MULTIPLE, NUTRIENT SENSING KINASES CONVERGE TO PHOSPHORYLATE AN ELEMENT OF Cdc34 THAT INCREASES *SACCHAROMYCES CEREVISIAE* LIFESPAN

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DEDICATION

This thesis is dedicated to the many teachers who have inspired me and taught me how to appreciate the learning process. Although I could never make a complete list of this group, those who first come to mind are my parents (Kim and Crystal), Ms. Fumie Bouvier, Mr. Rob Hartgrove, Mrs. Helen Sears, Ms. Pam Dawson, Dr. Bill Mahoney, Dr. Mu Wang, Dr. Bob Harris and Dr. Mark Goebl.

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ABSTRACT

Ross Roland Cocklin

Multiple, nutrient sensing kinases converge to phosphorylate an element of Cdc34 that increases *Saccharomyces cerevisiae* lifespan

Growth and division are tightly coordinated with available nutrient conditions. Cells of the budding yeast, *Saccharomyces cerevisiae*, grow to a larger size prior to budding and DNA replication when preferred carbon sources such as glucose, as opposed to less preferred sources like ethanol and acetate, are available. A culture's doubling time is also significantly reduced when the available carbon and nitrogen sources are more favorable. These physiological phenomena are well documented but the precise molecular mechanisms relaying nutrient conditions to the growth and division machinery are not well defined. I demonstrate here that Cdc34, the ubiquitin conjugating enzyme that promotes S phase entry, is phosphorylated upon a highly conserved serine residue which is part of a motif that defines the family of Cdc34/Ubc7 ubiquitin conjugating enzymes. This phosphorylation is regulated by multiple, nutrient sensing kinases including Protein Kinase A, Sch9 and TOR. Furthermore, this phosphorylation event is regulated through the cell cycle with the sole induction occurring in the G1 phase which is when nutrients are sensed and cells commit to another round of division. This phosphorylation likely activates Cdc34 and in turn propagates a signal to the cell division cycle machinery that nutrient conditions are favorable for commitment to a new round of division. This phosphorylation is critical for normal cell cycle progression but must be

carefully controlled when cells are deprived of nutrients. Crippling the activity of Protein Kinase A, SCH9 or TOR increases the proportion of cells that survive stationary phase conditions, which because of the metabolic conditions that must be maintained and the similarity to post-mitotic mammalian cells, is referred to as a yeast culture's chronological lifespan. Yeast cells expressing Cdc34 mutants that are no longer subject to this regulation by phosphorylation have a reduced chronological lifespan. A precise molecular mechanism describing the change in Cdc34 activity after phosphorylation of this serine residue is discussed.

Mark Goebl, Ph.D., Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACE2	Activator of CUP1 Expression
BCY1	Bypass of Cyclase mutations
BUR1	Bypass UAS Requirement
CDC34	Cell Division Cycle mutant
$CDC34^{tm}$	CDC34 S73K/S97D/Δ103-114 Triple Mutant
CLA4	CLn Activity dependent
CLB	CycLin B
CLN	CycLiN
CKA1-2	Casein Kinase Alpha subunit
CTK1-3	Carboxy-Terminal domain Kinase
CTS1	ChiTinaSe
CYR1	CYclic AMP Requirement
FAR1	Factor ARrest
GAP	GTPase Activating Protein
GCN2	General Control Nonderepressible
GEF	Guanine Nucleotide Exchange Factor
GPA2	G-Protein Alpha subunit
GPCR	G-Protein Coupled Receptor
GRR1	Glucose Repression Resistant
GUK1	GUanylate Kinase 1
HAA1	Homolog of Ace1 Activator
MAPK	Mitogen Activated Protein Kinase
MBF	Mlu1 cell cycle box Binding Factor
MDM30	Mitochondrial Distribution and Morphology
MET30	METhionine auxotroph
MKK1/2	Mitogen Activated Protein Kinase Kinase
NAT1	Nourseothricin AcetylTransferase
PCR	Polymerase Chain Reaction
PKA	Protein Kinase A

RAD23	RADiation sensitive protein
RIM	Regulator of IMe2 or Replication in Mitochondria
RPN10	Regulatory Particle (of the proteasome) Non-ATPase
RPO21	RNA POlymerase 21
S97	Cdc34 Serine 97
SBF	Swi4 cell cycle box Binding Factor
SGA	Synthetic Gene Array
SIC1	Substrate/Subunit Inhibitor of Cyclin-dependent protein kinase
SNF1	Sucrose Non-Fermenting
SWI5	mating type SWItching Deficient
TOR	Target Of Rapamycin
TPK1-3	Takashi's Protein Kinase
UBA	UBiquitin Associated domain
UBA1	UBiquitin Activating enzyme 1
UBL	UBiquitin Like domain
UBP14	Ubiquitin Binding Protein
UFD	Ubiquitin Fusion Degradation protein
UIM	Ubiquitin Interacting Motif
VPS15/34	Vacuolar Protein Sorting mutants
VWA	Von Willebrand Associated domain
YNK1	Yeast Nucleoside diphosphate Kinase 1

CHAPTER 1: INTRODUCTION

1.1 Cell Growth and Division

1.1.1 Yeast as a model system for the study of cell growth and division

The budding yeast, *Saccharomyces cerevisiae*, is an excellent model organism for the study of cell growth and division. It is a single celled eukaryote and its cell cycle stage can be monitored and estimated by the size of the bud. The budding yeast is also amenable to genetic analysis because of the relative ease of gene disruption (Hinnen, Hicks, & Fink, 1978) and a low nuclear DNA content (Bicknell & Douglas, 1970). Pioneering studies by Hartwell and coworkers (Hartwell, 1974) led to the identification of more than 50 gene products required for cell division. The essence of this work was a screen of mutagenized yeast strains for mutants which when shifted from a permissive temperature to a restrictive temperature arrested cell division in a particular and phenotypically distinguishable phase of the cell cycle. These studies allowed Hartwell and coworkers to construct a precise and well supported model of the cell division cycle and the cell cycle checkpoints which ensure its harmonious execution (Hartwell, 1974). In the time since these studies, different experimental techniques have identified other cell division cycle (CDC) genes (Stevenson, Kennedy, & Harlow, 2001) but Hartwell's original model of the cell division cycle has not required significant revision. However, a tremendous amount of effort has gone into characterizing the biochemical activities of the *CDC* gene products.

1.1.2 The G1 phase and commitment to a new cell division cycle

This discussion will focus on the G1 phase of the yeast cell division cycle. Much of the yeast cell division cycle is akin to that of other eukaryotic cells; however, there are notable differences. It is beyond the scope of this work to discuss the specifics of cell division for all eukaryotic cells so it is my hope to give a high level overview of the yeast G1 phase while making mention of its similarities with other eukaryotic cell cycles.

In contrast to many eukaryotes, yeast cells divide with the nuclear envelope intact and so the G1 phase of the yeast cell cycle is defined as the time between nuclear division and the initiation of DNA replication; therefore, cytokinesis and cell separation are two of the earliest G1 events (G. C. Johnston, Pringle, & Hartwell, 1977) (Fig. 1). Much of the early G1 phase is dedicated to the synthesis of building blocks such as nucleotides for DNA, amino acids for proteins and glucans for the cell wall. The G1 phase is the period where yeast cells ensure that conditions are favorable for another round of division. Alternative developmental fates, such as pseudohyphal differentiation, may occur if conditions are not sufficient for adequate growth and division. The landmark events of G1 are formation of the bud and septin ring, polarization of the actin cytoskeleton towards the new bud site and spindle pole body duplication. The critical decision point, termed START, occurs immediately prior to bud emergence, spindle pole body duplication and the initiation of DNA replication. Just prior to START, nutrients can be withdrawn and the cell will not proceed through a new round of division. If nutrients are withdrawn after START, the cell will proceed through a full round of division and arrest in the subsequent G1 phase (Williamson & Scopes, 1960).

Emergence of a new bud is the most visible event of the yeast cell cycle. The site for bud emergence is selected based on the mating type of the yeast cell. Haploids of mating type a or α select a bud site adjacent to the previous bud site. Diploids of mating type a/α form a new bud opposite the site used in the previous cycle. Upon bud site selection, the new septin ring is assembled just below the bud and the actin cytoskeleton is polarized and oriented toward the new bud so that cargo can be carried by the secretory system to the new bud site (reviewed in (Botstein et al., 1997; Cid, Adamikova, Sanchez, Molina, & Nombela, 2001)). While the bud is forming, the origins of DNA replication are being licensed and prepared to fire. This is a highly regulated process which ensures that the origins do not fire prematurely nor are they allowed to fire twice and make extra copies of the DNA (reviewed in (Kelly & Brown, 2000)). The spindle pole body, analogous to the mammalian centrosome, is duplicated during this time as well. Spindle pole body duplication occurs late in G1 and is quickly followed by separation to form the mitotic spindle. Like origin of replication firing, it is imperative that spindle pole body duplication occurs once and only once per cell cycle.

The cyclin and cyclin dependent kinase (CDK) gene products form a protein complex that governs progression through the cell cycle. This complex ensures an orderly and irreversible progression through the cell cycle. The cyclin/CDK complex was discovered independently in yeast and frog oocytes as a mitosis promoting factor (Beach, Durkacz, & Nurse, 1982; Lohka & Masui, 1983; Masui & Markert, 1971). In yeast, the primary mitosis promoting CDK, Cdc28, is independently activated by at least nine distinct cyclins (Cln1-3 and Clb1-6). Cln3 is the first cyclin to associate with Cdc28 in the early G1 phase. The Cln3/Cdc28 complex activates a transcriptional program,

dependent on the transcription factor SBF, that is critical for progression through the G1 phase. SBF is a heterodimer of Swi4 and Swi6 which directly binds to the promoter elements of the early G1 genes (Cosma, Panizza, & Nasmyth, 2001). The mechanism of SBF activation involves phosphorylation of the SBF inhibiting factor Whi5 (the functional equivalent of Rb) by Cln3/Cdc28 (de Bruin, McDonald, Kalashnikova, Yates, & Wittenberg, 2004; Wagner et al., 2009). Whi5 dissociates from SBF upon its phosphorylation and SBF is then capable of inducing the transcription of a suite of genes, including two cyclin genes, *CLN1* and *CLN2*. The Cln1 and Cln2 proteins share ~50% sequence identity, have overlapping function and associate independently with Cdc28. The Cln(1 or 2)/Cdc28 complex is responsible for assembly of the new septin ring (Cid et al., 2001) and polarization of the actin cytoskeleton towards the new bud site (reviewed in (Madden & Snyder, 1998; Pruyne, Legesse-Miller, Gao, Dong, & Bretscher, 2004)). Loss of the Cln cyclins results in failure to accumulate factors necessary for a polarized actin cytoskeleton and secretion at the incipient bud site (Lew & Reed, 1993). These polarization factors include the GTPase Cdc42, its GEF Cdc24 and its effector kinases Cla4 and Ste20, all of which are essential for budding and polarization (Butty et al., 2002; Cvrckova, De Virgilio, Manser, Pringle, & Nasmyth, 1995; D. I. Johnson & Pringle, 1990). The mechanism of factor recruitment to sites of polarized growth by Cln (1 or 2)/Cdc28 is not fully understood but recent evidence demonstrates that the Cdc42 GAPs, Bem2 and Bem3, are subject to Cln2/Cdc28 phosphorylation. This phosphorylation inhibits their GAP activity resulting in localized activation of Cdc42 at the site of bud emergence (Knaus et al., 2007). Furthermore, the Cln2/Cdc28 complex phosphorylates the GEF, Cdc24, triggering its relocalization from the nucleus to the polarization site.

Cdc24 reinforces the locally activated Cdc42 to promote polarization (Gulli et al., 2000). Like budding and septin ring formation, duplication of the spindle pole body depends of Cln/CDK activity and this involves CDK dependent phosphorylation of the spindle pole component Spc42 on two N-terminal sites (Jaspersen et al., 2004).

Eventually, Cln/CDK activity gives way to Clb/CDK activity at the G1/S phase transition. The B-type cyclins, Clb5 and Clb6, are transcribed at the same interval in G1 as Cln1 and Cln2. However, the Clb/Cdc28 complex is kept inactive by the cyclin dependent kinase inhibitor Sic1 until immediately prior to START. At that time, Sic1 is quickly degraded and the Clb5-6/Cdc28 complexes become active and initiate DNA replication which, via the firing of replication origins, is formally defined as the exit from the G1 phase. In the case of replication initiation, one of the essential targets of the Clb(5 or 6)/Cdc28 complex is known. Clb(5 or 6)/Cdc28 phosphorylates Sld5, an essential component of the replication complex, and this is required for functional loading of the replication complex (Masumoto, Muramatsu, Kamimura, & Araki, 2002). Other B-type cyclins, Clb1-4, are activated as cells progress through mitosis. Along with their roles in promoting DNA replication, the Clb/Cdc28 complexes inactivate the SBF transcription factor (Amon, Tyers, Futcher, & Nasmyth, 1993) and inhibit spindle pole body reduplication (Haase, Winey, & Reed, 2001). Ultimately, the exit from mitosis and entry into the next G1 phase is triggered by Clb/Cdc28 inactivation through proteasome mediated degradation of the Clb2 cyclin and accumulation of the cyclin dependent kinase inhibitor Sic1 (reviewed in (Sullivan & Morgan, 2007)).

The cyclin/Cdc28 complexes are highly regulated. Phosphorylation of Cdc28 on its activation loop by the CDK activating kinase, Cak1, is required for cyclin/Cdc28

activity both *in vivo* and *in vitro* and strains lacking *CAK1* are inviable (Chun & Goebl, 1997; Thuret, Valay, Faye, & Mann, 1996). Phosphorylation of Cdc28 on a conserved residue, tyrosine 19, by Swe1, negatively regulates Cdc28 activity and this phosphorylation ensures that the developing bud and events of the nuclear division cycle such as migration of the spindle into the daughter cell are appropriately coordinated (Booher, Deshaies, & Kirschner, 1993). Cdc28 Y19 phosphorylation can be reversed by action of the Mih1 phosphatase (Russell, Moreno, & Reed, 1989). The cyclin/CDK complexes also associate with cyclin dependent kinase inhibitors, namely Sic1 and Far1. Far1 specifically inhibits Cln/Cdc28 activity (Chang & Herskowitz, 1990; Peter & Herskowitz, 1994) while Sic1 inhibits Clb/Cdc28 activity (Mendenhall, 1993; Schwob, Bohm, Mendenhall, & Nasmyth, 1994). All of the G1 cyclins, Cln1-3, along with the cyclin dependent kinase inhibitors, Sic1 and Far1, are targeted for ubiquitin mediated degradation by an SCF/Cdc34 complex (Henchoz et al., 1997; Schwob et al., 1994; Tyers, Tokiwa, Nash, & Futcher, 1992). This layer of post-translational control is essential for cell cycle progression and will be discussed in more detail later (section 1.1.5).

1.1.3 Nutrients and nutrient sensing mechanisms necessary for cell division

As G1 is the time during the cell cycle that nutrients are sensed and a decision is made to commit to a new round of division, many of the nutrient sensing proteins are activated in G1 by nutrients (or the lack of nutrients). In yeast, the essential nutrients are a carbon source which can be used for energy and as a backbone for amino acids and other structural components. The preferred carbon source is glucose, which is the most abundant six carbon sugar on our planet. Yeast also require a nitrogen source for their

amino acids and nucleotides. Preferred nitrogen sources of the budding yeast include ammonium sulfate, glutamine and glutamate. There are many other sources of nitrogen which yeast can utilize but those listed above are the most preferred and used at the exclusion of others. Yeast also require sources of sulfur, phosphorous, potassium and essential metals. All complex vitamins, amino acids, nucleotides and structural components can be synthesized from simple sources of carbon, nitrogen, sulfur and phosphorous.

Glucose is the preferred carbon source of yeast and as such its presence represses the utilization of all other available carbon sources in a phenomenon known as glucose repression (recently reviewed in (Santangelo, 2006)). The glucose sensing mechanism is not fully understood but many of the components have been identified. An atypical G-protein signaling cascade is involved and begins with binding of glucose to the G-protein coupled receptor, Gpr1 (Lemaire, Van de Velde, Van Dijck, & Thevelein, 2004). Upon glucose binding to Gpr1, the G-alpha protein Gpa2 exchanges GDP for GTP and GTP-bound Gpa2 specifically interacts with the membrane tethered adenylyl cyclase, Cyr1 (Peeters et al., 2006). Genetic data suggests that Gpa2 directly activates Cyr1 (Colombo et al., 1998) but further biochemical and molecular studies are needed to substantiate this conclusion. The adenylyl cyclase, Cyr1, converts ATP to cAMP which binds the cAMP dependent kinase regulatory subunit, Bcy1, allowing the catalytic subunits, Tpk1, Tpk2 and Tpk3, to dissociate and become active (Corbin et al., 1978; Hixson & Krebs, 1980). Gpa2 also regulates the cAMP dependent protein kinase (also known as Protein Kinase A) by binding the kelch repeat proteins, Kel1 and Kel2, in a GTP dependent manner. Kel1 and Kel2 facilitate the interaction of Bcy1 with Tpk(1-3) and thus, Gpa2 serves to

titrate the Kel proteins away from the PKA catalytic and regulatory heterodimers ultimately reducing the Bcy1, Tpk(1-3) interaction and increasing PKA activity. The G-beta protein Asc1 is a negative regulator of the Gpr1/Gpa2 signaling pathway by i) preventing the dissociation of GDP from Gpa2 and ii) by interacting with and repressing adenylyl cyclase activity (Zeller, Parnell, & Dohlman, 2007). In yeast, as opposed to many mammalian cell types, protein kinase A is activated when glucose is available and conditions for growth and division are favorable (Wilson & Roach, 2002).

An intracellular, glucose sensing pathway is also able to activate adenylyl cyclase. The membrane tethered, small G protein, Ras, is activated by internal glucose via its GEF, Cdc25 (Colombo, Ronchetti, Thevelein, Winderickx, & Martegani, 2004). In the GTP bound state, Ras activates adenylyl cyclase, Cyr1, which again leads to activation of PKA (Toda et al., 1985). The glucose derived metabolite required for Ras activation appears to be glucose-6-phosphate which is produced as the first step in the glycolytic pathway (M. Rose, Albig, & Entian, 1991). It has been postulated that increased intracellular glucose-6-phosphate inhibits the activity of the Ras GTPase Activating Proteins (GAPs), Ira1 and Ira2, ultimately activating Ras (Colombo et al., 2004). Still, the exact mechanism of Ras activation by glucose remains to be elucidated. Notably, activated Ras and Gpa2 alleles can recapitulate nearly the entire glucose induced transcriptional response, independent of extracellular glucose (Wang et al., 2004). Ras and Gpa2 converge on adenylyl cyclase independently and yeast strains lacking both *RAS2* and *GPA2* grow very slowly (Kubler, Mosch, Rupp, & Lisanti, 1997). Interestingly, Sgt1, an essential component of the SCF complex that reduces the rate of Sic1 turnover *in vivo* and Cln1 ubiquitination *in vitro* (Kitagawa, Skowyra, Elledge,

Harper, & Hieter, 1999), interacts with the leucine-rich-repeats of Cyr1 and modulates the activity of the cAMP pathway (Dubacq, Guerois, Courbeyrette, Kitagawa, & Mann, 2002). *SGT1* is well conserved among eukaryotes and it is an essential gene in yeast. It is noteworthy, that temperature sensitive alleles of *SGT1* have been isolated that arrest as unbudded cells, early in G1 reminiscent of cells arrested due to nutrient deprivation or expression of a stable version of the cyclin dependent kinase inhibitor Far1 (Kitagawa et al., 1999).

What evidence exists for cell cycle regulation of the above nutrient sensing machines? A recent study from Steve McKnight's lab elegantly shows the metabolic changes that occur during a single yeast cell cycle. It is known that at high cell densities in a controlled environment, yeast cell cycle synchrony can be induced and monitoring of the transcriptional and metabolic changes revealed a "metabolic cycle". The key findings of this experiment were that yeast cells enter a non-oxidative metabolic state during DNA replication, likely to preserve genome integrity by preventing oxidative DNA damage (Chen, Odstrcil, Tu, & McKnight, 2007). Slowly growing yeast cells accumulate the storage carbohydrates glycogen and trehalose but break these down during G1 phase to provide the carbohydrates necessary for another round of division and to ensure that enough nutrients are available to complete the entire cycle. It has also been shown that cAMP levels fluctuate through the cell cycle with a peak in G1 and a trough late in mitosis (Müller, Exler, Aguilera-Vazquez, Guerrero-Martin, & Reuss, 2003; Tu, Kudlicki, Rowicka, & McKnight, 2005). Other intracellular metabolites such as GTP, ATP, ADP and AMP and their respective ratios also have a large impact on cell cycle progression although measurements have not yet been made to determine whether the

levels of these metabolites fluctuate through the cell cycle. The ratio of ATP:GTP appears to be approximately 1:1 in midlog phase cells and interconversion between ATP and GTP occurs enzymatically via Guk1 and Ynk1.

1.1.4 Cell cycle exit and entry into a G_0 state

When an essential nutrient is not available in quantities sufficient for the ensuing cell cycle, yeast cells enter a growth-arrested state termed G_0. Along with inhibition of DNA replication and cell division, G_0 entry involves increased synthesis of the storage carbohydrates glycogen and trehalose along with induction of stress responsive transcripts such as *HSP26, HSP12* and *GRE1* (Pedruzzi et al., 2003; Reinders, Burckert, Boller, Wiemken, & De Virgilio, 1998). Surviving nutrient deprivation requires an appropriate cell cycle arrest during the G1 phase and large scale reprogramming of the cell's metabolism. Nutrient deprivation in budding yeast is considered a model of chronological aging as assessed by the viability of cells driven into the G_0 state by nutrient depletion. It is clear that in many types of eukaryotic organisms (including yeast, mice and fruit flies), caloric restriction or inactivating mutations in conserved nutrient signaling pathways increases chronological lifespan (reviewed in (Longo & Finch, 2003; Longo, Mitteldorf, & Skulachev, 2005)).

Exact molecular mechanisms for G_0 entry in budding yeast are becoming clearer. The PAS family protein kinase Rim15 is required as cells lacking *RIM15* do not survive the G_0 state nearly as well as a wild type strain (Fabrizio, Pozza, Pletcher, Gendron, & Longo, 2001). In response to nutrient depletion, Rim15 moves from the cytoplasm to nucleus where it activates stress responsive transcription factors such as Msn2, Msn4 and Gis1 (Pedruzzi, Burckert, Egger, & De Virgilio, 2000). Rim15 is retained in the

cytoplasm by TORC1 and Sch9 kinases during periods of nutrient abundance but upon nutrient depletion TORC1 activity decreases thus reducing Sch9 activity. Rim15 activity is also repressed by PKA; however, under conditions of carbon starvation, PKA activity is absent and Rim15 becomes active (Pedruzzi et al., 2003).

Tight regulation of the cyclin/CDK complex is essential for proper G_0 entry. Cells overexpressing *CLN3* lose viability much quicker in the G_0 phase than an isogenic wild type strain (Weinberger et al., 2007). Normally Cln3 is down-regulated upon G_0 entry and its ectopic expression leads to a higher percentage of cells which arrest their growth in the S phase of the cell cycle rather than the G1 phase prior to G_0 entry. Ultimately, strains lacking control elements of *CLN3* have a shorter chronological lifespan along with age dependent increases in apoptosis and chromosome instability (Weinberger et al., 2007). The same is true for cells lacking the cyclin dependent kinase inhibitor Sic1 (Zinzalla, Graziola, Mastriani, Vanoni, & Alberghina, 2007). Both Cln3 and Sic1 are subject to ubiquitin mediated degradation and as such it seems likely that appropriate post-translational regulation of both Sic1 and Cln3 is essential for surviving nutrient deprivation.

1.2 The Mechanism of Ubiquitin Dependent Protein Degradation

1.2.1 Mechanism of SCF/Cdc34 ubiquitin conjugation

Ubiquitin is a small, 76 amino acid residue protein. The covalent attachment of ubiquitin to another protein often serves as the signal for the selective degradation of that protein by a complex protease, the 26S Proteasome (for review see (Glickman & Ciechanover, 2002)). The mechanism of protein ubiquitination begins with the ubiquitin activating (or E1) enzyme forming a high energy thiolester intermediate with ubiquitin in

an ATP dependent reaction (Fig. 2). The E1 enzyme transfers the ubiquitin molecule to an ubiquitin conjugating (or E2) enzyme which, like the E1, forms a thiolester with ubiquitin. In the case of a RING-type E3 ubiquitin ligase, the E2 transfers ubiquitin directly onto a lysine residue of a substrate. The transfer of ubiquitin to substrate typically requires the activity of an ubiquitin ligase (E3). The bond formed between ubiquitin and substrate is an isopeptide bond which links the COOH-terminal glycine residue of ubiquitin to the ε-amino group of a lysine residue of the substrate. Substrates may be monoubiquitinated or polyubiquitinated with the polyubiquitin chain being linked through one of the seven lysine residues of ubiquitin. Lysine-48 linked ubiquitin chains are the canonical signal for proteasomal degradation.

Cdc34 is an E2 which ubiquitinates histones *in vitro* (Goebl et al., 1988) and itself *in vitro* (Banerjee, Gregori, Xu, & Chau, 1993) and *in vivo* (Goebl, Goetsch, & Byers, 1994). Cdc34 mediated conjugation of lysine 48 linked polyubiquitin chains to the cyclin dependent kinase inhibitor Sic1 is essential for cell viability and the initiation of DNA replication in the yeast, *S. cerevisiae* (Skowyra, Craig, Tyers, Elledge, & Harper, 1997; Verma, Feldman, & Deshaies, 1997). Cdc34 genetically and physically interacts with the SCF family of ubiquitin ligases and this interaction is a requirement for Cdc34 to carry out its ubiquitin conjugating function *in vivo* (Mathias, Steussy, & Goebl, 1998). The SCF family of ubiquitin ligases is composed of at least four distinct proteins, including Skp1, Cdc53, Rbx1 and a member of a family of proteins known as F-box proteins. Rbx1 contains a Ring-H2-finger domain and is essential for SCF dependent attachment of ubiquitin to its substrates (Skowyra et al., 1999). Cdc53 is a scaffolding subunit or cullin which binds both Skp1 and Cdc34. Skp1 tethers the F-box protein to the SCF complex

through an interaction with the F-box motif. The F-box proteins are the component of the SCF ubiquitin ligase which determine substrate specificity (for review see, (Deshaies, 1999)). The F-box is also the unique component of each SCF complex and as such its name is written in superscript to designate a specific SCF complex (for example, SCF^{Cdc4}). Seventeen genes within the yeast genome encode proteins with predicted F-box motifs and although verifiable substrates have been identified for only six of these thirteen, thirteen unique SCF complexes have been reconstituted *in vitro* (Kus, Caldon, Andorn-Broza, & Edwards, 2004; Patton, Willems, & Tyers, 1998). SCF^{Grr1} is required for ubiquitination of the cyclins Cln1 and Cln2 while SCF^{Cdc4} is required for ubiquitination of the cyclin dependent kinase inhibitors Sic1 and Far1 (Skowyra et al., 1997; Skowyra et al., 1999; Verma et al., 1997)

Cdc34 self-associates and accumulating evidence suggests homodimerization is critical for its catalytic activity (Gazdoiu et al., 2005; Varelas, Ptak, & Ellison, 2003). The formation of the Cdc34~ubiquitin thiolester precedes and facilitates Cdc34 self-association. Formation of the Cdc34~ubiquitin thiolester also increases the rate of dissociation of Cdc34 from the SCF complex which is part of the catalytic cycle (Deffenbaugh et al., 2003). Dissociation of ubiquitin-charged Cdc34 from the SCF complex provides a satisfactory explanation for how ubiquitin can bridge the seemingly expansive space, uncovered in the SCF crystal structure, between Cdc34 and substrate (Zheng et al., 2002).

Recent work has extended our understanding of the mechanistic principles underlying polyubiquitin chain formation. At least three distinct mechanisms that result in lysine-48 linked polyubiquitin chains conjugated to substrate have been described.

What might be considered the traditional model involves only one E2 whereby the polyubiquitin chain is built upon the substrate in a series of reactions. Elegant *in vitro* reconstitution of Sic1 polyubiquitination by SCF^{Cdc4} demonstrates that conjugation of the first ubiquitin to the substrate is the rate limiting step in this process (Petroski & Deshaies, 2005). Seemingly many E2s are capable of catalyzing only the monoubiquitination or the polyubiquitin chain extension reactions but not both. Recently, it was demonstrated that Ubc4 monoubiquitinates substrates of the Anaphase Promoting Complex while Ubc1 serves to extend the chain of these substrates (Rodrigo-Brenni & Morgan, 2007). Finally, at least one E2 in mammals, Ube2g1, is capable of generating ubiquitin chains on its catalytic cysteine prior to transfer to the substrate (Li, Tu, Brunger, & Ye, 2007).

A single motif, unique to the Cdc34/Ubc7 family of ubiquitin conjugating enzymes, allows the Cdc34/Ubc7 family to catalyze both the monoubiquitination and ubiquitin chain extension reactions (Petroski & Deshaies, 2005). This motif is defined by two serines and a twelve amino acid acidic "loop", all of which lie in close physical proximity to the catalytic cysteine (Fig. 1). In contrast, the majority of E2s, of which Rad6 is a classic example, have a lysine and aspartic acid residue in lieu of the serine residues and lack the acidic "loop". Cdc34 mutants which lack the acidic "loop" monoubiquitinate Sic1 with the same kinetics as the wild type enzyme but extend ubiquitin chains at a negligible rate (Petroski & Deshaies, 2005) which is reflected *in vivo* as cells expressing Cdc34 mutants which lack the acidic "loop" are inviable (Y. Liu, Mathias, Steussy, & Goebl, 1995). More recently, Li et al. (Li et al., 2007) discovered that the polyubiquitin chain can be formed on the ubiquitin conjugating enzyme prior to

substrate ubiquitination. The acidic "loop" of the Cdc34-like ubiquitin conjugating enzyme Ube2g1 is essential for preforming the polyubiquitin chain on the ubiquitin conjugating enzyme itself and transfer of the "preformed" chain onto the known *in vivo* target HERPc, a short lived, ER-associated protein (Li et al., 2007). Interestingly, the polyubiquitin chain preformation and ubiquitin conjugation to HERPc *in vitro* assays, like Cdc34 enzyme activity *in vivo*, require the presence of a RING finger containing protein. In the *in vitro* reconstitution of Cdc34 autoubiquitination or Cdc34 dependent histone ubiquitination assays, which do not require the RING finger protein, acidic "loop" deletion mutants function as well if not better than WT Cdc34 (Pitluk, McDonough, Sangan, & Gonda, 1995; Varelas et al., 2003) suggesting that interactions between the acidic loop of Cdc34-like E2s and the RING finger protein facilitates SCF-dependent polyubiquitination.

1.2.2 Regulating the ubiquitin conjugation reaction

It is becoming increasingly clear that substrate recognition by the SCF complex is preceeded by substrate level phosphorylation (Hsiung et al., 2001; Nash et al., 2001; Skowyra et al., 1997; Song, Wang, Goebl, & Harrington, 1998). This mechanism provides a means to temporally control SCF activity against any particular substrate. For example, ubiquitin conjugation of Sic1 by the SCF^{Cdc4} complex during the transition to S phase requires multisite phosphorylation of Sic1 by the Cln/Cdc28 kinase complex (Nash et al., 2001; Orlicky, Tang, Willems, Tyers, & Sicheri, 2003; Skowyra et al., 1997; Tang et al., 2007). Furthermore, the G1 cyclin Cln2, which is an SCF^{Grr1} substrate, requires Cdc28 phosphorylation in order to be recognized by Grr1 (Lanker, Valdivieso, & Wittenberg, 1996). Other examples of SCF substrates whose phosphorylation is a

prerequisite for timely ubiquitination include Far1, Swi5, Gcn4, Tec1, Pcl5 and Rcn1 (Aviram, Simon, Gildor, Glaser, & Kornitzer, 2008; Chou, Zhao, Song, Liu, & Nie, 2008; Henchoz et al., 1997; Kishi, Ikeda, Koyama, Fukada, & Nagao, 2008; Kishi, Ikeda, Nagao, & Koyama, 2007; Shemer, Meimoun, Holtzman, & Kornitzer, 2002).

The abundance of some F-box proteins is also subject to modulation and it is likely that regulation of F-box protein steady state abundance contributes to SCF complex activity. The best example of F-box abundance regulation has been demonstrated for Met30. SCF^{Met30} ubiquitinates and targets Met4, a transcriptional inducer of the methionine biosynthetic genes, for degradation. When L-methionine is present, SCF^{Met30} ubiquitinates Met4. Work from our lab and the lab of Dr. Neal Mathias demonstrated that L-methionine stabilizes the Met30 protein thereby increasing Met4 ubiquitination (Smothers, Kozubowski, Dixon, Goebl, & Mathias, 2000). Changes in protein abundance have been also been demonstrated for Cdc4 and Grr1 but the physiological significance of these changes is not well understood (Fey & Lanker, 2007; Mathias, Johnson, Byers, & Goebl, 1999).

Both human and budding yeast Cdc34 enzymes are phosphorylated *in vivo* (Block, Boyer, & Yew, 2001; Goebl et al., 1994; Semplici, Meggio, Pinna, & Oliviero, 2002). Site directed mutagenesis of five serines in the C-terminal tail of the human Cdc34 distinguishes these residues as potential phosphorylation sites. These five serines are not conserved in budding yeast. Some of these serines are phosphorylated by Casein Kinase 2 (Block et al., 2001; Semplici et al., 2002). Phosphorylation of yeast Cdc34 on serines 130, 167, 207 and 216 by yeast casein kinase 2 modestly stimulates Cdc34 activity *in vitro* (Coccetti et al., 2008; Sadowski, Mawson, Baker, & Sarcevic, 2007).

Human Rad6 (hHR6A) is the only other ubiquitin conjugating enzyme known to be regulated by phosphorylation. The phosphorylation of human Rad6 on the highly conserved serine 120 by the cyclin A-CDK2 kinases occurs *in vivo* and this phosphorylation increases the *in vitro* ubiquitin conjugating activity of Rad6 four-fold. This phosphorylation is also cell cycle regulated and contributes to cell cycle progression through the G2/M phase (Sarcevic, Mawson, Baker, & Sutherland, 2002).

1.2.3 Transfer of ubiquitinated proteins to the proteasome

Following lysine-48 linked polyubiquitination, a substrate is shuttled to the proteasome where it is recognized by virtue of its polyubiquitin chain. Lysine-48 linked chains of four ubiquitin moieties are sufficient for a substrate to bind the proteasome (Thrower, Hoffman, Rechsteiner, & Pickart, 2000). It is not clear whether ubiquitinated substrates are "shuttled" from the ubiquitin conjugating enzyme to the proteasome or if ubiquitinated proteins are transferred directly from the ubiquitin conjugating system to the proteasome and recognized by an intrinsic receptor. There is evidence that both pathways may exist. Multiple proteasomal receptors which recruit ubiquitinated substrates to the proteasome have been discovered. S5a, the human ortholog of Rpn10, was the first proteasomal subunit implicated in polyubiquitin conjugate binding and was thought to be the sole ubiquitin receptor of the proteasome until it was found that yeast cells lacking *RPN10* are viable (van Nocker et al., 1996). Of the total cellular pool of Rpn10, only a small fraction can be found physically associated with the proteasome (van Nocker et al., 1996). Rpn10 possesses a UIM (**U**biquitin **I**nteracting **M**otif) which recruits substrates to the proteasome and a VWA (Von Willebrand Associated) domain that stabilizes the proteasome (Glickman et al., 1998).

Other polyubiquitin chain receptors, such as Rad23, Dsk2, Ddi1 and Rpn13 have been identified. Polyubiquitin chain length appears to be a major determinant of receptor specificity. *In vitro* binding assays show that Rad23 prefers substrates with approximately 2-4 ubiquitin moieties while Rpn10 will preferentially bind substrates with > 4 ubiquitin molecules per chain (Richly et al., 2005). Other work utilizing slightly different *in vitro* binding assays and different substrates confirms the finding that Rpn10 prefers long chains; however, in the same assay Rad23 enhances the proteasomal binding of long chained substrates (Elsasser, Chandler-Militello, Muller, Hanna, & Finley, 2004). It is unclear if long ubiquitin chains (> 6 ubiquitin moieties) exist *in vivo* or if there is even a specific receptor for such chains. Rad23 is not a stoichiometric subunit of the proteasome but is found loosely associated with the proteasome and binds polyubiquitinated substrates with its UBA (**UB**iquitin **A**ssociated) domain. Rad23 also contains a UBL (**UB**iquitin **L**ike) motif that is required for its interaction with the proteasome via the19S lid component Rpn1. Rad23 is believed to act as a shuttle by binding ubiquitinated substrates apart from the 26S Proteasome and subsequently bringing them to the proteasome. Rad23 can also be recruited to the proteasome via an interaction between the Rpn10 UIM domain and its UBL domain and this pathway is separate from its proteasomal recruitment via Rpn1 (Elsasser et al., 2004). It is currently unclear whether Rpn10 binds its substrates while associated with the proteasome or prior to proteasomal association and subsequently recruits them.

Rpn13, an integral subunit of the 19S regulatory particle of the proteasome, was recently found to bind ubiquitinated substrates (Husnjak et al., 2008; Schreiner et al., 2008). Purified proteasomes from *rpn10Δ rpn13Δ* mutants lack nearly all ubiquitin chain

binding activity although depending on the execution of the assays a small amount of residual binding can be detected (Husnjak et al., 2008). Like Rpn10, Rpn13 binds the UBL domains of the UBL-UBA family of proteins Rad23, Ddi1 and Dsk2 (Husnjak et al., 2008) and therefore may act to recruit these proteins and their substrates to the proteasome.

The discovery of an E4 enzyme, Ufd2, which can extend short ubiquitin chains is an additional layer of complexity within this system (Koegl et al., 1999). Ufd2 is a component of a pathway which extends from the ubiquitin conjugating enzyme to the homohexameric Cdc48 complex and finally to the proteasome. Ufd2 physically interacts with the Cdc48 complex which along with Cdc48 itself, also consists of two canonical adaptors, Ufd1 and Npl4. Ufd2 can extend short polyubiquitin chains to a length of 6 moieties *in vitro*. Rad23 indirectly interacts with Ufd2 and this interaction is mediated by Cdc48 (Rumpf & Jentsch, 2006). It is thought that Ufd2 creates ubiquitin chains which are ideally suited for Rad23 binding and subsequent shuttling to the proteasome. At least some substrates, for example the cyclin dependent kinase inhibitor Far1, which are dependent on Rad23 for degradation also rely on Cdc48 and likely Ufd2 (Fu, Ng, Feng, & Liang, 2003; Verma, Oania, Graumann, & Deshaies, 2004). The Cdc48, Ufd2, Rad23 pathway is known to be critical for the degradation of many ERAD substrates including Spt23, Hmg2 and the artificial ERAD model substrate ^{Deg1}SEC62 (Rumpf & Jentsch, 2006). Moreover, Rpn10 has been suggested to function redundantly with Ufd2 as the above mentioned ERAD substrates are moderately but significantly more stable in *ufd2Δ* strains and much more stable in *ufd2Δ rpn10Δ* strains, although this stability may simply

be due to defective assembly of the proteasome in the *rpn10Δ* mutant (Glickman et al., 1998; Richly et al., 2005).

1.2.4 Substrate deubiquitination and proteasomal degradation

Ubiquitin is a stable protein and therefore it is believed that substrates are deubiquitinated at the proteasome prior to or during their degradation (Haas & Bright, 1987). Ubp6 and Rpn11 are intrinsic subunits of the 26S proteasome and both possess deubiquitination activity (Guterman & Glickman, 2004; Verma et al., 2002; Yao & Cohen, 2002). Proteasomes possessing both Ubp6 and Rpn11 will deubiquitinate the unnatural ubiquitin-GFP fusion protein without subsequent degradation. Preventing deubiquitination either by addition of N-ethyl-maleimide or incubation of proteasomes lacking Ubp6 and Rpn11 results in the degradation of both ubiquitin and GFP moieties (Guterman & Glickman, 2004). Sic1 is deubiquitinated by Rpn11 and if this activity is blocked then Sic1 is stabilized although it does become ubiquitinated and binds the proteasome (Verma et al., 2002).

An elegant characterization of another deubiquitinating enzyme, Ubp14, demonstrated that Ubp14 is the major deubiquitination activity for free ubiquitin chains and *upb14Δ* cells accumulate free ubiquitin chains which inhibit degradation of the 26S Proteasomal substrates, Mat alpha2 and Ub-P-β-Galactosidase and L-β-Galactosidase (Amerik, Swaminathan, Krantz, Wilkinson, & Hochstrasser, 1997). Ubp14 does not deubiquitinate the proteasomal substrate but it disassembles the ubiquitin chains after substrate deubiquitination so that these chains do not rebind the proteasome. If they are not disassembled then these free ubiquitin chains become proteasomal inhibitors.

Once deubiquitinated, proteasomal substrates are degraded by the 26S Proteasome. The 26S Proteasome is an approximately 2 megadalton complex which unfolds the substrate in an ATP dependent manner and degrades the substrate in an ATP independent reaction (Hough, Pratt, & Rechsteiner, 1986, 1987; Rechsteiner, 1998). The 26S Proteasome can be electrophoretically resolved into two separate structures, namely the 20S Core particle and the 19S regulatory particle. The 20S core particle is a barrel-shaped protease that possesses at least three distinct endoproteolytic activities: a tryptic activity (cleaving after basic residues), a chymotryptic activity (cleaving after hydrophobic residues), and a Glu-C like activity (cleaving after acidic residues) (Kisselev, Callard, & Goldberg, 2006). Mutagenesis studies suggest that the 20S core contains all the proteolytic activities of the 26S complex while the 19S regulatory cap structure stimulates proteolysis by providing the ATP dependent unfolding activity which is required for proteolysis of the substrate (Braun et al., 1999; C. W. Liu et al., 2002; Seemuller et al., 1995). Much is known about the structure and assembly of the 26S proteasome but that is beyond the scope of this thesis and I refer the reader to reviews which are able to cover these subjects in detail (Hanna & Finley, 2007; Rechsteiner, 1998).

1.2.5 The role of ubiquitin-dependent protein degradation during the G1 phase

There are numerous substrates of the SCF/Cdc34 complexes that have been identified, but only a few have been implicated specifically in cell cycle progression with their degradation confined to a specific phase of the cell cycle. Known substrates degraded in a cell cycle dependent manner include Far1, Sic1, Cln1, Cln2 and Cdc6 (Fu et al., 2003; Schneider et al., 1998; Schwob et al., 1994). All of these substrates play

important roles in the G1 phase and the transcription rates of their genes are cell cycle regulated (Spellman et al., 1998). Transcription of these genes is induced late in mitosis and repressed in late G1. Their degradation also occurs during the G1 phase. Far1 and Sic1 are phosphorylated by the Cln(1-2)/Cdc28 complexes and recognized by SCF^{Cdc4} which catalyzes their ubiquitination (Henchoz et al., 1997; Tyers, 1996). Cln1 and Cln2, in collaboration with Cdc28, phosphorylate themselves, resulting in recognition by SCF^{Grr1} and subsequent degradation (Barral, Jentsch, & Mann, 1995; Lanker et al., 1996; Willems et al., 1996). By the end of G1, Cln1 and Cln2 are virtually absent (Schneider et al., 1998). The degradation of ubiquitinated Far1 depends on a pathway involving Cdc48, Ufd1 and Rad23 which most likely extends the initial ubiquitin chain conjugated by SCF^{Cdc4} and delivers modified Far1 to the proteasome (Fu et al., 2003; Verma et al., 2004). Far1 is degraded early in G1 and its degradation is required for cell cycle progression (Henchoz et al., 1997). Far1 is an inhibitor of Cln/CDK activity and its stabilization leads to an arrest much like the *cdc28* arrest (Henchoz et al., 1997). Sic1 is another cyclin dependent kinase inhibitor which serves to keep the Clb/Cdc28 kinase inactive in G1 so that origins of DNA replication do not fire prematurely. Sic1 is not an inhibitor of the Cln/Cdc28 complexes so the landmark events of G1 induced by the Cln/Cdc28 complex, namely septin ring formation, bud emergence and spindle pole body duplication are unaffected by the stabilization of Sic1 (Goebl et al., 1988). Sic1, like Far1, is phosphorylated by the Cln/Cdc28 complex and subsequently recognized by SCF^{Cdc4} which ubiquitinates Sic1 and targets it to the proteasome through an Rpn10 dependent pathway (Nash et al., 2001; Verma et al., 2004). A recent report has called into question the requirement of Sic1 phosphorylation and degradation for cell cycle

progression (Cross, Schroeder, & Bean, 2007). Cells containing Sic1 which lacks all phosphorylation sites are viable but with a delay in the budded portion of the cell cycle. The fact that these cells are viable is at odds with the *cdc34Δ*, *cdc4Δ* and *cln1Δ cln2Δ cln3Δ* strains which are inviable and require Sic1 destruction for appropriate progression through START. Certainly more work needs to be done to explain this paradox.

1.3 Research Objectives

The objective of this research is to expand our understanding of a highly conserved motif within the ubiquitin conjugating enzyme Cdc34 with the expectation that a better understanding of this enzyme, its function and regulation will enhance our knowledge of the eukaryotic cell division cycle. Cdc34 is well conserved in all eukaryotic species sequenced to date and it is necessary for targeting certain cell cycle promoting and inhibiting proteins for timely degradation. A motif which is highly conserved among eukaryotes and which is unique to the Cdc34/Ubc7 family of ubiquitin conjugating enzymes is not required for Cdc34 to fulfill its function *in vivo* and this paradox left us to wonder why this motif had been so strongly selected for throughout evolution. Cdc34 is particularly interesting because it serves as one of the key enzymes of the cell division cycle. Its activity in targeting the cyclin dependent kinase inhibitors, Far1 and Sic1 in yeast and $p27^{Kip}$ and $p21^{Cip}$ in mammalian cells, for degradation is a switch that nearly all eukaryotic cells need to flip in order to initiate DNA replication. Even the slightest alteration in the timing of cyclin dependent kinase inhibitor degradation has major implications for the fidelity of DNA replication and chromosome segregation. The motif within Cdc34 which is the subject of study here and elsewhere (Y. Liu et al., 1995; Silver, Gwozd, Ptak, Goebl, & Ellison, 1992; Varelas et al., 2003) is

also fascinating because its lies in close physical proximity (~5 angstroms) to the enzyme's active site and two separate portions of the motif are both essential for enzyme activity and cell viability. Furthermore, although separately these portions are essential, removal of both portions is tolerated. Intragenic suppression within a single enzyme has been observed during the study of numerous enzymes but to my knowledge this motif of Cdc34 is the only example of dual intragenic suppression where both components are separately essential and both can be made non-essential by mutation of the partner.

Recent advances in molecular biology allowed us to use genome wide analysis tools to better understand the contribution of this motif to Cdc34 function. We found these experiments to be a favorable methodology because it allowed me to first observe the global responses of the cell to perturbation of the motif without having to formulate *a priori* hypotheses. From these initial global screens, we were able to formulate specific hypotheses regarding the contribution of this motif to the mechanism of ubiquitin dependent protein degradation and then use more traditional molecular, genetic and biochemical assays to rigorously test these hypotheses. Previous work from our lab suggested that portions of this motif might also be subject to regulated phosphorylation but the reason for this phosphorylation was unclear. We began these studies with the ultimate goal of determining whether or not an amino acid within this motif was indeed phosphorylated and subsequently identifying the enzymes that catalyze the phosphorylation reaction.

CHAPTER 2: MATERIALS AND METHODS

2.1 Media, Strains and Plasmids

2.1.1 Bacterial growth media

Escherichia coli strains, DH5α, BL21 and XL10-Gold, were grown in LB, SOB or NZY+ media. Liquid LB media contains 10 g Bacto tryptone, 5 g Bacto yeast extract and 10 g NaCl per liter. Liquid NZY+ media contains 10 g NZ amine (casein hydrolysate), 5 g yeast extract and 5 g NaCl per liter and is adjusted to pH 7.5 using NaOH. Prior to use 12.5 ml of 1 M $MgCl_2$, 12.5 ml of 1 M $MgSO_4$ and 10 ml of 2 M glucose are added to complete the NZY+ broth. Liquid SOB media contains 20 g Bacto tryptone, 5 g Bacto yeast extract, 0.5 g NaCl, 25 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$ per liter and is adjusted to pH 7.0 with NaOH. LB, SOB and NZY+ plates also contain 20 g/L Difco agar. Ampicillin is used at a final concentration of 50 mg/L.

2.1.2 Plasmid DNA isolation from bacteria

In order to isolate approximately 0.5 μg of plasmid DNA, DH5α strains harboring the indicated plasmids were grown in 5 ml LB+Ampicillin for 12-16 hours at 37°C. Cells were then pelleted and the plasmids were isolated using a Qiagen Miniprep kit (Qiagen, California, USA) following the manufacturer's instructions.

2.1.3 Site directed mutagenesis

Only plasmids encoding mutations of the *CDC34* gene were constructed during the course of this work. All site directed mutagenesis was carried out using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, California, USA) and its associated protocol. Basically, complementary forward and reverse primers,

approximately 25 nucleotides in length, encoding the desired mutation(s) were used to amplify the appropriate plasmid. The plasmid pRC001 is derived from pYL029 and encodes the CDC34 (S97D/Δ103-114) mutant under control of the GAL10 promoter. To construct pRC001 the forward primer (5'-CCGGAAGACTTTCCCTTTTCTCCA-CCACAGTTTCGATTTACGCC) and the reverse primer (5'-GGCGTAAATCGAAA-CTGTGGTGGAGAAAAGGGAAAGTCTTCCGG) were utilized. In the construction of pTL008 which is derived from pYL150 and encodes CDC34 (R93D) mutation, the forward primer (5'-AACGTTTACAGGGATGGCGACCTTTGTATTTCT) and reverse primer (5'-AGAAATACAAAGGCTCCGATCCCTGTAAACGTT) were utilized. pTL012, encoding CDC34 (R90D/D91N/R93D) mutations and derived from pTL008, was constructed using forward primer (5'-ATCCAAACGTTTACGAGAA-TGGCGACCTTTGTATTTCTATTTT) and reverse primer (5'-AAAATAGAAATACA-AAGGCTCCGATTCTCGTAAACGTTTGGAT). To construct the pRC004 plasmid, the plasmid AD002 was used as template and primers 97AF2 (5'-GGGATGGCAGG-CTTTGTATTGCTATTTTACACCAAAGTGGG) and 97AF2 (5'-CCCACTTTG-GTGTAAAATAGCAATACAAAGCCTGCCATCCC) were used. Following PCR amplification, 1 μl of the *Dpn I* restriction enzyme was added to the reaction mix to digest the parental, methylated plasmid DNA and the reactions were incubated at 37°C for 1 hour. XL10-Gold Ultracompetent cells were transformed (see section 2.2.1) with two μl of the *Dpn* I-treated DNA. Transformants were selected on LB+Ampicillin plates. Multiple clones from each transformation were selected and the plasmid DNA was isolated and sequenced at the Indiana University DNA Sequencing Core Facility. Plasmids used in this thesis are listed in table 2.

2.1.4 Yeast growth media and genetic techniques

Standard rich (YPD) and defined minimal (SD) media were prepared as described previously (M. D. Rose, Winston, & Hieter, 1990). For analysis of Far1 and Cln1 abundance and Cln1 half-life, cells were grown in YPD buffered with 30 mM succinic acid. Standard sporulation and dissection procedures were used as described previously (M. D. Rose et al., 1990). For sulfite sensitivity assays, 2 mM of sodium sulfite and 75 mM tartaric acid was added to YPD media as previously described (Avram & Bakalinsky, 1996). Strains were mated, sporulated and dissected as described previously (M. D. Rose et al., 1990). If a tetrad containing a temperature sensitive allele was dissected, the zymolyase digestion and spore germination were done at room temperature.

2.1.5 Yeast strain construction

Standard methods were used for strain construction (M. D. Rose et al., 1990). Strains RC29, RRC73, RRC74, RRC76, RRC78 containing the *CDC34*^{tm} allele flanked by the nourseothricin N-acetyltransferase gene (*NAT1)*, which confers resistance to the aminoglycoside nourseothricin, were constructed as follows. The plasmid pAG25 containing the nourseothricin N-acetyltransferase gene (Goldstein & McCusker, 1999) was amplified with adaptamer primers CDC34F2 (5'- ACTTTTTTCAAGGCTGAGAA-TCCATCGACAGATTGTAACGAAGCAGCTGAAGCTTCGTACGC- 3') and CDC34R2 (5'- TGCTCTGTATAGTTCAATAGAATCTTACAGTACATCACGC-TGCAAGCATAGGCCACTAGTGGATCTG - 3') using the PCR cocktail and conditions as described previously (Goldstein & McCusker, 1999). The PCR product was transformed into the *CDC34*^{tm} containing strain KS418 using a previously described

transformation protocol (Gietz & Woods, 2002). Selection of transformants was carried out on YPD plus 80 mg/L of nourseothricin (Werner BioAgents, Germany). Insertion into the correct chromosomal locus was confirmed by PCR using the primers 34F2t(5'-CAAACTTGAGATGGAGTTGTTGATG-3') and pAG25Tr1(5'-GTCAATCGTATGTGAATGCT-3'). This strain was named RC6. DNA containing the *CDC34*^{tm} allele and the *NAT1* gene was amplified from RC6 genomic DNA using Phusion DNA Polymerase (Finnzymes, Finland) according to the manufacturer's instructions using primers 34F2t and 34R3 (5'-ATGAGTAGTCGCAAAAGCACCG-3'). To construct strains RRC73, RRC74, RRC76, RRC78 the PCR product from the RC6 genomic amplification was transformed into the appropriate BY4741 strain containing either *CLN1-TAP, CLN2-TAP, SIC1-TAP or FAR1-TAP* at their endogenous chromosomal locus described in (Ghaemmaghami et al., 2003). Transformation was carried out following the protocol of Johnston et al. (M. Johnston, Riles, & Hegemann, 2002) for gene disruption generated from yeast genomic template. Transformants were selected on YPD plus 80 mg/L of nourseothricin.

RC29, the query strain for the SGA screen, was made by a cross of RC6 and MT1901 creating the diploid RC21 which was sporulated and its tetrads dissected until a haploid with the desired markers was acquired. RC94, the control strain for the secondary SGA screen, was constructed by insertion of the nourseothricin N-acetyltransferase gene into strain MT1901 at the exact same chromosomal location as strain RC6. The PCR conditions and transformation were carried out exactly as described for construction of RC6. Strains used during the course of these studies are listed in table 3.

2.1.6 Spot dilution assays

For figure 14A, the *cdc34-2* temperature sensitive strain YL10-1 harboring one of the plasmids pYL150 (*CDC34),* pYL029 (*CDC34^{tm})*, or pYL027 (*CDC34 $^{\Delta 103-114}$*) (Y. Liu et al., 1995) were grown overnight at 28°C in 5 ml SD-Leu. Cells were diluted to equal densities and then 3 μl of each culture and two ten fold serial dilutions were spotted onto SD-Leu plates. Images were taken after 3 days at 30°C. For figure 14B, strains BL2 (WT) and RRC85 [*CDC34^{tm} (NAT1)*] were grown overnight in YPD, diluted to equal densities and then 3 μl of each culture and ten fold serial dilutions were spotted onto a YPD plate.

2.2 Transformations

2.2.1 Bacterial transformation

E. coli (DH5α, BL21, XL10-1 GOLD) were made competent for transformation by streaking an aliquot of frozen cells onto an LB plate. Ten colonies were picked and inoculated into 250 ml SOB media. Cells were grown by vigorously shaking at 18°C until an OD_{600} of 0.6 was achieved. The entire culture was placed on ice for 10 minutes and then centrifuged at 2,500xg for 10 minutes at 4°C. The cell pellet was resuspended in 80 ml TB (10mM Pipes, 15 mM $CaCl_2$, 250 mM KCl made to pH 6.7 with KOH and 55 mM $MnCl_2$). The TB resuspension was centrifuged at 2,500xg for 10 minutes at 4°C and the cell pellet was resuspended in 20 ml of TB containing 7% DMSO. The cell suspension was placed on ice for 10 minutes and then aliquoted into eppendorf tubes in 200 μl increments. The aliquots were frozen with liquid nitrogen and stored at -80°C until use.

Competent *E. coli* were transformed by adding approximately 20 nanograms of plasmid DNA to 50 μl of competent cells and incubating on ice for 25 minutes. Cells were heat shocked at 42°C for 90 seconds and then placed on ice for 90 seconds. Five hundred μl of SOB media was added to the cells and incubated at 37°C for 30 minutes. Cells were then spread on LB+ampicillin plates.

2.2.2 Yeast transformation

For gene disruption generated from a plasmid template, the lithium acetate method for yeast transformation as previously described (M. Johnston et al., 2002) was followed. To transform yeast with plasmid DNA, a quicker but less efficient method was utilized. Briefly, 0.5 μg of the plasmid DNA, 20 μl of 2 mg/ml carrier DNA and four volumes of PEG/Li-acetate/TE solution (PLATE; prepared by combining 90 ml of sterile 45% PEG 3350, 10 ml of 1 M lithium acetate, 0.2 ml of 0.5 M EDTA and 1 ml Tris-HCl, pH 7.5) were combined in an eppendorf tube along with a small swab of cells from a fresh plate or overnight culture. The mixture was quickly vortexed and left at room temperature for 24 hours at which time 100 μl was plated on selective media.

2.3 Protein Expression and Purification

2.3.1 Cdc34 expression and purification using bacteria

Cdc34ΔC^{6XHis} and Cdc34ΔC(S97A)^{6XHis} proteins encoding the first 244 amino acids of Cdc34 fused to a 6XHis tag at their C-terminus were expressed and purified for these studies. Basically, BL21(DE3) bacterial cells harboring either plasmid AD002 (Cdc34ΔC^{6XHis}) or pRC004 [Cdc34ΔC(S97A)^{6XHis}] were grown in 15 ml of LB+ampicillin for 16 hours at 37°C. Five ml of the starter culture was used to inoculate

500 ml LB+ampicillin. The 500 ml culture was grown with vigorous shaking at 37°C for 2 hours or until the OD_{600} reached 1.2 at which time IPTG was added to a final concentration of 0.5 mM. The cells were allowed to grow for an additional 3 hours and were then collected by centrifugation.

To purify the desired histidine tagged proteins, the bacterial pellet was resuspended in 5 ml of Lysis Buffer [50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole and one complete mini, EDTA-free protease inhibitor cocktail tablet (Roche, Mannheim, Germany) adjusted to pH 8 using NaOH]. Five hundred μl of ten mg/ml lysozyme was added and the cell suspension was sonicated for five cycles of ten seconds with a twenty-five percent duty cycle. The lysate was repeatedly drawn through a 23G1 needle to shear the DNA and then spun quickly in a clinical centrifuge. The supernantant was collected and mixed with 1.5 ml of Nickel Sepharose 6 Fast Flow resin (GE Healthcare-Biosciences, Pittsburgh, PA) that had been washed twice with Lysis Buffer. The nickel sepharose slurry was incubated on a rocker at 4°C for three hours. The beads were then washed twice with 1 ml of Wash Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole adjusted to pH 8 with NaOH) for 1 hour at 4°C. Bound proteins were eluted by adding 500 μl of Elution Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole adjusted to pH 8 with NaOH) and mixing at 4°C for 15 minutes. Two additional rounds of elution, accomplished by adding 750 μl of Elution Buffer to the beads and mixing at 4°C for 15 minutes, were necessary to recover the maximal amount of protein. The purified protein was then dialyzed overnight at 4°C in 1 L of Dialysis Buffer I (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 4 mM $MgCl_2$, 1 mM DTT, 55 mM NaCl and 20%

glycerol) and for an additional hour in Dialysis Buffer II (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 4 mM $MgCl_2$, 1 mM DTT, 55 mM NaCl and 50% glycerol).

2.3.2 ^{Gst}Kinase overexpression and purification using yeast

An array of strain EJ758 [*MAT***a** *his3-200, leu2-3,112, ura3-52, pep4::URA3*], harboring a unique plasmid expressing a single GST-kinase fusion protein under control of the P_{CUP1} promoter which has been described previously (Martzen et al., 1999) was used for expression of GST-tagged kinases which would subsequently be purified and as a tool to measure Cdc34 S97 phosphorylation in strains with a singular kinase over-expressed.

For analysis of Cdc34 S97 phosphorylation *in vivo*, each strain of the EJ758 array was grown overnight in 5 ml SD-Ura then diluted to $4x10^5$ cells/ml in 100 ml SD-Ura and grown for 8 hours at 30°C. After 8 hours of growth, copper sulfate was added to a final concentration of 0.5 mM to induce GST-Kinase expression. Induction lasted for 3 hours at which time the cells were pelleted in a Beckman Centrifuge at 4000xg, 4°C for 5 minutes and immediately frozen with liquid nitrogen. The level of Cdc34 S97 phosphorylation in each strain was measured qualitatively by western blot analysis as described in the western blotting section below.

To express and purify the ^{GST}Kinase from yeast, a patch of cells from the EJ758 strain harboring the plasmid encoding the desired kinase was inoculated into 50 ml SD-Ura to an OD_{600} ~0.2 and grown for the remainder of the day, shaking, at 30°C. At the end of the day, the 50 ml culture was diluted into 500 ml so that it reached an OD_{600} of 0.8 the following morning. The following morning, copper sulfate was added to a final concentration of 0.5 mM to induce transcription of the ^{GST}Kinase. The cultures were left

to shake at 30°C for 2 hours. After 2 hr, the culture was split into 250 ml fractions and each was pelleted by centrifugation in a Beckman Centrifuge at 4000xg. One tube was used for protein purification and the other was stored at -70°C for future purification.

Each yeast cell pellet was resuspended in 1 ml EXTRACTION buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 4 mM $MgCl_2$, 5 mM DTT, 10% (v/v) glycerol, 1 M NaCl) with one protease inhibitor cocktail (Roche Diagnostics, USA) added per 10 ml buffer.

The suspension was transferred to an eppendorf tube and glass beads were added up to the level of the meniscus. Cells were lysed at 4°C for 1 minute in a Biospec mini bead beater, using 10 cycles of shearing for 20 sec, followed by 1 minute cooling in an ice-water bath. The cell extract was removed from beads by puncturing the bottom of the tube with a hot (flamed) 25-gauge needle and the liquid was gently forced out with low-pressure air or light centrifugation. The remaining protein was washed off the beads with 0.5 ml EXTRACTION buffer. Two μl of one molar PMSF was added to the two ml of protein solution and insoluble cellular debris was removed by gentle centrifugation. The supernatant was transferred to a new microfuge tube and protein concentration was determined by the Bradford method (Bradford, 1976). Normally protein yields ranged from 15-25 mg/ml.

The crude protein extract was diluted with an equal volume of NO SALT WASH BUFFER (50 mM Tris-HCl, 4 mM $MgCl_2$, 1 mM DTT, 10% Glycerol) to bring the final salt concentration to 0.5 M NaCl. This salt concentration was chosen because it is high enough to prevent most nonspecific protein-ligand or protein-protein interactions, while still preserving required functional interactions. It may be necessary to alter this

concentration to preserve kinase+cyclin or kinase+targeting subunit interactions although a functional Pho85/Pcl7 complex was purified at this salt concentration (Tan, Morcos, & Cannon, 2003). One hundred μl of pre-equilibrated glutathione-Sepharose resin were added per two ml of extract (as measured after addition of No Salt Wash Buffer). The tube was mixed gently at 4°C for 3 hours. To remove non-specific proteins, the mixture was centrifuged for 20-30 seconds at low speed in a microfuge and the supernatant was decanted. The beads were washed twice with 1 ml of WASH BUFFER (0.5 M NaCl, 4 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 50 mM Tris-HCL, pH 7.5) by mixing for 15 minutes, then centrifuged and the wash buffer was decanted. Bound proteins were eluted by adding 2 ml ELUTION BUFFER (29.7 ml WASH BUFFER plus 0.3 ml of 2M NaOH and 0.23 g Glutathione. The Wash Buffer and NaOH were mixed first followed by addition of glutathione) to the resin, followed by 1 hour of mixing, and then low speed centrifugation. The elution was dialyzed overnight against DIALYSIS BUFFER I (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 4 mM $MgCl_2$, 1 mM dithiothreitol, 55 mM NaCl, 20% (v/v) glycerol) for 2 hours at 4°C and then overnight against DIALYSIS BUFFER II (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 4 mM $MgCl_2$, 1 mM dithiothreitol, 55 mM NaCl, 50% (v/v) glycerol) and stored at -20°C. Normal ^{GST}Kinase yield from a 250-ml culture is 0.5 ml of protein at ~250 μg/ml.

2.4 Antibody Production and Purification

2.4.1 Antigen production and rabbit immunization

In collaboration with Open Biosystems (Alabama, USA), antibodies were generated that recognize Cdc34 when serine 97 is phosphorylated. The phosphopeptide DGRLCI(pS)ILHQ (N- and C-termini are not blocked) was synthesized and then

conjugated to the Keyhole Limpit Hemocyanin (KLH) protein. On day one, 0.5 mg of KLH-phosphopeptide conjugate was emulsified with Freund's complete adjuvant and subsequently used to immunize two New Zealand white rabbits. On days 14, 28 and 42, the rabbits were injected with an additional 0.25 mg of KLH-phosphopeptide conjugate emulsified with Freund's incomplete adjuvant. On days 35 and 56, approximately 25 ml of serum was collected from each rabbit. Production bleeds of 50 ml were taken from each rabbit on day 70 at which time the animals were euthanized.

2.4.2 α-pS97 Antibody ELISA titers

Post-injection antibodies were detected by indirect ELISA against the passively coated peptides DGRLCI(pS)ILHQ and DGRLCISILHQ with anti-rabbit IgG-Horseradish peroxidase (HRP) conjugate and ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) which is a water-soluble HRP substrate that yields a green end product upon reaction with HRP. The green product has two major absorbance peaks, 410 nm and 650 nm. The peptides were coated directly onto an Immulon II plate without conjugation to the KLH which avoids articial increase in titer levels due to the presence of KLH-specific antibodies. Titers are reported as the reciprocal sum of serum dilution. Values are calculated by measuring the dilution point where the absorbance drops below 0.2 at OD_{405} which is four times the background absorption.

Table 1. ELISA titers of α-pS97 antisera. Values were established by indirect ELISA as described in section 2.4.2.

Animal	Peptide	Pre-bleed	Day 35	Day 58
PA0589	DGRLCI(pS)ILHQ	< 50	25,600	> 204,800
PA0589	DGRLCISILHQ	< 50	< 50	12,800
PA0590	DGRLCI(pS)ILHQ	< 50	> 204,800	> 204,800
PA0590	DGRLCISILHQ	<50	51,200	51,200

2.4.3 α-pS97 Antibody Purification

To ensure that the antibody specifically recognized Cdc34 phosphorylated on serine amino acid residue 97, 360 μg of purified, bacterially expressed Cdc34ΔC^{6XHis} was electrophoresed on a 10% SDS-PAGE gel. The protein was transferred to PVDF membrane for 2.5 hours at 30 volts at 4°C in Transfer Buffer (25mM Tris, 190mM Glycine, 15% methanol). The membrane was blocked with 5% milk in 1X KPBS-T for 1 hour at room temperature. After blocking, the membrane was exposed to 2 ml of crude anti-pS97 (Rabbit PA0590, 1 ml day 56 and 1 ml day 70) antisera which had been diluted with 13 ml of sterile 1X KPBS-T + 0.02% sodium azide. The membrane was incubated overnight at 4°C with the antisera. The unbound fraction was collected and 11.5 of the 13 ml were purified with protein A beads as previously described (Harlow & Lane, 1999). α-pS97 antibodies were eluted from the protein A column with 2 ml of 100 mM Glycine (pH 2.2) and immediately neutralized with 100 μl of 1 M Tris-HCl (pH 9.1). An A_{280} measurement estimates the α-pS97 antibody concentration to be 1.7 mg/ml. Sodium azide was added to a final concentration of 0.02% prior to storage at 4°C.

2.5 Protein Manipulation

2.5.1 Yeast protein extraction methods

Once grown to the desired density, cells were collected by centrifugation for 5 minute at 4000xg in a Beckman centrifuge which had been cooled to 4°C. The cell pellet was immediately frozen with liquid nitrogen and stored at -80°C until lysis. To extract the protein, the cell pellet was washed with water and then resuspended in 300 µl of Breaking Buffer [150 mM NaCl, 50 mM Tris-HCl pH7.5, 5 mM EDTA, 1% Triton X-100, 50 mM NaF and one Complete Mini protease inhibitor cocktail tablet (Roche Diagnostics, USA) per 10 ml of breaking buffer]. Five hundred micrometer acid washed, glass beads (Sigma, USA) were added to the cell suspension and the cells were broken by repeated rounds of glass bead beating until a protein concentration of approximately 5 mg/ml was achieved. Usually, this required three rounds of bead beating at one minute per round. Protein concentration was determined by the Bradford method (Bradford, 1976).

The Horvath-Rietzman protocol (Horvath & Riezman, 1994) is an alternate method for protein extraction from yeast cells. This protocol was used infrequently for these studies but when used it has been denoted. Briefly, 3.75 µl of Extraction Buffer (60mM Tris pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 5 mM EDTA, 50 mM NaF plus one Complete Mini protease inhibitor cocktail tablet (Roche Diagnostics, Germany) per 10 mL of extraction buffer) was added per milligram of wet cell pellet. Cells were resuspended in extraction buffer and boiled at 95°C for 5 min. Protein extract was then spun at 14,000 rpm in a cooled (4°C) microfuge for 5 minutes and the supernatant was saved as the protein extract.

2.5.2 SDS-Polyacrylamide gel electrophoresis and western blot analysis

Five times concentrated Laemmli Sample Buffer (20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue and 62.5 mM Tris, pH 6.8) was added in an appropriate amount to the protein (~50 μg of protein) made from the glass bead method. Protein from either extraction method was loaded in equal amounts onto a 10% or 12% SDS-PAGE gel. Separated proteins were transferred to a PVDF membrane using Transfer Buffer (25 mM Tris, 190 mM Glycine and 15% methanol). The membranes were washed with KPBS-T (135 mM NaCl, 2.5 mM KCl, 5.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.2% Tween-20, pH to 7.2) and blocked with 5% milk in KPBS-T. Antibodies were used at the following dilutions, affinity purified α-Cdc34 (1:10,000), affinity purified α-MBPSic1 (1:1000) (Babbitt et al., 2005), α-pS97 antibody (1:1000), α-Ace2 antisera (1:5000) (Sbia et al., 2008) and α-TAP (1:2000) (Open Biosystems, USA). Primary antibody was detected with an HRP conjugated goat α-rabbit secondary antibody at a 1:10,000 dilution (Santa Cruz Biotechnology, USA).

2.6 *In Vitro* Phosphorylation of Cdc34

2.6.1 Detecting Cdc34 phosphorylation using ^{32}P

Bacterially expressed $^{6XHis}Cdc34^{\Delta C}$ and $^{6XHis}Cdc34(S97A)^{\Delta C}$ (1.3 nanomoles) were incubated with 10 μl (1 unit/μl) of bovine Protein Kinase A (Sigma Aldrich, USA) or 10 μl of ^{GST}Tpk3 purified from yeast (section 2.3.2) in PKA phosphorylation buffer (35 mM Potassium Phosphate, pH 7.4, 0.25 mg/ml BSA) plus 10 μl of 5X ATP solution (1 mM ATP, 25 mM $MgCl_2$, 310 cpm/pmol [γ-^{32}P]ATP) in a total reaction volume of 100 μl. When PKA Inhibitor (Sigma-Aldrich, USA) was used, 1μl (1 mg/ml) was added to the

reaction. The reactions were incubated at 30°C for 20 minutes and stopped by addition of 25 μl of 5X Laemmli Buffer. Samples were heated at 95°C for 3 minutes and 30 μl of each reaction was loaded onto a 12% SDS-PAGE gel. The gel was run and then dried and exposed to film for 4 hours.

2.6.2 Detecting Cdc34 phosphorylation using α-pS97 antibody

Bacterially expressed $^{6XHis}Cdc34^{\Delta C}$ (165 picomoles) was incubated with 10 μl (1 unit/μl) of bovine Protein Kinase A (Sigma Aldrich, USA) or 5 μl of ^{GST}Tpk3 purified from yeast (section 2.3.2) in PKA phosphorylation buffer (35 mM Potassium Phosphate, pH 7.4, 0.25 mg/ml BSA) plus 10 μl of 5X ATP solution (1 mM ATP, 25 mM $MgCl_2$) in a total reaction volume of 50 μl. When PKA Inhibitor (Sigma-Aldrich, USA) was used, 1 μl (1 mg/ml) was added to the reaction. The reactions were incubated at 30°C for 10 minutes and the samples were placed on ice. Ten μl of each reaction was spotted onto a strip of PVDF membrane which had been pre-soaked in methanol. The spot was allowed to dry for 30 minutes. The membrane was re-wet with methanol and then processed as described in the western blotting section above (section 2.5.2).

2.7 Microarray Analysis

2.7.1 Yeast growth conditions

A *CDC34^{ts}* strain (RRC85) and a wild type strain (DBY2059) were inoculated from stationary phase cultures into a synthetic defined minimal medium containing 2% dextrose, 0.17% yeast nitrogen base minus amino acids and ammonium sulfate, 0.25% L-glutamine, 0.025% magnesium sulfate and 25.2 mg/L L-Leucine. Four separate cultures of each strain were grown at 30°C, allowing between three and four doublings and cells

were collected at approximately 8 x 10^6cells/ml. Cells were centrifuged and immediately frozen in liquid nitrogen.

2.7.2 RNA extraction and cRNA construction

Cells were centrifuged and immediately frozen in liquid nitrogen. Total RNA was extracted using a hot acid phenol-chloroform protocol as previously described (Schmitt, Brown, & Trumpower, 1990). RNA quality was verified with $OD_{260/280}$ readings and a 1.5% agarose gel. The following portion of this microarray study was carried out using the facilities of the Center for Medical Genomics at Indiana University School of Medicine. The Center for Medical Genomics is supported in part by the Indiana Genomics Initiative at Indiana University (INGEN®), which is supported in part by the Lilly Endowment, Inc. Briefly, cDNA was synthesized from the original RNA template by single cycle labeling using a T7 promoter-dT24 oligonucleotide as primer with the Invitrogen Life Technologies SuperScript Choice system (Invitrogen, California, USA) per the manufacturer's instructions. Following second strand cDNA synthesis and incubation with T4 DNA polymerase, the products were purified using an Affymetrix Cleanup Module (Affymetrix, California, USA) per the manufacturer's protocol. The cDNA was converted to cRNA using the Affymetrix IVT kit, again following the manufacturer's instructions. The cRNA was purified with Qiagen RNAeasy columns (Qiagen, California, USA), quantitated and then fragmented by incubating at high temperature with magnesium.

2.7.3 cRNA hybridization and data analysis

Fifteen µg of biotinylated cRNA was added to a total hybridization cocktail of 300 µl, and 200 µl was hybridized to an Affymetrix Yeast 2.0 GeneChip after adding control

oligonucleotides. The cRNA was hybridized at 45°C for 17 hours with constant rotation. The hybridization mixture was then removed and the Affymetrix Yeast 2.0 GeneChip were washed, stained with phycoerythrin-labeled Streptavidin, washed, incubated with biotinylated anti-streptavidin, and then restained with phycoerythrin-labeled Streptavidin for signal amplification. Balanced groups of samples were handled in parallel to reduce non-random error. The arrays were then scanned using the dedicated scanner, controlled by Affymetrix GCOS software. The Affymetrix Microarray Suite version 5 (MAS5) algorithm was used to analyze the hybridization intensity data from each array. The average intensity on each array was normalized by globally scaling to a target intensity of 1000. A student's t-test of the log base 2 transformed data was used to establish p-values. The q-value, or the false discovery rate, for each transcript was calculated based on the associated p values using the method of Storey et al. and setting π_0 equal to 0.661958910889993 (Storey & Tibshirani, 2003). A software package was downloaded from http://genomine.org/qvalue/ to assist with the calculations. The microarray data presented in this thesis have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible to the public through GEO Series accession numbers GSM210016-GSM210023.

2.8 Synthetic Gene Array

2.8.1 A screen for interactions with non-essential genes

Construction of RC29, the query strain for the primary SGA screen, is described in the "Yeast Strain Construction" (2.1.4) section of this thesis. Duplicate SGA screens of the *CDC34^{tm} (NAT1)* query strain, RC29, were performed against the approximately 4,700 single, non-essential gene deletion strains as previously described (Tong et al.,

2001). Computer-based image analysis of colony size allowed genetic interactions to be scored on a scale of 1-4 with 1=lethal, 2=severely sick, 3=sick, 4=mildly sick. Candidate synthetic genetic interactions were validated by a second SGA screen which was done manually. Strains RC29 and RC94, isogenic to RC29 except that the *NAT1* gene marks a wild type *CDC34* allele, were separately crossed to strains with the candidate gene deletion. Diploids were selected by replica-plating onto YPD + 100 mg/L nourseothricin + 200 mg/L G418 then isolated by streaking for single colonies on the same media. The isolated diploids were sporulated on Sporulation Medium (1% Potassium Acetate, 2% Agar, 0.1% yeast extract, 0.05% glucose supplemented with 1mM of uracil, histidine and leucine). Diploids were sporulated at room temperature for 7 days and then struck onto Haploid, Double Mutant Selection Media (2% Glucose, 0.17% Yeast Nitrogen base lacking ammonium sulfate and amino acids, 0.1% monosodium glutamatic acid, 0.2% Amino Acid Mix lacking histidine and arginine, 50 mg/L canavanine, 100 mg/L nourseothricin, 200 mg/L G418 and 2% Agar). The *CDC34*^{tm} (*NAT1*) *xxxΔ::KanR* genetic interactions were scored visually after 3 days growth at 30°C by comparing its growth to that of the *CDC34 (NAT1) xxxΔ::KanR* haploid.

To determine if deletion of *SIC1, ACE2* or *SWI5* was capable of suppressing the lethality of certain double mutants, *SIC1, SWI5* or *ACE2* were disrupted with the *K. lactis URA3* gene in a diploid strain which was heterozygous for the gene deletion of interest (for example *RAD23)* and heterozygous for the *CDC34*^{tm} allele. The primers used were as follows: SIC1(5'-CCACCAAGGTCCAGAGGGACTAGGTAC-CTTACTAGTGGATCTGATATCC-3' and 5' GCTCTTGATCCCTAGATTGAA-ACAATGCCTCGATTTAGGTGACACTAT-3'), ACE2 (5'-TGGATAACG-

TTGTAGATCCGTGGTATATAAACTAGTGGATCTGATATCAC-3' and 5'-TCAGAGAGCATCAGTTTCGTTTGAAAGGGTGCGATTTAGGTGACACTAT-3') and SWI5 (5'-GATGGATACATCAAACTCTTGGTTTGATGCACTAGTGGATCT-GATATCAC-3' and 5'-CTTTGATTAGTTTTCATTGGCGAAACCATAC-GATTTAGGTGACACTAT). The plasmid pUG72 (Gueldener, Heinisch, Koehler, Voss, & Hegemann, 2002) which contains the *K.lactis URA3* gene was amplified with adaptamers for *SIC1, ACE2 or SWI5* listed above using the PCR cocktail and conditions as described previously (Goldstein & McCusker, 1999). The PCR product was transformed into the desired diploids using a previously described transformation protocol (Gietz & Woods, 2002). Transformants were isolated and patched onto YPD and grown for 24 hours after which they were replicaplated onto the Sporulation Media described above. Diploids were sporulated at room temperature for 7 days and then struck onto the Haploid, Double Mutant Selection Media described above. Plates were imaged after 72 hours of incubation at 30°C.

2.8.2 A screen for interactions with essential genes

A screen for genetic interactions between the *NAT1* marked $CDC34^{tm}$ and essential genes was carried out much like the screen for genetic interactions between $CDC34^{tm}$ and the non-essential genes described in section 2.8.1. Basically strains RC29 and RC94 were mated to each of the ~575 strains containing a single essential gene under control of a $TetO_7$ promoter (Yu, Pena Castillo, Mnaimneh, Hughes, & Brown, 2006). The essential gene remains at its native chromosomal location but part of its promoter has been replaced with a doxycycline repressible element ($TetO_7$). The *KANR* gene marks the regulatable, essential allele and so diploids for this screen were selected on YPD +

G418(200 mg/L) + Nourseothricin (90 mg/L). The diploids were sporulated for five days at room temperature and then were replicaplated onto Haploid, Double Mutant Selection Media (2% Glucose, 0.17% Yeast Nitrogen base lacking ammonium sulfate and amino acids, 0.1% monosodium glutamatic acid, 0.2% Amino Acid Mix lacking histidine and arginine, 50 mg/L canavanine, 100 mg/L nourseothricin, 200 mg/L G418 and 2% Agar) without doxycycline. After 3 days, the haploids were replicaplated onto new Haploid, Double Mutant Selection Media plates containing 10 mg/L doxycycline. Growth of the *CDC34* (*NAT1)* and *CDC34*^{TM} *(NAT1)* haploids was scored after 3 days at 30°C.

CHAPTER 3: DISCOVERY AND CHARACTERIZATION OF THE ESSENTIAL PHOSPHORYLATION OF CDC34 SERINE 97

3.1 Structure/Function Studies of Cdc34 Serine 97 Mutants

A partial sequence alignment of the yeast ubiquitin conjugating enzymes and Cdc34 orthologs is shown in figure 3A. It is apparent that there is a relationship among S73, S97 and a twelve amino acid "loop" region which surrounds the catalytic cysteine. Ubc7, like Cdc34, contains serine residues at the positions equivalent to yeast Cdc34 S73 and S97. In contrast, the majority of E2s, of which Rad6 is a classic example, have a lysine and an aspartic acid residue, respectively, at these positions. Cdc34/Ubc7 family members also contain an insertion near S97 (residues 103-114 of Cdc34) that other E2s lack. This motif of serine/serine/insert or lysine/aspartic acid/no insert is conserved among most eukaryotic E2s (Y. Liu et al., 1995). The amino acid residues that constitute the motif are in close physical proximity. The crystal structure for Ubc7 shows serine residue S91 (Cdc34 S97 equivalent) to lie within 3 Å of the well conserved aspartic acid residue (D108) of the twelve amino acid "loop" while serine residue S67 (Cdc34 S73 equivalent) is within 4 Å of the invariant tryptophan residue (W110) (Cook, Martin, Edwards, Yamazaki, & Chau, 1997). Even with differences at these amino acid residues, the tertiary fold of the Cdc34/Ubc7 family resembles that of the Rad6 family (Fig. 3B).

Previously, our lab determined that mutation of Cdc34 which changes amino acid residue S97 to alanine or aspartatic acid renders the Cdc34 enzyme non-functional both *in vivo* and *in vitro*; however, the S97T mutation does not compromise Cdc34 activity *in vivo* (Y. Liu et al., 1995; Varelas et al., 2003). During the course of reproducing the growth assays, a condition was found in which the Cdc34 S97D mutation can

complement a *cdc34-2* temperature sensitive strain at a non-permissive temperature (Fig. 4A). Our lab's previous study used 36°C as the non-permissive temperature and used patch assays to assess complementation of the *cdc34-2* temperature sensitive strain by *CDC34* (S97) mutations (Y. Liu et al., 1995). For experiments reported here, the non-permissive temperature is set to 35°C and complementation was assessed by a spot dilution assay which is more sensitive to differences in growth and allows more cells to be deposited on a plate than a patch assay. Two notable discoveries resulted from this experiment. First, the Cdc34 S97D mutant can complement a *cdc34-2* temperature sensitive strain when overexpressed at the non-permissive temperature of 35°C while the Cdc34 S97A mutant does not complement at any temperature or any expression level (Fig. 4A). However, neither the S97D nor S97A mutant complements a *cdc34Δ* strain, while a mutation that feasibly permits phosphorylation, S97T, complements both the *cdc34-2* temperature sensitive and *cdc34Δ* strain in a spot dilution assay (Fig. 4B). Furthermore, this experiment reveals that overexpression of wild type *CDC34* on galactose medium inhibits growth compared to the *CDC34 S97T* mutant (Fig. 4B). Comparisons between wild type *CDC34* and an empty plasmid control or other *CDC34* mutants demonstrate that wild type *CDC34* overexpression on galactose media is growth inhibitory (for example, see Fig. 12). This phenotype was exploited in other experiments to explore the functional significance of Cdc34 amino acid residue S97 (Section 3.6).

3.2 Discovery of Cdc34 Amino Acid Residue S97 Phosphorylation

3.2.1. Cdc34 is phosphorylated in vivo on serine residue 97

Cdc34 is phosphorylated *in vivo* on serine residues (Goebl et al., 1994) and some of the serine phosphorylation sites have been mapped (Coccetti et al., 2008; Sadowski et

al., 2007). Due to the combined evidence that the Cdc34 S97T mutant is functional both *in vivo* and *in vitro* and amino acid residue S97 is highly conserved across eukaryotic species, we reasoned that S97 might be a site of phosphorylation. To test this hypothesis, antibodies capable of specifically recognizing Cdc34 phosphorylated on amino acid residue S97 were generated. This antibody, termed α-pS97, recognizes a protein from a whole cell yeast lysate that migrates at approximately the same position as the Cdc34 protein (Fig. 5A, lane 1). When full length Cdc34 is replaced by a C-terminally truncated Cdc34 that migrates noticeably faster through an SDS-PAGE gel, the α-pS97 antibody no longer recognizes a protein at the size of full length Cdc34 but now recognizes a protein which migrates at approximately the same location as the C-terminally truncated Cdc34 protein (Fig. 5A, lane 2) indicating that the α-pS97 antibody recognizes Cdc34 and that Cdc34 is the only protein contributing to the signal at the approximate molecular mass of 42 kDa which is where Cdc34 migrates. The α-pS97 antibody also does not recognize the Cdc34^{tm} (S73K/S97D/Δ103-114) mutant (Fig. 5A, lane 4) indicating that this antibody is specific to S97. Bacterially expressed Cdc34, which should not be phosphorylated, is also not recognized by this antibody even when loaded in excess of the endogenous yeast Cdc34 (Fig. 5A, lane 6). From these studies, we conclude that the α-pS97 antibody is specific for Cdc34 phosphorylated on serine amino acid residue 97 and that Cdc34 is phosphorylated *in vivo* on serine 97. Furthermore, Fig. 5A, lanes one and two, reveal that the tail of Cdc34 (amino acids 245-295) contributes to the level of Cdc34 S97 phosphorylation.

During the course of these experiments, it was discovered that S97 phosphorylation becomes undetectable when the protein extract is subject to a single

freeze/thaw cycle or when the protein extract is made by the Horvath method (Horvath & Riezman, 1994), a common method for yeast cell protein extraction which requires heating the sample in SDS for 5 minutes (Fig. 5B, lane 2 and data not shown). Breaking cells open by repeated rounds of bead beating in either the breaking buffer described in the materials and methods section or in 8M urea allows S97 phosphorylation to be detected by the western blot method. Previous studies have attempted to characterize all the phosphorylation sites of Cdc34 but did not report S97 phosphorylation (Sadowski et al., 2007). This discrepancy is likely due to the method of protein extraction.

3.2.3 Phosphorylation of S97 is induced in the G1 phase

Cdc34 in cooperation with the SCF complexes has a plethora of substrates which it targets for proteasome mediated degradation. Many of the substrates whose degradation is required for timely cell cycle progression, including Cln1, Cln2, Sic1, Far1 and Cdc6, are ubiquitinated and degraded in the G1 phase of the cell cycle. We reasoned that if Cdc34 S97 phosphorylation regulates Cdc34 activity then S97 phosphorylation might be regulated through the cell cycle. To test this hypothesis, cells were synchronized in G1 with alpha factor. After three hours, >95% of the cells were arrested with a mating proficient or schmoo-like morphology. The alpha factor was washed away and cells were resuspended in fresh medium which allowed cells to synchronously re-enter the cell cycle. S97 phosphorylation levels were monitored over the course of the ensuing one and a half cell cycles (Fig. 6). Analysis of the budding index revealed that the cells did indeed re-enter the cell cycle with a high level of synchrony and as expected after one division, the cells became asynchronous as the mothers progress through the second G1 phase faster than the daughter cells. This is because daughters typically

emerge smaller than the mother and spend extra time growing before bud emergence. This experiment revealed two notable discoveries regarding Cdc34 S97 phosphorylation. This first being that the level of Cdc34 S97 phosphorylation is induced in the G1 phase of the cell cycle (Fig. 6, 100 min.). Secondly, S97 phosphorylation is reduced by exposure of cell's to alpha factor. We confirmed this finding in a separate experiment (see Fig. 9) and the reasons for this decrease of S97 phosphorylation in response to alpha factor became clear when we discovered a kinase which phosphorylates Cdc34 S97 (see section 3.3.2 and 3.4).

3.3 Identification of Kinases which Affect the Level of S97 Phosphorylation

3.3.1 A screen for kinases which when overexpressed or deleted alter S97 phosphorylation

Suspecting that the kinase(s) responsible for the phosphorylation of S97 might be essential and thereby, preventing identification in a screen of strains lacking an individual kinase, a genomic ordered array of 124 strains, each over-expressing a unique GST-kinase fusion from a copper inducible promoter was used. Construction and utilization of this collection of strains was described previously (Martzen et al., 1999) as well as a previous utilization of the kinase collection (Burchett, Scott, Errede, & Dohlman, 2001). Strains were grown to mid-exponential phase (3-5x10^6 cells) in minimal media and the expression of the GST-kinase fusion protein was induced with the addition of copper sulfate. Cell lysates were made from each strain and the level of Cdc34 S97 phosphorylation was assessed by the western blot method. More than 90 unique kinases were screened for their effect on S97 phosphorylation in this manner. A number of kinases (Tpk1, Tpk2, Sch9, Vps15, Mkk2, Cla4, Mrk1, Mec1, Mps1, Snf1 and Gcn2),

which when overexpressed, increase the level of S97 phosphorylation (Fig. 7, red lettering). There are also two MAP kinases, Mkk1 and Ste7, which when overexpressed slightly reduce the level of S97 phosphorylation (Fig. 7, green lettering). There were some kinases which were not present in our array (Vps34, Tor1, Tor2 and Ste20 among others) and a number of others which did not grow to a density sufficient for analysis of S97 phosphorylation. It should also be noted that other kinases require targeting subunits and that without simulateous overexpression of both the catalytic and regulatory subunits, the activity of these kinases against certain substrates remains unchanged.

To extend the investigation of kinases affecting S97 phosphorylation, the level of S97 phosphorylation in strains lacking the individual kinases discovered by the initial, kinase overexpression, screen was analyzed. Many of the kinases which increase the level of S97 phosphorylation when overexpressed, the level of S97 is reduced in strains lacking these kinases (Fig. 8, see *sch9Δ, vps15Δ, mkk2Δ, snf1Δ and gcn2Δ*). Loss of other kinases does not noticeably reduce the level of S97 phosphorylation (Fig. 8, see *tpk1Δ. tpk2Δ, mrk1Δ, hsl1Δ, cla4Δ, mkk1Δ*). The level of Cdc34 S97 phosphorylation was also measured in a strain lacking the *VPS34* kinase. *VPS34* was not included in the initial set of strains overexpressing a particular kinase but Vps34 is a lipid kinase that dimerizes with Vps15 and these proteins depend on each other for their activity (Backer, 2008). The Vps15 kinase positively affects affects Cdc34 S97 phosphorylation (see Fig. 7 and Fig. 8); therefore, we reasoned that Vps34 protein activity would also positively affect Cdc34 S97 phosphorylation. Ultimately, this rationale proved sound as deletion of *VPS34* results in a reduction in Cdc34 S97 phosphorylation (Fig. 8). Contribution of the Tor kinases to Cdc34 S97 phosphorylation was also pursued for three reasons. First,

multiple, nutrient sensing kinases were found to control S97 phosphorylation and the TORC1 complex (Tor1, Kog1, Lst8, Tco89) is one of the major sensors of nitrogen availability in eukaryotic cells. Secondly, both Vps15, a kinase which activates TORC1 in some eukaryotic cell types, and Sch9, a kinase activated by TORC1, increase S97 phosphorylation when overexpressed which suggested that TORC1 could lie in the middle of the Vps15, Sch9, Cdc34 cascade. Finally, neither Tor1 nor Tor2 are included in the collection of copper inducible kinases. As predicted, S97 phosphorylation is reduced when cells are treated with the Tor kinase inhibitor rapamycin (Fig. 8, + rapamycin).

Many scenarios exist to explain how loss of kinases such as Tpk1 and Cla4 do not reduce the level of S97 phosphorylation. First, as demonstrated below for Tpk1 and Tpk2 (section 3.3.2), kinases of the same family with a high level of sequence similarity often phosphorylate the same substrates; therefore, deletion of only one member of a kinase family will not change the degree to which a substrate is phosphorylated *in vivo*. This rationale may also explain why deletion of Cla4 does not reduce Cdc34 S97 phosphorylation while its overexpression significantly increases the level of Cdc34 S97 phosphorylation. Cla4, Ste20 and Skm1 share a high degree of sequence similarity and are members of the PAK family of protein kinases which are activated by Cdc42 and Cdc42-like G-proteins. Mrk1 is one of four GSK-3 homologs in *S. cerevisiae* and Hsl1 shares a high degree of similarity with Kcc4 and Gin4.

Another possibility is that these kinases might be phosphorylating a very small population of the intracellular Cdc34 and reduction in the level of only this particular pool goes unnoticed when the entire pool of Cdc34 phospho-S97 is probed. Cdc34

associates with many SCF complexes which are unique in their F-box component and different kinases may phosphorylate Cdc34 S97 only in the context of certain SCF complexes.

3.3.2 Altered PKA activity affects S97 phosphorylation

An upstream component of the cAMP/PKA pathway, *RAS2*, genetically interacts with *CDC34* (Irniger, Baumer, & Braus, 2000). Compromising the activity of protein kinase A, either by deletion of the upstream component, *RAS2,* or growth on a non-fermentable carbon source which reduces intracellular cAMP levels, inhibits the growth of *cdc34-2* temperature sensitive strains at otherwise permissive temperatures (Irniger et al., 2000). This finding suggests that Cdc34 and PKA act in a common pathway and combined with our discovery that Cdc34 S97 phosphorylation increases when *TPK2* is overexpressed led us to test whether S97 phosphorylation levels are affected in other circumstances where PKA activity is altered. There are three isoforms of the catalytic subunit of PKA, Tpk1, Tpk2 and Tpk3. Bcy1 is the regulatory subunit which inhibits PKA catalytic activity in the absence of cAMP. Bcy1 directly binds to cAMP, causing its release from the catalytic subunits and resulting in an increase in PKA activity (Hixson & Krebs, 1980). Strains lacking *BCY1* have increased PKA activity and are sensitive to heat shock and nutrient deprivation due specifically to increased PKA activity (Toda, Cameron, Sass, Zoller, Scott et al., 1987). Our experiments reveal the level of S97 phosphorylation increases in cells harboring the crippled *bcy1-14* allele (Fig. 9, lane 2).

Strains lacking all three *TPK* genes are inviable (Toda, Cameron, Sass, Zoller, & Wigler, 1987) and strains lacking either *TPK1* or *TPK2* do not have altered levels of S97 phosphorylation (Fig. 8); therefore, a strain known as the tpk^{wee} strain, which was

isolated because of its ability to withstand heat shock in the absence of *BCY1,* was utilized to examine the level of S97 phosphorylation in a strain with highly reduced PKA activity (Cameron, Levin, Zoller, & Wigler, 1988). This strain lacks the *TPK2* and *TPK3* genes and Tpk1 activity is highly reduced due to a point mutation. Our studies show that S97 phosphorylation is reduced in the tpk^{wee} strain (Fig. 9, lane 4).

Early experiments dissecting the molecular mechanisms of the alpha factor response found that intracellular cAMP levels decrease in strains of the 'a' mating type when exposed to alpha factor (Liao & Thorner, 1980). This observation combined with our observation that alpha factor decreases S97 phosphorylation in an alpha factor arrest and release experiment (Fig. 6) led me to compare the levels of Cdc34 S97 phosphorylation in alpha factor treated and untreated cells. As predicted, alpha factor exposure leads to a reduction in S97 phosphorylation likely because of the reduction in cAMP and PKA activity (Fig. 9, lane 6). In all, these western blots demonstrate that during exponential growth in glucose and in response to alpha factor the level of S97 phosphorylation is controlled by PKA.

3.4 Reconstitution of Cdc34 S97 Phosphorylation *In Vitro*

PKA is an arginine directed protein kinase and at least two consensus sequences have been determined. PKA exhibits a definite preference for arginine amino acid residues at positions -2 and -3 and a preference for an amino acid with a hydrophobic side chain at the +1 position relative to the phosphorylation site (Denis, Kemp, & Zoller, 1991; Songyang et al., 1994; Tegge, Frank, Hofmann, & Dostmann, 1995). A second consensus sequence for PKA has been identified as R_{-6}-X-X-R_{-3}-X-X-(S/T_0)-B_{+1} with X referring to any amino acid and B referring to an amino acid with a hydrophobic side

chain (Smith, Radzio-Andzelm, Madhusudan, Akamine, & Taylor, 1999). Cdc34 S97 has arginine residues at positions -4 and -7 and an isoleucine residue at the +1 position (Fig. 10A). Although the Cdc34 S97 sequence doesn't align exactly with the PKA consensus sequence we reasoned that the sequence shared enough of a resemblence to test whether PKA can directly phosphorylate Cdc34 S97. Either PKA from bovine source or ^{GST}Tpk3 from yeast can phosphorylate ^{6XHis}Cdc34$^{\Delta C}$ (Fig. 10B, lanes 3 and 7). Bovine PKA and ^{GST}Tpk3 can also phosphorylate the ^{6XHis}Cdc34(S97A)$^{\Delta C}$ mutant but to a lesser degree (Fig. 10B, lanes 5 and 9). The activity of bovine PKA against ^{6XHis}Cdc34$^{\Delta C}$ can be inhibited by addition of Protein Kinase A Inhibitor while ^{GST}Tpk3 appears to be insensitive of this inhibitor (Fig. 10B, lanes 4 and 8). To confirm that phosphorylation of Cdc34 by PKA is specific to S97, the kinase reaction contents were probed with the α-pS97 antibody (Fig. 10C). This reagent revealed that both bovine PKA and ^{GST}Tpk3 can specifically phosphorylate Cdc34 S97. Taken together, these experiments demonstrate that PKA phosphorylates S97 and possibly other amino acids with Cdc34. The sequence surrounding Cdc34 amino acid residue T7 conforms to a PKA consensus sequence and may be another site of PKA phosphorylation which is being phosphorylated in these *in vitro* experiments.

Sch9 is another member of the AGC family of protein kinases. It shares some sequence similarity with the Tpk1-3 proteins and its overexpression can suppress the lethality of a *tpk1Δ tpk2Δ tpk3Δ* strain (Toda, Cameron, Sass, & Wigler, 1988). It serves a redundant role with the Tpk kinases to inhibit autophagy in rich medium and it is believed that both Sch9 and PKA must be inactivated for autophagy (Yorimitsu, Zaman, Broach, & Klionsky, 2007). Sch9 has been described as the yeast equivalent of AKT.

No consensus sequence has been defined for Sch9 as it has very few known substrates but a consensus sequence has been identified for AKT and it, like PKA, is an arginine directed kinase. A peptide library screen defined the AKT consensus sequence as R_{-7}-X-R_{-5}-X-R_{-3}-X-X-S-P_{+1} (Obata et al., 2000). The facts that Cdc34 S97 phosphorylation levels correlate with Sch9 activity *in vivo* (Fig. 7 and 8) and PKA, another arginine directed kinase, phosphorylates S97 led us to test whether Sch9 can also directly phosphorylate S97. We find that ^{Gst}Sch9 purified from yeast, like ^{GST}Tpk3, can directly phosphorylate Cdc34 S97 (Fig. 10C).

3.5 Structure/Function Studies of Cdc34 Serine 97, PKA Consensus Sequence Mutants

Upon closer observation, a pattern emerged from what might be considered the PKA/AKT consensus sequence surrounding S97. The Cdc34/Ubc7 family of ubiquitin conjugating enzymes has one of two sequences neighboring S97. Yeast Cdc34 encodes arginines at positions -7 and -4 and aspartic acids at positions -6 relative to S97. Ubc7 and human Cdc34 have an aspartic acid at position -7 and -4 and an asparagine at -6 (Fig. 3A). These residues were mutated within the yeast Cdc34 to their Ubc7/hCdc34 counterparts to test the contribution to the function of Cdc34 *in vivo*. The R93D and R90D/D91N/R93D mutants complement the *cdc34* temperature sensitive strains much more poorly than the WT enzyme at both high and low levels of Cdc34 expression (Fig 11A). However, the R93D and R90D/D91N/R93D complement the *cdc34Δ* strain as well as the wild type enzyme (Fig. 11B). This suggests that the presence of the endogenous Cdc34 enzyme in the temperature sensitive cells is inhibiting the activity of the Cdc34 R93D and R90D/D91N/R93D mutants. There is also the possibility that the R93D and R90D/D91N/R93D mutants are not stable proteins at 37°C. These data are reported

without extensive interpretation because the possible explanations are too numerous and speculative without further experimentation.

3.6 Genetic Interactions Between Cdc34 and the Kinases which Affect Cdc34 S97 Phosphorylation

Mutagenesis studies of S97 (Fig. 4 and Section 3.1) and the genetic interactions between *CDC34* and both *RAS2* and *SLT2* (Irniger et al., 2000; Varelas, Stuart, Ellison, & Ptak, 2006) suggest that the phosphorylation of S97 increases the activity of Cdc34. The growth inhibitory effects of *CDC34* overexpression on galactose (Fig. 4B) are likely the result of an overly active Cdc34 which might reduce the Cln1 and Cln2 protein levels to such a degree that progression through G1 is slowed. Furthermore, we hypothesized that the growth inhibition resulting from *CDC34* overexpression on galactose would be relieved in a strain(s) lacking the kinase(s) which increase S97 phosphorylation. This concept has been termed dosage suppression and has been documented for other kinase-substrate pairs. Reciprocal relationships of a kinase phosphorylating and reducing a substrate's activity have also been established in this way. This genetic phenomenon is termed synthetic dosage lethality and in the case of the kinase Pho85, its substrates Pho2, Gsy1 and Gsy2 inhibit yeast growth when overexpressed in cells lacking *PHO85* (Sopko et al., 2006).

To test for dosage suppression, *CDC34* was overexpressed in strains lacking a single candidate S97 kinase. Overexpression of *CDC34* on galactose inhibits cell growth in the BY4741 background while BY4741 strains overexpressing the $CDC34^{(S97D/\Delta 12)}$ mutant grow as well as the empty vector control BY4741 strain. Strains lacking either *gcn2Δ, vps15Δ or vps34Δ* allows cells overexpressing *CDC34* to grow at a rate

comparable to the same strain overexpressing *CDC34*$^{S97D/\Delta 12}$ or carrying an empty vector (Fig. 12). A strain lacking either *VPS15* or *VPS34* grow slowly on galactose but slow growth alone will not suppress the growth inhibitory effects of *CDC34* overexpression as *sch9Δ* strains grow slowly on galactose media but are not dosage suppressors of *CDC34* overexpression (Fig. 12). Deletion of *MKK2* slightly suppresses the slow growth phenotype of *CDC34* overexpression on galactose but it is not as robust a suppressor as *GCN2*, *VPS34* or *VPS15*. We interpret these results to mean that Gcn2, Vps34 and Vps15 positively activate Cdc34, likely by controlling the phosphorylation of Cdc34 S97, on galactose containing media. Furthermore, Sch9 exerts very little control over Cdc34 S97 phosphorylation on galactose containing media. We hypothesize that deletion of *GCN2*, *VPS15* and *VPS34* but not *SCH9* should reduce Cdc34 S97 phosphorylation when galactose is the sole carbon source. We were unable to test whether strains which had compromised PKA function could suppress the slow growth phenotype of CDC34 overexpression on galactose due to a conflict with the selectable auxotrophies required to maintain the CDC34-bearing plasmid. This experiment is possible but it would require further strain construction.

3.7 Summary and Model of S97 Phosphorylation

By screening nearly the entire collection of yeast kinases, a number of kinases that affect Cdc34 S97 phosphorylation were discovered. Attempting to explain all the findings of a global screen can be overwhelming so instead common themes among the various kinases which increase Cdc34 S97 phosphorylation were explored. In looking for relationships among these kinases it is apparent that many of them are responsive to intracellular nutrient conditions and in turn, loss of these signaling pathways results in G1

arrest. PKA, Sch9, Tor, Vps34, Vps15, Gcn2 and Snf1 all have a role in nutrient sensing and the resulting intracellular adaptations. PKA is activated by glucose through an increase in intracellular cAMP (Mbonyi, van Aelst, Arguelles, Jans, & Thevelein, 1990). The yeast Targets of Rapamycin, Tor1 and Tor2, also respond to nutrients. The best evidence for this comes from studies which demonstrate that the effects of nitrogen depletion can be closely mimicked by rapamycin treatment. In mammalian cells, and likely yeast cells as well, the Vps34/Vps15 complex is activated by amino acids while glucose starved cells have no detectable Vps34 activity (Byfield, Murray, & Backer, 2005; Nobukuni et al., 2005). Vps34 and Vps15 dimerize and pools of the complex can be found at both the vacuole and late golgi stacks (Kihara, Noda, Ishihara, & Ohsumi, 2001; Obara, Sekito, & Ohsumi, 2006).

Gcn2 and Snf1, on the other hand, are most well characterized for their functions when nutrient conditions less favorable. Gcn2 becomes active when yeast are starved for amino acids. Uncharged tRNAs accumulate upon amino acid starvation. The Gcn2 histidyl-tRNA binding domain binds the uncharged tRNAs and becomes active. This allows Gcn2 to phosphorylate eIF2α, in turn slowing the rate of general translation but increasing the rate of Gcn4 translation. Gcn4 is a transcriptional activator which induces the amino acid biosynthetic genes. Snf1 is the yeast equivalent of AMP Kinase. It is activated by a high AMP/ATP ratio but, unlike mammalian AMPK, is not directly allosterically activated by AMP (Wilson, Hawley, & Hardie, 1996). Low glucose and non-preferred carbon sources lead to high AMP/ATP ratios and therefore strains lacking *SNF1* struggle to grow on sucrose, galactose, acetate and ethanol. This is due to the fact

that Snf1 has a major role in activation of glucose-repressed genes such as genes encoding the gluconeogenic enzymes.

As nutrient sensing is an integral function of the G1 phase, it is not suprising that loss of some of these nutrient sensing kinases results in an arrest in the G1 phase. For example, a strain lacking all the PKA isoforms arrests in G1 as do strains lacking TOR function and *mec1Δ* strains arrest in G1 as well as in the S and G2/M phases (Toda, Cameron, Sass, Zoller, & Wigler, 1987).

Consistent with their established roles in nutrient sensing during the G1 phase, there are interrelationships among the kinases which control Cdc34 S97 phosphorylation. The primary example is phosphorylation of Sch9 by the TORC1 complex (Urban et al., 2007). Phosphorylation of Sch9 on its C-terminus by TORC1 increases Sch9 activity against the ribosomal S6 subunit, suggesting that Sch9 functions as the S6 kinase in yeast (Urban et al., 2007). Furthermore, the Vps15/Vps34 heterodimer activates TORC1 by an unknown mechanism (Nobukuni et al., 2005). From these studies, it appears that Vps34/Vps15 activates Tor in turn activating Sch9 which is consistent with the finding that each of these kinases controls Cdc34 S97 phosphorylation. Protein Kinase A is inactive when bound to the cAMP responsive protein, Bcy1. cAMP binds to Bcy1 and allows the PKA catalytic subunits to dissociate and become active. The kinases Mrk1 and Mkk2, which increase S97 phosphorylation when overexpressed, negatively regulate Bcy1 cytoplasmic accumulation. Regulation of Bcy1 by Mrk1 and Mkk2 has only been observed under conditions of heat stress (growth at 37°C) (Griffioen, Swinnen, & Thevelein, 2003). We hypothesize that overexpression of *MRK1* or *MKK2* may cause relocalization of Bcy1 and result in increased Tpk1-3 activity against Cdc34 S97. Mec1

has also been shown to activate PKA under conditions of DNA damage (Searle, Schollaert, Wilkins, & Sanchez, 2004) which offers a potential explanation for the increase in S97 phosphorylation in a strain overexpressing *MEC1*. As *MEC1* is an essential gene, it has been difficult to assess whether Mec1 contributes to PKA activation under steady state growth conditions.

It is unclear where the phosphorylation of Cdc34 S97 occurs in the cell. Cdc34 can be found in both the nucleus and cytoplasm, although it appears to be more highly concentrated in the nucleus (Goebl et al., 1994). The regulatory and catalytic subunits of PKA are also found in both the nucleus and cytoplasm and the regulation of their localization is important for execution of some of their known functions (Behrens & Mazon, 1988; Bharucha et al., 2008; Griffioen et al., 2003). A pool of Vps15/Vps34 complex and TORC1 resides at the vacuole and it is likely that this is the site of TORC1 activation by Vps15/Vps34.

Sch9 and the PKA catalytic subunits (Tpk1-3) are members of the AGC (Protein Kinase A/G/C) kinase family which consists of the cAMP activated protein kinases, the cGMP activated kinases and Protein Kinase C. Sch9 and PKA may function in redundant pathways as suggested by the findings that *SCH9* acts as a multicopy suppressor of PKA signaling defects and activation of PKA eliminates the slow growth defects of *sch9Δ* cells (Toda et al., 1988). Furthermore, Sch9 and PKA function seemingly redundantly to inhibit autophagy in rich media conditions (Yorimitsu et al., 2007). AGC family kinases have similar consensus sequences neighboring the amino acid to be phosphorylated. Most frequently, two or three basic amino acids are found N-terminal to the serine or threonine which accepts the phosphate group. Protein Kinase A was screened against a

peptide substrate library and was found to have a strong preference for arginine amino acid residues at positions -2 and -3 and a preference for an amino acid with a hydrophobic side chain at the +1 position (Denis et al., 1991; Songyang et al., 1994; Tegge et al., 1995). A second consensus site for PKA has been identified as R_{-6}-X-X-R_{-3}-X-X-(S/T_0)-B_{+1} with X referring to any amino acid and B referring to an amino acid with a hydrophobic side chain (Smith et al., 1999). Cdc34 S97 can be phosphorylated by both PKA and Sch9 and although atypical, Cdc34 S97 is part of a reasonable AGC-type consensus sequence with arginine residues at positions -4 and -7 and isoleucine at +1 relative to S97.

We have not yet explored the impact of S97 phosphorylation on Cdc34 ubiquitin conjugating activity; however, genetic and molecular experiments suggest that S97 phosphorylation activates the enzyme. Mutating S97 to alanine or aspartic acid inhibits the *in vivo* and *in vitro* activity of Cdc34; however, an S97T mutation is functional. There are published genetic interaction between the cAMP/Ras/PKA pathway and Cdc34 which suggests that PKA stimulates Cdc34 activity (Irniger et al., 2000). Furthermore, elimination of kinases that are positive effectors of S97 phosphorylation, appear to reduce Cdc34 activity *in vivo* (section 3.6). We hypothesize that S97 phosphorylation increases the ubiquitin conjugating activity of Cdc34. The mechanism is unclear but our working model is as follows. Self-association is required for Cdc34 function and Cdc34 S97D mutants do not self-associate (Y. Liu et al., 1995; Varelas et al., 2003). However, fusion of an artificial dimerization domain, like GST, to the Cdc34 S97D mutant restores its ability to polyubiquitinate a natural substrate (Gazdoiu et al., 2005). The Cdc34 S97D mutant can interact with the SCF complex (Varelas et al., 2003). The experiment

demonstrating this was an *in vivo* immunoprecipitation of ^{Flag}Cdc34(S97D) showing that it co-precipitates with Cdc53. The wild type Cdc34 enzyme was present in this assay and it is possible that the wild type Cdc34 dimerized with the Cdc34 S97D mutant allowing S97D to interact with the SCF. Our discovery that the Cdc34 S97D mutant can complement a *cdc34* temperature sensitive strain but not a null strain means that the residual activity of the temperature sensitive enzyme is supplying a component of the necessary activity to the S97D mutant which the S97A mutant can not take advantage of. In light of the above discussion, it is plausible that the Cdc34 S97D mutant and wild type enzyme are forming functional heterodimers and supplying the necessary Cdc34 activity to the cell. In a physiological setting, this model could be extended to a scenario where two Cdc34 molecules dimerize after one has been phosphorylated on S97 (Fig. 13). Extrapolating further, we postulate that these molecules of Cdc34, which are differentially modified at S97, have different activities, with one Cdc34 molecule conjugating the initial ubiquitin to the substrate and the other extending the polyubiquitin chain.

CHAPTER 4: THE MOTIF WHICH DEFINES THE CDC34/UBC7 FAMILY OF E2 ENZYMES IS REQUIRED FOR APPROPRIATE TRANSCRIPTIONAL AND POST-TRANSLATIONAL REGULATION OF CDC34 SUBSTRATES

4.1 Structure/Function Studies of the S73/S97/Loop Motif which Defines the Cdc34/Ubc7 Family

Three conserved elements, S73, S97 and an acidic loop, distinguish the catalytic core of Cdc34 from all other E2 ubiquitin-conjugating enzymes except Ubc7. The possibility that these three elements collaborate in a key aspect of Cdc34 function was first suggested by the finding that a substitution of residue S97, or deletion of the loop render yeast inviable, but these mutations act together with the S73K substitution, which by itself has no phenotype, as intragenic suppressors (Y. Liu et al., 1995). A defect in polyubiquitin chain extension could be responsible for lethality associated with lack of the loop alone, as biochemical *in vitro* analysis of the $Cdc34^{\Delta 103\text{-}114}$ construct showed that it cannot rapidly extend polyubiquitin chains (Petroski & Deshaies, 2005). In such a case, however, how could the Cdc34/Rad6 chimeric E2 (Silver et al., 1992), or the triple mutant $Cdc34^{tm}$ E2 (Y. Liu et al., 1995) constructs that lack the loop in the context of the S73K and S97D replacements rescue growth of *cdc34-2ts* yeast? The $Cdc34^{\Delta 103\text{-}114}$ construct has been evaluated in SCF dependent *in vitro* reactions (Petroski & Deshaies, 2005); however, the $Cdc34^{tm}$ construct has never been investigated in an SCF dependent *in vitro* reaction. Previously, our group found the $Cdc34^{\Delta 103\text{-}114}$ mutant supported growth much more poorly that the $Cdc34^{tm}$. This was confirmed with spot dilution assays using a low expression plasmid to suppress a *cdc34*-2 temperature sensitive strain (Fig. 14A). It is clear that the $CDC34^{tm}$ supports yeast growth much more robustly than the

CDC34$^{\Delta 103\text{-}114}$ at low expression levels but strains expressing the wild type *CDC34* show the most robust growth. Next, we wished to compare the function of *CDC34* and *CDC34*^{tm} expressed from the *CDC34* chromosomal locus under its native promoter. The level of colony growth is indistinguishable between strains harboring a chromosomal copy of the *CDC34*^{tm} allele relative to the wild type (Fig. 14B). Furthermore, integration of the *CDC34*^{tm} allele at the endogenous *CDC34* locus does not notably alter the cell cycle distribution as assessed by flow cytometry (Fig. 14C). Although very similar, the flow cytometric profiles of wild type and *CDC34*^{tm} strains are not identical and both peaks are shifted slightly to the left in CDC34^{tm} strains revealing less DNA-bound DAPI (Fig. 14C). In this type of experiment the majority of fluorescence arises from staining of the nuclear DNA but mitochondrial DNA also contributes to the overall fluorescence intensity of a single cell and we believe that the most likely explanation for the slight left-shift in the peaks of the *CDC34*^{tm} strain is a result of less mitochondrial DNA. Furthermore, Fzo1, a protein required for mitochondrial fusion, is a substrate of the SCF^{Mdm30} complex (Cohen, Leboucher, Livnat-Levanon, Glickman, & Weissman, 2008). Changes in its steady state abundance have been shown to increase the amount of mitochondrial aggregation (Escobar-Henriques, Westermann, & Langer, 2006) potentially altering the amount of mitochondrial DNA. A change in Fzo1 steady state abundance due to altered activity of the Cdc34^{tm} protein may explain the slight difference in fluorocytometric profiles of wild type and *CDC34*^{tm} strains.

4.2 Determining the Contribution of the S73/S97/Loop Motif to Substrate Abundance

The disparity between the less efficient, Cdc34$^{\Delta 103\text{-}114}$ catalyzed Sic1 polyubiquitination and the lack of a noticeable change in growth and viability in a

CDC34^{tm} strain led us to test whether the absence of the S73/S97/loop motif in Cdc34 affected the steady state levels of known Cdc34 substrates *in vivo*. Strikingly, the steady state levels of Cln1, Cln2, Sic1 and Far1 were altered in a *CDC34*^{tm} strain (Fig. 15A). The steady state abundances of Sic1, Cln1 and Cln2 are increased while the Far1 steady state level is reduced in the *CDC34*^{tm} expressing strain. Consistent with previous studies, Cln1 protein is visualized as two bands with the slower migrating species representing a modified species (Tyers, Tokiwa, & Futcher, 1993). A similar pattern of migration is seen when yeast extracts are probed for Cln2 protein and the slower migrating species are attributable, at least in part, to phosphorylation (Wittenberg, Sugimoto, & Reed, 1990). Protein half-life analyses verify that Cln1^{Tap} is stabilized in *CDC34*^{tm} yeast. Instead of a half-life of less than 10 minutes (Fig. 15B, *CDC34*, WB and graph; (Blondel & Mann, 1996)), Cln1^{Tap} has a half-life of ~20 minutes in *CDC34*^{tm} cells (Fig. 15B, *CDC34*^{tm}, WB and graph). This change is modest when compared to the half-life of Cln1 in *cdc34-2ts* yeast (Blondel & Mann, 1996), but it uncovers a defect in Cdc34 function that could explain the accumulation of Cln1^{Tap} and of the related Cln2^{Tap} (Fig. 15A, Cln1^{Tap} and Cln2^{Tap}). In contrast, Sic1^{Tap} protein is degraded much faster in *CDC34*^{tm} than in *CDC34* yeast. Instead of a half-life of 50 minutes, which is a typical Sic1 half-life in cycloheximide-treated wild type yeast (Bailly & Reed, 1999), Sic1^{Tap} has a half-life of less than 10 minutes in *CDC34*^{tm} yeast (Fig. 15C, *CDC34*^{tm}, WB and graph). The half-life of Far1 protein could not be measured in *CDC34*^{tm} yeast as they have undetectable Far1 protein (Fig. 15A, Far1^{Tap}).

The increased Sic1 protein turnover rate in *CDC34*^{tm} yeast could result from the accumulation of Cln1-2/Cdc28 activity which drives more rapid recruitment of Sic1 to

SCF^{Cdc4} (Nash et al., 2001) and, as a result, compensates for a modest defect in $Cdc34^{tm}$ function. Whether a similar effect could explain the disappearance of Far1 in *CDC34^{tm}* yeast is unclear. An alternative possibility is that $Cdc34^{tm}$ is defective in SCF^{Grr1}-mediated proteolysis, but that a yet unknown feature of SCF^{Cdc4} can compensate for the defect in $Cdc34^{tm}$, leading to a more rapid proteolysis of Sic1 and Far1 without a change in their recruitment. This model could explain the disappearance of $Far1^{Tap}$ providing that there is no inhibition in the *FAR1* gene expression, and would be consistent with the accumulation of $Sic1^{Tap}$ providing that an increase in the *SIC1* gene expression compensates for the abnormally rapid Sic1 protein turnover. These possibilities are addressed below.

4.3 Microarray Comparison of *CDC34^{tm}* and WT Yeast

4.3.1 The transcription factor Ace2 is responsible for increased transcription of the SIC1 *cluster of cell cycle regulated genes in* $CDC34^{tm}$ *cells*

To investigate the basis of the increase in Sic1 steady state abundance in *CDC34^{tm}* cells and to better understand the functional significance of the Cdc34 S73/S97/loop motif, a microarray analysis was performed. Four biological replicates were sampled for the both a *CDC34^{tm}* strain and a wild type strain. Sic1 mRNA levels are significantly increased 1.6 fold ($p < 0.01$) in the *CDC34^{tm}* strain. Examination of the cluster of genes co-regulated throughout the cell cycle with Sic1, as defined by Spellman et al. (Spellman et al., 1998), revealed that more than 60% of the genes in this cluster are significantly increased ($p < 0.05$) (Table 4). Evidence suggests that this effect can not be attributed to an altered cell cycle distribution of the *CDC34^{tm}* strain as asynchronous *CDC34^{tm}* cultures have a cell cycle distribution approximating that of wild type as assessed by

DNA content (Fig. 14C). The transcriptional flux of most genes in the *SIC1* cluster is attributed to the transcriptional activities of the homologous transcripion factors Ace2 and Swi5. Earlier work dissected the transcription factor dependence for the Sic1 co-regulated gene cluster (Doolin, Johnson, Johnston, & Butler, 2001). As shown in table 4, Ace2 dependent genes are the most highly upregulated in *CDC34^{tm}* cells. Furthermore, the *CLN3* transcript is repressed by Ace2 in daughter cells and this is thought to be a mechanism which allows daughter cells to grow to a larger size prior to START (Laabs et al., 2003). *CLN3* mRNA levels are decreased 1.41 fold ($p = 0.006$) in *CDC34^{tm}* cells.

Our microarray results suggested that in *CDC34*^{tm} cells, Ace2 activity is increased and that Ace2 could be responsible for the increase in *SIC1* transcription. To address this hypothesis, western blot analysis was used to determine whether Ace2 levels were elevated in *CDC34^{tm}* cells. The Ace2 protein levels are increased in *CDC34*^{tm} cells while the Ace2 mRNA levels are not significantly different (Fig. 16A; mRNA fold change of -1.21; pval=0.0616). It was next tested whether the increase in Sic1 protein could be attributed solely to Ace2. Disruption of *ACE2* in the context of *CDC34*^{tm} reduces Sic1 protein levels to below that detected in the wild type strain; deletion of *SWI5* in the context of *CDC34*^{tm} had only a minor effect on the steady state abundance of Sic1 (Fig. 16B). Thus, the increase in the steady state abundance of Sic1 in *CDC34*^{tm} cells is due to increased Ace2, which is the result of an increase in Ace2 protein abundance.

As the increased steady state abundance of Ace2 in *CDC34^{tm}* cells is due to a post-transcriptional mechanism, we reasoned that Ace2 might be targeted for degradation by an SCF/Cdc34 complex. Therefore, the steady state abundance of Ace2 was assessed in strains lacking each of the genes encoding F-box proteins except the essential genes,

CDC4 and *MET30*. The Ace2 levels remain unchanged in all strains tested except *grr1Δ* and *mdm30Δ* cells which both have increased levels of the Ace2 protein (Fig. 16C, lanes 2 and 4 and data not shown). This is consistent with the finding that both Cln1 and Cln2, SCF^{Grr1} substrates, accumulate in $CDC34^{tm}$ cells and our conclusion that the $CDC34^{tm}$ strain is defective in degradation of SCF^{Grr1} substrates. It is striking that the Swi5 transcription factor, a positive regulator of *SIC1* gene expression, is also an SCF^{Cdc4}-dependent substrate of Cdc34 (Kishi et al., 2008). If Ace2 is indeed an SCF^{Grr1}substrate, both transcription factors implicated in *SIC1* gene expression thus appear to be regulated in a manner dependent on Cdc34. SCF^{Grr1} and SCF^{Mdm30} both affect the stability of another transcription factor, Gal4. Gal4 is a transcription factor which induces a suite of genes required for growth on galactose. SCF^{Grr1} ubiquitinates Gal4 under conditions, such as raffinose-dependent growth, where Gal4 target genes are not induced. SCF^{Mdm30} ubiquitinates and destabilizes Gal4 when its target genes are being induced. Ubiquitination and degradation of Gal4 by SCF^{Mdm30} occurs somewhere during the transcriptional process and this actually stimulates translation of the Gal4-dependent transcripts. The conclusion from this study is that Gal4 must be degraded for efficient processing and translation of Gal4-dependent mRNA (Muratani, Kung, Shokat, & Tansey, 2005).

Our lab, in collaboration with Lilia Iakoucheva and Predrag Radivojac, recently published a manuscript entitled “Identification, Analysis and Prediction of Protein Ubiqutination Sites”. In this work, we constructed a computerized predictor of ubiquitination sites based on local sequence information. The predictor was developed from 283 known ubiquitination sites identified in two large-scale proteomics-based

studies along with our own experiments (Hitchcock, Auld, Gygi, & Silver, 2003; Peng et al., 2003). Lysines 82 and 556 of Ace2 are predicted with high confidence to be sites of ubiquitination based on our predictor, UbPred (Radivojac et al., 2009).

We measured the half-life of Ace2 in wild type and *CDC34*^{tm} cells but found no noticeable difference (data not shown). However, the transcription of *ACE2* and localization of Ace2 are regulated in a cell cycle dependent manner. *ACE2* mRNA is up-regulated during the G2/M transition and the protein product enters the daughter cell nucleus late in mitosis (Dohrmann et al., 1992). It can also be found in the mother nucleus and is involved in constitutive transcription of *CUP1* gene (Butler & Thiele, 1991). The pool of Ace2 in the daughter nucleus has been shown to be more unstable than the cytoplasmic pool but the mechanism by which Ace2 becomes unstable is not clear (O'Conallain, Doolin, Taggart, Thornton, & Butler, 1999). Because Ace2 is so highly regulated, we feel that further studies which can elucidate the stability of Ace2 in mother and daughter separately may be more informative regarding the exact mechanism by which Ace2 is up-regulated in *CDC34*^{tm} cells.

It is tempting to consider the possibility that Ace2 may be an SCF/Cdc34 substrate. The SCF ubiquitin ligase complex is responsible for the ubiquitination of numerous transcription factors. Recent evidence demonstrates that ubiquitination of these substrates can play both a positive and negative role in transcriptional regulation and can but does not always result in proteasome mediated degradation (Chandrasekaran & Skowyra, 2008; Lipford, Smith, Chi, & Deshaies, 2005). There are examples of transcription factors, like Tec1, which are polyubiquitinated and degraded resulting in reduced transcription of target genes (Chou, Huang, & Liu, 2004). There is also an

example of transcription factor polyubiquitination and subsequent degradation leading to increased transcriptional activation of that factor's targets (Salghetti, Caudy, Chenoweth, & Tansey, 2001). Another regulatory paradigm has been established by the study of Met4, which in late exponential yeast cultures in media lacking methionine, is polyubiquitinated but protected from degradation by a tight interaction with the F-box protein Met30 and its co-activator proteins. When returned to early exponential growth, the polyubiquitinated Met4 is rapidly degraded, which by an incompletely explained mechanism correlates with transcriptional activation (Chandrasekaran et al., 2006). Another interesting regulatory paradigm comes from studies of the Gal4 transcriptional activator. Gal4 transcriptional activity is enhanced by its monoubiquitination. The 19S regulatory particle of the 26S proteasome binds to promoter bound Gal4 and dissociates the Gal4-DNA complex (Ferdous et al., 2007). When monoubiquitinated, Gal4 is resistant to the stripping activity of the 19S regulatory particle. These studies reveal that there are numerous ways in which ubiquitination controls transcription factor activity and the length of the ubiquitin chain tethered to the transcription factor is known to be a critical component of this regulation.

4.3.2 Targets of the transcription factor Haa1 are down-regulated in CDC34^{tm} *cells apparently due to alterations in acetaldehyde metabolism*

A previous microarray comparison of *cdc34* and *cdc53* temperature sensitive strains found an increase in the activity of the Met4, Gcn4, Tec1 transcription factors (Varelas et al., 2006). Met4, Gcn4 and Tec1 are SCF/Cdc34 complex substrates and their stabilization in the *cdc34* and *cdc53* temperature sensitive strains leads to increased activation of their respective target genes. The analysis of *CDC34^{tm}* cells did not show a

comparable change in the Met4, Gcn4 or Tec1 dependent transcripts providing further support to the conclusion that the *CDC34^{tm}* is a neomorphic, rather than hypomorphic, allele.

In all, 55 gene transcripts were significantly (p value < 0.05) up-regulated at least two fold and 153 were significantly down-regulated by at least two fold in *CDC34^{tm}* cells. To determine the transcription factor(s) whose activity is altered in *CDC34^{tm}* cells the YEASTRACT (www.yeastract.com) database which relates *Saccharomyces cerevisiae* transcriptional regulators and their target genes was used (Teixeira et al., 2006). Ninety percent of documented Haa1 target genes are downregulated more than two fold in *CDC34^{tm}* cells (Table 5). Haa1 is critical for the transcriptional response to exogenous acetaldehyde and weak organic acids. Although insensitive to exogenous acetaldehyde, *haa1Δ* strains are sensitive to acetic, sorbic and propionic acids (Aranda & del Olmo, 2004; Fernandes, Mira, Vargas, Canelhas, & Sß-Correia, 2005).

Elevated levels of acetaldehyde can inhibit growth of *Saccharyomyces cerevisiae* cells (Stanley, Douglas, Every, Tzanatos, & Pamment, 1993). Excess acetaldehyde can be detoxified by allowing it to react with endogenous sulfite to form acetaldehyde hydroxysulfonate (Casalone et al., 1992). Acetaldehyde hydroxysulfonate production also mitigates the growth inhibitory effects of excess sulfite which is used as a food preservative and is produced during the intracellular reduction of sulfate to cysteine (Casalone et al., 1992). Microarray studies demonstrate that part of the response to exogenous acetaldehyde exposure is an increase of intracellular sulfite levels via up-regulation of the homocysteine production pathway and reduction of the sulfite exporter Ssu1(Aranda & del Olmo, 2004). Strains which can not up-regulate methionine

biosynthesis due to mutations in their transcriptional regulators (*MET4*, *CBF1*, *MET28*) are sensitive to exogenous acetaldehyde relative to isogenic wild type strains.

A strong inverse correlation was discovered between the *CDC34*^{tm} transcriptional profile and the transcriptional profile of cells exposed to exogenous acetaldehyde (Aranda & del Olmo, 2004). Many of the transcripts most highly upregulated by acetaldehyde are significantly down-regulated in a *CDC34*^{tm} strain (Table 5). *SSU1*, which encodes a sulfite extrusion pump, is down regulated 3.3-fold in cells exposed to acetaldehyde but is up-regulated 3-fold in *CDC34*^{tm} cells. Genes encoding the branch points of glucose fermentation which shunt glycolytic flux away from acetaldehyde production, namely the NAD-dependent glycerol-3-phosphate dehydrogenases genes (*GPD1* and *GPD2*), genes encoding the first and second steps of the pentose phosphate pathways (*ZWF1* and *SOL4*), and the pyruvate carboxylase genes (*PYC1* and *PYC2*) which convert pyruvate to oxaloacetate, are all significantly decreased in *CDC34*^{tm} cells (Fig. 17A and Table 5). The abundances of mRNAs encoding other glycolytic enzymes are unchanged in *CDC34*^{tm} cells.

The inverse correlation between cells exposed to acetaldehyde and *CDC34*^{tm} cells led us to consider whether *CDC34*^{tm} cells would be sensitive to sulfite because of an inability to detoxify it. Indeed, *CDC34*^{tm} cells are much more sensitive than their isogenic wild type counterparts (Fig. 17B). It has previously been shown that *grr1Δ* cells are sensitive to sulfite (Avram & Bakalinsky, 1996) which supports the conclusion that Cdc34^{tm} compromises SCF^{Grr1} activity. A defect in SCF^{Grr1}-mediated proteolysis of a yet unknown substrate, similar to that observed with the Cln1 substrate (Fig. 15A and B, Cln1^{Tap}), could thus be responsible for the sulfite sensitivity of *CDC34*^{tm} yeast and

explain the link between a change in Cdc34^{tm} function and the upregulation of the Haa1 gene cluster.

4.4 Synthetic Lethal Screens Uncover Genes Necessary for Cell Survival in the Presence of CDC34^{tm}

4.4.1 General comments on the CDC34^{tm} *SGA screen*

As an alternative approach to identifying the functional significance of the Cdc34 S73/S97/loop motif, a global synthetic lethal screen using the *CDC34^{tm}* allele as the query gene was performed. This was accomplished by tightly linking the *CDC34^{tm}* allele to a nourseothricin N-acetyltransferase gene (*nat1*) from *Streptomyces noursei*, which confers nourseothrcin resistance (Goldstein & McCusker, 1999). The *nat1* start codon was placed ~1700 nucleotide bases 5' of the *CDC34^{tm}* start codon. The *nat1* gene faithfully segregated with the *CDC34^{tm}* allele in more than 20 individual segregants derived from a *CDC34^{tm}(nat1)/CDC34* diploid (data not shown). The SGA screen was carried out in duplicate and genetic interactions were scored by computer-based image analysis of colony size (Fig. 18A). To my knowledge, this is the first synthetic lethal screen accomplished using a neomorphic mutant rather than a loss of function, gene deletion. Notably, fourteen genes proximal to the *CDC34* chromosomal locus scored as synthetically lethal in the primary screen. Reduced recombination between neighboring loci prevents facile generation of double mutant haploids. Genes neighboring *CAN1* and the *MFA1::pMFA1-HIS3* loci also appeared in the primary screen. These interactions result from reduced recombination frequency rather than true synthetic lethality and were not included in the secondary screen.

Candidate genetic interactions from the primary screen were confirmed by crossing both a *CDC34*^{tm}(*nat1*) strain and a *CDC34*(*nat1*) strain to strains carrying deletions of genes that scored as synthetically lethal in the primary screen. Diploids were struck on a media that selected for haploids with both the *NAT1* marked *CDC34* or *CDC34*^{tm} and the *KANR* marked gene deletion. To my knowledge this is the first time that the control strain, which determines the gene deletions that are lethal when crossed to a wild type strain, harbors the resistance gene in exactly the same genomic location as the query strain. In a typical synthetic lethal screen, *NAT1* marks the gene deletion rather than being present at the same position as in the control strain.

4.4.2 An altered mechanism of Sic1 degradation in CDC34^{tm} *cells is responsible for many of the synthetic lethal interactions*

In all, 86 genes were confirmed to be either synthetically lethal or sick with the *CDC34*^{tm} allele (Table 6). Seven genes with synthetic interaction with the *CDC34*^{tm} share a synthetic sick/lethal interaction with *SIC1* (Fig. 18B). This evidence indicates that although the steady state abundance of Sic1 protein and *SIC1* mRNA are increased, the effective concentration of Sic1 protein is decreased in *CDC34*^{tm} cells. *RPN10* is synthetically lethal with both *CDC34*^{tm} and *SIC1* (Fig. 19C). Rpn10 is a non-ATPase subunit of the 19S regulatory particle of the proteasome which binds medium to long, lysine-48 linked, polyubiquitin chains. Deletion of *RPN10* stabilizes Sic1 (Verma et al., 2004). The human Rpn10 ortholog, S5a, was the first 26S proteasome subunit implicated in the binding of polyubiquitinated substrates (Deveraux, Ustrell, Pickart, & Rechsteiner, 1994). Rad23, which is also synthetically lethal with the *CDC34*^{tm}, has a ubiquitin like domain (UBL) near its N-terminus and a UBA domain which binds K48-linked ubiquitin

chains *in vitro* (Bertolaet et al., 2001). In yeast, Rad23 can bind substrates with shorter polyubiquitin chains (4-6 ubiquitin molecules) through its UBA domain and recruit them to the proteasome through an interaction between its UBL domain and Rpn1 (Elsasser et al., 2002). Yeast strains lacking Rad23 are not impaired in their ability to degrade Sic1 *in vivo* but 26S proteasomes isolated from *rad23*Δ strains are defective in Sic1 deubiquitination and degradation *in vitro* (Verma et al., 2004). These genetic interactions between *CDC34^{tm}* and the polyubiquitin receptors *RAD23* and *RPN10* are particularly intriguing because utilization of polyubiquitin receptors in different contexts might explain the ability of *CDC34^{tm}* cells to survive while the polyubiquitin conjugating activity of the SCF complex is compromised as suggested by *in vitro* reconstitution of SCF ubiquitination using the Cdc34$^{\Delta 103\text{-}114}$ mutant (Petroski & Deshaies, 2005). Dsk2 and Ddi1 are also short chain polyubiquitin receptors of the 26S proteasome and like Rad23 have both UBL and UBA domains. Neither Dsk2 nor Ddi1 appeared in the initial *CDC34*^{tm} synthetic lethal screen and there is no detectable fitness defect in *CDC34^{tm} ddi1*Δ or *CDC34^{tm} dsk2*Δ haploids (Fig. 19C and data not shown).

4.4.3 Deletion of SIC1 *rescues the synthetic lethality of* CDC34^{tm} *with* RAD23 *and the RNA Pol II CTDK-I kinase genes*

We reasoned that the additional increase in *SIC1* transcription or compromise in Sic1 degradation might be the cause of synthetic lethality between *CDC34^{tm}* and each of the *RAD23*, *RPN10* and *CTK2* genes. Both *CDC34^{tm} rad23Δ* and *CDC34^{tm} rpn10Δ* cells die with a multiple, elongated bud phenotype (data not shown) indicative of defective Sic1 degradation. Disruption of *RPN10* reduces the rate of Sic1 degradation. Although, disruption of *RAD23* alone does not affect the rate of Sic1 degradation, a *rad23Δ rpn10Δ*

double mutant degrades Sic1 more slowly than the *rpn10Δ* mutant (Verma et al., 2004). The transcription factor, Swi5, which induces *SIC1* at the end of anaphase is targeted for proteasome mediated degradation by SCF^{Cdc4}. Furthermore, data suggests that the RNA Pol II CTD kinase, Srb10, phosphorylates Swi5 which stimulates binding to SCF^{Cdc4} (Kishi et al., 2008). While the Koyama lab concluded that Srb10 is involved in Swi5 degradation it is unlikely to be the only kinase which targets Swi5 to the SCF^{Cdc4} complex (Kishi et al., 2008). The wild type Swi5 protein is more stable in an *srb10Δ* strain than a Swi5 mutant in which all of the consenus CDK sites have been mutated to alanine. A different RNA Polymerase II C-terminal domain kinase, Ctk1, and the other members of the CTDK-1 kinase complex, Ctk2 and Ctk3, are all synthetically lethal with the $CDC34^{tm}$. The CTDK-1 complex coordinates transcriptional elongation, pre-mRNA 3'end processing and translational fidelity (reviewed in (Hampsey & Kinzy, 2007). Its role in transcriptional elongation is well characterized and its ability to phosphorylate the second serine of the repetitive C-terminal domain of Rpo21 increases the efficiency of transcriptional elongation (Lee & Greenleaf, 1997; Patturajan, Conrad, Bregman, & Corden, 1999). The CTDK-1 complex might be acting to degrade Swi5 because it is also a cyclin dependent kinase which binds RNA Pol II and can often be found near transcriptional activators such as Swi5 and Ace2. Therefore, *SIC1*, *ACE2* or *SWI5* were disrupted in the *rad23Δ/RAD23* $CDC34^{tm}$*/CDC34*, *ctk2Δ/CTK2* $CDC34^{tm}$*/CDC34* and *rpn10Δ/RPN10* $CDC34^{tm}$*/CDC34* diploids. These diploids were struck on haploid selection media. Deletion of *SIC1*, *ACE2* or *SWI5* was unable to suppress the lethality of the *rpn10Δ* $CDC34^{tm}$ haploid. Two previous large scale synthetic lethal screens found that a *sic1Δ rpn10Δ* strain grows poorly (Collins, Schuldiner, Krogan, & Weissman,

2006; Pan et al., 2006). The growth defect of the *rpn10Δ sic1Δ* strain is likely due to an inability to reduce Clb2 activity late in mitosis as Sic1 contributes to exit from mitosis by inhibiting Clb/Cdc28 activity and, although it has not been tested, Rpn10 might be involved in degradation of the Clb2 cyclin. There is no evidence to suggest that the *CDC34^{tm}* allele would alleviate the defect of the *rpn10Δ sic1Δ* strain, so it is not surprising that a *SIC1* deletion will not suppress the lethality of the *rpn10Δ CDC34^{tm}* strain. However, deletion of *SIC1* did suppress the lethality of *CDC34^{tm} rad23Δ* (Fig. 19D)

The synthetic lethality of the *ctk2Δ CDC34^{tm}* strain, and presumably the *ctk1Δ CDC34^{tm}* and *ctk3Δ CDC34^{tm}* strains, can be suppressed by deletion of *SIC1*, *SWI5* or *ACE2* (Fig. 20). This finding suggests that the CTDK-1 complex negatively regulates Sic1 activity by affecting Swi5 and Ace2 activity. Swi5 is known to be targeted for proteasome-mediated degradation by the SCF^{Cdc4} complex. Phosphorylation of at least one of the eight consensus CDK sites targets Swi5 to the SCF^{Cdc4} complex (Kishi et al., 2008). The Srb10 cyclin dependent kinase is partially responsible for phosphorylation of Swi5 but it is not the sole kinase involved. Swi5 is stabilized in an *srb10Δ* strain but not nearly to the same degree as in a strain in which all eight CDK consensus sites of Swi5 have been mutated to alanines (Kishi et al., 2008). We postulate that Ctk1 is also involved in targeting Swi5 to SCF^{Cdc4}. Both Srb10 and Ctk1 phosphorylate the CTD of RNA Pol II. Therefore they are often associated with the transcriptional machinery and thus both Swi5 and Ace2 may be in close physical proximity to these kinases. The fact that deletion of either *SWI5* or *ACE2* suppresses the lethality of *CDC34^{tm} ctk2Δ* cells

suggests that Ctk1 might be involved in the degradation of both of these transcriptional activators.

4.4.4 A screen for genetic interactions between CDC34^{tm} *and essential genes*

Approximately 12%, or 750, of the ORFs of *S. cerevisiae* are essential for growth and division under standard laboratory conditions (Goebl & Petes, 1986). To further our understanding of *CDC34^{tm}* yeast, we screened a collection of the ~750 essential genes for genetic interactions with the *CDC34^{tm}* allele. This array of strains, developed in the lab of Tim Hughes, places a single essential gene under the control of a doxycycline-repressible promoter. Addition of doxycycline to the media reduces the transcription of the essential gene. However, the expression of many essential genes can be dramatically reduced without compromising cell growth. This fact is evidence by the growth of strains from this collection as ~80% of the strains show at least some growth after addition of doxycycline to the media. Regardless, we uncovered a number of genetic relationships between essential genes and the *CDC34^{tm}* allele. This screen was executed much like the confirmation SGA screen discussed in section 4.4.1. The *NAT1* marked *CDC34* served as the control and allowed us to detect synthetic lethal relationships where reduced expression of the essential gene prevented colony growth in *CDC34^{tm}* but not *CDC34* haploid. This experiment also uncovered numerous synthetic rescue interactions where the inhibition of growth due to reduced expression of an essential gene expression could be suppressed by the *CDC34^{tm}* allele (Table 7).

The *CDC34^{tm}* allele interacts with multiple genes whose protein products are known to participate in ubiquitin-mediated protein degradation. Ufd1, an essential gene identified as synthetically lethal with *CDC34^{tm}*, is part of a complex containing Cdc48

and Npl4 that recognizes polyubiquitinated proteins and presents them to the proteasome. This complex is important for the degradation of many ERAD (Endoplasmic Reticulum Associated Degradation) substrates such as Spt23 and Hmg2. Another non-essential component of the Cdc48 complex, Dfm1, is also synthetically lethal with *CDC34^{tm}* (Table 6) (Goder, Carvalho, & Rapoport, 2008). This implies that the mechanism of protein degradation utilized by the Cdc34^{tm} enzyme relies on the Cdc48 pathway, at least for one of its substrates. As mentioned in the chapter one, Far1 is degraded in a Cdc48 dependent manner (Fu et al., 2003; Verma et al., 2004).

We also discovered a number of interactions between *CDC34^{tm}* and components of the RNA Polymerase machinery, including a synthetic lethal interaction with the large subunit of RNA Pol II, Rpo21 (Table 5). Considering the interactions with the CTDK-1 complex discussed in section 4.4.3, it appears that *CDC34^{tm}* yeast are very sensitive to alterations in the activity and regulation of RNA Polymerases, especially RNA Pol II. RNA Pol II is responsible for transcribing nearly all mRNAs in *S. cerevisiae*. The C-terminal domain (CTD) of Rpo21 is a repetitive seven-amino-acid sequence, Y-S_2-P-T-S_5-P-S. Both Ser_2 and Ser_5 are phosphorylated *in vivo* and the levels of phosphorylation at each residue fluctuate as Pol II moves through the body of an actively transcribed gene. Modulating the phosphorylation state of Pol II is important for transcriptional elongation and capping, polyadenylation and splicing of the primary transcript. *BUR1*, a cyclin-dependent kinase which phosphorylates the RNA Pol II CTD, is synthetically lethal with both the *CDC34^{tm}* and the CTDK-1 complex. *BUR1* mutants, much like CTDK-1 mutants, are defective in transcriptional elongation (Murray, Udupa, Yao, Hartzog, & Prelich, 2001). The combined action of the cyclin dependent kinases, Ctk1/Ctk2/Ctk3

and Bur1/Bur2 is the functional equivalent of mammalian P-TEFb which also regulates transcription elongation through phosphorylation of the CTD of RNA Pol II (Wood & Shilatifard, 2006).

Phosphorylation of RNA Pol II CTD (Serine 2) increases cells enter the diauxic phase of growth. This growth phase is characterized by a reduction in doubling time and a transition from fermentative to respiratory metabolism. Cells with a truncated RNA Pol II CTD grow at the same rate as wild type cells through the exponential growth phase but growth is retarded through the diauxic phase and ultimately reach a lower density than wild type cells when growth ceases. Although Ctk1 directly phosphorylates Ser5 of the CTD, the increase in S2 phosphorylation as cells enter the diauxic shift is dependent on CTK activity (Patturajan et al., 1999). In fact, a strain harboring an activated $RAS2^{G19V}$ allele is phenotypically similar to the strain with truncated RNA Pol II CTD regarding diauxic phase growth. The $RAS2^{G19V}$ allele is synthetically lethal when the elongating activity of RNA Pol II is compromised, either by deletion of the CTD itself or deletion of any of the CTK subunits (Patturajan et al., 1999). Mycophenolic acid (MPA) and 6-Azauracil (6-AU) reduce intracellular GTP levels and partially inhibit Pol II elongation ability. The RAS^{G19V} mutants do not grow on medias with 6-AU or MPA and also are synthetically lethal with mutations in *ctk1Δ* (Howard, Hester, & Herman, 2003). These interactions are reflected in the network depicted in figure 21. Interestingly, overexpression of any of the three G1 cyclins, *CLN1-3*, can suppress the G1 arrest associated with temperature sensitive *rpo21-4* allele suggesting that coordinated regulation of RNA Pol II activity and G1 cyclin function is imperative for G1 phase progression (Drebot, Johnston, Friesen, & Singer, 1993). Furthermore, *CLN3* and *FAR1*

are synthetically lethal with *CTK1*, which expands the relationship between SCF/Cdc34 substrates, RNA Pol II CTD phosphorylation and *CDC34*, specifically the S73/S97/loop motif.

The remainder of *CDC34*^{tm}-interacting, essential genes are not easily explained based on our current knowledge. A number of the genes, which are either synthetically lethal or rescued by the *CDC34*^{tm} allele, were also identified in a large-scale proteomics screen to identify ubiquitinated proteins. Figure 22 depicts those essential proteins which genetically interact with with *CDC34*^{tm} and are potentially ubiquitinated (Peng et al., 2003). We hypothesize that alterations in their rates of ubiquitin-mediated degradation are responsible for the genetic interactions detected. This hypothesis implies that these proteins are substrates of various SCF/Cdc34 complexes and can be extended to suggest that those genes which share a synthetic rescue relationship are more stable in the *CDC34*^{tm} cells while those having a synthetic lethal relationship are less stable in *CDC34*^{tm} cells.

4.5 Summary and a List of Candidate SCF Substrates Suggested by the *CDC34*^{tm} Microarray and Synthetic Lethal Screens

This work originated with the discovery that mutations to Cdc34 S97 or deletion of amino acid residues 103-114 in the N-terminal catalytic domain of Cdc34 make a non-functional enzyme but a combination of the mutations yields a functional enzyme, as assessed by *in vivo* complementation of a *cdc34* null strain. However, recent work has shown that the stretch of acidic residues in Cdc34 is necessary for timely and appropriate polyubiquitin chain extension of Sic1 (Petroski & Deshaies, 2005). Therefore, we reasoned that because a strain bearing the *CDC34*$^{\Delta 103\text{-}114}$ mutant does not support growth

while the *CDC34^{tm}* is viable and has a growth rate comparable to wild type there must be an *in vivo* mechanism to compensate for the defect in polyubiquitin chain extension. Two ubiquitin conjugating enzymes can function together to polyubiquitinate a single substrate with one E2 serving to monoubiquitinate and the other extending the ubiquitin chain (Rodrigo-Brenni & Morgan, 2007). No genetic interaction between *CDC34^{tm}* and any of the other ubiquitin conjugating enzymes were detected in the primary SGA screen suggesting that other E2s are not compensating for the defective polyubiquitination activity of the Cdc34^{tm}. In contrast, *CDC34*^{tm} exhibits strong genetic interactions with the polyubiquitin receptor genes *RAD23* and *RPN10*. Rad23 and Rpn10 have been shown to be required for Sic1 degradation; however, neither is an essential gene. In fact, *rad23Δ rpn10Δ* double mutants degrade Sic1 slower than either single mutant but are viable. This argues for an additional mechanism of recruiting Sic1 to the proteasome for degradation (Lambertson, Chen, & Madura, 1999; Verma et al., 2004). Both *CDC34^{tm} rad23Δ* and *CDC34^{tm} rpn10Δ* cells have a multiple, elongated bud phenotype (data not shown) indicative of defective Sic1 degradation.

The SCF^{Cdc4} substrate Sic1 (and likely Far1) has a shorter half life in *CDC34^{tm}* as compared to wild type cells while the SCF^{Grr1} substrate Cln1 is more stable in *CDC34^{tm}* cells. If steady state abundance is indicative of activity, Cln/CDK activity is increased in *CDC34^{tm}* cells. The Cln/Cdc28 complexes phosphorylate Sic1 and Far1 and based on the number of CDK sites, likely phosphorylate Cln1 as well (Henchoz et al., 1997; Mendenhall, Jones, & Reed, 1987; Peter, Gartner, Horecka, Ammerer, & Herskowitz, 1993). We postulate that increased Cln/Cdk phosphorylation of the substrates Sic1, Far1, Cln1 and Cln2 enables substrate recruitment to the individual SCF complexes; however,

upon encountering a Cdc34^{tm} bound SCF complex the substrate is ubiquitinated but with a polyubiquitin chain different from that formed by wild-type Cdc34. This could mean in Cdc34^{tm} cells the time between SCF association and dissociation may be shorter for each substrate and thereby available for proteasomal degradation earlier than substrates which encounter a normal wild-type Cdc34 containing SCF complex. Alternatively, the polyubiquitin receptors necessary for a substrate's proteasomal recognition may be different for each of these substrates when ubiquitinated by Cdc34^{tm}. Rad23 and Rpn10 both recruit Sic1 to the proteasome but there is likely a third pathway, possibly involving Rpt5, by which Sic1 is recognized by the proteasome. Rad23 and Rpn10 do not appear to be proteasomal receptors for Cln1 and Cln2 while Rad23 and Rpn10 to a lesser degree are involved in Far1 degradation (Verma et al., 2004). Our data do not uncover the contribution of each step (phosphorylation, ubiquitination, proteasome binding) to the overall differences in substrate steady state abundance and half lives in *CDC34*^{tm} cells. However our data do suggest that the Cdc34^{tm} be considered a useful tool for both *in vivo* and *in vitro* experiments as we continue to dissect the contribution of each step to the rate of the entire process.

It might be argued that the increase in the steady state abundance of the Sic1 protein in the *CDC34^{tm}* strain contradicts our finding that Sic1 protein half-life in a *CDC34^{tm}* strain is decreased by nearly 40 minutes. However, microarray analysis provides a partial explanation for the increase in Sic1 protein steady state abundance. The level of Sic1 mRNA is significantly increased in the *CDC34^{tm}* strain. Asynchronous batch cultures were analyzed for these microarrays. We expect that the relative difference in *SIC1* mRNA between *CDC34*^{tm} and wild type cells would be measurably

larger than 1.6-fold if measurements were made at the M/G1 transition when Ace2 is activated. Not only are *SIC1* transcript levels increased but so are the transcript levels of 17 of the 26 genes of the *SIC1* cluster of co-transcribed genes. This group of co-transcribed genes is controlled by the two homologous transcriptional activators Swi5 and Ace2 (Zhu et al., 2000). The gene products from this group of co-regulated genes are important for the final steps of mother-daughter separation and early G1 phase progression. Although homologous, Ace2 and Swi5 act at measurably different times in the cell cycle and have both common and unique target genes (McBride, Yu, & Stillman, 1999). It is apparent that the Ace2 targets are up-regulated to a greater degree than Swi5 targets in *CDC34^{tm}* cells. Therefore, we compared Ace2 protein levels (*ACE2* mRNA is not significantly different between WT and *CDC34^{tm}* cells) in wild type and *CDC34^{tm}* cells and discovered the steady state abundance of Ace2 protein to be increased in *CDC34^{tm}* cells. Further confirmation that Ace2 upregulates Sic1 protein in *CDC34*^{tm} cells can be derived from figure 16B which shows that *CDC34^{tm}* cells lacking *ACE2* do not accumulate Sic1 protein whereas *CDC34^{tm}* cells lacking *SWI5* still accumulate Sic1. Interestingly, the misregulation of Sic1 in *CDC34*^{tm} cells mirrors the aberrant regulation of the mammalian cyclin dependent kinase inhibitor, p27^{Kip1}, in many human breast cancer cells which often have an elevated steady state abundance of p27^{Kip1} (Fredersdorf et al., 1997).

4.6 The S73/S97/Loop Motif Increases Chronological Lifespan

Surprisingly, *S. cerevisiae* has served as a very good model for aging research. Before advances in the genetic manipulation of model organisms like yeast, worms and fruit flies, it was a commonly held belief that single genes would not dictate aging. The

process of aging is extremely complex and is influenced by diet and lifestyle. As such it was believed that the genetic components of aging would be multi-factorial. However, numerous, single gene mutations have been found in all model organisms including yeast, fruit flies, worms and mice which dramatically extend lifespan (for review see (Bitterman, Medvedik, & Sinclair, 2003)). Evidence for a conserved mechanism of aging can be derived from the fact that mutations in genes such as Sch9/AKT affect the lifespan of all model organisms mentioned above. Sch9 is 49% identical to the human, fly and worm AKT over a stretch of ~300 amino acids which encode the kinase domain. In these and other higher eukaryotes, AKT is activated by insulin and the insulin-like growth factor (IGF-1). Weak mutations in the IGF-1 pathway can extend the lifespan of worms more than two fold and that of fruit flies by 85% (Clancy et al., 2001; T. E. Johnson, 1990; Kenyon, Chang, Gensch, Rudner, & Tabtiang, 1993; Tatar et al., 2001). Furthermore, Prop-1 (-/-) and Pit-1 (-/-) dwarf mice have decreased levels of IGF-1 and live ~65% longer than wild type mice (Brown-Borg, Borg, Meliska, & Bartke, 1996; Flurkey, Papaconstantinou, & Harrison, 2002; Hsieh, DeFord, Flurkey, Harrison, & Papaconstantinou, 2002)

Aging studies of *S. cerevisiae* are classically conducted by two methods. The first method allows for the determination of replicative lifespan which is a study of mitotically dividing cells. An individual yeast cell will undergo a finite number of divisions before dying. There is a certain amount of strain specific variation in the number of divisions a new mother can undergo prior to senescence but on average a single yeast cell can give rise to ~20-25 daughters. Determination of the viability of non-dividing yeast cells in the G_0 or postdiauxic state, termed chronological lifespan, is another method by which aging

is assessed in *S. cerevisiae*. After approximately three days in synthetically defined media, yeast cells stop dividing and significantly slow their metabolic activity. Nutrients become limiting under these conditions but the cells are not starving and it has been postulated that this state resembles in some respects the conditions of postmitotic cells in higher organisms (Longo & Fabrizio, 2002).

Conditions, such as nutrient depletion or exposure to mating pheromone, which require timely cell cycle arrest in the G1 phase depend on destabilization of the G1 cyclins Cln1, Cln2 and Cln3 and stabilization of the cyclin dependent kinase inhibitors Sic1 and Far1. Overexpression of *CLN2* or loss of *FAR1* prevents mating pheromone induced cell cycle arrest (Chang & Herskowitz, 1990; Oehlen & Cross, 1994). And, as mentioned in the introduction, ectopic *CLN3* expression or loss of *SIC1* compromises the G1 arrest and ultimately a yeast cell's ability to withstand prolonged period of nutrient depletion (Weinberger et al., 2007; Zinzalla et al., 2007). Furthermore, overexpression of CLN3 or loss of SIC1 makes yeast sensitive to otherwise tolerable levels of the TORC1 inhibitor rapamycin, which mimics nutrient deprivation (Zinzalla et al., 2007). The Cdc34^{tm} increases the rate of Sic1 degradation while decreasing the rate of Cln1 degradation. Therefore, we hypothesized that the highly conserved S73/S97/loop motif of Cdc34 does contribute to a cell's ability to survive low dose rapamycin treatment and nutrient depletion. Indeed, we find that a normally permissive level of rapamycin inhibits growth of *CDC34^{tm}* cells (Fig. 23A) likely due to improper regulation of Sic1, Cln1 and Cln2.

To test survival of *CDC34^{tm}* yeast during a prolonged period of nutrient depletion, isogenic wild type and *CDC34^{tm}* strains were grown to stationary phase in liquid culture

and the number of individual cells capable of forming a colony was determined over a period of ten days. After 48 hours in defined liquid media, the cells have ceased dividing and some of the essential nutrients have been depleted (Bitterman et al., 2003). As the graph shows, loss of the S73/S97/loop motif does not initially compromise survival (Fig. 23B, day 0) but as the time in a quiescence state extends, CDC34^{tm} yeast are significantly less robust and by day 10 their survival rate is more than five fold worse than the WT strain (Fig. 23B, days 3-10). This simple experiment explicitly demonstrates the selective pressure on the yeast *S. cerevisiae* to retain the Cdc34 S73/S97/loop motif. In nature, nutrient limitation is commonly encountered; therefore, genetic elements, like the S73/S97/loop motif, which contribute to survival of such conditions are highly desirable.

Table 2. Plasmids used in this study.

Plasmid Name	Vector	Yeast Marker	Bacterial Marker	Insert Gene	Source
pYL150	pSJ101	LEU2	ampicillin	CDC34	(Y. Liu et al., 1995)
pYL029	pSJ101	LEU2	ampicillin	CDC34S73K/S97D/delta	Yun Liu
pSJ101	pSJ101	LEU2	ampicillin		S. Johnson
pYL027	pSJ101	LEU2	ampicillin	CDC34insert delta	Yun Liu
pRC001	pSJ101	LEU2	ampicillin	CDC34S97D/delta	this study
pTL008	pSJ101	LEU2	ampicillin	CDC34 R93D	this study
pTL012	pSJ101	LEU2	ampicillin	CDC34 R90D/D91N/R93D	this study
pAG25	pFA6	natMX4	ampicillin	Nourseothricin N-acetyltransferase	EUROSCARF
AD002	pET21	none	ampicillin	CDC34deltaC(1-244)-6XHis	D. Skowyra
pRC004	pET21	none	ampicillin	CDC34deltaC(1-244 (S97A)-6XHis	this study

Table 3. Yeast strains used in this study.

Strain	Genotype	Reference
2690	*MAT***a** *sic1::Kan^R his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Winzeler et al., 1999)
4080	*MAT***a** *swi5::Kan^R his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Winzeler et al., 1999)
4088	*MAT***a** *ace2::Kan^R his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Winzeler et al., 1999)
7137	*MAT***a** *haa1::Kan^R his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Winzeler et al., 1999)
5149	*MAT***a** *vps34::Kan^R his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Winzeler et al., 1999)
3236	*MAT***a** *vps15::Kan^R his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Winzeler et al., 1999)
3642	*MAT***a** *gcn2::Kan^R his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Winzeler et al., 1999)
6055	*MAT***a** *sst2::Kan^R his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Winzeler et al., 1999)
BY4741 (Cln1Tap)	*MAT***a** *CLN1-TAP(HIS3-MX6) his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Ghaemmaghami et al., 2003)
BY4741 (Cln2Tap)	*MAT***a** *CLN2-TAP(HIS3-MX6) his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Ghaemmaghami et al., 2003)
BY4741 (Far1Tap)	*MAT***a** *FAR1-TAP(HIS3-MX6) his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Ghaemmaghami et al., 2003)
BY4741 (Sic1Tap)	*MAT***a** *SIC1-TAP(HIS3-MX6) his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Ghaemmaghami et al., 2003)
DBY2059	*MATα leu2-3,112*	Hennessey
EJ758(YIL035c)	*MAT***a** *his3-Δ200 leu2-3,113 ura3-52 pep4::HIS3* pYEX4T-+rec ::YIL035c	(Martzen et al., 1999)
KS415	*MAT***a** *grr1::URA3 ura3-52 leu2Δ-1 his3Δ-200*	(Schweitzer, Cocklin, Garrett, Desai, & Goebl, 2005)
KS418	*MAT***a**, *CDC34^{tm} ura3 leu2 trp1 lys2 ade2 ade3*	This study
KS422	*MAT***a** *ura3 leu2 trp1 lys2 ade2 ade3*	(Schweitzer et

		al., 2005)
KT945	*MATα his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1*	(Cameron et al., 1988)
KT948	*MAT***a** *his3 leu2 ura3 trp1 ade8 bcy1::LEU2 tpk1-w tpk2::HIS3 tpk3::TRP1*	(Cameron et al., 1988)
KT1112	*MAT***a** *leu2 ura3-52 his3*	(Zaremberg & Moreno, 1996)
KT1126	*MAT***a** *leu2 ura3-52 bcy1-14*	(Zaremberg & Moreno, 1996)
MT1901	*MATα mfa1Δ::pMFA1-HIS3 can1Δ ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*	M. Tyers
RC21	*MAT***a**/α *CDC34^{tm}*(*NAT1*)/*CDC34 ura3/ura3 leu2/leu2 trp1/TRP1 lys2/LYS2 ade2/ADE2 ade3/ADE3 his3/HIS3 MFA1/mfa1Δ::pMFA1-HIS3 can1/CAN1*	This study
RC29	*MATα cdc34tm(NAT1) mfa1Δ::pMFA1-HIS3 his3Δ ura3Δ leu2Δ can1Δ*	This study
RC94	*MATα CDC34*(*NAT1*) *mfa1Δ::pMFA1-HIS3 his3Δ1 leu2Δ0 ura3Δ0 can1Δ*	This study
RC96	*MAT***a** *CDC34^{tm}*(*NAT1*) *ace2Δ::Kan^{R} pMFA1-HIS3 ura3Δ0 leu2Δ0 can1*	This study
RC100	*MAT***a** *CDC34^{tm}*(*NAT1*) *swi5Δ::Kan^{R} pMFA1-HIS3 ura3Δ0 leu2Δ0 can1 met15Δ0*	This study
RC106	*MAT***a**/α *CDC34*(*NAT1*)/*CDC34 rad23::Kan^{R}/RAD23 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC113	*MAT***a**/α *CDC34^{tm}*(*NAT1*)/*CDC34 rad23::Kan^{R}/RAD23 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC131	*MAT***a**/α *CDC34*(*NAT1*)/*CDC34 ctk2::Kan^{R}/CTK2 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC141	*MAT***a**/α *CDC34^{tm}*(*NAT1*)/*CDC34 ctk2::Kan^{R}/CTK2 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC150	*MAT***a** *bar1::LEU2 his3 leu2 trp1 ura3 lys met-* [p*SIC1*]	This study
RC153	*MAT***a** *CDC34^{tm} bar1::LEU2 his3 trp1 leu2 ura3 lys met-* [p*SIC1*]	This study
RC166	*MAT***a**/α *CDC34^{tm}*(*NAT1*)/*CDC34 rad23::Kan^{R}/RAD23 sic1::URA3/SIC1 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC167	*MAT***a**/α *CDC34^{tm}*(*NAT1*)/*CDC34 ctk2::Kan^{R}/CTK2 sic1::URA3/SIC1 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC168	*MAT***a**/α *CDC34^{tm}*(*NAT1*)/*CDC34 ctk2::Kan^{R}/CTK2 ace2::URA3/ACE2 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC169	*MAT***a**/α *CDC34^{tm}*(*NAT1*)/*CDC34 ctk2::Kan^{R}/CTK2 swi5::URA3/URA3 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC171	*MAT***a**/α *CDC34^{tm}*(*NAT1*)/*CDC34 ubp14::Kan^{R}/UBP14 mfa1Δ::pMFA1-HIS3/MFA1 his3Δ/his3Δ ura3/ura3Δ0 leu2/leu2Δ0 can1/CAN1 met15Δ0/MET15*	This study
RC172	*MAT***a**/α *CDC34*(*NAT1*)/*CDC34 ubp14::Kan^{R}/UBP14 mfa1Δ::pMFA1-HIS3/MFA1 his3Δ/his3Δ ura3/ura3Δ0 leu2/leu2Δ0 can1/CAN1 met15Δ0/MET15*	This study
RC173	*MAT***a**/α *CDC34^{tm}*(*NAT1*)/*CDC34 cka2::Kan^{R}/CKA2 mfa1Δ::pMFA1-HIS3/MFA1 his3Δ/his3Δ ura3/ura3Δ0 leu2/leu2Δ0 can1/CAN1 met15Δ0/MET15*	This study
RC174	*MAT***a**/α *CDC34*(*NAT1*)/*CDC34 cka2::Kan^{R}/CKA2 mfa1Δ::pMFA1-HIS3/MFA1 his3Δ/his3Δ ura3/ura3Δ0 leu2/leu2Δ0 can1/CAN1 met15Δ0/MET15*	This study
RC175	*MAT***a**/α *CDC34^{tm}*(*NAT1*)/*CDC34 rps7b::Kan^{R}/RPS7B mfa1Δ::pMFA1-HIS3/MFA1 his3Δ/his3Δ ura3/ura3Δ0 leu2/leu2Δ0 can1/CAN1 met15Δ0/MET15*	This study
RC176	*MAT***a**/α *CDC34*(*NAT1*)/*CDC34 rps7b::Kan^{R}/RPS7B mfa1Δ::pMFA1-*	This study

	HIS3/MFA1 his3Δ/his3Δ ura3/ura3Δ0 leu2/leu2Δ0 can1/CAN1 met15Δ0/MET15	
RRC43	*MAT***a**/α *CDC34^{tm}*(*NAT1*)/*CDC34 rim13::Kan^{R}/RIM13 mfa1Δ::pMFA1-HIS3/MFA1 his3Δ/his3Δ ura3/ura3Δ0 leu2/leu2Δ0 can1/CAN1 met15Δ0/MET15*	This study
RRC73	*MAT***a** *CLN1-TAP*(*HIS3-MX6*) *CDC34^{tm}*(*NAT1*) *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
RRC74	*MAT***a** *SIC1-TAP*(*HIS3-MX6*) *CDC34^{tm}*(*NAT1*) *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
RRC76	*MAT***a** *FAR1-TAP*(*HIS3-MX6*) *CDC34^{tm}*(*NAT1*) *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
RRC78	*MAT***a** *CLN2-TAP*(*HIS3-MX6*) *CDC34^{tm}*(*NAT1*) *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
RRC85	*MATα CDC34^{tm}*(*NAT1*)	This study
YL10-1	*MAT***a** *cdc34-2 leu2Δ1 ura3-52 trp1Δ63 his3Δ* Gal+	(Y. Liu et al., 1995)
YL18	*MAT***a** cdc34::HIS3 ura3-52 leu2delta1 trp1delta63 his3delta200 (pYL250)	(Y. Liu et al., 1995)

Table 4. The *SIC1* cluster of cell cycle regulated genes is up-regulated in *CDC34*^{tm} cells. The genes which are co-regulated through the cell cycle with *SIC1* as previously defined by (Spellman et al., 1998) are shown with their relative expression levels (labeled *CDC34*^{tm} FC), p values (labeled *CDC34*^{tm} pval) and q values (labeled *CDC34*^{tm} qval) in a *CDC34*^{tm} strain relative to an isogenic wild type as determined by microarray analysis. The column labeled "Transcription Factor" is derived from a separate study that determined the influence of the two main transcription factors of this gene cluster, Ace2 and Swi5 (Doolin et al., 2001). The genes are arranged in order of their relative expression and the rows are colored according to the transcription factor dependence. (*) Indicates that the gene was not originally identified to be co-regulated with Sic1 (Spellman et al., 1998) but was defined as having either Swi5 or Ace2 dependence (Doolin et al., 2001). The relative expression levels (labeled cdc34-2ts FC) and p values (*cdc34*-2ts pval) from a separate microarray experiment (Varelas et al., 2006) comparing a *cdc34*-2 temperature sensitive strain to a wild type strain are also listed.

ORF	ALIAS	Cdc34tm FC	Cdc34tm pval	Cdc34tm qval	Transcription Factor	cdc34-2ts FC	cdc34-2ts pval
YDR055W	*PST1*	-2.17	7.9e-05	0.0027	Slight Ace2/Swi5	-1.12	0.54
YKL163W	*PIR3*	-1.35	0.058111	0.1377	Slight Ace2/Swi5	1.31	0.44
YJL159W	*HSP150*	-1.1	0.001441	0.0134	Slight Ace2/Swi5	-1.16	0.29
YNR067C	*DSE4*	-1.06	0.635923	0.5558	not detected	-2.51	0.008
YBR083W	*TEC1*	-1.04	0.897662	0.637	not affected	1.37	0.395
YKL116C	*PRR1*	1.14	0.04453	0.115	not detected	1.12	0.299
YNL192W	*CHS1*	1.16	0.063773	0.146	not affected	-1.43	0.0167
YGR086C	*PIL1*	1.18	0.023168	0.075	not affected	1.15	0.416
YDL117W	*CYK3*	1.21	0.052732	0.128	not detected	1.24	0.44
YJL078C	*PRY3*	1.25	0.217825	0.319	not detected	-1.69	0.025
YNL327W	*EGT2*	1.34	0.003588	0.0228	Ace2/Swi5	-1.5	0.029
YBR158W	*AMN1*	1.34	0.000267	0.0053	Ace2/Swi5	-1.39	0.05
YIL009W	*FAA3*	1.38	0.001426	0.013	not affected	-1.11	0.516
YKL185W	*ASH1*	1.38	0.000885	0.0100	Ace2/Swi5	1.26	0.179
YKL164C	*PIR1*	1.41	7.2e-05	0.0026	Swi5	1.01	0.869
*YJL194W**	*CDC6*	1.41	0.04	0.123	Swi5	-	-
*YDL127W**	*PCL2*	1.47	0.03	0.105	Swi5	1.03	0.947
YNL078W	*NIS1*	1.51	0.005773	0.0308	Swi5	1.66	0.06
YDL179W	*PCL9*	1.52	0.000748	0.009	Swi5	1.92	0.083
YGR044C	*RME1*	1.54	0.001677	0.014	Ace2/Swi5	-1.04	0.862
YOR264W	*DSE3*	1.6	0.000353	0.006	Ace2/Swi5	-1.18	0.869
YLR079W	*SIC1*	1.63	0.000288	0.005	Ace2/Swi5	1.41	0.398
YLR286C	*CTS1*	1.8	2.3e-05	0.0015	Ace2	-1.22	0.05
YPL158C	---	1.87	6.1e-05	0.002	Swi5	1.44	0.455
YHR143W	*DSE2*	1.89	5.8e-05	0.002	Ace2	-1.58	0.015
*YNL046W**	---	2.13	5.00E-06	0.0008	Swi5	2.24	0.01
*YDL227C**	*HO*	2.2	9.20E-05	0.0029	Swi5	1.4	0.2598
YGL028C	*SCW11*	2.58	1e-06	0.0003	Ace2	-1.49	0.006
YER124C	*DSE1*	3.61	5e-06	0.0008	Ace2	-1.48	0.064

Table 5. Genes induced in response to acetaldehyde, including most of the targets of the transcription factor Haa1, are repressed in *CDC34^{tm}* cells. The genes that are both repressed in *CDC34^{tm}* cells (this work) and induced in *CDC34* cells in response to exogenous acetaldehyde (Aranda & del Olmo, 2004) are shown with their relative expression level (fold change, FC) and p value (pval). The relative expression levels (labeled *cdc34-2ts* FC) and p values (*cdc34-2ts* pval) from a separate microarray experiment (Varelas et al., 2006) comparing a *cdc34*-2 temperature sensitive strain to a wild type strain are also listed. Rows are colored and ordered according to the respective transcription factor(s) that was determined by the querying the Yeastract database (www.yeastract.com). Targets of the transcription factor Haa1 that were not detected in response to acetaldehyde are included and marked with an asterisk (*).

ORF	ALIAS	Cdc34tm FC	Cdc34tm pval	Transcription Factor	acetaldehyde FC	cdc34-2ts FC	cdc34-2ts pval
YPR157W	---	-8.78	2.60E-05	Haa1	101.01	-1.38442	0.345335
YER037W	*PHM8*	-8.68	0.005485	Haa1	8.54	-1.89659	0.0637
YLR297W	---	-3.38	0.000164	Haa1	7.86	-1.062	0.771
*YIR035C**	---	-2.74	4.00E-06	Haa1	Not Detected	-1.2589	0.3574
*YPR156C**	*TPO3*	-2.73	0.002466	Haa1	Not Detected	-1.168	0.346
YBR054W	*YRO2*	-17.55	0.00065	Haa1/Hsf1	14.23	-1.1402	0.938804
YER150W	*SPI1*	-3.67	0.051281	Haa1/Hsf1	78.26	1.097	0.582014
YNL160W	*YGP1*	-3.39	0.004359	Haa1/Hsf1	5.51	-1.072	0.6907
YGR138C	*TPO2*	-3.84	0.000148	Haa1/Sok2	111.65	-1.21147	0.4589
YER130C	---	-3.05	2.20E-05	Haa1/Sok2	6.05	-1.291	0.3937
YCR021C	*HSP30*	-34.85	0.000473	Hsf1	284.03	-1.25741	0.0592527
YDR171W	*HSP42*	-20.84	0.005181	Hsf1	20.58	1.05293	0.803385
YJL144W	---	-15.75	0.005232	Hsf1	48	1.27157	0.554221
YGR249W	*MGA1*	-8.04	0.005973	Hsf1	9.01	-1.13988	0.540401
YGR142W	*BTN2*	-7.81	0.055734	Hsf1	35.6	-1.29884	0.808935
YGR248W	*SOL4*	-7.33	0.022398	Hsf1	14.6	1.45075	0.15987
YDR258C	*HSP78*	-6.27	0.042359	Hsf1	6.4	-1.06028	0.978133
YOR134W	*BAG7*	-6.24	0.00011	Hsf1	4.31	1.00096	0.946521
YFL053W	*DAK2*	-5.17	0.013542	Hsf1	7.83	-1.16282	0.388227
YPR158W	---	-4.33	0.006109	Hsf1	4.48	-1.21017	0.684579
YPR015C	---	-3.78	0.000101	Hsf1	4.87	1.76389	0.00249866
YGL037C	*PNC1*	-3.72	0.006157	Hsf1	3.46	1.00461	0.782109
YLL026W	*HSP104*	-3.53	0.055282	Hsf1	12.71	-1.39409	0.276707
YNL077W	*APJ1*	-3.46	0.008297	Hsf1	5.7	-1.23354	0.631739
YJL082W	*IML2*	-3.45	2.10E-05	Hsf1	3.69	-1.31331	0.0639387
YBR214W	*SDS24*	-3.41	0.007507	Hsf1	5.48	-1.19071	0.598434
YPL247C	---	-2.66	0.007523	Hsf1	3.2	1.07658	0.810784
YNL007C	*SIS1*	-2.55	0.004259	Hsf1	3.77	-1.20468	0.263542
YOR267C	*HRK1*	-2.38	0.000483	Hsf1	4.96	-1.34363	0.206224
YFL040W	---	-2.32	0.000625	Hsf1	4.33	Not detected	
YOL032W	*OPI10*	-2.25	0.025852	Hsf1	5.19	1.17739	0.701631
YBR101C	*FES1*	-1.99	0.005175	Hsf1	6.11	-1.15911	0.410713
YER035W	*EDC2*	-2.55	0.002572	Hsf1/Sok2	4.76	-1.09357	0.409178
YHL021C	---	-2.37	0.015077	Hsf1/Sok2	7.59	-1.89444	0.0901108
YGR088W	*CTT1*	-1.94	0.005252	Hsf1/Sok2	10.08	1.76375	0.0952713
YER028C	*MIG3*	-4.48	0.000248	Sok2	7.18	5.29889	0.102304
YNR014W	---	-4.31	0.000868	Sok2	3.26	-1.5523	0.256663
YMR316W	*DIA1*	-3.31	0.001082	Sok2	10.62	-1.45601	0.312658
YER053C	*PIC2*	-3.21	0.046544	Sok2	12.13	-1.12369	0.461775
YOL016C	*CMK2*	-2.39	0.035474	Sok2	28.58	-1.51724	0.118288

YGL179C	*TOS3*	-2.14	0.00326	Sok2	3.4	1.08028	0.799538
YLR121C	*YPS3*	-2	0.009871	Sok2	3.02	1.06573	0.70035
YOR298C-A	*MBF1*	-2	0.010964	Sok2	3.09	-1.20198	0.0338532
YDL038C	---	-5.51	1.00E-05	Sok2/Mga1	4.68	-3.08668	0.000767269
YDL048C	*STP4*	-4.91	1.00E-05	Sok2/Mga1	6.18	-1.07975	0.754752
YKL043W	*PHD1*	-2.16	0.008645	Sok2/Mga1	11.51	1.05313	0.851588
YDR259C	*YAP6*	-2.1	0.000163	Sok2/Mga1	4.76	Not detected	
YOR273C	*TPO4*	-5.23	0.002478	Mga1	6.5	-1.17421	0.610451
YBR183W	*YPC1*	-3.08	0.001528	Mga1	3.6	-1.04891	0.966512
YMR181C	---	-2.64	1.20E-05	???	4.15	-1.15099	0.633694
YOL014W	---	-2.39	0.006914	???	13.86	-7.53793	1.97E-05
YFR022W	*ROG3*	-2.33	1.00E-06	???	10.53	-1.01005	0.999712
YLR343W	*GAS2*	-2.27	0.000181	???	8.48	1.77452	0.103383
YPL165C	*SET6*	-2.25	0.000919	???	4.41	Not detected	
YGR008C	*STF2*	-2	0.112446	???	4.32	-1.06963	0.653618

Table 6. *CDC34^{tm}* genetic interactions with non-essential genes. These genes were confirmed to be synthetically lethal with the *CDC34^{tm}* allele. Superscipted "1" indicates no growth and a superscripted "2" is very limited growth.

Gene Product Function	ALIAS
CELL GROWTH REGULATION	*FPR1*[2], *LAG2*[2], *RAS2*[2], *RIM13*[1], *RIM20*[1], *RIM8*[1], *SOK2*[1], *TIP41*[1]
CELL STRESS	*HAL1*[1], *NRG2*[2], *RIM13*[1], *RIM20*[1], *RIM8*[1], *SOY1*[2], *TIR1*[1]
CHROMATIN	*AHC1*[2], *CTK2*[1], *CTK1*[1], *CTK3*[1], *YAF9*[1], *IES2*[1], *IES5*[1], *HHT1*[2]
CYTOSKELETON	*BNI4*[2], *LSB3*[2], *SPH1*[2]
DNA DAMAGE	*MRE11*[1], *RAD16*[1], *RAD30*[1], *RAD51*[1]
METABOLISM	*ABZ1*[1], *ADE12*[1], *COQ6*[1], *COX17*[2], *OPI3*[2], *PGM3*[2], *SIP1*[2]
METAL HOMEOSTASIS	*FSF1*[1], *PPZ2*[2]
MICROTUBULE FUNCTION	*CIK1*[1], *DYN1*[2], *DYN2*[1], *GIM5*[1], *KAR3*[2]
PROTEIN TRAFFICKING	*SEC22*[2], *VAC8*[1], *VPS21*[1], *VPS45*[2], *SWA2*[2], *GVP36*[2], *NIR1*[2], *GET2*[1], *CHS5*[2], *MNN11*[2], *YUR1*[2], *PIB2*[2], *YIP2*[2],
SIGNALING-PHOSPHORYLATION	*BNI4*[2], *CKA2*[1], *CTK2*[1], *FPR1*[2], *PPZ2*[2], *DBF2*[2], *SIP1*[2], *TIP41*[1],
SIGNALING-PI	*PDR16*[2], *PDR17*[2], *PIB2*[2]
TRANSCRIPTION	*CTK2*[1], *NRG2*[2], *RIM13*[1], *RIM20*[1], *RIM8*[1], *RTR2*[2], *SOK2*[1], *STB1*[2],
TRANSLATION	*DOM34*[2], *GCN1*[1], *NCS2*[1], *RPL16B*[2], *RPL34A*[2], *RPL34B*[2], *RPL29*[1] *TIF4631*[2]
UBIQUITIN PATHWAY	*DFM1*[1], *RAD23*[1], *UBP14*[1]
MISCELLANEOUS	*FCY21*[2], *NUP100*[2], *PEX13*[1], *PRB1*[1]
UNKNOWN	*YCL049c*[2], *YEL043w*[1], *YHL042w*[1], *YHR151c*[2], *YMR102c*[1], *YNL034w*[1], *YNR070w*[2]

[1] Synthetic lethality
[2] Synthetic growth defect

Table 7. *CDC34^{tm}* genetic interactions with essential genes. These genes were found to have a genetic interaction with the *CDC34^{tm}* allele. Superscipted "1" indicates a strong interaction while a superscripted "2" is a noticeable but not as dramatic difference in growth between *CDC34* and *CDC34^{tm}* strains.

Category	*CDC34^{tm}* Synthetic Lethality	*CDC34^{tm}* Sythetic Rescue
Ubiquitin	*UFD1*[1] *RPT2*[2] *UBA1*[2] *RPT6*[2]	*UBI3*[1] *PRE1*[2]
RNA Polymerase	*BUR1*[1] *RPC34*[1] *RPB10*[1] *RPA43*[1] *TAF5*[1] *NCB2*[1] *TFA2*[1] *TFG2*[2] *RPO21*	*TAF6*[1] *CTK1*[2] *RP031*[2]
DNA Replication	*DNA2*[1]	*MCM3*[1] *POL1*[1] *MCM7*[1] *CDC6*[1]
Metabolism	*GUK1*[1] *ERG12*[1] *SAH1*[2]	*PGI1*[1] *VHT1*[1] *CDC19*[2] *ERG25*[2]
Transcription	*MCM1*[1] *ABF1*[1] *GCR1*[1]	*RAP1*[1]
tRNA splicing/ synthetase	*FRS1*[1] *SEN34*[2] *THS1*[2]	*TRL1*[1] *KRS1*[2]
Chromatin Remodeling	*RSC4*[1] *ABF1*[1]	*RVB1*[1] *RSC8*[1] *EPL1*[2]
Ribosomal RNA Processing	*RRP42*[1] *NOP4*[1] *RRP17*[1] *GAR1*[1] *UTP6*[1] *PXR1*[1] *DHR2*[1] *SME1*[1] *NOG1*[1] *SQT1*[1] *URB1*[1] *RRS1*[1] *SDO1*[1] *KRR1*[2] *UTP4*[2] *ECM16*[2]	*NAN1*[1] *RRP40*[1] *ESF1*[1] *IPI1*[1] *CBF5*[1] *UTP11*[2] *YGR251W*[2]
Translation	*SRP68*[1] *NAB2*[1] *HRP1*[1] *NAB3*[1] *RRP17*[1] *HYP2*[2] *KRR1*[2] *DRS1*[2]	*UBI3*[1] *RPL15A*[1] *TIF34*[1] *RPG1*[2]
Chromosome Segregation	*SMC4*[1] *ASK1*[1] *SPC34*[2] *CDC5*[2]	*MPS2*[1]
Secretion	*SED5*[1] *SEC17*[2]	*SEC12*[1] *SEC4*[2]
Uncharacterized	*SGD1*[1] *YHR122W*[2] *YBR190W*[2]	---
Miscellaneous	*ERO1*[1] *RFT1*[1] *CCT6*[1]	*CDC42*[1] *CCT4*[1] *TIM50*[1]

[1] Strong genetic interaction
[2] Noticeable but less dramatic difference in growth between *CDC34* and *CDC34^{tm}* strains.

FIGURES

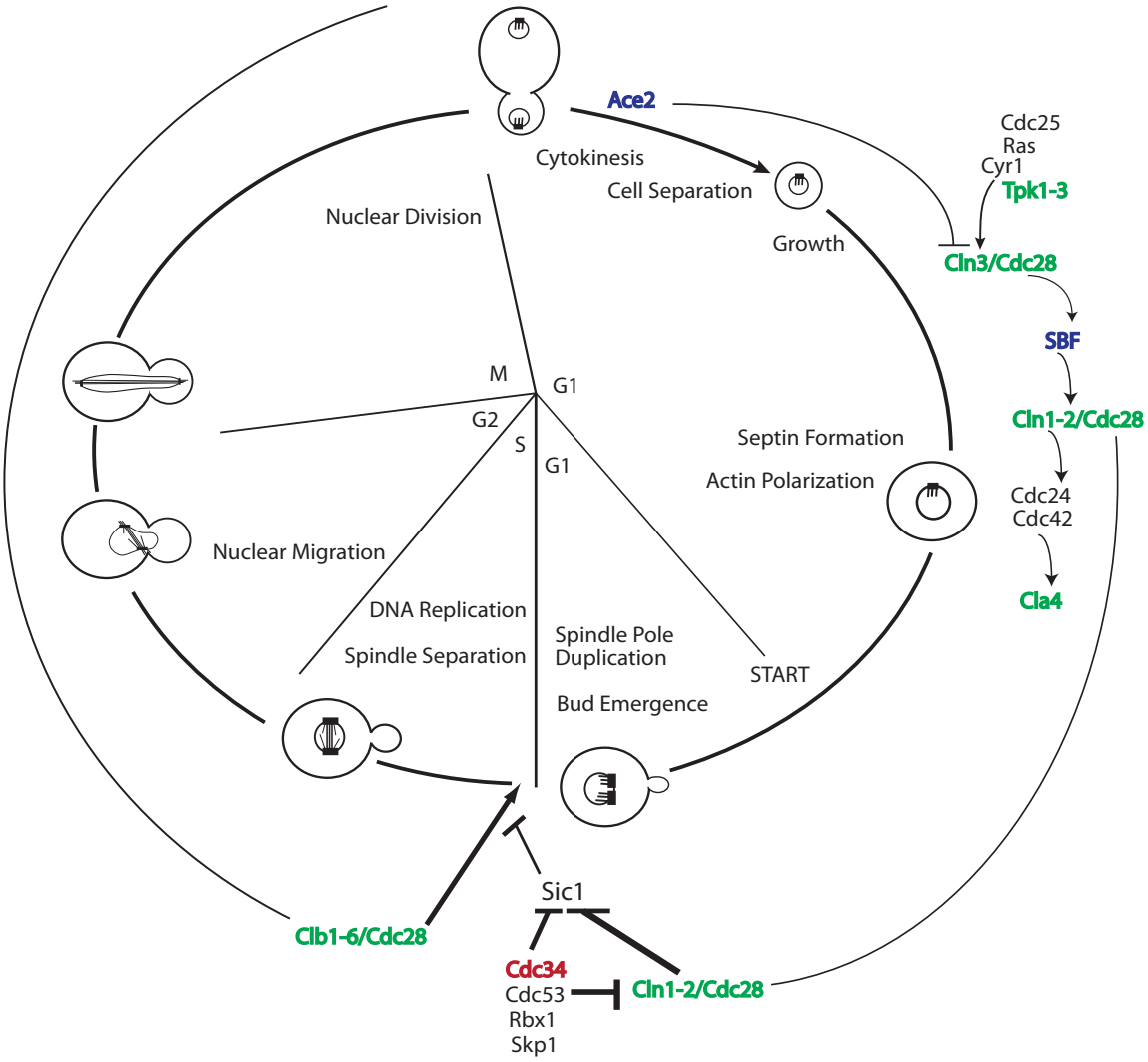

Figure 1. Model of the *S. cerevisiae* cell cycle. This model is derived from Hartwell (Hartwell, 1974) and emphasizes gene products discussed throughout this thesis. Kinases are represented in green and transcription factors are represented in blue. The spindle pole body and the attached microtubules are depicted. See introductory text for details and supporting references.

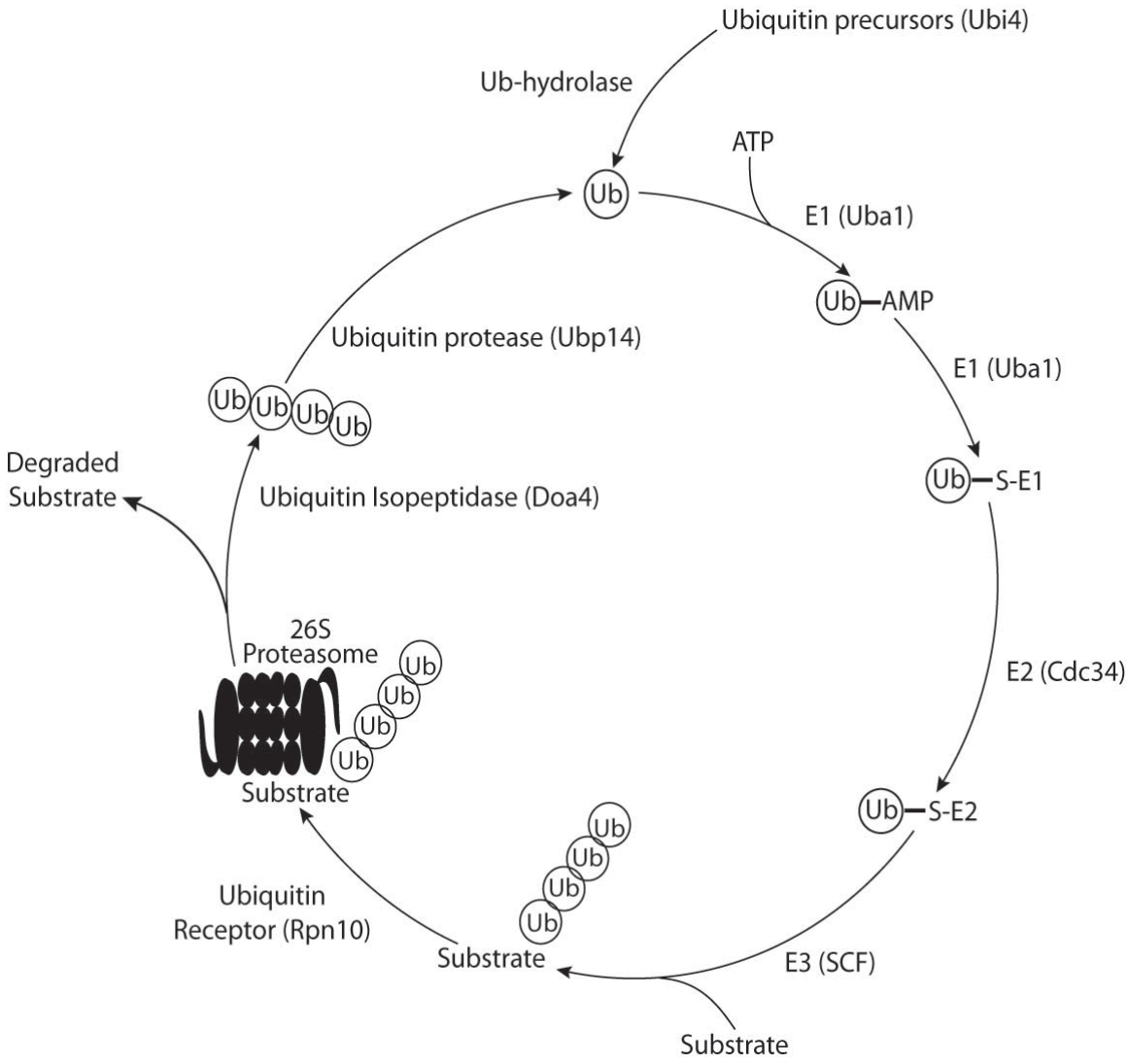

Figure 2. Ubiquitin conjugation and 26S Proteasome dependent degradation. Proposed pathway by which polyubiquitination targets substrates to the proteasome with emphasis on the SCF/Cdc34 ubiquitin ligase complex. Proteins and protein complexes listed in parentheses are examples of proteins with the type of activity specified.

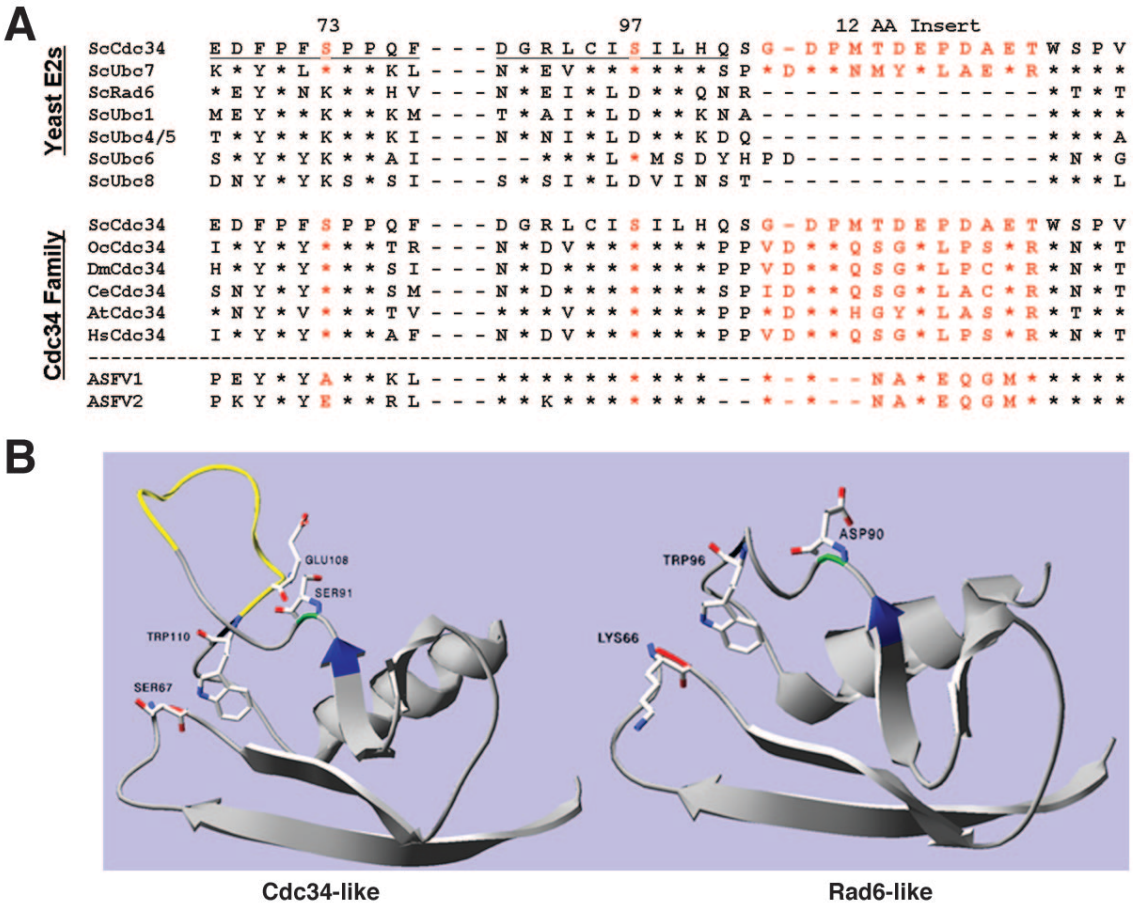

Figure 3. Structure and alignment of E2s: The serine/serine/loop motif is conserved in all Cdc34 family members. (A) Partial alignment of yeast E2s and Cdc34/Ubc7 family members. Red indicates amino acid residues unique to the Cdc34 family of E2s (the regulatory triad). Asterisks represent identities to Cdc34. Dashes represent gaps. Sc – *S. cerevisiae*; Oc – *O. cuniculus*; Dm – *D. melanogaster*; Ce – *C. elegans*; At – *A. thaliana*; Hs – *H. sapiens*; ASFV1 –African swine fever virus (GI:9628248); ASFV2 – African swine fever virus (GI:450743). **(B)** The structure shown at left is a stereo view of Ubc7 (Cook et al., 1997) depicting amino acids D51-S128. The catalytic cysteine is represented by the blue arrow. The serines (Ser67 and Ser91) of the triad are labeled and amino acid residues Gly97-Arg109 which constitute the insert are colored yellow. The invariant residue Trp110 and the highly conserved residue Asp108 of the loop are depicted in atomic detail to demonstrate proximity to each serine. The structure on the right is a stereo view of Rad6 (Worthylake, Prakash, Prakash, & Hill, 1998) depicting amino acids Asp50 - Asn114. The residues equivalent to Ser67 and Ser91 of Ubc7 are Lys66 and Asp90 which are shown in atomic detail.

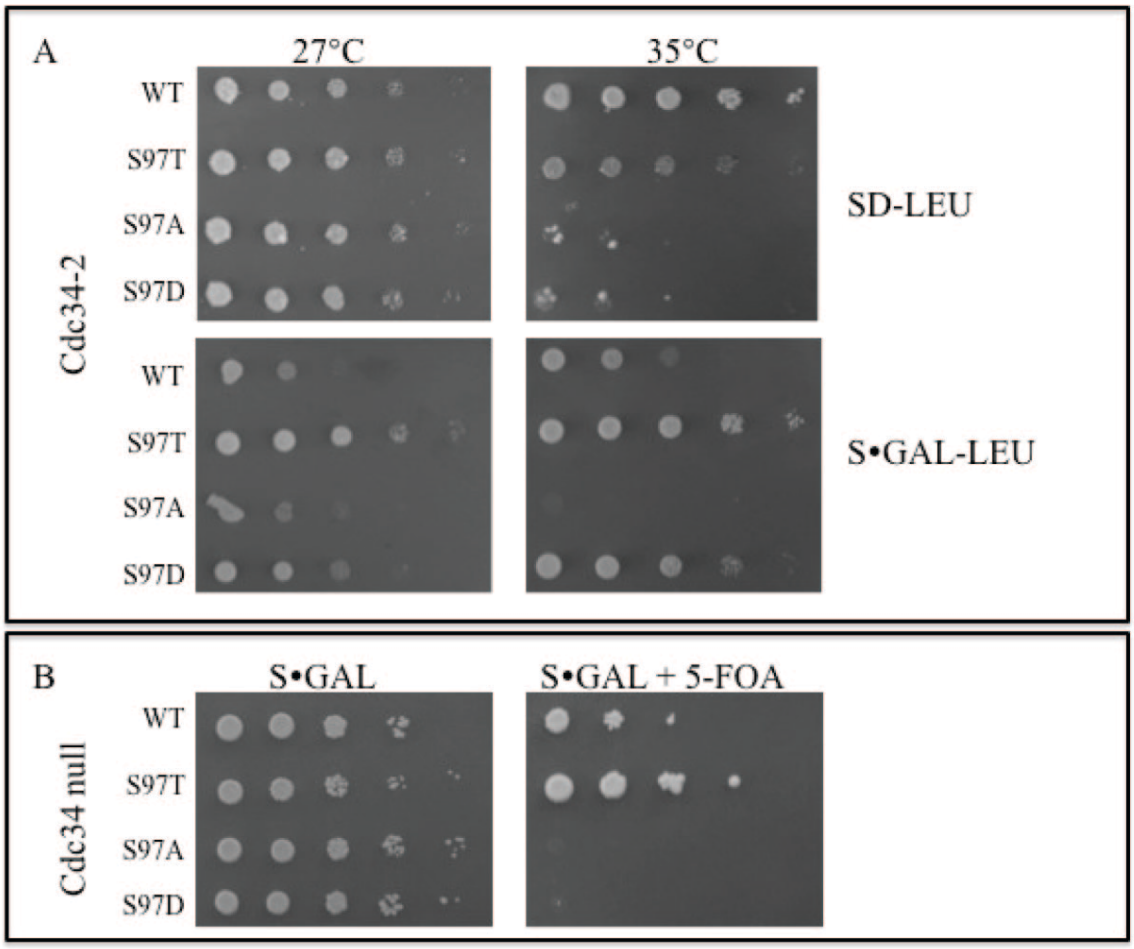

Figure 4. Complementation of *cdc34-2* and *cdc34Δ* strains by Cdc34 S97 Mutants. **(A)** YL10-1, a *cdc34-2* temperature sensitive strain, bearing 2-micron plasmids encoding the indicated *CDC34* mutant under control of the GAL10 promoter were spotted in ten fold serial dilution on the indicated medias and grown at the indicated temperatures for 3 days. **(B)** YL18, a *cdc34Δ* strain harboring a URA3 marked plasmid encoding wild type *CDC34* and a LEU2-marked plasmid encoding the indicated *CDC34* mutant were spotted in ten fold serial dilution on the indicated medias and grown at 30°C for 3 days. 5-FOA is toxic to cells containing a functional URA3 allele.

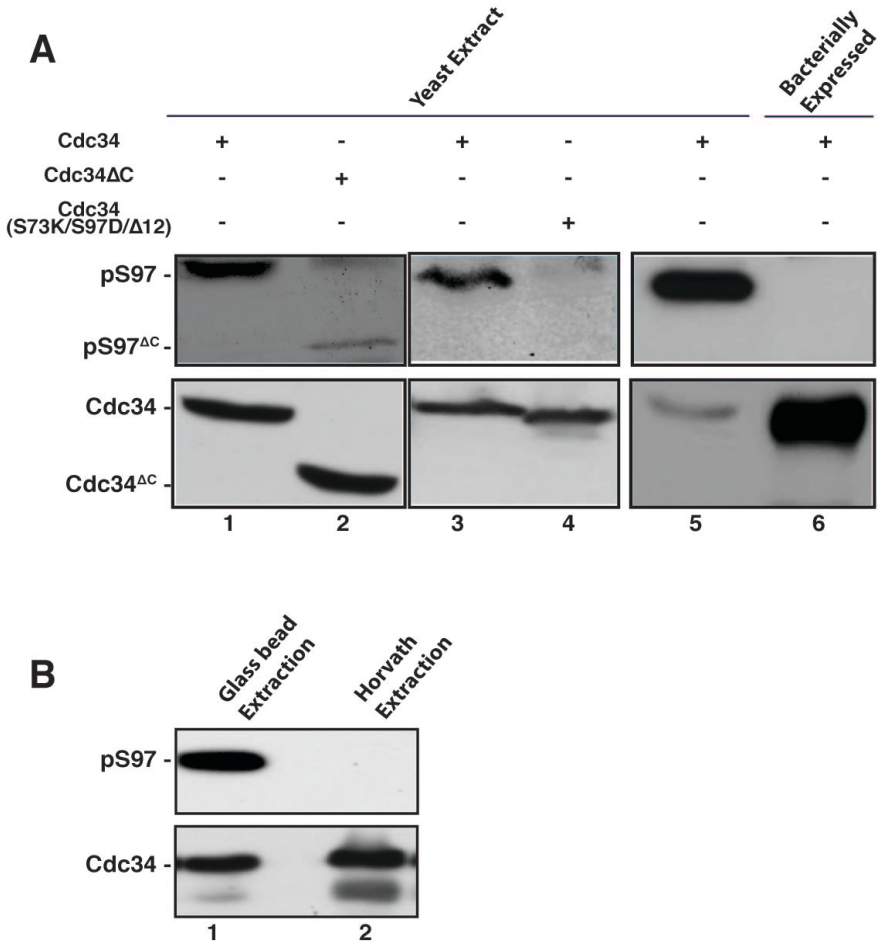

Figure 5. Cdc34 S97 is phosphorylated *in vivo* and detection is prohibited by certain protein extraction conditions. (A) Soluble protein from yeast cells expressing from the *CDC34* chromosomal locus either the wild type Cdc34 (lanes 1, 3 and 5), a C-terminally truncated Cdc34 encoding amino acids 1-244 (lane 2) or the Cdc34^{tm} mutant encoding the S97D mutation (lane 4) or bacterially expressed ^{6XHis}Cdc34 (lane 6) were analyzed by western blot using the α-pS97 phosphospecific and α-Cdc34 antibodies. **(B)** An equal amount of soluble yeast protein extract made by either the glass bead or Horvath extraction protocol (see Materials and Methods) was analyzed by western blot using the α-pS97 phosphospecific and α-Cdc34 antibodies.

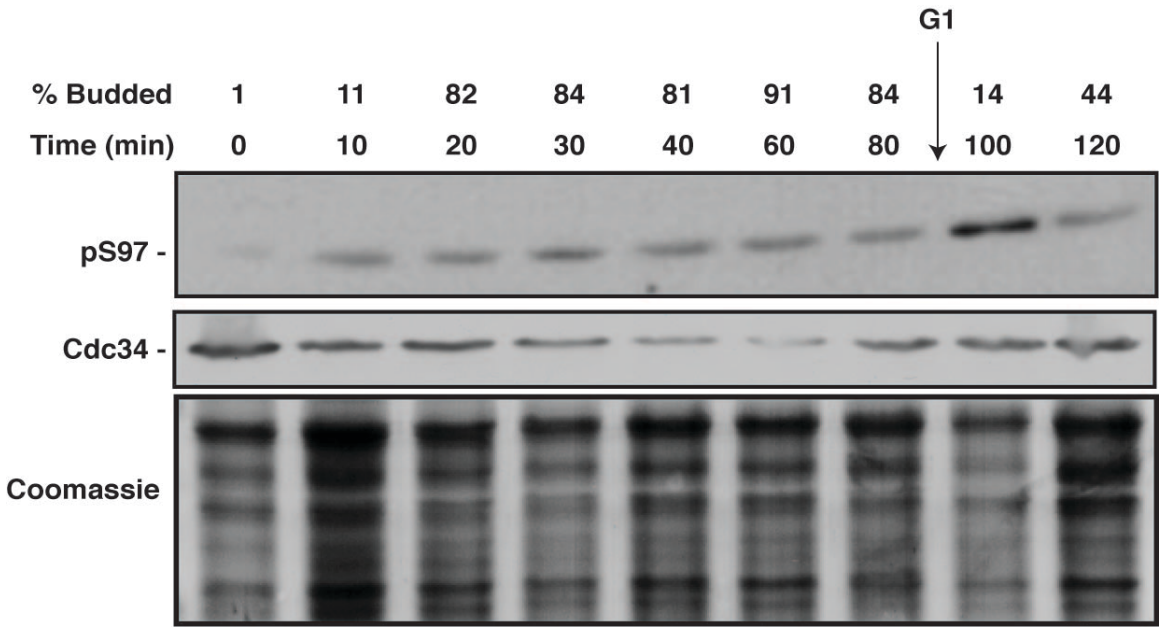

Figure 6. Cdc34 S97 phosphorylation increases in the G1 phase of the cell cycle. Strain RC150 was grown to mid-log phase and alpha factor was added to a final concentration of 0.5 μg/ml. After 3 hours, > 95% of the cells were arrested with a schmoo like morphology. Cells were washed and resuspended in fresh YPD. Cells were collected at the indicated time points and total soluble protein was analyzed by western blot using the α-pS97 phosphospecific and α-Cdc34 antibody. At least 100 cells were counted per time point to determine the budding index**.**

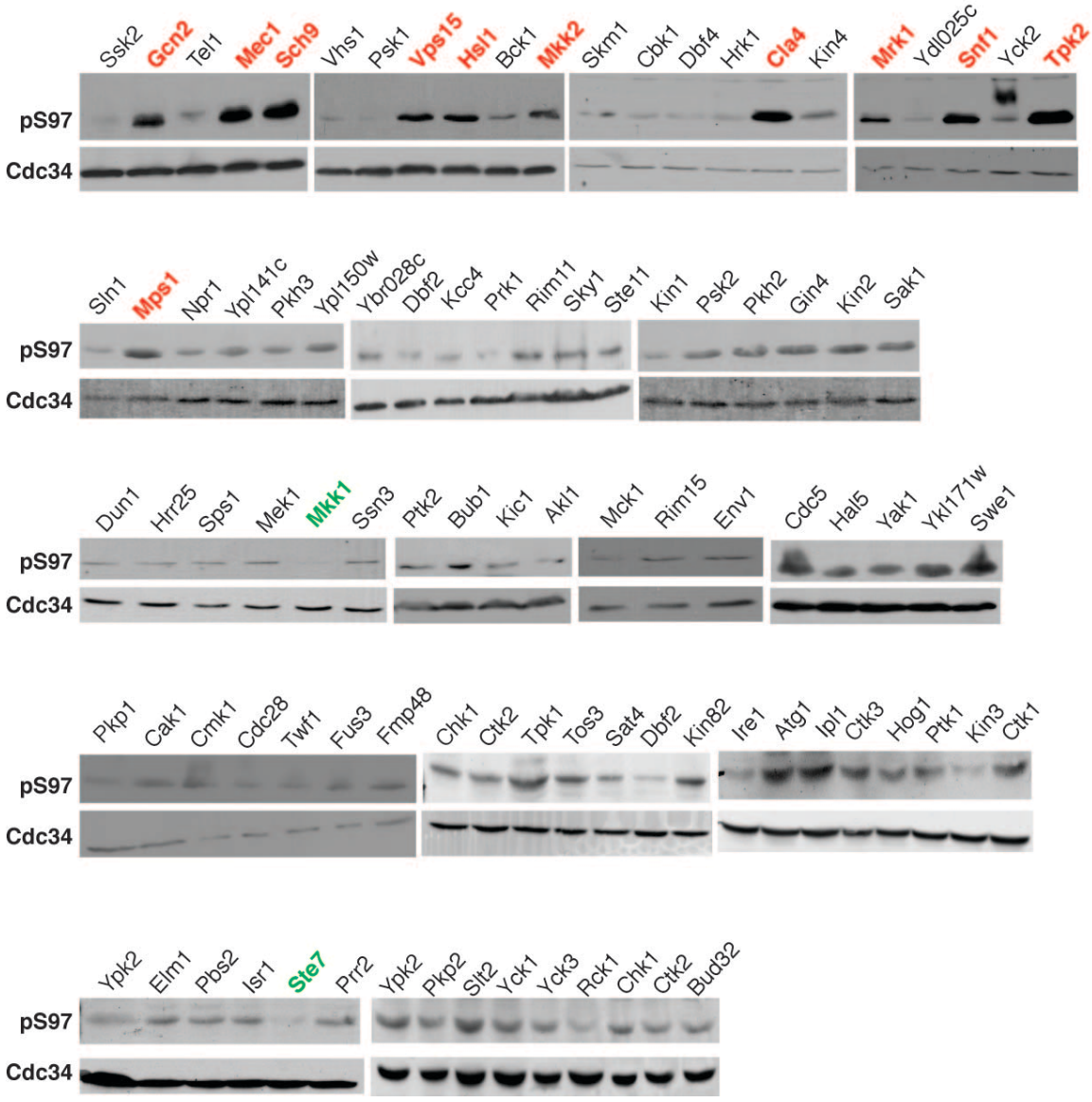

Figure 7. A screen for kinases which when overexpressed alter the level Cdc34 S97 phosphorylation. Total soluble protein from an array of strains each overexpressing the indicated kinase was analyzed by western blot using the α-pS97 phosphospecific and α-Cdc34 antibodies.

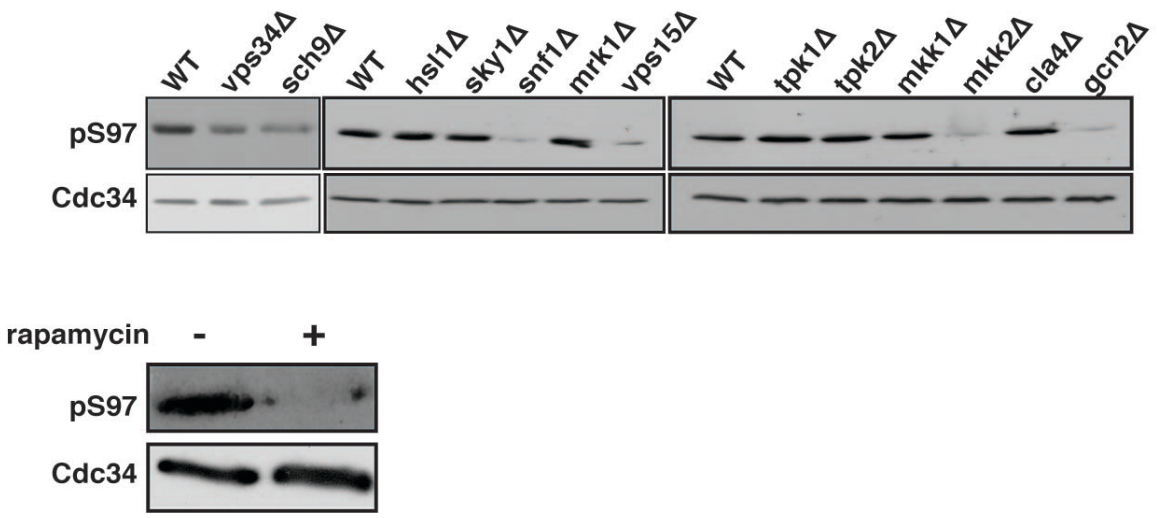

Figure 8. Loss of Vps34, Vps15, Sch9 and TORC1 activity reduce Cdc34 S97 phosphorylation. Total soluble protein from strains and lacking the indicated kinase or from a strain treated with 200 nM rapamycin for 1 hour was analyzed by western blot using α-pS97 phosphospecific and α-Cdc34 antibodies.

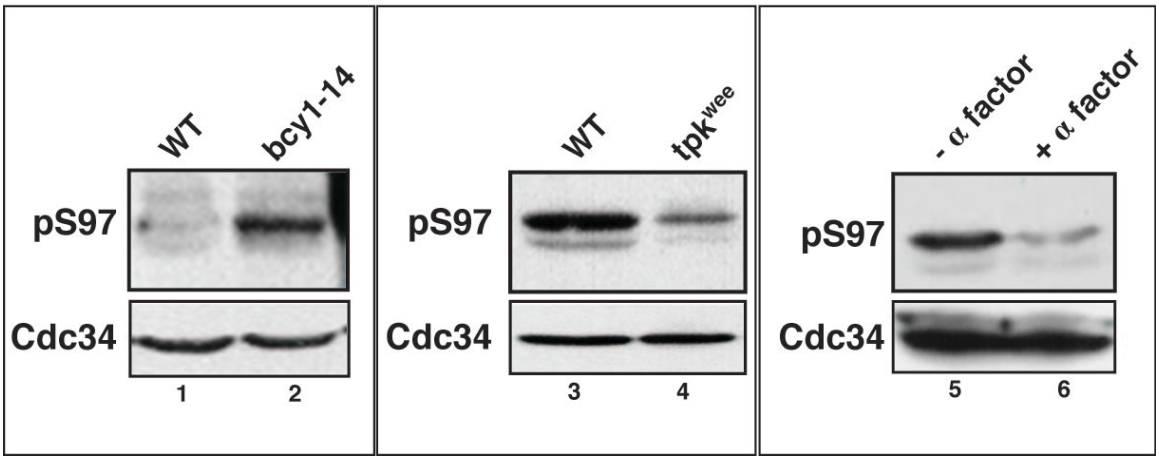

Figure 9. The cAMP/Protein Kinase A pathway regulates Cdc34 S97 phosphorylation. Total soluble protein from the strains KT945 (WT, lanes 1 and 3), KT948 (tpk^{wee}, lane 2), KT1126 (bcy1-14, lane 4), and 6055 untreated (lane 5) or exposed to alpha factor (lane 6) was analyzed by western blot using α-pS97 phosphospecific and α-Cdc34 antibodies.

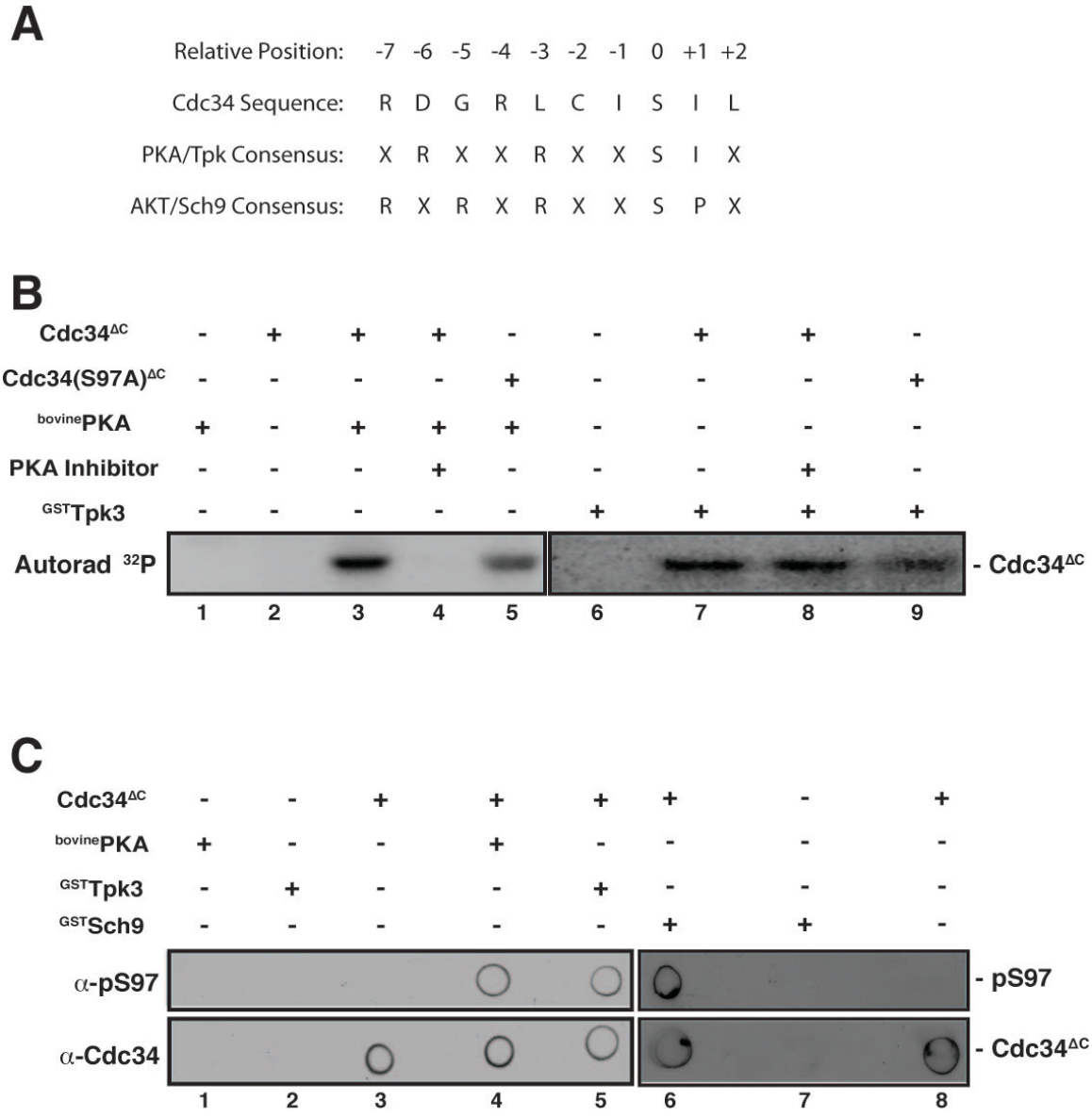

Figure 10. PKA and Sch9 directly phosphorylate Cdc34 upon serine 97. (A) Alignment of the sequence neighboring Cdc34 S97 and the consensus phosphorylation sites of PKA and AKT (Obata et al., 2000; Smith et al., 1999). **(B)** ^{6XHis}Cdc34$^{\Delta C}$ and ^{6XHis}Cdc34(S97A)$^{\Delta C}$ were phosphorylated *in vitro* by addition of either bovine PKA or ^{GST}Tpk3 and [γ-^{32}P]ATP. Protein kinase A inhibitor was added to the indicated reactions. The reaction components were separated by SDS-PAGE and autoradiographed. **(C)** ^{6XHis}Cdc34$^{\Delta C}$ was phosphorylated *in vitro* by either bovine PKA, ^{GST}Tpk3 or ^{GST}Sch9. The reaction was spotted onto a PVDF membrane and probed with either α-pS97 phosphospecific or α-Cdc34 antibody.

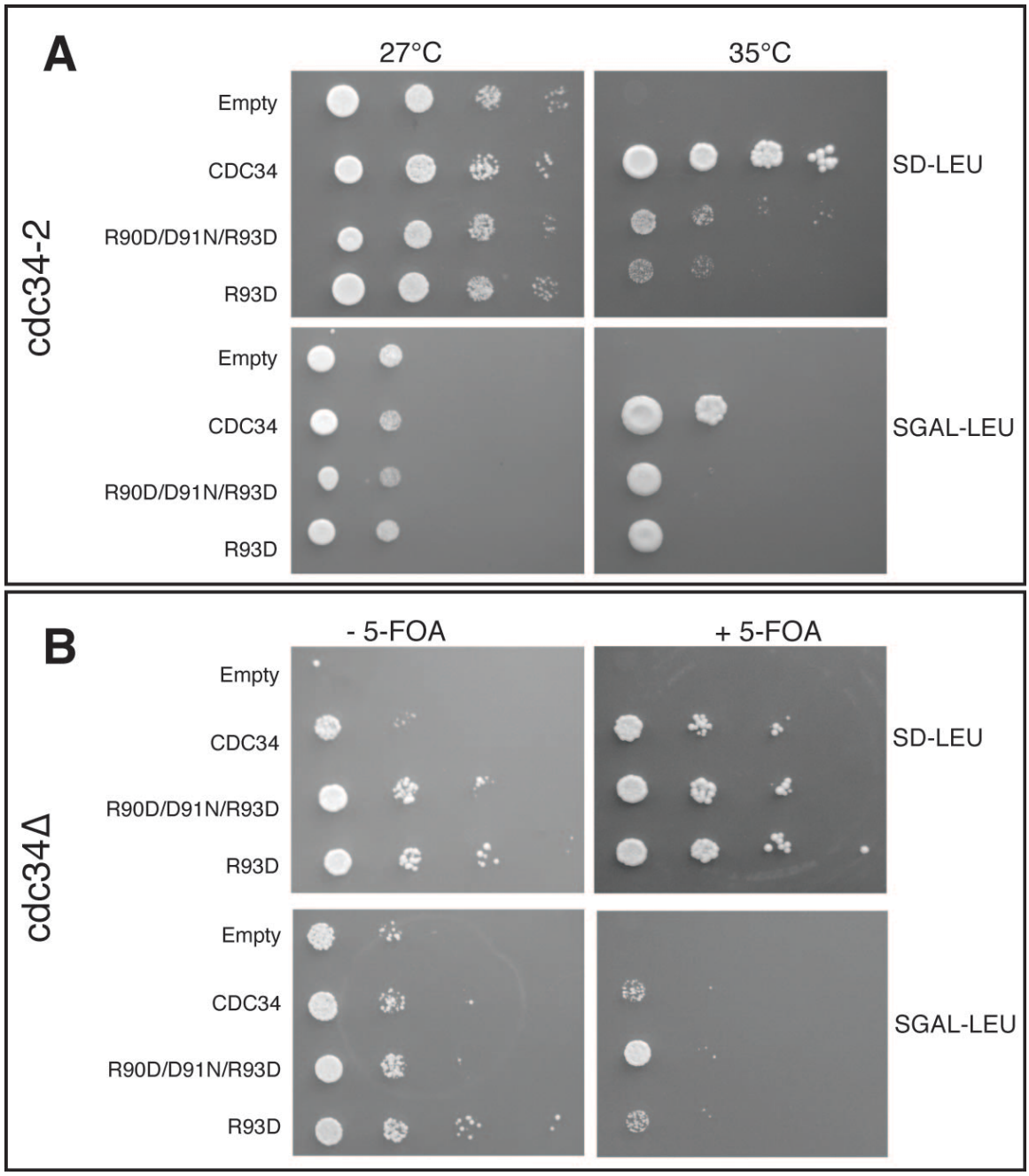

Figure 11. Functional studies of Cdc34 amino acid residues R90, D91 and R93. (A) YL10-1, a *cdc34-2* temperature sensitive strain, bearing 2-micron plasmids encoding the indicated *CDC34* mutants (pSJ101 = Empty, pYL150 = *CDC34*, pTL012 = R90D/D91N/R93D, pTL008 = R93D) were spotted in ten fold serial dilution on the indicated medias and grown at the indicated temperatures for 3 days. **(B)** YL18, a *cdc34Δ* strain harboring a URA3 marked plasmid encoding wild type *CDC34* and a LEU2-marked plasmid encoding the indicated *CDC34* mutant were spotted in ten fold serial dilution on the indicated medias and grown at 30°C for 3 days.

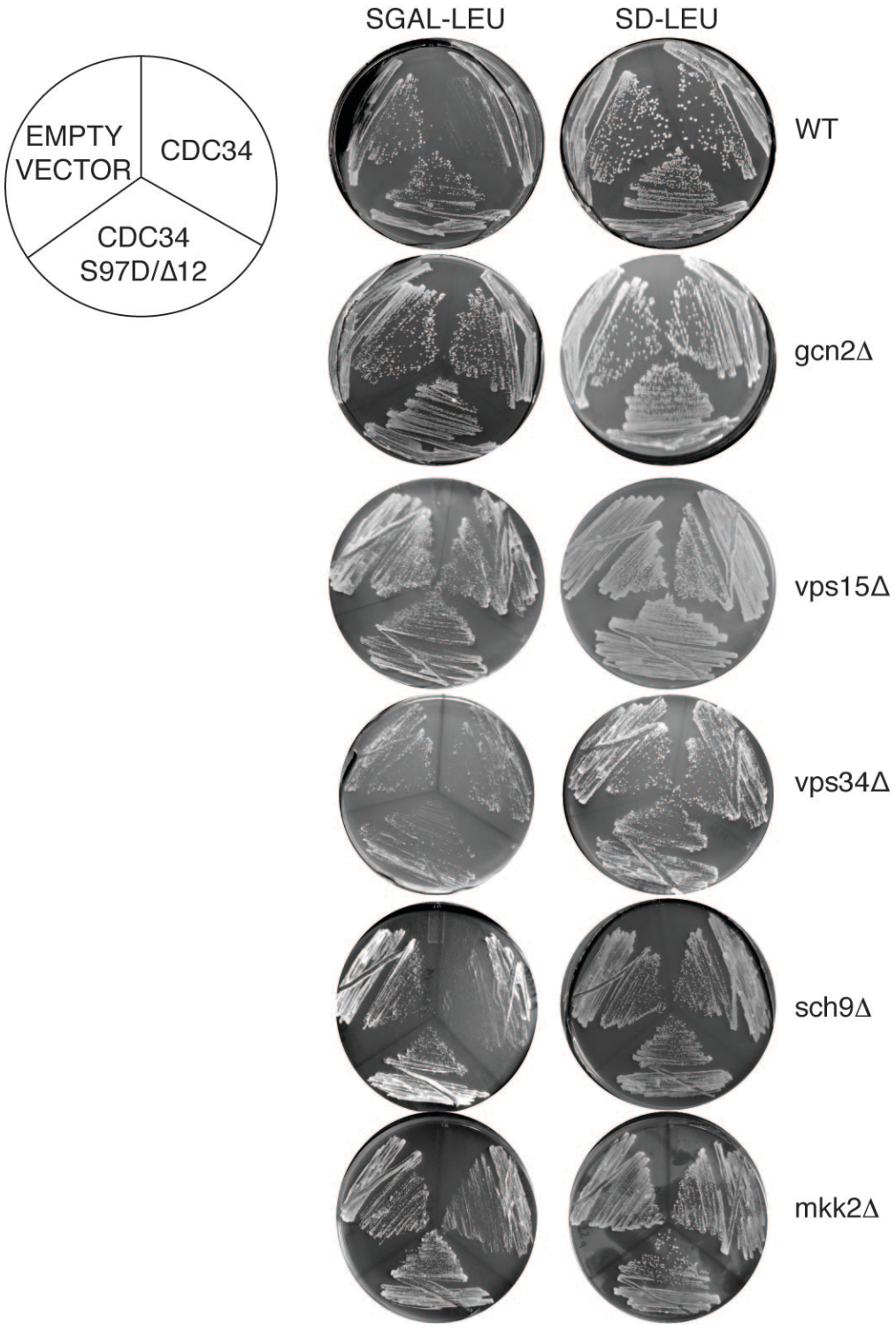

Figure 12. Deletion of *GCN2, VPS34* or *VPS15* suppresses the growth inhibition of *CDC34* overexpression on galactose. Isogenic strains lacking the indicated kinases were transformed with either pSJ101 (empty vector), pYL150 (*CDC34*) or pRC001 (*CDC34*$^{S97D/\Delta12}$). Expression is under control of the GAL10 promoter. Cells were struck on either SD-LEU or SGAL-LEU and incubated at 32°C for 4 days.

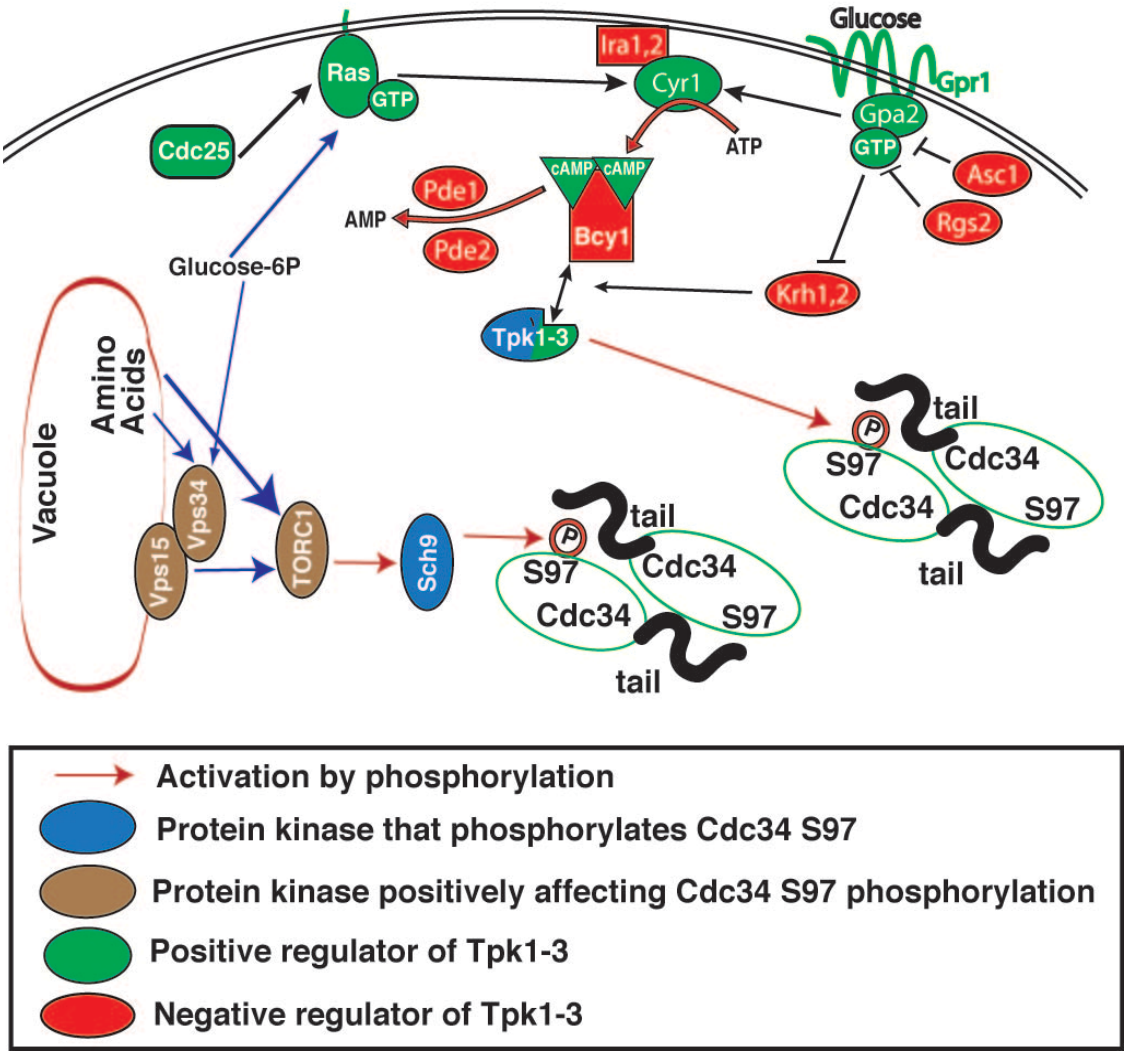

Figure 13. Model of Cdc34 S97 phosphorylation and dimerization.

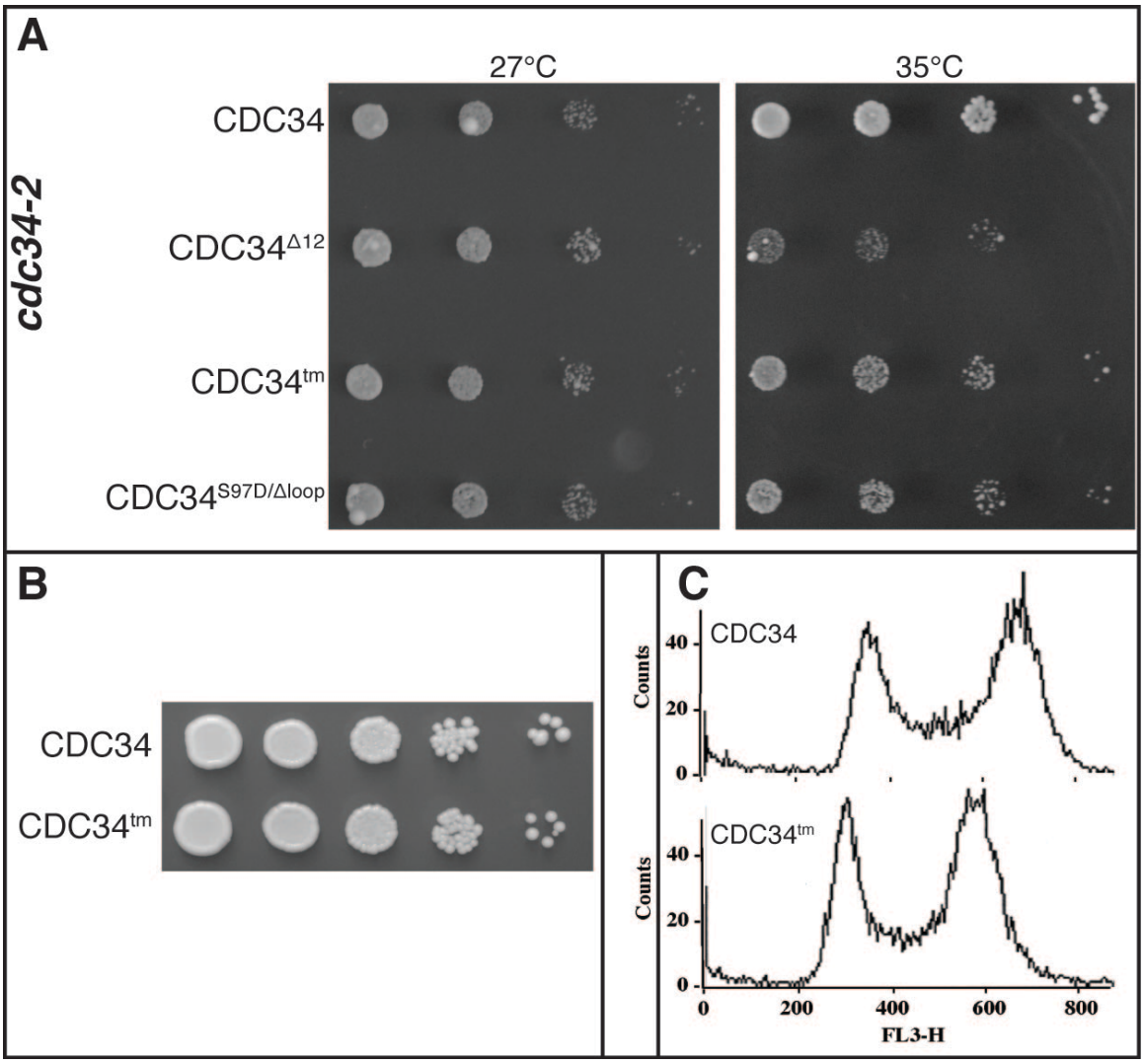

Figure 14. Either the *S73/S97/loop* motif, or the *K73/D97/no loop* arrangement of the catalytic E2 domain can support yeast growth when placed in the context of Cdc34. **(A)** Overnight cultures of *cdc34-2 ts* strain YL10-1 carrying the indicated constructs (pYL150 -*CDC34,* pYL27-*CDC34* $^{\Delta 103\text{-}114}$, pYL29 -*CDC34*^{tm}, pRC001-*CDC34*$^{S97D/\Delta loop}$) under the *GAL10* promoter on a 2 micron YEp51 plasmid were grown overnight at 27°C in 5 ml of SD-Leu, adjusted to a density of $1x10^8$ cells/ml, serially diluted, spotted onto SD-Leu plates and incubated at permissive (27°C) or non-permissive (37°C) temperature for 4 days. **(B)** Isogenic strains BL2 and RC85 encoding chromosomal copies of either *CDC34* or *CDC34*^{tm} were serially diluted as in C, spotted onto YPD and grown at 30°C for 4 days. **(C)** Isogenic strains encoding chromosomal copies of *CDC34* or *CDC34*^{tm} were grown to mid-log phase and analyzed by FACS for their DNA content indicative of cell cycle distribution.

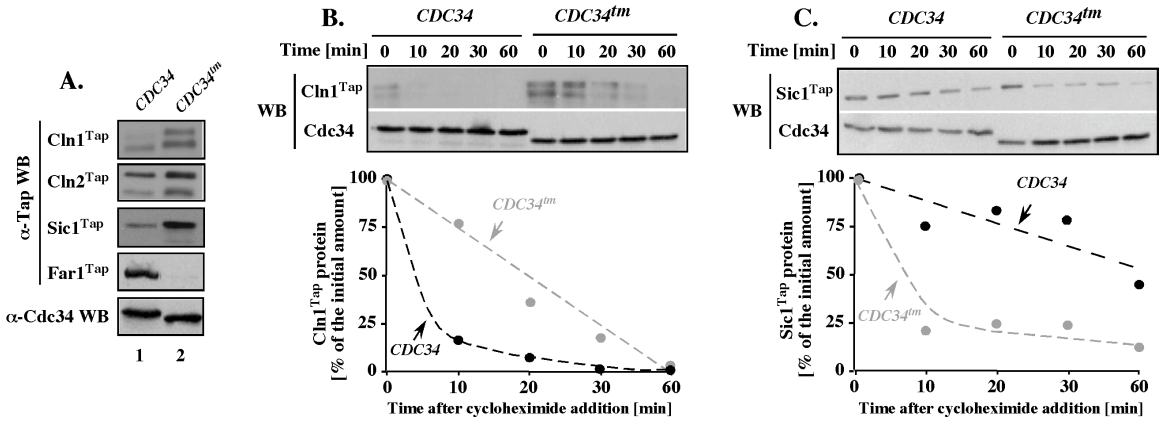

Figure 15. Replacement of *CDC34* with the *CDC34^{tm}* allele leads to unexpected changes in steady-state levels and half-lives of substrates that are recruited via SCF^{Cdc4} and SCF^{Grr1}. **(A)** Protein levels were analyzed by α-Tap western blot of extracts made from haploid cells containing a genomic copy of either *CDC34* (Ghaemmaghami et al., 2003), or *CDC34^{tm}* (strains RRC74, RRC76, RRC73, RRC78) allele and a genomic copy of one of the Sic1^{Tap}, Far1^{Tap}, Cln1^{Tap} and Cln2^{Tap} constructs, as indicated (see Table 3 for details). At least in the case of the Sic1^{Tap} construct, its cell cycle fluctuations were confirmed to be identical to untagged Sic1 (Ghaemmaghami et al., 2003). Like in the case of Cln1 and Cln2 (Tyers et al., 1993) two bands are visible in the α-Tap Cln^{Tap} western blots, representing different electrophoretic mobilities of the phosphorylated and unphosphorylated forms. Comparable levels of Cdc34 and Cdc34^{tm} proteins were detected by α-Cdc34 western blot in each type of an extract, but only the analysis of the Far1^{Tap} extract is shown. **(B)** Protein levels were analyzed as in A, except that extracts were prepared from mid-log-phase yeast cultures harvested at various times after cycloheximide treatment as indicated. Graphs show quantitation of the western blot results, as indicated.

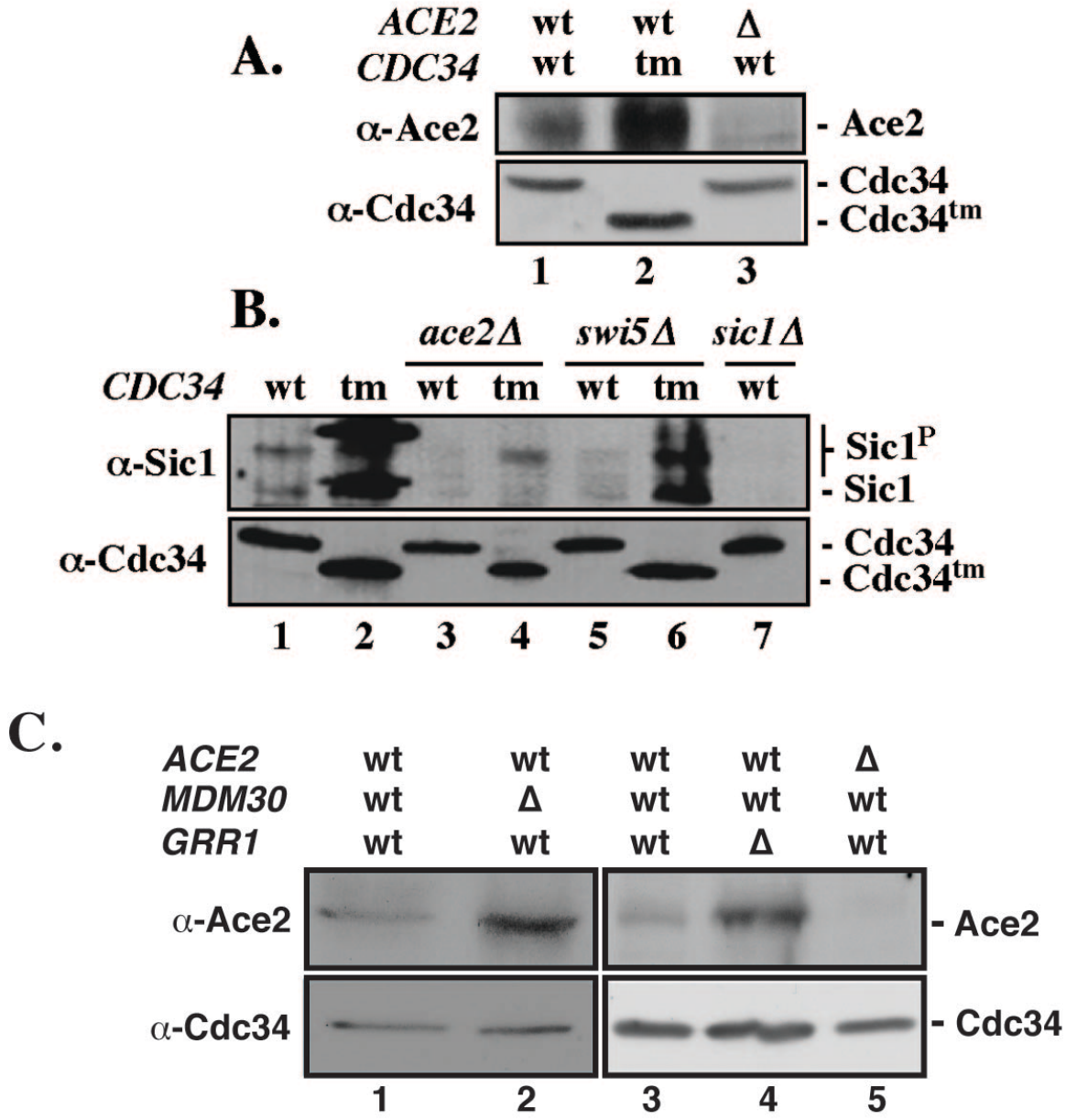

Figure 16. Ace2 is responsible for the increase in Sic1 steady state abundance in CDC34^{tm} cells and is potentially substrate of both SCF^{Grr1} and SCF^{Mdm30}. (A and C) Protein extracts were prepared by the Horvath and Riezman method from yeast of the indicated genotype and analyzed with affinity purified α-Ace2 or α-Cdc34 antibodies, as indicated. **(B and C)** Protein extracts were prepared from yeast of the indicated genotype and analyzed with affinity purified α-Sic1, α-Ace2 or α-Cdc34 antibodies, as indicated.

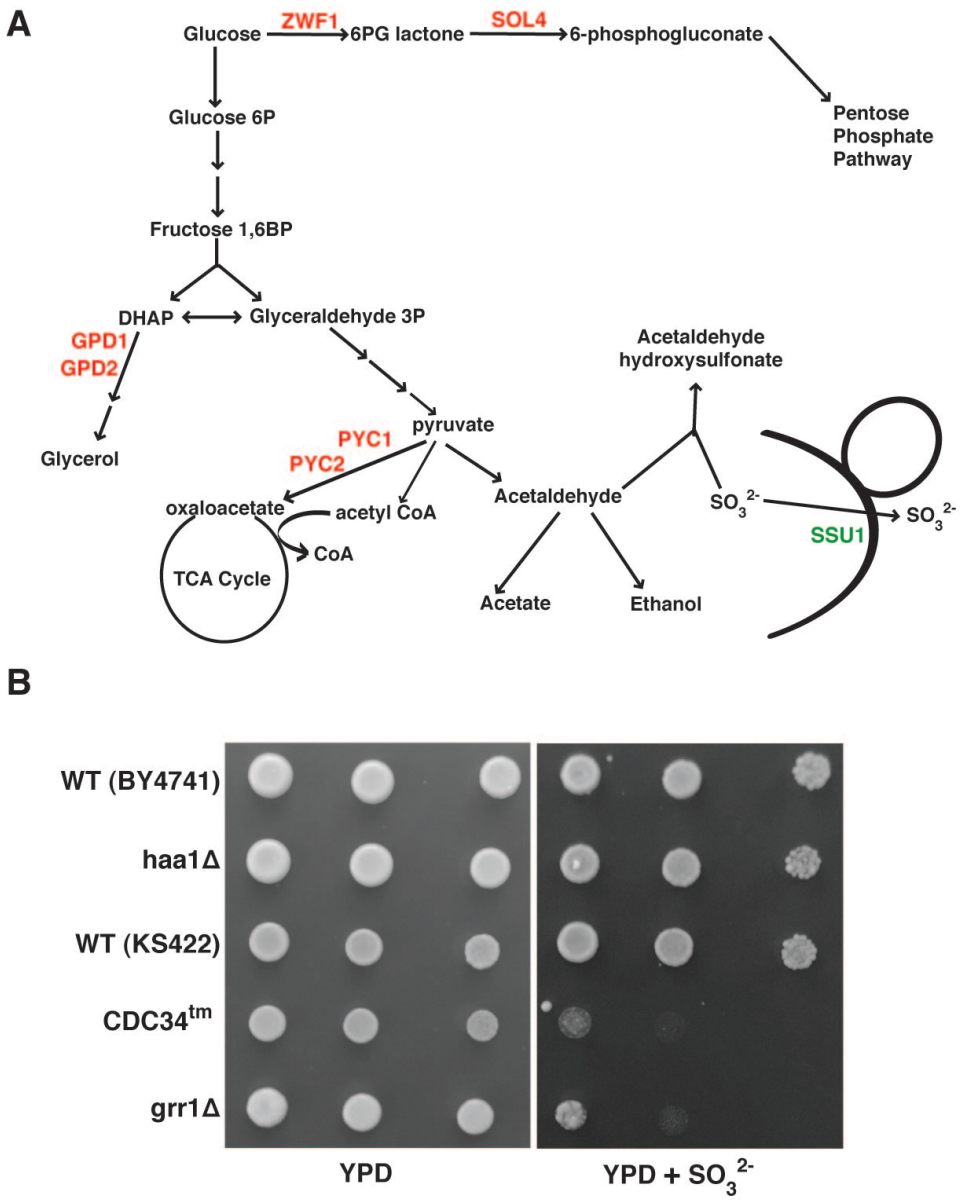

Figure 17. mRNAs encoding enzymes which reduce the metabolic flux through glycolysis are reduced in CDC34^{tm} cells and correlates with CDC34^{tm} and grr1Δ sulfite sensitivity. (A) Metabolic pathways of glycolysis and its shunts. The mRNAs which are significantly reduced (p val < 0.01) in *CDC34^{tm}* cells are shown in red. mRNAs which are not significantly different between WT and *CDC34^{tm}* cells are not shown. The mRNA encoding the sulfite extrusion pump, *SSU1*, is significantly up-regulated (p < 0.01) in *CDC34^{tm}* cells and is shown because it directly affects acetaldehyde levels by preventing the formation of the acetaldehyde hydroxysulfonate. **(B)** Strains BY4741 (WT), 7137 (*haa1Δ*), KS422 (WT), KS418 (*CDC34^{tm}*), KS415 (*grr1Δ*) were grown overnight adjusted to equal densities, serially diluted ten fold, spotted onto YPD plates, pH 3.5, either with or without 2 mM sulfite, and incubated at 37^{o}C for 2 days.

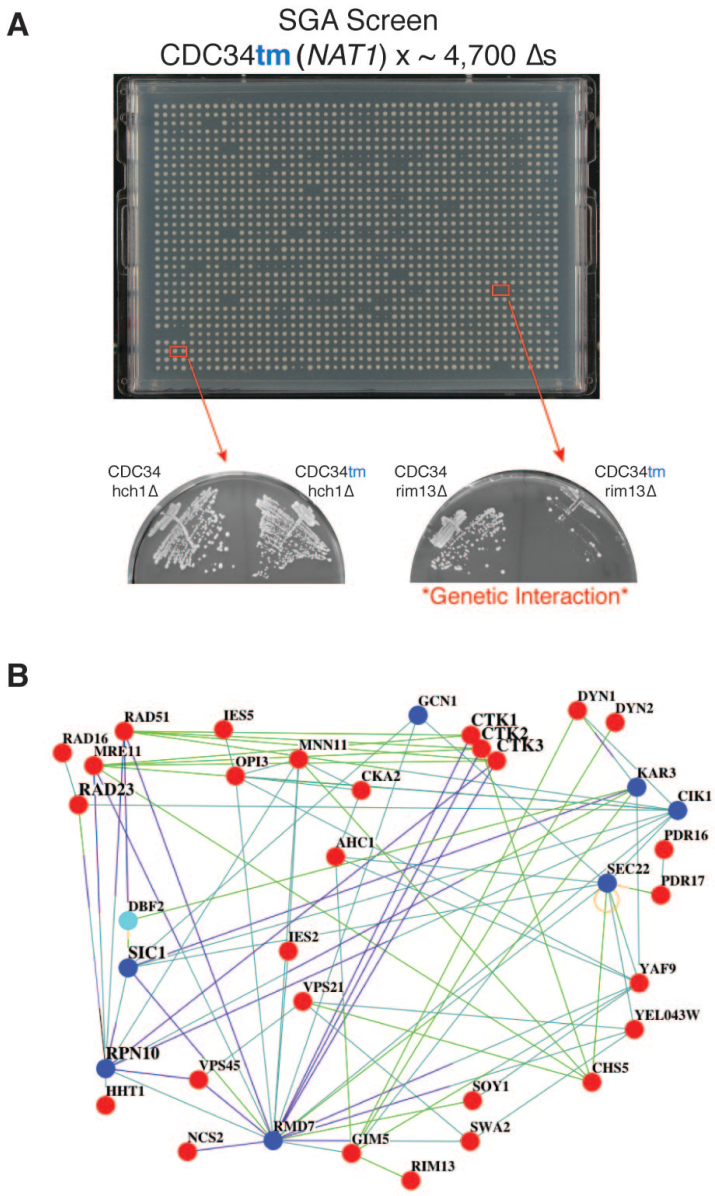

Figure 18. Synthetic lethality analysis with the *CDC34^{tm}* allele identifies several groups of genes that are critical for growth of *CDC34^{tm}* but not *CDC34* yeast. (A) General schematic of our SGA approach. An example of results of the primary (top) and secondary (bottom) screen using the *CDC34^{tm}* query allele, or the control *CDC34* allele, as indicated, each marked with *NAT1* in the same location (for details see text, and Methods). **(B)** Network analysis of genetic interactions. Osprey software was used to visualize interactions housed in the Biogrid database among genes which are synthetically lethal or sick with the *CDC34^{tm}*. Excluded from the analysis are genes identified as synthetically lethal with *CDC34^{tm}* but lacking known interactions with other genes. The analysis includes *SIC1* that is not synthetically lethal with *CDC34^{tm}*, but that nevertheless shares seven genetic interactions with *CDC34^{tm}* (blue nodes and lines). The analysis identifies synthetic lethality (green), dosage rescue (yellow), synthetic growth defect (purple) and phenotypic enhancement (aqua).

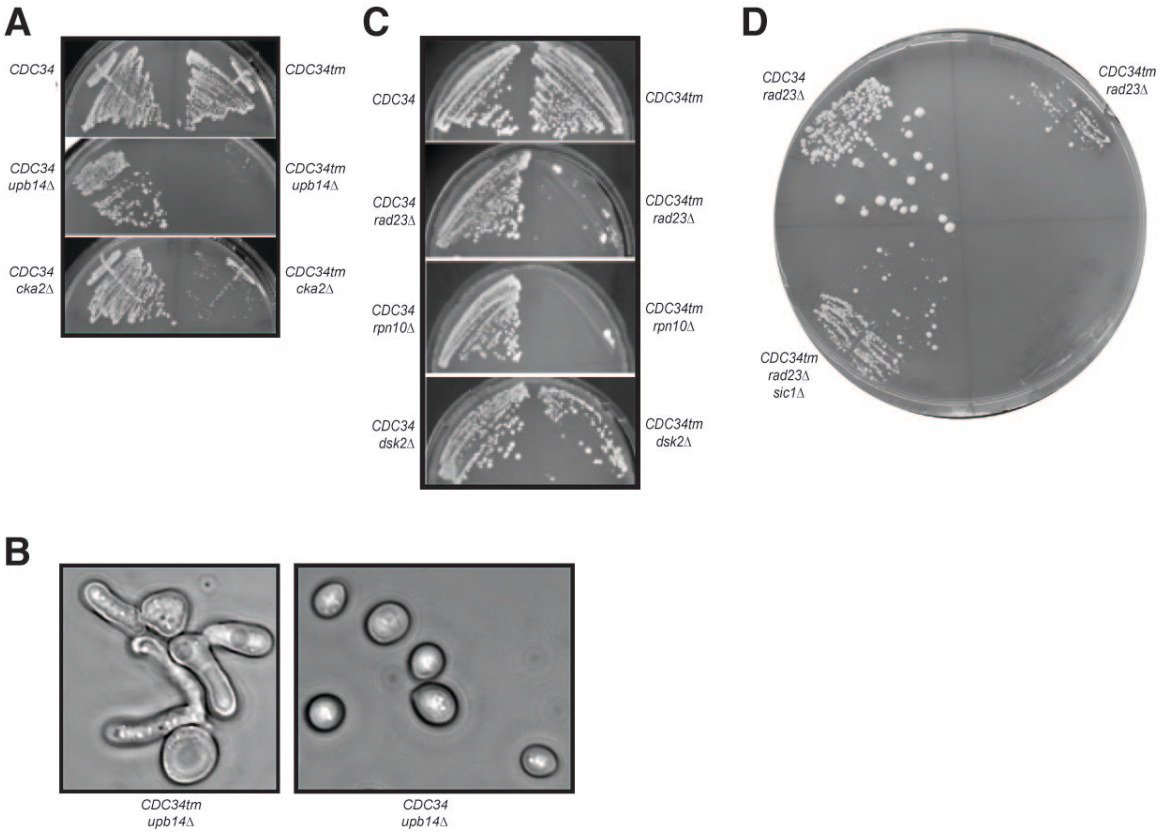

Figure 19. Growth of *CDC34*^{tm} but not of *CDC34* yeast depends on the intact functions of *UBP14*, *CKA2*, *RPN10* and *RAD23* genes. (A and C) Haploids with the indicated genotypes were selected by streaking heterozygous diploids on haploid selection media with G418 and nourseothricin. Plates were incubated at 30^{o}C for three days. **(B)** *CDC34*^{tm} *ubp14Δ* haploids have an elongated bud and enlarged vacuole. Haploids of the indicated genotype were isolated by tetrad dissection and imaged microscopically. **(D)** Deletion of *SIC1* bypass the requirement for *RAD23* in growth of *CDC34*^{tm} yeast. Diploid strains with the indicated genotypes (Table 3) were sporulated and then struck on haploid double mutant selection plate that allows for growth of only the indicated haploid. Plate was incubated at 30°C for three days.

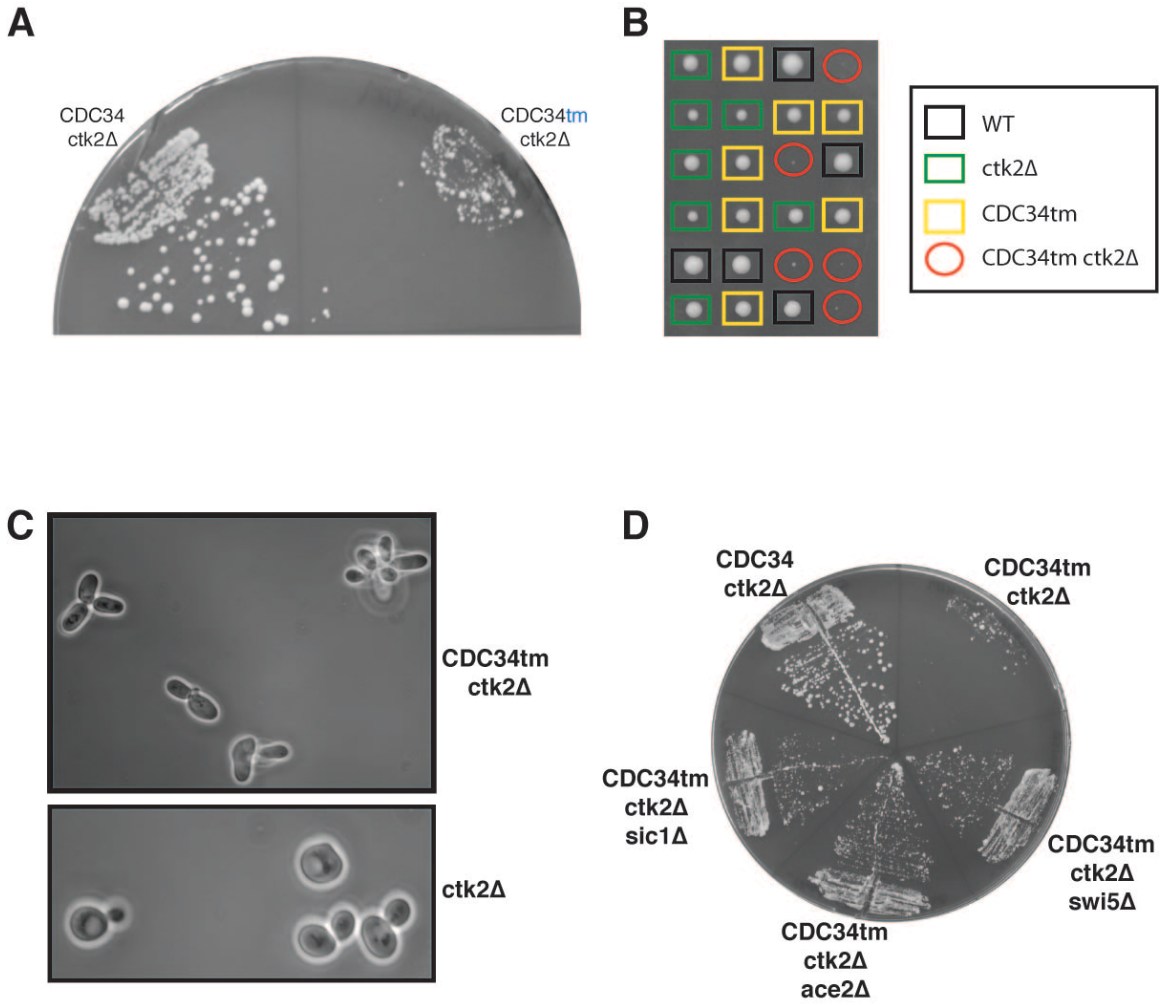

Figure 20. *CTK2* is synthetically lethal with *CDC34^{tm}*. (A) Haploids with the indicated genotypes were selected by streaking heterozygous diploids on haploid selection media with G418 and nourseothricin. Plates were incubated at 30°C for three days. **(B)** *CDC34^{tm}/CDC34 ctk2Δ/CTK2* diploids, RC141, were sporulated and dissected on YPD. **(C)** Haploids of the indicated genotype were isolated by tetrad dissection and imaged microscopically. **(D)** Diploid strains with the indicated genotypes (Table 3) were sporulated and then struck on haploid double mutant selection plate that allows for growth of only the indicated haploid. Plate was incubated at 30°C for three days.

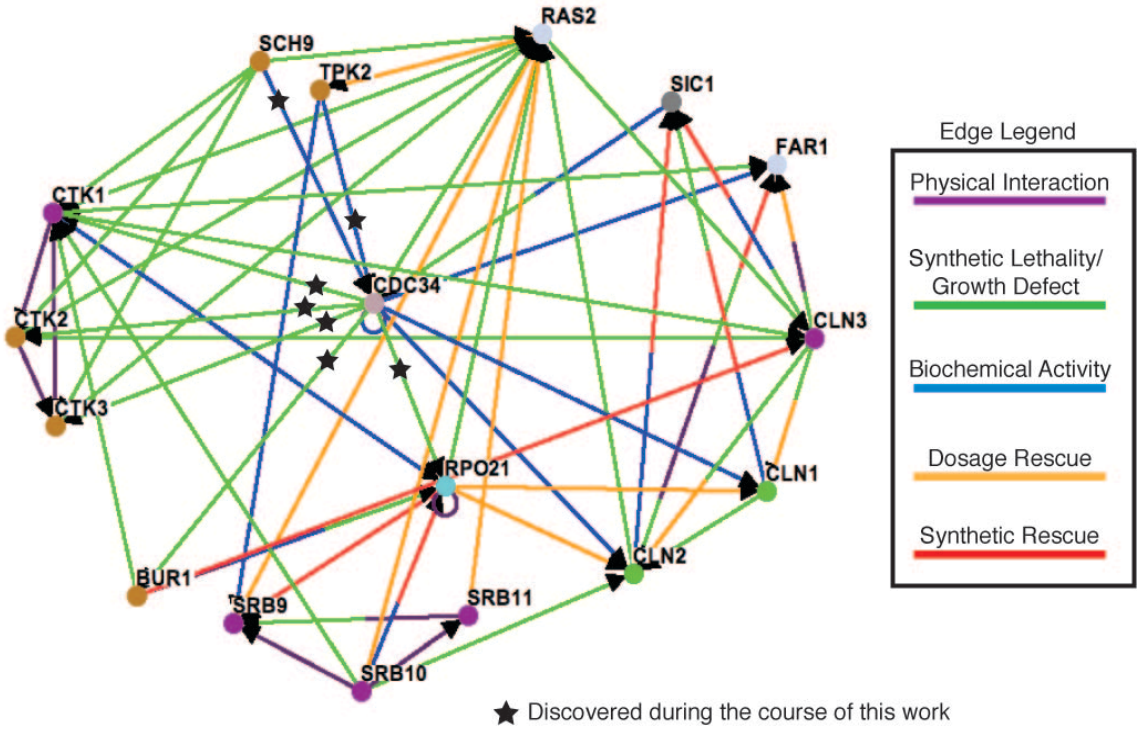

Figure 21. The highly connected network among nutrient sensing kinases, RNA Pol II, Cdc34 and Cdc34 substrates. Osprey software was used to visualize interactions housed in the Biogrid database along with interactions discovered during the course of this work (☆).

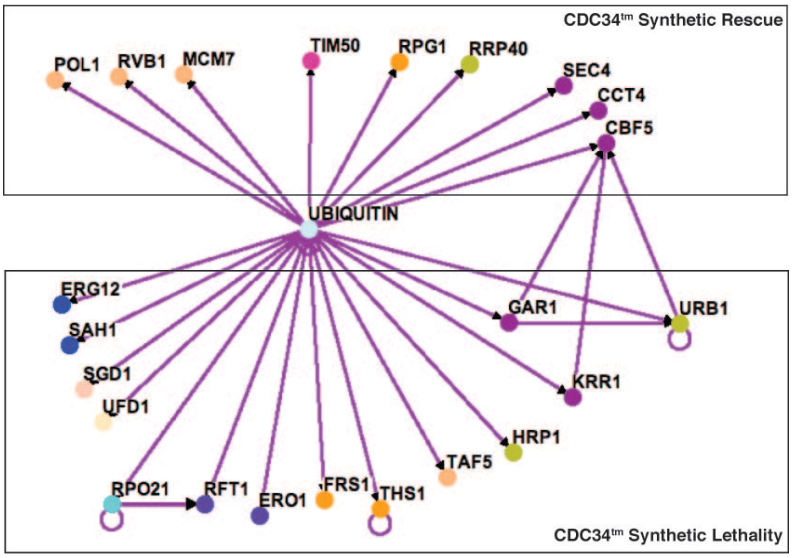

Figure 22. A network of essential genes which genetically interact with *CDC34^{tm}* and whose protein products are ubiquitinated. Osprey software was used to visualize physical interactions housed in the Biogrid database among essential genes which share a genetic relationship with *CDC34^{tm}* and were found in a global, proteomic screen (Peng et al., 2003) to physically associate with the *UBI4* gene product, ubiquitin.

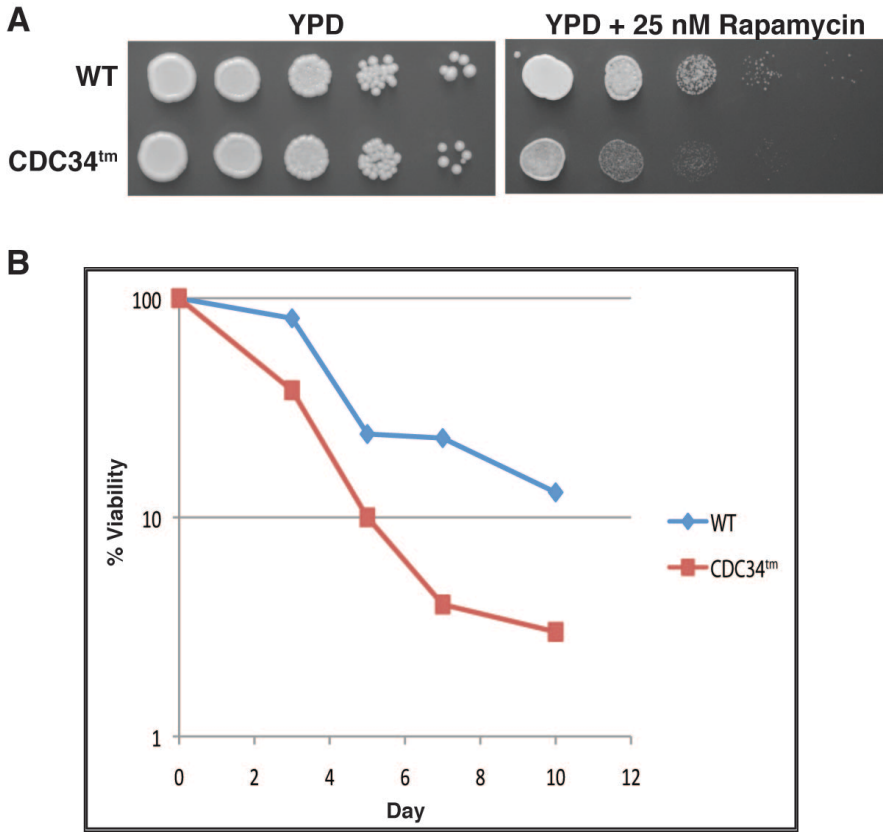

Figure 23. The Cdc34 S73/S97/loop motif increases chronological lifespan and is required for rapamycin resistance. (A) Strains BL2 and RRC85 were grown overnight in SD (2% glucose) lacking all amino acids, diluted to $5x10^7$ cells/ml and spotted in ten fold serial dilution on YPD and YPD+25 nM rapamycin plates. Plates were incubated at 32°C for 3 days. **(B)** Strains BL2 (WT) and RRC85 ($CDC34^{tm}$) were grown in 50 ml of SD (2% glucose) lacking all amino acids for the indicated number of days and then an appropriate dilution of cells was spotted onto a YPD plate. The plates were incubated for 3 days at 32°C and the percentage of viable cells was calculated by counting the number of colony forming units divided by the number of cells plated. Data shown are the average of at least three independent experiments.

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