A Conserved Pathway that Controls c-Myc Protein Stability Through Opposing Phosphorylation Events Occurs in Yeast

Julienne R. Escamilla-Powers1 and Rosalie C. Sears*1

1Department of Molecular and Medical Genetics, Oregon Health & Sciences University, Portland, OR. 97239 USA

Running Title: A pathway that controls c-Myc protein degradation in yeast

Address correspondence to: Rosalie C. Sears, Department of Molecular and Medical Genetics, Oregon Health & Sciences University, 3181 SW Sam Jackson Park Rd., L103A Portland, OR. 97239, Tel: (503) 494-6885; Fax: (503) 494-4411; Email: searsr@ohsu.edu

The c-Myc transcription factor is a key regulator of cell proliferation and cell fate decisions. c-Myc overexpression is observed in a variety of human tumors, revealing the importance of maintaining normal levels of c-Myc protein. c-Myc protein stability in mammalian cells is controlled by interdependent and sequential phosphorylation and dephosphorylation events on two highly conserved residues, Serine 62 and Threonine 58. Here we show that these sequential phosphorylation and dephosphorylation events, and their effect on c-Myc stability also occurs in the model system Saccharomyces cerevisiae. These results suggest the presence of a conserved pathway in yeast that controls protein turnover in response to a specific phospho-degron sequence. These findings have implications regarding conserved pathways for regulated protein degradation and they validate the use of genetically tractable yeast for the study of the turnover of proteins, such as c-Myc, that contain this motif.

The c-myc proto-oncogene encodes a helix-loop helix transcription factor that is involved in a number of crucial cellular processes, including cell proliferation, cell growth, differentiation and apoptosis. c-Myc heterodimerizes with its partner protein, Max, and together they regulate transcription at E box sequences (CAC A/GTG) of a variety of important genes. Recently, it has been reported that c-Myc also regulates transcription of PolII and PolIIIII-dependent genes (1,2). Given that c-Myc is involved in many vital cellular activities, it is not surprising that it is highly regulated at several levels, including transcription, mRNA stability, translation and protein stability (3-6). A number of animal models have shown that misregulation of c-Myc can result in tumorigenesis (7). Indeed, overexpression of c-Myc is observed in over 70% of human cancers. This can involve amplification or translocation of the c-myc gene (8). However, these genetic changes are observed only in a minority of the cases, suggesting that other mechanisms, such as a change in c-Myc protein stability, may play a role in tumorigenesis.

We and others have previously reported that the stability of c-Myc protein in mammalian cells is controlled by sequential phosphorylation and dephosphorylation events on two highly conserved residues, Threonine 58 and Serine 62 (9-11). Phosphorylation at these sites has opposing effects on c-Myc protein stability. An initial event, phosphorylation at residue Serine 62 by Ras-activated ERKs, stabilizes c-Myc, while a subsequent phosphorylation at residue Threonine 58 by the Glycogen Synthase Kinase 3β (GSK3β), destabilizes c-Myc. Prior to degradation, a cis to trans isomerization at the bond proceeding Serine 62 is catalyzed by the peptidyl prolyl isomerase, Pin1, allowing the stabilizing Serine 62 phosphate to be removed by the trans-specific phosphatase, PP2A. Singly Threonine 58 phosphorylated c-Myc can then be targeted for multi-ubiquitination by the E3 ligase SCF Fbw7 and degraded by the 26S proteasome (12,13).

Saccharomyces cerevisiae is frequently used as a model system to study mammalian proteins because of the ease of genetic manipulation, rapid doubling time, and the presence of conserved orthologs between a number of yeast and mammalian proteins, including a number of proteins required for proteasome-mediated degradation. For these reasons, it is an excellent model system to study interactions, function and turnover of mammalian proteins. Indeed, a number of groups have used the budding yeast, S. cerevisiae, to study the ubiquitination and subsequent destruction of...
important mammalian cell cycle proteins. (14-17). Likewise, c-Myc half-life has been studied in yeast cells and found to be very short (14,18). Additionally, it has been reported that c-Myc protein can be stabilized in this system by mutating core components of the yeast SCF E3 ubiquitin ligase complex. Specifically, c-Myc half-life has been shown to be increased in yeast strains containing mutations in Cdc53, Skp1 or the F-box protein, Grr1 (18). Interestingly, many of the mammalian proteins that regulate c-Myc phosphorylation and protein stability are conserved in yeast.

In this study we show that yeast orthologs of key proteins in the signaling pathways that control c-Myc phosphorylation are functionally conserved. These proteins are involved in the same sequential and interdependent phosphorylation and dephosphorylation events at Serine 62 and Threonine 58 in c-Myc that occur in mammalian cells. Additionally, these phosphorylation events have the same consequence on c-Myc protein stability. This is an important finding because it supports discoveries made in mammalian cells, it emphasizes the importance of this signaling pathway in regulating protein turnover through a conserved phospho-degron motif, it validates the use of the yeast S. cerevisiae as a model system to study c-Myc turnover, and it implicates this pathway in the degradation of yeast proteins and other mammalian proteins.

MATERIALS AND METHODS

Yeast media and reagents - All strains used were haploid and isogenic with BY4741 (Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) or W303 (Mata ade2-1 his3-1,15 ura3-1 leu2-3,112 trp1-1 can1-100). Cells were grown in selective media or YAPD (1% yeast extract, 2% peptone, adenine, 2% glucose) at 23, 30 or 37°C as indicated. Yeast strains were transformed with c-Myc expression constructs by lithium acetate-mediated transformation (19). To arrest cells in G1 or M phases, cells were treated with α factor (2ug/mL) or nocodazole (15ug/mL), respectively.

Strains and plasmids - Strains used in this study are BY4741, [Δrim11], [Δpph21], [Δts1], TAP-Rim11, TAP-Kss1 (Open Biosystems), W303, cdc4-1, grr1 (generous gift of S. Lanker) ess1H164R, and ess1A144T (generous gift of S. Hanes). The 2u plasmids pYESDEST52-MycWT, pYESDEST52-MycT58A, and pYESDEST52-MycS62A, containing V5-6xhis epitope-tagged c-Myc or c-Myc mutants, under the control of the GAL1 promoter were generated using TOPO cloning (Invitrogen).

Determination of Protein Stability - Cells were grown in a 2% raffinose synthetic complete medium overnight. Cells were then diluted to an optical density at 600 nm (OD600) of 0.3 and grown for an additional 2-4 hours at the temperature indicated. A sample was removed as a negative control prior to addition of galactose. Galactose was added to the media to a final concentration of 2% to induce expression of the c-myc gene from the GAL1 promoter for 1-3 hours at the indicated temperature. Glucose was added to a final concentration of 5% to stop gene expression, and samples were taken at the indicated timepoints. Protein extraction and western blotting were performed as described below. The optical density at 600 nm (OD600) of all samples was measured and the volume of each sample was adjusted to ensure that equal cell numbers were used.

Antibodies - The monoclonal V5 antibody used to detect total c-Myc protein was from Invitrogen. The c-Myc Serine 62 phospo-specific antisera were raised against the chemically synthesized phosphopeptide CKFELLPA/TPPLpSPRSSG in rabbits. The antisera were purified against this phosphopeptide conjugated to Sulfolink Coupling Gel (Pierce Biotechnology Inc.) as described (20). To deplete antibodies that recognized unphosphorylated c-Myc, the affinity-purified antibodies were re-purified by passing through Sulfolink Coupling Gel conjugated with the corresponding unphosphorylated peptide. The c-Myc Threonine 58 phospo-specific antibody was purchased from Cell Signaling Technology (Beverly, MA). The polyclonal Cdc28 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Western Blotting and quantitation - Yeast extracts were prepared by using the rapid protein extraction procedure (21). Protein from equal yeast cell numbers were separated by SDS-PAGE gel and transferred to Immobilon-FL membrane (Millipore, Billerica, MA). Membranes were blocked with Odyssey Blocking Buffer (LI-COR
Biosciences, Lincoln, Nebraska) or 5% non-fat milk in PBS (P-T58 antibody). Primary antibodies were diluted in 1:1 Odyssey Blocking Buffer:PBS with 0.05% Tween20 or in 2.5% non-fat milk PBS, 0.05% Tween (P-T58 antibody). Primary antibodies were detected with secondary antibodies labeled with the near-infrared fluorescent dyes IRDye800 (Rockland, Philadelphia, Pennsylvania) and Alexa Fluor 680 (Molecular Probes, Eugene, Oregon) to allow two-color imaging and band overlay. Secondary antibodies were diluted 1:10,000 in 1:1 Odyssey Blocking buffer: PBS, 0.05% Tween or in 1.25% nonfat milk PBS, 0.05% Tween (P-T58 antibody). Blots were scanned with a LI-COR Odyssey Infrared Imager (Lincoln, Nebraska) to visualize proteins. c-Myc and Cdc28 protein levels were quantitated using LI-COR Odyssey Infrared Imager software version 1.2. For degradation assays c-Myc total protein levels were normalized to total protein as measured by Cdc28 protein levels. Normalized c-Myc protein levels at each time point were calculated as a percentage of the first timepoint and graphed on a semi-log graph. Half-lives were calculated based on best-fit lines drawn using Microsoft Excel. Mean half-lives ± standard deviation were calculated based on three or more independent experiments.

In vitro kinase assay – c-Myc expression was induced in the Δkss1 or the Δrim11 strains as described above. Cells were lysed in Ni-NTA lysis buffer (5mM imidazole, 5mM IME and 0.5% NP-40) and c-Myc was extracted using Ni-NTA agarose (QIAGEN, Valencia, CA). c-Myc was eluted from agarose in Ni-NTA elution buffer (1:1 Ni-NTA lysis buffer:1M imidazole). The TAP-Kss1 and TAP-Rim11 strains were grown overnight in YAPD to an optical density at 600nm of 0.5. TAP-tagged proteins were extracted using Calmodulin Affinity Resin (Stragagene, La Jolla, CA.) in IPP150 Calmodulin binding buffer (22). Beads were washed three times in Calmodulin binding buffer and one time in kinase reaction buffer (50mM Tris-HCL pH 7.5, 0.1mM EDTA, 15mM DTT). The immobilized kinases were incubated with eluted c-Myc, kinase buffer and 12uCi [γ-32P] ATP (Perkin-Elmer, Boston, MA) in a final volume of 60 uL. Reactions were incubated at 30°C for 30 minutes. Unbound proteins were mixed with an equal volume of 2x SDS-PAGE sample buffer. Proteins were analyzed by SDS-PAGE and autoradiography.

RESULTS

Interdependent phosphorylation of c-Myc at Threonine 58 and Serine 62 occurs in yeast

We and others have previously used phospho-peptide mapping and phospho-specific antibodies to demonstrate an inter-relationship between phosphorylation at Serine 62 (S62) and Threonine 58 (T58) (9,10). Specifically, S62 phosphorylation is required prior to T58 phosphorylation, and T58 phosphorylation facilitates subsequent S62 dephosphorylation (9,11). In order to perform a yeast two-hybrid assay to identify new proteins that interact with phospho-T58 and phospho-S62, we first investigated whether mammalian c-Myc is phosphorylated at either of these sites in yeast. We expressed c-MycWT or one of two c-Myc phosphorylation mutants, c-Myc T58A or c-Myc S62A, in a wildtype yeast strain using the GAL1 promoter. This promoter is induced by addition of galactose to the yeast media. Following a one-hour induction of c-MycWT or the c-Myc mutants from the GAL1 promoter, total cell lysates from yeast expressing c-Myc WT, c-MycT58A or c-Myc S62A were visualized by western blot using antibodies specific for phosphorylated Threonine 58 or phosphorylated Serine 62. Using LI-COR technology we can double label for total c-Myc and phospho-c-Myc. This technology allows us to accurately quantitate the ratio of phospho-S62 or phospho-T58 c-Myc to total c-Myc (see methods). As shown in Figure 1A, c-MycWT is both T58 and S62 phosphorylated, with a weaker signal detected by the anti-phospho-S62 antibody (lane 1). The relative difference in T58 and S62 phosphorylation detected with the phospho-specific antibodies, is consistent with phosphopeptide mapping results showing a reduced amount of S62 phosphorylation and increased T58 phosphorylation under conditions...
where c-Myc is unstable (9). In contrast, c-Myc$^{T58A}$, which lacks phosphorylation at T58, showed a large increase, over 10-fold, in S62 phosphorylation levels compared to c-Myc$^{WT}$ (Fig. 1A, lane 2). This increase in S62 phosphorylation with the c-Myc$^{T58A}$ mutant is also observed in mammalian cells, both by phospho-peptide mapping and phospho-specific antibodies, and is due to T58 phosphorylation-dependent S62 dephosphorylation (9-11). Lastly, c-Myc$^{S62A}$ does not appear to be phosphorylated on either site in yeast cells (Fig. 1A, lane 3). This is again consistent with observations in mammalian cells in which c-Myc$^{S62A}$ lacks phosphorylation at T58, forming the basis for the reported dependent relationship between S62 and T58 (9,10,24).

These results are striking because they demonstrate the conservation of a pathway controlling two interdependent relationships between the S62 and T58 phosphorylation sites: 1) the hierarchical phosphorylation of S62 followed by T58, and 2) the role of T58 phosphorylation in promoting S62 dephosphorylation.

Given that the interdependent phosphorylation of c-Myc at S62 and T58 occurs in yeast, we next asked if phosphorylation at these sites also controls c-Myc protein stability in yeast as it does in mammalian cells. In order to study the stability of c-Myc protein in yeast, we used a galactose induction/glucose shut off system. Specifically, addition of galactose to yeast cells activates expression of genes under control of the GAL1 promoter while addition of glucose rapidly shuts down the promoter. Galactose was added to yeast cells carrying a plasmid with either c-myc$^{WT}$ or the phosphorylation mutants under the control of this promoter to induce expression. After one hour, expression was terminated by addition of glucose and samples were taken at various time points for western blot analysis. c-Myc protein levels were quantified and normalized to the control Cdc28 protein and c-Myc half-life was calculated. As shown in Figure 1B, c-Myc$^{WT}$ exhibited a short half-life in a wildtype yeast strain of 8.3 minutes, with a mean half-life of 9.5 ± 1.6 minutes based on multiple independent experiments. This short half-life has previously been reported for c-Myc in both yeast cells and mammalian cells (14,15,18,25), and it is consistent with the levels of detected T58 and S62 phosphorylation seen in Figure 1A. In contrast, c-Myc$^{T58A}$ showed a marked increase in half-life to 34.1 minutes, with a mean half-life of 33.9 ± 1.7 minutes. This approximate four-fold increase over wildtype c-Myc is consistent with results in mammalian cells where c-Myc$^{T58A}$ is 4-6 times more stable compared to c-Myc$^{WT}$ (9,14). The c-Myc$^{S62A}$ mutant demonstrated an intermediate mean half-life of 16.1 ± 1.5 minutes. The half-life of c-Myc$^{S62A}$ in mammalian cells is either reported to be short like c-Myc$^{WT}$, or somewhat longer, but less than c-Myc$^{T58A}$, consistent with our observations in yeast (9,13). Taken together our results not only show that the interdependent phosphorylation of T58 and S62 occurs in yeast, but also that phosphorylation of c-Myc at these sites appears to control c-Myc protein stability in yeast cells. Since there are no known functional yeast homologues to mammalian c-Myc this suggests the presence of a conserved pathway that controls protein degradation through a conserved phospho-degron.

Proteins controlling c-Myc S62 and T58 phosphorylation and c-Myc stability in mammalian cells are conserved in yeast

The previous results suggest that functional homologues to mammalian proteins that target c-Myc for degradation are conserved in yeast. Indeed, many of the key proteins that have been implicated in controlling c-Myc T58 and S62 phosphorylation and regulating c-Myc protein stability in mammalian cells have S. cerevisiae orthologs (Table 1). One of the advantages to working in yeast is its easy genetic manipulation. Thus, strains that are mutant in each of these proteins are available. We made use of these strains to ask whether deletion or mutation of yeast orthologs of proteins which destabilize c-Myc in mammalian cells affect c-Myc phosphorylation in yeast, and whether this correlates with changes in protein stability.

A role for yeast kinases Kss1p and Rim11p in controlling c-Myc phosphorylation and c-Myc stability

Kss1p is a yeast mitogen-activated protein kinase (MAPK) that has high sequence homology to the mammalian ERK kinase, and Rim11p is the yeast serine/threonine kinase that shares a conserved function to the GSK3 kinase (26,27).
In mammalian cells, ERK can phosphorylate c-Myc on S62, stabilizing the protein, while GSK3 is responsible for phosphorylating c-Myc on T58, thereby destabilizing c-Myc protein. Both KSS1 and RIM11 are non-essential yeast genes, presumably due to the presence of additional MAPK-like and GSK3-like kinases (28-31).

We asked whether the yeast kinase, Kss1p affects the phosphorylation of c-Myc at S62 in yeast. To examine this, we expressed V5-tagged c-Myc in a wildtype strain and a strain lacking the Kss1p gene (Δkss1) from the GAL1 promoter for one hour. Cells were lysed in SDS sample buffer and whole cell lysates were run on an SDS-PAGE gel. c-Myc protein was visualized by double labeling with one of the c-Myc phospho-antibodies, as well as an antibody to the V5 tag to measure total c-Myc. The intensity of each band was quantified and ratios of phosphorylated c-Myc to total c-Myc were calculated relative to c-Myc phosphorylation in the wildtype strain. As shown in Figure 2A, c-Myc expressed in the Δkss1 strain is decreased for phosphorylation at S62, compared to that of c-Myc expressed in a wildtype strain. This decrease in S62 phosphorylation is presumably due to the loss of Kss1p. Residual phosphorylation of S62 is likely due to other MAP-like kinases in the cell. This result suggests that Kss1p plays a role in phosphorylating c-Myc at S62 in yeast cells, as ERK does in mammalian cells.

We next examined whether the presence of Rim11p affects phosphorylation of T58 in yeast. To test this, V5-tagged c-Myc WT was again expressed from the GAL1 promoter for one hour in both a yeast strain lacking RIM11 (Δrim11) and an isogenic wildtype control strain. Whole cell lysates were prepared in SDS sample buffer and run on an SDS-PAGE gel. c-Myc protein was visualized by double labeling with one of the c-Myc phospho-specific antibodies and anti-V5 (Fig. 2B). We observed a substantial decrease in T58 phosphorylation and an increase in S62 phosphorylation in the Δrim11 strain compared to the wildtype control. This decrease in T58 phosphorylation is consistent with observations in mammalian cells where inhibition of GSK3 results in a decrease in phospho-T58 c-Myc (9,32). Likewise, the increase in S62 phosphorylation is consistent with the role of T58 phosphorylation in facilitating PP2A-mediated dephosphorylation of S62 seen in mammalian cells (11). Thus the inter-relationship between the two phosphorylation sites is again observed. There is some residual phosphorylation of T58 in the Δrim11 yeast strain. This is presumably due to the presence of three other GSK3-like kinases in the yeast (28-30). This result suggests that Rim11p plays a significant role in phosphorylating c-Myc at residue 58 in yeast just as GSK3 does in mammalian cells.

We next asked whether loss of Kss1p and/or Rim11p affects the half-life of c-Myc in yeast. As shown in Figure 1, mutation of the S62 residue of c-Myc results in a small increase in half-life compared to c-MycWT. Therefore, if the Kss1 kinase phosphorylates c-Myc at S62 we would expect a similar result from loss of Kss1p. To test this, c-Myc expression was induced in the Δkss1 strain for one hour and following termination of induction, cells were collected at the indicated timepoints. As shown in Figure 2C, the half-life of c-Myc in a wildtype strain was 11.7 minutes while the half-life in the Δkss1 strain was 12.2 minutes. Based on multiple experiments the mean half-life of c-Myc in the Δkss1 strain was 15.7 ± 3.1 minutes. This is similar to the c-MycS62A mutant mean half-life, which was measured to be 16.1 ± 1.5 minutes (see Fig 1B). The small increase in stability with loss of phosphorylation at S62 is also consistent with previously published results in mammalian cells and suggests that without phosphorylation at S62 or at T58 c-Myc is degraded by an alternate E3 ligase (13).

On the other hand, mutation of T58 to a non-phosphorylatable residue results in enhanced S62 phosphorylation and substantial stabilization of c-Myc (Fig. 1A and 1B). Thus, loss of Rim11p may result in a similar stabilization of c-Myc. To test this, c-Myc was expressed from a GAL1 promoter for one hour in the Δrim11 background. Following addition of glucose to stop induction of the promoter, samples were taken at various timepoints. c-Myc protein levels and half-lives are shown in Figure 2C. The half-life of c-Myc expressed in the Δrim11 background was 28.4 minutes. Based on multiple independent experiments the mean half-life of c-Myc in the Δrim11 strain is 28.1 ± 1.0 minutes, a threefold
increase when compared to mean c-Myc half-life in a wildtype strain. These results demonstrate that in *S. cerevisiae*, Rim11p participates in the phosphorylation of c-Myc at T58, and that this phosphorylation leads to c-Myc destabilization, similar to the activity of GSK3β on c-Myc in mammalian cells.

Since c-Myc is not a yeast protein, it is important to ask whether these yeast kinases are able to directly phosphorylate mammalian c-Myc protein. In order to answer this question we performed an in vitro kinase assay. Briefly, V5-6xhis-c-Myc expression was induced in either the kss1Δ strain or the rim11Δ strain and c-Myc protein was extracted using nickel agarose. We used c-Myc protein from these strains because (1) we have already shown them to have low PS62 or low PT58, respectively (see Figures 2A and 2B) and (2) in the case of Rim11-mediated phosphorylation, to ensure that c-Myc was properly primed with a phosphate at S62, since GSK3β kinases are reported to be processive kinases. c-Myc protein was eluted from the nickel agarose and incubated with either the Kss1 or Rim11 kinase in the presence of 32P-labeled ATP. As a negative control nickel agarose was incubated with non-induced yeast lysates, and those elutions were also incubated with either the Kss1 or Rim11 protein (Fig. 2D, lanes 1 and 3). The samples were analyzed by SDS-PAGE and autoradiography. As shown in Figure 2D, c-Myc extracted from the kss1Δ strain is phosphorylated in the presence of purified Kss1 (lane 2), while c-Myc extracted from the rim11Δ strain is phosphorylated in the presence of purified Rim11 (lane 4). As an additional control, c-Myc alone was incubated with 32P-labeled ATP to ensure that additional kinases were not being co-purified with Myc (lane 5). This data demonstrates that the Kss1 and Rim11 yeast kinases can directly phosphorylate mammalian c-Myc protein.

**c-Myc isomerization by Ess1 is not a significant limiting step in c-Myc degradation in yeast**

Ess1p is the yeast homologue of the peptidyl-prolyl isomerase, Pin1. In mammalian cells, Pin1 recognizes c-Myc phosphorylated at T58 and catalyzes a cis to trans isomerization of the bond preceding Proline 63 in c-Myc. This isomerization is thought to underlie the role of T58 phosphorylation in facilitating the dephosphorylation of S62 by the PP2A phosphatase, which dephosphorylates residues when the proceeding proline is in *trans*. In Pin1-null mouse embryo fibroblasts c-Myc S62 phosphorylation is increased and degradation is inhibited (11). *ESS1* is an essential yeast gene and cells mutated for this gene arrest in M phase of the cell cycle (33). It is also a highly conserved gene and mammalian Pin1 can substitute for Ess1 protein function in yeast (33).

We asked if the yeast peptidyl-prolyl isomerase, Ess1p, affects phosphorylation, and consequently protein stability of c-Myc in yeast. Since *ESS1* is an essential yeast gene, a temperature-sensitive yeast mutant strain with an H164R mutation in the active site of the enzyme was used (34). This strain arrests in M phase, however with slow kinetics. c-Myc expression was induced in the ess1H164R strain at the restrictive temperature of 37°C for three hours. Approximately 85% of the cells showed mutant phenotype microscopically (data not shown). Whole cell lysates were run on a gel and the western blot was duel labeled with antibodies specific for phospho-S62 or phospho-T58 and anti-V5 (Fig. 3A). Interestingly, c-Myc expressed in the ess1H164R strain showed only a small increase in T58 or S62 phosphorylation compared to the similarly treated wildtype strain. We also examined c-Myc half-life in the ess1H164R strain at the restrictive temperature, 37°C, and compared this to an isogenic wildtype control strain under the same temperature but with the addition of the microtubule inhibitor, nocodazole, to control for M phase arrest in the ess1H164R strain. c-Myc half-life overall did not appear to be significantly longer in the ess1H164R strain (13.8 minutes) compared to the wildtype strain arrested with nocodazole (12.5 minutes, Fig. 3B). Based on multiple experiments the mean half-life of c-Myc in a nocodazole-arrested wildtype strain was 13.0 ± 2.0 minutes while the mean overall half-life of c-Myc in the ess1H164R strain was 13.8 ± 1.3 minutes. Although the overall half-life for c-Myc did not appear to be significantly affected by loss of Ess1p, it is interesting to note that we consistently observed a biphasic decay, where there is an initial decrease in c-Myc levels, which then levels off between ten and twenty minutes in the ess1H164R strain.
strain (dashed lines). This suggests that loss of Ess1p does affect a subset of c-Myc protein, which could explain why we only saw a small increase in phosphorylation in c-Myc in the ess\textsuperscript{H164R} strain. It may be that a significant portion of c-Myc already exists in the \textit{trans} conformation in the yeast cells and therefore does not require Ess1p activity for dephosphorylation or degradation. However, after this portion is degraded a remaining subset of c-Myc protein exists in the \textit{cis} conformation, and it is this population that continues to be stable out to at least 60 minutes in the ess\textsuperscript{H164R} strain (data not shown).

To confirm the previous results and ensure that the biphasic nature of c-Myc decay was not specific to the ess\textsuperscript{H164R} yeast strain, we also tested c-Myc half-life in another \textit{ESS1} temperature-sensitive strain with an A144T mutation in the substrate-binding pocket (34). As shown in Figure 3C, c-Myc expressed in the ess\textsuperscript{A144T} strain has a half-life of 16.1 minutes with an overall mean half-life of 16.6 ± 1.8 minutes. Consistent with our observations in the ess\textsuperscript{H164R} strain, this is not a significant increase compared to c-Myc half-life in a wildtype strain under the same conditions (13.0 ± 2.0 minutes). However, again we observe a biphasic decay of c-Myc in this yeast strain (dashed lines), suggesting that this phenotype may be common to all \textit{ESS1} mutants and reflect a requirement for Ess1p to degrade a subset of c-Myc.

\section*{Yeast PP2A activity facilitates c-Myc S62 dephosphorylation and degradation}

Protein phosphatase 2A (PP2A) is a heterotrimeric enzyme that dephosphorylates Serine 62 of c-Myc thereby destabilizing the protein in mammalian cells (11,23). PP2A is a \textit{trans}-specific phosphatase, which explains the role of prior isomerization by Pin1 (35). The PP2A holoenzyme is composed of a catalytic (C) subunit, which is encoded by two genes in \textit{S. cerevisiae}, \textit{PPH21} and \textit{PPH22}, a scaffolding (A) subunit, encoded by the \textit{TPD3} gene in \textit{S. cerevisiae}, and a substrate-recognizing regulatory (B) subunit, of which there are two in yeast. \textit{RTS1} encodes the B’ family ortholog while \textit{CDC55} encodes the B family ortholog (36,37).

In mammalian cells, S62 phosphorylation is associated with c-Myc stabilization and removal of the S62 phosphate by PP2A occurs prior to polyubiquitination and degradation. Previous studies in mammalian cells have shown that inhibiting PP2A activity by addition of okadaic acid or SV40 small T antigen results in increased c-Myc half-life (11). To determine if yeast PP2A activity affects the phosphorylation of Serine 62, we used a mutant strain lacking one of the two main PP2A catalytic subunits in \textit{S. cerevisiae}, \textit{PPH21}. We asked whether loss of Pph21p ([\textit{pph21}]) changes the amount of c-Myc phosphorylation at Serine 62 compared to that of c-Myc in a wildtype yeast strain. As shown in Figure 4A, c-Myc in the mutant strain has increased Serine 62 phosphorylation compared to c-Myc expressed in the wildtype strain. This is presumably due to a reduction in the ability of the cell to dephosphorylate Serine 62. The increase in S62 phosphorylation in the \textit{pph21} strain is modest, likely due to redundancy by the other PP2A C subunit.

We next examined c-Myc half-life in the \textit{pph21} strain. As shown in Figure 4B, c-Myc half-life in this mutant strain was increased approximately two-fold (19.1 minutes) compared to c-Myc half-life in a wildtype control strain (7.8 minutes). Based on multiple independent experiments, the mean half-life of c-Myc in the \textit{pph21} strain was 18.0 ± 2.2 minutes compared to a mean half-life of 9.5 ± 1.6 minutes in the isogenic control. We also examined c-Myc protein stability in a strain lacking the yeast ortholog to the PP2A substrate-recognizing subunit from the B’ family, \textit{RTS1} ([\textit{rts1}]). We have recently reported that the B’ family subunit, B56\textendash, is responsible for targeting PP2A to c-Myc for dephosphorylation in mammalian cells (23). As shown in Figure 4B, c-Myc expressed in the \textit{rts1} strain had a little more than a two-fold increase in half-life compared to c-Myc expressed in the wildtype control, with a mean half-life of 22.1 ± 3.1 minutes. The modest effect in these PP2A mutant yeast strains is likely due to multiple PP2A C and B subunits. Taken together this data suggests that PP2A in yeast does dephosphorylate c-Myc at S62, and this leads to destabilization of c-Myc protein. We also tested c-Myc half-life in a strain deleted for the PP2A A subunit. However, we obtained variable results from quite stable (34.3 minutes) to unstable (data not shown). This is likely due to spurious activity by the C subunit.
in the absence of the structural A subunit as previously reported (23).

**The yeast F-box protein, Cdc4p, does not appear to be involved in controlling c-Myc degradation**

The ubiquitination machinery is highly conserved from yeast to mammals. The ubiquitin ligase complex, SCF, which targets phospho-proteins for ubiquitination, has been shown in yeast to target c-Myc and other cell cycle proteins for ubiquitination and subsequent degradation (17,18). The SCF complex is composed of four subunits, Skp1, Cdc53, Roc1 and a variable F-box protein, which determines substrate specificity. In mammalian cells, the F-box protein, Skp2 is reported to target c-Myc through MBII and the C-terminal domains in a phosphorylation independent manner (18,38). In contrast, the F-box protein, Fbw7, has been shown to target c-Myc that is phosphorylated at T58, suggesting that it is the E3 ligase which degrades c-Myc in response to regulated phosphorylation of S62 and T58 (12,13).

Fbw7 is also referred to as hCdc4 since it is the structural homologue of the *S. cerevisiae* F-box protein, Cdc4 (17). Therefore to examine c-Myc half-life in the absence of functional Cdc4, we expressed c-Myc in the temperature-sensitive *cdc4-1* strain from the GAL1 promoter for two hours at the restrictive temperature. It has been reported that *cdc4-1* cells arrest at the G1/S transition, therefore, c-Myc protein stability was also measured in an isogenic wildtype control strain at the restrictive temperature with the addition of α-factor to arrest the cells at G1 phase of the cell cycle (39). Mutant phenotype at the restrictive temperature was verified microscopically (data not shown). As shown in Figure 5A, c-Myc is equally unstable in both the *cdc4-1* strain and the wildtype control strain with the half-lives of 11.0 minutes and 12.6 minutes, respectively. This result is consistent with previous reports showing that c-Myc is not stabilized in a yeast strain mutant for Cdc4p, however this is a surprising result given the structural homology with Fbw7/hCdc4 (18).

The structural homology between Fbw7 and yeast Cdc4 is based on similar WD40 repeats (Fig 5B) (17). However, despite the fact that these proteins are structurally related their substrates do not have similar cell cycle roles. In mammalian cells, Fbw7 targets cyclin E and c-Jun, in addition to c-Myc; all of which are proteins known to drive cell cycle progression (17,40). In contrast, yeast Cdc4p targets the yeast cell cycle inhibitor, Sic1p, for ubiquitin-mediated degradation (41,42) (Fig. 5B). This suggests that while these proteins are structurally similar, they may not be functional homologues and it also argues for the presence of another yeast F-box protein capable of targeting c-Myc.

**DISCUSSION**

Previously, we and others have shown that phosphorylation of c-Myc at Serine 62 and Threonine 58 is sequential and interdependent and that these phosphorylation events have opposing effects on c-Myc protein stability, presumably by influencing the ability of c-Myc to bind its E3 ligase (9,10,12,13). Many investigators have used the genetically tractable budding yeast *S. cerevisiae* as a model system to study the ubiquitin-mediated degradation of mammalian proteins. This is because the ubiquitin-proteasome pathway is highly conserved from mammals to yeast. Several groups have examined the half-life of c-Myc in the *S. cerevisiae* and found it to be very short. However, it has never been determined whether c-Myc could be phosphorylated at either S62 or T58 in yeast. Additionally, while there are yeast orthologs to the mammalian proteins that phosphorylate c-Myc, it was unknown whether these proteins could phosphorylate mammalian c-Myc in the complex manner observed in mammalian cells. In this report we have shown that c-Myc is phosphorylated at T58 and S62 in yeast and these phosphorylation events are interrelated like they are in mammalian cells. Specifically, phosphorylation of T58 required prior phosphorylation of S62 and S62 dephosphorylation was facilitated by T58 phosphorylation, just as in mammalian cells. This is a striking observation and points to the importance and conserved nature of the signaling pathway that can control phosphorylation of the T58-S62 phospho-domain found in mammalian c-Myc.
We have shown that phosphorylation of c-Myc at S62 and T58 occurs in yeast through a conserved signaling pathway which is likely to target a specific phospho-degron motif similar to that found in c-Myc (Fig. 6A). As in mammalian cells, phosphorylation at S62 stabilizes c-Myc and phosphorylation at T58 destabilizes c-Myc. This suggests that the mode of recognition of c-Myc by an E3 ubiquitin ligase and its subsequent proteasomal degradation is conserved in yeast. The T58 and S62 residues are located in a highly conserved region of c-Myc designated Myc Box I (MBI). Consistent with our findings, it has previously been reported that deletion of MBI, or a region encompassing MBI, results in increased c-Myc protein stability in yeast (14,15,43).

Serine 62 phosphorylation by ERK stabilizes c-Myc in mammalian cells. However, without S62 phosphorylation T58 cannot be phosphorylated and c-Myc degradation is likely mediated by an alternate E3 ligase not involving these phosphorylation sites, thus a somewhat longer half-life is observed in mammalian cells with the S62A mutant as well as in yeast with wild-type c-Myc.

Our data supports a role for yeast Rim11p in mediating T58 phosphorylation similar to GSK3β, leading to c-Myc destabilization. Likewise, Pph21p/Rts1p appears to dephosphorylate S62 similar to PP2A-B56α and destabilizes c-Myc. Our data, however, did not support a strong role for Ess1p in regulating c-Myc turnover in yeast, nor do we observe a role for the Fbw7 structural homologue, Cdc4p, in regulating c-Myc ubiquitin-mediated degradation. The F-box protein, Grr1p, is, however, reported to play a role in c-Myc degradation in yeast, as described below.

The requirement for c-Myc isomerization is not conserved in yeast

While the phosphorylation and dephosphorylation events that control c-Myc protein stability appear to be conserved from yeast to mammals, isomerization by a peptidyl prolyl isomerase does not appear to be a limiting step in c-Myc degradation in yeast cells, as it is in mammalian cells. Since the dephosphorylation step leading towards c-Myc degradation is conserved in yeast, there are two possibilities that would explain why Ess1p does not appear to be required for c-Myc destruction, 1) yeast PP2A does not require c-Myc to be in the trans conformation prior to dephosphorylation or 2) c-Myc is already in the trans conformation allowing for dephosphorylation of S62 by PP2A without Ess1p activity. The former is a less likely explanation since it has been previously shown that Pin1 and PP2A have a reciprocal genetic interaction in yeast, in that Pin1 has been able to partially rescue a yeast temperature sensitive mutant that lacks PP2A activity and overexpression of one of the yeast PP2A catalytic subunits almost completely rescues an Ess1 temperature sensitive mutation (35). Thus, while there is accumulation of the cis conformation of c-Myc in mammalian cells prior to its degradation, in yeast cells the majority of c-Myc may remain in the trans conformation. Given that in mammalian cells, MAP kinases phosphorylate Ser/Thr-Pro motifs in the trans conformation, and PP2A requires Pin1 to efficiently dephosphorylate S62, it is likely that an intermediary trans to cis isomerization step exists in mammalian cells but not in yeast (35,44). At this time it is unknown which enzyme catalyzes this initial conversion, although Pin1 is a likely candidate, however it appears that this step may not occur in yeast, perhaps due to the fact that c-Myc is not functioning as a transcription factor in yeast.

Structurally related E3 ligase F-box proteins in mammals, Fbw7, and yeast, Cdc4, are not functionally conserved

Another interesting aspect of this work is the lack of stabilization of c-Myc in a yeast strain mutated for the F-box Cdc4, the reported yeast homologue to mammalian Fbw7 (17). This observation has been previously reported, Kim et al has shown that mutation of the F-box protein Grr1, but not Cdc4, results in the stabilization of c-Myc in yeast (18). This led to the finding that the mammalian F-box, Skp2, which has a similar structure to Grr1, can target c-Myc for ubiquitination in mammalian cells. At the same time, Skp2 can also enhance c-Myc transcriptional activity (18,38). However, it was also shown that the Skp2-c-Myc interaction was via MBII, a second highly conserved region in c-Myc, and through the c-Myc C-terminal domain; and recognition was not phosphorylation dependent.

Phosphorylation by conserved proteins controls c-Myc stability in yeast

We have shown that phosphorylation of c-Myc at S62 and T58 occurs in yeast through a conserved signaling pathway which is likely to target a specific phospho-degron motif similar to that found in c-Myc (Fig. 6A). As in mammalian cells, phosphorylation at S62 stabilizes c-Myc and phosphorylation at T58 destabilizes c-Myc. This suggests that the mode of recognition of c-Myc by an E3 ubiquitin ligase and its subsequent proteasomal degradation is conserved in yeast. The T58 and S62 residues are located in a highly conserved region of c-Myc designated Myc Box I (MBI). Consistent with our findings, it has previously been reported that deletion of MBI, or a region encompassing MBI, results in increased c-Myc protein stability in yeast (14,15,43).
(18,38). Shortly after this, Fbw7 was identified as a second mammalian F-box that targets c-Myc for ubiquitination, and binding of Fbw7 to c-Myc was dependent on the phosphorylation of T58 in MBI (12,13). Since we now have shown that these phosphorylation events are conserved and they are important for controlling c-Myc stability in yeast, the question arises why does mutation of the Fbw7 homologue, Cdc4, not result in c-Myc stability in yeast? While c-Myc contains a reported optimal Cdc4 phospho-degron (CPD) (45), some of Cdc4p substrates in yeast, like Sic1p, require multiple phosphorylation events for recognition, and perhaps a singly T58 phosphorylated c-Myc is not well recognized (46,47).

In yeast cells, Grr1p targets the G1 cyclin, Cln2p, for degradation (48). Since mammalian Fbw7 has similar substrates, such as Cyclin E, it is possible that Grr1p, but not Cdc4p, may be more functionally homologous to Fbw7. At this time it is unknown whether recognition of c-Myc for ubiquitination by Grr1p occurs through phosphorylation at T58 in yeast cells, but our data strongly suggests that either Grr1p or another yet unknown F-box targets c-Myc for ubiquitination through this phospho-residue in yeast cells.

A conserved pathway to control protein degradation

In this work we have shown a number of similarities between the complex control of c-Myc protein stability in mammalian cells and in the model system S. cerevisiae. This conservation supports the importance of this pathway that targets c-Myc for degradation and it validates the use of S. cerevisiae as a model system to study c-Myc. Additionally, this work suggests that this pathway may act on other target proteins with a similar phosphorylation consensus sequence of T/S-P-X-X-T/S-P in both mammalian and yeast cells, as illustrated in Figure 6A.

Figure 6B shows a selected list of mammalian and yeast proteins with this potential phospho-degron sequence. As we and others have shown in mammalian cells, and now in yeast cells, the sequence TPPLSP of c-Myc controls c-Myc stability presumably by changing its affinity for its E3 ligase (11-13). In yeast cells the transcription factor Ash1, the kinase Elm1, and the pre-replication complex protein, Cdc6, all contain a similar phospho-degron sequence. While little work has been done elucidating the control of stability of Elm1 and Ash1, there has been a great deal of work dedicated to describing the mechanism of Cdc6 degradation. It has been shown that there are three modes of degradation for Cdc6 (49). Two of the modes, which occur in either S Phase or M phase, appear to be controlled by the two sequences indicated in Figure 6B. For example, Perkins et al. demonstrated that mutation of T368 of Cdc6 completely stabilizes the Cdc6 protein while mutation of S372 of Cdc6 resulted in a partially stable protein (50). These results are very similar to our observations of mutations of the corresponding residues of c-Myc (see Figure 1B), suggesting that this pathway in yeast may normally target Cdc6 for degradation.

There are also other proteins that may be targeted by this degradation pathway in mammalian cells, these include Kruppel-like factor KLF7, the anti-apoptotic protein Bcl-2, the cell cycle protein Cyclin E and the proto-oncogene c-Jun. While we were unable to find any data concerning regulation of KLF7 stability, it has been shown that ERK-dependent phosphorylation of Thr74 of Bcl-2 has a stabilizing effect on the protein (51). This is similar to the effect of the ERK-dependent phosphorylation of S62, the corresponding residue of c-Myc in the phospho-degron sequence. Cyclin E does not have a perfect consensus sequence, however, its degradation is controlled by many of the same players as c-Myc, including GSK3β and FBW7 (17,52). Additionally, it has recently been shown that PIN1 may be important in regulating Cyclin E levels, although at this time it appears to require phosphorylated S284, the residue that corresponds to c-Myc S62, for binding to PIN1 (53). This is in contrast to c-Myc, which requires a phosphorylation at T58 for binding to PIN1 (54). Both proteins have also been reported to be substrates of the F-box SCF<sub>Fbw7</sub>(12,13,54). While testing all of these proteins is not within the scope of this paper, currently studies are underway to determine if this degradation pathway controls the stability of other mammalian and yeast proteins.
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Fig. 1 Interdependent phosphorylation of c-Myc at T58 and S62 in yeast. A, V5-tagged Myc\textsuperscript{WT}, Myc\textsuperscript{T58A} or Myc\textsuperscript{S62A} expression was induced from a GAL1 promoter in the BY4741 yeast strain by addition of galactose for one hour at 30°C. Equal cell numbers were visualized by western blot analysis with V5, P-Ser 62, or P-Thr 58 using dual probing and overlay with the Odyssey Imaging System. Protein levels were quantitated and ratios of phosphorylated c-Myc to total c-Myc were calculated as described in methods. Fold change compared to c-Myc\textsuperscript{WT} is shown. B, V5-tagged Myc\textsuperscript{WT}, Myc\textsuperscript{T58A} or Myc\textsuperscript{S62A} expression was induced for one hour in the BY4741 yeast strain at 30°C by addition of galactose. Glucose was added to inhibit expression of c-Myc. Cells were harvested at the timepoints indicated after glucose addition and lysed in SDS sample buffer. Equal cell numbers were visualized by western blot analysis with V5 and Cdc28. c-Myc protein levels and Cdc28p levels were quantitated by the Odyssey Imaging System.
Imaging System and c-Myc levels were normalized to total protein as determined by the amount of Cdc28 protein. c-Myc levels at each time point are shown as a percent of the first time point and are plotted on a semi-log graph. Best-fit lines were calculated using Microsoft Excel. Experiments were repeated three or more times and representative data is shown. Mean half-lives are indicated in bold.

**Fig. 2** The yeast kinases Kss1 and Rim11 phosphorylate c-Myc and thereby control c-Myc protein stability. **A**, V5-tagged c-Myc<sup>WT</sup> was expressed in the BY4741 and Δkss1 yeast strains for one hour at 30°C. Cells were lysed in SDS sample buffer. Equal cell numbers were analyzed by western blotting with the indicated antibodies as described in Figure 1A. Ratios of phosphorylated c-Myc<sup>WT</sup> to total c-Myc<sup>WT</sup> were calculated. Fold change of ratios in the Δkss1 strain compared to the BY4741 strain are shown. **B**, V5-tagged c-Myc<sup>WT</sup> was expressed in the BY4741 and Δrim11 yeast strains for one hour at 30°C. Cells were lysed in SDS sample buffer. Equal cell numbers were analyzed by western blotting as described in Figure 1A. Ratios of phosphorylated c-Myc<sup>WT</sup> to total c-Myc<sup>WT</sup> were calculated. Fold change of ratios in the Δrim11 strain compared to the BY4741 strain are shown. **C**, V5-tagged c-Myc<sup>WT</sup> expression was induced in the BY4741, Δkss1 or Δrim11 yeast strains for one hour at 30°C. Following addition of glucose, cells were harvested at the timepoints indicated and cells were lysed in SDS sample buffer. Western Blotting and quantitation were performed as described in Fig. 1B. Experiments were repeated three or more times and representative data is shown. Mean half-lives are indicated in bold. **D**, Lysates from Δkss1 or Δrim11 strains expressing or not expressing V5-6xhis tagged c-Myc protein were incubated with Nickel agarose to purify c-Myc. Kss1 and Rim11 kinases were purified from yeast strains with knocked-in TAP tags using calmodulin purification as described in methods. c-Myc or lysates not expressing c-Myc were eluted from the Nickel agarose and then incubated with either the immobilized TAP-Kss1 or TAP-Rim11 kinases in the presence of <sup>32</sup>P-labeled ATP (lanes 1-4). As an additional control c-Myc protein was incubated with <sup>31</sup>P-labeled ATP in the absence of either TAP-tagged kinase (lane 5). Representative results are shown.

**Fig. 3** Mutation of the yeast peptidyl prolyl isomerase, Ess1, does not significantly affect c-Myc phosphorylation or overall stability. **A**, W303 and ess1<sup>H164R</sup> cells were grown in 2% raffinose medium at 30°C overnight. Cells were diluted to OD<sub>600</sub> = 0.3, nocodazole was added to the W303 strain and cells were grown at 37°C for 4 hours. Galactose was added and c-Myc<sup>WT</sup> expression was induced for 3 hours at 37°C. Following addition of glucose, cells were harvested and lysed in SDS sample buffer. Equal cell numbers were visualized by western blot analysis with the indicated antibodies and quantitated as described in Fig.1A. Ratios of phosphorylated c-Myc<sup>WT</sup> to total c-Myc<sup>WT</sup> were calculated. Fold change of ratios in the ess1<sup>H164R</sup> strain compared to the W303 strain are shown. **B**, c-Myc<sup>WT</sup> expression in the W303 or ess1<sup>H164R</sup> yeast strain was induced as described above. Following addition of glucose, cells were harvested at the indicated timepoints and lysed in SDS sample buffer. Western blot analysis and quantitation were performed as described in Fig. 1B. Experiments were repeated three or more times and representative data is shown. Mean half-lives are indicated in bold. Dashed lines indicate the biphasic degradation of c-Myc in the ess1<sup>H164R</sup> strain. C, c-Myc expression was induced in the ess1<sup>A144T</sup> yeast strain as described in A. Following addition of glucose, cells were harvested at the indicated timepoints and lysed in SDS sample buffer. Western blot analysis and quantitation were performed as described in Fig. 1B. Experiments were repeated three or more times and representative data is shown. Mean half-life is indicated in bold. Dashed lines indicate the biphasic degradation of c-Myc in the ess1<sup>A144T</sup> strain.

**Fig. 4** Loss of yeast PP2A activity increases c-Myc S62 phosphorylation and c-Myc half-life. c-Myc<sup>WT</sup> expression was induced at 30°C for one hour in the BY4741 or Δpph21 yeast strains. Following addition of glucose, cells were harvested and lysed in SDS sample buffer. Equal cell numbers were visualized by western blot analysis with the indicated antibodies and quantitated as described in Fig.1A. Ratios of phosphorylated c-Myc<sup>WT</sup> to total c-Myc<sup>WT</sup> were calculated. Fold change of ratios in the Δpph21 strain...
compared to the BY4741 strain are shown. B, c-MycWT expression was induced for one hour at 30°C in the BY4741, Δpph21 or Δrts1 yeast strain. Following addition of glucose, cells were harvested at the indicated timepoints and lysed in SDS sample buffer. Western blot analysis and quantitation were performed as described in Fig. 1B. Experiments were repeated three or more times and representative data is shown. Mean half-lives are indicated in bold.

Fig. 5 Mutation of the Fbw7 structural homolog, Cdc4, does not result in c-Myc stabilization. A, W303 cells, in the presence of α factor, and cdc4-1 cells were grown in 2% raffinose medium at 37°C for two hours. c-Myc expression was induced for one hour at 37°C by addition of galactose. Following termination of expression by addition of glucose, cells were harvested at the indicated timepoints and lysed in SDS sample buffer. Western blotting and quantitation were performed as described in Figure 1B. B, Schematics of the human F-box protein, Fbw7, and the S. cerevisiae F-box protein, Cdc4. Key structural domains are indicated, as well as target proteins of the F-boxes that are important in regulating the cell cycle (see text).

Fig. 6 A conserved pathway to control protein degradation. A, Summary of the conserved pathway that controls protein stability through a specific phospho-degron present in c-Myc, in mammalian cells and yeast cells. B, Other mammalian and yeast proteins with predicted phospho-degron sequence that would be affected by this pathway (see text for details).
Table 1
Mammalian proteins known to regulate c-Myc protein stability and their *S. cerevisiae* orthologs

| Mammalian Protein       | Effect on c-Myc Protein stability | *S. cerevisiae* orthologs |
|-------------------------|----------------------------------|---------------------------|
| ERK kinase              | Stabilizing (6)                  | Kss1                      |
| Gsk3β kinase            | Destabilizing (9)                | Rim11                     |
| Pin1 isomerase          | Destabilizing (11)               | Ess1                      |
| PP2A C subunit          | Destabilizing (11,23)            | Pph21                     |
| PP2A B subunit (B’ type)| Destabilizing (23)               | Rts1                      |
| F-box hCdc4 (Fbw7)      | Destabilizing (12,13)            | Cdc4                      |
| F-box Skp2              | Destabilizing (18,38)            | Grr1                      |
Figure 1

A

| Myc WT | Myc T58A | Myc S62A |
|--------|----------|----------|
| αPT58-Myc |         |          |
| αV5-Myc   |         |          |
| 1.0 11.4x 0 |     |          |
| αPS62-Myc |         |          |
| αV5-Myc   |         |          |

Fold change phospho-S62 Myc/Total Myc

B

c-MycWT

| αV5-Myc | αCdc28 |
|---------|--------|
| - 0 10 20 30 min. | |

c-MycT58A

| αV5-Myc | αCdc28 |
|---------|--------|
| - 0 10 20 30 min. | |

c-MycS62A

| αV5-Myc | αCdc28 |
|---------|--------|
| - 0 10 20 30 min. | |
Figure 3

A

| Condition | MycWT | ess1H164R + MycWT |
|-----------|-------|-------------------|
| αPT58-Myc | 1.0   | 1.3x              |
| αV5-Myc   |       |                   |
| αPS62-Myc | 1.0   | 1.1x              |
| αV5-Myc   |       |                   |

Fold change phospho-T58 Myc/Total Myc

Fold change phospho-S62 Myc/Total Myc

B

| Condition          | MycWT + nocodazole | ess1H164R + MycWT |
|--------------------|---------------------|-------------------|
| αV5-Myc            |                     |                   |
| αCdc28             |                     |                   |


C

| Condition | MycWT |
|-----------|-------|
| αV5-Myc   |       |
| αCdc28    |       |

Percent c-Myc remaining

Time after addition of glucose (min.)

Wildtype + MycWT + nocodazole

ess1H164R + MycWT

Wildtype + MycWT + nocodazole

ess1H164R + MycWT

(11.7 ± 1.8 min.)
Figure 4

A

Wildtype + c-Myc WT
Δph21 + c-Myc WT
Δts1 + c-Myc WT

1.0

-1.1x

Fold change phospho-
PT58 Myc/Total Myc

αPT58-Myc

αV5-Myc

1.0

1.7x

Fold change phospho-
S62 Myc/Total Myc

αPS62-Myc

αV5-Myc

1 2

B

Wildtype + c-Myc WT
Δph21 + c-Myc WT
Δts1 + c-Myc WT

αV5-Myc

αCdc28

- 0 10 20 30 min

- 0 10 20 30 min

100

10

1

Percent c-Myc remaining

0 5 10 15 20 25 30

Time after addition of glucose (min.)

● Wildtype + c-Myc

(\(t_{1/2} = 7.8\) min.)

(9.5 ± 1.6 min.)

■ Δph21 + c-Myc WT

(\(t_{1/2} = 19.1\) min.)

(18.0 ± 2.2 min.)

▲ Δts1 + c-Myc WT

(\(t_{1/2} = 20.9\) min.)

(22.1 ± 3.1 min.)
Figure 6

A

**Mammalian**

**Yeast**

| Target Protein | T/S-P-X-X-T/S-P |
|----------------|----------------|
| Erk            |                |
| Gsk3β          |                |
| Pin1           | cis           |
| SCF Fbox       | Proteasome Degradation |
| Target Protein | T/S-P-X-X-T/S-P |
| Target Protein | (Stable)       |
| Target Protein | pT/S-P-X-X-pT/S-P |
| Target Protein | (Unstable)     |

B

| Protein | Species    | Sequence |
|---------|------------|----------|
| c-Myc   | H. Sapiens | pplsp_{62} |
| c-Jun   | H. Sapiens | pplsp_{243} |
| Bcl-2   | H. Sapiens | splqp_{75} |
| KLF7    | H. Sapiens | pplsp_{142} |
| CyclinE | H. Sapiens | pppqsg_{385} |
| Cdc6    | S. Cerevisiae | pessp_{44} |
| Ash1    | S. Cerevisiae | pttsp_{173} |
| Elm1    | S. Cerevisiae | spqqsp_{475} |
|         |            | sppitp_{95} |
A conserved pathway that controls c-Myc protein stability through opposing phosphorylation events occurs in yeast
Julienne R. Escamilla-Powers and Rosalie C. Sears

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