University of Alberta

Genetic and Biochemical Studies of the CDC34/SCF complex.

By

Rajeet Singh Pannu ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

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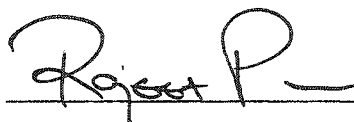

Rajeet Singh Pannu
12620 66th Avenue
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"It's nothing," the doctor they had called declared a little later. "He is working too much. In a week he will be on his feet again." "You are sure he will get well?" asked Louise with a distorted face. "He will get well." In the other room Rateau was looking at the canvas, completely blank, in the center of which Jonas had merely written in very small letters a word that could be made out, but without any certainty as to whether it should be read *solitary* or *solidary*.

- ***Albert Camus***

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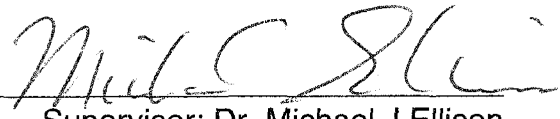

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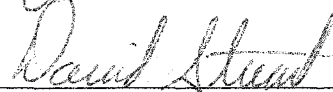

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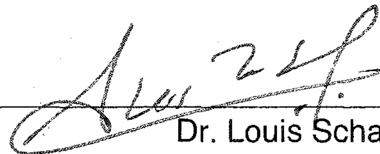

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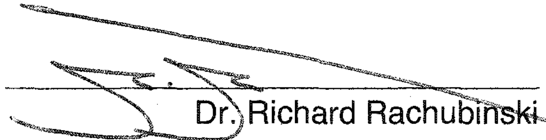

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ABSTRACT

The regulatory mechanisms which govern progression through the eukaryotic cell cycle are highly complex and adaptable. In recent years a great deal of scientific scrutiny has been directed towards the understanding of such processes at a molecular level. In the budding yeast *Saccharomyces cerevisiae*, the Cdc34/SCF complex regulates physiological processes within the cell by specifically targeting proteins for ubiquitin-mediated degradation by 26S proteasome. Its essential cell cycle function at the G1/S transition involves coordinating the timely degradation of the cyclin-kinase inhibitor Sic1, thus allowing cells to proceed into S phase. In this study, we identify a new role for the Cdc34/SCF complex at the G1/S transition: the positive regulation of the yeast cell integrity pathway. Using a variety of physiological, genetic, and biochemical techniques, we provide data strongly suggesting that yeast cells bearing temperature-sensitive mutant alleles of Cdc34, Cdc4, and Cdc53 are specifically defective in the cell integrity pathway, and that this defect is fundamental to their inability to enter S-phase at restrictive growth temperatures. This study therefore provides unique insight into the molecular mechanisms governing the interplay between a cell's response to its extracellular environment and its ability to regulate the cell cycle.

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LIST OF ABBREVIATIONS

Å- Angstrom

aa – amino acid

Ade – Adenine

Ala – Alanine

AMP – Adenosine monophosphate

Arg – Argenine

ATP – Adenosine triphosphate

°C – degrees Celsius

cpm – counts per minute

Cdc – cell division cycle

Cdk – Cyclin-dependent kinase

CW – Calcafluor White

Cys – Cysteine

DNA – Deoxyribonucleic acid

DTT - Dithiothreotol

GAP – GTP-ase activating protein

GEF – GTP exchange factor

GFP – green fluorescent protein

GlcNAc – N-acetylglucosamine

Gly – Glycine

GST – Glutathione-s-transferase

GTP – Guanosine triphosphate

His – Histidine

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HRP – Horseradish peroxidase

IA - Iodoacetamide

Ile – Isoleucine

kD – kilodalton

Leu – Leucine

LRR – Leucine-rich-repeat

Lys or K – Lysine

M – molar

MAPK – mitogen-associated protein kinase

MBF – MCB-binding factor

MCB – *Mlu1*-cell cycle box

mg – milligram

Mg – Magnesium

min - minute

mM – millimolar

nm – nanometer

O.D. – Optical Density

ORF – Open Reading Frame

PAGE – Polyacrylamide gel electrophoresis

PEST – Proline-Glutamate-Serine-Threonine

Pro – Proline

SBF – SCB-binding factor

SCB – Swi4-cell cycle box

SCF – Skp1-Cullin-F-box protein

SD – Synthetic Dropout

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SDS – Sodium dodecyl sulfate

TBS – Tris-buffered saline

Thre – Threonine

Trp – Tryptophan

ts – temperature-sensitive

Ub – ubiquitin

Ubc – ubiquitin conjugating enzyme

UCS – Uncharacterized cell integrity suppressor

Ura - Uracil

UV – Ultraviolet

Val - Valine

VHL – von Hippel-Lindau

YPD – Yeast extract Peptone Dextrose

Δ - delta or deletion

μ - micron

μg – microgram

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CHAPTER I – BIOCHEMICAL REGULATION OF THE G1-S TRANSITION IN BUDDING YEAST

1. Introduction

In the budding yeast *Saccharomyces cerevisiae*, progression from the G1 to S phases of the cell cycle requires passage through a critical interval termed START, where three essential cell cycle events are initiated: budding, spindle pole body duplication, and DNA replication. Once the START interval is breached, a cell becomes committed to cell cycle entry and a myriad of biochemical processes become activated orchestrating both nuclear and cellular division.

The central regulator of the yeast cell cycle engine is a single essential cyclin-dependent kinase (Cdk) encoded by the *CDC28* gene. Activation of Cdc28 catalyzes progression through START, initiation of DNA replication, and the onset of mitosis. At each of these transitions, Cdc28 is activated by its association with a specific group of periodically-expressed proteins known as cyclins. The association of Cdk with different cyclins allows for the timely phosphorylation of a variety of substrates critical to cell cycle progression. Thus, due to the pivotal importance of the cyclin-Cdk complexes in controlling cell cycle events, the timing and duration of their activity is a highly regulated process that involves signal transduction, transcriptional activation, and protein degradation. This chapter will review the some of the important functions of cyclin-Cdk complexes at the G1-S transition in yeast, and discuss the important regulatory networks that both activate and inhibit these complexes.

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2. Cyclins and Cyclin-dependent Kinases

Cdc28 is activated by at least nine distinct cyclins: 3 G1 cyclins (Clns), and six B-type cyclins (Clbs). The G1 cyclins Cln1, Cln2, and Cln3 are essential for cell cycle initiation during G1 phase. Two of the B-type cyclins, also known as S-phase cyclins, Clb5 and Clb6, promote the transition from the G1 to S phase of the cell cycle, while the remaining four cyclins, Clb1-4, are required for completion of mitosis. In addition, the Cdk Pho85 contributes to cell cycle progression at G1 through its association with the G1 cyclins Pcl1 and Pcl2. For progression through the START interval to be achieved, the precisely coordinated combinatorial effects of different cyclin-Cdk complexes are required.

2.1 The G1 cyclins

While Cdc28-Cln complexes function by catalyzing the START interval, not all of these complexes function equivalently. Deletion of all three Clns (*cln*$^-$) results in cell cycle arrest as unbudded cells with a 1C DNA content. Replacement of any one Cln in *cln*$^-$ cells suppresses the cell cycle arrest, suggesting that these genes are, at least in part, functionally redundant. However, primary sequence analysis of yeast Clns reveals that Cln3 is considerably different from the two highly related cyclins Cln1 and Cln2. The similarity between Cln3 and Cln1,2 is restricted to the cyclin box, a region of the cyclin involved in the physical interaction with Cdc28 (Hadwiger et al., 1989). Furthermore, Cln3 expression is relatively constitutive during the cell cycle, with a two to three-fold peak where cells finish the cell cycle and enter G1. Cln1 and Cln2 transcripts, on the other hand, are cell cycle regulated, and are virtually absent in early G1 and then increase dramatically in late G1 (Tyers et al., 1993; Wittenberg et al., 1990). Genetic and biochemical studies have neatly demonstrated that the function of Cln3 during early G1 is

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to monitor cell size and to induce the late G1 expression of Cln1 and Cln2, driving cells into S phase. Cdc28-Cln3 induces Cln1 and Cln2 expression by activating the SBF and MBF transcriptional complexes (Tyers et al., 1993; Dirick et al., 1995; Stuart and Wittenberg., 1995; reviewed here in Section 3). These complexes promote the expression of Cln1, Cln2, Clb5, Clb6, Pcl1, Pcl2, as well as a host of other genes required for S phase entry, bud formation, and cell wall synthesis (reviewed by Koch and Nasmyth, 1994; Spellman et al., 1998). The precise mechanism by which Cln3 activates these transcriptional complexes is not clear. Cln3 does not directly interact with the proteins within these complexes, resulting in the speculation that Cln3-Cdc28 may activate SBF and MBF indirectly, perhaps by phosphorylating an unknown intermediary protein (Wijnen et al., 2002). Interestingly Cln1 and Cln2 can also activate MBF and SBF-mediated transcription, which implies a positive-feedback loop driving Cln1 and Cln2 expression. The genetic evidence suggests, however, that the primary role of Cln3 at the G1-S transition is to activate the SBF and MBF-mediated transcription driving cells into S-phase (Stuart and Wittenberg., 1995)

Being highly related proteins, it is not surprising that Cln1 and Cln2 function redundantly. They act to promote polarized cell growth, spindle pole body duplication, to repress the anaphase-promoting complex (APC), and to phosphorylate Cdc28 inhibitors (Lew and Reed, 1993; Haase et al., 2001; Cross, 1995; Schwob et al., 1994; Tyers et al., 1993). It is this last function that is likely their most important. Insight into Cln1,2 function during G1 came from the observation that deletion of the *SIC1* gene suppressed the cell cycle arrest in a *cln*$^{-}$ deletion (Tyers, 1996). Sic1 was initially identified as an *in vitro* substrate for Cdc28 (Reed et al., 1985). It is expressed from anaphase until START, and potently inhibits Clb-Cdc28 but not Cln-Cdc28 kinase activity. Its persistence precludes DNA replication in pre-START G1 by its inhibitory association with Clb5/6-

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Cdc28 complexes (Schwob et al., 1994). As Sic1 is a START antagonist, Cln1,2 activate START by phosphorylating Sic1 which, in turn, targets Sic1 for degradation by the ubiquitin-proteosome pathway (Verma et al., 1997; reviewed here in Section 4). Once Sic1 is degraded, Clb5/6-Cdc28 complexes are liberated, allowing for S phase progression.

The G1 cyclins Pcl1 and Pcl2 also play an important role in the G1-S phase cell cycle transition. Their Cln3-dependent transcription peaks in G1, and their deletion in a *cln1,2Δ* strain results in a G1 arrest, suggesting an important cell cycle role for these proteins (Espinoza et al., 1994; Measday et al., 1994). They complex with Pho85 Cdk, a nonessential Cdk that associates with 10 cyclins and phosphorylates a variety of substrates involved in processes as diverse as glycogen accumulation during fermentation, inhibition of phosphate-starvation induced gene expression, and cell cycle regulation (reviewed by Lenburg and O' Shea, 1996). A possible cell cycle function of Pcl1,2/Pho85 is the phosphorylation of Rvs167, a cell polarity protein involved in actin polymerization (Lee et al., 1998). However, the most clearly delineated function for this complex at the G1-S transition is its redundant role in Sic1 phosphorylation (Nishizawa et al., 1998). Both Cln1,2-Cdc28 and Pcl1,2-Pho85 complexes have been demonstrated capable of phosphorylating Sic1 *in vivo* and *in vitro*, and both complexes are transcriptionally regulated in late G1 phase by the SBF transcriptional complex in a Cln3 dependent manner. Why do two different cyclin-Cdk complexes play redundant roles in Sic1 phosphorylation? The significance of this observation was not clear until recently with the demonstration that multisite phosphorylation of Sic1 sets a kinase activity threshold for passage into S-phase (Nash et al., 2002). More broadly, Sic1 phosphorylation seems to act as a gauge monitoring levels of START gene expression. As Cln1,2 and Pcl1,2 transcription occurs as part of the START program, it appears that

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the Sic1 cell cycle blockage is only removed once an acceptable amount of kinase activity accumulates. The kinase activity seems therefore to act as a reporter for gene expression, indicating to the cell cycle machinery that sufficient transcription has occurred for irreversible commitment to a new round of cell division. The G1 cyclins therefore regulate the G1 to S phase cell cycle transition by monitoring internal growth conditions within the cell, activating the transcription of key genes involved in the multifaceted components of cell division, and by phosphorylating important substrates necessary for cell cycle progression most notably the Clb-Cdc28 inhibitor Sic1 (Figure 1-1).

2.2 The S Phase Cyclins

The S-phase or B-type cyclins Clb5 and Clb6 are temporally expressed during the cell cycle as part of the late G1 transcriptional burst discussed previously. Their expression is largely mediated by the MBF transcriptional complex, which is activated by Cln3-Cdc28 just prior to START (reviewed here in Section 3.1). Once translated, however, these proteins remain inactive in G1 due to the presence of Sic1, which binds Clb5,6-Cdc28 complexes stoichiometrically and prevents them from activating substrates by phophorylation (Schwob et al., 1994). Relief from Sic1 inhibition by the ubiquitin proteasome pathway results in Clb5,6-Cdc28 activation and the subsequent induction of yeast S phase (Schwob et al., 1994; Verma et al., 1998). The observation that these genes are required for S phase activation comes from penetrating genetic studies of *CLB5,6* deletion mutants. It was observed that while deletion of *CLB6* has little or no effect on the vegetative growth of yeast cells, deletion of *CLB5* greatly extends S phase due to the specific failure of activation of late-firing origins of DNA replication (Epstein and Cross., 1992; Koch and Nasmyth, 1993; Donaldson et al., 1998). In the complete absence of

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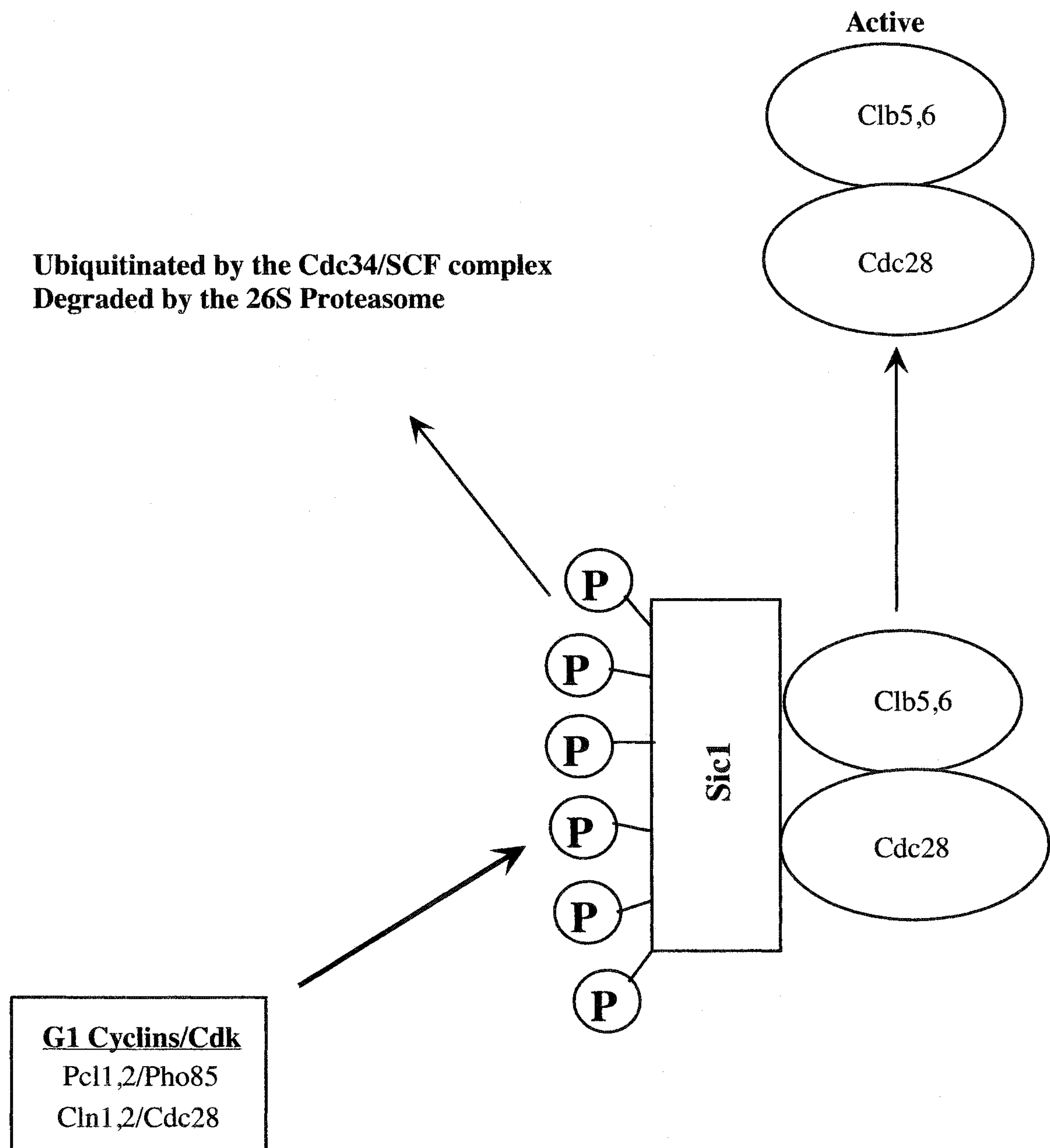

Figure 1-1. Phosphorylation of Sic1 at the G1-S transition.
G1 cyclin/CDK complexes induce S-phase by phosphorylating the Clb5,6/Cdc28 inhibitor Sic1. Sic1 is thus targeted for ubiquitin-mediated degradation by the Cdc34/SCF complex, liberating activated Clb5,6/Cdc28

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both *CLB5* and *CLB6*, initiation of DNA synthesis is delayed but ultimately activated due to the redundant activity of Clb1,2,3,4-Cdc28 complexes (Schwob and Nasmyth, 1993).

A tremendous amount of information has been generated demonstrating the vital importance of cyclin-Cdk complexes in the coordination and regulation of cell cycle events. Interestingly, the substrates of many of the cyclin-Cdk complexes have not as of yet been determined. No doubt the focus of future research in this area will be on the identification and functional characterization of these target proteins.

3. Transcriptional Activators

As previously described, the transition from the G1 to S phases of the cell cycle is particularly important in budding yeast for coordinating cell growth with cell division. When cells reach a critical size, they duplicate their spindle pole bodies, form buds, and commence DNA synthesis. All these events are simultaneously initiated at the START interval and require the activation of Cdc28 by the G1 cyclins Cln1, Cln2, and Cln3. Transcripts for the G1 cyclins *CLN1*, *CLN2*, *CLB5*, *CLB6*, *PCL1*, and *PCL2* are absent in early G1 phase, but appear abruptly at START. This precisely timed transcriptional burst is directly coupled to cell size and nutritional status and is required for transition into S-phase. The activation of the SBF and MBF transcriptional complexes is largely responsible for initiating this timely burst of transcription, though the activity of a second transcription factor, Rme1, may also play an important role at this juncture.

3.1 The SBF and MBF complexes

The SBF (SCB-binding factor) is a heteromeric complex containing the Swi4 and Swi6 proteins. It functions by binding repeated *cis*-acting SCB elements (Swi4,6 cell cycle box; CACGAAA) found within the promoters of SBF-regulated genes (Nasmyth, 1985; Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989; Andrews and

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Moore, 1992) It shares a common subunit with the MBF or DSC1 transcription factor (MCB-binding factor), which is also heterodimeric, composed of Swi6 and Mbp1. MBF functions by its association with MCB promoter elements (*MluI* cell cycle box; ACGCGTNA; Koch and Nasmyth, 1993). The Swi4 component of the SBF is its DNA binding component, and its N-terminal domain is sufficient for SCB-binding *in vitro*. As such, its DNA binding domain is highly related to that of Mbp1, which serves as the MCB recognition element through a helix-turn-helix DNA-binding structural feature (Primig et al., 1992; Koch et al., 1993). Swi6 acts as the regulatory component of these complexes and associates with Swi4/Mbp1 C-terminal domains (Andrews and Moore, 1992; Sidorova and Breeden, 1993).

The activation of SBF and MBF-mediated transcription is a fundamental event regulating the G1-S transition in the yeast cell cycle (reviewed by Koch and Nasmyth, 1993). These complexes induce the late G1 expression of the G1 cyclins Cln1, Cln2, Pcl1 and Pcl2; the S-phase cyclins Clb5 and Clb6; a variety of DNA synthesis-related genes; and, as previously mentioned, genes involved in cell wall biosynthesis (Spellman et al., 1998). Activation of these complexes is poorly understood, though the Cln3-Cdc28 cyclin kinase complex, the Bck2 kinase and the cell integrity MAPK Slt2 have all been demonstrated capable of activating SBF and MBF directly or indirectly by phosphorylation (Wijnen et al., 2002; Wijnen and Futcher, 1998 ; Madden et al., 1997; Figure 1-2). The SBF and MBF are also regulated by changes in the subcellular localization of the Swi6. Cytoplasmic Swi6 is translocated to the nucleus during late M and G1 phase upon phosphorylation of an internal serine residue (Ser-160) which reveals a cryptic nuclear localization sequence. Once inside the nucleus, SBF and MBF complexes coalesce and bind to their respective promoter elements but do not initiate transcription until their activation in late G1 phase. Upon completion of its

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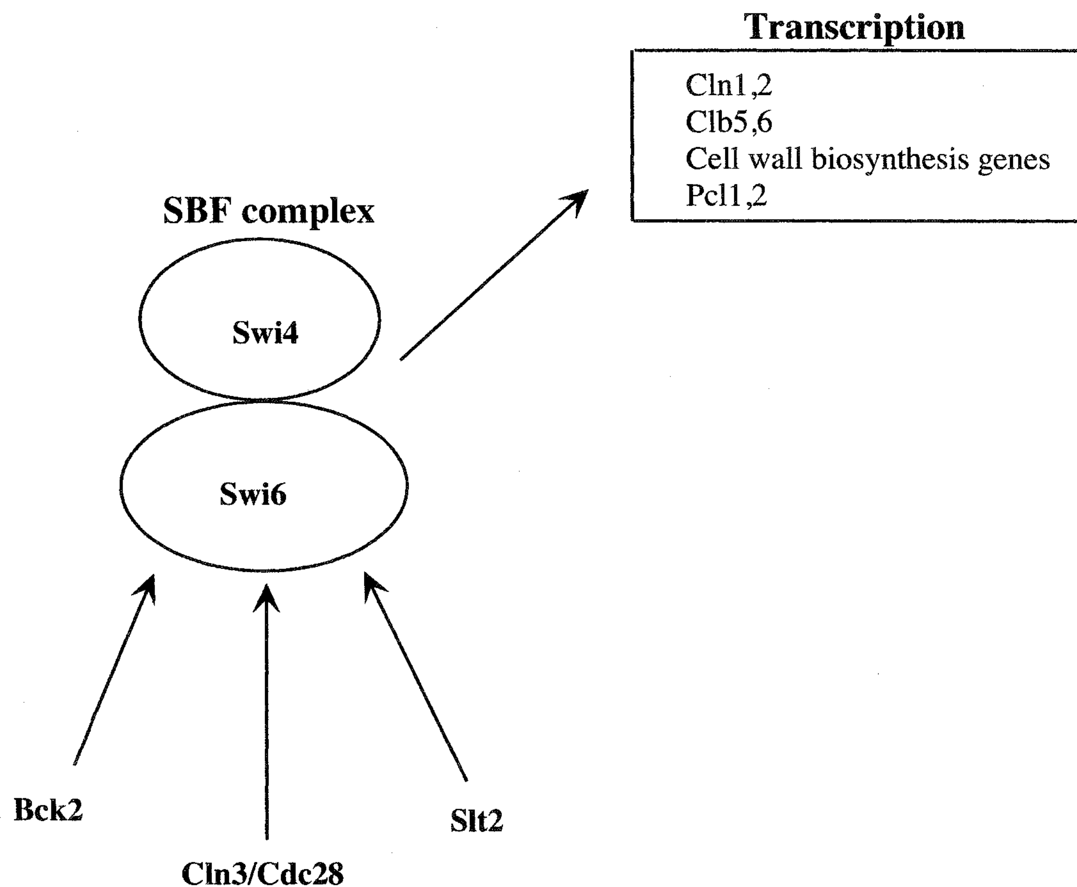

Figure 1-2. Transcriptional activation at the G1/S transition.
Three independent signaling pathways intersect to activate the SBF complex at the G1-S transition. Upon activation, the SBF activates the transcription of genes involved in cell cycle and cell wall biosynthesis.

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transcriptional function at START, the SBF complex is repressed by the G2 activity of Clb/Cdc28 complexes, which interact with the central ankyrin-repeat domain of Swi4 and deactivate it by phosphorylation (Amon et al., 1993).

3.2 Rme1

The *RME1* gene encodes a zinc-finger protein that was originally identified in a screen for mutations that were synthetically lethal with a *SWI4* temperature sensitive (ts) mutant (Toone et al., 1995). It was found that *RME1* overexpression bypassed the requirement for an active SBF complex, and deletion of *RME1* in a *SWI6Δ SWI4*ts strain strongly accentuated the temperature sensitivity defects associated with the mutant strain. These data indicated a redundancy in the essential function between the SBF/MBF complexes and *RME1*. It was further observed that *RME1* deletion by itself, while phenotypically unremarkable, resulted in a 30% decrease in *CLN2* transcription. This result was complemented by the fact that Rme1 can directly bind the *CLN2* promoter, perhaps indicating its function as a transcription factor. Last year, Frenz and colleagues (2001) determined that Rme1 localizes to the nucleus, and is transcribed in a cell cycle-specific manner, beginning at the end of mitosis and peaking in G1 phase. They also identified two Rme1 upstream response elements in the Cln2 promoter, which were sufficient to drive expression of the Cln2 gene in an Rme1-dependent fashion. While the exact role of this protein in cell cycle regulation is not known as yet, its identification adds further complexity to a complete understanding of the G1-S transition.

The key elements of the START program come from the activation of transcription mediated by these three transcription factors. The focus of the ensuing sections of this chapter will be the additional regulatory pathways that influence S phase

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progression by their direct and indirect effects on these transcription factors and on cyclin-Cdk activity.

4. The Cdc34/SCF complex

The timely degradation of short-lived regulatory proteins by ubiquitin-dependent proteolysis plays a vital role in controlling critical physiological pathways within the cell. Upon covalent modification with a poly-ubiquitin chain, a substrate protein is captured and rapidly degraded by the 26 S proteasome. Ubiquitin (Ub) is a 76 amino acid heat-stable polypeptide present in all eukaryotes, and its conjugation to protein substrates is orchestrated by a series of well-defined enzymatic reactions. Initially, Ub is activated from a free intracellular Ub pool in an ATP-dependent manner by the essential Ub-activating enzyme E1 or Uba1. This results in the formation of a high-energy thiolester linkage between the active site cysteine residue of E1 and the extreme carboxy-terminal glycine (Gly76) of Ub. Upon activation by E1, Ub is then passed via a trans-thiolation reaction to the active site cysteine of a second enzyme known as an E2 or Ub-conjugating enzyme (Ubc). The E2 either alone or in concert with a specialized Ub-ligase or E3, then transfers the activated Ub to a substrate protein, resulting in the formation of an iso-peptide bond between an internal lysine residue on the substrate and gly76 of Ub. Once a substrate becomes covalently modified in this manner, the substrate-appended Ub then serves as a substrate itself for further ubiquitination on an internal lysine residue by the same mechanism. The second Ub, then becomes the substrate for the attachment of a third, the third Ub for a fourth etc., resulting in the formation of a substrate-attached poly-Ub chain (reviewed by Pickart et al., 2001; Hochstrasser, 1995; Figure 1-3).

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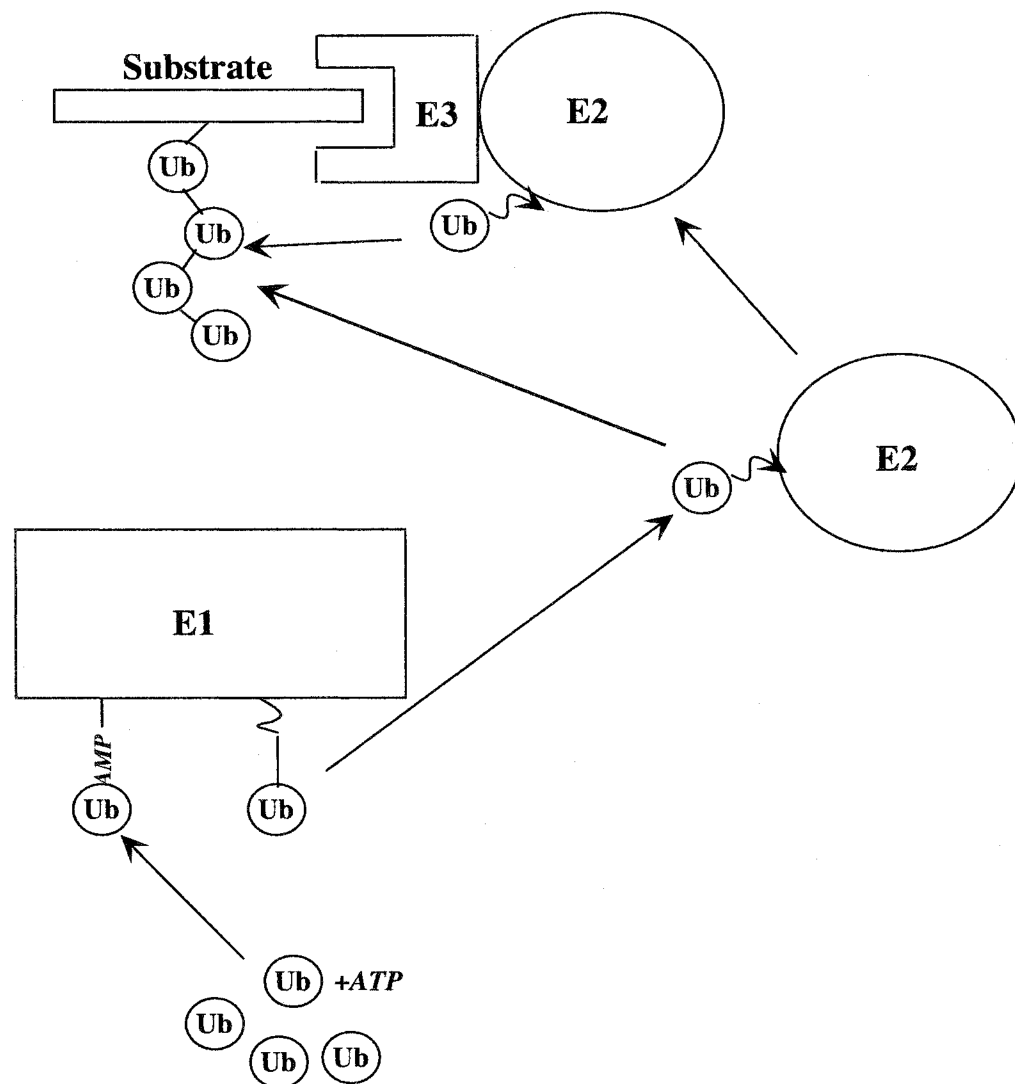

Figure 1-3. The Ubiquitin conjugation system.
The 76 amino acid protein ubiquitin (Ub) is activated in an ATP-dependent manner by E1. It is transferred from the active site of E1 to the active site of E2, which either alone or in conjunction with an E3, catalyzes the covalent modification of a substrate with poly-Ub chain

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4.1 Cdc34

While a single E1 gene controls the activation of Ub in yeast, eleven different E2s have been identified regulating processes as varied as signal transduction, DNA repair, cell cycle progression, protein folding, sporulation, chromatid separation, peroxisome biogenesis, and metabolism. E2s are highly structurally conserved within a core catalytic domain of approximately 150 amino acids, which contains the active-site cysteine residue required for Ub transfer. While some E2s are composed only of the core domain, others bear N- and C-terminal extensions that provide functional specificity. Despite their similarity to one another, E2s execute distinct biological functions, a phenomenon that is likely due to the specificity of E2-E3 interactions (reviewed by Pickart et al., 2001; King et al., 1996).

Cdc34 or Ubc3, however, is one of only two essential E2s in yeast, functioning in the regulation of the G1-S phase cell cycle transition. The *CDC34* gene encodes a 295-residue protein with a 125 amino acid COOH-terminal extension important for its function. Molecular cloning of the *CDC34* gene provided the first observation that the activity of a ubiquitin conjugating enzyme was required prior to the onset of DNA replication (Goebl et al., 1988; reviewed by King et al., 1996). Extracts made from *CDC34* temperature sensitive (ts) mutants were found to inhibit S-phase CDK activity, implying that *CDC34* may be required for the degradation of a CDK negative regulator (Schwob et al., 1994). A candidate gene to mediate this activity was thought to be *SIC1*, a high affinity S-phase CDK inhibitor. Consistent with this hypothesis was the observation that Sic1 was rapidly degraded within wild-type cells entering S-phase, but stabilized in CDC34ts mutants growing at the restrictive temperature. Thus Sic1 appeared to be the essential target for blocking G1-S phase cell cycle progression. Further genetic studies clarified this point, as *CDC34*ts *sic1*Δ cells were found to initiate DNA synthesis

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at the non-permissive temperature. Furthermore, hyperstablized Sic1 mutants, with mutations in three key CDK phosphoacceptor sites, were found to block S-phase entry in wild-type cells (Verma et al., 1997). Taken together, these results demonstrated that progression into S-phase is dependent on the degradation of Sic1 by Cdc34.

4.2 The SCF complex

While not all Ub-mediated degradation requires an E3, E3's appear to be the primary source of substrate specificity within the ubiquitination cascade (reviewed by DeSalle and Pagano, 2001). The E3 regulating the function of Cdc34 in budding yeast is a heterotetrameric protein complex known as the SCF complex (Figure 1-4). The idea that Cdc34 functions through a multi-protein complex initially came from the observation that Cdc34 physically associates with the Cdc4 and Cdc53 proteins (Willems et al., 1996; Mathias et al., 1996). A third component of the E3 complex was identified genetically with the observation that the *SKP1* gene suppressed defects in *CDC4*ts mutants at high copy (Bai et al., 1996). Mutations in the *SKP1*, *CDC53* (a Cullin family member), and *CDC4* (an F-box containing protein) genes – hence SCF – exhibit a variety of recognizable mutant phenotypes identical to *CD34*ts mutants demonstrating their requirement in the same biochemical pathways. These include: highly polarized growth morphologies, G1 cell cycle arrest, multiple buds emerging from the same bud site, and Sic1 accumulation, when grown for prolonged periods at restrictive temperatures (DeSalle and Pagano., 2001). Furthermore, in each case, deletion of the *SIC1* gene was found to suppress the S-phase entry defects of temperature sensitive mutants. A fourth SCF component, the RING-H2 protein Rbx1/Hrt1, was later identified by mass spectroscopic analysis of proteins copurifying with Cdc53 (Seol et al., 1999). Rbx1/Hrt1 was found to associate with the SCF complex stoichiometrically and with high efficiency

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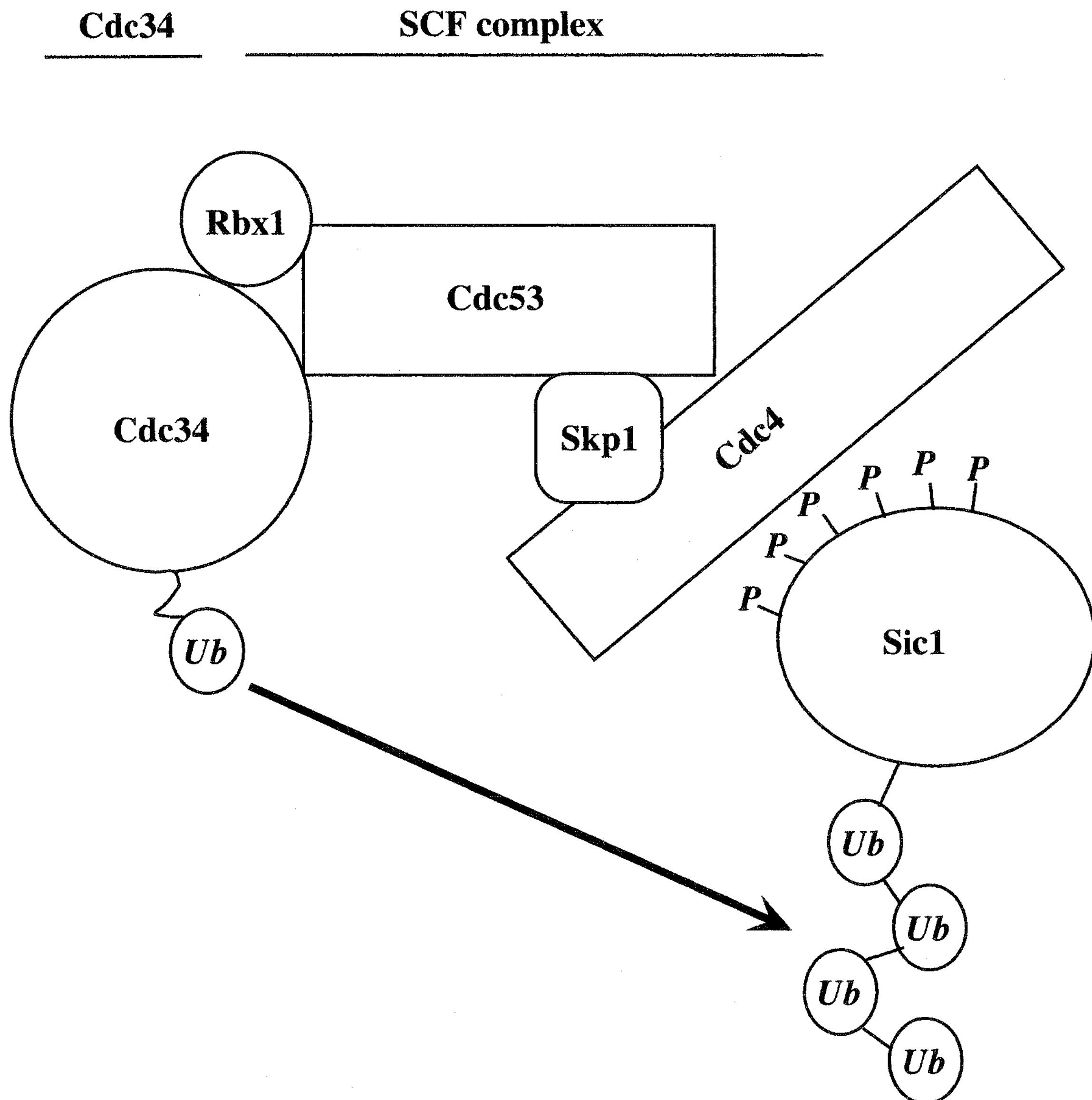

Figure 1-4. The Cdc34/SCF^{Cdc4} complex ubiquitinates phosphorylated Sic1. Cdc34 is the E2 which acts in concert with its E3, the SCF complex, to ubiquitinate the phosphorylated cyclin-kinase inhibitor Sic1. Cdc34 is the catalytic component of the complex and the SCF served to bridge the interaction between Cdc34 and Sic1. For Sic1 degradation, the SCF is composed of four proteins: Rbx1, Cdc53, Skp1, and the modular F-box component Cdc4.

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in vivo and *in vitro*, and *RBX1* ts mutants exhibited identical G1-S phase progression defects due to Sic1 accumulation observed with mutants of *CDC34* and the other SCF components.

4.3 Activity and Substrate Specificity

While the structural determinants important for substrate recognition by the Cdc34/SCF complex are at present unclear, three distinct parameters are known to be important for determining SCF activity and substrate specificity. The first is the nature of the F-box protein component of the SCF. Primary sequence alignment of three known mammalian Skp1-interacting proteins, cyclin F, Skp2 and Cdc4, revealed the presence of a shared degenerate hydrophobic motif of approximately 40 amino acids, which has come to be known as an F-box motif – after cyclin F (Bai et al., 1996). Subsequent genomic analysis within *S. cerevisiae*, has revealed that at least 17 open reading frames (ORFs) code for F-box containing genes, and all the genes thus far analyzed have been shown to be components of SCF or SCF-like complexes. These observations have been coherently distilled into a single theoretical framework termed the F-box hypothesis. The two basic tenets of this hypothesis are as follows: first, F-box proteins associate with the Skp1 subunit of the SCF via the F-box motif; second, F-box proteins recruit substrates for ubiquitination through specialized protein-protein interaction domains present in the COOH-terminal portion of the F-box protein (Bai et al., 1996). Thus the F-box subunit of the SCF is in fact modular, and substrates for ubiquitination are determined by the identity of the F-box protein in the SCF complex. Two different F-box proteins are known to play important roles in the yeast G1-S transition: Cdc4 and Grr1. The SCF^{Cdc4} complex is required for Cdc6 and Sic1 degradation, while SCF^{Grr1} mediates the degradation of a variety of cell cycle regulators, including Cln2, and the Cdc42 effector

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proteins, Gic1 and Gic2. The relevance of these targets to cell cycle progression will be discussed in detail in ensuing sections.

A second factor determining substrate specificity in the ubiquitin pathway involves the phosphorylation state of the target protein. A shared feature of all known SCF complexes is their dependence on phosphorylation for substrate recognition. For some SCF targets, such as the cyclin kinase inhibitor Far1, monophosphorylation appears to be sufficient for its recognition by the ubiquitin system (Henchoz et al., 1997). For other substrates, such as Cln2 and Sic1, phosphorylation on multiple redundant sites is required (Lanker et al., 1996; Verma et al., 1997). As mentioned in Section 2.1 of this review, an important paper by Nash and colleagues (2001) recently demonstrated a requirement for multi-site phosphorylation on Sic1 in order for it to be recognized as a target by the SCF^{Cdc4} complex. They further showed that at least six of nine low-affinity phosphoacceptor sites on Sic1 must be phosphorylated in order for substrate recognition to occur. They created a mutant yeast strain expressing a Sic1 derivative that served as a high affinity substrate for ubiquitination independent of the phosphorylation threshold, which dramatically illustrated the catastrophic consequences of premature Sic1 degradation by exhibiting unregulated S-phase progression and severe genomic instability. The mechanism by which phosphorylated substrates are recognized by F-box proteins, however, is not at all clear. There do not appear to be consensus motifs for either phosphorylation or ubiquitination among SCF targets, and whether phosphorylation aids directly in substrate association with the F-box proteins through charge interactions, or indirectly by inducing substrate conformational changes is also unknown (Willems et al., 1999). In combination with phosphorylation sites, most SCF substrates contain so called 'PEST-motifs', which are sequences rich in proline (P), glutamate (E), serine (S), and threonine (T) residues. Rechsteiner et al, (1996) suggested

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that the PEST domain may in fact be an important determinant for protein instability. Due to the unusually large number of proteins with PEST-like sequences however, it seems likely that the PEST domains are likely not obligatory on SCF substrates, but may, in some way, be contributing factors to protein degradation.

A third parameter governing the activity of the Cdc34/SCF complex is the post-translational modification of the Cdc53 or Cullin component with the ubiquitin homologue Nedd8/Rub1. The *CDC53* or *CULA* gene is a member of the Cullin protein family (Kipreos et al., 1996). It is homologous to the *CUL1* gene, which was originally identified in the nematode *C. elegans* in a screen for genes inhibiting cellular hyperplasia. Since then, four additional Cul genes have been identified in *C. elegans*, four in yeast, and six in human cells. Nedd8 was originally identified as an abundant mRNA present in the fetal mouse brain (Neural precursor cell expressed developmentally downregulated; Kumar et al., 1992). It was found to be an 81 amino acid ubiquitin homologue, one of a growing number of known ubiquitin-like molecules. Nedd8 was consequently found to be 59% identical to a ubiquitin-like protein identified by the yeast genome project dubbed Rub1. In an analogous manner to ubiquitin, Nedd8/Rub1 was found to be activated and conjugated to substrates in a series enzymatic reactions carried out by enzymes closely related to *UBA1* and the Ubcs of the ubiquitin system. In mammalian cells, Nedd8 is activated by a heterodimer of two *UBA1* homologues, *UBA3* and *APP-BP1* while in yeast *AOS1* and *ENR2* activate it (Osaka et al., 2000; Gong et al., 1999). Moreover, conjugation of Nedd8/Rub1 occurs in both organisms via the *UBC12* enzyme. The targets for Nedd8 conjugation have been found to be members of the Cullin family, and Nedd8 modification modulates the function of Cullin-based ligases. In yeast, while *RUB1* is not essential for vegetative growth, cells deleted for *RUB1* are slow to complete the cell cycle. Furthermore, mutation of SCF components in the *rub1Δ* genetic

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background is lethal, suggesting an important role for Rub1-modification in SCF activity. Interestingly, in *S. pombe*, Nedd8 is essential for cell viability, and critical to the function of the Cullin protein Pcu1. While a substantial portion of Cdc53 appears to be modified by Rub1 *in vivo*, the consequences of this modification to the activity of the ubiquitin system are unclear (Lammer et al., 1998). Several theories have emerged speculating on the role that neddylation may play in modulating SCF activity. Some possible functions include recruitment of Cdc34 to the SCF, facilitation in the polyubiquitination of target proteins, stabilization of the SCF complex, or perhaps influencing SCF activity towards its substrates (reviewed by Hochstrasser, 1995).

Recently Zheng et al., (2002) provided an unprecedented boost to SCF structure/function studies by reporting the 3.2Å resolution crystal structure of a complete human SCF tetrameric complex containing Cul1, Rbx1, Skp1, and the F-box protein Skp2. They further generated a computer-modeled association between this complex and Ubch7, a human ubiquitin conjugating enzyme. They found the SCF structure to be highly elongated and polarized, with Rbx1 and Ubch7 segregated to one end and tSkp1 and the F-box protein Skp2 to the other. Their data nicely complemented previous genetic studies, demonstrating that the Cul1/Cdc53 component serves as the scaffolding for the entire complex. The Cul1 C- terminal domain was found to associate with the enzymatically active Rbx1 and Ubc components, while the N-terminal domain bound the Skp1/F-box moiety. They also observed that the positioning of the neddylation site (lysine 720 in Cul1) with respect to the Rbx1 and Ubc binding-domains of Cul1 support the notion that modification of a Cullin with Nedd8/Rub1 modulates the binding and positioning of the Ubc in the complex.

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4.4 Cdc34/SCF targets at the G1-S transition

As previously discussed, the essential target of the Cdc34/SCF complex at the G1-S transition is known to be the cyclin kinase inhibitor Sic1, and deletion of the *SIC1* gene bypasses the requirement for an active SCF complex at this interval (Figure 1-4). In recent years, several other key substrates of this complex have also been identified, further establishing the important role of regulatory pathways in coordinating the timing of cell cycle events.

Apart from Sic1, perhaps the most significant Cdc34/SCF substrates controlling progression through the cell cycle are the G1 cyclins Cln1, Cln2, and Cln3 (Lanker et al., 1996; Willems, 1996; Tyers et al., 1992). The instability of Cln1 and Cln2 is essential for coupling Cln-Cdc28 activity to changes in *CLN* gene expression at START (Tyers et al., 1992; Tyers 1993). This coupling is important for proper cell cycle regulation because stabilized Cln mutants are insensitive to factors that arrest cells before commitment to the cell cycle, such as alpha factor and a lack of appropriate nutrients (Cross, 1988; Nash et al., 1988; Hadwiger et al., 1989). Cdc34 was found to be required for Cln2 ubiquitination *in vitro* and *in vivo* (Deshaies et al., 1995; Lanker et al., 1996). Cdc53 was also implicated directly in Cln2 turnover, having been identified in a screen for Cln2 associated proteins (Willems et al., 1996). It was determined that the Cdc53-Cln2 interaction was phosphorylation dependent, because mutation of CDK phosphoacceptor sites on *CLN2* abrogated its association with Cdc53 and resulted in its stabilization. Cln2 was also found to be ubiquitinated and rapidly turned over in wild type cells, while accumulating in *CDC34ts* and *CDC53ts* mutant strains at the restrictive temperature (Tyers et al., 1992). Lastly, in a genetic screen for mutants defective in Cln1 degradation, it was discovered that the leucine-rich repeat containing F-box protein Grr1 was required for Cln proteolysis (Barral et al., 1995). Thus, the Cdc34/SCF^{Grr1} complex regulates

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Cln1 and Cln2 turnover thereby ensuring a regulated transition into S-phase. Cln3 proteolysis has also been shown to be Cdc34-dependent (Tyers et al., 1992). Cln3 was found to be an unstable protein in relatively low cellular abundance. C-terminal truncation of Cln3, effectively removing its PEST-motifs, was found to dramatically stabilize the protein *in vivo*. While these researchers were unable to demonstrate an increased half-life for Cln3 in a *cdc34-2* mutant cell strain, they demonstrated that the kinase activity of Cln3 was upregulated in the mutant strain. These results suggest that Cln3 activity is influenced by Cdc34/SCF-mediated ubiquitination.

Another recently defined SCF target at the G1-S transition is the Cdc48 effector protein Gic2. Cdc48 is a Rho-related, GTP-binding protein that initiates polarization of the actin cytoskeleton towards the incipient bud site during bud emergence. In the absence of Cdc42, cells fail to grow in a polarized manner and instead increase in size isotropically. Activation of Cdc48 at the G1-S transition leads to the activation of numerous downstream effector molecules involved in the regulation of polarized growth, including: Gic2, Ste20, and Bni1 to name a few (Chen et al., 1997). Gic2 expression is cell cycle regulated, peaking in late G1 just prior to bud emergence (Brown et al., 1997). Its degradation *in vivo* is phosphorylation dependent and is mediated via the $Cdc34/SCF^{Grr1}$ complex. Mutation of Gic2 phosphoacceptor sites causes stabilization of the protein and results in hyperpolarized growth defects in the mutant cell strains. In a similar manner to the cyclins, Gic2 proteolysis requires the Grr1 F-box subunit and not Cdc4, demonstrating the versatility of the F-box protein in the recognition of multiple structurally unrelated proteins as Cdc34/SCF substrates.

Another regulatory molecule whose abundance is controlled by the Cdc34/SCF complex is the DNA replication initiation factor Cdc6. Just prior to the onset of DNA replication, Cln/Cdc28 complexes trigger the onset of DNA replication from origins

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containing assembled pre-replicative complexes, and simultaneously inhibit the assembly of new complexes. The Cdc6 protein is required for the proper assembly of pre-replication complexes, and its levels are tightly controlled in a Cln/Cdc28-dependent manner (Cocker et al., 1996; Detweiler and Li, 1997). The Cdc34/SCF^{Cdc4} complex is responsible for the degradation of Cdc6 *in vivo* (Drury et al., 1997). As previously seen with other Cdc34/SCF targets, mutation of phosphoacceptor sites on Cdc6 increase the stability of the protein throughout the cell cycle, but unlike many other targets, the enhanced stability of Cdc6 mutants does not result in a discernable phenotype with regards to DNA replication. In *S. pombe*, however, a Cdc6 homologue, Cdc18 is also degraded in the same manner, and hyperstabilized Cdc18 mutants result in re-replication of DNA (Jallepalli et al., 1997). Thus it is possible that multiple redundant mechanisms exist in *S. cerevisiae* to prevent DNA re-replication events.

The enormous role of ubiquitin-dependent proteolysis in yeast physiology is just beginning to be understood. Not only does the Cdc34/SCF complex regulate the cell cycle at the G1-S transition, it plays an essential yet undefined role at the G2-M checkpoint as well. The functional characterization of uncategorized F-box proteins will no doubt deconvolute the intricacies of this biochemical system. Also, structural studies on SCF-target interactions are required to provide insight into the determinants of substrate identification and specificity.

5. The Cell Integrity Pathway

Another regulatory system in *Saccharomyces cerevisiae* that controls progression through the G1-S transition is the cell integrity pathway. It is a complex signaling cascade consisting of various cytoplasmic and nuclear components that regulates yeast cellular morphology and cell division under conditions of stress. It is not, as the name

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suggests, one single pathway. It is, in fact, a multiply redundant highly complex and very poorly understood series of interconnected signaling pathways that share roles in coordinating the timing of large scale morphologic occurrences in the cell with the specific requirements of both the nuclear and the cell cycle. It is induced during periods of polarized cell growth, such as budding and mating, and in response to any environmental exposure that compromises the stability of the cell wall or interferes with its synthesis (Zarzov et al., 1996, Ketela et al., 1999). Correspondingly, impairment of this pathway results in a breach of the functional architecture of the cell wall, resulting in cell lysis under specific environmental stresses that can be ameliorated by osmotic stabilization (Jung and Levin, 1999). The cell integrity pathway originates at the cell membrane and signals to a wide variety of effector molecules that directly influence the polymerization of cell wall carbohydrates, and upregulate specific gene transcription essential for morphologic homeostasis and cell cycle progression. It is a tightly regulated system and signaling through this pathway is controlled at different levels by the activities of numerous unrelated proteins. This section will focus on some of the important molecules, both effector and regulatory, which control this pathway and discuss their influence on S-phase progression in yeast.

5.1 Important effector molecules of the cell integrity pathway

Cell wall or plasma membrane disturbance induced by heat shock or hypotonic stress provides the activating stimulus for signaling through this complex signal transduction cascade. Two similar membrane proteins Slg1 and Mid2 act in parallel to recognize extracellular membrane stress and to initiate the concomitant cell integrity signaling (Figure 1-5). Primary structure analysis of these proteins predicts them to be integral membrane proteins with a single putative transmembrane region, a cysteine and

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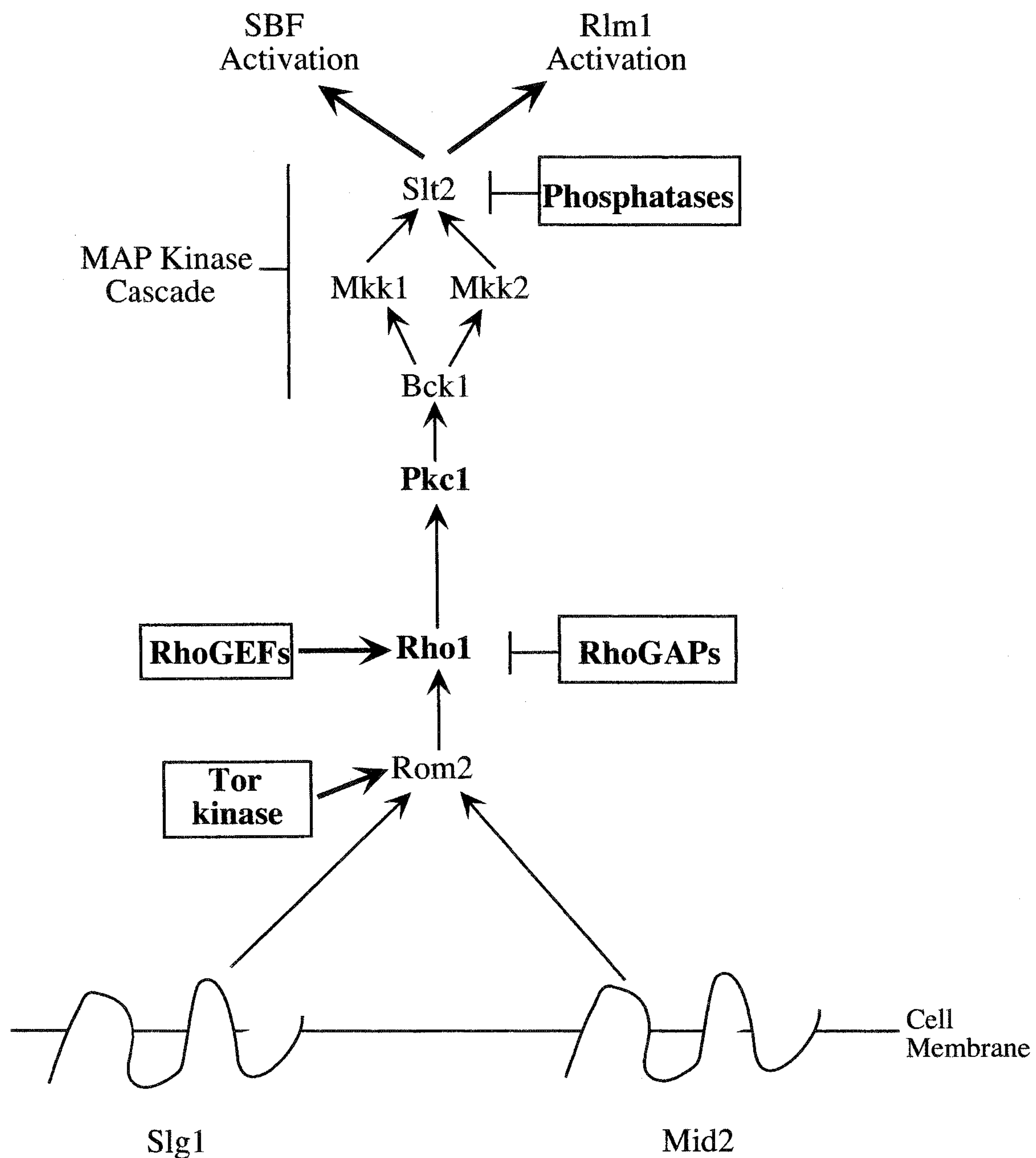

Figure 1-5. The yeast cell integrity signaling pathway.
The yeast cell integrity signaling pathway is activated at the G1-S transition and in response to cell wall stress. It involves the activities of numerous signaling proteins. The essential components of this pathway are Rho1 and Pkc1 (in bold) which have other important cellular functions apart from their roles in cell integrity signaling. Activators and inhibitors of this pathway are also shown (boxed).

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serine/threonine-rich extracellular domain, and short unique intracellular domains unrelated to any other known proteins. The serine/threonine-rich regions are highly O-mannosylated resulting, it is thought, in extension and stiffening of the extracellular domain. Thus, these molecules are proposed to act as rigid probes of the extracellular environment. Interestingly, despite their broad similarity, these proteins are not considered to be related. Upon sensing a disturbance at the cell membrane, they transmit a signal through a specific interaction between their C-terminal intracellular domains and a protein interaction domain on the GTP exchange factor (GEF) Rom2, directly stimulating its catalytic ability (Philip and Levin, 2001). Deletion of either of these genes greatly reduces signaling through the cell integrity pathway, suggesting their importance in the activation of the cascade. Furthermore, the phenotypes associated with deletion of the *SLG1* and *MID2* genes are consistent with all other cell integrity pathway mutants, most demonstrably characterized by a temperature-dependent cell lysis that is suppressed by high-osmolarity medium. Thus Slg1 and Mid2 function as upstream stress sensors that affect cell behavior by transforming mechanical membrane disturbances into biochemical signals (Jacoby et al., 1998; Rajavel et al., 1999).

Downstream of the membrane-sensing components of the cell integrity pathway is a small GTP-binding protein of the Ras-superfamily called Rho1 (Figure 1-5). Rho1 is an essential multifunctional GTPase that localizes to sites of polarized cell growth and exists in dynamic equilibrium between two distinct and interconvertible biochemical forms: an inactive GDP-bound form and an active GTP-bound form. Localizing to sites of cell growth, including the presumptive budding site, the bud tip and the cytokinesis site, its function is to mediate bud growth by controlling the polarization of the actin cytoskeleton, and by regulating the synthesis of the cell wall (Cabib et al., 1998). It is activated by at least two separate inputs, signaling by the phosphatidylinositol kinase

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homologue *TOR2*, and by mechanical defects in the cell wall. Both of these mechanisms activate Rho1 via the aforementioned GEF Rom2, which stimulates Rho1 by promoting its catalytic transformation into the active GTP-bound state. Once activated, GTP-Rho1 in turn, binds and activates at least four downstream effector molecules: Fks1, Bni1, Skn7 Pkc1. Through its interaction with Fks1, Rho1 directly controls the polymerization of the yeast cell wall. β-1,3 glucan, a major component of the cell wall, is synthesized at the cell surface by a pair of differentially expressed glucan synthases, Fks1 and Fks2. GTP-bound Rho1 is required for the catalytic activity of Fks1, and it colocalizes and co-immunoprecipitates with Fks1 *in vivo* (Drogovna et al., 1996., Qatoda et al., 1996). Genetic evidence also implicates Rho1 as a key player in the organization of the actin cytoskeleton through its interaction with Bni1 (Kohno et al., 1996). *BNI1* is a member of a gene family that plays a regulatory role in cytokinesis, establishment of cell polarity, and cell morphology. It has two distinct protein interaction domains called formin domains that bind the actin monomer-binding protein profilin. The precise role of Rho1 in modulating Bni1 function is not however known (Fujiwara et al., 1998). Skn7, another Rho1 target, is a response-regulator transcription factor that becomes activated by different stress pathways within the cell. Upon activation, Skn7 upregulates the transcription of several genes that are involved in cell wall synthesis, most notably the α-1,6-mannosyltransferase *OCH1*, a gene involved in the production of cell wall mannoprotein (Cui et al., 2002). Lastly, Rho1 binds and activates Pkc1, a homologue of mammalian protein kinase C, resulting the induction of a mitogen associated protein kinase (MAPK) cascade that has varied consequences for cell behavior, including the transcriptional activation of cell wall synthesis components and genes important for cell cycle progression (Igual et al., 1996)

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A central component of the cell integrity pathway is the essential serine/threonine specific protein kinase Pkc1. Activated by Rho1, it functions by initiating a MAPK cascade consisting of the MEKK (Map kinase kinase kinase) Bck1, the redundant MEKs (MAP kinase kinase) Mkk1 and Mkk2, and finally the MAPK Slt2 (Figure 1-5; reviewed by Cid et al., 1995). Temperature sensitive mutants within the *PKC1* signaling pathway have thin cell walls and a well described osmoremedial sensitivity to a variety of cell wall stresses such as heat shock. Yeast strains deleted for *BCK1*, *SLT2*, or both *MKK1* and *MKK2* while inviable at high temperatures are able to grow at 25°C, whereas a *PKC1* deletion is lethal, suggesting that PKC1 plays another essential role apart from activation of the MAPK cascade (Lee and Levin, 1992). This is substantiated by genetic evidence implicating Pkc1 in the MAPK-independent depolarization and repolarization of the actin cytoskeleton in response to cell integrity stress (Delley and Hall, 1999).

The key contribution that Pkc1 signaling makes to cell integrity is the activation of both the SBF transcriptional complex and the MADS-box transcription factor Rlm1, both of which control the transcription of numerous genes involved in cell wall synthesis (Figure 1-5; reviewed by Cid et al., 1995). As previously mentioned, the SBF is required for normal expression of a variety of genes involved in both cell wall synthesis and cell cycle progression including the G1 cyclins Pcl1 and Pcl2. It is an *in vivo* substrate of the Slt2 kinase, and its activation by the Pkc1 signaling pathway results in the transcriptional activation of a subset of genes under its control (Madden et al., 1997). Rlm1 is an *in vitro* substrate for Slt2 and also exhibits heat stress-induced *SLT2* dependent phosphorylation *in vivo* (Watanabe et al., 1997). One of the common phenotypes exclusive to mutants in the cell integrity pathway is sensitivity to low millimolar concentrations of caffeine. The precise mechanism by which caffeine affects cells at a molecular level is unknown, however it enhances the cell lysis phenotype of *SLT2*

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mutants. Caffeine likely affects some aspect of cell wall synthesis because numerous other proteins with altered cell wall construction are also sensitive to caffeine. Interestingly, the caffeine sensitivity phenotype of cell integrity mutants specifically results from the reduced activation of Rlm1. The temperature-dependent cell lysis phenotype, on the other hand, is thought to be conferred by decreased signaling through the SBF component of the pathway (reviewed by Madden and Snyder 1998). The DNA consensus sequence for Rlm1 binding has been identified and promoters for containing these sequences have been shown to be regulated by Rlm1 and Slt2. Some of the genes known to be regulated by this transcription factor are: the β-glucan synthesis regulator *HKR1*, the mannosyltransferase Ktr2, and the flocculation protein Flo1. The expression of these genes in the cell integrity pathway, however, has not yet been determined, though the potential role of *HKR1* in this pathway will be discussed in detail in another chapter (Dodou et al., 1997). Deletion mutants of either Rlm1 or components of the SBF exhibit cell integrity defects shared by *PKC1* pathway mutants. The Pkc1 pathway, therefore, exerts its influence on cell integrity indirectly by contributing to the specific transcriptional upregulation of genes important for morphological homeostasis.

Apart from these signaling molecules, many other genes, too numerous to review here, have also been implicated in cell integrity maintenance. The activity of one particular kinase, however, while poorly understood, is directly relevant to this thesis and thus will be mentioned briefly. The essential Ste20/PAK kinase Kic1 is a member of a conserved family of serine/threonine protein kinases that play multiple important roles in cell morphology and cell cycle progression (Sullivan et al., 1998). Kic1 was originally cloned as *CWH30*, a suppressor of a calcafluor white hypersensitivity yeast mutant, suggesting that it plays a direct role in cell integrity. This initial characterization became somewhat confused when Kic1 was later identified as a Cdc31 interacting protein by

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two-hybrid screen. Cdc31 is a calmodulin homologue that localizes to the spindle pole body and is essential for its duplication at the G1/S transition. Cdc31 was found to bind and activate Kic1 kinase activity *in vitro*, and interestingly Cdc31 mutants were also found to exhibit cell integrity defects. Interestingly, while Kic1 mutants were found to be defective in cell integrity, they exhibited no obvious difficulties in spindle pole body duplication (Sullivan et al., 1998). The links between duplication of the spindle pole body and cell integrity are not obvious, and these relationships suggest an important convergence of morphological pathways during the cell cycle. A potential clue to the function of Kic1 comes from genome-wide co-immunoprecipitation studies which have determined that Kic1 associates with Slt2, the terminal kinase in the Pkc1 MAPK cascade, suggesting an interaction between the Pkc1 pathway and Kic1 (Ho et al., 2002). Thus it is tempting to speculate that Kic1 may act as a parallel activator of Slt2 linking cell integrity and spindle pole body duplication. Extensive genetic interactions between the Pkc1 signaling pathway and components of the spindle pole body have also been observed, further illustrating this point (Stirling and Stark, 2000; Khalfan et al., 2000).

5.2 Important regulatory molecules of the cell integrity pathway

As previously indicated, Rho1 exists in equilibrium between its GDP-bound form and its GTP-bound form. Influencing the dynamics of this biochemical conversion are RhoGEFs (GTP-exchange factors) and RhoGAPs (GTP-ase activating proteins) (Figure 1-5; Cid et al., 1998). RhoGEFs are positive regulators of Rho1 that facilitate its transition into the GTP-bound active state and are encoded by the redundant genes *ROM1* and *ROM2* (Ozaki et al., 1996). Once activated by the Tor2 kinase, Rom2 in turn activates Rho1 and thus stimulates downstream signaling. Conversely, the RhoGAPs act as key negative regulators of cell integrity signaling in yeast. RhoGAPs comprise a

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family of related Rho-interacting proteins that function by stimulating intrinsic Rho GTPase activity, thus generating the GDP-bound inactive Rho1 (Cid et al., 1998). So far seven different RhoGAPs have been found to influence the activity of Rho1: Sac7, Bem2, Bem3, Rga1, Lrg1, Bag7 and Tus1 (Roumanie et al., 2001). While none of these genes is essential, deletion or mutation of any one of them results in broad changes in Rho1 activity and aberrant signaling to downstream effector molecules as a consequence. It is indeed remarkable that so many different proteins are required to modulate Rho1 activity. The current hypothesis is that different GAP proteins may be required for the regulation of specific Rho1 functions. In addition, RhoGAPs themselves are thought to be regulated in some manner. In mammalian cells, phosphorylation of RhoGAPS has been shown to influence their activity *in vivo* (Wolf et al., 2001). Also, the presence of PEST motifs in RhoGAP primary sequences may indicate their regulation by the ubiquitin-proteasome pathway (Roumanie et al., 2001). While some of these proteins appear play redundant roles, some RhoGAPs appear to act pleotropically, regulating more than one Rho-related protein. It is therefore clear that signaling through Rho1 depends on the complex interplay between its activators and inhibitors.

An emerging class of regulatory proteins integral to the cell integrity pathway are protein phosphatases. MAPK cascades are activated by phosphorylation, and are similarly deactivated by dephosphorylation. The MAPK in the Pkc1 cell integrity pathway is Slt2, and it is activated by the phosphorylation of two conserved tyrosine and threonine residues on its subdomain VIII activation loop. The mutation of either one of these activation determinants results in the inactivation of this kinase. Several different phosphatases are known to negatively regulate Slt2 activity. The two phosphatases Ptp2 and Ptp3 are involved in maintaining low basal activities of Slt2 and for adaptation following osmotic stress and heat shock (Jacoby et al., 1997). Similarly the dual specific

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phosphatase Msg5 has also demonstrated specific activity towards Slt2 *in vivo* (Martin et al., 2000). More recently another phosphatase Sdp1 has been show to interact with Slt2 and regulate its activity in the cell integrity pathway as well (Hahn and Thiele, 2002). Opposing the regulatory effects of these molecules is the phosphatase Glc7, which encodes the catalytic subunit of the serine/threonine phosphatase PP1. Glc7 acts as a positive regulator of the cell integrity pathway, and Glc7 mutants exhibit similar cell integrity defects to mutants in the Pkc1 pathway (Andrews and Stark., 2000). Protein phosphatases, therefore, play both regulatory and effector roles in maintaining cell integrity under stress conditions.

5.3 Cell integrity and the G1-S transition

Mazzoni and coworkers (1993) provided the first evidence that the cell integrity pathway plays a role during the G1-S transition. These authors observed that *SLT2* mutant strains growing at the nonpermissive temperature exhibit numerous cell cycle growth defects. They observed accumulation of abnormally large or small cells during the cell cycle, an alteration in the pattern of chitin deposition, an abnormal distribution of actin, an accumulation of secretory vesicles, and generally a phenotype that resembles mutations in genes required for polarized cell growth. Moreover, the same authors isolated an *SLT2* mutant as an enhancer of the cell division defects of the *CDC28-109* mutant strain. These observations taken together imply that either the *SLT2* pathway is regulated by *CDC28*, or alternatively, both *SLT2* and *CDC28* act in parallel to promote the budding process. These authors further demonstrate that the defective phenotype of a G1-specific *CDC28* mutant is enhanced by deletion of the *SLT2* gene, but a G2-M mutant of *CDC28* is not, suggesting that the role of *SLT2* in cell cycle regulation occurs in G1 and not in G2.

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More evidence directly implicating the cell integrity pathway in S-phase progression comes from genetic and biochemical studies characterizing the *in vivo* targets of the Slt2 kinase. Several proteins have been identified as downstream substrates of Slt2, most notably the two subunits of the SBF transcription factor Swi4 and Swi6. As described previously, the SBF promotes cell cycle progression by inducing a transcriptional burst at the G1/S transition of numerous genes including G1 cyclins *CLN1*, *CLN2*, *PCL1* and *PCL2* at the G1/S transition (Spellman et al., 1998; reviewed here in Section 3). One mechanism by which the cell integrity pathway may be regulating the cell cycle is through modulation of SBF activity. Phenotypic similarities between cell integrity pathway mutants and SBF mutants suggest a role for the SBF in the cell integrity pathway. Igual and colleagues (1996) demonstrated the coordinated regulation of gene expression by the SBF and the Pkc1 pathway by showing that a functional SBF complex is required for viability of Pkc1 mutants. Furthermore, they showed that deletion of either the *SW14* or *SWI6* genes resulted in hypersensitivity to Calcafluor White, and SDS, as well as osmo-redeemable cell lysis defects similar to mutants in the cell integrity pathway. They showed that a number of cell integrity genes contain promoter sequences for binding the SBF complex, including *KNR4/SMI1* which will be discussed later in this thesis. Madden and colleagues (1997) added to these findings with biochemical studies of SBF and Slt2. They showed that Slt2 and Pkc1 mutants exhibit a reduction in SBF-regulated gene transcription in late G1 and early S-phase including the G1 cyclins *PCL1* and *PCL2*. They further demonstrated that Slt2 modulates SBF function through the phosphorylation of both SBF subunits. These authors also reported that Slt2 phosphorylation stimulates the SBF-mediated activation of only a subset of genes controlled by this transcription factor, for example, it was observed that *CLN1* and *CLN2* expression was unaltered by deletion of the *SLT2* gene.

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Apart from Pkc1 and Slt2, other components of the cell integrity pathway have also been implicated in the regulation of the G1-S transition. Yeast strains carrying a mutation of either *SLG1* or *RHO1* exhibit phenotypes consistent with impaired cell cycle progression. *SLG1*, the most upstream component in the cell integrity pathway, was found to play a role in the regulation of the START interval (Ivanovska and Rose, 2000). *SLG1* deletion mutants growing at the nonpermissive temperature, were found to initiate bud emergence but were unable to duplicate their spindle pole bodies or initiate DNA replication. Furthermore, *SLG1* was found to exhibit genetic interactions with the ubiquitin conjugating enzyme Cdc34, suggesting its involvement in the G1-S transition. Similarly, genetic studies also indicate a G1-S role for *RHO1*. Examination of yeast cells bearing a specific $rho1^{E45I}$ mutation demonstrated that the *RHO1* mutants accumulate as large unbudded cells in G1 at 37°C (Drgonova et al., 1999). Furthermore, these cells were also severely impaired in the nuclear cycle, with very slowly proceeding DNA replication and a total inability to complete nuclear replication. Taken together, both these studies indicate a cell cycle role for individual components of the yeast cell integrity pathway upstream of Pkc1, further delineating the relationship between cell morphology and progression through the cell cycle.

A final piece of evidence linking the cell integrity pathway to the G1/S transition was the identification of the Bck2 kinase as a suppressor of mutations in either SLT2 or *PKC1* (Lee et al., 1993). Bck2, a protein kinase with no other yeast homologues, was originally identified as a high copy suppressor of Pkc1 pathway mutants, suggesting that it is a signaling component in the cell integrity pathway. Interestingly, parallel observations with Bck2 identified it as a gene whose overexpression bypasses the *CLN* requirement at START, demonstrating that it acts in a separate pathway to Cln3 to promote passage into the S-phase of the cell cycle (Epstein and Cross, 1994). Subsequent

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studies on Bck2 have demonstrated that it activates *CLN1* and *CLN2* transcription via the SBF at the G1/S transition, and that it plays a shared role with *CLN3* to activate SBF/MBF mediated transcription at the G1/S transition (Di Como et al.,1995; Wijnen and Futcher., 1999). Whether Bck2 functions directly in the cell integrity pathway or not is unclear. As it is a SBF activator like Cln3, its effects on cell integrity may simply be a result of its ability to activate the transcription of cell integrity genes via the SBF in a manner unrelated to morphological signaling.

Given the abundance of genetic and biochemical evidence, one can thus posit a potential mechanism by which the cell integrity pathway contributes to cell cycle progression. Cdc28 activation during late G1 triggers numerous growth events, including an increase in membrane flux, thus stimulating the downstream activation of Slg1, Rho1, Pkc1 and the Slt2 kinase. While both Pkc1 and Rho1 likely play multiple roles in the regulation of cell morphology and the cell cycle, phosphorylation of Slt2 results in the propagation of the growth response, by activating the SBF transcriptional complex and stimulating the expression of numerous genes involved in both cell wall biogenesis and in the regulation of polarized cell growth and cell cycle progression. In mammalian cells, a precisely analogous pathway has been directly implicated in G1-S progression in the manner just described. The mammalian Rho1-counterpart RhoA in response to growth signals from the membrane, activates a MAPK cascade leading to the downstream phosphorylation of the cyclin kinase inhibitor Kip1, which is then rapidly degraded in a ubiquitin–dependent manner (Hu et al., 1999; Rivard et al., 1999). It is intriguing to speculate that the cell integrity pathway may in some way be ancestral to this important mammalian growth pathway.

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6. Conclusion

The numerous interconnected signaling pathways and the sheer number of redundant mechanisms by which S-phase entry is controlled attests to the remarkable complexity of regulatory systems in living cells. The purpose of this thesis is to perhaps muddy the waters a little bit by demonstrating previously uncharacterized associations among some of these important pathways.

The focus of this thesis is on expanding the known physiological and biochemical roles of the Cdc34/SCF complex and the ubiquitin proteolytic system. In Chapter 2, a novel role of the Cdc34/SCF complex in the positive regulation of the yeast cell integrity pathway will be demonstrated genetically. In Chapter 3 a novel genetic suppressor of cell integrity and cell cycle defects of Cdc34/SCF mutants is identified and its potential role as a cell cycle regulator is examined. Chapter 4 is an *in vitro* biochemical analysis of dimerization between ubiquitin-conjugating enzymes, and the potential role it plays in E1 association. Chapter 5, the final chapter, will summarize and discuss the data and arguments presented in this thesis and allude to the potential for future work in this area.

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CHAPTER II – POSITIVE REGULATION OF YEAST CELL INTEGRITY BY THE CDC34/SCF COMPLEX

1. Introduction

In the budding yeast *Saccharomyces cerevisiae*, the maintenance of cell integrity is crucial to the execution of morphogenic events and survival under conditions of stress. Commonly referred to as the cell integrity pathway, it is induced during periods of polarized cell growth, such as budding and mating, and in response to any environmental exposure that compromises the stability of the cell wall or interferes with its synthesis (Zarzov et al., 1996, Ketela et al., 1999). Correspondingly, impairment of this pathway results in a breach of the functional architecture of the cell wall, resulting in cell lysis under specific environmental stresses that can be ameliorated by osmotic stabilization (Levin and Bartlett- Heubusch, 1992).

In yeast, the cell wall accounts for approximately 25% of the dry mass of the cell. It is a rigid structure that affords mechanical protection to the cells, controls cell shape, and modulates the selective uptake of macromolecules (reviewed by Cabib et al., 2001). It consists mainly of polysaccharides composed of three sugars: glucose, mannose, and N-acetylglucosamine. The most abundant glucose polysaccharide within the cell wall is β-1,3-glucan, and also present in significantly less quantity is β-1,6 glucan which plays an important role in crosslinking. The mannose polysaccharides are tethered to proteins termed mannoproteins, that mainly localize to the external surface and function as a filter for large molecular weight materials (Zlotnik et al., 1984). Lastly, 1-3% of the cell wall is composed of a β-1,4-linked GlcNAc known as chitin. Chitin is present in small amounts in vegetative cells, and is essential for survival, localizing specifically to a ring that circles the septum in budding cells and the bud scar in mother cells (Shaw et al., 1991). Despite the rigidity of the structure, the cell wall itself is extremely dynamic, as it

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is susceptible to numerous modifications during various stages of the yeast growth cycle. The composition of the wall fluctuates during bud emergence, bud growth, septum formation, sporulation, and conjugation. The mechanisms that regulate the architecture and composition of the wall are diverse and complex, balancing synthetic and degradation pathways for the polymers that constitute the wall. While it is well known that the genes involved in this pathway must be cell cycle regulated in some manner, the interrelationships among cell wall synthetic components and cell cycle components have not been clearly defined (reviewed by Cid et al., 1995).

More than 100 genes involved in cell wall synthesis have been isolated and characterized in large genetic studies of cell wall synthesis-defective mutants (Ram et al., 1994; Lussier et al., 1997). The genes isolated in these screen can be broadly grouped into four basic categories: structural genes involved directly in cell polymer synthesis, genes encoding GPI anchor proteins that are required for cell wall remodeling, genes involved in signaling and morphogenic pathways, and lastly genes involved in other pathways such as metabolic, transcriptional, or cell cycle pathways that have been shown also to have morphogenic functions (Martin et al.,1999).

In this chapter, genetic evidence demonstrating of a novel role for the Cdc34/SCF complex in the positive regulation of the cell integrity pathway is presented. The Cdc34/SCF complex is an essential ubiquitin conjugation/ligation complex that specifically targets cellular protein substrates for degradation by the 26S proteasome. It functions by catalyzing the covalent modification of a surface lysine residue on a particular substrate with a polyubiquitin chain. Entry into S-phase requires activation of the Cdc28/Clb protein kinase, which is assembled but inactive in G1 due to the presence of high levels of the Sic1 inhibitor (Schwob et al., 1994). At the G1-S transition, due to SBF-mediated transcriptional activation of G1 cyclins, Sic1 becomes phosphorylated and

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abruptly degraded by the Cdc34/SCF complex, liberating Cdc28/Clb and triggering DNA synthesis (reviewed by Desalle and Pagano., 2001). Thus the Cdc34/SCF complex functions as an important cell cycle regulator, playing an essential role in *S. cerevisiae* S-phase progression.

In this study, we show that a variety of mutant alleles of the Cdc34/SCF complex are specifically defective in the cell integrity pathway. The observed cell integrity defects are separable from the temperature-sensitivity cell cycle defects associated with these mutants and can be specifically suppressed by overexpression of important regulators of β-1,3-glucan synthesis. Moreover, we demonstrate a novel physical interaction between a previously identified genetic suppressor of a *CDC34* ts mutant strain (*UBS1*) and the Hkr1 protein, an essential component of the β-1,3-glucan synthesis machinery. Taken together, these data demonstrate genetically that the Cdc34/SCF complex plays a role in the positive regulation of the yeast cell integrity pathway.

2. Materials and Methods

2.1 Yeast Strains and Plasmids

The *Saccharomyces cerevisiae* strains used in this study and their relevant genotypes are listed in Table 2-1. YES71 was constructed by disrupting the *cdc34-2* allele with the HIS3 gene using the ApaI/EcoRI fragment from pGEM34 H/S as previously described (Goebl et al., 1988). Viability of the strain is maintained by a *CDC34* expression plasmid carrying the URA3 marker (Silver et al., 1992). CWY231, DSY795, DSY841, DSY1105 are all congenic with BF264-15D and the CDC strains were generated by backcrossing with the parental strain CWY231 eight times.
Plasmid pGBTUBS1 was made by PCR amplification of the *UBS1* gene with a 5' primer that containing an EcoRI restriction endonuclease site flanking the initiation codon and a 3' primer that with an in-frame STOP codon flanked by a PstI restriction endonuclease

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site. The PCR product was subsequently cloned in frame into EcoRI/PstI sites of the pGBT9 two-hybrid vector (Clonetech Inc., Pao Alto, CA). The pGADGH control vector was also obtained from Clonetech Inc.

Yeast expression plasmid pUBS1 was generated in the same manner as pGBTUBS1 but instead cloned into the EcoRI/PstI sites of a copper-inducible yeast expression pCUP1 vector containing a Trp1 selectable marker.

Yeast expression plasmid pUBS1-GFP was generated by PCR of pUBS1 with Sal1/Pst1 flanking restriction sites and directed cloning into pGFP-C-fuse driven by a methionine repressible promoter, and containing a Ura3 selectable marker

The pSK05 plasmid containing a 6.5 kb BamHI-BamHI fragment of the HKR1 gene (including the 5'- noncoding sequence) cloned into the BamHI site of pUC18 was generously provided by Dr. Tomio Yabe (Dept. of Applied Biochemistry, Tohoku University, Japan).

Yeast expression plasmid pHKR1ΔC was made first by digestion of pSK05 with KpnI/HindIII to give a 2.8 kb KpnI/HindIII fragment which was subcloned into a copper-inducible pCUP1 yeast expression plasmid with STOP codons in all three reading frames 3' to the gene insertion, containing a URA3 selectable marker to generate pUHK. PCR primers with flanking 5' and 3' KpnI restriction endonuclease sites were then made to amplify the 513 bp product from the start codon of *HKR1* to the internal KpnI site. The PCR product was then cloned in the proper orientation into the 5' KpnI site of pUHK to generate pHKR1ΔC containing the first 3.3 kb of the *HKR1* gene.

Yeast expression vector pKNR4/SMI1 was made by PCR amplification of the *KNR4/SMI1* gene with a 5' primer that containing a flanking Sac1 restriction endonuclease site and a 3' primer containing an in frame STOP codon and a flanking Kpn1 restriction endonuclease site. The PCR product was subsequently cloned in frame

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into Sac1/Kpn1sites of a copper-inducible yeast expression pCUP1 vector containing a Trp1 selectable marker.

The sequences of all the plasmids used in this study were confirmed by automated sequencing.

2.2 Media and Microbiological Techniques

YPD/sorbitol is yeast extract peptone-dextrose media (YPD) supplemented with 1M sorbitol. Calcafluor sensitivity was analyzed on plates containing YPD supplemented with 50 μg/ml fluorescent brightener 28 (Calcafluor White; Sigma Chemical, St. Louis, MO). SDS sensitivity was analyzed on YPD plates supplemented with 0.0025% sodium dodecyl sulphate. Caffeine sensitivity was analyzed on plates containing YPD supplemented with 8mM caffeine. Yeast transformations were performed by the lithium acetate method.

Cell Viability Assays – Yeast cells were picked as individual colonies or transformants and grown at room temperature to an O.D. between 0.8 and 1.0 in Synthetic Dropout media. The cells were then pelleted, washed twice with distilled water, and then diluted in distilled water and plated in tenfold serial dilutions starting at $1x10^5$ cells on supplemented or unsupplemented YPD plates, and grown for 72 hours at a variety of temperatures. The sensitivity of cells to a particular reagent was determined by lack of growth.

Calcafluor White Staining– Microscopic examination of the bud site was done by staining with Calcafluor White. Mid log phase wild type and *cdc34-2* cells were first grown with rotation at 30°C then switched to 37°C for six hours until most of the *cdc34-2* cells exhibited an elongated bud. The cells were then fixed in 3.7% formaldehyde at

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37°C, washed with water, resuspended in 50 mM Tris pH 7.5, containing 20 μg/ml Calcafluor White, and examined by light microscopy under UV light.

GFP localization– Microscopic examination of GFP-tagged Ubs1 was done by confocal microscopy under previously described conditions (Hellmuth et al., 1998).

Two-Hybrid Screen - The yeast two-hybrid screen for Ubs1 interacting proteins was done using the method of James *et al.* (1996). Briefly, PJ69-4a cells carrying pGBTUBS1 were transformed with a mixture of equal concentrations of the 3-reading frame pGAD-C1, p-GADC2, and pGADC3 libraries (generously provided by Phillip James, University of Wisconsin). Transformants were initially grown for two weeks on synthetic dropout medium (SD) lacking Leu, Trp, and His. After sufficient growth was observed, the transformants were replica plated onto Synthetic Dropout (SD) medium lacking Leu, Trp, and Ade. Colonies that were observed to be Ade^+ were retested for growth on SD lacking Leu, Trp, and His, and then screened for β-galactosidase activity using both filter assay and a liquid culture assay. $1X10^5$ colonies were screened in total.

3. Results

3.1 *UBS1* associates with *APG12* and *HKR1* in a two-hybrid screen

Previously, we reported the discovery of *UBS1*, a novel gene with slight similarity to *CDC34* whose overexpression partially suppressed the temperature sensitivity defects of the Cdc34ts mutant allele *cdc34-2* (Pendergast et al., 1996). In that study, High copy (2μ) overexpression of Ubs1 from a copper-inducible CUP promoter allowed for partial growth of YL10 cells (*cdc34-2*) at 37°C and a notable suppression of the hyperpolarized growth phenotype associated with *CDC34* ts mutants grown for prolonged periods at restrictive temperatures. Curiously, *UBS1* was found also to function in an allele-specific manner, allowing for the temperature-sensitivity suppression of only Yl10 cells and not

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other cells expressing different Cdc34 mutant alleles though the significance of this observation is not clear.

To further study the role of *UBS1* in the positive regulation of Cdc34 function, we conducted a yeast two-hybrid screen with *UBS1* as bait to characterize its intracellular binding-partners. Of $1X10^5$ colonies screened, we identified two gene products that specifically associated with Ubs1: the ubiquitin analogue Apg12, and the essential β-1,3–glucan synthesis and bud site selection protein Hkr1.

Apg12 is a component of the yeast autophagy response. Autophagy is a proteolytic system separate from proteasomal degradation, which is induced during periods of stress such as of starvation and radiation damage. It results in engulfment and digestion of cytoplasm and internal cellular organelles (reviewed by Hochstrasser, 2000). *APG12* was one of several genes pulled out of a genetic screen for autophagy-defective yeast mutants (Mizushima et al., 1998). It was subsequently found to be a component of a protein conjugation system highly similar to the ubiquitin proteolytic system. In an enzymatic reaction highly similar to ubiquitination, the 21 kD Apg12 serves as a protein modifier and becomes covalently attached to the lysine residue of a substrate protein (Apg5) via a C-terminal glycine residue in a manner analogous to ubiquitin. The conjugation of Apg12 is accomplished by the action of the yeast E1 homologue Apg7, which was also cloned and characterized in the same study. We observed a strong two-hybrid interaction between *UBS1* and amino acids 1-180 of the Apg12 protein. The final 6 amino acids containing the extreme carboxy-terminal glycine residue, required for its covalent conjugation, were not found in the GAL4 fusion sequence, and subsequent re-testing of the 2-hybrid interaction with a fully intact *APG12* gene was also positive. This strongly suggests that Ubs1 is not a substrate for Apg12, but rather that the association is non-covalent (Figure 2-1).

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HKR1 was one of several genes isolated in a genetic screen for factors which provide resistance to the effects of the specific β–1,3 –glucan synthesis inhibitor HM-1 killer toxin (Kasahara et al., 1994). It was found to encode a 189 kD type I membrane protein containing a hydrophobic amino terminal, a central serine-rich repeat region, and a C-terminal Ca^{2+}-binding EF-hand motif (Figure 2-2). Clues to the function of *HKR1* came from genetic studies done by the same group (Yabe et al., 1996). Overexpression of *HKR1* resulted in upregulated β-1,3-glucan deposition in the cell wall, suggesting that *HKR1* might function in the regulation of β-glucan synthesis. While *HKR1* is an essential gene, truncation of the entire C-terminal half of the protein results in no obvious growth defects or sensitivity to HM-1 toxin. However, yeast cell strains expressing only a C-terminal truncation of *HKR1* (*HKR1ΔC*) were found to exhibit an atypical bud scar distribution, suggesting a further role for *HKR1* in bud site selection (Figure 2-2). Interestingly, *MSB2*, a homologue of *HKR1*, has been implicated in cell cycle regulation, suppressing budding defects of Cdc24 mutants.

The serine-rich repeat region of Hkr1, amino acids 453-788, consists of 12 repeats of a 28 amino acid motif (SAPVAVSSTYTSSPSAPAAISSTYTSSP). Experiments involving the insertion disruption of the *HKR1* gene at various restriction endonuclease sites demonstrate that the region of *HKR1* essential for the viability of yeast cells is located near or within this repeat region (Yabe et al., 1996). We found that *UBS1* binds to a region of *HKR1* that includes only the serine-rich repeat domain with minimal flanking sequence (amino acids 310-803, Figure 2-2).

Having identified two different Ubs1-binding proteins, we then proceeded to examine if either of them had a role in Cdc34 function. We observed that knocking out *APG12* in *cdc34-2* cells did not result in synthetic growth defects. Furthermore, we found that plasmid overexpression (2μ) of *APG12* under the control of a CUP promoter did not suppress *cdc34-2* temperature sensitivity defects. While these results in no way

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exclude the possibility that *UBS1* and *APG12* form a functional complex, and that *APG12* is important for Cdc34 function, we did not proceed with an examination of this phenomenon even though the result provides a tantalizing association between two known protein degradation systems.

We attempted to confirm biochemically by co-immunoprecipitation studies the association between Ubs1 and Hkr1. Despite repeated efforts, however, we were unable to tag HKR1 *in vivo*, and as such a biochemical confirmation of this intriguing association was never obtained. Due to the unexpected nature of the two-hybrid interaction, we decided to investigate if the association of Ubs1 and Hkr1 was relevant to Cdc34 function, we proceeded to test if Cdc34 mutant cells have observable defects in cell integrity. We rationalized that if Ubs1 promotes β-1,3-glucan synthesis through its association with Hkr1, then it may suppress *cdc34* ts mutants by functioning in a similar manner. Yeast mutants with impaired cell integrity exhibit well characterized lysis defects when exposed to environmental circumstances or pharmacological agents that damage the cell wall or interfere with its synthesis. We therefore tested *cdc34-2* cells to see if they exhibited phenotypes consistent with this hypothesis.

3.2 Cdc34 mutants exhibit allele-specific cell wall synthesis defects

We analyzed this phenomenon by testing the sensitivity of *cdc34-2* cells to sodium dodecyl sulfate (SDS), Calcafluor White, and caffeine. A low concentration of the anionic detergent SDS is selectively toxic for yeast cells with compromised cell wall synthesis because it solubilizes the unprotected plasma membrane and results in cell lysis (Shimizu et al., 1994). Analogously, Calcafluor White is also toxic to yeast cells with cell wall synthesis defects because of its interaction with chitin and its interference with cell wall assembly (Roncero et al., 1988). Furthermore, sensitivity to caffeine is a phenotype often associated with defects within the *PKC1* cell integrity pathway. The

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cdc34-2 mutant strain exhibited extreme sensitivity to 0.0025% SDS as compared to an isogenic wild type strain under identical growth conditions (Figure 2-3). It also demonstrated a clear sensitivity to 50 μg/ml Calcafluor White. In addition, we observed that *cdc34-2* cells were also sensitive to caffeine at a concentrations up to 8mM.

Mutant alleles of the Cdc34/SCF complex are unusual in that they exhibit cell cycle defects when grown at restrictive temperatures, and as a result of these cell cycle abnormalities, also demonstrate severe morphological defects. These morphological defects are thought to stem from an inability to degrade cell cycle regulatory proteins in these mutants at restrictive growth temperatures, resulting in the formation of elongated or hyperpolarized buds and multiple buds emerging from the same budsite (Goebl et al., 1988). In the late G1 phase of the cell cycle, concomitant with bud emergence, cells enter into an apical or polarized growth phase where the structural and secretory apparatus of the cell is focussed specifically on the site of budding. *cdc34-2* cells grown at restrictive temperatures are trapped in a state of constitutive apical growth, hence, the elongated bud phenotype. To examine if the cell integrity defects we observed with the *cdc34-2* mutant were due to the well characterized morphological defects of this same mutant at restrictive growth temperatures, we first tested to see of the cell integrity defect of *cdc34-2* cells was dependent on the growth temperature. When grown at 25°C, *cdc34-2* cells are considered to be morphologically normal and are capable of normal cell polarity induction during processes such as mating (Bidlingmair and Snyder, 2002). We observed that even under fully permissive growth conditions, *cdc34-2* cells were sensitive to 0.0025% SDS suggesting that the cell integrity defects are independent of the hyperpolarized growth defects (Figure 2-4). We also examined the morphology of our *cdc34-2* mutants grown at 30°C by light microscopy and did not observe any significant morphological defects at that growth temperature.

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Often when yeast cells experience difficulty in the regulation of their integrity, a compensatory pathway becomes activated to shore up the cell wall and ensure survival of the cell under conditions of stress (Lagorce et al., 2002; Ram et al., 1998). Activation of this pathway results in an observable increase in chitin deposition at the mother-bud neck during budding. We stained *cdc34-2* cells and an isogenic wild-type strain with Calcafluor White, a UV fluorescent chitin-binding stain, to visualize the chitin rich bud scars that mark the site of previous budding. When grown at 37°C for six hours, *cdc34-2* cells exhibited elongated and multibudded morphology classically associated with defects in the Cdc34/SCF pathway (Figure 2-5). They also demonstrated highly irregular and abnormally enhanced chitin staining at the site of budding, as well as diffuse staining at secondary sites within the presumptive bud. Wild type cells, conversely, exhibited a discretely localized pattern of chitin staining typical of normal yeast cells.

Finally, we examined the effects of these pharmacologic agents on a different mutant allele of Cdc34 to determine if the observed cell integrity defects were allele-specific. We chose to examine a yeast cell strain expressing the *cdc34-3* mutant allele, which bears a different mutational load than *cdc34-2* (extensively reviewed in Chapter V of this thesis). *cdc34-3* cells, while also inviable at 37°C, did not exhibit a noticeable SDS sensitivity as compared to either *cdc34-2* cells or an isogenic wild type strain (Figure 2-6).

3.3 Osmotic suppression rescues the G1/S cell cycle arrest of *cdc34-2* cells

Having observed allele-specific cell integrity defects with the *cdc43-2* mutant allele, we then subjected these same cells to a sorbitol suppression assay to examine what effect, if any, stabilization of the yeast cell wall would have on this mutant. Sorbitol is a sugar alcohol that at high extracellular concentrations suppresses cell lysis defects of yeast cell integrity mutants by causing a decrease in intracellular turgor pressure (Cid et

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al., 1995). While stabilization of the cell wall should theoretically prevent lysis of the *cdc34-2* mutant at high temperatures, one would expect that it would have no effect on the cell cycle arrest phenotype of these cells at restrictive growth temperatures, as this defect is caused by the cells inability to degrade the Sic1 cyclin kinase inhibitor and hence progress into S-phase. We were surprised to observe, however, that osmotic stabilization of *cdc34-2* mutants with 1M sorbitol suppressed the G1/S cell cycle defect of the *cdc34-2* mutant allele, allowing for cell growth to proceed at the restrictive growth temperature (Figure 2-7). This result was entirely unexpected, given the current understanding of the cell cycle defects of Cdc34 mutants and seems to indicate a relationship between cell integrity and cell cycle progression at restrictive growth temperatures. From this experiment we can conclude that osmotic stabilization of the yeast cell wall is a suppressor of the *cdc34-2* cell cycle defect.

3.4 SCF mutant alleles also exhibit cell wall synthesis defects and cell cycle arrest suppression by osmotic stabilization

Cdc34 functions in association with an E3 complex known as the SCF. In yeast, the SCF complex consists of at least four proteins: Skp1, Hrt1, Cdc53, and a fourth protein that contains a structural motif termed an F-box (Bai et al.,1996). Cdc53 is a multi-domain protein that forms the scaffolding of the SCF complex and mediates the interactions among all the subunits (Zheng et al., 2002). Skp1 associates very tightly with the F-box motif, and functions by recruiting into the SCF complex different F-box containing proteins. F-box proteins, in turn, recognize ubiquitination targets, while Skp1 links these F-box/target complexes to the ubiquitination machinery (Zheng,et al., 2002). Cdc53, Skp1, Hrt1, and the F-box protein Cdc4 represent the prototypical SCF, involved

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in the degradation of a variety of proteins including the Clb5,6/Cdc28 inhibitor Sic1 (reviewed by Desalle and Pagano., 2001).

Having observed cell integrity defects associated with the *cdc34-2* mutant allele, we speculated that SCF mutants might exhibit similar defects. We therefore tested temperature-sensitive mutants of the SCF components Cdc53 (*cdc53-1*) and Cdc4 (*cdc4-3*) for sensitivity to SDS, Calcafluor White and caffeine. Both *cdc53-1* cells and *cdc4-3* cells exhibited at least some of the defects associated with impaired cell integrity, Interestingly the *cdc53-1* phenotype was very strong whereas the *cdc4-3* phenotype was the weakest of the three alleles tested (Figure 2-8). We observed that both mutant strains exhibited sensitivity to the anionic detergent SDS (0.0025%). While sensitive to Calcafluor White, the *cdc4-3* cells were not, however, sensitive to caffeine at concentrations up to 8mM, whereas *cdc53-1* cells were found to be exquisitely sensitive to both agents. As expected, an isogenic wild type strain exhibited no obvious toxicity to any of the reagents tested. From these experiments it is clear that the SCF mutant alleles *cdc4-3* and *cdc53-1* are both sensitive to exposure to low concentrations of cell integrity antagonists.

As with the *cdc34-2* allele, we confirmed that the cell growth conditions of the *cdc4-3* and *cdc53-1* cells were not inducing hyperpolarized growth phenotypes (Figure 2-9) indicating that these defects were not a result of aberrant growth morphologies associated with these mutants at high temperatures. Furthermore, we also tested these same mutants for osmotic suppression of their cell cycle defects (Figure 2-10). Similar to our results with the *cdc34-2* allele, we observed a strong suppression of the *cdc4-3* and *cdc53-1* G1/S cell cycle arrest upon osmotic stabilization of the cell wall with 1M sorbitol.

3.5 *UBS1* is a suppressor of the Cdc34/SCF cell integrity defect

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As previously indicated, we observed that the partial temperature-sensitivity suppression conferred on *cdc34-2* cells by *UBS1* overexpression was allele-specific. Of multiple Cdc34 mutant alleles tested, only *cdc34-2* was rescued by *UBS1* (Prendergast et al., 1995). In this study we have also observed a similar allele-specificity phenomenon with respect to the Cdc34 cell integrity defect. These data combined with the two-hybrid interaction between *UBS1* and *HKR1* suggested to us that *UBS1* might play a role in the positive regulation of the *cdc34-2* cell integrity defect.

We tested this hypothesis by examining if plasmid overexpression of *UBS1* could suppress the cell integrity defects associated with the Cdc34/SCF mutant strains. We chose to assay the SDS-sensitivity as it was the most severe phenotype exhibited by all three mutant strains (Figure 2-11). We observed, as before, that *UBS1* overexpression partially suppressed the temperature-sensitivity defect associated with the *cdc34-2* strain, though its overexpression had no discernable effect on the temperature sensitivity of either *cdc4-3* cells or *cdc53-1* cells. More interestingly, we observed that overexpression of *UBS1* fully suppressed the cell integrity defects associated with the *cdc34-2* cells and the *cdc4-3* cells while having no effect on the cell integrity defects of the *cdc53-1* cells. These data indicated to us that *UBS1* indeed functions in the positive regulation of cell integrity.

3.6 Ubs1 localizes to the nucleus and to the site of polarized bud growth

Due to our inability to demonstrate a biochemical association between Ubs1 and its budsite-associated binding partner Hkr1, we felt that localization of the Ubs1 might provide some level of validation of our hypothesis concerning its role in cell integrity. We constructed a C-terminal GFP-fusion construct of *UBS1* under the control of a methionine-repressible promoter, and examined the localization of Ubs1-GFP in wild-type cells and *cdc34-2* mutants (Figure 2-12). We observed a strong predominantly

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nuclear signal of Ubs1-GFP in wild-type cells grown at 37°C by confocal microscopy consistent with the observation made by another research group (Baumer et al., 2000). Interestingly, in *cdc34-2* mutants growing at the restrictive growth temperature and exhibiting hyperpolarized budding defects, we found that Ubs1-GFP localized predominantly to the site of polarized bud growth (i.e. the tip of the growing bud, and at various punctate sites within the yeast cell cytoplasm). To confirm the biological activity of the Ubs1-GFP construct, we tested it for its ability to partially suppress the temperature-sensitive growth defect of the *cdc34-2* cell strain and found it to be biologically active. From this experiment, we therefore conclude that Ubs1 localizes both to the nucleus and to the site of polarized cell growth in yeast cells.

3.7 *HKR1* and *KNR4* suppress the cell integrity defects of *cdc34-2* mutants

Having confirmed the cell membrane association of Ubs1 by GFP-localization, we next wanted to test if *HKR1* had any influence on the cell integrity defects of Cdc34/SCF mutants. As *HKR1* was found previously to be unstable in expression plasmids (Dr. Tomio Yabe, personal communication), we generated a functional derivative of *HKR1* called *HKR1ΔC* which was previously found to be sufficient for performing the essential function of *HKR1* in yeast cells (Figure 2-2; Yabe et al., 1996). We tested isogenic wild type cells, and *cdc34-2* cells transformed with either an empty vector or *HKR1ΔC* expressed from a CUP1 promoter, for growth on YPD supplemented with 0.0025% SDS. We observed that the truncated form of *HKR1* was capable of partially suppressing the SDS toxicity phenotype exhibited by *cdc34-2* cells (Figure 2-13). In a similar manner to *UBS1*, *HKR1ΔC* was also capable of suppressing the SDS sensitivity of *cdc4-3* cells but not *cdc53-1* cells.

As previously mentioned, *HKR1* was originally identified as a gene whose expression confers upon yeast cells resistance to HM-1 killer toxin (Kasahara et al.,

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1994). HM-1 killer toxin is a small polypeptide toxin that has severe cytocidal effects on yeast by acting on the budding region of proliferating yeast cells, resulting in pore formation, leakage of cell material, and cell death (Komiyama et al., 1994). Apart from *HKR1*, a variety of other genes have been reported that confer resistance to this toxin including the *KNR4/SMI* gene (Kimura et al., 1997; Hong et al., 1994b). *KNR4* encodes for a non-essential 57 kD gene involved in β-1,3-glucan synthesis thought to be involved in the same biochemical pathway as *HKR1* (Hong et al., 1994b; Yabe et al., 1996). Deletion of *KNR4*, while not lethal, results in decreased β-1,3-glucan synthesis and increased sensitivity to Calcafluor White and SDS. Knr4 has since been shown to be a component of the yeast cell integrity signaling pathway, likely modulating the effects of the Slt2 kinase on cell integrity transcription (Matin-Yken et al., 2002a; 2002b).

Due to the genetic relationship between *KNR4* and *HKR1*, we surmised that *KNR4* overexpression might also have suppressive effects on Cdc34/SCF cell integrity mutants. We, therefore, tested *cdc34-2*, *cdc53-1* and *cdc4-3* mutants transformed with either an empty vector or a yeast expression vector driving *KNR4* expression form a CUP1 plasmid for their ability to grow on SDS and at their restrictive temperatures (Figure 2-14). As previously observed with *HKR1*, *KNR4* exhibited strong suppressive effects on the *cdc34-2* and *cdc4-3* SDS-sensitivity defects while having no apparent effect on the cell integrity defects associated with the *cdc53-1* allele. Furthermore, overexpression of *KNR4* had no obvious effects on the temperature sensitivities of the mutant strains. Taken together, these data provide strong genetic evidence for positive regulation of cell integrity by the Cdc34/SCF complex, and novel physical and genetic associations among and a cadre of genes involved in β-1,3-glucan synthesis and cell cycle regulation.

4. Discussion

4.1 The Cdc34/SCF positively regulates cell integrity

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Given the critical role of Cdc34/SCF in S-phase entry through the degradation of Sic1 and the variety of substrates recognized by the complex, it is not surprising that further evidence expanding its known roles is constantly accumulating. In this chapter, we have demonstrated a role for Cdc34/SCF complex in the positive regulation of yeast cell wall biosynthesis and have identified allele-specific suppressors of defects in the novel Cdc34/SCF cell integrity pathway.

We found that cells expressing the temperature-sensitive *cdc34-2* allele exhibit several hallmarks of cell wall synthesis defects, including hypersensitivity to the detergent SDS and to the cell wall synthesis inhibitor Calcafluor White. These cells also demonstrated a significant amelioration of their G1/S cell cycle arrest phenotype when grown in the presence of the osmotic stabilizer 1M sorbitol. Interestingly, we also found that the cell integrity defect was allele-specific, as a second temperature-sensitive allele of *CDC34* did not exhibit an SDS cell lysis phenotype. Furthermore, we showed that temperature-sensitive alleles of other SCF components, namely Cdc53 and Cdc4, also exhibit similar cell wall synthesis deficits. With all the mutant strains tested, we found these cell integrity defects were unrelated to hyperpolarized growth morphologies often attributed to these mutant strains, which argues strongly for a specific cell integrity defect associated with these mutant strains. The volume of genetic evidence in this study suggests that the Cdc34/SCF plays a positive role in the maintenance of cell wall integrity.

Examination of our data indicates that the cell cycle and cell integrity defects of Cdc34/SCF mutants, while potentially related, are separable entities, and suppressors of one pathway are not necessarily suppressors of the other. We know that these mutants fail to progress into S-phase due to an inability to degrade Sic1 at the restrictive temperature, accounting for their temperature sensitivity. *UBS1*, however, suppresses both the cell cycle defects and the cell integrity defects of the *cdc34-2* mutant as well as

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the cell integrity defects of the *cdc4-3* mutant strain. Its two-hybrid binding partner, *HKR1*, suppresses only the *cdc34-2/cdc4-3* cell integrity defects. This might indicate that *UBS1* plays multiple suppressive roles in complementing Cdc34/SCF activity, or perhaps it might indicative of the different roles that these molecules play in the pathway that links the cell cycle functions of Cdc34 to its cell integrity functions. This data strongly suggests that the association of Ubs1 and Hkr1 function in a previously uncharacterized cell integrity pathway associated with the Cdc34/SCF complex.

4.2 What is the function of *UBS1*?

There is scant genetic and biochemical information on the *in vivo* role of *UBS1*. Our lab had previously implicated this gene in the positive regulation of Cdc34 function, though no direct interaction between these two proteins was observed by two-hybrid analysis (Prendergast et al., 1996). A very interesting recent paper identifying UBS1 as a suppressor of nuclear transport defects in a Yrb1 mutant strain may provide some clues to its function (Baumer et al., 2000). Yrb1 binds to the small Ras-like GTPase Ran and facilitates dissociation of molecules exported from the nucleus and association of molecules destined for the nucleus with the nuclear transport machinery. This group localized GFP-Ubs1 to the nucleus and to the cytosol, and demonstrated the requirement of nuclear transport for proteolysis of Cdc34/SCF cell cycle targets including Sic1. We also observed nuclear and cytosolic localization of C-terminally tagged Ubs1 in this study, but we also observed a dramatic re-distribution of Ubs1-GFP to the site of polarized cell growth in *cdc34-2* cells grown at the restrictive temperature. This result may indicate that Ubs1 shuttles between the nucleus and cell membrane, and further it may indicate that under conditions of apical growth Ubs1 is predominantly localized to the budsite.

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In this study we demonstrate associations between Ubs1 and the cell surface protein Hkr1, as well Apg12 a protein modifier required for autophagy and cytosol-vacuole transport, by yeast two-hybrid screen. The identification of Apg12 as a Ubs1-binding partner is suggestive of a role in intracellular transport as *APG12* has been implicated as a component of the cytosol-to-vacuole transport machinery (Hochstrasser et al., 1999). It would be interesting to test Ubs1 intracellular transport in the context of both *APG12* deletion and Apg12 localization. One can hypothesize that the non-covalent association between Ubs1 and Apg12 might facilitate its transport throughout the cell. Furthermore, the potential role of *UBS1* the regulation of cellular transport is also supported by a study done in our lab identifying the secretory protein alpha-COP in a synthetic lethal screen with a *UBS1* knockout strain (John Prendergast, unpublished data). Interestingly, alpha-COP was recently identified as a suppressor of β-1,3-glucan synthesis defects in a genetic screen for novel genes regulating the synthesis of the cell wall (Lee et al., 1999). Finally, a recent paper has identified genetic interactions between the cell integrity pathway and the nuclear import machinery (Nanduri and Tartakoff, 2001). Components of the cell integrity pathway, including *PKC1*, were found to influence nuclear transport processes in yeast. It is possible that *UBS1* may somehow be involved in this pathway as well.

4.3 What are the functions of *HKR1* and *KNR4*?

The function of *HKR1* is not known at present. *In vitro* studies suggest that *HKR1* does not enzymatically participate in the synthesis of β-1,3-glucan, but rather regulates the process in some manner (Kasahara et al., 1994). Its role in the maintenance of cell integrity has been further demonstrated by the observation that *HKR1* transcription was identified as one of six genes whose transcription is rapidly induced following spheroplasting, indicating a potential role for it in the early stages of cell wall synthesis.

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Interestingly this early-stage transcriptional activation of cell wall synthesis components was not sufficient to initiate the replacement of the cell wall in liquid medium (Braley et al., 1999).

KNR4 is also a gene whose precise function is unknown and many aspects of its intracellular role have been controversial. It was originally named *SMI1* and identified in a biochemical screen for nuclear-matrix associated proteins (Fishel et al., 1993). In that study, its disruption was found to produce a temperature-sensitive growth arrest just prior to the onset of S-phase, perhaps suggesting a role for it in the regulation of the cell cycle. It has a hydrophilic profile and contains several PEST-sequences usually found on rapidly degraded proteins (reviewed in Chapter I). It was later re-named *KNR4* by a second group that identified it in a genetic screen for genes involved in resistance to HM-1 killer toxin (Hong et al., 1994a; Hong et al., 1994b). Functional characterization of *KNR4* suggested a role in cell wall synthesis as its deletion resulted in a considerable reduction in both β-1,3-glucan synthase activity and β-1,3-glucan deposition in the cell wall. A third group, found *KNR4* to be a suppressor of Calcafluor White-hypersensitive mutants, and using C-terminal GFP-tagged Knr4, localized it to the presumptive bud sites of unbudded cells and the incipient bud site during bud emergence (Martin et al., 1999). It was observed that *KNR4* overexpression inhibits the transcription of chitin synthase genes in both wild-type and Calcafluor White hypersensitive mutants. This same group has recently identified *KNR4* as a component of the cell integrity signaling pathway, modulating the activity of the Slt2 kinase, an observation which will be discussed in greater detail in the ensuing chapters of this thesis (Martin-Yken et al., 2002a; 2002b). Our results are clearly consistent with the role of *KNR4* in the regulation of cell wall synthesis and further support the speculation that *HKR1* and *KNR4* function within the same physiological pathway.

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This study raises several significant issues with respect to protein ubiquitination and cell integrity. As the Cdc34/SCF complex is involved in targeting proteins for degradation by the 26S proteasome, one can speculate that it plays a regulatory role in the cell integrity pathway by degrading a negative regulator of cell integrity signaling of β-1,3-glucan synthesis. It is interesting to note that the *cdc4-3* mutant strain was least demonstrative of cell integrity defects, while *cdc34-2* and *cdc53-1* cells appeared to be highly defective in this pathway. This might suggest a role for a different F-box protein in the cell integrity function of the Cdc34/SCF complex. The identification of Gic2, which localizes both to the cytoskeleton and the presumptive bud site, as a Cdc34/SCF^{Grr1} target suggests the possibility of multiple targets for this complex in the cytosol and at the at the cell membrane (Jacquenaud et al., 1998). A more detailed analysis of this pathway will undoubtedly illuminate on some of these important questions.

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Strain	Genotype	Reference
YL10	*MAT*a *ura3-52 trp1*D*63 leu2*D*1 his3*D *cdc34-2*	M. Goebl
YES71	*MAT*a *ura3-52 trp1*D*63 leu2*D*1 his3*D *cdc34-2::HIS3*	Ptak *et al.*, 1994
CWY231	*MATa ura3Dns trp1 leu2-3,112 his2 ade1 bar1*	Richardson *et al.*, 1989
DSY795	*MATa ura3Dns trp1 leu2-3,112 his2 ade1 bar1 cdc34-3*	This study
DSY1105	*MATa ura3Dns trp1 leu2-3,112 his2 ade1 bar1 cdc53-1*	This study
DSY841	*MATa ura3Dns trp1 leu2-3,112 his2 ade1 bar1 cdc4-3*	This study

Table 2-1. Genotypes of yeast strains used in this study

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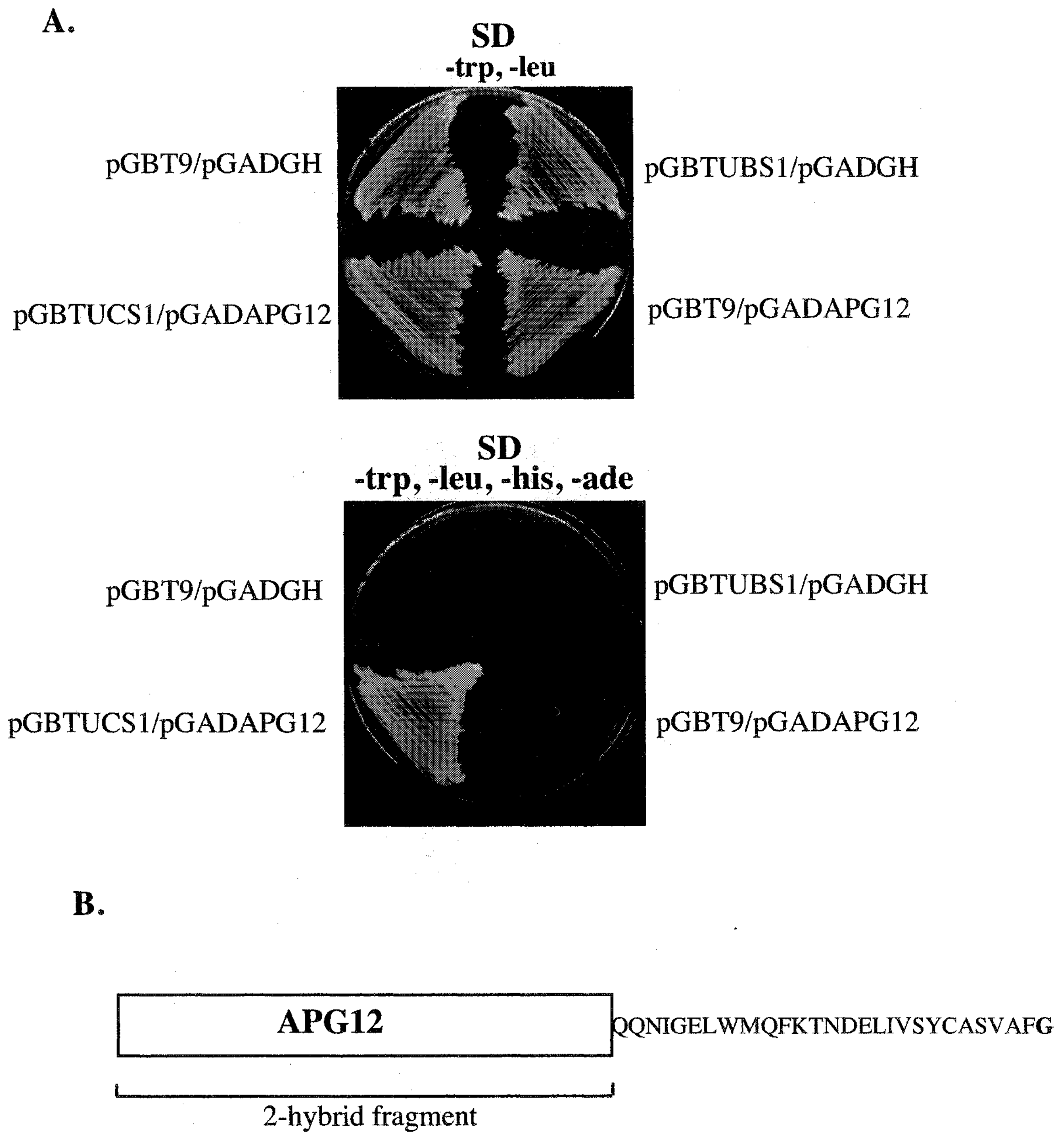

Figure 2-1. Ubs1 interacts with Apg12 by yeast two-hybrid screen. Ubs1 interacts with the autophagy protein Apg12 in a two hybrid screen (Panel A). The area of interaction was found to include the entire open reading frame with the exception of the final 28 amino acids (Panel B).

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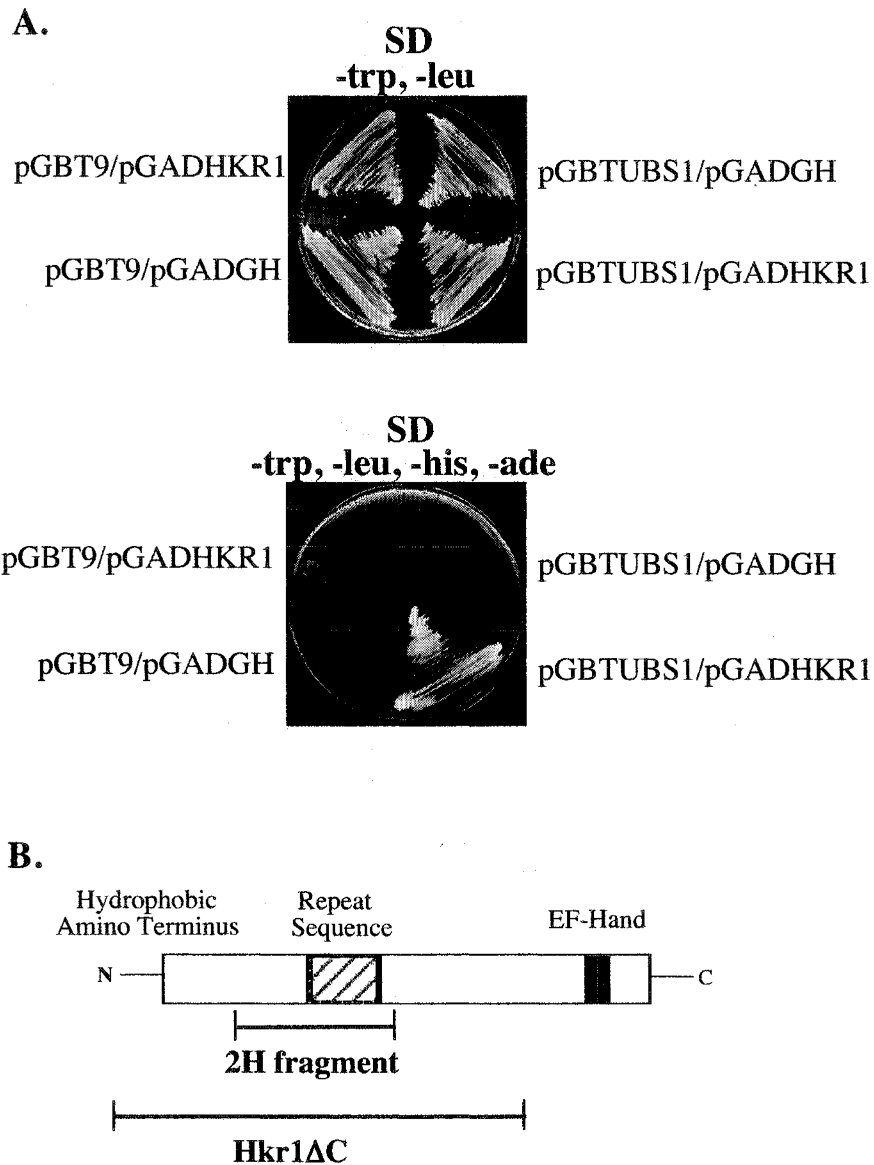

Figure 2-2. Ubs1 interacts with the β-glucan synthesis protein Hkr1 by yeast two-hybrid screen.Two-hybrid analysis of intracellular Ubs1-binding partners reveals an interaction between Ubs1 and the repeat domain of Hkr1 (Panel A). Hkr1 is a 189 kD multi-domain protein involved in β-1,3 glucan synthesis and budsite selection (Panel B).

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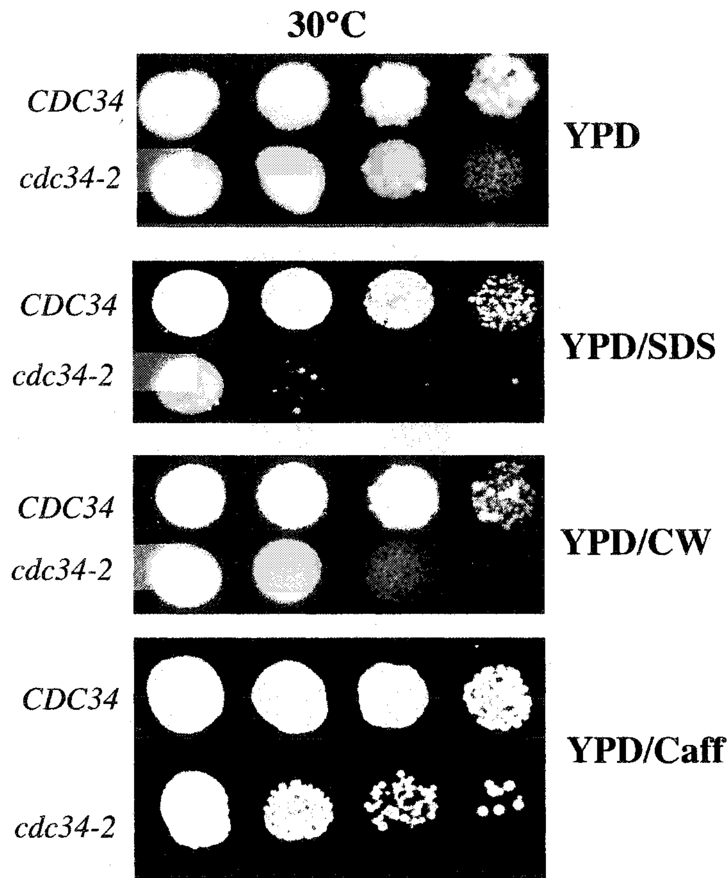

Figure 2-3. *cdc34-2* cells exhibit sensitivity to cell integrity antagonists. Under permissive growth conditions, *cdc34-2* cells exhibit severe growth sensitivity to 0.0025% SDS and Calcafluor White (50 μg/ml), and caffeine (8 mM) relative to an isogenic wild type strain.

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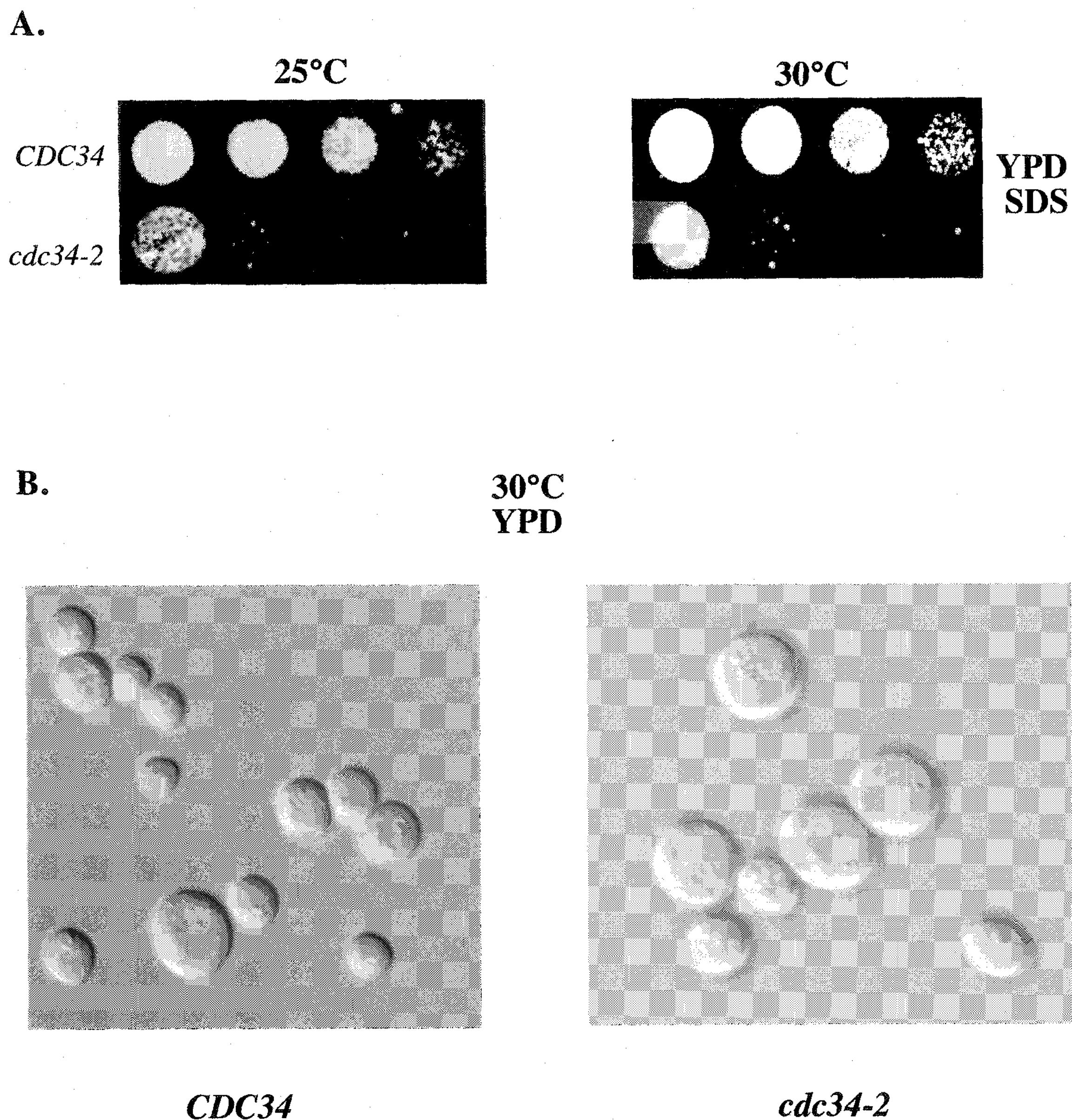

Figure 2-4. The lysis defect of *cdc34-2* cells occurs at permissive growth temperatures and is independent of hyperpolarized growth morphology. *cdc34-2* cells plated in 10-fold serial dilutions and incubated at 25°C and 30°C exhibit sensitivity to SDS relative to an isogenic wild type strain (Panel A). *cdc34-2* cells do not exhibit hyperpolarized growth defects were grown at 30°C for 6 hours in rich medium (YPD)(Panel B; 1000X magnification under oil).

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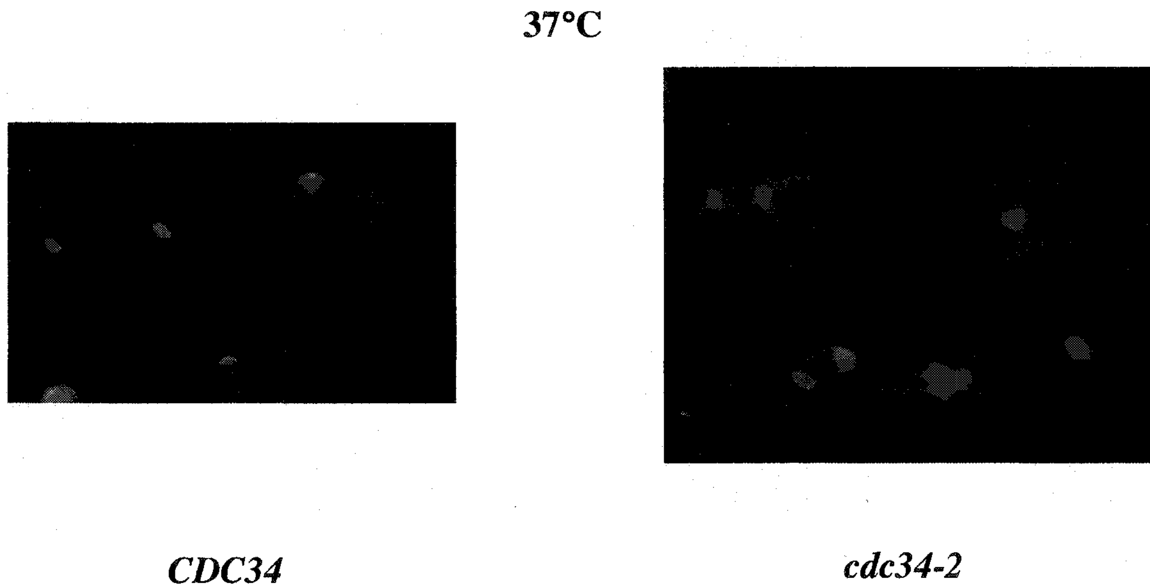

Figure 2-5. *cdc34-2* mutants exhibit enhanced chitin deposition at restrictive growth temperatures. Calcafluor white staining of *CDC34* and *cdc34-2* cells grown for 6 hours at 37°C. The *cdc34-2* mutant strain exhibits a marked accumulation of chitin at the mother-bud neck and within the growing bud as compared to an isogenic wild type control strain (1000X magnified image in UV light under oil).

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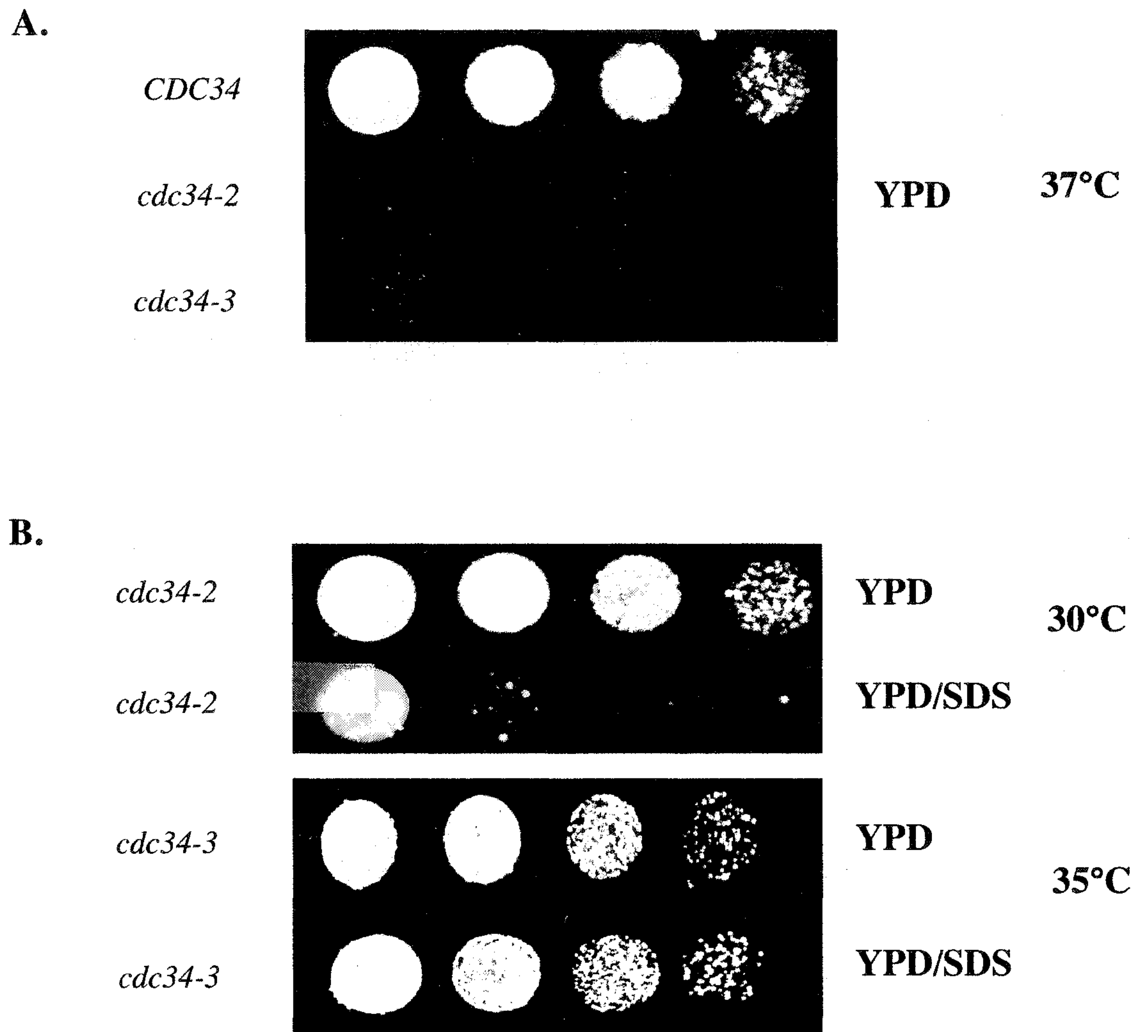

Figure 2-6. Cdc34 cell integrity sensitivity is allele specific.
Both *cdc34-2* and *cdc34-3* cells exhibit temperature sensitivity growth defects (Panel A). Only *cdc34-2* and not *cdc34-3* cells are sensitive to 0.0025% SDS under permissive growth conditions (Panel B).

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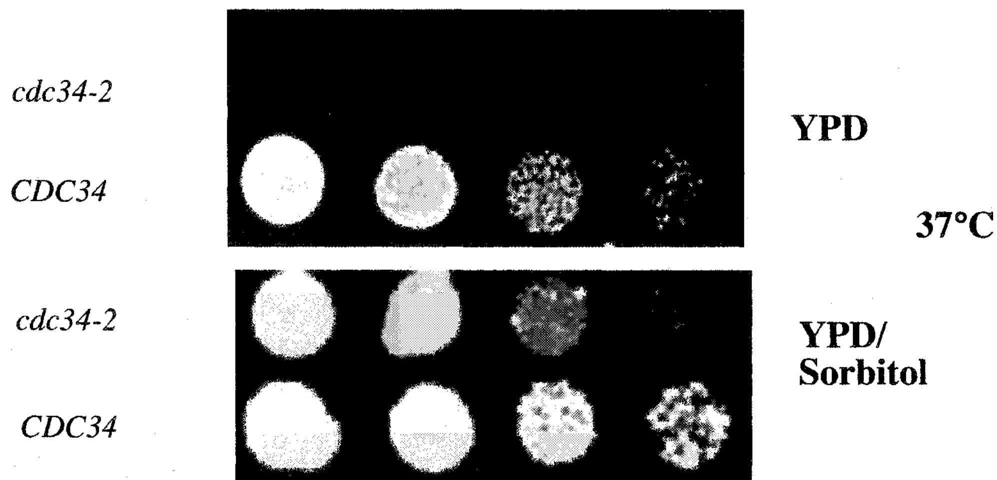

Figure 2-7. Osmotic stabilization of the cell wall suppresses the cell cycle arrest of the *cdc34-2* mutant. *cdc34-2* mutant cells and an isogenic wild type strain (CDC34) were plated in 10-fold serial dilutions on rich medium (YPD) or rich medium supplemented with 1M sorbitol(YPD/Sorbitol) and grown for 3 days at the indicated temperature.

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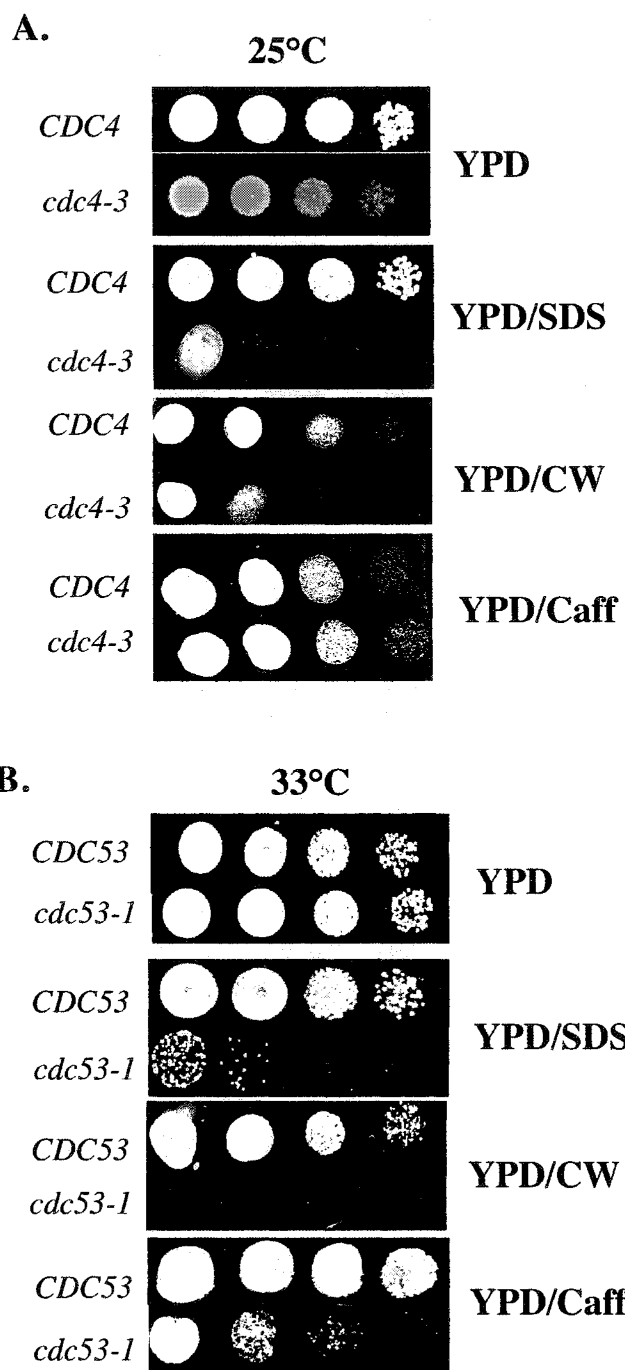

Figure 2-8. SCF mutant strains exhibit sensitivity to cell integrity antagonists. The *cdc4-3* mutant strain exhibits growth sensitivity to 0.0025% SDS and 50 μg/ml Calcafluor white (Panel A). The *cdc53-1* mutant strain also exhibits sensitivity to 8mM caffeine (Panel B).

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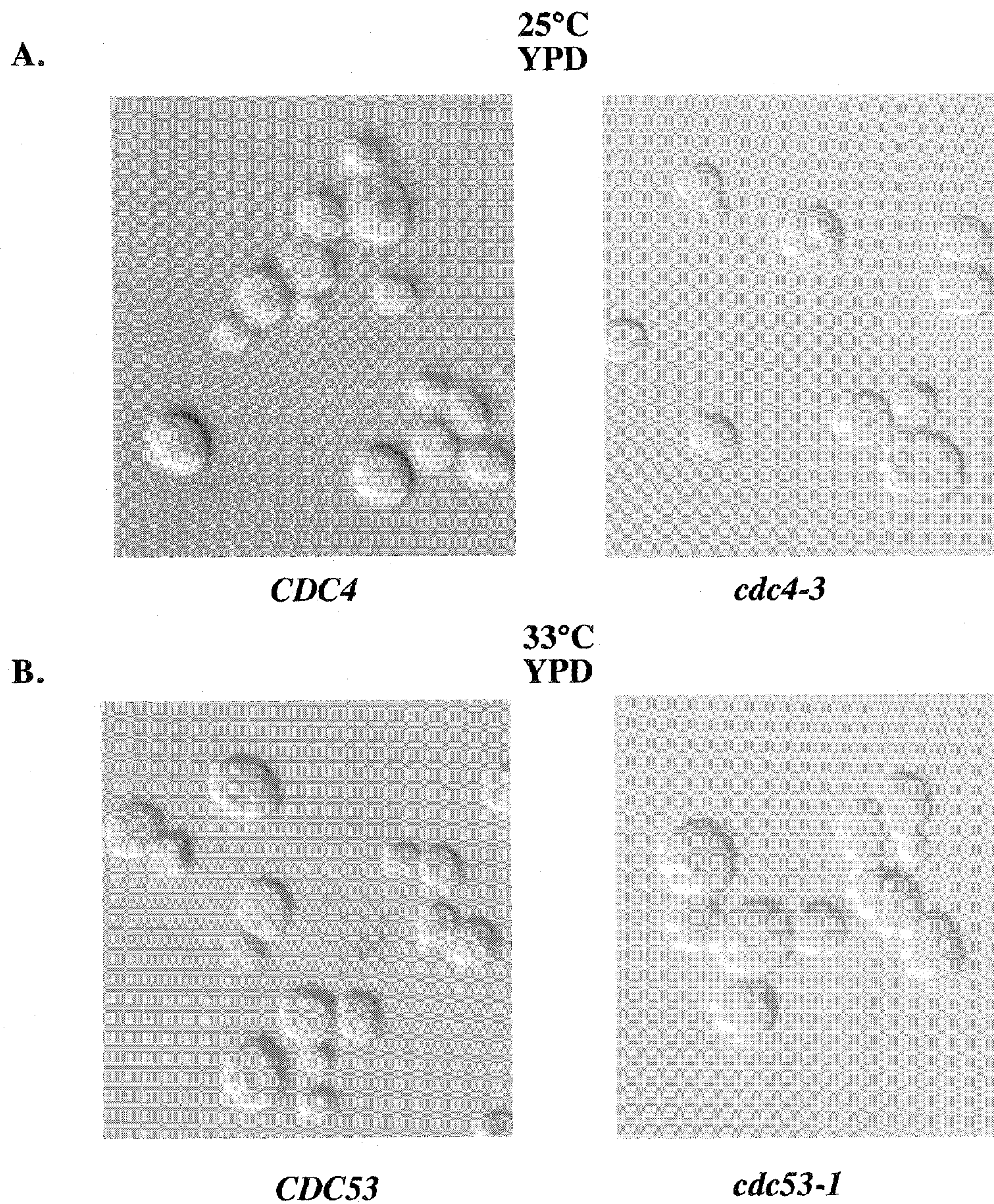

Figure 2-9. *cdc4-3* mutants grown at 25°C and *cdc53-1* mutants grown at 33°C for 6 hours do not exhibit morphologic defects in rich medium. *cdc4-3* (Panel A) and *cdc53-1* (Panel B) yeast strains and isogenic wild type strains were grown in YPD for 6 hours at the indicated temperatures and examined microscopically (1000X magnification).

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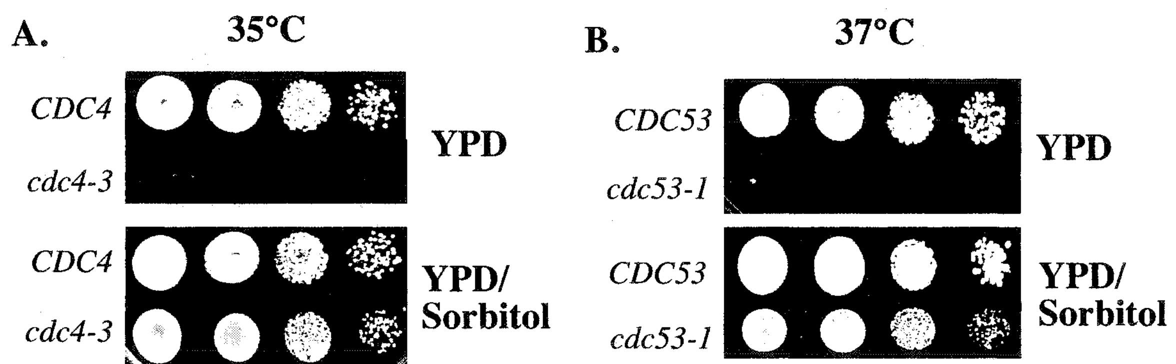

Figure 2-10. Osmotic stabilization of the cell wall suppresses the cell cycle arrest of the *cdc4-3* and *cdc53-1* mutants. *cdc4-3* mutant cells (Panel A) and *cdc53-1* mutant cells (Panel B) and an isogenic wild type strain were plated in 10-fold serial dilutions on rich medium (YPD) or rich medium supplemented with 1M Sorbitol(YPD/Sorbitol) and grown for 3 days at their restrictive growth temperatures.

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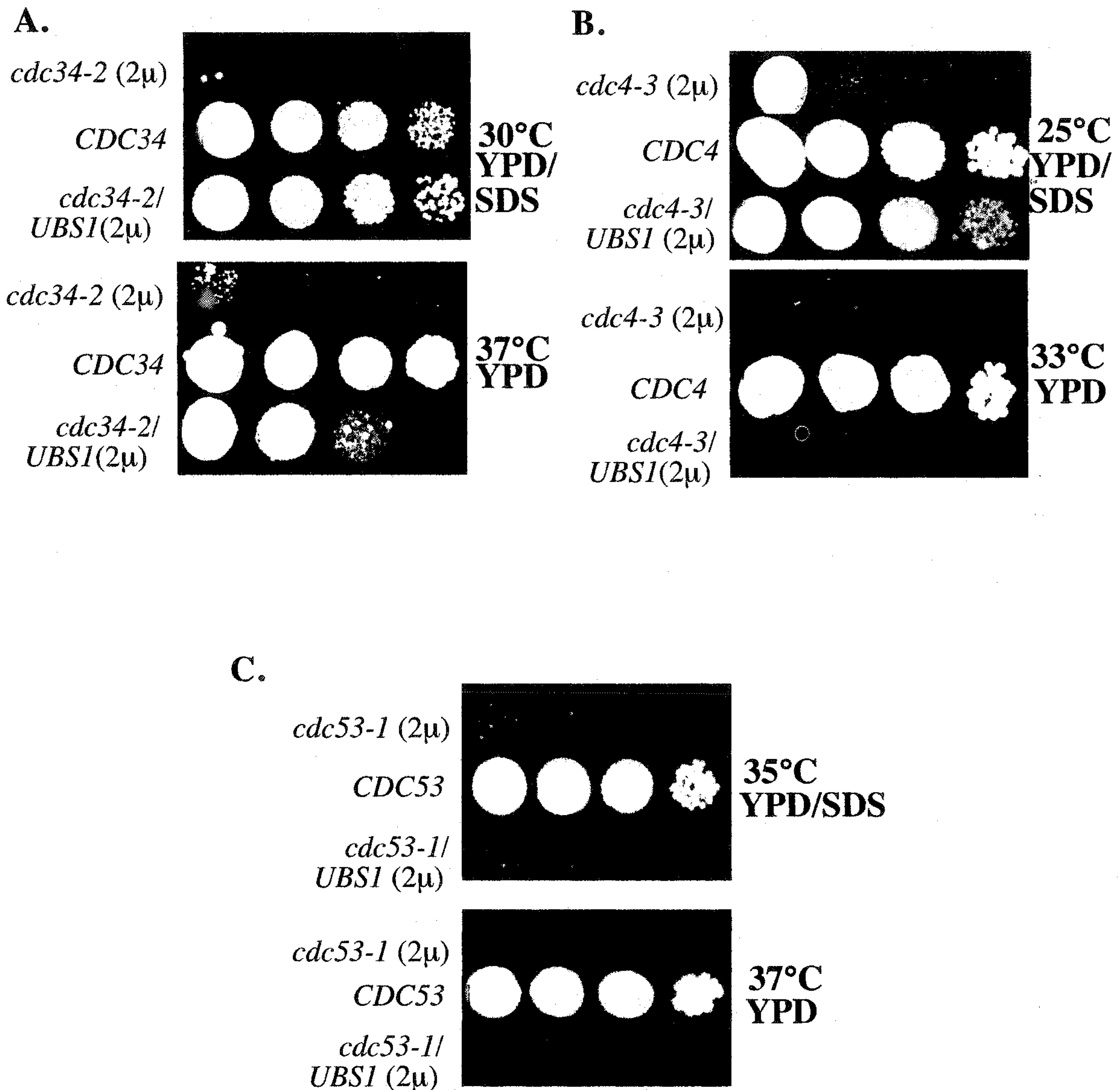

Figure 2-11. *UBS1* suppresses selective Cdc34/SCF cell integrity defects. Plasmid overexpression of *UBS1* partially suppresses the *cdc34-2* temperature-sensitivity defect (Panel A), and fully suppresses the cell lysis defects of *cdc34-2* and *cdc4-3* mutants (Panel A,B) grown in the presence of 0.0025% SDS. Overexpression of *UBS1* had no effect on cell cycle or cell lysis defects of the *cdc53-1* mutant strain (Panel C).

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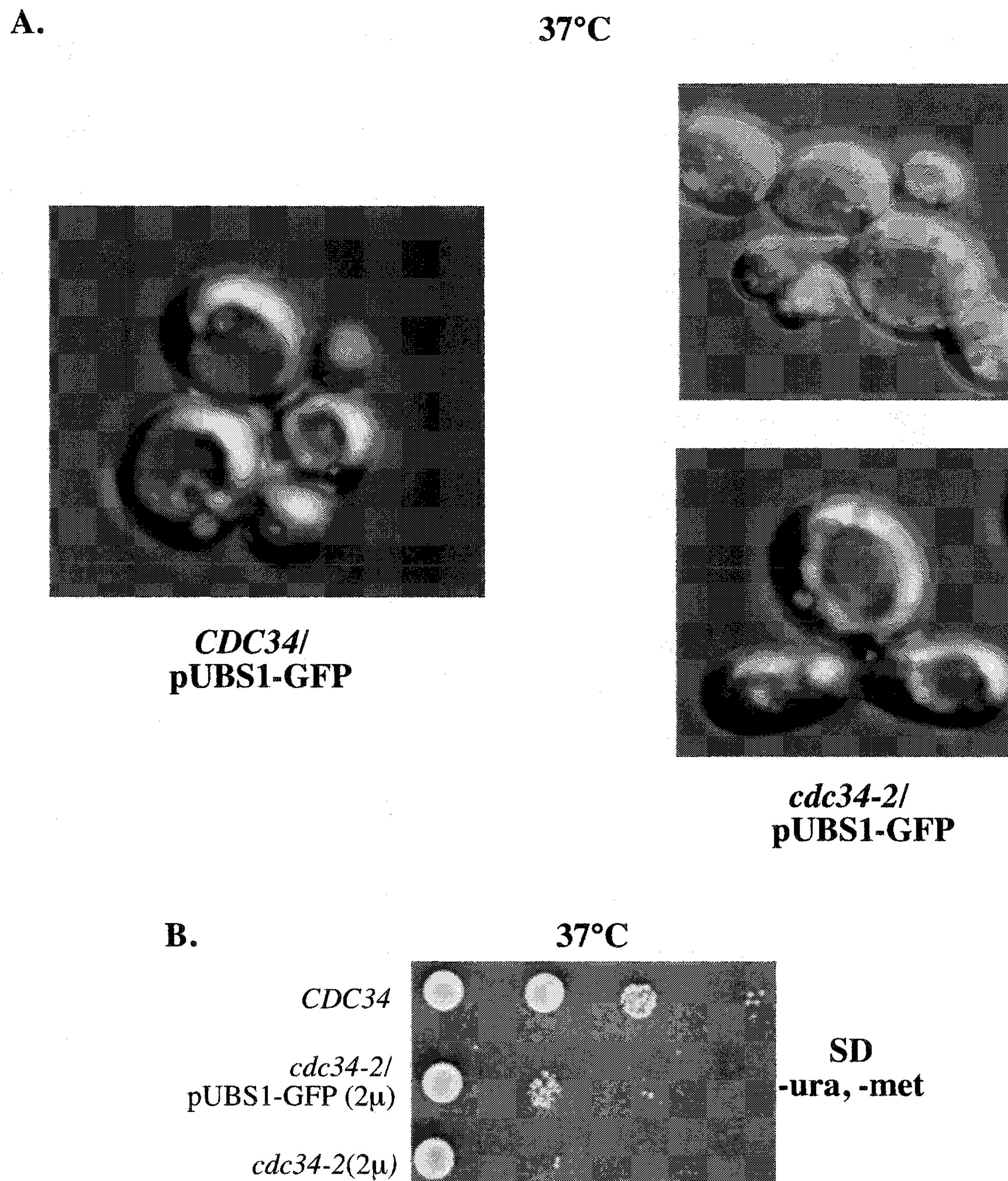

Figure 2-12. Ubs1-GFP localizes both to the nucleus and to the site of polarized growth. *cdc34-2* mutants and an isogenic wild type strain (*CDC34*) were transfected with the pUBS1-GFP construct, and grown at 37°C for 6 hours and analyzed by fluorescence confocal microscopy. Ubs1-GFP localized to the nucleus of wild type cells, and to multiple spots within *cdc34-2* mutants, including tip of growing bud (Panel A) pUBS1-GFP partially suppresses the *cdc34-2* cell cycle defect (Panel B).

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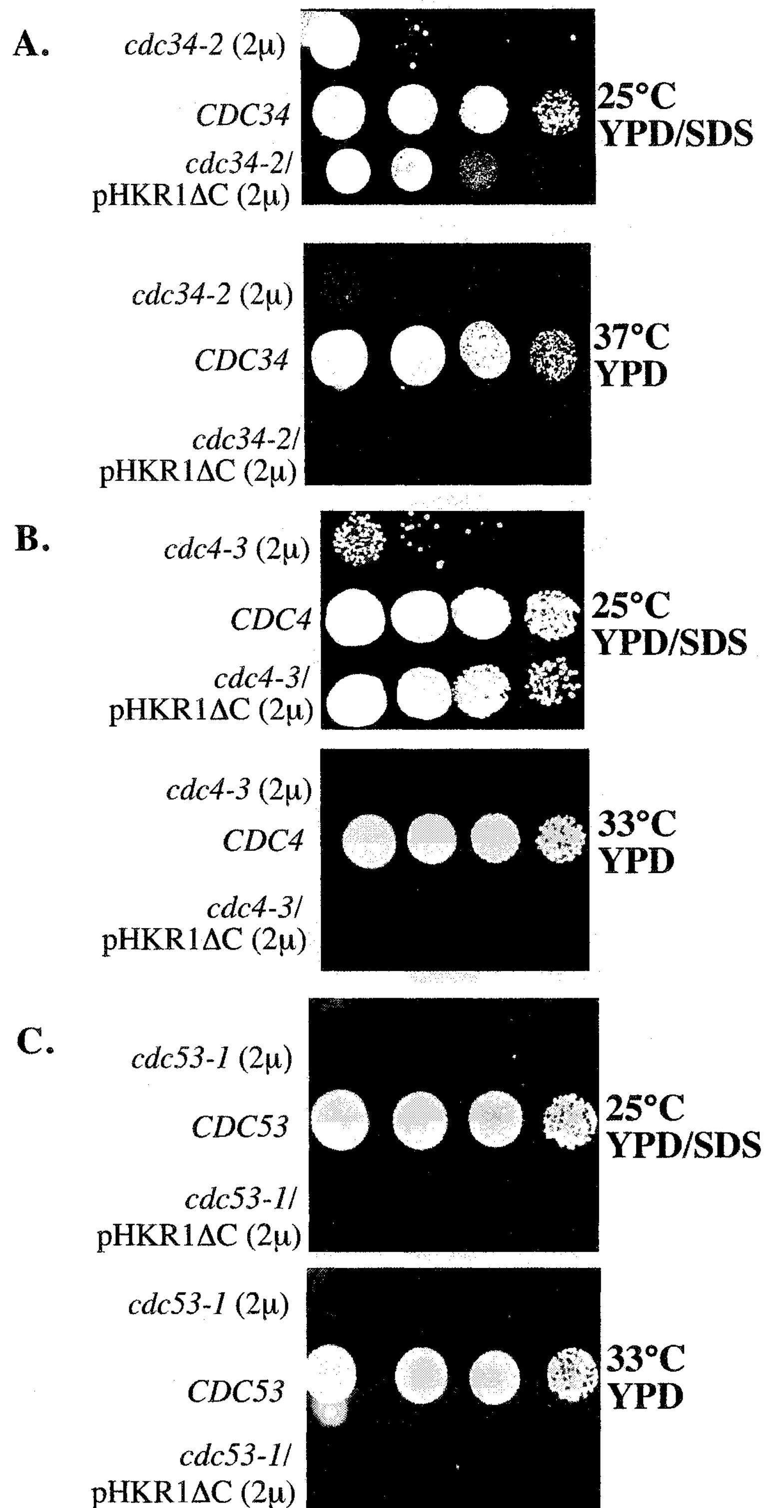

Figure 2-13. *HKR1ΔC* suppresses selective Cdc34/SCF cell integrity defects. Plasmid overexpression of *HKR1ΔC* suppresses the cell lysis defects of *cdc34-2* (Panel A), *cdc4-3* (Panel B), but not *cdc53-1* (Panel C) mutant strains grown in the presence of 0.0025% SDS.

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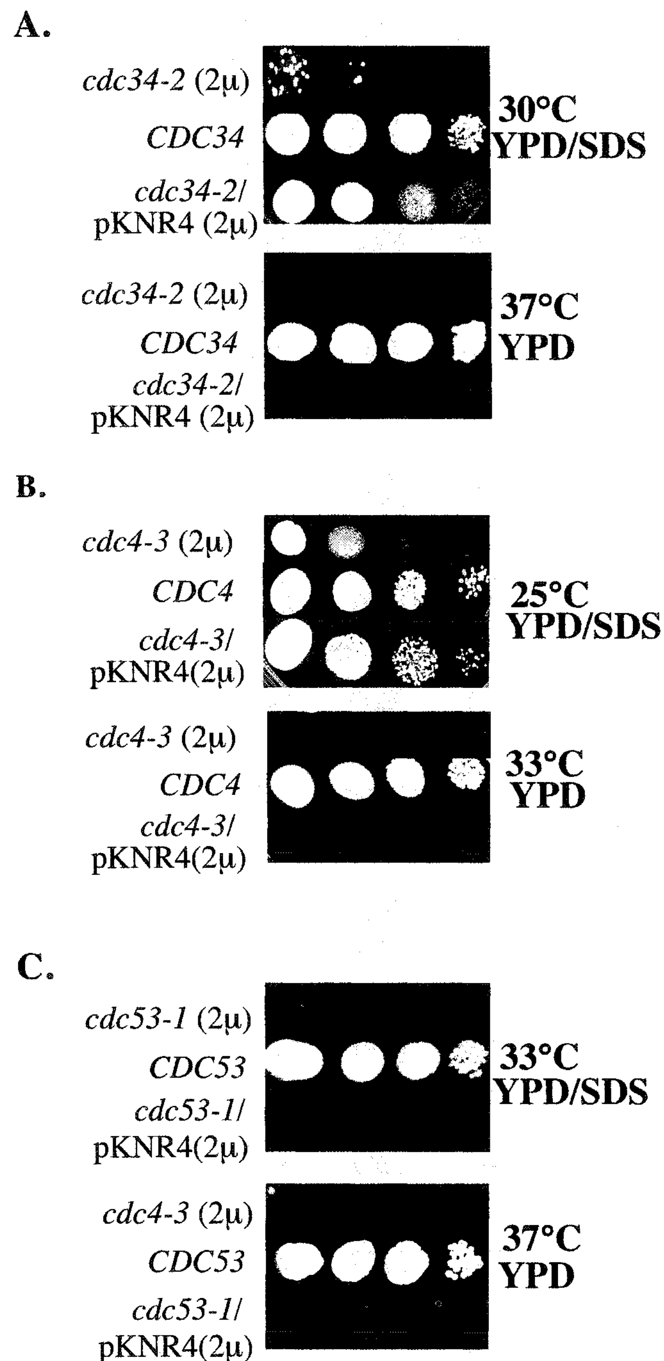

Figure 2-14. *KNR4* suppresses selective Cdc34/SCF cell integrity defects. Plasmid overexpression of *KNR4* suppresses the cell lysis defects of *cdc34-2* (Panel A), *cdc4-3* (Panel B), but not *cdc53-1* (Panel C) mutant strains grown in the presence of 0.0025% SDS.

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CHAPTER III – A NOVEL YEAST GENE LINKS CDC34/SCF CELL INTEGRITY AND CELL CYCLE FUNCTIONS

1. Introduction

Yeast cell integrity signaling is thought to originate at the cell membrane, from the cell surface sensors Slg1 and Mid2, which, in response to high temperatures or cell wall disturbances, transmit a signal to the small cytoplasmic GTP-ase Rho1 (extensively reviewed in Chapter I; Madden and Snyder, 1998). Rho1, in turn, activates four downstream effectors: Fks1, Pkc1, Bni1, and Skn7. The activation of Skn7, Fks1 and Pkc1 results in the regulation of cell wall biogenesis. Skn7 is a transcription factor that regulates the expression of the *OCH1* gene encoding α-1,6- mannosyltransferase (Horecka and Sprague, 2000). Fks1 is a β-1,3-glucan synthase, an integral membrane protein that directly controls the synthesis and polymerization of cell wall carbohydrates. Pkc1 controls cell wall synthesis indirectly, by activating a MAPK phosphorylation cascade involving several downstream protein kinases, including the MAPK Slt2, which in turn activates the transcription factor Rlm1 and the SBF and MBF transcriptional complexes to upregulate the synthesis of cell cycle progression and cell wall biosynthesis genes (Madden et al., 1997; Igual et al.,1996).

The activation of SBF and MBF-mediated transcription is a fundamental event regulating the G1-S transition in the yeast cell cycle (Koch et al., 1993). These complexes induce the late G1 expression of the G1 cyclins Cln1, Cln2, Pcl1, Pcl2; the S-phase cyclins Clb5 and Clb6; and a variety of DNA synthesis and cell integrity-related genes (Spellman et al., 1998). The precise requirements for activation of these complexes is incompletely understood as three different kinases, all active at the G1/S transition in yeast, are known to stimulate SBF and MBF-mediated transcription. The Cdc28/Cln3 cyclin kinase complex is responsive to cell size and nutritional status (Stuart and Wittenberg, 1995). It

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is considered to the principal engine of SBF and MBF activation in the yeast cell cycle and its activity is important in driving cells into the DNA synthesis phase (Tyers et al., 1993). The Bck2 kinase also activates the SBF and MBF complexes independently of Cdc28/Cln3, and may play a different but shared role in mediating progression through the cell cycle (Wijnen and Futcher, 1999). Finally, SBF-mediated transcription is also stimulated by the *PKC1* cell integrity pathway (Igual et al., 1996). The terminal kinase of this pathway, Slt2, induces gene-specific SBF-mediated transcription by phophorylating Swi4 and Swi6 (Madden et al., 1997). Thus the cell integrity signaling is directly integrated into the cell cycle machinery at the G1-S transition, a critical point in the cell cycle coordinating bud emergence, DNA replication and duplication of the spindle pole body duplication (Khalfan et al., 2000).

The Cdc34/SCF complex is also an important regulator of the G1-S transition in yeast targeting a variety of proteins including, Sic1, Cln2, Gic2 for timely degradation by the 26S proteasome (reviewed by Desalle and Pagano, 2001). In Chapter 2 of this thesis, genetic evidence indicating a role for the Cdc34/SCF complex in the regulation of yeast cell integrity was presented, and *HKR1* and *KNR4*, two genes involved in cell wall biogenesis and cell integrity maintenance were identified as specific suppressors of this defect. Interestingly, both *HKR1* and *KNR4* contain transcriptional response elements within their promoters indicating their regulation by the SBF transcriptional complex and Rlm1, both substrates of the yeast cell integrity pathway at the G1-S transition (Igual et al., 1996; Jung and Levin, 1999). Also, genetic associations between cell integrity signaling and Cdc34 have previously been reported for the *SLG1* gene (Ivanovska and Rose, 2000). Moreover, a study linking the Cdc34/SCF complex, the SBF, and cell integrity signaling has recently been published, making the observation that a Cdc4 mutant yeast strain exhibits hyperactivated transcription of the *OCH1* gene in a Swi4-dependent manner (Cui et al., 2002).

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In this chapter we report the cloning of a novel gene whose overexpression suppresses both the cell integrity and cell cycle defects associated with the Cdc34/SCF complex. We also provide evidence that the *cdc34*-2 mutant strain is START-defective, and its temperature-sensitivity and cell integrity defects are suppressed by overexpression of signaling components of the yeast cell integrity pathway.

2. *Materials and Methods*

2.1 Yeast strains and plasmids

All yeast strains in this study are identical to those listed in Table 2-1.
pGST-UCS1 and pGST-KIC1 were obtained as N-terminal GST-fusions from a yeast ORF GST-fusion library. They contain a URA3 selectable marker for yeast expression, and their transcription is driven by the copper-inducible CUP1 promoter.
pUCS1 and pKIC1 was generated by PCR amplification of the yeast ORF YOR353c and KIC1 from a yeast genomic DNA prep with 5' and 3' primers containing flanking SacI and KpnI restriction endonuclease sites. The PCR fragments were then cloned into a yeast expression plasmid with TRP selectable markers, with their transcription under the control of a copper-inducible CUP1 promoter.
pRHO1 and pSLT2 were initially isolated as N-terminal GST-fusions from a yeast ORF GST-fusion library (Research Genetics), they were subsequently cut with EcoR1/SmaI and subcloned into the EcoR1/Sma1 sites of a copper-inducible yeast expression pCUP1 vector containing a URA3 selectable marker.
YlpLZC2 and YlpLZCYC were generously provided by Dr. David Stuart. These are integrating reporter plamids with either the CLN2 promoter of the CYC terminator driving expression of the LacZ gene. These plasmids were linearized by BstEII restriction endonuclease digestion and transformed into yeast cells. Integration

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reconstitutes LEU2 expression in cells and cells carrying the insertion are selected on SD –Leu dropout medium.

pSIC1 3XHA was generously provided by Dr. David Stuart. It is a YCplac111 LEU2, CEN plamid with a 2631 bp genomic fragment that includes the SIC1 ORF along with 423 basepairs of upstream sequence and 1193 basepairs of downstream sequence as an EcoR1- Hind III genomic fragment (Gietz and Sugino, 1988). Site directed mutagenesis was used to create a unique NotI site immediately prior to the Stop codon of *SIC1*. Then three tandem repeats of the HA epitope were inserted as a single NotI cassette (Tyers et al., 1993).

2.2 Media and Microbiological techniques

Genetic methods and growth media were as described by Sherman and colleagues (1986). SDS sensitivity was analyzed on YPD plates supplemented with 0.0025% sodium dodecyl sulphate (SIGMA). Yeast transformations were performed by a lithium acetate transformation protocol.

Isolation of a high-copy suppressor of the *cdc34-2* cell integrity defect - To identify a high-copy suppressor of the *cdc34-2* cell integrity defect, YL10 cells were transformed with a genomic library on the high-copy vector pMA3A. A suppressor plasmid was identified by its ability to allow *cdc34-2* mutants to form colonies on YPD media supplemented with 0.0025% SDS at room temperature. Under these stringent conditions *cdc34-2* mutants were found to be exquisitely sensitive and unable to grow, as such, weak suppressors were likely eliminated from this screen. A suppressor plasmid was isolated from a single colony that resulted from the screen and transformed into *E. coli* cells. The plasmid was propagated in bacteria, amplified, purified, re-introduced into *cdc34-2* mutant cells, and re-tested for SDS-resistance to confirm the specificity of the suppressor phenotype. Once the phenotype was confirmed it, plasmid was then sequenced by the

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dideoxy chain-termination method on an Applied Biosystems automated Sequenator in the University of Alberta DNA Sequencing and Synthesis facility.

The suppressor plasmid was found to contain a 9.9kb fragment of *S. cerevisiae* chromosome XV from 998394 to 1008391 inclusive, and contained four yeast ORFs. Each ORF was independently isolated as a GST-fusion from a yeast ORF GST-fusion library (Research Genetics), and tested for its ability to compliment the cell lysis defect of *cdc34-2* cells

Cell Viability Assays – Yeast cells were picked as individual colonies or transformants and grown at room temperature to an O.D. between 0.8 and 1.0 in Synthetic Dropout media. The cells were then pelleted, washed twice with distilled water, and then diluted in distilled water and plated in tenfold serial dilutions starting at $1x10^5$ cells on supplemented or unsupplemented YPD plates, and grown for 72 hours at a variety of temperatures. The sensitivity of cells to a particular reagent was determined by lack of growth.

β-galactosidase assays – Yeast cells were grown as 5 mL cultures overnight in Synthetic Dropout media at 30 °C with agitation. OD_{600} was then taken, and fresh YPD cultures were made to OD_{600}= 0.3 and the cells were further cultured at 30°C or 37°C for 3 hours before harvesting 1 mL of culture fluid for assay. B-galactosidase measurements were performed using O-nitrophenol β-D-galactopyranoside (ONPG) as a substrate according to the manufacturer's protocol (CLONETECH Laboratories, Inc). Bar heights in the figures represent Miller units (Miller, 1972) averaged from six independent assays of each strain; standard deviations are indicated.

Nacodazole arrest – Yeast cells cultures were grown at 30°C in Synthetic dropout media to an OD_{600} of 0.5 at which time 15μg/mL Nacodazole (SIGMA chemicals) was added to the media and the cells were allowed to grow for an additional two hours at the same temperature. The cells were harvested by centrifugation, microscopically visualized for a

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mitotic arrest phenotype, and resuspended in 100mL of YPD media pre-warmed to 37°C, and grown at 37°C for the duration of the experiment. Time points were taken every 30 minutes for 2 hours, and then again at 3 hours.

Yeast extract preparation– A crude yeast extract was prepared from cells for Western blotting analysis according to the methods of Foiani and colleagues (1996). 10 mls of cell culture from each time point were spun down at 4000 rpm and resuspended in 20% TCA. The cells were re-harvested in a microfuge at 14,000 rpm for one minute, and then resuspended in 200μL of 20% TCA. Glass beads were added to match the volume of the cell suspension and the cells were then vortexed for 4 minutes in 1 minute intervals at 4°C. 400μL of 5% TCA was then added and the aqueous extract was removed to a new tube and microfuged at 14,000 for 10 minutes. The supernatant was removed , and the pellet was resuspended in 100μl of Laemmli buffer and 50μL of Tris-Base 1M. The extract was then incubated at 95°C for 5 minutes, spun in a microfuge tube at 3000 rpm for 10 minutes, and the crude extract supernatant was transferred to a microfuge tube for SDS PAGE and Western blotting.

Western Blotting analysis – 10ml of each sample was loaded onto a 12% polyacrylamide gel and subjected to SDS-PAGE. The blot was then transferred onto PVDF paper (Immobilon) at 300mA for 1 hour in a Mini-Protean gel apparatus (BIORAD). Western Blot analysis was carried out with using αHA-3F10 antibody conjugated to Horseradish peroxidase (SIGMA) and visualization was done by enhanced chemiluminescence (ECL). The gels were stripped by incubation at 65°C in stripping buffer (TBS, 1%SDS, 10mM DTT) and re-probed with an αCdc28 monoclonal primary antibody and a goatαmouse-HRP conjugate secondary antibody, generously provided by Dr. David Stuart.

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3. Results

3.1 A novel yeast gene suppresses the *cdc34-2* cell integrity defect

We previously demonstrated an allele-specific cell integrity defect associated with the *cdc34-2* mutant strain that could be specifically suppressed by overexpression of the *UBS1*, *HKR1* and *KNR4* genes (Chapter 2). In order to identify more genes in this pathway, we screened *cdc34-2* cells with a high copy pMA3A yeast expression library for genes whose overexpression suppressed the strong SDS lysis defect associated with the mutant strain. Interestingly, of $1X10^4$ colonies screened, we obtained only a single transformant of the mutant strain that grew in the presence of 0.0025% SDS. Following confirmation of the phenotype, we sequenced the suppressing plasmid and found that it contained an approximately 10kb fragment of *S. cerevisiae* chromosome XV containing four intact open reading frames: *YOR353c*, *MCS6*, *GDS1*, and *YOR356W*.

All of the four ORFs were obtained as N-terminal GST-fusion 2μ plasmids from a GST-fusion yeast expression library, and each individual ORF was tested for its ability to rescue the *cdc34-2* mutant. Only one ORF was capable of suppressing the *cdc34-2* SDS sensitivity phenotype: the previously uncharacterized gene *YOR353c*. We observed that transformation of *cdc34-2* cells with GST-*YOR353c* resulted in a strong suppression of the SDS-sensitivity phenotype (Figure 3-1). We therefore named the gene *UCS1* (uncharacterized cell integrity supressor-1). *UCS1* is an essential gene encoding a 791 amino acid protein with a predicted molecular weight of 87.3 kD. Analysis of its primary sequence using the NCBI conserved domain database reveals one predominant structural feature, the presence of an N-terminal domain consisting of 6 tandem leucine-rich repeats (LRRs) and a centrally located BC-box. A BLAST search for homologues to this gene in yeasts and other organisms revealed that the LRR domain is highly similar to the LRR domain of the *S. cerevisiae CYR1* gene encoding the cAMP-generating adenylate cyclase enzyme (Figure 3-2). In this region the two proteins were found to be 55% similar and

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35% identical. Their region of similarity, however, is restricted to their LRR domains, and there is no significant similarity between the two proteins outside of this region. While it is interesting to note that the centrally located LRR domain of Cyr1 modulated its interaction with the Ras oncoprotein, LRR domains are an extremely common and highly versatile structural feature that are found in numerous proteins of unrelated function in every eukaryotic genome (Kobe and Deisenhofer., 1994). *SPBC887*, an uncharacterized gene from the yeast *Schizosaccharomyces pombe*, encodes a hypothetical protein of similar size to Ucs1 and contains protein sequence with homology to it, both including and extending beyond its LRR domain (Figure 3-3). While this may indicate a functional relationship between these genes, at present there is no available data to suggest as much.

3.2 High copy expression of GST-*UCS1* suppresses the cell integrity defects of SCF mutant alleles

In Chapter 2, we reported that the *cdc4-3* and *cdc53-1* SCF mutant cell lines also exhibit cell integrity defects similar to those of the *cdc34-2* strain. In that study the *cdc53-1* strain was more severely affected in the pathway, and was not rescued by overexpression of the *UBS1*, *HKR1* or *KNR4* genes. Having observed that overexpression of GST-*UCS1* suppressed the cell integrity defects of *cdc34-2* cells, we decided to test the *cdc4-3* and *cdc53-1* strains for SDS-sensitivity suppression by GST-*UCS1*. We observed that *cdc4-3* cells transformed with GST-*UCS1* were indeed strongly rescued from their SDS-sensitivity phenotype in comparison to the same cells transformed with an empty vector (Figure 3-4). Furthermore, we also observed that GST-*UCS1* also suppressed the cell integrity defect of the *cdc53-1* mutant strain. As previously observed, the isogenic wild-type strains of exhibited no obvious lysis defects. Having previously observed that the temperature-sensitivity defects of the *cdc34-2* cells

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were partially suppressed by overexpression of the UBS1 gene, we tested *cdc34-2*, *cdc53-1*, and *cdc4-3* mutant strains for suppression of their temperature-sensitivity by GST-*UCS1* (Chapter 2, Pendergast et al., 1995). We also observed that transformation of these mutant strains with the GST-*UCS1* plasmid had no effect on the temperature-sensitivity phenotypes of exhibited by the strains.

3.3 pUCS1 suppresses the cell integrity and cell cycle defects of the Cdc34/SCF mutants

The LRR domain of Ucs1 is located at the extreme N-terminal region of the protein, occurring approximately between amino acids 30-180. While LRRs are found in functionally and evolutionarily diverse proteins, all characterized LRR domains serve as protein-protein interaction modules and nearly half of known LRR containing proteins are involved in signal transduction (Kobe and Deisenhofer, 1994). We therefore speculated that the presence of an N-terminal GST appended to the LRR domain might be impairing the function of Ucs1. We therefore generated pUCS1, a high copy yeast expression plasmid containing the *UCS1* gene without the GST moiety, and tested it in the same cell integrity and cell cycle suppression assays as previously described for GST-*UCS1*. We observed as before that all three of the mutant cell strains (*cdc34-2*, *cdc4-3*, *cdc53-1*) transformed with pUCS1 exhibited strong suppression of their cell integrity defects as evidenced by their ability to grow in the presence of 0.0025% SDS. Surprisingly, we also observed that overexpression of pUCS1 fully suppressed the temperature-sensitivity phenotypes of both the *cdc53-1* and *cdc34-2* strains, but not on the *cdc4-3* strain (Figure 3-5). Taken together, these data suggest strongly that the cell integrity and cell cycle pathways regulated by the Cdc34/SCF intersect at some level, and the activity of a novel gene directly suppresses defects in this pathway.

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3.4 Cell integrity genes suppress *cdc34-2* cell integrity and cell cycle defects

Activation of the SBF transcriptional complex by the cell integrity pathway results in the transcriptional upregulation of cell cycle and cell wall synthesis genes (Madden et al., 1997; Igual et al., 1996). In particular, the transcription of the G1 cyclins Pcl1 and Pcl2 is clearly responsive to *PKC1*/MAPK signaling. The Pcl1,2/Pho85 complex, in turn, phosphorylates a variety of protein substrates including Sic1, resulting in its degradation by the Cdc34/SCF complex (Nishizawa et al., 1998). As our results so far have suggested that the Cdc34/SCF complex positively regulates signaling through a cell integrity pathway, we surmised that overexpression of key components of the *PKC1*/MAPK pathway should have suppressive effects on the cell cycle and cell integrity defects of the *cdc34-2* mutant strain.

To test this hypothesis, we transformed *cdc34-2* cells with high copy yeast expression vectors containing the *SLT2* and *RHO1* genes under the control of the copper-inducible CUP1 promoter. We observed that overexpression of *RHO1* and *SLT2* clearly rescued growth defects of *cdc34-2* cells on both 0.0025% SDS and on YPD growing at the 37°C as compared to the mutant strain transformed with an empty control vector (Figure 3-6). We did not, however, observe any cell integrity or cell cycle rescue in the *cdc4-3* and *cdc53-1* mutant strains.

Microscopic examination of *cdc34-2* cells either expressing or not expressing *RHO1* and *SLT2* and grown for 3 hours the 37°C demonstrates that high copy expression of both cell integrity signaling molecules results in amelioration of the hyperpolarized and multiple-bud phenotypes exhibited by the mutant strain (Figure 3-7). The polarized growth phenotype is thought to result from a failure to activate the isotropic growth program due to Sic1 accumulation. We therefore tested nacodazole-synchronized cell cultures of the *cdc34-2* mutant strain and the same strain expressing *RHO1* and *SLT2*, for their ability to degrade Sic1 at 37°C. Consistent with the our previous results, we

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observed that Sic1 was degraded far more efficiently in *cdc34-2* cells expressing *RHO1* and *SLT2* than it was in the same strain transformed with an empty control vector (Figure 3-8). Taken together, these data demonstrate that reconstitution of cell integrity signaling results in the restoration of the cell cycle progression of the *cdc34-2* mutant cell strain.

4. Discussion

4.1 *UCS1* is a novel cell integrity and cell cycle regulator

We report here the cloning and characterization of a novel genetic suppressor of growth and lysis defects exhibited *cdc34-2* mutant cell strain. The essential *YOR353c/UCS1* gene of *S. cerevisiae* encodes an 87.3 kD protein with N-terminal leucine-rich repeats. It associates *in vivo* with the essential Pak kinase Kic1, which in turn associates with the Mapk Slt2. The function of Ucs1 is at present unknown, as it does not have any obvious primary structural features that are suggestive of any obvious activity. Interestingly, its binding partner Kic1p belongs to a subset of PAK kinases that contain an eleven amino acid motif (AKPXSILXD/ELI) that is found just COOH-terminal to the kinase domain that is known to modulate its interactions with regulatory components (Leeuw et al., 1998). Given the strength of Kic1-Ucs1 interactions, it is possible that they are some form of functional complex that targets proteins for phosphorylation. Further studies on the localization of Ucs1, its intracellular binding partners, and its effects of SBF activity and Sic1 turnover will undoubtedly prove useful in determining its *in vivo* role.

4.2 Ucs1 interacts with the essential PAK-kinase Kic1

Our initial thought upon observing the strong suppressive effects of pUCS1 on Cdc34/SCF mutant pathways was that Ucs1 might somehow interact directly with the proteins in the complex, possibly stabilizing their interaction. We were, however, unable

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to identify such an interaction by coimmunoprecipitation experiments with tagged versions of Cdc34 or other components of the SCF. Genome-wide analysis of protein-protein interactions in *S. cerevisiae* have been done by two-hybrid analysis and coimmunoprecipitiation studies (Uetz et al., 2000; Ito et al., 2001; Ho et al., 2002). In all three independent studies, Ucs1 was found to interact with the essential yeast PAK kinase Kic1.

PAK kinases are a large and poorly understood family of related protein kinases. Kic1p, *SOK1*, *CELT19A5*, *MST1*, *MST2*, and Ste20p, are examples of PAK kinases that share a common 11-amino acid motif (AKPXSILXD/ELI) just COOH-terminal to the kinase domain (Sullivan et al., 1998). This domain is unique to PAK kinases and is similar to a recently identified Ste20/PAK kinase sequence shown to interact with G protein -subunits (Leeuw et al., 1998). Beyond these regions, Kic1p shows no significant homology to any other known proteins. Clues as to the function of Kic1 *in vivo* have come from a single study indicating a role for this kinase in the maintenance of cell integrity. Kic1 was found to interact with and become activated by Cdc31, a calmodulin-homologue essential for spindle pole body duplication (Sullivan et al., 1998). While both Cdc31 mutants and kinase defective mutants of Kic1 were found to be severely defective in cell integrity, Kic1 mutants were not found to be spindle pole body defective. Futhermore, Kic1 mutants exhibited lysis defects that were readily suppressed by osmotic stabilization with 1M sorbitol. Taken together, these data suggest that Kic1 activation of Kic1 by Cdc31 somehow results in cell integrity signaling. Interestingly, genome-wide co-immunoprecipitation studies have demonstrated an interaction between the MAPK Slt2 and Kic1, which strongly suggest that the cell integrity effects of Kic1 may be mediated through its ability to activate Slt2 (Ho et al., 2002).

The potential significance of the interaction between Kic1 and Ucs1 was not lost on us, and we generated a pKIC1 high copy yeast expression plasmid to determine if

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Kic1 overexpression could influence the cell integrity defects of Cdc34/SCF mutants. Curiously, we observed that Kic1 had no effect on either the SDS-sensitivity or temperature-sensitivity phenotypes of all the mutant strains.

4.3 *UCS1*: a component of a novel ubiquitin ligase?

Our identification of *YOR353c/UCS1* as a high copy suppressor of cell integrity and cell cycle defects is particularly interesting given the unusual primary structure of the Ucs1 protein. The first third of protein consist of a Leucine-Rich–Repeat region which is a well characterized protein-protein interaction domain found on numerous proteins including the yeast adenylate cyclase Cyr1 as well as the F-box protein Grr1. Within the central region of the protein however is the presence of a sequence motif called a BC-box that has not been characterized within yeast proteins but is common in higher eukaryotes. In mammalian cells, the yeast homologue to Cdc34 is the human Ubc3 protein. Ubc3 interacts with the SCF complex in mammalian cells, but is also able to interact with a second type of E3 known as the VHL-Elongin BC complex (Lisztwan et al., 1999). VHL-Elongin BC is a multisubunit E3 consisting of the heterodimeric Elongin BC complex and a third protein termed the von Hippel-Lindau (VHL) tumor suppressor protein (Kibel et al, 1995). Interaction of Elongin BC with VHL is governed by a 10 amino acid degenerate sequence motif termed a BC-box which has the following consensus sequence: (Ala,Pro,Ser,Thre) LeuXXXCysXXX (Ala,Ile,Leu,Val). Analysis of the crystal structure of the VHL-Elongin BC complex is dependent on the association of the highly conserved Leu residue in position 2 of the VHL BC-box with a hydrophobic pocket created by C-terminal residues on Elongin C (Stebbins et al., 1999). Within this ubiqutin ligase system, the BC-box bearing VHL subunit functions as the substrate-binding subunit of the complex in a similar manner to the F-box protein of the SCF complex (Kamura et al., 1998). Elongin BC serves to link the VHL component to Cul5

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(a Cdc53 homologue) and Rbx1 (also part of the SCF complex) which function to recruit the ubiquitin conjugating enzyme to the complex for ubiquitination. Thus the Elongin BC/VHL complex is a component of a ubiquitin ligase that shares submits with and functions in a similar manner to the SCF in higher eukaryotes.

Interestingly, while such a complex has yet to be discovered in yeast, we have found that the Ucs1 contains a BC-box within its primary sequence (Figure 3-1). Carboxy-terminal to the leucine-rich repeat region of Ucs1 we found the sequence motif: **Ala Leu** Ile Lys Leu **Cys** Leu Thre Ile **Ile** which matches the consensus sequence for known BC-boxes. Notably a similar protein to Ucs1 termed Muf1 was recently purified from rat liver (Kamura et al., 2001). It was found to function by recruiting a Cullin/Rbx1 module to form an intact ubiquitin ligase. Like Ucs1, Muf1 was found to contain seven leucine-rich-repeats, albeit C-terminal repeats instead of N-terminal repeats in addition a BC-box domain. It was found to be able to interact with different Cullin proteins and facilitate the conjugation of ubiquitin to different artificial substrate proteins *in vitro*. What is most striking about the suppressive effects of the *UCS1* expression in the *cdc34-2*, *cdc53-1* and, *cdc4-3* temperature-sensitive mutant strains is that no gene so far discovered in yeast is capable of muti-allelic SCF suppression *except* components of the SCF itself. The *cdc34-2* and *cdc53-1* mutant yeast strains are capable of being cross-suppressed by overexpression of either *CDC34* or *CDC53* (Mathias et al., 1994). Overexpression of neither gene was found to be capable of the cell cycle arrest defect of the *cdc4-3* strain at 37°C. Having observed precisely the same phenotype with *UCS1* overexpression this leads us to conjecture that Ucs1 is the substrate-binding component of an as of yet undiscovered ubiquitin ligase that may include Cdc34 and Cdc53 as subunits but likely not Cdc4.

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4.4 The cell integrity pathway and the Cdc34/SCF complex

We observed that overexpression of the *SLT2* and *RHO1* signaling components of the cell integrity pathway suppressed the cell cycle and cell integrity defects of the *cdc34-2* mutant strain, but not the *cdc4-3* and *cdc53-1* strains. This was truly an interesting and unexpected result that directly links cell integrity signaling to cell cycle progression. While differences in the strain genetic backgrounds may account for the differing suppressive effects of these genes, it is curious that overexpression of these components had no effect on the cell integrity pathway defects of the Cdc53 and Cdc4 mutant strains, as our previous results identified several genes including *KNR4*, *HKR1* and *UBS1* which suppressed the SDS-sensitivity phenotype of the Cdc4 mutant strain. These results, however, further confirm our previous observations that the Cdc34/SCF complex in some way positively regulates signaling through the cell integrity pathway. Interestingly, the *KNR4* gene has recently been demonstrated to be a component of the *PKC1* cell integrity signaling pathway and an Slt2-binding partner, modulating its activity away from SBF-activation and toward cell integrity signaling through Rlm1 (Martin-Yken et al., 2002a; 2002b). This may indicate why, in Chapter II, it was observed that *KNR4* suppressed only the cell integrity and not the cell cycle defects of the *cdc34-2* and *cdc4-3* mutants, while Slt2 overexpression suppresses both in the *cdc34-2* allele.

This data is further supported by an interesting genetic observation made in our lab (C. Ptak, unpublished results). We previously observed that the temperature-sensitivity phenotype of *cdc34-2* mutant cells at 37°C could be partially suppressed by overexpression of the *cdc34Δ185* truncation mutant (Ptak et al., 1994). We exploited this partial suppression phenotype to screen for potential cell cycle targets of the Cdc34/SCF complex. We transfected these partially rescued cells with a high copy yeast expression library and looked for genes whose overexpression enhanced the temperature sensitivity defects of the *cdc34-2/cdc34Δ185* strain. From this the RhoGAP *SAC7* was isolated as a

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specific enhancer of the *cdc34-2* temperature-sensitivity defect. Sac7 is a principal negative regulator of Rho1, and Sac7 overexpression results in decreased signaling through the yeast cell integrity pathway (reviewed by Cid et al., 1995). Currently investigations are underway to determine if in fact Sac7 is a target of the Cdc34/SCF complex *in vivo*. At the very least, however, these results independently confirm the results presented here by demonstrating that Rho1 signaling suppresses *cdc34-2* ts defects.

4.5 Are *cdc34-2* mutants defective in G1 cyclin transcription?

Having observed a convergence between the cell cycle and cell integrity pathways regulated by the Cdc34/SCF complex, we began to conjecture about the role of this complex in the regulation of cell integrity. Numerous studies on *cdc34-2* mutants growing at restrictive temperatures have demonstrated clearly observable cell cycle defects associated with the strain including: a G1-S phase cell cycle arrest, a polarized growth and multiple-bud phenotype, temperature sensitivity, and most notably accumulation of the Clb5/Cdk suppressor Sic1 (reviewed by King et al., 1996). As our studies demonstrate that *cdc34-2* cells are also defective in the cell integrity pathway, we speculated that one of the functions of the Cdc34/SCF complex may be to positively regulate cell integrity signaling at the G1-S transition. Previous studies have demonstrated that *PKC1*/MAPK signaling is activated at this juncture, resulting in, activation of the SBF-transcriptional complex and the transcription of G1 cyclins (Igual et al., 1996). Moreover, deletion of SBF subunits (Swi4 or Swi6) results in cell integrity defects and cell cycle effects when done in combination with other mutations (Madden et al., 1997).

We therefore decided to examine if the transcription of the G1 cyclin *CLN2*, considered to be the cyclin important for Sic1 phosphorylation, is in any way different

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between wild type cells and *cdc34-2* cells growing at permissive and restrictive temperatures. To accomplish this, we transformed *cdc34-2* mutant cells and an isogenic wild-type control strain with a reporter construct composed of the *CLN2* gene promoter driving expression of the LacZ gene. As a control for background LacZ signal, we used promoter-less LacZ vector and the same two yeast strains. We then assayed *CLN2* gene transcription in cells grown for three hours at 30°C and 37°C in both strains by liquid β-galactosidase assay as a measure of *CLN2* transcription.

We observed that the *cdc34-2* mutant strain was defective in *CLN2* transcription at 37°C while at the permissive growth temperature, no obvious defect was observed (Figure 3-9). Heat shock of yeast cells at 42°C is known to induce cell cycle arrest at the G1-S transition and results in decreased Cln1,2 transcription despite the presence of Cln3/Cdc28, possibly indicating a reduction Cln3 activity at higher temperatures (Li and Cai., 1999). Correspondingly, the yeast cell integrity pathway is activated in response to temperature upshift resulting in increased phosphorylation of Slt2 and increased transcription of cell wall biosynthesis genes (de Nobel et al., 2000). Our results seem to suggest that the *cdc34-2* mutant strain is unable to compensate for loss of *CLN2* transcription upon temperature upshift, and as a result *CLN2* transcription is notably diminished. Cell division under conditions of cell wall stress would likely increase the amount of signaling through the cell integrity pathway relative to conditions of vegetative growth under permissive growth conditions.

We recognize, however, that there are numerous caveats with this experiment which include the fact that Cdc34 itself may influence the longevity of the β-galactosidase enzyme product, and that Cdc34 may have some influence on *CLN2* transcription irrespective of its known role in Sic1 degradation. Furthermore, the comparison between cycling cells and cells arresting at the G1/S transition is not a biologically fair comparison and as such numerous secondary effects cannot be ruled out.

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This experiment, however, does support the concept that at high temperatures transcription of *CLN2* is impaired in *cdc34-2* cells.

While the results presented in this chapter are compelling, they are incomplete in the sense that they do not indicate the mechanism by which the Cdc34/SCF complex regulates yeast cell integrity. Recently, Cui and colleagues (2002) demonstrated a role for the Cdc34/SCF complex in the regulation of SBF-mediated transcription of cell wall synthesis genes. There they reported the isolation of a Cdc4 mutant strain that exhibited hyperactivated transcription of the *OCH1* gene in an SBF-dependent manner. While these results are somewhat confusing they do draw similar genetic relationships between the SBF and the Cdc34/SCF complex to those demonstrated in this thesis. Taken together then, these results serve as an initial introduction to what should prove to be an interesting avenue of future research.

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A.

MVATSSKRTLDPKEEHLPADKTSTNSSNTIISELATQEKSSSSGTTLK**LIALNIK**SISDEDVGYIQN**VERLSLRKNHLTS**
LPASFKRLSR**LQYLDL**HNNNFKEIPYILTQCPQ**LEILDL**SSNEIEALPDEISSFWQDN**IRVLSL**KDNNVTSIRNLKSITK
LNK**LSILDL**EDNKIPKEELDQVQSYTPFHTGIPKEEYWAIAISRYLKDHPNLPTPEPKISRAAKRMGFINTNLSNGAMNE
NNIISLAPSANTTISASTAMVSSNQTSATSFSGTVNAESEQSGAVNGTELYNHTKYNDYFKRLSILPEESMSNGHQKISH
AELVVSCRKLLFSFTECQQAIRKIASFCKEKAVAVNVVSLLYSVRSHTDNLVEVLQQTENEDESHDQALIKLCLTIITNF
KQIITLLRKNFEIFFKEDDLCFIRMFYMTLMCAYMEMYNAWSFIKEDDQVSGSASKAPKKHSFSRHETSSSSITSGGGPA
ASTTSTHCSGNIKLLPKTRSTRTPSASALLSNSNILTGDTTAVPLLSPNLNGAHTHGPILGHQNAISNGSSQTNMNEVKT
TSDTIPRQQLLQHNKSISDSKKESQAHEPKQHPVMTSSIINASNSNNVSNVNITPPPMNGGGAANSSANVVETNIDIQLY
QTLSTVVKMVSVVYNQLTSEISKIAIASTMGKQILTDSLAPKIRDLTETCRQAMDLSKQLNERLNVLIPNDSNSEKYLTS
LEKLKTWEIMNSFLKVIISILANTKIVMSDVPNLNELRPNLANLAKITKDVTVILDLSSYKAVSVSANSPEZ

B.

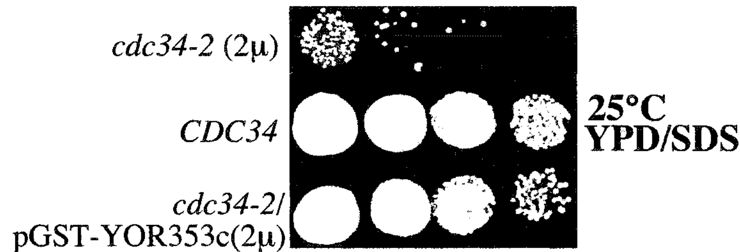

Figure 3-1. *YOR353c* is a high copy suppressor of the *cdc34-2* cell lysis defect. A genetic screen for high copy suppressors of the *cdc34-2* growth defect in the presence of 0.0025% SDS is suppressed by the uncharacterized chromosome XV ORF *YOR353c*. Yor353 has 7 N-terminal leucine-rich repeats (in bold) and a centrally located BC-box (underlined; Panel A). High copy expression of GST-YOR353 suppressed the SDS-sensitivity defect of *cdc34-2* cells (Panel B).

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```
Ucs1    65  IQNVERLSLRKNHLTSLPASFKRLSRLQYLDLHNNNFKEIPYILTQCPQLEILDLSSNEI 124
            + N+  L+L+ N L SLPA F  L  LQ LDL +N F   P ++  C  L  +DLS N+I
Cyr1  : 213 LSNLTILNLQCNELESLPAGFVELKNLQLLDLSSNKFMHYPEVINYCTNLLQIDLSYNKI 272

Ucs1  : 125 EALPDEISSFWQDNIRVLSLKDNNVTSIRNLKSITKLNKLSI 166
            ++LP   S+ +   +  ++L  N +  I +L  +T L  L++
Cyr1  : 273 QSLPQ--STKYLVKLAKMNLSHNKLNFIGDLSEMTDLRTLNL 312
```

Figure 3-2. The LRR domain of Ucs1 is similar to the LRR domain of adenylate cyclase. The N-terminal LRR domain of UCS1 is 35% identical and 55% similar to the central ras-binding LRR domain of the yeast adenylate cyclase gene *CYR1*.

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```
Ucs1 : 37  QEKSSSSGTTLKLIALNIKSISDEDVGYIQN-VERLSLRKNHLTSLPASFKRLSRLQYLD 95
           ++    +  TL L  LN++ +  E +  IQ  + RL+L  N + S+     + +RL+YLS+
Sp887: 22  EDAGPENALTLDLSHLNLRELPYEQLERIQGRIARLALGHNFIKSIGPEILKFTRLRYLN 81

Ucs1 : 96  LHNNNFKEIPYILTQCPQLEILDLSSNEIEALPDEISSFWQDNIRVLSLKDNNVTSIRNL 155
           + +N  +E P  L +   LEILD+S N+I+ LP+    +   N++VLS+  N +  +
Sp887: 82  IRSNVLREFPESLCRLESLEILDISRNKIKQLPESFGALM--NLKVLSISKNRLFELPTY 139

Ucs1 : 156 KSITKLNKLSILDLEDNKI 174
             I +   L IL +E+N I
Sp887: 140 --IAHMPNLEILKIENNHI 156


Ucs1: 286  NGTELYNHTKYNDYFKRLSILPEESMSNGHQKISHAELVVSCRKLLFSFTECQQAIRKIA 345
           NG+ L N +  N YF  +     E   +  +K      + S R +LFS ++ QQA+R+
Sp887:475  NGSNLTNDS-VNSYFSNIGGSEVEMKHSAFEKT-----LESSRGILFSLSQVQQALRQQL 528

Ucs1 : 346 SFCKEKAVAVNVVSLLYSVRSHTDNLVEVLQQTE--NEDESHDQALIKLCLTIITNFKQI 403
            FC    V  ++  +L++       L+   + T+  N+  ++  +++   L+ I++F+++
Sp887: 529 LFCSNPVVLDSMRHVLHTANVQIKRLILCFEDTQQSNDGTANINSIVNASLSCISSFRKL 588

Ucs1:  404 ITLLRKNFEIFFKEDDLCFIRMFYMTLMCAYMEMYNA 440
           I + +K         D+ ++R+  + L  A  E+ NA
Sp887: 589 IEVTKKFLNELTSRADVRYVRLLLLILFDAAKELQNA 625
```

Figure 3-3. A BLAST sequence search for Ucs1 homologues from different eukaryotic databases reveals a Ucs1 homologue in the yeast *S. pombe*.
The *S. pombe* protein Spbc887 is an uncharacterized homologue of Ucs1.

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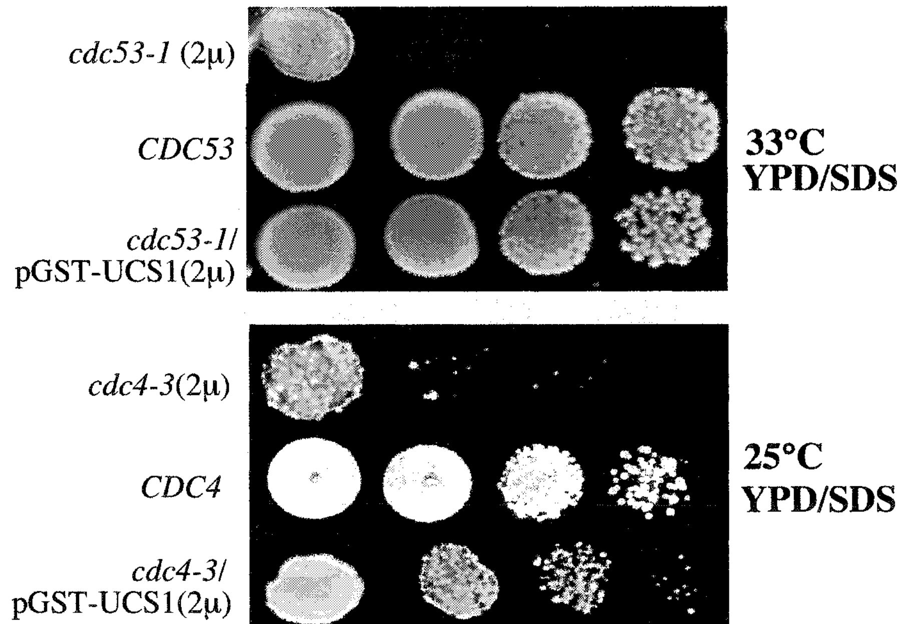

Figure 3-4. Cell lysis defects of SCF mutants are suppressed by pGST-UCS1. Plasmid overexpression of *GST-UCS1* in *cdc4-3* and *cdc53-1* strains alleviates the cell lysis phenotype of *cdc4–3* and *cdc53-1* cells in the presence of 0.0025% SDS.

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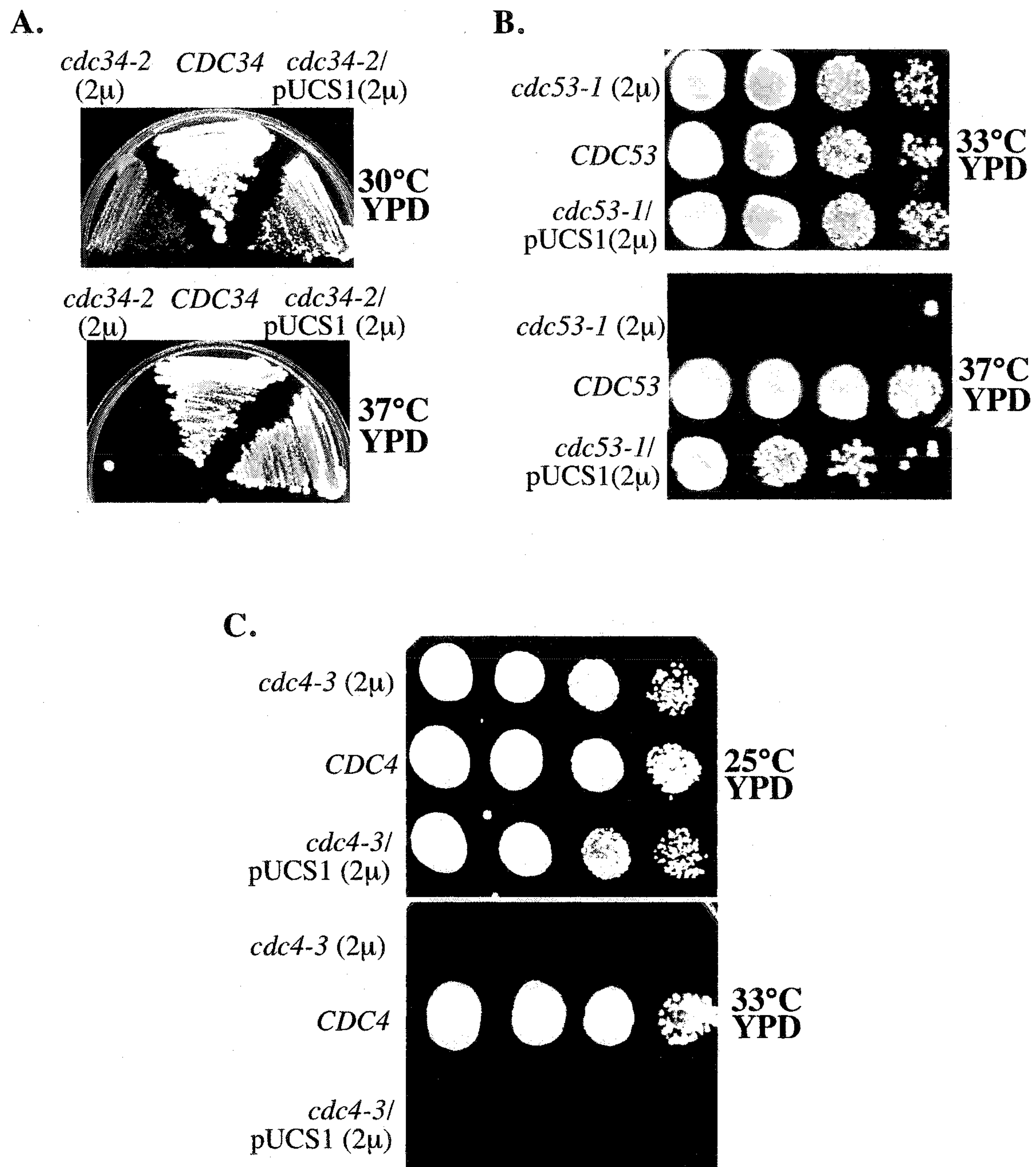

Figure 3-5. pUCS1 suppresses selective Cdc34/SCF cell cycle defects. Plasmid overexpression of *UCS1* alleviates the temperature-dependent growth defects of *cdc34-2* (Panel A), *cdc53-1* (Panel B), but not *cdc4-3* mutants grown at restrictive temperatures (Panel C).

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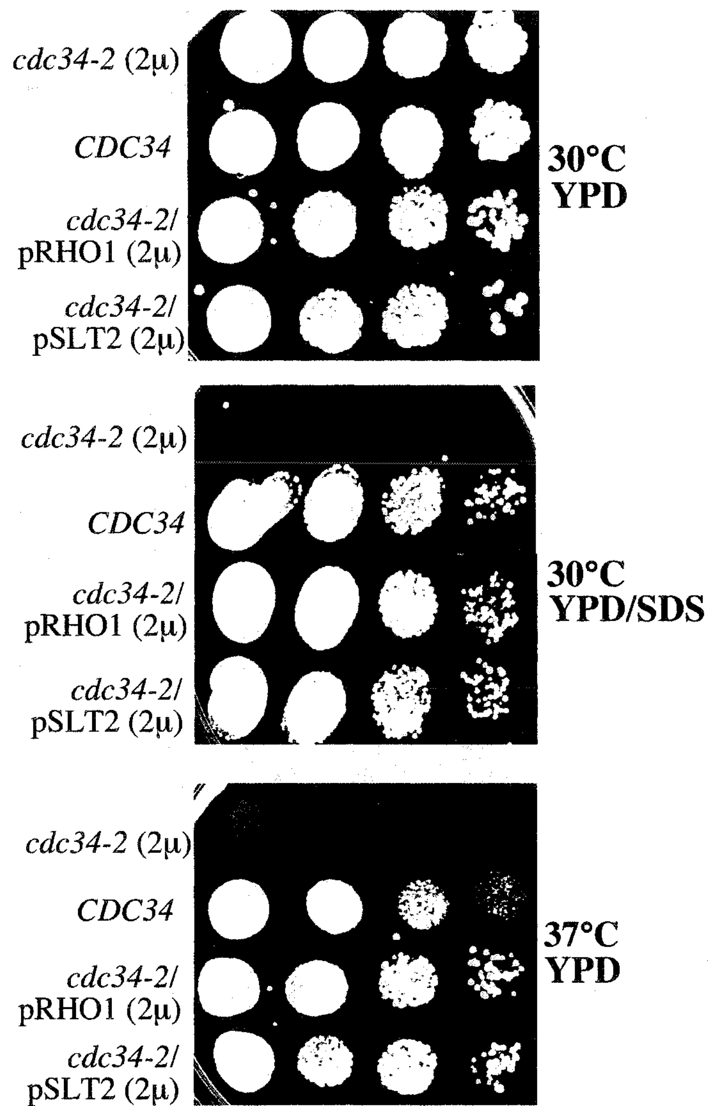

Figure 3-6. Cell integrity genes suppress *cdc34-2* cell lysis and cell cycle defects. Plasmid overexpression of *RHO1* and *SLT2* suppresses the cell lysis and temperature-dependent growth defects of *cdc34-2* cells

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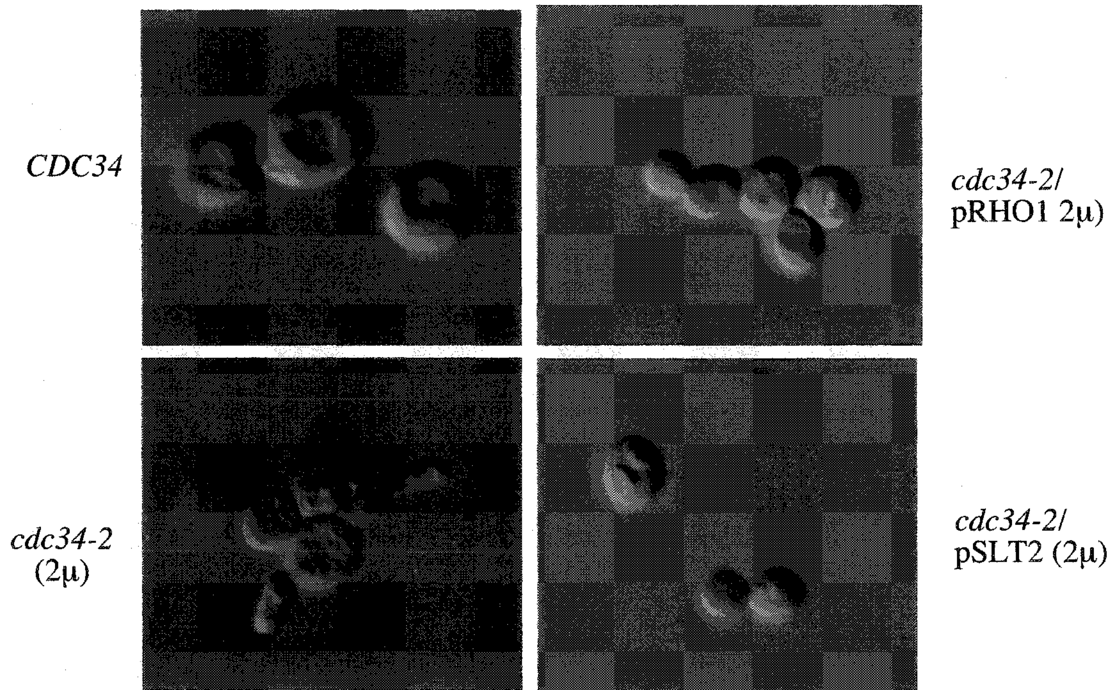

Figure 3-7. Cell integrity genes suppress *cdc34-2* morphogenic defects. Plasmid overexpression of *RHO1* and *SLT2* suppress the temperature-dependent hyperpolarized growth morphology of *cdc34-2* cells grown for 6 hours at 37°C.

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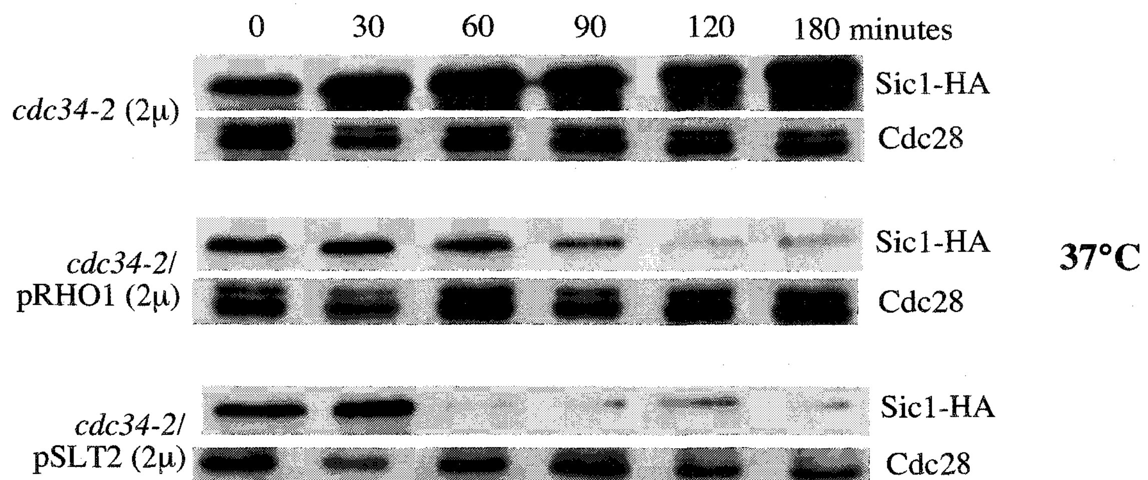

Figure 3-8. cell integrity genes contribute to Sic1 turnover in *cdc34-2* cells. Western blotting against Sic1-HA from cell lysates after nacodozole synchronization demonstrates that plasmid overexpression of *RHO1* and *SLT2* induces Sic1 turnover in *cdc34-2* cells grown at 37°C. Western blotting against Cdc28 is used as a loading control.

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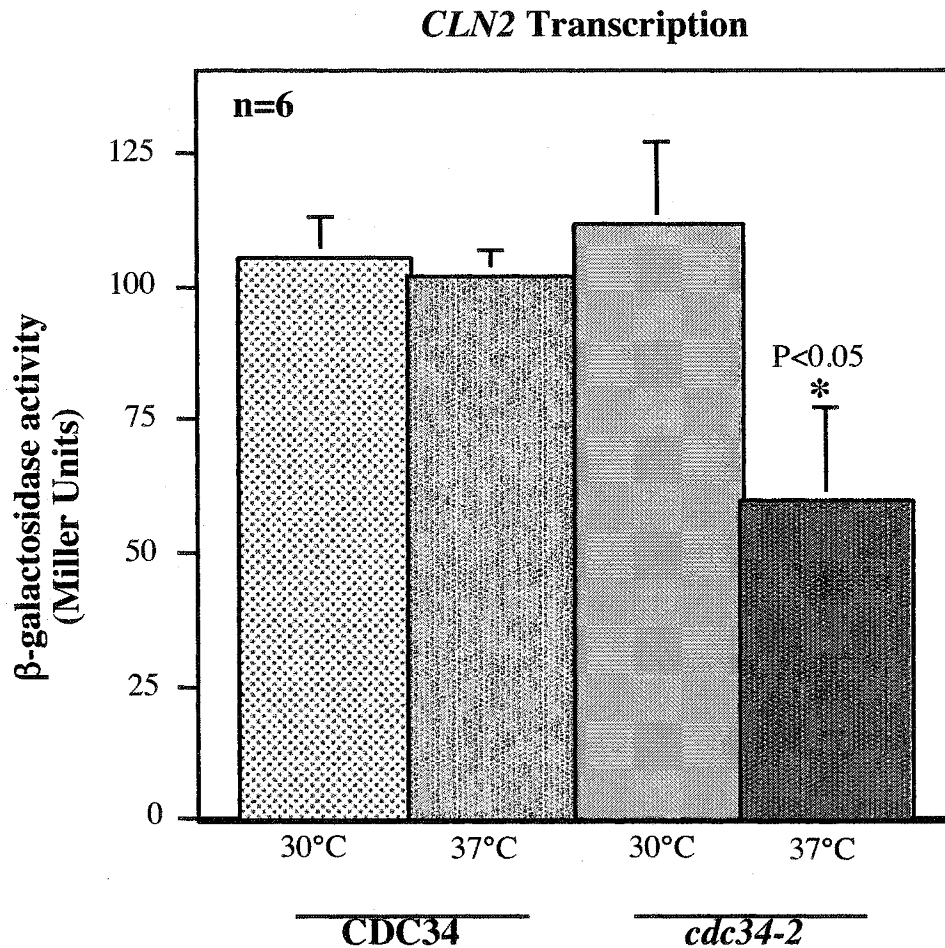

Figure 3-9. *cdc34-2* cells exhibit reduced *CLN2* transcription at 37°C . *cdc34-2* cells and an isogenic wild type strain were transfected with *CLN2* reporter constructs driving the expression of the β-galactosidase gene. The cells were cultured at 30°C and then incubated at either 30°C or 37°C for 3 hours. The cells were then rapidly lysed and assayed for β-galactosidase activity by an ONPG calorimetric assay. A promoterless β-galactosidase expression vector was used as a background control and yielded no signal.

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CHAPTER IV – A BIOCHEMICAL STUDY OF E2 SELF-ASSOCIATION *IN VITRO*: EVIDENCE FOR ITS ROLE IN E1-E2 INTERACTION

1. Introduction

In most organisms, including humans and the yeast *S. cerevisiae*, a single E1 enzyme is responsible for the activation of ubiquitin from the free intracellular pool (McGrath et al., 1991). While the structure of E1 has still not yet been determined, the chemistry of ubiquitin activation this enzyme is very well understood (Haas and Rose, 1982). The reaction mechanism commences with the binding of MgATP to E1, followed by ubiquitin. ATP-hydrolysis results in formation of an E1 tethered ubiquitin-adenylate intermediate that serves as a donor of ubiquitin to the E1 active site cysteine residue. Following the transfer of ubiquitin to the active site cysteine residue, the adenylate-site of E1 is then free to activate another ubiquitin in an ATP-dependent manner. In this manner, a fully activated E1 is an enzyme that is doubly charged with ubiquitin, one bound as a thiolester intermediate at the active site, and another as an adenylate-intermidate at the adenylate site. As activation of ubiquitin by E1 is a far more rapid process than conjugation of ubiquitin by ubiquitin conjugating enzymes (E2s), a sub-stoichiometrich concentration of E1 is generally sufficient for the activation requirements of this pathway (reviewed by Pickart., 2001). E1 has demonstrated itself to be largely impervious to structure-function analysis. Domain mapping of the enzyme has not been successfully accomplished, and very little is known about the structural determinants that facilitate its interaction with E2s.

E2s, or Ubcs, comprise a very well characterized family of related proteins, both structurally and functionally. In *Saccharomyces cerevisiae*, there are eleven known E2s which regulate a dizzying variety of biochemical pathways. All E2s share a highly

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conserved core domain of approximately 150 amino acids, which is embellished on some E2 family members with the addition of N- or C-terminal extensions important for their function. A variety of crystal and solution structures of E2s have been solved to date and they have all revealed a highly conserved core domain structure composed of four standard helices, a short 3_{10} helix, and a four stranded anti-parallel β-sheet. The active-site cysteine residue of the E2 is located in a shallow groove on the highly conserved catalytic face of the molecule. While many of the residues on this surface are required for the interaction of E2 with ubiquitin, others are clearly involved in mediating E1-E2 interactions, as well as interactions between E2s and E3s, and E2s and target proteins (reviewed by Pickart, 2001).

An interesting point of contention within the E2 structure-function field has been whether the Ubc enzymes form functional dimers. Evidence supporting the notion of E2 dimerization is abundant but somewhat ambiguous. Pickart and Rose (1985) initially observed homodimerization of E2s from reticulocyte extracts upon gel filtration chromatography, though the implication of this was not clear. Subsequently, Girod and Vierstra (1993) observed that the Ubc4 enzyme from *Arabidopsis thaliana* also migrated in dimeric form upon gel filtration. This was supported by studies done in our lab that revealed that yeast Ubc4 can ubiquitinate itself in intermolecular conjugation reactions and self-associate in dimeric and higher order complexes in the presence of a chemical crosslinker (Gwozd et al., 1995). Another study also demonstrated dimerization of Ubc4 upon gel filtration chromatography (Haldeman et al., 1997). In that study functional evidence for self-association was provided by the observation that artificial GST-induced dimerization of the core domain of the mammalian E2-25K allowed for novel ubiquitin-chain assembly properties that did not occur in the absence of dimerization. Also , gel filtration studies of recombinant Ubc-1 from *C. elegans* demonstrated that the protein

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occurred in two forms, a dimeric and a monomeric form (Leggett and Candido., 1997) Ubc6 and Ubc7 have exhibited homo-and heterotypic associations by genetic studies, as has the yeast Ubc9 gene, a Ubc homologue involved in SUMO conjugation (Chen et al., 1993; Jiang and Koltin, 1996). Moreover, studies done on the Cdc34 enzyme in our lab have demonstrated self-associative properties *in vitro* in the presence of a chemical crosslinker, and *in vivo* by immunoprecipitation (Ptak et al., 1994; X. Varelas personal communication). Lastly, a crystal structure of the E2-C, a Ubc derived from clam, was solved as a dimer to 2.0 A resolution (Jiang and Basavappa., 1999). There, the interface of the theoretical dimer involved a surface area of 1318 A^2 and included residues found predominantly within the N-terminal region of the core catalytic domain.

While dimerization has been observed as a physical phenomenon, the importance of this to E2 function has not been clairified. In this study, we generate a stable disulfide cross-linked dimer of the core catalytic domain of the *S. cerevisiae* E2 Ubc1. We show that this dimer interacts stably with E1 upon gel filtration chromatography, and upon E1 interaction, the dimer is catalytically reduced. The implications of E1-E2 interactions in light of these observations is discussed.

2. Materials and Methods

2.1 Plasmids

E. coli overexpression plasmids for ubiquitin and UBC1Δ450 have been previously described (Hodgins et al., 1996). The yeast over expression vectors derived from pUBA1, created by Dr. Stefan Jentch (University of Heildelberg), consists of a CUP promoter, UBA1-6 3' His gene and CYC transcriptional terminator.

2.2 Bacterial and Yeast Strains

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The *E. coli* strain used for the overexpression of UBC1Δ450 and ubiquitin was BL21(DE3)pLysS (F- ompT hsdSB (rB-mB-) gal dcm (DE3) pLysS (CamR)) (Invitrogen). The E. coli strain MC1061 (F- araD139 (ara-leu)7696 galE15 galK16 (lac)X74 rpsL (Strr) hsdR2 (rk-mk+) mcrA mcrB1) was used for the PCR mutagenesis and plasmid production (New England Biolabs). Wild type UBA1 was obtained as a stable plasmid PJD-325 in the yeast strain JD-773A. The UBA1 deletion strain JD77-1A (MATa leu2-3, 112 lys2-801 his3-_200 trp1-_63 ura3-5 uba1 ::HIS3 expressing Uba1p-6His) was generously provided by Dr. Seth Sadis (Harvard University) and was maintained on YPD plates. Wild type UBA1 and mutant forms of UBA1 plasmids were cloned and UBA1 was expressed from the *Saccharomyces cerevisiae* strain MHY-501 (MATa his3-_200 leu2-3 ,112 ura3-52 lys2-801 trp1-1, gal2) (constructed by Dr. Mark Hochstrasser, University of Chicago). Transformants were selected on -LEU dropout plates.

2.3 Protein expression and Purification

Overexpression and purification of Ub and UBC1Δ450 - The methods used for the labeling recombinant S^{35}-Ubc1Δ450 were described previously in our laboratory (Ptak et al., 1994). Overexpression and purification of recombinant ubiquitin and Ubc1Δ450 were done as described previously in our laboratory (Hodgins et al., 1996).

Uba1 overexpression - The yeast expression plasmid for UBA1 was transformed into the yeast strain MHY-501 and were plated onto SD –LEU plates. Individual colonies were then used to innoculate 100 ml SD –LEU liquid media and the cultures grown overnight OD_{590} of 1.0. These cultures were then used to seed larger cultures to an initial cell density OD_{590} of 0.1 which were grown at 30°C with shaking to an OD of 0.15 at which point copper sulfate was added to a final concentration of 100 to stimulate expression of

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the UBA1 derivatives from the CUP promoter. The cultures were then grown for an additional 12 hours at 30°C with vigorous shaking.

Cells were harvested by centrifugation and the cell pellet was re-suspended in 2 volumes of zymolyase buffer (50 mM Tris pH 7.5, 10 mM MgCl2, 1M Sorbitol, and 1 mM DTT). The cells were then pelleted a second time and re-suspended in 2 volumes of zymolyase buffer containing 1 mg Zymolyase (Seikagaka Corp Cat#120493 104000 Units/g) per gram of packed yeast cells. The cells were incubated at 30°C with gentle rocking for 1 hour to aid in the formation of spheroblasts. Spheroblasts were then pelleted and re-suspended in zymolyase buffer lacking zymolyase. This process was repeated two times to remove any contaminating proteases. Finally, the pellet was re-suspended in 2 volumes of phosphate buffer (Na2HP04, NaH2PO4, and NaCl Ph 7.4) and the spheroblasts were lysed due to osmotic shock.. To ensure complete cell lysis1:5 volume of acid washed glass beads (0.5 mm Biospec Cat # 11079105, washed with concentrated Nitric acid) was added to the spheroblast suspension in a 50 mL conical Falcon tube and the cells were vortexed ten times for one minute with one minute on ice between vortexing steps. The lysate was then spun at 3000 RPM to remove the glass beads and the bulk of the cell wall debris. The lysate was then clarified further by centrifugation at 40,000 RPM followed by filtration through a 0.45 μm, low protein binding, syringe tip filter (Millipore).

Clarified lysates were then passed over a 1mL Pharmacia HiTrap Chelating column (Pharmacia Biotech Part 17-0408-01) which had been pre-charged with 2mL of 100mM Nickel Sulfate and washed with de-ionized, distilled water. The lysate was recirculated over the column for 1 hour at 4 C at a flow rate of 1 ml/min using a peristaltic pump P-1 (Pharmacia Biotech). To remove any non-specific interacting proteins, the column was washed with 5 ml of Buffer A (Na2HPO4, NaH2PO4 and NaCl

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pH 7.4) containing 10mM Imidazole, followed be 5 mL of Buffer A containing 100mM Imidazole and finally an additional 10 mL Buffer A containing10 mM imidazole. The UBA1 was then eluted from the column with 5 mL of Buffer A containing 500 mM Imidazole. Peak fractions were pooled and were immediately loaded on to a High Load Superdex 75 16/60 FPLC column (Pharmacia) which had been previously equilibrated with buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA and 50ug/ml BSA. Peak fractions were then pooled and run on an SDS polyacrylamide gel containing BSA standards to determine the concentration. The gel was scanned and densitomitry was performed on the UBA1 and BSA standards using the Macintosh program MacBas (Fuji Film corporation). After the concentration was determined the sample was diluted to 150 ug/ml having a final concentration of BSA at 50 ug/ml and Glycerol at 10% v/v. The sample was then aliquoted, flash frozen in liquid nitrogen and stored at -80°C.

E1-Dimer association studies – E1-dimer association was done at 30°C with 8.76 μg of purified S^{35}-labelled E2-dimer, 2.5 μg of purified E1 in Reaction Buffer (30 mM Hepes, 1mM EDTA, protease inhibitors, pH 7.5). When DTT was added, it was added to a final concentration of 50mM.

Gel filtration chromatography – Gel filtration chromatography was done on either a Hiload Superdex 75 column or a Superdex 200 sizing column. The columns were run with Superdex running buffer (50mM, Hepes, 1mM EDTA, 150 mM Nacl, 50 mg/ml BSA at 1ml/minute and 1 mL samples were collected. An elution profile was generated by counting 150 μl of each fraction by liquid scintillation programmed for S^{35} detection.

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E1-ubiquitination reaction – The ubiquitination reactions were carried out according to the methodology outlined by Hodgins and colleagues. The reaction was allowed to proceed for 15 minutes at 30°C in the presence of a 10X excess of ubiquitin. After 15 minutes, the dimer was added to the reaction, it was incubated an additional 15 minutes at 30°C and then loaded on the Highload superdex 75 column.

3. Results

3.1 Ubc1Δ450 forms a disulfide-linked dimer

Previous studies in our laboratory demonstrated that truncation of the yeast Ubc1 75 amino acid C-terminal extension all the way to the core catalytic domain results in a variant of the enzyme that autoubiquitinates itself in a canonical fashion in the presence of E1, ubiquitin and ATP (Hodgins et al., 1996). We have since adopted UBC1Δ450 as a model system for studying ubiquitination *in vitro* and have recently reported the crystal and solution structures of free and ubiquitin-thioester bound derivatives of the enzyme (Hamilton et al., 2001). Upon purification of S^{35}- labelled recombinant UBC1Δ450, we unexpectedly observed that the enzyme eluted upon gel filtration chromatography in two distinct peaks, one running at approximately 17 kD, and another running at approximately 34 kD (Figure 4-1). SDS-PAGE analysis of fractions taken from the Superdex 75 column under non-denaturing conditions demonstrated that the purified recombinant protein indeed ran in both 34 and 17kD forms (Figure 4-1). We pooled the 34kD fractions and the 17 kD fractions separately, and then ran SDS-PAGE gels under reducing and non-reducing conditions. With the addition of 10mM DTT to the Laemmi buffer, we observed a reduction of the dimer, with a concomitant collapse of the high molecular weight band to a band of 17 kD (Figure 4-2). Taken together these data

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demonstrate that recombinant Ubc1Δ450 forms a covalent disulfide-linked dimer which becomes specifically reduced in the presence of 10mM DTT.

3.2 E1 forms a high molecular weight complex with the E2 dimer

Many proteins form dimers when purified at high concentrations through oxidation of surface exposed cysteine residues. These interactions can be interpreted as artifactual because the reducing conditions within the cell would likely preclude such interactions from occurring *in vivo*. There is only one cysteine residue within the primary sequence of UBC1Δ450, that being C88, the cysteine at the active site of the molecule, to which the ubiquitin becomes attached upon activation by E1. Our observation of a reducible homodimerization of E2s shows that the two E2 molecules have become covalently attached via their respective C88 amino acids. Our tertiary structure determination of UBC1Δ450 is in agreement with other published structural solutions of E2 core catalytic domains, where the active site cysteine occurs within a cleft on the active site face of the molecule somewhat recessed within the overall surface topology of the enzyme (Jiang and Basavappa., 2001; Hamilton et al., 2001). We conjectured that while the covalent nature of the dimer is clearly artifactual,, the surface occluded by the dimerization may in fact be reflective of the true interactive surface of the molecules.

If dimerization is in fact relevant to E2 function, it is likely weak in nature, as these molecules must be free to interact with numerous different proteins in order to carry out their functions (Jiang and Basavappa., 1999). The active site face of the molecule is clearly critical to protein-protein interaction because the active site cysteine must both receive the ubiquitin on this face as well as transfer to the target protein from this face. The determinants for E1 interaction would likely then be found on this surface (Pickart, 2001). This being the case, we speculated that dimerization may be important for E1-E2

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interaction. E1 –E2 interactions are undetectable by gel filtration chromatography, presumably due to the rapidity and transient nature of the interaction. Thus, we thought that the covalent association of the E2 dimer might in fact be a useful tool to trap a structural intermediate in E1-E2 interactions. We obtained 6XHis-tagged Uba1 or E1 from yeast by nickel column chromatography. SDS-PAGE analysis followed by Coomasie staining revealed that Uba1 was present as a highly pure 110kD band on the gel with no observable contaminating proteins (Figure 4-3)

We therefore tested purified E2 dimer in the presence and absence of purified recombinant yeast E1 to see if an E1-E2 interaction could be detected (Figure 4-4). We observed that in the absence of E1, a 0.27 nM concentration of the tracer S^{35}- labelled UBC1Δ450 dimer was stable, eluting as a single peak with an apparent molecular weight of 40 kD. Upon the addition of a 0.1 molar equivalent of yeast E1, a portion of the peak corresponding to E2 dimer noticeably shifted to a higher molecular weight is clearly observable. SDS-PAGE followed by autoradiographic analysis of the peak fractions clearly demonstrated that upon addition of purified E1, an observable proportion of the peak only containing E2 dimer was shifted into the High Molecular Weight peak. Also observable is the presence of a smaller peak corresponding to E2 monomer. Sizing of the high molecular weight peak by Superdex 200 gel filtration chromatography revealed an apparent molecular weight of 150 kD, approximately corresponding to two E2 molecules (34 kD), and one 110 kD E1 molecule.

3.3 E1-binding reduces disulfide-linked dimer to monomer

Having observed an *in vitro* association between E1 and E2, we proceeded to study the interaction as a time course in an effort to examine the stability of the complex (Figure 4-5). In the last experiment we observed that the addition of E1 resulted in

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reduction of the dimer to monomer. In this experiment, after 30 minutes of E1-dimer incubation, the third labelled peak, corresponding to E2 monomer, becomes prominent with a corresponding reduction in the size of the labelled dimer peak. When left overnight, the dimer becomes completely reduced to monomer, with little neither E1-E2 or E2-E2 complexes left. This is clearly observable both by gel filtration chromatography and by SDS-PAGE analysis of the peak fractions followed by autoradiography. From this data, we conclude that the addition of purified E1 to the dimer results in the catalytic reduction of the disulfide linked dimer to monomer.

3.4 Ubiquitination not derivitization of the E1-active site stabilizes the E1-dimer interaction

The catalytic reduction of the E2 dimer, we thought, was likely due to the presence of the highly reactive active site cysteine residue present on the E1 molecule, disrupting the disulfide-bond beween the two E2s. We therefore speculated that stabilization of the complex might occur if prior to the addition of E1 to the incubation, we doubly charged it with ubiquitin (Figure 4-6). We also thought that perhaps that E1 with two ubiquitins is likely a more relevant biological substrate for the dimer and for that reason as well the interaction might be more stable. We observed that the incubation of pre-charged E1-ubiquitin with labeled dimer resulted in a dramatic proportional increase in the amount of dimer bound within the high molecular weight complex with no observable reduction of the dimer to monomer.

To test if derivitization of the E1 active site cysteine residue might in some way stabilize the E1-dimer interaction, we also pre-treated the E1 with 10 mM iodoacetamide an alkylating agent that is specifically reactive against free thiol groups. We observed that iodoacetamide-treated E1 was entirely unable to interact with the disulfide-linked

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dimer, and no observable dimer reduction was seen (Figure 4-6). These results seem to indicate that the active site surface of E1 is directly responsible for interaction with the dimer, and further confirmed the fact that the E1 cysteine residue was a contributing factor in the observed reduction of the disulfide-linked E2.

4. Discussion

The evidence presented in this study seems to indicate that E2s covalently linked within a dimeric form can serve as a surface for E1 interaction. This is an interesting observation given the number of reports regarding E2 dimerization and the general lack of knowledge regarding the functional significance of such an interaction.

Previously in our lab, crosslinking studies on Cdc34 from yeast demonstrated a requirement for the C-terminal tail mediating homotypic interactions (Ptak et al., 1994). We have recently demonstrated, however, that while the tail may be involved in stabilizing dimeric interactions, the core catalytic domain of Cdc34 is sufficient for dimer formation *in vivo* (Varelas et al., manuscript in preparation). Results from that study further confirm the results presented here with the observation that surface determinants for thiolester production and self-association overlap, indicating that dimerization occurs through interaction of the conserved active site surface of the E2. It also might indicate that ubiquitination of the active site cysteine on E2 might abolish the dimeric interaction, which makes intuitive sense if the dimer form is required for E1-binding. Also, the fact that charging E1 prior to incubation with E2 dimer facilitates formation of the high molecular weight complex seems to indicate that ubiquitin interactions with the dimer might also play a role in recognition of E2 by E1. The role of N-or C-terminal extensions in dimerization has also been observed in another study (Leggett and Candido., 1997). In results similar to those generated in our lab, these authors demonstrated that the core

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catalytic domain of the *C. elegans* Ubc-1 is both sufficient and necessary for dimerization, whereas the C-terminal extension was found to be an important factor in contributing to the stability of the complex. Our own molecular modeling efforts based on the crystal structure of Ubc1Δ450 and the observed disulfide-bond formation between two monomers suggest an interaction between monomers in a tail-to-head configuration (Figure 4-7; T. Huzil unpublished observations). This interaction creates a hydrophobic pocket on the catalytic surface of E2 for the potential E1-dimer interaction.

Interestingly, the previously mentioned dimeric crystal structure solution of a Ubc-1 from clam did not interact via residues found on its catalytic surface. In fact the determinants for dimerization were found to localize to a region on precisely the opposite face of the molecule (Jiang and Basavappa., 1999). While these authors are unclear whether or not the observed dimer was a crystallographic artifact, another possibility is that different Ubcs dimerize differently. If this is indeed the case, then a functional explanation for dimerization would likely be more complex than simply mediating E1-E2 interaction. While these results are *in vitro* results using recombinant forms of the proteins, they do provide the basis to speculate that E2 dimerization is a functional phenomenon.

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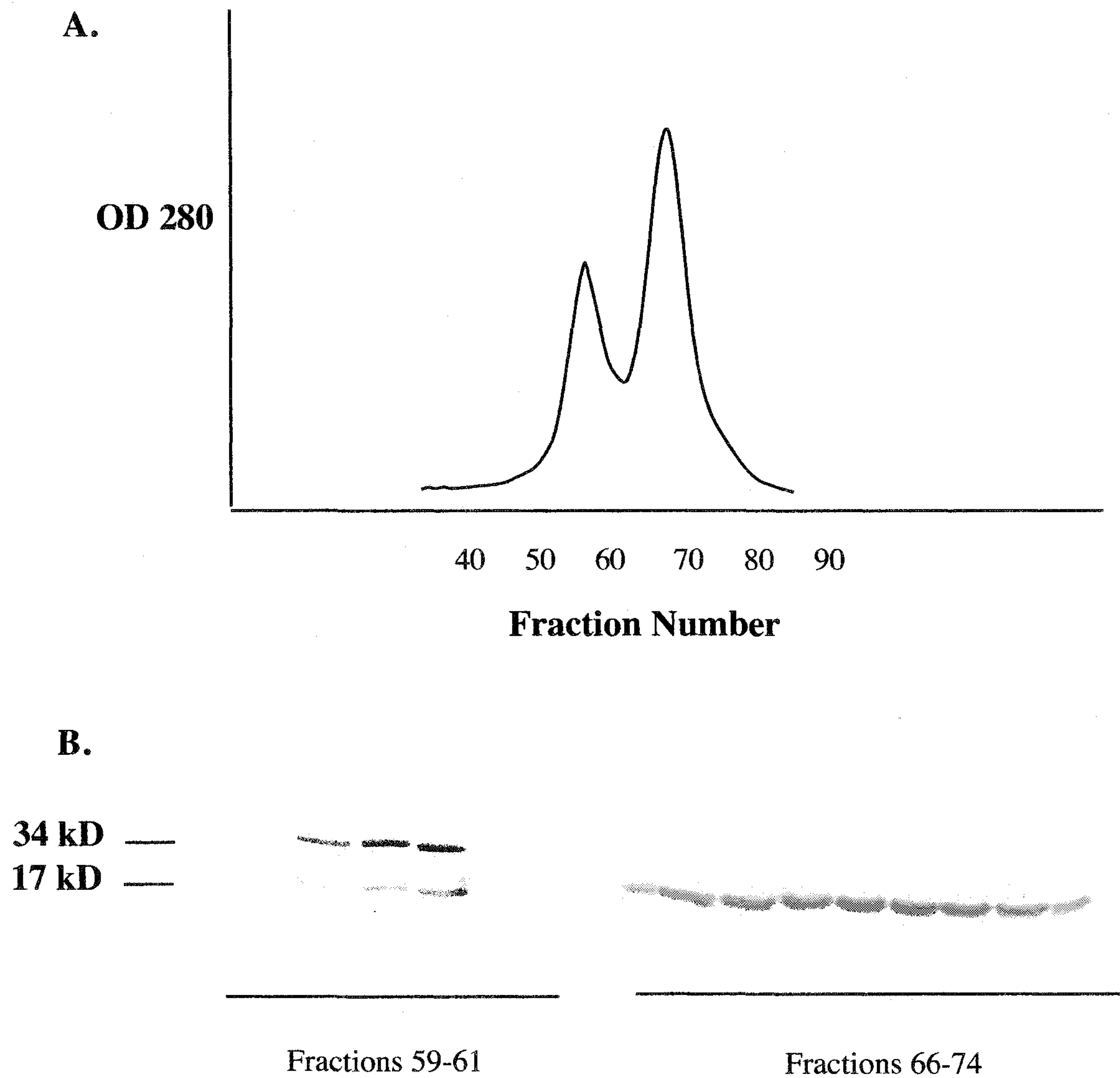

Figure 4-1. Ubc1Δ450 elutes as a dimer.
Hiload Superdex 75 gel filtration chromatography of purified recombinant Ubc1Δ450 demonstrates that it elutes in two distinct protein peaks (Panel A). SDS-PAGE analysis and Coomasie Blue staining of the indicated fractions indicate two distinct species of 34 kD and 17 kD (Panel B).

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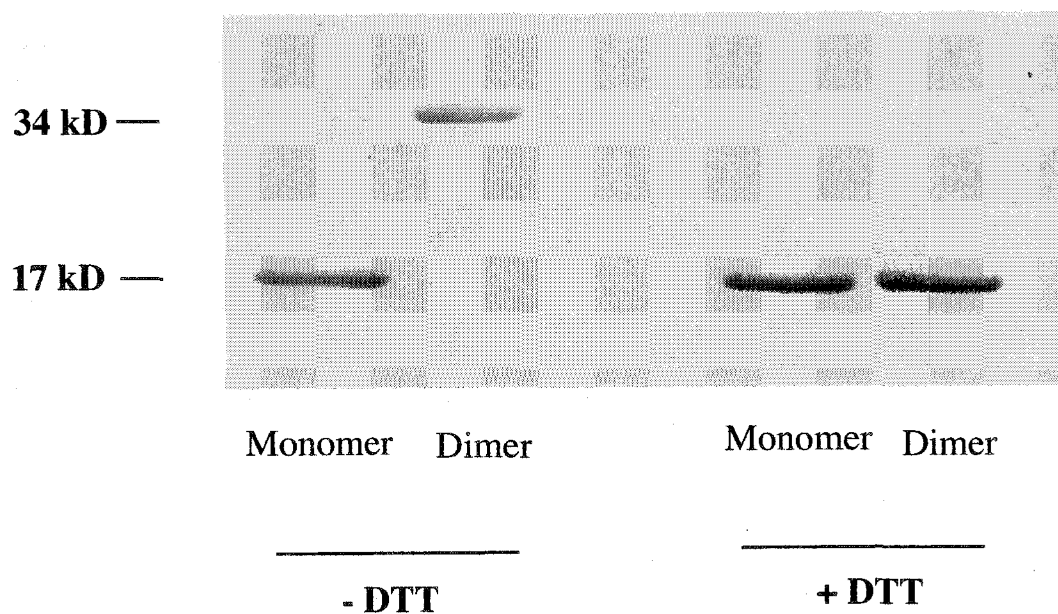

Figure 4-2. Ubc1Δ450 dimer is DTT sensitive.
SDS-PAGE and Coomasie Blue staining of the Ubc1Δ450 dimer under reducing and non-reducing conditions demonstrates that the dimerization is formed by disulfide-bond formation.

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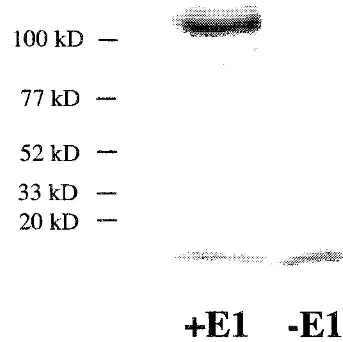

Figure 4-3. Uba1 purification.
6XHis-tagged yeast Uba1 was purified to near homogeneity from a yeast cell lysate by nickel column chromatography and visualized by SDS-PAGE followed by Coomasie Blue staining. Uba1 runs as a highly pure 110 kD band. A yeast lysate not expressing 6XHis-Uba1 was used as a control.

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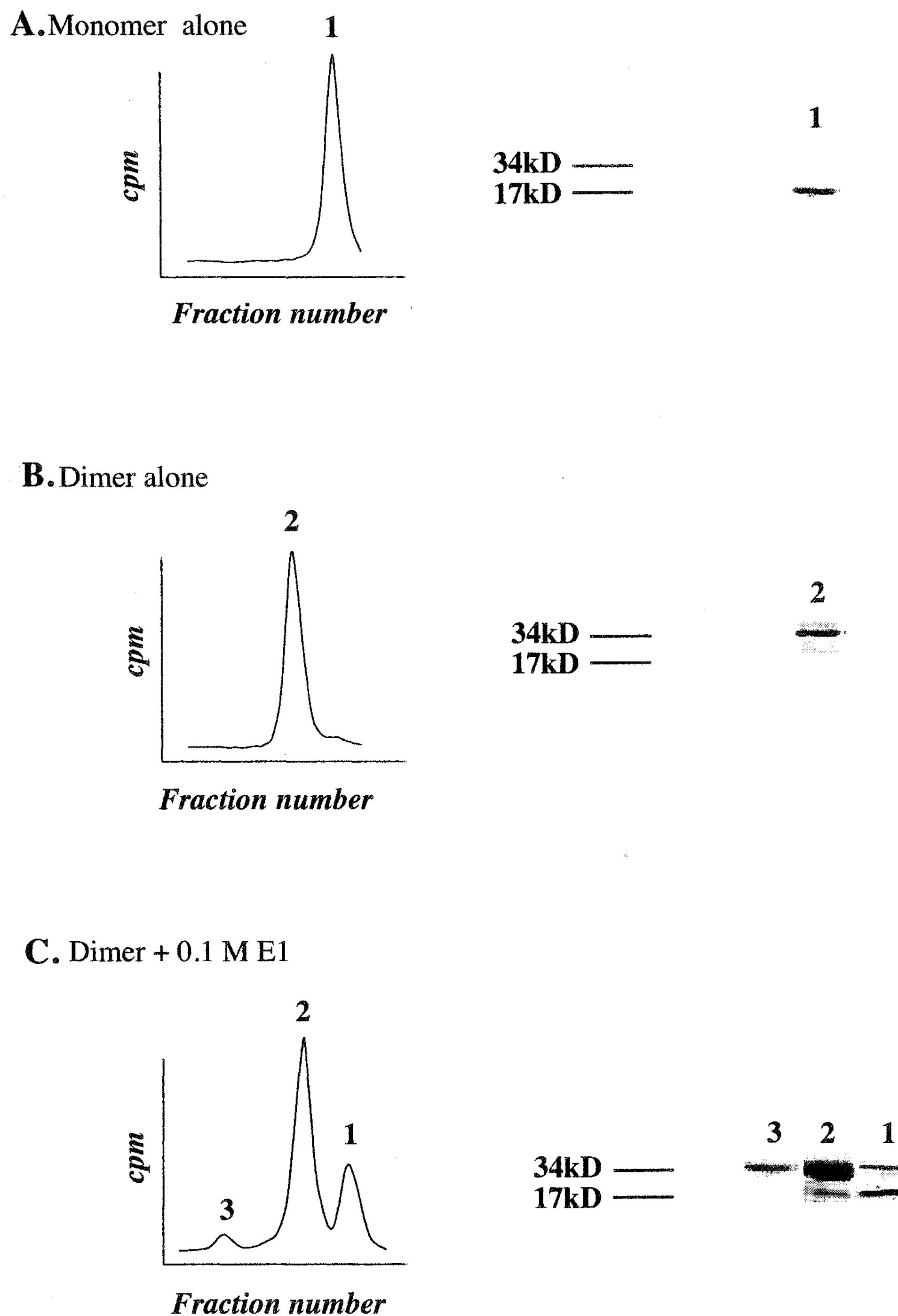

Figure 4-4. Addition of purified E1 to purified Ubc1Δ450 dimer results in a high molecular weight complex containing dimeric E2. Purified S^{35}-labelled Ubc1Δ450 dimer (Panel A), monomer (Panel B) and dimer plus 0.1 molar equivalent of E1 (Panel C) were subjected to Hiload Superdex 75 gel filtration chromatography. 15 μl of each peak fraction was run on SDS-PAGE under non-reducing conditions and visualized by autoradiography.

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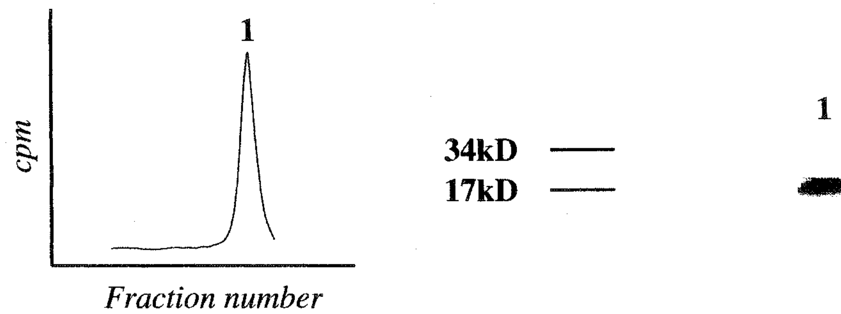
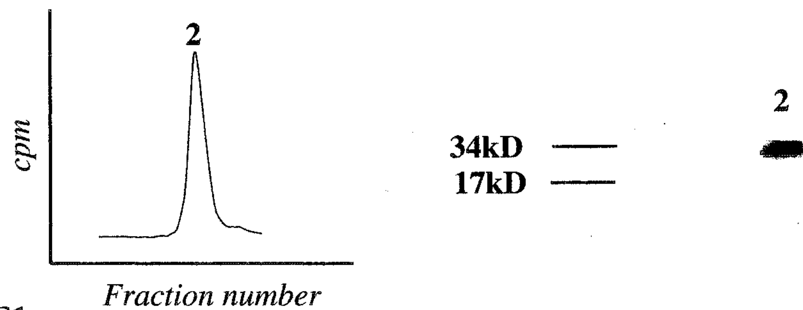
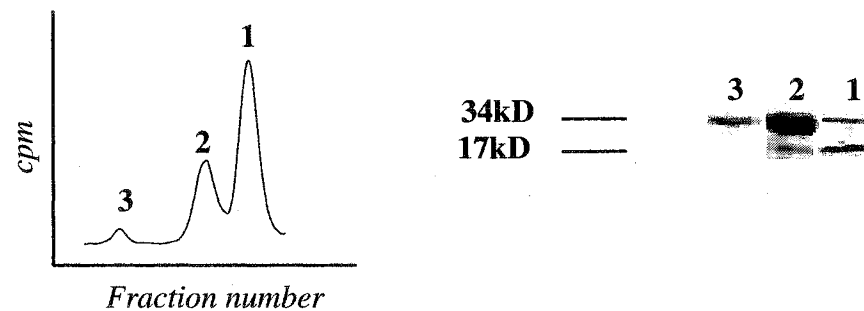
A. Monomer alone



B. Dimer alone



C. Dimer + 0.1M E1
30 mins 30°C



D. Dimer + 0.1M E1
Overnight 30°C

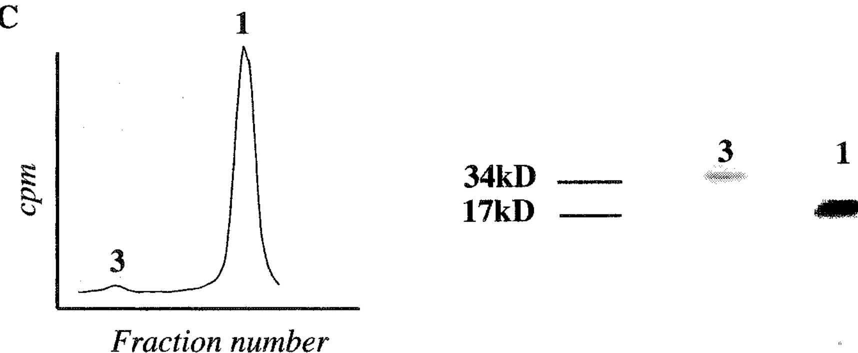


Figure 4-5. E1 catalytically reduces Ubc1Δ450 dimer to monomer.
Purified S^{35}-labelled Ubc1Δ450 monomer (Panel A), dimer (Panel B), dimer incubated with 0.1M E1 for 15 mins at 30°C (Panel C), and dimer incubated with 0.1M purified E1 overnight at 30°C (Panel D) were subjected to Hiload Superdex 75 gel filtration chromatography. 15 μl of each peak fraction was run on SDS-PAGE under non-reducing conditions and visualized by autoradiography.

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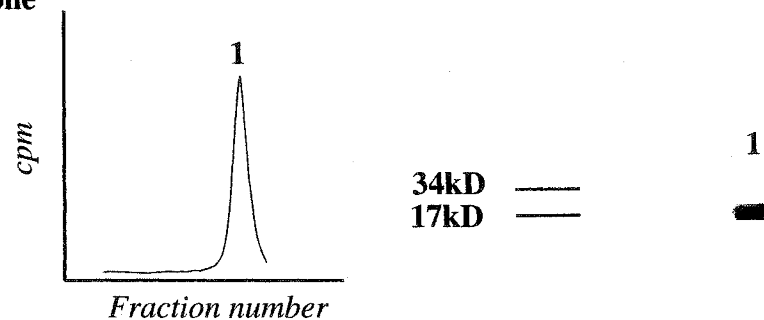
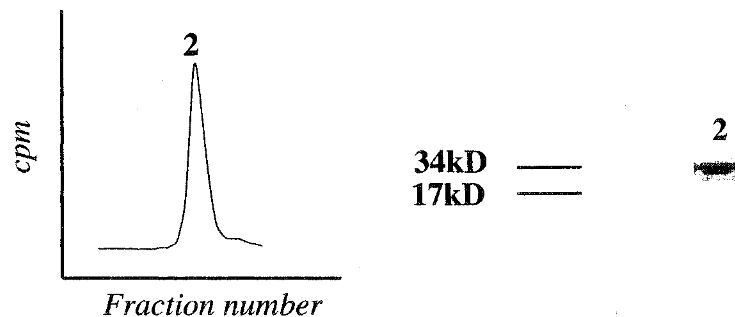
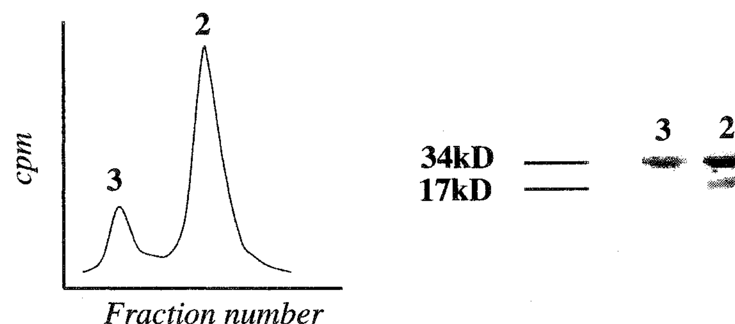
A. Monomer alone



B. Dimer alone



C. Dimer + E1 (Ubiquitin)



D. Dimer + E1 (10mM IA)

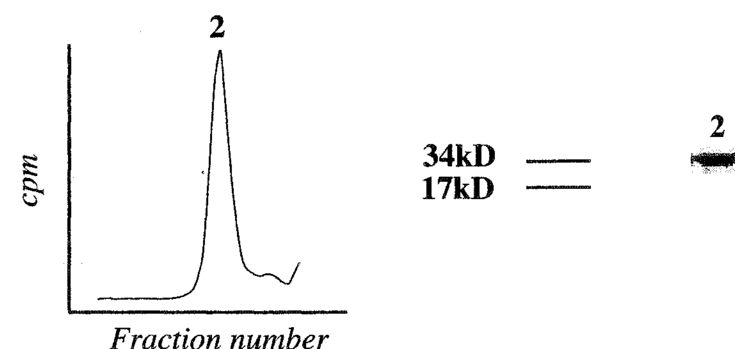


Figure 4-6. Ubiquitinating but not alkylating E1 stabilizes E1-Ubc1Δ450 dimer interactions. Purified S^{35}-labelled Ubc1Δ450 monomer (Panel A), dimer (Panel B), dimer incubated with 0.1M E1 which had been pre-ubiquitinated for 15 mins at 30°C (Panel C), and dimer incubated with 0.1M purified E1 pre-treated with 10mM iodoacetamide for 15 minutes at 30°C (Panel D) were subjected to Hiload Superdex 75 gel filtration chromatography. 15 μl of each peak fraction was run on SDS-PAGE under non-reducing conditions and visualized by autoradiography.

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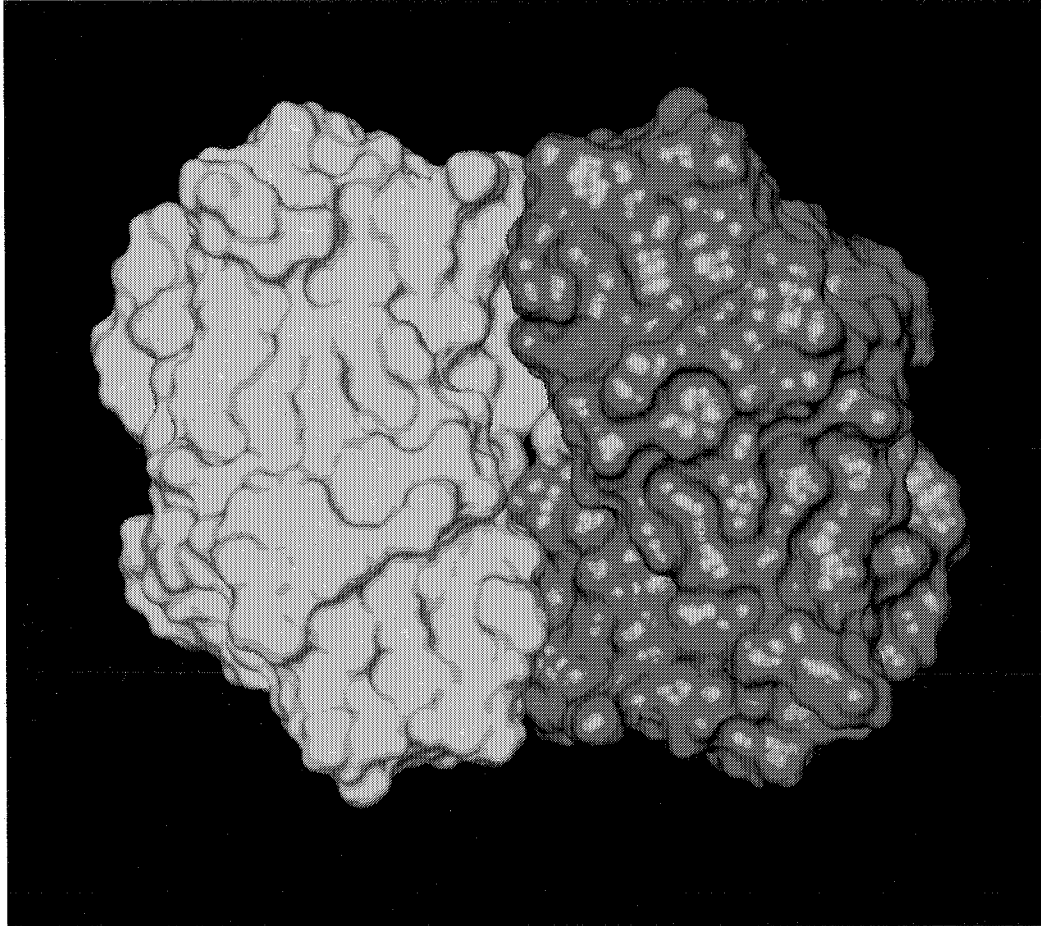

Figure 4-7. Molecular Modeling of the E2 dimer. Modeling of the Ubc1Δ450 dimer based on the crystal structure of the monomer indicates a possible conformation where the monomers associate in a tail-to-head manner (Connolly surfaces of each monomer are rendered in dark blue and light blue), with the active site Cys88 residues (in yellow) covalently interact via a disulfide bond.

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CHAPTER V – SUMMARY AND DISCUSSION

Introduction

The Cdc34/SCF complex is an important regulator of cell cycle progression in the yeast *Saccharomyces cerevisiae*. It is a modular, multiprotein, ubiquitin conjugation and ligation complex that functions by mediating the covalent attachment of poly-ubiquitin chains to specific substrate proteins at required intervals within the cell growth cycle. Once a protein is modified in this manner, it is generally but not exclusively degraded by the 26S proteasome (reviewed by Desalle and Pagano, 2001). The Cdc34/SCF complex often acts as a secondary level of regulation, positively influencing progression through a biochemical pathway by causing the degradation of short lived regulatory proteins such as Sic1 and Far1, or proteins that have specific short term functions such as Cdc6 and the G1 cyclins. It generally recognizes phosphorylated targets, and as such is itself subject to tight regulatory controls. In this thesis novel genetic and biochemical roles for the essential yeast ubiquitin conjugating enzyme Cdc34 have been presented. This chapter will review and discuss the results presented in this thesis within the context of the current literature, and conjecture avenues of future work in this area.

2. Cdc34/SCF complex, cell integrity and the cell cycle

The principal arguments presented in Chapters II and Chapter III this thesis are that the Cdc34/SCF complex plays an important regulatory role in the maintenance of cell integrity under stress, and this role is directly integrated into its ability to regulate the cell cycle at the G1/S transition. Our discovery and analysis of this idea is based on genetic work with temperature-sensitive mutant alleles of *CDC34* and the SCF components *CDC4* and *CDC53*. Moreover, we have identified numerous genes whose overexpression suppresses either the cell integrity defects, the cell cycle defects, or both

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defects simultaneously in these same mutants alleles. While the mechanism by which cell cycle progression, cell integrity signaling, and protein ubiquitination is not clarified by these studies, this thesis provides novel hypothesis generating insight into all three of these important fields.

2.1 Cdc34/SCF mutant alleles

The foundation of this work principally rests on the unusual biological and biochemical properties of the *cdc34-2*, *cdc34-3*, *cdc53-1* and *cdc4-3* mutant alleles. These alleles have been well characterized and have been used very often in biochemical and genetic studies of the cell cycle and the ubiquitin system. Phenotypic characterization of numerous yeast strains bearing point and truncation mutations of *CDC34* have demonstrated a variety of growth abnormalities when these strains are incubated at restrictive temperatures. Specifically, these mutant strains accumulate Cdc34/SCF targets including Sic1, Cln1, and Cln2, exhibit a post-START cell cycle arrest prior to the onset of DNA replication, and exhibit severe morphological defects including hyperpolarized growth and multiple budding from the same budsite (Goebl et al., 1988; Kolman et al., 1992; Pitluk et al., 1995). Furthermore, temperature-sensitive mutant alleles *cdc53-1* and *cdc4-3* exhibit identical growth defects to *CDC34* mutant alleles and as such were consequently identified as components of the same functional complex (Goebl et al., 1988; Mathias et al., 1996). Cross suppression studies have demonstrated that while plasmid overexpression of *CDC34* and *CDC53* will suppress the morphological and cell cycle arrest phenotypes of the *cdc34-2* and *cdc53-1* alleles, the *cdc4-3* allele is impervious to suppression by any gene except *CDC4*, presumably due to substrate-binding function of Cdc4 within the SCF complex (Mathias et al., 1996). From a biochemical standpoint, point mutations in *CDC34* are likely to have one of three possible effects: they may render the protein structurally unstable due to the alteration of

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key residues, they may render the enzyme catalytically unstable, where at high temperatures either the acceptance or transfer of ubiquitin is impaired, or they may impair key protein-protein interactions important for the function of the molecule. Based on our own alignment, crystallographic, and NMR studies of the core catalytic domain of ubiquitin conjugating enzymes, we have been able to determine the position of various Cdc34 point mutations with respect to the catalytic face of this molecule containing the active site C95 residue (Hamilton et al., 2001). The mutations associated with the *cdc34-3* allele, which bears the substitutions in Arg65, Phe72, and Ser73, map to a loop on the catalytic surface overhanging the most highly conserved region the molecule (Figure 5-1; Ptak et al., 2001). Mutations present on the active site face of the molecule are more inherently likely to impair the function of the enzyme, as it is this surface that is required for E1 interaction, ubiquitin interaction, catalytic activity, and substrate ubiquitination. One can therefore speculate that the *cdc34-3* allele is temperature-sensitive due to the impairment of the catalytic face of the molecule.

By contrast, the *cdc34-2* allele bears a single Gly58Arg point mutation. Interestingly, the Gly58 residue, a highly conserved glycine residue in yeast ubiquitin conjugating enzymes (Ubcs), is found on a relatively unconserved non-catalytic face of the molecule (Figure 5-2; Ptak et al., 2001). The functional ramifications of the *cdc34-2* allele are not at all clear. The conserved Gly58 residue is a surface residue on yeast Ubcs, and is therefore unlikely to make a structural contribution. In agreement with this, our own molecular dynamic studies of this mutant have suggested that such a mutation does not induce thermolability of the tertiary structure of the Cdc34 protein at high temperatures (T. Huzil personal communication). The possibility that Gly58 may in some way participate in Cdc34 thiolester formation is also unlikely. NMR studies on thiolester formation with the core catalytic domain of yeast Ubc1 have demonstrated that the residues involved in E1 and ubiquitin binding surround the active site cysteine residue

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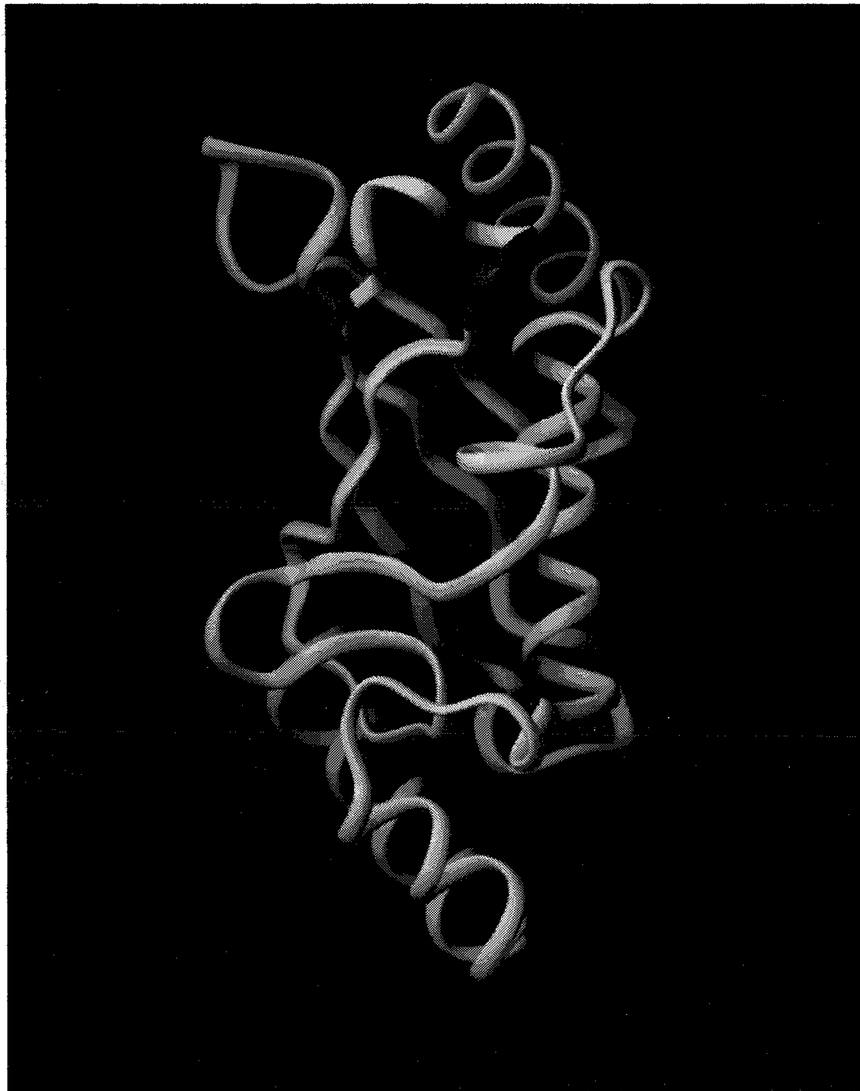

Figure 5-1. The *cdc34-3* allele bears mutations in conserved residues on the catalytic face of the molecule. Molecular modeling of the core catalytic domain of Cdc34 based on the crystal structure of the core catalytic domain of Ubc1 reveals that the conserved residues Arg65, Phe72 and Ser73 (in red) which are mutated in the *cdc34-3* allele flank a loop on the conserved catalytic face of the enzyme above the active site Cysteine residue (in yellow).

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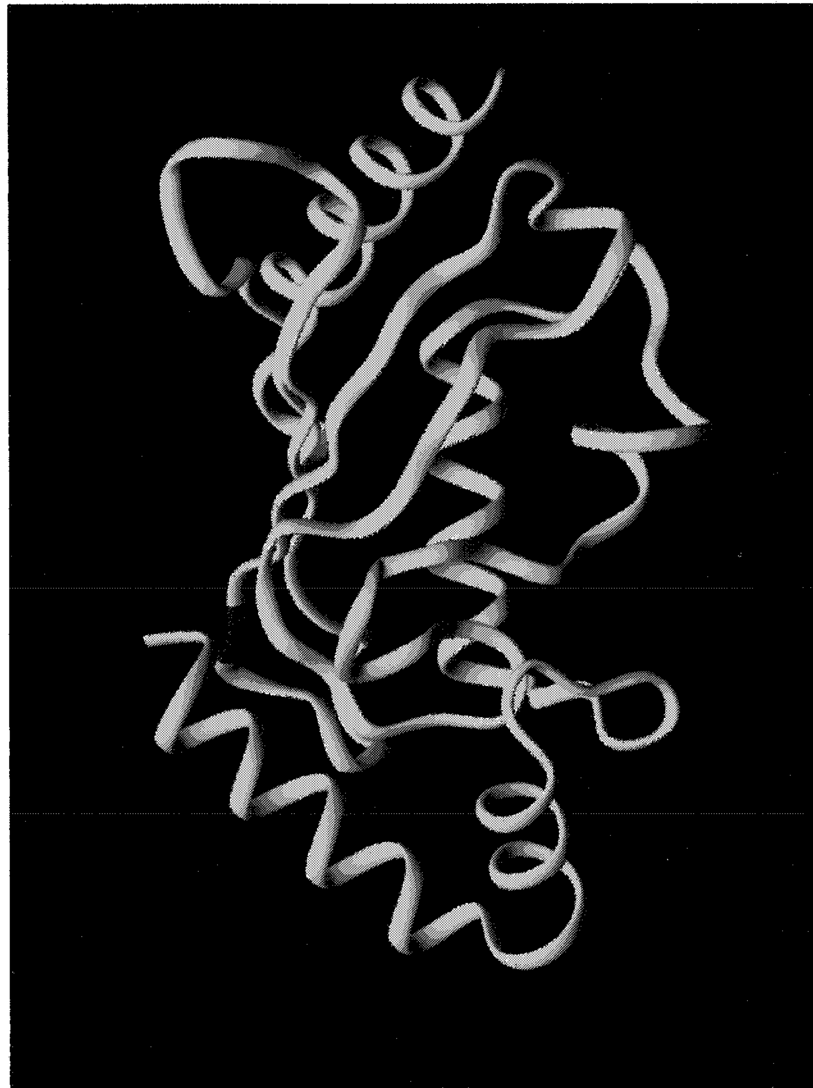

Figure 5-2. The Gly58Arg mutation of the *cdc34-2* allele is on a non-catalytic face of Cdc34. Molecular modeling of the core catalytic domain of Cdc34 based on the crystal structure of the Ubc1 core catalytic domain reveals the conserved Gly58 of Cdc34 (in red) is located on the opposite side of the molecule from catalytic C95 residue of the active site (in yellow; Ptak et al., 2001).

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and occur on the catalytic face of the molecule, significantly removed from the Gly58 residue (Hamilton et al., 2001; Figure 5-3). A third possibility, that Cdc34-SCF association in some way involves the Gly58 residue, is also inconsistent with the structural evidence. Biochemical studies of Cdc34/SCF interactions have revealed that the important SCF-binding determinant is located within the 295-residue carboxy-terminal tail domain (Mathias et al., 1998). This binding determinant, which occurs from amino acids 170-209, is required for Cdc34-SCF interaction and Sic1 degradation *in vivo*. As such, a Cdc34 mutant truncated to amino acid 209 can fully complement a Cdc34 deletion strain. In addition, molecular modeling of Ubc7 in association with the SCF suggest that the surface on which G58 is found does not participate in E2- SCF interactions (Zheng et al., 2002). Cdc34 molecules are also known to dimerize *in vivo* and *in vitro*, thus the possibility arises that Gly58 may in some way participate in dimerization. Recent immunoprecipitation results in our laboratory have revealed the surface determinants for Cdc34 self-association occur within the core catalytic domain of the molecule and overlap with those of thiolester formation (X. Varelas, manuscript in preparation). These *in vivo* results are also consistent with the *in vitro* results presented in Chapter IV of this thesis, where we demonstrate that artificial dimerization of Ubc1Δ450 via its catalytic surface forms a complex that associates with E1. Therefore mutation of the surface Gly58 residue, which does not appear to be required for Cdc34 structural integrity, catalytic activity, SCF interaction, or ubiquitin interaction, impairs the function of Cdc34 by an unknown mechanism.

The functional basis of the *cdc4-3* and *cdc53-1* allelic mutations is much less speculative than the *cdc34-2* allelic mutation. While the three dimensional structure of the Cdc4 protein has not yet been solved, analysis of its primary structure suggests that like other F-box proteins, it consists of two distinct domains: an N-terminal F-box domain required for its interaction with the SCF, and a C-terminal protein-binding

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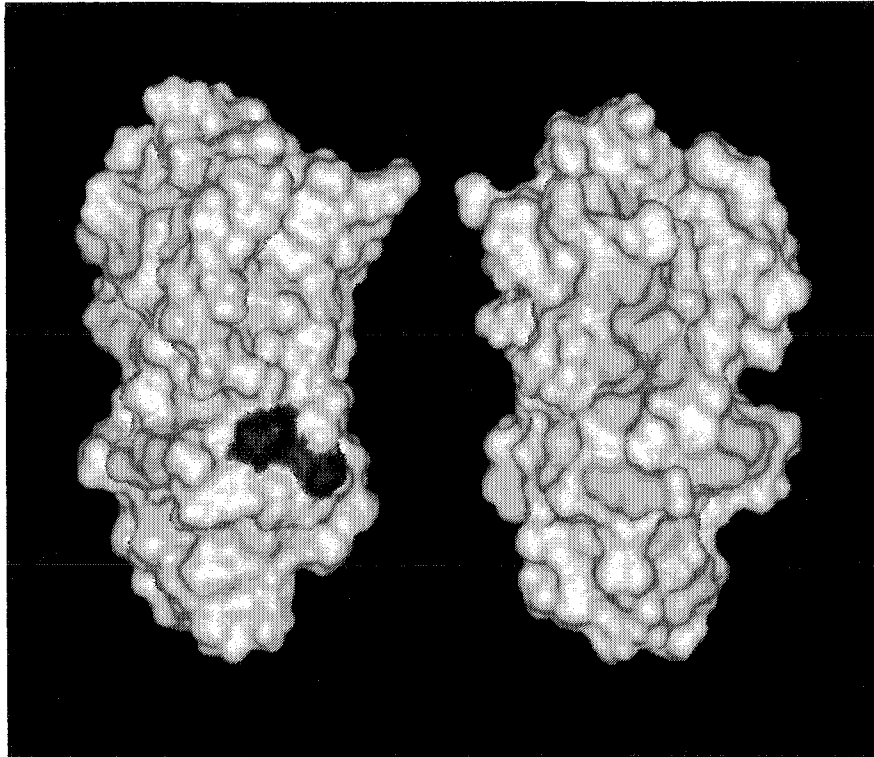

Figure 5-3. Gly58Arg does not likely impair the ability of an E2 to form thiolester with ubiquitin. Based on the NMR structure of Ubc1Δ450 thiolester, modeling of the key residues involved in E2-ubiquitin interaction (in green) reveals that they surround the active site cysteine residue (in yellow) and are removed from the conserved Gly58 residue (in red; Hamilton et al., 2001).

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domain consisting of WD-40 repeats by which it recruits substrates to the SCF (Feldmann et al., 1997). The mutation in the *cdc4-3* allele has been mapped to the WD-40 repeat region, and hence is thought to destabilize Cdc4-substrate interaction (Mathias et al., 1998). The *cdc53-1* allele bears mutations in its conserved Cul1 domain, which occurs at the extreme C-terminus of the molecule. The recently solved structure of human Cul1 bound to Rbx1 reveals that the yeast *cdc53-1* mutations map to a long α-helical segment of the Cul1 domain that is thought to stabilize both Rbx1 and Ubc interactions (Zheng et al., 2002; Figure 5-4). It is therefore reasonable to infer that the *cdc53-1* mutant is impaired in its abilities to bind Cdc34 or Rbx1, which is consistent with the genetic observation that Cdc34 overexpression rescues the *cdc53-1* mutant at high temperatures as previously described (Mathias et al., 1996).

2.2 Cell integrity defects and the *CDC34*, *CDC4*, and *CDC53* mutant alleles

When considering the argument of defective cell integrity with Cdc34/SCF mutants, one must consider very carefully the abnormal morphological characteristics of these mutants when grown at restrictive growth temperatures. In the late G1 phase of the cell cycle, apical growth is initiated, where the structural and secretory apparatus of the cell is polarized towards the budsite (Casamayor and Snyder, 2002). The failure of Sic1 degradation in *cdc34-2*, *cdc53-1* and *cdc4-3* cells at the restrictive temperature results in a constitutive period of apical growth with these mutants, generating the well described elongated and multibudded phenotype characteristic of these mutants. Genetic insertion/disruption mutagenesis within a *cdc34-2* background has recently identified numerous genes involved in the maintenance of hyperpolarized growth (Bidlingmaier and Snyder, 2002). Genes involved in intracellular transport, actin polymerization, cell wall biogenesis, and signal transduction were among the genes identified in this study. As such, one might argue that our observation that the *cdc34-2*, *cdc53-1* and *cdc4-3* mutant

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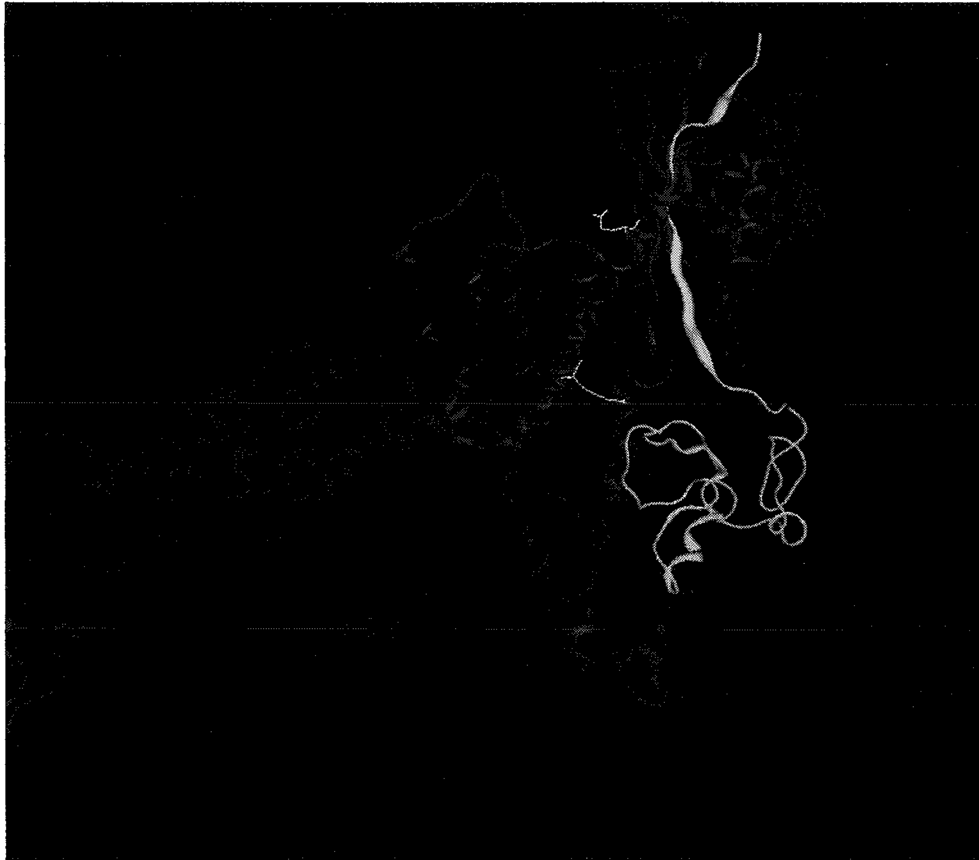

Figure 5-4. The *cdc53-1* mutant bears mutations in the conserved C-terminal Cul domain. The crystal structure of the human SCF complex reveals that Arg472 and Glu485 (in yellow) which are mutated in the yeast *cdc53-1* mutant occur on an alpha helix shown here in the conserved C-terminal domain of human Cul1 (in red) involved in stabilizing its interactions with both Rbx1 (in green) and the ubiquitin conjugating enzyme (based on the Cul1-Rbx1structure by Zheng et al., 2002).

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alleles appear to have cell integrity defects might simply be a consequence of the hyperpolarized growth morphology, completely independent of a direct regulation of signaling through this pathway. We controlled for this likelihood by conducting our experiments under growth conditions where such growth hyperpolarization is not evident, and the cells maintain normal growth morphologies. At permissive growth temperatures, *cdc34-2* cells do not exhibit apical bud growth abnormalities and as such are not impaired in processes such as mating (Bidlingmaier and Snyder, 2002). As Sic1 deletion has been found to suppress the cell cycle arrest phenotype and growth morphology defects of *CDC34* temperature-sensitive mutants, it would be informative to conduct similar experiments in a *sic1Δ* strain bearing the same mutant alleles to determine the role, if any, of growth polarization in the observed Cdc34/SCF cell integrity defects.

Another key observation that suggests a specific cell integrity defect associated with Cdc34/SCF mutants is our demonstration that the cell integrity defects of the *cdc34-2* cells are specific to that mutant allele. The *cdc34-3* mutant also exhibits hyperpolarized growth defects do to the accumulation of Sic1. However, even when growing on YPD/SDS plates within two degrees of its restrictive growth temperature the *cdc34-3* mutant did not exhibit sensitivity to the detergent. This argues that the *cdc34-2* mutant is in some way unique in its sensitivity to cell integrity stress. We have previously observed unusual properties of this allele in relation to the suppressive effects of the *UBS1* gene (Prendergast et al., 1996). In that study, *UBS1* overexpression specifically suppressed cell cycle defects associated with the *cdc34-2* allele only, and not the *cdc34-3* or *cdc34-1* alleles. Taken together these results argue strongly that the *cdc34-2* allele is specifically defective in the cell integrity pathway, apart from its clear involvement in cell cycle regulation.

The cell integrity impairment we observed with these mutant alleles we found to be suppressed by high copy overexpression of various yeast cell integrity genes. Our first

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observation in this study was an observed two-hybrid interaction between Ubs1 and Hkr1, a protein involved in β-glucan synthesis and budsite selection. Unfortunately due to an inability to tag Hkr1 *in vivo*, we were unable to demonstrate a biochemical association between Ubs1 and Hkr1, a standard of proof that would demonstrate unequivocally an interaction between these two proteins. This standard of proof becomes even more relevant when we consider that Ubs1 was previously shown to be a predominantly nuclear protein nuclear that suppresses defects associated with the nuclear transport protein Yrb1 (Baumer et al., 2000). In that study, localization of Ubs1 was done by N-terminal GFP tagging the protein followed by *in vivo* fluorescence localization. The authors indicate in the text of their paper that the GFP-tagged protein was functional in its ability to suppress the temperature sensitivity of the *yrb1-51* mutant yeast strain. We addressed this same issue by GFP-tagging the C-terminus of Ubs1 and followed its localization by *in vivo* fluorescence confocal microscopy. We observed a nuclear accumulation of Ubs1-GFP in wild-type cells at 37°C, but we observed a punctate cytoplasmic and bud tip accumulation of the protein in *cdc34-2* cells at 37°C. Furthermore, we also observed partial suppression of the *cdc34-2* temperature-sensitivity phenotype by Ubs1-GFP at the restrictive temperature, which suggests that it is a functional protein. We argue therefore that Ubs1 functions within nucleus and within the cytoplasm and it is specifically transported into the nucleus. This speculation is substantiated by the observation that Ubs1 fails to accumulate in the nucleus of *yrb1-51* mutants at the restrictive temperature (Baumer et al., 2000). This may also indicate that *cdc34-2* cells are defective in nuclear transport, though no other evidence for this has been demonstrated. The localization of Ubs1 to the site of polarized cell growth is significant, in that Hkr1 was previously identified as a membrane protein involved in the remodeling of the cell wall, therefore in the absence of direct biochemical data, we have demonstrated that Ubs1 localizes and may function within the site of the growing bud.

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Finally, we were able to demonstrate that *UBS1*, *HKR1*, and *KNR4* overexpression resulted in the strong suppression of cell integrity defects associated with the *cdc4-3* and *cdc34-2* mutant alleles. The inability of Ubs1 to suppress the cell cycle defects of the *cdc4-3* is very significant to us because it indicates that Ubs1 functions very strongly as a cell integrity suppressor and weakly as a cell cycle suppressor. As previously indicated, the *cdc4-3* allele is defective in substrate recognition, therefore we would argue that the substrate recognized by Cdc4 in the cell integrity pathway is independent of its cell cycle function (i.e. Cdc4 recognizes a specific cell integrity substrate apart from Sic1). Given that the *cdc53-1* allele exhibits the same polarized growth phenotype as *cdc4-3* and *cdc34-2* and is not suppressed in its cell integrity or cell cycle defects by overexpression of any of these genes suggests to us that these genes are not generally stabilizing cells with polarized growth phenotypes, but rather in some way facilitating the recognition of a substrate by the SCF complex, or bypassing a specific defect present in yeast Cdc34/SCF mutants. Both possibilities indicate that the Cdc34/SCF complex ubiquitinates a specific cell integrity target, most likely a negative regulatory factor as our data demonstrates clearly that impaired Cdc34/SCF function results in impaired cell integrity. Also the identification of *KNR4* as a strong cell integrity suppressor of the *cdc34-2* and *cdc4-3* cell integrity defects is extremely interesting. Knr4 has recently been identified as a component of the Pkc1 cell integrity signaling pathway (Martin-Yken et al., 2002a). The same group has also demonstrated that Knr4 immunoprecipitates with the Slt2 kinase and modulates its activity towards Rlm1 stimulation and away from SBF activation (Martin-Yken et al., 2002b). Thus Knr4 is a regulatory component of a key cell integrity signaling pathway that is activated by cell integrity stress and cell cycle progression. While the case we have made for positive regulation of cell integrity by the Cdc34/SCF complex is circumstantial, it would be very difficult to argue against the data by suggesting that it is simply a generic artifact of an

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unstable cellular morphology in these mutants given the sheer abundance of genetic data. Rather it is our assertion that the data taken together indicate that the Cdc34/SCF complex regulates cell integrity specifically and independently of its effects on the regulation of the cell cycle.

2.3 Dual regulation of cell integrity and the cell cycle by the Cdc34/SCF complex

Perhaps the most intriguing observations of this thesis involve the dual regulation of cell cycle and cell integrity by the Cdc34/SCF complex. The initial unexpected result linking these two distinct pathways came from our genetic result demonstrating that osmotic stabilization of the cell wall with1M sorbitol allows the *cdc34-2*, *cdc4-3*, and *cdc53-1* yeast mutant strains to divide at non-permissive temperatures. This is a striking result these mutants are thought to be impaired in cell cycle progression, and as such one would not expect sorbitol suppression to influence these mutants in such a dramatic manner. Sorbitol is a sugar alcohol that is thought to function as an osmotic stabilizer, decreasing the outward turgor pressure exerted by the membrane on the cell wall (Cid et al., 1995). *In vitro*, however, sorbitol has been shown to facilitate molecular crowding and increase the efficiency of biomolecular reactions though there is no evidence that such a mechanism occurs within living cells. While one might argue that sorbitol has non-specific intracellular effects, the fact that is not a generalized suppressor of yeast temperature sensitive mutations, and very definitely plays a role as a high osmolarity cell wall stabilizer in a large number of biological systems argues that it performs a cell integrity function rather than a molecular stabilization function within cells. Thus the fact that cell wall stabilization results in the G1/S cell cycle progression for Cdc34/SCF temperature-sensitive yeast mutants at high temperatures is consistent with the idea that an essential function of this complex is directly linked to its ability to regulate cell integrity.

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Interestingly, yeast growth on high osmolarity medium is known to activate a specific MAP kinase signaling pathway known as the HOG pathway (high osmolarity growth pathway). Activation of the HOG pathway by osmotic stress results in a protein kinase cascade involving the Ste11 kinase, the Pbs2 kinase, the Hog1 kinase, ultimately resulting in cellular production of glycerol (Brewster et al., 1993). The HOG signaling cascade has been thought to participate in cell cycle progression due to the observation that yeast cells grown in the presence of osmotic stress that are deficient within the HOG pathway generate similar hyperpolarized buds to Cdc34/SCF mutant cell strains grown at restrictive temperatures. The precise cause of the hyperpolarized growth defect is unknown, though some work has suggested that it may be due to the activation of a filamentation/invasion MAP kinase pathway (Brewster and Gustin 1994; Davenport et al., 1999). Thus osmotic stabilization of cells with high osmolarity growth medium clearly has physiological consequences for cells that may be important for cell cycle progression. The suppressive effects of sorbitol in *cdc34-2 Δhog1* and *cdc34-2 Δpbs2* strains would delineate the importance of this signaling pathway in G1/S progression.

The second key observation linking cell integrity and cell cycle progression in yeast came from our identification of the novel yeast gene *YOR353c*, which we have re-named *UCS1*, as a strong suppressor of both defects exhibited by the *cdc34-2* and *cdc53-1* mutant strains. Particularly curious about these results is the fact that appending the N-terminal of Ucs1 with GST results in a partially functional version of the gene, unable to rescue the G1/S arrest phenotype of the *cdc34-2*, *cdc53-1*, and *cdc4-3* alleles, but fully capable of rescuing their cell integrity defects. Removal of the GST tag, however, resulted in a gain-of-function phenotype from *UCS1* overexpression where *cdc34-2* and *cdc53-1* cells became fully competent to grow at restrictive growth temperatures. We interpreted this result to mean that the cell integrity and cell cycle functions of the Cdc34/SCF complex are distinct but directly related to one another, where strong

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suppression of the cell integrity defect results in cell cycle progression. Interestingly, the *cdc4-3* allele was suppressed in its cell integrity defects but not in its cell cycle defects by *UCS1* overexpression which strongly argues that if the Cdc34/SCF complex regulates the cell integrity pathway by ubiquitinating a target protein independently of its ubiquitination of Sic1. The fact that the *CDC4* mutant exhibits cell integrity defects may also indicates that the cell integrity target protein is a nuclear protein as Cdc4 is known to be restricted to the nucleus (Blondel et al., 2000).

The sorbitol and *UCS1* suppression results indicated to us that Cdc34 may regulate both cell integrity and cell cycle pathways together. As the yeast cell integrity signaling pathway had previously been shown to dually coordinate cell cycle and cell integrity transcription through the SBF complex, we tested the *RHO1* and *SLT2* genes for their ability to suppress the lysis and cell cycle progression defects of the mutant strains (Madden et al., 1997). Consistent with our previous findings, we observed that overexpression of either gene fully suppressed both the temperature sensitivity and cell integrity defects of the *cdc34-2* mutant strain. We further demonstrated that overexpression of *RHO1* and *SLT2* suppressed the morphological and biochemical defects (ie Sic1 accumulation) of the *cdc34-2* mutant strain, directly demonstrating the importance of cell integrity signaling in cell cycle progression at high temperatures. We feel that these results illustrate the importance of cell integrity signaling in the progression of the cell cycle. As indicated in the introductory chapter of this thesis, the role of the cell integrity signaling pathway in cell cycle progression has not as of yet been clearly delineated. Previous studies have implicated *SLG1*, *RHO1*, and *SLT2* as prospective regulators of cell cycle progression, but clearly multiple redundant mechanisms are responsible both for SBF activation and Sic1 phosphorylation. Sic1 phophorylation, however is crucial to its ubiquitination, and as previously indicated, underphosphorylation of Sic1 causes its stabilization in the presence of a fully intact

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Cdc34/SCF complex (Nash et al., 2001). Thus the conditions under which signaling through this the cell integrity pathway contributes to cell cycle progression may be important. In this study, with the unusual *cdc34-2* mutant, the involvement of cell integrity signaling with G1/S progression became apparent only at restrictive growth temperatures. Under permissive temperatures, cell cycling continued even though cell integrity was clearly impaired. Numerous previous studies have clearly demonstrated that the yeast cell integrity signaling pathway becomes activated under both under cell wall stress conditions, and at the G1/S transition when budding is initiated (Cid et al, 1995).

Based on the work in this thesis, we can propose a model by which cell integrity signaling is required for G1/S progression under conditions of cell integrity stress (Figure 5-5). Our data suggests that at the G1/S transition, in addition to its role in the degradation of Sic1, the Cdc34/SCF complex simultaneously regulates the degradation of a second unknown protein, we've called Protein X, whose function is the negative regulation of the cell integrity signaling pathway. In our model, degradation of Protein X is essential to cell cycle regulation under stress conditions, as cell integrity signaling takes on a key cell cycle regulatory role, importantly initiating G1 cyclin transcription which in turn results in the phosphorylation of Sic1. Thus Sic1 turnover becomes dependent on cell integrity signaling. By simultaneously regulating both of these processes, the Cdc34/SCF complex ensures that cell wall stress information is directly integrated into the START decision under stress conditions. Our model predicts that under vegetative growth conditions, the two pathways would be uncoupled due to the redundant mechanisms of SBF activation such as the Bck2 and Cln3/Cdc28 signaling pathways, but upon increasing the incubation temperature the yeast cell adapts by switching to the cell integrity pathway as a key modulator of cell cycle progression. As previously indicated, recent work in our laboratory further corroborated our model, as we

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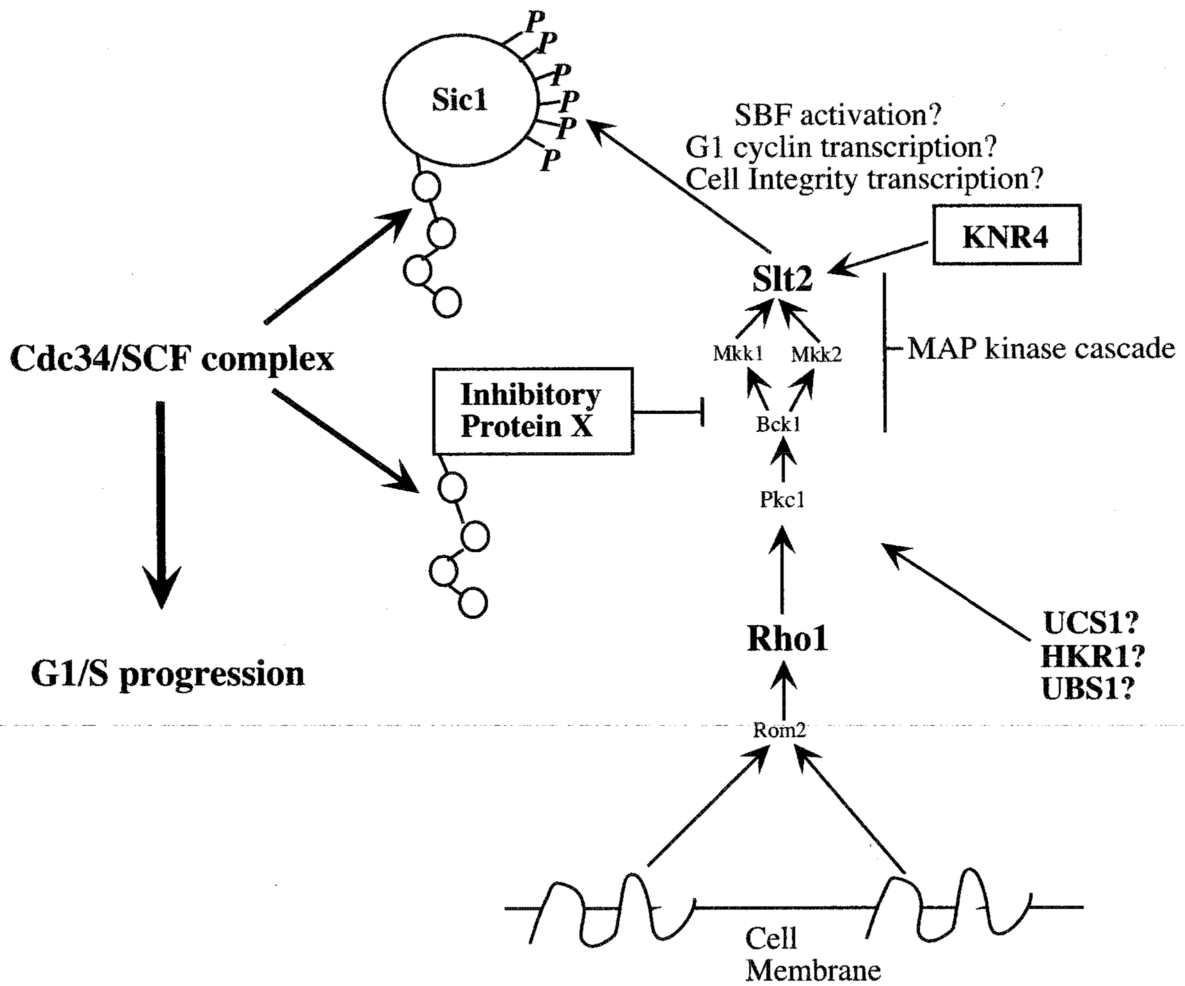

Figure 5-5. A model for dual cell cycle/cell integrity regulation by the Cdc34/SCF complex under stress conditions. We hypothesize that the Cdc34/SCF complex regulates both G1/S progression and cell integrity signaling by ubiquitin mediated degradation of target proteins. Protein X is an unknown cell integrity signaling inhibitory protein that is simultaneously degraded with Sic1 at the G1/S transition to allow for Sic1 phosphorylation and cell cycle progression at high temperatures. The suppressing genes used in this study are shown in bold.

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have discovered that the RhoGAP Sac7, a key negative regulator of the cell integrity signaling pathway is a specific enhancer of the *cdc34-2* temperature sensitivity growth defect at 37°C (C. Ptak, unpublished results). Sac7 is a specific inhibitor of the small G-protein Rho1, so this observation nicely complements the *RHO1/SLT2* suppression data in Chapter III of this thesis suggesting the essential role of the cell integrity signaling pathway of this complex in cell cycle progression at high growth temperatures. These results may also indicate that Sac7 and possibly other RhoGAPs are targets of the Cdc34/SCF complex, as Sac7 is known to contain PEST-sequences that are often found in ubiquitinated proteins.

2.4 Future Directions

The data presented in this thesis relating to the role of the Cdc34/SCF complex in cell integrity signaling and cell cycle progression is novel, unexpected, and a departure from what has previously been understood about the function this well studied enzymatic complex. This study provides a strong genetic basis on which to direct future biochemical and genetic studies of this complex problem. One key avenue of future work should involve a direct biochemical analysis of cell integrity activation in *cdc34-2* mutants at permissive and non-permissive growth temperatures. Signaling through this pathway can be directly assayed by examination of the phosphorylation state of the Slt2 protein. One would expect that in *cdc34-2* cells one should see a clear impairment of Slt2 activation as measured by anti-phospho Slt2 immunoblotting relative to wild type cells grown at 37°C. Secondly, as one would also expect that *cdc34-2 Δslt2* would significantly enhance the temperature sensitivity defect of the *cdc34-2* mutant allele. To determine if the suppressive effects of sorbitol, *SLT2*, and *RHO1* on the *cdc34-2* allele are due to transcriptional activation of the SBF complex, one would expect that *cdc34-2 Δswi4* cells would be impervious to sorbitol rescue and *SLT2/ RHO1* suppression.

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Conversely, it might be useful to try to suppress the *cdc34-2* allele by *SWI4* overexpression which would very clearly demonstrate that Swi4 activation is lacking in *cdc34-2* mutants. Finally, if the *cdc34-2* cell cycle defect in fact is due to its inability to properly phosphorylate Sic1, one would expect to observe decreased phosphorylation of nuclear Sic1 in *cdc34-2* cells at 37°C. These experiments would provide further biochemical and genetic substantiation to the results presented in this thesis. Examination of the roles of *UBS1* and *HKR1* in cell integrity signaling might prove rewarding also. Increased diligence regarding their co-immunoprecipitation would add to the two-hybrid result from this thesis, and the *UBS1/APG12* interaction might also be important both to verify by immunoprecipitation, and to study independently. As Apg12 is known to be an essential protein required for autophagy, it might be worthwhile to study the effects of a *Δubs1* strain on the autophagic response of yeast cells. Furthermore, the linkage between nucleocytoplasmic transport and Ubs1 would also be interesting to pursue by examination of its transport features (i.e. does it shuttle between the membrane and the nucleus? What is the mechanism of this shuttling? Is its intracellular transport involved with Cdc34 function?). Also, Cdc34/SCF regulation of the cell integrity signaling pathway suggests that a ubiquitin substrate protein exists that negatively regulates cell integrity signaling. Studies on the RhoGAPs and known phosphatases that regulate the cell integrity signaling pathway such as *GLC7* might yield the target protein, though this is no mean task and ubiquitination targets are elusive and difficult to identify due very often to their short half-life upon modification with ubiquitin.

A final and extremely promising area for future research relates to the biochemical and genetic characterization of the *UCS1* gene. Screening for a temperature-sensitive allele of this essential gene would provide valuable genetic tools to study its function *in vivo*. *In vivo* tagging of the protein would allow for its immunolocalization,

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and northern blots on synchronized cells would determine its expression throughout the cell cycle. Two-hybrid analysis and co-immunoprecipitation studies should also result in further discrimination of its intracellular binding partners. Due to its known association with Kic1 *in vivo* it would also be very useful to construct *cdc34-2 kic1-2* double mutants to examine if the suppressive effects of Ucs1 on Cdc34/SCF mutants is in any way mediated though Kic1. Also mutagenesis of its BC-box might be useful in determining the importance of this structural feature in its function and may potentially indicate a role for Ucs1 as a component of a novel ubiquitin ligase. It is most certainly an interesting and likely important cellular protein an its further study will be of interest to both the cell integrity and cell cycle fields.

The data presented in Chapters II and III of this thesis are strongly suggestive of the notion that cell integrity signaling is positively regulated by the Cdc34/SCF complex. This is not, however, the first work that has implicated this complex in cell integrity regulation. Cdc4 mutants exhibiting chitin delocalization in the mitotic cycle was reported nearly 20 years ago (Roberts et al., 1983). And recent work has implicated both Cdc34 and Cdc4 in both cell integrity signaling and SBF-mediated cell integrity transcription (Ivanovska and Rose, 2000 ; Cui et al., 2002). This is, however, the first report suggesting the direct involvement of this protein ubiquitination system in cell integrity maintenance and future work in this area should prove both fruitful and rewarding.

3. E2 dimerization

In Chapter IV we demonstrated using purified recombinant Ubc1Δ450, that the core catalytic domain of an E2 can self-associate *in vitro*. We observed this self-association to be covalent, through the formation of an intermolecular disulfide bond between Cys88 of each monomer, and readily disruptable upon exposure to 10mM DTT.

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Based on numerous published reports on E2 dimerization, and research in our own lab indicating the involvement of the active site face of the molecule in dimerization, we speculated that while the covalent association of the two monomers may be an artifact, it may be an accurate representation of transient E2 dimerization that occurs within the cell. We therefore used the covalently bound E2 as a tool to study E1-E2 interactions *in vitro*. We observed that incubation of the purified dimer with purified recombinant yeast E1 resulted in the formation of a high molecular weight complex of with a molecular weight of 150 kD by gel filtration chromatography. Furthermore, we successfully demonstrated by autoradiography that this high molecular weight peak consists of dimerically associated E2, and hence, by inference, E1 as well. This then is the first ever demonstration of E1/E2 interaction *in vitro*, and it may indicate a trapped intermediate where E1 exhibits a high affinity for E2 artificially crosslinked in a dimeric state. The presence of a reactive cysteine residue on the interaction interface of E1 resulted in reduction of the dimer to monomer upon exposure to E1. We found that alkylating E1 with 10mM iodoacetamide abolished the interaction between the two molecules entirely, but pre-activating E1 with ubiquitin resulted in a stabilization of the high molecular weight complex, possibly indicating a role for ubiquitin in E1-E2 interactions.

These experiments are novel in that they represent the first demonstration of E1-E2 interaction *in vitro*. Furthermore, they demonstrate a functional role for E2 dimerization as a structural interface for E1-recognition. A previous study with an artifactually dimerized E2 demonstrated enhanced functions for the enzyme complex when co-associated (Haldeman et al., 1997). Thus it seems likely that E2 self-association plays biological role.

The possible biological importance of such dimers forming *in vivo* is not clear. Our own studies of Cdc34 dimerization by analytical ultracentifugation have suggested that the native state of the protein is a monomer (Ptak et al., 1994). However, *in vitro* and

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in vivo crosslinking of Cdc34 is readily done, with these molecules forming both dimers and higher order complexes when observed by SDS-PAGE (Ptak et al., 1996; X. Varelas, personal communication). Thus, if these dimers, are in fact biologically relevant, they are very likely either transient in nature or facilitated *in vivo* by as of yet unknown proteins. Because of these facts, and our demonstration that the core catalytic domain only, and not N- or C-terminal appendages found on different E2s are required for such a process to take place, we argue that dimerization is necessary for the process of E1-E2 interaction: the only specific intermolecular interaction shared by all E2s with the exception of their association with ubiquitin.

A model of E1/E2 dimer interaction can therefore be proposed (Figure 5-6). A possible explanation for our *in vitro* observation is that once E1 recognizes a transient E2 dimer it transfers a ubiquitin to the active site cysteine of an E2 monomer. The dimeric surface would then likely be occluded by the presence of ubiquitin, preventing further association of E2 thiolester with both free E2 and ubiquitin-charged E1. This might be a mechanism by which thiolester-bound E2 is prevented from further interactions with E1, allowing for an uninhibited catalytic process. Our observation that E1 charged with ubiquitin stabilizes the high molecular weight complex is also interesting, in that it suggests that charged E1 has a higher affinity for E2 than uncharged E1, a hypothesis which makes intuitive sense. Recent work in our lab has demonstrated that E1 can transfer ubiquitin to E2 from both the active site cysteine and the adenylate site. If E1 recognizes a dimeric E2 this presents the formal possibility that ubiquitins are simultaneously transferred from both sites to both E2 active sites (Figure 5-6; T. Huzil, manuscript in preparation). It is easy to understand that the simultaneous transfer of ubiquitin to dimerically associated E2s would be far more efficient than one at a time: the currently hypothesized as the mechanism. Such a double transfer might also explain why

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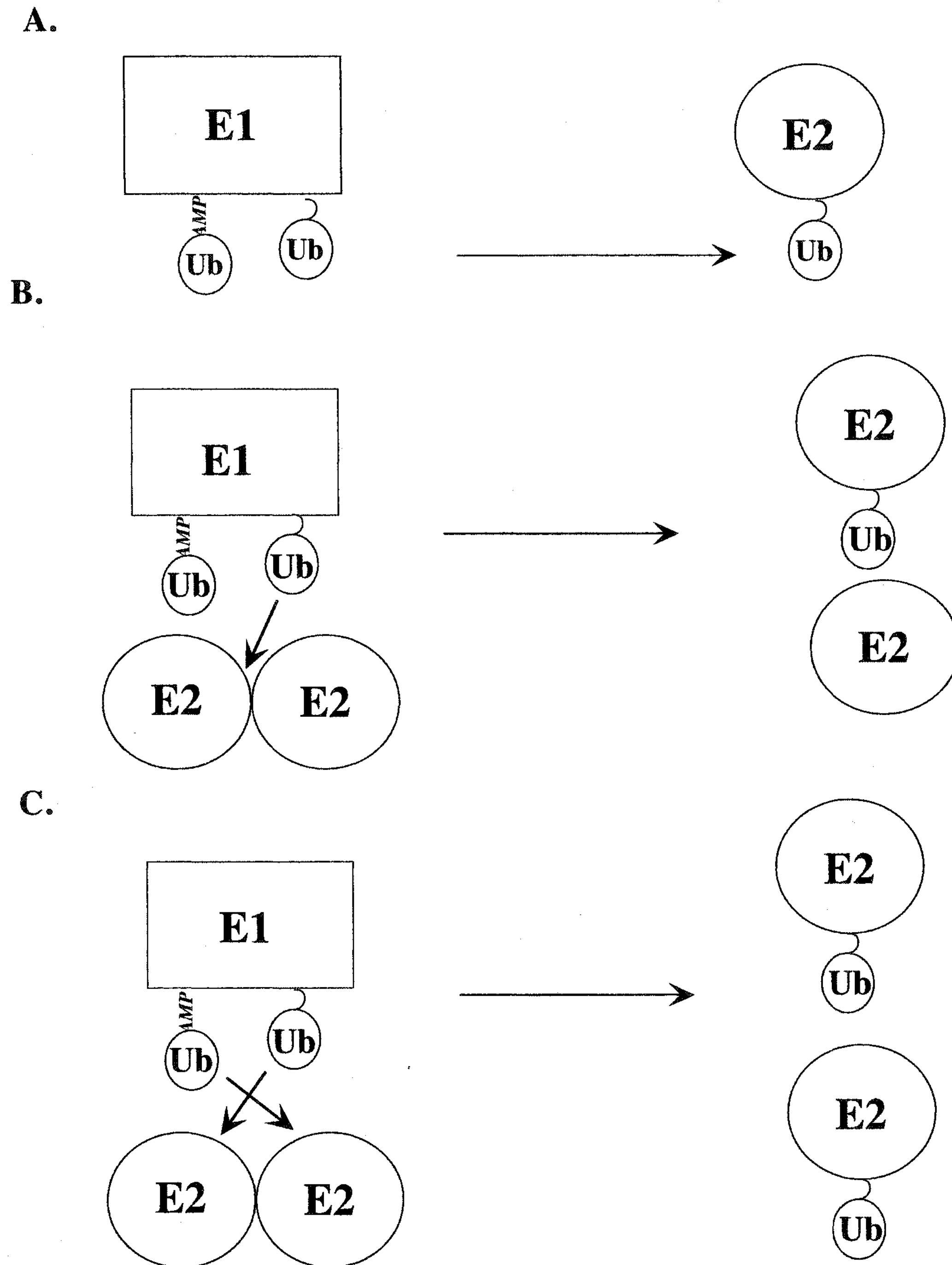

Figure 5-6. A model for the biological role of E2 dimerization.
The current model of E1/E2 interaction, for which there is no empirical evidence, involves a single E2 interacting with a doubly ubiquitinated E1 resulting in the formation of an E2 thiolester (Panel A). Our data demonstrates that purified E2 dimer interacts strongly with purified and doubly ubiquitinated E1 suggesting that dimerization may facilitate thiolester formation for one E2 (Panel B), or the simultaneous transfer of ubiquitin to both E2s from both the adenylate site and the E1 active site (Panel C) doubling the efficiency of the reaction.

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such substoichiometric amounts of E1 are required for highly efficient E2 ubiquitination reactions.

This study could be extended to include more biophysical data to substantiate the observations in this thesis. For example, the titrated addition of different concentrations of E1 to a dimeric E2, which would be predicted to increase the size of the E2 dimer/E1 peak, would add further credibility to our observations, as would Coomasie Blue staining of the radioactive High Molecular Weight peak bands to confirm the presence of E1 in the peak. What is really necessary at this stage, however, is dissection of this phenomenon *in vivo*. Site-directed mutagenesis of individual residues on the E2 catalytic surface might provide a mutant that is specifically defective in dimerization. This molecule can be tested *in vitro* for its efficiency in thiolester formation (a good test for E1 interaction), as well as *in vivo* for its ability functionally complement a knockout strain or perform a biological function. Such an *in vivo* demonstration of the functional aspects of E2 dimerization would clarify what has been a contentious issue in this field for many years and would provide extremely valuable insight into E2 structure and function studies.

4. Conclusion

The Cdc34/SCF complex is an extremely versatile system that regulates cell cycle transitions at both the G1/S and G2/M boundaries of the yeast cell cycle. It is involved in processes as diverse as budding, kinetochore assembly, cyclin-kinase activation, transcriptional control, and DNA synthesis regulation to name but a few. The data presented in this thesis provide novel and provocative insight to the vast and continuously growing body of knowledge in this complex and interesting field.

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