The novel proteasomal substrate Far10 contributes to control of mitotic exit in yeast

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ABBREVIATIONS

mA	milli-Ampere
amp	Ampicillin
APS	Ammoniumpersulphate
AA	Amino acid
ATP	Adenosinetriphosphate
bp	Base pairs
BSA	Bovine serum albumin
cm	Centimeter
CM	Synthetic whole medium
CTP	Cytidinetriphosphate
dATP	de-oxy adenosinetriphosphate
dCTP	de-oxycytidinetriphosphate
dGTP	de-oxyguanosinetriphosphate
dTTP	de-oxythymidinetriphosphate
dNTP	de-oxynucleosidetriphosphate
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	De-oxy ribonucleic acid
E.coli	*Escherichia coli*
EDTA	Ethylenedi-aminetetraacetic acid
EGTA	Ethyleneglycol-bis-(2-aminoethyl)-tetraacetic acid
etc.	et cetera
EtOH	Ethanol
5-FOA	5-Fluoro-orotate
(m,µ,p)g	(milli, micro, pico) gram
Gal	Galactose
Glu	Glucose
H	hour
HCl	Hydrochloric acid
his/HIS	Histidine
IgG	Immunoglobulin G
KAN	Kanamycin
kb	Kilo base
kDa	Kilo Dalton
mL	milliliter

LB	Luria-Bertani medium
leu/LEU	Leucine
lys/LYS	Lysine
mM	millimolar
min	minutes
MW	Molecular weight
MV	Mineral medium
nm	Nano meter
OD_{600}	Optical density at 600nm
ORF	open reading frame
PAGE	Poly-acrylamide gel electrophoresis
PEG	Poly-ethylene glycol
PMSF	Phenyl methyl sulfonyl fluoride
RNA	Ribonucleic acid
rpm	rotations per minute
RT	room temperature
S.cerevisiae	*Saccharomyces cerevisiae*
SCF complex	Skp1-Cullin/Cdc53-F box complex
SDS	Sodiumlaurylsulfate
TCA	Trichloroaceticacid
TEMED	N,N.N',N'-Tetramethylenediamine
Tris	Tris- (hydroxymethyl)-aminomethane
trp/TRP	Tryptophan
Tween 20	Polyoxyethylenesorbitalmonolaureate
U	Units (Enzyme activity)
ura/URA	Uracil
V	Volt
W	Watt
WT	Wild type
YPD	Glucose whole medium

Zusammenfassung

Das Ubiquitin-Proteasom System ist verantwortlich für die Regulierung einer Fülle von physiologischen Prozessen in der Zelle. Dazu gehört der gezielte Abbau von verschiedenen zellulären Proteinen, die über Ubiquitinmarkierung für die Proteolyse gekennzeichnet werden. Dieser spezifische regulatorische Mechanismus spielt in vielen grundlegenden Abläufen der Zelle eine bedeutende Rolle [Hilt, 2004]. So reguliert das Ubiquitin-Proteasom-System zum Beispiel Abläufe im Zellzyklus, z.B. bei der Zellteilung oder der Trennung von Mutter und Tochterzelle oder in der Entwicklung der Zelle. Es erfüllt außerdem wichtige Aufgaben in der Stressantwort der Zelle, z.B. durch Anpassung von Rezeptoren an der Zelloberfläche oder der Steuerung der Reparatur von DNA-Schäden. Zu den weiteren Aufgaben gehören die Regulation der Transkription, die Steuerung der Entzündungsreaktion, die Kontrolle der Biosynthese von Organellen in der Zelle. Zu wichtigen, an der Kontrolle des Zellzyklus beteiligten Substraten des Ubiquitin-Proteasom Systems zählen die Cycline, bestimmte Inhibitoren cyclinabhängiger Kinasen wie Sic1 sowie Regulator und Strukturproteine, die für die Trennung der Schwesterchromatiden und den Aufbau der Mitosespindel verantwortlich sind. Bestimmte Tumorsuppressoren sowie Transkriptionsaktivatoren und Inhibitoren werden auch durch Ubiquitin-abhängige Proteolyse kontrolliert.

Das Ubiquitin-Proteasom-System hat außerdem eine zentrale Bedeutung in der Proteinqualitätskontrolle. Es entfernt gezielt mutierte, falschgefaltete oder denaturierte Proteine. Dazu gehören abnormale Proteine des Zytoplasmas und des Zellkerns sowie geschädigte Proteine aus dem endoplasmatischen Retikulum (ER), die über eine speziellen Rücktransportmechanismus ins Zytoplasma zurückgeschleust und damit dem Abbausystem zugeführt werden

.

Um zelluläre Funktionen des Ubiquitin-Proteasom Systems weiter aufklären zu können, war es interessant und wichtig, neue Substrate zu identifizieren und zu charakterisieren. Dazu war in einer vorangegangenen Arbeit über eine spezielle genetische Methode nach Proteinen gesucht worden, deren Überexpression in Zellen mit einem Defekt des proteasomalen Abbaus eine Störung des Wachstums oder Letalität erzeugt (HEL-Phänotyp: high expression lethality) [Ledig,1996; Velten, 1996; Velten, 2000]. Die Suche basierte auf folgendem Ansatz: Werden in proteasomalen Mutanten (*pre1-1 pre4-1*) Proteine überexprimiert, deren Abbau über das Ubiquitin-Proteasom System für das Überleben der Zelle erforderlich ist, so sollte diese angehäuft werden und messbare Wachstumsdefekte auslösen. Mit dieser Methode gelang es, unter anderen ein zu diesem Zeitpunkt unbekanntes Protein zu identifizieren, das alle Bedingungen des Screens erfüllte und zusätzlich Zellzyklus-spezifische Effekte induzierte. Dieses Protein, ursprünglich Hel48 genannt, wird jetzt allgemein in der Literatur als Far10 bezeichnet [Kemp et al., 2001].

Cycloheximid-Chase-Proteolysestudien bestätigten, dass Far10 tatsächlich ein proteasomales Substrat ist. Hierbei wurde der Umsatz von unter ihren endogenen Promotor exprimierten, N-terminal mit einem 19-Myc-Epitop markierten oder C-terminal mit einem HA-Tridem markierten Far10 Versionen verfolgt. Während Far10 in Wildtypzellen bemerkenswert schnell abgebaut werden, erfolgte in proteasomalen Mutanten (*pre1-1 pre4-1*) eine klare Stabilisierung. Expressions-Untersuchungen der Epitop-markierten Versionen von Far10 hatten ergeben, dass diese wie Wildtypzellen in der Lage sind das Wachstum von Hefezellen zu blockieren. Die Markierung scheint damit keine Beeinträchtigung der Funktion von Far10 auszulösen. Die mit den Epitop-markierten Versionen gewonnen Abbaudaten spiegeln daher mit hoher Wahrscheinlichkeit die normale physiologische Situation wieder.

Proteolyse ist ein zentraler Regulationsmechanismus des Zellzyklus in Eukaryontenzellen. Wichtige Teilschritte wie die Initiation der DNA-Replikation, die Trennung der Schwesterchromatiden und die Kontrolle des Austritt aus der Mitose hängen vom proteolytischen Abbau spezifischer Zielproteine ab [Hershko, 1997; Jan-Michael Peters, 1998; Hilt, 2004]. Die Ubiquitin-Protein Ligase APC (anaphase promoting complex) ist ein essenzieller Proteinkomplex, der für die Ubiquitin-abhängige Proteolyse wichtiger Regulatoren und Strukturkomponenten der Mitose verantwortlich ist. Der in allen Eukaryonten hoch konservierte APC-Komplex besteht in der Hefe *Saccharomyces cerevisiae* aus 12 bekannten Untereinheiten [Morgan, 1998; Harper et al., 2002; Jan-Michael Peters, 2002]. Beim dem Metaphase-Anaphase Übergang triggert APC in der Hefe *Saccharomyces cerevisiae* den proteolytischen Abbau des Anaphaseinhibitorproteins Pds1 [Cohen-Fix, 1996]. Pds1 blockiert bis zu diesem Zeitpunkt Esp1, eine hochspezifische Protease, die die Cohesinkomplexe zwischen den Schwesterchromatiden prozessiert [Uhlmann, 1999] und damit die Auftrennung der Chromosomen ermöglicht. Solange Pds1 vorhanden ist wird die Dissoziation der Chromosomen verhindert und somit auch der Eintritt in die Anaphase blockiert [Michaelis, 1997; Ciosk, 1998; Nasmyth, 1999]. Der Abbau von Pds1 wird durch Aktivieren des APCs durch den Regulator Cdc20 in der Metaphase induziert.

Der Austritt aus der Mitose benötigt die Inaktivierung B-Typ-Cyclin-abhängiger Kinase Komplexe (Clb2-CDKs), die als zentrale Steuereinheiten die einzelnen Zellzyklusphasen definieren. Dazu wird einerseits ein spezifisches Clb-CDK Inhibitorprotein, Sic1, aktiviert, andererseits der Hct1-APC-vermittelte Abbau des Cyclinmoleküls Clb2 in Gang gesetzt. Diese Mechanismen werden durch ein komplexes Signalverarbeitungssystem, das so genannte mitotische Exit Netzwerk (MEN) induziert. Dazu wird das G-Protein Tem1 bei Eintritt des Tochterspindelpolkörpers in die neu generierte Knospe aktiviert. Tem1 löst dann in einer Signalkaskade über Dbf2 die Freisetzung der Phosphatase Cdc14 aus dem Nukleolus aus. An der Signalverarbeitung durch MEN sind zusätzlich Cdc5 und Cdc15

Kinase beteiligt. Freigesetztes Cdc14 stimuliert schließlich die Inaktivierung der CDK Komplexe durch Aktivierung von Hct1 und Sic1. Diese Befunde machten klar, dass Cdc20 und Cdh1 als substratspezifische Aktivatoren des APC den Abbau definierter Zielproteine in zeitlich abgestimmter Reihenfolge während der Mitose steuern.

Überexpressionexperimente hatten gezeigt, dass die Überproduktion von Far10 führte in *cdc23-1*, *cdc20-1* und *hct1-Δ1* Mutanten mehr oder weniger starke Wachstumsdefekte auslöst. Damit war eine Beteiligung des APC-Komplexes beim Abbau von Far10 wahrscheinlich. Cycloheximid-Chase Experimente mit N-terminal 19-Myc markiertem Far10 bestätigten dies: Sowohl in *cdc23-1* Mutanten als auch in *cdc20-1* Mutanten trat eine fast vollständige Stabilisierung von Far10 auf. Im Gegensatz dazu zeigte die Deletion von *HCT1* keinen Effekt. Die Ergebnisse konnten damit bestätigen, dass Far10 spezifisch über den Cdc20-APC-vermittelten Abbauweg degradiert wird.

Alle bisher identifizierten Substratproteine des Cdc20-APC Wegs besitzen ein konserviertes Erkennungsmotiv, die so genannte D-Box („destruction box“). Dieses Motiv besteht aus neun Aminosäuren mit der Konsensussequenz **R**XX**L**XX**V**X**N**/ **D**/ **E**. In der Far 10 Sequenz konnte in der Nähe des C-Terminus Motiv mit ähnlicher Sequenz (^{340}**R**RK**L**SGKY**E**^{348}) entdeckt werden. Zur Kontrolle der Relevanz dieser potenziellen D-Box wurden die beiden ersten Aminosäuren Arginin$^{340/341}$ des Motivs in Alanin^{340} sowie Leucin^{341} mutiert. In einem zweiten Konstrukt wurde auch die Aminosäure Leucin^{343} an der Position vier des D-Box Motivs durch Alanin ausgetauscht. Entgegen der Erwartung zeigten beide Konstrukte bei Überexpression in Wildtypzellen jedoch keine toxische Wirkung. Abbauexperimente mit einer N-terminal 19-Myc markierten Version des in der potenziellen D-Box mutierten N(Myc)$_{19}$Far10(L343A) zeigte nach Expression des Konstrukts unter seinem endogenen Promotor in Wildtypzellen normale Degradationsraten. Die Veränderungen in der potenziellen D-Box reichen demnach nicht aus um das Protein zu stabilisieren. Möglicherweise sind andere oder zusätzliche Determinanten des Far10 Proteins als Abbausignal wirksam.

Die Sequenzanalyse ergab außerdem dass *FAR10* neben einer N-terminalen FHA Domäne (fork head associated; diesen wird eine Funktion in der Vermittlung durch Phosphorylierung getriggerter Protein-Protein-Interaktionen zugeschrieben [Li et al., 2000; Durocher and Jackson, 2002] eine C-terminale Transmembrandomäne besitzt. Fraktionierungsexperimente sowie immunofluoreszenzmikroskopische Untersuchungen lokalisierten Far10 an der Kernhülle [Velten, 2000]. Um die Funktion der C-terminalen Transmembrandomäne zu untersuchen, wurde ein Far10 Deletionskonstrukt generiert, bei dem die Transmembrandomäne fehlt. Im Gegensatz zu Far10 Wildtypprotein konnte die verkürzte Version Far10ΔTM bei Überexpression in *pre1-1 pre4-1*, *cdc23-1* und *cdc20-1* Mutanten keine toxischen Effekte auslösen. Immunofluoreszenzexperimente zeigten, dass

das mutierte Protein nicht mehr korrekt platziert wird. Daraus kann geschlossen werden, dass die Lokalisierung von Far10 an der nukleären Membran zur Ausführung seiner inhibitorischen Funktion notwendig ist.

Zur weiteren Aufklärung der funktionellen Wechselwirkung von Far10 mit den regulatorischen Komponenten des den Austritt aus der Mitose steuernden MEN Netzwerks wurde ein genetischer Ansatz verwendet. Dazu wurde die Auswirkungen der Überexpression und Deletion von *FAR10* in Mutanten mit Defekten im MEN-Netzwerk als auch in Mutanten gestörter Clb2-CDK Inaktivierung untersucht.

Die Überexpression von Far10 löste bei einer Reihe von MEN Mutanten, speziell in *tem1-3, dbf2-2*, *cdc14-3*, Mutanten stark toxische Effekte aus. In *cdc15-2* und *cdc5-1* Mutanten konnten schwache und in *lte1Δ* and *mcd1* Mutanten keine Effekte gefunden werden. Das heißt wenn MEN inaktiv ist, die Zelle sich sozusagen in einem antiproliferativen Status befindet, wirkt sich der inhibitorische Effekt von überexprimiertem Far10 besonders stark aus. Im Gegensatz dazu wird bei einer Hyperaktivierung des MEN-Wegs, das heißt wenn die Zelle proaktive Signale zur Beendigung der Mitose empfängt, der Far10 Effekt teilweise überdeckt. Zellen mit einer Bub2 Deletion, bei denen Tem1 nicht mehr korrekt in den inaktiven Zustand überführt werden kann, zeigten bei Überexpression von Far10 im Vergleich zu Wildtyp Zellen verbessertes Wachstum. Zusätzlich wurde in MEN Mutanten bei Abwesenheit von Far10 (*far10*Δ Deletionsmutante) eine Verbesserung des Wachstums beobachtet. Das heißt, der antiproliferative Effekt einer fehlenden Aktivierung von MEN ist zumindest teilweise von der Anwesenheit von Far10 abhängig. Diese Ergebnisse werden am besten durch die Annahme erklärt, dass MEN und Far10 Mitglieder von zwei unterschiedlichen Prozessen sind, die parallel, redundant oder gemeinschaftlich den Austritt aus der Mitose steuern.

Um die funktionelle Verbindung zwischen Far10 und der Inaktivierung der Clb-CDKs am Ende der Mitose weiter zu untermauern, wurde die Auswirkung der *FAR10* Überexpression und Deletion auf Zellen, in denen die CDK-Inaktivierung direkt gestört ist, untersucht. Dazu wurde Far10 sowohl in *sic1*Δ Mutanten, sowie auch *hct1* Knock-out-Zellen überexprimiert bzw. deletiert. In beiden Fällen wurden signifikante Wachstumsdefekte (synthetische Dosierungsdefekte, synthetische Letalität) beobachtet. Bei partiell gestörter Clb2-CDK Inaktivierung ist offensichtlich das Vorhandensein von Far10 in der richtigen Konzentration notwendig; beziehungsweise wird bei nicht-physiologischer Expression von Far10 eine perfekte Clb2-CDK Inaktivierung am Ende der Mitose benötigt. Diese Ansicht wurde durch Experimente, bei denen die Störung der Clb2-CDK Inaktivierung durch Überexpression des Clb2-CDK-Inhibitorproteins Sic1 (partiell) ausgeglichen war, bestätigt. Zellen, mit nicht-abbaubarem Clb2 können bei Sic1 Überexpression normal wachsen. Entsprechend wird ein Defekt der MEN-Aktivierung in *cdc14* oder *tem1* Mutanten durch eine verstärkte

Sic1 Expression überdeckt. Die durch Hyperaktivierung von Far1, bzw. dessen Abwesenheit ausgelösten Defekte konnten aber durch eine verstärkte Expression von Sic1 nicht vollständig kompensiert werden. Wird Far10 nicht oder in zu hoher Menge produziert, benötigt die Zelle für ihr Wachstum offensichtlich den perfekt ausbalancierten Mechanismus der natürlichen Clb2-CDK Inaktivierung.

Zusammengefasst zeigen die hier vorgelegten Daten, dass Far1 offensichtlich eine Komponente ist, die kooperativ zu den zentralen Taktgebern der Mitose, den Cyclin-abhängigen Kinase Komplexen eine wichtige Rolle in der Kontrolle des mitotischen Exits spielt. Sein proteolytischer Abbau via Cdc20-APC ist dabei offensichtlich ein wichtiges regulatorisches Moment.

ABSTRACT

Ubiquitin-Proteasome System (UPS) mediated proteolysis of an array of cellular proteins plays an important role in many basic physiological processes. Among these are control of cell cycle and division, differentiation and development, response to stress, transcriptional regulation, circadian rhythms, regulation of the immune and inflammatory responses, and biogenesis of organelles. Some of the well-known substrates of this system are cell cycle regulators such as cyclins, cyclin dependent kinase inhibitors, and proteins involved in sister chromatid separation, tumor suppressors, as well as transcriptional activators and their inhibitors [Glickman, 2002; Hilt, 2004; Wolf, D.H, 2004].

Due to these facts, identification and characterization of new substrates of the ubiquitin-proteasome system is important to reveal its cellular functions. For this purpose a high expression lethality [HEL] screen had been developed [Ledig, 1996; Velten, 1996, Velten, 2000]. This screen was based on the hypothesis that overexpression of a protein whose degradation by the ubiquitin-proteasome system is required for viability or growth, will cause a strong growth defect in cells where proteasome function is impaired, as for instance in *pre1-1 pre4-1* mutants. An unknown protein originally designated as Hel48 now commonly termed as Far10 was identified, [Velten, 2000; Kemp and Sprague, Jr., 2003].

In this work cycloheximide chase experiments were undertaken to prove that Far10 is a *novel* substrate of the proteasome. Far10 expressed from its endogenous promoter on the chromosome either as N-terminally 19Myc tagged or as C-terminally 3Ha-tagged version was rapidly degraded in wild type cells and stabilized in *pre1-1 pre4-1* proteosome mutants. Based on the ability of HA-tagged Far10 to cause lethality it was concluded that the tagged version of this protein is functional. Therefore the degradation rates seen with different tagged versions are supposed to be as wild type. The identical behavior of N-terminally and C-terminally tagged Far10 strongly support this idea.

Regulatory proteolysis is an important mechanism for major cell cycle transitions such as the initiation of DNA replication, separation of sister chromatids and exit from mitosis [Jan-Michael Peters, 1998; Hilt, 2004]. APC, an ubiquitin-protein ligase, consisting of 12 known subunits in *Saccharomyces cerevisiae* is essential for ubiquitin-dependent proteolysis during mitosis [Harper et al., 2002; Jan-Michael Peters, 2002]. It

requires two substrate specific co-activators: Cdc20 and Cdh1/Hct1. Substrates of APC^{Cdc20} complex include non-cyclins such as Pds1 [Cohen-Fix et al., 1996; Michaelis et al., 1997; Ciosk et al., 1998; Nasmyth, 1999] and cyclins such as Clb2 and Clb5 [Bäumer et al., 2000; Wäsch, 2002; Irniger, 2002; Cross, 2003]. APC^{Cdh1} complex initiates degradation of the mitotic cyclin Clb2 in telophase and also mediates proteolysis of other proteins such as the spindle-associated protein Ase1, Cdc20 and the polo-like kinase Cdc5 [Schwab et al., 1997; Visintin et al., 1997; Shirayama et al, 1998]. Thus, Cdc20 and Cdh1 ensure that different target proteins of the APC are degraded in a proper temporal order during mitosis.

The participation of the anaphase-promoting complex and its co-activators in the degradation of Far10 was demonstrated by the observation of synthetic dosage effects in *cdc23-1*, *cdc20-1* and *hct1-Δ1* mutants. Cycloheximide decay analysis of 19Myc tagged Far10 in *cdc23-1* APC mutants as well as *cdc20-1* and proteasome mutants uncovered a clear proteolytic stabilization of $N(myc)_{19}$Far10. On the contrary, a deletion of *HCT1* had no effect on the degradation of Far10. These results confirm that Far10 is a genuine substrate of the APC and requires the specificity factor Cdc20 for its degradation. In addition to this, analysis of *in-vivo* ubiquitination experiments of Far10$(HA)_3$ in wild type (WCG4) and *pre1-1 pre4-1* proteasome mutants revealed that the polyubiquitinated forms of Far10$(HA)_3$ accumulate in the *pre1-1 pre4-1* proteasome mutants.

Substrates of APC and Cdc20 in particular identified till date have a nine amino acid conserved motif called the destruction [D] box which has a consensus sequence: RXXLXXVXN/D/E. Far10 being a substrate of APC^{Cdc20} has a nine amino-acid sequence similar to the D box motif, ^{340}**R**RK**L**SGKY**E**^{348} residing in the C-terminal region. To check the relevance of this motif in the degradation of Far10, site directed mutagenesis of 1) first two arginines (340, 341) to alanine and leucine and 2) leucine (343) to alanine was carried out. Overexpression of these two different mutant versions of Far10 in the wild type yeast strains did not result in toxicity. Moreover, cycloheximide chase analyses of $N(Myc)_{19}$Far10(L343A) expressed from the endogenous promoter on the chromosome showed that this mutant protein was not stabilized in wild type yeast strains. These data suggest that this sequence in Far10 may not confirm to a classical D-box and that the degradation signals might be located else where in the protein. It could also be possible that mutations in this D-box have to be collective in order for the desired effect(s) to be seen.

Database analysis of *FAR10* revealed an N-terminal FHA (**f**ork **h**ead **a**ssociated) domain and a C-terminal transmembrane domain. Cell fractionation experiments as well as immunofluorescence studies proved that Far10 localizes to the nuclear envelope [Velten, 2000]. To investigate the function of the C-terminal transmembrane domain, a deletion construct containing Far10 lacking the transmembrane domain, *far10ΔTM* was generated. In contrast to wild type Far10 this mutant protein was unable to cause synthetic dosage effects in *pre1-1 pre4-1*, *cdc23-1* and *cdc20-1* mutants. Immunofluorescence studies of Far10ΔTM$(HA)_2$ revealed that this mutant protein was indeed mislocalized. These results provide evidence that the ability of Far10 to induce lethality depends on its correct localization to the nuclear membrane [Murray, 2001].

An investigation into the synthetic interactions of *FAR10* with *cdc20-1* mutant revealed that *cdc20-1 far10Δ* double mutants displayed a synthetic growth defect at 25°C. On the other hand *far10Δ cdc23-1* double mutants showed no obvious growth effects when compared to *cdc23-1* single mutants. The data imply that APC is fully active at 25°C in *far10Δ cdc23-1* mutants. On the contrary *cdc20-1 far10Δ* double mutants at the same temperature may show a defective APC activity. These results propose that when APC-Cdc20 activity is disturbed, presence of Far10 is required.

The mitotic exit network [MEN] in budding yeast is a complex signaling cascade consisting of Tem1 (a GTPase); Cdc15, Dbf2 and Cdc5 (protein kinases); Cdc14 (a protein phosphatase); Mob1 (a Dbf2 associated factor); Bub2-Bfa1/Byr4 (a two component GTPase-activating Protein; GAP); Lte1 (a guanine nucleotide exchange factor; GEF) and a scaffold protein, Nud1 [Amon, 2001]. Tem1 is a positive regulator of MEN. The ultimate effector of MEN is Cdc14 and it is held inactive in the nucleolus by its inhibitor Cfi1/Net1 during G_1, S, G_2 and early M phase. MEN is activated when the spindle pole body reaches daughter cell where Lte1 (GEF) exchanges a GDP for GTP on Tem1. Thus activated, Tem1-GTP binds to and activates Cdc15, which in turn activates Mob1-Dbf2 complex. Dbf2 facilitates release of Cdc14 from the nucleolus. The freed Cdc14 functions to shut down mitotic Cdk activity by promoting expression of the Cdk inhibitor Sic1 and stimulation of degradation of the essential mitotic cyclins.

To outline the relation(s) of *FAR10* with regulatory modules of the mitotic exit network a genetic method was executed. For this purpose, effects of *FAR10* overexpression and inactivation were studied in MEN mutants. Overexpression of *FAR10* in a string of MEN mutants was found to cause toxicity in *cdc14-3*, *dbf2-2*, and *tem1-3* mutants, with

cdc15-2 and *cdc5-1* mutants displaying a mild effect and *lte1Δ* mutant showing no effect at all. These results prove that when MEN activation is defective cells become sensitive to Far10 overexpression. In contrast when MEN is hyperactive as in the case of *bub2Δ* mutants, the effect of overexpression of *FAR10* is suppressed.

FAR10 is not an essential gene and its deletion causes no obvious growth defects when compared to wild type (W303) strain. Though *far10Δ cdc14-3* double mutants showed no detectable growth effects at either 25°C or 30°C when compared to *cdc14-3* single mutants, deletion of *FAR10* in *dbf2-2* mutants had a moderate suppression effect at 25°C. In the case of *far10Δ tem1-3* double mutants this suppression effect was enhanced at 32°C revealing that *FAR10* may be an inhibitor of mitotic exit.

Overexpression of *FAR10* causes synthetic dosage effects in mutants that are defective in Clb-CDK inactivation such as *hct1-Δ1* and *sic1-Δ1*. These results suggest that a defective Clb-CDK inactivation either to due impaired degradation or absence of inhibition by Sic1 makes cells susceptible to Far10 overexpression. In addition, *hct1-Δ1 far10Δ* and *sic1-Δ1 far10Δ* double mutants showed synthetic growth defect at 30°C, which was markedly enhanced at 37°C. The data prove that presence of Far10 is required under these conditions.

The ultimate function of the mitotic exit network in budding yeast is inactivation of the mitotic Clb2-CDK activity, which is followed by cytokinesis resulting in the formation of two daughter cells [Visintin et al., 1998].

In relevance of these findings it was rationalized that an enhancement in Clb-CDK inactivation through ectopic overexpression of Sic1 might alleviate the toxic effects associated with *FAR10* overexpression. Henceforth, *SIC1(HA)1X* was co-overexpressed with *FAR10* in *cdc23-1*, *cdc14-3*, *dbf2-2* and *tem1-3* mutants. Results in this case show that co-overexpression of *SIC1(HA)1X* along with *FAR10* did not restore wild type growth rates. An analogous result was obtained when Sic1 was overexpressed in MEN mutants that harbored a deletion of *FAR10*. The data here propose an ill-defined role for Far10 as an inhibitor of mitotic exit. Additionally, these results also provide evidence that *FAR10* may act in parallel to MEN and/or co-operate in triggering exit from mitosis.

INTRODUCTION

The Ubiquitin-Proteasome System.

Ubiquitin is ubiquitous in biology. This small 76 amino acid residue polypeptide regulates virtually all aspects of cell biology, including cell division, growth, communication/signaling, movement and death/apoptosis [Pickart, 2001; Johnson, 2002]. Ubiquitin carries out all of these functions by becoming covalently attached to specific target proteins through a three-step enzymatic cascade [Pickart, 2001]. The most, well worked out aspect of the function of ubiquitin is regulatory proteolysis and ubiquitin mediated proteolysis of a variety of cellular proteins plays an important role in many basic cellular processes. Among these are regulation of cell cycle and division, differentiation and development, involvement in the cellular response to stress and extracellular effectors, morphogenesis of neuronal networks, modulation of cell surface receptors, ion channels and the secretory pathway, DNA repair, transcriptional regulation, transcriptional silencing, long-term memory, circadian rhythms, regulation of the immune and inflammatory responses, and biogenesis of organelles. Among the prominent substrates are cell cycle regulators such as cyclins, cyclin dependent kinase inhibitors, and proteins involved in sister chromatid separation, tumor suppressors, as well as transcriptional activators and their inhibitors. Cell surface receptors and endoplasmic reticulum (ER) proteins are also targeted by the system. Finally, mutated and denatured/misfolded proteins are recognized specifically and are removed efficiently. In this capacity, the system is a key player in the cellular quality control and defense mechanisms [Glickman, 2002; Hirsch et al., 2004; Wolf D.H, 2004 and **Fig. I**].

Degradation of a protein via the ubiquitin-proteasome pathway involves two discrete and successive steps: 1) tagging of the substrate by covalent attachment of multiple ubiquitin molecules and 2) degradation of the tagged protein by the 26S proteasome complex with release of free and reusable ubiquitin. This last process is mediated by ubiquitin recycling enzymes [deubiquitinating enzymes (DUBs)]. Conjugation of ubiquitin, to the protein substrate proceeds via a three-step cascade mechanism. Initially, the ubiquitin activating enzyme E1, which in *S. cerevisiae* is encoded by a single essential gene *UBA1* [McGrath et al., 1991] activates ubiquitin in an ATP-requiring reaction to generate a high energy thiol ester intermediate, E1-S-ubiquitin. One of several E2 enzymes [ubiquitin-carrier proteins or ubiquitin-conjugating enzymes (UBCs)] transfers the activated ubiquitin moiety from E1, via an additional high energy

thiol ester intermediate, E2-S-ubiquitin. Activated ubiquitin is then transferred to the substrate that is specifically bound to a member of the ubiquitin-protein ligase family, E3. There are a total of 13 E2 and E2 like enzymes in yeast out of which Ubc9 transfers SUMO [**s**mall **u**biquitin related **mo**difier] and Ubc12 functions to conjugate Rub1 [**r**elated to **ub**iquitin] to the target proteins with the rest involved in the transfer of ubiquitin. However, most of the Ubc's share overlapping functions [Jentsch, 1992; Hochstrasser, 1996; Sommer, 2000]. There are different classes of E3 enzymes. For the HECT (**h**omologous to the **E**6-AP **C**OOH **t**erminus) domain E3s, ubiquitin is transferred once again from the E2 enzyme to an active site Cys residue on the E3, to generate a third high energy thiol ester intermediate, ubiquitin-S-E3, before its transfer to the ligase-bound substrate [Huibregtse et al., 1995]. RING (**r**eally **i**nteresting **n**ew **g**ene) finger-containing E3s catalyze direct transfer of the activated ubiquitin moiety from the specific E2 to the E3-bound substrate. E3s catalyze the last step in the conjugation process: covalent attachment of ubiquitin to the substrate and consequently determine the specificity of this system. The ubiquitin molecule is generally transferred to an ε-NH_2 group of an internal Lys residue in the substrate to generate a covalent isopeptide bond. In some cases, however, ubiquitin is conjugated to the NH_2-terminal amino group of the substrate [Breitschopf, K et al. 1998; Aviel, S et al. 2000; Reinstein, E et al. 2000]. Ubiquitin has seven internal lysine residues: K6, K11, K27, K29, K33, K48 and K63. One of these, typically K48, is used for conjugation by additional ubiquitin moieties in a highly processive manner to form a polyubiquitin chain which is then recognized and therefore mediates degradation of the modified substrate by the downstream 26S proteasome complex [Pickart, 2000; Glickman, 2002; **Fig. II**].

The proteasome is a large, 26S, multi catalytic protease, which degrades poly-ubiquitinated proteins to small peptides. It is composed of two sub complexes: a 20S core particle (CP) that carries the catalytic activity and a 19S regulatory particle (RP). The 20S CP from yeast is a barrel-shaped structure composed of four stacked rings of 14 different subunits consisting of two identical outer rings and two identical inner rings of α- or β type. The eukaryotic α and β -rings are composed each of a set of seven distinct subunits, giving the 20S complex a pseudo-seven fold symmetry and general structure of α1–7β1–7β1–7α1–7 [Groll et al., 1999; Groll et al., 2001]. In eukaryotes the threonine protease active sites are localized to three of the seven β–subunits: β1/*PRE3*, β2/*PUP1* and β5/*PRE2*, which harbor peptidyl-glutamyl peptide bond hydrolyzing (PGPH), trypsin-like, and chymotrypsin-like activities respectively [Heinemeyer et al., 1993; Arendt and Hochstrasser, 1997; Heinemeyer et al., 1997]. Thus each proteasome has a total of 6 (3 different) proteolytically active sites. Each extremity of the 20S barrel

can be capped by a 19S RP [Baumeister et al., 1998]. The 19S RP, which is made up of at least 18 different subunits can be subdivided into lid and base sub-complexes. The base is composed of 9 subunits, six of them being ATPases of the AAA family, which are designated as Rpt1-6 (for **r**egulatory **p**article **t**riple-A protein). The other subunits of the base are Rpn1, Rpn2 and Rpn10 (for **r**egulatory **p**article **n**on-ATPase). The lid consists of a set of eight subunits, all of them being non-ATPases. These subunits function to recognize ubiquitinated proteins and/or play a structural role in defining the cavity of the RP. One important function of the 19S RP is to select and bind ubiquitinated proteins and other potential substrates of the proteasome. A second function of the 19S RP is to open an orifice in the α-ring that will allow entry of the substrate into the proteolytic chamber, referred to as gating function [Groll, M et al., 1997; Groll, M et al., 2000; Bajorek and Glickman, 2004]. Moreover, because a folded protein would not be able to fit through the narrow proteasomal channel, it is assumed that the 19S particle unfolds substrates and inserts them into the 20S CP. Both the channel opening function and the unfolding of the substrate require metabolic energy, and indeed, the 19S RP contains six different ATPase subunits. After degradation of the substrate, short peptides derived from the substrate are released, as well as free and reusable ubiquitin.

Cell Cycle Control through Ubiquitin - Proteosome Mediated Proteolysis.

In eukaryotes, cyclin-dependent kinases (CDKs) induce key cell cycle events, such as DNA replication and chromosome segregation [Morgan, 1997]. In budding yeast, a single CDK, Cdc28, governs progression through the cell cycle. Cdc28 is activated by the Clb1-Clb6 cyclins in S through M phase and the Cln1-Cln3 [G_1] cyclins in G_1 phase. When Clb-CDKs are active, cells are either in S phase or in mitosis; when Clb-CDKs are inactive, cells are in G_1 phase. The switches from a low to a high Clb-CDK state at the G_1-S phase transition and from a high to a low Clb-CDK state at the end of mitosis are the essence of the cell cycle engine. Permissive conditions for Clb-CDK activities are established at the G_1-S phase transition by the Cln-CDKs, which inactivate the proteolytic machinery responsible for degradation of Clb cyclins [Amon et al., 1994] and trigger degradation of the Clb kinase inhibitor Sic1. Ubiquitination of Sic1 and Cln1/2/3 (G_1 cyclins) is mediated by a multi subunit E3 enzyme complex known as SCF (**S**kp1-**C**ullin-**F** box) complex. This triggers degradation of these regulators via the proteosome [Lanker et al., 1996; Skowyra et al., 1997; Verma et al., 1997]. One way of Clb-CDK inactivation is by the degradation of the cyclin molecule by ubiquitin-dependent proteolysis. This is mediated by another ubiquitin protein ligase known as

the cyclosome or **a**naphase-**p**romoting **c**omplex (APC) [King et al., 1995; Sudakin et al., 1995].

Proteolysis is an important mechanism for regulation of the eukaryotic cell cycle. Major cell cycle transitions such as the initiation of DNA replication, the separation of sister chromatids and the exit from mitosis depend on proteolytic degradation of specific proteins [Hershko, 1997; Jan-Michael Peters, 1998]. The anaphase-promoting complex (APC) is an ubiquitin-protein ligase essential for ubiquitin-dependent proteolysis during mitosis. This large complex, consisting of 12 subunits in *Saccharomyces cerevisiae*, appears to be highly conserved in all eukaryotes [Morgan, 1998; Harper et al., 2002; Jan-Michael Peters, 2002]. At the metaphase to anaphase transition, APC triggers proteolytic degradation of Pds1, which acts as anaphase inhibitor protein in budding yeast [Cohen-Fix, 1996]. Pds1 blocks the sister chromatid separating protease Esp1 and thereby prevents the dissociation of chromosomes and entry into anaphase [Michaelis, 1997; Ciosk, 1998; Nasmyth, 1999]. Pds1 degradation, initiated in metaphase, by APC activation by Cdc20, liberates Esp1 which then induces the cleavage of the cohesin subunit Scc1 ensuing the separation of sister chromatids [Uhlmann, 1999]. The activity of the APC-Cdc20 complex is inhibited by the spindle assembly checkpoint, which is activated upon defects in the mitotic spindle or by unattached kinetochores and thereby prevents the separation of sister chromatids until correct assembly of the mitotic spindle [Fang et al., 1998; Hwang et al., 1998]. Cdh1 (also termed Hct1) like Cdc20, is an activator of the APC. Upon binding of Cdh1 in telophase, degradation of the mitotic cyclin Clb2 is initiated. APC-Cdh1 mediates proteolysis of other proteins as well such as the spindle-associated protein Ase1 and the polo-like protein kinase Cdc5 [Schwab et al., 1997; Visintin et al., 1997]. Due to this behavior it is thought that Cdc20 and Cdh1 act as substrate-specific activators of the APC and thereby ensure that different target proteins of the APC are degraded in a proper temporal order during mitosis.

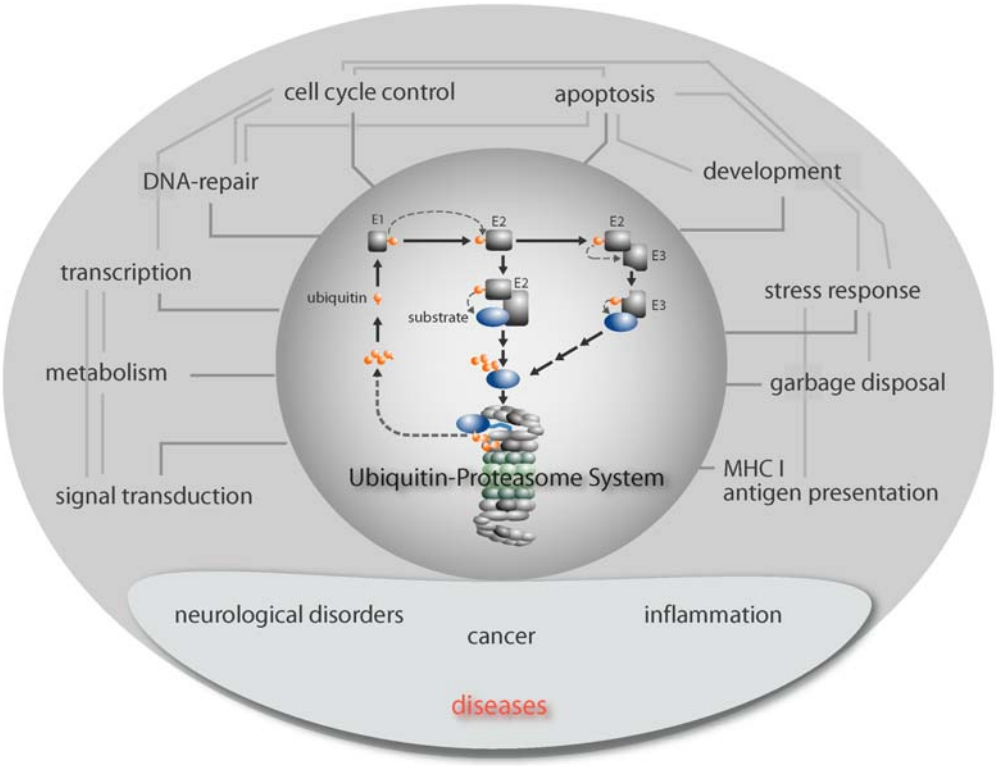
MEN and the FEAR Pathways Control Exit from Mitosis in Yeast.

In as much as cells under favorable conditions, commit themselves to mitosis, it is imperative that they exit from mitosis so that two daughter cells are formed, each containing one complement of every chromosome. The process of exit from mitosis in budding yeast, is regulated by a complex signal transduction cascade - the **m**itotic **e**xit **n**etwork (MEN). Successful exit from mitosis in yeast requires movement of one spindle pole body into the bud and inactivation of the Clb-CDK activity [Visintin et al., 1998] This is governed by the spindle positioning check point which ensues that cytokinesis is

initiated only after arrival of one spindle pole body in the newly formed bud [Bardin et al., 2000; McCollum and Gould, 2001]. The ultimate effector of MEN is Cdc14. Cdc14, a protein phosphatase, is held inactive in the nucleolus by its inhibitor Cfi1/Net1 during G_1, S, G_2 and early M phase. Cdc14, when released upon activation of MEN, acts in three ways [Visintin et al., 1998; Shou et al., 1999; Visintin e al., 1999]. Firstly, the phosphatase activity of Cdc14 directly reverses Cdc28 phosphorylation of substrates [Visintin et al., 1998]. Second, it promotes the destruction of B-type cyclins by activating anaphase-promoting complex, through de-phosphorylation of Hct1 to form the active E3 ligase, APC^{Cdh1}. Third, it activates Sic1 by two means: proteolytic stabilization through de-phosphorylation and induction of its transcription [Knapp et al., 1996; Visintin et al., 1998]. The critical regulator of MEN is Tem1, a small spindle pole body located G protein, which acts on the top of this pathway [Shirayama, et al., 1994; Lee et al., 2001]. Tem1 is negatively regulated by a highly unusual two component GAP (**G**TPase **a**ctivating **p**rotein) consisting of Bub2 and Bfa1 [Fesquet et al., 1999; Lee et al., 2001]. Bub2 and Bfa1 also form components of the spindle-positioning checkpoint and hence function to negatively regulate Tem1. In the mother cell, Tem1 exists in an inactive GDP bound form but binds GTP when the spindle pole body enters the bud and is brought into contact with its presumptive **g**uanine nucleotide **e**xchange **f**actor (GEF) Lte1, which exists only in the daughter cell [Bardin et al., 2000; Pereira et al., 2000]. Activated Tem1-GTP then binds to and activates the Cdc15 kinase, which in turn activates Mob1-Dbf2 finally resulting in the release of Cdc14 from the nucleolus. The order in which, the MEN pathway components function is Tem1-Cdc15-Mob1-Dbf2-Cdc14 [Sarah E. Lee et al., 2001]. The role of Cdc5 in MEN is more complex and impinges at two levels. First, it phosphorylates Bfa1/Bub2 complex thus restraining MEN [Hu et al., 2001] and also possibly phosphorylates Cfi1/Net1 facilitating the release of Cdc14 from its nucleolar prison [Shou et al., 1999; Visintin et al., 1999]. Cdc14 once released de-phosphorylates its targets in the nucleus as well as cytoplasm [**Fig. III**]. When the process of exit from mitosis is completed Cdc14 shuttles back to the nucleolus.

The newly discovered FEAR (Cdc **f**ourteen **e**arly **a**naphase **r**elease) network regulates transient release of Cdc14 during early anaphase [Stegmeier et al., 2002]. The FEAR network dependent release of Cdc14 is independent of MEN but requires the function of Esp1, Slk19, Spo12 and Cdc5. The FEAR dependent release of Cdc14 is neither sufficient nor essential for exit from mitosis. Moreover, in an MEN conditional mutant held at restrictive temperature, Cdc14 is re-sequestered into the nucleolus after the early release in anaphase showing that the MEN activity is essential to maintain Cdc14

in a released state at later stages of anaphase and telophase. The FEAR network is therefore critical only for the timely exit from mitosis. When the MEN activity is decreased, however the FEAR network becomes indispensable for mitotic exit as suggested by the fact that in cells deleted for Lte1, removal of *ESP1*, *SPO12* or *SLK19* is lethal. What triggers re-localization of Cdc14 to the nucleolus remains a mystery as does the ultimate physiological function of the FEAR network. Also the order of functioning of the various components is not known. One interesting possibility is that the FEAR network monitors other essential events besides spindle positioning. FEAR induced release of Cdc14 is confined to the nucleus and its export to the cytoplasm may require MEN activity. Cdc14 released by the FEAR network is catalytically active and de-phosphorylates one of its substrates, the Cdc15. A reasonable model for the significance of early release of Cdc14 is that this step is necessary for the complete and timely activation MEN. A positive feed back loop would then ensure that sufficient Cdc14 is maintained in a liberated and active state to drive the cells out of mitotic exit [Saunders, preview in Molecular Cell, 2002; Geymonat et al., 2002; Jensen et al., 2002].



Courtesy of Dr. Wolfgang Hilt

Figure I: Physiological functions of the ubiquitin-proteasome system. Protein degradation via the ubiquitin-proteasome system plays an important role in the entire gamut of physiological pathways.

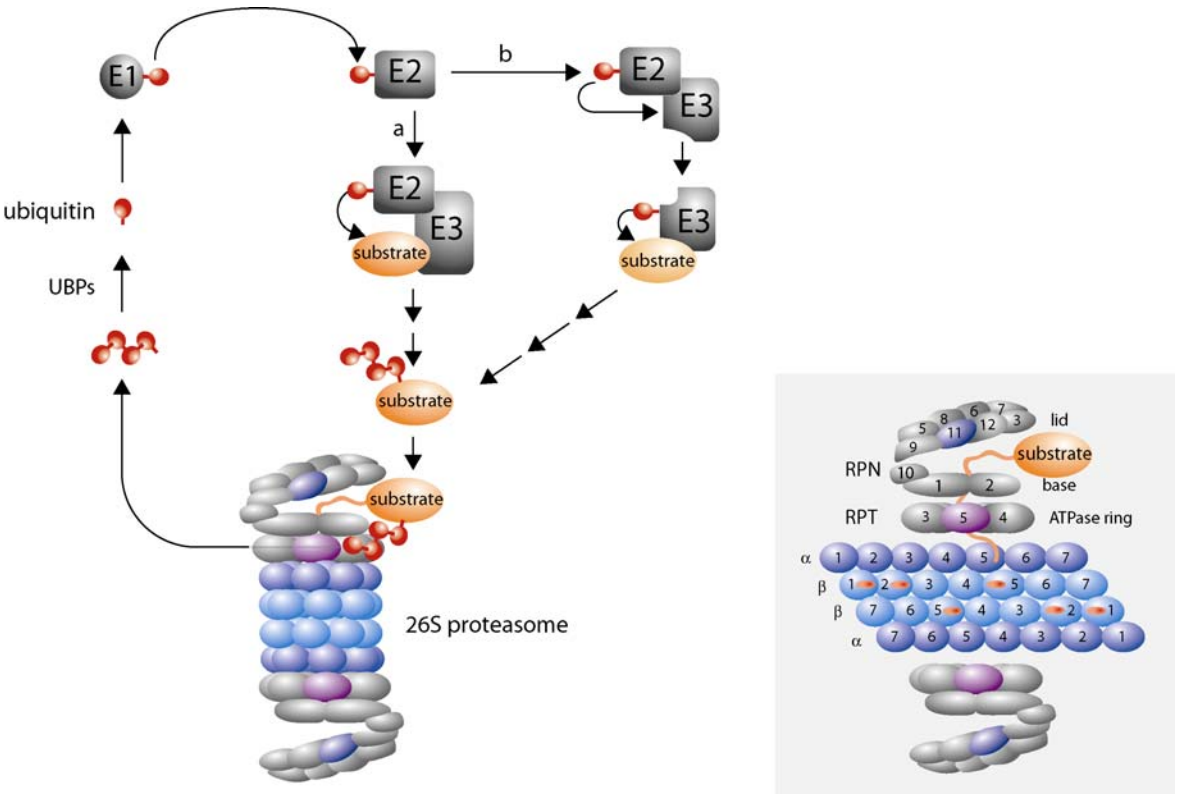


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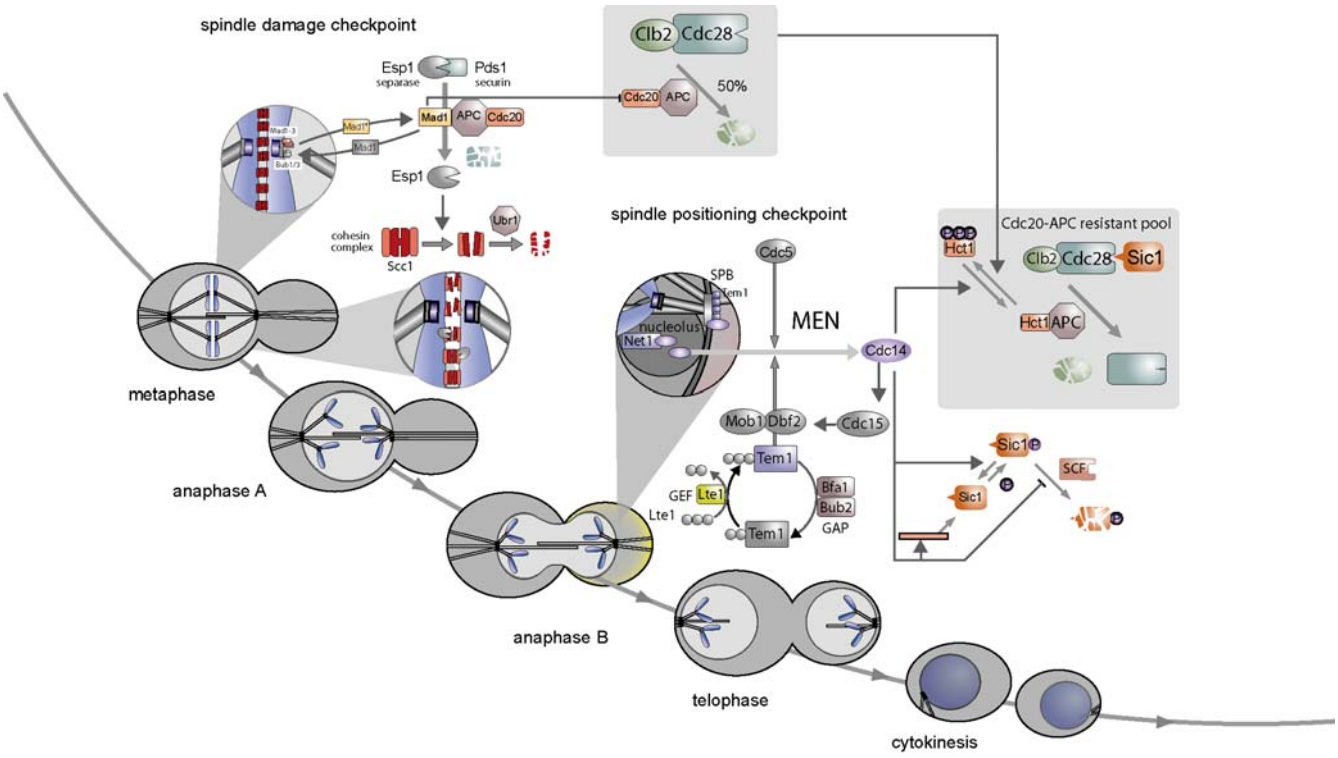
Figure II: Overview of the ubiquitin-proteasome system. Substrates of this system are tagged by the sequential and coordinated action of E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and E3 ubquitin ligases. Polyubiquitinated substrates are recognized by the 19S cap of the proteasome and finally degraded by the proteolytic activity of the 20S core particle of the 26S proteasome.



Courtesy of Dr. Wolfgang Hilt

Figure III: Outline of mitosis in budding yeast. Anaphase entry is governed by Pds1, which "secures" Esp1 protease. APC^{Cdc20} targets proteolytic degradation of Pds1 thereby liberating Esp1. Free Esp1 then cleaves Scc1 leading to the resolution of cohesion between sister chromatids. The C-terminal Scc1 fragment is removed by a Ubr1-dependent proteasomal pathway. Entry into anaphase is inhibited by the spindle damage checkpoint, which functions in response to incomplete kinetochore-microtubule attachment or lack of tension at kinetochores. This checkpoint produces a "wait anaphase" signal presumably generated by an activated form of Mad2 protein, which functions to inhibit APC^{Cdc20} activity. About 50% of Clb2 is proteolytically removed by APC^{Cdc20} pathway in early mitosis. Upon arrival of the spindle pole body in the bud cell, the mitotic exit network is activated. Tem1 is activated by its GTP exchange factor Lte1, which is restricted to the bud cortex. Tem1.GTP then binds to and activates Cdc15, which then stimulates Dbf2 kinase to activate release of Cdc14 from its nucleolar prison. Liberated Cdc14 antagonizes Clb-CDK activity. [See text for more details].

SCOPE OF THIS WORK

Ubiquitin-Proteasome System is involved in a host of physiological processes. The many substrates of proteasome have numerous functions. The main purpose of this work was to divulge more about the role of the proteasomal substrate Far10 in the cell cycle. In addition to this, some of the domains found in this protein were characterized. To detail its role in cell cycle, an overexpression based genetic approach was carried out. In this method a "reference gene", in this case Far10 was overproduced in a series of yeast strains having "target mutations" which could be a deletion or a point mutation having a growth defect at 37°C. The observed phenotype was scored as causing a synthetic dosage effect if there was an inhibition in growth at permissive temperature or as synthetic dosage suppression if it results in rescue of growth defect. Additionally, combining deletion of *FAR10* with mutations in various components of MEN and Clb-CDK inactivation machinery were done to look for if any synthetic growth effects in the double mutants. Results obtained in this work suggest that Far10 is part of a system that acts parallel to mitotic exit network and/or co-operates with various components of MEN in facilitating exit from mitosis.

2. MATERIALS AND METHODS

Methods specifically used or modified for this study are described in this section. Appropriate references are quoted in special cases.

2.1. CHEMICALS AND MISCELLANEOUS MATERIALS

Amersham-Pharmacia Braunschweig.	Hyper film ECL kits
Biolabs, Beverly USA.	Vent DNA Polymerase
Difco, Detroit USA.	Media for cell cultures.
DuPont, Nemours.	Glusulase
Merck, Darmstadt.	TEMED, APS, SDS, PMSF.
MWG Biotech, Ebersberg.	Oligo-nucleotide primers.
Promega.	Herring sperm-DNA
Qiagen, Hilden.	Plasmid Miniprep kits and QUIA quick spin columns, PCR purification kits.
Roche Diagnostics, Mannheim.	Restriction Endo-nucleases and compatible Buffers, RNAseA, DNA loading standards, Ampicillin, Complete Protease Inhibitor Mix, T4 DNA ligase, Taq Polymerase.
Roth, Karlsruhe and Genaxxon.	Chemicals and Agarose.
Pall Gelman, Rossdorf.	Nitrocellulose.
Seikagaku, Tokyo, Japan.	Zymolyase100T
Serva, Heidelberg.	Coomassie Brilliant Blue 250, Bromophenol Blue.

Sigma Diesenhofen.	Aminoacids, BSA, DAPI, Cycloheximide, Ethidiumbromide.
TRC, North York, Canada.	5-FOA.

2.1.1. ANTIBODIES USED

Dianova, Hamburg.	Goat Anti-Mouse conjugated with HRPO.
Hiss Diagnostics, Freiburg.	Mouse Anti-Ha, Clone 16B12
Roche Diagnostics, Mannheim.	Mouse Anti-Myc, Clone 9E10.
Molecular Probes, USA.	Mouse Anti-Pgk.

2.2. INSTRUMENTS

Bio Rad, Munich.	PAGE electrophoresis equipment.
Eppendorf, Hamburg.	Photometer 1101M for measuring the optical density of yeast cell cultures, Table-Top Centrifuge type 5415.
Froebel, Lindau.	Transfer-Blot semi-dry system.
Jasco, Gross Umstadt.	Photometer V530
Kühner, Basel.	Mini-shakers and lab shaker for yeast cell cultures
MWG, Ebersberg.	Gel-Documentation system.
Pharmacia, Freiburg.	Agarose-gel Electrophoresis equipment and PCR machines.
Stratagene, LaJolla, USA.	Robo-Cycler Gradient-40 PCR machine.

Zeiss, Oberkochen.	Fluorescence microscope.
Nikon, Tokyo, Japan.	Micromanipulator.

2.3. MEDIA

To make solid medium, Bacto-Agar to a final concentration of 2% was added. Mineral medium contained the following amino acids: 0.3mM Adenine, 0.3mM Histidine, 1.7mM Leucine, 1.0mM Lysine, 0.4mM Tryptophan and 0.2mM Uracil. Any of the supplemented amino acids were omitted depending on the selection conditions used. All the media were prepared in de-ionized water and autoclaved at 121°C for 20 min. The supplements were constituted in weight/volume ratios.

2.3.1. MEDIA FOR YEAST CELL CULTURES (Sherman et al., 1974)

Whole Medium [YPD]

1.0% Yeast-Extract
2.0% Bacto-Peptone
2.0% Glucose. pH was set to 5.5 by using 1M Hydrochloric acid.

Synthetic Whole Medium [CM]

0.67% Yeast-Nitrogen Base w/o Amino Acids
2.0% D-Glucose
0.2% Dropout Powder Mixture. pH was set to 5.5 using 1M Hydrochloric acid.

Dropout Powder mixture contained 2g each of Alanine, Arginine, Asparagine, Aspartic acid, Cysteine, Glutamic acid. Glycine, Isoleucine, Methionine, Phenyl-Alanine, Proline, Serine, Threonine, Tyrosine, Valine and myo-Inositol together with 0.2g of para-Amino Benzoic acid.

Mineral Medium [MV]

0.67% Yeast-Nitrogen Base w/o Amino Acids
2.0% Glucose. pH was set to 5.5 using 1M Hydrochloric acid.

5-FOA Selection Medium [5-FOA]

0.67% Yeast-Nitrogen Base w/o Amino Acids
2% D-Glucose
1g/L 5-FOA
50mg/L Uracil. pH was set to 5.5 using 1M Hydrocholoric acid.

Minimal Sporulation Medium

1% Potassium Acetate

2.3.1. Media for *Escherichia coli* Cultures

Whole Medium [LB]

1% Yeast extract
1% Bacto-Tryptone
0.5% Sodium chloride. pH was set to 7.5 using 1M NaOH.

To make cultures of *E.coli* containing ampicillin marked plasmids, the whole medium [LB] was supplemented with the antibiotic ampicillin to a final concentration of 50mg/L.

SOC Medium

2% Bacto-Tryptone
0.5% Yeast Extract
0.4% D-Glucose
10mM Sodium chloride
2.5mM Potassium chloride
10mM Magnesium chloride
10mM Magnesium sulfate. pH was set to 7.4 using 1M NaOH

2.4. GROWTH CONDITIONS

Cultures were typically grown in Erlenmeyer flasks of at least 4-5 times the volume of the medium used to culture yeast/*E.coli*. Yeast cell cultures were shaken at 180*rpm* at 30°C/25°C as required whereas *E.coli* cultures were incubated at 37°C and simultaneously were shaken at 220*rpm*.

2.4.1. Glycerol Stock Cultures

E.coli stock cultures were made by suspending 500µL of overnight culture in autoclaved 60% (V/V) Glycerol and stored at –80°C.

Yeast stock cultures were made by suspending a loop-full of fresh overnight culture from solid agar medium in autoclaved 15% (V/V) Glycerol and stored at –80°C.

2.4.2. Measurement of Optical Density of Cultures

1mL of the cell culture was taken in a cuvette and its optical density (O.D.) was measured at a wave length of 600nm in a visible spectrophotometer.

2.4.3. Yeast Pre-Cultures and Main-Cultures

To make pre-cultures, yeast cells were grown in 2mL of the required medium in a capped test tube for about 16-24 hours at 30°C/25°C on a shaker. For yeast cells containing plasmid(s), appropriately supplemented CM medium was used. Once the cultures were in stationary phase, these were subsequently diluted to appropriate concentration into a much bigger volume of the same medium for subsequent experiments.

2.5. STRAINS and PLASMIDS

2.5.1. YEAST STRAINS

Strain Name	Genotype	Donor
WCG4a *	*MAT*a *his3-11,15 ura3 leu2-3,112* Can^s Gal^+	W. Heinemeyer
WCG4α *	*MAT* α *his3-11,15 ura3 leu2-3,112* Can^s Gal^+	W. Heinemeyer
WCG4a/α *	*MAT*a/*MAT α*	W. Hilt
YHi29/14a *	*MATa pre1-1 pre4-1*	W. Hilt
YIV017 *	*MAT*a *FAR10-3HA::HIS5*	I. Velten
YIV036 *	*MAT*a *FAR10-3HA::HIS5; pre1-1 pre4-1*	I. Velten
YL268 *	*MAT*a *19MYC-FAR10*	M. Ligr
YL272 *	*MAT*a *19MYC-FAR10; pre1-1 pre4-1*	M. Ligr
YIV076	*MAT*a *far10Δ::KAN*^{R}	I. Velten
YIV081	*MAT*α *far10Δ::KAN*^{R}	I. Velten
W320	*MAT*α *hct1-Δ1::LEU2*	M. Schwab
W321	*MAT*a *hct1-Δ1::LEU2*	M. Schwab
W267	*MAT*a *cdc20-1*	W. Seufert
W312	*MATa cdc20-1*	W. Seufert
W190	*MAT*a *sic1-Δ1::HIS5*	W.Seufert
E304	*MAT*a *URA3::GAL1:SIC1(HA)1X*	E.Schwob
K699	*MAT*a Wild type W303	K.Nasmyth
FYS29	*MAT*α *cdc23-1*	K. Nasmyth
Y795	*MATa cdc14-3*	A. Amon
Y851	*MAT*a *dbf2-2*	A. Amon
Y859	*MAT*a *cdc5-1*	A. Amon
Y1740	*MAT*a *tem1-3*	A. Amon

Y1863	*MAT*a *bub2Δ::HIS3*	A. Amon
Y2596	*MAT*a *cdc15-2*	A. Amon
Y3963	*MAT*a *lte1Δ::KanMX6*	A.Amon
YL271	*MATa 19MYC-FAR10*	M. Ligr
YL281	*MAT*a *19MYC-FAR10* in *hct1-Δ1*	M. Ligr
HarY004	*MAT*a *far10Δ::KAN^R ; sic1-Δ1 :: HIS5*	This work
HarY010	*MAT*a *far10Δ::KAN^R; cdc23-1 ;URA3::Gal1:SIC1(HA)1X*	This work
HarY015	*MATa cdc14-3; far10Δ::KAN^R; URA3::Gal1:SIC1(HA)1X*	This work
HarY016	*MAT*a *cdc23-1; far10Δ::KAN^R*	This work
HarY019	*MATa cdc14-3; far10Δ::KAN^R*	This work
HarY020	*MAT*a *dbf2-2; far10Δ::KAN^R*	This work
HarY022	*MAT*a *tem1-3; far10Δ::KAN^R*	This work
HarY028	*MAT*a *far10Δ::KAN^R ; hct1-Δ1 :: LEU2*	I. Velten
HarY029	*MAT*a *far10Δ::KAN^R; dbf2-2; URA3::Gal1:SIC1(HA)1X*	This work
HarY031	*MAT*a *far10Δ::KAN^R; tem1-3; URA3::Gal1:SIC1(HA)1X*	This work
HarY034	MATa *19MYC-FAR10* {L - A in the D-box} in W303	This work
HarY035	*MAT*a 19MYC-*FAR10* {L - A in the D-box} in WCG4	This work
HarY036	*MATa 19MYC-FAR10; cdc23-1*	This work
HarY037	MATa *19MYC-FAR10*; *cdc20-1*	This work
HarY038	MATa *19MYC- FAR10; dbf2-2*	This work
HarY040	MATa*19MYC-FAR10; tem1-3*	This work
YR312	*MAT*a Tester strain	R.Hitt
YR320	*MATa* Tester strain	R. Hitt

Table I: **Strains used in this study**. *: Isogenic to WCG4 strain background *his3-11, 15 leu2-3, 112 ura3 Can^S Gal$^+$*. All the other strains are isogenic to W303 background *ade2-1oc leu2, 3-112 his3-11, 15 trp1-1 ura3-1 Can1-100*

2.5.2. *Esherichia coli* strains

DH5α

The *E.coli* strain DH5α was used to amplify the plasmid DNA (*F- endA1 recA1 gyrA96 thiΔ (argF-lacZYA) U169 (80ΔlacZDM15) l- hsdR17 (rK- mK-) supE44 (Nalr) relA1*) Hanahan, 1983

2.5.3. Plasmids used

pYES2 [P_{GAL1} *URA3*]

The high copy 2μ plasmid pYES2 (Invitrogen, Groningen, The Netherlands) consists of an inducible *GAL1* promoter with a *URA3* selection marker for the yeast and an ampicillin selection marker for the *E.coli*. There is a multiple cloning site (MCS) in this vector where yeast genes can be cloned. The genes thus cloned are directly under the control of the *GAL1* promoter whereby their expression can be regulated.

pL001 [P_{GAL1} *2-HA URA3*]

The plasmid pL001 (Personal Communication, M. Ligr) is based on pYES2. It contains a tandem sequence for the epitope of human influenza haemagglutinin protein, called the HA epitope. Genes cloned in the multiple cloning site (MCS) of this vector contain a C-terminal 2-HA epitope and are under the direct control of the *GAL1* promoter.

BR697 [P_{GAL1} *LEU2*]

This multi-copy 2μ plasmid consists of an inducible *GAL1* promoter with a *LEU2* selection marker for the yeast and an ampicillin selection marker for the *E.coli* [Mumberg et al., 1994]. There is a multiple cloning site (MCS) in this vector where yeast genes can be cloned. The genes thus cloned are directly under the control of the *GAL1* promoter whereby their expression can be regulated.

pGB48 [P_{GAL1}*::FAR10 URA3*]

The description for this plasmid can be found in the PhD dissertation (Velten 2000).

pIV017 [P_{GAL1}::*FAR10-2HA URA3*]

The method of construction and a suitable description for this plasmid can be found in the PhD dissertation (Velten, 2000).

pC001 [P_{GAL1}::*FAR10ΔTM*$_{441-478}$ *URA3*]

This plasmid was made my PCR based method. The oligo-nucleotide primers Hpr004F and Hpr005R (containing a stop codon *TAG*) were used to amplify *FAR10* without the C-terminal trans-membrane domain by using pGB48 (P_{GAL1}::*FAR10*) as the template. The PCR product obtained contained an N-terminal *HINDIII* restriction enzyme cleavage site and a C-terminal *SphI* site. The PCR product was then double digested with *HINDIII* and *SphI* restriction enzymes. The digested PCR product was then ligated into the *HINDIII*/*SphI* digested pYES2 vector yielding *FAR10ΔTM*$_{441-478}$ (with a stop codon) under the control of the *GAL1* promoter (P_{GAL1}::*FAR10ΔTM*$_{441-478}$).

pC002 [P_{GAL1}::*FAR10ΔTM*$_{441-478}$*-2HA::URA3*]

PCR based strategy was used to contruct this plasmid. Oligo-nucleotide primers Hpr004F and Hpr007R were used to amplify *FAR10ΔTM*$_{441-478}$ excluding the stop codon from pC001. The PCR product obtained contained an N-terminal *HINDIII* restriction enzyme cleavage site and a C-terminal *NotI* cleavage site. The PCR product was then digested with *HINDIII* and *NotI* restriction enzymes. The cut PCR product was then ligated into the *HINDIII*/*NotI* restricted pYES2-HAC plasmid yielding *FAR10ΔTM*$_{441-478}$*-2HA* under the control of the *GAL1* promoter (P_{GAL1}::*FAR10ΔTM*$_{441-478}$*-2HA*).

pC012 [P_{GAL1}::*FAR10 (RR340, 341AL) URA3*]

This plasmid contains *FAR10* with point mutations in the D-box. The codons for the two arginine residues in the positions 340 (*AGA*) and 341 (*AGG*) were changed to alanine (*GCA*) and leucine (*CTT*) respectively. Additionally, the codon for lysine at 342 was changed to AAG from AAA. The specific mutations were introduced by following a two-stage PCR protocol using QuikChange ™ site-directed mutagenesis method. Two extension reactions were performed in separate tubes using a single template plasmid pIV48 in both cases; one containing the forward primer Hpr011F and the other containing the reverse primer Hpr011R. These primers included the following base changes: *GCACTTAAG* for Hpr011F and *CTTAAGTGC* for Hpr011R. In the single primer

extension reaction, 50-200ng of the template pIV48 plasmid along with 10pmol of the particular primer were extended using 1U of *Pfu* DNA polymerase in 1X *Pfu* buffer/dNTP cocktail in a 50µL reaction. The extension reaction was initiated by preheating the reaction mixture to 95°C for 30s: 1, 3 or 10 cycles of 95°C for 30s, 55°C for 1 min and 68°C for 8 min. Following the completion of the extension reactions, 25µL of the each pair were mixed in one tube along with 1 U of *Pfu* DNA polymerase and subjected to the standard QCM PCR procedure.

Cycling parameters for the Quik Change XL Method

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	18	95°C	50 seconds
		60°C	50Seconds
		68°C	2minute/kb
3	1	68°C	7 minutes

Following the PCR, 10 U of *DpnI* were added (as per QCM protocol), mixed well and incubated at 37°C for another hour before checking for the plasmid yield on agarose gel electrophoresis. This was then subsequently introduced into *E.coli* followed by mini-prep and restriction enzyme analysis. The point mutations introduced an *AflII* cleavage site in *FAR10*, which was confirmed accordingly.

pC015 [*19MYC-FAR10(L343A)::URA3*]

This yeast integrating plasmid based on pL085 contains *FAR10* with point mutations in the D-box. The codon (*CTG*) for leucine at position 343 was mutated to alanine (*GCT*) and the codon for serine at 344 was changed to AGC from TCG. The procedure used to make this plasmid was the same as that used to construct the plasmid pC012. The forward primer used in the site specific mutagenesis PCR was Hpr012F and the reverse primer was Hpr012R. The template plasmid used for this reaction was pL085, which contains *19MYC-FAR10* under the control of its endogenous promoter. The whole procedure yielded *19MYC-FAR10(L343A)* under the control of its own endogenous promoter.

pC017 [*P_{GAL1}::FAR10(L343A) URA3*]

To construct this plasmid, ORF *FAR10* (*L343A*) was amplified from pC015 using the primers Hpr004F and Hpr016R. The primer Hpr016R contained a stop codon *TAG* to terminate the reading frame. The PCR product obtained contained an N-terminus *HINDIII* and a C-terminus *SphI* restriction enzyme cleavage sites. This was then double digested using *HINDIII*/ *SphI* enzymes and inserted into the multi-cloning site [MCS] of similarly digested pYES2 plasmid. The whole procedure yielded the plasmid pC015 which contained *FAR10*(*L343A*) under the control of the *GAL1* promoter [P_{GAL1}*::FAR10*(*L343A*)].

pC021 [*19MYC-FAR10(RR340, 341 AL)::URA3*]

To construct this plasmid, ORF *FAR10 (RR340, 341AL)* was amplified by PCR using pC012 as the template. The forward primer Hpr017F and the reverse primer Hpr016R were used in this reaction. The PCR product obtained contained an N-terminal NotI site and a C-terminal SphI site. The digested PCR product was then inserted into similarly restricted pL086. The final product of this ligation was the plasmid pC021 which contained *FAR10(RR340, 341 AL)* under the control of its endogenous promoter.

pC026 [*P_{GAL1}::FAR10 LEU2*]

This plasmid contains *FAR10* under the control of *GAL1* promoter. *FAR10* was amplified by PCR using pIV48 as the template. Forward primer Hpr019F, which contained a *SpeI* site and the reverse primer Hpr019R, which has an *XhoI* site were used in this reaction. The PCR product was digested with *SpeI* and *XhoI* restriction enzymes and was ligated into similarly digested BR697 plasmid. The whole procedure yielded FAR10 uunder the control of the *GAL1* promoter. [P_{GAL1}*::FAR10 LEU2]*

MOLECULAR BIOLOGY METHODS

2.6.1. Isolation of plasmid DNA from *E.coli*

1.5 mL of stationary culture of cells was centrifuged, washed with water and re-suspended in 100µL of Buffer I (50mM Glucose, 25mM Tris/HCl pH 8, 10mM EDTA pH8). 200µL Buffer II (200mM NaOH, 1% SDS) was added to the cell suspension and gently mixed well and incubated for 5 min on ice. Then 150µL Buffer III (5M Potassium acetate, 25% Acetic acid) was added and incubated for a further 15 min on ice. Then the samples were centrifuged for 10 min and the supernatant was taken into a fresh eppendorf and 700µL of 100% ethanol was added and the samples were kept at –80°C for about 15 min to precipitate the DNA. Subsequently, the samples were centrifuged for 10 min at 13K, supernatant was removed and the pellet was washed with 70% ethanol, dried in a vaccum drier and later dissolved in 50µL TE-buffer (10mM Tris/HCl pH 7.5, 1mM EDTA) containing 100µg of RNaseA.

2.6.2. Isolation of extra-chromosomal DNA from Yeast

A 5mL culture of yeast was centrifuged, washed with water and re-suspended in a 200µL Breaking Buffer (2% TritonX-100, 1% SDS, 100mM NaCl, 10mM Tris/HCl pH 8, 1mM EDTA). To this 200µL of acid washed glass beads (0.5mm, Braun, Melsungen) and 200µL Phenol/Chloroform mixture were added and vortexed continuously for about 5 min followed by centrifugation for 5 min at 13K. About 100µL of the water phase was removed to a fresh eppendorf and 10µL 3M NaoAc (pH 6.0) was added followed by the addition of 280µL of 100% ethanol. The DNA was precipitated at –80°C for about 15 min. This was followed by centrifugation at 13K for 5 min. The supernatant was removed and the pellet was washed with 70% ethanol and later dried and suspended in 25µL TE buffer (10mM Tris/HCl pH 7.5, 1mM EDTA). Later 5-10µL was used for transformation into 50µL of competent *E.coli*.

2.6.3. Whole DNA isolation from Yeast

A 2mL yeast culture was centrifuged, washed with water and re-suspended in 200µL of Breaking Buffer (2% TritonX-100, 1% SDS, 100mM NaCl, 10mM Tris/HCl pH 8, 1mM EDTA). To this 200µL of acid washed glass beads (0.5mm, Braun, Melsungen) and 200µL Phenol/Chloroform mixture were added and vortexed continuously for about 5 min followed by addition of 200µL of water. The samples were then centrifuged for 5

min at 13K. 300µL of water phase was removed to a fresh tube followed by the addition of 1mL of 100% ethanol. DNA was precipitated at –20°C for about 20 min. The samples were then centrifuged for 5 min at 13 K. Supernatant was removed and the pellet dissolved in 400µL of water containing 4µL of RNase (10µg/ml) and samples were kept at 37°C for 5 min. Then 10µL of ammonium acetate (5M, pH 6.0) and 1mL of 100% ethanol were added and the samples were kept at –80°C for 10 min. Samples were then centrifuged for 5 min at 13K and the pellet was washed with 70% ethanol and dried in a vaccum drier and later dissolved in 25µL water or TE buffer (10mM Tris/HCl pH 7.5, 1mM EDTA).

2.6.4. DNA digestion with Restriction Endonucleases

Restriction endonucleases cleave DNA at specific sequences and have applications in cloning and nucleotide sequencing of DNA molecules. Each standard DNA digestion reaction included 0.3 – 1.0 µg of plasmid DNA (2-5µL from plasmid mini-prep) and 2-5 units of restriction enzyme used for the cleavage reaction (Roche Diagnostics, Mannheim). All the compatible buffers used in the final reaction volume of 20µL were of 1X concentration, diluted from an initial conc. of 10X. The samples for digestion were incubated at 37°C for about 2 hours.

2.6.5. Agarose Gel electrophoresis

DNA samples were run in agarose gels made in 1X TAE buffer (40mM Tris/Sodium acetate pH 7.0 2mM EDTA) and were identified by comparing with the DNA molecular weight markers. The DNA probes were run along with loading standards (Roche Diagnostics, Mannheim) in freshly casted 0.8%-1.0% agarose gels with 0.5µg/ml of ethidium bromide, in electrophoresis apparatus (Amersham-Pharmacia, Freiburg). TAE buffer was used as both the gel as well as the running buffer. Upon completion of electrophoresis gel documentation was done using a gel documentation system (MWG-Biotech, Ebersberg) in UV light (302nm) and later photographed.

2.6.6. Ligation of DNA fragments

A standard ligation reaction of a final volume 10µL contained 300-600ng DNA, 1X ligase buffer and 1 U of T4-DNA ligase. The reaction was carried out over-night at 18°C.

2.6.7. Yeast and *E.coli* transformations

2.6.7.1. Transformation of yeast cells

Extra chromosomal self replicating plasmids or to make mutation sin the chromosome of yeast genes through recombination was done by Lithiumacetate method (Ito et al., 1983). 1mL of an overnight stationary culture was diluted in 25mL of fresh culture medium and the cells were grown at 25°C/30°C to logarithmic phase to an O.D.600 of 0.5-0.8. 10mL of this main culture was centrifuged at 3000rpm and washed with 5mL of autoclaved water. These cells were again washed with 5mL of TE/LiAc buffer (10mM Tris/HCl, 100mM Lithiumacetate, 1M EDTA, pH 7.5) and later re-suspended in 50µL TE/LiAc buffer. To this cell suspension, 2-5µL of plasmid-DNA (approx. 1µg), 5µL of denatured Herring sperm DNA and 300µL of freshly prepared 40% PEG-4000 solution in TE/LiAc buffer were added. The cell suspension was kept for 30 min at 30°C and was followed by a heat-shock for at least 15 min at 42°C. Then the cells were centrifuged at 3000 rpm for 4 min and later re-suspended in 100µL TE buffer followed by plating on selection medium (Gietz and Schiestl, 1991; Gietz et al., 1995). Agar plates were incubated at the permissive temperature for 2-4 days. ** All the buffers used in this method were autoclaved at 121°C for 30 min.***

2.6.7.2. Preparation of frozen competent *E.coli* cells

A single colony of DH5α was inoculated into 5mL of LB medium to make an overnight pre-culture. 1mL of this was inoculated in 100mL of LB medium in a 500mL Erlenmyer flask. The culture was allowed to grow at 37°C to an $O.D._{600}$ of 0.3-0.5. Later, cells were harvested in pre-chilled 50mL falcon tubes by centrifuging at 3000 rpm for 5 min at 4°C. Supernatant was removed and cells were washed with 50mL of ice-cold 100mM $MgCl_2$, followed by gently re-suspending the cells in 30mL of TC buffer (75mM $CaCl_2$, 10mM Tris/HCl pH 7.5). Cells were let in ice for a further 20-30 min and later harvested as done before. The cell pellet was gently re-suspended in 2mL of TC:Glycerol 85:15 (V/V) and about 100µL of these was aliquoted into sterile pre-chilled eppendorfs. The aliquoted cells were then stored at –80°C. ** All the buffers used in this method were autoclaved at 121°C for 30 min.***

2.6.7.3. *E.coli* transformation with frozen competent cells

Frozen competent cells were thawed on ice. About 5-10µL DNA was added and incubated on ice for 30 min. This was followed by heat shock at 42°C for about 80-90 seconds. Cells were again kept on ice for a further 20 min and later were mixed in a sterile glass tube containing 1mL of LB medium and incubated at 37°C for 1 hour with shaking. After this, the cells were collected by centrifugation at 3000 rpm for 4 min and re-suspended in 100µL of fresh LB medium and plated on to LB-agar plates containing ampicillin followed by incubation at 37°C O/N.

** All the buffers used in this method were autoclaved at 121°C for 30 min.***

2.6.8. Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is used to amplify specific DNA fragments using synthetic oligonucleotides called primers. These hybridize to specific regions on the DNA template and facilitate extension reaction. This PCR reaction was carried out by using either the Vent DNA polymerase (Biolabs, USA) or with Taq DNA polymerase (Roche Diagnostics, Mannheim)
A typical 50µL PCR mixture contains
0.4mM each of dATP, dCTP, dGTP and dTTP.
1mM each of the starting oligonucleotide primers.
1pg of the DNA template
and 2 or 8mM of $MgSO_4$ (for Vent polymerase) or $MgCl_2$ (For Taq polymerase) respectively.

The standard reaction included a “hot start” starting with denaturing of the DNA at 94°C for 3 min. To this 1.0 U of the polymerase was added and DNA was allowed to be amplified for 20-30 cycles. Each typical cycle included a 45 sec hybridizing phase at 54-68°C during which the primers hybridize with the template. This was followed by an extension phase at 72°C (1min/kb of the DNA amplified) and a final phase of denaturing the DNA at 94°C before starting the whole cycle afresh.

Optimal temperature for the hybridization of the primers, the number of cycles and the concentration of various components were chosen accordingly. When the reaction was complete the samples were kept at 4°C and later analysed by agarose-gel electrophoresis.

2.6.9. Two-Step Gene Replacement Strategy

Method of contruction of yeast strains yL268, yL271, yL272 and yL281 are described elsewhere [Velten, 2000]. However strains HarY034, HarY035, HarY036 and HarY037 were made as follows. Plasmids pL085 and pC015 were linearized by digestion with *SspI*. The linear fragments were checked on an agarose gel and isolated by using agarose-gel extraction kit. Linear pL085 was introduced into *cdc20-1* and *cdc23-1* strains where as linearized pC015 was introduced into W303 and WCG4a by standard method of yeast transformation. Single colonies from CM-URA plates were streaked out on YPD plates. Individual colonies from this step were streaked out on 5-FOA containing plates from which viable clones were picked up and confirmed for the presence of Myc tag by Western Blotting procedure. Strains were also verified by PCR analysis.

2.7. Spot dilution tests.

All the spot dilution tests mentioned in this work were carried out as follows. Appropriate strains were cultured at either 25°C/30°C till logarithmic phase. 1 $O.D._{600}$ cells were collected, and later re-suspended in 100µL autoclaved 1X TE buffer from which serial dilutions till 10^{-5} were made by taking 10µL of the cell suspension from previous dilution and diluting in fresh 90µL TE buffer. Finally 2.5µL of each dilution was spotted onto required culture media containing 2% agar.

2.6.9. Synthetic Oligonucleotide primers

Hpr004F
ATCACTGACGAAGCTTATGACTGGTCCTGGACC

Hpr005R
GATAGTACAGGCATGC **GAT**CTTAGGATTCATGCCCGG

Hpr007R
GATAGTACAGGCGGCCGCCTTAGGATTCATGCCCGG

Hpr011F
GGTAAAGCTGCAAAATGGATTAGCACTTAAGCTGTCGGGGAAATACGAAAAGTTATCG

Hpr011R
CGATAATCTTTTCGTATTTCCCCGACAGCTTAAGTGCTAATCCATTTTGCAGCTTTACC

Hpr012F
GCAACTGGTAAAGCTGCAAAATGGATTAAGAAGGAAAGCTAGCGGGAAATACGAAAAGAT
TATCG

Hpr012R
CGATAATCTTTTCGTATTTCCCGCTAGCTTTCCTTCTTAATCCATTTTGCAGCTTTACCAGTT
GC

Hpr013F
GCTGCAAAATGGATTAAGAAGGAAAGCTAGCGGGAAATACGAAAAGATTATCG

Hpr013R
CGATAATCTTTTCGTATTTCCCGCTAGCTTTCCTTCTTAATCCATTTTGCAGC

Hpr014F
GGATTAAGAAGGAAAGCTAGCGGGAAATACGAAAAGATTATCG

Hpr014R
CGATAATCTTTTCGTATTTCCCGCTAGCTTTCCTTCTTAATCC

Hpr016R

GATAGTACAGGCATGCCTAGTTGGGGGAAAGGATC

Hpr017F

GATAGTACAGGCGGCCGCATGACTGGTCCTGGACC

Hpr019F

CGTAGACTAGTATGACTGGTCCTGGACCTG

Hpr019R

CCGCCGCTCGAGCTAGTTGGGGGAAAGGATC

Hpr021

CGGATCGGACTACTAGC

Hpr022

TCCTTTTCGGTTAGAG

Hpr023

CGGTCAAAGAATTGGCTC

Hpr024

GGTTAGAGCGGATGTGG

2.7. Protein Biochemistry

2.7.1. Preparation of yeast cell extracts

2.7.1.1. Denaturing cell lysis (Alkaline Lysis)

For the denaturing cell lysis harvested yeast cells were washed with 1mL of cold water and later re-suspended in the same amount of cold water. To this 150µL of the lysis solution (1.85M NaOH, 7.5% β –ME) was added and vortexed at RT for 4 min followed by incubation on ice for 15 min. Later 150µL of 50% TCA was added and incubated for a further 10 min on ice. The protein precipitates were centrifuged for 10 min at 14K. Supernatant was removed and samples were given a short spin at 14K and the rest of the supernatant was removed. The protein pellet was re-suspended in 50/100µL of urea buffer (8M Urea, 200mM Tris/HCl, pH6.8, 1mM EDTA, 5% SDS, 0.03% Bromophenol Blue, 1% (V/V) β–ME or 1.5% DTT) by shaking at 37°C. Finally, proteins were denatured by boiling at 98°C for 3 min. Samples were later centrifuged at 13K for 5 min and appropriate amount of the protein samples (5µL/10µL) were loaded on to pre-casted SDS-polyacrylamide gels.

2.7.1.2. Native cell lysis

To make native cell extracts, harvested yeast cells were washed with 1mL cold water and later re-suspended in 400µL Breaking buffer (50mM Tris/HCl, pH 7.5) containing Complete Inhibitor mix (Roche Diagnostics, Mannheim) and 1mM PMSF. To this 200µL of acid washed glass beads were added and cells were lysed by vortexing followed by cooling on ice. Samples were centrifuged at 2000 rpm for 5 min at 4°C. Supernatant was removed to fresh tubes and used for further experiments.

2.7.2. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Gelelectrophoresis of the proteins was carried out by a discontinuous SDS-PAGE (Laemmlli, 1970) in Mini-Protean II Dual Slab Cells (Biorad, Muenchen). The gels were of 7.5% - 9.0% conc. and the protein samples along with the loading marker (Biorad) were electrophoresed at the RT. When the bromophenol band was electrophoresed out, the proteins were transferred to the nitrocellulose membrane or were detected in the cell by Coomassie staining.

2.7.3. Protein/Immuno Blotting (Western Blotting)

The protein samples electrophoresed by SDS-PAGE were transferred from the gel to nitrocellulose membrane(s) (Gelman Laboratory, Roßdorf) in a Semi-Dry blotting apparatus (Fröbel, Lindau) using the Blotting Buffer (25mM Tris, 190mM Glycine, 20% Methanol). The transfer was for 90 min at 75mA per gel. To detect proteins on the membrane ECL™-Detection system (Amersham-Pharmacia, Freiburg) was used following manufacturers instructions.

Non-specific sites on the nitrocellulose membrane were blocked using 10% nonfat dry milk powder in TBS-T (20mM Tris/HCl, pH 7.6 137mM NaCl, 0.1% (w/v) Tween-20) or PBS-T (80mM Na_2HPO_4, 20mM NaH_2PO_4, 100mM NaCl, 0.1% (w/v) Tween-20) by incubating the membranes overnight at 8°C. To detect the proteins, membranes were briefly washed with TBS-T/PBS-T buffer and incubated for 1 hour at RT with primary antibody suitably diluted (1:10,000) in TBS-T/PBS-T. This was followed by incubation of the blots with HRPO conjugated secondary antibody suitably diluted (1:10,000) in the same buffer as that used for primary antibody for a further 1 hour. Later the blots were washed 6 times with fresh TBS-T/PBS-T with a gap of 10 min between each wash. Later the proteins were detected using ECL™ chemiluminiscence detection system by following manufacturers instructions.

2.7.3. Stripping and re-probing of the membranes

Antibodies bound to the proteins immobilized on the nitrocellulose membranes were stripped by using a harsh stripping buffer (88mM β-ME, 1% SDS and 62.5 mm Tris/HCl pH 6.7) for 30 min at 50°C with occasional agitation. This was followed by a washing step with either TBS-T/PBS-T depending on the requirement for about 1 hour and treated as above for further analysis.

2.7.3. Coomassie staining of Polyacryamide gels

To detect proteins in the polyacryamide gel, these were stained with Coomassie Blue. The gel was agitated at low speed in the Staining Solution (0.02% Coomassie Brilliant Blue 250 (Serva, Heidelberg), 25% (v/V) Ethanol, 8% Acetic acid) for 30 min at RT. To remove excess stain, the gel was de-stained with De-staining Solution (Methanol: Water: Acetic acid in 5:5:1) till the protein bands were seen clear.

2.7.4. Cycloheximide chase

Yeast strain(s) were cultured till an $O.D._{600}$ of 0.2 - 0.25 in 25mL of the YPD medium. They were later harvested by centrifuging the cultures at 3500 rpm for 5 min. Cells were later re-suspended in 2.5mL of fresh YPD medium and 125µL of CHX stock (10mg/ml) was added and immediately 500µL sample was taken into fresh tube and additionally 500µL of 30mM NaN_3 solution was added. This sample served as the initial zero time point control and was immediately freezed at –80°C. This process was repeated each time cells were collected at every time point. After the chase, the frozen cells were thawed, centrifuged for 3 min at 2500 rpm, washed with ice-cold water for two times and then proceeded to alkaline lysis as referred to before in the section 2.7.1.1.

2.7.5. Indirect Immunoflourescence microscopy

This method is based on the selective binding of specific antibody to a protein in the cell thereby indicating its localization. In this method (Pringle et al., 1989), 1mL of the logarithmic cell culture was fixed by adding 125µL Potassium phosphate buffer (1M, pH 6.5) and 125µL of 37% formaldehyde solution and rotated for 1 hour at RT on an eppendorf rotator. Cells were later centrifuged at 1000 rpm for 5 min and washed three times with SP buffer (1.2M Sorbitol, 100mM Potassium phosphate, pH 6.5). Sphaeroblasts of these cells were made by re-suspending them in 1mL of SP buffer containing 20mM β-ME and 10µL Zymolyase 100T (15mg/ml) and incubating at 30°C for 30 min. The sphaeroblasts were washed three times with SP buffer and 10µL of this were put on each well of a diagnostic slide (Serolab, Aidenbach), which was previously coated with Poly-L-Lysine. Sphaeroblasts were allowed to settle in the wells for 15 min and later washed three times with 20µL PBS buffer (53mM Na2HPO4, 13mM NaH2PO4, 75mM NaCl) and later incubated for 5 min with 20µL PBT (1% BSA, 0.1% (w/v) Triton X-100 in PBS). Later 20µL of suitably diluted primary antibody was added and incubated in a humid chamber for 2 hours. Subsequently, washed with PBT and incubated for a further 90 min with secondary antibody. Finally, these were washed with PBS and 2µL mounting solution (80% Glycerol, 0.025µg/ml DAPI, 0.1% p-Phenlynediamine in PBS) was added and later covered the slide with a cover slip. Cells were observed under a fluorescence microscope (Axioskop 100MC, Zeiss, Oberkochen).

3. RESULTS

3.1. A genetic screen to search for new substrates of the proteasome in yeast, *Saccharomyces cerevisiae* and further studies on *FAR10*.

To identify new substrates of the proteasome, an extensive cDNA based overexpression screen was done. This screen was based on the hypothesis that overproduction of potential proteasomal substrates would cause lethality in a yeast strain with a defect in the proteasome, the *pre1-1 pre4-1* mutants (yHi29/14) [Hilt et al., 1993; Ligr. M et al 2001; Velten, 2000]. One of the identified candidate genes, *FAR10* was subjected to further study.

3.2. Overexpression of *FAR10* and *FAR10(HA)$_2$* is toxic to *pre1-1 pre4-1* proteasome mutants.

To confirm the toxic effect of *FAR10* overexpression demonstrated in the screen and, in addition to check the functionality of an epitope tagged version, *FAR10* was inserted into the 2μ-plasmids pYES2 and pYES2-HAC using a PCR based strategy. This approach yielded plasmid encoded *GAL1::FAR10* and *GAL1::FAR10(HA)$_2$* respectively [Velten, 2000]. The corresponding plasmids were subsequently introduced into *pre1-1 pre4-1* mutants (yHi29/14) by transformation. The transformants were then checked by spot dilution tests. *pre1-1 pre4-1* mutants (yHi29/14) containing empty pYES2 and pYES2-HAC vectors were used as controls. *pre1-1 pre4-1* mutants encoding *GAL1::FAR10* and *GAL1::FAR10(HA)$_2$* showed a severe growth defect after induction of *FAR10* expression on galactose containing medium [Fig. 1]. These results show that overexpression of *FAR10* and *FAR10(HA)$_2$* as compared to pYES2 and pYES2-HAC empty vectors causes a strong growth effect. As expected no effect was observed under repression conditions (Glucose medium). Additionally, these observations confirm that tagging doesn't influence lethality and that the C-terminal 2-*HA* tagged version of *FAR10* is functional.

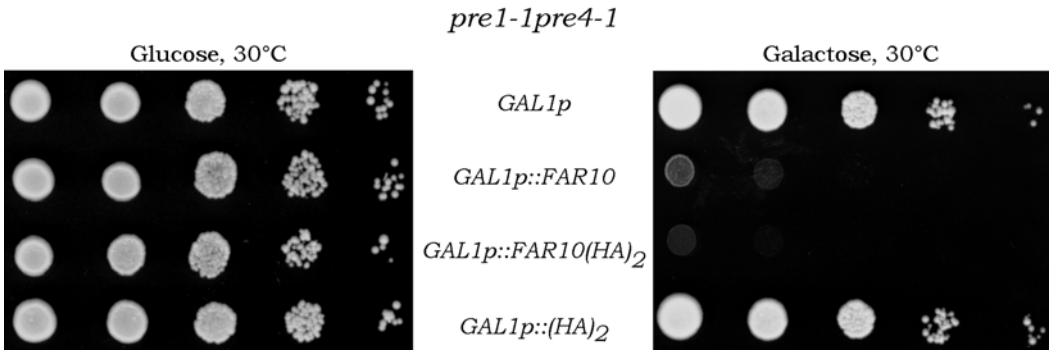


Figure 1: Overexpression of *FAR10* and *FAR10(HA)$_2$* is toxic to *pre1-1 pre4-1* proteasome mutants. *pre1-1 pre4-1* mutants (yHi29/14) expressing pYES2 vector derived *GAL1::FAR10* or *GAL1::FAR10(HA)$_2$* were grown in raffinose medium to logarithmic phase. Ten fold serial dilutions were spotted onto SC media containing either glucose or galactose. The plates were then incubated at 30°C for three days.

3.2.1. Far10 is stabilized in *pre1-1 pre4-1* proteasome mutants.

Previous data indicated that Far10 was a novel substrate of the proteasome. To confirm this wild type [yL268, yIV017] and *pre1-1 pre4-1* proteasome mutants [yL272, yIV036] containing chromosomally encoded N-terminal 19-Myc tagged Far10 [Ligr. M. personal communication] or C-terminal 3-HA-tagged Far10 [Velten, 2000] under the native *FAR10* promoter were grown to logarithmic phase in YPD medium. The stability of Far10 was followed by cycloheximide chase analysis. Both the N-terminal 19-Myc tagged and the C-terminal 3-HA tagged versions of Far10 were rapidly degraded in the wild type yeast strain. In contrast in *pre1-1 pre4-1* mutants marked stability was observed [Fig. 2] reinforcing the fact that it's a *novel* substrate of the proteasome.

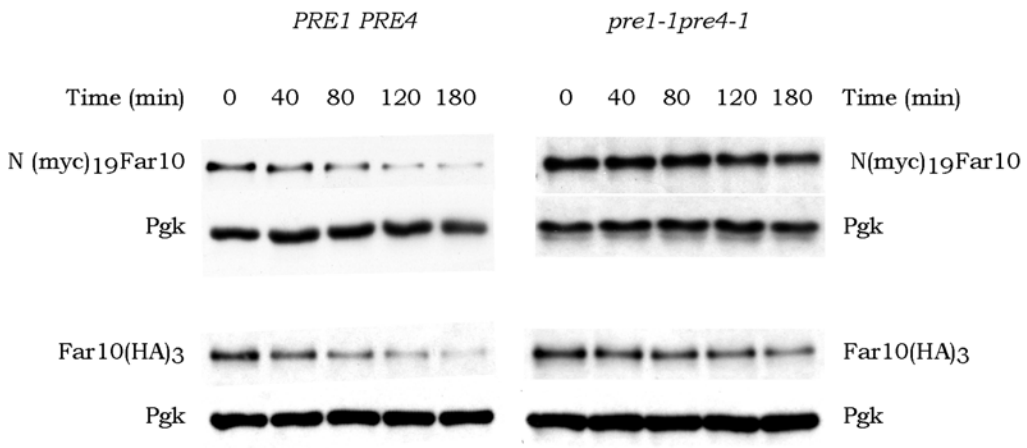


Figure 2: Chromosomally encoded Far10 is stabilized in *pre1-1 pre4-1* proteasome mutants. N(Myc)$_{19}$Far10 and Far10(HA)$_3$ expressed from the native *FAR10* promoter were subjected to cycloheximide chase analysis. The strains, yL268 [*19MYC-FAR10*], yL272 [*19MYC-FAR10 pre1-1 pre4-1*], yIV017 [*FAR10-3HA::HIS5*] and yIV036 [*FAR10-3HA::HIS5 pre1-1pre4-1*] were grown to logarithmic phase in YPD medium. Approximately 2 O.D.$_{600}$/ml cells were taken and cycloheximide was added to a final concentration of 0.5 mg/ml. At indicated time-points 500 µl of the culture was taken and subjected to alkaline lysis followed by western blot analysis. The proteins were detected by Anti-Myc and Anti-HA antibodies as required (dilution of 1:10000 in TBS-T). Phosphoglycerate kinase (Pgk) was used as a loading control and detected by Anti-Pgk antibodies (dilution 1:10000 in PBS-T).

3.3. Far10 stability is regulated through APC-Cdc20 complex.

The majority of the proteasomal substrates are marked for degradation through poly-ubiquitination. This process in yeast requires a ubiquitin-activating enzyme (E1), one of the several ubiquitin-conjugating enzymes (E2s) and a substrate specific ubiquitin protein ligase (E3) [Ciechanover, 1998; Glickmann and Ciechanover, 2002]. The anaphase-promoting complex (APC) is a substrate specific ubiquitin ligase with a vital function in mitosis. The APC is a high molecular mass complex composed of at least 11 different subunits [Zachariae et al., 1998; Zachariae and Nasmyth, 1999; Page and Hieter, 1999]. To be fully active this complex requires the binding of substrate specific co-activators such as Cdc20 or Hct1 resulting in distinct assemblies called APC^{Cdc20} or APC^{Hct1}. Interestingly recent studies have shown that at least in case of frog egg extracts

the substrates seem to be recognized directly by the APC core complex [Yamano et al., 2004]. Cdc20 and Hct1 bind and recruit their substrates to the APC [Burton and Solomon, 2001; Pfleger et al., 2001]. Ubiquitination of target proteins by this activated complex depends on the presence of defined sequence elements in the substrate, the destruction box (D box) [Glotzer et al., 1991; King et al., 1996] and the KEN box [Pfleger and Kirschner, 2000]. Prominent substrates of the APC include B-type cyclins like Clb2 [Murray 1995; King et al., 1996] mitotic regulators such as Pds1 [Cohen-Fix et al., 1996] and structural elements of the mitotic machinery like the spindle-associated protein Ase1 [Juang et al., 1997].

Previous results showed that overexpression of *FAR10* in *pre1-1 pre4-1* double mutants results in a defined cell cycle arrest. Cells stop growth as large budded cells containing 2N DNA content and intact central spindle indicating an arrest in late anaphase. Reasoning this, involvement of Far10 in cell cycle and a possible role of APC or SCF complex in its degradation was suggested. To further support this idea the effect of overproduction of *FAR10* was investigated in APC and SCF mutants. Thus *FAR10* was overexpressed in *cdc16-123* (FYS28) and *cdc23-1* (FYS29) APC mutants and *cdc53-1*, *skp1-11* and *skp1-12* SCF mutants [Velten, 2000]. Overexpression of *FAR10* had no discernable effect in the mentioned SCF complex mutants [data not shown]. However a relatively moderate effect was observed in *cdc16-123* mutants where as a strong growth defect was observed in *cdc23-1* (APC) mutants.

3.3.1. Overexpression of *FAR10* and *FAR10(HA)$_2$* is toxic to *cdc23-1* APC mutants.

To substantiate the lethal effect in *cdc23-1* mutants (FYS29), plasmids encoding *GAL1::FAR10* and *GAL1::FAR10(HA)$_2$* were introduced into *cdc23-1* mutants by transformation. The transformants were checked for growth by spot dilution tests using in addition *cdc23-1* mutant cells containing empty pYES2 and pYES2-HAC vector as controls. Overproduction of *FAR10* and *FAR10(HA)$_2$* in *cdc23-1* mutants (FYS29) resulted in a severe growth defect as shown on galactose containing medium at 25°C [Fig. 3].

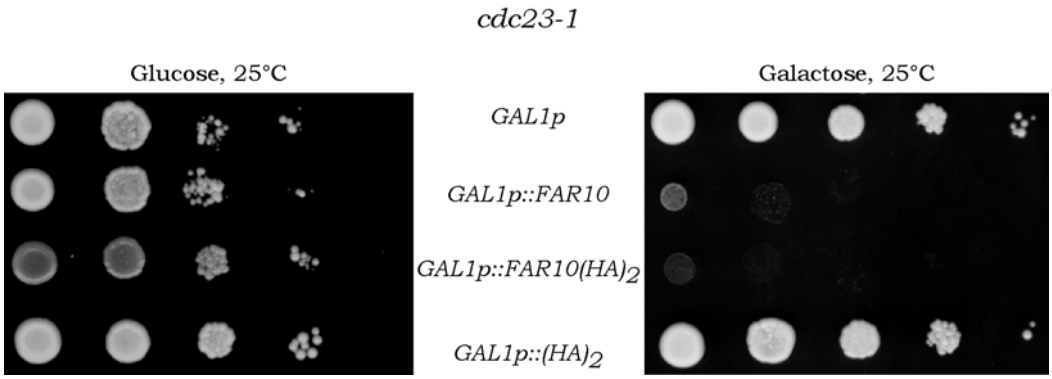


Figure 3: **Overexpression of *FAR10* and *FAR10(HA)₂* is toxic to *cdc23-1* APC mutants.** *cdc23-1* mutants (FYS29) expressing pYES2 vector derived *GAL1::FAR10* or *GAL1::FAR10(HA)₂* and controls containing empty vector pYES2/pYES2-HAC were grown in raffinose medium at 25°C to logarithmic phase. Ten fold serial dilutions were then spotted onto SC media containing either glucose (repression conditions) or galactose (induction of *FAR10*). The plates were then incubated at 25°C for three days.

3.3.2. Far10 is stabilized in *cdc23-1* APC mutants.

To confirm a possible role of the APC in Far10 degradation the stability of Far10 was studied in APC mutant cells. Wild type (yL271, Ligr. M. personal communication) and *cdc23-1* (HarY036) mutants containing chromosomally encoded N-terminal tagged $(Myc)_{19}$Far10, under the native *FAR10* promoter, made by two-step gene replacement strategy [see Materials and Methods] were grown to logarithmic phase in YPD medium. The stability of Far10 was then followed by cycloheximide chase analysis. $N(myc)_{19}$Far10 was rapidly degraded in the wild type yeast strain, whereas it was clearly stabilized in *cdc23-1* mutants confirming the role of APC in degradation of Far10 [Fig. 4].

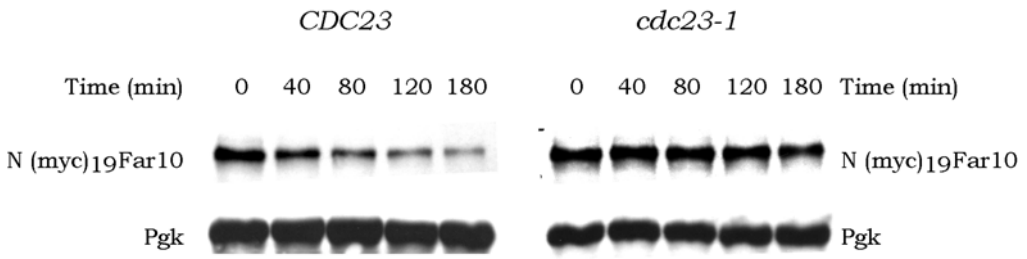


Figure 4: Chromosomally encoded Far10 is stabilized in *cdc23-1* mutants. N(myc)$_{19}$Far10 expressed from native *FAR10* promoter was subjected to cycloheximide chase analysis. The strains, yL271 [*19MYC-Far10*] and HarY036 [*19MYC-FAR10 cdc23-1*] were grown to logarithmic phase in YPD medium. Approximately 2 O.D.$_{600}$/ml cells were taken and cycloheximide was added to a final concentration of 0.5 mg/ml. At the indicated time-points 500 µl of the culture was taken and subjected to alkaline lysis followed by western blot analysis. N(myc)$_{19}$Far10 was detected by Anti-Myc antibodies (dilution of 1:10000 in TBS-T). Pgk was used as a loading control and detected by Anti-Pgk antibodies (dilution 1:10000 in PBS-T).

3.3.3. Degradation of Far10 depends on Cdc20.

The anaphase-promoting complex requires the substrate specific co-activators Cdc20 and Hct1/Cdh1 for regulatory proteolysis. Since the involvement of APC in the degradation of Far10 was demonstrated, we were interested to know whether mutants defective in one of these co-activators were susceptible to HEL effect and which co-activator was required for the degradation of Far10. To check this, *FAR10* and *FAR10(HA)$_2$* were overexpressed. *cdc20-1* point mutants (W267) which at the restrictive temperature of 37°C arrest in metaphase and *hct1-Δ1::LEU2* null mutant strain (W320) were used. *HCT1* is not an essential gene although it is required for Clb2 degradation in late mitosis and maintenance of a low Clb-CDK level in the G_1 phase of the cell cycle.

3.3.3.1. Overexpression of *FAR10* and *FAR10(HA)$_2$* is toxic to *cdc20-1 and hct1-Δ1::LEU2* mutants.

To demonstrate a possible synthetic dosage effect of Far10 overexpression in cells defective in Cdc20 or Cdh1/Hct1, plasmids encoding *GAL1::FAR10* and *GAL1::FAR10(HA)$_2$* were introduced into *cdc20-1* (W267) and *hct1-Δ1::LEU2* mutants (W320) by transformation. The transformants were then checked for growth by serial spot dilution tests using in addition mutant cells containing empty pYES2 and pYES2-HAC vector as controls. As seen in the Fig. 5 induction of *FAR10* and *FAR10(HA)$_2$* on galactose containing medium resulted in strong toxic effects in *cdc20-1* (A) whereas a slight synthetic dosage effect was seen in *hct1-Δ1::LEU2* mutants [see Fig. 19A]. As expected no effect was observed under repression conditions (glucose medium).

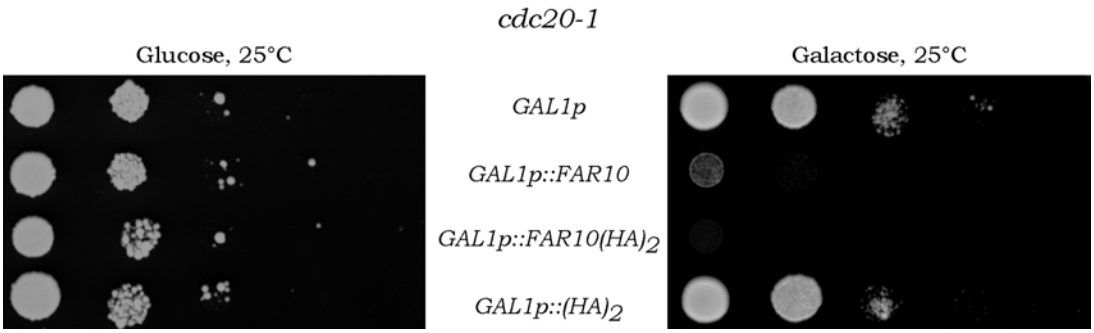


Figure 5: Overexpression of *FAR10* and *FAR10(HA)$_2$* is toxic to *cdc20-1* mutants. *cdc20-1* mutants (W267) expressing pYES2 vector derived *GAL1::FAR10* or *GAL1::FAR10(HA)$_2$* and controls containing pYES2/pYES2-HAC vector were grown in raffinose medium to logarithmic phase. Ten fold serial dilutions were spotted onto SC media containing either glucose or galactose. The plates were incubated at 25°C for three days. See Fig. 19A for overexpression based toxicity in *hct1-Δ1* mutants.

3.3.3.2. Far10 is stabilized in *cdc20-1* mutants whereas a deletion of *HCT1* has no effect on its degradation.

To investigate a possible role of Cdc20 or Hct1 in the degradation of Far10, wild type [yL271 Ligr. M. personal communication], *cdc20-1* mutants (HarY037) and *hct1-Δ1::LEU2* [yL281 Ligr. M. personal communication] were grown to logarithmic phase in YPD medium. These were then subjected to cycloheximide chase analysis. $N(myc)_{19}$Far10 was rapidly degraded in the wild type strain and in *hct1-Δ1::LEU2* mutant whereas it was stabilized in *cdc20-1* mutants [Fig. 6] confirming the specific role of Cdc20 in the degradation of Far10.

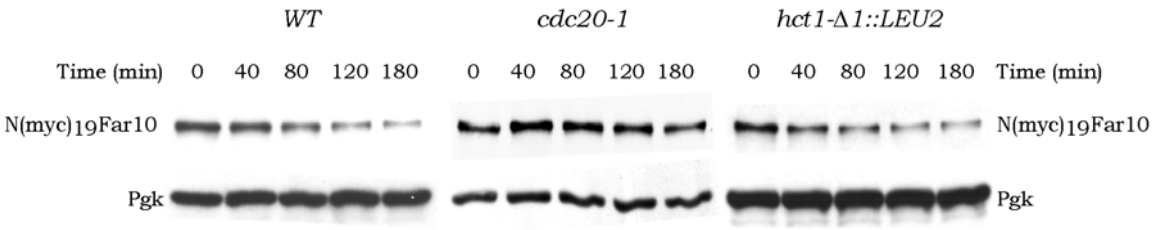


Figure 6: Chromosomally encoded Far10 is stabilized in *cdc20-1* mutants but not in *hct1-Δ1::LEU2* mutants. The strains, yL271 [*19MYC-FAR10*], HarY037 [*cdc20-1 19MYC-FAR10*] and yL281 [*19MYC-FAR10 hct1-Δ1::LEU2*] expressing $N(Myc)_{19}$Far10 from native *FAR10* promoter were grown to logarithmic phase in YPD medium. Approximately 2 $O.D._{600}$/ml cells were taken and cycloheximide was added to a final concentration of 0.5 mg/ml. At the indicated time-points 500 µl of the culture was taken and subjected to alkaline lysis followed by western blot analysis. $N(myc)_{19}$Far10 was detected by Anti-Myc antibodies (dilution of 1:10000 in TBS-T). Pgk was used as a loading control and detected by Anti-Pgk antibodies (dilution 1:10000 in PBS-T).

3.4. Ubiquitinated forms of Far10 accumulate in *pre1-1 pre4-1* proteasome mutants.

Substrates selected for proteasome mediated degradation are usually marked by poly-ubiquitin chains. Previous data indicated that higher molecular weight forms of Far 10 are accumulated in *pre1-1 pre4-1* mutants [Ligr. personal communication]. To demonstrate ubiquitination of Far10 *in vivo*, wild type, yIV17 (*FAR10-3HA::HIS5*), yIV36 (*FAR10-3HA::HIS35 pre1-1 pre4-1*) and yHi29/14 (*pre1-1 pre4-1*) strains were grown to logarithmic phase. About 10 $O.D._{600}$ cells were collected and subjected to glass bead lysis at RT in 100% ethanol containing freshly dissolved NEM. The ethanol/NEM lysates were further processed and later subjected to immunoprecipitation followed by immunodetection [Laney and Hochstrasser, 2002]. As can be seen in the Fig. 7, ubiquitinated forms of $Far10(HA)_3$ accumulate in *pre1-1 pre4-1* proteasome double mutants.

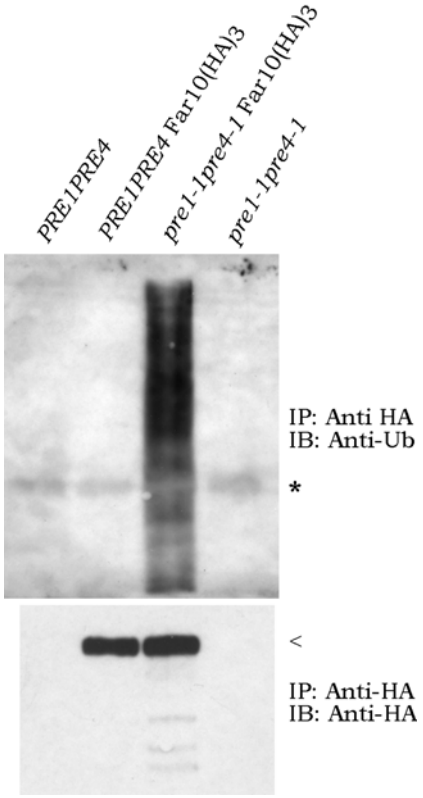


Figure 7: $Far10(HA)_3$ is polyubiquitinated *in vivo*. Ubiquitination was assayed by immuno-precipitation of $Far10(HA)_3$ from ethanol/NEM lysates with anti-HA antibodies (mouse monoclonal 16B12, Covance Research Products) followed by immunoblotting

with anti-Ub antibodies (1:1000 dilution; top: mouse monoclonal P4G7, Covance Research Products) and anti-HA antibodies (1:5000 dilution; bottom: mouse monoclonal 16B12, Covance Research Products). Extracts were made from cells expressing Far10$(HA)_3$ under the native promoter in the wild type and *pre1-1 pre4-1* mutants. Untagged wild type and *pre1-1 pre4-1* mutants were used as controls. The anti-HA immunoreactivity that extends below the position of full length Far10$(HA)_3$ (marked by an arrow head) is suggested to represent degradation intermediates accumulated in proteasome mutants. ***** Indicates IgG cross-reactivity.

3.5. Degradation of Far10 is independent of a potential destruction box [D-box].

Numerous cell cycle related domains/motifs are found within the Far10 sequence, the most prominent being a distinct destruction box [D-box], a CDK recognition element [CDRE] and two consensus CDK phosphorylation sites which may indicate that Far10 is a CDK substrate as well [Fig. 8]. All known substrates of Cdc20 identified till date show an obvious D-box, which is a nine amino acid conserved motif. The consensus sequence of the destruction box is RXXLXXVXN/D/E where R is arginine, X can be any amino acid residue, L is leucine, V is valine and N/D/E are glutamine/aspartate/glutamate, respectively. Arginine in the first position followed by leucine at the fourth position are known to be the most conserved. It is known that the mutation of these conserved amino acids of the D-box is sufficient to prevent both ubiquitination and proteolysis of cyclins A and B suggesting that the D-box serves as a ubiquitination signal [King et al., 1996] though it lacks any potential lysine residue as the ubiquitination site. Far10 being a *bonafide* Cdc20 substrate we found a potential D-box motif with the sequence: ^{340}RRKLSGKYE^{348}. Henceforth, the role of this motif in the degradation of Far10 was investigated by selectively mutating 1) the first two arginine residues to alanine and leucine respectively and 2) leucine at the fourth position to alanine. If mutation of this D-box leads to stabilization a synthetic dosage effect is expected after overexpression in wild type. Based on this idea overexpression effect was studied in the wild type yeast strain. Moreover, proteolytic stability of N$(Myc)_{19}$Far10 containing D-box mutations was also investigated in the wild type yeast strains, W303 and WCG4.

3.5.1. Protein sequence and associated features of Far10

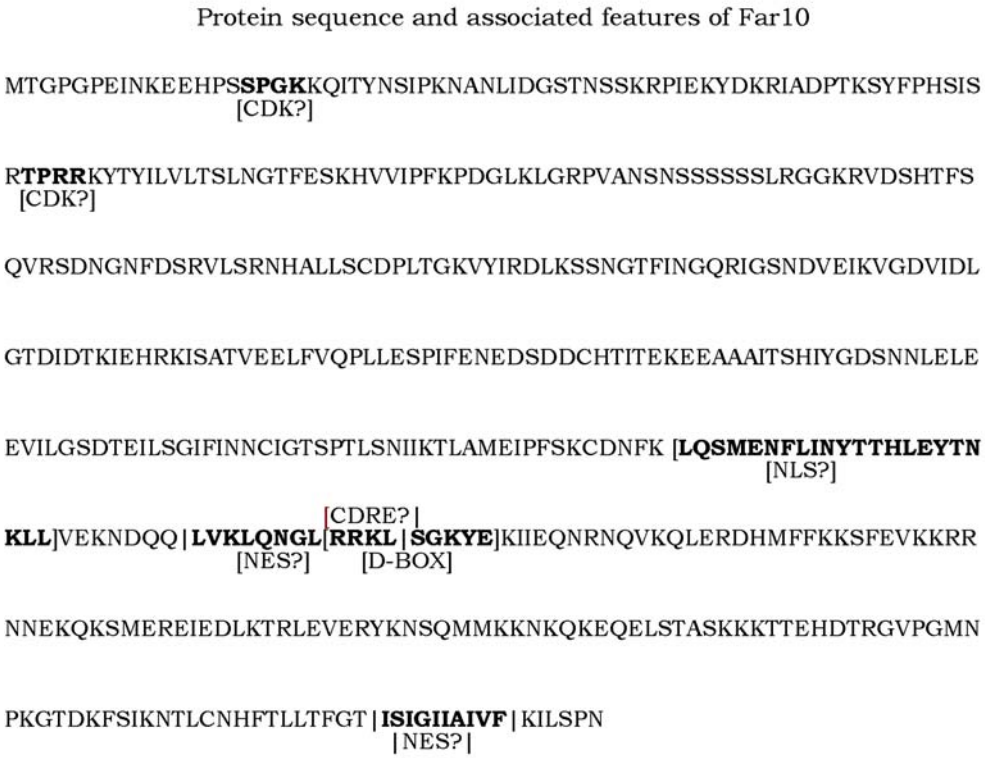


Figure 8: Protein sequence and associated features of Far10. Abbreviations in the figure indicate the following:

CDK	CDK consensus site
CDRE	CDK recognition element
D-box	Destruction box
NLS	Nuclear localization sequence
NES	Nuclear export sequence

3.5.2. Overexpression of Far10 harboring mutations in the potential D-box has no effect on cell growth in wild type background.

To construct the required D-box mutant versions, site specific mutagenesis of the D-box of pYES2 vector encoded *GAL1::FAR10* resulting in *RR340, 341AL* and *L343A* exchange was carried out by following a two stage PCR protocol [see Materials and Methods]. To investigate the effect of overexpression of D-box mutant versions of Far10, the plasmids encoding either *GAL1::FAR10(RR340, 341AL)* or *GAL1::FAR10(L343A)* were introduced into wild type W303 and WCG4 yeast cells by transformation. The transformants were then checked for growth by spot dilution tests using in addition cells containing *GAL1::FAR10* as a control. In both cases overexpression of the mutant versions of *FAR10* had no detectable effect on the growth of wild type yeast strains as it was the case with *GAL1::FAR10* [Fig. 9].

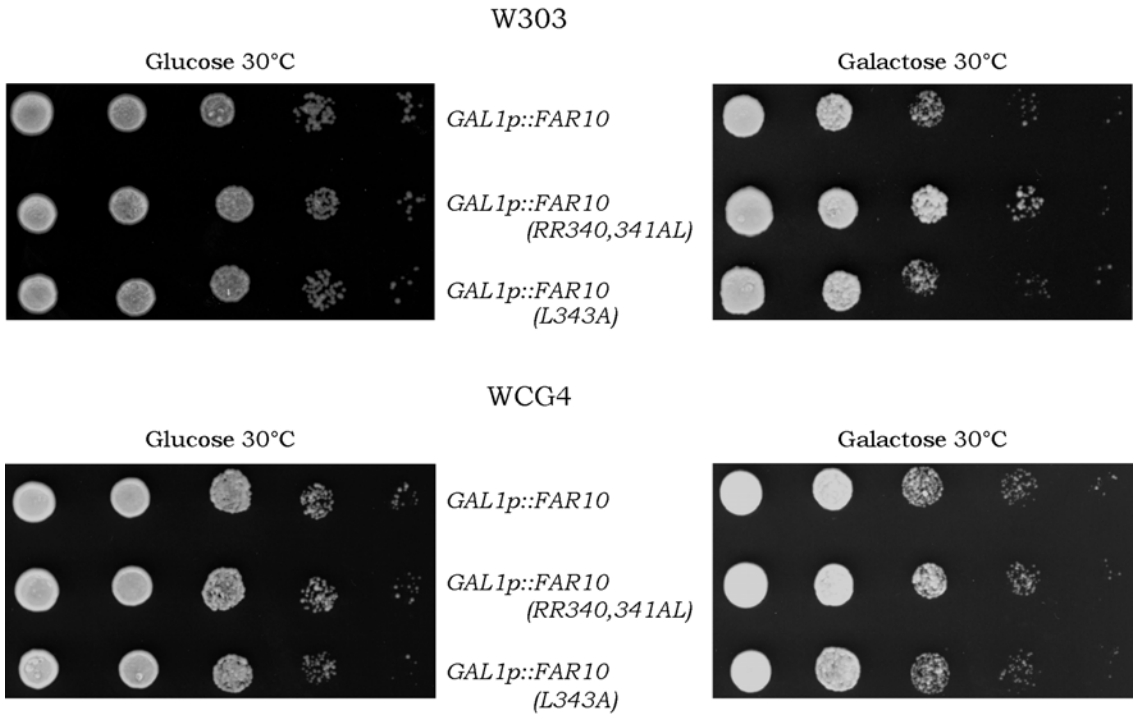


Figure 9: Overexpression of *FAR10* with RR340AL and L343A mutations in the D-box has no effect on the growth of wild type yeast strains. Wild type yeast strains, W303 and WCG4, expressing pYES2 vector derived *GAL1::FAR10, GAL1::FAR10(RR340,341AL) or GAL::FAR10(L343A)* were grown in raffinose medium to

logarithmic phase. Ten fold serial dilutions were spotted onto SC media containing either glucose or galactose. The plates were then incubated at 30°C for two days.

3.5.3. L343A mutation in the D-box of Far10 does not confer stability to Far10 in the wild type yeast strains.

The role of D-box in degradation of Far10 was investigated in wild type yeast strains harboring chromosomally encoded *19MYC-FAR10*(*L343A*), under the native *FAR10* promoter. The following strains yL268 [WCG4, *19MYC-FAR10*)], yL271 [W303, *19MYC-FAR10*)] [Ligr. M, Personal Communication], HarY034 [W303 *19MYC-FAR10(L343A*)] and HarY035 [WCG4 *19MYC-FAR10*(*L343A*)] were made by two-step gene replacement method [see Materials and Methods]. To follow stability of mutated Far10 the cells were grown to logarithmic phase in YPD medium. These were then subjected to cycloheximide chase analysis. As can be seen in the Fig. 10 A and B, the site-specific mutation L343A in the D-box of Far10 did not significantly influence the stability of the mutant protein when compared to the wild type version.

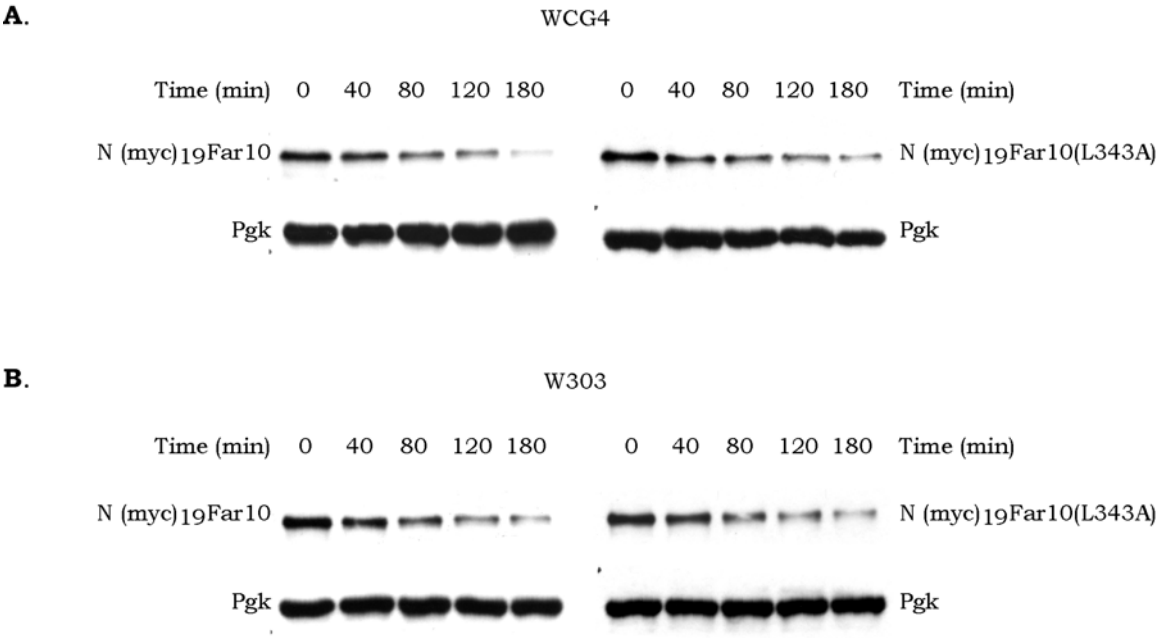


Figure 10: Chromosomally encoded D-box mutant version of Far10 is degraded at wild type rates. The strains, yL268 [*19MYC-FAR10*], HarY035 [*19MYC-FAR10*(*L343A*)], yL271 [*19MYC-FAR10*] and HarY034 [*19MYC-FAR10(L343A*)] expressing chromosomally

encoded, N-terminally tagged native and mutant proteins under control of the native *FAR10* promoter were grown to logarithmic phase in YPD medium. Approximately 2 $O.D._{600}$/ml cells were supplemented with cycloheximide to a final concentration of 0.5 mg/ml. At the indicated time-points 500 µl of the culture were taken and subjected to alkaline lysis followed by western blot analysis. $N(myc)_{19}$Far10 was detected by Anti-Myc antibodies (dilution of 1:10000 in TBS-T). Pgk was used as a loading control and detected by Anti-Pgk antibodies (dilution 1:10000 in PBS-T).

3.5.4. Deletion of the C-terminal single transmembrane domain does abrogate Far10 induced growth inhibition.

Database analysis of *FAR10* uncovered an N-terminal fork head associated [FHA] domain and a C-terminal transmembrane domain. Previous localization studies based on cell fractionation as well as immunofluorescence studies proved that Far10 resides to the nuclear envelope [Velten, 2000].

To investigate the function of the C-terminal transmembrane domain of Far10, plasmid encoded *FAR10* constructs lacking the transmembrane domain were generated by a PCR based strategy (see Materials and Methods). Two constructs were made: pC001 containing C-terminally truncated *FAR10ΔTM* (*GAL1::FAR10ΔTM*$_{441-478}$) and plasmid pC002 harboring *FAR10ΔTM(HA)*$_2$ with the same truncation but in addition HA-epitopes for immunodetection (*GAL1::FAR10ΔTM*$_{441-478}$ *(HA)*$_2$). These were subsequently introduced into *pre1-1 pre4-1*, *cdc23-1* and *cdc20-1* mutants by transformation. Spot dilution tests of the respective transformants were carried out on SC media containing glucose or galactose. As seen in Figs. 11-13 overexpression of the untagged as well as C-terminal 2-HA tagged forms of the transmembrane truncated version of *FAR10* in cells defective in the APC-Cdc20 proteasomal pathway does not cause any growth defect as compared with cells harboring empty vectors. These results show that the growth inhibitory effect of FAR10 overexpression requires transmembrane domain. Far10 localization appears to be important for the effect.

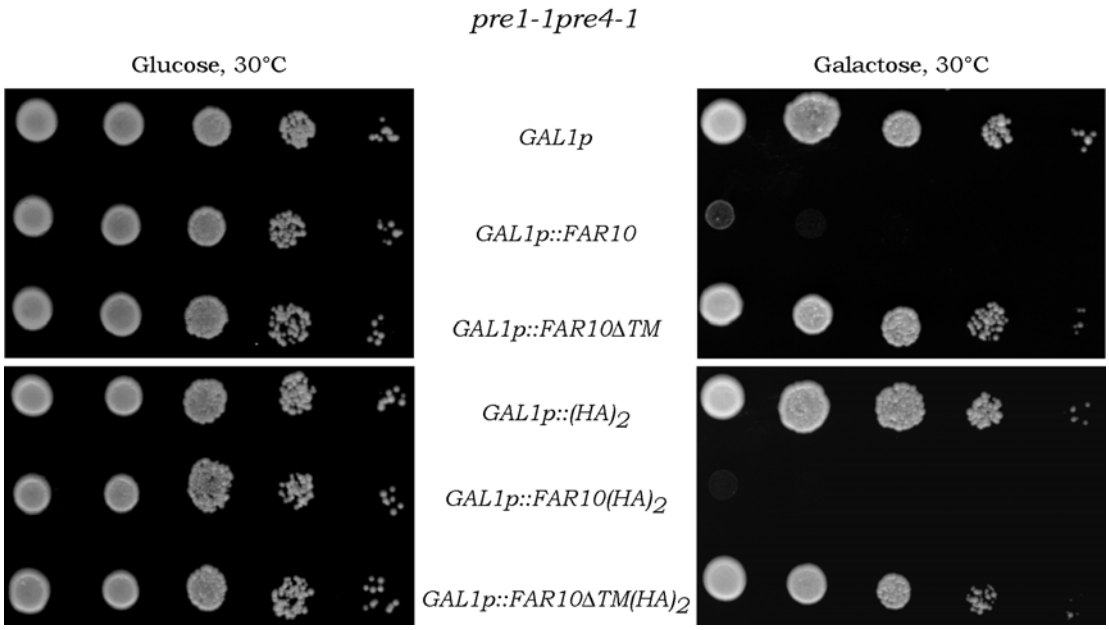


Figure 11: **Overexpression of *FAR10ΔTM*$_{441\text{-}478}$ and *FAR10ΔTM*$_{441\text{-}478}$*(HA)*$_2$ does not cause lethality in *pre1-1 pre4-1* cells.** *pre1-1 pre4-1* mutants expressing pYES2 vector derived *GAL1::FAR10, GAL1::FAR10ΔTM*$_{441\text{-}478}$ or *GAL::FAR10ΔTM*$_{441\text{-}478}$*(HA)*$_2$ and controls containing empty pYES2 vector were grown at 30°C in raffinose medium to logarithmic phase. Ten fold serial dilutions were spotted onto SC media containing either glucose or galactose. The plates were then incubated at 30°C for two days.

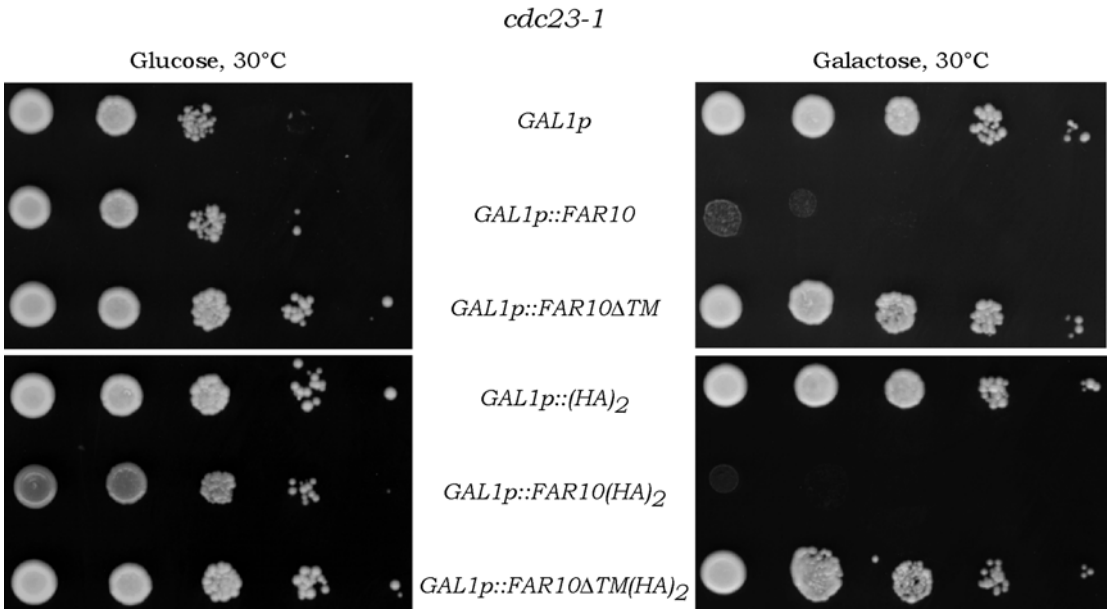


Figure 12: **Overexpression of *FAR10ΔTM*$_{441\text{-}478}$ and *FAR10ΔTM*$_{441\text{-}478}$*(HA)*$_2$ cause no toxicity in APC mutant cells.** *cdc23-1* mutants expressing pYES2 vector derived *GAL1::FAR10, GAL1::FAR10ΔTM*$_{441\text{-}478}$ *or GAL::FAR10ΔTM*$_{441\text{-}478}$*(HA)*$_2$ and controls containing empty pYES2 vector were grown at 25°C in raffinose medium to logarithmic phase. Ten fold serial dilutions were spotted onto SC media containing either glucose or galactose. The plates were incubated at 30°C for three days.

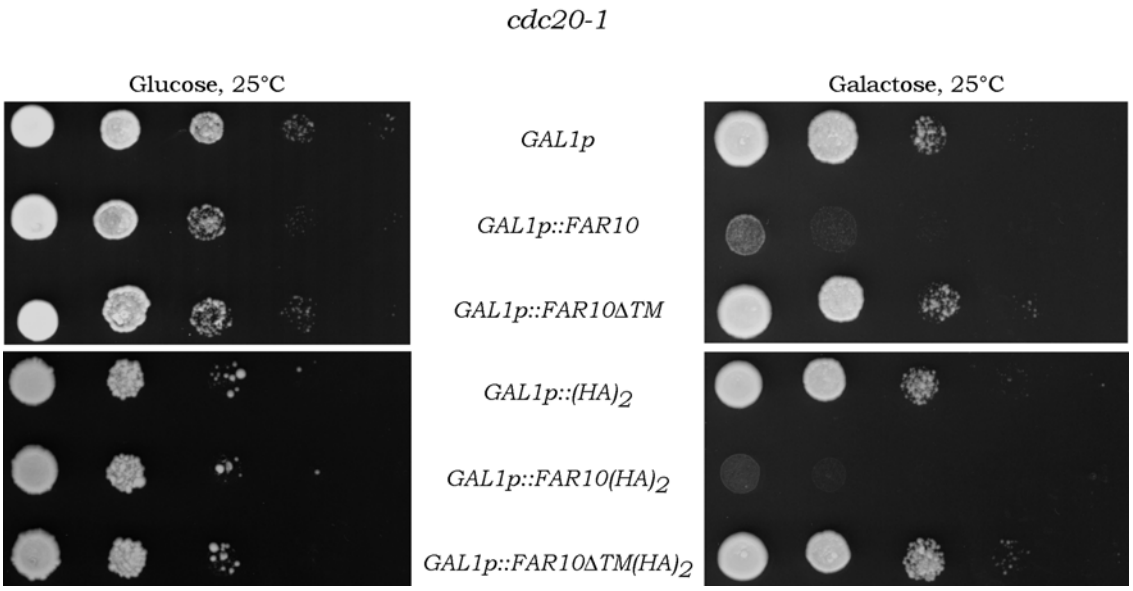


Figure 13: **Overexpression of *FAR10ΔTM*$_{441\text{-}478}$ and *FAR10ΔTM*$_{441\text{-}478}$*(HA)*$_2$ cause no toxicity in *cdc20-1* mutant strain.** *cdc20-1* mutants expressing pYES2 vector derived *GAL1::FAR10, GAL1::FAR10ΔTM*$_{441\text{-}478}$ *or GAL::FAR10ΔTM*$_{441\text{-}478}$*(HA)*$_2$ and controls containing empty pYES2 vector were grown at 25°C in raffinose medium to logarithmic phase. Ten fold serial dilutions were spotted onto SC media containing either glucose or galactose and incubated at 25°C for three days.

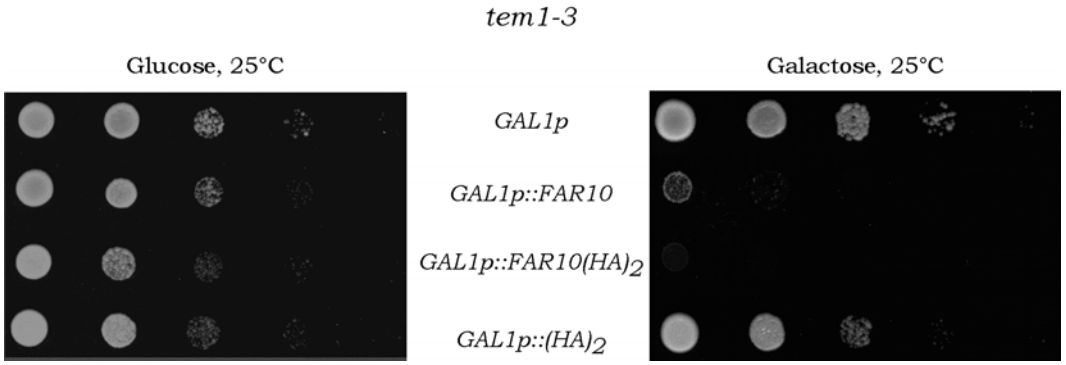
3.6. *FAR10* is genetically linked to mitotic exit pathways.

The mitotic exit network [MEN] in budding yeast is a complex signaling cascade triggering the release of Cdc14 phosphatase. Cdc14 is sequestered and kept inactive by Net1 in the nucleolus. Activation of Cdc14 ultimately results in the inactivation of mitotic CDKs and cytokinesis. The freed Cdc14 functions to shut down mitotic Cdk activity by promoting expression of the Cdk inhibitor Sic1 and stimulation of degradation of the essential mitotic cyclins. The process of exit from mitotis occurs only after sister chromatid separation has occurred and the genetic material has been distributed into the mother and the daughter cell. Correct course of these processes is surveyed by the mitotic exit network, which thereby functions as the core-signaling pathway of the spindle-positioning checkpoint. The MEN signaling cascade consists of Tem1 (a GTPase); Cdc15, Dbf2 and Cdc5 (protein kinases); Cdc14 (a protein phosphatase); Mob1 (a Dbf2 associated factor); Bub2-Bfa1/Byr4 (a two component GTPase-activating Protein; GAP); Lte1 (a guanine nucleotide exchange factor; GEF) and a scaffold protein, Nud1 [Amon, 2001]. When the spindle pole body reaches the bud, Lte1 (GEF) exchanges a GDP for a GTP on Tem1. Thus activated, Tem1-GTP then binds to and activates Cdc15, which in turn activates Mob1-Dbf2 finally resulting in the release of Cdc14 from the nucleolus.

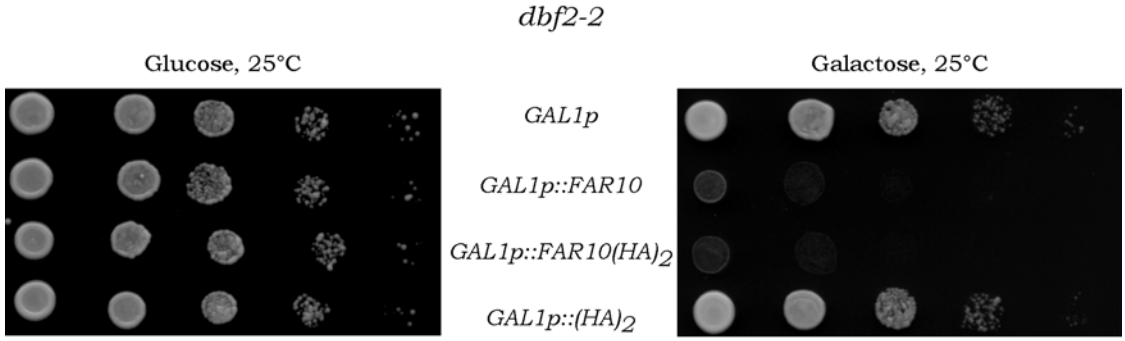
3.6.1. Overexpression of *FAR10* and *FAR10(HA)$_2$* is toxic to *tem1-3*, *dbf2-2* and *cdc14-3* mutants.

To find out whether overexpression is toxic to cells that have a defect in activating the MEN, plasmids encoding *GAL1::FAR10* and *GAL1::FAR10(HA)$_2$* were introduced into *tem1-3* (y1740), *dbf2-2* (y851) and *cdc14-3* (y795) mutants by transformation. Respective transformants were then checked by spot dilution tests using cells containing empty pYES2 and pYES2-HAC vector as controls. Overproduction of *FAR10* and *FAR10(HA)$_2$* upon induction on galactose containing medium resulted in a severe growth defect in *tem1-3* [Fig. 14A], *dbf2-2* [Fig. 14B] and *cdc14-3* [Fig. 14C] mutants. These results prove that impairment of the core-signaling cascade of the MEN makes the cell sensitive to Far10 overexpression.

A.



B.



C.

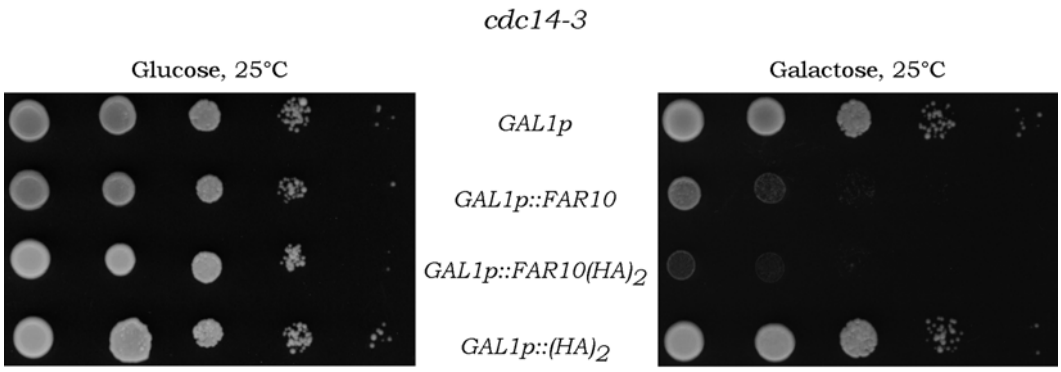


Figure 14: **Overexpression of *FAR10* and *FAR10(HA)*$_2$ is toxic to *tem1-3*, *dbf2-2* and *cdc14-3* mutants.** *tem1-3* (y1740) [**A**], *dbf2-2* (y851) [**B**], and *cdc14-3* (y795) [**C**] mutants expressing pYES2 vector derived *GAL1::FAR10* or *GAL1::FAR10(HA)*$_2$ along with corresponding mutants harboring empty pYES2 as controls were grown at 25°C in

raffinose medium to logarithmic phase. Ten fold serial dilutions were then spotted onto SC media containing either glucose or galactose. The plates were incubated at 25°C for three days.

3.6.2. Overexpression of *FAR10* causes a moderate growth inhibition in *cdc5-1* and *cdc15-2* mutants.

To investigate the effects of *FAR10* overexpression, pIV48 [*GAL1::FAR10*] was introduced into *cdc5-1* (y859) and *cdc15-2* (y2596) mutants by transformation. Spot dilution tests of the respective transformants were performed using wild type (W303) and *tem1-3* (y1740) strains expressing pYES2 vector based *GAL1::FAR10* as controls. Induction of *FAR10* on galactose containing medium in *cdc5-1* (y859) and *cdc15-2* (y2596) mutants resulted in a moderate growth defect [Fig. 15]. These results support the idea that impairment of MEN results in enhanced sensitivity to Far10 overexpression.

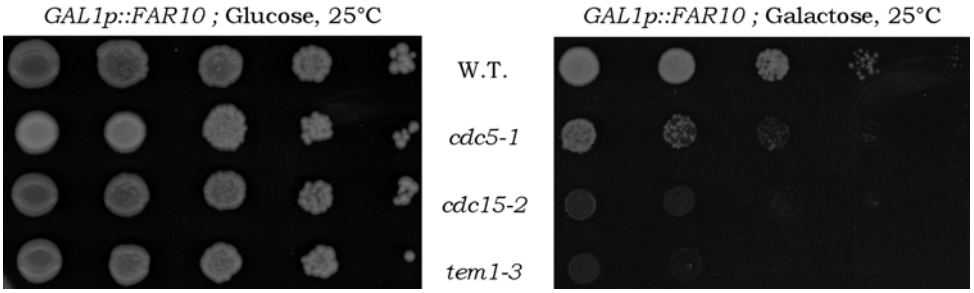


Figure 15: ***FAR10* overexpression causes a moderate growth defect in *cdc5-1* and *cdc15-2* mutants.** W303, *cdc5-1* (y859), *cdc15-2* (y2596) and *tem1-3* (y1740) mutants expressing pYES2 vector derived *GAL1::FAR10* were grown at 25°C in raffinose medium to logarithmic phase. Ten fold serial dilutions were spotted onto SC media containing glucose or galactose. The plates were incubated at 25°C for three days.

3.6.2. Far10 induced synthetic dosage effects are suppressed by *BUB2* deletion whereas *LTE1* knock out has no detectable effect.

We were interested to check the effect of Far10 overexpression in a mutant that in opposite to cells bearing mutations in MEN components was defective in MEN inactivation. For this purpose, pIV48 [*GAL1::FAR10*] was introduced into *bub2Δ::HIS3* (y1863) cells. Spot dilution tests of the transformants were performed using wild type

(W303) expressing pYES2 vector based *GAL1::FAR10* as control. Induction of *FAR10* on galactose containing medium in *bub2Δ::HIS3* (y1863) resulted in an improved growth when compared to the wild type control [Fig. 16]. Bub2 is a negative regulator of mitotic exit network and MEN is hyperactive in *bub2Δ* mutants. A hyperactive MEN is suggested to suppress the Far10 dosage effect.

In addition, the effect of Far10 overexpression in cells defective in Tem1 activation due to the absence of its GEF Lte1 was studied. Far10 overexpression caused no detectable effect in *LTE1* deleted cells [Fig. 16]. The data show that if MEN is hyperactive Far10 overexpression is better tolerated indicating that the inhibitory effect on mitotic exit induced by Far10 is partially cured by a constitutive stimulation of MEN.

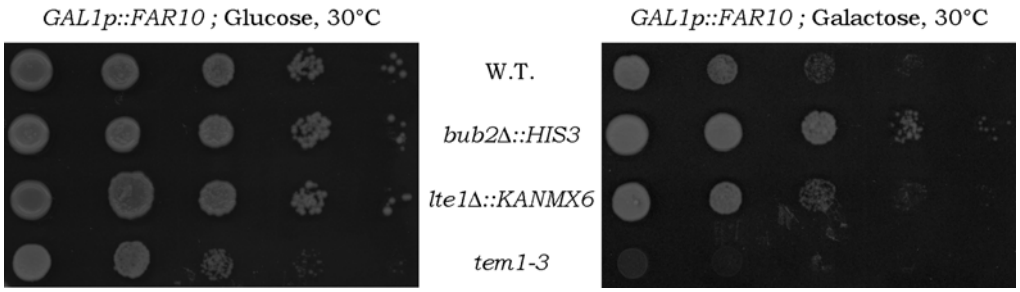


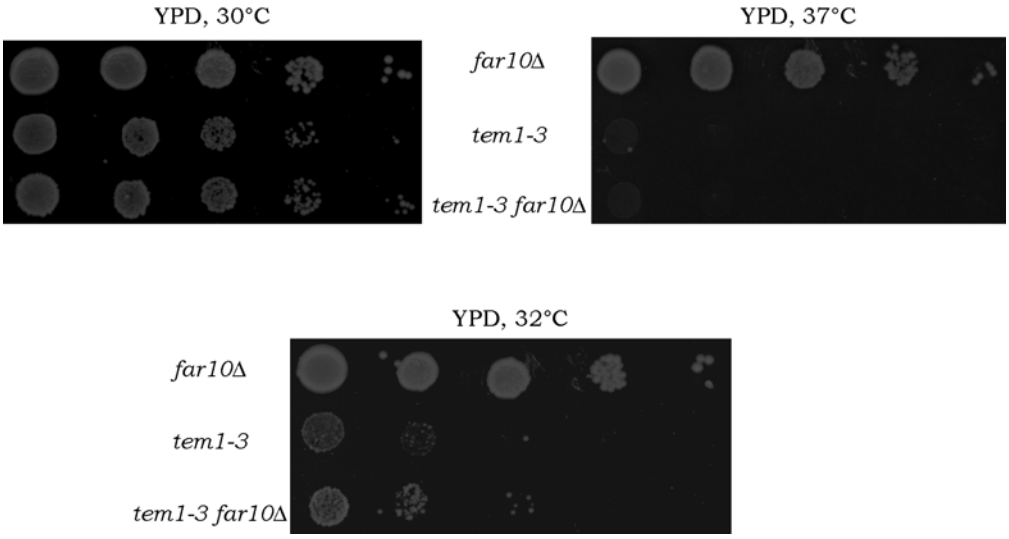
Figure 16: **Deletion of *BUB2* suppresses growth defects associated with *FAR10* overexpression whereas a deletion of *LTE1* has no appreciable effect.** W303, *bub2Δ::HIS3* (y1863), *lte1Δ::KANMX6* (y2003) and *tem1-3* (y1740) mutants expressing pYES2 vector derived *GAL1::FAR10* were grown at 30°C in raffinose medium to logarithmic phase. Ten fold serial dilutions were spotted onto SC media containing glucose or galactose. The plates were incubated at 30°C for three days.

3.6.3. Deletion of *FAR10* causes synthetic growth suppression effects when combined with mutations in the components of the mitotic exit network.

To investigate the contribution of *far10Δ* with mutations in MEN, deletion of *FAR10* in combination with mutations in various genes involved in the process of mitotic exit network were investigated. Wild type yeast strain carrying a *far10* deletion [yIV76/81] was crossed with *tem1-3* [Y1740], *dbf2-2* [Y851], and *cdc14-3* [Y795] mutant strains.

The diploids were isolated and allowed to sporulate on minimal sporulation medium at 25°C for about a week. Tetrad dissections were then performed. All the associated markers in the double mutants were accounted, like *KAN*^{R} for the presence of *far10::KANMX6* and temperature sensitivity at 37°C for *tem1*-3, *dbf2-2*, and *cdc14-3* alleles. Spot dilution tests were performed on YPD-agar plates for *far10Δ tem1-3* (HarY022), *far10Δ dbf2-2* (HarY020), and *far10Δ cdc14-3* (HarY018) using *far10Δ* (yIV076) and the appropriate single mutant strain as controls. As shown in Fig. 17A, *tem1-3 far10Δ* double mutants show a slight suppression effect at 30°C, which is more evident at 32°C. *far10Δ dbf2-2* double mutants show a moderate synthetic suppression effect at 25°C [Fig. 17B]. In contrast *far10Δ cdc14-3* double mutants showed no obvious synthetic effect [Fig. 18] when compared to *cdc14-3* single mutants. None of the double mutants as well as *tem1-3*, *dbf2-2*, and *cdc14-3* single mutants was viable at 37°C. These results show that at the semi-permissive temperature, absence of *FAR10* cures the negative effect of MEN inactivation and effect of MEN impairment partially depends on presence of Far10.

A.



B.

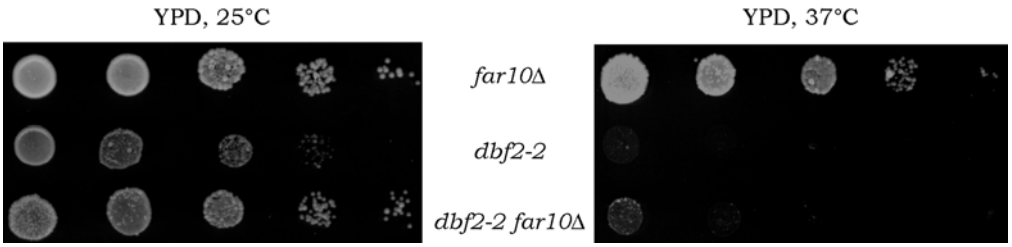


Figure 17: **Synthetic growth suppression observed upon deletion of *FAR10* in combination with *tem1-3* and *dbf2-2* mutant strains**. The strains *far10Δ* (yIV76), *tem1-3* (Y1740), *tem1-3 far10Δ* (HarY022), *dbf2-2* (y851), *far10Δ dbf2-2* (HarY020), were grown to logarithmic phase at 25°C. Ten fold serial dilutions were spotted on to YPD-agar plates and incubated at the appropriate temperature as indicated in the figures.

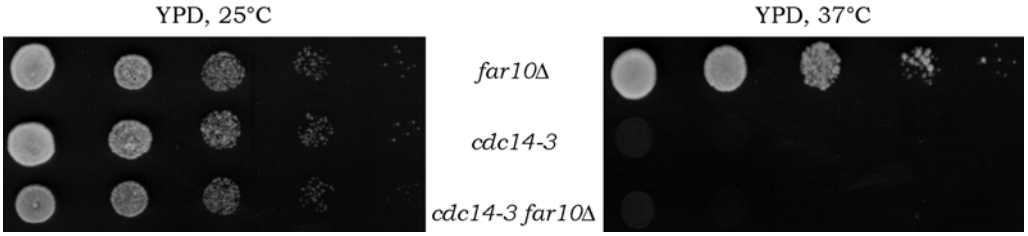


Figure 18: **Deletion of *FAR10* causes no obvious growth effects in *cdc14-3* mutant strain**. The strains *far10Δ* (yIV76), *cdc14-3* (Y795), *far10Δ cdc14-3* (HarY019) were grown to logarithmic phase at 25°C. Ten fold serial dilutions were spotted on to YPD-agar plates and incubated at the 25°C and 37°C for two days.

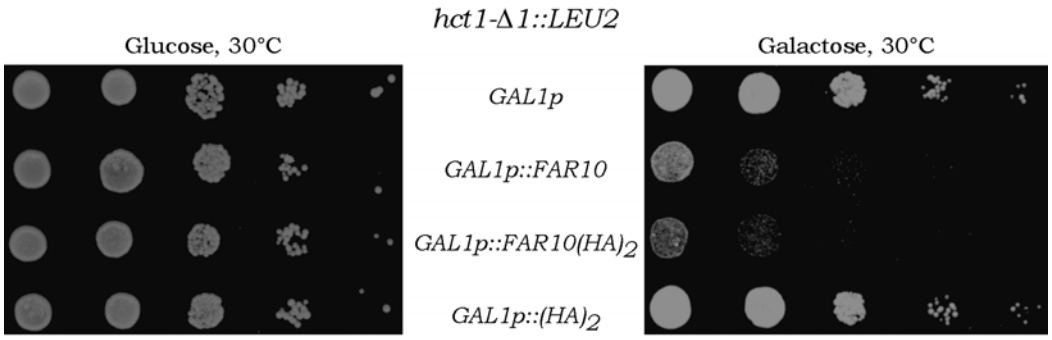
3.7. Far10 is genetically linked to components of the Clb-CDK inactivation machinery.

Mitotic Clb-CDK activity is inactivated by two means: 1) Inhibition of Clb-CDK activity through a specific inhibitor Sic1, which is active from late anaphase till G1 phase. 2) Proteolytic destruction of the cyclin subunit during early anaphase by the APC-Cdc20 complex [Wäsch, 2002] and during late mitosis by active APC-Cdh1 [Schwab et al., 1997]. APC-Cdh1 activity is in addition important to maintain a low Clb-CDK activity during G1 phase.

3.7.1. *FAR10* overexpression causes moderate growth defects in *hct1-Δ1* and *sic-Δ1* mutants.

To demonstrate a possible synthetic dosage effect of *FAR10* overexpression in cells defective in Clb-CDK inactivation, plasmids encoding *GAL1::FAR10* and *GAL1::FAR10(HA)$_2$* were introduced into *hct1-Δ1::LEU2* (W320) and *sic1-Δ1::HIS3* mutants (W190) by transformation. The transformants were checked for growth by serial spot dilution tests using in addition, mutant cells containing empty pYES2 and pYES2-HAC vector as controls. Induction of *FAR10* and *FAR10(HA)$_2$* on galactose containing medium resulted in moderate albeit significant synthetic dosage effects in *hct1-Δ1::LEU2* mutants [Fig. 19 A] and *sic-Δ1::HIS3* mutants [Fig. 19 B]. As expected no effect was observed under repression conditions (glucose medium). These results suggest that cells become sensitive to Far10 overexpression when Clb-CDK inactivation is defective.

A.



B.

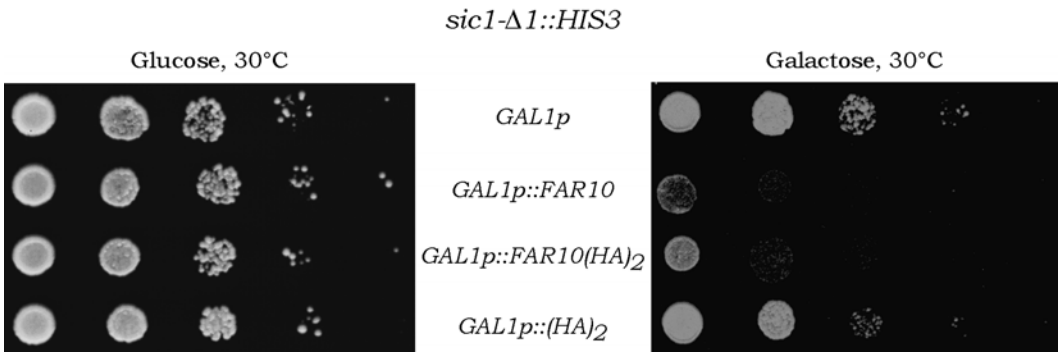


Figure 19: **Overexpression of *FAR10* and *FAR10(HA)$_2$* is toxic to mutants defective in Clb-CDK inactivation.** *hct1-Δ1::LEU2* (W320) and *sic-Δ1::HIS3* mutants (W190) expressing pYES2 vector derived *GAL1::FAR10* or *GAL1::FAR10(HA)$_2$* and controls

containing empty vector pYES2/pYES2-HAC were grown in raffinose medium at 30°C to logarithmic phase. Ten fold serial dilutions were then spotted onto SC media containing either glucose (repression conditions) or galactose (induction of *FAR10*). The plates were incubated at 30°C for three days.

3.7.2. Deletion of *FAR10* causes synthetic growth defects when combined with a deletion of either *HCT1* or *SIC1*.

To demonstrate the potential requirement of Far10 in a situation where Clb-CDK inactivation is impaired, the effect of combining a deletion of *FAR10* with mutations in genes involved in the mitotic Clb-CDK inactivation machinery were investigated. To generate double mutants, *far10*Δ strain (yIV76/81) was crossed with *hct1-Δ1::LEU2* (W320) and *sic1-Δ1::HIS3* (W190) mutant strains. Double mutants were checked for the presence of KAN^R marker for *far10* deletion and either *LEU2* or *HIS3* for *hct1* or *sic1* deletions respectively. Spot dilution tests were performed on YPD-agar plates with the double mutants *far10Δ hct1-Δ1::LEU2* (yIV065) and *far10Δ sic1-Δ1::HIS3* (HarY004) using *far10Δ* (yIV076) and the appropriate single mutant strains *hct1-Δ1* or *sic1-Δ1* as controls. In both cases, *far10Δ hct1-Δ1::LEU2* [Fig. 20] and *far10Δ sic1-Δ1::HIS3* [Fig. 21] double mutants a moderate synthetic growth defect at 37°C was observed. These results suggest that absence of Far10 is disadvantageous for a cell, which is unable to effectively inactivate Clb2-CDK activity in late mitosis.

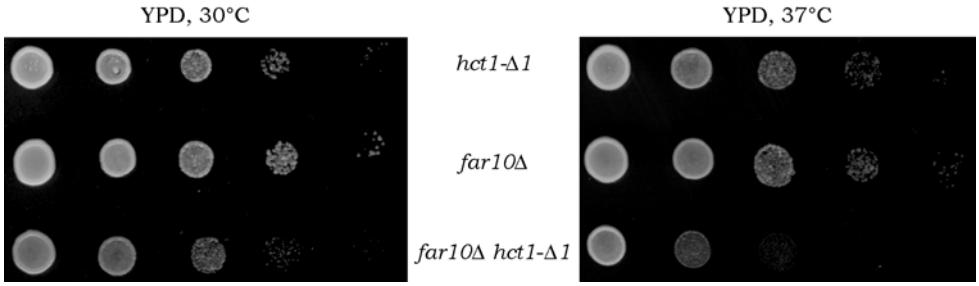


Figure 20: **Synthetic growth effects observed upon double deletion of *FAR10* and *HCT1***. The strains *far10Δ* (yIV76), *hct1-Δ1* (W320) and *hct1-Δ1 far10Δ* (yIV065) were grown to log phase at 30°C. Subsequently, 10 fold serial dilutions were spotted on to YPD-agar plates and incubated at 30°C and 37°C.

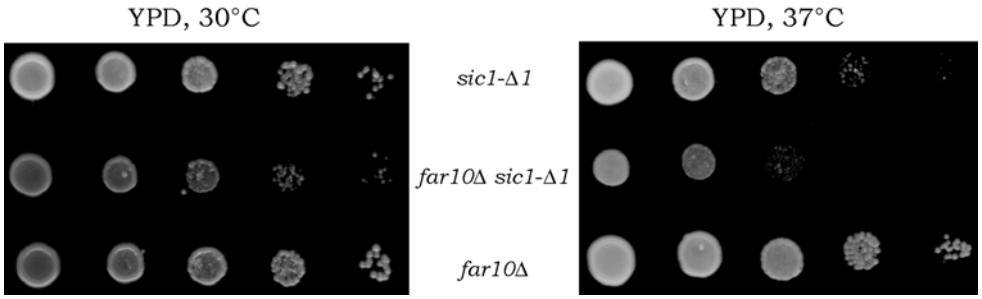


Figure 21: **Synthetic growth defects observed upon double deletion of *FAR10* and *SIC1***. The strains *far10Δ* (yIV76), *sic1-Δ1::HIS3* (W190) and *sic1-Δ1 far10Δ* (HarY004) were grown to log phase at 30°C. Ten fold serial dilutions were spotted on to YPD-agar plates and incubated at 30°C and 37°C.

3.8. *far10* null mutation when combined with *cdc20-1* leads to a synthetic growth defect.

To further investigate the linkage of *FAR10* and *CDC20* the effect of deletion of *FAR10* in *cdc20-1* mutant background was studied. *far10Δ* strain [yIV76/81] was crossed with *cdc20-1* mutant [W267/312]. Tetrad spores were dissected from diploids and haploid double mutants were checked for the presence of KAN^R marker for *far10* deletion and temperature sensitivity at 37°C for the *cdc20-1* allele. Spot dilution tests were performed on YPD-agar plates with *far10Δ cdc20-1* (yIV088) using *far10Δ* (yIV076) strain and *cdc20-1* single mutant strain as controls. As shown in Fig. 20 *far10Δ cdc20-1* double mutants show a moderate synthetic growth defect at 25°C [Fig. 22]. As expected *cdc20-1* and *cdc20-1 far10Δ* mutants are not viable at 37°C. The data obtained reveal that *FAR10* contributes to normal growth of *cdc20-1* mutants.

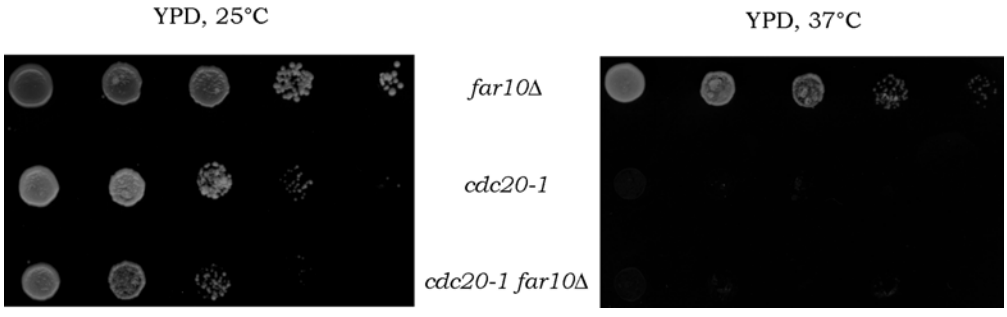


Figure 22: *cdc20-1 far10Δ* double mutants show synthetic growth defects. The strains *far10Δ* (yIV76), *cdc20-1* (W267) and *cdc20-1 far10Δ* (yIV088) were grown to log

phase at 25°C. Subsequently, 10 fold serial dilutions were spotted on to YPD-agar plates and incubated at 25°C and 37°C.

3.9. Deletion of *FAR10* causes no obvious growth effects in *cdc23-1* mutant strain.

To check whether presence of Far10 is required when the APC is disturbed the effect of deletion of *FAR10* in *cdc23-1* background was examined. *far10Δ cdc23-1* (HarY016) was constructed by crossing of single mutants. Spot dilution tests were performed with *far10Δ cdc23-1* double mutants using *far10Δ* and *cdc23-1* single mutants as control. *cdc23-1 far10Δ* double mutant display no detectable growth defects when compared to the single mutant *cdc23-1* at 25°C [Fig. 23]. On the other hand *cdc23-1* and *cdc23-1 far10Δ* mutants exhibit temperature sensitivity at 37°C. The data reveal that absence of Far10 may not be important when the APC is partially inactive.

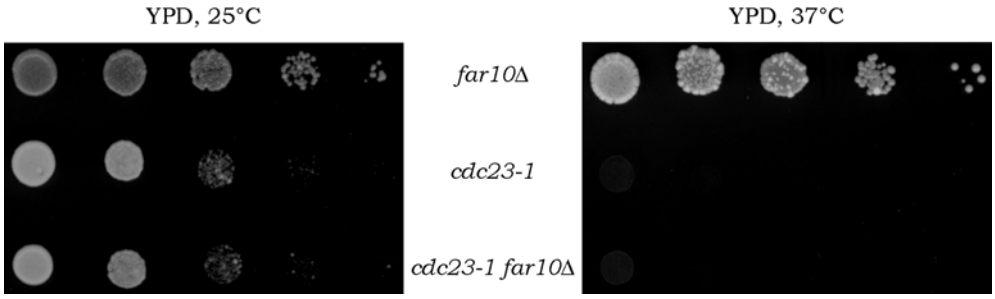


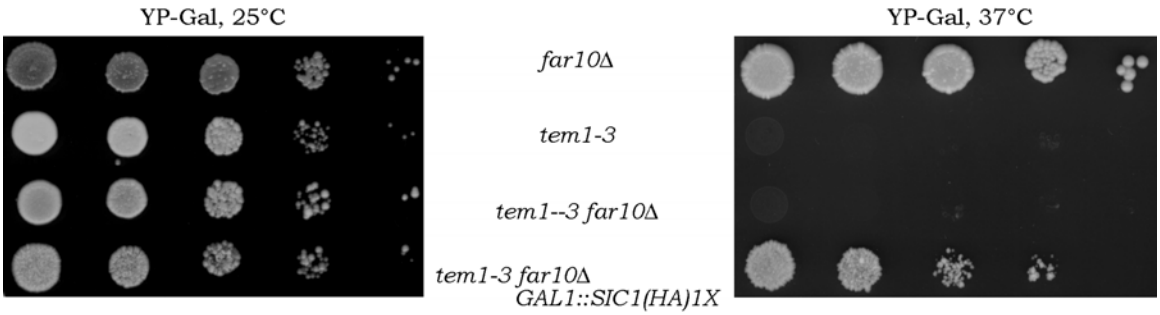
Figure 23: Deletion of *FAR10* causes no detectable growth defects when combined with *cdc23-1* mutation. The strains *far10Δ* (yIV76), *cdc23-1* (FYS29) and *cdc23-1 far10Δ* (HarY016) were grown to log phase at 25°C. Subsequently, 10 fold serial dilutions were spotted on to YPD-agar plates and incubated at 25°C and 37°C for two days.

4.0. *SIC1* overexpression does not restore normal growth at non-permissive temperature in MEN mutants harboring a deletion of *FAR10*.

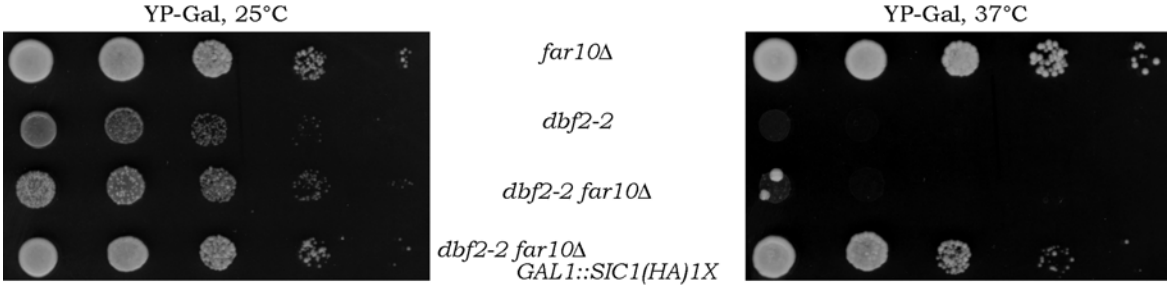
Overexpression of *SIC1* from a multi-copy plasmid was shown to be a suppressor of temperature sensitive lethality in *tem1-3*, *dbf2-2* and *cdc14-3* strains [Sue L. Jaspersen et al., 1998]. In addition to this, overexpression of Sic1 from *GAL1* promoter rescues mutants expressing non-degradable Clb2 [Wasch, 2002]. To check whether Sic1

overexpression rescues temperature sensitivity of MEN mutants containing a deletion of *FAR10*, *tem1-3 far10Δ*, *dbf2-2 far10Δ* and *cdc14-3 far10Δ* strains containing chromosomally encoded *SIC1(HA)1X* under the control of *GAL1* promoter were constructed by crossing of *URA3::GAL1::SIC1(HA)1X* (E304) with each of the individual double mutants. The triple mutants were checked for the presence of KAN^R marker, temperature sensitivity in non-inducing conditions and *URA3* marker. Additionally presence of $Sic1(HA)_{1X}$ was confirmed by immunoblotting. Spot dilution tests were carried out on YP-Gal plates using respective MEN single and double mutants as well as *far10Δ* cells as controls. Induction of Sic1(HA)1X rescued temperature sensitive lethality of *tem1-3 far10Δ* [Fig. 24A], *dbf2-2 far10Δ* [Fig. 24B] and *cdc14-3 far10Δ* mutants [Fig. 24C]. However, cells did not reach normal growth of *far10Δ* mutants under same conditions. These results demonstrate that presence of *FAR10* is necessary when *cdc14*, *dbf2* and *tem1* defects are cured by Sic1 overexpression.

A.



B.



C.

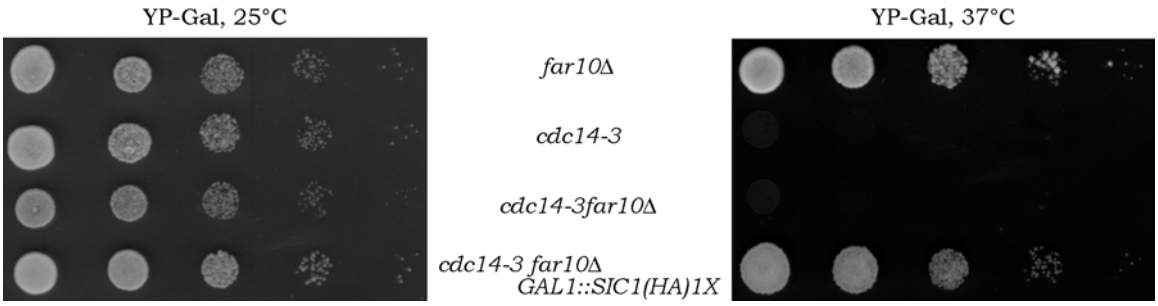
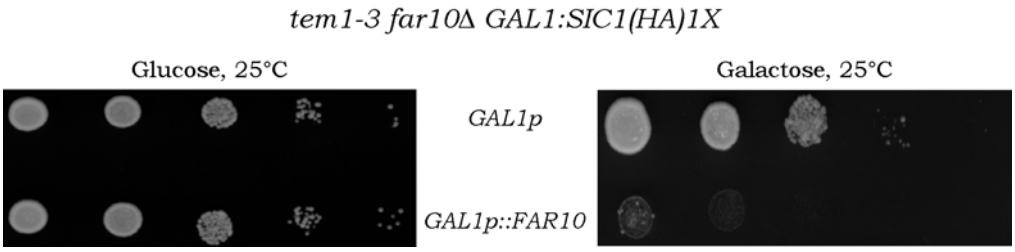


Figure 24: *SIC1* overexpression does not restore normal growth at non-permissive temperature in MEN mutants harboring a deletion of *FAR10*. The strains *tem1-3 far10Δ URA3::GAL1:SIC1(HA)1X* (HarY031), *dbf2-2 far10Δ URA3::GAL1:SIC1 (HA)1X* (HarY029) and *cdc14-3 far10Δ URA3::GAL1:SIC1(HA)1X* (HarY015) along with respective controls were grown to logarithmic phase at 25°C. Subsequently, 10 fold serial dilutions were spotted on to YP-Gal plates and incubated at 25°C and 37°C for two days.

4.1. Co-overexpression of *SIC1* doesn't rescue synthetic growth defects associated with overexpression of *FAR10*.

To find out whether co-overexpression of *SIC1(HA)1X* could rescue growth defects associated with Far10 overexpression, plasmid encoding *GAL1::FAR10* was introduced into *tem1-3 far10Δ URA3::GAL1:SIC1(HA)1X* (HarY031), *dbf2-2 far10Δ URA3::GAL1:SIC1 (HA)1X* (HarY029), *cdc14-3 far10Δ URA3::GAL1:SIC1(HA)1X* (HarY015) and *cdc23-1 far10Δ URA3::GAL1:SIC1(HA)1X* (HarY010 made by crossing strains E304 and HarY017) mutants by transformation. The transformants were checked by spot dilution tests using cells containing empty pYES2 vector as controls. Overexpression of *SIC1(HA)1X* along with *FAR10* upon induction on galactose containing medium did not result in rescue of *tem1-3 far10Δ URA3::GAL1:SIC1(HA)1X* [Fig. 25A], *dbf2-2 far10Δ URA3::GAL1:SIC1(HA)1X* [Fig. 25B], *cdc143 far10Δ URA3::GAL1:SIC1(HA)1X* [Fig. 25C] and *cdc231 far10Δ URA3::GAL1:SIC1 (HA)1X* [Fig. 25D] mutants. These results suggest that growth defects induced by *FAR10* overexpression in cells that are impaired in MEN cannot be cured by restoring Clb-CDK inactivation by ectopic expression of Sic1.

A.



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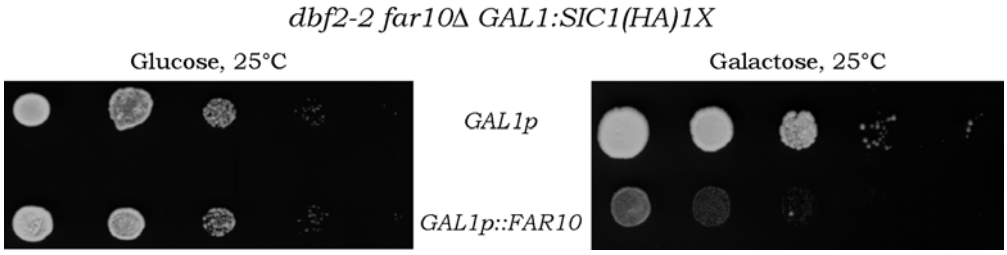
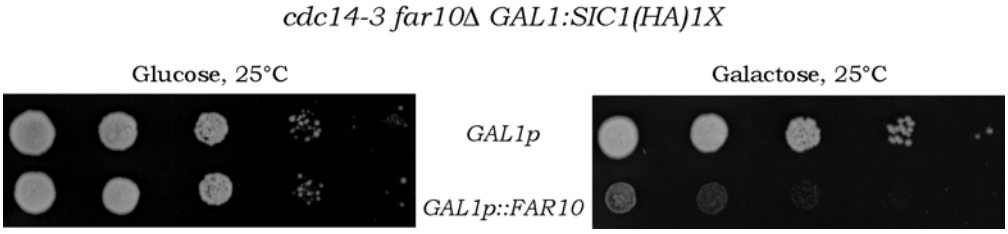


C.



D.

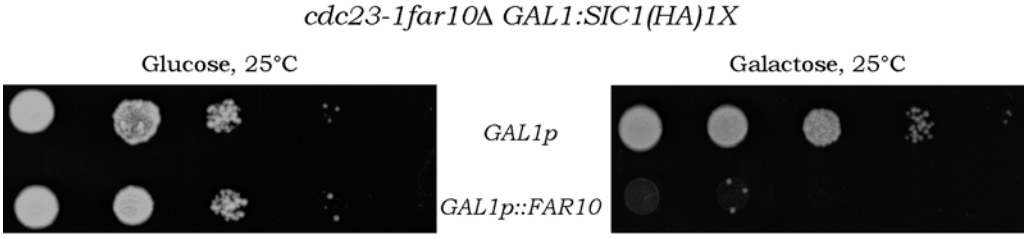


Figure 25: **Co-overexpression of *SIC1(HA)1X* does not rescue growth defects associated with *FAR10* overexpression.** *tem1-3 far10Δ URA3::GAL1:SIC1(HA)1X* [Fig. 24A], *dbf2-2 far10Δ URA3::GAL1:SIC1(HA)1X* [Fig. 24B], *cdc143 far10Δ*

URA3::GAL1:SIC1(HA)1X [Fig. 24C] and *cdc231 far10Δ URA3::GAL1:SIC1 (HA)1X* [Fig. 24D] mutants expressing pYES2 vector derived *GAL1::FAR10* along with mutants harboring empty pYES2 as controls were grown at 25°C in raffinose medium to logarithmic phase. Ten fold serial dilutions were then spotted onto SC media containing either glucose or galactose. The plates were incubated at 25°C for three days.

DISCUSSION

The ubiquitin-proteasome system is involved in a multitude of physiological processes occurring in the cell. One of the ways this system regulates physiology of a cell is through ubiquitin mediated regulated proteolysis and this plays an important role in many basic cellular processes [Glickman, 2002; Hilt, 2004; Hilt and Wolf, 2004].

To uncover new substrates of the proteasome, a genetic screen termed the high expression lethality (HEL) screen was done in the yeast *Saccharomyces cerevisiae*. This screen was based on the hypothesis that overexpression of a protein whose degradation by the ubiquitin-proteasome system is required for viability or growth, will cause a strong growth defect in cells where proteasome function is impaired, as for instance in *pre1-1 pre4-1* mutants. An unidentified protein Hel48 now commonly termed as Far10 was identified [Velten, 2000; Kemp and Sprague, Jr., 2003].

Overexpression of both the untagged and C-terminally 2Ha tagged versions of *FAR10* caused toxicity in *pre1-1 pre4-1* mutants providing evidence that tagging doesn't influence the functionality of the protein. Additionally, cycloheximide chase experiments proved that *FAR10* expressed from its endogenous promoter on the chromosome as $N(Myc)_{19}$Far10 and Far10$(HA)_3$ were rapidly degraded in wild type yeast strains and stabilized in *pre1-1 pre4-1* mutants. The identical behavior of N- and C-terminally tagged versions lend credence to the idea that the degradation rates observed are supposed to be as that of the native protein. These results provide comprehensive evidence that Far10 is indeed a novel substrate of the proteasome.

Buttressing the above evidence, analysis of *in-vivo* ubiquitination experiments of Far10$(HA)_3$ in wild type (WCG4) and *pre1-1 pre4-1* mutants revealed that poly-ubiquitinated forms of Far10$(HA)_3$ accumulate in *pre1-1 pre4-1* mutants highlighting the involvement of the ubiquitin-proteasome system in its degradation. Due to (possible) extensive degradation and/or de-ubiquitination, polyubiquitinated species of Far10$(HA)_3$ were not observed in the wild type (WCG4) yeast strain.

Overexpession of *FAR10* in *pre1-1 pre4-1* proteasome mutants resulted in defined cell cycle arrest in late anaphase. Thereby, an investigation of the role of *FAR10* in the cell cycle and the components of cell cycle involved in its degradation was done.

The ubiquitin protein ligase APC is a multi-component E3 enzyme complex which plays an important role in targeting many regulators of mitosis such B-type cyclins like Clb2, Clb5, and non-cyclins such as Pds1, Ase1, Cdc5 etc for degradation [Murray, 1995; Cohen-Fix et al., 1996; Juang et al., 1997; Shirayama et al., 1998; Shirayama et al., 1999; Wasch and Cross 2001]. The APC requires two substrate specific adaptor proteins Cdc20 and Hct1 to ensure that different target proteins are degraded in a proper temporal order during mitosis [Schwab et al., 1997; Visintin et al., 1997].

The potential involvement of the anaphase-promoting complex and its regulators in the degradation of Far10 was substantiated by the observation of synthetic dosage effects in *cdc23-1*, *cdc20-1* and *hct1-Δ1* mutants. In addition to this, cycloheximide chase analysis of 19Myc tagged Far10 in *cdc23-1* APC mutants as well as in *cdc20-1* and proteasome mutants uncovered a clear proteolytic stabilization of $N(Myc)_{19}$Far10. In contrast a deletion of *HCT1* had no effect on the degradation of Far10. These results confirm that Far10 is an authentic substrate of the APC and requires the specificity factor Cdc20 for its degradation whereas Hct1 is not involved.

All reported substrates of APC^{Cdc20} identified till date contain a 9 amino acid degradation motif called the destruction [D] box, which has a consensus sequence **R**XX**L**XXXX**N**/**D**/**E**. Prominent substrates of APC containing this motif include B type cyclins like Clb2, Clb5 and also non-cyclins like Pds1 [Glotzer et al., 1991]. Deletion or mutation of the conserved residues in this motif resulted in the stabilization of the respective proteins [Cohen-Fix et al., 1996; King et al., 1996; Juang et al., 1997]. Far10 being a substrate of APC^{Cdc20} has a nine amino-acid sequence similar to the D box motif, ^{340}**R**RK**L**SGKY**E**^{348} residing in the C-terminal region. To check the relevance of this motif in the degradation of Far10, the first two arginine residues (340, 341) were mutated to alanine and leucine respectively. Additionally, leucine in the fourth position (343) was changed to alanine. Overexpression of these two different mutant versions of Far10 in the wild type yeast strains did not result in toxicity. Moreover, cycloheximide chase analyses of the N-terminal 19Myc-tagged version of one of these mutant proteins, $N(Myc)_{19}$Far10(L343A) expressed from the endogenous promoter on the chromosome showed that this mutant protein was not stabilized in wild type yeast strains. These findings may mean that this motif in Far10 is not a classical D box. It may also be possible that the mutations in this D-box have to be cumulative in order for the desired effect(s) to be seen.

Database analysis of *FAR10* revealed an N-terminal FHA (**f**ork **h**ead **a**ssociated) domain and a C-terminal transmembrane domain. Fractionation experiments as well as immunofluorescence studies proved that Far10 localizes to the nuclear envelope [Velten, 2000]. To investigate the function of the C-terminal transmembrane domain, a deletion construct containing Far10 lacking the transmembrane domain, *far10ΔTM* was generated. In contrast to wild type Far10 this mutant protein was unable to cause synthetic dosage effects in *pre1-1 pre4-1*, *cdc23-1* and *cdc20-1* mutants. Immunofluorescence studies of Far10ΔTM$(HA)_2$ revealed that this mutant protein was indeed mislocalized [data not shown]. These results provide support that the ability of Far10 to induce lethality requires its correct localization to the nuclear membrane [Murray, 2001].

FAR10 is not an essential gene and its deletion causes no obvious growth defects when compared to wild type strain [data not shown]. However, an investigation into the synthetic interactions with *cdc20-1* mutant revealed that *cdc20-1 far10Δ* double mutants demonstrated synthetic growth defect at 25°C. On the other hand *far10Δ cdc23-1* double mutants showed no clear growth effects when compared to *cdc23-1* single mutants. The data suggest that APC may be fully active at 25°C in *far10Δ cdc23-1* mutants and hence no growth effect is displayed. This question can be addressed by an additional growth test for *far10Δ cdc23-1* mutants at the semi-permissive temperature (e.g. 30°C). In contrast, *cdc20-1 far10Δ* double mutants at 25°C may show a defective APC activity, which culminates in an obvious growth defect. These observations reveal that when APC-Cdc20 activity is distressed probably, leading to defective control of the other APC mediated cell cycle processes, presence of Far10 is necessary.

To delineate the interactions of *FAR10* with regulatory modules of the mitotic exit network a genetic approach was performed. For this purpose the effects of *FAR10* overexpression and inactivation were studied in mutants defective in MEN. *FAR10* when overexpressed in a series of MEN mutants was found to cause toxicity specifically in *cdc14-3*, *dbf2-2*, and *tem1-3* mutants. Whereas Far10 overexpression in *cdc15-2* and *cdc5-1* mutants displayed a mild effect, *lte1Δ* mutants showed no effect at all. This proves that defective activation of MEN results in cells becoming sensitive to *FAR10* overexpression. However, when the mitotic exit network is hyperactive as in the case of *bub2Δ* mutants (Bub2 is a negative regulator/in-activator of Tem1), then *FAR10* dosage

effect is suppressed. These data suggest that MEN activation (at least partially) counteracts *FAR10* induced inhibition of mitotic exit.

Deletion of *FAR10* in *dbf2-2* mutants had a moderate suppression effect at 25°C when compared to *dbf2-2* single mutants. In case of *far10Δ tem1-3* double mutants this suppression effect was only visible at 32°C. No synthetic growth effect was perceptible in *far10Δ cdc14-3* double mutants. The slight suppressor effect of *far10Δ* in MEN mutants may, however, only be visible at defined semi-permissive conditions (a temperature that allows growth of the MEN single mutant but causes a partial inactivation of the respective MEN component). Nevertheless, the data clearly indicate that *FAR10* is required for effective inhibition of mitotic exit induced by inactive MEN. These data may lead to the idea that MEN may be required to inactivate Far10 by stimulating its degradation. However this was not the case. At least in case of *dbf2-2* and *tem1-3* mutants Far10 is degraded at normal rates at both permissive and non-permissive temperatures [data not shown]. Altogether the data clearly show that MEN and *FAR10* are part of two processes, which are parallel/redundant and/or act to co-operate in triggering exit from mitosis.

The ultimate function of the mitotic exit network in budding yeast is inactivation of the mitotic Clb2-CDK activity, which is followed by cytokinesis resulting in the formation of two daughter cells [Visintin et al., 1998].

Overexpression of *FAR10* causes synthetic dosage effects in mutants that are directly defective in Clb-CDK inactivation such as *hct1-Δ1* and *sic1-Δ1*. Results in this case show that weakening of Clb-CDK inactivation either due to impaired Clb2 degradation or absence of Sic1 leads to a situation where *FAR10* overexpression is not tolerated. Further more, a strain deleted for *FAR10* and *HCT1* showed synthetic growth defect at 30°C, which was noticeably enhanced at 37°C. Similar data was obtained with *sic1-Δ1 far10Δ* double mutants. The data recommend that presence of Far10 is required under these conditions.

Moreover, due to these findings it was reasoned that improvement in Clb-CDK inactivation through Sic1 overexpression might alleviate the toxic effects associated with overexpression of *FAR10*, as it was the case if the cell expresses a non-degradable Clb2 [Wasch, 2002]. In fact, overexpression of *SIC1* from a multi-copy plasmid suppresses temperature sensitivity of *cdc14-3*, *dbf2-2* and *tem1-3* mutants at 37°C

[Jaspersen et al., 1998]]. However, when *SIC1(HA)1X* was co-overexpressed with *FAR10* in *cdc23-1*, *cdc14-3*, *dbf2-2* and *tem1-3* mutants growth was not restored to normal rates. A similar observation was made when *SIC1(HA)1X* was overexpressed in MEN mutants that harbored a *far10* deletion. Taken together the data provide evidence that perfect Clb-CDK inactivation is required when Far10 activity is disturbed and vice-versa indicating that Far10 is part of a pathway/system that acts parallel to the program of CDK regulation involved in promoting exit from mitosis.

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Erklärung

Hiermit versichere ich, dass ich diese Arbeit selbst verfasst und dabei keine andern als die angegebenen Quellen und Hilfsmittel verwendet habe

Stuttgart, den 20.06.2005

Harish Kumar Karnam