but possesses instead a region of leucine-rich repeats (LRR) that binds substrates, targeting them for ubiquitination (Kobe and Deisenhofer, 1994).

A third budding yeast F-box protein, Met30, has been characterized and shown to possess a WD-40 domain similar to that of Cdc4. Met30 plays a role in methionine biosynthesis, probably by regulating the transcriptional activator Met4 (Kuras et al., 1996; Patton et al., 1998a), and has been shown to mediate the stability of the cell cycle checkpoint kinase Swe1(Kaiser et al., 1998). Although Swe1 is not essential in budding

yeast, it plays a more prominent role in the cell cycle of other eukaryotes. Degradation of Xenopus Wee1 in oocyte extracts is essential for cell cycle progression, suggesting that a homolog of SCF^{Met30} may play a key role in this reaction (see below).

Regulation of F-box Protein Activity

A recent flurry of interest surrounding F-box proteins and their substrates has led to some conclusions about the general behavior of F-box proteins. The ability of F-box proteins to recognize and substrate appears to hinge on two critical factors: the phosphorylation state of the substrate, and the abundance of the F-box protein.

That Sic1's phosphorylation is a prerequisite for its binding Cdc4 and its subsequent ubiquitination was firmly established by elegant biochemical studies from Verma et al. (1997b). As mentioned previously, all other known substrates of Cdc4—Far1, Gcn4, Cdc6—must be phosphorylated before they can be targeted by Cdc4. The human homolog of Ccd4, β-TrCP, binds the substrate IκB only when IκB is phosphorylated. Furthermore, phosphorylated peptides that span a binding epitope of IκB--but not their unphosphorylated cousins--can compete with full-length IκB for binding to β-TrCP (Winston et al., 1999; Yaron et al., 1998). What's true for Cdc4 seems to be true for other F-box proteins as well; the LRR domain of Grr1 also recognizes only phosphorylated Sic1 (Skowyra et al., 1997). The F-box hypothesis predicts that other protein-protein interaction domains identified in F-box proteins, such as the tetratricopeptide repeat (TPR) domains, will likewise bind exclusively phosphorylated substrates.

Using phosphorylation as a prerequisite for F-box protein binding is an excellent regulatory mechanism since it provides an additional layer of complexity in the SCF pathway. For example, cells can potentially employ different kinases to phosphorylate substrate and these kinases can themselves by regulated temporally and spatially within the cell to control the targeting of potential SCF substrates. As mentioned previously, Sic1 is initially phosphorylated in late G1 by Cln/Cdc28 but can also be phosphorylated by Clb/Cdc28 complexes (Feldman et al., 1997). This arrangement ensures first that Cln/Cdc28 levels be sufficiently high to have executed G1 functions before targeting Sic1, and then allows then newly liberated Clb/Cdc28 complexes to accelerate the G1-S transition by a positive feedback mechanism. Another Cdc4 substrate, Cdc6, is targeted by Clb/Cdc28 kinase complexes, while a third substrate, Gcn4, requires phosphorylation by a still-undefined kinase (Y. Chi, personal communication). Likewise, the Cln proteins destined for ubiquitination by Grr1 are autophosphorylated by their Cdc28 binding partner, while the Grr1 substrate Gic2 appears to require the binding of active Cdc42 to promote its phosphorylaion (Jacquenoud et al., 1998).

The relative abundance of F-box proteins also plays a critical role in regulating overall SCF activity. Several experiments suggest that F-box proteins compete for a limiting amount of core ubiquitin ligase machinery (Li and Johnston, 1997; Mathias et al., 1996; Patton et al., 1998a). Interestingly, the short constitutive half-lives of F-box proteins so far surveyed may contribute to the diversity of SCF complexes available in vivo. Cdc4, Grr1, and Met30 are unstable throughout the entire cell cycle and are stabilized by *cdc53*, *cdc34*, *skp1*, and proteasome mutants (Galan and Peter, 1999; Mathias et al., 1999; Zhou and Howley, 1998). In contrast, Skp1, Cdc53, and Cdc34 are stable throughout the entire cell cycle (JH Seol, personal communication). Furthermore, Cdc4 is not known to undergo any additional post-translational modifications, and Cdc4 produced in E. coli is competent to drive the ubiquitination of Sic1 (Feldman et al., 1997). The short half-lives of F-box protein are important for two reasons. First, they allow the cell to

constantly adjust its SCF repertoire to match the current degradation needs at any given moment. Second, they permit a degree of sloppiness in the system: a cell need not closely regulate how much of each F-box protein it produces, since excess receptors are quickly degraded and eliminated. This argument assumes that primarily empty or idle F-box receptors are degraded, as opposed to those occupied with substrate; proof of this point awaits experimental validation.

Diversity of SCF Function

Control of the G1-S transition. Our ever-increasing knowledge of SCF's subunits and substrates reveals that SCF complexes participate in a wide array of cellular processes. Given that over 400 F-box proteins are now known, including over 100 in C. elegans, elegans, it is likely that SCF complexes will regulate related processes in all eukaryotes. For example, control of the G1-S transition is a key task of SCF^{Cdc4} complexes. In addition to budding yeast, the fission yeast S. pombe most likely employs an SCF complex to degrade the cell cycle inhibitor Rum1 and the replication origin component Cdc18, a homolog of the budding yeast SCF^{Cdc4} substrate Cdc6 (Kominami and Toda, 1997; Kominami et al., 1998). Phosphorylation-dependent recognition of Rum1 and Cdc18 is mediated by a heterodimer of the F-box/WD-40 proteins Pop1 and Pop2, which together function as the receptor module of an as-yet undefined SCF complex. The G1-S transition is likely controlled by SCF in other organisms as well. In Xenopus oocyte extracts, a Cdc34 homolog is required to initiate DNA synthesis, most likely because of the requirement to degrade Wee1 (Michael and Newport, 1998). The H. sapiens SCF^{Skp2} can bind and ubiquitinate several key cell cycle targets, including the transcriptional activator E2F1 (Marti et al., 1999), cyclins D1 and E, and the CDK inhibitors p21 and p27 (Pagano et al., 1995; Yu et al., 1998).

Other roles for SCF. Work published within the past two years has uncovered exciting cellular roles for SCF complexes, particularly those SCFs containing a Cdc4 homolog. Particularly noteworthy is the reconstitution of IκB ubiquitination (Winston et al., 1999; Yaron et al., 1998). The inducible degradation of IκB and concomitant liberation of the transcriptional activator NFκB is a critical pathway by which cells respond to a variety of stresses. Two avenues of work recently converged to reveal the kinase complex responsible for phosphorylating IκB as well as the SCF that promotes its subsequent ubiquitination. The F-box protein responsible--β-TrCP or E3RS-- is homologous to Cdc4, with seven tandem WD-40 repeats that have been shown to recruit IκB as well as the substrates CD4 and β-catenin (Fuchs et al., 1999; Hart et al., 1999; Margottin et al., 1998; Yaron et al., 1998). Drosophila exhibits a parallel use of SCF, as the F-box/WD-40 protein slimb mediates the stability of the transcription factor Armadillo, a homolog of mammalian β-catenin (Jiang and Struhl, 1998). In comparison to SCF^{Cdc4}, however, $SCF^{\beta\text{-}TrCP}$ utilizes a different subset of the available E2 enzymes-- ubiquitination of IκB occurs via one of three related hUbc5 enzymes (Spencer et al., 1999; Yaron et al., 1998). This suggests that the full repertoire of E2—SCF combinations has not yet been completely elucidated. In support of this, regulation of glucose-repressible genes by Grr1 is not Cdc34-dependent (Li and Johnston, 1997). Exactly what determines E2—SCF specificity and utilization remains an unresolved question.

The roles of SCF complexes in other biological pathways are still being elucidated. SCF^{Met30} regulates methionine synthesis in budding yeast and appears to do the same in Neurospora and Aspergillus via the F-box proteins Scon2 and SconB, respectively (Kumar

and Paietta, 1998; Natorff et al., 1998). In Arabadopsis, SCF complexes have been shown to regulate the auxin response (Ruegger et al., 1998) and tissue patterning (Lee et al., 1997). Drosophila also most likely use SCF to regulate tissue patterning: as mentioned previously, the downstream transcriptional activators of the Hedgehog/Wingless pathway, Cubitus interruptus and Armadillo, are regulated by the F-box protein slimb, and cell fate specification in the developing eye is regulated by another F-box protein, ebi (Dong et al., 1999). Likewise, in C. elegans the F-box protein sel-10 regulates cell fate by controlling the level of the Notch receptor family member *lin-12* (Hubbard et al., 1997). Furthermore, the first metazoan cullin protein, Cul-1, was identified in C. elegans as a mutant exhibiting excess cell proliferation in all tissues (Kipreos et al., 1996). An inability of *cul-1* mutant cells to degrade a positive-acting factor like cyclin might explain this hyperplastic phenotype, but this remains to be tested directly. Finally, Bai et al. (1996) point out the relatively large number of F-box protein encoded by viruses. The ability of human papillomavirus to commandeer the cellular ligase component E6-AP and thereby eliminate the genomic guardian p53 has already been discussed (see above). The human immunodeficieny virus (HIV) provides another example of a virus subverting the genuine role of an SCF complex and redirecting it to nefarious ends. Once it is phosphorylated, the HIV Vpu protein can interact with β-TrCP, causing it to bind and degrade the T-helper cell receptor CD4 (Margottin et al., 1998). No doubt ongoing research will continue to elucidate the devious means viruses employ to redirect the efforts of cellular SCF complexes.

Future Directions

Important issues and challenges await the eager researcher exploring SCF complexes. The mechanistic underpinnings of SCF remain shrouded in mystery; six key issues present themselves. First, how is ubiquitin transferred from E2 to substrate? Does

this reaction indeed involve a conformational change in E2, and is such an allosteric activation gated by the presence of substrate bound to the F-box subunit? Second, how does a lengthy multiubiquitin chain form? The site of subsequent ubiquitin additions (the "growing end") of a rapidly lengthening chain would be expected to be rapidly out of range of a spatially restricted E2 enzyme. Either some factor keeps the growing end of a ubiquitin chain tethered near the E2 enzyme's active site, or the E2 enzyme has a greater degree of spatial freedom or ability to migrate along a growing chain than is currently understood. Work on other ubiquitin ligases has uncovered a so-called "E4" activity thought to be responsible for promoting chain elongation (Koegl et al., 1999) although its mechanism of action is not yet well characterized. Third, do cullin plus RING-H2 subunits act as a general E2 stimulatory domain in all SCF complexes? RING-H2 domains have been identified in a wide array of proteins, including some not thought to be involved in ubiquitin-mediated proteolysis, such as caspase inhibitors and the breast cancer gene BRCA1. Furthermore, what determines the specificity of the cullin/RING-H2—E2 interaction? Can different conjugating enzymes be utilized depending on the associated F-box protein, as may be the case for SCF^{Cdc4} versus SCF^{Grr1}? Fourth, can a consensus substrate recognition sequence or "SCF-recognin" be defined? How unrelated protein-protein interaction domains present in F-box proteins identify and bind their corresponding substrates is an open question whose solution may await the solution of the crystal structure of an F-box protein. Fifth, can the activity of SCF complexes be regulated, particularly in response to cellular signals? Since Sic1 is not absolutely essential in unchallenged cells, evolution must have some reason to conserve Sic1 and the pathways needed to eliminate it. Haploid yeast cells exhibit a *RAD9*-dependent DNA-damage checkpoint in G1 phase at or immediately after the genetically-defined *CDC4* step (Siede et al., 1994; Siede et al., 1993). This may reflect the inhibition of SCF activity by the damage checkpoint, thereby stabilizing Sic1 and allowing cells time to repair chromosomal damage. Intriguingly, the APC is known to be regulated a mitotic spindle checkpoint. The

APC's activity can be inhibited by direct binding of Mad2 to the F-box protein Cdc20 (Hwang et al., 1998; Kim et al., 1998). Cdc20 acts analagously to Cdc4, controling turnover of the anaphase inhibitor Pds1 (Visintin et al., 1997) and inhibition of Pds1 destruction would allow the cell time to correct errors in the mitotic spindle before catastrophic missegregation. Sixth, do SCF complexes play additional roles in promoting the destruction of substrate? Although not directly tested in our current in vitro assay system, SCF may be involved in protecting a ubiquitinated substrate from attack by ubiquitin hydrolases, or in delivering a ubiquitinated substrate to the proteasome. Current data suggest that Sic1 bound to SCF^{Cdc4} can tightly associate with the proteasome and may reside there until Sic1 has been eliminated (R. Verma, personal communication).

In parallel with these mechanistic questions, much work remains to define the laundry list of SCF substrates and the F-box proteins responsible for their turnover. Currently, no systematic means exists to identify substrates; perhaps clever genetic screens or a novel pharmacological agent will speed up the search. Because of the high specificity of F-box proteins for binding substrates, they are attractive targets for therapeutic intervention, in contrast to the pleiotropically-acting components of the core ubiquitin ligase machinery. Given the rapid pace of and intense excitement surrounding the SCF field, answers to the questions highlighted above should be forthcoming.

References

Bachmair, A., Finley, D., and Varshavsky, A. (1986). In vivo half-life of a protein is a function of its amino-terminal. Science *234*, 179-86.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., and Elledge, S. J. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell *86*, 263-274.

Bailly, V., Lauder, S., Prakash, S., and Prakash, L. (1997). Yeast DNA repair proteins Rad6 and Rad18 form a heterodimer that has ubiquitin conjugating, DNA binding, and ATP hydrolytic activities. J. Biol. Chem. *272*, 23360-5.

Banerjee, A., Gregori, L., Xu, Y., and Chau, V. (1993). The bacterially expressed yeast *CDC34* gene product can undergo autoubiquitination to form a multiubiquitin chain-linked protein. Journal of Biological Chemistry *268*, 5668-5675.

Barral, Y., Jentsch, S., and Mann, C. (1995). G(1) cyclin turnover and nutrient-uptake are controlled by a common pathway in yeast. Genes & Development *9*, 399-409.

Bartel, B., Wunning, I., and Varshavsky, A. (1990). The recognition component of the N-end rule pathway. EMBO J. *9*, 3179-3189.

Bordallo, J., Plemper, R. K., Finger, A., and Wolf, D. H. (1998). Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. Mol Biol Cell *9*, 209-22.

Ciechanover, A., Elias, S., Heller, H., and Hershko, A. (1982). "Covalent affinity" purification of ubiquitin-activating enzyme. J. Biol. Chem *257*, 2537-2542.

Ciechanover, A., Hod, Y., and Hershko, A. (1978). A heat-stable polypeptide component of an AP-dependent proteolytic system from reticulocytes. Biochem. Biophys. Res. Comm. *81*, 1100-1105.

Connelly, C., and Hieter, P. (1996). Budding yeast SKP1 encodes an evolutionary conserved kinetochore protein required for cell cycle progression. Cell *86*, 275-285.

Deshaies, R. J. (1995). The self-destructive personality of a cell cycle in transition. Current Opinion in Cell Biology *7*, 781-789.

Deshaies, R. J., Chau, V., and Kirschner, M. (1995). Ubiquitination of the G1 cyclin Cln2p by a Cdc34p-dependent pathway. EMBO J. 14, 303-312.

Deshaies, R. J., and Kirschner, M. (1995). Reconstitution of p34CDC28 activity with G1 and mitotic cyclins. Proc. Natl. Acad. Sci USA *92*, 1182-1186.

Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (1994). A 26S protease subunit that binds ubiquitin conjugates. Journal of Biological Chemistry *269*, 7059-7061.

Dong, X. Z., Tsuda, L., Zavitz, K. H., Lin, M., Li, S. H., Carthew, R. W., and Zipursky, S. L. (1999). ebi regulates epidermal growth factor receptor signaling pathways in Drosophila. Genes Dev. *13*, 954-965.

Donovan, J. D., Toyn, J. H., Johnson, A. L., and Johnston, L. H. (1994). P40SDB25, a putative CDK inhibitor, has a role in the M/G1 transition in Saccharomyces cerevisiae. Genes Dev *8*, 1640-53.

Drury, L. S., Perkins, G., and Diffley, J. F. (1997). The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. Embo J *16*, 5966-76.

Feldman, R., Correll, C., Kaplan, K., and Deshaies, R. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell *91*, 221-230.

Finley, D., Ciechanover, A., and Varshavsky, A. (1984). Thermolability of ubiquitin-activating enzyme from the mammalian cell cycle mutant ts85. Cell *37*, 43-55.

Fuchs, S., Chen, A., Xiong, Y., Pan, Z., and Ronai, Z. (1999). HOS, a human homolog of Slimb, forms an SCF complex with Skp1 and Cullin1 and targets the phosphorylation-dependent degradation of IκB and β-catenin. Oncogene *18*, 2039-2046.

Galan, J., and Peter, M. (1999). Ubiquitin dependent degradation of multiple F-box proteins by an autocatalytic mechanism. Proc. Natl. Acad. Sci. USA *96*, 9124-9129.

Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991). Cyclin is degraded by the ubiquitin pathway. Nature *349*, 132-138.

Goebl, M. G., Goetsch, L., and Byers, B. (1994). The Ubc3 (Cdc34) ubiquitin-conjugating enzyme is ubiquitinated and phosphorylated in vivo. Molecular and Cellular Biology *14*, 3022-3029.

Goebl, M. J., Yochem, J., Jentsch, S., McGrath, J. P., Varshavsky, A., and Byers, B. (1988). The yeast cell cycle gene cdc34 encodes a ubiquitin-conjugating enzyme. Science *241*, 1331-1334.

Goldknopf, I. L., and Busch, H. (1977). Isopeptide linkage between nonhistone and histone 2A polypeptides of chromosomal conjugate protein A24. Proc. Natl. Acad. Sci. USA *74*, 864-868.

Goldstein, G., Scheid, M., Hammerling, U., Boyse, E. A., Schlesinger, D. H., and Niall, H. D. (1975). Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. Proc. Natl. Acad. Sci. USA *72*, 11-15.

Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtler, M., Bartunik, H. D., and Huber, R. (1997). Structure of 20S proteasome from yeast at 2.4 A resolution Nature *386*, 463-71.

Haas, A. L., Reback, P. B., and Chau, V. (1991). Ubiquitin conjugation by the yeast RAD6 and CDC34 gene products. Comparison to their putative rabbit homologs, E2(20K) AND E2(32K). J Biol Chem *266*, 5104-12.

Hampton, R. Y., Gardner, R. G., and Rine, J. (1996). Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3- methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. Mol Biol Cell *7*, 2029-44.

Hart, M., Concordet, J.-P., Lassot, I., Albert, I., del los Santos, R., Durand, H., Perret, C., Rubinfeld, B., Margottin, F., Benarous, R., and Polakis, P. (1999). The F-box protein β-TrCP associates with phosphorylated β-catenin and regulates its activity in the cell. Curr Biol *in press*.

Henchoz, S., Chi, Y., Catarin, B., Herskowitz, I., Deshaies, R. J., and Peter, M. (1997). Phosphorylation- and ubiquitin-dependent degradation of the cyclin-dependent kinase inhibitor Far1p in budding yeast. Genes Dev *11*, 3046-60.

Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983). Components of ubiquitin-protein ligase system: resolution, affinity purification, and role in protein breakdown. J Biol Chem *258*.

Hochstrasser, M. (1996). Ubiquitin-dependent protein degradation. Annual Review of Genetics *30*, 405-439.

Honda, R., Tanaka, H., and Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett *420*, 25-27.

Hu, G., and Fearon, E. R. (1999). Siah-1 N-terminal RING domain is required for proteolysis function, and C-terminal sequences regulate oligomerization and binding to target proteins. Mol Cell Biol *19*, 724-32.

Hubbard, E., Wu, G., Kitajewski, J., and Greenwald, I. (1997). sel-10, a negative regulator of lin-12 activity in Caenorhabditis elegans, encodes a member of the CDC4 family of proteins. Genes Dev *11*, 3182-3193.

Jacquenoud, M., Gulli, M.-P., Peter, K., and Peter, M. (1998). The Cdc42p effector Gic2p is targeted for ubiquitin-dependent degradation by the SCFGrr1 complex. EMBO J *17*, 5360-5373.

Jentsch, S. (1992). The ubiquitin-conjugation system. Annu. Rev. Genet. *26*, 179-207.

Jentsch, S., and Schlenker, S. (1995). Selective protein degradation: a journey's end within the proteasome. Cell *82*, 881-884.

Johnson, S. L. (1991). Structure and function analysis of the yeast CDC4 gene product: PhD thesis, University of Washington.

Kaiser, P., Sia, R. A., Bardes, E. G., Lew, D. J., and Reed, S. I. (1998). Cdc34 and the F-box protein Met30 are required for degradation of the Cdk-inhibitory kinase Swe1. Genes Dev *12*, 2587-97.

Kamura, T., Koepp, D. M., Conrad, M. N., Skowyra, D., Moreland, R. J., Iliopoulos, O., Lane, W. S., Kaelin, W. G., Jr., Elledge, S. J., Conaway, R. C., Harper, J. W., and Conaway, J. W. (1999). Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science *284*, 657-61.

Kipreos, E. T., Lander, L. E., Wing, J. P., He, W. W., and Hedgecock, E. M. (1996). cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family. Cell *85*, 1-20.

Kobe, B., and Deisenhofer, J. (1994). Trends Biochem. Sci. *19*, 415-421.

Kolman, C. J., Toth, J., and Gonda, D. K. (1992). Identification of a portable determinant of cell cycle function within the carboxyl-terminal domain of the yeast CDC34 (UBC3) ubiquitin conjugating (E2) enzyme. EMBO J *11*, 3081-90.

Kominami, K., and Toda, T. (1997). Fission yeast WD-repeat protein Pop1 regulates genome ploidy through ubiquitin-proteasome-mediated degradation of the CDK inhibitor Rum1 and the S-phase initiator Cdc18. Genes Dev *11*, 1548-60.

Kominami, K.-I., Ochotorena, I., and Toda, T. (1998). Two F-box/WD-repeat proteins Pop1 and Pop2 form hetero-and homo-complexes together with cullin-1 in the fission yeast SCF (Skp1-Cullin-1-F-box) ubiquitin ligase. Genes Cells *3*, 721-735.

Kumar, A., and Paietta, J. V. (1998). An additional role for the F-box motif: gene regulation within the Neurospora crassa sulfur control network. Proc Natl Acad Sci USA *95*, 2417-22.

Kuras, L., Cherest, H., Surdin-Kerjan, Y., and Thomas, D. (1996). EMBO J. *15*, 2519-2529.

Kwon, Y. T., Reiss, Y., Fried, V. A., Hershko, A., Yoon, J. K., Gonda, D. K., Sangan, P., Copeland, N. G., Jenkins, N. A., and Varshavsky, A. (1998). The mouse and human genes encoding the recognition component of the N- end rule pathway. Proc Natl Acad Sci USA *95*, 7898-903.

Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996). The 2.0 Å crystal structure of a heterotrimeric G protein. Nature *379*, 311-319.

Lammer, D., Mathias, N., Laplaza, J. M., Jiang, W., Liu, Y., and al., e. (1998). Modification of yeast Cdc53p by the ubiquitin-related protein Rub1p affects function of the SCFCdc4 complex. Genes Dev. *12*, 914-926.

Lanker, S., Valdivieso, M. H., and Wittenberg, C. (1996). Rapid degradation of the G1 cyclin Cln2 induced by CDK-dependent phosphorylation. Science *271*, 1597-1601.

Lee, I., Wolfe, D., Nilsson, O., and Weigel, D. (1997). A LEAFY co-regulator encoded by UNUSUAL FLORAL ORGANS. Curr Biol *7*, 95-104.

Li, F. N., and Johnston, M. (1997). Grr1 of Saccharomyces cerevisiae is connected to the ubiquitin proteolysis machinery through Skp1: coupling glucose sensing to gene expression and the cell cycle. Embo J *16*, 5629-38.

Lisztwan, J., Marti, A., Sutterluty, H., Gstaiger, M., Wirbelauer, C., and Krek, W. (1998). Association of human CUL-1 and ubiquitin-conjugating enzyme CDC34 with the F-box protein p45(SKP2): evidence for evolutionary conservation in the subunit composition of the CDC34-SCF pathway. EMBO J. *17*, 368-383.

Lyapina, S., Correll, C., Kipreos, E., and Deshaies, R. (1998). Human CUL1 forms an evolutionarily conserved ubiquitin ligase complex (SCF) with SKP1 and an F-box protein. Proc. Natl. Acad. Sci. USA *95*, 7451-7456.

Madura, K., Dohmen, R. J., and Varshavsky, A. (1993). N-recognin/Ubc2 interactions in the N-end rule pathway. Journal of Biological Chemistry *268*, 12046-12054.

Margottin, F., Bour, S., Durand, H., Selig, L., Benichou, S., Richard, V., Thomas, D., Strebel, K., and Benarous, R. (1998). A novel human WD protein, h-beta TrCp, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif. Mol. Cell *1*, 565-574.

Marti, A., Wirbelauer, C., Scheffner, M., and Krek, W. (1999). SCFSKP2 ubiquitin ligase/E2F-1 transcription factor complex formation underlies regulation of E2F-1 protein stability and S phase promoting function. Nature Cell Biol *in press*.

Mathias, N., Johnson, S., Byers, B., and Goebl, M. (1999). The abundance of cell cycle regulatory protein Cdc4p is controlled by interactions between its F box and Skp1p. Mol. Cell. Biol. *19*, 1759-67.

Mathias, N., Johnson, S. L., Winey, M., Adams, A. E., Goetsch, L., Pringle, J. R., Byers, B., and Goebl, M. G. (1996). Cdc53 acts in concert with Cdc4 and Cdc34 to control the G1 to S phase transition and identifies a conserved family of proteins. Molecular and Cellular Biology *16*, 6634-6643.

Mathias, N., Steussy, C. N., and Goebl, M. G. (1998). An essential domain within Cdc34p is required for binding to a complex containing Cdc4p and Cdc53p in Saccharomyces cerevisiae. J Biol Chem *273*, 4040-5.

Mendenhall, M. D. (1993). An inhibitor of p34^{CDC28} protein kinase activity from *Saccharomyces cerevisiae*. Science *259*, 216-219.

Michael, W. M., and Newport, J. (1998). Coupling of mitosis to the completion of S phase through Cdc34-mediated degradation of Wee1. Science *282*, 1886-9.

Michel, J. J., and Xiong, Y. (1998). Human CUL-1, but not other cullin family members, selectively interacts with SKP1 to form a complex with SKP2 and cyclin A. Cell Growth Differ *9*, 435-49.

Natorff, R., Piotrowska, M., and Paszewski, A. (1998). The Aspergillus nidulans sulphur regulatory gene sconB encodes a protein with WD40 repeats and an F-box. Mol Gen Genet *257*, 255-63.

Neer, E. J., Schmidt, C. J., Nambudripad, R., and Smith, T. F. (1994). The ancient regulatory-protein family of WD-repeat proteins. Nature *371*, 297-300.

Ng, R. W., Arooz, T., Yam, C. H., Chan, I. W., Lau, A. W., and Poon, R. Y. (1998). Characterization of the cullin and F-box protein partner Skp1. FEBS Lett *438*, 183-9.

Nugroho, T. T., and Mendenhall, M. D. (1994). An inhibitor of yeast cyclin-dependent protein kinase plays an important role in ensuring the genomic integrity of daughter cells. Molecular and Cellular Biology *14*, 3320-3328.

Ohta, T., Michel, J. J., Schottelius, A. J., and Xiong, Y. (1999). ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. Mol Cell *3*, 535-41.

Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G., and Rolfe, M. (1995). Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science *269*, 682-685.

Papa, F. R., and Hochstrasser, M. (1993). The yeast *DOA4* gene encodes a deubiquitinating enzyme related to a product of the human *tre-2* oncogene. Nature *366*, 313-319.

Patton, E. E., Willems, A. R., Sa, D., Kuras, L., Thomas, D., Craig, K. L., and Tyers, M. (1998a). Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box protein complexes that regulate cell division and methionine biosynthesis in yeast. Genes Dev *12*, 692-705.

Patton, E. E., Willems, A. R., and Tyers, M. (1998b). Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis. Trends Genet *14*, 236-43.

Peterson, T. A., Yochem, J., Byers, B., Nunn, M. F., Duesberg, P. H., Doolittle, R. F., and Reed, S. I. (1984). A relationship between the yeast cell cycle genes *CDC4* and *CDC36* and the ets sequence of oncogenic virus E26. Nature *309*, 556-558.

Ptak, C., Prendergast, J. A., Hodgins, R., Kay, C. M., Chau, V., and Ellison, M. J. (1994). Functional and physical characterization of the cell cycle ubiquitin- conjugating enzyme CDC34 (UBC3). Identification of a functional determinant within the tail that facilitates CDC34 self-association. J Biol Chem *269*, 26539-45.

Ruegger, M., Dewey, E., Gray, W. M., Hobbie, L., Turner, J., and Estelle, M. (1998). The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast Grr1p. Genes Dev *12*, 198-207.

Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell *75*, 495-505.

Scheffner, M., Nuber, U., and Huibregtse, J. M. (1995). Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. Nature *373*, 81-83.

Schneider, B. L., Yang, Q. H., and Futcher, A. B. (1996). Linkage of replication to START by the CDK inhibitor Sic1. Science *272*, 560-562.

Schwob, E., Böhm, T., Mendenhall, M., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor $p40^{SIC1}$ controls the G1/S transition in *Saccharomyces cerevisiae*. Cell *79*, 233-244.

Seol, J., Feldman, R., Zachariae, W., Shevchenko, A., Correll, C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., Shevchenko, A., Nasmyth, K., and Deshaies, R. (1999). Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. Genes Dev *13*, 1614-1626.

Silver, E. T., Gwozd, T. J., Ptak, C., Goebl, M., and Ellison, M. J. (1992). A chimeric ubiquitin conjugating enzyme that combines the cell cycle properties of CDC34 (UBC3) and the DNA repair properties of RAD6 (UBC2): implications for the structure, function and evolution of the E2s. Embo J *11*, 3091-8.

Skowyra, D., Craig, K., Tyers, M., Elledge, S., and Harper, J. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell *91*,.209-219.

Skowyra, D., Koepp, D. M., Kamura, T., Conrad, M. N., Conaway, R. C., Conaway, J. W., Elledge, S. J., and Harper, J. W. (1999). Reconstitution of G1 cyclin ubiquitination with complexes containing SCFGrr1 and rbx1. Science *284*, 662-5.

Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E., and Sigler, P. B. (1996). Crystal structure of a G protein beta-gamma dimer at 2.1 angstrom resolution. Nature *379*, 369-374.

Spencer, E., Jiang, J., and Chen, Z. J. J. (1999). Signal-induced ubiquitination of I kappa B alpha by the F-box protein Slimb/beta-TrCP. Genes Dev *13*, 284-294.

Suarin, A., Borden, K., Boddy, M., and Freemont, P. (1996). Does this have a familiar RING? Trends Biochem Sci *21*, 208-214.

Tan, P., Fuchs, S. Y., Chen, A., Wu, K., Gomez, C., Ronai, Z., and Pan, Z. Q. (1999). Recruitment of a ROC1-CUL1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of I kappa B alpha. Mol Cell *3*, 527-33.

Tyers, M. (1996). The cyclin-dependent kinase inhibitor p40*SIC1* imposes the requirement for *CLN* G1 cyclin function at Start. Proceedings of the National Academy of Sciences of the United States of America *93*, 7772-7776.

van Nocker, S., Sadis, S., Rubin, D. M., Glickman, M., Fu, H., Coux, O., Wefes, I., Finley, D., and Vierstra, R. D. (1996). The multiubiquitin-chain-binding protein Mcb1 is a component of the 26S proteasome in Saccharomyces cerevisiae and plays a nonessential, substrate-specific role in protein turnover. Mol Cell Biol *16*, 6020-8.

Varshavsky, A. (1992). The N-end rule. Cell *69*, 725-735.

Varshavsky, A. (1996). The N-end rule: functions, mysteries, uses. Proc Natl Acad Sci USA *93*, 12142-9.

Verma, R., Annan, R. S., Huddleston, M. J., Carr, S. A., Reynard, G., and Deshaies, R. J. (1997b). Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. Science *278*, 455-60.

Verma, R., Feldman, R., and Deshaies, R. J. (1997a). SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. Mol Biol Cell *8*, 1427-1437.

Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Llhui, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995). The structure of the G protein heterotrimer $G_{i\alpha 1}\beta_1\gamma_2$. Cell *83*, 1047-1058.

Wilkinson, K. D., Urban, M. K., and Haas, A. L. (1980). Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes. J. Biol. Chem. *255*, 7529-7532.

Willems, A. R., Lanker, S., Patton, E. E., Craig, K. L., Nason, T. F., Kobayashi, R., Wittenberg, C., and Tyers, M. (1996). Cdc53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway. Cell *86*, 453-463.

Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999). The SCF beta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in I kappa B alpha and beta-catenin and stimulates I kappa B alpha ubiquitination in vitro. Genes Dev. *13*, 270-283.

Yam, C. H., Ng, R. W. M., Yi Siu, W., Lau, A. W. S., and Poon, R. Y. C. (1999). Regulation of cyclin A-cdk2 by SCF component Skp1 and F-Box protein Skp2. Mol Cell Biol *19*, 635-45.

Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A. M., Andersen, J. S., Mann, M., Mercurio, F., and Ben-Neriah, Y. (1998). Identification of the receptor component of the IκBα-ubiquitin ligase. Nature *396*, 590-4.

Yu, H., Peters, J. M., King, R. W., Page, A. M., Hieter, P., and Kirschner, M. W. (1998). Identification of a cullin homology region in a subunit of the anaphase-promoting complex. Science *279*, 1219-22.

Yu, Z.-K., Gervais, J., and Zhang, H. (1998). Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21CIP1/WAF1 and cyclin D proteins. Proc Natl Acad Sci USA *95*, 11324-29.

Zachariae, W., Shevchenko, A., Andrews, P. D., Ciosk, R., Galova, M., Stark, M. J., Mann, M., and Nasmyth, K. (1998). Mass spectrometric analysis of the anaphase-promoting complex from yeast: identification of a subunit related to cullins. Science *279*, 1216-9.

Zhang, H., Kobayashi, R., Galaktionov, K., and Beach, D. (1995). p19(SKP1) and p45(SKP2) are essential elements of the cyclin A-CDK2 S phase kinase. Cell *82*, 912-925.

Zhou, P., and Howley, P. M. (1998). Ubiquitination and degradation of the substrate recognition subunits of SCF ubiquitin-protein ligases. Mol Cell *2*, 571-80.

Chapter 2. A Complex of Cdc4p, Skp1p, and Cdc53p/Cullin Catalyzes Ubiquitination of the Phosphorylated CDK Inhibitor Sic1p

The work presented in this chapter represents an equal collaborative effort with Craig Correll and includes contributions from Ken Kaplan. Specifically, Craig Correll generated the data shown in Figures 3C, 4, and 5; Ken Kaplan provided all Skp1-related reagents. These experiements have been previously published (Feldman et al., 1997; see Appendix II).

Introduction

A number of cellular processes in both prokaryotes and eukaryotes are controlled by selective proteolysis. Proteolysis in the eukaryotic cytosol typically involves the assembly of a substrate-linked ubiquitin chain, which targets the protein for degradation by the 26S proteasome (Hochstrasser, 1995) . Ubiquitin is first activated at its C-terminus through the formation of a thioester bond with the ubiquitin-activating enzyme, E1. Ubiquitin is subsequently transesterified to a member of a family of ubiquitin conjugating (E2) enzymes. Finally, ubiquitin is transferred from the E2 enzyme to a lysine residue of the target protein, either directly or with the assistance of a ubiquitin ligase (E3). E3 was originally defined as an activity that is both necessary and sufficient for the transfer of ubiquitin to a model substrate in the presence of a ubiquitin-charged E2 enzyme (Hershko et al.,1983). E3s appear to be the primary source of substrate specificity in the ubiquitination cascade, as some E3s have been shown to bind directly to substrate. Recent work suggests that the E6-AP E3 (Huibregtse et al., 1993) also forms a thioester with ubiquitin as an intermediate in the transfer of ubiquitin from E2 to substrate (Scheffner et al., 1995). Since E3s do not possess detectable sequence homology, and since few have been characterized mechanistically, it remains to be seen whether all physiological ubiquitination reactions require E3s, and whether all E3s will participate directly in both substrate recruitment and ubiquitin transfer.

Entry into S phase in budding yeast requires the activity of S phase-promoting Clb/Cdc28 protein kinase complexes (Schwob et al., 1994). Although these complexes are assembled during G1 phase, they are initially inactive due to the presence of high levels of the Clb/Cdc28 inhibitor Sic1 (Nugroho and Mendenhall, 1994; Schwob et al., 1994). At the G1/S transition, Sic1 is abruptly degraded and the liberated Clb/Cdc28 complexes trigger DNA synthesis. *CLN1-3*, *CDC4*, *CDC34*, *CDC53*, and *SKP1* are required for the timely disappearance of Sic1 in vivo (Schwob et al., 1994; Bai et al., 1996; Schneider et al., 1996). Since *CDC34* encodes an E2 enzyme, it was proposed that Sic1 degradation is mediated by ubiquitin-dependent proteolysis (Schwob et al., 1994). Indeed, Sic1 is efficiently ubiquitinated in yeast extract, and Cdc34, Cdc4, and Cln/Cdc28 are required for this process (Verma et al., 1997b). Biochemical and genetic experiments have revealed that the main role of Cln/Cdc28 in the G1/S transition is to phosphorylate Sic1, which triggers its ubiquitination by the Cdc34 pathway (Verma et al., 1997, Tyers, 1996; Schneider et al., 1996).

Although Cdc4 and Cdc34 have been implicated in Sic1 ubiquitination in yeast extract, it is not known whether Skp1 and Cdc53 are also directly involved in this process. The functional roles of these proteins are of great interest, since Skp1 and Cdc53 are members of multigene families that are conserved from yeast to humans (Kipreos et al., 1995; Zhang et al., 1995; Bai et al., 1996; Mathias et al., 1996). Skp1 and Cdc53 homologs have been implicated in diverse processes, including kinetochore assembly in yeast (Connelly and Hieter, 1996), withdrawal from the cell cycle in nematodes (Kipreos et al, 1995), and both S phase progression (Zhang et al., 1995) and transcription elongation (Pause et al., 1997) in animal cells. Here, we report the reconstitution of Sic1 ubiquitination with a set of purified proteins, including Cdc4, Cdc53 and Skp1. We also show that Cdc4, Cdc53, and Skp1 assemble into an E3 complex that selectively recognizes phosphorylated Sic1 via its Cdc4 and Skp1 subunits. These findings suggest that phosphorylation-triggered ubiquitin-dependent proteolysis may be mediated by a conserved cullin-dependent pathway in eukaryotic cells.

RESULTS

Reconstitution of Sic1 ubiquitination in insect cell lysates

In vitro reconstitution of Sic1 ubiquitination using fractionated yeast extract revealed a direct requirement for Cdc34, Cdc4, and cyclin–dependent kinase (Cdk) activities (Verma et al., 1997b) . However, yeast extract contributed a host of unidentified components, and a defined set of proteins sufficient to sustain Sic1 ubiquitination remained elusive. To define the biochemical requirements for Sic1 ubiquitination, several proteins that had been implicated in Sic1 destruction – including Cln2/Cdc28 complexes (Reynard and Deshaies, submitted) , Cdc53, Cdc4 (Verma et al., 1997b), and Skp1 – were expressed separately in Sf9 insect cells via infection with recombinant baculoviruses. Previously described recombinant expression systems were used to prepare the yeast E1 enzyme ^{His6}Uba1 (Dohmen et al., 1995; Figure 2A, lane 1), the E2 enzyme Cdc34 (Banerjee et al., 1993; see Figure 2A lane 2), and a maltose-binding protein-Sic1 chimera containing a C-terminal bipartite myc epitope-hexahistidine tag (MBP-Sic1^{MH6}) (Verma et al., 1997a) . Purified, metabolically-radiolabeled [^{35}S]-MBP-Sic1^{MH6} is ubiquitinated in fractionated yeast extract similarly to wild-type Sic1 produced by in vitro translation (Verma et al., 1997b). We used monomeric [^{35}S]-MBP-Sic1^{MH6} as a substrate for most of the experiments reported here because previous work indicated that association of Sic1 with Clb5/Cdc28 was not required for its ubiquitination (Verma et al., 1997b).

To test whether this set of recombinant proteins was sufficient to direct ubiquitination of Sic1 in the absence of other yeast proteins, we mixed unfractionated insect cells lysates containing Cdc4, Cdc53, and Skp1 with purified ^{His6}Uba1, Cdc34, ubiquitin, and an ATP-regenerating system. For the direct phosphorylation of Sic1, which is essential for its ubiquitination (Verma et al., 1997), we also added affinity-purified Cln2/Cdc28^{HA}/Cks1 protein kinase complexes to the reaction. In work to be published elsewhere (Reynard and Deshaies, submitted), we demonstrated that

Cln2/Cdc28 protein kinase activity is dependent upon the budding yeast $p13^{suc1}$ homolog Cks1 (Hadwiger et al., 1989). When mixed with the components listed above, [^{35}S]-MBP-$Sic1^{MH6}$ was converted to a ladder of high molecular weight (HMW) species (Figure 1A, lane 8). The HMW MBP-$Sic1^{MH6}$ species generated in this reaction represent the assembly of substrate-linked multiubiquitin chains, since their formation was markedly reduced by the omission of ubiquitin (lane 1; see also Figure 3). Reactions lacking Cdc4, Cdc34, Cdc53, or Skp1 (lanes 3-6) failed to generate HMW ubiquitin conjugates, and instead yielded phosphorylated Sic1. In contrast, Sic1 was neither phosphorylated nor ubiquitinated in reactions lacking Cln2/Cdc28/Cks1 (lane 2). The requirement for Cdk activity could be satisified by adding either Cln2/Cdc28/Cks1 or Clb5/Cdc28 complexes (Figure 1B, lane 1 vs. lane 4). This suggests that newly liberated Clb5/Cdc28 complexes may contribute to the destruction of Sic1 in vivo, thereby sharpening the G1/S transition (Verma et al., 1997b). Surprisingly, HMW derivatives of MBP-$Sic1^{MH6}$ were efficiently generated in the absence of ^{His6}Uba1 (lane 7). This presumably reflects the fact that a conserved Uba1-like activity is most likely present in insect cell lysates, since reactions utilizing purified components demonstrated a direct requirement for ^{His6}Uba1 (see below, Figure 3). Note that the C-terminal 60 amino acids of Cdc53, which are highly conserved among eukaryotic cullins (Kipreos et al, 1995, Mathias et al., 1996), were not required for the ubiquitination-promoting activity of Cdc53 (lane 9).

Although the ubiquitination of Sic1 leads to its destruction and the subsequent release of Clb5/Cdc28 from inhibition, it is unclear if the ubiquitination of Sic1 is sufficient to dislodge it from Clb5/Cdc28. To address this question, the reactions shown in Figure 1B, lanes 1 and 4, were incubated with anti-HA antibody to immunoprecipitate $Cdc28^{HA}$ and associated proteins. As expected, MBP-$Sic1^{MH6}$ did not bind Cln2/$Cdc28^{HA}$/Cks1 (lane 3); in contrast, both unmodified and multiubiquitinated MBP-$Sic1^{MH6}$ were both efficiently co-immunoprecipitated with Clb5/$Cdc28^{HA}$/Cks1(lane 6).

Reconstitution of MBP-Sic1^{MH6} ubiquitination with purified proteins

The experiment shown in Figure 1A clearly established that Cdc4, Cdc53, Skp1, and Cdc34 were required for the ubiquitination of MBP-Sic1^{MH6}. However, these experiments were conducted with unfractionated insect cell lysates that contained E1 activity, ubiquitin, and potentially other, unidentified ubiquitination factors. To define a set of components which are both necessary and sufficient for the ubiquitination of Sic1, we individually expressed and purified the recombinant Cdc4, Cdc53, and Skp1 proteins. Glutathione-S-transferase-Skp1 (GST-Skp1) was expressed in E. coli and purified by glutathione affinity chromatography (Figure 2A, lane 3). ^{PH}Cdc4 (Cdc4 tagged at its N-terminus with a bipartite Polyoma antigen-Hemagglutinin epitope, hereafter denoted as PH), Cdc53^{PH} (Cdc53 tagged with a PH epitope at its C-terminus), and Skp1^{His6} were expressed in Sf9 insect cells via infection with recombinant baculoviruses and affinity purified on either anti-polyoma monoclonal antibody or Ni^{+}-NTA matrices (Figure 2B, lanes 1-3). Cln2/GST-Cdc28^{HA} complexes were produced in Sf9 insect cells via co-infection with recombinant baculoviruses, activated by addition of purified Cks1, and affinity purified on glutathione resin (Figure 2C, lane 2). Purification of GST-Cdc28^{HA} alone from Sf9 cells is shown for comparison (lane 1). Similar results were obtained with Cln2/Cdc28^{HA}/Cks1 complexes purified on an anti–HA monoclonal antibody matrix (data not shown).

The availability of purified proteins allowed us to test whether this set of components was sufficient to direct ubiquitination of MBP-Sic1^{MH6}. Since the principal function of Cdk complexes in this reaction is to phosphorylate Sic1 (Verma et al., 1997), we prepared competent substrate by mixing purified [^{35}S]-MBP-Sic1^{MH6} with ATP and Cln2/GST-Cdc28^{HA}/Cks1 complexes that were bound to glutathione agarose beads. Following a brief incubation, the soluble pool phosphorylated substrate was separated from the beads. When phosphorylated [^{35}S]-MBP-Sic1^{MH6} was mixed with purified ^{PH}Cdc4, Cdc34, Cdc53^{PH}, GST-Skp1, ^{His6}Uba1, ubiquitin and an ATP-regenerating

system, it was extensively ubiquitinated (Figure 3A, lane 8). Substituting ubiquitin with the chain-terminating derivatives methyl–ubiquitin (Hershko and Heller, 1985) or K48R–ubiquitin (Chau et al., 1989; Figure 3B), or omitting ubiquitin from the reaction entirely (Figure 3A, lane 6) dramatically altered the profile of MBP-Sic1^{MH6} modification. These results confirm that the HMW forms observed in Figure 3A, lane 8 correspond to multiubiquitinated MBP-Sic1^{MH6}.

As was observed with crude components (Figure 1A), no ubiquitination was detected when ^{PH}Cdc4, Cdc34, or Cdc53^{PH} were omitted from the reaction (Figure 3A, lanes 2-4), or when unphosphorylated MBP-Sic1^{MH6} was used as substrate (lane 1). In contrast to reactions containing crude lysate, reactions carried out with purified components were absolutely dependent upon ^{His6}Uba1 (lane 7).

Unexpectedly, omitting GST-Skp1 from the reaction mixture did not affect the ubiquitination of MBP-Sic1^{MH6} (Figure 3A, lane 5). This was in marked contrast to the clear requirement for Skp1 seen in the previous experiments using crude insect cell lysates (Figure 1A). Previous studies have identified a specific interaction between Cdc4 and Skp1 that is mediated by a domain in Cdc4 known as the F-box (Bai et al., 1996) Skp1 appears to be conserved in eukaryotes, as homologues have been identified in A. thaliana, C. elegans, and humans (Zhang et al., 1995; Bai et al., 1996). We reasoned that if an insect Skp1-like protein was present, it might efficiently bind to and co-purify with ^{PH}Cdc4, and be present in sufficient quantity to provide a detectable activity in our ubiquitination assay.

To produce Cdc4 devoid of contaminating Skp1 activity, an MBP-Cdc4 fusion was expressed in E. coli and purified by amylose affinity chromatography (Figure 2A, lane 4). Purified MBP-Cdc4 was able to substitute for ^{PH}Cdc4 in a ubiquitination reaction conducted with purified components (Figure 3C, lane 3). However, in contrast to the results obtained with ^{PH}Cdc4 (Figure 3A, lane 5), reactions conducted with MBP-Cdc4 were absolutely dependent upon GST-Skp1 (Figure 3C, lane 2). Since Skp1^{His6} remains

active in promoting ubiquitination even after being heated at 95 °C (data not shown), we hypothesized that any insect Skp1 that co-purified with ^{PH}Cdc4 would dissociate from heat-denatured ^{PH}Cdc4, but would retain its activity. Whereas a heat-treated preparation of ^{PH}Cdc4 no longer possessed Cdc4 activity (data not shown), it partially fulfilled the requirement for Skp1 activity (lane 4). Thus, ^{PH}Cdc4 co-purifies from insect cells with a heat-stable Skp1-like activity that can promote the ubiquitination of Sic1. It remains unclear why this Skp1-like activity failed to sustain MBP-Sic1^{MH6} ubiquitination in crude Sf9 cell lysates in the absence of exogenously added Skp1. Nevertheless, our data suggest that Cdc34, Cdc4, Cdc53, and Skp1, along with ^{His6}Uba1, ubiquitin and ATP, are necessary and sufficient to support efficient multi-ubiquitination of phosphorylated Sic1. Since Cdc4, Cdc53, and Skp1 are required to direct the Cdc34-dependent ubiquitination of phospho-Sic1, these proteins appear to function collectively as an E3.

Phosphorylated MBP-Sic1^{MH6} is Specifically Targeted for Ubiquitination by Interaction with Cdc4/Skp1

In the two ubiquitination pathways that have been best characterized to date – the N-end rule and the E6/E6-AP pathway – substrate selectivity is conferred by a specific interaction between the substrate and an E3 (Bartel et al., 1990; Scheffner et al., 1993). Since Cdc4, Cdc53, and Skp1 collectively serve as an E3, we hypothesized that one of these proteins might interact selectively with phosphorylated Sic1. To test this possibility, we performed co-immunoprecipitation experiments using either unphosphorylated or phosphorylated MBP-Sic1^{MH6} (Figure 2D) as bait. Sf9 cells singly infected with baculoviruses expressing either Cdc4, Cdc53, or Skp1 were metabolically labeled with ^{35}S-amino acids, and lysates derived from the labeled cells (Figure 4A, lanes 1 and 4; Figure 4B, lane 1) were incubated separately with either phosphorylated or unmodified MBP-Sic1^{MH6}, after which MBP-Sic1^{MH6} and associated proteins were recovered with anti-MBP antibodies. This analysis revealed that ^{PH}Cdc4 (Figure 4A, lanes 2, 3), but not Cdc53 (lanes 5 and 6) or Skp1^{HA} (Figure 4B, lanes 2 and 3), selectively bound to phosphorylated MBP-Sic1^{MH6}. Combining Cdc53- and Skp1^{HA}-

containing lysates did not result in enhanced interaction of these components with either form of MBP-Sic1^{MH6} (data not shown). In contrast, when Skp1^{HA} lysate was mixed with ^{PH}Cdc4 lysate, both proteins were selectively co-immunoprecipitated with phosphorylated MBP-Sic1^{MH6}, and the level of co-precipitated ^{PH}Cdc4 was substantially increased (compare Figure 4B, lane 5, with. Figure 4A, lane 3).

Mutational analysis of Cdk phosphoacceptor sites in Sic1 revealed that phosphorylation of a specific set of residues triggers Sic1 destruction. An epitope-tagged version of Sic1 lacking these key Cdk phosphoacceptor sites (Sic1^{HAHis6}-T5G, T33A, S76A; Sic1^{HAHis6}-Δ3P) was neither ubiquitinated in vitro nor degraded in vivo, but retained the ability of the wild-type protein to bind to Clb5/Cdc28 complexes (Verma et al., 1997). Given the results described above, we surmised that the stability of the Sic1^{HAHis6}-Δ3P mutant might arise from a failure to bind to Cdc4/Skp1. To test this hypothesis, we affinity-purified ^{PH}Cdc4 from insect cell lysates using an anti-polyoma antibody matrix, and incubated the immobilized ^{PH}Cdc4 complexes with various [^{35}S]-labeled forms of Sic1. Incubation of the bead–bound ^{PH}Cdc4 with either unphosphorylated or phosphorylated MBP-Sic1^{MH6} confirmed that only the phosphorylated form bound to the ^{PH}Cdc4 matrix (Figure 5A). Sic1^{HAHis6} produced by in vitro translation in rabbit reticulocyte lysate also bound to ^{PH}Cdc4-containing beads in a phosphorylation-dependent manner (Figure 5B), but Sic1^{HAHis6}-Δ3P was not retained by the ^{PH}Cdc4 matrix even though it was clearly phosphorylated by Cln2/Cdc28^{HA}/Cks1 on sites whose modification is not sufficient to promote ubiquitination (Verma et al., 1997; Figure 5C). These data establish two key points: first, the stability of Sic1^{HAHis6}-Δ3P can be explained by its inability to bind Cdc4/Skp1; second, the failure of Cdc4/Skp1 to interact with stable (yet phosphorylated) Sic1^{HAHis6}-Δ3P suggests that the interaction between Cdc4/Skp1 and phosphorylated wild-type Sic1 is physiologically significant, and is not a non-specific ionic interaction stabilized by the phosphate groups.

Recruitment of phosphorylated Sic1 to a Skp1/Cdc53/Cdc4 E3 complex

Cdc4 and Skp1 associate with one another in reticulocyte lysate (Bai et al., 1996). Consistent with this observation, recombinant Cdc4 and Skp1 expressed in insect cells also associated with each other, as judged by co-immunoprecipitation of Skp1 with Cdc4 (Figure 4C, lane 5) and vice versa (lane 6). Since Cdc4 assembled into complexes with both Skp1 and phosphorylated MBP-Sic1^{MH6} in insect cell lysates, we also examined whether it could associate with Cdc53. Sf9 cells that were either singly- or co-infected with baculoviruses expressing Cdc4, Cdc53 or Cdc53ΔC were metabolically labeled with ^{35}S-amino acids, and lysates derived from the labeled cells were immunoprecipitated with anti–Cdc4 antibodies. As shown in Figure 4D, both Cdc53 (lane 4) and Cdc53-ΔC (lane 5) specifically co-precipitated with Cdc4. Since Cdc4 appeared to associate with a Skp1-like activity in insect cell lysates (Figure 3C), it remains unclear whether Cdc4 and Cdc53 directly bound each other, or interacted indirectly via Skp1.

Given that Cdc4 can associate with Skp1, Cdc53, and phosphorylated substrate, and that the interaction between phosphorylated MBP-Sic1^{MH6} and Cdc4 was strongly stimulated by Skp1, we hypothesized that Cdc4/Skp1 might serve as a receptor module that tethers phosphorylated substrate to an E3 complex containing Cdc53. To test this possibility, we combined ^{35}S-radiolabeled Sf9 cell lysates containing Cdc4, Cdc53, and Skp1^{HA}, and incubated these mixtures with either phosphorylated or unmodified MBP-Sic1^{MH6}, followed by immunoprecipitation with anti-MBP antibodies (Figure 4E). Whereas Cdc4, Cdc53, and Skp1^{HA} did not co-immunoprecipitate with unphosphorylated MBP-Sic1^{MH6}, all three proteins stably associated with phosphorylated MBP-Sic1^{MH6}. Since Cdc53 by itself failed to bind selectively to phosphorylated MBP-Sic1^{MH6} (Figure 4A, lane 6), the observed interaction was most likely due to the ability of both Cdc53 and phosphorylated MBP-Sic1^{MH6} to bind independently to Cdc4/Skp1 to form a single complex. Cdc53 has been reported to bind to Cdc34 (Willems et al., 1996). Thus, phosphorylated MBP-Sic1^{MH6} bound to the Cdc4/Cdc53/Skp1 complex would be well-positioned to serve as a substrate for Cdc34-dependent ubiquitination.

Discussion

Reconstitution of Sic1 ubiquitination with purified proteins

Ubiquitin-mediated degradation of the S phase Cdk inhibitor Sic1 plays a key role in triggering the initiation of DNA synthesis in budding yeast (Verma et al., 1997; Schwob et al., 1994). Here, we report the ubiquitination of Sic1 using nine purified proteins: Uba1, ubiquitin, Cdc34, Cdc4, Cdc53, Skp1 and Cln2/Cdc28/Cks1 complexes. Based on experiments reported here and elsewhere (Verma et al., 1997; Willems et al., 1996), we envision the following sequence of events for Sic1 ubiquitination (Figure 6). Active Cln2/Cdc28/Cks1 complexes phosphorylate Sic1 on a set of Cdk consensus phosphorylation sites (Verma et al., 1997), thereby priming it to serve as a substrate for ubiquitination. The phosphorylated Sic1 then binds to a complex containing Cdc4, Cdc53, and Skp1 subunits. We have agreed with Skowyra et al. (1997) to designate this assemblage SCF, which stands for Skp1/Cdc53 (or Cullin)/F-box ubiquitin ligase complex, with the identity of the associated F-box protein indicated by a superscript (e.g., SCF^{Cdc4}). Upon binding phosphorylated Sic1, we propose that the Cdc53 subunit of SCF^{Cdc4} recruits the ubiquitin-charged E2 enzyme Cdc34 to initiate Sic1 ubiquitination (Willems et al., 1996).

Several aspects of our model for Sic1 ubiquitination and degradation remain speculative. First, the interactions between Cdc4, Cdc53, Skp1, Cdc34, and phosphorylated Sic1 remains to be characterized in detail. Second, it is unclear how ubiquitin is transferred from Cdc34 to lysine residues in the SCF-bound substrate. Third, whereas SCF^{Cdc4} , Cdc34, E1 enzyme, and ubiquitin appear to be sufficient to sustain the ubiquitination of phosphorylated Sic1 in vitro, it is possible that additional proteins may regulate Sic1 ubiquitination in vivo. Such accessory factors might serve to activate known SCF^{Cdc4} subunits or to overcome negative regulation that is not reconstituted in our in vitro system. Finally, it is unclear if SCF^{Cdc4} rapidly dissociates from

multiubiquitinated substrate or remains bound, perhaps interacting with the proteasome to facilitate the degradation of multiubiquitinated Sic1.

Cdc4/Skp1 recruits phosphorylated substrates for ubiquitination

A hallmark of SCF^{Cdc4} –dependent ubiquitination is that it is selective for phosphorylated substrates. Cdc6, Gcn4, Far1, and Sic1 are ubiquitinated in crude yeast extract in a Cdc34- and Cdc4-dependent manner, and in each case ubiquitination requires a kinase that phosphorylates the substrate (Verma et al., 1997; Y. Chi and RJD, unpublished data). We show here that this requirement derives from a selective interaction between phosphorylated substrate and Cdc4/Skp1. Skp1 by itself cannot bind to phospho-Sic1. Whereas Cdc4 can interact with phospho-Sic1 in insect cell lysates, this interaction is enhanced considerably by Skp1. The existence of a Skp1-like activity in insect cells that co-purifies with Cdc4 obscures whether phospho-Sic1 can be recognized by Cdc4 alone or only by a Cdc4/Skp1 complex.

What are the individual roles of Cdc4 and Skp1 in substrate binding? Based on the observation that the WD-40 domains of β-transducin fold into a β-propeller structure with an extensive surface area suitable for protein-protein interaction (Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996), we propose that the WD-40 domain of Cdc4 binds directly to phospho-Sic1, and that this interaction is facilitated by Skp1. An unambiguous test of this hypothesis will likely require the generation of point mutations in the WD-40 repeats, since crude deletions in this domain appear to destabilize Cdc4; Cdc4-ΔWD mutants translated in reticulocyte lysate bind poorly to Skp1 and exhibit high non-specific adsorption to matrices (C. Correll, unpublished data). Regardless of the identity of the substrate interaction domain in Cdc4, Skp1 most likely promotes substrate recruitment to SCF^{Cdc4} either by forming direct contacts with bound substrate, or by evoking a conformational change in Cdc4. The ability of Skp1 to enhance Cdc4's substrate binding activity may prevent "free" Cdc4 from competing with SCF^{Cdc4} for association with phosphorylated substrates. This notion is supported by our observation

that recombinant Cdc4 does not competitively inhibit Sic1 ubiquitination in crude yeast extract even when added in amounts 200-fold greater than those needed to achieve efficient complementation of the ubiquitination defect of *cdc4* mutant extract (Verma et al., 1997b; data not shown).

Whereas the phosphorylation of Sic1 triggers its ubiquitination by enhancing its association with Cdc4/Skp1, it remains unclear how Cdc4/Skp1 discriminates between phosphorylated and unmodified Sic1. We can envision two distinct models. Phosphorylation of Sic1 may cause a conformational rearrangement that exposes a cryptic Cdc4/Skp1-binding determinant in Sic1. Alternatively, Cdc4/Skp1 may bind directly to the phosphate groups on Sic1, much like SH2 and 14-3-3 domains bind directly to phospho–amino acid epitopes (Songyang et al., 1993; Muslin et al., 1996). Mutagenesis experiments have shown that no single phosphorylation site on Sic1 is necessary or sufficient to specify its ubiquitination (Verma et al., 1997). Instead, the mutagenesis data suggest that there is a set of key phosphorylation sites on Sic1, and that several distinct Sic1 phospho-isomers that are modified in a subset of these sites are competent to serve as substrates for SCF^{Cdc4}-dependent ubiquitination. These data lead us to favor the idea that Cdc4/Skp1 interacts directly with phosphorylated serine or threonine. Perhaps selective interaction with phosphorylated targets will prove to be a general property of proteins containing WD-40 repeats.

Willems et al. (1996) argued that Cdc53 selectively targets phosphorylated Cln2 for Cdc34–dependent ubiquitination. However, these authors failed to demonstrate that the interaction they observed between Cdc53 and phospho-Cln2 was direct and not promoted by intermediary proteins. Our data suggest that this interaction was actually sustained by either Cdc4 or Grr1 (see below). Alternatively, Cdc53 may directly bind phosphorylated Cln2, but this interaction may serve to recruit Cln2/Cdc28 kinase activity to SCF, rather than to target Cln2 for ubiquitination.

Conservation and Generality of the SCF pathway

Phosphorylation triggers the degradation of a broad range of key regulatory proteins in metazoans, including G1 cyclins (Clurman et al., 1996; Won et al., 1996; Diehl et al., 1997), IκB, which is a global negative regulator of the immune response (Verma et al., 1995), CACTUS, which is required for dorsal-ventral patterning in Drosophila embryos (Belvin and Anderson, 1996), and B-catenin, which is involved in developmental patterning and acts as a tumor suppressor gene (Aberle et al., 1997). For IκB, CACTUS, and β-catenin, it is thought that phosphorylation-triggered degradation operates as a switch that governs the ultimate biological response. Based on our observations with Sic1 (Verma et al., 1997; this paper), we speculate that regulated protein kinases and SCF-like complexes coordinately regulate diverse biological processes including the immune response, organismal development, and cell proliferation/tumor progression. Based on the known diversity of protein kinases and the potential diversity of SCF pathways (see below), an attractive model is that the ubiquitination of different SCF substrates is controlled by distinct, independently regulated kinases, or by multiple kinases acting in a combinatorial fashion (King et al., 1996).

Given the diverse range of substrates whose degradation is regulated by phosphorylation, a key question is, are there different SCF pathways in eukaryotic cells? Whereas Sic1 degradation requires Cdc34, Cdc53, Skp1, and Cdc4, Cln2 degradation requires Cdc34, Cdc53, Skp1, and Grr1 (Barral et al., 1995; Deshaies et al., 1995; Bai et al., 1996; Willems et al., 1996), but not Cdc4 (RJD, unpublished data). Both Cdc4 and Grr1 contain an F-box, which is a conserved domain that is thought to mediate binding to Skp1 (Bai et al., 1996) . Thus, Cdc4 and Grr1 might serve as interchangeable modules that interact with a core ubiquitination machinery comprised of Skp1, Cdc53, and Cdc34 (Bai et al., 1996). This would allow for the assembly of two different ubiquitin ligase complexes with distinct substrate preferences; SCF^{Cdc4} would target Sic1 for ubiquitination, whereas SCF^{Grr1} would target Cln2. We envision that the F-box-containing receptor module would be recruited from a pool of inactive receptor subunits, only to be activated upon docking with Skp1. In light of this model, it is perhaps

surprising that Grr1 and Cdc4 share no homology outside of the F-box, even though Cln2, like Sic1, contains PEST sequences and must be phosphorylated to be recognized as a substrate for degradation (Lanker et al., 1996).

Besides Grr1 and Cdc4, at least 30 F-box-containing proteins have been detected in DNA sequence databases (Bai et al., 1996). Coupled with the diversity of Cdc53/cullin- and Skp1-like proteins in humans, the broad pallette of F-box-containing proteins would allow for the assembly of an array of SCFs with distinct substrate-binding specificities and modes of regulation. Thus, SCF-based ubiquitination pathways may play a broad role in eukaryotic regulatory biology.

Experimental Procedures

Insect Cells, Yeast Strains, and Reagents

Bacterial expression vectors for K48R-Ub and Cdc34 originated from J. Callis (U.C. Davis) and V. Chau (Wayne State Univ., Detroit), respectively. The mouse monoclonal cell line expressing anti-polyoma antibodies was a generous gift from M. Peter (Swiss Institute for Experimental Cancer Research, Epalinges). A bacterial expression vector for GST-Skp1, along with baculovirus constructs which express Skp1, Skp1^{HA}, or Skp1^{His6} were all generous gifts of P. Sorger (MIT, Cambridge). *CDC4*, *CDC53*, and SIC1^{HAHis6}-*T2A, T5G, T33A, S76A* plasmids were obtained from B. Jensen (University of Washington, Seattle), M. Goebl (Indiana University, Indianapolis), and R. Verma (this laboratory), respectively. Baculoviruses that express GST-Cdc28^{HA} (J. W. Harper, Baylor), Clb5 (M. Weinreich, Cold Spring Harbor), Cln2 and Cdc28^{HA} (Greg Reynard, this laboratory) were kindly provided by the indicated investigators.

Baculovirus Construction

Baculoviruses expressing Cln2 and $Cdc28^{HA}$ are described elsewhere (Reynard and Deshaies, submitted). The construction of a baculovirus encoding Cdc4 has been described previously (Verma et al., 1997b). Epitope-tagged versions of both *CDC4* and *CDC53* were constructed via insertion of a DNA cassette which contains two tandem repeats of a peptide epitope (MEYMPME) derived from the polyoma virus middle T protein (Grussenmeyer et al., 1985) followed by three tandem repeats of the hemagglutinin epitope (Field et al., 1988). This bipartite epitope is hereafter designated PH. An epitope-tagged version of Cdc4 was constructed by introducing via PCR a single copy of the PH cassette immediately after the start codon of Cdc4 present in pVL1392-CDC4 (Verma et al., 1997b).

To generate viral vectors for expression of Cdc53, a portion of *CDC53* lacking its 60 C-terminal residues was amplified by PCR from pGEM53-8 (Mathias et al., 1996) and the resulting PCR product subcloned into pVL1392 (Pharmingen), yielding the vector pVL-53ΔC. A fragment containing the last 60 codons of *CDC53* was amplified by PCR from pGEM53-8 and subcloned into pVL-53ΔC to yield pVL-53, which contains the full length open reading frame of *CDC53*. To generate an epitope-tagged version of Cdc53, a fragment encoding the C-terminal 95 residues of Cdc53 was amplified by PCR and engineered to contain the PH cassette immediately upstream of the termination codon. This epitope-tagged version of CDC53 was sublconed into the vector pFastbac (GibcoBRL), yielding $pFBCdc53^{PH}$. pVL-53, pVL-53ΔC and $pFBCdc53^{PH}$ were used to generate baculovirus stocks, as per the manufacturers' instructions. All portions of *CDC4* and *CDC53* plasmids generated by PCR were sequenced.

Antibodies

Anti-Cdc53 antibodies were generated in rabbits immunized with an MBP fusion protein containing the last 23 residues of Cdc53. Antibodies against MBP and Cdc53 were sequentially purified by affinity chromatography using antigen essentially as described (Harlow and Lane, 1988). Monoclonal anti-polyoma antibodies were purified

out of tissue culture supernatant using protein A-Sepharose beads. Purified anti-polyoma antibodies were cross-linked to protein A with dimethylpimilimadate (Harlow and Lane, 1988) at a concentration of 2 mg antibodies/mL of protein A resin.

Baculovirus Expression

Sf9 cells were infected with the appropriate baculovirus at an MOI of 10. Forty-two hours post-infection, cells were collected and lysed by resuspension in 1 ml of Buffer A per 10^7 cells (Buffer A: 20 mM Hepes pH 7.4, 100 mM NaCl, 0.1% Triton X-100, 5 mM $MgCl_2$, 5 mM EDTA, 2 mM DTT, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin). Lysates were centrifuged at 14,000 x*g* for 15 min, and the supernatant (typically 5 mg protein/ml) was adjusted to 10% glycerol, frozen in liquid nitrogen, and stored at -80 °C.

^{PH}Cdc4 and Cdc53^{PH} were affinity-purified as follows: 25 μl of anti-polyoma protein A beads were added to 500 μL of lysate and incubated at 4 °C for 1h. The beads were then washed twice with 1 mL of Buffer A fortified with 0.2 M KOAc, and then twice with 1 mL of Buffer B (30 mM Hepes pH 7.4, 300 mM KOAc, 0.1% octylglucoside, 10% glycerol, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM DTT, 20 μg/mL Arg-insulin, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin). Bound polyoma-tagged proteins were specifically eluted by incubation of the anti-polyoma beads in 50 μL of Buffer B containing 100 μg/mL polyoma peptide (MEYMPME) for 2 h at 4 °C.

Clb5/Cdc28^{HA}/Cks1 complexes were generated by co-infecting Sf9 cells with viruses encoding Clb5 and Cdc28HA. Cell lysates derived from these infections were incubated with Cks1 and immunopurified exactly as described for Cln2/Cdc28^{HA}/Cks1 complexes. Cln2/GST-Cdc28^{HA} kinase expressed in insect cells was purified by incubation of 200 μL of crude lysate with 25 μL of glutathione agarose beads for 30 minutes at 4 °C. The beads were washed twice with the above lysis buffer and twice with Kinase Assay Buffer (KAB: 10 mM Hepes pH 7.2, 50 mM NaCl, 10 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin). The kinase complex was either left immobilized on the beads, thereby allowing for the subsequent

removal of the complex after a kinase reaction, or was eluted by incubation of the resin in 30 μL of KAB containing 5 mM glutathione for 30 min at 4 °C.

To purify a hexahistidine-tagged version of Skp1 ($Skp1^{His6}$), Sf9 cells infected with a Skp1-expressing baculovirus were harvested and lysed in Buffer C (25 mM Hepes pH 7.4, 100 mM KCl, 10 mM imidazole, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin) supplemented with 50 mM NaF, 50 mM β-glycerophosphate, and 0.1% Triton X-100. After centrifugation (15 min, 14,000 x*g*), $Skp1^{His6}$ was adsorbed from the lysate onto Ni^{+}-NTA resin (Qiagen) and eluted in Buffer C plus 120 mM imidazole.

Bacterial/Yeast Expression and Purification of Proteins

Cdc34 was expressed and purified from E. coli as described previously (Banerjee et al., 1993). MBP-$Sic1^{MH6}$ was expressed and radiolabelled in E. coli and purified by successive steps of Ni^{+}-NTA and amylose affinity chromatography as described (Verma et al., 1997a). Other fusion proteins containing either maltose binding protein (MBP; New England BioLabs) or glutathione-S transferase (GST; Pharmacia) moieties were expressed and purified as specified by the supplier, and stored in 25 mM Hepes pH 7.2, 100 mM NaCl, 2 mM DTT, 1 mM EDTA, and 10% glycerol at -80 °C.

S. cerevisiae Uba1 was purified from the yeast strain JD77 (Dohmen et al., 1995) transformed with the plasmid pJD325 (J. Dohmen, personal communication), which carries a copper-inducible promoter driving expression of hexahistidine-tagged Uba1 (^{His6}Uba1). Log-phase JD77[pJD325] cells grown in 1L YPD were induced with $CuSO_4$ to 1 mM, harvested 24 h after induction, washed, and frozen in liquid nitrogen. The frozen cell mass was ground with a chilled mortar and pestle, and the resulting powder was resuspended in Buffer D (50 mM Tris pH 7.5, 0.1% Triton X-100, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin) and then clarified by centrifugation (30 min, 35,000 x*g*). ^{His6}Uba1 was purified from the lysate by Ni^{+}-NTA affinity chromatography followed ubiquitin-affinity chromatography

(Ciechanover et al., 1982). Purified ^{His6}Uba1 was dialyzed twice against 1 L of 50 mM Tris pH 7.6, 0.1 M KCl, 0.2 mM DTT, 15% glycerol and then adjusted to 0.2 mg/mL BSA and stored in aliquots at -80 °C.

Ubiquitination reactions

All ubiquitination reactions contained: 4 μg ubiquitin, 60 ng Cdc34, 25 ng ^{His6}Uba1, 1 μL of a 10X ATP-regenerating system (20 mM Hepes pH 7.2, 10 mM ATP, 10 mM MgOAc, 300 mM creatinine phosphate, 0.5 mg/mL creatinine phosphokinase), 1 μL of 10X reaction buffer (40 mM MgOAc, 10 mM DTT, 1 mM PMSF), and ca. 10,000 cpm [^{35}S]-MBP-Sic1^{MH6} (~20 ng). Reactions performed with crude lysates included: 1 μL each of Sf9 cell lysate containing Cdc4, Cdc53, or Skp1; and 2 μl of affinity purified Cln2/Cdc28^{HA}/Cks1 kinase. Reactions utilizing pure protein components contained 25 ng of either GST-Skp1 or Skp1^{His6}, plus 0.5 μL each of eluted ^{PH}Cdc4 and Cdc53^{PH} (from a 50 μL immunopurification as described above). For the experiments shown in Figure 3B, wild-type ubiquitin was replaced with 1.5 μg of either methyl ubiquitin or K48R ubiquitin. Reactions using MBP-Cdc4 contained 9 μg of the preparation shown in Figure 2A. Each ubiquitination component was present at saturating levels relative to substrate (data not shown). Reactions were brought to a final volume of 10 μL with 20 mM Hepes pH 7.4, 100 mM KOAc, 1 mM DTT. All components were mixed and incubated for at 25 °C 90 minutes (unless otherwise indicated). Reactions were terminated by addition of Laemmeli sample buffer, resolved by SDS-PAGE, and visualized by autoradiography.

To phosphorylate [^{35}S]-MBP-Sic1^{MH6}, 85 μL of purified radiolabeled substrate (approximately 1 μg) were mixed with 8 μL of glutathione agarose-bound Cln2/GST-Cdc28^{HA}/Cks1, and the reactions were diluted to a final volume of 100 μL with KAB and then adjusted to 1 mM ATP. The mixture was gently rocked at room temperature for 2 h, after which the beads were pelleted by centrifugation (5 sec, 13,000 x*g*) and the supernatant was used in reactions as described.

Immunoprecipitations

At 40 h post-infection, baculovirus-infected Sf9 cells were metabolically labeled for 3 h in methionine-deficient media by the addition of Tran[^{35}S]-label (10 μCi/mL), and then lysates were prepared as described above. Co-immunoprecipitation experiments with MBP-Sic1^{MH6} were carried out by combining 25 μL of the appropriate Sf9 cell lysate with 1 μg of either unphosphorylated or phosphorylated MBP-Sic1^{MH6} plus sufficient Buffer A to yield a final volume of 100 μl. The mixtures were incubated for 1 h at 4 °C, and immunoprecipitated by addition of anti-MBP antibodies Following a final 1 h incubation at 4 °C, immunoprecipitates were collected by centrifugation and washed with Buffer A containing 5 mM NaF. The resulting samples were analyzed by SDS-PAGE and autoradiography.

PCR templates of Sic1 carrying a tandem hemagglutinin epitope plus hexahistidine (HAHis6) tag at its C-terminus (Sic1^{HAHis6}), and a mutant form of Sic1^{HAHis6} (Sic1-T5G, T33A, S76A; Sic1^{HAHis6}-Δ3P) were translated in rabbit reticulocyte lysate to produce [^{35}S]-labeled proteins (Verma et al., 1997). Phosphorylation of both constructs by Cln2/Cdc28^{HA}/Cks1 was done essentially as described for [^{35}S]-MBP-Sic1^{MH6}. To produce samples that were not phosphorylated, substrates were incubated with immobilized kinase in the absence of exogenously added ATP. To test the ability of these proteins to bind ^{PH}Cdc4, 1 mL of insect cell lysate containing ^{PH}Cdc4 was incubated with 30 μL of anti-polyoma resin for 1h at 4 °C, and then washed three times with Buffer A. Cdc4:Sic1 binding reactions were carried out in a final volume of 100 μL and contained 10 μL of a 16% slurry of ^{PH}Cdc4-containing beads, 15 μL of protein A beads (pretreated with 1% bovine serum albumin), and either 3 μL ([^{35}S]-MBP-Sic1^{MH6}) or 5 μL ([^{35}S]-Sic1^{HAHis6} and [^{35}S]-Sic1^{HAHis6} -Δ3P) of ligand. Following a 1 h incubation at 4 °C, the beads were washed 3 times with Buffer A containing 5 mM NaF, boiled in Laemmeli sample buffer and subjected to SDS-PAGE and autoradiography.

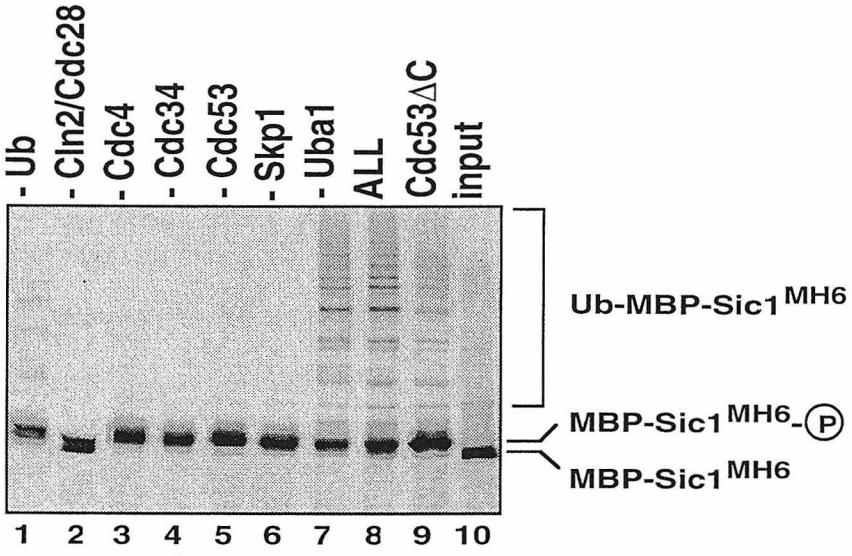
Acknowledgements

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Figure 1. Ubiquitination of MBP-Sic1 in vitro Requires Cdc4, Cdc34, Cdc53, Skp1, and Cyclin-Dependent Kinase.

(A) Unfractionated lysates from baculovirus-infected Sf9 cells expressing Cdc4, Cdc53 and Skp1, and purified Cdc34, ^{His6}Uba1, and Cln2/Cdc28^{HA}/Cks1 complexes were prepared as described in Experimental Procedures. The substrate MBP-Sic1^{MH6} was expressed and metabolically labeled in E. coli and purified via affinity chromatography (lane 10). Substrate was mixed with all of the above components plus ubiquitin and an ATP-regenerating system (lane 8). For lanes 1-7 the component indicated above each lane was omitted from the reaction mixture. In lane 9, Cdc53 lysate was replaced by an insect cell lysate containing Cdc53ΔC, a mutant of Cdc53 lacking its 60 C-terminal residues. All reactions were incubated at 25 °C for 90 minutes, stopped by addition of SDS-PAGE buffer, resolved by SDS-PAGE (8%), and visualized by autoradiography. The positions of unmodified, phosphorylated, and ubiquitinated MBP-Sic1^{MH6} are indicated.

(B) In vitro ubiquitination reactions containing either immunopurified Cln2/Cdc28^{HA}/Cks1 (lane 1) or Clb5/Cdc28^{HA} (lane 4) as a source of kinase were assembled as described in Figure 1. Lane 7 contains input MBP-Sic1^{MH6} used in the ubiquitination reactions. After incubation at 25 °C for 1 hour, Cdc28^{HA} and associated proteins were immunoprecipitated with the anti-HA antibody 12CA5. Equivalent amounts of the total reactions (T, lanes 1, 4) unbound (U, lanes 2, 5) and 12CA5-bound (B, lanes 3, 6) fractions were resolved by SDS-PAGE and MBP-Sic1^{MH6} was visualized by autoradiography.


- Ub
- Cln2/Cdc28
- Cdc4
- Cdc34
- Cdc53
- Skp1
- Uba1
ALL
Cdc53ΔC
input
Ub-MBP-Sic1MH6
MBP-Sic1MH6-P
MBP-Sic1MH6
1 2 3 4 5 6 7 8 9 10

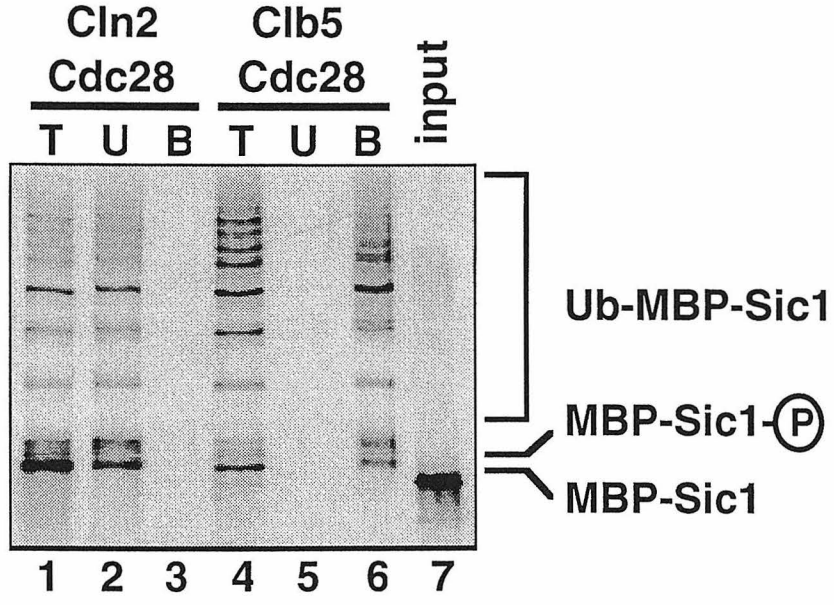

Cln2
Cdc28
Clb5
Cdc28
input
T U B T U B
Ub-MBP-Sic1
MBP-Sic1-P
MBP-Sic1
1 2 3 4 5 6 7

Figure 1.

Figure 2. Purified Ubiquitination Components.

(A, B, C, D) Recombinant proteins purified from yeast (^{His6}Uba1), E. coli (GST-Skp1, MBP-Cdc4, MBP-Sic1^{MH6}), and baculovirus-infected Sf9 cells (^{PH}Cdc4, Cdc53^{PH}, Cln2/GST-Cdc28^{HA}, GST-Cdc28^{HA}, Skp1^{His6}) as described in Experimental Procedures were separated on SDS-polyacrylamide gels and stained with Coomassie blue. In panel (A), the asterisks and arrowhead denote full-length GST-Skp1 (lane 3) and MBP-Cdc4 (lane 4) respectively. In panel (C), Cln2 is designated by an arrow. In panel (D), purified MBP-Sic1^{MH6} was incubated with bead-bound Cln2/Cdc28^{HA}/Cks1 kinase in the absence (lane 1) or presence (lane 2) of ATP. These forms of substrate were used for the experiments shown in Figure 4.

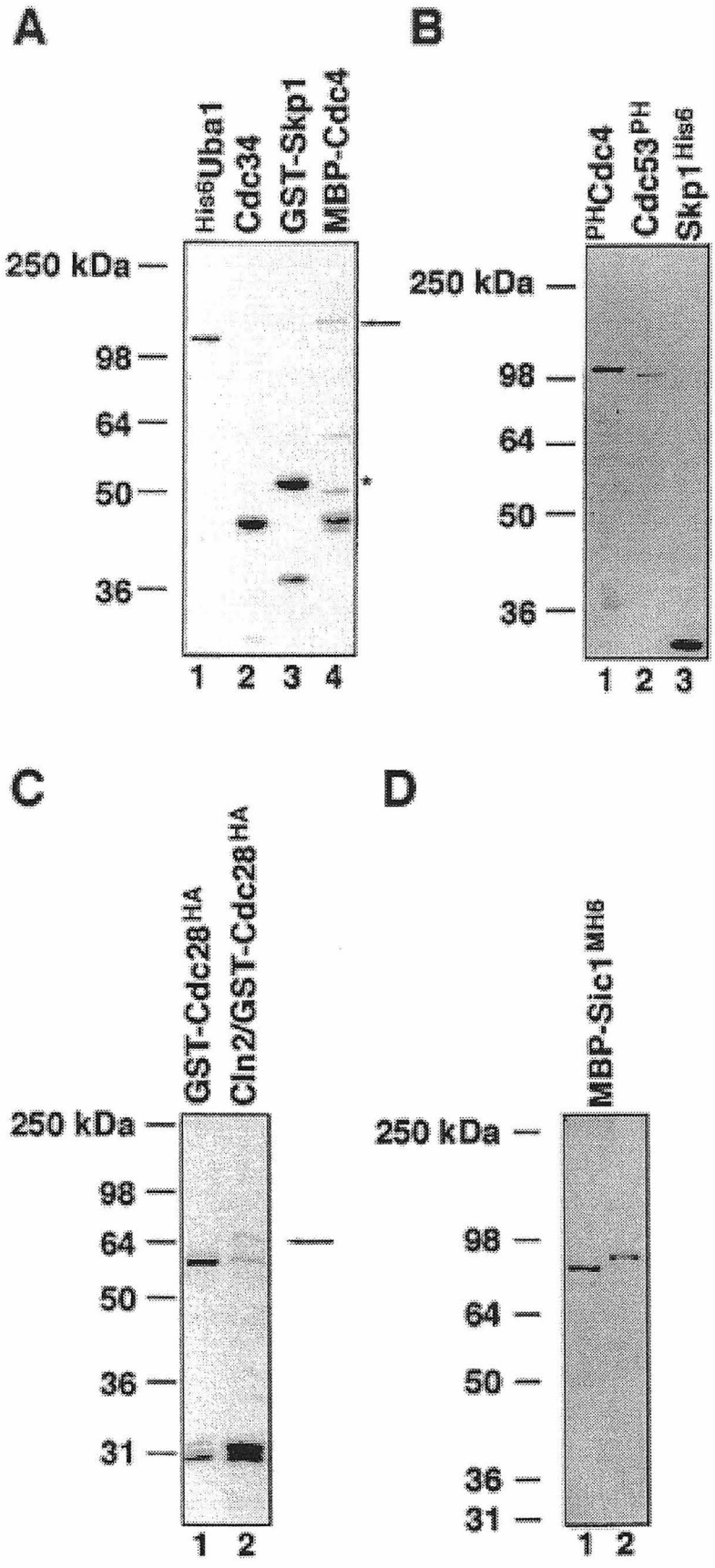

A
His6Uba1
Cdc34
GST-Skp1
MBP-Cdc4
250 kDa
98
64
50
36
*
1 2 3 4
B
PHCdc4
Cdc53PH
Skp1His6
250 kDa
98
64
50
36
1 2 3
C
GST-Cdc28HA
Cln2/GST-Cdc28HA
250 kDa
98
64
50
36
31
1 2
D
MBP-Sic1MH6
250 kDa
98
64
50
36
31
1 2

Figure 2.

Figure 3. Reconstitution of MBP-Sic1^{MH6} Ubiquitination with Purified Proteins.

(A) Purified ^{PH}Cdc4, Cdc34, Cdc53^{PH}, GST-Skp1, ^{His6}Uba1, ubiquitin, [^{35}S]-MBP-Sic1^{MH6} that had been phosphorylated by incubation with immobilized Cln2/GST-Cdc28^{HA}/Cks1 complexes, and an ATP-regenerating system were mixed together (lane 8). Reaction shown in lanes (2-7) lack the indicated component. For lane 1, all components listed above were added to MBP-Sic1^{MH6} that had not been pre-incubated with Cln2/GST-Cdc28^{HA}/Cks1. Reactions were incubated at 25 °C for 90 min, stopped by addition of SDS-PAGE buffer, resolved by SDS-PAGE (8%), and visualized by autoradiography. The positions of MBP-Sic1^{MH6}, phosphorylated MBP-Sic1^{MH6}, and ubiquitinated MBP-Sic1^{MH6} are indicated.

(B) Reactions were assembled using purified proteins as described in part (A), except that reactions contained either mutant K48R ubiquitin (lanes 1, 2), methyl ubiquitin (lanes 3, 4) or wild-type ubiquitin (lane 5). Reactions were incubated and processed as described above.

(C) In all reactions, [^{35}S]-MBP-Sic1^{MH6} was incubated with soluble Cln2/GST-Cdc28^{HA}/Cks1, ^{His6}Uba1, Cdc34, and Cdc53^{PH} along with ubiquitin and an ATP-regenerating system as described above. In addition, the following components were added to the indicated reactions: GST-Skp1 (lane 1), MBP-Cdc4 (lane 2), MBP-Cdc4 plus GST-Skp1 (lane 3), and the soluble fraction recovered from heat-denatured preparations of purified ^{PH}Cdc4 either with (lane 4) or without MBP-Cdc4 (lane 5). All reactions were incubated at 24 °C for 45 min and then evaluated by SDS-PAGE and autoradiography.

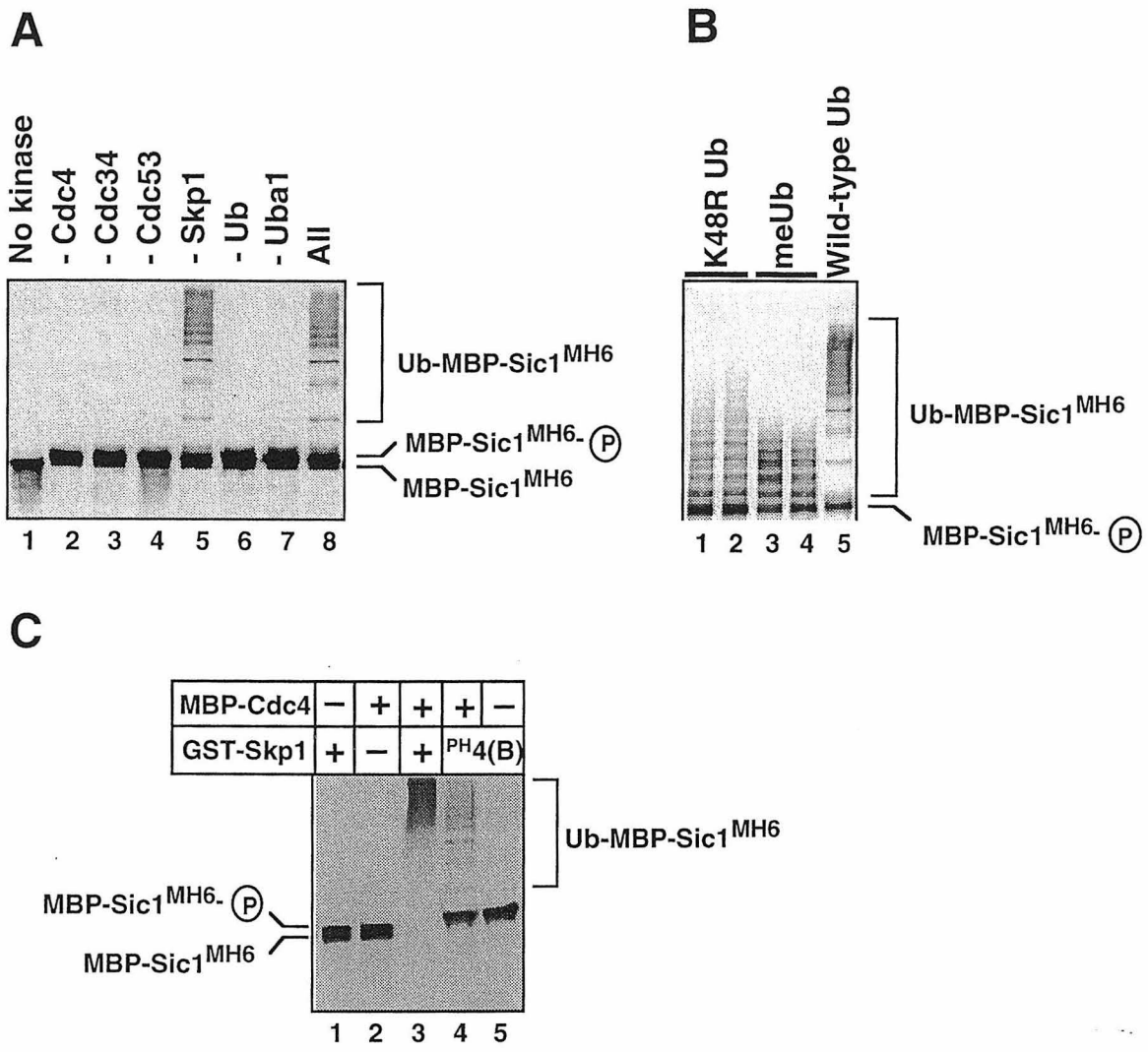

A
No kinase
- Cdc4
- Cdc34
- Cdc53
- Skp1
- Ub
- Uba1
All
Ub-MBP-Sic1MH6
MBP-Sic1MH6-P
MBP-Sic1MH6
1 2 3 4 5 6 7 8
B
K48R Ub
meUb
Wild-type Ub
Ub-MBP-Sic1MH6
MBP-Sic1MH6-P
1 2 3 4 5
C
MBP-Cdc4 − + + + −
GST-Skp1 + − + PH4(B)
Ub-MBP-Sic1MH6
MBP-Sic1MH6-P
MBP-Sic1MH6
1 2 3 4 5

Figure 3.

Figure 4. Phosphorylated MBP-Sic1^{MH6} Specifically Binds to Cdc4/Skp1 and Assembles into a Complex with Cdc4, Skp1, and Cdc53.

Sf9 cells were infected with the indicated baculovirus and metabolically labeled with Tran[^{35}S]-label for 3 h. For panels A, B, and E, the derived radiolabeled lysates were then incubated with either unphosphorylated (U) or phosphorylated (P) MBP-Sic1^{MH6}, and following a 1 h incubation at 4 °C MBP-Sic1^{MH6} and associated proteins were immunoprecipitated with MBP antibodies and analyzed by SDS-PAGE followed by autoradiography. The input (I) lanes were underloaded 2.5-fold relative to the immunoprecipitates. Panels A and B were from a single exposure of the same gel and so can be compared directly with each other.

(A) Radiolabeled lysates containing ^{PH}Cdc4 (lanes 2 and 3) or Cdc53 (lanes 5 and 6) were used for analysis. The relative position of each protein is indicated by (→).

(B) Radiolabeled lysate containing Skp1^{HA} (lanes 1-3), or an equal mixture of Skp1^{HA} plus ^{PH}Cdc4-containing lysates (lanes 4,5) was used.

(C) Radiolabeled lysates containing Cdc4, Skp1^{HA}, or a mixture of the two were immunoprecipitated with either anti-Cdc4 or anti-HA antibodies as indicated.

(D) Sf9 cells were singly infected with baculoviruses expressing Cdc4 (lane 1), Cdc53 (lane 2), and Cdc53ΔC (lane 3), or co-infected with Cdc4 and Cdc53 (lane 4) or Cdc4 and Cdc53ΔC (lane 5) baculoviruses. [^{35}S]-radiolabeled lysates were prepared from each infection and immunoprecipitated with anti-Cdc4 antibodies. The immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography.

(E) Radiolabeled lysates containing Cdc4 (lanes 1-6) were mixed with radiolabeled Skp1^{HA} (lanes 3-6) or Cdc53 (lanes 5, 6) lysates prior to addition of MBP-Sic1^{MH6}, followed by immunoprecipitation using anti-MBP antibodies.

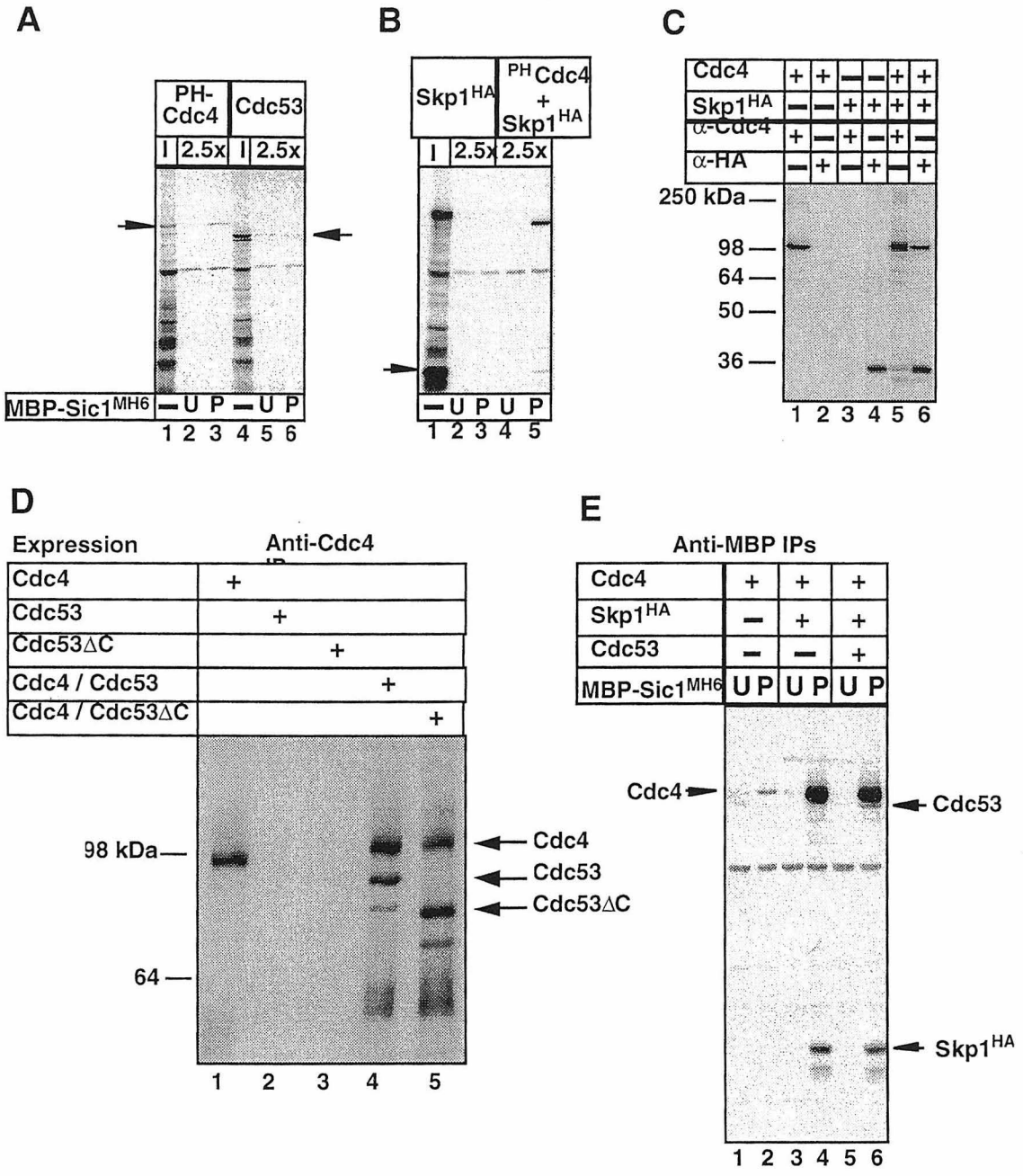

A
PH-Cdc4
Cdc53
I 2.5x I 2.5x
MBP-Sic1MH6
U P U P
1 2 3 4 5 6
B
Skp1HA
PH Cdc4 + Skp1HA
I 2.5x 2.5x
U P U P
1 2 3 4 5
C
Cdc4
Skp1HA
α-Cdc4
α-HA
250 kDa
98
64
50
36
1 2 3 4 5 6
D
Expression
Anti-Cdc4
Cdc4
Cdc53
Cdc53ΔC
Cdc4 / Cdc53
Cdc4 / Cdc53ΔC
98 kDa
64
Cdc4
Cdc53
Cdc53ΔC
1 2 3 4 5
E
Anti-MBP IPs
Cdc4
Skp1HA
Cdc53
MBP-Sic1MH6
U P U P U P
Cdc4
Cdc53
Skp1HA
1 2 3 4 5 6

Figure 4.

Figure 5. Purified Cdc4 Specifically Binds Phosphorylated Sic1 but not a Stabilized Mutant Form of Sic1.

[^{35}S]-Radiolabeled Sic1 proteins were incubated with purified bead-bound Cln2/GST-Cdc28^{HA}/Cks1 in the absence (-) or presence (+) of ATP to produce either an unphosphorylated or phosphorylated form of each protein. Following the kinase reactions, the supernatant fractions containing [^{35}S]-Sic1 were withdrawn and mixed with bead-bound purified ^{PH}Cdc4. After a 1 h incubation, the ^{PH}Cdc4 beads were recovered and analyzed by SDS-PAGE followed by autoradiography. The input lanes were underloaded 1.5-fold relative to the bead-bound fractions.

(A) [^{35}S]-MBP-Sic1^{MH6} was purified from metabolically-labeled *E. coli*.

(B) [^{35}S]-Sic1 was produced by in vitro translation in rabbit reticulocyte lysate.

(C) [^{35}S]-Sic1-Δ3P was produced by in vitro translation in rabbit reticulocyte lysate. The Sic1-Δ3P mutant lacks 3 Cln2/Cdc28 phosphoacceptor sites, and is neither ubiquitinated in vitro nor degraded in vivo (Verma et al., 1997).

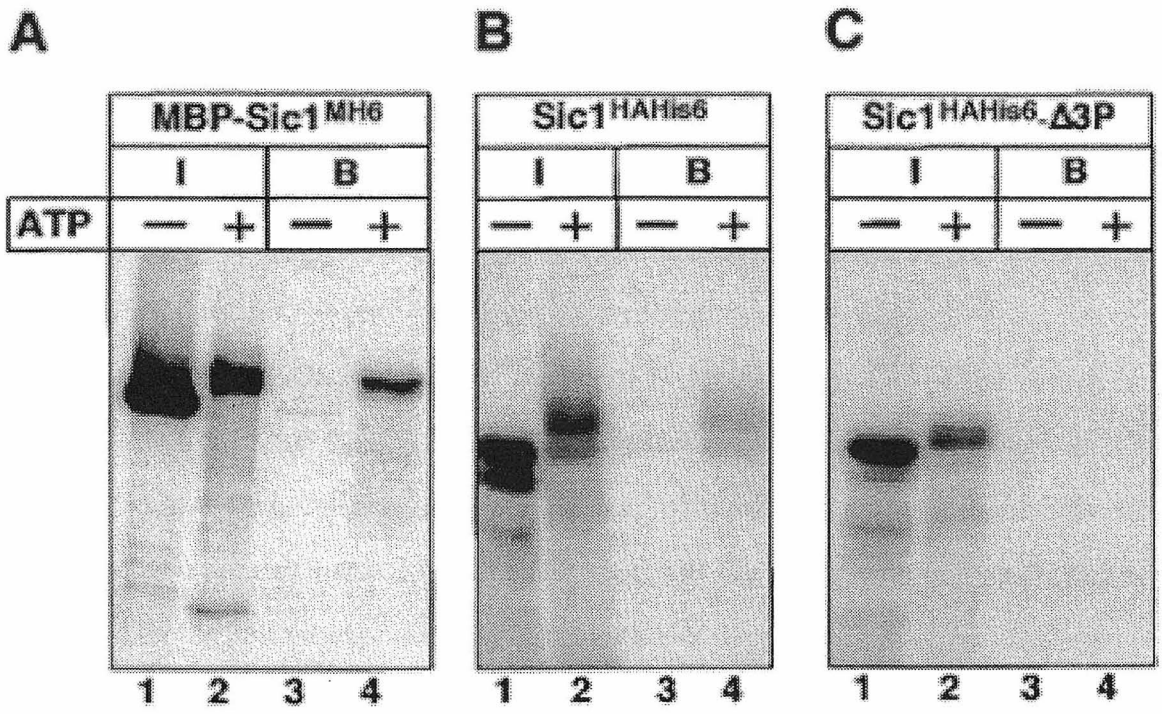


Figure 5.

Figure 6. A Model for the Ubiquitination of Sic1.

In early G1 phase, Sic1 is bound to S-phase cyclin/Cdk complexes. For Sic1 to be destroyed, it must first be phosphorylated on several phosphoacceptor sites by an active cyclin/Cdk complex. Once phosphorylated, Sic1 is recognized by Cdc4/Skp1, which can interact with Cdc53 and thereby promote the transfer of a ubiquitin moiety from Cdc34 to substrate. Once ubiquitination has been initiated, multiubiquitin chains are processively elongated by a process that is not well understood, and the multiply ubiquitinated Sic1 is destroyed by the 26S proteasome. The exact nature of the protein-protein interactions between Cdc4, Skp1, Cdc53, Cdc34 implied by this figure has not yet been fully delineated.

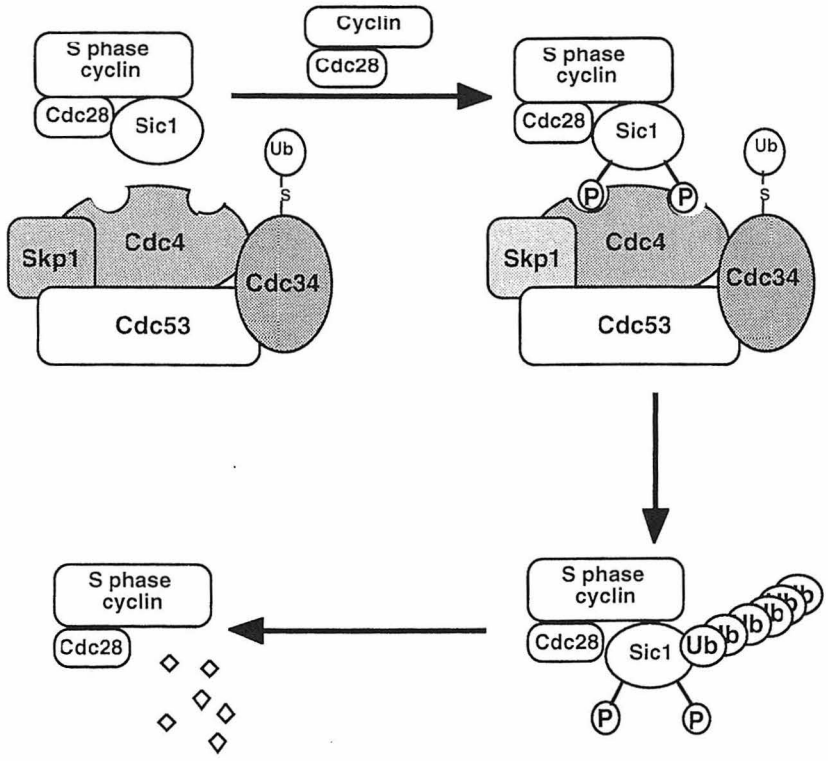

S phase cyclin
Cdc28
Sic1
Cyclin
Cdc28
Ub
S
Skp1
Cdc4
Cdc34
Cdc53
P
S phase cyclin
Cdc28
Sic1
Ub
S
Skp1
Cdc4
Cdc34
Cdc53
S phase cyclin
Cdc28
Sic1
Ub
P
S phase cyclin
Cdc28

Figure 6.

Literature Cited

Aberle, H., Baueer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). B-catenin is a target for the ubiquitin-proteasome pathway. EMBO J. *16*, 3797-3804.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., and Elledge, S. J. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell *86*, 263-274.

Banerjee, A., Gregori, L., Xu, Y., and Chau, V. (1993). The bacterially expressed yeast *CDC34* gene product can undergo autoubiquitination to form a multiubiquitin chain-linked protein. J. Biol. Chem. *268*, 5668-5675.

Barral, Y., Jentsch, S., and Mann, C. (1995). G(1) cyclin turnover and nutrient-uptake are controlled by a common pathway in yeast. Genes Dev. *9*, 399-409.

Bartel, B., Wunning, I., and Varshavsky, A. (1990). The recognition component of the N-end rule pathway. EMBO J. *9*, 3179-3189.

Belvin, M. P. and Anderson, K. V. (1996). A conserved signaling pathway - the Drosophila Toll-Dorsal pathway. Ann. Rev. Cell Dev. Biol. *12*, 393-416.

Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science *243*, 1576-1583.

Ciechanover, A., Elias, S., Heller, H., and Hershko, A. (1982). "Covalent affinity" purification of ubiquitin-activating enzyme. J. Biol. Chem. *257*, 2537-2542.

Clurman, B. E., Sheaff, R. J., Thress, K., Groudine, M., and Roberts, J. M. (1996). Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by CDK2 binding and cyclin phosphorylation. Genes Dev. *10*, 1979-1990.

Connelly, C., and Hieter, P. (1996). Budding yeast SKP1 encodes an evolutionary conserved kinetochore protein required for cell cycle progression. Cell *86*, 275-285.

Deshaies, R. J., Chau, V., and Kirschner, M. (1995). Ubiquitination of the G1 cyclin Cln2p by a Cdc34p-dependent pathway. EMBO J. *14*, 303-312.

Diehl, J. A., Zindy, F., and Sherr, C.J. (1997) Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. Genes Dev. *11*, 957-972.

Dohmen, R. J., Stappen, R., McGrath, J. P., Forrova, H., Kolarov, J., Goffeau, A., and Varshavsky, A. (1995). An essential yeast gene encoding a homolog of ubiquitin-activating enzyme. J. Biol. Chem. *270*, 18099-18109.

Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I., Lerner, R. A., and Wigler, M. (1988). Purification of a ras-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. *8*, 2159-2165.

Grussenmeyer, T., Scheidtmann, K. H., Hutchinson, M. A., Eckhart, W., and Walter, G. (1985). Complexes of polyoma virus medium T antigen and cellular proteins. Proc. Natl. Acad. Sci. USA *82*, 7952-7954.

Hadwiger, J. A., Wittenberg, C., Mendenhall, M. D., and Reed, S. I. (1989). The Saccharomyces cerevisiae CKS1 gene, a homolog of the *Schizosaccharomyces pombe* suc1+ gene, encodes a subunit of the Cdc28 protein. Mol. Cell. Biol. *9*, 2034-41.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory: Cold Spring Harbor, NY.

Hershko, A., and Heller, H. (1985). Occurrence of a polyubiquitin structure in ubiquitin-protein conjugates. Biochem. Biophys. Res. Commun. *128*, 1079-1086.

Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983). Components of ubiquitin-protein ligase system: resolution, affinity purification, and role in protein breakdown. J. Biol. Chem. *258*, 8206-8214.

Hochstrasser, M. (1995). Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. Curr. Opin. Cell Biol. *7*, 215-223.

Huibregtse, J. M., Scheffner, M., and Howley, P. M. (1993). Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. Mol. Cell. Biol. *13*, 4918-4927.

King, R. W., Deshaies, R.J., Peters, J.-M., and Kirschner, M.W. (1996). How proteolysis drives the cell cycle. Science *274*, 1652-1659.

Kipreos, E., He, W.-W., and Hedgecock, E. (1995). *lin-19* and *lin-23*: novel regulators of the cell cycle. 10th International C. elegans meeting Abstracts, p83.

Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996). The 2.0 Å crystal structure of a heterotrimeric G protein. Nature *379*, 311-319.

Lanker, S., Valdivieso, M. H., and Wittenberg, C. (1996). Rapid degradation of the G1 cyclin Cln2 induced by Cdk-dependent phosphorylation. Science *271*, 1597-1601.

Mathias, N., Johnson, S. L., Winey, M., Adams, A. E., Goetsch, L., Pringle, J. R., Byers, B., and Goebl, M. G. (1996). Cdc53 acts in concert with Cdc4 and Cdc34 to control the G1 to S phase transition and identifies a conserved family of proteins. Mol. Cell. Biol. *16*, 6634-6643.

Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996). Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. Cell *84*, 889-897.

Nugroho, T. T., and Mendenhall, M. D. (1994). An inhibitor of yeast cyclin-dependent protein kinase plays an important role in ensuring the genomic integrity of daughter cells. Mol. Cell. Biol. *14*, 3320-3328.

Pause, A., Lee, S., Worrell, R. A., Chen, D. Y. T., and Burgess, W. H. (1997). The Von Hippel-Lindau tumor suppressor gene product forms a stable complex with human CUL2, a member of the CDC53 family of proteins. Proc. Natl. Acad. Sci. USA *94*, 2156-2161.

Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell *75*, 495-505.

Scheffner, M., Nuber, U., and Huibregtse, J. M. (1995). Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. Nature *373*, 81-83.

Schneider, B. L., Yang, Q. H., and Futcher, A. B. (1996). Linkage of replication to START by the Cdk inhibitor Sic1. Science *272*, 560-562.

Schwob, E., Böhm, T., Mendenhall, M., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor $p40^{SIC1}$ controls the G1/S transition in *Saccharomyces cerevisiae*. Cell *79*, 233-244.

Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J., and Harper, J. W. (1997). F-box proteins function as receptors to recruit phosphorylated substrates to E3 ubiquitin ligase complexes. Cell *91*, 209-219.

Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E., and Sigler, P. B. (1996). Crystal structure of a G protein beta-gamma dimer at 2.1 angstrom resolution. Nature *379*, 369-374.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993). SH2 domains recognize specific phosphopeptide sequences. Cell *72*, 767-778.

Tyers, M. (1996). The cyclin-dependent kinase inhibitor p40SIC1 imposes the requirement for CLN G1 cyclin function at Start. Proc. Natl. Acad. Sci. USA *93*, 7772-7776.

Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D., and Miyamoto, S. (1995). Rel/NF-kB family: intimate tales of association and dissociation. Genes Dev. 9, 2723-2735.

Verma, R., Chi, Y., and Deshaies, R. J. (1997a). Ubiquitination of cell cycle regulatory proteins in yeast extract. Meth. Enzymol. *283*, 366-376.

Verma, R., Feldman, R., and Deshaies, R. J. (1997b). SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34, and cyclin/Cdk activities. Mol. Biol. Cell *8*, 1427-1437.

Verma, R., Annan, R., Huddleston, M., Carr, S., Reynard, G., and Deshaies, R. J. (1997c). Phosphorylation of Sic1p by G1 cyclin/Cdk required for its degradation and entry into S phase. Science *278*, 455-60.

Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Llhui, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995). The structure of the G protein heterotrimer $G_{i\alpha 1}\beta_1\gamma_2$. Cell *83*, 1047-1058.

Willems, A. R., Lanker, S., Patton, E. E., Craig, K. L., Nason, T. F., Kobayashi, R., Wittenberg, C., and Tyers, M. (1996). Cdc53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway. Cell *86*, 453-463.

Won, K.-A. and Reed, S. I. (1996). Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. EMBO J. *15*, 4182-4193.

Zhang, H., Kobayashi, R., Galaktionov, K., and Beach, D. (1995). p19(SKP1) and p45(SKP2) are essential elements of the cyclin A-Cdk2 S phase kinase. Cell *82*, 912-925.

Chapter 3: Cdc53/cullin and the Essential Hrt1 RING-H2 Subunit of SCF Define a Ubiquitin Ligase Module That Activates the E2 Enzyme Cdc34.

The work presented in this chapter has already been published (Seol et al., 1999; see Appendix III). It is an equal collaborative effort with J.-H. Seol and W. Zachariae and owes much to the help of the other authors listed. Specifically, J.-H. Seol perfromed the experiments shown in Figures 1A,B,C, and E; W. Zachariae contributed the data in Figures 1D and 2B-F.

Introduction

Proteolysis in the eukaryotic cytosol is triggered by the assembly of a substrate-linked multiubiquitin chain, which targets the protein for degradation by the 26S proteasome (Banerjee et al. 1993). Ubiquitin is activated for transfer to substrate through the ATP-dependent formation of a thioester bond between the C-terminus of ubiquitin and the ubiquitin-activating enzyme, E1. Ubiquitin is subsequently transferred to a member of a family of ubiquitin conjugating (E2) enzymes. Finally, thioesterified ubiquitin is transferred from E2 to a lysine residue of the target protein, either directly or with the assistance of a ubiquitin ligase (E3). E3s directly bind substrate, suggesting that they provide specificity in ubiquitination reactions. Whereas the budding yeast genome encodes 13 E2-like proteins, it is not known how many E3s exist, because these enzymes do not share a common sequence motif. So far, four different classes of E3 enzyme have been defined: Ubr1 (Bartel et al. 1990), the HECT domain family (Huibregtse et al. 1995), of which Mdm2 has been proposed to be a member (Honda et al. 1997), the SCF family (Feldman et al.,1997; Skowyra et al. 1997), and the Anaphase-Promoting Complex/Cyclosome (King et al.,1995).

SCF^{Cdc4}, the prototype of the SCF (for Skp1, Cdc53/Cullin, F box receptor) family of ubiquitin ligases, was first defined by in vitro reconstitution (Feldman et al. 1997; Skowyra et al. 1997; Verma et al. 1997b). Identification of SCF^{Cdc4} stemmed from a genetic analysis of the G1/S transition (Schwob et al. 1994). Budding yeast cells harboring *ts* mutations in either the Cdc34 E2 enzyme or in any one of the subunits of SCF^{Cdc4} fail to enter S phase at the nonpermissive temperature because they fail to

degrade the S phase cyclin/cyclin-dependent kinase (CDK) inhibitor Sic1 (Bai et al. 1996; Schwob et al. 1994). In wild type cells, G1 cyclin/CDK phosphorylates Sic1 at the end of G1 phase, thereby targeting it for Cdc34-dependent ubiquitination and degradation (Verma et al. 1997). Phosphorylated Sic1, but not the unmodified protein or a phosphorylation-site mutant, binds tightly to the Cdc4 subunit of SCF^{Cdc4} (Feldman et al. 1997; Skowyra et al. 1997). Cdc34 is also recruited to SCF^{Cdc4}, possibly via interaction with the Cdc53 subunit (Willems et al. 1996). Upon assembly with the SCF^{Cdc4} complex, phosphorylated Sic1 is extensively ubiquitinated as a prelude to its degradation.

Besides SCF, the Anaphase-Promoting Complex/Cyclosome (APC/C) ubiquitin ligase also plays a key role in cell cycle control. APC/C controls the separation of sister chromatids and the exit from mitosis by specifying the destruction of Pds1 and cyclin B, respectively (King et al. 1996). Although SCF and APC/C are distinctly regulated and differ greatly in size, subunit composition and apparent complexity, APC/C contains a subunit (Apc2) that bears homology to the C-terminal half of Cdc53/cullin (Yu et al. 1998; Zachariae et al. 1998).

A remarkable property of SCF ubiquitin ligases is that their specificity can be programmed via an array of distinct substrate receptors (Patton et al. 1998b; Skowyra et al. 1997). These receptors are recruited to SCF by the conserved F box domain they share. The discovery of the F box stemmed from the observation that human cyclin F and Cdc4 share a conserved sequence that mediates their interaction with Skp1 (Bai et al. 1996). The budding yeast genome encodes 17 proteins that contain an obvious F box motif (Patton et al. 1998b), and at least three of these proteins assemble into distinct SCF complexes in vivo (Patton et al. 1998a).

Over the past few years, F box proteins and other components of the SCF/Cdc34 pathway have been identified in many eukaryotes and linked to diverse cellular processes, ranging from the auxin response in plants (Ruegger et al. 1998), withdrawal from the cell cycle in nematodes (Kipreos et al. 1996), signaling via the Wnt and hedgehog pathways in Drosophila (Jiang and Struhl, 1998), and activation of the immunoregulatory protein NF-kB in human cells (Yaron et

al. 1998). Although SCF ubiquitin ligases clearly regulate diverse physiological pathways in a variety of eukaryotes, little is known about their structure, regulation or mechanism of action.

The HECT domain proteins are the only ubiquitin ligases whose mechanism of action is understood. The charter member of the HECT domain family of proteins, E6-Associated Protein (E6-AP), forms a thioester bond with ubiquitin as an intermediate in the transfer of ubiquitin from E2 to substrate (Scheffner et al. 1995). Mutation of the thioester-forming cysteine of E6-AP destroys its E3 activity. It remains unclear whether other (non-HECT) E3 enzymes catalyze substrate ubiquitination via a similar thioester cascade.

We report the discovery of a heretofore undetected fourth essential subunit of the SCF complex. This new subunit, Hrt1, is a member of the RING-H2 family of proteins (Suarin et al, 1996). Hrt1 is required for degradation of SCF substrates in vivo, and stimulates the ubiquitin ligase activity of both SCF^{Cdc4} and SCF^{Grr1} complexes in vitro. A Cdc53/Hrt1 subcomplex potently activates autoubiquitination of the Cdc34 E2 enzyme, suggesting that these two proteins define a minimal ubiquitin ligase. Based on analysis of Cdc34 autoubiquitination, we suggest that Cdc53/Hrt1 promotes substrate ubiquitination by a novel mechanism. Remarkably, the SCF and APC/C ubiquitin ligase complexes both contain essential subunits with cullin-homology and RING-H2 domains. We propose that these seemingly unrelated ubiquitin ligases define a broad family of cullin-containing E3s that share a common enzymatic mechanism.

Results

Purification of SCF complexes from yeast cells

To determine if SCF contains subunits other than the minimal set that suffices for reconstitution of ubiquitin ligase activity, we investigated its native composition in yeast. The chromosomal copies of *CDC53* and *SKP1* were modified by a PCR-based tagging procedure (see Materials and Methods) to encode proteins with either 6 or 9 copies of the myc epitope (myc6, myc9) appended to their C termini. *CDC53myc6* and control untagged cells were pulse-

radiolabeled with [^{35}S]-methionine, and proteins bound specifically to Cdc53myc6 were isolated on an α-myc 9E10 monoclonal antibody matrix (Figure 1A). Among other proteins, Cdc53myc6 immunoprecipitates contained a prominent ~17 kD band (lane 2) that was not detected in affinity-purified APC/C (data not shown). To isolate SCF-associated proteins in sufficient scale for protein sequencing, Skp1myc9 and Cdc53myc9 were affinity purified on 9E10 beads and associated proteins were resolved by SDS-PAGE and stained with silver (Figure 1B). Although several proteins were identified in both preparations, Cdc53, YJL149w (an F box protein), and the 17 kD polypeptide were the only species that were consistently detected in both Skp1myc9 and Cdc53myc9 immunoprecipitates (J.H.S. and A.S., unpublished data). The 17 kD protein was identified as the product of the *HRT1* (YOL133w) locus (Figure 1C) by a combination of high mass accuracy MALDI peptide mapping and nanoelectrospray tandem mass spectrometric sequencing (Shevchenko et al. 1996). *HRT1* was first identified as a gene whose high-level expression reduces transposition by the retrotransposon Ty3 (cited in the Saccharomyces Genome Database at http://genome-www.stanford.edu/Saccharomyces/; J.C. and S.S., submitted)

Interestingly, the yeast protein most closely related to Hrt1 was the Apc11 subunit of APC/C (Figure 1D) (Zachariae et al., 1998). These proteins are 51% related and 28% identical to each other, and both contain highly related RING-H2 domains (Suarin et al., 1996). Hrt1 has even closer relatives in other organisms, including humans. The human *hHRT1* (GenBank D52876) and *hHRT2* (HGI THC257076) genes are 62.5% identical (79% similar) and 41% identical (63% similar) to yeast *HRT1*, respectively.

To verify the interaction between Hrt1 and SCF, we modified the chromosomal *HRT1* locus to encode *HRT1myc9*. The resulting strain specifically expressed a 9E10-reactive polypeptide of the expected molecular weight (Figure 1E, lane 2) and was fully viable at 25 °C, but not 37 °C (see next section). In co-immunoprecipitation experiments, Cdc53 (lane 4), Cdc4 (lane 6), Skp1 (lane 8) and Cdc34 (lane 10) were specifically detected in 9E10 immunoprecipitates prepared

from *HRT1myc9* cells, but not in those prepared from a control untagged strain (odd-numbered lanes).

HRT1 is required for SCF activity in vivo

To evaluate the role of Hrt1 in SCF function, an *HRT1/hrt1::HIS3* heterozygote was constructed, sporulated and dissected into tetrads. Only two viable, His^- spores were recovered from each tetrad on rich medium (Figure 2A, top panel). However, *HRT1* overlaps extensively with the *YOL134c* locus on the opposite strand, such that *hrt1::HIS3* disrupted both genes. Two lines of evidence indicate that *HRT1* is essential, and *YOL134c* is dispensable. First, co-segregation of a *GAL-HRT1* plasmid complemented *hrt1::HIS3* on either galactose medium (second panel) or glucose medium (not shown) as evidenced by the appearance of tetrads with more than 2 viable spores, at least one of which was His^+. Second, insertion of a myc9 epitope immediately upstream of the *HRT1* stop codon yielded a temperature-sensitive (ts) growth phenotype, whereas insertion of the same epitope immediately downstream of the stop codon (*HRT1-stop-myc9*) had no effect on growth (e.g., Figure 2B). A similar effect of the myc9 epitope was observed with *APC11* (Zachariae et al. 1998). *hHRT1* expressed from the *GAL1* promoter also complemented the lethality of *hrt1::HIS3* on galactose medium (third panel), but not on glucose medium (data not shown). Thus, human and yeast Hrt1 most likely perform the same biochemical function. In contrast, the more distantly related *hHRT2* did not complement *hrt1::HIS3* on galactose medium (bottom panel). Nevertheless, hHRT2 is likely to perform a function related to that of hHRT1, since a cDNA encoding amino acids 9-112 of hHRT2 was recovered in a two-hybrid screen for CUL-1 interacting proteins (data not shown).

The fortuitous *ts* growth phenotype of *HRT1myc9* strains allowed us to evaluate the roles of Hrt1 in cell division and degradation of SCF substrates. Cells with *ts* mutations in SCF/Cdc34 pathway components are sensitive to overexpression of SCF substrates (Bai et al. 1996, Deshaies et al. 1995, Willems et al. 1996). Overproduction of either $SIC1^{HA}$ or $CLN2^{HA3}$ from the *GAL* promoter prevented growth at 25 °C of *HRT1myc9* cells, but not wild type or *HRT1-stop-myc9*

cells (data not shown). A second phenotype of cells harboring *ts* mutations in SCF/Cdc34 pathway components is that they bud but fail to initiate DNA replication at the non-permissive temperature (Bai et al. 1996; Schwob et al. 1994). *HRT1myc9* and *HRT1-stop-myc9* cells grown in YP-glucose medium were elutriated to obtain a population of small, unbudded G1 cells, which were re-inoculated into fresh YP-glucose medium at 37 °C. Cells harvested at 15 min. intervals were evaluated for DNA content, cell volume, and budding index (Figure 2B). Whereas most *HRT1-stop-myc9* cells initiated budding and DNA replication at ~90-105 min, *HRT1-myc9* cells did not enter S phase or form mitotic spindles, and exhibited a strong delay in bud emergence. Like *HRT1myc9*, *cdc34*^{ts} cells are also delayed in bud emergence (Schwob et al. 1994), and arrested cultures of *skp1-11* mutants contain unbudded cells (Bai et al. 1996). These observations suggest that SCF antagonizes an inhibitor of bud emergence. Consistent with their failure to duplicate DNA, Sic1 persisted at high levels in elutriated *HRT1myc9* cells, but was degraded concomitant with bud emergence in *HRT1* cells (Figure 2C).

To investigate the role of *HRT1* in turnover of substrates independent of its effects on cell cycle progression, *SIC1* and *CLN2* expression were transiently induced in G2-arrested wild type and *HRT1myc9* cells incubated at a semi-restrictive temperature, and the decline in Sic1 (Figure 2D) and Cln2 (Figure 2E) levels were monitored by immunoblotting. Comparison of protein levels (Figure 2D and 2E, top panels) at different time points after repression of transcription (middle panels) indicates that both Sic1 and Cln2 were stabilized in *HRT1myc9* cells. For unknown reasons, *HRT1myc9* had a stronger effect on Sic1 turnover than on Cln2 turnover. Densitometric scanning of the experiment in Figure 2E revealed that the half-life of Cln2 was 8 min in wild-type cells and 19 min in mutant cells. Flow cytometry confirmed that both wild type and mutant cells remained arrested in G2/M throughout the duration of the time courses (bottom panels). Although a protein target of the SCF^{Met30} complex has not yet been described, mutations in *CDC53*, *SKP1* and *MET30* prevent repression of *MET25*mRNA in response to high extracellular methionine (Patton et al., 1998a). Likewise, *HRT1myc9* cells were also deficient in

MET25 repression (Figure 2F). Thus, the activities of three distinct SCF complexes – SCF^{Cdc4}, SCF^{Grr1} and SCF^{Met30} – were diminished in *HRT1myc9* cells.

Assembly of recombinant Hrt1 with SCF^{Cdc4} subunits

To evaluate the assembly of Hrt1 with SCF subunits in vitro, glutathione-S-transferase-Hrt1 (GST-Hrt1) purified from E. coli was mixed with insect cell lysates containing recombinant SCF^{Cdc4} subunits, and proteins recovered on glutathione-agarose were detected by immunoblotting (Figure 3A). GST-Hrt1 presumably folded correctly, since it stimulated SCF^{Cdc4} E3 activity (Figure 4A). Whereas Skp1 (Figure 3A, lane 4) did not bind GST-Hrt1, ~20% of ^{PyHA}Cdc4 (lanes 1,2) and ~20% of $Cdc53^{PyHA}$ (lane 3) bound to GST-Hrt1 (middle panel), but not GST (bottom panel). The high efficiencies of binding suggest that interaction of GST-Hrt1 with Cdc4 and Cdc53 was direct and did not require intermediary insect cell proteins. MBP-$Cdc34\Delta C^{HA}$ purified from E. coli also bound purified GST-Hrt1 (lanes 5), but not GST. Similar protein interactions were observed upon co-expression of Hrt1 with SCF^{Cdc4} subunits in insect cells (Figure 3B). Hrt1 was retrieved on an anti-polyoma antibody matrix (α-Py beads) from insect cell lysates containing co-expressed SCF^{Cdc4} (lane 4; Cdc4 was tagged with PyHA epitope), ^{PyHA}Cdc4 (lane 1) or $Cdc53^{PyHA}$ (lane 2). Unexpectedly, recovery of Hrt1 bound to ^{PyHA}Cdc4 was diminished upon co-expression of Skp1, suggesting that Skp1 and Hrt1 may compete for binding to overlapping sites on Cdc4. Hrt1 was not recovered on α-Py beads upon co-expression with an untagged control protein ($Skp1^{HA}$; lane 3).

Hrt1 stimulates SCF^{Cdc4} activity in vitro

Ubiquitin ligase activity specific for phosphorylated Sic1 can be reconstituted with recombinant Skp1, Cdc53 and Cdc4 (Feldman et al. 1997; Skowyra et al. 1997). The identification of Hrt1 as a fourth essential subunit of the SCF complex required for normal rates of Sic1 and Cln2 proteolysis in vivo raised the question as to whether Hrt1 functions in substrate ubiquitination. To address this issue, we evaluated the effect of recombinant Hrt1 on SCF^{Cdc4}-dependent ubiquitination of Sic1 in vitro. Sf9 cells were infected with baculoviruses encoding

Skp1, Cdc53 and Cdc4, and recombinant SCF complexes were retrieved on α-Py beads. A deliberate excess of phosphorylated MBP-Sic1 substrate was incubated with immobilized SCF^{Cdc4} in the presence of E1 enzyme, Cdc34, ubiquitin, ATP and either GST or GST-Hrt1 purified from *E. coli*. As shown in Figure 4A, GST-Hrt1 potently stimulated ubiquitination of phospho-MBP-Sic1. A similar result was obtained by evaluating SCF^{Cdc4} activity obtained from insect cells co-expresseing Hrt1 and SCF subunits (Figure 4B). The potent (> 5-fold) stimulatory effect of Hrt1 upon Sic1 ubiquitination was not due to increased expression of SCF^{Cdc4} subunits (lanes 12, 13). Notably, Hrt1 augmented both the rate (as measured by percent conversion) and extent (as measured by MW of ubiquitin conjugates) of ubiquitination.

The F box protein Grr1 is required for Cln1 and Cln2 proteolysis (Barral et al., 1995; Patton et al., 1998a). Whereas recombinant SCF complexes assembled with Grr1 (SCF^{Grr1}) bind phospho-Cln1, they do not support its ubiquitination (Skowyra et al., 1997). Since Hrt1 is required for SCF function in vivo and stimulates SCF^{Cdc4} activity in vitro, we reasoned that Hrt1 might be essential for SCF^{Grr1} function. Recombinant SCF^{Grr1} complexes that either contained (Figure 4C, lane 3) or lacked (lane 2) yeast Hrt1 were expressed in insect cells. Complexes retrieved on α-Py beads were then mixed with Sf9 cell lysate that contained $Cln2^{myc}$/GST-Cdc28/Cks1 substrate. Whereas SCF^{Grr1} complexes were essentially inactive (lane 2), those that contained Hrt1 sustained Cdc34-dependent ubiquitination of Cln2 (lanes 3,4). Taken together with the previous data, these observations suggest that Hrt1 is an essential core component that contributes to the E3 activity of all SCF complexes.

Cdc53/Hrt1 defines a novel ubiquitin ligase module

To characterize the mechanism of action of Hrt1/SCF^{Cdc4}, we sought to identify a minimal ubiquitin ligase domain. Purified Cdc34 ubiquitinates itself upon incubation with E1 and ubiquitin, but this reaction proceeds slowly (Banerjee et al. 1993). Cdc34 lacking24 residues from the C-terminus (Cdc34ΔC) no longer detectably ubiquitinates itself (R.M.R.F., unpublished data), but nevertheless complements *cdc34Δ* (Kolman et al. 1992). We reasoned that the ubiquitin ligase activity of SCF might promote autoubiquitination of either intact Cdc34 or

Cdc34ΔC; this simplified system would allow us to monitor SCF^{Cdc4} ubiquitin ligase activity independently of substrate targeting requirements. Strikingly, SCF^{Cdc4} complexes reconstituted with yeast Hrt1 potently activated Cdc34 (data not shown) and Cdc34ΔC (Figure 5A, top panel, lane 4) autoubiquitination, whereas SCF^{Cdc4} complexes lacking yeast Hrt1 had much less activity (lane 5). Hrt1 did not act by simply enhancing the levels of SCF^{Cdc4} subunits recovered from insect cells (Figure 4B, lanes 12-13). To determine if this activity of SCF^{Cdc4} resides in a subdomain of the complex, individual subunits were affinity purified from baculovirus-infected Sf9 cells and tested for activity. $Cdc53^{PyHA}$ (Figure 5A, lane 2), but not equivalent amounts of ^{PyHA}Cdc4 (lane 3) or $Skp1^{HA}$ (data not shown), promoted Cdc34ΔC autoubiquitination. The activity (top panel), but not the yield (bottom panel) of $Cdc53^{PyHA}$ was substantially enhanced by assembly with Hrt1 (lane 6). Hrt1 bound to ^{PyHA}Cdc4, however, was not sufficient to catalyze Cdc34ΔC autoubiquitination (top panel, lane 7) even though it was present in large amounts (middle panel). The activity observed for Cdc53/Hrt1 subcomplexes was consistently less than that of Hrt1-containing SCF^{Cdc4} complexes. Thus, although the ubiquitin ligase activity of SCF^{Cdc4} is intrinsic to the Cdc53/Hrt1 subcomplex, this activity may be enhanced upon assembly of Cdc53/Hrt1 with Cdc4/Skp1.

E3 enzymes of the HECT domain family catalyze substrate ubiquitination by providing a catalytic cysteine residue that accepts ubiquitin from E2 enzyme, and subsequently transfers the thioesterified ubiquitin to the ε-amino group of a substrate lysine residue (Scheffner et al. 1995). To address whether the Cdc53/Hrt1 ligase domain of SCF^{Cdc4} operates by a similar mechanism, we preincubated ^{PyHA}Cdc53/Hrt1 with increasing amounts of the sulfhydryl-modifying reagent NEM, added DTT to quench further cysteine modification, and mixed the resulting samples with Cdc34, E1, ubiquitin and ATP. As a control, we evaluated the NEM-sensitivity of E1 enzyme. Whereas E1 was completely inactivated by pretreatment with 0.2 mM NEM (but not when the NEM was pre-quenched with DTT, compare lanes 4 and 5 of Figure 5B), ^{PyHA}Cdc53/Hrt1 was resistant to pretreatment with up to 5 mM NEM. Similarly, ubiquitin ligase activity of intact SCF^{Cdc4} towards either Cdc34ΔC or phosphorylated Sic1 was not affected by NEM (R.M.R.F.,

unpublished data). To test the possibility that exposure of the putative thioester-forming cysteine of Cdc53/Hrt1 is gated by association of Cdc53/Hrt1 with E2 enzyme, we mixed together E1, Cdc34, ubiquitin, ATP, and either mock beads or beads containing ^{PyHA}Cdc53/Hrt1. After a brief incubation, NEM was added to derivitize reactive cysteines, followed by DTT to quench the NEM. Treated beads were sedimented, washed, and mixed with fresh E1, Cdc34, ubiquitin, and ATP. As a control, a parallel two-stage reaction was carried out in which DTT was added prior to NEM during the first stage. As shown in Figure 5C, ^{PyHA}Cdc53/Hrt1 beads retained high autoubiquitination-promoting activity if incubated with NEM followed by DTT (lane 2), or vice versa (lane 4) during the first stage.

In the course of this work, we made the unexpected observation that polycations influence Cdc34 activity. Remarkably, polylysine (Figure 6A, lane 5), but not PEG (lanes 2-4) or lysine (lane 1) markedly stimulated Cdc34ΔC autoubiquitination. As an independent test, we evaluated the effect of polycations on Gcn4 ubiquitination. Gcn4 is stabilized in *cdc34^{ts}* cells (Kornitzer, et al. 1994), and Gcn4 ubiquitination can be reconstituted in vitro with recombinant SCF^{Cdc4} and Cdc34 (Y.C., unpublished data). Gcn4 was not detectably modified in the presence of E1, Cdc34, ATP and ubiquitin (Figure 6B, lane 2), but high molecular weight K48-linked multiubiquitin chains were assembled on Gcn4 upon inclusion of polyethyleneimine (lanes 6,7). Thus, polyethyleneimine acts like a classic E3. The ability of polycations to substitute for SCF strongly suggests that an E3-linked ubiquitin thioester intermediate is not required to evoke the intrinsic ability of Cdc34 to assemble K48-linked multiubiquitin chains.

We considered two possible artifactual roles of polycations. First, it is unlikely that polycations serve simply as macromolecular crowding reagents, since their stimulatory effects were observed at relatively low concentrations (300 nM for polylysine and 0.0005% for polyethyleneimine), and were not mimicked by PEG (Figure 6A). An alternative possibility is that polycations serve to link together ubiquitin donor and ubiquitin acceptor molecules. We

sought to address this by fusing the ubiquitin-acceptor domain of Sic1 to the C-terminus of Cdc34ΔC. The rationale underlying this experiment is that if SCF^{Cdc4} or E3 mimetics simply bring Sic1 and Cdc34 into close proximity, then direct fusion of substrate to E2 might bypass the requirement for E3. As shown in Figure 6C, both SCF^{Cdc4} and $Cdc53^{PyHA}$ potently stimulated Cdc34ΔC-Sic1 ubiquitination, whereas $Skp1^{HA}$ had no effect beyond what is observed in the presence of E1, ubiquitin, and Cdc34ΔC (data not shown). Thus, bringing a substrate into close proximity to Cdc34 is not sufficient to activate its ubiquitination.

Discussion

Hrt1, a new subunit of SCF ubiquitin ligase complexes

We have identified by mass spectrometric sequencing of proteins that co-immunoprecipitate with Cdc53 a new subunit of the SCF ubiquitin ligase complex, Hrt1. Hrt1 and the homologous Apc11 protein of APC/C both contain RING-H2 domains (Zachariae et al. 1998). *HRT1* is essential, and $hrt1^{ts}$ mutants – like $cdc34^{ts}$, $cdc4^{ts}$, $cdc53^{ts}$, and $skp1^{ts}$ mutants – fail to (i) tolerate overexpression of Cln2 and Sic1, (ii) transit from G1 to S phase, (iii) properly degrade the SCF^{Cdc4} substrate Sic1 and the SCF^{Grr1} substrate Cln2, and (iv) regulate properly the expression of *MET25*mRNA. Human *HRT1* (*hHRT1*) complements the lethality of *hrt1Δ*, and human HRT2 binds human CUL-1 in a two hybrid assay. Hrt1 independently binds to Cdc4, Cdc53 and Cdc34, but not to Skp1. SCF^{Cdc4} complexes assembled with yeast Hrt1 have much higher ubiquitin ligase activity towards phospho-Sic1. Moreover, SCF^{Grr1} complexes containing yeast Hrt1, but not those lacking the protein, promote ubiquitination of the G1 cyclin Cln2.

SCF^{Cdc4} ubiquitin ligase activity toward phospho-Sic1 can be reconstituted upon expression of the yeast Skp1, Cdc53 and Cdc4 proteins in insect cells (Feldman et al. 1997; Skowyra et al. 1997). This raises a simple question: is Hrt1 absolutely required for SCF^{Cdc4} function, or does it merely stimulate the basal activity of the heterotrimeric complex? Unambiguous resolution of this question will require the expression of SCF subunits in a system that lacks Hrt1 homologs (e.g., E. coli), or assembly of SCF complexes with mutant subunits that are selectively defective

in their ability to recruit Hrt1. We favor the notion that Hrt1 is essential for the E3 activity of all SCF complexes; an insect cell homolog of the highly conserved Hrt1 can weakly substitute for the yeast protein in the SCF^{Cdc4} complex, but perhaps not in SCF^{Grr1}.

Cdc53 and Hrt1 comprise a ubiquitin ligase module

To identify a minimal ubiquitin ligase module in SCF^{Cdc4}, we exploited the observation that Cdc34 can conjugate ubiquitin to itself (Banerjee et al., 1993). This reaction is not thought to be of physiological significance (Goebl et al., 1994). Nevertheless, we reasoned that Cdc34 autoubiquitination might serve as a surrogate to monitor the ubiquitin ligase activity of SCF in the absence of substrate targeting requirements. Although it is formally possible that Cdc34 autoubiquitination does not faithfully mimic the ubiquitin ligase activity of SCF towards natural substrates, this seems unlikely given the potent effects of SCF and Cdc53/Hrt1 in this assay. Furthermore, activated Cdc34 assembles multiubiquitin chains upon itself with the appropriate K48 linkage (R.M.R.F., unpublished data). Whereas efficient transfer of ubiquitin from Cdc34 to phospho-Sic1 requires all four subunits of SCF^{Cdc4}, either Cdc53 or a subcomplex of Cdc53/Hrt1 can partially substitute for intact SCF^{Cdc4} to promote assembly of multiubiquitin chains on Cdc34; in contrast, Hrt1 retrieved in a complex with Cdc4 is inactive.

How does the Cdc53/Hrt1 ubiquitin ligase module work?

Four distinct classes of ubiquitin ligases have been defined; the mechanism of action of only one - the HECT domain proteins - has been described (Scheffner et al., 1995). The HECT domain ubiquitin ligase E6-AP collaborates with a member of the UbcH5 family of E2s and human papillomavirus E6 protein to specify ubiquitination of p53 (Scheffner et al., 1993). Ubiquitin destined for attachment to p53 is transferred from E2 to the conserved cysteine 833 residue of E6-AP (Scheffner et al., 1995). The ubiquitin linked to E6-AP is subsequently transferred to a lysine residue of p53. Mutation of cysteine 833 destroys E6-AP's E3 activity, indicating that the E6-AP~ubiquitin thioester is an essential intermediate in the transfer of

ubiquitin from E2 to p53. Since the SCF, APC and Ubr1 E3s do not contain obvious HECT domains, it is unclear whether they catalyze ubiquitination by a similar mechanism.

Three lines of evidence lead us to suggest that SCF promotes Cdc34-dependent ubiquitination of substrates by a novel mechanism that does not involve an E3~ubiquitin thioester intermediate. First, Cdc34 is able to assemble K48-linked multiubiquitin chains in the absence of an E3 (Banerjee et al. 1993). Thus, the active site of Cdc34 is inherently capable of assembling appropriate multiubiquitin chains in the absence of an additional catalytic center. Second, whereas enzymes that use cysteine residues for catalysis (including ubiquitin pathway components E1 and E2) are typically sensitive to alkylating agents such as N-ethylmaleimide (NEM) and iodoacetamide (Hershko et al. 1983), Cdc53/Hrt1-dependent autoubiquitination of Cdc34 is indifferent to NEM. The NEM resistance of Cdc53/Hrt1 is consistent with previous studies showing that mutation of all cysteine residues within Cdc53 does not impair its biological function (Patton et al. 1998a). Third, compounds lacking sulfhydryls-polylysine and polyethyleneimine- can substitute for SCF^{Cdc4} and promote the assembly of K48 linked multiubiquitin chains on either Cdc34 or the SCF^{Cdc4}/Cdc34 pathway substrate Gcn4.

If Cdc53/Hrt1 does not activate ubiquitination of Cdc34 substrates by providing a catalytic thiol residue, how does it promote ubiquitin transfer? We propose that Cdc53/Hrt1 stabilize the oxyanion that is formed in the transition state as the carboxyl terminus of ubiquitin is transferred from Cdc34 to the ε-amino group of a substrate lysine. Cdc53/Hrt1 (or polycations) may directly provide a positive charge to neutralize the oxyanion, or may elicit a favorable conformational change in Cdc34. Hrt1 directly binds both Cdc34 and components of the SCF complex, placing it in an optimal position to coordinate the transfer of ubiquitin from Cdc34 to substrate.

SCF and APC/C: archetypes of a family of cullin-based ubiquitin ligases

SCF complexes can be subdivided into three distinct functional domains: the F box subunit recruits substrate (Feldman et al. 1997; Skowyra et al. 1997); the Skp1 subunit links the F box substrate receptor to the remaining subunits; and Cdc53 (Willems et al. 1996) and Hrt1 subunits

recruit the E2 enzyme and comprise the ubiquitin ligase core. Of the known members of the cullin family, the Apc2 subunit of APC/C is the most diverged from Cdc53 (Zachariae et al. 1998; Yu et al. 1998). Since APC/C nevertheless also contains an Hrt1-like protein (Apc11) (Zachariae et al. 1998), we suggest that SCF and APC/C define an extended family of multisubunit E3s that are elaborated from a basic ubiquitin ligase module comprised of a cullin (CUL-1 through CUL-5, APC2) and a RING-H2 finger protein (HRT1, HRT2 or APC11). The RING-H2/cullin module in turn can be linked to families of putative substrate receptors, three of which have been defined so far: the F box proteins for SCF (Bai et al., 1996), the SOCS box proteins for the presumed CUL-2 based ubiquitin ligases (Kamura et al. 1998) and the WD-40 repeat proteins Cdc20 and Hct1/Cdh1 for APC/C (Schwob et al. 1997; Visintin et al. 1997; Fang et al. 1998). Thus, many distinct ubiquitin ligase complexes can be assembled from the RING-H2/cullin ubiquitin ligase module.

Several other proteins implicated in ubiquitin-dependent proteolysis, including SINA, Der3/Hrd1 and the ubiquitin ligases Ubr1 and Mdm2, contain a putative RING finger (Kwon et al. 1998; Suarin et al. 1996). Both Ubr1 (F. Du and A. Varshavsky, personal communication) and Mdm2 (Honda et al. 1997) are thought to function without benefit of a cullin subunit. Intriguingly, E3a, which is the mammalian homolog of Ubr1, is resistant to NEM treatment (Hershko et al. 1983). Although Cys464 has been proposed to define a divergent HECT-like catalytic center within Mdm2 (Honda et al. 1997), this residue also occupies a conserved position within Mdm2's putative RING finger (Suarin et al. 1996). Without a definitive demonstration that ubiquitin thioesterified to Mdm2 can be chased to substrate, it is judicious to keep open the possibility that Mdm2 is in fact a member of a distinct family of RING-containing ubiquitin ligases. The RING finger may have been recruited early during eukaryotic evolution to serve as a basis for the elaboration of a broad range of cullin-dependent and –independent ubiquitin ligases.

Materials and Methods

Strains, Media, and Reagents

All yeast strains were derivatives of W303 (*HMLα* or *HMRa ho ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 ssd1*). Yeast strains were grown in YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) supplemented with 2% of glucose (YPD), raffinose, or raffinose plus galactose. Nocodazole (15 μg/ml) was added to arrest cells in mitosis. Rabbit polyclonal antisera against Sic1 (Mike Tyers), Cdc4 and Cdc34 (Zach Pitluck), and rat α-tubulin monoclonal antibody YOL1/34 (John Kilmartin) were generously provided by the indicated investigators. Rabbit polyclonal α-Skp1 and α-Hrt1 antibodies were raised against bacterially-expressed GST-Skp1 and His6-Hrt1, respectively. Production of polyclonal α–Cdc53 and cross-linked α-polyoma monoclonal antibody beads were described previously (Feldman et al. 1997). Methyl ubiquitin was purchased from Boston Biochem.

Strain and plasmid constructions

To epitope tag yeast genes, cassettes encoding an epitope tag, a 3' UTR, and a selectable marker were amplified by PCR with target gene-specific primers and integrated at the respective genomic locus; detailed descriptions of the tagging cassettes are available upon request. Strains containing one or five copies of a *GAL* promoter-*SIC1-HA1* fusion integrated at the *ura3* locus were generously provided by Etienne Schwob. *GAL-SIC1-HA1 HRT1-myc9* and *GAL-SIC1-HA1 HRT1-stop-myc9* strains were obtained by genetic crosses.

To generate plasmids expressing yeast or human Hrt1 in bacteria, yeast or insect cells, the appropriate open reading frames were amplified by PCR and inserted into expression vectors for E. coli (pGEX-4T-1 or pMAL-c2), S. cerevisiae (pRD54), or a baculovirus transfer vector (pFastBac1). Cdc34ΔC and MBP-Cdc34^{HA} were created by replacing the 24 C-terminal residues of Cdc34 with six histidines or the HA epitope and cloning the resulting reading frames into pET11b or pMAL-c2, respectively. A baculovirus expressing PyHA epitope-tagged Grr1 was obtained by annealing a set of four oligos containing a Drosophila codon-optimized sequence

encoding the N-terminal region of Grr1. This segment was followed by the remainder of the yeast *GRR1* sequence and the entire gene cloned into pFastbac. Sequences of the oligos and the details of cloning strategies used are available upon request.

Preparation of yeast cell extracts and immunoprecipitations

Exponentially growing yeast cells were pelleted, washed with water, and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl, 0.1 mM EDTA, 0.2% Triton X-100) containing protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1μg/ml aprotinin). Cells were lysed with glass beads by 10 cycles of vortexing for 40 sec followed by a 1 min incubation on ice. After centrifugation (30 min, 100,000g), extracts (7 mg) were incubated with monoclonal antibody 9E10 cross-linked to protein A-Sepharose (9E10 beads, 20 μL) for 90 min at 4 °C. Beads were washed four times with 1 ml of lysis buffer lacking protease inhibitors, and once with 1 ml of 50 mM Tris-HCl, pH 7.8. Proteins were eluted from the beads in a minimal amount of 50 mM Tris-HCl, pH 7.8 plus 0.2% SDS, lyophilized, resuspended in SDS-PAGE sample buffer, and applied to a 8-16% gradient SDS-polyacrylamide gel. Protein bands were visualized by silver staining (Harlow and Lane 1988). The larger-scale prep used to obtain Hrt1 for mass spectrometry-based sequencing was performed as described above, except that 600 mg of crude extract protein from *CDC53myc9* cells was applied to a 30 ml DEAE column, and the 0.35 M NaCl eluate was collected essentially as described (Deshaies et al. 1995). The resulting 'fraction II' (100 mg) was mixed with 250 μl 9E10 beads, and the immunoprecipitates were processed as described above except that proteins were visualized by Coomassie Blue stain following SDS-PAGE.

Mass Spectrometry

A 17 kD Coomassie stained band present in Cdc53myc9 immunoprecipitates was excised and in-gel digested with trypsin (Boehringer Mannheim, unmodified, sequencing grade) as described (Shevchenko et al. 1996). MALDI MS analysis of an aliquot withdrawn from the digest failed to identify proteins and therefore the recovered pool of tryptic peptides was subsequently analyzed by nanoelectrospray tandem mass spectrometry (Nano ES MS/MS)

(Wilm et al. 1996). Nano ES MS/MS was performed on a API III triple quadrupole mass spectrometer (PE Sciex, Ontario, Canada) equipped with a nanoelectrospray ion source (Wilm and Mann 1996) developed at EMBL. Short sequence stretches were determined from tandem mass spectra of individual tryptic peptides and, in combination with the masses of the intact peptides, were assembled into peptide sequence tags (Mann and Wilm 1994) and used to search a comprehensive sequence database with PeptideSearch v.3.0 software developed at EMBL.

Analysis of Hrt1 function in vivo

Small G1 cells were isolated by centrifugal elutriation from cultures grown at 25 °C in YPD medium as described (Schwob and Nasmyth 1993). A Becton-Dickinson FACScan was used for flow cytometric measurement of cellular DNA content (Epstein and Cross 1992). Spindles were detected by indirect immunofluorescence using anti-tubulin antibodies (YOL1/34) and FITC-conjugated secondary antibodies. To measure cell volume, cells were fixed in YPD medium containing formaldehyde (3.8%) for 4 h on ice. About 20,000 cells were counted with a CASY1 Cell Counter Plus Analyser (Model TTC, Schaerfe System, Reutlingen, Germany) and peak cell volume was measured in triplicate.

Swi6 and Sic1-HA1 were detected with rabbit polyclonal antibodies. Detection with monoclonal α-HA antibodies gave similar results for Sic1-HA1. RNA isolation and Northern blot analysis were performed as described (Cross and Tinkelenberg 1991; Price et al. 1991). Fragments from the coding regions of *SIC1* and *CMD1* were used as probes.

Protein Expression

GST-Hrt1, Cdc34ΔC^{His6}, and MBP-Cdc34ΔC^{HA} proteins were expressed in E. coli BL21(DE3)+pLysS cells and purified by glutathione, Ni-NTA and amylose affinity chromatography as directed by the resin suppliers, respectively. MBP-Cdc34ΔC^{HA} formed thioesters with ubiquitin, served as an E2 in Sic1 ubiquitination reactions, and could autoubiquitinate in the presence of Cdc53 (R.M.R.F., unpublished observations). Baculoviral expression of SCF subunits was as described (Feldman et al. 1997). Cln2^{myc}/GST-Cdc28/Cks1/Cak1 proteins were expressed in Sf9 insect cells infected with the respective recombinant baculoviruses in a 10:5:3:1 ratio.

GST pull-downs, α-Py IP from insect cells

GST pull-downs contained 2.5 μg of either GST or GST-Hrt1, 50 μL (~200 μg) lysate from insect cells infected with the indicated baculoviruses, and 100 μL of BVLB containing 1 mg/mL BSA. Reactions were incubated 15 min at 4 °C, supplemented with 7 μL of glutathione-agarose beads (Sigma), and incubated a further 15 min at 4 °C. Beads were collected by brief centrifugation and washed four times in BVLB fortified with 100 mM NaCl (250 mM final). After washing, beads were aspirated dry and resuspended in 1xSDS-PAGE buffer, boiled, and analyzed by SDS-PAGE and Western blotting.

Experiments utilizing baculovirus-expressed Hrt1 (Figure 3B) contained 100 μL of lysate from insect cells coinfected with Hrt1-expressing and other viruses as indicated. Lysates were incubated with 7 μL α-Py beads for 20 min at 4 °C, and were washed and analyzed as described above.

In vitro assays

To analyze Cdc34ΔC^{His6} autoubiquitination, 200 μL of lysate (~4 mg/ml) from insect cells infected with the indicated baculovirus(es) was incubated with 7 μL of α-Py beads for 1 h at 4°C. The beads were then washed three times in BVLB (30 mM Hepes pH 7.4, 150 mM NaCl, 0.1% Triton X-100), three times in KAB (50 mM Hepes pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, aspirated to dryness, and resuspended in reaction buffer (30 mM Tris, pH 7.4, 5 mM $MgCl_2$, 2 mM ATP) supplemented with 100 ng Uba1 (Feldman et al. 1997), 5 μg ubiquitin and 600 ng Cdc34Δ^{His6} (20 μl total volume). Reactions were incubated at 24 °C for 1 h with gentle mixing, and were stopped by addition of an equal volume of 2xSDS-PAGE sample buffer and boiled for 3 minutes. Samples were analyzed by SDS-PAGE and Western blotting using α-Cdc34 followed by HRP-conjugated α-rabbit secondary antibodies.

Sic1 ubiquitination reactions were performed as described (Feldman et al. 1997), except that 100 ng of phosphorylated MBP-Sic1 was used per reaction, and 200 μL (ca. 800 μg) of insect

cell lysate that contains polyoma-tagged SCF was immunoprecipitated with 7 μL Py beads and washed as described above, typically yielding 150-200 ng of SCF components per sample as estimated from Coomassie stained gels. For Figure 4A, 2.5 μg of purified GST or GST-Hrt1 was added to reactions that contained 4 μg of phospho-MBP-Sic1.

To test NEM sensitivity, α-Py immunoprecipitates were prepared as described above. NEM (5 mM unless otherwise indicated) was added to beads (or to Uba1 in the presence of an equal amount of beads) and incubated at 24 °C for 10 min, at which point a two-fold molar excess of DTT was added and the incubation was continued for 10 min. As a control, beads were treated with NEM that had been pre-mixed with two-fold excess DTT. After incubations, beads were washed with KAB and used in reactions as described; Uba1 treated in the presence of beads was used as is.

Sequence analysis

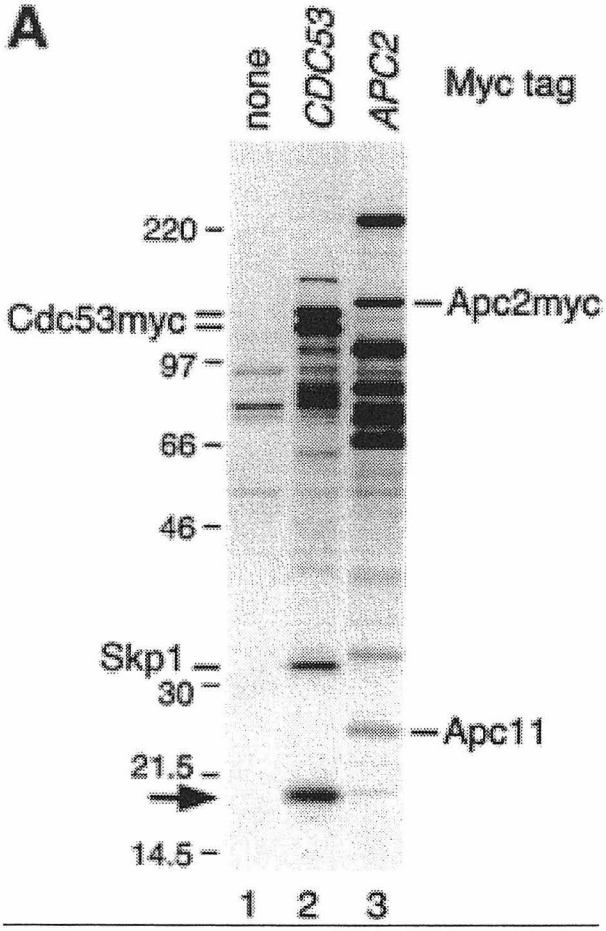
Databases were searched with Gapped BLAST (Altschul et al. 1997). ESTs were assembled using AssemblyLIGN (Oxford Molecular, Oxford, UK) and protein sequences were aligned with CLUSTAL W (Thompson et al. 1994) using the Blosum 62 matrix with default parameters.

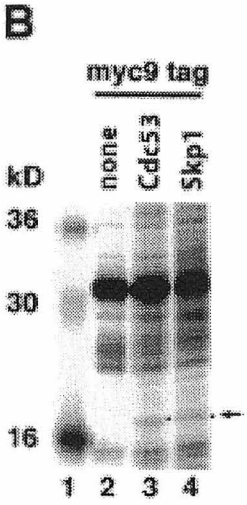
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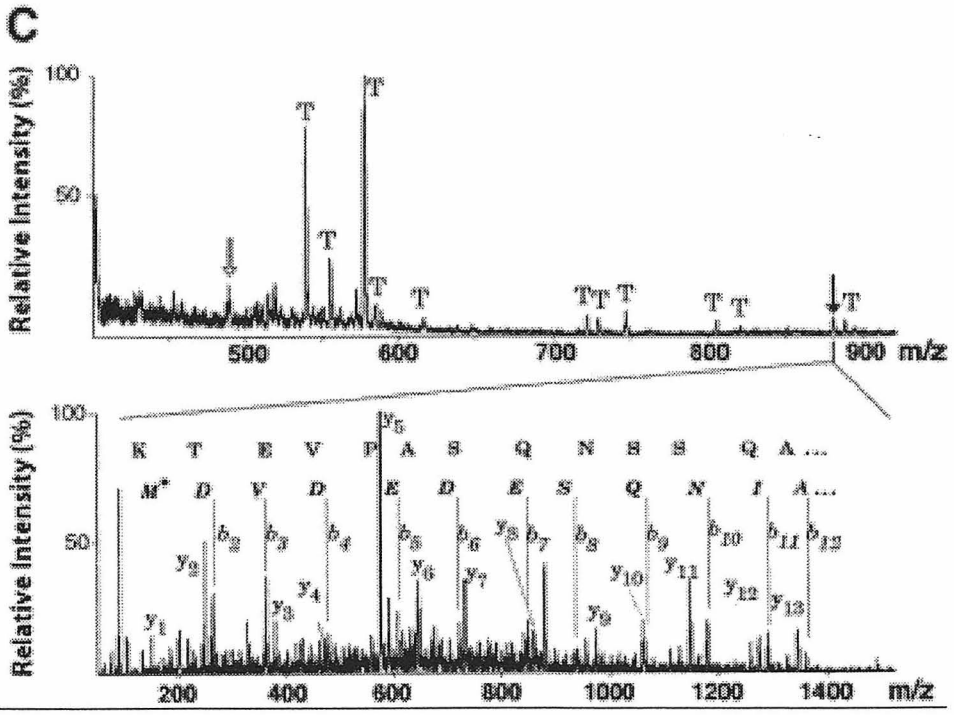
We thank S. Elledge, J. Kilmartin, Z. Pitluck, E. Schwob, and M. Tyers for supplying strains, baculoviruses, antibodies, libraries, and plasmids. We thank Y. Xiong for communicating unpublished data and V. Chau for stimulating discussion regarding the mechanism of SCF action. This work was supported by a grant from the NIH (GM52466-01, RJD), a Burroughs-Wellcome New Investigator Award in Basic Pharmacological Sciences (RJD), a Howard Hughes Medical Institute Predoctoral Fellowship (R.M.R.F.), and a Leukemia Society Postdoctoral Fellowship (C.C.).

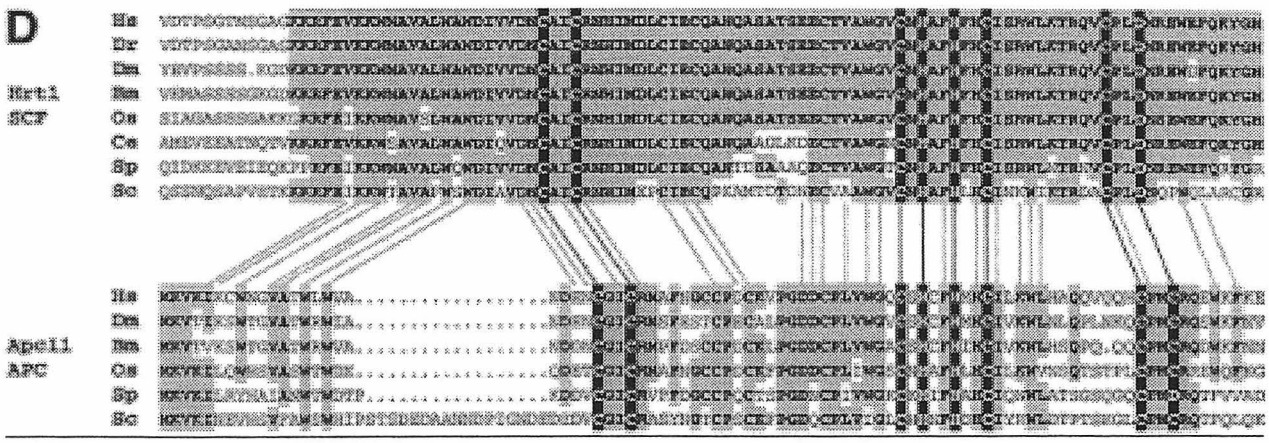
Figure 1: Identification of Hrt1 as a Cdc53-associated protein. **(A):** ^{35}S-methionine-labeled proteins that specifically co-immunoprecipitate with Cdc53myc6 (lane 2) or Apc2myc6 (lane 3) were visualized by SDS-PAGE followed by autoradiography. **(B):** Proteins co-immunoprecipitating with Cdc53myc9 (lane 3) or Skp1myc9 (lane 4) were separated by SDS-PAGE, and stained with silver. Both preparations consistently contained a 17 kD protein (arrowheads in panels A and B). **(C):** Identification of YOL133w by nanoelectrospray tandem mass spectrometry (Nano ES MS/MS). Upper panel: partial mass spectrum of a pool of tryptic peptides recovered after *in-gel* digestion of 17 kD band. Peaks designated with T were identified as trypsin autolysis products. Tandem mass spectrum acquired from the ion with m/z 488.2 (unfilled arrow) identified peptide AFLIEEQK from ribosomal protein RPL34B (data not shown). Tandem mass spectrum (lower panel) of the ion with m/z 880.4 (filled arrow) revealed two non-overlapping series of fragment y-ions and b-ions (Biemann, 1988) which both identified the same Hrt1-derived peptide M*DVDEDESQNIAQSSNQSAPVETK (M* stands for methionine sulfoxide). C-terminal part of the peptide sequence (...AQSSNQSAPVETK) is covered by continuous series of y-ions. N-terminal part of the sequence (M*DVDEDESQNIA) is covered by b-ion series. Since every amino acid residue in the peptide sequence can be accounted for, identification of Hrt1 is certain despite the complexity of the spectrum and that only a single peptide was identified. **(D):** Hrt1 is highly conserved between species and related to the APC subunit Apc11. Budding yeast Hrt1 and Apc11 were each aligned with related sequences from different species. Putative Zinc-coordinating residues are highlighted black. Residues identical in at least 4 (Apc11) or 5 (Hrt1) sequences are shaded. Lines connecting the alignments indicate residues conserved between Apc11 and Hrt1. Hs, Homo sapiens; Dm, Drosophila melanogaster; Dr, Danio rerio; Bm, Bombyx mori; Os, Oryza sativa; Ce, Caenorhabditis elegans; Sp, Schizosaccharomyces pombe; Sc, Saccharomyces cerevisiae. **(E):** Hrt1 is an authentic subunit of SCF. Crude extracts from untagged control (lane 1) and *HRT1myc9* (lane 2) strains were evaluated in parallel by immunoblotting with 9E10 antibodies. Extracts from untagged (odd numbered lanes) or *HRT1myc9* (even-numbered lanes) cells were

immunoprecipitated with 9E10 antibodies, fractionated by SDS-PAGE, and immunoblotted with α-Cdc53, α-Cdc4, α-Skp1 or α-Cdc34 as indicated to reveal the corresponding proteins.


A
none
CDC53
APC2
Myc tag
220
Cdc53myc
97
66
46
Skp1
30
21.5
14.5
Apc2myc
Apc11
1 2 3


B
myc9 tag
kD
none
Cdc53
Skp1
36
30
16
1 2 3 4


C
Relative Intensity (%)
100
50
500
600
700
800
900
m/z
K T E V P A S Q N S S Q A ...
M* D V D E D E S Q N I A ...
200
400
600
800
1000
1200
1400


D
Hs
Dr
Dm
Mm
Os
Ce
Sp
Sc
Hs
Dm
Mm
Os
Sp
Sc
Hrt1
SCF
Apc11
APC

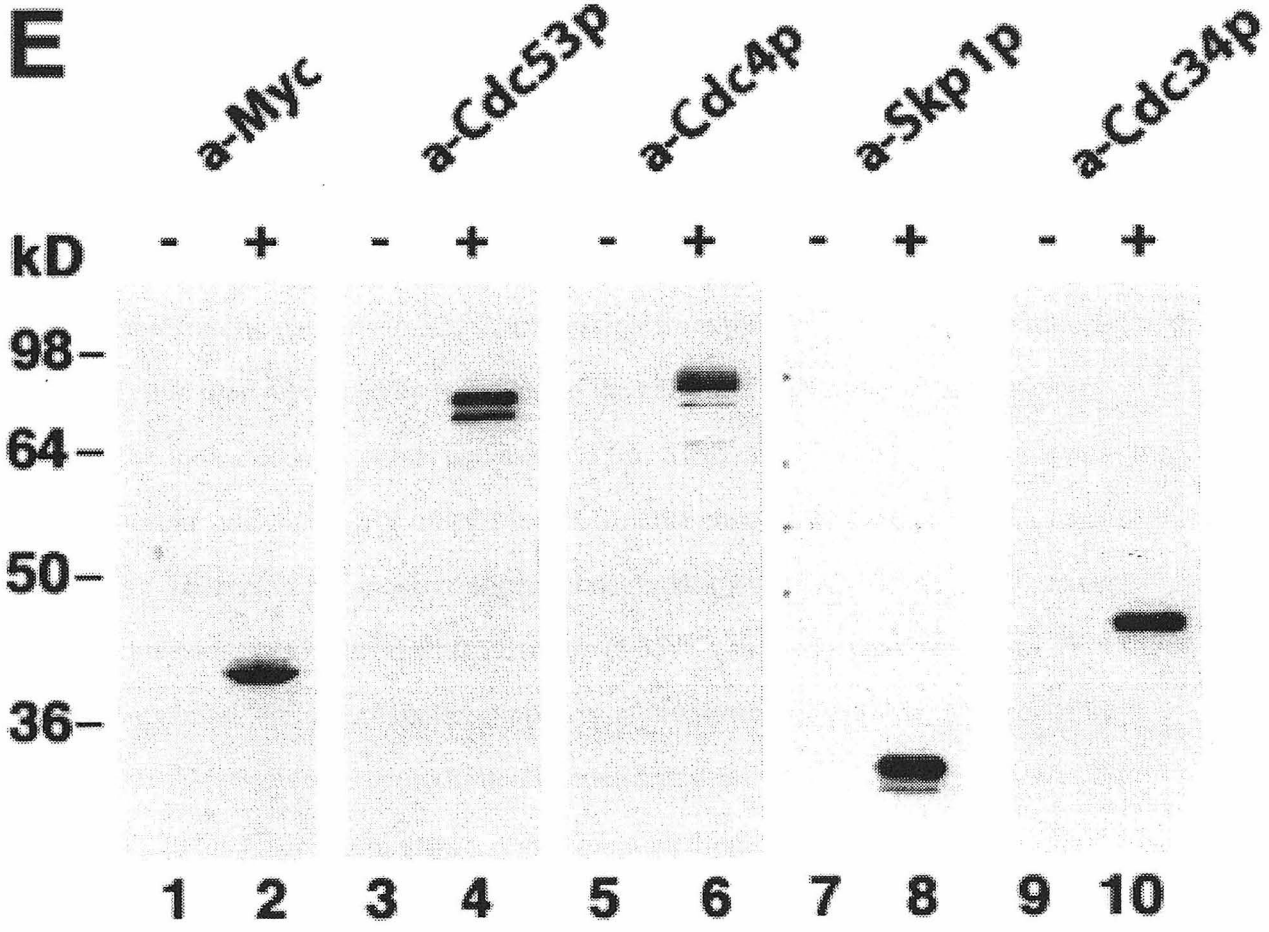
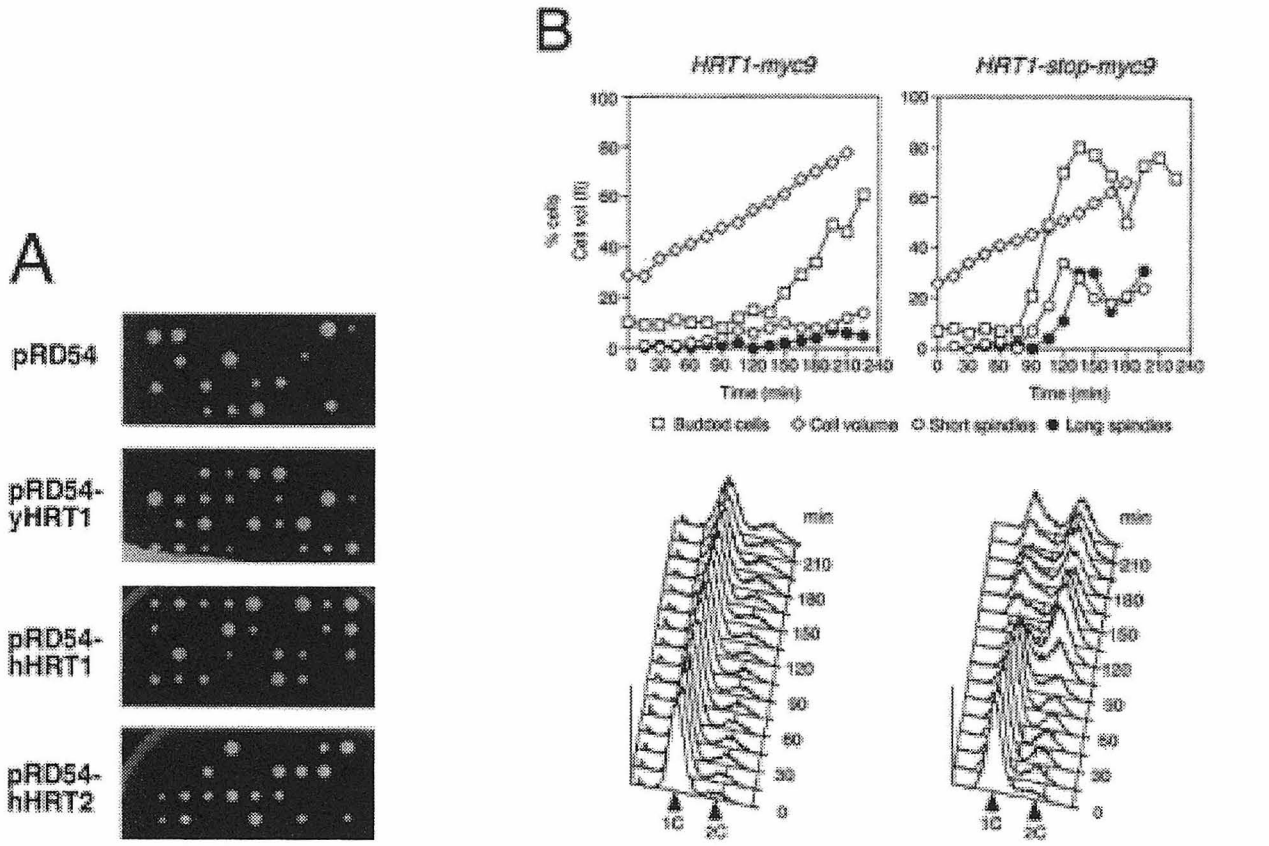
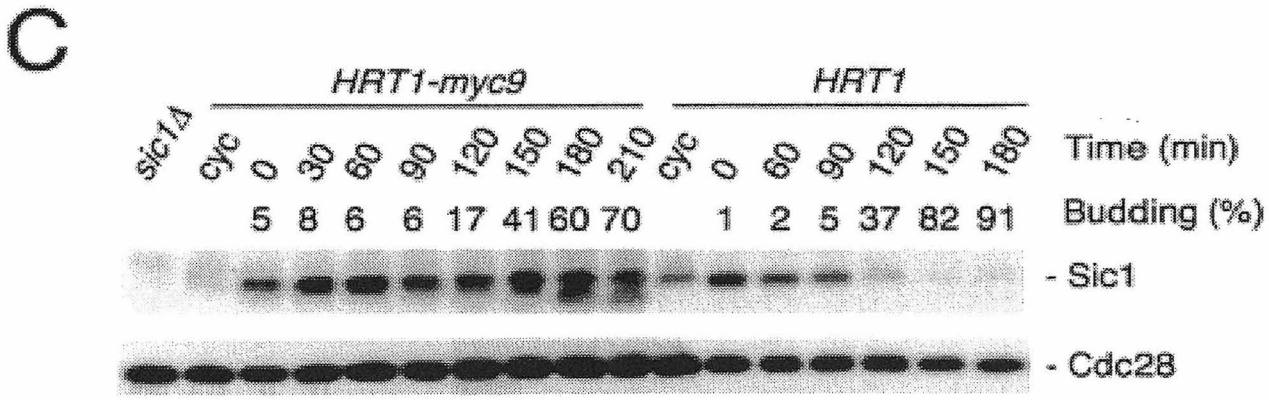
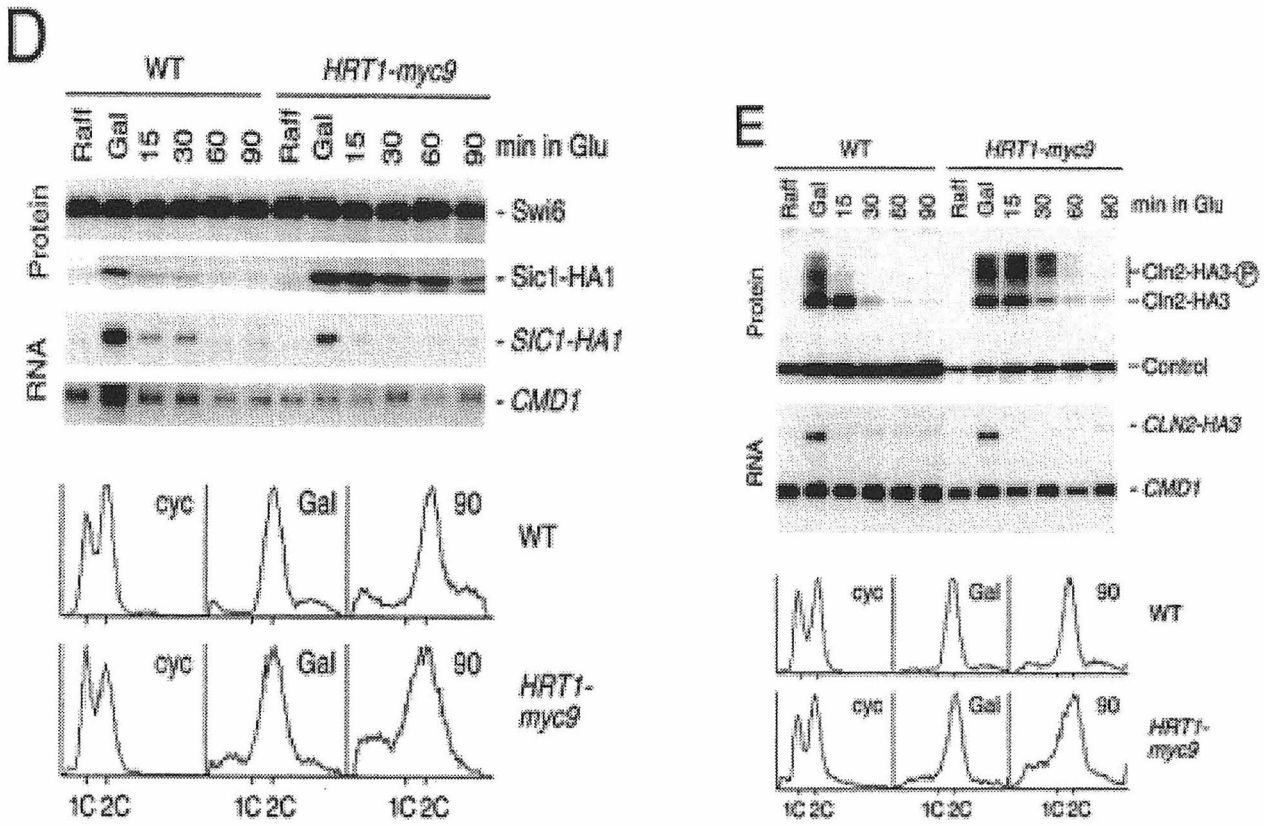

E
a-Myc
a-Cdc53p
a-Cdc4p
a-Skp1p
a-Cdc34p
kD
- + - + - + - + - +
98–
64–
50–
36–
1 2 3 4 5 6 7 8 9 10

Figure 1.

Figure 2: (A): *HRT1* is essential. A diploid *hrt1::HIS3/HRT1* strain was transformed with pRD54-based plasmids (pRS316 *CEN*, *URA3*) containing either yeast *HRT1*, human *HRT1*, or human *HRT2* under the control of the galactose-inducible *GAL1* promoter. Sporulated diploids were dissected on complete galactose medium, followed by incubation at 24 °C for 5 days. **(B, C):** *HRT1* is required for both elimination of Sic1 and for S phase. A myc9 epitope cassette was integrated at *HRT1* either immediately upstream (*HRT1-myc9*, left) or downstream (*HRT1-stop-myc9*, right) of the stop codon. Small G1 cells were isolated by centrifugal elutriation from cultures grown in YPD at 25 °C and inoculated into fresh medium at 37 °C at time 0. In (B), mean cell volume (fl), budding index, formation of mitotic spindles (top panels) and DNA contents (bottom panels) were assessed at the indicated times. In (C), budding index, Sic1 levels, and Cdc28 levels were monitored. **(D):** *HRT1* is required for proteolysis of Sic1. *HRT1* cells containing five integrated copies of *GAL-SIC1-HA1* and *HRT1-myc9 GAL-SIC1-HA1* (one copy) cells were grown in YP-raffinose at 25 °C (cyc) and arrested in mitosis with nocodazole (Raff). After shifting the cultures to 32 °C, expression from the *GAL* promoter was induced with galactose (Gal) and then repressed by transferring the cells to YP-glucose. Samples were withdrawn at the indicated time points and assayed for: Swi6 and Sic1-HA1 protein levels (top panels); calmodulin and *SIC1-HA1* mRNA levels (middle panels); and cellular DNA content (bottom panels). **(E):** *HRT1* is required for rapid degradation of Cln2. Same as (D), except *CLN2-HA3* expression was induced with galactose at 35 °C, and cells were shifted to 37 °C upon transfer to YP-glucose. The band labeled control is an unknown polypeptide recognized by 12CA5. **(F):** *HRT1* is required for methionine-dependent repression of *MET25* mRNA. Yeast strains indicated in the figure were grown in complete medium containing glucose and lacking methionine at 25 °C and were then shifted to 37 °C for two hours. Methionine was added to the medium and samples were withdrawn at the indicated times. *MET25* and *CMD1* mRNA levels were assayed by Northen blotting.


A
pRD54
pRD54-yHRT1
pRD54-hHRT1
pRD54-hHRT2
B
HRT1-myc9
HRT1-stop-myc9
% cells
Cell vol (fl)
Time (min)
Budded cells
Cell volume
Short spindles
Long spindles
min
1C
2C


C
HRT1-myc9
HRT1
sic1Δ
cyc
0
30
60
90
120
150
180
210
cyc
0
60
90
120
150
180
Time (min)
5 8 6 6 17 41 60 70
1 2 5 37 82 91
Budding (%)
- Sic1
- Cdc28


D
WT
HRT1-myc9
Raff
Gal
15
30
60
90
min in Glu
Protein
- Swi6
- Sic1-HA1
RNA
- SIC1-HA1
- CMD1
cyc
Gal
90
WT
HRT1-myc9
1C 2C
E
- Cln2-HA3-P
- Cln2-HA3
- Control
- CLN2-HA3
- CMD1


F
WT
HRT1-myc9
HRT1-stop-myc9
0
10
20
40
60
+ Met (min)
- MET25
- CMD1

Figure 2.

Figure 3: Hrt1 binds Cdc4, Cdc53, and Cdc34. **(A):** lanes 1-4: GST-Hrt1 (middle panel) or GST (bottom panel) purified from E. coli were mixed with crude insect cell lysates (top panel) that contained the proteins indicated above each lane. Proteins retrieved on glutathione agarose were fractionated by SDS-PAGE and revealed by immunoblotting with α-HA. Lane 5: same as lanes 1-4, except MBP-Cdc34ΔC^{HA} purified from E. coli was evaluated for its ability to bind GST-Hrt1. The asterisks denote a proteolytic fragment of MBP-Cdc34ΔC^{HA}. "Input" lanes contain 5% and "bound" lanes contain 25% of the material from a single binding reaction. **(B):** Lysates from Sf9 insect cells that co-expressed Hrt1 plus ^{PyHA}Cdc4 (lane 1), Cdc53^{PyHA} (lane 2), Skp1^{HA} (lane 3) or ^{PyHA}Cdc4/Cdc53/Skp1^{HA} (lane 4) were adsorbed to α-Py beads, and bound proteins were fractionated by SDS-PAGE and revealed by immunoblotting with α-HA (3rd row of panels) or α-Hrt1 (bottom row of panels) antibodies. Unfractionated insect cell lysates were directly immunoblotted to evaluate relative expression of the individual proteins (top row of panels, α-HA blot; second row of panels, α-Hrt1 blot).

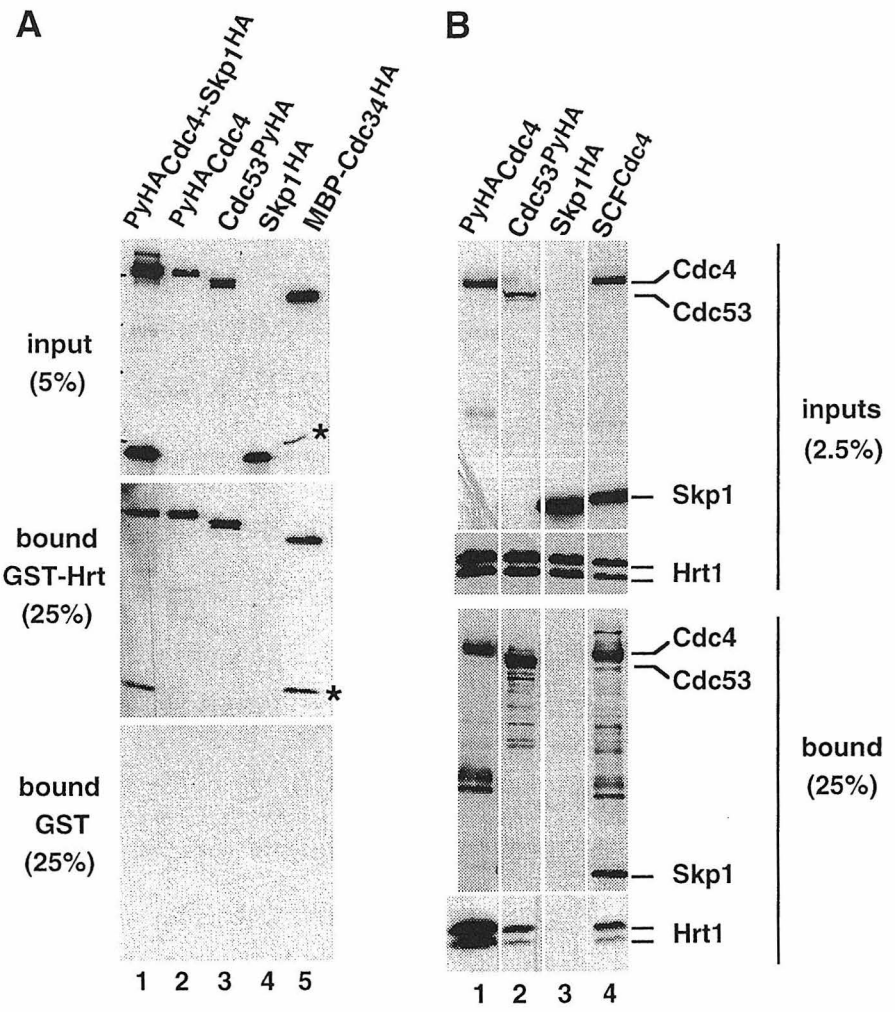

A
PyHACdc4+Skp1HA
PyHACdc4
Cdc53PyHA
Skp1HA
MBP-Cdc34HA
input
(5%)
bound
GST-Hrt
(25%)
bound
GST
(25%)
*
*
1 2 3 4 5
B
PyHACdc4
Cdc53PyHA
Skp1HA
SCFCdc4
Cdc4
Cdc53
Skp1
Hrt1
inputs
(2.5%)
Cdc4
Cdc53
Skp1
Hrt1
bound
(25%)
1 2 3 4

Figure 3.

Figure 4: Hrt1 potently stimulates ubiquitin ligase activity of SCF^{Cdc4}. **(A):** Recombinant SCF^{Cdc4} was produced in Sf9 insect cells upon co- infection with baculovirus vectors that express Skp1, $Cdc53^{PyHA}$, and $^{PyHA}Cdc4$, and the complex was purified by adsorption to a protein A resin containing covalently linked anti-polyoma monoclonal antibody (α-Py beads). Parallel samples were supplemented with E1 enzyme, Cdc34, ATP, ubiquitin, purified phosphorylated maltose binding protein-Sic1 chimera (PP-MBP-Sic1) and either glutathione-S-transferase (GST, lanes 1-5) or GST-Hrt1 (lanes 6-10). At the indicated times, samples were evaluated for ubiquitination of phospho-MBP-Sic1 by SDS-PAGE followed by immunoblotting with α-Sic1. **(B):** lanes 1-11: Same as (A), except that recombinant SCF^{Cdc4} complexes that either contained (lanes 6-10) or lacked (lanes 1-5) yeast Hrt1 were generated by either triple or quadruple infection of insect Sf9 cells (quadruple infection contained an Hrt1-expressing baculovirus). Input substrate is shown in lane 11. Lanes 12,13: Levels of SCF subunits in each preparation were evaluated by immunoblotting. **(C):** Same as (B), except that recombinant SCF^{Grr1} complexes containing (lane 3) or lacking (lane 2) Hrt1 were expressed in insect cells, purified, and mixed with $1/20^{th}$ volume insect cell lysate that contained recombinant $Cln2^{myc}$/GST-Cdc28/Cks1 complexes ($Cln2^{myc}$). In lanes 3 and 4, Cdc34 and substrate, respectively, were omitted from the final reaction. PP-$Cln2^{myc}$ refers to the phosphorylated substrate.

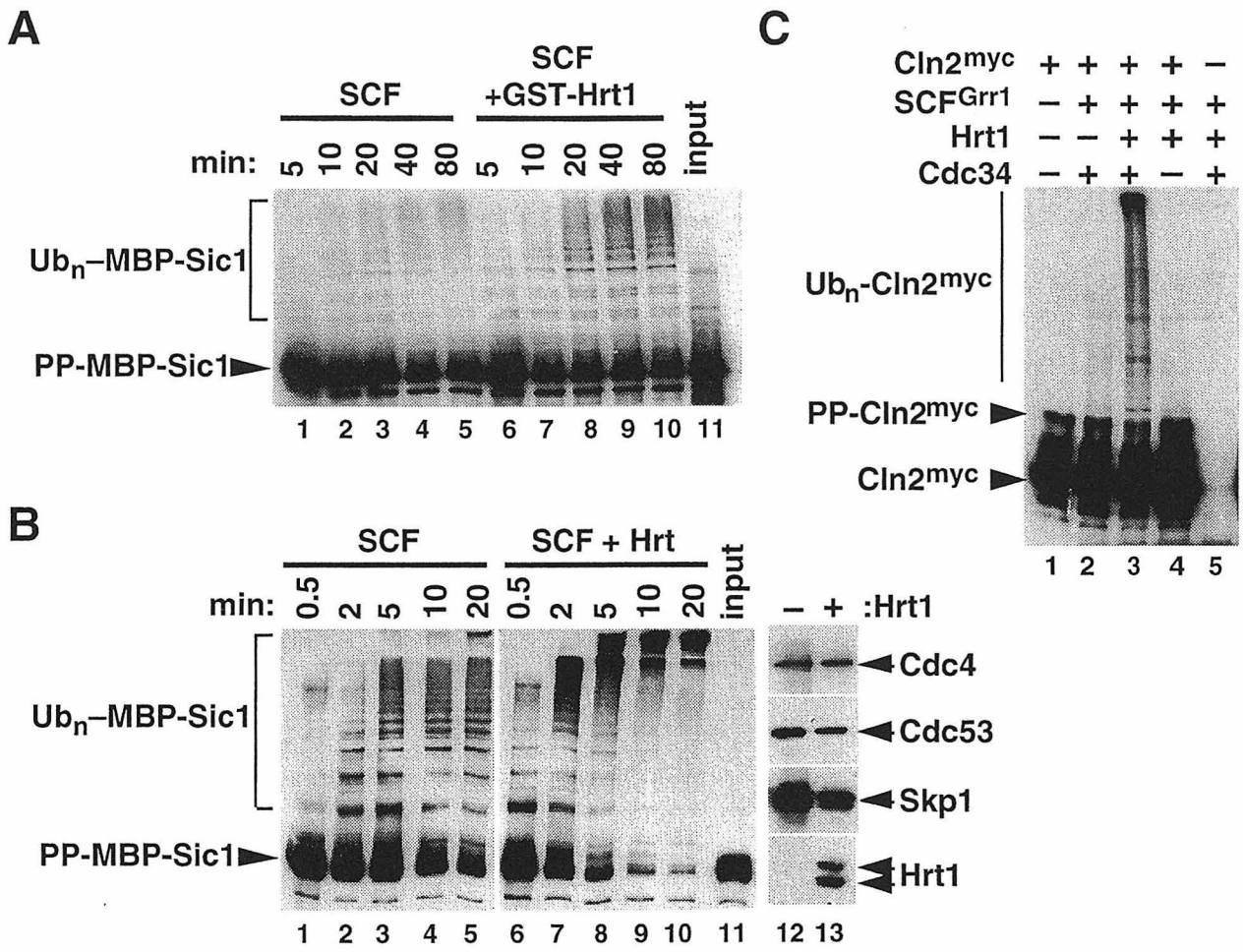

A
SCF
SCF +GST-Hrt1
min: 5 10 20 40 80 5 10 20 40 80 input
Ub$_n$–MBP-Sic1
PP-MBP-Sic1
1 2 3 4 5 6 7 8 9 10 11
C
Cln2^{myc} + + + + –
SCF^{Grr1} – + + + +
Hrt1 – – + + +
Cdc34 – + + – +
Ub$_n$-Cln2^{myc}
PP-Cln2^{myc}
Cln2^{myc}
1 2 3 4 5
B
SCF
SCF + Hrt
min: 0.5 2 5 10 20 0.5 2 5 10 20 input
– + :Hrt1
Ub$_n$–MBP-Sic1
PP-MBP-Sic1
Cdc4
Cdc53
Skp1
Hrt1
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 4.

Figure 5: Cdc53/Hrt1 comprise an NEM-resistant ubiquitin ligase module. **(A):** The proteins indicated were retrieved from baculovirus-infected insect cells on α-Py beads, and mixed with E1, Cdc34ΔC, ubiquitin and ATP. Following a 60 min incubation at 20 °C, reactions were evaluated for Cdc34ΔC autoubiquitination (top panel), levels of Hrt1 (middle panel), and levels of $Cdc53^{PyHA}$ or $^{PyHA}Cdc4$ (bottom panel) by SDS-PAGE following by immunoblotting. SCF^{Cdc4} complexes (lanes 4, 5) contained $^{PyHA}Cdc4$ and untagged Cdc53. Thus, only $^{PyHA}Cdc4$ is visible in these lanes in the α-HA blot (bottom panel; see asterisks). **(B):** Either $Cdc53^{PyHA}$/Hrt1 (lanes 1-3) or E1 enzyme (lane 4) were pretreated with the indicated amount of NEM for 10 min at 20°C. NEM was quenched with DTT, and complete reactions containing E1, Cdc34ΔC, ubiquitin, ATP and α-Py bead-bound $Cdc53^{PyHA}$/Hrt1 were incubated for 60 min at 20 °C. For the sample shown in lane 5, DTT was mixed with NEM prior to incubation with E1 to confirm the efficacy of the DTT quench. All reactions were subjected to SDS-PAGE followed by immunoblotting with α-Cdc34. **(C):** Same as (B), except that complete reactions were first assembled and then treated with 5 mM NEM followed by 10 mM DTT (lanes 1,2) or vice versa (lanes 3,4). Afterwards, α-Py bead-bound $Cdc53^{PyHA}$/Hrt1 was recovered, washed, and supplemented with either buffer (lanes 1,3) or fresh E1, Cdc34ΔC, ubiquitin and ATP (lanes 2,4). The signal in lanes 1 and 3 represents Cdc34ΔC from the first stage incubation that was retained on the $Cdc53^{PyHA}$/Hrt1 matrix.

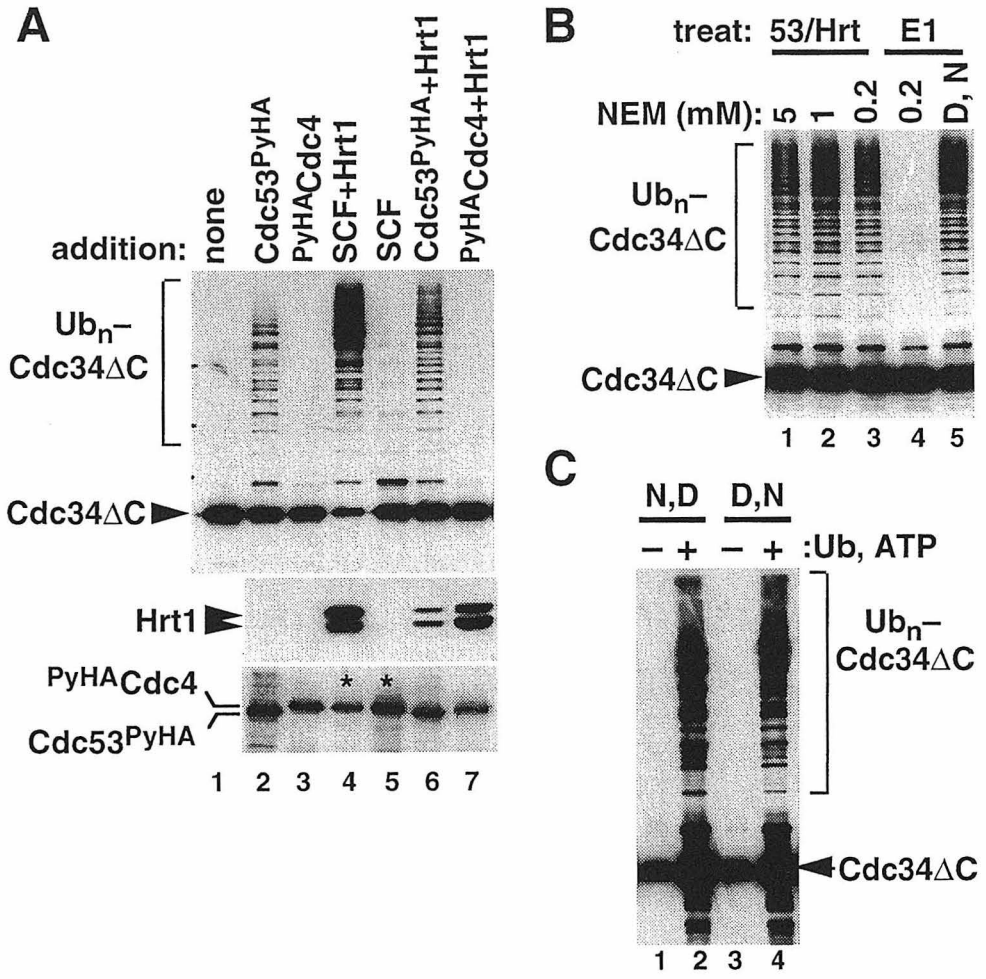

A
addition:
none
Cdc53PyHA
PyHACdc4
SCF+Hrt1
SCF
Cdc53PyHA+Hrt1
PyHACdc4+Hrt1
Ubn–
Cdc34ΔC
Cdc34ΔC
Hrt1
PyHACdc4
Cdc53PyHA
1 2 3 4 5 6 7
B
treat: 53/Hrt E1
NEM (mM): 5 1 0.2 0.2 D, N
Ubn–
Cdc34ΔC
Cdc34ΔC
1 2 3 4 5
C
N,D D,N
– + – + :Ub, ATP
Ubn–
Cdc34ΔC
Cdc34ΔC
1 2 3 4

Figure 5.

Figure 6: Polycations can substitute for SCF. **(A):** Polylysine activates Cdc34ΔC autoubiquitination. Reactions containing Cdc34ΔC, ATP, ubiquitin, and E1 enzyme were adjusted to either 10 mM lysine (lane 1), 0.01, 0.1, or 1% polyethylene glycol (PEG, lanes 2-4), or 0.01% (~300 nM) polylysine (lane 5), and incubated at 20 °C for 60 min. Reactions were evaluated as described for Figure 5A. **(B):** Polyethyleneimine is an 'E3' for Gcn4. [^{35}S]-Gcn4 produced by in vitro translation in reticulocyte lysate and enriched by DEAE chromatography was mixed with E1, Cdc34, ATP, the indicated ubiquitin derivatives (lanes 4-6; H6 stands for His6) and 0.0005% polyethyleneimine (PEI), and incubated at 25 °C for 60 min. Reactions conducted in the absence of either Cdc34, PEI or ubiquitin are depicted in lanes 1-3. Gcn4 was visualized by SDS-PAGE followed by autoradiography. **(C):** Fusion of Sic1 to Cdc34 does not activate its ubiquitination. Cdc34ΔC-Sic1 (arrowhead) was mixed with E1, ubiquitin, and ATP in the presence of immobilized SCF^{Cdc4} (lane 1), $Cdc53^{PyHA}$ (lane 2) or (as a negative control) $Skp1^{HA}$ (lane 3). Samples were incubated and processed as described for Figure 5A.

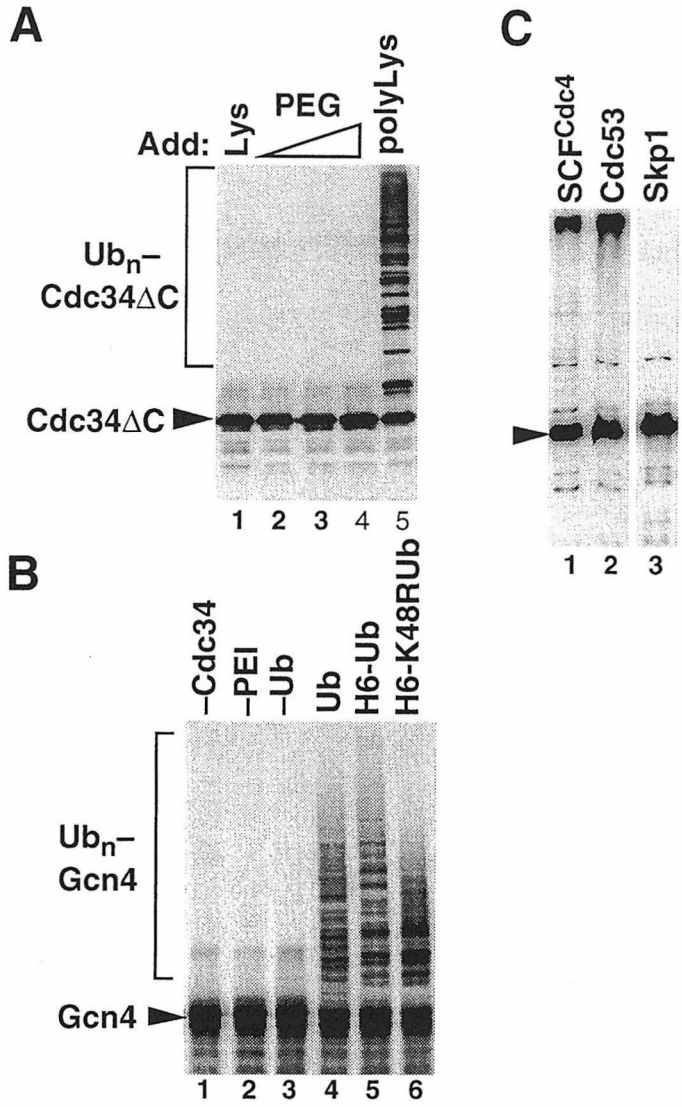

A
Add:
Lys
PEG
polyLys
Ub$_n$–
Cdc34ΔC
Cdc34ΔC
1 2 3 4 5
B
–Cdc34
–PEI
–Ub
Ub
H6-Ub
H6-K48RUb
Ub$_n$–
Gcn4
Gcn4
1 2 3 4 5 6
C
SCF^{Cdc4}
Cdc53
Skp1
1 2 3

Figure 6.

References

Altschul, S., T. Madden, A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25:** 3389-3402.

Bai, C., P. Sen, K. Hofmann, L. Ma, M. Goebl, J.W. Harper, and S.J. Elledge. 1996. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* **86:** 263-274.

Banerjee, A., L. Gregori, Y. Xu, and V. Chau. 1993. The bacterially expressed yeast *CDC34* gene product can undergo autoubiquitination to form a multiubiquitin chain-linked protein. *J. Biol. Chem.* **268:** 5668-5675.

Bartel, B., I. Wunning, and A. Varshavsky. 1990. The recognition component of the N-end rule pathway. *EMBO J.***9:** 3179-3189.

Cross, F.R. and A.H. Tinkelenberg. 1991. A potential positive feedback loop controlling CLN1 and CLN2 gene expression at the start of the yeast cell cycle. *Cell* **65:** 875-883.

Deshaies, R.J., V. Chau, and M. Kirschner. 1995. Ubiquitination of the G1 cyclin Cln2p by a Cdc34p-dependent pathway. *EMBO J.***14:** 303-312.

Epstein, C.B. and F.R. Cross. 1992. CLB5: a novel B cyclin from budding yeast with a role in S phase. *Genes Dev.* **6:** 1695-1706.

Fang, G.W., Yu, H.T., and Kirschner, M.W. 1998. Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. *Molec Cell* **2**: 163-171.

Feldman, R.M.R., C. Correll, K. Kaplan, and R. Deshaies. 1997. A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* **91:** 221-230.

Goebl, M.G., L. Goetsch, and B. Byers. 1994. The Ubc3 (Cdc34) ubiquitin-conjugating enzyme is ubiquitinated and phosphorylated in vivo. *Mol. Cell. Biol.* **14:** 3022-3029.

Hershko, A., H. Heller, S. Elias, and A. Ciechanover. 1983. Components of ubiquitin-protein ligase system: resolution, affinity purification, and role in protein breakdown. *J Biol Chem* **258:**

Honda, R., H. Tanaka, and H. Yasuda. 1997. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* **420:** 25-27.

Hu, G. and E. Fearon. 1999. Siah-1 N-terminal RING domain is required for proteolysis function, and C-terminal sequences regulate oligomerization and binding to target proteins. *Mol Cell Biol* **19:**

Huibregtse, J.M., M. Scheffner, S. Beaudenon, and P.M. Howley. 1995. A family of proteins structurally and functionally related to the E6-AP ubiquitin protein ligase. *Proc. Natl. Acad. Sci* USA.**92:** 2563-2567.

Jiang, J. and G. Struhl. 1998. Regulation of the Hedgehog and Wingless signalling pathways by the F- box/WD40-repeat protein Slimb. *Nature* **391:** 493-6.

Kamura, T., Sato, S., Haque, D., Liu, L., Kaelin, W.G., Conaway, R.C. and Conaway, J.W. 1998. The elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin families. *Genes & Dev* **12**:3872-3881.

King, R.W., R.J. Deshaies, J.M. Peter, and M. Kirschner. 1996. How proteolysis controls cell division. *Science* **274:** 1652-1659.

King, R.W., J.M. Peters, S. Tugendreich, M. Rolfe, P. Hieter, and M.W. Kirschner. 1995. A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* **81:** 279-288.

Kipreos, E.T., L.E. Lander, J.P. Wing, W.W. He, and E.M. Hedgecock. 1996. cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family. *Cell* **85:** 1-20.

Kolman, C.J., J. Toth, and D.K. Gonda. 1992. Identification of a portable determinant of cell cycle function within the carboxyl-terminal domain of the yeast CDC34 (UBC3) ubiquitin conjugating (E2) enzyme. *Embo J* **11:** 3081-90.

Kornitzer, D., B. Raboy, R.G. Kulka, and G.R. Fink. 1994. Regulated degradation of the transcription factor GCN4. *EMBO J.***13:** 6021-6030.

Kwon, Y., Y. Reiss, V. Fried, A. Hershko, J. Yoon, D. Gonda, P. Sangan, N. Copeland, N. Jenkins, and A. Varshavsky. 1998. The mouse and human genes encoding the recognition component of the N-end rule pathway. *Proc. Natl. Acad. Sci. USA* **95:** 7898-7903.

Lyapina, S., C. Correll, E. Kipreos, and R. Deshaies. 1998. Human CUL1 forms an evolutionarily conserved ubiquitin ligase complex (SCF) with SKP1 and an F-box protein. *Proc. Natl. Acad. Sci. USA* **95:** 7451-7456.

Mann, M. and M. Wilm. 1994. Error tolerant identification of peptides in sequence databases by peptide sequence tags. *Anal Chem* **66:** 4390-4399.

Patton, E.E., A.R. Willems, D. Sa, L. Kuras, D. Thomas, K.L. Craig, and M. Tyers. 1998a. Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box protein complexes that regulate cell division and methionine biosynthesis in yeast. *Genes Dev* **12:** 692-705.

Patton, E.E., A.R. Willems, and M. Tyers. 1998b. Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis. *Trends Genet* **14:** 236-43.

Price, C., K. Nasmyth, and T. Schuster. 1991. A general approach to the isolation of cell cycle-regulated genes in the budding yeast Saccharomyces cerevisiae. *J Mol Biol* **218:** 543-556.

Ruegger, M., E. Dewey, W.M. Gray, L. Hobbie, J. Turner, and M. Estelle. 1998. The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast Grr1p. *Genes Dev* **12:** 198-207.

Scheffner, M., U. Nuber, and J.M. Huibregtse. 1995. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* **373:** 81-83.

Schwob, E., T. Böhm, M. Mendenhall, and K. Nasmyth. 1994. The B-type cyclin kinase inhibitor $p40^{SIC1}$ controls the G1/S transition in *Saccharomyces cerevisiae*. *Cell* **79:** 233-244.

Schwob, E. and K. Nasmyth. 1993. CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae*. *Genes Dev.* **7:** 1160-1175.

Shevchenko, A., O. Jensen, A. Podtelejnikov, F. Sagliocco, M. Wilm, O. Vorm, P. Mortensen, A. Shevchenko, H. Boucherie, and M. Mann. 1996. Linking genome and proteome by mass spectrometry: large scale identification of yeast proteins from two-dimensional gels. *Proc. Natl. Acad. Sci. USA* **93:** 14440-14445.

Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996. Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. *Anal Chem* **68:** 850-858.

Skowyra, D., K. Craig, M. Tyers, S. Elledge, and J. Harper. 1997. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* **91:** 209-219.

Suarin, A., K. Borden, M. Boddy, and P. Freemont. 1996. Does this have a familiar RING? *Trends Biochem Sci* **21:** 208-214.

Thompson, J., D. Higgins, and T. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalities and weight matrix choice. *Nucleic Acids Res* **22:** 4673-4680.

Verma, R., R.S. Annan, M.J. Huddleston, S.A. Carr, G. Reynard, and R.J. Deshaies. 1997. Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. *Science* **278:** 455-60.

Verma, R., R. Feldman, and R.J. Deshaies. 1997b. SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. *Mol Biol Cell* **8:** 1427-1437.

Willems, A.R., S. Lanker, E.E. Patton, K.L. Craig, T.F. Nason, R. Kobayashi, C. Wittenberg, and M. Tyers. 1996. Cdc53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway. *Cell* **86:** 453-463.

Wilm, M. and M. Mann. 1996. Analytical properties of the nano electrospray ion source. *Anal Chem* **66:** 1-8.

Wilm, M., A. Shevchenko, T. Houthaeve, S. Breit, L. Schweigerer, T. Fotsis, and M. Mann. 1996. Femtomole sequencing of proteins from polyacrylamide gels by nanoelectrospray mass spectrometry. *Nature* **379:** 466-469.

Yaron, A., A. Hatzubai, M. Davis, I. Lavon, S. Amit, A.M. Manning, J.S. Andersen, M. Mann, F. Mercurio, and Y. Ben-Neriah. 1998. Identification of the receptor component of the IκBα-ubiquitin ligase. *Nature* **396:** 590-4.

Yu, H., J.M. Peters, R.W. King, A.M. Page, P. Hieter, and M.W. Kirschner. 1998. Identification of a cullin homology region in a subunit of the anaphase-promoting complex. *Science* **279:** 1219-22.

Zachariae, W., A. Shevchenko, P.D. Andrews, R. Ciosk, M. Galova, M.J. Stark, M. Mann, and K. Nasmyth. 1998. Mass spectrometric analysis of the anaphase-promoting complex from yeast: identification of a subunit related to cullins. *Science* **279:** 1216-9.

Appendix I: Analysis of Sic1 Lysine Utilization by SCF^{Cdc4}

Introduction

One outstanding question in the field of ubiquitin-mediated proteolysis is how lysines within a substrate molecule are selected by ubiquitin ligase as sites of attachment for multiubiquitin chains. Interest in this question was probably first stimulated by work from the Varshavsky lab establishing the principle of "trans-ubiquitination" (Johnson et al., 1990). This work utilized a multimeric complex of β-galactosidase-derived molecules that served as substrates of the N-end rule and demonstrated that the property of binding the N-end rule E3, Ubr1, could be separated from the ability of a molecule to receive ubiquitin moieties. Since then, many researchers have investigated the ability of lysines within a substrate molecule to act as the initial site of attachment. Interestingly, no prevailing model has emerged: some systems require the presence of a specific lysine within the substrate, whereas in other molecules, a great degree of structural freedom appears to be tolerated. An example of the former situation has been elucidated recently by exploring the ubiquitination of IκB (Spencer et al., 1999; Winston et al., 1999; Yaron et al., 1998). The ubiquitin ligase $SCF^{\beta\text{-}TrCP}$ competes with the SUMO-1 conjugating system for access to lysine 21 of IκB (Desterro et al., 1999). SUMO-1 attachment

prevents ubiquitination and stabilizes the molecule, suggesting that Lys21 is the key site of ubiquitin attachment on IκB. An opposing view emerges from analyzing cyclin destruction. Cyclins contain a conserved, nine residue domain called the destruction box that is sufficient and necessary to confer a short half-life (Glotzer et al., 1991). However, the cyclin box does not contain any conserved lysine, and a careful analysis of mitotic cyclin destruction revealed that no single lysine is required for its ubiquitination and destruction (King et al., 1996).

The ubiquitination of Sic1 is efficient and highly processive. Experiments using forms of ubiquitin (methyl ubiquitin or K48R ubiquitin) suggest that up to eight lysines are utilized as sites of multiubiquitin chain formation in vitro by purified SCF^{Cdc4} (Feldman et al., 1997). How many of these sites are actually utilized, and are any sites essential for Sic1's subsequent destruction by the 26S proteasome? Sic1 contains 20 lysine residues distributed throughout its length, but previous work suggested that the first 160 amino acids of Sic1, containing only six lysines, constitute a sufficient and necessary domain for SCF^{Cdc4} recruitment (Verma et al., 1997a). We constructed versions of the first 160 residues of Sic1 that contained different numbers of lysine residues to analyze which lysines are important for the ubiquitination of Sic1.

Materials and Methods

DNA constructs. Sequences encoding the first 160 amino acids of Sic1 followed by sequences encoding the HA epitope plus six histidine residues (HAHis6) were amplified by PCR and cloned into the vector pBS-KS+ (Stratagene). To generate mutants in which various lysine codons were mutated to those signifying arginine, successive rounds of QuikChange mutagenesis strategy (Stratagene) were employed. Briefly, oligos encoding the desired mutation were synthesized as complementary pairs and used as primers in a Pfu-driven PCR reaction. Mutations were confirmed by DNA sequencing or by assaying for incorporation of novel restriction enzyme sites included in the design of the oligo primers. All completed clones were confirmed by DNA sequencing to contain no unintentional errors. Resulting DNA fragments were cloned into the vector pMAL-C2 to yield clones that express in-frame fusions of maltose binding protein (MBP) to the first 160 amino acids of Sic1 followed by the HA epitope tag plus His6.

Protein expression. To express the encoded proteins, correct clones were transformed into BL21(DE3)+pLysS cells, and a resulting colony was grown at 37 °C in the presence of ampicillin and chloramphenicol to an OD_{600} of 0.5. Plasmid-borne

protein expression was induced by addition of IPTG to 0.4 mM and cells allowed to grow for an additional 2 hrs at 30 °C. Cells were collected by centrifugation, growth media was removed, and the cell pellet resuspended in 25 mM Tris pH 7.4, 0.5M NaCl, 10 mM imidazole, 0.2% Triton X-100, 0.1 mM PMSF, 1 μg/mL leupeptin and pepstatin and then frozen in liquid nitrogen. After thawing, the solution was sonicated 3 times for 1 minute and then clarified by centrifugation (20 minutes, 30,000xg). MBP-Sic1 fusion proteins were purified by successive steps of Ni-NTA affinity chromatography followed by amylose affinity chromatography essentially as described (Feldman et al., 1997). Purified proteins were analyzed by SDS-PAGE followed by staining with Coomassie blue, and protein concentration determined by the Bio-Rad protein assay.

In vitro assays. In vitro ubiquitination assays using various mutant forms of MBP-Sic1 were performed essentially as described (Seol et al., 1999). Briefly, to generate phosphorylated substrate, MBP-Sic1 fusion proteins were incubated with glutathione bead-bound cyclin/GST-Cdc28 complexes purified out of baculovirus-infected Sf9 cells. Phosphorylated or untreated Sic1 molecules were then removed from kinase complexes and incubated with Py antibody-coupled beads that had been previously incubated with insect cell lysates expressing ^{Py}Cdc4, Cdc53^{Py}, and Skp1. Sic1 and the beads containing SCF were incubated with Cdc34, Uba1, Ub and ATP and allowed to incubate for 1 hour at room temperature. Reaction products were analyzed by

SDS-PAGE followed by Western blotting with antibodies against either Sic1 or the HA epitope.

Interactions between truncated forms of Sic1 and SCF^{Cdc4} were assessed by incubating approximately equal amounts of mutant Sic1 proteins with SCF^{Cdc4} complexes purified from approximately 200 μL of cell lysate, produced from approximately $2 \bullet 10^6$ cells. Under these conditions, Sic1 proteins were present in approximately 20-fold molar excess. Reactions were adjusted to a final volume of 250 μL with wash buffer (25 mM Hepes 7.4, 150 mM NaCl, 0.1% Triton X-100) and the beads were incubated for 30 minutes at room temperature. The beads were then washed four times with wash buffer and then boiled in SDS-PAGE sample buffer to release bound proteins, which were analyzed by SDS-PAGE and Western blotting.

Results

The initial in vitro reconstitution of Sic1 utilized a full-length Sic1 fused in-frame to the C-terminus of maltose binding protein (MBP). The resulting MBP-Sic1 fusion protein was expressed in bacteria and shown to be an efficient substrate of SCF^{Cdc4} (Feldman et al., 1997). However, the first 160 amino acids of Sic1 are sufficient to

confer SCF^{Cdc4} recognition and ubiquitination (Verma et al., 1997a). Conveniently, this portion of Sic1 contains only six lysine residues, simplifying the analysis of their utilization. These lysines occur in three clusters; within a cluster, each pair of lysines is no more than 4 residues away from the other. Thus, we treated each cluster as a single candidate site, mutating both lysines of a cluster simultaneously. To differentiate full-length MBP-Sic1 from the 160 residue version, the truncated forms are denoted by a subscripted "t" (MBP-$Sic1_t$). The various mutants generated by PCR and their corresponding designations are shown in Table 1.

The mutant forms of Sic1 were expressed in E. coli and purified as described in Materials and Methods. To assess the ability of these proteins to accept ubiquitin, we used two forms of these proteins as substrates in our in vitro assays: MBP-$Sic1_t$-WT and MBP-$Sic1_t$-Δ32,49,84 (hereafter, the MBP-Sic1t portion of the name will be omitted for clarity). As shown in Figure 1, full length MBP-Sic1 and the truncated WT form served as equally competent substrates for SCF^{Cdc4}, while the Δ32,49,84 form did not appreciably acquire covalent ubiquitin attachments. As expected, the ability of the wild-type molecules to be ubiquitinated depended on their previous phosphorylation. Note that all three forms of the molecules used in these reactions are generated as MBP fusion proteins; the MBP portion contributes numerous Lys residues to the chimeric fusion protein. Although not shown here, these lysines may in fact accept some small amount of

ubiquitin; an enhanced exposure of the Western blot shown in Figure 1 indicates a trace amount of multiubiquitin chain attachment, approximately 20-50 fold less than is present on either wild-type construct.

The inability of the lysine-lacking Sic1 derivative to be ubiquitinated could stem from a general misfolding defect: the substitution of six residues might have significantly altered the three-dimensional structure of the protein rendering it incompetent to be recognized by Cdc4. Furthermore, the lysines themselves may constitute an essential part of the as-yet undefined Sic1 epitope recognized by Cdc4; mutation of these lysines may therefore directly abrogate binding by Cdc4. To address these possibilities, we first looked at the ability of WT and Δ32,49,84 molecules to bind Cdc4. As shown in Figure 2, both forms of truncated molecule could bind SCF^{Cdc4} (Figure 2, lanes 3 and 9). Furthermore, this binding required that the Sic1 molecules first be phosphorylated by treatment with active Cln/Cdc28 complexes; failure to phosphorylate either form of truncated Sic1 prevented significant interactions with SCF^{Cdc4} (lanes 6, 12).

Next, we assayed the ability of these truncated Sic1 molecules to affect the activity of SCF^{Cdc4} toward full-length MBP-Sic1. MBP-Sic1 was phosphorylated and incubated with SCF^{Cdc4} plus varying amounts of phosphorylated WT or Δ32,49,84, ranging from a 5- to a 125-fold molar excess of the truncated molecules over MBP-Sic1. As shown in Figure 3, truncated WT protein permitted continued SCF activity, directed

towards either full length MBP-Sic1 or the truncated WT molecule (the reactions were probed with anti-Sic1 antibodies, making it impossible to discriminate which substrate is receiving ubiquitin conjugates). By contrast, the Δ32,49,84 protein effectively inhibited the activity of available SCF complexes (Figure 3, lanes 6-8). Furthemore, this inhibitory effect is dependent on Δ32,49,84 being phosphorylated by active Cln/Cdc28 kinase (data not shown).

Since the most severe allele of mutated truncated Sic1 (Δ32,49,84) is still competent to bind Cdc4, we investigated the in vitro ubiquitination phenotype of the intermediate alleles listed in Table 1. The various mutants were incubated with SCF and either wild-type ubiquitin or methyl ubiquitin, a derivative that prevents chain elongation and thus reveals the number of lysines being utilized as the number of high weight conjugates visible in the lane. All of the mutants appeared to efficiently generate high weight multiubiquitin conjugates (Figure 4, lanes 3, 6, 8, 11). One mutant, Δ32,84, did display a mild defect in incorporating methyl ubiquitin, suggesting that the pair of lysines beginning at position 32 may be relatively preferentially utilized (compare lane 5 with lanes 2, 5, and 12).

Discussion

The analysis presented above suggests that no single lysine is absolutely essential for ubiquitin conjugation on the first 160 amino acids of Sic1. This mechanism of lysine utilization appears to share more in common with that of mitotic cyclins than that of IκB which utilizes one or two specific lysines as sites of ubiquitin chain formation. The experiments described in this section utilized Sic1 produced as a fusion protein with MBP; MBP contains many lysines, but these do not appear to be utilized efficiently by SCF, as the number of bands present in reactions containing methyl ubiquitin is roughly equal to the number of lysines available in the Sic1 portion of the chimera (see Figure 4). Furthermore, the Δ32, 4, 84 mutant is unable to accept ubiquitins, suggesting that SCF or Cdc34 cannot (at least initially) target a lysine in an inappropriate context (i.e. outside of Sic1). SCF may show a slight preference for delivering ubiquitin to the pair of lysines beginning at position 32, since this mutant (Δ32,84) exhibited a slight decrease in the number of ubiquitin adducts formed in the presence of methyl ubiquitin. However, a possible caveat may be caused by lysine misincorporation in ubiquitin. This phenomenon is observed in E. coli challenged to produce a heterologous protein containing a high number of arginine codons. This defect can be rescued by enhancing the bacterium's ability to synthesize arginine-containing tRNAs, and strains that can do so are now commercially available.

Nevertheless, the Δ32,49,84 mutant appears to be able to eliminate SCF activity towards full-length Sic1 substrate. Presumably it does so by competing with full-length Sic1 for access to Cdc4. While it is unclear if the inhibition is competitive or irreversible, the excess mutant protein effectively constipates SCF^{Cdc4}. This property makes it a potentially useful tool for the investigator, allowing the opportunity to "trap" SCF in a bound and potentially active state to permit investigations of SCF or E2 structure and activity. Extending this idea, it will be extremely useful to examine the in vivo behavior of these mutant constructs in yeast when placed under the control of an inducible promoter. A form of Sic1 that cannot be phosphorylated causes cells to arrest in late G1 with a *cdc4* -like phenotype due to inhibition of Clb/Cdc28 activity (Verma et al., 1997b). A non-degradable form of Sic1 that can bind Cdc4 might also cause a G1 arrest by virtue of its ability to inactive SCF. Since no systematic means of identifying SCF substrates has yet been reported, the non-ubiquitinatable Sic1 mutants could form the basis for a genetic screen to identify SCF substrates. For example, a GFP- or β-gal fusion library could be expressed in yeast and screened for clones that are stabilized in when non-degradable Sic1 is produced. Regardless, the mutants presented in this section represent a convenient starting point to further analyze SCF, its mechanism and substrates.

Table 1. MBP-Sic1 Truncation Mutants and the Location of Lys→Arg changes.

A cDNA encoding the first 160 amino acids of Sic1 was cloned to yield an in-frame N-terminal fusion to maltose binding protein (MBP) followed by a C-terminal fusion to the HA epitope plus six histidine residues (HAHis6). The locations of the pairs of lysines present in this portion of Sic1 and the resulting mutants generated are indicated.

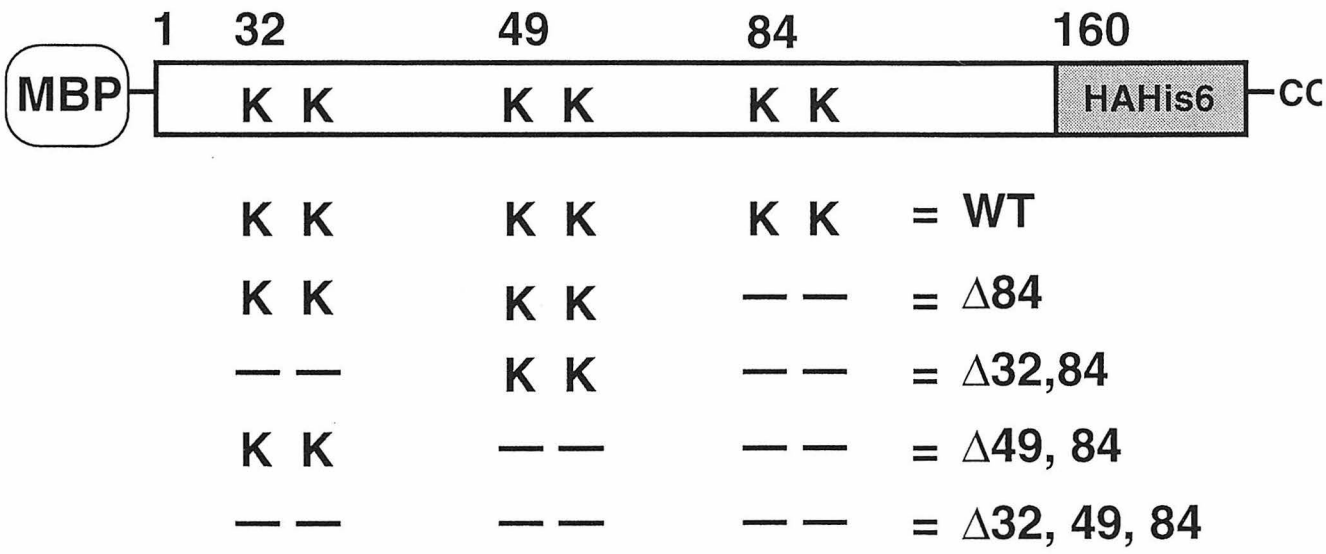

1
32
49
84
160
MBP
K K
K K
K K
HAHis6
CC
K K
K K
K K
= WT
K K
K K
— —
= Δ84
— —
K K
— —
= Δ32,84
K K
— —
— —
= Δ49, 84
— —
— —
— —
= Δ32, 49, 84

Table 1.

Figure 1. A Truncated Form of Sic1 Lacking All Lysines Cannot Be Efficiently Ubiquitinated In Vitro.

Ubiquitination reactions were assembled using SCF^{Cdc4} produced in insect cells and including full-length MBP-Sic1 (lanes 1-3), a fusion of MBP to the first 160 amino acids of Sic1 (MBP-$Sic1_t$-WT, lanes 4-6) or a mutant form of the latter molecule in which all lysines have been mutated to arginine residues (MBP-Sic1t-Δ32,48,84). Lanes 1,4, and 7 contain aliquots of the purified proteins, and lanes 2,5, and 8 show these same proteins after treatment with active Cln/Cdc28 kinase. The proteins shown in lanes 2, 5, and 8 were then used as substrates in ubiquitination reactions (lanes 3, 6, and 9) as described in Materials and Methods. All samples were resolved by SDS-PAGE and proteins detected by Western blotting with polyclonal antibodies made against Sic1.

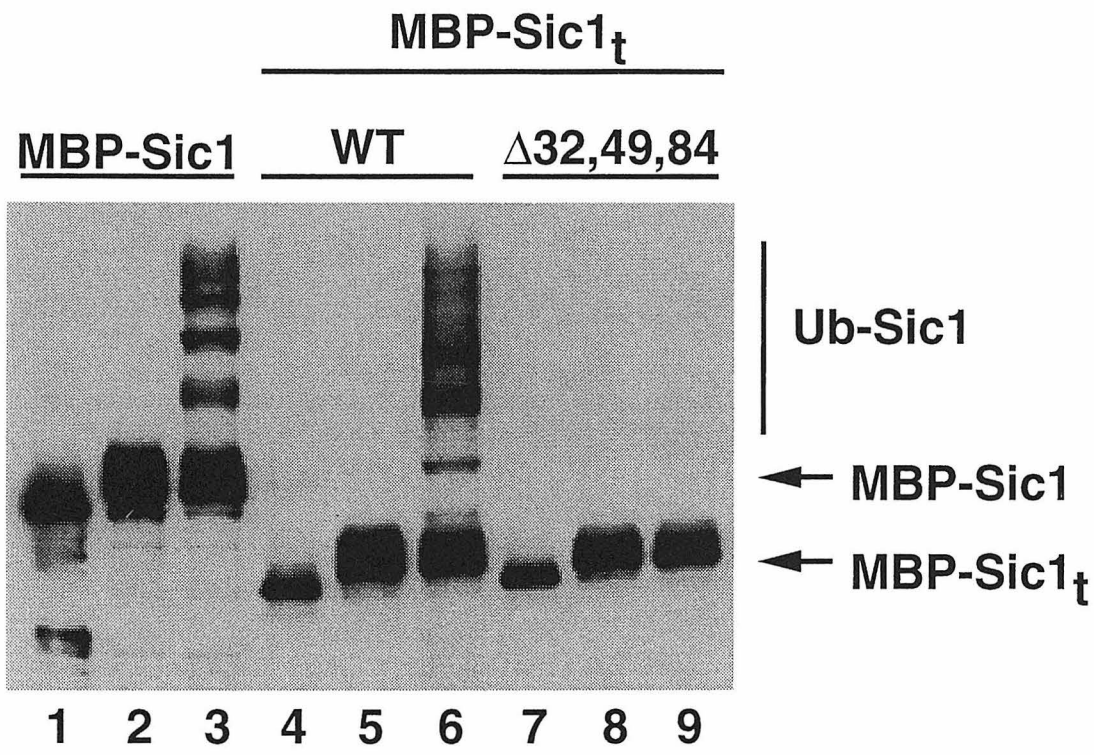

MBP-Sic1t
MBP-Sic1
WT
Δ32,49,84
Ub-Sic1
MBP-Sic1
MBP-Sic1t
1 2 3 4 5 6 7 8 9

Figure 1.

Figure 2. Phosphorylated Truncated Versions of Sic1 Can Bind SCF^{Cdc4}.

The first 160 amino acids of Sic1 (WT) or a mutant form where all lysine residues have been changed to arginine (Δ32,49,84) were produced in E. coli as a fusion protein to MBP. Proteins in lanes 1-3 and 7-9 were incubated with active Cln/Cdc28 kinase. Anti-polyoma antibody coupled to beads were used to retrieve epitope-tagged SCF^{Cdc4} complexes from baculovirus-infected insect cells and the resulting beads were incubated with an ca. 5-fold molar excess of the Sic1 fusion proteins, shown in lanes 1, 4, 7, and 10. After an incubation, unbound Sic1 proteins were collected (lanes 2, 5, 8, and 11) and the beads were washed and then boiled in SDS-PAGE sample buffer to release bound Sic1 molecules, shown in lanes 3, 6, 9, and 12. Samples were analyzed by SDS-PAGE followed by Western blotting with anti-Sic1 antibody. An equal fraction of each aliquot is loaded in all lanes so amount retrieved can be directly compared.

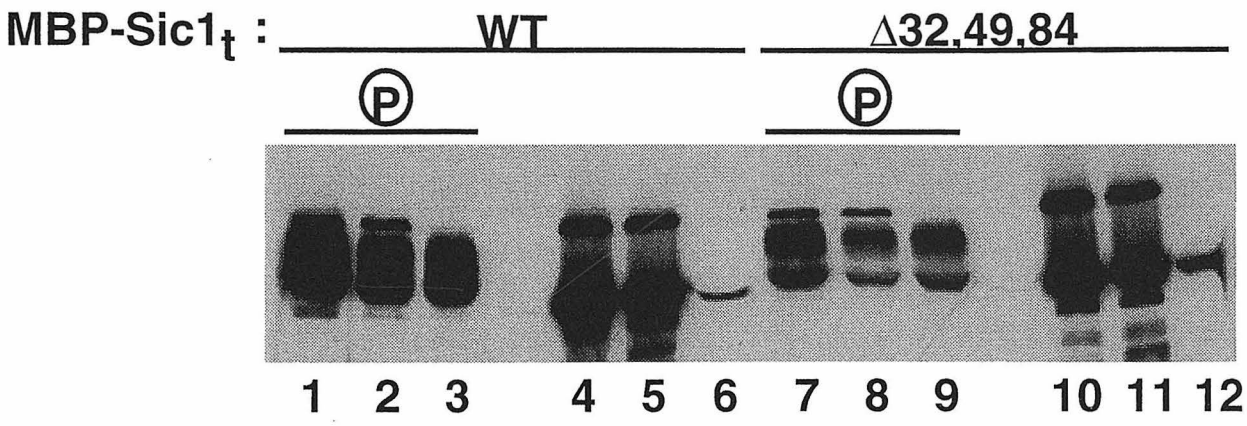


Figure 2.

Figure 3. A Form of Sic1 Lacking All Lysines Can Effectively Inhibit the Activity of SCF^{Cdcd4} Complexes.

Full length, phosphorylated MBP-Sic1 was incubated either with phosphorylated MBP-$Sic1_t$-WT (lanes 2-4) or with the lysine-lacking version, MBP-$Sic1_t$-Δ32,49,84 (lanes 6-8) where the truncated protein was in 125-, 25-, or 5- fold molar excess as indicated on top. These molecules were then incubated with SCF^{Cdc4} from baculovirus-infected insect cells plus other standard ubiquitination components. Resulting reaction products were resolved by SDS-PAGE and analyzed by Western blot using an anti-Sic1 antibody. The migration positions of full length MBP-Sic1 and its truncated derivatives are shown at right. Lanes 1 and 5 contain truncated MBP-Sic1 proteins run in the absence of SCF or full-length MBP-Sic1.

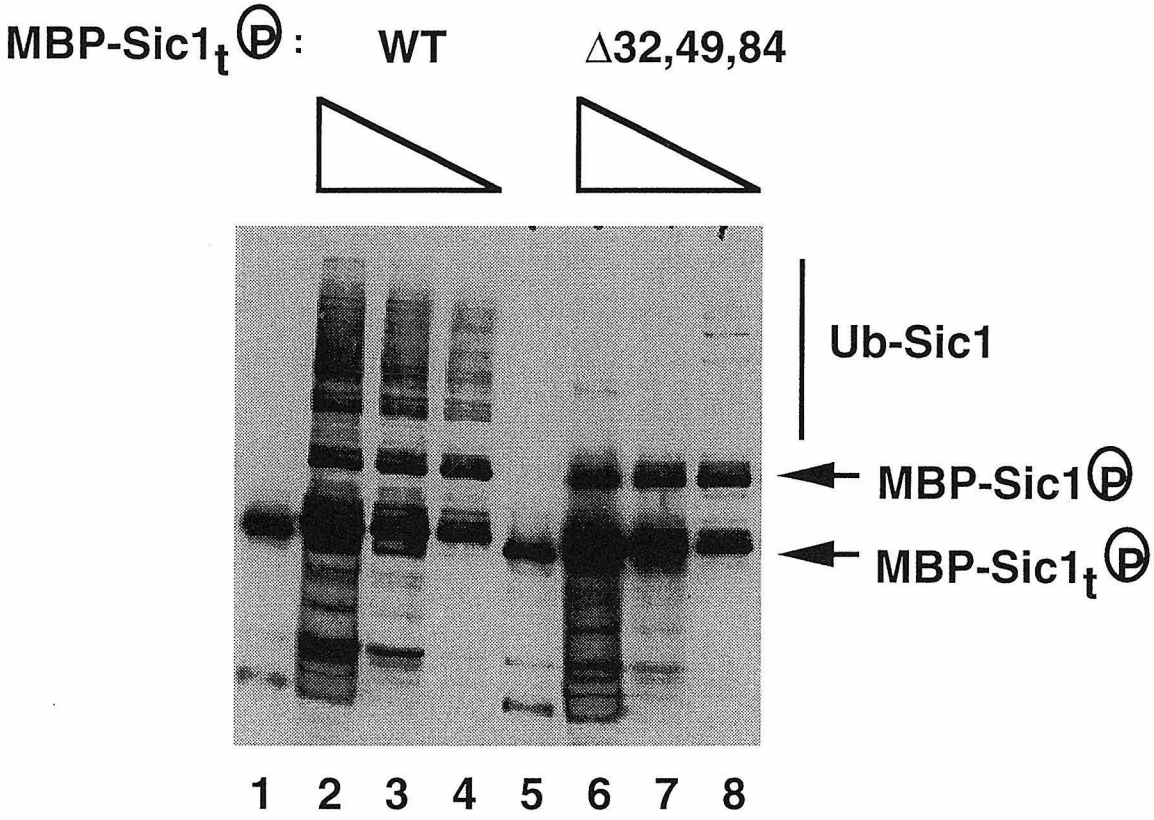


Figure 3.

Figure 4. Sic1 Mutants Reveal That No Single Lysine Is Absolutely Required for Ubiquitin Chain Attachment.

The Sic1 mutants described in Table 1 were purified from E. coli and phosphorylated by treatment with active Cln/Cdc28 kinase; samples are shown in lanes 1, 4, 7, and 10. These kinased molecules were used as substrates in ubiquitination reactions mediated by SCF^{Cdc4} that utilized either wild-type ubiquitin (lanes 3, 6, 8, and 11) or the chemical derivative methyl ubiquitin that prevents chain elongation after initial isopeptide bond formation (lanes 2, 5, 9, and 12). Reactions were analyzed by SDS-PAGE followed by Western blotting with anti-Sic1 antibody.

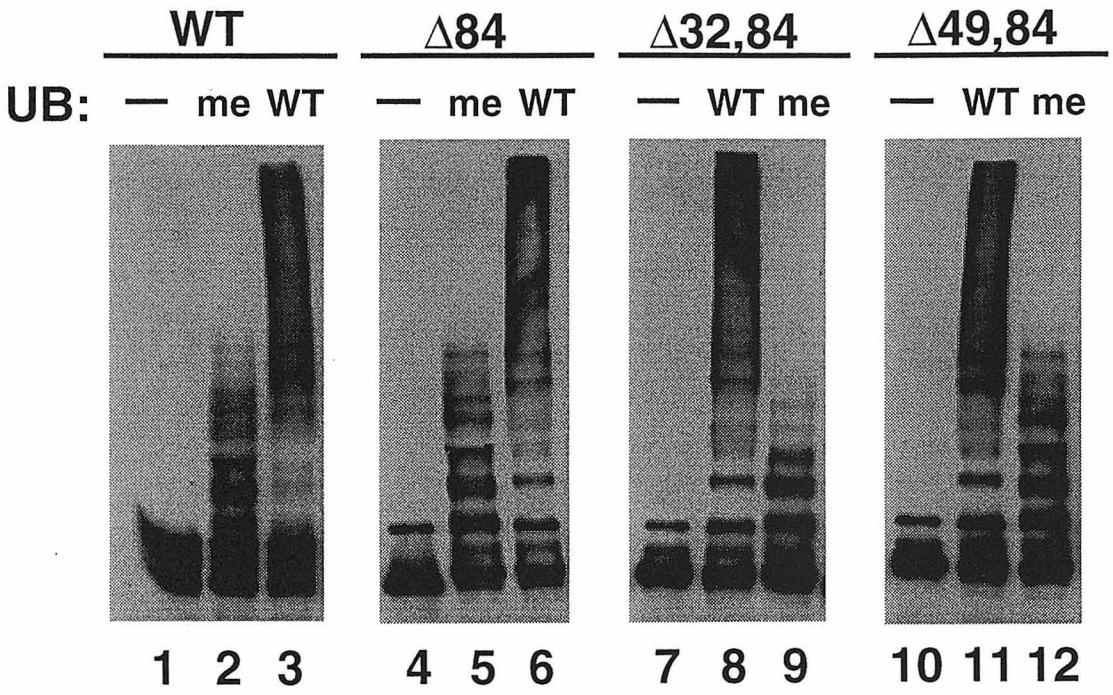


Figure 4.

References

Desterro, J. M. P., Rodriguez, M. S., and Hay, R. T. (1999). SUMO-1 modification of I kappa B alpha inhibits NF-kappa B activation. Molec. Cell **2**: 233-239.

Feldman, R., Correll, C., Kaplan, K., and Deshaies, R. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell *91*, 221-230.

Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991). Cyclin is degraded by the ubiquitin pathway. Nature *349*, 132-138.

Johnson, E. S., Gonda, D. K., and Varshavsky, A. (1990). cis-trans recognition and subunit-specific degradation of short-lived proteins. Nature *346*, 287-91.

King, R. W., Glotzer, M., and Kirschner, M. W. (1996). Mutagenic analysis of the destruction signal of mitotic cyclins and structural characeterization of ubiquitinated intermediates. Molecular Biology of the Cell *7*, 1343-1357.

Spencer, E., Jiang, J., and Chen, Z. J. J. (1999). Signal-induced ubiquitination of I kappa B alpha by the F-box protein Slimb/beta-TrCP. Genes Dev *13*, 284-294.

Verma, R., Annan, R. S., Huddleston, M. J., Carr, S. A., Reynard, G., and Deshaies, R. J. (1997b). Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. Science *278*, 455-60.

Verma, R., Feldman, R., and Deshaies, R. J. (1997a). SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. Mol Biol Cell *8*, 1427-1437.

Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999). The SCF beta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in I kappa B alpha and beta-catenin and stimulates I kappa B alpha ubiquitination in vitro. Genes Dev. *13*, 270-283.

Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A. M., Andersen, J. S., Mann, M., Mercurio, F., and Ben-Neriah, Y. (1998). Identification of the receptor component of the IκBα-ubiquitin ligase. Nature *396*, 590-4.

Appendix II

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A Complex of Cdc4p, Skp1p, and Cdc53p/Cullin Catalyzes Ubiquitination of the Phosphorylated CDK Inhibitor Sic1p

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Summary

In S. cerevisiae, the G1/S transition requires Cdc4p, Cdc34p, Cdc53p, Skp1p, and the Cln/Cdc28p cyclin-dependent kinase (Cdk). These proteins are thought to promote the proteolytic inactivation of the S-phase Cdk inhibitor Sic1p. We show here that Cdc4p, Cdc53p, and Skp1p assemble into a ubiquitin ligase complex named SCF^{Cdc4p}. When mixed together, SCF^{Cdc4p} subunits, E1 enzyme, the E2 enzyme Cdc34p, and ubiquitin are sufficient to reconstitute ubiquitination of Cdk-phosphorylated Sic1p. Phosphorylated Sic1p substrate is specifically targeted for ubiquitination by binding to a Cdc4p/Skp1p subcomplex. Taken together, these data illuminate the molecular basis for the G1/S transition in budding yeast and suggest a general mechanism for phosphorylation-targeted ubiquitination in eukaryotes.

Introduction

A number of cellular processes in both prokaryotes and eukaryotes are controlled by selective proteolysis. Proteolysis in the eukaryotic cytosol typically involves the assembly of a substrate-linked ubiquitin chain, which targets the protein for degradation by the 26S proteasome (Hochstrasser, 1995). Ubiquitin is first activated at its C terminus through the formation of a thioester bond with the ubiquitin-activating enzyme, E1. Ubiquitin is subsequently transesterified to a member of a family of ubiquitin conjugating (E2) enzymes. Finally, ubiquitin is transferred from the E2 enzyme to a lysine residue of the target protein, either directly or with the assistance of a ubiquitin ligase (E3). E3 was originally defined as an activity that is both necessary and sufficient for the transfer of ubiquitin to a model substrate in the presence of a ubiquitin-charged E2 enzyme (Hershko et al., 1983). E3s appear to be the primary source of substrate specificity in the ubiquitination cascade, as some E3s have been shown to bind directly to substrate. Recent work suggests that the E6-AP E3 (Huibregtse et al., 1993) also forms a thioester with ubiquitin as an intermediate in the transfer of ubiquitin from E2 to substrate (Scheffner et al., 1995). Since E3s do not possess detectable sequence homology, and since few have been characterized mechanistically, it remains to be seen whether all physiological ubiquitination reactions require E3s, and whether all E3s will participate directly in both substrate recruitment and ubiquitin transfer.

Entry into S phase in budding yeast requires the activity of S phase–promoting Clb/Cdc28p protein kinase complexes (Schwob et al., 1994). Although these complexes are assembled during G1 phase, they are initially inactive due to the presence of high levels of the Clb/Cdc28p inhibitor Sic1p (Nugroho and Mendenhall, 1994; Schwob et al., 1994). At the G1/S transition, Sic1p is abruptly degraded and the liberated Clb/Cdc28p complexes trigger DNA synthesis. *CLN1–3*, *CDC4*, *CDC34*, *CDC53*, and *SKP1* are required for the timely disappearance of Sic1p in vivo (Schwob et al., 1994; Bai et al., 1996; Schneider et al., 1996). Since *CDC34* encodes an E2 enzyme, it was proposed that Sic1p degradation is mediated by ubiquitin-dependent proteolysis (Schwob et al., 1994). Indeed, Sic1p is efficiently ubiquitinated in yeast extract, and Cdc34p, Cdc4p, and Cln/Cdc28p are required for this process (Verma et al., 1997b). Biochemical and genetic experiments have revealed that the main role of Cln/Cdc28p in the G1/S transition is to phosphorylate Sic1p, which triggers its ubiquitination by the Cdc34p pathway (Verma et al., 1997c; Schneider et al., 1996; Tyers, 1996).

Although Cdc4p and Cdc34p have been implicated in Sic1p ubiquitination in yeast extract, it is not known whether Skp1p and Cdc53p are also directly involved in this process. The functional roles of these proteins are of great interest, since Skp1p and Cdc53p are members of multigene families that are conserved from yeast to humans (Kipreos et al., 1995; Zhang et al., 1995; Bai et al., 1996; Mathias et al., 1996). Skp1p and Cdc53p homologs have been implicated in diverse processes, including kinetochore assembly in yeast (Connelly and Hieter, 1996), withdrawal from the cell cycle in nematodes (Kipreos et al., 1995), and both S phase progression (Zhang et al., 1995) and transcription elongation (Pause et al., 1997) in animal cells. Here, we report the reconstitution of Sic1p ubiquitination with a set of purified proteins, including Cdc4p, Cdc53p, and Skp1p. We also show that Cdc4p, Cdc53p, and Skp1p assemble into an E3 complex that selectively recognizes phosphorylated Sic1p via its Cdc4p and Skp1p subunits. These findings suggest that phosphorylation-triggered ubiquitin-dependent proteolysis may be mediated by a conserved cullin-dependent pathway in eukaryotic cells.

Results

Reconstitution of Sic1p Ubiquitination in Insect Cell Lysates

In vitro reconstitution of Sic1p ubiquitination using fractionated yeast extract revealed a direct requirement for Cdc34p, Cdc4p, and cyclin-dependent kinase (Cdk) activities (Verma et al., 1997b). However, yeast extract contributed a host of unidentified components, and a defined set of proteins sufficient to sustain Sic1p ubiquitination remained elusive. To define the biochemical

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requirements for Sic1p ubiquitination, several proteins that had been implicated in Sic1p destruction—including Cln2p/Cdc28p complexes (Reynard and Deshaies, unpublished data), Cdc53p, Cdc4p (Verma et al., 1997b), and Skp1p—were expressed separately in Sf9 insect cells via infection with recombinant baculoviruses. Previously described recombinant expression systems were used to prepare the yeast E1 enzyme ^{His6}Uba1p (Dohmen et al., 1995; Figure 2A, lane 1), the E2 enzyme Cdc34p (Banerjee et al., 1993; see Figure 2A lane 2), and a maltose-binding protein-Sic1p chimera containing a C-terminal bipartite Myc epitope–hexahistidine tag (MBP-Sic1p^{MH6}) (Verma et al., 1997a). Purified, metabolically radiolabeled [^{35}S]-MBP-Sic1p^{MH6} is ubiquitinated in fractionated yeast extract similarly to wild-type Sic1p produced by in vitro translation (Verma et al., 1997b). We used monomeric [^{35}S]-MBP-Sic1p^{MH6} as a substrate for most of the experiments reported here because previous work indicated that association of Sic1p with Clb5p/Cdc28p was not required for its ubiquitination (Verma et al., 1997b).

To test whether this set of recombinant proteins was sufficient to direct ubiquitination of Sic1p in the absence of other yeast proteins, we mixed unfractionated insect cell lysates containing Cdc4p, Cdc53p, and Skp1p with purified ^{His6}Uba1p, Cdc34p, ubiquitin, and an ATP-regenerating system. For the direct phosphorylation of Sic1p, which is essential for its ubiquitination (Verma et al., 1997c), we also added affinity-purified Cln2p/Cdc28p^{HA}/Cks1p protein kinase complexes to the reaction. In work to be published elsewhere (Reynard and Deshaies, unpublished data), we demonstrated that Cln2p/Cdc28p protein kinase activity is dependent upon the budding yeast p13^{suc1} homolog Cks1p (Hadwiger et al., 1989). When mixed with the components listed above, [^{35}S]-MBP-Sic1p^{MH6} was converted to a ladder of high molecular weight (HMW) species (Figure 1A, lane 8). The HMW MBP-Sic1p^{MH6} species generated in this reaction represent the assembly of substrate-linked multiubiquitin chains, since their formation was markedly reduced by the omission of ubiquitin (lane 1; see also Figure 3). Reactions lacking Cdc4p, Cdc34p, Cdc53p, or Skp1p (lanes 3–6) failed to generate HMW ubiquitin conjugates and instead yielded phosphorylated Sic1p. In contrast, Sic1p was neither phosphorylated nor ubiquitinated in reactions lacking Cln2p/Cdc28p/Cks1p (lane 2). The requirement for Cdk activity could be satisified by adding either Cln2p/Cdc28p/Cks1p or Clb5p/Cdc28p complexes (Figure 1B, lane 1 versus lane 4). This suggests that newly liberated Clb5p/Cdc28p complexes may contribute to the destruction of Sic1p in vivo, thereby sharpening the G1/S transition (Verma et al., 1997b). Surprisingly, HMW derivatives of MBP-Sic1p^{MH6} were efficiently generated in the absence of ^{His6}Uba1p (lane 7). This presumably reflects the fact that a conserved Uba1p-like activity is most likely present in insect cell lysates, since reactions utilizing purified components demonstrated a direct requirement for ^{His6}Uba1p (see below, Figure 3). Note that the C-terminal 60 amino acids of Cdc53p, which are highly conserved among eukaryotic cullins (Kipreos et al., 1995; Mathias et al., 1996), were not required for the ubiquitination-promoting activity of Cdc53p (lane 9).

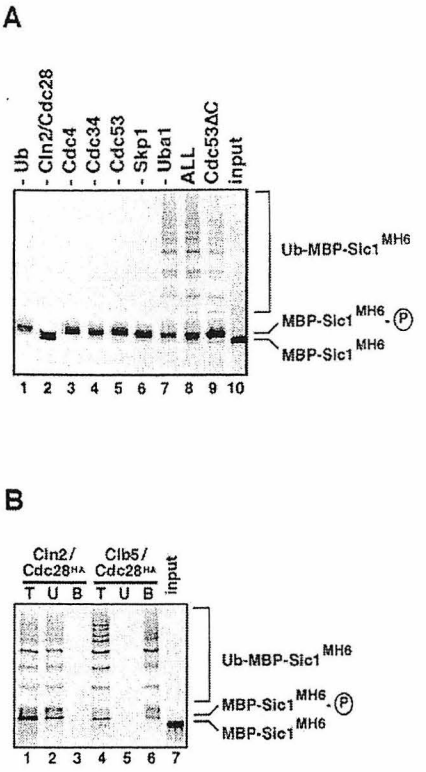


Figure 1. Ubiquitination of MBP-Sic1p In Vitro Requires Cdc4p, Cdc34p, Cdc53p, Skp1p, and Cyclin-Dependent Kinase

(A) Unfractionated lysates from baculovirus-infected Sf9 cells expressing Cdc4p, Cdc53p, and Skp1p, and purified Cdc34p, ^{His6}Uba1p, and Cln2p/Cdc28p^{HA}/Cks1p complexes were prepared as described in Experimental Procedures. The substrate MBP-Sic1p^{MH6} was expressed and metabolically labeled in E. coli and purified via affinity chromatography (lane 10). Substrate was mixed with all of the above components plus ubiquitin and an ATP-regenerating system (lane 8). For lanes 1–7, the component indicated above each lane was omitted from the reaction mixture. In lane 9, Cdc53p lysate was replaced by an insect cell lysate containing Cdc53pΔC, a mutant of Cdc53p lacking its 60 C-terminal residues. All reactions were incubated at 25°C for 90 minutes, stopped by addition of SDS–PAGE buffer, resolved by SDS–PAGE (8%), and visualized by autoradiography. The positions of unmodified, phosphorylated, and ubiquitinated MBP-Sic1p^{MH6} are indicated.

(B) In vitro ubiquitination reactions containing either immunopurified Cln2p/Cdc28p^{HA}/Cks1p (lane 1) or Clb5p/Cdc28p^{HA} (lane 4) as a source of kinase were assembled as described in Figure 1. Lane 7 contains input MBP-Sic1p^{MH6} used in the ubiquitination reactions. After incubation at 25°C for 1 hour, Cdc28p^{HA} and associated proteins were immunoprecipitated with the anti-HA antibody 12CA5. Equivalent amounts of the total reactions ([T], lanes 1 and 4) unbound ([U], lanes 2 and 5) and 12CA5-bound ([B], lanes 3 and 6) fractions were resolved by SDS–PAGE, and MBP-Sic1p^{MH6} was visualized by autoradiography.

Although the ubiquitination of Sic1p leads to its destruction and the subsequent release of Clb5p/Cdc28p from inhibition, it is unclear if the ubiquitination of Sic1p is sufficient to dislodge it from Clb5p/Cdc28p. To address this question, the reactions shown in Figure 1B, lanes 1 and 4 were incubated with anti-HA antibody to immunoprecipitate Cdc28p^{HA} and associated proteins. As expected, MBP-Sic1p^{MH6} did not bind Cln2p/Cdc28p^{HA}/

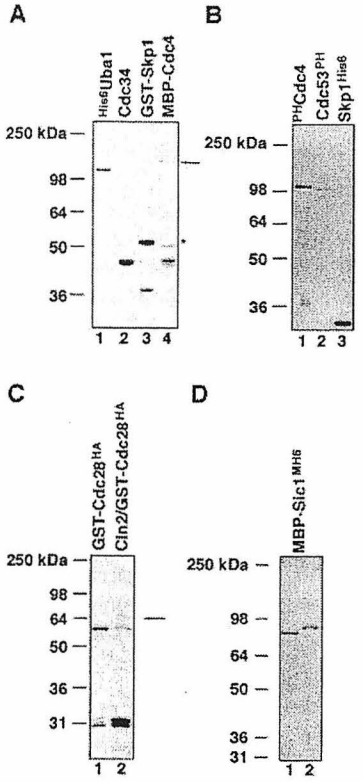


Figure 2. Purified Ubiquitination Components

(A–D) Recombinant proteins purified from yeast (^{His6}Uba1p), E. coli (GST-Skp1p, MBP-Cdc4p, MBP-Sic1p^{MH6}), and baculovirus-infected Sf9 cells (^{PH}Cdc4p, Cdc53p^{PH}, Cln2p/GST-Cdc28p^{HA}, GST-Cdc28p^{HA}, Skp1p^{His6}) as described in Experimental Procedures were separated on SDS–polyacrylamide gels and stained with Coomassie blue. In (A), the asterisks and arrowhead denote full-length GST-Skp1p (lane 3) and MBP-Cdc4p (lane 4), respectively. In (C), Cln2p is designated by an arrow. In (D), purified MBP-Sic1p^{MH6} was incubated with bead-bound Cln2p/Cdc28p^{HA}/Cks1p kinase in the absence (lane 1) or presence (lane 2) of ATP. These forms of substrate were used for the experiments shown in Figure 4.

Cks1p (lane 3); in contrast, both unmodified and multi-ubiquitinated MBP-Sic1p^{MH6} were both efficiently coimmunoprecipitated with Clb5p/Cdc28p^{HA} (lane 6).

Reconstitution of MBP-Sic1p^{MH6} Ubiquitination with Purified Proteins

The experiment shown in Figure 1A clearly established that Cdc4p, Cdc53p, Skp1p, and Cdc34p were required for the ubiquitination of MBP-Sic1p^{MH6}. However, these experiments were conducted with unfractionated insect cell lysates that contained E1 activity, ubiquitin, and potentially other, unidentified ubiquitination factors. To define a set of components that are both necessary and sufficient for the ubiquitination of Sic1p, we individually expressed and purified the recombinant Cdc4p, Cdc53p, and Skp1p proteins. Glutathione-S-transferase-Skp1p (GST-Skp1p) was expressed in E. coli and purified by glutathione affinity chromatography (Figure 2A, lane 3). ^{PH}Cdc4p (Cdc4p tagged at its N terminus with a bipartite Polyoma antigen–Hemagglutinin epitope, hereafter denoted as PH), Cdc53p^{PH} (Cdc53p tagged with a PH epitope at its C terminus), and Skp1p^{His6} were expressed in Sf9 insect cells via infection with recombinant baculoviruses and affinity-purified on either anti-polyoma monoclonal antibody or Ni^{+}-NTA matrices (Figure 2B, lanes 1–3). Cln2p/GST-Cdc28p^{HA} complexes were produced in Sf9 insect cells via coinfection with recombinant baculoviruses, activated by addition of purified Cks1p, and affinity-purified on glutathione resin (Figure 2C, lane 2). Purification of GST-Cdc28p^{HA} alone from Sf9 cells is shown for comparison (lane 1). Similar results were obtained with Cln2p/Cdc28p^{HA}/Cks1p complexes purified on an anti-HA monoclonal antibody matrix (data not shown).

The availability of purified proteins allowed us to test whether this set of components was sufficient to direct ubiquitination of MBP-Sic1p^{MH6}. Since the principal function of Cdk complexes in this reaction is to phosphorylate Sic1p (Verma et al., 1997c), we prepared competent substrate by mixing purified [^{35}S]-MBP-Sic1p^{MH6} with ATP and Cln2p/GST-Cdc28p^{HA}/Cks1p complexes that were bound to glutathione-agarose beads. Following a brief incubation, the soluble pool of phosphorylated substrate was separated from the beads. When phosphorylated [^{35}S]-MBP-Sic1p^{MH6} was mixed with purified ^{PH}Cdc4p, Cdc34p, Cdc53p^{PH}, GST-Skp1p, ^{His6}Uba1p, ubiquitin, and an ATP-regenerating system, it was extensively ubiquitinated (Figure 3A, lane 8). Substituting ubiquitin with the chain-terminating derivatives methyl-ubiquitin (Hershko and Heller, 1985) or K48R-ubiquitin (Chau et al., 1989; Figure 3B), or omitting ubiquitin from the reaction entirely (Figure 3A, lane 6) dramatically altered the profile of MBP-Sic1p^{MH6} modification. These results confirm that the HMW forms observed in Figure 3A, lane 8 correspond to multiubiquitinated MBP-Sic1p^{MH6}.

As was observed with crude components (Figure 1A), no ubiquitination was detected when ^{PH}Cdc4p, Cdc34p, or Cdc53p^{PH} were omitted from the reaction (Figure 3A, lanes 2–4), or when unphosphorylated MBP-Sic1p^{MH6} was used as substrate (lane 1). In contrast to reactions containing crude lysate, reactions carried out with purified components were absolutely dependent upon ^{His6}Uba1p (lane 7).

Unexpectedly, omitting GST-Skp1p from the reaction mixture did not affect the ubiquitination of MBP-Sic1p^{MH6} (Figure 3A, lane 5). This was in marked contrast to the clear requirement for Skp1p seen in the previous experiments using crude insect cell lysates (Figure 1A). Previous studies have identified a specific interaction between Cdc4p and Skp1p that is mediated by a domain in Cdc4p known as the F box (Bai et al., 1996). Skp1p appears to be conserved in eukaryotes, as homologs have been identified in A. thaliana, C. elegans, and humans (Zhang et al., 1995; Bai et al., 1996). We reasoned that if an insect Skp1p-like protein was present, it might efficiently bind to and copurify with ^{PH}Cdc4p, and be present in sufficient quantity to provide a detectable activity in our ubiquitination assay.

To produce Cdc4p devoid of contaminating Skp1p activity, an MBP-Cdc4p fusion was expressed in E. coli and purified by amylose affinity chromatography (Figure 2A, lane 4). Purified MBP-Cdc4p was able to substitute for ^{PH}Cdc4p in a ubiquitination reaction conducted with purified components (Figure 3C, lane 3). However, in

contrast to the results obtained with ^{PH}Cdc4p (Figure 3A, lane 5), reactions conducted with MBP-Cdc4p were absolutely dependent upon GST-Skp1p (Figure 3C, lane 2). Since Skp1p^{His6} remains active in promoting ubiquitination even after being heated at 95°C (data not shown), we hypothesized that any insect Skp1p that copurified with ^{PH}Cdc4p would dissociate from heat-denatured ^{PH}Cdc4p but would retain its activity. Whereas a heat-treated preparation of ^{PH}Cdc4p no longer possessed Cdc4p activity (data not shown), it partially fulfilled the requirement for Skp1p activity (lane 4). Thus, ^{PH}Cdc4p copurifies from insect cells with a heat-stable Skp1p-like activity that can promote the ubiquitination of Sic1p. It remains unclear why this Skp1p-like activity failed to sustain MBP-Sic1p^{MH6} ubiquitination in crude Sf9 cell lysates in the absence of exogenously added Skp1p. Nevertheless, our data suggest that Cdc34p, Cdc4p, Cdc53p, and Skp1p, along with ^{His6}Uba1p, ubiquitin, and ATP, are necessary and sufficient to support efficient multiubiquitination of phosphorylated Sic1p. Since Cdc4p, Cdc53p, and Skp1p are required to direct the Cdc34p-dependent ubiquitination of phospho-Sic1p, these proteins appear to function collectively as an E3.

Phosphorylated MBP-Sic1p^{MH6} Is Specifically Targeted for Ubiquitination by Interaction with Cdc4p/Skp1p

In the two ubiquitination pathways that have been best characterized to date—the N-end rule and the E6/E6-AP pathway—substrate selectivity is conferred by a specific interaction between the substrate and an E3 (Bartel et al., 1990; Scheffner et al., 1993). Since Cdc4p, Cdc53p, and Skp1p collectively serve as an E3, we hypothesized that one of these proteins might interact selectively with phosphorylated Sic1p. To test this possibility, we performed coimmunoprecipitation experiments using either unphosphorylated or phosphorylated MBP-Sic1p^{MH6} (Figure 2D) as bait. Sf9 cells singly infected with baculoviruses expressing either Cdc4p, Cdc53p, or Skp1p were metabolically labeled with ^{35}S-amino acids, and lysates derived from the labeled cells (Figure 4A, lanes 1 and 4; Figure 4B, lane 1) were incubated separately with either phosphorylated or unmodified MBP-Sic1p^{MH6}, after which MBP-Sic1p^{MH6} and associated proteins were recovered with anti-MBP antibodies. This analysis revealed that ^{PH}Cdc4p (Figure 4A, lanes 2 and 3), but not Cdc53p (lanes 5 and 6) or Skp1p^{HA} (Figure 4B, lanes 2 and 3), selectively bound to phosphorylated MBP-Sic1p^{MH6}. Combining Cdc53p- and Skp1p^{HA}-containing lysates did not result in enhanced interaction of these components with either form of MBP-Sic1p^{MH6} (data not shown). In contrast, when Skp1p^{HA} lysate was mixed with ^{PH}Cdc4p lysate, both proteins were selectively coimmunoprecipitated with phosphorylated MBP-Sic1p^{MH6}, and the level of coprecipitated ^{PH}Cdc4p was substantially increased (compare Figure 4B, lane 5 with Figure 4A, lane 3).

Mutational analysis of Cdk phosphoacceptor sites in Sic1p revealed that phosphorylation of a specific set of residues triggers Sic1p destruction. An epitope-tagged version of Sic1p lacking these key Cdk phosphoacceptor sites (Sic1p^{HAHis6}-T5G, T33A, S76A; Sic1p^{HAHis6}-Δ3P)

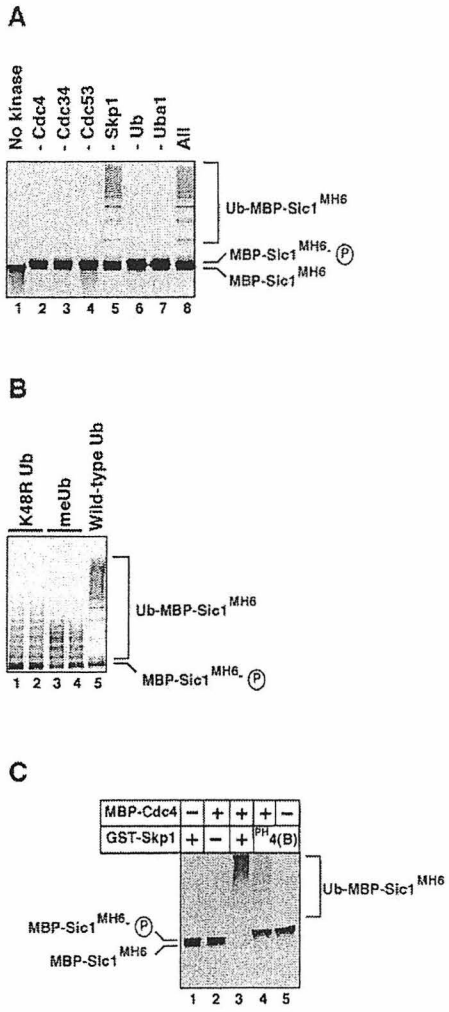


Figure 3. Reconstitution of MBP-Sic1p^{MH6} Ubiquitination with Purified Proteins.

(A) Purified ^{PH}Cdc4p, Cdc34p, Cdc53p^{PH}, GST-Skp1p, ^{His6}Uba1p, ubiquitin, [^{35}S]-MBP-Sic1p^{MH6} that had been phosphorylated by incubation with immobilized Cln2p/GST-Cdc28p^{HA}/Cks1p complexes, and an ATP-regenerating system were mixed together (lane 8). Reactions shown in lanes 2–7 lack the indicated component. For lane 1, all components listed above were added to MBP-Sic1p^{MH6} that had not been preincubated with Cln2p/GST-Cdc28p^{HA}/Cks1p. Reactions were incubated at 25°C for 90 minutes, stopped by addition of SDS–PAGE buffer, resolved by SDS–PAGE (8%), and visualized by autoradiography. The positions of MBP-Sic1p^{MH6}, phosphorylated MBP-Sic1p^{MH6}, and ubiquitinated MBP-Sic1p^{MH6} are indicated.

(B) Reactions were assembled using purified proteins as described in part (A), except that reactions contained either mutant K48R ubiquitin (lanes 1 and 2), methyl ubiquitin (lanes 3 and 4), or wild-type ubiquitin (lane 5). Reactions were incubated and processed as described above.

(C) In all reactions, [^{35}S]-MBP-Sic1p^{MH6} was incubated with soluble Cln2p/GST-Cdc28p^{HA}/Cks1p, ^{His6}Uba1p, Cdc34p, and Cdc53p^{PH} along with ubiquitin and an ATP-regenerating system as described above. In addition, the following components were added to the indicated reactions: GST-Skp1p (lane 1), MBP-Cdc4p (lane 2), MBP-Cdc4p plus GST-Skp1p (lane 3), and the soluble fraction recovered from heat-denatured preparations of purified ^{PH}Cdc4p either with (lane 4) or without MBP-Cdc4p (lane 5). All reactions were incubated at 24°C for 45 min and then evaluated by SDS–PAGE and autoradiography.

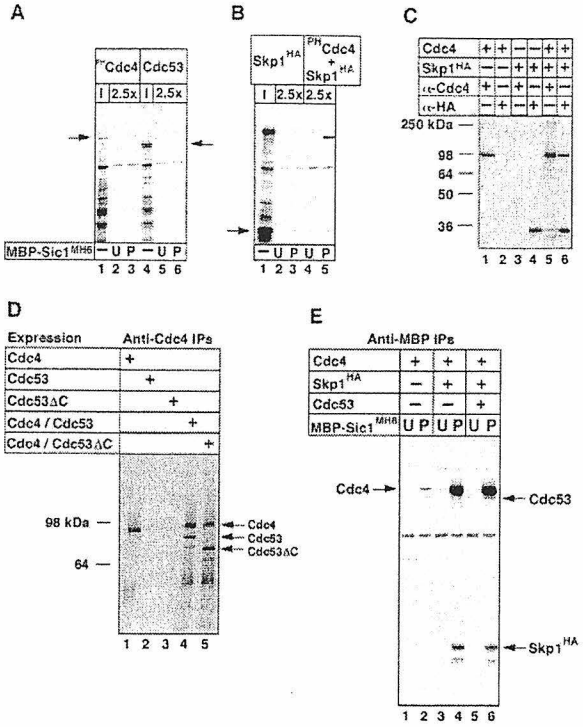


Figure 4. Phosphorylated MBP-Sic1p^{MH6} Specifically Binds to Cdc4p/Skp1p and Assembles into a Complex with Cdc4p, Skp1p, and Cdc53p

Sf9 cells were infected with the indicated baculovirus and metabolically labeled with Tran[^{35}S]-label for 3 hr. For (A), (B), and (E), the derived radiolabeled lysates were then incubated with either unphosphorylated (U) or phosphorylated (P) MBP-Sic1p^{MH6}, and following a 1 hr incubation at 4°C MBP-Sic1p^{MH6}-associated proteins were immunoprecipitated with anti-MBP antibodies and analyzed by SDS–PAGE, followed by autoradiography. The input (I) lanes were underloaded 2.5-fold relative to the immunoprecipitates. (A) and (B) were from a single exposure of the same gel and so can be compared directly with each other.

(A) Radiolabeled lysates containing ^{PH}Cdc4p (lanes 2 and 3) or Cdc53p (lanes 5 and 6) were used for analysis. The relative position of each protein is indicated by an arrow.

(B) Radiolabeled lysate containing Skp1p^{HA} (lanes 1–3) or an equal mixture of Skp1p^{HA} plus ^{PH}Cdc4p-containing lysates (lanes 4 and 5) was used.

(C) Radiolabeled lysates containing Cdc4p, Skp1p^{HA}, or a mixture of the two were immunoprecipitated with either anti-Cdc4p or anti-HA antibodies as indicated.

(D) Sf9 cells were singly infected with baculoviruses expressing Cdc4p (lane 1), Cdc53p (lane 2), and Cdc53pΔC (lane 3), or coinfected with Cdc4p and Cdc53p (lane 4) or Cdc4p and Cdc53pΔC (lane 5) baculoviruses. [^{35}S]-radiolabeled lysates were prepared from each infection and immunoprecipitated with anti-Cdc4p antibodies. The immunoprecipitates were analyzed by SDS–PAGE followed by autoradiography.

(E) Radiolabeled lysates containing Cdc4p (lanes 1–6) were mixed with radiolabeled Skp1p^{HA} (lanes 3–6) or Cdc53p (lanes 5 and 6) lysates prior to addition of MBP-Sic1p^{MH6}, followed by immunoprecipitation using anti-MBP antibodies.

was neither ubiquitinated in vitro nor degraded in vivo, but retained the ability of the wild-type protein to bind to Clb5p/Cdc28p complexes (Verma et al., 1997c). Given the results described above, we surmised that the stability of the Sic1p^{HAHis6}-Δ3P mutant might arise from a failure to bind to Cdc4p/Skp1p. To test this hypothesis, we

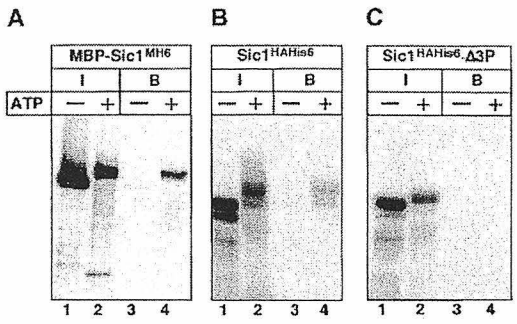


Figure 5. Purified Cdc4p Specifically Binds Phosphorylated Sic1p but Not a Stabilized Mutant Form of Sic1p

[^{35}S]-radiolabeled Sic1p proteins were incubated with purified bead-bound Cln2p/GST-Cdc28p^{HA}/Cks1p in the absence (−) or presence (+) of ATP to produce either an unphosphorylated or phosphorylated form of each protein. Following the kinase reactions, the supernatant fractions containing [^{35}S]-Sic1p were withdrawn and mixed with bead-bound purified ^{PH}Cdc4p. After a 1 hr incubation, the ^{PH}Cdc4p beads were recovered and analyzed by SDS–PAGE followed by autoradiography. The input lanes were underloaded 1.5-fold relative to the bead-bound fractions.

(A) [^{35}S]-MBP-Sic1p^{MH6} was purified from metabolically labeled E. coli.

(B) [^{35}S]-Sic1p was produced by in vitro translation in rabbit reticulocyte lysate.

(C) [^{35}S]-Sic1p-Δ3P was produced by in vitro translation in rabbit reticulocyte lysate. The Sic1p-Δ3P mutant lacks three Cln2p/Cdc28p phosphoacceptor sites and is neither ubiquitinated in vitro nor degraded in vivo (Verma et al., 1997c).

affinity-purified ^{PH}Cdc4p from insect cell lysates using an anti-polyoma antibody matrix and incubated the immobilized ^{PH}Cdc4p complexes with various [^{35}S]-labeled forms of Sic1p. Incubation of the bead-bound ^{PH}Cdc4p with either unphosphorylated or phosphorylated MBP-Sic1p^{MH6} confirmed that only the phosphorylated form bound to the ^{PH}Cdc4p matrix (Figure 5A). Sic1p^{HAHis6} produced by in vitro translation in rabbit reticulocyte lysate also bound to ^{PH}Cdc4p-containing beads in a phosphorylation-dependent manner (Figure 5B), but Sic1p^{HAHis6}-Δ3P was not retained by the ^{PH}Cdc4p matrix even though it was clearly phosphorylated by Cln2p/Cdc28p^{HA}/Cks1p on sites whose modification is not sufficient to promote ubiquitination (Verma et al., 1997c; Figure 5C). These data establish two key points: first, the stability of Sic1p^{HAHis6}-Δ3P can be explained by its inability to bind Cdc4p/Skp1p; second, the failure of Cdc4p/Skp1p to interact with stable (yet phosphorylated) Sic1p^{HAHis6}-Δ3P suggests that the interaction between Cdc4p/Skp1p and phosphorylated wild-type Sic1p is physiologically significant and is not a nonspecific ionic interaction stabilized by the phosphate groups.

Recruitment of Phosphorylated Sic1p to a Skp1p/Cdc53p/Cdc4p E3 Complex

Cdc4p and Skp1p associate with one another in reticulocyte lysate (Bai et al., 1996). Consistent with this observation, recombinant Cdc4p and Skp1p expressed in insect cells also associated with each other, as judged by coimmunoprecipitation of Skp1p with Cdc4p (Figure 4C, lane 5) and vice versa (lane 6). Since Cdc4p assembled into complexes with both Skp1p and phosphorylated MBP-Sic1p^{MH6} in insect cell lysates, we also exam-

ined whether it could associate with Cdc53p. Sf9 cells that were either singly infected or coinfected with baculoviruses expressing Cdc4p, Cdc53p, or Cdc53pΔC were metabolically labeled with ^{35}S-amino acids, and lysates derived from the labeled cells were immunoprecipitated with anti-Cdc4p antibodies. As shown in Figure 4D, both Cdc53p (lane 4) and Cdc53p-ΔC (lane 5) specifically coprecipitated with Cdc4p. Since Cdc4p appeared to associate with a Skp1p-like activity in insect cell lysates (Figure 3C), it remains unclear whether Cdc4p and Cdc53p directly bound each other or interacted indirectly via Skp1p.

Given that Cdc4p can associate with Skp1p, Cdc53p, and phosphorylated substrate, and that the interaction between phosphorylated MBP-Sic1p^{MH6} and Cdc4p was strongly stimulated by Skp1p, we hypothesized that Cdc4p/Skp1p might serve as a receptor module that tethers phosphorylated substrate to an E3 complex containing Cdc53p. To test this possibility, we combined ^{35}S-radiolabeled Sf9 cell lysates containing Cdc4p, Cdc53p, and Skp1p^{HA} and incubated these mixtures with either phosphorylated or unmodified MBP-Sic1p^{MH6}, followed by immunoprecipitation with anti-MBP antibodies (Figure 4E). Whereas Cdc4p, Cdc53p, and Skp1p^{HA} did not coimmunoprecipitate with unphosphorylated MBP-Sic1p^{MH6}, all three proteins stably associated with phosphorylated MBP-Sic1p^{MH6}. Since Cdc53p by itself failed to bind selectively to phosphorylated MBP-Sic1p^{MH6} (Figure 4A, lane 6), the observed interaction was most likely due to the ability of both Cdc53p and phosphorylated MBP-Sic1p^{MH6} to bind independently to Cdc4p/Skp1p to form a single complex. Cdc53p has been reported to bind to Cdc34p (Willems et al., 1996). Thus, phosphorylated MBP-Sic1p^{MH6} bound to the Cdc4p/Cdc53p/Skp1p complex would be well-positioned to serve as a substrate for Cdc34p-dependent ubiquitination.

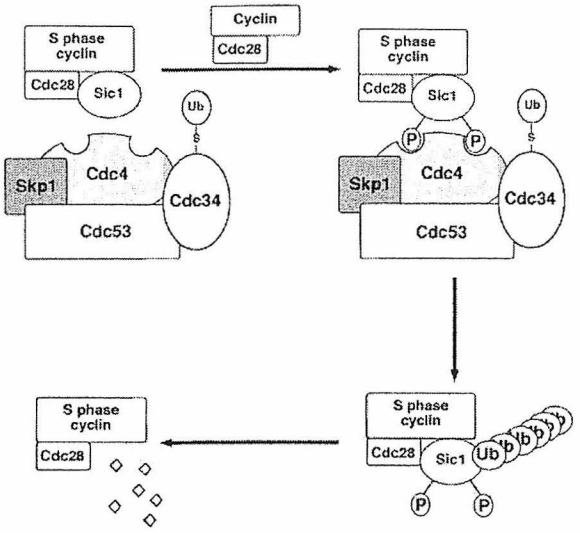


Figure 6. A Model for the Ubiquitination of Sic1p

In early G1 phase, Sic1p is bound to S-phase cyclin/Cdk complexes. For Sic1p to be destroyed, it must first be phosphorylated on several phosphoacceptor sites by an active cyclin/Cdk complex. Once phosphorylated, Sic1p is recognized by Cdc4p/Skp1p, which can interact with Cdc53p and thereby promote the transfer of a ubiquitin moiety from Cdc34p to substrate. Once ubiquitination has been initiated, multiubiquitin chains are processively elongated by a process that is not well understood, and the multiply ubiquitinated Sic1p is destroyed by the 26S proteasome. The exact nature of the protein–protein interactions between Cdc4p, Skp1p, Cdc53p, and Cdc34p implied by this figure has not yet been fully delineated.

Discussion

Reconstitution of Sic1p Ubiquitination with Purified Proteins

Ubiquitin-mediated degradation of the S phase Cdk inhibitor Sic1p plays a key role in triggering the initiation of DNA synthesis in budding yeast (Verma et al., 1997c; Schwob et al., 1994). Here, we report the ubiquitination of Sic1p using nine purified proteins: Uba1p, ubiquitin, Cdc34p, Cdc4p, Cdc53p, Skp1p, and Cln2p/Cdc28p/Cks1p complexes. Based on experiments reported here and elsewhere (Verma et al., 1997c; Willems et al., 1996), we envision the following sequence of events for Sic1p ubiquitination (Figure 6). Active Cln2p/Cdc28p/Cks1p complexes phosphorylate Sic1p on a set of Cdk consensus phosphorylation sites (Verma et al., 1997c), thereby priming it to serve as a substrate for ubiquitination. The phosphorylated Sic1p then binds to a complex containing Cdc4p, Cdc53p, and Skp1p subunits. We have agreed with Skowyra et al. (1997 [this issue of *Cell*]) to designate this assemblage SCF, which stands for Skp1p/Cdc53p (or Cullin)/F-box ubiquitin ligase complex, with the identity of the associated F-box protein indicated by a superscript (e.g., SCF^{Cdc4p}). Upon binding phosphorylated Sic1p, we propose that the Cdc53p subunit of SCF^{Cdc4p} recruits the ubiquitin-charged E2 enzyme Cdc34p to initiate Sic1p ubiquitination (Willems et al., 1996).

Several aspects of our model for Sic1p ubiquitination and degradation remain speculative. First, the interactions between Cdc4p, Cdc53p, Skp1p, Cdc34p, and phosphorylated Sic1p remains to be characterized in detail. Second, it is unclear how ubiquitin is transferred from Cdc34p to lysine residues in the SCF-bound substrate. Third, whereas SCF^{Cdc4p}, Cdc34p, E1 enzyme, and ubiquitin appear to be sufficient to sustain the ubiquitination of phosphorylated Sic1p in vitro, it is possible that additional proteins may regulate Sic1p ubiquitination in vivo. Such accessory factors might serve to activate known SCF^{Cdc4p} subunits or to overcome negative regulation that is not reconstituted in our in vitro system. Finally, it is unclear if SCF^{Cdc4p} rapidly dissociates from multiubiquitinated substrate or remains bound, perhaps interacting with the proteasome to facilitate the degradation of multiubiquitinated Sic1p.

Cdc4p/Skp1p Recruits Phosphorylated Substrates for Ubiquitination

A hallmark of SCF^{Cdc4p}-dependent ubiquitination is that it is selective for phosphorylated substrates. Cdc6p, Gcn4p, Far1p, and Sic1p are ubiquitinated in crude yeast extract in a Cdc34p- and Cdc4p-dependent manner, and in each case ubiquitination requires a kinase that phosphorylates the substrate (Verma et al., 1997c; Y. Chi and R. J. D., unpublished data). We show here that

this requirement derives from a selective interaction between phosphorylated substrate and Cdc4p/Skp1p. Skp1p by itself cannot bind to phospho-Sic1p. Whereas Cdc4p can interact with phospho-Sic1p in insect cell lysates, this interaction is enhanced considerably by Skp1p. The existence of a Skp1p-like activity in insect cells that copurifies with Cdc4p obscures whether phospho-Sic1p can be recognized by Cdc4p alone or only by a Cdc4p/Skp1p complex.

What are the individual roles of Cdc4p and Skp1p in substrate binding? Based on the observation that the WD-40 domains of β-transducin fold into a β-propeller structure with an extensive surface area suitable for protein–protein interaction (Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996), we propose that the WD-40 domain of Cdc4p binds directly to phospho-Sic1p and that this interaction is facilitated by Skp1p. An unambiguous test of this hypothesis will likely require the generation of point mutations in the WD-40 repeats, since crude deletions in this domain appear to destabilize Cdc4p; Cdc4p-ΔWD mutants translated in reticulocyte lysate bind poorly to Skp1p and exhibit high nonspecific adsorption to matrices (C. C. C., unpublished data). Regardless of the identity of the substrate interaction domain in Cdc4p, Skp1p most likely promotes substrate recruitment to SCF^{Cdc4p} either by forming direct contacts with bound substrate, or by evoking a conformational change in Cdc4p. The ability of Skp1p to enhance Cdc4p's substrate binding activity may prevent "free" Cdc4p from competing with SCF^{Cdc4p} for association with phosphorylated substrates. This notion is supported by our observation that recombinant Cdc4p does not competitively inhibit Sic1p ubiquitination in crude yeast extract, even when added in amounts 200-fold greater than those needed to achieve efficient complementation of the ubiquitination defect of *cdc4* mutant extract (Verma et al., 1997b; data not shown).

Whereas the phosphorylation of Sic1p triggers its ubiquitination by enhancing its association with Cdc4p/Skp1p, it remains unclear how Cdc4p/Skp1p discriminates between phosphorylated and unmodified Sic1p. We can envision two distinct models. Phosphorylation of Sic1p may cause a conformational rearrangement that exposes a cryptic Cdc4p/Skp1p-binding determinant in Sic1p. Alternatively, Cdc4p/Skp1p may bind directly to the phosphate groups on Sic1p, much like SH2 and 14-3-3 domains bind directly to phospho–amino acid epitopes (Songyang et al., 1993; Muslin et al., 1996). Mutagenesis experiments have shown that no single phosphorylation site on Sic1p is necessary or sufficient to specify its ubiquitination (Verma et al., 1997c). Instead, the mutagenesis data suggest that there is a set of key phosphorylation sites on Sic1p and that several distinct Sic1p phospho-isomers that are modified in a subset of these sites are competent to serve as substrates for SCF^{Cdc4p}-dependent ubiquitination. These data lead us to favor the idea that Cdc4p/Skp1p interacts directly with phosphorylated serine or threonine. Perhaps selective interaction with phosphorylated targets will prove to be a general property of proteins containing WD-40 repeats.

Willems et al. (1996) argued that Cdc53p selectively targets phosphorylated Cln2p for Cdc34p-dependent ubiquitination. However, these authors failed to demonstrate that the interaction they observed between Cdc53p and phospho-Cln2p was direct and not promoted by intermediary proteins. Our data suggest that this interaction was actually sustained by either Cdc4p or Grr1p (see below). Alternatively, Cdc53p may directly bind phosphorylated Cln2p, but this interaction may serve to recruit Cln2p/Cdc28p kinase activity to SCF, rather than to target Cln2p for ubiquitination.

Conservation and Generality of the SCF pathway

Phosphorylation triggers the degradation of a broad range of key regulatory proteins in metazoans, including G1 cyclins (Clurman et al., 1996; Won et al., 1996; Diehl et al., 1997); IκB, which is a global negative regulator of the immune response (Verma et al., 1995); CACTUS, which is required for dorsal-ventral patterning in Drosophila embryos (Belvin and Anderson, 1996); and B-catenin, which is involved in developmental patterning and acts as a tumor suppressor gene (Aberle et al., 1997). For IκB, CACTUS, and B-catenin, it is thought that phosphorylation-triggered degradation operates as a switch that governs the ultimate biological response. Based on our observations with Sic1p (Verma et al., 1997c; this paper), we speculate that regulated protein kinases and SCF-like complexes coordinately regulate diverse biological processes including the immune response, organismal development, and cell proliferation/tumor progression. Based on the known diversity of protein kinases and the potential diversity of SCF pathways (see below), an attractive model is that the ubiquitination of different SCF substrates is controlled by distinct, independently regulated kinases, or by multiple kinases acting in a combinatorial fashion (King et al., 1996).

Given the diverse range of substrates whose degradation is regulated by phosphorylation, a key question is, are there different SCF pathways in eukaryotic cells? Whereas Sic1p degradation requires Cdc34p, Cdc53p, Skp1p, and Cdc4p, Cln2p degradation requires Cdc34p, Cdc53p, Skp1p, and Grr1p (Barral et al., 1995; Deshaies et al., 1995; Bai et al., 1996; Willems et al., 1996), but not Cdc4p (R. J. D., unpublished data). Both Cdc4p and Grr1p contain an F box, which is a conserved domain that is thought to mediate binding to Skp1p (Bai et al., 1996). Thus, Cdc4p and Grr1p might serve as interchangeable modules that interact with a core ubiquitination machinery comprised of Skp1p, Cdc53p, and Cdc34p (Bai et al., 1996). This would allow for the assembly of two different ubiquitin ligase complexes with distinct substrate preferences; SCF^{Cdc4p} would target Sic1p for ubiquitination, whereas SCF^{Grr1p} would target Cln2p. We envision that the F box–containing receptor module would be recruited from a pool of inactive receptor subunits, only to be activated upon docking with Skp1p. In light of this model, it is perhaps surprising that Grr1p and Cdc4p share no homology outside of the F-box, even though Cln2p, like Sic1p, contains PEST sequences and must be phosphorylated to be recognized as a substrate for degradation (Lanker et al., 1996).

Besides Grr1p and Cdc4p, at least 30 F box–containing proteins have been detected in DNA sequence databases (Bai et al., 1996). Coupled with the diversity of

Cdc53p/cullin- and Skp1p-like proteins in humans, the broad pallette of F box–containing proteins would allow for the assembly of an array of SCFs with distinct substrate-binding specificities and modes of regulation. Thus, SCF-based ubiquitination pathways may play a broad role in eukaryotic regulatory biology.

Experimental Procedures

Insect Cells, Yeast Strains, and Reagents

Bacterial expression vectors for K48R-Ub and Cdc34p originated from J. Callis (University of California, Davis) and V. Chau (Wayne State University, Detroit, MI), respectively. The mouse monoclonal cell line expressing anti-polyoma antibodies was a generous gift from M. Peter (Swiss Institute for Experimental Cancer Research, Epalinges). A bacterial expression vector for GST-Skp1p, along with baculovirus constructs that express Skp1p, Skp1p^{HA}, or Skp1p^{His6}, were all generous gifts of P. Sorger (Massachusetts Institute of Technology, Cambridge). *CDC4*, *CDC53*, and SIC1^{HAHis6}-*T2A, T5G, T33A, S76A* plasmids were obtained from B. Jensen (University of Washington, Seattle), M. Goebl (Indiana University, Indianapolis), and R. Verma (this laboratory), respectively. Baculoviruses that express GST-Cdc28p^{HA} (J. W. Harper, Baylor College of Medicine, Houston, TX), Clb5p (M. Weinreich, Cold Spring Harbor), Cln2p, and Cdc28p^{HA} (Greg Reynard, this laboratory) were kindly provided by the indicated investigators.

Baculovirus Construction

Baculoviruses expressing Cln2p and Cdc28p^{HA} are described elsewhere (Reynard and Deshaies, unpublished data). The construction of a baculovirus encoding Cdc4p has been described previously (Verma et al., 1997b). Epitope-tagged versions of both *CDC4* and *CDC53* were constructed via insertion of a DNA cassette that contains two tandem repeats of a peptide epitope (MEYMPME) derived from the polyoma virus middle T protein (Grussenmeyer et al., 1985) followed by three tandem repeats of the hemagglutinin epitope (Field et al., 1988). This bipartite epitope is hereafter designated PH. An epitope-tagged version of Cdc4p was constructed by introducing via PCR a single copy of the PH cassette immediately after the start codon of Cdc4p present in pVL1392-CDC4 (Verma et al., 1997b).

To generate viral vectors for expression of Cdc53p, a portion of *CDC53* lacking its 60 C-terminal residues was amplified by PCR from pGEM53-8 (Mathias et al., 1996) and the resulting PCR product subcloned into pVL1392 (Pharmingen), yielding the vector pVL-53ΔC. A fragment containing the last 60 codons of *CDC53* was amplified by PCR from pGEM53-8 and subcloned into pVL-53ΔC to yield pVL-53, which contains the full-length open reading frame of *CDC53*. To generate an epitope-tagged version of Cdc53p, a fragment encoding the C-terminal 95 residues of Cdc53p was amplified by PCR and engineered to contain the PH cassette immediately upstream of the termination codon. This epitope-tagged version of CDC53 was subcloned into the vector pFastbac (GIBCO-BRL), yielding pFBCdc53p^{PH}. pVL-53, pVL-53ΔC and pFBCdc53p^{PH} were used to generate baculovirus stocks, per the manufacturers' instructions. All portions of *CDC4* and *CDC53* plasmids generated by PCR were sequenced.

Antibodies

Anti-Cdc53p antibodies were generated in rabbits immunized with an MBP fusion protein containing the last 23 residues of Cdc53p. Antibodies against MBP and Cdc53p were sequentially purified by affinity chromatography using antigen essentially as described (Harlow and Lane, 1988). Monoclonal anti-polyoma antibodies were purified out of tissue culture supernatant using protein A–Sepharose beads. Purified anti-polyoma antibodies were cross-linked to protein A with dimethylpimilimadate (Harlow and Lane, 1988) at a concentration of 2 mg of antibodies/mL of protein A resin.

Baculovirus Expression

Sf9 cells were infected with the appropriate baculovirus at an MOI of 10. Forty-two hours post-infection, cells were collected and lysed by resuspension in 1 ml of Buffer A per 10^7 cells (Buffer A: 20 mM HEPES [pH 7.4], 100 mM NaCl, 0.1% Triton X-100, 5 mM $MgCl_2$, 5 mM EDTA, 2 mM DTT, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin). Lysates were centrifuged at 14,000 × g for 15 min, and the supernatant (typically 5 mg of protein/ml) was adjusted to 10% glycerol, frozen in liquid nitrogen, and stored at −80°C.

^{PH}Cdc4p and Cdc53p^{PH} were affinity-purified as follows: 25 μl of anti-polyoma protein A beads was added to 500 μL of lysate and incubated at 4°C for 1 hr. The beads were then washed twice with 1 mL of Buffer A fortified with 0.2 M KOAc, and then twice with 1 mL of Buffer B (30 mM HEPES [pH 7.4], 300 mM KOAc, 0.1% octylglucoside, 10% glycerol, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM DTT, 20 μg/mL Arg-insulin, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin). Bound polyoma-tagged proteins were specifically eluted by incubation of the anti-polyoma beads in 50 μL of Buffer B containing 100 μg/mL polyoma peptide (MEYMPME) for 2 hr at 4°C.

Clb5p/Cdc28p^{HA}/Cks1p complexes were generated by coinfecting Sf9 cells with viruses encoding Clb5p and Cdc28p^{HA}. Cell lysates derived from these infections were incubated with Cks1p and immunopurified exactly as described for Cln2p/Cdc28p^{HA}/Cks1p complexes. Cln2p/GST-Cdc28p^{HA} kinase expressed in insect cells was purified by incubation of 200 μL of crude lysate with 25 μL of glutathione agarose beads for 30 minutes at 4°C. The beads were washed twice with the above lysis buffer and twice with Kinase Assay Buffer (KAB: 10 mM HEPES [pH 7.2], 50 mM NaCl, 10 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin). The kinase complex either was left immobilized on the beads, thereby allowing for the subsequent removal of the complex after a kinase reaction, or was eluted by incubation of the resin in 30 μL of KAB containing 5 mM glutathione for 30 min at 4°C.

To purify a hexahistidine-tagged version of Skp1p (Skp1p^{His6}), Sf9 cells infected with a Skp1p-expressing baculovirus were harvested and lysed in Buffer C (25 mM HEPES [pH 7.4], 100 mM KCl, 10 mM imidazole, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin) supplemented with 50 mM NaF, 50 mM β-glycerophosphate, and 0.1% Triton X-100. After centrifugation (15 min, 14,000 × g), Skp1p^{His6} was adsorbed from the lysate onto Ni^+-NTA resin (Qiagen) and eluted in Buffer C plus 120 mM imidazole.

Bacterial/Yeast Expression and Purification of Proteins

Cdc34p was expressed and purified from E. coli as described previously (Banerjee et al., 1993). MBP-Sic1p^{MH6} was expressed and radiolabeled in E. coli and purified by successive steps of Ni^+-NTA and amylose affinity chromatography as described (Verma et al., 1997a). Other fusion proteins containing either maltose binding protein (MBP; New England BioLabs) or glutathione-S transferase (GST; Pharmacia) moieties were expressed and purified as specified by the supplier, and stored in 25 mM HEPES [pH 7.2], 100 mM NaCl, 2 mM DTT, 1 mM EDTA, and 10% glycerol at −80°C.

Saccharomyces cerevisiae Uba1p was purified from the yeast strain JD77 (Dohmen et al., 1995) transformed with the plasmid pJD325 (J. Dohmen, personal communication), which carries a copper-inducible promoter driving expression of hexahistidine-tagged Uba1p (^{His6}Uba1p). Log-phase JD77[pJD325] cells grown in 1L YPD were induced with $CuSO_4$ to 1 mM, harvested 24 hr after induction, washed, and frozen in liquid nitrogen. The frozen cell mass was ground with a chilled mortar and pestle, and the resulting powder was resuspended in Buffer D (50 mM Tris [pH 7.5], 0.1% Triton X-100, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF, 1 μg/mL leupeptin and pepstatin) and then clarified by centrifugation (30 min, 35,000 × g). ^{His6}Uba1p was purified from the lysate by Ni^+-NTA affinity chromatography followed by ubiquitin-affinity chromatography (Ciechanover et al., 1982). Purified ^{His6}Uba1p was dialyzed twice against 1 L of 50 mM Tris [pH 7.6], 0.1 M KCl, 0.2 mM DTT, 15% glycerol and then adjusted to 0.2 mg/mL BSA and stored in aliquots at −80°C.

Ubiquitination Reactions

All ubiquitination reactions contained: 4 μg ubiquitin, 60 ng Cdc34p, 25 ng ^{His6}Uba1p, 1 μL of a 10× ATP-regenerating system (20 mM HEPES [pH 7.2], 10 mM ATP, 10 mM MgOAc, 300 mM creatinine phosphate, 0.5 mg/mL creatinine phosphokinase), 1 μL of 10× reaction buffer (40 mM MgOAc, 10 mM DTT, 1 mM PMSF), and ~10,000

cpm [^{35}S]-MBP-Sic1p^{MH6} (~20 ng). Reactions performed with crude lysates included: 1 μL each of Sf9 cell lysate containing Cdc4p, Cdc53p, or Skp1p; and 2 μl of affinity-purified Cln2p/Cdc28p^{HA}/Cks1p kinase. Reactions utilizing pure protein components contained 25 ng of either GST-Skp1p or Skp1p^{His6}, plus 0.5 μL each of eluted ^{PH}Cdc4p and Cdc53p^{PH} (from a 50 μL immunopurification as described above). For the experiments shown in Figure 3B, wild-type ubiquitin was replaced with 1.5 μg of either methyl ubiquitin or K48R ubiquitin. Reactions using MBP-Cdc4p contained 9 μg of the preparation shown in Figure 2A. Each ubiquitination component was present at saturating levels relative to substrate (data not shown). Reactions were brought to a final volume of 10 μL with 20 mM HEPES [pH 7.4], 100 mM KOAc, 1 mM DTT. All components were mixed and incubated at 25°C for 90 minutes (unless otherwise indicated). Reactions were terminated by addition of Laemmeli sample buffer, resolved by SDS–PAGE, and visualized by autoradiography.

To phosphorylate [^{35}S]-MBP-Sic1p^{MH6}, 85 μL of purified radiolabeled substrate (approximately 1 μg) was mixed with 8 μL of glutathione-agarose-bound Cln2p/GST-Cdc28p^{HA}/Cks1p, and the reactions were diluted to a final volume of 100 μL with KAB and then adjusted to 1 mM ATP. The mixture was gently rocked at room temperature for 2 hr, after which the beads were pelleted by centrifugation (5 sec, 13,000 × g) and the supernatant was used in reactions as described.

Immunoprecipitations

At 40 hr post-infection, baculovirus-infected Sf9 cells were metabolically labeled for 3 hr in methionine-deficient media by the addition of Tran[^{35}S]-label (10 μCi/mL), and then lysates were prepared as described above. Coimmunoprecipitation experiments with MBP-Sic1p^{MH6} were carried out by combining 25 μL of the appropriate Sf9 cell lysate with 1 μg of either unphosphorylated or phosphorylated MBP-Sic1p^{MH6} plus sufficient Buffer A to yield a final volume of 100 μl. The mixtures were incubated for 1 hr at 4°C and immunoprecipitated by addition of anti-MBP antibodies Following a final 1 hr incubation at 4°C, immunoprecipitates were collected by centrifugation and washed with Buffer A containing 5 mM NaF. The resulting samples were analyzed by SDS–PAGE and autoradiography.

PCR templates of Sic1p carrying a tandem hemagglutinin epitope plus hexahistidine (HAHis6) tag at its C-terminus (Sic1p^{HAHis6}), and a mutant form of Sic1p^{HAHis6} (Sic1p-T5G, T33A, S76A; Sic1p^{HAHis6}-Δ3P) were translated in rabbit reticulocyte lysate to produce [^{35}S]-labeled proteins (Verma et al., 1997c). Phosphorylation of both constructs by Cln2p/Cdc28p^{HA}/Cks1p was done essentially as described for [^{35}S]-MBP-Sic1p^{MH6}. To produce samples that were not phosphorylated, substrates were incubated with immobilized kinase in the absence of exogenously added ATP. To test the ability of these proteins to bind ^{PH}Cdc4p, 1 mL of insect cell lysate containing ^{PH}Cdc4p was incubated with 30 μL of anti-polyoma resin for 1 hr at 4°C and then washed three times with Buffer A. Cdc4p:Sic1p binding reactions were carried out in a final volume of 100 μL and contained 10 μL of a 16% slurry of ^{PH}Cdc4p-containing beads, 15 μL of protein A beads (pretreated with 1% bovine serum albumin), and either 3 μL ([^{35}S]-MBP-Sic1p^{MH6}) or 5 μL ([^{35}S]-Sic1p^{HAHis6} and [^{35}S]-Sic1p^{HAHis6}-Δ3P) of ligand. Following a 1 hr incubation at 4°C, the beads were washed three times with Buffer A containing 5 mM NaF, boiled in Laemmeli sample buffer, and subjected to SDS–PAGE and autoradiography.

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References

Aberle, H., Baueer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). B-catenin is a target for the ubiquitin-proteasome pathway. EMBO J. *16*, 3797–3804.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W., and Elledge, S.J. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell *86*, 263–274.

Banerjee, A., Gregori, L., Xu, Y., and Chau, V. (1993). The bacterially expressed yeast *CDC34* gene product can undergo autoubiquitination to form a multiubiquitin chain-linked protein. J. Biol. Chem. *268*, 5668–5675.

Barral, Y., Jentsch, S., and Mann, C. (1995). G(1) cyclin turnover and nutrient-uptake are controlled by a common pathway in yeast. Genes Dev. *9*, 399–409.

Bartel, B., Wunning, I., and Varshavsky, A. (1990). The recognition component of the N-end rule pathway. EMBO J. *9*, 3179–3189.

Belvin, M.P., and Anderson, K.V. (1996). A conserved signaling pathway—the Drosophila Toll-Dorsal pathway. Annu. Rev. Cell Dev. Biol. *12*, 393–416.

Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science *243*, 1576–1583.

Ciechanover, A., Elias, S., Heller, H., and Hershko, A. (1982). "Covalent affinity" purification of ubiquitin-activating enzyme. J. Biol. Chem. *257*, 2537–2542.

Clurman, B.E., Sheaff, R.J., Thress, K., Groudine, M., and Roberts, J.M. (1996). Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by CDK2 binding and cyclin phosphorylation. Genes Dev. *10*, 1979–1990.

Connelly, C., and Hieter, P. (1996). Budding yeast SKP1 encodes an evolutionarily conserved kinetochore protein required for cell cycle progression. Cell *86*, 275–285.

Deshaies, R.J., Chau, V., and Kirschner, M. (1995). Ubiquitination of the G1 cyclin Cln2p by a Cdc34p-dependent pathway. EMBO J. *14*, 303–312.

Diehl, J.A., Zindy, F., and Sherr, C.J. (1997). Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. Genes Dev. *11*, 957–972.

Dohmen, R.J., Stappen, R., McGrath, J.P., Forrova, H., Kolarov, J., Goffeau, A., and Varshavsky, A. (1995). An essential yeast gene encoding a homolog of ubiquitin-activating enzyme. J. Biol. Chem. *270*, 18099–18109.

Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I., Lerner, R.A., and Wigler, M. (1988). Purification of a ras-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. *8*, 2159–2165.

Grussenmeyer, T., Scheidtmann, K.H., Hutchinson, M.A., Eckhart, W., and Walter, G. (1985). Complexes of polyoma virus medium T antigen and cellular proteins. Proc. Natl. Acad. Sci. USA *82*, 7952–7954.

Hadwiger, J.A., Wittenberg, C., Mendenhall, M.D., and Reed, S.I. (1989). The *Saccharomyces cerevisiae* CKS1 gene, a homolog of the *Schizosaccharomyces pombe* suc1+ gene, encodes a subunit of the Cdc28 protein. Mol. Cell. Biol. *9*, 2034–2041.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Hershko, A., and Heller, H. (1985). Occurrence of a polyubiquitin structure in ubiquitin-protein conjugates. Biochem. Biophys. Res. Commun. *128*, 1079–1086.

Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983). Components of ubiquitin-protein ligase system: resolution, affinity purification, and role in protein breakdown. J. Biol. Chem. *258*, 8206–8214.

Hochstrasser, M. (1995). Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. Curr. Opin. Cell Biol. *7*, 215–223.

Huibregtse, J.M., Scheffner, M., and Howley, P.M. (1993). Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. Mol. Cell. Biol. *13*, 4918–4927.

King, R.W., Deshaies, R.J., Peters, J.-M., and Kirschner, M.W. (1996). How proteolysis drives the cell cycle. Science *274*, 1652–1659.

Kipreos, E., He, W.-W., and Hedgecock, E. (1995). *lin-19* and *lin-23*: novel regulators of the cell cycle. 10th International C. elegans Meeting Summaries, p. 83.

Lambright, D.G., Sondek, J., Bohm, A., Skiba, N.P., Hamm, H.E., and Sigler, P.B. (1996). The 2.0 Å crystal structure of a heterotrimeric G protein. Nature *379*, 311–319.

Lanker, S., Valdivieso, M.H., and Wittenberg, C. (1996). Rapid degradation of the G1 cyclin Cln2 induced by Cdk-dependent phosphorylation. Science *271*, 1597–1601.

Mathias, N., Johnson, S.L., Winey, M., Adams, A.E., Goetsch, L., Pringle, J.R., Byers, B., and Goebl, M.G. (1996). Cdc53 acts in concert with Cdc4 and Cdc34 to control the G1 to S phase transition and identifies a conserved family of proteins. Mol. Cell. Biol. *16*, 6634–6643.

Muslin, A.J., Tanner, J.W., Allen, P.M., and Shaw, A.S. (1996). Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. Cell *84*, 889–897.

Nugroho, T.T., and Mendenhall, M.D. (1994). An inhibitor of yeast cyclin-dependent protein kinase plays an important role in ensuring the genomic integrity of daughter cells. Mol. Cell. Biol. *14*, 3320–3328.

Pause, A., Lee, S., Worrell, R.A., Chen, D.Y.T., and Burgess, W.H. (1997). The Von Hippel-Lindau tumor suppressor gene product forms a stable complex with human CUL2, a member of the CDC53 family of proteins. Proc. Natl. Acad. Sci. USA *94*, 2156–2161.

Scheffner, M., Huibregtse, J.M., Vierstra, R.D., and Howley, P.M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell *75*, 495–505.

Scheffner, M., Nuber, U., and Huibregtse, J.M. (1995). Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. Nature *373*, 81–83.

Schneider, B.L., Yang, Q.H., and Futcher, A.B. (1996). Linkage of replication to START by the Cdk inhibitor Sic1. Science *272*, 560–562.

Schwob, E., Böhm, T., Mendenhall, M., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor $p40^{SIC1}$ controls the G1/S transition in *Saccharomyces cerevisiae*. Cell *79*, 233–244.

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J., and Harper, J.W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin–ligase complex. Cell, this issue, *91*, 209–219.

Sondek, J., Bohm, A., Lambright, D.G., Hamm, H.E., and Sigler, P.B. (1996). Crystal structure of a G protein beta-gamma dimer at 2.1 angstrom resolution. Nature *379*, 369–374.

Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J., et al. (1993). SH2 domains recognize specific phosphopeptide sequences. Cell *72*, 767–778.

Tyers, M. (1996). The cyclin-dependent kinase inhibitor p40SIC1 imposes the requirement for CLN G1 cyclin function at Start. Proc. Natl. Acad. Sci. USA *93*, 7772–7776.

Verma, I.M., Stevenson, J.K., Schwarz, E.M., Van Antwerp, D., and Miyamoto, S. (1995). Rel/NF-κB family: intimate tales of association and dissociation. Genes Dev. *9*, 2723–2735.

Verma, R., Chi, Y., and Deshaies, R.J. (1997a). Ubiquitination of cell cycle regulatory proteins in yeast extract. Meth. Enzymol. *283*, 366–376.

Verma, R., Feldman, R., and Deshaies, R.J. (1997b). SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34, and cyclin/Cdk activities. Mol. Biol. Cell *8*, 1427–1437.

Verma, R., Annan, R., Huddleston, M., Carr, S., Reynard, G., and Deshaies, R.J. (1997c). Phosphorylation of Sic1p by G1 cyclin/Cdk required for its degradation and entry into S phase. Science, in press.

Wall, M.A., Coleman, D.E., Lee, E., Iniguez-Llhui, J.A., Posner, B.A., Gilman, A.G., and Sprang, S.R. (1995). The structure of the G protein heterotrimer $G_{i\alpha1}\beta_1\gamma_2$. Cell *83*, 1047–1058.

Willems, A.R., Lanker, S., Patton, E.E., Craig, K.L., Nason, T.F., Kobayashi, R., Wittenberg, C., and Tyers, M. (1996). Cdc53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway. Cell *86*, 453–463.

Won, K.-A., and Reed, S.I. (1996). Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. EMBO J. *15*, 4182–4193.

Zhang, H., Kobayashi, R., Galaktionov, K., and Beach, D. (1995). p19(SKP1) and p45(SKP2) are essential elements of the cyclin A-Cdk2 S phase kinase. Cell *82*, 912–925.

Appendix III

Cdc53/cullin and the essential Hrt1 RING–H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34

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SCF^{Cdc4} (Skp1, Cdc53/cullin, F-box protein) defines a family of modular ubiquitin ligases (E3s) that regulate diverse processes including cell cycle, immune response, and development. Mass spectrometric analysis of proteins copurifying with Cdc53 identified the RING–H2 finger protein Hrt1 as a subunit of SCF. Hrt1 shows striking similarity to the Apc11 subunit of anaphase-promoting complex. Conditional inactivation of *hrt1^{ts}* results in stabilization of the SCF^{Cdc4} substrates Sic1 and Cln2 and cell cycle arrest at G_1/S. Hrt1 assembles into recombinant SCF complexes and individually binds Cdc4, Cdc53 and Cdc34, but not Skp1. Hrt1 stimulates the E3 activity of recombinant SCF potently and enables the reconstitution of Cln2 ubiquitination by recombinant SCF^{Grr1}. Surprisingly, SCF and the Cdc53/Hrt1 subcomplex activate autoubiquitination of Cdc34 E2 enzyme by a mechanism that does not appear to require a reactive thiol. The highly conserved human *HRT1* complements the lethality of *hrt1Δ*, and human HRT2 binds CUL-1. We conclude that Cdc53/Hrt1 comprise a highly conserved module that serves as the functional core of a broad variety of heteromeric ubiquitin ligases.

[*Key Words*: Roc1; Rbx1; cullin; ubiquitin; RING finger]

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Proteolysis in the eukaryotic cytosol is triggered by the assembly of a substrate-linked multiubiquitin chain, which targets the protein for degradation by the 26S proteasome (Banerjee et al. 1993). Ubiquitin is activated for transfer to substrate through the ATP-dependent formation of a thioester bond between the carboxyl terminus of ubiquitin and the ubiquitin-activating enzyme, E1. Ubiquitin is transferred subsequently to a member of a family of ubiquitin-conjugating (E2) enzymes. Finally, thioesterified ubiquitin is transferred from E2 to a lysine residue of the target protein, either directly or with the assistance of a ubiquitin ligase (E3). E3s bind substrate directly, suggesting that they provide specificity in ubiquitination reactions. Whereas the budding yeast genome encodes 13 E2-like proteins, it is not known how many E3s exist, because these enzymes do not share a common sequence motif. So far, four different classes of E3 enzymes have been defined: Ubr1 (Bartel et al. 1990), the HECT (homology to E6-AP carboxyl terminus) domain family (Huibregtse et al. 1995), of which Mdm2 has been proposed to be a member (Honda et al. 1997), the SCF family (Feldman et al. 1997; Skowyra et al. 1997), and the anaphase-promoting complex/cyclosome (APC/C) (King et al. 1995).

SCF^{Cdc4}, the prototype of the SCF (for Skp1, Cdc53/Cullin, F-box receptor) family of ubiquitin ligases, was first defined by in vitro reconstitution (Feldman et al. 1997; Skowyra et al. 1997; Verma et al. 1997b). Identification of SCF^{Cdc4} stemmed from a genetic analysis of the G_1/S transition (Schwab et al. 1994). Budding yeast cells harboring temperature-sensitive mutations in either the Cdc34 E2 enzyme or in any one of the subunits of SCF^{Cdc4} fail to enter S phase at the nonpermissive temperature because they fail to degrade the S phase cyclin/cyclin-dependent kinase (CDK) inhibitor Sic1 (Bai et al. 1996; Schwab et al. 1994). In wild-type cells, G_1 cyclin/CDK phosphorylates Sic1 at the end of G_1 phase, thereby targeting it for Cdc34-dependent ubiquitination and deg-

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radation (Verma et al. 1997a). Phosphorylated Sic1, but not the unmodified protein or a phosphorylation-site mutant, binds tightly to the Cdc4 subunit of SCF^{Cdc4} (Feldman et al. 1997; Skowyra et al. 1997). Cdc34 is also recruited to SCF^{Cdc4}, possibly via interaction with the Cdc53 subunit (Willems et al. 1996). Upon assembly with the SCF^{Cdc4} complex, phosphorylated Sic1 is extensively ubiquitinated as a prelude to its degradation.

Besides SCF, the APC/C ubiquitin ligase also plays a key role in cell-cycle control. APC/C controls the separation of sister chromatids and the exit from mitosis by specifying the destruction of Pds1 and cyclin B, respectively (King et al. 1996). Although SCF and APC/C are distinctly regulated and differ greatly in size, subunit composition and apparent complexity, APC/C contains a subunit (Apc2) that bears homology to the carboxy-terminal half of Cdc53/cullin (Yu et al. 1998; Zachariae et al. 1998).

A remarkable property of SCF ubiquitin ligases is that their specificity can be programmed via an array of distinct substrate receptors (Patton et al. 1998b; Skowyra et al. 1997). These receptors are recruited to SCF by the conserved F-box domain they share. The discovery of the F box stemmed from the observation that human cyclin F and Cdc4 share a conserved sequence that mediates their interaction with Skp1 (Bai et al. 1996). The budding-yeast genome encodes 17 proteins that contain an obvious F-box motif (Patton et al. 1998b), and at least three of these proteins assemble into distinct SCF complexes in vivo (Patton et al. 1998a).

Over the past few years, F-box proteins and other components of the SCF/Cdc34 pathway have been identified in many eukaryotes and linked to diverse cellular processes, ranging from the auxin response in plants (Ruegger et al. 1998), withdrawal from the cell cycle in nematodes (Kipreos et al. 1996), signaling via the Wnt and Hedgehog pathways in *Drosophila* (Jiang and Struhl 1998), and activation of the immunoregulatory protein NF-κB in human cells (Yaron et al. 1998). Although SCF ubiquitin ligases clearly regulate diverse physiological pathways in a variety of eukaryotes, little is known about their structure, regulation, or mechanism of action.

The HECT domain proteins are the only ubiquitin ligases whose mechanism of action is understood. The charter member of the HECT domain family of proteins, E6-associated protein (E6-AP), forms a thioester bond with ubiquitin as an intermediate in the transfer of ubiquitin from E2 to substrate (Scheffner et al. 1995). Mutation of the thioester-forming cysteine of E6-AP destroys its E3 activity. It remains unclear whether other (non-HECT) E3 enzymes catalyze substrate ubiquitination via a similar thioester cascade.

We report the discovery of a heretofore undetected fourth essential subunit of the SCF complex. This new subunit, Hrt1, is a member of the RING–H2 family of proteins (Suarin et al. 1996). Hrt1 is required for degradation of SCF substrates in vivo, and stimulates the ubiquitin ligase activity of both SCF^{Cdc4} and SCF^{Grr1} complexes in vitro. A Cdc53/Hrt1 subcomplex potently activates autoubiquitination of the Cdc34 E2 enzyme, suggesting that these two proteins define a minimal ubiquitin ligase. Based on analysis of Cdc34 autoubiquitination, we suggest that Cdc53/Hrt1 promotes substrate ubiquitination by a novel mechanism. Remarkably, the SCF and APC/C ubiquitin ligase complexes both contain essential subunits with cullin homology and RING–H2 domains. We propose that these seemingly unrelated ubiquitin ligases define a broad family of cullin-containing E3s that share a common enzymatic mechanism.

Results

Purification of SCF complexes from yeast cells

To determine if SCF contains subunits other than the minimal set that suffices for reconstitution of ubiquitin ligase activity, we investigated its native composition in yeast . The chromosomal copies of *CDC53* and *SKP1* were modified by a PCR-based tagging procedure (see Materials and Methods) to encode proteins with either six or nine copies of the myc epitope (myc6, myc9) appended to their carboxyl termini. *CDC53myc6* and control untagged cells were pulse radiolabeled with [^{35}S]-methionine, and proteins bound specifically to Cdc53myc6 were isolated on an α-myc 9E10 monoclonal antibody matrix (Fig. 1A). Among other proteins, Cdc53myc6 immunoprecipitates contained a prominent ~17-kD band (lane 2) that was not detected in affinity-purified APC/C (lane 3). To isolate SCF-associated proteins in sufficient scale for protein sequencing, Skp1myc9 and Cdc53myc9 were affinity purified on 9E10 beads and associated proteins were resolved by SDS-PAGE and stained with silver (Fig. 1B). Although several proteins were identified in both preparations, Cdc53, YJL149w (an F-box protein), and the 17-kD polypeptide were the only species that were detected consistently in both Skp1myc9 and Cdc53myc9 immunoprecipitates (J.H. Seol and A. Sherchenko, unpubl.). The 17-kD protein was identified as the product of the *HRT1* (YOL133w) locus (Fig. 1C) by a combination of high-mass-accuracy MALDI (matrix-assisted laser desorption ionization mass spectrometry) peptide mapping and nanoelectrospray tandem mass spectrometric sequencing (Shevchenko et al. 1996a). *HRT1* was first identified as a gene whose high-level expression reduces transposition by the retrotransposon Ty3 (cited in the *Saccharomyces* Genome Database at http://genome-www.stanford.edu/Saccharomyces/; J. Claypool and S. Sandmeyer, in prep.)

Interestingly, the yeast protein most closely related to Hrt1 was the Apc11 subunit of APC/C (Fig. 1D) (Zachariae et al., 1998). These proteins are 51% related and 28% identical to each other, and both contain highly related RING–H2 domains (Suarin et al. 1996). Hrt1 has even closer relatives in other organisms, including humans. The human *hHRT1* (GenBank accession no. D52876) and *hHRT2* (TIGR accession no. HGI THC257076) genes are 62.5% identical (79% similar)

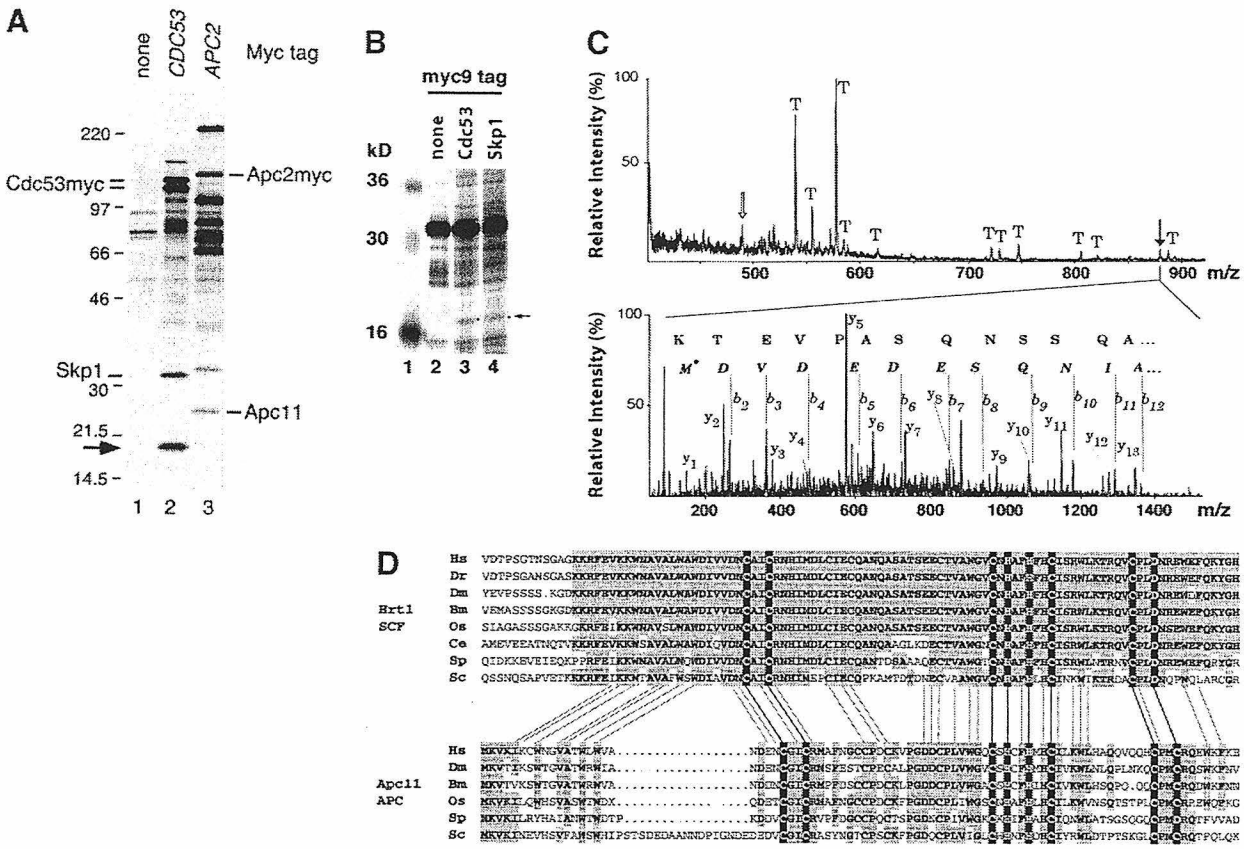


Figure 1. Identification of Hrt1 as a Cdc53-associated protein. (*A*) [^{35}S]Methionine-labeled proteins that specifically coimmunoprecipitate with Cdc53myc6 (lane *2*) or Apc2myc6 (lane *3*) were visualized by SDS-PAGE followed by autoradiography. (*B*) Proteins coimmunoprecipitating with Cdc53myc9 (lane *3*) or Skp1myc9 (lane *4*) were separated by SDS-PAGE, and stained with silver. Both preparations consistently contained a 17-kD protein (arrow in *A* and *B*). (*C*) Identifiecation of YOL133w by nano-electrospray tandem mass spectrometry (Nano ES MS/MS). (*Top*) Partial mass spectrum of a pool of tryptic peptides recovered after in gel digestion of 17-kD band. (T) Peaks identified as trypsin autolysis products. Tandem mass spectrum acquired from the ion with m/z 488.2 (open arrow) identified peptide AFLIEEQK from ribosomal protein RPL34B (data not shown). Tandem mass spectrum (bottom) of the ion with m/z 880.4 (solid arrow) revealed two nonoverlapping series of fragment y-ions and b-ions (Biemann 1988) that both identified the same Hrt1-derived peptide M*DVDEDESQN-IAQSSNQSAPVETK (M* stands for methionine sulfoxide). Carboxy-terminal part of the peptide sequence (...AQSSNQSAPVETK) is covered by continuous series of y-ions. Amino-terminal part of the sequence (M*DVDEDESQNIA) is covered by b-ion series. As every amino acid residue in the peptide sequence can be accounted for, identification of Hrt1 is certain despite the complexity of the spectrum and that only a single peptide was identified. (*D*) Hrt1 is highly conserved between species and related to the APC subunit Apc11. Budding yeast Hrt1 and Apc11 were each aligned with related sequences from different species. Putative zinc-coordinating residues are highlighted black. Residues identical in at least 4 (Apc11) or 5 (Hrt1) sequences are shaded. Lines connecting the alignments indicate residues conserved between Apc11 and Hrt1. (Hs) *Homo sapiens*, (Dm) *Drosophila melanogaster*; (Dr) *Danio rerio*; (Bm) *Bombyx mori*; (Os) *Oryza sativa*; (Ce) *Caenorhabditis elegans*; (Sp) *Schizosaccharomyces pombe*; (Sc) *Saccharomyces cerevisiae*. (E) Hrt1 is an authentic subunit of SCF. Crude extracts from untagged control (lane *1*) and *HRT1myc9* (lane *2*) strains were evaluated in parallel by immunoblotting with 9E10 antibodies. Extracts from untagged (odd-numbered lanes) or *HRT1myc9* (even-numbered lanes) cells were immunoprecipitated with 9E10 antibodies fractionated by SDS-PAGE, and immunoblotted with α-Cdc53, α-Cdc4, α-Skp1, or α-Cdc34 as indicated to reveal the corresponding protein.

and 41% identical (63% similar) to yeast *HRT1*, respectively.

To verify the interaction between Hrt1 and SCF, we modified the chromosomal *HRT1* locus to encode *HRT1myc9*. The resulting strain specifically expressed a 9E10-reactive polypeptide of the expected molecular weight (Fig. 1E, lane 2) and was fully viable at 25°C, but not 37°C (see next section). In coimmunoprecipitation experiments, Cdc53 (lane 4), Cdc4 (lane 6), Skp1 (lane 8), and Cdc34 (lane 10) were detected specifically in 9E10

immunoprecipitates prepared from *HRT1myc9* cells, but not in those prepared from a control untagged strain (odd-numbered lanes).

HRT1 *is required for SCF activity in vivo*

To evaluate the role of Hrt1 in SCF function, an *HRT1/hrt1::HIS3* heterozygote was constructed, sporulated, and dissected into tetrads. Only two viable, His^- spores were recovered from each tetrad on rich medium (Fig. 2A, top). However, *HRT1* overlaps extensively with the *YOL134c* locus on the opposite strand, such that *hrt1::HIS3* disrupted both genes. Two lines of evidence indicate that *HRT1* is essential, and *YOL134c* is dispensable. First, cosegregation of a *GAL–HRT1* plasmid complemented *hrt1::HIS3* on either galactose medium (second panel) or glucose medium (not shown) as evidenced by the appearance of tetrads with more than two viable spores, at least one of which was His^+. Second, insertion of a myc9 epitope immediately upstream of the *HRT1* stop codon yielded a temperature-sensitive growth phenotype, whereas insertion of the same epitope immediately downstream of the stop codon (*HRT1–stop–myc9*) had no effect on growth (e.g., Fig. 2B). A similar effect of the myc9 epitope was observed with *APC11* (Zachariae et al. 1998). *hHRT1* expressed from the *GAL1* promoter also complemented the lethality of *hrt1::HIS3* on galactose medium (third panel), but not on glucose medium (data not shown). Thus, human and yeast Hrt1 most likely perform the same biochemical function. In contrast, the more distantly related *hHRT2* did not complement *hrt1::HIS3* on galactose medium (bottom panel). Nevertheless, hHRT2 is likely to perform a function related to that of hHRT1, because a cDNA encoding amino acids 9–112 of hHRT2 was recovered in a two-hybrid screen for CUL-1-interacting proteins (data not shown).

The fortuitous temperature-sensitive growth phenotype of *HRT1myc9* strains allowed us to evaluate the roles of Hrt1 in cell division and degradation of SCF substrates. Cells with temperature-sensitive mutations in SCF/Cdc34 pathway components are sensitive to overexpression of SCF substrates (Deshaies et al. 1995; Bai et al. 1996; Willems et al. 1996). Overproduction of either $SIC1^{HA}$ or $CLN2^{HA3}$ from the *GAL* promoter prevented growth at 25°C of *HRT1myc9* cells, but not wild-type or *HRT1–stop–myc9* cells (data not shown). A second phenotype of cells harboring temperature-sensitive mutations in SCF/Cdc34 pathway components is that they bud but fail to initiate DNA replication at the nonpermissive temperature (Schwab et al. 1994; Bai et al. 1996). *HRT1myc9* and *HRT1–stop–myc9* cells grown in YP-glucose medium were elutriated to obtain a population of small, unbudded G_1 cells, which were reinoculated into fresh YP-glucose medium at 37°C. Cells harvested at 15-min intervals were evaluated for DNA content, cell volume, and budding index (Fig. 2B). Whereas most *HRT1–stop–myc9* cells initiated budding and DNA replication at ~90–105 min, *HRT1–myc9* cells did not enter S phase or form mitotic spindles, and exhibited a strong delay in bud emergence. Like *HRT1myc9*, $cdc34^{ts}$ cells are also delayed in bud emergence (Schwab et al. 1994), and arrested cultures of *skp1-11* mutants contain unbudded cells (Bai et al. 1996). These observations suggest that SCF antagonizes an inhibitor of bud emergence. Consistent with their failure to duplicate DNA, Sic1 persisted at high levels in elutriated *HRT1myc9* cells, but was degraded concomitant with bud emergence in *HRT1* cells (Fig. 2C).

To investigate the role of *HRT1* in turnover of substrates independent of its effects on cell-cycle progression, *SIC1* and *CLN2* expression were induced transiently in G_2-arrested wild-type and *HRT1myc9* cells incubated at a semirestrictive temperature, and the decline in Sic1 (Fig. 2D) and Cln2 (Fig. 2E) levels were monitored by immunoblotting. Comparison of protein levels (Fig. 2D,E, top panels) at different time points after repression of transcription (middle panels) indicates that both Sic1 and Cln2 were stabilized in *HRT1myc9* cells. For unknown reasons, *HRT1myc9* had a stronger effect on Sic1 turnover than on Cln2 turnover. Densitometric scanning of the experiment in Figure 2E revealed that the half-life of Cln2 was 8 min in wild-type cells and 19 min in mutant cells. Flow cytometry confirmed that both wild-type and mutant cells remained arrested in G_2/M throughout the duration of the time courses (bottom panels). Although a protein target of the SCF^{Met30} complex has not been described yet, mutations in *CDC53*, *SKP1*, and *MET30* prevent repression of *MET25* mRNA in response to high extracellular methionine (Patton et al. 1998a). Likewise, *HRT1myc9* cells were also deficient in *MET25* repression (Fig. 2F). Thus, the activities of three distinct SCF complexes—SCF^{Cdc4}, SCF^{Grr1} and SCF^{Met30}—were diminished in *HRT1myc9* cells.

Assembly of recombinant Hrt1 with SCF^{Cdc4} *subunits*

To evaluate the assembly of Hrt1 with SCF subunits in vitro, glutathione *S*-transferase–Hrt1 (GST–Hrt1) purified from *Escherichia coli* was mixed with insect cell lysates containing recombinant SCF^{Cdc4} subunits, and proteins recovered on glutathione–agarose were detected by immunoblotting (Fig. 3A). GST–Hrt1 presumably folded correctly, as it stimulated SCF^{Cdc4} E3 activity (Fig. 4A). Whereas Skp1 (Fig. 3A, lane 4) did not bind GST–Hrt1, ~20% of ^{PyHA}Cdc4 (lanes 1,2) and ~20% of $Cdc53^{PyHA}$ (lane 3) bound to GST–Hrt1 (middle panel), but not GST (bottom panel). The high efficiencies of binding suggest that interaction of GST–Hrt1 with Cdc4 and Cdc53 was direct and did not require intermediary insect cell proteins. MBP–$Cdc34\Delta C^{HA}$ purified from *E. coli* also bound purified GST–Hrt1 (lane 5), but not GST. Similar protein interactions were observed upon coexpression of Hrt1 with SCF^{Cdc4} subunits in insect cells (Fig. 3B). Hrt1 was retrieved on an antipolyoma antibody matrix (α-Py beads) from insect cell lysates containing coexpressed SCF^{Cdc4} (lane 4; Cdc4 was tagged with PyHA epitope), ^{PyHA}Cdc4 (lane 1) or $Cdc53^{PyHA}$ (lane 2). Unexpectedly, recovery of Hrt1 bound to ^{PyHA}Cdc4 was

diminished upon coexpression of Skp1, suggesting that Skp1 and Hrt1 may compete for binding to overlapping sites on Cdc4. Hrt1 was not recovered on α-Py beads upon coexpression with an untagged control protein ($Skp1^{HA}$; lane 3).

Hrt1 stimulates SCF^{Cdc4} activity in vitro

Ubiquitin ligase activity specific for phosphorylated Sic1 can be reconstituted with recombinant Skp1, Cdc53, and Cdc4 (Feldman et al. 1997; Skowyra et al. 1997). The

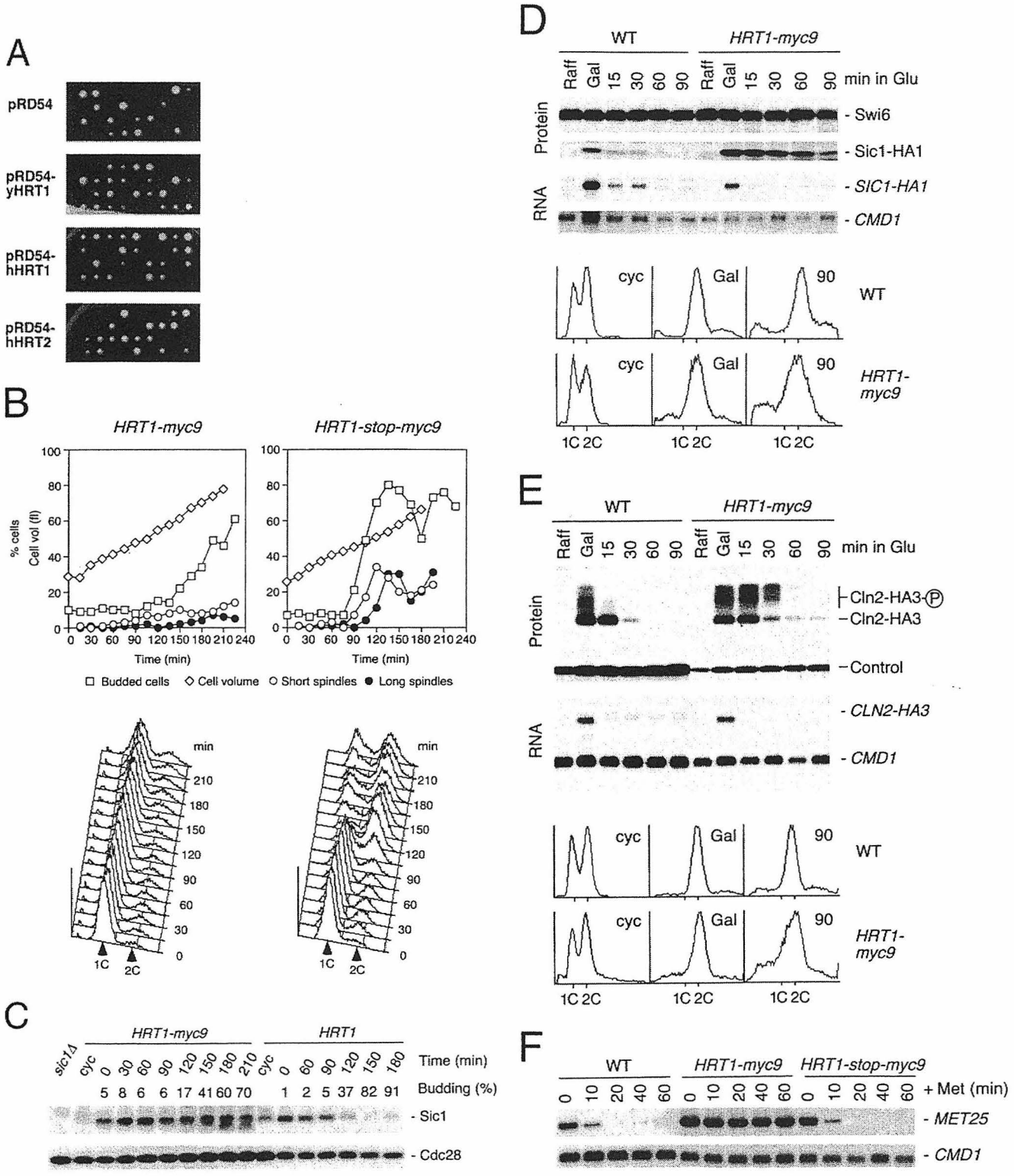


Figure 2. *(See facing page for legend.)*

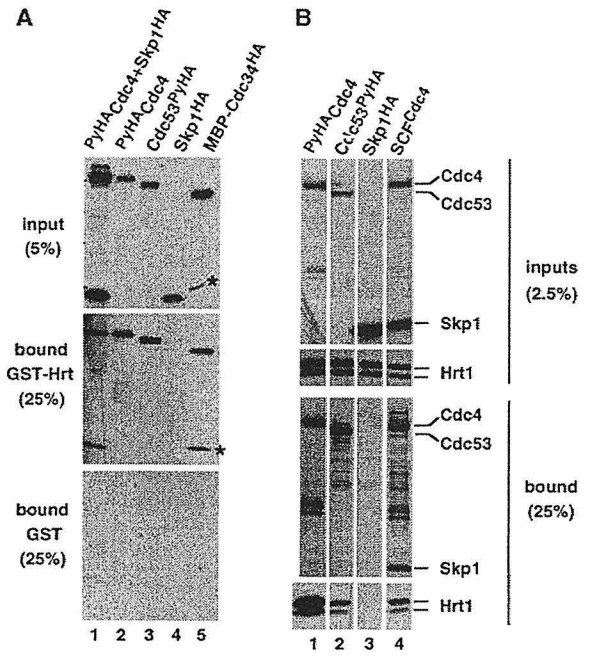


Figure 3. Hrt1 binds Cdc4, Cdc53, and Cdc34. (*A*) (Lanes *1–4*) GST–Hrt1 (*middle* panel) or GST (*bottom* panel) purified from *E. coli* were mixed with crude insect cell lysates (*top* panel) that contained the proteins indicated above each lane. Proteins retrieved on glutathione-agarose were fractionated by SDS-PAGE and revealed by immunoblotting with α-HA. (Lane *5*) Same as lanes *1–4*, except MBP–Cdc34ΔC^{HA} purified from *E. coli* was evaluated for its ability to bind GST-Hrt1. The asterisks denote a proteolytic fragment of MBP–Cdc34ΔCHA. Input lanes contain 5%; bound lanes contain 25% of the material from a single binding reaction. (*B*) Lysates from Sf9 insect cells that coexpressed Hrt1 plus ^{PyHA}Cdc4 (lane *1*), Cdc53^{PyHA} (lane *2*), Skp1^{HA} (lane *3*), or ^{PyHA}Cdc4/Cdc53/Skp1^{HA} (lane *4*) were adsorbed to α-Py beads, and bound proteins were fractionated by SDS-PAGE and revealed by immunoblotting with α-HA (third row of panels) or α-Hrt1 (*bottom row* of panels) antibodies. Unfractionated insect cell lysates were directly immunoblotted to evaluate relative expression of the individual proteins (*top row* of panels, α-HA blot; *second row* of panels, α-Hrt1 blot).

identification of Hrt1 as a fourth essential subunit of the SCF complex required for normal rates of Sic1 and Cln2 proteolysis in vivo raised the question as to whether Hrt1 functions in substrate ubiquitination. To address this issue, we evaluated the effect of recombinant Hrt1 on SCF^{Cdc4}-dependent ubiquitination of Sic1 in vitro. Sf9 cells were infected with baculoviruses encoding Skp1, Cdc53, and Cdc4, and recombinant SCF complexes were retrieved on α-Py beads. A deliberate excess of phosphorylated MBP–Sic1 substrate was incubated with immobilized SCF^{Cdc4} in the presence of E1 enzyme, Cdc34, ubiquitin, ATP, and either GST or GST–Hrt1 purified from *E. coli*. As shown in Figure 4A, GST–Hrt1 potently stimulated ubiquitination of phospho–MBP–Sic1. A similar result was obtained by evaluating SCF^{Cdc4} activity obtained from insect cells coexpressing Hrt1 and SCF subunits (Fig. 4B). The potent (>fivefold) stimulatory effect of Hrt1 upon Sic1 ubiquitination was not because of increased expression of SCF^{Cdc4} subunits (lanes 12,13). Notably, Hrt1 augmented both the rate (as measured by percent conversion) and extent (as measured by molecular weight of ubiquitin conjugates) of ubiquitination.

The F-box protein Grr1 is required for Cln1 and Cln2 proteolysis (Barral et al. 1995; Patton et al. 1998a). Whereas recombinant SCF complexes assembled with Grr1 (SCF^{Grr1}) bind phospho-Cln1, they do not support its ubiquitination (Skowyra et al. 1997). As Hrt1 is required for SCF function in vivo and stimulates SCF^{Cdc4} activity in vitro, we reasoned that Hrt1 might be essential for SCF^{Grr1} function. Recombinant SCF^{Grr1} complexes that either contained (Fig. 4C, lane 3) or lacked (lane 2) yeast Hrt1 were expressed in insect cells. Complexes retrieved on α-Py beads were then mixed with Sf9 cell lysate that contained Cln2^{myc}/GST–Cdc28/Cks1 substrate. Whereas SCF^{Grr1} complexes were essentially inactive (lane 2), those that contained Hrt1 sustained Cdc34-dependent ubiquitination of Cln2 (lanes 3,4). Taken together with the previous data, these observations suggest that Hrt1 is an essential core component that contributes to the E3 activity of all SCF complexes.

Figure 2. (*A*) *HRT1* is essential. A diploid *hrt1::HIS3/HRT1* strain was transformed with pRD54-based plasmids (pRS316 *CEN, URA3*) containing either yeast *HRT1*, human *HRT1*, or human *HRT2* under the control of the galactose-inducible *GAL1* promoter. Sporulated diploids were dissected on complete galactose medium, followed by incubation at 24°C for 5 days. (*B,C*) *HRT1* is required for both elimination of Sic1 and for S phase. A myc9 epitope cassette was integrated at *HRT1* either immediately upstream (*HRT1–myc9, left*) or downstream (*HRT1–stop–myc9, right*) of the stop codon. Small G_1 cells were isolated by centrifugal elutriation from cultures grown in YPD at 25°C and inoculated into fresh medium at 37°C at time 0. In *B*, mean cell volume (fl), budding index, formation of mitotic spindles (*top* panels), and DNA contents (*bottom* panels) were assessed at the indicated times. In *C*, budding index, Sic1 levels, and Cdc28 levels were monitored. (*D*) *HRT1* is required for proteolysis of Sic1. *HRT1* cells containing five integrated copies of *GAL–SIC1–HA1* and *HRT1–myc9 GAL–SIC1–HA1* (one copy) cells were grown in YP–raffinose at 25°C (cyc) and arrested in mitosis with nocodazole (Raff). After shifting the cultures to 32°C, expression from the *GAL* promoter was induced with galactose (Gal) and then repressed by transferring the cells to YP–glucose. Samples were withdrawn at the indicated time points and assayed for: Swi6 and Sic1-HA1 protein levels (*top* panels); calmodulin and *SIC1–HA1* mRNA levels (*middle* panels); and cellular DNA content (*bottom* panels). (*E*) *HRT1* is required for rapid degradation of Cln2. Same as *D*, except *CLN2–HA3* expression was induced with galactose at 35°C, and cells were shifted to 37°C upon transfer to YP–glucose. The band labeled control is an unknown polypeptide recognized by 12CA5. (*F*) *HRT1* is required for methionine-dependent repression of *MET25* mRNA. Yeast strains indicated were grown in complete medium containing glucose and lacking methionine at 25°C and were then shifted to 37°C for 2 hr. Methionine was added to the medium and samples were withdrawn at the indicated times. *MET25* and *CMD1* mRNA levels were assayed by Northern blotting.

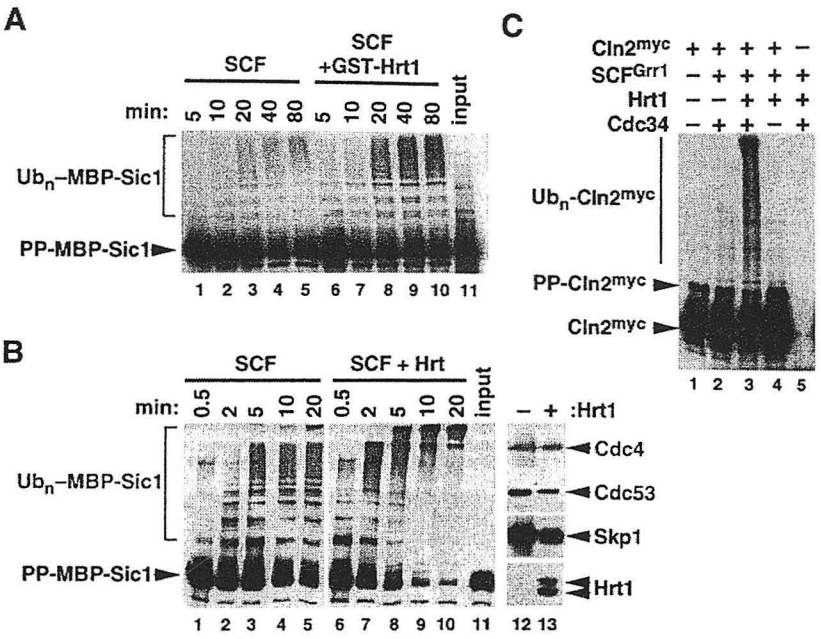


Figure 4. Hrt1 potently stimulates ubiquitin ligase activity of SCF^{Cdc4}. (*A*) Recombinant SCF^{Cdc4} was produced in Sf9 insect cells upon coinfection with baculovirus vectors that express Skp1, Cdc53^{PyHA}, and ^{PyHA}Cdc4, and the complex was purified by adsorption to a protein A resin containing covalently linked antipolyoma monoclonal antibody (α-Py beads). Parallel samples were supplemented with E1 enzyme, Cdc34, ATP, ubiquitin, purified phosphorylated maltose binding protein–Sic1 chimera (PP–MBP–Sic1), and either GST (lanes *1–5*) or GST–Hrt1 (lanes *6–10*). At the indicated times, samples were evaluated for ubiquitination of phospho–MBP–Sic1 by SDS-PAGE followed by immunoblotting with α-Sic1. (*B*) (Lanes *1–11*) Same as *A*, except that recombinant SCF^{Cdc4} complexes that either contained (lanes *6–10*) or lacked (lanes *1–5*) yeast Hrt1 were generated by either triple or quadruple infection of insect Sf9 cells (quadruple infection contained an Hrt1-expressing baculovirus). Input substrate is shown in lane *11*. (Lanes *12,13*) Levels of SCF subunits in each preparation were evaluated by immunoblotting. (*C*) Same as *B*, except that recombinant SCF^{Grr1} complexes containing (lane *3*) or lacking (lane *2*) Hrt1 were expressed in insect cells, purified, and mixed with 1/20 volume insect cell lysate that contained recombinant Cln2^{myc}/GST–Cdc28/Cks1 complexes (Cln2^{myc}). In lanes *3* and *4*, Cdc34 and substrate, respectively, were omitted from the final reaction. PP–Cln2^{myc} refers to the phosphorylated substrate.

Cdc53/Hrt1 defines a novel ubiquitin ligase module

To characterize the mechanism of action of Hrt1/SCF^{Cdc4}, we sought to identify a minimal ubiquitin ligase domain. Purified Cdc34 ubiquitinates itself upon incubation with E1 and ubiquitin, but this reaction proceeds slowly (Banerjee et al. 1993). Cdc34 lacking 24 residues from the carboxyl terminus (Cdc34ΔC) no longer detectably ubiquitinates itself (R.M.R. Feldman, unpubl.), but nevertheless complements *cdc34Δ* (Kolman et al. 1992). We reasoned that the ubiquitin ligase activity of SCF might promote autoubiquitination of either intact Cdc34 or Cdc34ΔC; this simplified system would allow us to monitor SCF^{Cdc4} ubiquitin ligase activity independently of substrate targeting requirements. Strikingly, SCF^{Cdc4} complexes reconstituted with yeast Hrt1 potently activated Cdc34 (data not shown) and Cdc34ΔC (Fig. 5A, top panel, lane 4) autoubiquitination, whereas SCF^{Cdc4} complexes lacking yeast Hrt1 had much less activity (lane 5). Hrt1 did not act by simply enhancing the levels of SCF^{Cdc4} subunits recovered from insect cells (Fig. 4B, lanes 12,13). To determine if this activity of SCF^{Cdc4} resides in a subdomain of the complex, individual subunits were affinity purified from baculovirus-infected Sf9 cells and tested for activity. Cdc53^{PyHA} (Fig. 5A, lane 2), but not equivalent amounts of ^{PyHA}Cdc4 (lane 3) or Skp1^{HA} (data not shown), promoted Cdc34ΔC autoubiquitination. The activity (top panel), but not the yield (bottom panel) of Cdc53^{PyHA} was substantially enhanced by assembly with Hrt1 (lane 6). Hrt1 bound to ^{PyHA}Cdc4, however, was not sufficient to catalyze Cdc34ΔC autoubiquitination (top panel, lane 7) even though it was present in large amounts (middle panel). The activity observed for Cdc53/Hrt1 subcomplexes was consistently less than that of Hrt1-containing SCF^{Cdc4} complexes. Thus, although the ubiquitin ligase activity of SCF^{Cdc4} is intrinsic to the Cdc53/Hrt1 subcomplex, this activity may be enhanced upon assembly of Cdc53/Hrt1 with Cdc4/Skp1.

E3 enzymes of the HECT domain family catalyze substrate ubiquitination by providing a catalytic cysteine residue that accepts ubiquitin from E2 enzyme, and subsequently transfers the thioesterified ubiquitin to the ε-amino group of a substrate lysine residue (Scheffner et al. 1995). To address whether the Cdc53/Hrt1 ligase domain of SCF^{Cdc4} operates by a similar mechanism, we preincubated ^{PyHA}Cdc53/Hrt1 with increasing amounts of the sulfhydryl-modifying reagent NEM, added DTT to quench further cysteine modification, and mixed the resulting samples with Cdc34, E1, ubiquitin, and ATP. As a control, we evaluated *N*-ethylmaleimide (NEM) sensitivity of the E1 enzyme. Whereas E1 was inactivated completely by pretreatment with 0.2 mM NEM [but not when the NEM was prequenched with dithiothreitol (DTT), cf. lanes 4 and 5 of Fig. 5B], ^{PyHA}Cdc53/Hrt1 was resistant to pretreatment with up to 5 mM NEM. Similarly, ubiquitin ligase activity of intact SCF^{Cdc4} towards either Cdc34ΔC or phosphorylated Sic1 was not affected by NEM (R.M.R. Feldman, unpubl.). To test the possibility that exposure of the putative thioester-forming cysteine of Cdc53/Hrt1 is gated by association of Cdc53/Hrt1 with E2 enzyme, we mixed together E1, Cdc34, ubiquitin, ATP, and either mock beads or beads containing ^{PyHA}Cdc53/Hrt1. After a brief incubation, NEM was added to derivitize reactive cysteines, followed by DTT to quench the NEM. Treated beads were sedimented,

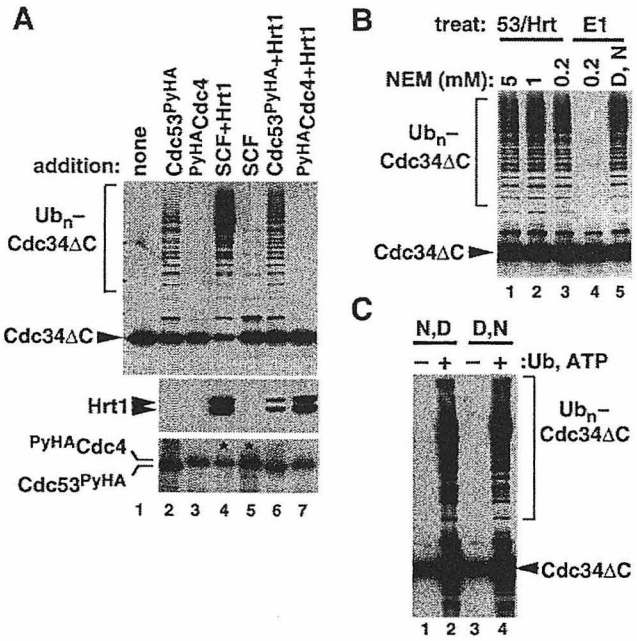


Figure 5. Cdc53/Hrt1 comprise an NEM-resistant ubiquitin ligase module. (*A*) The proteins indicated were retrieved from baculovirus-infected insect cells on α-Py beads, and mixed with E1, Cdc34ΔC, ubiquitin, and ATP. Following a 60-min incubation at 20°C, reactions were evaluated for Cdc34ΔC autoubiquitination (*top* panel), levels of Hrt1 (*middle* panel), and levels of $Cdc53^{PyHA}$ or $^{PyHA}Cdc4$ (*bottom* panel) by SDS-PAGE following by immunoblotting. SCF^{Cdc4} complexes (lanes *4,5*) contained $^{PyHA}Cdc4$ and untagged Cdc53. Thus, only $^{PyHA}Cdc4$ is visible in these lanes in the α-HA blot (*bottom* panel; see asterisks). (*B*) Either $Cdc53^{PyHA}$/Hrt1 (lanes *1–3*) or E1 enzyme (lane *4*) was pretreated with the indicated amount of NEM for 10 min at 20°C. NEM was quenched with DTT, and complete reactions containing E1, Cdc34ΔC, ubiquitin, ATP, and α-Py bead-bound $Cdc53^{PyHA}$/Hrt1 were incubated for 60 min at 20°C. For the sample shown in lane *5*, DTT was mixed with NEM prior to incubation with E1 to confirm the efficacy of the DTT quench. All reactions were subjected to SDS-PAGE followed by immunoblotting with α-Cdc34. (*C*) Same as *B*, except that complete reactions were first assembled and then treated with 5 mM NEM followed by 10 mM DTT (lanes *1,2*) or vice versa (lanes *3,4*). Afterwards, α-Py bead-bound $Cdc53^{PyHA}$/Hrt1 was recovered, washed, and supplemented with either buffer (lanes *1,3*) or fresh E1, Cdc34ΔC, ubiquitin and ATP (lanes *2,4*). The signal in lanes *1* and *3* represents Cdc34ΔC from the first stage incubation that was retained on the $Cdc53^{PyHA}$/Hrt1 matrix.

washed, and mixed with fresh E1, Cdc34, ubiquitin, and ATP. As a control, a parallel two-stage reaction was carried out in which DTT was added prior to NEM during the first stage. As shown in Figure 5C, $^{PyHA}Cdc53$/Hrt1 beads retained high autoubiquitination-promoting activity if incubated with NEM followed by DTT (lane 2), or vice versa (lane 4) during the first stage.

In the course of this work, we made the unexpected observation that polycations influence Cdc34 activity. Remarkably, polylysine (Fig. 6A, lane 5), but not PEG (lanes 2–4) or lysine (lane 1) markedly stimulated Cdc34ΔC autoubiquitination. As an independent test, we evaluated the effect of polycations on Gcn4 ubiquitination. Gcn4 is stabilized in $cdc34^{ts}$ cells (Kornitzer et al. 1994), and Gcn4 ubiquitination can be reconstituted in vitro with recombinant SCF^{Cdc4} and Cdc34 (Y.Chi, unpubl.). Gcn4 was not modified detectably in the presence of E1, Cdc34, ATP, and ubiquitin (Fig. 6B, lane 2), but high-molecular-mass K48-linked multiubiquitin chains were assembled on Gcn4 upon inclusion of polyethyleneimine (lanes 6,7). Thus, polyethyleneimine acts like a classic E3. The ability of polycations to substitute for SCF strongly suggests that an E3-linked ubiquitin thioester intermediate is not required to evoke the intrinsic ability of Cdc34 to assemble K48-linked multiubiquitin chains.

We considered two possible artifactual roles of polycations. First, it is unlikely that polycations serve simply

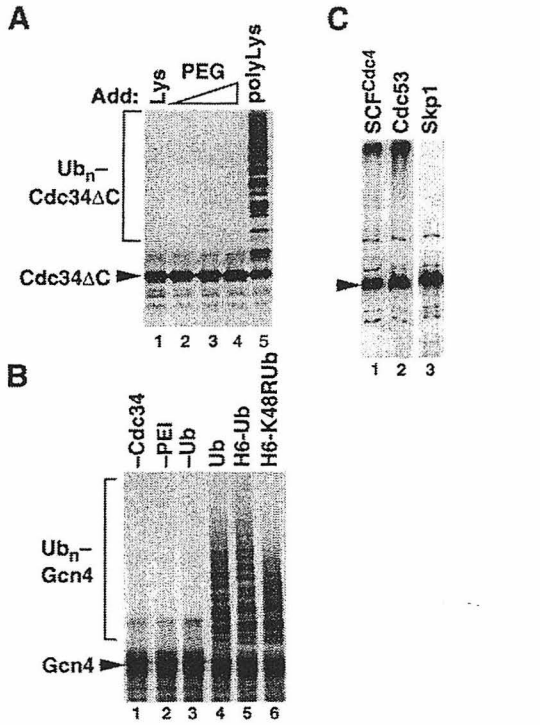


Figure 6. Polycations can substitute for SCF. (*A*) Polylysine activates Cdc34ΔC autoubiquitination. Reactions containing Cdc34ΔC, ATP, ubiquitin, and E1 enzyme were adjusted to either 10 mM lysine (lane *1*), 0.01, 0.1, or 1% polyethylene glycol (PEG, lanes *2–4*), or 0.01% (~300 nM) polylysine (lane *5*), and incubated at 20°C for 60 min. Reactions were evaluated as described for Fig. 5A. (*B*) Polyethyleneimine is an E3 for Gcn4. ^{35}S labeled Gcn4 produced by in vitro translation in reticulocyte lysate and enriched by DEAE chromatography was mixed with E1, Cdc34, ATP, the indicated ubiquitin derivatives (lanes *4–6*; H6 stands for His6) and 0.0005% polyethyleneimine (PEI), and incubated at 25°C for 60 min. Reactions conducted in the absence of either Cdc34, PEI, or ubiquitin are depicted in lanes *1–3*. Gcn4 was visualized by SDS-PAGE followed by autoradiography. (*C*) Fusion of Sic1 to Cdc34 does not activate its ubiquitination. Cdc34ΔC–Sic1 (arrowhead) was mixed with E1, ubiquitin, and ATP in the presence of immobilized SCF^{Cdc4} (lane *1*), $Cdc53^{PyHA}$ (lane *2*), or (as a negative control) $Skp1^{HA}$ (lane *3*). Samples were incubated and processed as described for Fig. 5A.

as macromolecular crowding reagents, as their stimulatory effects were observed at relatively low concentrations (300 nM for polylysine and 0.0005% for polyethyleneimine), and were not mimicked by PEG (Fig. 6A). An alternative possibility is that polycations serve to link together ubiquitin donor and ubiquitin acceptor molecules. We sought to address this by fusing the ubiquitin-acceptor domain of Sic1 to the carboxyl terminus of Cdc34ΔC. The rationale underlying this experiment is that if SCF^{Cdc4} or E3 mimetics simply bring Sic1 and Cdc34 into close proximity, then direct fusion of substrate to E2 might bypass the requirement for E3. As shown in Figure 6C, both SCF^{Cdc4} and $Cdc53^{PyHA}$ potently stimulated Cdc34ΔC–Sic1 ubiquitination, whereas $Skp1^{HA}$ had no effect beyond what is observed in the presence of E1, ubiquitin, and Cdc34ΔC (data not shown). Thus, bringing a substrate into close proximity to Cdc34 is not sufficient to activate its ubiquitination.

Discussion

Hrt1, a new subunit of SCF ubiquitin ligase complexes

We have identified by mass spectrometric sequencing of proteins that coimmunoprecipitate with Cdc53 a new subunit of the SCF ubiquitin ligase complex, Hrt1. Hrt1 and the homologous Apc11 protein of APC/C both contain RING–H2 domains (Zachariae et al. 1998). *HRT1* is essential, and *hrt1^{ts}* mutants—like *cdc34^{ts}*, *cdc4^{ts}*, *cdc53^{ts}*, and *skp1^{ts}* mutants—fail to (1) tolerate overexpression of Cln2 and Sic1, (2) transit from G_1 to S phase, (3) properly degrade the SCF^{Cdc4} substrate Sic1 and the SCF^{Grr1} substrate Cln2, and (4) regulate properly the expression of *MET25* mRNA. *hHRT1* complements the lethality of *hrt1*Δ, and human HRT2 binds human CUL-1 in a two-hybrid assay. Hrt1 independently binds to Cdc4, Cdc53, and Cdc34, but not to Skp1. SCF^{Cdc4} complexes assembled with yeast Hrt1 have much higher ubiquitin ligase activity towards phospho-Sic1. Moreover, SCF^{Grr1} complexes containing yeast Hrt1, but not those lacking the protein, promote ubiquitination of the G_1 cyclin Cln2.

SCF^{Cdc4} ubiquitin ligase activity toward phospho-Sic1 can be reconstituted upon expression of the yeast Skp1, Cdc53, and Cdc4 proteins in insect cells (Feldman et al. 1997; Skowyra et al. 1997). This raises a simple question: Is Hrt1 absolutely required for SCF^{Cdc4} function, or does it merely stimulate the basal activity of the heterotrimeric complex? Unambiguous resolution of this question will require the expression of SCF subunits in a system that lacks Hrt1 homologs (e.g., *E. coli*), or assembly of SCF complexes with mutant subunits that are selectively defective in their ability to recruit Hrt1. We favor the notion that Hrt1 is essential for the E3 activity of all SCF complexes; an insect cell homolog of the highly conserved Hrt1 may weakly substitute for the yeast protein in the SCF^{Cdc4} complex, but perhaps not in SCF^{Grr1}.

Cdc53 and Hrt1 comprise a ubiquitin ligase module

To identify a minimal ubiquitin ligase module in SCF^{Cdc4}, we exploited the observation that Cdc34 can conjugate ubiquitin to itself (Banerjee et al. 1993). This reaction is not thought to be of physiological significance (Goebl et al. 1994). Nevertheless, we reasoned that Cdc34 autoubiquitination might serve as a surrogate to monitor the ubiquitin ligase activity of SCF in the absence of substrate targeting requirements. Although it is formally possible that Cdc34 autoubiquitination does not faithfully mimic the ubiquitin ligase activity of SCF towards natural substrates, this seems unlikely given the potent effects of SCF and Cdc53/Hrt1 in this assay. Furthermore, activated Cdc34 assembles multiubiquitin chains upon itself with the appropriate K48 linkage (R.M.R. Feldman, unpubl.). Whereas efficient transfer of ubiquitin from Cdc34 to phospho-Sic1 requires all four subunits of SCF^{Cdc4}, either Cdc53 or a subcomplex of Cdc53/Hrt1 can partially substitute for intact SCF^{Cdc4} to promote assembly of multiubiquitin chains on Cdc34; in contrast, Hrt1 retrieved in a complex with Cdc4 is inactive.

How does the Cdc53/Hrt1 ubiquitin ligase module work?

Four distinct classes of ubiquitin ligases have been defined; the mechanism of action of only one—the HECT domain proteins—has been described (Scheffner et al. 1995). The HECT domain ubiquitin ligase E6–AP collaborates with a member of the UbcH5 family of E2s and human papillomavirus E6 protein to specify ubiquitination of p53. Ubiquitin destined for attachment to p53 is transferred from E2 to the conserved cysteine 833 residue of E6–AP (Scheffner et al. 1995). The ubiquitin linked to E6–AP is transferred subsequently to a lysine residue of p53. Mutation of cysteine 833 destroys E6–AP's E3 activity, indicating that the E6-AP~ubiquitin thioester is an essential intermediate in the transfer of ubiquitin from E2 to p53. Because the SCF, APC, and Ubr1 E3s do not contain obvious HECT domains, it is unclear whether they catalyze ubiquitination by a similar mechanism.

Three lines of evidence lead us to suggest that SCF promotes Cdc34-dependent ubiquitination of substrates by a novel mechanism that does not involve an E3~ubiquitin thioester intermediate. First, Cdc34 is able to assemble K48-linked multiubiquitin chains in the absence of an E3 (Banerjee et al. 1993). Thus, the active site of Cdc34 is inherently capable of assembling appropriate multiubiquitin chains in the absence of an additional catalytic center. Second, whereas enzymes that use cysteine residues for catalysis (including ubiquitin pathway components E1 and E2) are typically sensitive to alkylating agents such as NEM and iodoacetamide (Hershko et al. 1983), Cdc53/Hrt1-dependent autoubiquitination of Cdc34 is indifferent to NEM. The NEM resistance of Cdc53/Hrt1 is consistent with previous studies showing that mutation of all cysteine residues within Cdc53 does not impair its biological function (Patton et al. 1998a). Third, compounds lacking sulfhydryls—polylysine and polyethyleneimine—can substitute for SCF^{Cdc4} and

promote the assembly of K48 linked multiubiquitin chains on either Cdc34 or the SCF^{Cdc4}/Cdc34 pathway substrate Gcn4.

If Cdc53/Hrt1 does not activate ubiquitination of Cdc34 substrates by providing a catalytic thiol residue, how does it promote ubiquitin transfer? We propose that Cdc53/Hrt1 stabilizes the oxyanion that is formed in the transition state as the carboxyl terminus of ubiquitin is transferred from Cdc34 to the ϵ-amino group of a substrate lysine. Cdc53/Hrt1 (or polycations) may directly provide a positive charge to neutralize the oxyanion, or may elicit a favorable conformational change in Cdc34. Hrt1 binds both Cdc34 and components of the SCF complex directly, placing it in an optimal position to coordinate the transfer of ubiquitin from Cdc34 to substrate.

SCF and APC/C: archetypes of a family of cullin-based ubiquitin ligases

SCF complexes can be subdivided into three distinct functional domains: the F-box subunit recruits substrate (Feldman et al. 1997; Skowyra et al. 1997); the Skp1 subunit links the F-box substrate receptor to the remaining subunits; and Cdc53 (Willems et al. 1996) and Hrt1 subunits recruit the E2 enzyme and comprise the ubiquitin ligase core. Of the known members of the cullin family, the Apc2 subunit of APC/C is the most diverged from Cdc53 (Yu et al. 1998; Zachariae et al. 1998). As APC/C nevertheless also contains an Hrt1-like protein (Apc11) (Zachariae et al. 1998), we suggest that SCF and APC/C define an extended family of multisubunit E3s that are elaborated from a basic ubiquitin ligase module comprised of a cullin (CUL-1 through CUL-5, APC2) and a RING–H2 finger protein (HRT1, HRT2, or APC11). The RING–H2/cullin module in turn can be linked to families of putative substrate receptors, three of which have been defined so far: the F-box proteins for SCF (Bai et al. 1996), the SOCS box proteins for the presumed CUL-2-based ubiquitin ligases (Kamura et al. 1998), and the WD-40 repeat proteins Cdc20 and Hct1/Cdh1 for APC/C (Schwab et al. 1997; Visintin et al. 1997; Fang et al. 1998). Thus, many distinct ubiquitin ligase complexes can be assembled from the RING–H2/cullin ubiquitin ligase module.

Several other proteins implicated in ubiquitin-dependent proteolysis, including SINA (Hu and Fearon 1999), Der3/Hrd1 (Hampton et al. 1996; Bordallo et al. 1998), and the ubiquitin ligases Ubr1 and Mdm2, contain a putative RING finger (Kwon et al. 1998; Suarin et al. 1996). Both Ubr1 (F. Du and A. Varshavsky, pers. comm.) and Mdm2 (Honda et al. 1997) are thought to function without benefit of a cullin subunit. Intriguingly, E3a, which is the mammalian homolog of Ubr1, is resistant to NEM treatment (Hershko et al. 1983). Although Cys-464 has been proposed to define a divergent HECT-like catalytic center within Mdm2 (Honda et al. 1997), this residue also occupies a conserved position within Mdm2's putative RING finger (Suarin et al. 1996). Without a definitive demonstration that ubiquitin thioesterified to Mdm2 can be chased to substrate, it is judicious to keep open the possibility that Mdm2 is in fact a member of a distinct family of RING-containing ubiquitin ligases. The RING finger may have been recruited early during eukaryotic evolution to serve as a basis for the elaboration of a broad range of cullin-dependent and -independent ubiquitin ligases.

Materials and methods

Strains, media, and reagents

All yeast strains were derivatives of W303 (*HMLα* or *HMRa ho ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3-1 ssd1*). Yeast strains were grown in YEP medium (1% yeast extract, 2% Bacto-Peptone, 50 mg/liter adenine) supplemented with 2% of glucose (YPD), raffinose, or raffinose plus galactose. Nocodazole (15 μg/ml) was added to arrest cells in mitosis. Rabbit polyclonal antisera against Sic1 (Mike Tyers, Samuel Lunenfeld Research Institute, Toronto, Canada), Cdc4 and Cdc34 (Zach Pitluck), and rat α-tubulin monoclonal antibody YOL1/34 (John Kilmartin, MRC, Cambridge, UK) were generously provided by the indicated investigators. Rabbit polyclonal α-Skp1 and α-Hrt1 antibodies were raised against bacterially expressed GST–Skp1 and His6–Hrt1, respectively. Production of polyclonal α-Cdc53 and cross-linked α-polyoma monoclonal antibody beads were described previously (Feldman et al. 1997). Methyl ubiquitin was purchased from Boston Biochem.

Strain and plasmid constructions

To epitope tag yeast genes, cassettes encoding an epitope tag, a 3′ UTR, and a selectable marker were amplified by PCR with target-gene-specific primers and integrated at the respective genomic locus; detailed descriptions of the tagging cassettes are available upon request. Strains containing one or five copies of a *GAL* promoter–*SIC1–HA1* fusion integrated at the *ura3* locus were generously provided by Etienne Schwab (CNRS, Montpellier, France). *GAL–SIC1–HA1 HRT1–myc9* and *GAL–SIC1–HA1 HRT1–stop–myc9* strains were obtained by genetic crosses.

To generate plasmids expressing yeast or human Hrt1 in bacteria, yeast, or insect cells, the appropriate open reading frames were amplified by PCR and inserted into expression vectors for *E. coli* (pGEX-4T-1 or pMAL-c2), *Saccharomyces cerevisiae* (pRD54), or a baculovirus transfer vector (pFastBac1). Cdc34ΔC and MBP–$Cdc34^{HA}$ were created by replacing the 24 carboxy-terminal residues of Cdc34 with six histidines or the HA epitope and cloning the resulting reading frames into pET11b or pMAL-c2, respectively. A baculovirus expressing PyHA epitope-tagged Grr1 was obtained by annealing a set of four oligos containing a *Drosophila* codon-optimized sequence encoding the amino-terminal region of Grr1. This segment was followed by the remainder of the yeast *GRR1* sequence and the entire gene cloned into pFastbac. Sequences of the oligos and the details of cloning strategies used are available upon request.

Preparation of yeast cell extracts and immunoprecipitations

Exponentially growing yeast cells were pelleted, washed with water, and resuspended in lysis buffer (50 mM Tris-HCl at pH 7.8, 100 mM NaCl, 0.1 mM EDTA, 0.2% Triton X-100) containing protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin). Cells were lysed with glass beads by 10 cycles of vortexing for 40 sec followed by a 1 min incubation on ice. After centrifugation (30 min at 100,000*g*), extracts

(7 mg) were incubated with monoclonal antibody 9E10 cross-linked to protein A–Sepharose (9E10 beads, 20 µl) for 90 min at 4°C. Beads were washed four times with 1 ml of lysis buffer lacking protease inhibitors, and once with 1 ml of 50 mM Tris-HCl at pH 7.8. Proteins were eluted from the beads in a minimal amount of 50 mM Tris-HCl at pH 7.8 plus 0.2% SDS, lyophilized, resuspended in SDS-PAGE sample buffer, and applied to a 8%–16% gradient SDS–polyacrylamide gel. Protein bands were visualized by silver staining (Harlow and Lane 1988). The larger-scale prep used to obtain Hrt1 for mass spectrometry-based sequencing was performed as described above, except that 600 mg of crude extract protein from *CDC53myc9* cells was applied to a 30-ml DEAE column, and the 0.35 M NaCl eluate was collected essentially as described (Deshaies et al. 1995). The resulting fraction II (100 mg) was mixed with 250 µl of 9E10 beads, and the immunoprecipitates were processed as described above except that proteins were visualized by Coomassie Blue stain following SDS-PAGE.

Mass spectrometry

A 17-kD Coomassie-stained band present in Cdc53myc9 immunoprecipitates was excised and in-gel digested with trypsin (Boehringer Mannheim, unmodified, sequencing grade) as described (Shevchenko et al. 1996b). MALDI mass spectrometry (MS) analysis of an aliquot withdrawn from the digest failed to identify proteins and therefore the recovered pool of tryptic peptides was analyzed subsequently by nanoelectrospray tandem MS (Nano ES MS/MS) (Wilm et al. 1996). Nano ES MS/MS was performed on a API III triple quadrupole mass spectrometer (PE Sciex, Ontario, Canada) equipped with a nanoelectrospray ion source (Wilm and Mann 1996) developed at European Molecular Biology Laboratory (EMBL). Short sequence stretches were determined from tandem mass spectra of individual tryptic peptides and, in combination with the masses of the intact peptides, were assembled into peptide sequence tags (Mann and Wilm 1994) and used to search a comprehensive sequence database with PeptideSearch version 3.0 software developed at EMBL.

Analysis of Hrt1 function in vivo

Small G_1 cells were isolated by centrifugal elutriation from cultures grown at 25°C in YPD medium as described (Schwab and Nasmyth 1993). A Becton-Dickinson FACScan was used for flow cytometric measurement of cellular DNA content (Epstein and Cross 1992). Spindles were detected by indirect immunofluorescence using anti-tubulin antibodies (YOL1/34) and FITC-conjugated secondary antibodies. To measure cell volume, cells were fixed in YPD medium containing formaldehyde (3.8%) for 4 hr on ice. About 20,000 cells were counted with a CASY1 Cell Counter Plus Analyser (Model TTC, Schaerfe System, Reutlingen, Germany) and peak cell volume was measured in triplicate.

Swi6 and Sic1–HA1 were detected with rabbit polyclonal antibodies. Detection with monoclonal α-HA antibodies gave similar results for Sic1–HA1. RNA isolation and Northern blot analysis were performed as described (Cross and Tinkelenberg 1991; Price et al. 1991). Fragments from the coding regions of *SIC1* and *CMD1* were used as probes.

Protein expression

GST–Hrt1, Cdc34ΔC^{His6}, and MBP–Cdc34ΔC^{HA} proteins were expressed in *E. coli* BL21(DE3)+pLysS cells and purified by glutathione, Ni-NTA, and amylose affinity chromatography as directed by the resin suppliers, respectively. MBP–Cdc34ΔC^{HA} formed thioesters with ubiquitin, served as an E2 in Sic1 ubiquitination reactions, and could autoubiquitinate in the presence of Cdc53 (R.M.R. Feldman, unpubl.). Baculoviral expression of SCF subunits was as described (Feldman et al. 1997). Cln2^{myc}/GST–Cdc28/Cks1/Cak1 proteins were expressed in Sf9 insect cells infected with the respective recombinant baculoviruses in a 10:5:3:1 ratio.

GST pull-downs, α-Py IP from insect cells

GST pull-downs contained 2.5 µg of either GST or GST–Hrt1, 50 µl (~200 µg) lysate from insect cells infected with the indicated baculoviruses, and 100 µl of BVLB (30 mM HEPES at pH 7.4, 150 mM NaCl, 0.1% Triton X-100) containing 1 mg/ml BSA. Reactions were incubated 15 min at 4°C, supplemented with 7 µl of glutathione–agarose beads (Sigma), and incubated a further 15 min at 4°C. Beads were collected by brief centrifugation and washed four times in BVLB fortified with 100 mM NaCl (250 mM final). After washing, beads were aspirated dry and resuspended in 1× SDS-PAGE buffer, boiled, and analyzed by SDS-PAGE and Western blotting.

Experiments utilizing baculovirus-expressed Hrt1 (Fig. 3B) contained 100 µl of lysate from insect cells coinfected with Hrt1-expressing and other viruses as indicated. Lysates were incubated with 7 µl α-Py beads for 20 min at 4°C, and were washed and analyzed as described above.

In vitro assays

To analyze Cdc34ΔC^{His6} autoubiquitination, 200 µl of lysate (~4 mg/ml) from insect cells infected with the indicated baculovirus (es) was incubated with 7 µl of α-Py beads for 1 hr at 4°C. The beads were then washed three times in BVLB, three times in KAB (50 mM HEPES at pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA), aspirated to dryness, and resuspended in reaction buffer (30 mM Tris at pH 7.4, 5 mM $MgCl_2$, 2 mM ATP) supplemented with 100 ng of Ubal (Feldman et al. 1997), 5 µg ubiquitin and 600 ng of Cdc34Δ^{His6} (20 µl total volume). Reactions were incubated at 24°C for 1 hr with gentle mixing, and were stopped by addition of an equal volume of 2× SDS-PAGE sample buffer and boiled for 3 min. Samples were analyzed by SDS-PAGE and Western blotting using α-Cdc34 followed by HRP-conjugated α-rabbit secondary antibodies.

Sic1 ubiquitination reactions were performed as described (Feldman et al. 1997), except that 100 ng of phosphorylated MBP–Sic1 was used per reaction, and 200 µl (~ 800 µg) of insect cell lysate that contains polyoma-tagged SCF was immunoprecipitated with 7 µl Py beads and washed as described above, typically yielding 150–200 ng of SCF components per sample as estimated from Coomassie-stained gels. For Figure 4A, 2.5 µg of purified GST or GST–Hrt1 was added to reactions that contained 4 µg of phospho-MBP–Sic1.

To test NEM sensitivity, α-Py immunoprecipitates were prepared as described above. NEM (5 mM unless otherwise indicated) was added to beads (or to Ubal in the presence of an equal amount of beads) and incubated at 24°C for 10 min, at which point a twofold molar excess of DTT was added and the incubation was continued for 10 min. As a control, beads were treated with NEM that had been premixed with twofold excess DTT. After incubations, beads were washed with KAB and used in reactions as described; Ubal treated in the presence of beads was used as is.

Sequence analysis

Databases were searched with Gapped BLAST (Altschul et al.

1997). ESTs were assembled using AssemblyLIGN (Oxford Molecular, Oxford, UK), and protein sequences were aligned with CLUSTAL W (Thompson et al. 1994) using the Blosum 62 matrix with default parameters.

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Note

While this manuscript was under review, Ohta et al. (1999), Tan et al. (1999), Kamura et al. (1999), and Skowyra et al. (1999) reported the identification of yeast and human HRT1.

References

Altschul, S., T. Madden, A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. Lipman. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **25:** 3389–3402.

Bai, C., P. Sen, K. Hofmann, L. Ma, M. Goebl, J.W. Harper, and S.J. Elledge. 1996. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* **86:** 263–274.

Banerjee, A., L. Gregori, Y. Xu, and V. Chau. 1993. The bacterially expressed yeast *CDC34* gene product can undergo autoubiquitination to form a multiubiquitin chain-linked protein. *J. Biol. Chem.* **268:** 5668–5675.

Barral, Y., S. Jentsch, and C. Mann. 1995. G(1) cyclin turnover and nutrient-uptake are controlled by a common pathway in yeast. *Genes & Dev.* **9:** 399–409.

Bartel, B., I. Wunning, and A. Varshavsky. 1990. The recognition component of the N-end rule pathway. *EMBO J.* **9:** 3179–3189.

Biemann, K. 1988. Contributions of mass spectrometry to peptide and protein structure. *Biomed. Environ. Mass Spectrom.* **16:** 99–111.

Bordallo, J., R.K. Plemper, A. Finger, and D.H. Wolf. 1998. Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. *Mol. Biol. Cell* **9:** 209–222.

Cross, F.R. and A.H. Tinkelenberg. 1991. A potential positive feedback loop controlling CLN1 and CLN2 gene expression at the start of the yeast cell cycle. *Cell* **65:** 875–883.

Deshaies, R.J., V. Chau, and M. Kirschner. 1995. Ubiquitination of the G1 cyclin Cln2p by a Cdc34p-dependent pathway. *EMBO J.* **14:** 303–312.

Epstein, C.B. and F.R. Cross. 1992. CLB5: A novel B cyclin from budding yeast with a role in S phase. *Genes & Dev.* **6:** 1695–1706.

Fang, G.W., H.T. Yu, and M.W. Kirschner. 1998. Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. *Mol. Cell* **2:** 163–171.

Feldman, R.M.R., C. Correll, K. Kaplan, and R. Deshaies. 1997. A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* **91:** 221–230.

Goebl, M.G., L. Goetsch, and B. Byers. 1994. The Ubc3 (Cdc34) ubiquitin-conjugating enzyme is ubiquitinated and phosphorylated in vivo. *Mol. Cell. Biol.* **14:** 3022–3029.

Hampton, R.Y., R.G. Gardner, and J. Rine. 1996. Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Mol. Biol. Cell* **7:** 2029–2044.

Harlow, E. and D. Lane. 1988. *Antibodies: a laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Hershko, A., H. Heller, S. Elias, and A. Ciechanover. 1983. Components of ubiquitin-protein ligase system: Resolution, affinity purification, and role in protein breakdown. *J. Biol. Chem.* **258:** 8206–8214.

Honda, R., H. Tanaka, and H. Yasuda. 1997. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett.* **420:** 25–27.

Hu, G. and E. Fearon. 1999. Siah-1 N-terminal RING domain is required for proteolysis function, and C-terminal sequences regulate oligomerization and binding to target proteins. *Mol. Cell Biol.* **19:** 724–732.

Huibregtse, J.M., M. Scheffner, S. Beaudenon, and P.M. Howley. 1995. A family of proteins structurally and functionally related to the E6-AP ubiquitin protein ligase. *Proc. Natl. Acad. Sci.* **92:** 2563–2567.

Jiang, J. and G. Struhl. 1998. Regulation of the Hedgehog and Wingless signalling pathways by the F- box/WD40-repeat protein Slimb. *Nature* **391:** 493–496.

Kamura, T., S. Sato, D. Haque, L. Liu, W.G. Kaelin, R.C. Conaway, and J.W. Conaway. 1998. The elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin families. *Genes & Dev.* **12:** 3872–3881.

Kamura, T., D.M. Koepp, M.N. Conrad, D. Skowyra, R.J. Moreland, O. Iliopoulos, W.S. Lane, W.G. Kaelin, Jr., S.J. Elledge, R.C. Conaway et al. 1999. Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. *Science* **284:** 657–661.

King, R.W., R.J. Deshaies, J.M. Peter, and M. Kirschner. 1996. How proteolysis controls cell division. *Science* **274:** 1652–1659.

King, R.W., J.M. Peters, S. Tugendreich, M. Rolfe, P. Hieter, and M.W. Kirschner. 1995. A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* **81:** 279–288.

Kipreos, E.T., L.E. Lander, J.P. Wing, W.W. He, and E.M. Hedgecock. 1996. cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family. *Cell* **85:** 1–20.

Kolman, C.J., J. Toth, and D.K. Gonda. 1992. Identification of a portable determinant of cell cycle function within the carboxyl-terminal domain of the yeast CDC34 (UBC3) ubiquitin conjugating (E2) enzyme. *EMBO J.* **11:** 3081–3090.

Kornitzer, D., B. Raboy, R.G. Kulka, and G.R. Fink. 1994. Regulated degradation of the transcription factor GCN4. *EMBO J.* **13:** 6021–6030.

Kwon, Y., Y. Reiss, V. Fried, A. Hershko, J. Yoon, D. Gonda, P. Sangan, N. Copeland, N. Jenkins, and A. Varshavsky. 1998. The mouse and human genes encoding the recognition component of the N-end rule pathway. *Proc. Natl. Acad. Sci.* **95:** 7898–7903.

Mann, M. and M. Wilm. 1994. Error tolerant identification of peptides in sequence databases by peptide sequence tags. *Anal. Chem.* **66:** 4390–4399.

Ohta, T., J.J. Michel, A.J. Schottelius, and Y. Xiong. 1999. ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquiting ligase activity. *Mol. Cell* **3:** 535–541.

Patton, E.E., A.R. Willems, D. Sa, L. Kuras, D. Thomas, K.L. Craig, and M. Tyers. 1998a. Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box protein complexes that regulate cell division and methionine biosynthesis in yeast. *Genes & Dev.* **12:** 692–705.

Patton, E.E., A.R. Willems, and M. Tyers. 1998b. Combinatorial control in ubiquitin-dependent proteolysis: Don't Skp the F-box hypothesis. *Trends Genet.* **14:** 236–243.

Price, C., K. Nasmyth, and T. Schuster. 1991. A general approach to the isolation of cell cycle-regulated genes in the budding yeast *Saccharomyces cerevisiae. J. Mol. Biol.* **218:** 543–556.

Ruegger, M., E. Dewey, W.M. Gray, L. Hobbie, J. Turner, and M. Estelle. 1998. The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast Grr1p. *Genes & Dev.* **12:** 198–207.

Scheffner, M., U. Nuber, and J.M. Huibregtse. 1995. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* **373:** 81–83.

Schwab, E. and K. Nasmyth. 1993. CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae. Genes & Dev.* **7:** 1160–1175.

Schwab, E., T. Böhm, M. Mendenhall, and K. Nasmyth. 1994. The B-type cyclin kinase inhibitor p40SIC1 controls the G1/S transition in *Saccharomyces cerevisiae. Cell* **79:** 233–244.

Schwab, M. A.S. Lutum, and W. Seufert. 1997. Yeast Hct1 is a regulators of Clb2 cyclin proteolysis. *Cell* **90:** 683–693.

Shevchenko, A., O. Jensen, A. Podtelejnikov, F. Sagliocco, M. Wilm, O. Vorm, P. Mortensen, A. Shevchenko, H. Boucherie, and M. Mann. 1996a. Linking genome and proteome by mass spectrometry: Large scale identification of yeast proteins from two dimensional gels. *Proc. Natl. Acad. Sci.* **93:** 14440–14445.

Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996b. Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. *Anal. Chem.* **68:** 850–858.

Skowyra, D., K. Craig, M. Tyers, S. Elledge, and J. Harper. 1997. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* **91:** 209–219.

Skowyra, D., D.M. Koepp, T. Kamura, M.N. Conrad, R.C. Conaway, J.W. Conaway, S.J. Elledge, and J.W. Harper. 1999. Reconstitution of G1 cyclin ubiquitination with complexes containing SCFGrr1 and rbx1. *Science* **284:** 662-665.

Suarin, A., K. Borden, M. Boddy, and P. Freemont. 1996. Does this have a familiar RING? *Trends Biochem. Sci.* **21:** 208–214.

Tan, P., S.Y. Fuchs, A. Chen, K. Wu, C. Gomez, Z. Ronai, and Z.Q. Pan. 1999. Recruitment of a ROC1-Cul1 ubiquitin ligase by Skp1 and HOS to catalyze the ubitquitination of I kappa B alpha. *Mol. Cell.* **3:** 527–533.

Thompson, J., D. Higgins, and T. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalities and weight matrix choice. *Nucleic Acids Res.* **22:** 4673–4680.

Verma, R., R.S. Annan, M.J. Huddleston, S.A. Carr, G. Reynard, and R.J. Deshaies. 1997a. Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. *Science* **278:** 455–460.

Verma, R., R. Feldman, and R.J. Deshaies. 1997b. SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. *Mol. Biol. Cell.* **8:** 1427–1437.

Visintin, R., S. Prinz, and A. Amon. 1997. CDC20 and CDH1: A family of substrate-specific activators of APC-dependent proteolysis. *Science* **278:** 460–463.

Willems, A.R., S. Lanker, E.E. Patton, K.L. Craig, T.F. Nason, R. Kobayashi, C. Wittenberg, and M. Tyers. 1996. Cdc53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway. *Cell* **86:** 453–463.

Wilm, M. and M. Mann. 1996. Analytical properties of the nano electrospray ion source. *Anal. Chem.* **66:** 1–8.

Wilm, M., A. Shevchenko, T. Houthaeve, S. Breit, L. Schweigerer, T. Fotsis, and M. Mann. 1996. Femtomole sequencing of proteins from polyacrylamide gels by nanoelectrospray mass spectrometry. *Nature* **379:** 466–469.

Yaron, A., A. Hatzubai, M. Davis, I. Lavon, S. Amit, A.M. Manning, J.S. Andersen, M. Mann, F. Mercurio, and Y. Ben-Neriah. 1998. Identification of the receptor component of the IκBα-ubiquitin ligase. *Nature* **396:** 590–594.

Yu, H., J.M. Peters, R.W. King, A.M. Page, P. Hieter, and M.W. Kirschner. 1998. Identification of a cullin homology region in a subunit of the anaphase-promoting complex. *Science* **279:** 1219–1222.

Zachariae, W., A. Shevchenko, P.D. Andrews, R. Ciosk, M. Galova, M.J. Stark, M. Mann, and K. Nasmyth. 1998. Mass spectrometric analysis of the anaphase-promoting complex from yeast: Identification of a subunit related to cullins. *Science* **279:** 1216–1219.