Nutrient Sensing Kinases PKA and Sch9 Phosphorylate the Catalytic Domain of the Ubiquitin-Conjugating Enzyme Cdc34

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Abstract

Cell division is controlled in part by the timely activation of the CDK, Cdc28, through its association with G1 and G2 cyclins. Cdc28 complexes are regulated in turn by the ubiquitin conjugating enzyme Cdc34 and SCF ubiquitin ligase complexes of the ubiquitin-proteasome system (UPS) to control the initiation of DNA replication. Here we demonstrate that the nutrient sensing kinases PKA and Sch9 phosphorylate S97 of Cdc34. S97 is conserved across species and restricted to the catalytic domain of Cdc34/Ubc7-like E2s. Cdc34-S97 phosphorylation is cell cycle regulated, elevated during active cell growth and division and decreased during cell cycle arrest. Cell growth and cell division are orchestrated to maintain cell size homeostasis over a wide range of nutrient conditions. Cells monitor changes in their environment through nutrient sensing protein kinases. Thus Cdc34 phosphorylation by PKA and Sch9 provides a direct tether between G1 cell division events and cell growth.

Introduction

The ubiquitin proteasome system (UPS) controls cellular functions through the targeted degradation of key regulatory proteins. The covalent attachment of ubiquitin often serves as a signal for the degradation of these regulatory proteins by the 26S proteasome (for review see [1]). The first step in ubiquitination is the formation of a high energy intermediate between ubiquitin and a conserved cysteine of the ubiquitin activating (or E1) enzyme. E1 then transfers the ubiquitin via a thiolester linkage to a conserved cysteine of an ubiquitin conjugating (or E2) enzyme. The final transfer of ubiquitin to a specific substrate typically requires both an activated E2 as well as a particular ubiquitin ligase (E3), which provides specific substrate modifying capacity, forming an isopeptide linkage between the COOH-terminal glycine residue of ubiquitin and the ε-amino group of a lysine residue of the substrate. A substrate is often targeted for degradation upon the addition of a polyubiquitin chain to the lysine 48 residue of ubiquitin.

Cdc34 encodes a ubiquitin conjugating enzyme that is essential for cell viability and the initiation of DNA replication in the yeast, Saccharomyces cerevisiae [2]. Cdc34 conjugates ubiquitin with target proteins in conjunction with the SCF family of E3-ubiquitin ligases [3]. A functional SCF complex consists of at least four distinct proteins, Skp1, Cdc53, Rbx1 and an F-box protein, the component that determines substrate specificity (for review, see [4]). When Cdc4 is present in the SCF complex, Cdc34 and SCF^Cdc4^ mediate ubiquitylation and subsequent degradation of the cyclin dependent kinase inhibitors Sic1 and Far1 [5,6,7]. On the other hand, Cdc34 and SCF^Sic1^ ubiquitylate the cyclins Cln1 and Cln2 [6,7,8].

The Cdc34/Ubc7 family of ubiquitin conjugating enzymes is defined by a conserved motif within the catalytic domain that consists of two serines and a twelve amino acid acidic loop, which lie in close physical proximity to the catalytic cysteine. In contrast, the majority of E2s, of which Rad6 is a classic example, have a lysine and aspartic acid residue in lieu of these serine residues and lack the acidic loop. This motif allows the Cdc34/Ubc7 family to catalyze both monoubiquitylation and ubiquitin chain extension [9]. Accumulating evidence suggests the formation of the Cdc34−ubiquitin thiolester precedes self-association of Cdc34, which in turn is critical for Cdc34 catalytic activity [10,11]. Interestingly, Cdc34^S97D^ mutants are unable to homodimerize and are nearly inviable [11]. Elegant in vitro reconstitution of Sic1 polyubiquitylation by SCF^Cdc4^ has demonstrated that conjugation of the first ubiquitin to the substrate is the rate limiting step in this process. In vitro, Cdc34 mutants that lack the acidic loop (Cdc34^A12^) monoubiquitylate Sic1 with kinetics comparable to wild type Cdc34, but extend ubiquitin chains at a negligible rate [9]. However, during in vitro Cdc34 autoubiquitylation or histone ubiquitylation assays which do not require RING finger containing proteins, Cdc34^A12^ mutants function as well as, if not better than, Cdc34 [11,12]. Cells solely expressing Cdc34^A12^ mutants are nearly inviable, as are cells harboring Cdc34^S97D^ or Cdc34^S73K/S97D^ mutants [13]. Paradoxically, deletion of the acidic loop, residues 103−114, in combination with S73K and S97D mutations (hereafter referred to as the Cdc34 triple mutant, Cdc34^TM^) shows only subtle defects on cell growth in vivo
[11,12,13]. However, in vivo SClCdc4-dependent Cdc34* polyubiquitylation of Sic1 is defective similar to Cdc34*12 [14]. Recent data indicates that Cdc34* expressing cells show key differences from wild-type cells. Importantly, Cln1 and Cln2 proteins are more stable, while Sic1 has a decreased t1/2 and Far1 becomes undetectable. Further, the steady state level of the Ace2 and Swi5 transcription factors, as well as the abundance of their transcriptional targets, is altered. A subsequent Synthetic Gene and Swi5 transcription factors, as well as the abundance of their expression of RAD23 due to an increase in toxic, free ubiquitin chains [14,15]. Interestingly, loss of the Cdc34-Ubc7 specific motif causes cells to become dependent on Cka2 and Ubp14, most likely due to an increase in toxic, free ubiquitin chains [14,15].

This study demonstrates that the Cdc34/Ubc7 specific motif is also a key target of signaling pathways coordinating the regulation of cell growth in response to changes in environmental conditions such as nutrient levels. Here we demonstrate that Cdc34+S97 can be directly phosphorylated by either cAMP-dependent protein kinase or the AKT protein kinase homolog Sch9. We also demonstrate that Cdc34+S97 phosphorylation is elevated under conditions of active cell growth and division while it is decreased under conditions causing cell cycle arrest. These results provide a direct link between the growth regulating protein kinases PKA and Sch9 and early cell division events through post-translational modification of the ubiquitin-conjugating enzyme Cdc34.

Results

Genetic interactions among S73/S97/loop residues that define Cdc34/Ubc7-like class of E2s

A partial sequence alignment of the yeast ubiquitin conjugating enzymes and Cdc34 orthologs is shown in Figure 1. This partial alignment centers around two highly conserved regions of the E2 enzymes. S73 is centered within the highly conserved motif – [FY][FLY][ST]PP - while S97 is centered within the highly conserved motif – [LVI]C[IL]S[IMV][IL]. These two sequences are almost completely restricted to the E2 enzymes. S73 is centered within the highly conserved motif –[FY]P[FLY][ST]PP – while S97 is centered within the highly conserved motif –[FY]P[FLY][ST]PP. These two sequences are almost completely restricted to the E2 enzymes. The acidic loop region begins seven amino acid residues COOH-terminal to the active site cysteine at position 95 in Cdc34. As described above, there are robust genetic and functional relationships among S73, S97 and a twelve amino acid “loop” region (residues 103-114 of Cdc34) that surround the catalytic cysteine (C95). 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 residue and an aspartic acid residue, respectively, within these conserved regions and lack the “loop” region. This motif of serine/serine/insert or lysine/aspartic acid/no insert is conserved among most eukaryotic E2s [13].

Previously, we and others determined that the Cdc34*73K/S97D or Cdc34*73K/S97A enzymes are non-functional both in vivo and in vitro and the Cdc34* mutant supports growth much more poorly than Cdc34 [11,13]. Interestingly though, a mutant Cdc34 combining the S73K, S97D, and A103–114 (hereafter referred to as Cdc34*) mutations is viable, even when introduced at the normal CDC34 chromosomal locus under the CDC34 promoter [11,13]. In fact, Cdc34* can be rescued by combining this mutation with the A103–114 mutation (hereafter referred to Cdc34*). Thus there is a very strong genetic relationship between S97 and the loop region of Cdc34.

During the course of reproducing viability assays, we found that the Cdc34*103–114 temperature sensitive strain at a non-permissive temperature (Fig. 2B). Previous studies utilized 36°C as the non-permissive temperature and used patch assays during complementation experiments. By reducing the non-permissive temperature to 35°C and testing complementation by a spot dilution assay, several observations were made. Cdc34*103–114 can complement a cdc34-2 temperature sensitive strain at 35°C while CDC34*103–114 does not (Fig. 2B and C). However, neither CDC34*103–114 nor CDC34*103–114 can complement cdc34-4. Thus while neither cdc34-2 (CDC34*103–114) nor CDC34*103–114 retain Cdc34 function at 35°C, each can suppress the growth defect of the other. This intragenic suppression suggests that multiple Cdc34 proteins work together during ubiquitylation events. On the other hand, CDC34*103–114, a mutation that ostensibly permits phosphorylation at this residue, complements both the cdc34 temperature sensitive and cdc34 strains in spot dilution assays (Fig. 2). The viability of CDC34*103–114, coupled with the previous result that Cdc34 can be found phosphorylated in vivo [16], provided the rationale for the experiments described below to determine whether Cdc34*S97 is a

![Figure 1. Alignment of E2s: The serine/serine/insert motif is conserved in all Cdc34 family members.](image-url)
**Figure 2.** Complementation of *cdc34-2* and *cdc34-4* strains by Cdc34 S97 Mutants. A and B) YL10-1, a *cdc34-2* temperature sensitive strain, bearing 2 μm plasmids encoding the indicated *CDC34* mutant under control of the *GAL10* promoter were spotted in tenfold serial dilutions on the indicated media and grown at the indicated temperatures for 3 days. C) YL18, a *cdc34-2* strain harboring a URA3 marked plasmid encoding wild type *CDC34* and a LEU2-marked plasmid encoding the indicated *CDC34* allele under control of the *GAL10* promoter were spotted in tenfold serial dilution on the indicated media and grown at 30°C for 3 days.

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Cdc34 is phosphorylated *in vivo* on S97

Cdc34 is phosphorylated *in vivo* on serine residues [16] and several of the serine phosphorylation sites have been mapped [17,18]. Due to the fact that *CDC34 F497T* is functional both *in vivo* and *in vitro* and that Cdc34-S97 is highly conserved across eukaryotic species, we hypothesized that Cdc34-S97 is a site of phosphorylation. To test this hypothesis, we generated antibodies that specifically recognize Cdc34 phosphorylated at S97 (S97p). This antibody, termed α-pS97, recognizes a protein from whole cell yeast lysate which migrates at the same position as Cdc34 (Fig. 3A, lane 1). When full length Cdc34 is replaced by a COOH-terminally truncated Cdc34 that migrates noticeably faster through an SDS-PAGE gel, the α-pS97 antibody recognizes a protein which migrates at the same location as COOH-terminally truncated Cdc34 (Fig. 3A, 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. The α-pS97 antibody does not recognize Cdc34tm (Fig. 3A, lane 4) indicating that this antibody is specific to S97. Moreover, even when loaded in excess of endogenous yeast Cdc34, bacterially expressed Cdc34, which is not phosphorylated, is not recognized by α-pS97 (Fig. 3A, lane 6). From these studies, we conclude that the α-pS97 antibody is specific for Cdc34-S97p and that Cdc34 is phosphorylated *in vivo* on S97. Furthermore, these data indicate that the COOH-terminus of Cdc34 (amino acids 245–295) is an important determinant of Cdc34-S97 phosphorylation, since the level of Cdc34-S97 phosphorylation is dramatically reduced on COOH-truncated Cdc34 (Fig. 3A, lane 2).

An important methodological consideration for these experiments is that Cdc34-S97 phosphorylation becomes undetectable when the protein extract is made by the Horvath-Riezman method [19], a common method for yeast cell protein extraction which requires heating the sample in SDS for 5 minutes (Fig. 3B, lane 2). However, cell disruption by the glass bead method in either the breaking buffer described in materials and methods or 8 M urea allows S97 phosphorylation to be detected by western blotting.

Phosphorylation of S97 is induced in the G1 phase

Cdc34 in conjunction with SCF complexes has a plethora of substrates which are targeted for proteasome mediated degradation. The known substrates whose degradation is required for timely cell cycle progression are all ubiquitylated and degraded in the G1 phase of the cell cycle. Thus, we hypothesized that Cdc34-S97 phosphorylation is regulated through the cell cycle. To test this hypothesis, cells were synchronized in late G1 with α-factor. After three hours, >95% of the cells were arrested with a mating projection, or shmoo, morphology. The α-factor was washed out; cells were resuspended in fresh medium; and cells were then collected at the indicated times (Fig. 4). At the initial removal of α-factor, Cdc34-S97 phosphorylation is very low, but quickly rises to a level that remains constant through most of the cell cycle until the second G1 phase is reached. At the second G1 phase the level of Cdc34-S97 phosphorylation increases dramatically (Fig. 4). α-factor arrests cells late in G1, just prior to the initiation of DNA replication and bud emergence. When incubated at a non-permissive temperature, cells harboring temperature sensitive alleles of *cdc28* or *cdc25* arrest at a point (or points) prior to α-factor induced arrest and remain sensitive to α-factor when returned to a permissive temperature [20,21]. The fact that induction of S97 phosphorylation does not occur immediately after α-factor release and only after a full cell division cycle suggests that the S97 phosphorylation increase occurs early in G1, prior to the α-factor arrest point. This finding is consistent with Cdc34-S97 phosphorylation occurring on active Cdc34 when its cell cycle substrates are degraded.

Identification of kinases that may phosphorylate Cdc34-S97

Upon establishing that Cdc34 is phosphorylated on S97 in a cell cycle dependent manner, we focused on identifying the protein kinase(s) responsible for this event. We began our screen for candidate Cdc34-S97 kinases by an in vivo over-expression assay. This was done to avoid overlooking candidate protein kinase genes that are essential as would have been the case if we had begun our analysis with a collection of yeast containing viable protein kinase deletion mutants. To identify the kinase(s) responsible for phosphorylating Cdc34-S97, we utilized a genomic ordered array of 124 yeast strains, each expressing a unique GST-kinase fusion under the control of a copper inducible promoter. Construction and utilization of the yeast ORF collection has been described previously [20]. Strains were grown to mid exponential phase in minimal media and GST-kinase expression was induced with the
Figure 3. Cdc34 S97 is phosphorylated in vivo and its detection is prohibited by certain protein extraction conditions. A) Soluble protein from yeast cells, expressing from the CDC34 chromosomal locus, either wild type Cdc34 (lanes 1, 3 and 5), a COOH-terminally truncated Cdc34 encoding amino acids 1–244 (lane 2) or a Cdc34<sup>tm</sup> mutant encoding the S97D mutation (lane 4) or bacterially expressed Cdc34<sup>6XHis</sup> (lane 6) were analyzed by western blot using the α-pS97 phospho-specific or 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 phospho-specific and α-Cdc34 antibodies.
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Figure 4. Cdc34 S97 phosphorylation increases in the G1 phase of the cell cycle. Strain RC150 was grown to mid-log phase and arrested with a final concentration of 0.5 μg/ml α-factor. After 3 hours, >95% of the cells had arrested with a shmoo-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 phospho-specific and α-Cdc34 antibody. At least 100 cells were counted per time point to determine the budding index.
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addition of copper sulfate. In total, more than 100 unique kinases were screened for their effect on Cdc34-S97 phosphorylation in this manner (Fig. 5). Using this assay, a number of kinases (Tpk2, Sch9, Vps15, Mkk2, Cla4, Mrk1, Mec1, Mps1, Snf1 and Gcn2), when over-expressed, caused the greatest increase in the level of Cdc34-S97 phosphorylation in vivo. There are also two MAP kinase kinases, Mkk1 and Ste7, which when over-expressed noticeably reduce the level of Cdc34-S97 phosphorylation (Fig. 5). While we considered this to be a comprehensive screen of the yeast kinome, it did not include the full complement of kinase activity in yeast. Several kinase encoding genes were not present in our array including VPS34, TOR1, TOR2 and STE20. Furthermore, a number of strains did not grow to sufficient density for analysis of Cdc34-S97 phosphorylation. We also realize that other kinases require targeting subunits and that without simultaneous over-expression of both the catalytic and regulatory subunits, the activity of these kinases against certain substrates will remain unchanged. Interestingly though, most of the candidate kinases for Cdc34-S97 phosphorylation are known regulators of nutritional status and growth control.

To extend the investigation of kinases affecting Cdc34-S97 phosphorylation, we analyzed the level of Cdc34-S97 phosphorylation in strains lacking the individual kinases whose over-expression causes an increase in Cdc34-S97 phosphorylation. This analysis did not include strains lacking MEC1 or MPS1 as these genes are essential. Based on this analysis, the level of S97 is reduced in strains lacking Gcn2, Mkk2, Sch9, Snf1, and Vps15/ Vps34 (Fig. 6). We also examined the effect of a VPS34 deletion on Cdc34-S97 phosphorylation. VPS34 was not included in our set of protein kinase overexpressing strains; however, Vps34 and Vps15 function as a complex [21]. Ultimately, loss of either Vps15 or Vps34 results in a reduction in Cdc34-S97 phosphorylation (Fig. 6).

Finally, the absence of a number of these protein kinases (Tpk1, Tpk2, Mrk1, Hsl1, Cla4, or Mkk1) does not noticeably reduce the level of S97 phosphorylation (Fig. 6). While there are multiple reasons that might explain why the loss of these kinases does not reduce the level of S97 phosphorylation, one likely explanation is that each of these remaining protein kinases is a member of a structurally related subfamily of protein kinases whose members are thought to have overlapping functions (the cAMP-dependent protein kinases-Tpk1/Tpk2/Tpk3, the Gsk3-like protein kinases-Mrk1/Rim11/ Mck1/Ygk3, the PAK kinases-Cla4/Ste20/Skm1, and the Hsl1/Kcc4/Gin4 family). Addi-

Figure 5. A screen for kinases whose overexpression alters the level of Cdc34-S97 phosphorylation. A) Total soluble protein from an array of strains each overexpressing the indicated kinase was analyzed by western blot using the α-pS97 phospho-specific and α-Cdc34 antibodies. doi:10.1371/journal.pone.0027099.g005
tionally, we cannot eliminate the possibility that a particular kinase might only influence the phosphorylation of a small pool of Cdc34 whose reduced phosphorylation is undetectable when the entire pool of Cdc34-S97p is probed. Cdc34 associates with many SCF complexes which are unique in their F-box component and it is also possible that different kinases might influence the phosphorylation of Cdc34-S97 only in the context of specific SCFs.

For several reasons, we also chose to examine specifically whether the Tor kinases controlled Cdc34-S97 phosphorylation for several reasons. First, as shown above, several other nutrient sensing kinases alter Cdc34-S97 phosphorylation. A function of the TORC1 complex (Tor1, Kog1, Lst8, and Tco89) is as a sensor of nitrogen availability in eukaryotic cells. Secondly, both Vps15, a kinase which activates TORC1, and Sch9, a kinase activated by TORC1, increase Cdc34-S97 phosphorylation when over-expressed and strains lacking either of these kinases have reduced Cdc34-S97 phosphorylation. Thus TORC1 could be a part of a Vps15/Vps34-TORC1-Sch9 cascade leading to Cdc34-S97 phosphorylation. Finally, neither Tor1 nor Tor2 are part of the collection of copper inducible kinases used in the initial screen; however, Tor activity can be inhibited by the natural compound rapamycin. As predicted, Cdc34-S97 phosphorylation is reduced when cells are treated with the Tor kinase inhibitor rapamycin (Fig. 6).

Reconstitution of Cdc34-S97 phosphorylation in vitro

While genetic and physiological manipulation of Cdc34-S97 phosphorylation provides compelling evidence that specific protein kinases can control this event, these experiments do not allow us to distinguish between a direct Cdc34-S97 phosphorylating activity and indirect effects. Therefore in vitro phosphorylation assays were performed to determine which protein kinase(s)

Altered PKA activity affects S97 phosphorylation

An upstream component of the cAMP/PKA pathway, RAS2, genetically interacts with CDC34 [22]. Compromising the activity of protein kinase A, either by deleting RAS2 or by growing cells on a non-fermentable carbon source which reduces intracellular cAMP levels, inhibits the growth of cdc34-2 temperature sensitive strains at otherwise permissive temperatures [22]. These findings suggest that Cdc34 and PKA act in a common pathway and together with the fact 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, Tpk1–3 (reviewed in [23]). Bcy1 is the regulatory subunit which inhibits PKA catalytic activity in the absence of cAMP. Cyclic AMP binds Bcy1 causing its release from the catalytic subunits, resulting in increased PKA activity [24]. Strains lacking BCT1 have increased PKA activity and are sensitive to heat shock and nutrient deprivation due specifically to increased PKA activity [25]. We find that the level of Cdc34-S97 phosphorylation is increased in cells harboring the crippled bcy1-14 allele (Fig. 7, lane 2), consistent with the conclusion that PKA controls Cdc34-S97 phosphorylation.

Strains lacking all three TPK genes are inviable [26] and strains lacking either TPK1 or TPK2 do not have altered levels of Cdc34-S97 phosphorylation (Fig. 6). Therefore, we utilized a strain lacking both TPK2 and TPK3 genes and harboring a weak TPK1 allele, termed tpk1"", whose protein product shows reduced cAMP-dependent protein kinase activity [27]. We find that Cdc34-S97 phosphorylation is reduced in this strain lacking TPK2 and TPK3 and harboring the tpk1"" allele (Fig. 7, lane 4). In all, these experiments demonstrate that during exponential growth in glucose the level of Cdc34-S97 phosphorylation can be controlled by protein kinase A.

![Figure 6](image6.png)

**Figure 6. Loss of Vps34, Vps15, Sch9 and Torc1 activity reduce Cdc34 S97 phosphorylation.** Total soluble protein from strains lacking the indicated kinase or from a strain treated with 200 nM rapamycin for 1 hour was analyzed by western blot using the α-pS97 phospho-specific and α-Cdc34 antibodies.

![Figure 7](image7.png)

**Figure 7. The cAMP/Protein Kinase A pathway regulates Cdc34 S97 phosphorylation.** A) Total soluble protein from the strains KT945 (WT, lanes 1 and 3), RS13-58A-1 (tpk1"", lane 2) and KT1126 (bcy1-14, lane 4) was analyzed by western blot using α-pS97 phospho-specific and α-Cdc34 antibodies.
implicated in Cdc34-S97 phosphorylation directly phosphorylate Cdc34 on S97. Protein Kinase A has a strong preference for arginine residues at positions −2 and −3 and a preference for an amino acid residue with a hydrophobic side chain at the +1 position relative to the phosphorylation site [28,29,30]. A second consensus sequence for PKA has been identified as R−x−x−x−x−(S/T)/0−B−1 with x referring to any amino acid residue and B referring to an amino acid residue with a hydrophobic side chain [31]. While Cdc34-S97 is not part of a PKA consensus sequence, Cdc34 has arginine residues at positions −4 and −7 and an isoleucine residue at the +1 position relative to S97. Therefore we tested whether PKA is able to phosphorylate Cdc34-S97 in vitro. Indeed, Cdc34 can be phosphorylated by bovine PKA while the Cdc34S97A mutant is phosphorylated to a lesser degree (Fig. 8A). Cdc34 phosphorylation is completely abolished by addition of Protein Kinase A Inhibitor to the reaction (Fig. 8A). To confirm these findings, we probed the in vitro phosphorylation reaction products with the α-pS97 antibody which shows that both bovine PKA and GSTTpk3, purified from yeast, are able to phosphorylate Cdc34-S97 (Fig. 8B). Furthermore, GSTSch9 purified from yeast is also able to phosphorylate Cdc34 on S97 in vitro in similar assays (Fig. 8B). The Sch9/Akt family members also modify an arginine-directed consensus sequence (for review, see [32]). Thus we conclude that Protein Kinase A and Sch9, both AGC family protein kinases, directly phosphorylate Cdc34-S97 in vitro and support our findings that alterations of these protein kinases dramatically affect S97 phosphorylation in vivo.

The S73/S97/Loop Motif is Critical for Chronological Lifespan and rapamycin resistance

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 CLN3 or loss of FAR1 prevents mating pheromone induced cell cycle arrest [33,34]. And, as mentioned above, ectopic CLN3 expression or loss of SIC1 compromises G1 arrest and ultimately the capability of a yeast cell to withstand prolonged periods of nutrient depletion [35,36]. Furthermore, over-expression of CLN3 or loss of SIC1 makes yeast sensitive to otherwise tolerable levels of the TORC1 inhibitor rapamycin, which mimics nutrient deprivation [36]. Cdc34 consists increases the rate of Sic1 degradation while decreasing the rate of Cln1 degradation [15]. Therefore, we hypothesized that the highly conserved S73/S97/loop motif of Cdc34 contributes to the ability of cells to survive low dose rapamycin treatment or nutrient depletion. Indeed, we find that a normally permissive level of rapamycin inhibits growth of CDC34tm cells (Fig. 9A) likely due to improper regulation of Sic1, Cln1 and Cln2.

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 degree 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
It has been postulated that this state resembles in some respects the limit under these conditions but the cells are not starving and significantly slow their metabolic activity. Nutrients become synthetically defined media, yeast cells stop dividing and depleted [39]. As Figure 9 shows, have ceased dividing and some of the essential nutrients have been individual cells capable of forming a colony was determined over a grown to stationary phase in liquid culture and the number of assessed in chronological lifespan, is another method by which aging is extended, compromised for survival (Fig. 9B, day 0) but as the quiescent state is their survival rate is more than fivefold lower than the WT strain tm yeast are significantly less robust; and by day 10 as nutrient sensing is an integral function of the G1 phase, it is not surprising that loss of nutrient sensing kinases can result in an arrest in the G1 phase. For example, strains lacking all the PKA isoforms or TOR arrest in G1. Consistent with their established roles in nutrient sensing during the G1 phase, there are inter-relationships among the kinases that control Cdc34-S97 phosphorylation. The primary example is phosphorylation of Sch9 by both Snf1 and the TORC1 complex [30,51]. Phosphorylation of Sch9 on its COOH-terminus by TORC1 increases Sch9 activity against the ribosomal S6 subunit, suggesting that Sch9 functions as the S6 kinase in yeast [50]. Furthermore, the Vps15/Vps34 heterodimer activates TORC1 by an unknown mechanism [47]. 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. Snf1 also directly phosphorylates and likely activates Sch9. This phosphor-
ylation occurs in older cells of a population and appears to promote yeast cell senescence [31].

Protein Kinase A is inactive when bound to the cAMP responsive protein, Bcy1. The signaling molecule 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, its GSK-3 orthologs and Mkk2 has only been observed under conditions of heat stress (growth at 37°C) [52]. One possibility is that overexpression of MRK1 or MKK2 may cause re-localization of Bcy1 and result in increased Tpk1–3 activity towards Cdc34-S97. Mec1 activates PKA under conditions of DNA damage [53] which offers a possible explanation for the increase in Cdc34-S97 phosphorylation in a strain overexpressing MECL. Since MECL is an essential gene, it is difficult to assess whether Mec1 contributes to PKA activation under steady state growth conditions.

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 dependent protein kinases, the cGMP dependent protein kinases and Protein Kinase C. Sch9 and PKA may function in redundant pathways as suggested by the findings that SCH9 acts as a multi-copy suppressor of PKA signaling defects and activation of PKA eliminates the slow growth defects of sch9Δ cells [54]. Furthermore, Sch9 and PKA function redundantly to inhibit autophagy in rich media [55]. 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 [28,29,30]. A second consensus site for PKA has been identified as R−6-X-X-R−3-X-X-(S/T0)-B−5 with X referring to any amino acid residue and B referring to an amino acid residue with a hydrophobic side chain [31]. We find that Cdc34-S97 is a substrate for PKA and, although atypical, Cdc34-S97 is part of a reasonable AGC-type sequence with arginine residues at positions −4 and −7 and isoleucine at +1 relative to S97.

Although we have not yet tested the impact of S97 phosphorylation on Cdc34 ubiquitin conjugating activity, genetic and molecular results suggest that Cdc34-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 interactions between the cAMP/Ras/PKA pathway and Cdc34 suggesting that PKA and Sch9 function redundantly to inhibit autophagy in rich media [55]. 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 [28,29,30]. A second consensus site for PKA has been identified as R−6-X-X-R−3-X-X-(S/T0)-B−5 with X referring to any amino acid residue and B referring to an amino acid residue with a hydrophobic side chain [31]. We find that Cdc34-S97 is a substrate for PKA and, although atypical, Cdc34-S97 is part of a reasonable AGC-type sequence with arginine residues at positions −4 and −7 and isoleucine at +1 relative to S97.

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The process of aging is extremely complex, influenced by diet and lifestyle. However, single gene mutations have been found in all model organisms including yeast, fruit flies, worms and mice which dramatically extend lifespan (for review see [39]). Evidence for a conserved mechanism of aging can be derived from the fact that mutations in genes including Sch9/AKT affect the lifespan of all model organisms mentioned above. Sch9 is 49% identical to 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% [57,58,59,60]. Furthermore, Prop-1 (−/−) and Pit-1 (−/−) dwarf mice have decreased levels of IGF-1 and live ~65% longer than wild type mice [61,62,63]. The aging phenotype of Cdc34Δ cells is consistent with Cdc34Δ cells becoming at least partially independent of PKA or Sch9 activity and no longer responding properly to growth arrest signals normally associated with the inactivation of these protein kinases, much like hyperactivation of the PKA pathway by loss of IRA1 or hyperactivation of Ras [64].

Materials and Methods

Site directed mutagenesis

All site directed mutagenesis was carried out using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, California, USA) and associated protocol. Complementary forward and reverse primers, approximately 25 nucleotides in length, encoding the desired mutation(s) were used to amplify the appropriate plasmid. Following PCR amplification, 1 μl of 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 Ultra-competent cells were transformed with two μl of Dpn I-treated DNA. Transformants were selected on LB+Ampicillin plates. Multiple clones from each transformation were verified by sequencing. The plasmids used in this work can be found in Table 1.

Yeast growth media and genetic techniques

Standard rich (YPD) and defined minimal (SD) media were prepared as described previously. To transform yeast with plasmid DNA, a quick 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 microfuge tube along with a small swap 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.
For spot dilution assays, cells were grown overnight in the indicated media, diluted to equal densities and then 3 μl of each culture and tenfold serial dilutions were spotted onto the indicated plates.

**Cdc34 expression and purification using bacteria**

Cdc34ΔC<sub>6XHis</sub> and Cdc34ΔC(S97A)<sub>6XHis</sub> proteins, encoding the first 244 amino acids of Cdc34 fused to a 6XHis tag at their COOH-terminus, were expressed and purified for these studies. BL21(DE3) bacterial cells (Invitrogen, California USA) harboring plasmid AD002 (Cdc34ΔC<sub>6XHis</sub>) or pRC004 (Cdc34ΔC(S97A)<sub>6XHis</sub>) 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<sub>600</sub> reached 1.2 at which time IPTG was added to a final concentration of 500 mM. The cells were allowed to grow for an additional 3 hours and were then collected by centrifugation. The bacterial pellet was resuspended in 5 ml of Lysis Buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, 10 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 10 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.

| Table 1. Plasmids used in this study. |
|--------------------------------------|
| **Plasmid Name** | **Vector** | **Yeast Marker** | **Bacterial Marker** | **Insert Gene** | **Source** |
| pYL150 | pSJ101 | LEU2 | ampicillin | CDC34 | [13] |
| pYL029 | pSJ101 | LEU2 | ampicillin | CDC34/S73K/S97D | [13] |
| pSJ101 | pSJ101 | LEU2 | ampicillin | CDC34/S97D/Δ103–114 | Yun Liu |
| pYL027 | pSJ101 | LEU2 | ampicillin | CDC34insert delta | Yun Liu |
| pRC001 | pSJ101 | LEU2 | ampicillin | CDC34/S97D/delta | this study |
| pAG25 | pFA6 | natMX4 | ampicillin | Nourseothricin | EUROSCARF |
| AD002 | pET21 | none | ampicillin | CDC34deltaC(1–244)-6XHis | D. Skowyra |
| pRC004 | pET21 | none | ampicillin | CDC34deltaC(1–244) (S97A)-6XHis | this study |

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supernatant 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 NaH₂PO₄, 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 NaH₂PO₄, 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 1 (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 4 mM MgCl₂, 1 mM DTT, 55 mM NaCl and 20% glycerol) and for an additional hour in Dialysis Buffer 2 (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 4 mM MgCl₂, 1 mM DTT, 55 mM NaCl and 50% glycerol).

**GST Kinase over-expression and purification using yeast**

An array of strain EJ758 [MATa his3-200, leu2-3,112, ura3-52, pep4::URA3], harboring a unique plasmid expressing a single GST-kinase fusion gene under control of the P_CDC7 promoter [20] was used for determining Cdc34-S97 phosphorylation levels after single kinase over-expression (Table 2).

For analysis of Cdc34-S97 phosphorylation in vivo, each strain was grown overnight in 5 ml SD-Ura then diluted to 4 x 10⁶ 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 500 μM to induce GST-Kinase expression. Induction lasted for 3 hours at which time the cells were pelleted in a Beckman Centrifuge at 4000 x g, 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.

To express and purify the GST Kinase from yeast, a patch of cells was inoculated into 50 ml SD-Ura to an OD₆₀₀ ~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 OD₆₀₀ of 0.8 the following morning. The following morning, copper sulfate was added to a final concentration of 500 μM 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 harvested by centrifugation in a Beckman Centrifuge at 4000 x g. One tube was used for protein purification and the other was stored at −70°C for future purification. The cell pellet was resuspended in 1 ml EXTRAC- TION buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 4 mM MgCl₂, 5 mM DTT, 10% (v/v) glycerol, 1 mM NaCl) with one protease inhibitor cocktail tablet (Roche Diagnostics, Germany) added per 10 ml buffer. Suspension was transferred to a microtube and glass beads were added to the top of the tube. Cells were lysed at 4°C for 200 sec 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 flamed 25G needle and the extract 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 1.0 M PMSE was added to two μl of protein solution and insoluble cellular debris was removed by gentle centrifugation. The supernatant was transferred to a fresh microtube and protein concentration was determined by the Bradford method [65].

The crude protein extract was diluted with an equal volume of No Salt Wash Buffer (50 mM Tris-HCl, 4 mM MgCl₂, 1 mM DTT, 10% Glycerol) to bring the final salt concentration to 0.5 M NaCl. This salt concentration was chosen because it prevents most nonspecific protein-ligand or protein-protein interactions, while still preserving required functional interactions. 100 μl of pre-equilibrated glutathione-Sepharose resin were added per 2 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 sec at low speed in a microtube and the supernatant was decanted. The beads were washed twice with 1 ml of WASH BUFFER by mixing for 15 minutes, centrifuged and wash buffer was decanted. Bound proteins were eluted by adding 2 ml ELUTION BUFFER to the resin, followed by 1 hour of mixing, and then low speed centrifugation. The elution was dialyzed overnight against

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**Table 2. Strains used in this study.**

| Strain | Genotype | Reference |
|--------|----------|-----------|
| S149   | MATa vps34-Kan₆ his3-1 leu2-3,10 met15-10 ura3-10 | [67] |
| 3236   | MATa vps15-Kan₆ his3-1 leu2-3 met15-10 ura3-10 | [67] |
| 3642   | MATa gcn2-Kan₆ his3-1 leu2-3 met15-10 ura3-10 | [67] |
| BL2    | MATs prototroph | This study |
| EJ758(YIL035c) | MATa his3-200 leu2-3,113 ura3-52 pep4::URA3, pYEX4T-rec::YIL035c | [20] |
| KS418 | MATa, CDC34⁴⁴ ura3 leu2 trp1 lys2 ade2 ade3 | This study |
| KS422 | MATa ura3 leu2 trp1 lys2 ade2 ade3 | [68] |
| KT945 | MATs his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1 | [27] |
| RS13-58A-1 | MATa his3 leu2 ura3 trp1 ade8 bcy1::LEU2 tpk1::TRP1 tpk2::HIS3 tpk3::TRP1 | [27] |
| KT1112 | MATa leu2 ura3-52 his3 | [69] |
| KT1126 | MATa leu2 ura3-52 bcy1-14 | [69] |
| RRC85 | MATs CDC34⁴⁴ (NATI) | [15] |
| VL10-1 | MATa cdc34-2 leu2-11 ura3-52 trp1-163 his3-1 Gal⁺ | [13] |
| VL18  | MATa cdc34::HIS3 ura3-52 leu2-11 trp1-163 his3-1200 (pYL250) | [13] |

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DIALYSIS BUFFER I (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 4 mM MgCl₂, 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 MgCl₂, 1 mM dithiothreitol, 35 mM NaCl, 50% (v/v) glycerol) and stored at −20°C. Normal Kninase yield from a 250-ml culture is 0.5 ml of protein at ~250 µg/ml.

Antigen production and rabbit immunization

Antibodies were generated that recognize Cdc34 when serine 97 is phosphorylated (Open Biosystems, Alabama USA). The phosphopeptide DGRILC6XHis(pS)ILHQ was synthesized and conjugated to Keyhole Limpet Hemocyanin (KLH). On day one, 500 µg 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 250 µg of KLH-phosphopeptide conjugate emulsified with Freund’s incomplete adjuvant. On days 35 and 56, approximately 25 ml of serum was collected each from rabbit. Production bleed of 50 ml were taken from each rabbit on day 70 at which time the animals were euthanized.

α-pS97 Antibody Purification

To ensure that the antibody specifically recognized Cdc34 phosphorylated on serine 97 is phosphorylated (Open Biosystems, Alabama USA). The phosphopeptide DGRILC6XHis(pS)ILHQ was electrophoresed on a 10% SDS-PAGE gel. The protein was transferred to PVDF membrane for 2.5 hours at 4°C in Transfer Buffer (25 mM Tris, 190 mM Glycine and 15% methanol). The membrane was incubated overnight at 4°C with 5% milk in KPBS-T for 1 hour at room temperature. After blocking, the membrane was exposed to 2 ml of crude anti-pS97 antisera which had been diluted with 15 ml of sterile 1× 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 [66]. 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 A280 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.

Yeast protein extraction methods

Once grown to the desired density, cells were collected by centrifugation for 5 minutes at 4000 x g in a Beckman centrifuge at 4°C. The cell pellet was immediately frozen in 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 pH 7.5, 5 mM EDTA, 1% Triton X-100, 50 mM NaF and one Complete Mini protease inhibitor cocktail tablet (Roche Diagnostics, Germany) per 10 ml of breaking buffer). 500 µm 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 ~5 µg/ml was achieved. Typically, this required three rounds of bead beating at one minute per round. Protein concentration was determined by the Bradford method [63]. The Horvath-Riezman protocol [19] is an alternate method for protein extraction from yeast cells. This protocol was used infrequently here but when used it has been denoted. Briefly, 3.75 µl of Extraction Buffer (60 mM 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 spun at 14,000 rpm for 5 minutes at 4°C and the supernatant was saved as protein extract.

SDS-PAGE and western blot analysis

5× Laemmli Buffer (20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue and 62.5 mM Tris, pH 6.8) was added to the protein extract (~50 µg of protein) made via 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 Na2HPO4, 1.5 mM KH2PO4, 0.2% Tween-20, pH 7.2) and blocked with 5% milk in KPBS-T. Antibodies were used at the following dilutions, affinity purified α-Cdc34 (1:10,000) 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).

Detection of Cdc34 phosphorylation using 32P

Bacterially expressed Cdc34C6XHis and Cdc34/S97A/C6XHis (1.3 pmoles) were incubated with 10 µl (1 µl/µl) of bovine Protein Kinase A (Sigma Aldrich, USA) in PKA phosphorylation buffer (35 mM Potassium Phosphate, pH 7.4, 0.25 mg/ml BSA) plus 10 µl of 5× ATP solution (1 mM ATP, 25 mM MgCl₂, 310 cpm/pmole ATP) in a total reaction volume of 100 µl. When PKA Inhibitor (Sigma-Aldrich, USA) was used, 1 µl of 1 mg/ml solution was added to the reaction. The reactions were incubated at 30°C for 20 minutes and stopped by addition of 25 µl of 5× 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. To quantitate the relative amounts of 32P incorporation, bands were cut from the dried gel and analyzed by a liquid scintillation counter.

Detecting Cdc34 phosphorylation using α-pS97 antibody

Bacterially expressed Cdc34C6XHis (165 picomoles) was incubated with 10 µl (1 unit/µl) of bovine Protein Kinase A (Sigma Aldrich, USA) or 5 µl of GSTTpk3 or GST-Sch9 purified from yeast in PKA phosphorylation buffer (35 mM Potassium Phosphate, pH 7.4, 0.25 mg/ml BSA) plus 10 µl of 5× ATP solution (1 mM ATP, 25 mM MgCl₂) in a total reaction volume of 30 µl. When PKA Inhibitor (Sigma-Aldrich, USA) was used, 1 µl (1 mg/ml) solution was added to the reaction. The reactions were incubated at 30°C for 10 minutes and the samples were placed on ice. 10 µl of each was spotted onto a piece 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.

Author Contributions

Conceived and designed the experiments: RC MG. Performed the experiments: RC. Analyzed the data: RC MG. Contributed reagents/materials/analysis tools: RC MG. Wrote the paper: RC MG.
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