Molecular Networks that Intersect With Anaphase Promoting Complex-dependent Longevity and Stress Response

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By

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ABSTRACT

The molecular mechanisms controlling longevity have been subject to intense scrutiny in recent years. It is clear that genomic stability, stress response and nutrient signaling all play critical roles in lifespan determination, but the precise molecular mechanisms and their often subtle influence on cellular function remain largely unknown. The Anaphase Promoting Complex (APC) is an evolutionarily conserved ubiquitin-protein ligase composed of 13 subunits in yeast, required for M and G1 cell cycle progression, and is associated with cancer and premature aging in many model systems when defective. The APC targets substrates for proteasome-dependent degradation, yet the full range of APC substrates and their role in mediating genomic stability, stress response and longevity are largely unknown. In this study, we use the model organism *Saccharomyces cerevisiae* to investigate the results of two screens designed to identify novel APC targets, regulators and/or modifiers, in an effort to better understand the function of the APC. Both of these screens made use of the Apc5 subunit. This subunit is likely an important structural component of the APC and may be targeted by many APC regulatory enzymes. This subunit is essential, but a temperature sensitive (*ts*) allele of Apc5 was available for these studies.

First, a Yeast 2-Hybrid (Y2H) screen utilizing Apc5 as bait recovered the lifespan determinant Fob1 as a potential APC substrate. We hypothesized that the APC targets Fob1 for proteasome- and ubiquitin-dependent degradation. Authenticating Fob1 as a novel APC substrate makes up the first part of this thesis. We have found that Fob1 is unstable specifically in G1, and cycles throughout the cell cycle in a manner similar to Clb2, an APC target. Consistent with the APC mediating Fob1 degradation, Fob1 is stabilized in APC and proteasome mutants. Disruption of *FOB1* in WT cells increased replicative lifespan, a measure of how many daughter cells a single mother will produce prior to senescence; moreover, *FOB1* disruption improved APC mutant replicative lifespan defects. Increased *FOB1* expression decreased replicative lifespan in WT cells, while increased expression in APC mutant cells did not reduce replicative lifespan further, suggesting an epistatic interaction. *FOB1* deletion also suppressed cell cycle progression, and rDNA recombination defects observed in $apc5^{CA}$ cells. Mutation to a putative Destruction Box-like motif ($Fob1^{E420V}$) disrupted Fob1 modification, stabilized the protein and increased rDNA recombination. These results support our hypothesis that Fob1 is a novel APC target and that Fob1

dosage may be regulated by the APC in response to cell cycle and environmental cues to regulate APC-dependent genomic stability and longevity.

Second, an aptamer (small peptide) based screen identified peptides capable of suppressing the *ts* defect of the *apc5*^{CA} mutant. One aptamer of interest is Y65, which has homology to the ubiquitin ligase Elc1. A Y2H found that this peptide Y65 binds the unstable stress response transcription factor Cin5. We hypothesized that this peptide may stabilize Cin5 by masking ubiquitin-dependent degradation. Stabilized Cin5 may in turn alleviate some *apc5*^{CA} mutant defects. Characterizing Cin5 and confirming that Cin5 is subject to proteasome and ubiquitin-dependent degradation makes up the second portion of this thesis. During our investigation of Cin5 we identify a phospho-inhibited degradation motif within Cin5 that prevents ubiquitination and subsequent degradation when phosphorylated. We also provide evidence suggesting Cin5 may be targeted by a previously unidentified ubiquitin ligase subcomplex including Elc1 and Grr1. These data have helped elucidate the ubiquitin dependent regulation of Cin5.

In summary, this research demonstrates the feasibility of using the Y2H and aptamer screens to identify and characterize molecular networks that interplay with the APC. Additionally, identifying and characterizing proteins where APC activity or function can be modified by aptamer binding has the potential to classify drug targets for therapeutic use in higher eukaryotes. Further understanding of the role the APC plays in cell cycle progression, chromatin assembly, genomic stability, stress response and longevity will be valuable to fundamental biological science, and may also have applications in health science and medicine.

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LIST OF ABBREVIATIONS

aa	amino acid
ade	adenine
ADE2	phosphoribosylaminoimidazole carboxylase (gene/protein designation)
ŬF	Ŭ-Factor, yeast mating pheromone
Amp	ampicilin
AMP	Adenosine monophosphate
APC	Anaphase-Promoting Complex
APS	ammonium persulphate
ARS	Autonomous Replicating Sequence (DNA locus)
ATP	Adenosine triphosphate
BBB	bead-beat buffer
bp	base pair
CA	chromatin assembly
CAN	canavanine
Cdc	Cell division cycle (gene/protein designation)
CDK	Cyclin-dependent protein kinase
CEN	centimere
cfu	colony forming unit
CHX	cycloheximide
CIP	calf intestinal alkaline phosphatase
CLS	Chronological lifespan
CM	complete media
CP	Core particle of 26S proteasome
CoIP	Co-immunoprecipitation
CR	caloric restriction
CRL	Cullin-RING Ligase
CuSO2	copper sulfate
Cys	Cystine
ddH2O	double distilled water
ECL	Electrochemiluminescence (reaction solution)
æ	defines gene deletion
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
DSB	double-stranded DNA break
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin-ligase
dsRNA	double-stranded RNA
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ERC	extrachromsome rDNA circle
EtBr	ethidium bromide
EtOH	ethanol

FACS	Fluorescence Activated Cell Sorting
FOA	5-Fluoroorotic Acid
Fob1	replication fork blocking protein (gene/protein designation)
G0	non-dividing or quiescent state within G1 phase of the cell cycle
G1	growth phase 1, first of four cell cycle stages
G2	growth phase 2, third of four cell cycle stages
G418	Geneticin
GAD	Gal4 activating domain (transcription factor motif)
Gal	Galactose
GAL4	Galactose activated transcription factor (gene/protein designation)
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GBD	Gal4 DNA-binding domain (transcription factor motif)
Glu	Glucose
GST	Glutathione S-transferase epitope
HA	hemagglutinin epitope
HECT	Homologous to E6AP Carboxy Terminus (class of E3s)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	histamine
HIF1Ŭ	hypoxia inducible factor Ŭl
HU	Hydoxyurea
IgG	immuneglobulin G
IP	immunoprecipitation
IRESs	internal ribosome entry sites
KAc	potassium acetate
KAN	Kanamycin
kanMX	cassette which confers kanamycin resistance (gene/protein designation)
kDa	kilodalton
KH2PO4	potassium hydrogen phosphate
Lb	Lysogeny broth
Leu	leucine
LiCl	lithium chloride
M	Mitotic phase, last of four cell cycle stages
MAT	Mating type locus
MgAc	magnesium acetate
ml	milliliter
µl	microliter
MonoUb	mono-ubiquitin
MYC	c-myc epitope
NaAc	sodium acetate
NaCl	sodium chloride
NaOH	sodium hydroxide
NaH2PO4	sodium hydrogen phosphate
NOC	Nocodazole
NP40	nonyl phenoxypolyethoxylethanol
NTS	Non-transcribed Spacer (DNA locus)
OD	optical density

ON	over night
PABP	poly (A) binding protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	polyethylene glycol
PI	Propidium iodide
PMSF	phenylmethylsulfonyl fluoride
Poly-Ub	poly-ubiquitin
rDNA	ribosomal deoxyribonucleic acid
RENT	*re*gulators of *n*ucleolar silencing and *t*elophase exit
RFB	replication fork block (DNA locus)
RING	Really Interesting New Gene
RLS	Replicative lifespan
RNase A	Ribonuclease A
RP	regulatory particle of the 26S proteasome
rRNA	ribosomal ribonucleic acid
RT	room temperature
S	synthesis phase (DNA replication), second of four cell cycle stages
S. cerevisiae	*Saccharomyces cerevisiae*
SCF	Skp1/Culin/F-box protein
SD	synthetic dextrose
SDS	sodium dodecyl sulfate
SMC	Structural Maintenance of Chromosomes (gene/protein designation)
TAE	Tris-base, glacial acetic acid, EDTA
TAP	Tandem affinity purification epitope
TCA	trichloroacetic acid
TE	Tris-HCl, EDTA
TrxA	thioredoxin A (used as aptamer scaffold)
ts	temperature sensitive
Ub	ubiquitin
USCE	Unequal Sister Chromatid Exchange
UV	ultraviolet light
VHL	Von Hippel-Lindau
Y2H	Yeast ï 2 ï hybrid
YPD	Yeast extract, peptone, dextrose
Y65	Aptamer 65 (short peptide)

1. MOTIVATION

The primary objective of this study was to identify molecular networks that intersect with the Anaphase Promoting Complex (APC) and characterize how these networks impinge on longevity and stress response. The APC is a ubiquitin ligase that targets proteins for degradation. The anaphase promoting function of the APC have been exemplified in the literature, yet the precise range of APC substrates and their often subtle influence on cellular function remain largely unknown. Our current working model defines the APC as a vital warden that coordinates mechanisms that impinge on chromatin metabolism in responses to various internal and environmental signals. How various signals such as nutrient availability and stress responses convey APC target specificity, and how the degradation or modifications of those specific protein targets affect downstream processes are topics of ongoing research.

This endeavor makes use of two screens to identify novel APC targets, regulators and/or modifiers. The results of these screens have hatched two parallel projects that were initiated prior to this study and were continued in this thesis. First, a Yeast-2 Hybrid (Y2H) screen was used to detect potential APC binding partners or substrates. This screen identified the protein Fob1, among others. Authenticating Fob1 as a novel APC substrate and characterizing a molecular mechanism that describe how this interaction impinges on genomic stability and longevity makes up the first project in this thesis.

Secondly, an aptamer (small peptide) based screen was employed to identify and characterize additional novel APC targets, regulators and/or modifiers. In contrast to the Y2H approach, the aptamer screen selected for suppressors that were able to recover APC mutant defects. This screen yielded several aptamers, from millions screened, able to suppress the temperature sensitive (*ts*) defect of the $apc5^{CA}$ mutant. One such aptamer, Y65 has homology to the ubiquitin ligase Elc1 and was thus investigated further. In an effort to characterize the mechanism by which this aptamer is able to suppress APC mutant defects, the aptamer was cloned into a Y2H vector to screen for binding partners. This Y2H identified the stress response transcription factor Cin5 as a binding partner for Y65. Formulating a possible mechanism by which this aptamer Y65 and its interaction with Cin5 is able to suppress APC mutant defects make up the second project of this thesis.

2. INTRODUCTION AND BACKGROUND

2.1. Ubiquitin

The modiýcation of proteins by the covalent attachment of ubiquitin (Ub) is a highly conserved regulatory process in all eukaryotic cells. Ub is a protein of 76 amino acids where only four amino-acids differ among yeast, plants, and animals (CATIC and PLOEGH 2005). In general, ubiquitination targets proteins to be degraded by the proteasome, a process that shapes the proteome (BELLE *et al.,* 2006). Critical cellular events, including cell cycle progression, DNA replication, and chromosome segregation are subject to regulation by proteolytic destruction of important regulators (NASMYTH 1996; PINES 2006; DIFFLEY 2010). Such regulators include the cyclin-dependent kinase complexes Clb2/Cdc28, which inhibits cell separation (HERSHKO, 1999; KRAFT *et al.,* 2006), and Clb5/Cdc28, which aids in the required removal of rDNA specific cohesin during chromosome segregation (SULLIVAN *et al.,* 2008), where Clb2 and Clb5 are the cyclins and Cdc28 is the Kinase. The ubiquitin-dependent degradation of securin (Pds1 in yeast), an inhibitor of chromosome segregation, is critical for anaphase progression (SUDAKIN *et al.,* 1995; GUACCI *et al.,* 1997; MICHAELIS *et al.,* 1997). In short, the ubiquitin-proteasome degradation pathway is intimately coupled to the cell cycle, and cellular health and function.

2.1.1. Monoubiquitin and polyubiquitin signaling and degradation

Ub is attached to other proteins via lysine residues. Ub itself has seven lysines, all of which can be conjugated to multiple ubiquitin molecules to form a polyubiquitin chain (PENG *et al.,* 2003). Diverse sequences and combinations of ubiquitination allow for structurally distinct polyubiquitin chains for diverse signaling modes. The nature of the ubiquitin modification can control the fate of the ubiquitinated protein. For instance, substrates modified by the addition of a single Ub, termed monoubiquitination, can impinge on non proteolytic mechanisms, such as in DNA repair and chromatin dynamics. Substrates modified by the addition of multiple ubiquitins, termed polyubiquitination, are preferentially targeted to the proteasome (reviewed in FINLEY *et al.,* 2012). Ub receptors, many of which display specificity or preference for ubiquitin chain linkage

type or length play a key role in decoding the signals embedded in the structure of ubiquitin chains (DIKIC *et al.,* 2009; reviewed in FINLEY *et al.,* 2012).

2.1.2. Ubiquitination reaction and components

The conjugation of Ub to substrates is completed through three steps involving three different enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-ligase enzyme (E3) (Figure 2.1). In yeast there is only one E1, 13 E2s and a growing list of E3s (reviewed in FINLEY *et al.,* 2012; PICKART and EDDINS, 2004). First, a thioester bond between the C-terminal glycine of Ub and the active cysteine of E1 is formed in an ATP-dependent manner. Ub is then transferred to a ubiquitin-protein ligase (E2), again through a thioester linkage. Finally, an E3 catalyzes the formation of an isopeptide bond between the C-terminus of Ub and a lysine residue of a specific target protein (DESHAIES and JOAZEIRO 2009; VARSHAVSKY 2012). Additional Ub moieties can be conjugated to the previous Ub to form polyubiquitin chains (Figure 2.1).

2.1.3. E1, ubiquitin-activating enzyme

For ubiquitination, the C-terminus of Ub is first activated to facilitate its conjugation to a substrate. This reaction is catalyzed by an E1 enzyme in two steps. First, an Ub-adenylate intermediate is formed in which the C-terminal carboxyl group of Ub is covalently linked to AMP. Breakage of the Ub-AMP linkage is followed by the formation of an E1-Ub thioester with the C-terminus of Ub linking to a cysteine residue on E1. There is only one E1 in yeast, Uba1 (CIECHANOVER *et al.,* 1982; McGRATH *et al.,* 1991), which suggests that the first step in ubiquitination is conserved although there are many ubiquitination substrates.

2.1.4. E2s, ubiquitin-conjugating enzymes

The next step in the ubiquitination process is the transfer of Ub from the E1 cysteine residue to an E2 cysteine. The ubiquitin-conjugating enzymes are at the center of the E1ï E2ï E3 cascade. They interact with E1 and E3 but also ensure unidirectional handoff of ubiquitin from E1 to the substrate. E1 and E3 use a shared binding site on E2s, preventing recharging of E2s while bound to E3s and forcing their dissociation before the next round of conjugation (ELETR *et al.,* 2005). There is a large family of E2s dedicated to ubiquitination, including 13 enzymes in *S. cerevisiae* and many more in higher organisms (FINLEY *et al.,* 2012). Each E2 may serve several E3s and several E2s may serve a single E3 (CHEN *et al.,* 1993; SOMMER and WOLF, 1997). Different combinations of E2s and E3s lead to the large diversity of ubiquitination products. Among the 13 ubiquitin-conjugating enzymes, Cdc34/Ubc*3* is the only one essential for viability (GOEBL *et al.,* 1988). Temperature sensitive *cdc34* mutants arrest at the G1-to S-phase transition of the cell cycle due to a defect in degradation of the cyclin-dependent kinase inhibitor Sic1 (SCHWOB *et al.,* 1994). Cdc34 has many other substrates and most are selected by the Skp1ï Cdc53ï F-box (SCF) ubiquitin ligase family for which Cdc34 serves as the main, if not only, E2 enzyme (PETROSKI and DESHAIES 2005).

The E2 active site cysteine, which is absolutely conserved, sits in a shallow cleft on the protein surface. Not surprisingly, many of the most highly conserved E2 residues surround the active cysteine (COOK *et al.,* 1994; WORTHYLAKE *et al.,* 1998; JIANG and BASAVAPPA, 1999). Some of these residues interact with Ub, and others presumably interact with E1. Most of the poorly conserved E2 residues cluster on the opposite side of the active cysteine. Some E2s also have N-terminal or C-terminal extensions, which are believed to mediate interactions between E2s and downstream factors: E3s or substrates (PICKART, 2001). The extensions make these E2s distinct, thus achieving the specificity and diversity of E2-E3 interaction in spite of the core structure similarity of all E2s. Some E2s can target substrates in the absence of an E3 (DAVID *et al.,* 2010).

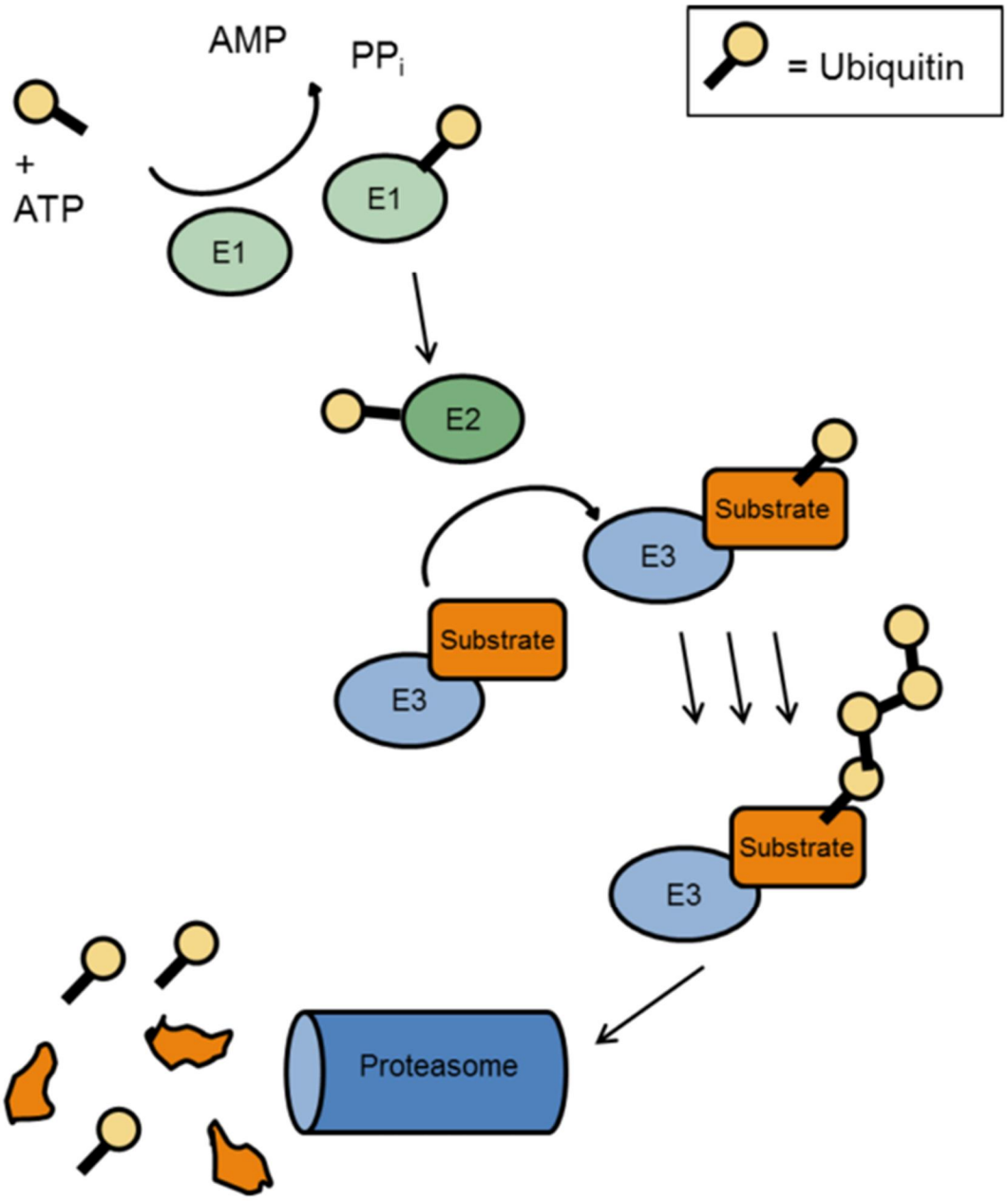


Figure 2.1 Ubiquitination
Ubiquitin must first be activated by adenylation using ATP as a donor. The activated ubiquitin molecule is then covalently attached to the E1 through a thioester bond. The ubiquitin molecule is then transferred from the E1 to an E2. The E3 associates with the E2 to facilitate substrate ubiquitination. Polyubiquitination then results in the substrate being targeted to the proteasome and subsequently degraded. Information obtained and adapted from HOCHSTRASSER, 1996; HERSHKO and CIECHANOVER, 1998.

2.1.5. E3s, ubiquitin-ligases

The last step in the ubiquitination cascade is the covalent ligation of one or more Ub molecules to the substrate. E3s bind E2s and substrate proteins to facilitate substrate-speciýc ubiquitination. The large number of E3s reflects the breadth of ubiquitination involved in biological functions. Different from E2s, E3s are structurally diverse and may function alone or as large multi-subunit protein complexes. E3s belong to two major classes; RING (Really Interesting New Gene) domain E3s, including the structurally related U-box domain E3s, and HECT (Homologous to E6AP Carboxy Terminus) domain E3s (reviewed in in FINLEY *et al.,* 2012; JOHNSON *et al.,* 1995; LIU, 2004; ARDLEY and ROBINSON, 2005).

2.1.5.1. RING finger E3s

It is not clear whether all RING finger proteins play roles in ubiquitination. However, a large number of these proteins are E3s and they comprise the largest known class of E3s. The RING finger domain can be defined by existence of the consensus sequence Cys-X2-Cys-X(9/39)-Cys-X-1/3-His-X2/3-Cys/His-X2-Cys-X4-48-Cys-X2- Cys, where X is any amino acid, and Cys and His represent zinc binding residues (JOAZEIRO and WEISSMAN, 2000). RING E3s include the SCF (Skp1/Culin/F-box protein) and the APC and both are composed of several proteins in which Cullin and RING domain subunits are core members (KAMURA *et al.,* 1999; OHTA *et al.,* 1999; SEOL *et al.,* 1999; TAN *et al.,* 1999). Human cells express seven different Cullins (Cul1, 2, 3, 4A, 4B, 5 and 7; PETROSKI and DESHAIES, 2005); each of them functions as a scaffold protein to recruit the RING protein and adapter protein(s) to form a RING E3 complex. The adapter protein binds to the N-terminal region of Cullins, whereas the RING protein which recruits E2, binds to the C-terminal globular domain (ZHENG *et al.,* 2002). This E3 complex recruits the substrate through an adapter protein or a substrate receptor which binds to an adapter protein. For example, in the SCF, Cul1 binds to Skp1 which in turn binds the F-box substrate receptor (SCHULMAN *et al.,* 2000). F-box proteins contain an F-box motif, which is required for binding to Skp1, as well as a specific substrate-recognition motif. F-box proteins are highly variable and interchangeable. Thus, Cul1 can assemble with numerous substrate receptors to form CRLs (Cullin-RING Ligase)

that share a common catalytic core yet recruit different substrates (NAKAYAMA *et al.,* 2001). Other Cullins have a similar mechanism to assemble multi-subunit RING E3s (PETROSKI and DESHAIES, 2005). The APC is a high molecular mass complex composed of at least 13 subunits, but it is only fully active as an E3 once it has bound to Cdc20 or Cdh1 adaptors or activators (PETERS, 2002). Two of its subunits, APC2 and APC11, are distant members of the Cullin and RING domain families, respectively (YU *et al.,* 1998; ZACHARIAE *et al.,* 1998b).

2.1.6. The 26S proteasome

The 26S proteasome is a highly conserved complex of 33 distinct subunits in eukaryotes (FINLEY 2009). Its principal function is to degrade ubiquitinï protein conjugates. The proteasome consists of two principal components, the 19S regulatory particle (RP) and the 20S core particle (CP). The RP recognizes substrates to be degraded, while the CP contains the proteolytic active sites. The proteasome recognizes substrates by two subunits, Rpn10 and Rpn13 that are within the RP and bind polyubiquitin chains (ELSASSER *et al.,* 2004; VERMA *et al.,* 2004; MAYOR *et al.,* 2007; HUSNJAK *et al.,* 2008). Ubiquitinated protein substrates are fed by the RP into the interior cavity of the barrel shaped CP where they are unfolded and degraded in an ATP-dependent manner (reviewed in FINELY *et al.,* 2012). Unstable proteins that are turned over in a ubiquitin/proteasome-dependent manner will be stabilized and accumulate in the absence of RP components such as Rpn10 (ISLAM *et al.,* 2011; FLEMING *et al.,* 2002; LIU *et al.,* 2007). In this study we utilize a *RPN10* deletion mutant to confirm ubiquitin/proteasome-dependent turnover.

2.2. The APC and implications on stress response, genomic stability and longevity

2.2.1. The Anaphase Promoting Complex

The APC is a highly conserved E3 ubiquitin ligase crucial for progression through mitosis, mitotic exit and G1 maintenance (SKAAR and PAGANO 2009; CONAWAY *et al.,* 2002; PASSMORE 2004; THORNTON and TOCZYSKI 2006; HARPER *et al*., 2002; McLEAN *et al.,* 2011; BARFORD 2011). The primary function of the APC is to ubiquitinate protein substrates that block sister chromatid separation and exit from mitosis. To initiate anaphase, the APC targets securin

(Pds1 in yeast), an inhibitor of chromosome segregation, and Scc1/Mcd1, a protein required for sister chromatid cohesion, for degradation (SUDAKIN *et al.,* 1995; GUACCI *et al*., 1997; MICHAELIS *et al.,* 1997). There are other cell cycle dependent APC targets, such as the cyclin Clb2 (IRNIGER and NASMYTH 1997; WASCH and CROSS 2002; MANCHADO *et al*., 2010; WASCH *et al.,* 2010). The cyclin-dependent kinase Cdc28 is activated by Clb2 to promote mitotic progression (SKAAR and PAGANO 2009; MORGAN 2007; HOLT 2012). Ase2, a protein required for elongation of the mitotic spindle during mitosis, is also an APC target (UFANO *et al.,* 2004). However, there is mounting evidence that the APCôs role is not exclusive to the progression of mitosis but impinges on a wide range of cellular processes, including processes upstream and downstream of stress response, genomic maintenance, and nutrient sensing and utilization (EGUREN *et al.,* 2011; reviewed in QIAO *et al.,* 2010; MANCHADO *et al.,* 2010; GARCIA-HIGUERA *et al.,* 2008; HARTWELL and SMITH 1985; POSTNIKOFF and HARKNESS 2012; POSTNIKOFF *et al.,* 2012).

The APC is necessary for genomic stability, as a number of APC mutants lose plasmids at an accelerated rate (HARTWELL and SMITH 1985; PALMER *et al*., 1990; HARKNESS *et al.,* 2002), consistent with a role in chromosome segregation. The yeast APC appears to be required for chromatin metabolism, as APC mutants lack the ability to effectively assemble chromatin *in vitro* and properly regulate histone post-translational modifications (HARKNESS *et al*., 2002; HARKNESS *et al*., 2005; ARNASON *et al*., 2005; TURNER *et al*., 2010; ISLAM *et al.,* 2011). Cancer and reduced lifespan are both tightly linked to genomic instability (MCMURRAY and GOTTSCHLING 2004; SEVIOUR and LIN 2010). The defects observed in APC mutants manifest as reduced lifespan in yeast and mice (BAKER *et al*., 2004; HARKNESS *et al.,* 2004; HARKNESS 2006; POSTNIKOFF *et al*., 2012; POSTNIKOFF and HARKNESS 2012, LI *et al.*, 2008; MALUMBRES and BARBACID 2009). The yeast APC appears to respond to signals from the transcription factor Forkhead Box proteins Fkh1 and Fkh2, to regulate chronological and replicative lifespan, as well as stress response (POSTNIKOFF *et al.,* 2012). These Fkh proteins drive the expression of histones, which when defective, reduce yeast replicative lifespan (DANG *et al.,* 2009; FESER *et al*., 2010). Consistent with a role for the APC in histone metabolism, genomic stability and lifespan, human cancer cells are continually being described that express aberrant APC activity (reviewed in WÄSCH *et al*., 2010; SMOLDERS and TEODORO 2011,

WIRTH *et al*., 2004; MALUMBRES and BARBACID 2009; GARCIA-HIGUERA *et al.,* 2008; HEILMAN *et al.,* 2005).

2.2.2. APC subunits

The APC in yeast is composed of 13 core subunits (Figure 2.2). Eight of the 13 yeast APC subunits are essential, with conditional alleles resulting in cell cycle arrest at the anaphase/metaphase junction (PETERS 2006; ZACHARIAE and NASMYTH 1999; FANG *et al.,* 1998a, b; KALLIO *et al.,* 1998; ZACHARIAE *et al.,* 1998a; COOPER *et al.,* 2000; MORGAN 2007; THORNTON and TOCZYSKI 2006). Apc2 and Apc11 form the catalytic core, responsible for the ubiquitination of target molecules (LEVERSON *et al.,* 2000; TANG *et al.,* 2001), while Cdc16, Cdc27 and Apc1 are regulatory subunits, as they are the targets of activating and inhibitory phosphorylation (KOTANI *et al.,* 1998; RUDNER and MURRAY 2000). Apc10/Doc1 encodes a conserved protein that is required for efficient ubiquitination of substrates and thought to contribute to the substrate binding site of the APC (CARROLL and MORGAN 2002; GROSSBERGER *et al.,* 1999; AU *et al.,* 2000; HWANG and MURRAY 1997). The *apc10*Δ mutants show *ts* growth with defects in chromosome segregation (UFANO *et al.,* 2004).

Cdc23 is an essential protein required for cell cycle progression through mitosis (ICHO and WICKNER, 1987; SIKORSKI *et al.,* 1990, 1991, 1993). Cdc23 has been shown to interact with other APC subunits including Mnd2, Cdc16 and Cdc27, as well as the N-terminus of Clb2 (HALL *et al.,* 2003; MEYN *et al.,* 2002). Mnd2 (meiotic nuclear division protein-2) inhibits Ama1 activated APC in pre-meiotic S phase and prophase (OELSCHLAEGEL *et al.,* 2005; PENKNER *et al.,* 2005). Similarly, Apc13/Swm1 (spore wall mutation) is required for late stages of sporulation. Both Cdc26 and Apc9 are nonessential APC subunit at permissive (30ºC) temperatures. However, Cdc26 was largely absent in precipitates from *apc9*Δ strains. Apc9 might stabilize the interaction of Cdc26 with the APC, which may underscore the requirement for Apc9 for efficient entry into anaphase (ZACHARIAE *et al.,* 1998b). Apc4 does not share significant homology to any previously identified proteins (PAGE and HIETER, 1999; CASTRO, 2005).

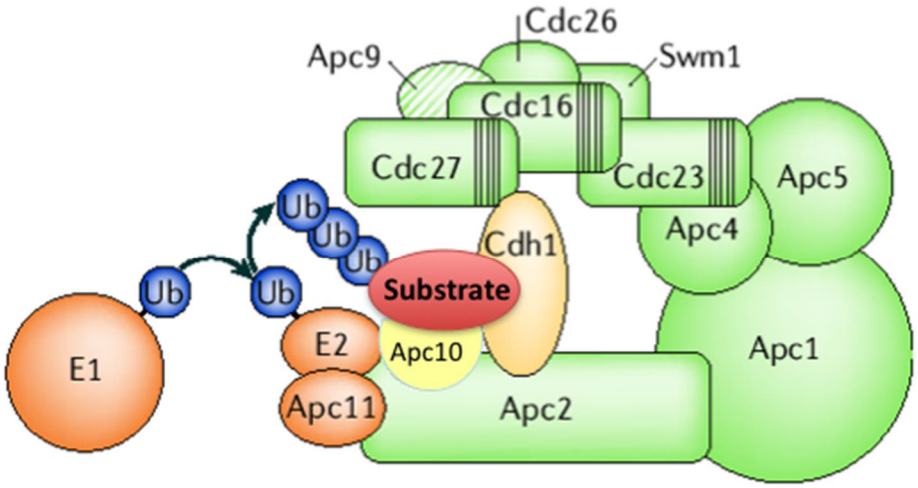


Figure 2.2 The Anaphase Promoting Complex
Schematic of APC structure. Biochemical and genetic analysis of APC structure indicates the APC is composed of 2 arms held together by Apc1. The catalytic core of Apc2, Apc11 and Apc10 compose one arm. Information obtained and adapted from (THORNTON and TOCZYSKI 2006).

2.2.3. Three activators, Cdc20, Cdh1, and Ama1 required for APC substrate recognition

APC activity and substrate specificity is regulated by the binding of two conserved cofactors, Cdc20 and Cdh1/Hct1 (SCHWAB *et al.,* 1997; 2001; VISINTIN *et al.,* 1997; FANG *et al.,* 1998b; KITAMURA *et al.,* 1998; LORCA *et al.,* 1998). Cdc20 activates the APC to initiate the metaphase to anaphase transition whereas Cdh1 activates the APC for mitotic exit and G1 maintenance (YU 2007; LIM and SURANA, 1996; VISINTIN *et al.,* 1997; SHIRAYAMA *et al*., 1999; KRAMER *et al.,* 2000; RUDNER and MURRAY 2000; GOLAN *et al.,* 2002). In addition, there is a third, meiotic specific cofactor Ama1 that is only found in yeast (COOPER *et al.,* 2000). Ama1 is required for sporulation and contributes to the degradation of Pds1 and the cyclin Clb5 in anaphase of meiosis I (OELSCHLAEGEL *et al.,* 2005).

These activator proteins are characterized by the presence of sequence elements, known as the C-box and the IR-tail that mediate their binding to the APC (SCHWAB *et al.,* 2001; PASSMORE *et al.,* 2003; VODERMAIER *et al.,* 2003). Also, all APC co-activators contain a C-terminal WD40 domain that is believed to recognize APC substrates by interacting with specific recognition elements in these substrates called D-boxes and KEN-boxes (KRAFT *et al.,* 2005; GLOTZER *et al.,* 1991; PFLEGER and KIRSCHNER 2000; PETERS 2006; MANCHADO *et al.,* 2010; YU, 2007; THORNTON and TOCZYSKI, 2006). The Destruction (D-box) motif contains a complete consensus sequence, Rï xï xï Lï xï xï xï xï N/D/E or at least Rï xï xï L (KING *et al.,* 1996). Similarly the KEN-boxes have a K-E-N consensus sequence (PFLEGER and KIRSCHNER 2000). Deletion or modification of D-boxes and KEN boxes can result in the stabilization of the APC substrate (PARK *et al.,* 2008).

2.2.4. The Apc5 subunit and the *apc5*^{ca} *ts* allele

The Apc5 subunit is relatively uncharacterized, but is highly conserved from yeast to humans (YU *et al*., 1998; BENTLEY *et al.,* 2002; SCHREIBER *et al.,* 2011). A specific APC mutant commonly used in our lab is a *ts* allele of the *APC5* gene, coined *apc5*^{CA}; ñCAò for its chromatin assembly defect (HARKNESS *et al.,* 2002). This allele has served as a valuable tool in genetic studies, as it is easily modified by mutations that alter APC function (HARKNESS *et al.,* 2002,

2004, 2005; HARKNESS 2005; ARNADON *et al.,* 2005; ISLAM *et al.,* 2011; TURNER *et al.,* 2010). This allele confers regular growth at 30°C but causes growth defects at 37°C as demonstrated by spot dilutions (Supplementary Figure 6.1A). Serial spot dilutions are a qualitative measure of growth. Small volumes of culture, usually 3 or 5 µl, are spotted alongside each other in a left to right direction on an agar plate, with each spot diluted 10 fold to that of the previous spot. The *apc5*^{ca} mutant is commonly used because it is a mild APC mutant; many other APC mutants have severe growth and cell cycle defects and many are not viable, for example the *apc10*Δ is severely *ts* (Supplementary Figure 6.1A). The *apc5*^{CA} allele has a two base pair (bp) deletion, bp A37 and T38 in the codon encoding the twelfth phenylalanine (F_{12}). The resulting frame shift generates 18 nonspecific amino acids followed by a premature stop codon (Supplementary Figure 6.1B, HARKNESS *et al.,* 2002). The *apc5Δ* strain is inviable and a plasmid expressing the first 78 bp of the *apc5*^{CA}*,* does not rescue *apc5Δ* (HARKNESS *et al.,* 2002); additionally, western analysis of tagged alleles show that the *apc5*^{CA} product is smaller than the WT *APC5* product but still produced an intact C-terminal TAP tag (Supplementary Figure 6.1C, STAHL, DYKES, and HARKNESS, unpublished). Here we isolated multiple C-terminally TAP tagged *apc5*^{CA} strains of which the most severe *ts* isolate was used for further experiments (isolate indicated by **, Supplementary Figure 6.1C). This data suggests the *apc5*^{CA} allele possibly produces an N-terminally truncated protein initiated at an internal methionine. Moreover, less of the smaller *apc5*^{CA} product was detected which may suggest the truncated protein is functional but in short supply.

The temperature sensitivity of this allele is, for all intents and purposes, the important feature of this *apc5*^{CA} mutant in regard to the experiments performed in this study. However, understanding the nature of this *apc5*^{CA} allele and how it conveys temperature sensitivity may implicate the specific influence Apc5 has on APC function and help us better interpret data generated by this mutant. To this end we investigated the *apc5*^{CA} allele further and tested our hypothesis that the *apc5*^{CA} allele is producing an N-terminal truncation of the Apc5 subunit (see section 4.3.).

2.2.5. Longevity in yeast: replicative and chronological lifespan

In the model organism *Saccharomyces cerevisiae* there are two separate but overlapping assays to study factors that contribute to aging. Replicative aging, or the replicative lifespan assay, is a measure of the number of daughter cells, mother cells can produce before they senesce (MORTIMER and JOHNSTON 1959). This phenomenon is known as ñreplicative senescesò or the ñHayflick limitò and is relevant to aging of somatic cells of complex eukaryotes (HAYFLICK and MOORHEAD 1961). The asymmetrical division of yeast may be particularly informative as a model for aging of asymmetrical dividing stem cells in these organisms. Chronological aging, or the chronological lifespan assay, is a measure of the survival and reproductive capacity of cells that have entered a stationary or quiescence state due to the depletion of glucose and other nutrients in the growth medium (LONGO *et al.,* 1996). This model is relevant to the study of aging post mitotic differentiated cells of complex eukaryotes.

These two lifespan assays are different since they take physiologically distinct processes into account. The replicative lifespan assay measures senesces, where cells have lost the ability to reproduce. The chronological lifespan assay measures the capacity to maintain reproductive competence during quiescence. There is often confusion regarding the definition of quiescence, which is physiologically distinct from senesces. Although neither quiescent nor senescent cells divide, quiescent cells retain the ability to respond to growth signals and divide, whereas senescent cells have lost the ability to divide. Both of these assays have been instrumental in understanding the processes that contribute to aging. The different processes considered by these two assays have highlighted the overlap between factors that contribute to aging; such as, DNA damage, DNA replication stress, genomic stability, and nutrient sensing and allocation (reviewed in BURHANS W.C. and WEINBERGER M. 2012).

2.3. Identifying Novel APC Targets and Regulators

2.3.1. Genetic screens

Saccharomyces cerevisiae is a powerful model organism for studying fundamental molecular biology. This distinction is due to the ease, precision, and diversity of genetic manipulations that

can be performed on yeast. The genetic malleability of yeast make it exceptional for genetic analysis and screens.

Genetic screens are experimental techniques used to identify and select for individuals who possess a phenotype of interest in a mutagenized population (HARTWELl *et al.,* 2008). Genetic screens can provide insight into gene function as well as the molecular events that underlie a biological process or pathway. There are two basic types of genetic screens; forward and reverse screens. Forward genetic screens identify gene disruptions responsible for a particular phenotype. Reverse genetic screens identify the phenotypes of a known gene disruption. In addition, there are screens to identify physical interactions among proteins, such as the Y2H. In an effort to identify novel APC substrates, regulators and/or modifiers a variety of screening techniques described below were utilized in this study. The data presented was completed by prior researchers in Dr. Troy Harkness lab.

2.3.2. Yeast 2 hybrid

The Yeast 2 hybrid (Y2H) is a molecular biology technique used to discover proteinï protein interactions (YOUNG 1998). The system consists of three elements all present in one cell: a reporter gene, in our case the *ADE2* gene under the control of an inducible promoter, such as the *GAL2* promoter (JAMES *et al.,* 1996), and one of each, bait and prey selectable plasmid borne constructs. The reporter gene is activated by the binding of a transcription factor that is separated into two fragments, the *GAL2* binding domain (GBD) and the *GAL2* activating domain (GAD). The bait construct produces a fusion protein product of a known protein of interest fused to the GBD, which will bind the *GAL2* promoter sequence just upstream of the *ADE2* reporter gene. Similarly, the prey construct produces a fusion protein of the GAD and an unknown protein or prey protein. If bait and prey proteins physically interact they bring the GBD and GAD into close proximity resulting in reporter gene transcription. Transcription of the reporter gene allows for that particular cell to grow on selective media lacking adenine (Ade^-). The prey-GAD fusion construct is derived from a library of plasmids, each construct containing a unique gene fragment. The library can approximate the entire genome, including all reading frames. Plasmids from positive colony forming units (cfu) are isolated and sequenced to identify unknown binding partners.

2.3.3. Identification of Fob1 as an Apc5 binding partner in a Y2H screen

In an effort to identify novel APC substrates, regulators and/or modifiers a Y2H screen was conducted using the APC subunit Apc5 as bait. The Y2H plasmid pGBD-*APC5* was functional as it complemented the *apc5*^{CA} *ts* growth phenotype (HARKNESS, unpublished). A prey plasmid encoding nucleotides 5-1263 of the replication *fo*rk *b*lock binding protein (Fob1) was recovered in this screen. This was of particular interest to us because Fob1 has been identified as an important player in the molecular mechanisms involved in determining lifespan in yeast (reviewed in ZUIN *et al.,* 2010; KOBAYASHI 2011a; PAN 2011; SINCLAIR *et al.,* 1997; DEFOSSEZ *et al*., 1999).

2.3.4. Identification of a Fob1 putative modified D-box that is important for Apc5 binding in a Y2H screen

To identify regions within Fob1 responsible for Apc5 interactions *FOB1* fragments were subcloned into the Y2H prey vector, pGAD·C2, and tested for interactions with pGBD-*APC5* (Supplementary Figure 6.2A and Figure 6.2B). The original *FOB1* clone we identified in the Y2H screen contained *FOB1* nucleotides 5-1263. *FOB1* gene fragments containing nucleotides 1-299 (D1), 300-1258 (D2), 1259-1701 (D3), 1-1258 (D1/D2), or 1-849 (N-half) (Supplementary Figure 6.2A) did not interact with Apc5. The only other fragments capable of interacting with Apc5 were the full length *FOB1* gene and the fragment 850-1701 (C-term), which displayed a very weak interaction (Supplementary Figure 6.2B). This implies that *FOB1* nucleotides 1259-1263 are important but not exclusive for this Fob1-Apc5 interaction. Additional motifs or structural features likely contribute to a full robust interaction. Upon close inspection, the region flanking the basepairs 1258-1263 encodes $_{417}$LANEADN$_{423}$ similar to the known modified D box, (LXEXXXN) referred to as the L box, a sequence found in APC substrates (see section 2.2.3). Given that the main function of the APC is to target substrates for ubiquitin- and proteasome-dependent degradation, the possibility that the Fob1/Apc5 interaction defined Fob1 as a bonafide APC substrate was tested using a biochemical approach (see result section 4.1).

2.3.5. Aptamers

Aptamers are short peptide-based agents that provide an innovative approach to forward and reverse genetic screens that can be used to identify and analyze signaling networks by inhibiting protein function (COLAS, *et al.,* 1996). Aptamers are small variable peptide sequences that can interact with proteins in an antibody-like manner. Combinatorial libraries of aptamers typically contain members that bind almost any protein target. Constrained peptides displayed on the surface of a scaffold protein such as the *E. coli* thioredoxin A (TrxA) are preferred as they generally bind stronger and are more stable than linear peptides (COLAS *et al.,* 1996; DAVIDSON and SAUER 1994; GEYER and BRENT 2000). TrxA mounted aptamers have been used extensively to interfere with cellular functions (CUI *et al.,* 2005; GILCH *et al.,* 2007; LOPEZ-OCHOA *et al.,* 2006; ZHAO and HOFFMANN 2006). Forward screens can select aptamers that suppress phenotypes. Aptamers that can suppress a phenotype of interest can then be cloned into an Y2H bait vector and used to identify binding partners. This approach can identify and decipher signaling networks and potential druggable protein targets. Reverse screens select aptamers that specifically bind target proteins. These aptamers can then be screened for phenotypes since this binding may potentially interfere with or modify the function of the target protein. The aptamers that bind a specific target can be isolated using the Y2H system. This Y2H aptamer system has been used for reverse screening, where aptamers against specific proteins are sought that block biological processes involving cell cycle (COLAS *et al.,* 1996; FABBRIZIO *et al.,* 1999), apoptosis (BUTZ *et al.,* 2000; NAUNENBERG *et al.,* 2001) and viral coat protein formation (BUTZ *et al.,* 2001).

2.3.6. Identifying aptamers that suppress APC mutant phenotypes

As the APC has been implicated in cancer development and longevity (CUI *et al.,* 2005; HARKNESS *et al.,* 2004; SMOLDERS and TEODORO 2011; TURNELL and MYMRYK 2006; WASCH and ENGELBERT 2005; WÄSCH *et al.,* 2010; POSTNIKOFF *et al*., 2012; POSTNIKOFF and HARKNESS 2012), it was proposed that identification of drugs or aptamers that interfere with APC activity through specific interaction with the APC subunits and/or APC regulators could provide an effective therapeutic intervention (WASCH and ENGELBERT 2005). Similarly, this approach can be used to identify unknown APC targets or regulators. Identifying

aptamers able to modulate APC activity may help identify novel molecular networks or pathways that intersect with the APC. To test this idea, aptamers were screened for their ability to suppress the *apc5*^{CA} *ts* phenotype (Harkness, unpublished). This screen was done prior to my arrival in the lab.

2.3.7. Identifying the aptamer Y65, which resembles Elc1 and binds to Cin5

A forward screen was performed with a library of aptamers in cells harboring the *apc5*^{CA} *ts* allele. The aptamers were expressed on a plasmid borne TrxA scaffold under the control of a galactose inducible promoter. Over 200 aptamers, from millions screened, suppressed the *apc5*^{CA} *ts* phenotype. Many of the aptamers that suppressed the *apc5*^{CA} *ts* phenotype were recovered, retested and sequenced (Supplementary Figure 6.3). The sequence of the aptamer Y65 is shown below: MSDKIILTDDSFDTDVLKADGAILVDFWAEWCGPVRQ**KSDKEYE**RVLGLGLRRLVRAK (bold sequence has homology to the Elc1 sequence KDDKEYE). The aptamer Y65 was of interest to us because it had homology to Elongin C (Elc1), a ubiquitin protein ligase conserved among eukaryotes which forms a complex with the cullin family member Cul3 (HYMAN *et al.,* 2002; RIBAR *et al.,* 2007). In humans, the *ELC1* orthologue is part of the Von Hippel-Lindau (VHL) tumor suppressor E3 complex that targets the hypoxia inducible factor 1 Ŭ(HIF1Ŭ), an oncogene, for degradation under normal conditions (BOTUYAN *et al.,* 1999; KIBEL *et al.,* 1995). This recovered Y65 aptamer was then cloned into the Y2H bait vector for a reverse screen to find binding partners. Cin5, an evolutionarily conserved stress response transcription factor was identified as a binding partner in this Y2H screen (data not shown). This was particularly interesting because Cin5 was also identified in the Y2H screen which identified Fob1 as a binding partner for Apc5. We hypothesize that Y65 binding to Cin5 may mask a degradation signal. Stabilized Cin5 may elicit a stress response that in turn may help alleviate the *apc5*^{CA} *ts* defects. (see section 4.2).

2.3.8. The stress response transcription factor Cin5

Cin5, otherwise known as Yap4, is a basic leucine zipper (bZIP) stress response transcription factor of the yAP-1 family that mediates pleiotropic drug resistance and salt tolerance

(FERNANDES *et al.,* 1997; FURUCHI *et al.,* 2001; MENDIZABAL *et al.,* 1998). Under reducing conditions Cin5 is sequestered in the cytoplasm by Lot6-20S proteasome complex and protected from degradation (SOLLNER *et al.,* 2009). Lot6 is a quinone reductase that behaves like a redox switch that senses oxidative stress. Under conditions where Lot6 is oxidized including mild hyperosmotic and oxidative stress, temperature shift or metal exposure, Lot6 releases Cin5 which is then localized to the nucleus where it is unstable but also physically interacts with the repressor Tup1-Cyc8 complex to drive expression of stress response target genes (SOLLNER *et al.,* 2009; HANLON *et al.,* 2011). Oxidized Lot6 also affects the physiological function of the proteasome (SOLLNER *et al.,* 2009). Cin5 has several putative phosphorylation sites, but only the mutation of residues T192 and S196 impairs its phosphorylation under different stress conditions (PEREIRA *et al.,* 2009). Phosphorylation of these sites appears to stabilize the protein but does not affect localization. Whether these sites impinge on Cin5 activity is unclear.

2.4. Fob1 and the APC

2.4.1. Fob1 and ribosomal DNA metabolism

2.4.1.1. Nucleolus and ribosomal DNA

The nucleolus is a non-membrane bound structure found within the nucleus of eukaryotic cells. Its function is to transcribe ribosomal DNA (rDNA) and begin assembling the resulting ribosomal RNA (rRNA) and appropriate proteins into ribosomes (WATSON *et al*., 2004). In *S. cerevisiae* the rDNA region consists of 100-200 copies of rDNA units arrayed tandemly in a central position on chromosome XII. Each repeat of rDNA (9.1 kb) consists of a 5S and 35S rRNA coding region which is further processed into 5.8S, 18S and 25S RNAs. The 35S rRNA is transcribed by RNA polymerase I, and the 5S rRNA coding region transcribed by RNA polymerase III (NOMURA, 2001). The 35S and 5S rRNAs are transcribed in directions opposite to each other. The rDNA locus also contains two non-transcribed spacers (NTS) i.e., NTS1 and NTS2 (SKRYABIN *et al.,* 1984; BREWER and FANGMAN, 1988; LINSKENS and HUBERMAN, 1988). These two spacers contain multiple *cis*-acing DNA elements that control replication initiation and pausing, the *r*eplication *f*ork *b*lock (RFB) site in NTS1, and the *a*utonomously *r*eplicating *s*equence (ARS) in NTS2 (Figure 2.3A). During S phase of the cell cycle, replication starts at the ARS

bidirectionally and the rightward-moving replication forks are arrested at the RFB located near the 3' end of the 35S rRNA in NTS1. This unidirectional blocking of the replication fork ensures that rDNA is replicated in one direction, the same direction with that of transcription of 35S rRNA (BREWER *et al.,* 1992).

Ribosomal proteins (RP) account for approximately 50% of the total protein, and rRNA represents approximately 80% of the total RNA in a yeast cell (WARNER 1999). Due to the highly repetitive structure and active transcription, the rDNA region is highly recombinogenic and is the most fragile site in the chromosome (reviewed in KOBAYASHI 2010). Genomic stability correlates with lifespan, particularly stability in the rDNA region due to its sheer size and high transcriptional activity. This region is the dominant source of genomic instability and is believed to be a major contributing factor to aging in yeast (reviewed in KOBAYASHI 2008; KOBAYASHI 2011; KOBAYASHI 2010; GANLEY *et al.,* 2009).

The nucleolus is also an important player in cell cycle regulation as it sequesters a key regulator of mitotic exit, the phosphatase Cdc14, and releases it in a cell cycle dependent manner (D'AMOURS and AMON, 2004; PEREIRA and SCHIEBEL, 2004). Mitotic exit is coupled with the late segregation of the rDNA locus through the timely release of Cdc14. This phosphatase acts to reset the cell cycle to the G1 state through the inactivation of mitotic cyclin-dependent kinases (CDK) and the reversal of CDK phosphorylation events (STEGMEIER and AMON, 2004; VISINTIN *et al.,* 1998).

2.4.1.2. Fob1, the RENT Complex and rDNA Stability

Fob1 is an important player in rDNA maintenance and metabolism (MOHANTY and BASTIA 2004; KOBAYASHI and HORIUCHI 1996; KOBAYASHI *et al.,* 1998; STRAIGHT 1999; HUANG 2006; HUANG 2003; KOBAYASHI 2005; JOHZUKA and HORIUCHI 2009). One function of Fob1 is to bind the RFB site within NTS1 (MOHANTY and BASTIA 2004) and recruit and anchor the RENT (*re*gulators of *n*ucleolar silencing and *t*elophase exit) complex within the nucleolus. The RENT complex consists of Cdc14, the Cdc14 inhibitor Net1/Cfi1, the silencing factor Sir2, and also Tof2, Csm1, and Lrs4 (SHOU *et al.,* 1999; STRAIGHT *et al.,* 1999;

VISINTIN *et al.,* 1999; HUAG and MOAZED 2003; HUANG *et al.,* 2006; HUANG 2003; KOBAYASHI 2005; JOHZUKA and HORIUCHI 2009). The RENT complex has multiple functions: it sequesters Cdc14 within the nucleolus in a cell cycle dependent manner, promotes DNA silencing within the rDNA, and inhibits hyper-recombination between repeats, thus promoting overall stability of the array (SHOU *et al.,* 1999; STRAIGHT *et al.,* 1999; VISINTIN *et al.,* 1999; HUANG and MOAZED 2003; HUANG *et al.,* 2006). Specifically, components of the RENT complex Tof2, Lrs4, and Csm1 interact with cohesin to promote stability of the rDNA array (Figure 2.3B; HUANG *et al.,* 2006). Cohesin is a clamp-like protein that holds together sister chromatids until all chromatids have been attached to mitotic spindles prior to the onset of anaphase (NEWMAN *et al.,* 2000; GRAUMANN *et al.,* 2004; MICHAELIS *et al.,* 1997; BRITO *et al.,* 2010). Furthermore, Cdc14 promotes the late segregation of the rDNA array through the anaphase recruitment of condensin, which serves to both resolve cohesin-independent linkages and compact the array (D'AMOURS *et al.,* 2004; SULLIVAN *et al.,* 2004; TORRES-ROSELL *et al.,* 2004; MACHIN *et al.,* 2005). Condensin is a conserved protein belonging to a large family of chromosomal ATPases, known as Structural Maintenance of Chromosomes (SMC) proteins (HIRANO 2012; WOOD *et al.,* 2010). Condensin recruitment to the RFB sites is critical in facilitating higher order structure and rDNA integrity, conceivably by holding the tandem rDNA repeats in loops by an array of interacting condensins (Figure 2.3C; JOHZUKA and HORIUCHI, 2009). This condensed Fob1-dependent structure may reduce rDNA recombination and transcription, and may contribute to the regulation of rDNA activity. This function may be particularly important during nutrient starvation and quiescence (TSANG *et al.,* 2007).

2.4.1.3. Fob1 and rDNA recombination

Despite the Fob1-recruited RENT complex promoting overall stability of the rDNA array when Fob1 binds the RFB site and unidirectionally blocks and stalls DNA replication forks, the stalled replication machinery can result in double stranded DNA breaks (DSBs) (Figure 2.3D; BAIRWA *et al.,* 2010; KOBAYASHI and HORIUCHI 1996; KOBAYASHI *et al.,* 1998; KOBAYASHI, 2003). Due to the repeating structure of the rDNA, homologous DNA repair of these DSBs can result in unequal sister chromatid exchange (USCE), where one or more tandem repeat is either lost or duplicated, generating substantial genomic instability within the rDNA region

(KOBAYASHI *et al.,* 1998; IDE *et al.,* 2010; SINCLAIR *et al.,* 1997). Although *FOB1* encodes a fungal specific gene that is not conserved in higher eukaryotes, DSBs, resulting from stalled DNA replication forks, and USCE within the rDNA region is a conserved process (MOHANTY *et al.,* 2009; GANLEY *et al.,* 2009). This process may be beneficial in an evolutionary capacity, by maintaining a high copy number of rDNA repeats and ensuring a high degree of homology among tandem repeats (reviewed in KOBAYASHI 2010; SINCLAIR *et al.,* 1997; RICHARD *et al.,* 2008). High copy numbers of these critical rDNA genes provide a cell robustness and competiveness. It is probable that this Fob1 function is conserved and accomplished by other factors in higher eukaryotes. One possible candidate is a conserved protein called Geminin. Geminin is involved in preventing DNA replication and is targeted for degradation by the APC during anaphase and is absent in G1 of the cell cycle (THOMAS *et al.,* 1998). Taken together, Fob1 plays a multifaceted role in rDNA metabolism, contributing to rDNA stability through the RENT complex, as well as generating instability by creating DSB within this region.

2.4.2. Fob1 and the APC interact genetically

The physical interaction between Fob1 and the APC, as identified by the Y2H (see section 2.3), and the role both the APC and Fob1 play with regard to genomic stability and longevity prompted an investigation to test for genetic interactions with respect to growth and replicative lifespan (RLS), a measure of how many daughter cells a single mother will produce prior to senescence (KENNEDY *et al*., 1994). The data generated by Dr. Troy Harkness, and former M.Sc. student Jing Chen (CHEN, C. 2006), has led to our hypothesis that the APCôs role in maintaining genomic stability and longevity is achieved, at least in part, through the degradation of Fob1.

2.4.2.1. Deletion of *FOB1* in APC mutants has little impact on growth, but a positive influence of APC mutant replicative lifespan.

Dr. Harkness and Jing Chen deleted *FOB1* in $apc5^{CA}$ and *apc10*Δ cells to ask whether this restored $apc5^{CA}$ or *apc10*Δ associated *ts* growth phenotypes using serial spot dilutions. Serial spot dilutions are a measure of growth. Small volumes of culture, usually 3 or 5 µl, are spotted alongside each other on an agar plate, each successive spot is diluted 10 fold to that of the previous spot. These

plates may be made with various media and grown at various temperatures. Spot dilution results show that deletion of *FOB1* had little impact on *apc5*^{CA} *ts* growth and mildly suppressed *apc10*Δ *ts* growth (Supplementary Figures 6.4A and 6.4B). Removal of *FOB1* from the cell clearly has little benefit on growth in cells harboring an impaired APC.

Next they asked whether *FOB1* deletion was of any benefit to *apc5*^{CA} or *apc10*Δ defective RLS. Conventionally, RLS results are interpreted at the 50% viability or the mean RLS, the number of daughter cells produced by 50% of the population studied. Their results demonstrate that deletion of *FOB1* increased both *apc5*^{CA} and *apc10*Δ RLS (Supplementary Figures 6.4C and 6.4D). The increase or partial recovery in RLS associated with the deletion of *FOB1* was approximately equal in WT, *apc5*^{CA}, and *apc10*Δ cells; nevertheless, disruption of *FOB1* did alleviate the mutant APC RLS defects. These data imply that the growth and RLS defects of APC mutants are broad in range and are not solely due to excess Fob1. However, an intact APC may be required for removal of Fob1 for normal RLS. This observation still lends support for our theory that the APC regulates lifespan, at least in part, by affecting Fob1 protein levels.

2.4.2.2. *FOB1* can be toxic to cells and reduce replicative lifespan

Prior to my arrival to Dr. Harknessôs lab the effects of increased *FOB1* expression on RLS and cell cycle progression was assessed (Chen, M.Sc. Thesis). Increased *FOB1* expression, via a 2μ plasmid, reduced RLS in WT cells. This suggests that increased Fob1 activity reduced lifespan. Moreover, increased *FOB1* expression in *apc5*^{CA} cells did not reduce RLS any further (Supplementary Figure 6.5A). This suggested that Fob1 levels may already be elevated to a functional saturation in *apc5*^{CA} cells and any further increase through expression from a 2μ plasmid had no further negative effect of RLS.

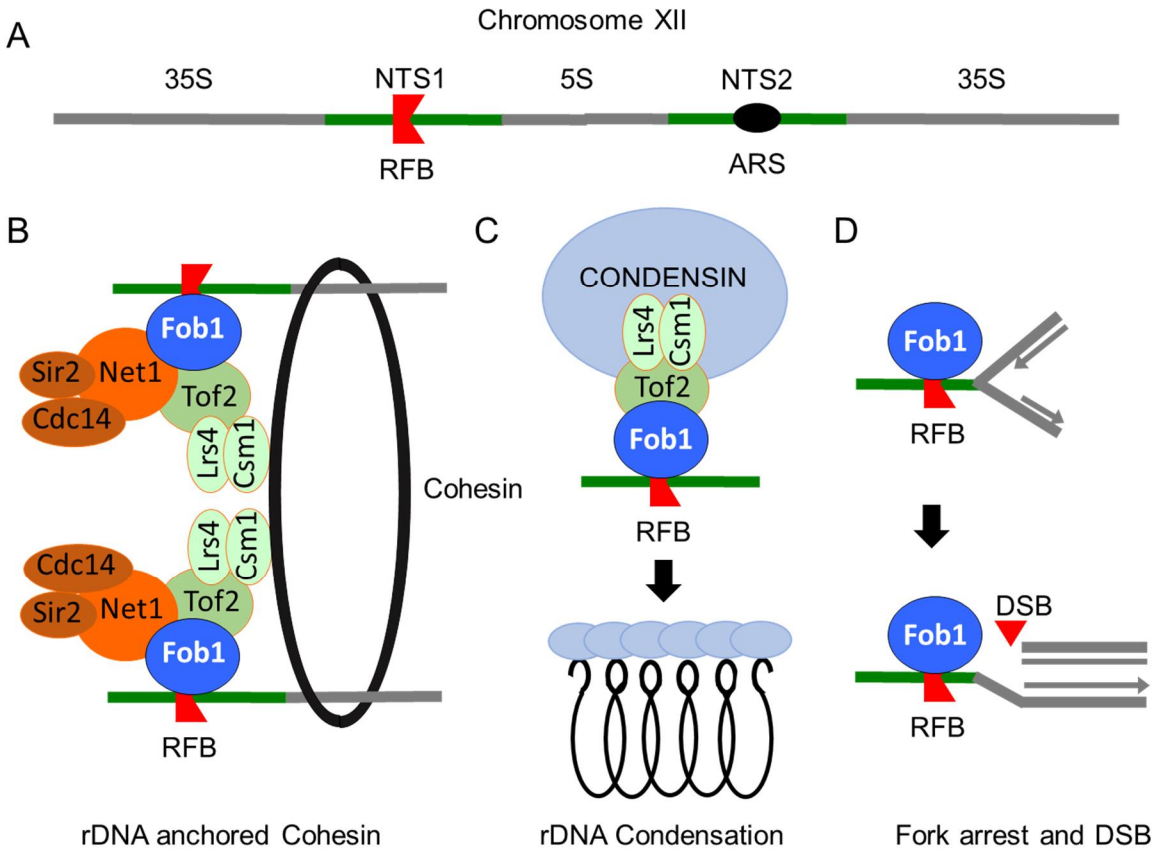


Figure 2.3 Functions of Fob1 within rDNA

(A) Schematic of the rDNA array in chromosome XII, each rDNA unit has 35S and 5S-encoding sequences interspersed by two non-transcribed spacers, NTS1 and NTS2, shown in green. Note Fob1 binds to the replication fork block (RFB) site within NTS1, and NTS2 contains the autonomously replicating sequence (ARS). (B) Schematic of Fob1 anchoring the RENT complex (containing Net1, Cdc14, and Sir2), Tof2, Csm1, Lrs4 and cohesin within the rDNA array. Note that cohesin, which clamps rDNA sister chromatids together, is prevented from sliding, thereby aiding in the suppression of unequal sister chromatid exchange (USCE). (C) Schematic of Fob1 recruiting condensin which in turn facilitates higher order structure of the rDNA by holding tandem repeats in loops by an array of interacting condensins. (D) Schematic representation of Fob1-mediated DNA replication fork arrest which can result in a double stranded DNA break (DSB). Repair of the DSB by homologous recombination, using an adjacent tandem repeat as a template, can result in USCE. Information obtained and adapted from BAIRWA *et al.,* 2010; JOHZUKA and HORIUCHI 2009; HUANG *et al.,* 2006.

On the other hand, overexpression of *FOB1* via the *GAL$_{pro}$-FOB1-HA* construct was toxic to growth in WT cells and even more so to *apc5^{CA}* cells (Supplementary Figure 6.5B). Fob1 expression from the 2μ plasmid did not impair WT or *apc5^{CA}* growth. The slow growth in the presence of excess Fob1 suggested the removal of Fob1 may be required for efficient cell cycle progression.

To test whether Fob1 levels were indeed elevated in *apc5^{CA}* cells Fob1-HA was expressed from a *GAL*-inducible promoter for 6 hours in WT and *apc5^{CA}* asynchronous cells and samples were removed every hour to observe Fob1-HA induction (Supplementary Figure 6.5C, Chen M.Sc.). After induction, the cells were washed and resuspended in 2% glucose to reduce Fob1-HA expression, with samples taken every hour for FACS (Fluorescence Activated Cell Sorting) to assess cell cycle progression and Western analysis to measure protein levels (Supplementary Figure 6.5D). FACS data is a measure of cellular DNA content where the first peak represent cells in G1 and the second peak represents G2. It was demonstrated that Fob1-HA did accumulate faster, and degraded slower, in *apc5^{CA}* cells compared to WT cells (Supplementary Figures 6.5C and 6.5D). The FACS data showed that overexpression of *FOB1* caused cell populations to accumulate in G1. WT cells re-entered the cell cycle within a few hours, whereas in *apc5^{CA}* cells, cell cycle re-entry was delayed (Supplementary Figure 6.5D). It is unlikely that accumulated Fob1 levels, in the *apc5^{CA}* cells, alone is responsible for the delayed re-entry into the cell cycle as additional APC targets will have likely accumulated. Still this data does support a model where Fob1 must be removed during G1 by the action of the APC. Failure to do so results in G1 impairment and reduced RLS.

2.5. Rationale

The work introduced in the above sections sheds light on the APC's role in longevity and stress response, and demonstrates the feasibility of using Y2H and aptamer screens in *S. cerevisiae* to identify APC targets, regulators and/or modifiers. Identification of targets, regulators and/or modifiers will uncover novel molecular networks that interplay with the APC and will help us better understanding of the role the APC plays in cell cycle progression, chromatin assembly, genomic stability, stress response and longevity. As many of the proteins involved in these

processes are evolutionarily conserved the findings from studies on *S. cerevisiae* should be relevant to higher organisms.

The Y2H screen identified the protein Fob1 as a potential target of the APC and the presence of a putative modified D-box within Fob1 supports this notion (Supplementary Figure 6.2). Furthermore, there is a clear genetic interaction between *FOB1* and the *APC5* and *APC10* with regard to RLS (Supplementary Figure 6.4). Overexpression of Fob1 does reduce RLS in WT cells but does not reduce RLS any further in the defective APC mutant *apc5*^{CA} (Supplementary Figure 6.5). These data suggest that the removal of excess Fob1 requires an intact APC. These data have led us to hypothesize that the APCôs role in maintaining longevity is, at least in part, through the degradation of Fob1.

The aptamer Y65, which has the ability to suppress *apc5*^{CA} *ts* defects, was chosen because it has homology to the ubiquitin ligase Elc1 and binds to the stress response transcription factor Cin5. This is confirmation that the molecular networks of the APC do interplay with stress response pathways. We hypothesize that the aptamer Y65 masks a degradation signal in the stress response transcription factor Cin5. In turn, the stabilized Cin5 elicits stress responses that help alleviate *apc5*^{CA} *ts* defects. Furthermore, this study demonstrates that aptamers or small peptides, able to modify stress response elements, have the potential to discover drug targets for therapeutic use in higher eukaryotes.

Finally, the severe *ts* isolate of the *apc5*^{CA} allele appear to be truncated yet still produce an intact C-terminal TAP tag (Supplementary Figures 6.1). To better understand the nature of the *apc5*^{CA} allele and how it conveys temperature sensitivity will help us better interpret data generated by this mutant. To this end we investigated the *apc5*^{CA} allele further and tested our hypothesis that the *apc5*^{CA} allele is producing an N-terminal truncation of the Apc5 subunit (see section 4.3.).

2.6. Hypotheses and Experimental Objectives

This study is broken down into two primary projects:

2.6.1. Authenticate Fob1 as a novel APC substrate

We hypothesize that the APCôs role in maintaining longevity is, at least in part, through the degradation of Fob1.

- Confirm and characterize the physical interaction between Fob1 and the Apc5
- Confirm APC-dependent Fob1 degradation
- Investigate Fob1 and APC interaction with regard rDNA metabolism and longevity

2.6.2. Identify possible mechanisms for the ability of Y65/Cin5 to suppress *apc5*^{CA} *ts* defects

We hypothesize that the aptamer Y65 masks a degradation signal in the stress response transcription factor Cin5. In turn, the stabilized Cin5 elicits stress responses that helps alleviate *apc5*^{CA} *ts* defects.

- Confirm proteasome- and ubiquitin-dependent degradation of Cin5
- Characterize the ubiquitin dependent regulation of Cin5

2.6.3. Characterize the *ts apc5*^{CA} allele.

We hypothesize that the *ts apc5*^{CA} allele is producing an N-terminal truncated Apc5 subunit.

- Construct an APC5 allele that is N-terminally truncated at methionine 99 to see whether this *apc5*^{M99} allele can mimic *apc5*^{CA} defects.

3. MATERIALS AND METHODS

3.1. Yeast genetics and screens

3.1.1. Yeast strains and cell culture

The yeast strains used in the Fob1 study are listed in Table 3.1 and strains used in the Cin5/Y65 study are listed in Table 3.2. Yeast cells were grown either in YPD (1% yeast extract, 2% peptone, 2% glucose) or synthetic defined (SD) lacking the appropriate amino acids (1.7g/L yeast nitrogen base, 5 g/L $(NH_4)_2SO_4$, 1.3 g/L drop out powder minus appropriate amino acids, 1 tablet NaOH). Glucose, galactose or sucrose were used at a final concentration of 2%. SD medium was used for selective growth of yeast auxotrophs. The necessary auxotrophic supplements included 20 mg/L adenine hemisulfate salt, 20 mg/L L-histidine HCl monohydrate, 100 mg/L L-leucine, 20 mg/L L-tryptophan, 20 mg/L L-uracil. Any of the above auxotrophic supplements can be omitted to provide a selection media for yeast transformation (ROSE *et al.,* 1990). The auxotrophic supplements were made in 100 X stocks. For plates, 2% agar was added to either YPD or SD medium prior to autoclaving. For long term storage, 800 µl of liquid culture grown overnight at 30ºC were mixed with 200 µl of 80% glycerol then stored at -80ºC.

Table 3.1. Strains used in Fob1 study

Strain	Genotype	Source/Reference
YTH5	*MAT*α *ade2 his3 lys2 ura3*	W. Neupert; S288C
YTH6	*MAT***a** *ade2 his3 lys2 ura3*	W. Neupert; S288C
YTH225 (PJ69-4A)	*MAT***a** *trp1-901 leu2-3 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lac*	E. Craig
YTH370 (DSY925)	*MAT***a** *ade1 bar1 his3 leu2 trp1 ura3 cdc16-1*	D. Stuart; *BF264-15Du* background
YTH457	*MAT***a** *ade2 his3 leu2 ura3 apc5^{CA}*	Harkness *et al*. 2002; S288C, 8 backcrosses with YTH5/6
YTH1029	*MAT***a** *his3Δ1 Δleu2 Δmet15 Δura3*	Research Genetics
YTH1033	*MAT***a** *his3Δ1 Δleu2 Δmet15 Δura3 apc10::KanMX6*	Research Genetics
YTH1037	*MAT***a** *his3Δ1 Δleu2 Δmet15 Δura3 fob1::KanMX6*	Research Genetics
YTH1049	*MAT*α *cdc16-1 leu2 his3 ura3*	This study; YTH370, 5 backcrosses with YTH5/6
YTH1235	*MAT***a** *ade2 his3 leu2 lys2 ura3*	Harkness *et al.* 2004; isogenic with YTH5/6
YTH1636	*MAT***a** *ade2 his3 leu2 lys2*(?) *ura3*	Harkness *et al.* 2004
YTH1637	*MAT*α *ade2 his3 leu2 lys2*(?) *ura3 apc5^{CA}*-PA*::His5*	Harkness *et al.* 2004
YTH1693	*MAT*α *ade2 his3 leu2 ura3 apc10::KanMX6*	This study; S288C
YTH2425	*MAT*? *ade2 his3 leu2 lys2*(?) *ura3*	This study; S288C
YTH2454	*MAT*? *ade2 his3 leu2 ura3 fob1::KanMX6*	This study; 7 backcrosses with YTH5/6

YTH2456	*MAT*? *ade2 his3 leu2 ura3 lys2*(?) *apc5^{CA}*-PA*::His5^{+} fob1::KanMX6*	This study; S288C
YTH3175	*MAT*? *ade2 his3 leu2 ura3 lys2*(?) *apc5^{CA}*-PA*::His5^{+} fob1::KanMX6*	This study; S288C
YTH3179	*MAT*? *ade2 his3 leu2 ura3 fob1::KanMX6*	This study; S288C
YTH3181	*MAT*? *ade2 his3 leu2 ura3 lys2*(?) *apc5^{CA}*-PA*::His5^{+} fob1::KanMX6*	This study; S288C
YTH3334	*MAT*? *ade2 his3 leu2 ura3 apc10::KanMX6 fob1::KanMX6*	This study; S288C
YTH3335	*MAT*? *ade2 his3 leu2 ura3 apc10::KanMX6 fob1::KanMX6*	This study; S288C
YTH3367	*MAT*? *ade2 his3 leu2 ura3 apc10::KanMX6*	This study; S288C
YTH3880	*MAT***a** *his3 leu2 met15 ura3 APC5-TAP::HIS3*	A. Ghavidel
YTH4001	as YTH1235, but *bar1::KanMX6*	This study; S288C
YTH4004	as YTH457, but *bar1::KanMX6*	This study
YTH4028	As YTH5, but *APC5-TAP::HIS3*	This study
YTH3863	w303, *cdc20-1*	A. Ghavidel
YTH3638	*MAT*α*, rpn10:: KanMX6*	Wei Xiao; Research Genetics
YTH3785	*MAT*? *cdh1:: KanMX6*	Wei Xiao; Research Genetics
YTH4104	*MAT***a** *ade2 leu2 ura3 FOB1-TAP::HIS3*	A. Ghavidel
YTH4269	*MAT*α *ade2 his3 leu2 ura3*	This study; isogenic with YTH5/6, S288C
YTH4267	*MAT***a** *ade2 his3 leu2 ura3*	This study; isogenic with YTH5/6, S288C
YTH4414	*MAT*? *ade2 his3 leu2 ura3*	This study; S288C
YTH4415	*MAT*? *ade2 his3 leu2 ura3 fob1::KanMX6*	This study; S288C
YTH4416	*MAT*? *ade2 his3 leu2 ura3 apc5^{CA}*-PA*::His5^{+}*	This study; S288C
YTH4417	*MAT*? *ade2 his3leu2 ura3 apc5^{CA}*-PA*::His5^{+} fob1::KanMX6*	This study; S288C
YTH4422	CCFY100 triple silencer W303ï 1A *MATa ade2–1 ura3–1 trp1–289 leu2–3,112 his3–11,15 can1–100*	K. Runge
YTH4444	as 4422, but *apc5^{CA}*-PA*::His5^{+}*	This study
YTH4447	as 4422, but *fob1::KanMX6*	This study
YTH4450	as 4422, but *apc5^{CA}*-PA*::His5^{+} fob1::KanMX6*	This study
YTH4519	*MAT***a** *ade2 his3 leu2 ura3 FOB1^{DB1}-TAP::HIS3*	This study; S288C
YTH4520	*MAT***a** *ade2 his3 leu2 ura3 FOB1^{E420V}-TAP::HIS3*	This study; S288C
YTH4525	*MAT***a** *ade2 his3 leu2 ura3 FOB1-TAP::HIS3*	This study; S288C
YTH4539	*MAT*α*, rpn10:: KanMX6 FOB1-TAP::HIS3*	This study; Research Genetics
YTH4540	*MAT*α*, rpn10:: KanMX6 FOB1^{DB1}-TAP::HIS3*	This study; Research Genetics
YTH4541	*MAT*α*, rpn10:: KanMX6 FOB1^{E420V}-TAP::HIS3*	This study; Research Genetics
YTH4546	Diploid of YTH457 and YTH3 *APC5/apc5^{CA}*	This study; S288C
YTH4547	Diploid as 4546 and M99 N-terminal truncation with *leu2-GAL10pro-M99apc5 APC5/apc5^{M99} (A)*	This study; S288C
YTH4548	Diploid as 4546 and M99 N-terminal truncation with *leu2-GAL10pro-M99apc5 apc5^{M99}/ apc5^{CA} (C)*	This study; S288C
YTH4615	*MAT***a** *ade2 his3 leu2 ura3 FOB1^{E420V}-TAP::HIS3*	This study; S288C
YTH4616	as 4447 and *FOB1^{E420V}-TAP::HIS3*	This study
YTH4625	as 4422 and *FOB1-TAP::HIS3*	This study
YTH4691	*MAT***a** *ade2 his3 leu2 ura3 fob1::KanMX6*	This study; S288C
YTH4692	*MAT***a** *ade2 his3 leu2 ura3 fob1::KanMX6*	This study; S288C
YTH4696	*MAT***a** *ade2 his3 leu2 ura3 FOB1-Stopcodon-TAP::HIS3*	This study; S288C
YTH4698	*MAT***a** *ade2 his3 leu2 ura3 FOB1^{E420V} -Stopcodon-TAP::HIS3*	This study; S288C

Unknown mating type: *MAT*?

Table 3.2. Strains used in Cin5 and Y65 study

Strain	Genotype	Source/Reference
YTH2894	*MAT***?** *ade2 his3 lys2 ura3*	This study; S288C
YTH2896	*MAT***?** *ade2 his3 lys2 ura3*	This study; S288C
YTH4237	*MATα ade2 his3 lys2 ura3 trp1::KanMX6*	This study; S288C
YTH4238	*MAT***a** *ade2 his3 leu2 ura3 apc5^{CA} trp1::KanMX6*	This study; S288C
YTH4239	*MAT***a** *his3 leu2 met15 ura3 CIN5-TAP-HIS3 trp1::KanMX6*	A. Ghavidel
YTH4240	as 4237, and *CIN5-TAP-HIS3*	This study; S288C
YTH4241	as 4238, and *CIN5-TAP-HIS3*	This study; S288C
YTH4242	as 4001, *CIN5-TAP-HIS3*	This study; S288C
YTH4243	as 4004, *CIN5-TAP-HIS3*	This study; S288C
YTH4626	*MAT***?** *ade2 his3 lys2 ura3 CIN5^{K194R}-TAP*	This study; S288C
YTH4627	*MAT***?** *ade2 his3 lys2 ura3 CIN5$^{T192E, S196E}$-TAP*	This study; S288C
YTH4628	*MAT***?** *ade2 his3 lys2 ura3 CIN5$^{T192A, S196A}$ -TAP*	This study; S288C
YTH4629	*MAT***?** *ade2 his3 lys2 ura3 CIN5$^{T192E, K194R, S196E}$ -TAP*	This study; S288C
YTH4630	*MAT***?** *ade2 his3 lys2 ura3 CIN5$^{T192A, K194R, S196A}$-TAP*	This study; S288C
YTH4631	*Mata ade2 his3 leu2 trp1 ura3 cdc34-1 CIN5-TAP-HIS3*	This study; S288C
YTH4632	*Mata ade2 his3 leu2 trp1 ura3 dia2::KanMX6 CIN5-TAP-HIS*	This study; S288C
YTH4632	*Mata ade2 his3 leu2 trp1 ura3 grr1::KanMX6 CIN5-TAP-HIS*	This study; S288C
YTH4634	*Mata ade2-1 ura3 leu2-3 trp1 his3 can1 CIN5-TAP-HIS*	Amon lab
YTH4635	*Mata ade2-1 ura3 leu2-3 trp1 his3 can1 cdc20-1 CIN5-TAP*	Amon lab
YTH4636	*MAT***a** *ade2 his3 leu2 ura3 apc10::KanMX6 CIN5-TAP-HIS*	This study; S288C
YTH4637	*MAT***?** *ade2 his3 leu2 ura3 cdh1::KanMX6 CIN5-TAP-HIS*	This study; S288C
YTH4638	*MATα, ade2 his3 leu2 ura3 rpn10:: KanMX6 CIN5-TAP-HIS*	This study; S288C
YTH4639	*MATα, ade2 his3 leu2 ura3 snf1:: KanMX6 CIN5-TAP-HIS*	This study; S288C
YTH4641	*MAT***?** *ade2 his3 lys2 ura3 elc1::KanMX6 CIN5-TAP-HIS*	This study; S288C
YTH4699	*MAT***?** *ade2 his3 lys2 ura3 CIN5$^{T192E, S196E}$-TAP grr1::KanMX6*	This study; S288C
YTH4700	*MAT***?** *ade2 his3 lys2 ura3 CIN5$^{T192A, S196A}$ -TAP grr1::KanMX6*	This study; S288C

Unknown mating type: *MAT*?

3.1.2. Yeast transformation

Briefly, a liquid culture of *S. cerevisiae* was grown overnight at 30ºC in YPD or appropriate drop out media. The next day the cells were diluted to an OD_{600} (optical density of 600 nm wavelength) of 0.5 in fresh media, and allowed to grow until an OD_{600} of 1.0 was reached. Yeast cells were then collected by centrifugation and resuspended in 500 µl of 100 mM LiOAc solution (0.1 M lithium acetate, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and incubated for 15 minutes at 30ºC. 5 µl of carrier DNA (salmon sperm DNA) and 0.5 ï 2 µl of transforming plasmid DNA or 5 ï 15 µl PCR product DNA were added. Then, 300 µl of a PEG4000 solution (5% polyethylene glycol 4000 in LiOAc solution) was added. The transformation mixture was incubated for 20 minutes at 30ºC, followed immediately by a heat shock at 42ºC for 15 minutes. Yeast cells were then

centrifuged for 30 seconds and resuspended in 100 µl of 1 M sorbital. The resuspended cells were plated on the appropriate drop out media and incubated at 30ºC for 3-5 days. For transformation with the geneticin (G418) resistant marker, the transformed culture was grown in YPD for a minimum of 3 hours or overnight, to allow integration and expression, prior to plating on 0.2 mg/ml G418 media.

3.1.3. Yeast two-hybrid system

Full length *APC5* was cloned into the 2-hybrid bait plasmid pGBT9 (HOCKMAN and HARKNESS, unpublished). The 2-hybrid yeast strain PJ69-4A (generously provided by E. Craig) was transformed with pGBT-*APC5*. Transformants were then transformed with a library of yeast fragments (0.5 to 2-3 kb) ligated into the prey vectors pGAD-C1, pGAD-C2 and pGAD-C3 (the library was kindly provided by E. Craig; see JAMES *et al.* 1996 for details). The three pGAD vectors represent the three different reading frames. A combination of all three libraries was used to transform pGBT-*APC5* expressing PJ69-4A cells. Transformants were grown on SD -trp -leu plates to select for pGBT-*TRP1* and pGAD-*LEU2* containing cells. Roughly 50,000 colonies were recovered on each of 30 separate transformation plates, for a total of approximately 1,500,000 transformants. After 2 to 3 days of growth, the plates were replica plated to SD -trp -leu -ade and SD -trp -leu -his plates to select for interactions with Apc5. Colonies required 1 to 3 weeks to grow on the triple drop out plates. Positives grew on both sets of selection plates. A total of 80 positive colonies were recovered from the screen. Plasmids were recovered from 36 of the positives and sequenced. Two types of plasmids were recovered. First, as expected, plasmids in which gene-coding sequences were fused in frame to the *GAL* activation domain were recovered. This group included *FOB1* and *CIN5*, which were recovered twice each, and *SRS2*, *IQG1*, *RMD7* and *GAL1*, which were each recovered once.

The second group of unexpected sequences included the promoter regions of a select number of genes. This set included the intergenic region between *MATalpha1* and *BUD5* (recovered 14 times), the *TUP1* promoter (recovered 5 times), and the promoters to *SSN6*, *PEX28*, *PRP2*, and *RDN25-2* (each recovered once). We suspect that this group of sequences represents a 1-hybrid

type of interaction where Apc5 is recruiting a transcription factor to the *GAL* promoter, that when bound to these pGAD-based sequences, is capable of driving the transcription of the reporter gene.

3.1.4. Sporulation and yeast tetrad dissection

Two haploid strains with opposite mating types were cross-streaked on YPD plates to allow diploid formation. After overnight growth at 30ºC, cells from the overlapping section of the streaks were restreaked onto sporulation media (1% potassium acetate, 0.1% yeast extract, 0.05% glucose/dextrose, 2% agarose and 5 ml adenine after autoclaving), and incubated at room temperature for 7-14 days. Sporulation was checked by visual inspection of the cells with a light microscope for the formation of tetrads. Dissection of tetrads was carried out as follows: a small amount of cells from the sporulation media plate were resuspended in 100 µl of ddH2O, to which 20 εl of 12.5 units/εl of lyticase solution was added. The tetrads were dissected on YPD plates using a Singer MSM micromanipulator (Singer Instrument Co. Sumerset, England). Markers were scored by testing the growth of each spore on the appropriate drop out media. Segregation of mutants created by gene replacement using the KanMX6 cassette was followed using YPD media supplemented with 0.2 mg/ml G418. Double mutants where KanMX6 was used for each deletion were selected by identifying tetrads that segregated 2:2 on G418 plates. The plates were incubated for 2-3 days at 30ºC.

3.1.5. Replicative lifespan determination

Replicative lifespan (RLS), or generational lifespan of the strains tested in this study was based on previously published protocols (KENNEDY *et al*., 1994; HARKNESS *et al.,* 2004; POSTNIKOFF *et al*., 2012). Briefly, cells from fresh overnight cultures were struck out onto the appropriate media prepared fresh. The plates were then grown overnight at 30°C. The next day, approximately 30 to 50 cells containing small buds were micro-manipulated to isolated areas of the plate. The small daughter buds were kept as the starting mother cells. All additional buds from the starting mother cells were periodically scored and discarded until mothers no longer produced buds. The plates were kept at 30° during working hours and stored at 4°C overnight. Data is plotted as viability

(percentage of mothers) verses number of daughters. Conventionally RLSs are compared at viability at 50%.

3.1.6. Chronological lifespan determination

Chronological lifespan of the strains tested in this study was based on previously published protocols (POSTNIKOFF *et al*., 2012; POSTNIKOFF and HARKNESS 2012). Cultures were grown for 3 to 5 days, nearing cell arrest and quiescence. Set volumes of sample were removed periodically, serially diluted and plated, on YPD plates and grown at 30°C for 3 days, to determine cfu density. The maximum density was set to 100%, consecutive cfu densities were calculated as a percentage of the maximum.

3.1.7. Spot dilutions

Spot dilution assays were performed on fresh overnight cultures. All cultures were diluted to a starting OD_{600} of 0.5 then 10-fold serially diluted 4 times. Five µl of each dilution was spotted on to various media plates and grown at the temperatures indicated, for 2 ï 5 days. The starting spot generally contained 10^4 cells. Plates were periodically scanned

3.1.8. Cell cycle dependent analysis of protein stability

Actively growing overnight cell cultures with an OD_{600} of 0.5 were arrested in G1 with 0.1 µl/ml Ŭ-factor (ŬF), S with 300 mM Hydoxyurea (HU), or M with 50 ng/ml Nocodazole (NOC). Cultures were exposed to arresting agents for 2 to 3 hours until approximately 80 to 100 percent of cells were arrested in desired cell cycle phase. Cell cycle arrest was first assessed visually under a microscope; in addition, arrest was confirmed with Fluorescence Activated Cell Sorting (FACS). If applicable, cultures were treated with 2% galactose or 0.5 mM $CuSO_4$ for 2 ï 4 hours, either before or during cell cycle arrest to induce expression vectors. For release of cell cycle arrest, cells were washed twice with fresh media. To determine protein stability or turnover rate 2 µg/ml cycloheximide (CHX) was added post cell cycle arrest to stop protein synthesis. 1 ml samples were collected periodically for protein extraction and/or 0.5 ml samples for FACS.

3.1.9. FACS

FACS was used to confirm cell cycle arrest and release into a new round of cell growth. 0.5 ml samples were collected and washed with 0.5 ml of 50 mM Tris-HCl (pH 8.0), and resuspended in 0.5 ml of 70% EtOH. The mixture was incubated for a minimum of 1 hour at 4ºC or stored indefinitely. For FACS analysis cells were resuspended in 500 µl of 50 mM Tris-HCl (pH 8.0). Next, 10 µl of RNase A (10 mg/ml) was added and the mixture was incubated for 2 hours at 37ºC. The mixture was centrifuged and washed with 1 ml of 50 mM Tris-HCl (pH 8.0), and resuspended in 500 µl Propidium iodide staining solution (PI solution; 1 mg/ml Propidium iodide in PBS (phosphate-buffered saline: 8% (w/v) NaCl, 0.2% (w/v) KCl, 1.44% (w/v) Na_2HPO_4, and 0.24% (w/v) KH_2PO_4)), and incubated for 1 hour at RT in the dark. The samples were transferred to FACS tubes (Falcon polystyrene round-bottom tubes, 12 X 75 mm) and sent to the Health Research Division Cancer Research Unit for FACS analysis (Thanks to Mr. Boyd for FACS assistance).

3.1.10. Determination of rDNA recombination frequency and plasmid loss assay.

The triple silencer CCFY100 strain containing a phenotypic marker, a double gene cassette in the 25S rRNA gene within the rDNA tandem array, was used to measure the recombination frequency within the rDNA region (Dr. K. Runge; Cleveland Clinic Lerner Research Institute; is thanked for providing this strain). This strain will grow red instead of white colonies on YPD when the *CAN1-ADE2* cassette is lost due to recombination events within the rDNA locus. The ratio between red to white colonies are representative of rDNA recombination frequency. Similar to the rDNA frequency measurement, the loss of an empty vector *CEN-ADE2* plasmid in non-selective media will result in a red, instead of a white, colony on YPD. Here the ratio between red to white colonies is an approximate measure of chromosome loss and is representative of more broad genomic instability.

For rDNA frequency measurements, an overnight culture grown in YPD, inoculated by an individual white colony, was serially diluted to approximately 500 cfu/plate and plated on YPD. Similarly, colonies harboring the *CEN-ADE2* plasmid, picked off of selective plates, were grown in non-selective YPD overnight then diluted and plated on YPD as above. Plates were grown for

4 ï 7 days to allow red colour to form in recombinants. Red and total cfu were counted. The experiments were repeated at least 3 times.

In addition to the *CAN1-ADE2* cassette in the rDNA locus, this strain has the *TRP1* gene in the *hmrΔE* mating type locus and the *URA3* gene followed by $TG_{1ï3}$ repeats replacing the right telomere of chromosome V (ROY and RUNGE, 2000). The three reporter genes cloned into this strain are used to measure silencing of these three regions by spot dilutions on Trp^-, Can^+, and FOA^+ plates for silencing determination of the *hmrΔE*, rDNA, and the telomere loci respectively (see 3.1.7). Relative growth on these selective plates compared to YPD is an indication of the level of silencing at the respective loci. For Trp^- plates more growth means less silencing at the *hmrΔE* locus; Can^+ plates selects against *CAN* expression, more growth means more silencing at the rDNA locus; FOA^+ plates select against *URA3* expression, more growth means more silencing at the telomere.

3.2. Molecular biology techniques

3.2.1. Bacterial culture

The *Escherichia coli* (*E. coli*) strains DH5Ŭand K12 were used for bacterial transformations. All plasmids used in this study contained the ampicillin resistance marker gene, amp^R and are listed in Table 3.3. Transformed strains were cultured in Luria broth (LB: 1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl) containing 50 µg/ml of Ampicillin. Transformed cells were stored at -80ºC in 20% glycerol for long term storage.

3.2.2. Bacterial transformation

Electro-competent *E. coli* cells were prepared as indicated in the BioRad *E. coli* Pulser manual. Briefly, 0.5 ï 2 µl of transforming DNA was added to 50 µl of competent cells. The cell mixture was transferred to a 1 mm width electroporation cuvette (BioRad). The cells were then exposed to a voltage of 1.7 kV using the *E. coli* Pulser (BioRad). After electroporation, 1 ml of pre-warmed LB was added to the cuvette, and the cells were transferred to a 1.5 ml Eppendorf tube. The cells

were incubated for 60 minutes at 37ºC with constant shaking and plated onto LB + Amp plates for incubation at 37ºC overnight.

3.2.3. **DNA extraction**

3.2.3.1. LiCl plasmid mini-prep

A 5 ml overnight transformed bacterial *E. coli* culture was centrifuged, the pellet was resuspended in 200 µl cold GTE (50 mM glucose, 25 mM Tris-HCl PH 8.0 and 10 mM EDTA) and incubated at room temperature for 5 minutes. 400 µl of fresh lysis buffer (2% 10 N NaOH, and 1% SDS) was added, mixed fully by inverting several times and put on ice for 5 minutes. 300 µl of 3 M NaAc (pH 4.8) was added, mixed fully by inverting several times and incubated on ice for 5 minutes. The mixture was centrifuged and the supernatant was transferred to a new tube. 450 µl of isopropanol was added, mixed thoroughly and incubated on ice for 5 minutes. After centrifugation for 5 minutes, the supernatant was discarded, and the pellet was resuspended in 100 µl of 1 X TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0). 100 µl of 10 M LiCl was added and the tube was mixed. Next, 100 µl of chloroform was added, mixed by vortexing and incubated for 10-20 minutes at room temperature. The mixture was then centrifuged and 200 µl of the upper layer of supernatant was transferred to a new tube, added with 600 µl of 95% EtOH and incubated for 15-30 minutes at -80ºC. The pellet was then collected by centrifugation, washed with 500 µl of 70% EtOH, resuspended in 50 µl dH_2O and stored at -20ºC.

3.2.3.2. Phenol/chloroform genomic smash and grab

This protocol is designed for rapid genomic yeast DNA extraction. In a 1.5ml Eppendorf tube one yeast colony was resuspended in 100 µl breaking buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% SDS, 2% Triton X-100). To this tube 100 µl Phenol/Chloroform/Isoamyl Alcohol (25:25:1) and 30 ï 50 µl of 0.1 mm glass beads were added. Tubes were then vortexed for 1 ï 2 minutes then centrifuged at high speed for 5 minutes. 80 ï 100 µl of aqueous phase (top layer) was removed to a new tube and 10 µl of 3 M NaAc (pH 4.8) and 300 µl of 98 % EtOH was added, the rest was discard. This was centrifuged at high speed for 10 minutes, supernatant was discarded and the tube was carefully washed with 300 µl 70% EtOH without dislodging the very small clear

pellet. The pellet was resuspended in 30 ï 50 µl dH_2O. For a PCR reaction 0.5 ï 1 µl per 50 µl of this solution was used.

3.2.3.3. Yeast plasmid smash and grab

Overnight 5 ml cultures grown in appropriate drop out media at 30ºC were centrifuged and resuspended into 200 µl SCE (1 M sorbital, 0.1 M sodium citrate, and pH 5.8, 0.01 M EDTA) plus lyticase (high-yield purification of yeast lytic enzymes; SCOTT and SCHEKMAN, 1980) (0.0033 g lyticase from Sigma in 1 ml SCE). After the cells were incubated for 1 hour at 37ºC, 400 µl fresh SDS (sodium dodecyl sulfate)/NaOH lysis buffer (10 N NaOH, 10% SDS and dH_2O) was added, and mixed by inverting 5 times. Then on ice 300 µl of 3 M NaAC (PH 4.8) was added, then centrifuged for 5 minutes and the aqueous layer was transferred into a new 1.5ml microcentrifuge tube with 600 µl iospropanol. After 10 minutes on ice, the mixture was centrifuged for 10 minutes. After discarding the supernatant, the pellet was washed in 500 µl 70% ethanol and then resuspended in 100 µl of dH_2O. In order to obtain a pure DNA preparation, the precipitated DNA was treated with 5 µl of RNaseA (10 mg/ml stock) at 37ºC for 30 minutes. After the RNaseA treatment, 100 µl of phenol/chloroform (1:1) was added, and the mixture was centrifuged and the upper layer was transferred to a fresh tube, and mixed with 100 ul dH_2O and 20 µl 3 M NaAC. Next, 500 µl of 95% ethanol was added, mixed and incubated on ice for 10 minutes. The mixture was centrifuged for 10 minutes, and the pellet was resuspended in 100 µl dH_2O.

3.2.4. PCR and cloning

PCR was used to amplify DNA used for transformation of yeast. Genomic yeast DNA obtained by Smash and Grab was used as template (described in 3.2.3). Primers of 18 ï 22 bp were designed against sequences 500 bp upstream and downstream of the DNA sequence of interest. See Table 3.4 for a list of primers used in this study. Gene sequence data used to design primers were acquired from the Saccharomyces Genome Database website (www.yeastgenome.org/cgi-bin/blastsgd.pl). A typical 25 εl PCR reaction mixture contained 1 εl dNTPs (10 mM each deoxyribonucleotide triphosphate; Bio Basic Inc., #D0056), 1 ng of each primer, 1 εl of genomic DNA, 2.5 εl 10X PCR Buffer (Sigma, #P2317), 4 εl of 15 mM MgCl2 (Sigma, #M8787), 16.5 εl of distilled water and

0.5 ε l high fidelity Phusion® polymerase (New England Biolabs, #M0267). Volumes of reagents were adjusted to optimize reactions as necessary. A programmable thermocycler (Eppendorf Mastercycler) was used to perform the amplifications. The standard PCR protocol was as follows: 98ęC for 60 seconds, followed by thirty repeats of 10 to 60 seconds of denaturing at 95ęC, 10 seconds of annealing at 55ęC, and 30 seconds to 5 minutes of elongation at 72ęC. A final 5-10 minute incubation of 72ęC was followed by long-term storage at 4ęC. Denaturing and amplification times were adjusted to optimize for product length as necessary.

Table 3.3. Plasmids used in this study

Plasmid	Markers	Source/Reference
pVA3	p53 2μ-*TRP1* 2 hyb·$GAL4_{BD}$	W. Neupert
pTD1	SV40 large T-antigen 2μ-*LEU2* 2 hyb·$GAL4_{AD}$	W. Neupert
pGBT9	2μ-*TRP1* 2 hyb·$GAL4_{BD}$	W. Neupert
pGBT-*APC5*	2µ-*TRP1* 2 hyb·$GAL4_{BD}$	This study
YCp50	*CEN-URA3*	W. Neupert
pTH54	YCp50-$APC5^{\Delta 3'69}$	Harkness *et al.* 2002
YEplac195-*FOB1*	2μ-*URA3*	T. Kobayashi
pGAD·C2	2μ-*LEU2* 2 hyb·$GAL4_{AD}$	E. Craig
pRIN12, 20	$FOB1^{5\text{-}1263}$ 2μ-*LEU2* 2 hyb·$GAL4_{AD}$	This study
pCR4-TOPO	Bacterial PCR cloning vector	Invitrogen
topo-*FOB1*	pCR4-TOPO	This study
pGAD-*FOB1*	2μ-*LEU2* 2 hyb·$GAL4_{AD}$	This study
pGAD-$FOB1^{D1}$	2μ-*LEU2* 2 hyb·$GAL4_{AD}$	This study
topo-$FOB1^{D2}$	pCR4-TOPO	This study
pGAD-$FOB1^{D2}$	2μ-*LEU2* 2 hyb·$GAL4_{AD}$	This study
topo-$FOB1^{D3}$	pCR4-TOPO	This study
pGAD-$FOB1^{D3}$	2μ-*LEU2* 2 hyb·$GAL4_{AD}$	This study
pGAD-$FOB1^{D1/2}$	2μ-*LEU2* 2 hyb·$GAL4_{AD}$	This study
topo-$FOB1^{N\text{-}term}$	pCR4-TOPO	This study
pGAD-$FOB1^{N\text{-}term}$	2μ-*LEU2* 2 hyb·$GAL4_{AD}$	This study
topo-$FOB1^{C\text{-}term}$	pCR4-TOPO	This study
pGAD-$FOB1^{C\text{-}term}$	2μ-*LEU2* 2 hyb·$GAL4_{AD}$	This study
pBG1805	$GAL_{pro.}$6X His·HA·Prot A 2μ-*URA3*	W. Xiao
GAL_{pro}-*SIR2-HA*	$GAL_{pro.}$6X His·HA·Prot A 2μ-*URA3*	W. Xiao
GAL_{pro}-*FOB1-HA*	$GAL_{pro.}$6X His·HA·Prot A 2μ-*URA3*	W. Xiao
GAL_{pro}-$FOB1^{E420V}$-*HA*	$GAL_{pro.}$6X His·HA·Prot A 2μ-*URA3*	This study
GAL_{pro}-$FOB1^{DB1}$-*HA*	$GAL_{pro.}$6X His·HA·Prot A 2μ-*URA3*	This study
GAL_{pro}-$FOB1^{DB2}$-*HA*	$GAL_{pro.}$6X His·HA·Prot A 2μ-*URA3*	This study
GAL_{pro}-$FOB1^{DB1/DB2}$-*HA*	$GAL_{pro.}$6X His·HA·Prot A 2μ-*URA3*	This study
GAL_{pro}-$FOB1^{E420V/DB1}$-*HA*	$GAL_{pro.}$6X His·HA·Prot A 2μ-*URA3*	This study
pES7-*MYC-UB*	2μ-*URA3 CuP*	This study
pYEX-*GST*	*URA3/AMP CuP*	This study
pYEX-*APC5-GST*	*URA3/AMP CuP*	This study
pPAS-*ADE2*	*CEN-ADE2*	W. Xiao
pYEX-*APC10-GST*	*URA3/AMP CuP*	This study
pFA6a-*13MYC-KanMx6*	*KAN/AMP*	This study
pFA6a-*3HA-KanMx6*	*KAN/AMP*	This study

3.2.4.1. Construction of gene constructs and plasmids

All plasmids used or constructed in this study are listed in Table 3.3. All constructs, including insertions of genes, markers, promoters, N-terminal or C-terminal tag, or gene replacements or deletions were created using a PCR-based method to direct homologous recombination within yeast cells (ITO *et al.,* 2008). Briefly, PCR fragments were designed to have homologous overlap to genomic DNA on both ends with a minimum of 50 bps of homology to DNA flanking the target sequence. These PCR products were directly transformed into yeast. The yeast recombination machinery inserts the PCR products at sites of homology. The original genomic DNA sequencing between the two homologous regions is lost. Similarly, two or more PCR fragments can be integrated at the same locus. In this case the PCR fragments are designed to have homologous overlap with each other on one end, and be homologous to genomic DNA on the other opposing ends.

Likewise, for plasmid reconstruction, the PCR fragments are designed to have homologous overlap with linearized plasmid DNA on both ends, or with each other on one end, and with linearized plasmid DNA on the other in the case of two fragments. These PCR products along with linearized plasmid DNA were directly transformed into yeast were the circular plasmid is reconstructed by the yeast recombination machinery.

3.2.4.2. Site directed mutagenesis

All site specific point mutations were introduced using a standard PCR based approach (SHORTLE *et al.,* 1981). Briefly, primers were designed to be homologous to site of interest except the desired mutations with at least 18bp homology at the 3ôend. Two such mutant primers running in opposite directions, overlapping each other at the desired mutation site, would be used along with flanking primers upstream and downstream of gene to generate two gene fragments that overlap at the mutation site. For plasmids, these two fragments would be transformed directly into yeast along with linearized plasmid as explained above (see section 3.2.4.1). For genomic integrations, at least one gene fragment would include a selectable marker. Generally, markers and/or tags were derived from genomic template DNA from a strain that already contained a

Table 3.4. Primers used in this study

Primer Name	Primer Sequence
E420V 3´	ACGTACGTGGAATTC CATTATTGTTACAGCTATTATCCGCAACGTTAGCAAGGG
Cin5F-K194R	CCTCAAATAGTACCCCAGCTACGAGAAGAAATAGTGCCACGACTAACCTTCCTTC
Cin5F-T192E-S196E	CCTCAAATAGTACCCCAGCTGAGAGAAAAAATGAAGCCACGACTAACCTTCCTTC
Cin5F-T192A-S196A	CCTCAAATAGTACCCCAGCTGCGAGAAAAAATGCTGCCACGACTAACCTTCCTTC
Cin5-T192E-K194R-S196E	CCTCAAATAGTACCCCAGCTGAGAGAAGAAATGAAGCCACGACTAACCTTCCTTC
Cin5F-T192A-K194R-S196A	CCTCAAATAGTACCCCAGCTGCGAGAAGAAATGCTGCCACGACTAACCTTCCTTC
Cin5R-Mutant	AGCTGGGGTACTATTTGAGG
Cin5stop +500	TTCAGACGTTGATCGCTG
Cin5start -500	AGTAATGCAACCAAGTGC
Fob1-ReStop	AGGGTAATGGAGATCAAACAAGAGACTTTGGCACATCAATGGAATTGTAACGGAT CCCCGGGTTAATTAA
F2 Fob1	AACAGGGTAATGGAGATCAAACAAGAGACTTTGGCACATCAATGGAATTGCGGA TCCCCGGGTTAATTAA
R1 Fob1	TTTTTTTTTCACCTATGGTGACTCCTCCTTTCATTCTATCCTACATATTAGAATTCG AGCTCGTTTAAAC
FOB1 start-250	AACATTGTGATCGAGGTTTC
Fob1 -500TAP	CCAGGGACTAAATGGATTC
Fob1 E420ï V420 primer (reverse)	TATTATGAAGAATTCCATTATTGTTACAGCTATTATCCGCAACGTTAGCAAGGG
Fob1 E420V forward	GATGTCAATACTTTTGCCCTTGCTAACGTTGCGGATAAT
M99-rev (APC5)	TAAATCCATCATTAGTCTGTTGATTGAATCCAGTCCATTAATTGCTTCCAAATAGC CCATTGGATGGACGCAAAGAAG
M115-rev (APC5)	TGTACGCATTTTCATTGACCTGTAATTATTAACCAAACAGTTCTTTTCTAAATCCAT CATTGGATGGACGCAAAGAAG
M141-rev (APC5)	AATTTGATACTTGCGTATACAAGTTGACAAAAATGTTCCTAAAAAACTCGCTCTTG TCATTGGATGGACGCAAAGAAG
APC5startsite-500	GGAAACCTAGCAATCTTTTCG
APC5 -600	GATGCAAAGGTCAAGTCC
APC10-500UP	GGGACTCATGGACGTGAAG
APC10+500TAP	CAGAGTCAGCAATACTGG
Grr1 start -150	CTTGTGTCCAACTAGCAGGG
Grr1 stop -250	GGCAAACGATACAAGTAAC
Grr1 stop +150	TTCGTTGATACCGCAAGTC
F2 Grr1-HA(KAN)	TTGATTCTCAAATGGATGAAGCATCAGGAACGCCCGATGAAGATATGTTA CGGATCCCCGGGTTAATTAA
R1 Grr1-HA(KAN)	ATAAGTAAGAAAAGTGGGATTTTGAAAATAAAGGTGTAGTAGGACAGTAA GAATTCGAGCTCGTTTAAAC

Underlined bps are modified to introduce a different amino acid codon sequence for site directed mutagenesis.

marker or tag attached to the gene of interest. The two gene fragments would be transformed into strains where the gene of interest has been deleted and replaced by another marker, effectively reintroducing a mutant version of the gene of interest. This gene replacement method insures that introduced mutations are not repaired using WT gene alleles during transformation.

3.2.4.3. Agarose gel electrophoresis and DNA fragment isolation

For analysis of plasmid and PCR DNA, a 1 ï 2% agarose gel was used. Electrophoresis was performed at 90 mV in 1 X TAE (24% Tris-base, 5.7% glacial acetic acid, 10% EDTA pH 8.0) and the gel was stained in 0.5 µg/ml EtBr (ethidium bromide) for viewing under UV light. Isolation of DNA fragments from an agarose gel was done using a DNA extraction kit (MBI Fermentas). The agarose gel containing the DNA fragment was cut out, placed into an eppendorf tube and weighed. According to the mass of agarose slice (mg), 3 volumes binding solution was added and incubated for 5 minutes at 55ºC to dissolve the agarose slice. Next, the solution was added to the kit column and centrifuged for 2 minutes then washed 2 times with 500 ul of cold wash buffer. The DNA was collect by addition of 10 ï 30 µl of dH_2O at 55ºC to the column followed by centrifugation into a fresh tube.

3.2.4.4. DNA sequencing

All DNA sequencing was performed at the National Research Council (NRC) Plant Biotechnology Institute (PBI) DNA Technologies Unit using the primers provided.

3.2.5. Protein purification

3.2.5.1. Bead beat extract

For immunoprecipitations (IPs), yeast whole cell protein lysates were prepared from 5 ml cultures by bead beat lysis. The 5ml cultures were grown overnight to an OD_{600} of 0.5-1.2. The cells were pelleted and washed with 5 ml sterile media. The pellet was resuspended in the remaining liquid and transferred to a fresh eppendorf. The cells were then resuspended in 250 µl bead-beat buffer (BBB; 50 mM HEPES pH 7.8, 5 mM MgAc, 10% glycerol, 0.005% NP40, 60 mM KAc, with 1

mM DTT, 0.5 mM PMSF and 10 εl/ml protease inhibitor cocktail from Sigma added fresh). Cells were then added to tubes containing 200 µl 0.5 mm glass beads. The cells were subjected to bead-beating using a Disruptor Genie (Scientific Industries) for 3 pulses of 20 seconds, with 1 minute ice incubation in between pulses. The samples were then immediately centrifuged for 15 minutes at 4°C, with the supernatant stored at ï 80°C.

3.2.5.2. TCA extract

Briefly, 1 ml samples were removed from experimental cultures and the cells were pelleted and resuspended in 250 µl 1.85 M NaOH/7.4% β-mercaptoethanol. After a 5-minute incubation on ice, 250 µl of 100% trichloroacetic acid (TCA) was added, followed by another 5-minute incubation on ice. The sample was centrifuged for 10 minutes, the supernatant was removed and the pellet was gently washed with H_2O. The pellet was then resuspended in 25 µl Buffer A (13% SDS, 1 M unbuffered Tris) and 25 µl Buffer B (30% glycerol, bromophenol blue) and stored at ï 80°C. The samples were heated at 65°C for 15 minutes prior to running 5-10 µl by SDS-PAGE.

3.2.6. Immunoprecipitation

3.2.6.1. Antibody and Protein A bead immunoprecipitation

Protein concentrations of beat bead lysates were determined via the Bradford assay. Lysates were kept on ice at all times. Briefly, 200 εl of Bradford Reagent (BioRad) plus 800 εl of sterile distilled water was pipetted into a disposable cuvette (VWR) and 1 εl of the protein lysate was mixed into the Bradford reagent. For a control no protein was added. Cuvettes were mixed via pipetting and allowed to sit at room temperature for two minutes. After two minutes, the protein concentration was determined using a spectrophotometer set at OD_{595} (BioRad SmartSpecTM 3000). A previously prepared calibration curve was used to quantify protein concentrations.

For each Immunoprecipitation (IP), lysate was mixed in to IP buffer (BBB; 50 mM HEPES pH 7.8, 5 mM MgAc, 10% glycerol, 0.005% NP40, 60 mM Kac, with 1 mM DTT, 0.5 mM PMSF and 10 εl/ml protease inhibitor cocktail from Sigma added fresh) to make up a total of 1 mg protein in 300 εl buffer. The lysate was pre-cleared by the addition of 10 µl of washed salmon sperm

DNA/Protein A agarose beads (Upstate) and rotated at 4ęC for 1 hour. The suspension was centrifuged at 4ęC for 30 seconds at 10,000 rpm and the lysate was recovered. 10 ï 50 µl of lysate was removed and added to an equal volume of 2x SDS loading buffer (100 mM Tris-HCl pH6.8, 2% ß-mercaptoethanol) and boiled for 5 minutes and stored at -80ęC; this was used as ñinputò. Then 1 µl of the required antibody was added to the remainder and rotated at 4ęC for 2 hours or overnight. Next, washed protein-A beads were added to the lysate antibody mixture and rotated at 4ęC for 90 minutes. The samples were centrifuged as before and the recovered supernatant serving as the ñunboundò sample, to which equal volume of 2x SDS loading buffer was added as the above input samples. The pellet of beads served as the bound sample, which was washed 3 times with 300 µl IP buffer. To these ñboundò samples, 30 µl 2X SDS loading buffer was added and the samples were boiled for 5 minutes and stored at ï 80°C until Western analysis.

3.2.6.2. IgG Sepharose bead IP

The TAP or Protein A antigen can be precipitated directly by IgG sepharose beads. IP protocols on lysates containing these antigens are the same as in the above section (see 3.2.6.1) with the following exceptions: the lysates are not precleared and the antibody incubation step is omitted; lysates are incubated with washed IgG sepharose beads and rotated at 4ęC for 20 minutes. Beads and supernatant are treated as above and stored at ï 80°C until Western analysis.

3.2.7. SDS-PAGE and Western blotting

Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Depending on the degree of protein separation needed, 10-15% separating gels [4ï 2.4 ml distilled water, 3.3ï 5 ml 30% Acrylamide (BioRad), 2.5 ml 1.5% Tris-HCl pH8.8, 100 ε l 10% SDS (VWR), 100 ε l ammonium per sulfate (APS; Sigma), 10 ε l tetramethylethylenediamine (TEMED; VWR)] were used, each topped with a 5% stacking gel (4.2 ml distilled water, 1 ml 30% Acrylamide, 76 ε l 1.0% Tris-HCl pH 6.8, 60 ε l SDS, 60 ε l APS, 6 ε l TEMED). The separating gel solution was mixed and then immediately poured between two glass plates immobilized in a casting apparatus. Gels were cast and run using the BioRad Mini-PROTEAN®-2 and -3 Electrophoresis systems (BioRad). The gel solution was topped with a thin layer of isopropanol to

ensure a level surface. Once the separating gel solidified, the isopropanol was poured off and a 5% stacking gel solution was poured on top of the separating gel between the plates and a comb was inserted to create wells. Once the stacking gel had solidified, the gels were removed from the casting apparatus, locked into the electrophoresis apparatus and then placed in the buffer tank. Combs were removed once the gel and connecting wires were covered with 1 X SDS-Page running buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS). Samples were kept on ice prior to loading into wells. A 2 ε l aliquot of pre-stained protein ladder (Fermentas) was pipetted into the left most lane. Unused wells were filled with 2X SDS loading buffer to a volume equaling that of the samples to prevent sample spreading. Gels were run at a constant 150 Volts until desired proteins were sufficiently separated, approximately one to two hours. Gels were removed from the electrophoresis apparatus and glass plates.

To examine protein load gels were then removed from the glass plates and proteins were visualized by agitating in Coomassie blue staining solution [(0.25% (w/v) Coomassie brilliant blue R250 dissolved in 40% (v/v) methanol, 10% (v/v) acetic acid in water)] for minimum of thirty minutes. Excess stain was removed by agitating in de-stain solution (40% methanol, 10% acetic acid) until protein bands were visible (one hour to overnight).

For Western analysis gels were then assembled into the transfer unit in between a sandwich of Wattman paper, nitrocellulose membrane and another layer of Wattman paper. Sandwiches used in wet-transfer were themselves sandwiched between two fiber pads and inserted into the BioRad Mini Trans-Blot Electrophoretic Transfer Cell Cassette. The cassette was placed into the buffer tank and covered with transblot buffer (1.8% glycine, 0.4% Tris and 40% ml methanol). Transfers were performed at 400 milliAmps for two hours. Membranes were removed from the transfer apparatus and stained with Ponceau S staining solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) by agitating until proteins were visible, approximately one minute, to confirm equal protein load. Ponceau S strained blots were scanned for a permanent record and the stain then removed by rinsing with distilled water. Membranes were then incubated in 5% PBST blocking milk (PBS, 0.01% Tween (v/v), 5% non-fat milk) for one hour at room temperature or overnight at 4ęC. Following blocking, membranes were incubated with primary antibody diluted in 1% PBST/non-fat milk powder for one hour at room temperature or overnight at 4ęC, depending on antibody.

Primary antibodies were typically diluted at 1:1000 in 1% PBST blocking milk which was diluted in 5% bovine serum albumin in PBS. After incubation, primary antibody was removed and membranes were washed three times for five to fifteen minutes with PBST. Membranes were then incubated with secondary antibodies conjugated with horseradish peroxidase for one or two hours at 4ęC. Secondary antibodies were diluted at 1:10,000 in 1% PBST/non-fat milk powder. Membranes were then washed with PBST for five minutes, twice. Proteins were detected by rinsing the membranes with a 1:1 dilution of Chemiluminescence Substrate (PerkinElmer, Inc.) that allows visualization of horseradish peroxidase-conjugated secondary antibodies by exposing the membranes to X-ray film (Kodak, BioMax, Primary antibodies used in this study were purchased from the indicated suppliers: rabbit anti-Clb2 (Santa Cruzsc-9071), mouse anti-GAPDH (Sigma-Aldrich G8795), rat anti-HA (Roche 3F10), rabbit anti-tandem affinity purification (TAP) (Genscript Cat# A00683), rabbit anti-GST
(generated by T.A.A. Harkness and M.C. Schultz), mouse anti-myc (Stressgen MSA110), and mouse anti-Ub (Cell signaling P4D1). Secondary antibodies used in this study were goat anti-mouse (BioRad #170-6516), goat anti-rabbit (BioRad #170-6515) and rabbit anti-rat (Sigma #A5795).

4. RESULTS

4.1. APC-dependent degradation of Fob1

A Y2H screen conducted in an effort to identify novel APC substrates and/or regulators identified Fob1 as a binding partner for Apc5 (see section 2.3.3). Fob1 plays an important role at the yeast rDNA locus, and the maintenance and regulation of this locus impinges heavily on lifespan (KOBAYASHI and HORIUCHI 1996; KOBAYASHI *et al.,* 1998; KOBAYASHI 2011a; KOBAYASHI 2011b). Considering the involvement of the APC in genomic stability and lifespan (BAKER *et al*., 2004; HARKNESS *et al.,* 2004; HARKNESS 2006; POSTNIKOFF *et al.,* 2012; POSTNIKOFF and HARKNESS 2012; MALUMBRES and BARBACID 2009), we asked whether the Apc5/Fob1 Y2H interaction defined a molecular mechanism for APC-dependent longevity. We hypothesized that Fob1 may be an APC target and that APC-dependent genomic stability and longevity is achieved, at least in part, through the degradation of Fob1.

To this end, Dr. Troy Harkness and former graduate student Jing Chen analyzed the genetic interactions between Fob1 and the APC with regard to growth and RLS (see section 2.4.2). They found that the deletion of *FOB1* in APC mutants has little impact on growth, but a positive influence of APC mutant RLS. The deletion of *FOB1* did partially recover $apc5^{CA}$ and *apc10*Δ RLS defectives (Supplementary Figure 6.4). In addition, increased expression of *FOB1* can be toxic to cells and reduce replicative lifespan (Supplementary Figure 6.5). My results presented in the following sections provide additional evidence that supports our hypothesis that the APCôs role in maintaining genomic stability and longevity is achieved, at least in part, through the degradation of Fob1.

4.1.1. Confirmation of a physical interaction between Fob1 and the Apc5

First, to corroborate the Apc5/Fob1 Y2H interaction, coimmunoprecipitation (CoIP) experiments were performed to verify a physical interaction between Fob1 and Apc5. Cells expressing TAP-tagged *FOB1* were transformed with a plasmid expressing *GST-APC5* under the control of the *CUP1* promoter. The CUP1 promoter induces expression in the presence of Cu^{+2}. Fob1-TAP was

precipitated with IgG-beads. The corresponding Western analysis was probed against GST-Apc5 and Fob1-TAP with antibodies against the GST and TAP epitopes (Figure 4.1). The results show that Fob1 and Apc5 specifically interact in cell lysates using this CoIP approach. The mild band present in the sample containing GST-Apc5 alone defines cross-reactivity between the IgG beads, which are specific for TAP, and the GST epitope. Despite the cross reactivity, since the band present when both tagged proteins are in the sample has significantly higher levels of GST-Apc5 we believe this to be a positive CoIP. This experiment was repeated three times with similar results.

4.1.2. Deletion of *FOB1* alleviates cell cycle and rDNA recombination defects in $apc5^{CA}$ cells

Genetic interactions between the APC and *FOB1* are also apparent with regard to cell cycle profiles (Figures 4.2A and 4.2B) and rDNA recombination frequency (Figure 4.2D). FACS analysis show that in an asynchronous population of $apc5^{CA}$ cells, over 60% of the cells accumulate in G2 of the cell cycle (Figure 4.1A), whereas only 50% of the WT cells are in G2. We suspected that difficulties in progressing through G2/M observed in $apc5^{CA}$ cells may be due to untimely degradation of APC substrates, such as the cell cycle regulator Clb2 or Pds1, an inhibitor of chromosome segregation. Interestingly, the deletion of *FOB1* in $apc5^{CA}$ cells restored a WT cell cycle profile and reduced the accumulation of Clb2 in $apc5^{CA}$ cells (Figures 4.2A and 4.2B). One possible explanation for our data could involve rDNA segregation defects in $apc5^{CA}$ cells due to slow or untimely cohesin cleavage, that are relieved in *fob1Δ* cells by the reduction of cohesin recruitment to the rDNA. Recall that Fob1 recruits and anchors a variety of proteins to the rDNA including Tof2, Csm1, and Lrs4 which bind cohensin, a clamp-like protein that holds together sister chromatids (see section 2.4.1.2). This runs contrary to APC function, which is required for sister chromatid segregation.

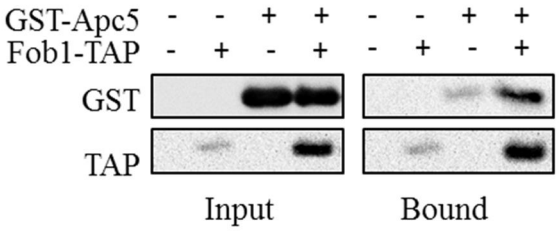


Figure 4.1. Fob1 physically interacts with Apc5
CoIP of Fob1-TAP using IgG beads in untagged WT cells, in cells expressing plasmid borne *CUP1$_{pro}$-GST-APC5*, tagged *FOB1-TAP,* or a combination of both *CUP1$_{pro}$-GST-APC5* and *FOB1-TAP*. Whole cell lysates were prepared using the bead beat method in RIPA buffer: 150 mM NaCl, 50 mM Tris pH8, 5 mM EDTA, 1 mM EGTA, 1% v/v NP-40, 0.5% deoxycholate, 40 mM Na Vanadale, 40 mM Na F, 20 mM beta-glycerphosphate; 1 mg of protein was incubated with 20 εl IgG sepharose beads for 30 min to immunoprecipitate Fob1-TAP, beads were washed 2X with 100 ul buffer, then boiled in 30 ul 2x SDS buffer; 20 ul of bound sample along with 10 εg of input in 2x SDS buffer was separated by SDS PAGE, transferred to membrane and detected with antibodies against TAP and GST. The lab technician Mackenzie Malo performed these experiments.

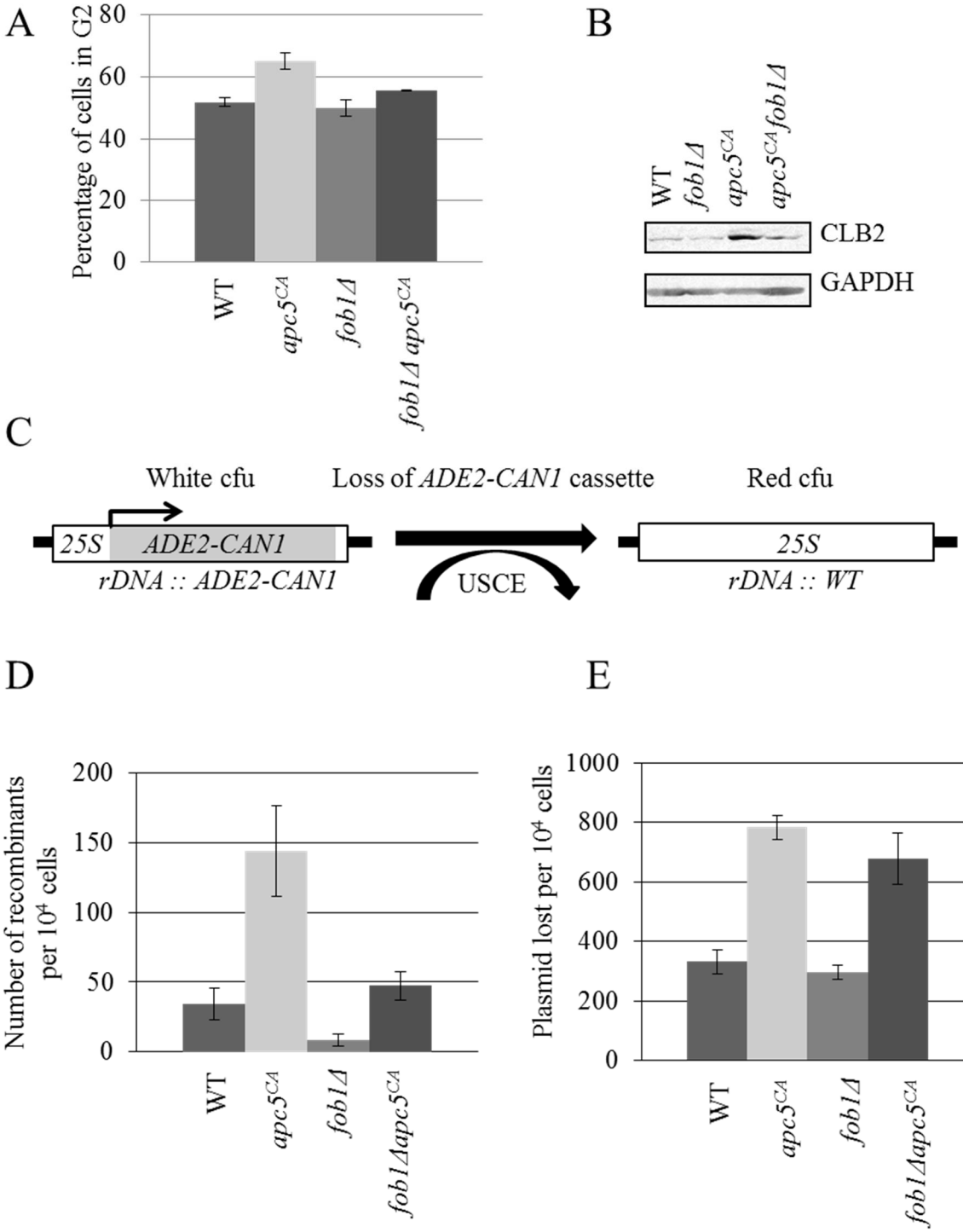

A
Percentage of cells in G2
80
60
40
20
0
WT
apc5CA
fob1Δ
fob1Δ apc5CA
B
WT
fob1Δ
apc5CA
apc5CA fob1Δ
CLB2
GAPDH
C
White cfu
Loss of ADE2-CAN1 cassette
Red cfu
25S
ADE2-CAN1
rDNA :: ADE2-CAN1
USCE
25S
rDNA :: WT
D
Number of recombinants per 10⁴ cells
200
150
100
50
0
WT
apc5CA
fob1Δ
fob1Δapc5CA
E
Plasmid lost per 10⁴ cells
1000
800
600
400
200
0
WT
apc5CA
fob1Δ
fob1Δapc5CA

Figure 4.2. Deletion of *FOB1* alleviates cell cycle and rDNA recombination defects in *apc5^{CA}* cells

(A) FACS analysis of mitotically active WT, *fob1Δ, apc5^{CA}*, and *apc5^{CA} fob1Δ* cells grown overnight in YPD at 30ºC. Three biological replicate cell cultures, 10^4 cells each, stained with propidium iodide were analyzed with FACS. FACS data is a measure of cellular DNA content to determine the cell cycle phase cells are in. Standard error of the mean is shown.

(B) Western analysis of the cells used above with antibodies against Clb2 and GAPDH as a loading control. Samples from the 3 replicates were pooled.

(C) A schematic of the *CAN1-ADE2* construct cloned into one of the 25S rDNA repeats and the corresponding recombinant WT form when the construct is lost due to unequal sister chromatid exchange (USCE). The strain was a generous gift from Dr. K. Runge (ROY and RUNGE 2000).

(D) The average frequency of recombination in the rDNA for five individual cultures of WT, *fob1Δ, apc5^{CA}*, and *apc5^{CA} fob1Δ*, as judged by the loss of the *ADE2* gene. Cultures were inoculated with 5 individual colonies and allowed to grow in YPD until an OD_{600} of 1.0, then cells were diluted and plated on YPD and grown for 5 days at 30ºC. Approximately 10^3 cells were plated from each sample. Recombination frequency is expressed as the number of recombinant red colony forming units (cfu) per 10^4 cells. Standard error of the mean is shown.

(E) The average frequency of plasmid loss for five individual cultures of WT, *fob1Δ, apc5^{CA}*, and *apc5^{CA} fob1Δ*, as judged by the loss of the *CEN-ADE2* plasmid. The frequency is expressed as the number of red cfu per 10^4 cells. Standard error of the mean is shown. Cells were grown overnight in non-selective YPD then plated on YPD for 5 days at 30ºC.

Considering the role Fob1 plays in rDNA metabolism we tested whether *fob1Δ* genetically interacts with *apc5*^{CA} at the rDNA locus. Recombination events within the rDNA locus occur frequently relative to other regions in the genome rendering this region a predominant source of genomic instability. Genomic instability in heterochromatic regions such as the rDNA and telomeres is correlated with decreased longevity (KOBAYASHI 2011a). Recombination events can result in the loss of tandem rDNA repeats (ROY and RUNGE 2000; KOBAYASHI 2011b). This allowed us to assess the recombination frequency within the rDNA region by measuring the loss of a phenotypic marker cloned into an rDNA repeat. A strain containing the *CAN1-ADE2* cassette within the rDNA locus will grow red instead of white colonies when this cassette is lost due to recombination events (Figure 4.2C). The ratio between red to white colonies are representative of rDNA recombination frequency. Similarly, the loss of an empty vector *CEN-ADE2* plasmid in non-selective media is an approximate measure of chromosome loss and is representative of more broad genomic instability. The *CEN-ADE2* plasmid contains a centromere which allows this plasmid to segregate into daughter cells during cell division, thus approximating a chromosome (HARTWELL and SMITH 1985; PALMER *et al*., 1990; HARKNESS *et al.,* 2002). Both *apc5*^{CA} (Figure 4.2D), and *apc10Δ* (data not shown) mutants have significantly increased frequency of rDNA recombination and plasmid loss (Figures 4.2D and 4.2E), indicating increased genomic instability (Plasmid loss was previously reported in *apc5*^{CA} mutants; HARKNESS *et al.,* 2002). If rDNA instability and reduced RLS are truly linked, then this is in agreement with the reduced replicative lifespan in APC mutants. Deletion of *FOB1* strongly reduces recombination frequency to that of WT levels in the *apc5*^{CA} mutant (Figure 4.2D). This suggests that Fob1 levels are perhaps elevated in APC mutants and require an intact APC for removal. Here we demonstrate a link between the *APC5*, *FOB1* and rDNA recombination, a process known to impinge on lifespan (RIESEN and MORGAN 2009; KOBAYASHI 2011a). In contrast, the *apc5*^{CA} and *apc5*^{CA} *fob1Δ* cells have similar levels of plasmid loss suggesting that *apc5*^{CA} genomic instability is not exclusive to the rDNA locus. This genetic interaction at the rDNA locus strongly supports our hypothesis that the APCôs role in maintaining genomic stability is achieved, at least in part, through the degradation of Fob1.

4.1.3. Fob1 levels oscillate throughout the cell cycle and require APC^{Cdh1} for turnover

If Fob1 is indeed a target of the APC, then Fob1 protein levels may fluctuate as the cell progresses through the cell cycle, as many known APC targets do, and may peak in M and be lowest in G1. To test Fob1 cycling we arrested cells harboring *FOB1-TAP* in G1 using yeast α factor pheromone for two hours. The mating pheromone Ŭ factor prompts preparations for mating in yeast and induces G1 arrest. The cells were then washed and released into fresh YPD media. Periodic samples were collected for Western analysis to monitor protein levels, and FACS to monitor cell cycle progression. Western analysis of TAP, Clb2 (a known APC target), and GAPDH as a loading control, showed that Fob1 protein levels are low in G1 of the cell cycle and rise again as the cells progress to mitosis, resembling Clb2 cycling (Figure 4.3A). To address concerns of Fob1 cycling being an artifact due to Ŭfactor arrest we corroborate our results by arresting cells in G1 or mitosis, using Ŭfactor or nocodazole respectively. Nocodazole causes mitotic arrest by interfering with the polymerization of microtubules of the mitotic spindles required for mitosis (JACOBS *et al.,* 1988). We collected protein samples at 20 and 40 minutes, and 180 and 210 minutes after release to demonstrate that Fob1 cycling is a trend observed well into the next cell cycle and independent of the arresting agent used (Figure 4.3B). To solidify our results and establish whether Fob1 degradation is a cell cycle specific event, we again arrested Fob1-TAP cells in G1, M, and S phase using Ŭfactor, nocodazole, or hydroxyurea respectively. Hydroxyurea arrests cells in S phase by interfering with nucleotide synthesis resulting in DNA replication fork stalling due to nucleotide depletion (KOÇ *et al.,* 2004). Following the arrest, cycloheximide (CHX) was added to stop protein synthesis and samples were removed every 30 minutes for Western analyses using antibodies against TAP and GAPDH. CHX stops protein synthesis by interfering with protein translation, specifically CHX prevents tRNA translocation within the ribosome resulting in the stop of protein elongation (SCHNEIDER-POETSCH *et al.,* 2010). The results show that Fob1-TAP is specifically unstable during G1 (Figure 4.3C).

To further examine APC-dependent Fob1 turnover, we utilized cells expressing a mutation to *CDC20* or a deletion of *CDH1*, which encode temporal APC activators that are required for APC target specificity. The APC utilizes these cell cycle specific adaptors which recruit substrates for ubiquitination and subsequent degradation (BARFORD 2011, see section 2.2.3). The mitotic APC

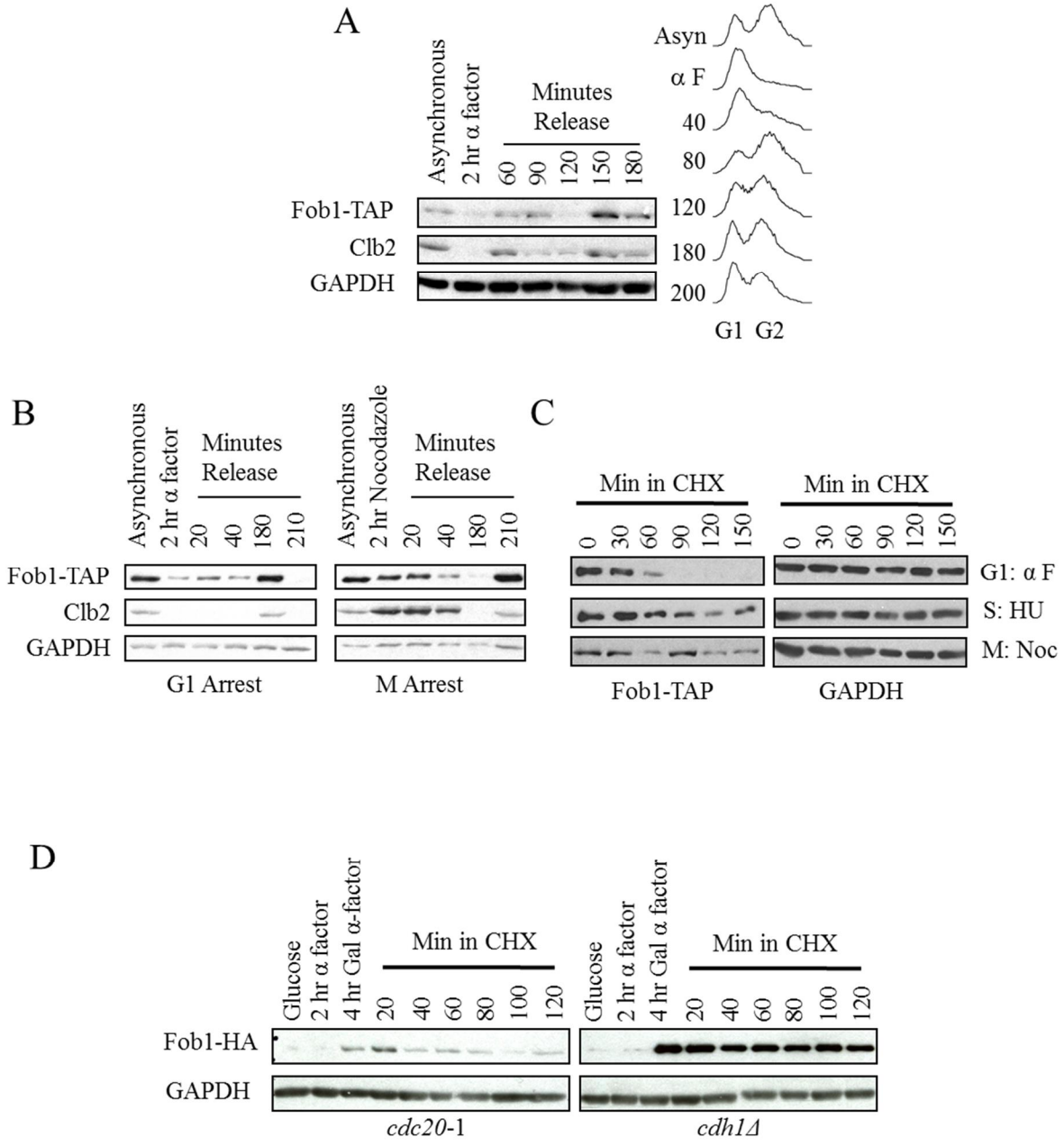

A
Asynchronous
2 hr α factor
Minutes Release
60
90
120
150
180
Fob1-TAP
Clb2
GAPDH
Asyn
α F
40
80
120
180
200
G1 G2
B
Asynchronous
2 hr α factor
Minutes Release
20
40
180
210
Asynchronous
2 hr Nocodazole
Minutes Release
20
40
180
210
Fob1-TAP
Clb2
GAPDH
G1 Arrest
M Arrest
C
Min in CHX
0
30
60
90
120
150
Min in CHX
0
30
60
90
120
150
G1: α F
S: HU
M: Noc
Fob1-TAP
GAPDH
D
Glucose
2 hr α factor
4 hr Gal α-factor
Min in CHX
20
40
60
80
100
120
Glucose
2 hr α factor
4 hr Gal α factor
Min in CHX
20
40
60
80
100
120
Fob1-HA
GAPDH
cdc20-1
cdh1Δ

Figure 4.3. Fob1 is specifically unstable during G1, with levels oscillating throughout the cell cycle and requiring APC^{Cdh1} for turnover

(A) WT cells expressing tagged *FOB1-TAP* were arrested in G1 with Ŭ factor for 2 hours then washed and released into fresh media. Periodic samples were collected for FACS and Western analysis. Westerns were performed using antibodies against TAP, Clb2, and GAPDH as a loading control. FACS represent cellular DNA content where the first peak are cells in G1 and the second peak are cells in G2. This experiment was repeated 3 times.

(B) As in (A) cells were arrested using Ŭ factor or nocodazole and samples were collected to demonstrate Fob1 cycling is not an artifact of arresting agent but a trend observed well into the next cell cycle. This experiment was repeated 3 times.

(C) WT cells expressing Fob1-TAP were arrested in G1 with α factor, S phase with hydroxyurea or M phase using Nocodazole for 2 hours. The cells were washed and resuspended in fresh media containing cycloheximide. Samples were removed at the times shown and analyzed by Western blotting with antibodies against TAP and GAPDH. This experiment was repeated 3 times.

(D) *cdh1Δ* and *cdc20-1* cells expressing plasmid borne GAL_{pro}-*FOB1-HA* were arrested in G1 with Ŭfactor for 2 hours followed by induction of the *FOB1* construct with galactose for 4 hours. Cells were then washed and released into fresh media containing cycloheximide (CHX) and periodic samples were collected for protein stability analysis. Samples were analyzed by Western analysis with antibodies against HA and GAPDH as a loading control. This experiment was repeated 3 times.

adaptor Cdc20 binds to the APC in G2 to promote the metaphase/anaphase transition. The APC adaptor Cdh1 binds the APC in late mitosis and promotes APC substrate degradation specific for mitotic exit and G1 maintenance. The absence of these adaptors stabilizes APC targets. Since Fob1 protein levels are low in G1 we wished to test whether the G1 specific Cdh1 adaptor is required for Fob1 turnover. We performed arrest/release experiments in *cdh1Δ* and *cdc20-1* cells expressing vector borne *GAL$_{pro}$-FOB1-HA*. WT and *apc5^{CA}* cells expressing *GAL$_{pro}$FOB1-HA* or were grown in 2% glucose to an OD_{600} of 0.5 then α factor was added for 2 hours to arrest cells in G1. After 2 hours, the cells were washed and 2% Gal + α factor was added for 4 hours to express the *FOB1* constructs while keeping the cells in G1. Next, the cells were washed and resuspended in 2% glucose and CHX to stop protein synthesis. Samples were taken every 30 minutes for two hours for protein analysis with antibodies against HA and GAPDH as a load control. We observed that Fob1-HA is stabilized in the absence of Cdh1, but not when Cdc20 is mutated (Figure 4.3D). These results are in agreement with Fob1 protein levels peaking during mitosis with a subsequent APC^{Cdh1}-dependent drop in G1.

4.1.4. A Fob1 putative modified D box is required for Fob1 modification

As already described in section 2.3.4, a modified D box $_{417}$**L**AN**E**AD**N**$_{423}$ referred to as the L box, was identified within Fob1. *FOB1* gene fragments which lack or have this L box locus disrupted fail to have a robust Fob1/Apc5 Y2H interaction (Supplementary Figure 6.2). These motifs are thought to be required for interactions between the APC and substrates boxes (KRAFT *et al.,* 2005; GLOTZER *et al.,* 1991; PFLEGER and KIRSCHNER 2000; PETERS 2006; MANCHADO *et al.,* 2010; YU, 2007; THORNTON and TOCZYSKI, 2006), (see section 2.2.3). We hypothesize that this L box is required for Fob1/Apc5 interaction, possibly mediating Fob1 ubiquitination. This would define this putative L box as a destruction motif. Under the premise that this region does represent an APC interaction motif, we mutated the Fob1 amino acid E, at position 420 to V, using site directed mutagenesis, to disrupt the Fob1/Apc5 interaction and consequently Fob1 modification. Destruction and KEN boxes are conserved and often require mutations to more than one motif to stabilize the APC substrate (PARK *et al.,* 2008). Therefore, in addition to the L Box, we searched for other potential APC interaction motifs within *FOB1* and identified D box 1, $_{306}$YGL**R**IG**L**G$_{313}$ (DB1) and D box 2, $_{542}$SKL**R**QK**L**D$_{549}$ (DB2). We altered these motifs by

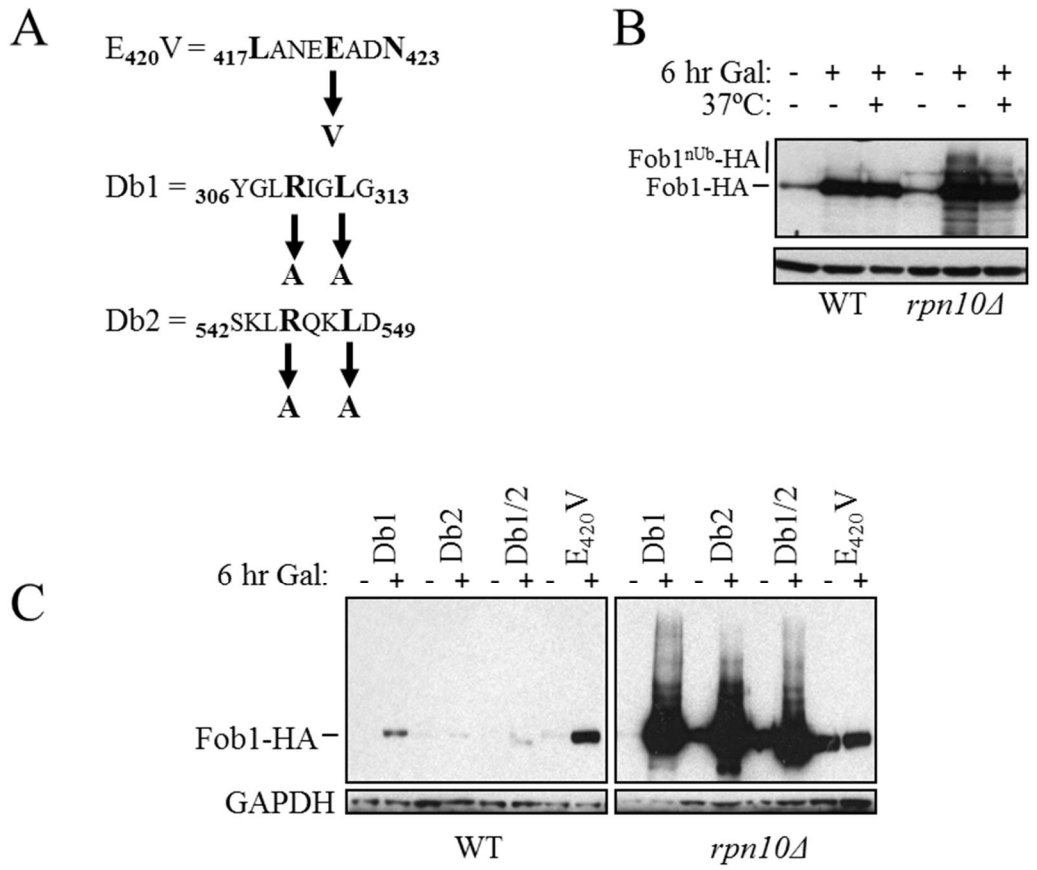


Figure 4.4. Fob1 modification requires the D box-like motif

(A) Creation of point mutations within *FOB1* that alter the putative APC interaction motifs: the L box, $E_{420}V$; DB1, $R_{309}A$, $L_{312}A$; and DB2, $R_{545}A$, $L_{548}A$.

(B) Modified Fob1 bands accumulate in *rpn10Δ* cells defective in proteosome poly-ubiquitin binding. WT and *rpn10Δ* cells expressing *GAL$_{pro}$-FOB1-HA* grown in glucose or galactose, at 30ºC or 37ºC, to induce expression for 6 hours.

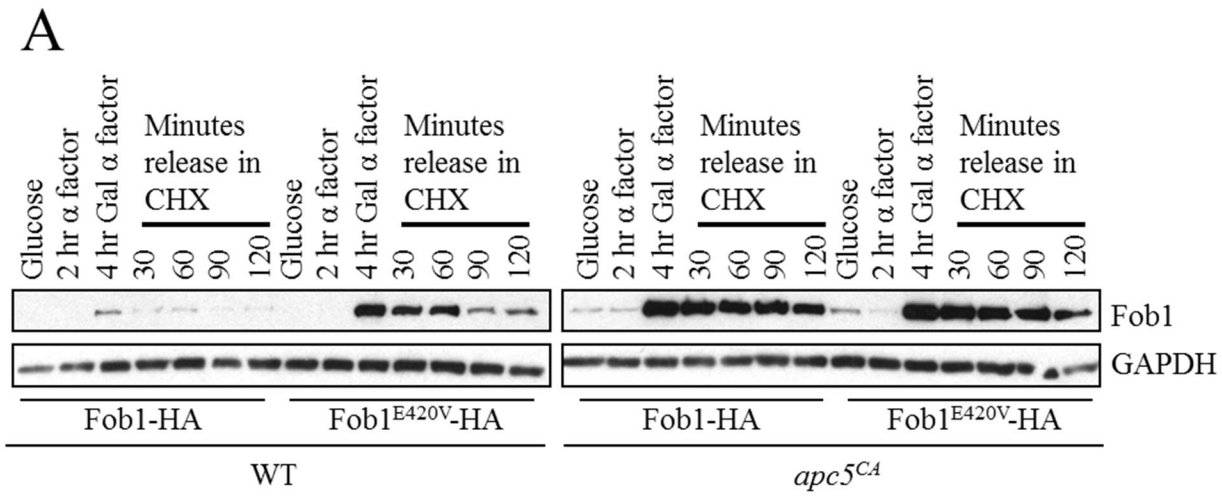
(C) *GAL$_{pro}$-FOB1-HA* mutant plasmid constructs DB1, DB2, DB1DB2, and $E_{420}V$ in WT or *rpn10Δ* cells grown at 30ºC in glucose or galactose for 6 hours. Protein samples were analysis by Western analysis with antibodies against HA and GAPDH as a loading control. This experiment was repeated 3 times.

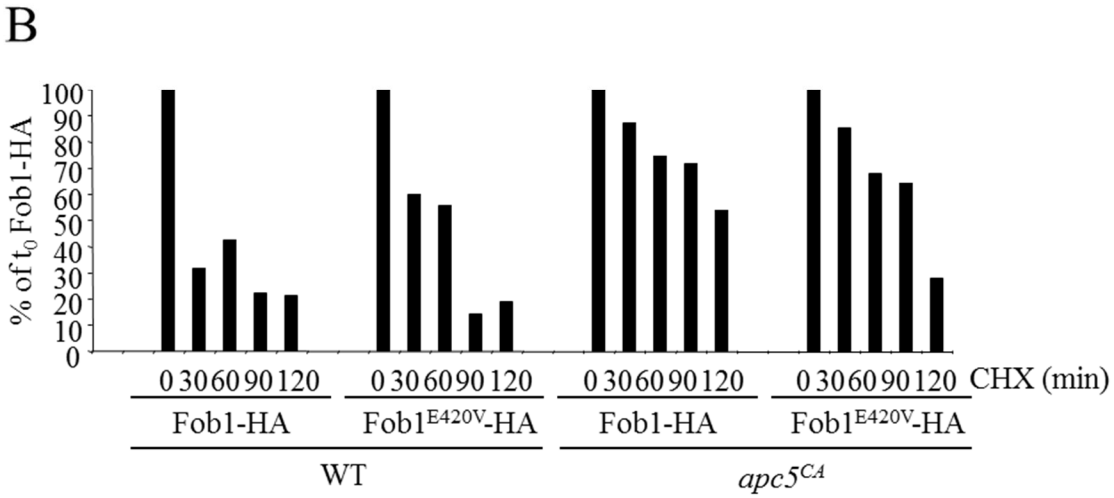
mutating the **R** and **L** amino acids within the motifs to **A** (Figure 4.4A). We expected that the disruption of one or more of these motifs may result in reduction of Fob1 modification.

We were unable to readily visualize ubiquitinated Fob1 directly. However, we were able to visualize Fob1 modifications and accumulation using a strain lacking *RPN10*, which encodes the proteasome ubiquitin receptor (ISASA *et al.,* 2010, see section 2.1.2). In the absence of Rpn10 we demonstrated that proteins accumulate within the cell (ISLAM *et al.,* 2011). Consistent with Fob1 ubiquitination, we observed an accumulation of higher molecular weight bands when we expressed Fob1-HA from the *GAL*-inducible promoter in asynchronous *rpn10*Δ cells (Figure 4.4B). Next, we expressed Fob1-HA mutant vectors DB1, DB2, DB1/DB2, and E420V in *rpn10*Δ cells and found a similar accumulation of higher molecular weight bands in all but the Fob1^{E420V}-HA construct (Figure 4.4C). These data show that Fob1 is modified to produce higher molecular weight species, consistent with ubiquitination, and these modification requires glutamate at position 420. Taken together with the Y2H data, which suggests the region around glutamine 420 of Fob1 is part of an APC interaction motif, these data are in support of the L box motif being a bona fide destruction box.

4.1.5. Fob1 is an unstable protein that requires the APC and the L box for turnover

Our results demonstrate that the Fob1 modification requires an intact L box motif (see section 4.1.4). Since Fob1 and Apc5 physically interact, we sought to determine whether Fob1 is targeted for degradation in an APC-dependent manner and whether the L box influences Fob1 turnover. We arrested cells in G1 using α factor since Fob1 is specifically unstable in G1 and performed protein stability assays as above to characterize Fob1 and Fob1^{E420V} turnover in WT and APC defective *apc5*^{CA} cells (see section 4.1.3). The results show that in WT cells, Fob1-HA is weakly detected after a 4-hour induction, with levels declining over the 2-hour release into CHX (Figure 4.5A). Fob1^{E420V}-HA accumulated to higher levels after 4 hours in galactose, with protein levels continuing to decrease after the 2-hour release. Our observations in WT cells indicate that Fob1 is indeed turned over and that the E420V mutation increases Fob1 accumulation and slows turnover. In *apc5*^{CA} cells, Fob1-HA accumulated to yet higher levels after the 4-hour galactose induction and remained elevated throughout the 2-hour release. Similarly the Fob1^{E420V} protein accumulated


A
Glucose
2 hr α factor
4 hr Gal α factor
Minutes release in CHX
30
60
90
120
Fob1
GAPDH
Fob1-HA
Fob1E420V-HA
WT
apc5CA


B
% of t0 Fob1-HA
0 30 60 90 120
CHX (min)
Fob1-HA
Fob1E420V-HA
WT
apc5CA

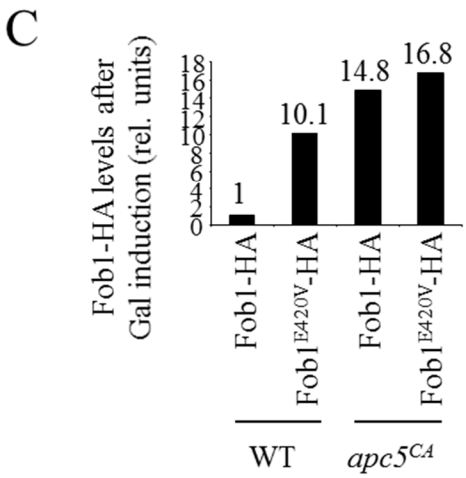

C
Fob1-HA levels after Gal induction (rel. units)
1
10.1
14.8
16.8
Fob1-HA
Fob1E420V-HA
WT
apc5CA

Figure 4.5. Fob1 is an unstable protein that requires the APC and the L box for turnover
(A) WT and *apc5*^{ca} cells expressing either *GAL*$_{pro}$*-FOB1-HA* or *GAL*$_{pro}$*-FOB1*^{E420V}*-HA* were arrested in G1 with Ŭ factor for 2 hours, followed by induction of the *FOB1* constructs with galactose for 4 hours. Cells were then washed and released into fresh media containing cycloheximide (CHX) and periodic samples were collected for protein stability analysis. Samples were analyzed by Western analysis with antibodies against HA and GAPDH as a loading control. Films were scanned for analysis with ImageJ. Experiments were repeated 3 times with similar results.
(B) Bands intensities from (A) were normalized to their corresponding GAPDH signal and then compared to band intensities after the 4 hour galactose induction.
(C) The band intensity of WT Fob1 in WT cells after the 4 hour induction was set to 1. The band intensities from each sample the after 4 hour induction were compared to WT Fob1 in WT cells and plotted. The *GAL*$_{pro}$*-FOB1*^{E420V}*-HA* vector was constructed by Chelsea Stahl.

at equally high levels (Figure 4.5A). An analysis of Fob1 protein content shows that Fob1 turnover is slowed down in the E420V mutant, and slowed even further in *apc5*^{CA} cells compared to WT (Figure 4.5B). Next, we compared total Fob1-HA levels after the 4 hour induction in galactose in WT and *apc5*^{CA} cells expressing WT or the E420V mutant Fob1 (Figure 4.5C). Fob1^{E420V} accumulated approximately 10-fold more than Fob1 in WT cells. In *apc5*^{CA} cells, Fob1^{E420V} accumulated approximately 15-17 fold more than the WT condition. Our results show that both intact Apc5 and the Fob1 E420 residue are required for efficient Fob1 turnover.

4.1.6. Mutation of the L box increases rDNA recombination frequency

Presumably a more stable Fob1 should influence rDNA recombination and life span, in a manner comparable to the negative effect increased *FOB1* expression had on RLS (Supplementary Figure 6.5A, section 2.4.4.2). To discern whether the more stable *FOB1*^{E420V} has any impact on rDNA recombination frequency we introduced a genomic copy of *FOB1*^{E420V} within the recombination strain and found that this mutation did in fact increase rDNA recombination (Figure 4.6). This result supports our notion that the insufficient removal of Fob1 by the defective *apc5*^{CA} mutant is what results in increased recombination and perhaps genomic instability within the rDNA region.

4.1.7. Summary and Conclusion

In summary, we hypothesized that the APCôs role in maintaining longevity is, at least in part, through the degradation of Fob1. This hypothesis was based on work done prior to this study (see section 2.0). In this study we have found that Fob1 is unstable specifically in G1, and cycles throughout the cell cycle in a manner similar to Clb2, an APC target. Consistent with the APC mediating proteasome- and ubiquitin-dependent Fob1 degradation, Fob1 is stable in APC and proteasome mutants. *FOB1* deletion also suppressed cell cycle progression, and rDNA recombination defects observed in *apc5*^{CA} cells. Mutation of a putative Destruction Box-like motif (Fob1^{E420V}) disrupted Fob1 modification, stabilized the protein and increased rDNA recombination. Taking into consideration the dual functions of Fob1, the genomic destabilizing replication fork blocks and subsequent DNA breaks, and the genomic stabilizing condensin-

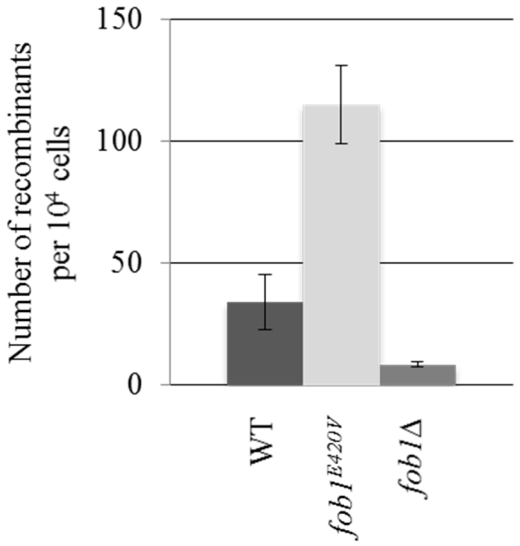


Figure 4.6. Disruption of the L box, the E420V mutation, increases rDNA recombination frequency

(A) The average frequency of recombination in the rDNA for five individual cultures of WT, *FOB1*^{E420V}, and *fob1*Δ as judged by the loss of the *ADE2* gene. Cultures were inoculated with 5 individual colonies and allowed to grow in YPD until an OD_{600} of 1.0, then cells were diluted and plated on YPD and grown for 5 days at 30ºC. Approximately 10^3 cells were plated from each sample. Recombination frequency is expressed as the number of recombinant red cfu per 10^4 cells. Standard error of the mean is shown.

dependent rDNA condensation and silencing; it is conceivable that Fob1 dosage may be regulated by the APC in response to cell cycle and environmental cues.

4.2. Investigating Cin5

As introduced in section 2.3.7, a screen for aptamers capable of suppressing the *apc5*^{CA} *ts* phenotype, was used in an effort to identify novel molecular networks or pathways that intersect with the APC. The screen yielded the aptamer Y65, among others not investigated in this report. Aptamer Y65 was investigated further because it has homology to Elongin C (Elc1), a ubiquitin protein ligase conserved among eukaryotes which forms a complex with the cullin family member Cul3 (HYMAN *et al.,* 2002; RIBAR *et al.,* 2007). This recovered Y65 aptamer was then cloned into the Y2H bait vector for a reverse screen to find binding partners. Cin5, a stress response transcription factor was identified as a binding partner in this Y2H screen (data not shown). Presented below is data that describes the ubiquitin-dependent degradation of Cin5 and a phosphor-inhibited degradation motif within Cin5. This data contributes to our understanding of how the APC or other ubiquitin ligases may regulate this transcription factor.

4.2.1. Cin5 is an unstable protein that is stabilized by glucose starvation and salt stress.

First, we wanted to corroborate that Cin5 is an unstable protein that is stabilized by salt stress (FERNANDES *et al.,* 1997; FURUCHI *et al.,* 2001; MENDIZABAL *et al.,* 1998). We endogenously TAP tagged *CIN5* and performed a protein stability assay to measure protein turnover. We added CHX to stop protein synthesis to fresh growing culture and periodically removed samples, every 15 minutes for 45 minutes, for Western analysis. Similarly, we washed and replaced the media with media lacking glucose, or added NaCl to a final concentration of 0.4 M to two identical cultures (Figure 4.7). These data indicate that Cin5 is an unstable protein that is actively turned over in approximately 45 minutes; moreover, Cin5 is stabilized both by salt exposure and starvation. In addition, mobility shifts are observed under stabilizing conditions, consistent with Cin5 phosphorylation (Figure 4.7; PEREIRA *et al.,* 2009). This suggested phosphorylated Cin5 may be protected from degradation.

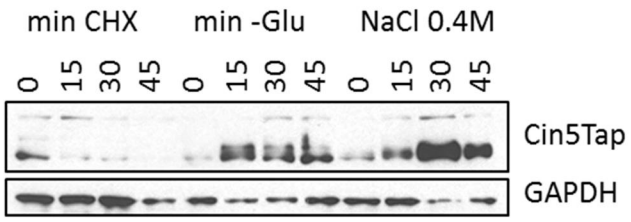


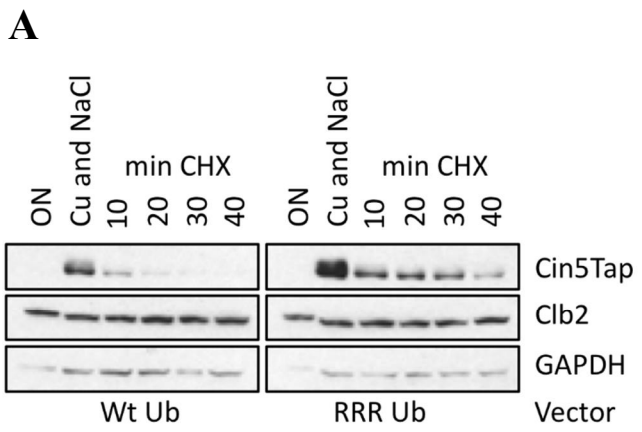
Figure 4.7. Cin5 is an unstable protein that is stabilized by glucose starvation and salt stress. Cin5-TAP cells were grown in YPD overnight then split into 3 cultures (time ñ0ò), washed and re-suspended into fresh media with either CHX, or media lacking a carbon source (0% glucose), or 0.4 M NaCl for 15, 30, 45 minutes. The CHX treatment stops protein synthesis to assess Cin5 stability of turnover rate. Treatment with 0% glucose or 0.4M NaCl appear to increase cin5 stability. Westerns were performed using antibodies against TAP, and GAPDH as a loading control.

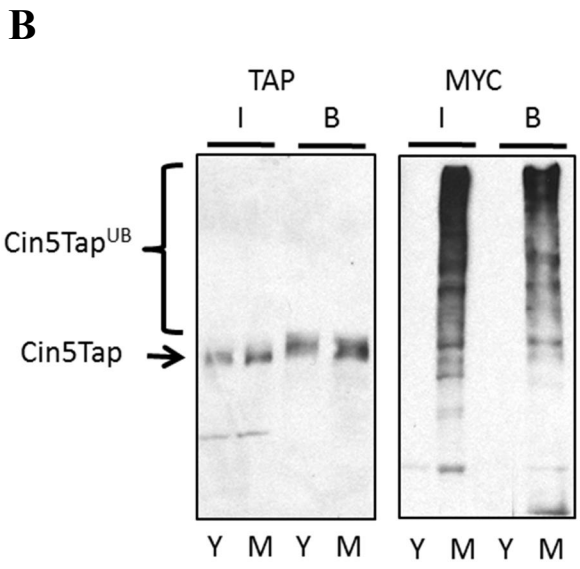
4.2.2. Proteasome- and ubiquitin-dependent degradation of Cin5

To demonstrate that Cin5 is subject to proteasome- and ubiquitin-dependent degradation we performed a variety of protein stability assays and IPs. First a stability assay, similar to the one in the previous section, except prior to the addition of CHX we expressed a copper inducible vector expressing a mutant Ub unable to form polyubiquitin chains. This RRR Ub has lysines 29, 48 and 63, required to construct polyubiquitin chains replaced with arginine (R), hence RRR, which disrupts the ability of this Ub molecule to form polyubiquitin chains. Protein substrates modiýed by a polyubiquitin chain are preferentially targeted to the proteasome; whereas, monoubiquitin chains and short or incomplete Ub chains have a lower avidity for preoteasome binding and are less susceptible to degradation (reviewed in FINLEY *et al.,* 2012). Under this premise, incorporation of this mutant RRR Ub into a growing Ub chain should prevent further ubiquitination and partially stabilize or slow the degradation of ubiquitinated substrates.

In our stability assay we induced expression of either a copper inducible plasmid borne WT Ub or the RRR Ub for 2.5 hours by addition of 100 εM $CuSO_4$, followed by an additional 20 minutes of 0.4 M NaCl to stabilize Cin5, followed by several washes to removed salt prior to release into fresh media containing CHX to stop protein synthesis. Samples were collected every 10 minutes for Western analysis. Westerns were probed against Cin5-TAP and GAPDH as a loading control. The presence of the RRR Ub slowed the turnover of Cin5-TAP compared to WT Ub, suggesting that Cin5 degradation is ubiquitin dependent (Figure 4.8A).

Next, we demonstrated that Cin5 is likely ubiquitinated by immunoprecipitating Cin5-TAP and probing for Myc-Ub expressed from a copper inducible vector (Figure 4.8B). Cells harboring tagged Cin5-TAP and the copper inducible vector *$CUP1_{pro}$-Myc-UB* (M) or empty vector Ycp50 (Y) were grown overnight followed by addition of 100 εM Cu^+ for 2.5 hr to express vector, followed by an additional 20 min Cu^+ and 0.4 M NaCl to induce Cin5 stabilization. Then the cells were washed with fresh media for 15 minutes. Samples were prepared via the bead beat method and immunoprecipitated with IgG beads for Western analysis (Figure 4.8B). The anti-TAP blot confirms that Cin5-TAP is present in the input and was successfully immunoprecipitated in the bound lanes. Both input and bound lanes containing Myc-Ub, probed with anti-Myc antibodies,





Y = Ycp50 (empty Vector)

M = Myc-Ub

I = Input

B = bound

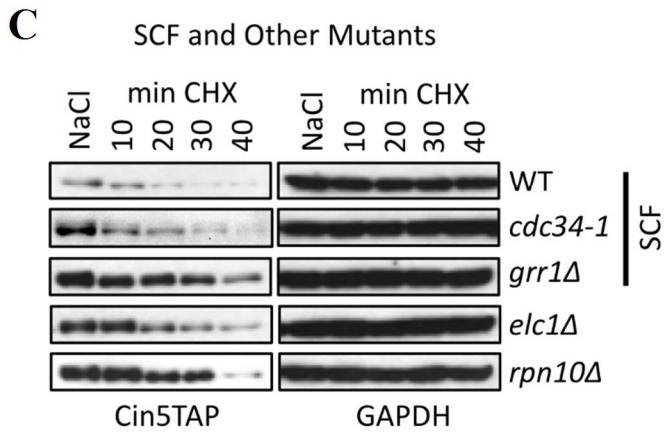


Figure 4.8. Proteasome- and ubiquitin-dependent degradation of Cin5.
(A) Cin5 degradation requires formation of polyubiquitin chains. Cin5-TAP cells were grown overnight (ON), followed by addition of 100 εM $CuSO^4$ for 2.5hr to express vector borne WT Ub or mutant RRR Ub (R29, R48, and R63) which is unable to form polyubiquitin chains. Then an additional 20 min 100 εM $CuSO^4$ and 0.4 M NaCl was added for an additional 20 minutes to stabilize Cin5, followed by a wash and release into fresh media with CHX for 10, 20, 30, 40 minutes. Westerns were performed using antibodies against TAP, Clb2, and GAPDH as a loading control.
(B) Cin5 is likely ubiquitinated. Cin5-TAP cells harboring either *$CUP1_{pro}$-Myc-UB* (M) or empty vector Ycp50 (Y) were grown ON, followed by addition of 100 εM $CuSO^4$ for 2.5 hr to express vectors, followed by an additional 20 min 100 εM $CuSO^4$ and 0.4 M NaCl to induce Cin5 stabilization, then wash into fresh media for 15 minutes. Whole cell lysates were prepared using the bead beat method in RIPA buffer: 150 mM NaCl, 50 mM Tris pH8, 5 mM EDTA, 1 mM EGTA, 1% v/v NP-40, 0.5% deoxycholate, 40 mM Na Vanadale, 40 mM Na F, 20 mM beta-glycerphosphate; 1 mg of protein was incubated with 20 εl IgG sepharose beads for 30 min to immunoprecipitate Cin5-TAP, beads were washed 2X with 100 ul buffer, then boiled in 30 ul 2x SDS buffer; 20 ul of bound sample along with 10 εg of input in 2x SDS buffer was separated by SDS PAGE, transferred to membrane and detected with anti MYC PL-14 mouse or anti TAP antibodies.
(C) Cin5 is stabilized in the absence of *RPN10, ELC1, GRR1,* and when *CDC34* is compromised. *RPN10* is a component of the ubiquitin-dependent proteasome; *ELC1*, which the aptamer Y65 has homology to, is required for polyubiquitination of RNA polymerase II, and *GRR1* member of the SCF ubiquitin ligase complex, and the E2 *CDC34* are where *cdc34-1* is a *ts* mutant. These mutants strains with tagged Cin5-TAP were grown ON, followed by 0.4 M NaCl for 20 minutes to induce Cin5 stabilization, then washed and released into fresh media + CHX for 10, 20, 30, 40 minutes. Westerns were performed using antibodies against TAP, and GAPDH as a loading control.

produced an upward smear banding pattern that is indicative of polyubiquitination (Figure 4.8B). Such upward bands are present on the anti-TAP blots and are visible under higher exposure (data not shown). The more prominent bands seen in the bound lanes immediately above Cin5-TAP are spaced approximately 8 kDa apart from one another, consistent with the size of a single Ub. Although this data does suggest Cin5 is ubiquitinated, it is possible that ubiquitinated proteins other than Cin5 immunoprecipitated and are also present within the upward smear banding pattern.

Finally, we performed protein stability assays on mutants tagged with Cin5-TAP (Figure 4.8C). The mutants include: the SCF mutants *cdc34-1* and *grr1*Δ, *elc1*Δ (shares homology with Elc1), and *rpn10*Δ. All these mutants appear to stabilize Cin5 to some degree, particularly *grr1*Δ and *rpn10*Δ (Figure 4.8C). Grr1 is an adaptor that gives the SCF ubiquitin ligase substrate specificity. This data suggests that the Grr1 bound SCF may target Cin5 for ubiquitination. Elc1 is also a ubiquitin protein ligase that appears to be involved in Cin5 degradation. Cin5 stability in the *rpn10*Δ mutant supports that Cin5 degradation is proteasome- and ubiquitin-dependent.

4.2.3. Cin5 does not appear to be an APC target

Here we provide evidence to suggest that Cin5 turnover is not APC dependent. As described in section 4.1.3, the APC targets substrates in a cell cycle dependent manner and if Cin5 were in fact an APC target, Cin5 protein levels could be expected to fluctuate or cycle as the cell cycle progresses. To see if this is the case we arrested cells in either G1, S or M phase using Ŭ-factor, hydroxyurea, and nocodazole respectively and collected samples for Western analysis for Cin5-TAP, Clb2 (a known APC target degraded in G1), and GAPDH as a load control (Figure 4.9A). The results show that Cin5 is not preferentially degraded at any particular phase of the cell cycle. However, in G1 Cin5 does appear to be lacking a small band, second from the bottom, that we believe to be phosphorylated Cin5. In addition, we followed Cin5 levels in a synchronized culture arrested in G1 with Ŭ-factor, then released into fresh media to allow them to reenter the cell cycle (Figure 4.9B). Here it is clear Cin5 levels do not fluctuate throughout the cell cycle, such as the known APC target Clb2.

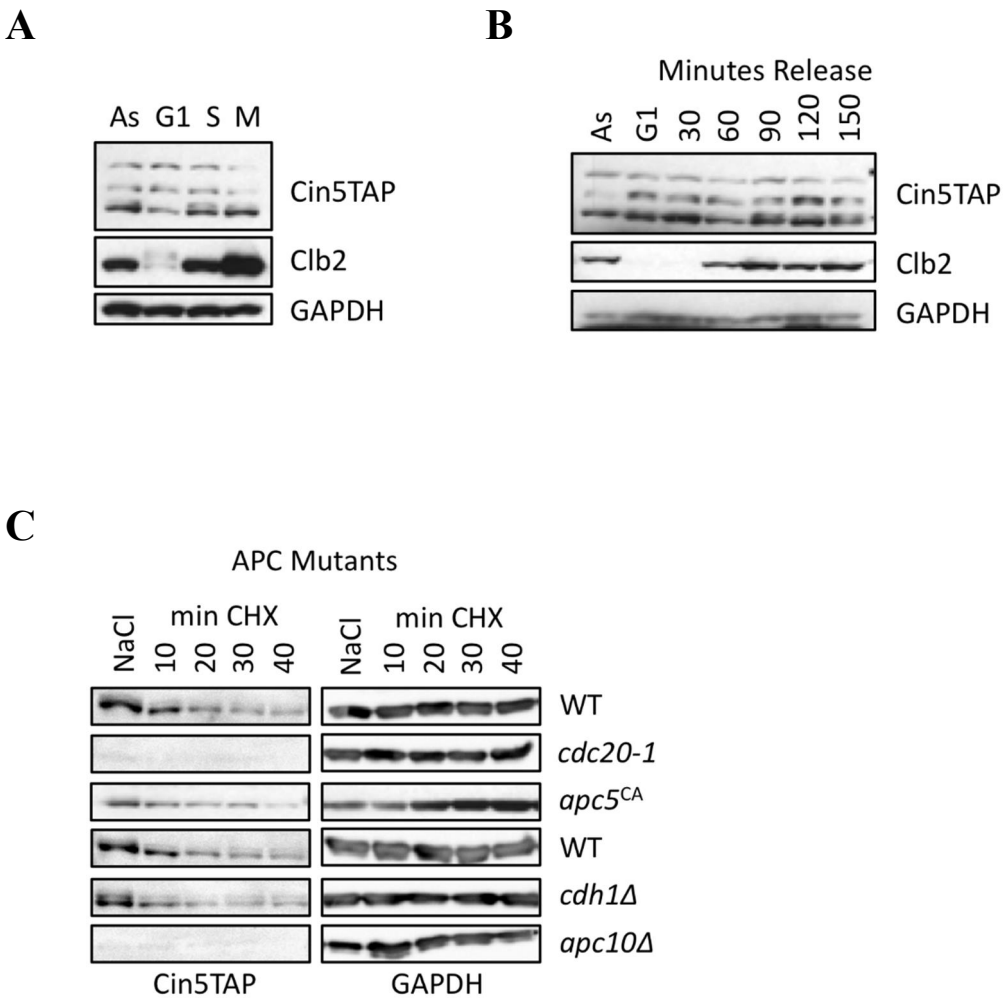


Figure 4.9. Cin5 does not appear to be an APC target.
(A) Cin5-TAP levels do not fluctuate with the cell cycle. Cin5TAP cells asynchronous (As) or arrested in G1, S, or M phase of the cell cycle with Ŭ factor, hydroxyurea, or Nocodazole respectively for 2.5 hours.
(B) Cin5TAP 4242 cells asynchronous (As) or arrested in G1 with Ŭfactor for 2.5 hours, followed by wash and release into fresh media. Periodic samples were collected for Western analysis. Cell cycle phase confirmed by visual inspection and Clb2 western. Westerns were performed using antibodies against TAP, Clb2 and a cell cycle indicator, and GAPDH as a loading control.
(C) APC mutants *cdc20-1* and *apc10Δ* appear to destabilize Cin5-TAP. Various APC mutants with tagged Cin5-TAP grown in 0.4 M NaCl for 20 minutes to induce Cin5 stabilization, followed by a wash and release into fresh media with CHX for 10, 20, 30, 40 minutes. Westerns were performed using antibodies against TAP, and GAPDH as a loading control.

If the Cin5 were an APC target we could expect Cin5 turnover to be reduced in APC mutants, similar to what we observed for Fob1 (section 4.1.3). We performed protein stability assays, as in the previous section, in APC mutants: *apc5*^{CA}, *apc10*Δ, and activators *cdc20-1* and *cdh1*Δ. APC mutant *apc5*^{CA} and *cdh1*Δ do not appear to impact Cin5 stability or turnover (Figure 4.9C). Interestingly, *cdc20-1* and *apc10*Δ appear to destabilize Cin5. This result may be a clue that links regulatory crosstalk between the SCF and the APC, as we know some SCF activators are subject to APC-dependent degradation (BENANTI 2012, SKAAR and PAGANO 2009; IRNIGER *et al,.* 2000). Perhaps the failure of defective APC to target particular SCF activators or regulators for degradation may result in increased targeting and degradation of Cin5 by the SCF. Taken together, the APC appears to not be directly responsible for Cin5 turnover; however, there may be some indirect regulation through other ubiquitin ligases such as the SCF that may be critical under specific cell cycle or stress conditions.

4.2.4. Phosphorylation sites T192 and S196, and ubiquitination site K194 regulate Cin5 stability.

Cin5 has several putative phosphorylation sites, but only the mutation of T192 and S196 impairs its phosphorylation under different stress conditions (PEREIRA *et al.,* 2009). Interestingly, a proteomic screen designed to identify ubiquitinated lysines found Cin5 K194 to be ubiquitinated (STARITA *et al.,* 2012). This lysine is situated between the known phosphorylation sites, amino acid residues T192 and S196. Ubiquitination sites are often regulated by phosphorylation events, such as the phospho-inhibited degron, a site that is targeted for ubiquitination in the absence of phosphorylation (HOLT 2012). Considering this, Cin5 may be protected from ubiquitination and consequently degradation when T192 and S196 are phosphorylated.

Using site directed mutagenesis we mutated T192 and S196 to E to mimic phosphorylation, or to A to abolish the phosphorylation sites. In addition, we mutated K194 to R to abolish the ubiquitination site. We also put these mutations into combinations to amplify their potential impact. The results were quite pronounced, as the phospho-mimic T192E, S196E double mutant and K194R stabilized Cin5 as did the triple mutant (Figure 4.10A). In contrast, as we predicted, abolishing the phosphorylation sites T192A, S196A severely destabilized Cin5. The

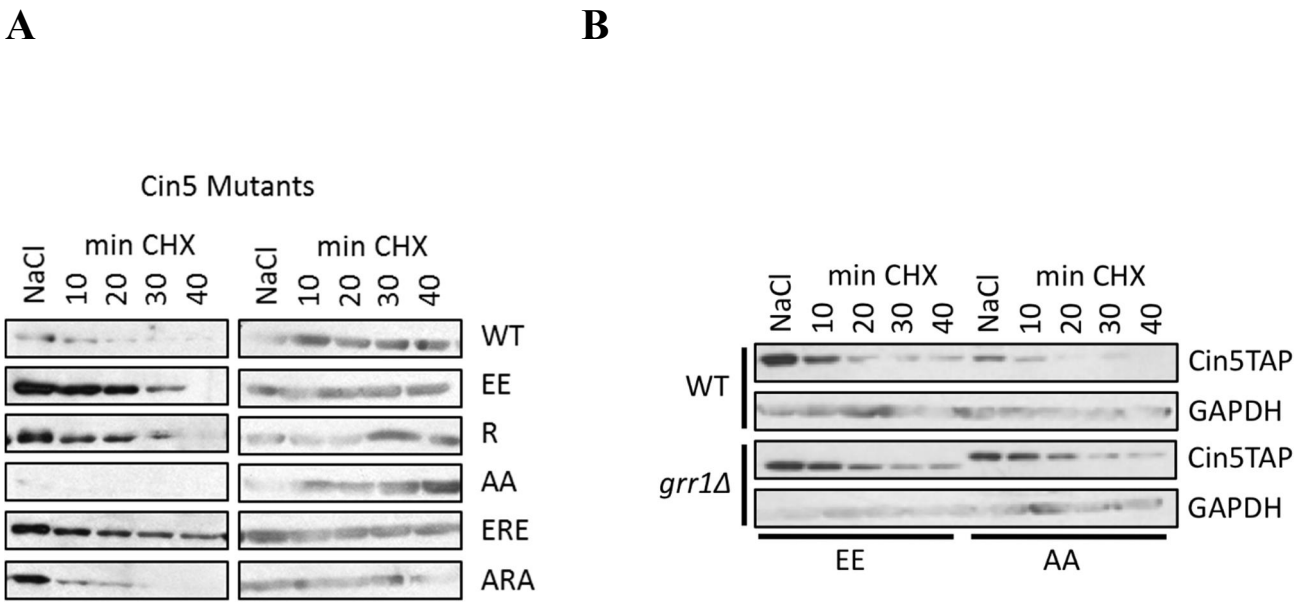


Figure 4.10. Phosphorylation sites T192 and S196, and ubiquitination site K194 regulate Cin5 stability.

(A) Cin5 amino acids T192 and S196 were mutated to E to mimic phosphorylation (EE), or A to abolish phosphorylation (AA), and amino acid K194 was mutated to R to abolish ubiquitination (R), or both mutation combinations (ERE and ARA). These tagged Cin5-TAP cells were grown in 0.4 M NaCl for 20 minutes to induce Cin5 stabilization, followed by a wash and release into fresh media with CHX for 10, 20, 30, 40 minutes. Westerns were performed using antibodies against TAP, and GAPDH as a loading control.

(B) The unstable AA Cin5 mutant is stabilized in a *grr1*Δ background, suggesting that Grr1 is involved in Cin5 degradation. Note the distinct mobility shift of approximately 8 kDa, consistent with monoubiquitin, in the AA *grr1*Δ cells. Cells were grown and treated as in A.

combination of T192A, S196A with the stabilizing K194R mutation produced a Cin5 with intermediate stability (Figure 4.10A). Taken together, these results strongly support our hypothesis that this region including residues T192, K194 and S196 form a phospho-inhibited degron site.

Finally, to provide further evidence supporting that the SCF, or at least the activator Grr1 is involved in Cin5 degradation, we introduced the unstable Cin5 T192A, S196A and the stable Cin5 T192E, S196E into *grr1*Δ. Deletion of *GRR1* does appear to recover the stability of the unstable Cin5 T192A, S196A somewhat (Figure 4.10B). This result supports our notion that Grr1 is involved in Cin5 degradation; however, Cin5 is still turned over which suggests there are additional regulatory and/or ubiquitination sites involved in its degradation. We made a noteworthy observation in *grr1*Δ cells harboring Cin5 T192A, S196A. Cin5 T192A, S196A in the *grr1*Δ background shows a distinct mobility shift of about 8 kDa which is consistently present throughout the time course (Figure 4.10). This shift is consistent with a single ubiquitin. This may indicate that a single ubiquitin may be involved in the regulation of Cin5 stability or activity.

4.2.5. Summary and Conclusion

In summary, we hypothesized that the aptamer Y65 masks a degradation signal in the stress response transcription factor Cin5. This hypothesis was based on data generated prior to this study (see section 2.0). We did not collect evidence to directly support this hypothesis; nevertheless, in this study we did find that Cin5 is targeted for proteasome- and ubiquitin-dependent degradation. Furthermore, we identified a phospho-inhibited degron motif within Cin5, which includes the aa residues T192, K194 and S196. We have evidence to suggest that the APC is not the ubiquitin ligase directly responsible for Cin5 degradation; however, some APC mutants destabilized Cin5. Cin5 is stabilized in the absence of Grr1 and Elc1, components of other ubiquitin ligase complexes. Taken together, this data suggest that the APC may indirectly influence Cin5 stability through other ubiquitin ligases such as the SCF, at least under specific cell cycle or stress conditions.

4.3. Characterizing the *apc5^{ca} ts* allele

The *apc5*^{CA} allele has served as a valuable tool in genetic studies, as the behavior of this allele is easily modified by mutations to genes that regulate APC function (HARKNESS *et al.,* 2004; HARKNESS *et al.,* 2005; ISLAM *et al.,* 2011; TURNER *et al.,* 2010; POSTNIKOFF *et al.* 2012). The temperature sensitivity of this allele is, for all intents and purposes, the important feature of this APC mutant in regard to the experiments performed in the Fob1 and aptamer study. However, understanding the nature of this *apc5^{CA}* allele and how it conveys temperature sensitivity may reveal the specific influence the Apc5 has on APC function and help us better interpret data generated with this mutant. Here, we conduct experiments designed to characterize the *apc5^{CA}* allele and test our hypothesis that the *apc5^{CA}* allele is producing an N-terminal truncation of the Apc5 subunit (see section 2.2.4).

4.3.1. Construction of an N-terminal Apc5 truncation

As discussed in section 2.2.4, the *ts apc5^{CA}* allele has a two base pair deletion, AT, at bp 37 and 38 in the codon encoding phenylalanine, F12, resulting in the generation of 18 additional nonspecific amino acids followed by a nonsense stop codon. This premature stop codon is within the N-terminal region of the protein; however, the C-terminally TAP tagged *apc5^{CA}* produces a smaller protein with an intact C-terminal TAP tag (see section 2.2.4). We hypothesize that the *apc5^{CA}* allele is utilizing an internal methionine start site to generate an N-terminally truncated protein that still has an intact C-terminal tag. This would be consistent with the *ts apc5^{CA}* allele producing a smaller product. Moreover, less of the smaller *apc5^{CA}* product was detected which suggests the truncated protein is functional but may be in short supply. Thus, perhaps the *ts* phenotype is due to low Apc5 levels. Apc5 is, after all, an essential protein (MCLEAN *et al.,* 2011; FINLEY *et al.,* 2012).

To test whether an internal methionine is being used as a translational start site to generate a functional N-terminal truncated protein as a result of the *apc5^{CA}* mutation, an *APC5* allele was generated that starts at the internal methionine M99. This is the next available methionine within the *APC5* gene. To construct this truncation a *LEU* marked *GAL10* promoter was cloned into the

start of the genomic *APC5* allele to replace the natural promoter and the *APC5* coding sequence up to the indicated methionine, effectively truncating the allele (Figure 4.11A). This was achieved by standard yeast transformations and homologous integration of a *LEU-GAL10-apc5^{M99}* PCR product consisting of the *LEU* marked *GAL10* promoter flanked by 50bp sequences homologous to 50bps upstream and downstream of desired integration site at M99 of the *APC5* gene. We henceforth use the notation *apc5^{M99}* to denote this *APC5* allele N-terminally truncated at M99.

4.3.2. The N-terminal truncated *apc5^{M99}* allele is not viable

As described above the *apc5^{M99}* allele is N-terminally truncated to the second available methionine M99 to mimic what we hypothesize may be the translational product of the *apc5^{CA}* allele. Unfortunately, we were unable to recover viable haploid *apc5^{M99}* colonies. However, we did recover two viable diploid colonies when we transformed the *LEU-GAL10-apc5^{M99}* PCR product into *APC5*/*apc5^{CA}* diploid cells, containing both an intact *APC5* allele and the *ts apc5^{CA}* allele. We confirmed the presence of both, an original intact *APC5* or *apc5^{CA}* allele, in addition to a truncated *apc5^{M99}* allele using PCR in both of these isolated diploid colonies (Figure 4.11B). Using primers designed to amplify a 1000 bp region +/- 500 bp of the *APC5* start codon we were able to detect and confirm the homologous integration of the larger *LEU-GAL10-apc5^{M99}* PCR product (Figure 4.11B).

Serial spot dilutions are a measure of competitive growth. Spot dilutions of the isolated *apc5^{M99}* diploid strains show that isolate ñAò is *ts* while the other ñCò, is not (Figure 4.11C). This suggests that the healthy WT *APC5* allele in this diploid isolate ñAò was likely replaced with the truncated *apc5^{M99}* construct; whereas isolate ñCò likely had the *ts apc5^{CA}* allele replaced with *apc5^{M99}*.

Diploid yeast cells contain two sets of chromosomes. These cells can be induced to undergo sporulation whereby meiosis generates a tetrad, four different haploid spores. The use of modern microscopy and micromanipulation techniques allows the four haploid spores of a yeast tetrad to be separated and germinated individually to form isolated spore colonies. This technique called tetrad analysis is used to isolate recombined haploid stains and is useful for assessing whether a particular phenotype is caused by a specific mutation. In our case, we wish to isolate the *apc5^{M99}*

allele and ask whether it is phenotypically equivalent to the *apc5*^{CA} allele. The sporulation analysis of our two diploid isolates both produce a 2:2 tetrad segregation of two viable and 2 non-viable haploid cells, meaning two of the haploid genotypes are unable to grow and have a lethal mutation (Figure 4.11D). Since we know the WT *APC5* and the *apc5*^{CA} allele are viable this data suggests that the *apc5*^{M99} allele is lethal. Taken together with the spot dilution data, we are able to conclude that we have constructed two diploid strains: an *APC5*/*apc5*^{M99} that grows as WT and a *ts* *apc5*^{M99}/*apc5*^{CA} strain. The truncated *apc5*^{M99} allele is not viable since it is unable to sustain growth of a haploid stain.

Since our *apc5*^{M99} allele was constructed using the *GAL10* promoter, which strongly promotes transcription in the presence to galactose, it is possible that transcription of our *apc5*^{M99} allele under normal 2% glucose conditions was inadequate for viability. To test if increased expression of the truncated *apc5*^{M99} allele can recover function we spotted both the *APC5*/*apc5*^{M99} and the *ts* *apc5*^{M99}/*apc5*^{CA} strains on increasing amounts of galactose containing media (Figure 4.11E). None of the galactose concentrations were able to recover the *ts* defect of the *apc5*^{M99}/*apc5*^{CA} strain, which supported our previous conclusion that the truncated *apc5*^{M99} allele is not viable. Furthermore, we performed tetrad analysis on plates with various concentrations of galactose and were not able to recover viable *apc5*^{M99} haploids (data not shown).

4.3.3. Summary and conclusion

In conclusion, our hypothesis that the *apc5*^{CA} allele is utilizing an internal methionine start site at position 99 to generate an N-terminally truncated protein was not supported. The *apc5*^{M99} allele did not mimic the *apc5*^{CA} allele and this truncation produced a non-viable allele. However, to further corroborate these results the *apc5*^{M99} allele should be sequenced to insure it was constructed correctly and free of other extraneous mutations that may have interfered with our results. Similarly, truncating the *APC5* allele using *LEU-GAL10* promoter may have impinged on the viability of this *apc5*^{M99} allele. Constructing the *apc5*^{M99} allele using the natural promoter may be warranted.

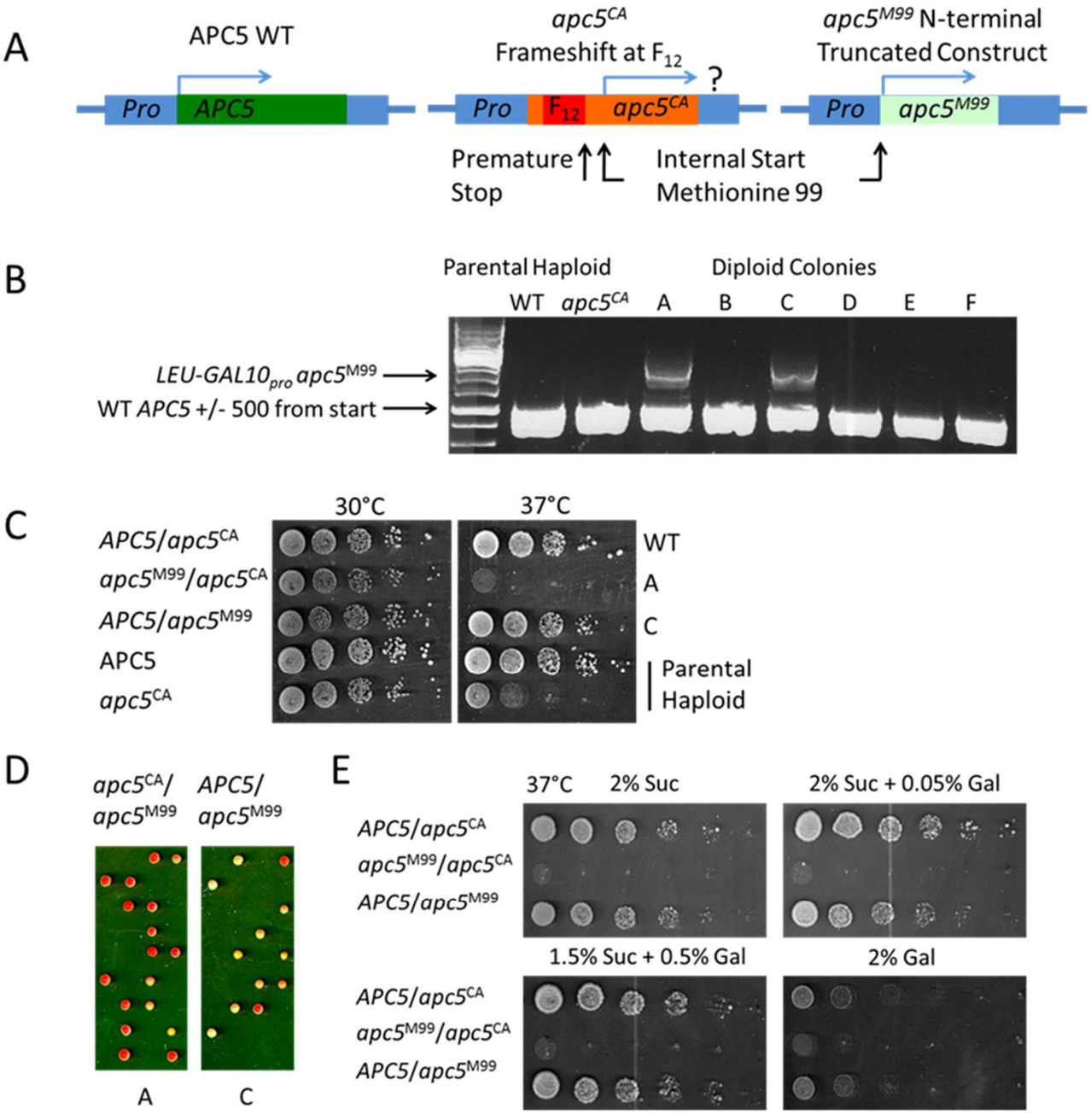

A
APC5 WT
Pro
APC5
apc5CA
Frameshift at F12
?
Pro
F12
apc5CA
apc5M99 N-terminal
Truncated Construct
Pro
apc5M99
Premature
Stop
Internal Start
Methionine 99
B
Parental Haploid
Diploid Colonies
WT
apc5CA
A
B
C
D
E
F
LEU-GAL10pro apc5M99
WT APC5 +/- 500 from start
C
30°C
37°C
APC5/apc5CA
apc5M99/apc5CA
APC5/apc5M99
APC5
apc5CA
WT
A
C
Parental
Haploid
D
apc5CA/
apc5M99
APC5/
apc5M99
A
C
E
37°C
2% Suc
2% Suc + 0.05% Gal
APC5/apc5CA
apc5M99/apc5CA
APC5/apc5M99
1.5% Suc + 0.5% Gal
2% Gal
APC5/apc5CA
apc5M99/apc5CA
APC5/apc5M99

Figure 4.11 The M99 N-terminal Apc5 truncation is lethal and does not suppress the *apc5^{CA} ts* phenotype when overexpressed.

(A) Schematic illustrating the WT *APC5* allele, the *ts apc5^{CA}* allele with a frameshift and consequential premature stop codon shown in red and proposed internal methionine start codon, and the constructed *apc5^{M99}* allele. Region in blue represents non-coding DNA and promoter sequences (Pro and Gal10pro). In the constructed *apc5^{M99}* allele, the natural promoter and the gene sequence up until the methionine 99 codon was replaced by the *GAL10* promoter. Blue right angle arrows indicate assumed translational start site and direction.

(B) Construction of the M99 N-terminal Apc5 truncation. Confirmation of *LEU-GAL10pro apc5^{M99}* insertion. PCR products were generated using primers +/- 500 bp of *APC5* start codon from parental haploid cells and isolated diploid colonies (A-F) that were transformed with the *LEU-GAL10pro-MET99* PCR product to generate the *apc5^{M99}* endogenous N-terminal truncation. DNA products were separated on 1% agarose gels. All lanes contain a WT 1000 bp DNA product derived from the full length allele, while lanes A and C contain an additional 2000 bp product, the *LEU-GAL10pro* provided an additional 1000 bps, indicative of a positive *LEU-GAL10pro apc5^{M99}* insertion at one of the two *APC5* alleles.

(C) Serial spot dilutions of diploid isolates A and C and parental WT (YTH3) and *apc5^{CA}* (YTH457) haploid strains, and diploid *APC*/*apc5^{CA}* cells were grown to an OD_{600} of 0.5 and then 10-fold serially diluted and spotted onto YPD plates and incubated at 30ºC and 37ºC for 3 to 5 days. Based on the *apc5^{CA}* temperature sensitive phenotype, isolate A carries the *apc5^{M99}*/*apc5^{CA}* alleles and isolate C carries *APC5*/*apc5^{M99}* alleles.

(D) Sporulation of diploid isolates A (*apc5^{M99}*/*apc5^{CA}*) and C (*APC5*/*apc5^{M99}*). Colonies indicate viable haploid progeny. Sporulation was induced by plating diploids onto sporulation media (see section 3.1.4) for 5 to 7 days. Isolated tetrads were treaded with 0.5 mg/mL lyticase for 15 minutes and dissected into four haploid cells (left to right). The tetrads were dissected on YPD plates using a Singer MSM micromanipulator (Singer Instrument Co. Sumerset, England) and grown on YPD plates at room temperature for 3 days. Both isolates A and C have a 2:2 segragation with two viable and two nonviable progeny. Since the *apc5^{M99}* allele is under the control of the *GAL10* promoter, expression on glucose may be insufficient resulting in nonviable progeny. Therefore, tetrad analyses were performed on various concentrations of galactose and/or sucrose with identical 2:2 segregation results (data not shown). Data generated by Dr. Troy Harkness.

(E) Serial spot dilutions of parental diploid *APC*/*apc5^{CA}* and isolate A (*apc5^{M99}*/*apc5^{CA}*) and C (*APC*/*apc5^{M99}*) cells were grown to an OD_{600} of 0.5 and then 10-fold serially diluted and spotted onto YPD plates with various concentrations of galactose and/or sucrose, to induce increased expression of *LEU-GAL10pro-apc5^{M99}*, and incubated at 30ºC and 37ºC for 3 to 5 days. Increased expression of *apc5^{M99}* did not suppress *ts* phenotype.

5. DISCUSSION AND FUTURE WORK

5.1. Fob1 is a novel APC target

In a Y2H screen designed to discover potential targets of the APC, a Fob1/Apc5 interaction and a domain within Fob1 that specifically interacted with Apc5 was identified. The domain encompassed nucleotides 1259-1263, which encoded a motif that resembled a motif found in APC targets, the Destruction (D) Box. We refer to this motif as the L box (KIM and BONNI 2007; SULLIVAN and MORGAN 2007). Mutation to the Fob1 L box ($Fob1^{E420V}$) abolished accumulation of slower migrating species, indicative of poly-ubiquitination, when overexpressed in *rpn10Δ* cells (Figure 4.4). Predictably, the E420V mutation stabilized the Fob1 protein (Figures 4.5) and increased rDNA recombination frequency (Figure 4.6A). Finally, the fact that Fob1 protein levels fluctuate throughout the cell cycle (Figure 4.3), and require an intact APC5 (Figure 4.5) and the APC activator Cdh1 for removal (Figure 4.3C) is compelling evidence supporting our claim that Fob1 is targeted by the APC^{Cdh1} in G1.

We also identified two additional motifs in Fob1 that resembled D box sequences, DB1 and DB2 (Figure 4.4A). Since mutation to DB1 and DB2 did not significantly contribute to Fob1 stabilization (data not shown) or abolish the accumulation of slower migrating species (Figure 4.4C), it is unlikely that either motif alone is required for APC interaction. However, since the L Box mutation (E420V) did not completely abolish Fob1 turnover (Figure 4.5), it remains possible that the DB motifs play a contributory role when combined with the L Box.

5.1.1. Reduction of Fob1 reduces rDNA recombination and genomic instability

The rDNA region is a considerable source of genomic instability due to its repetitive structure (VADER *et al.,* 2011; KOBAYASHI 2011). The short lived $apc5^{CA}$ mutant has a broad range of genomic instability issues including chromatin assembly and histone metabolism defects (HARKNESS *et al.,* 2002; HARKNESS *et al.,* 2005; ARNASON *et al.,* 2005; TURNER *et al.,* 2010), increased rDNA recombination (Figure 4.2D), as well as increased plasmid loss (Figure 4.2E; HARKNESS *et al.,* 2002) which is an approximation of chromosome loss.

One function of Fob1 is to block and stall oncoming DNA replication forks within the rDNA region. The stalled replication machinery can result in DSBs and promote recombination (KOBAYASHI *et al.,* 1996; KOBAYASHI *et al.,* 1998). Homologous DNA repair of these DSBs can result in USCE due to the repeating structure of the rDNA. USCE can result in duplication or deletion of one or more rDNA tandem repeats (KOBAYASHI *et al.,* 1998; IDE *et al.,* 2010). This process may be beneficial in an evolutionary sense, by maintaining a high copy number of rDNA repeats and insuring a high degree of homology among tandem repeats, but the increased frequency of stalled replication forks and DSBs within the rDNA region generates detrimental genomic instability in individual cells (SINCLAIR *et al.,* 1997). The removal of Fob1 and the reduction of this process would be beneficial to the genomic stability of the cell, particularly in APC mutants. The deletion of *FOB1* significantly reduced rDNA recombination events and restored recombination frequency to WT levels in the *apc5*^{CA} background (Figure 4.2D). In contrast, the stabilized Fob1^{E420V}, which presumably has reduced interaction with the APC, increased rDNA recombination (Figure 4.6). This strongly supports our notion that the APC is required for the removal of Fob1 and that its removal is required for normal rDNA metabolism. However, the deletion of *FOB1* exclusively reduced rDNA recombination and did not affect the other broad ranging genomic stability defects of the *apc5*^{CA} mutant. This is reflected by the inability of the *FOB1* deletion to reduce plasmid loss in the *apc5*^{CA} background (Figure 4.2E). Our results presented here do expose one particular pathway in which the APC impinges on genomic stability and consequently longevity by reducing Fob1 levels and in turn reducing rDNA recombination.

5.1.2. Reduction of cohesin within the rDNA locus may alleviate *apc5*^{CA} defects.

The APCôs primary function is to promote a timely separation of sister chromatids during anaphase. Sister chromatids are held together by cohesin, a clamp-like protein, and held intact until all chromatids have been attached to mitotic spindles. The removal of cohesin by separase is what allows the separation of sister chromatids. The APC^{Cdc20} triggers the removal of cohesins by degrading securin, which in turn releases the cohesin cleaving separase. The efficient rapid switch-like removal of cohesins is achieved by several positive feedback loops involving the simultaneous degradation of cyclin-dependent kinases and release of the phosphatase Cdc14 from the nucleolus.

The observation that *apc5*^{CA} cells accumulate in G2 (Figure 4.2A and 4.2B), suggests that the robustness of this rapid switch-like removal of cohesins is compromised. This is likely due to delayed degradation of cyclins, observed in *apc5*^{CA} cells (Figure 4.2B; POSTNIKOFF *et al.,* 2012), which in turn affects the synchrony of the positive feedback mechanisms. Degradation of other APC substrates, such as Clb5 and possibly Dbf4, aid in the required removal of rDNA specific cohesin during chromosome segregation (SULLIVAN *et al.,* 2008). Failure to remove the rDNA specific cohesins will result in mitotic arrest or fatal unfaithful rDNA segregation (JOHZUKA and HORIUCHI 2009).

The primary function of Fob1 is to bind the RFB within the rDNA to recruit and anchor a variety of proteins within the nucleolus (KOBAYASHI and HORIUCHI 1996; KOBAYASHI *et al.,* 1998; JOHZUKA and HORIUCHI, 2002; HUANG *et al.,* 2006). Fob1 recruits Tof2, Lrs4, and Csm1 to the rDNA locus (see section 2.4.1.2; HUANG *et al.,* 2006). The interaction of Csm1 with cohesins (NEWMAN *et al.,* 2000; GRAUMANN *et al.,* 2004; MICHAELIS *et al.,* 1997), suggests that Fob1 mediates the association of cohesins with rDNA. Lrs4 and Csm1 are released from the nucleolus during anaphase (HUANG *et al.,* 2006), which likely weakens the interaction between the rDNA and cohesins, providing an opportunity to segregate chromosomes. It is conceivable that the APC is targeting Fob1 as early as anaphase to initiate release of Lrs4 and Csm1 from the nucleolus and aid in the segregation of the rDNA region during mitosis.

The deletion of *FOB1* in *apc5*^{CA} cells restored a WT cell cycle profile (Figures 4.2A and 4.2B). One possible explanation for this is that *apc5*^{CA} cells may have rDNA segregation defects due to slow or untimely cohesin cleavage. Deletion of *FOB1* may relieve these defects due to less rDNA specific cohesin being recruited to the rDNA locus resulting in less need for cohesin removal. This improved rDNA segregation during mitosis may contribute to the improved RLS in the *FOB1* deletion (Supplementary Figure 6.4C and 6.4D).

5.1.3. The APC may play an important role in maintaining rDNA silencing through degradation of Fob1

Two recent studies have linked the APC with rDNA silencing and segregation (SULLIVAN *et al.,* 2008; DUBEY *et al.,* 2009), suggesting that Fob1 degradation may play an important role in APC-dependent rDNA silencing. We have shown that deletion of *FOB1* suppresses $apc5^{CA}$ RLS, rDNA recombination, and cell cycle defects (Supplementary Figures 6.4C, 6.4D, Figures 4.2A, 4.2B and 4.2D), suggesting that Fob1 is not beneficial, at least not under conditions favorable to growth such as during a RLS assay. But consider, while Fob1 is bound to the RFB site it recruits the Sir2 containing RENT complex to rDNA (HUANG and MOAZED 2003). This mechanism can facilitate silencing of rDNA intrachromatid recombination; in contrast to the DNA replication fork blocking function of Fob1 (BAIRWA *et al.,* 2010). In addition, Tof2, Lrs4, and Csm1 recruit condensin to the RFB site in a Fob1-dependent manner. Condensin recruitment to the RFB sites is critical in facilitating higher order structure and rDNA integrity, conceivably by holding the tandem rDNA repeats in loops by an array of interacting condensins (JOHZUKA and HORIUCHI, 2009). This condensed, Fob1-dependent structure may reduce rDNA recombination and transcription, which may be particularly important during nutrient starvation and quiescence (TSANG *et al.,* 2007).

In consideration of the beneficial role Fob1 may have in establishing condensed transcriptionally silenced rDNA, it is possible that under conditions not favorable to growth the absence of Fob1 and Fob1-dependent recruitment of condensin could result in improper rDNA silencing, hyperactive rDNA transcription and recombination, and consequently improper energetic homeostasis. Such conditions are achieved during a chronological lifespan (CLS) assay, which is a measure of the cells mitotic competency or ability to reenter the cell cycle after quiescence. Under this premise, it is conceivable that the deletion of *FOB1* may diminish CLS, in contrast to the increased RLS. This possibility could be investigated further by future members of the Harkness Lab.

In summary, under optimum growth conditions, such as during an RLS assay, cells are continuously growing and reentering the cell cycle. Under these conditions, mechanisms that generate replication stress, such as Fob1-dependent replication fork blocks, DSBs, and perhaps rDNA cohesin retention, may have overpowering negative effects in comparison to the genomic stabilizing Fob1/condensin-dependent rDNA condensation and silencing, which is beneficial

predominantly during G1 and cellular quiescence. Conceivably, Fob1 dosage may be regulated by the APC as needed, to impinge on rDNA silencing, in response to cell cycle and environmental cues.

5.2. Cin5 regulation and future work

5.2.1. Identification of a phospho-inhibited degron motif within Cin5

Regulated degradation and phosphorylation are complimentary mechanisms that modulate the activity of proteins (HOLT 2012). A phosphodegron describes a protein where the phosphorylation of that protein initiates its ubiquitin-dependent degradation. Integrated mechanisms of phosphorylation and ubiquitination can provide robust control of a proteinôs activity, stability, and localization. These parameters are often not exclusive to one another, where localization may dictate stability or activity for example. There are a number of different motifs that link phosphorylation to protein stability. For example, an inhibitor of the Cyclin-Dependent Kinase (Cdk1), Sic1, is bound by Cdc4, an F-box subunit of the SCF, and targeted for degradation when a Cdk1 consensus motif is phosphorylated (FELDMAN *et al.,* 1997; SKOWYRA *et al.,* 1997). This is an example of a phosphodegron, where the phosphorylation of a protein initiates its ubiquitin-dependent degradation. In contrast, a phospho-inhibited degron are motifs that prevent ubiquitination when phosphorylated; for example, turnover of the microtubule-associated protein Tau, is inhibited by phosphorylation by GSK3ϐ (POPPEK *et al.,* 2006).

Our data do confirm that Cin5 is an unstable protein that is targeted for proteasome- and ubiquitin-dependent degradation and that Grr1 and Elc1 play a role in its degradation (Figures 4.7 and 4.8). Furthermore, Cin5 residues T192, K194 and S196 appear to form a phospho-inhibited degron motif, where the phosphorylation mimic T192E, S196E stabilized Cin5, and the phosphorylation knockout T192A, S196A destabilized the protein (Figure 4.10A). It is conceivable that phosphate groups on residues T192 and S196 may prevent the binding of a ubiquitin ligase such as Elc1 or the F-box Grr1, and the ubiquitination of K194. In addition, the 8 kDa mobility shift observed when *GRR1* is deleted in the Cin5 T192A, S196A (Figure 4.10B), hints at the possibility that the ligation of a single ubiquitin may be involved in Cin5 activity.

To further understand the dynamics of Cin5 regulation by phosphorylation and ubiquitination, future endeavors may wish to investigate additional lysine residues or potential ubiquitin ligase binding motifs. How our identified phospho-inhibited degron motif and other potential residues, or the aptamer Y65 impinge on Cin5 localization or DNA and protein complex binding are worthwhile inquiries.

5.2.2. Elc1 and Grr1 may form a yet uncharacterized E3 ubiquitin ligase complex

Elc1 and its orthologs have been identified as components of several E3 ubiquitin ligase complexes, including the von-Hippel Lindau tumor suppressor complexes, the Elc1-Rad7-Rad16 complex which shares several characteristic features of the SCF (Skp1-Cullin-F box)-type Ub ligases (HOCHSTRASSER 1996 and 1998; KREK, 1998), and an Elc1-Ela1-Cul3-Roc1/Rbx1 complex that mediates polymerase II ubiquitination (RIBAR *et al.,* 2007). In the Rad7-Rad16-Elc1 complex, Rad7 is an F-box protein, Rad16 is a RING protein, and Elc1 is a Skp1 homolog (CONNELLY and HIETER 1996; KREK, 1998). In the SCF complex, the Skp1 subunit acts as an adaptor which connects the F-box protein to the scaffold protein Cdc53/cullin, to which the RING finger protein Rbx1/Roc1 binds. The F-box protein binds the protein substrates targeted for ubiquitination by the Ub-conjugating enzyme (E2) which assembles with the Cdc53/cullin subunit.

Our data suggests that Cin5 may be the target of a ubiquitin ligase complex that includes Elc1. This would be consistent with the *elc1*Δ stabilizing Cin5 (Figure 4.8C). Since *grr1*Δ also stabilized Cin5 (Figure 4.8C), there is perhaps an uncharacterized complex that includes both Elc1, acting as the Skp1 adaptor and Grr1 as an F-box. This would be consistent with our hypothesis that the aptamer Y65, which has homology to Elc1, potentially displacing Elc1 from, or prevents Cin5 binding to, such a complex which in turn reduces Cin5 degradation. To further test this hypothesis, comprehensive CoIPs of Elc1, Grr1 and Cin5 may confirm the presence of such a Ub ligase complex.

However, there is data in the literature which suggests that Elc1 binding does not support protein degradation via a Ub ligase complex but rather does the opposite and stabilizes stress response proteins (HYMAN *et al.,* 2002). Here the authors propose Elc1 acts as the SCF activator Skp1 but

with a proposed model where Skp1 confers stability to the F-box protein, thereby regulating the abundance of Skp1 interacting proteins (MATHIAS *et al.,* 1999). This would be consistent with our hypothesis that Y65 may mask a degradation signal by binding to Cin5. But then again, this is not consistent with *elc1*Δ stabilizing Cin5 (Figure 4.8C). Identifying and analyzing Cin5/Elc1 interaction motifs, as we did for Fob1/APC, may shed light on the precise nature of this interaction. It appears that the function of Elc1 and the impact it has on its binding partners may be multifaceted and dynamic.

5.2.3. Possible mechanism for Y65/Cin5 ability to suppress *apc5*^{CA} *ts* defects

Our data suggest that the APC is not directly involved in Cin5 degradation (Figures 4.9). It is unlikely that Cin5, Elc1 or Y65 have any direct involvement in the function of the APC or the Apc5 subunit. However, Elc1 and Y65 do physically interact with Cin5 in a Y2H (JACKSON *et al.,* 2000, see section 2.3.7), and the deletion of *ELC1* stabilizes Cin5 (Figure 4.8C). Most likely, the ability of Y65 to suppress the *apc5*^{CA} *ts* defects are indirect, and due to the increased stress tolerance elicited as a result of the stabilization of the stress response transcription factor Cin5.

We have done preliminary experiments to examine this possibility, where we overexpressed the aptamer Y65 and observed Cin5 stability (Data not shown; Stahl and Harkness, unpublished). These data do suggest that Y65 stabilizes Cin5, but unfortunately more work will be required to complete this line of inquiry. Similarly, the effect increased levels of Elc1 has on Cin5 can be investigated. Moreover, the more stable Cin5 T192E, S196E could be cloned into *apc5*^{CA} and tested for temperature sensitivity and other stress tolerance. Such experiments would provide compelling evidence that increased stress tolerance elicited as a result of stabilized Cin5 is Y65ôs mode of action.

5.3. Conclusions

The primary objective of this study was to identify molecular networks that intersect with the APC and characterize how these networks impinge on longevity and stress response. The precise range of APC substrates and their often subtle influence on cellular function remain largely unknown.

To address this shortcoming, the Y2H and aptamer screens were used, prior to this study, to identify the proteins Fob1 and Cin5, respectively, as potential APC targets, regulators and/or modifiers.

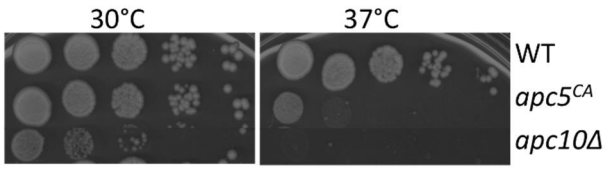
In this thesis, we were able to confirm that Fob1 is targeted by the APC^{Cdh1} for ubiquitin and proteasome-dependent degradation in G1 of the cell cycle, and that the efficient turnover requires an intact L-box motif within Fob1. Furthermore, the APC-dependent removal of Fob1 is required for a normal rDNA metabolism and RLS. In addition, we were able to further characterize the regulation of Cin5. We identified a phospho-inhibited degron motif within Cin5, which includes the aa residues T192, K194 and S196. We have data that suggest that the APC may indirectly influence Cin5 stability through other ubiquitin ligases such as the SCF, at least under specific cell cycle or stress conditions. In addition, Cin5 is stabilized in the absence of Elc1 and Grr1 which suggests these two proteins may contribute to its degradation.

In summary, this research demonstrates the feasibility of using the Y2H and aptamer screens to identify and characterize molecular networks that interplay with the APC. Further understanding of the role the APC plays in cell cycle progression, chromatin assembly, genomic stability, stress response and longevity will be valuable to fundamental biological science, and may also have applications in health science and medicine.

6. SUPPLEMENTARY FIGURES

This thesis was a continuation of work by Dr. Troy Harkness, former student Jing Chen, and former laboratory technician Chelsea Stahl. These supplementary figures are included here to complement and provide background to the data presented in this thesis. Figures 6.2, 6.4, 6.5 were previously published in Jing Chen MSc. thesis: An anaylsis of APC5p/FOB1 Interaction in Yeast: Implications for Extended Lifespan, MSc Thesis (2006). Figures 6.1 and 6.3 include unpublished data generated by Dr. Troy Harkness and lab technician Chelsea Stahl, and data from a peer reviewed publication HARKNESS *et al.,* 2002.

A



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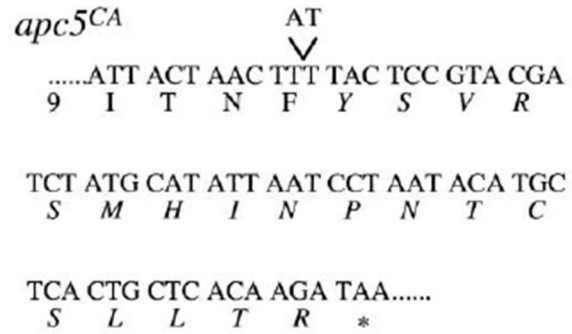


C

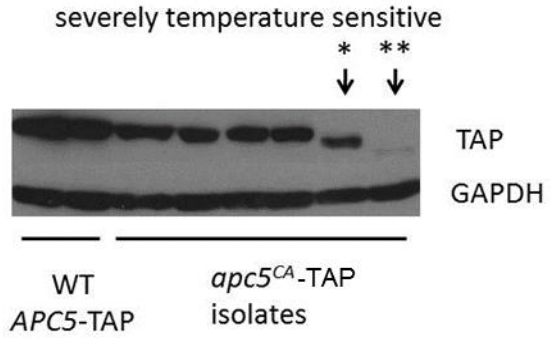


Figure 6.1. The *apc5*^{CA} allele is *ts* and appears to be N-terminally truncated.

(A) WT, *apc5*^{CA} and *apc10Δ* cells were grown to an OD_{600} of 0.5 and then 10-fold serially diluted and spotted onto YPD plates and incubated at 30ºC and 37ºC for 3 days (data from HARKNESS *et al.,* 2002).
(B) Schematic of the *apc5*^{CA} allele illustrating the AT deletion and resulting non-specific amino acids and premature stop codon (data from HARKNESS *et al.,* 2002).
(C) Western of *APC5* and *apc5*^{CA} cell transforments tagged with C-terminal TAP epitope on the endogenous APC5 locus. Protein extracts were separated on 10% acrylamide gels transferred to nitrocellulose membrane and probed with anti-TAP rabbit and anti-GAPDH mouse primary antibodies followed by anti-rabbit or anti-mouse HRP secondary antibodies. GAPDH is a protein load control. The isolates in the last two lanes appear to be truncated and are mildly *ts* (*) and severely *ts* (**). The *apc5*^{CA} allele frequently used in our lab is the severely *ts* isolate in the last lane (STAHL, DYKES, and HARKNESS, unpublished).

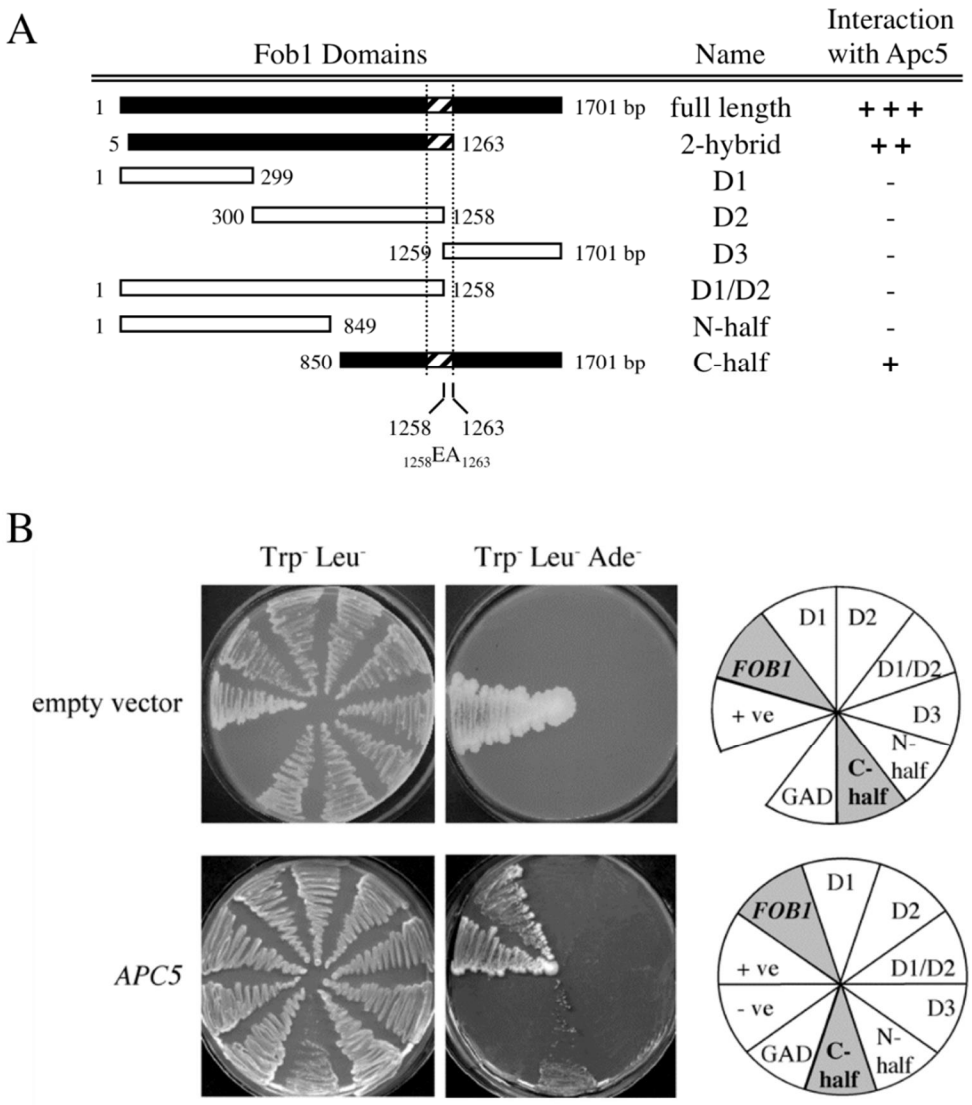


Figure 6.2. Apc5 interacts with Fob1 in a Yeast 2-hybrid assay

(A) A schematic of the *FOB1* fragments cloned into pGAD-C2 to identify a Fob1 binding motif responsible for Apc5 interactions. +++ denotes best growth, + denotes weak growth and ï indicates no growth. The hatched area denotes a putative Apc5 binding domain.
(B) Growth analysis of *FOB1* derivatives and full length *APC5* in a 2-hybrid assay. Top panels show the *FOB1* derivatives (cloned into pGAD-C2) paired with pGBT9 empty vector. The bottom panel shows the *FOB1* derivatives paired with full length *APC5* cloned into pGBT9. The cells were grown on SD ï trp ï leu to select for plasmids and on SD ï trp ï leu ï ade to select for interactions. The positive control plasmids encode the SV40 large T-antigen (pTD1) and p53 (pVD3). Data generated by Jing Chen, (2006) An anaylsis of APC5p/FOB1 Interaction in Yeast: Implications for Extended Lifespan, MSc Thesis

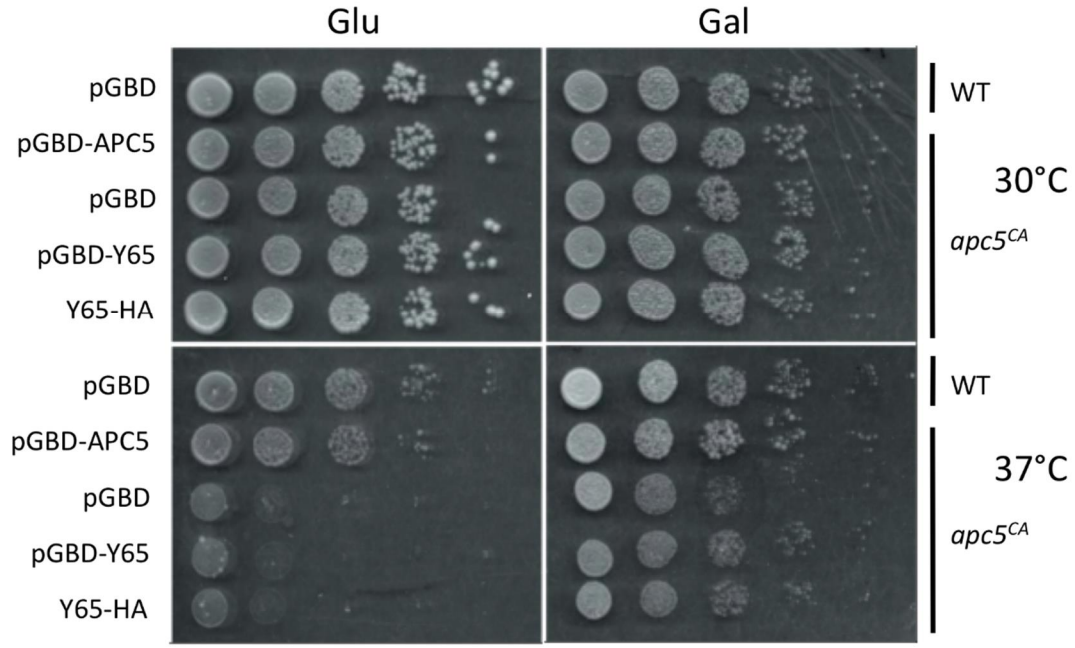


Figure 6.3. The aptamer Y65 suppresses the *apc5*^{CA} *ts* phenotype

YTH WT cells transformed with the pGBD plasmid, and YTH *apc5*^{CA} cells transformed with plasmids pGBD-APC5, pGBD, pGBD-Y65, and Y65-HA were grown to an OD_{600} of 0.5 and then 10-fold serially diluted and spotted onto Trp- plates with 2% Glu or 2% Gal to induce expression of the vector and incubated at 30ºC and 37ºC for 3 to 5 days. The plasmids pGBD are Y2H plasmids whereas the Y65-HA plasmid expresses an HA epitope. Data generated by Chelsea Stahl.

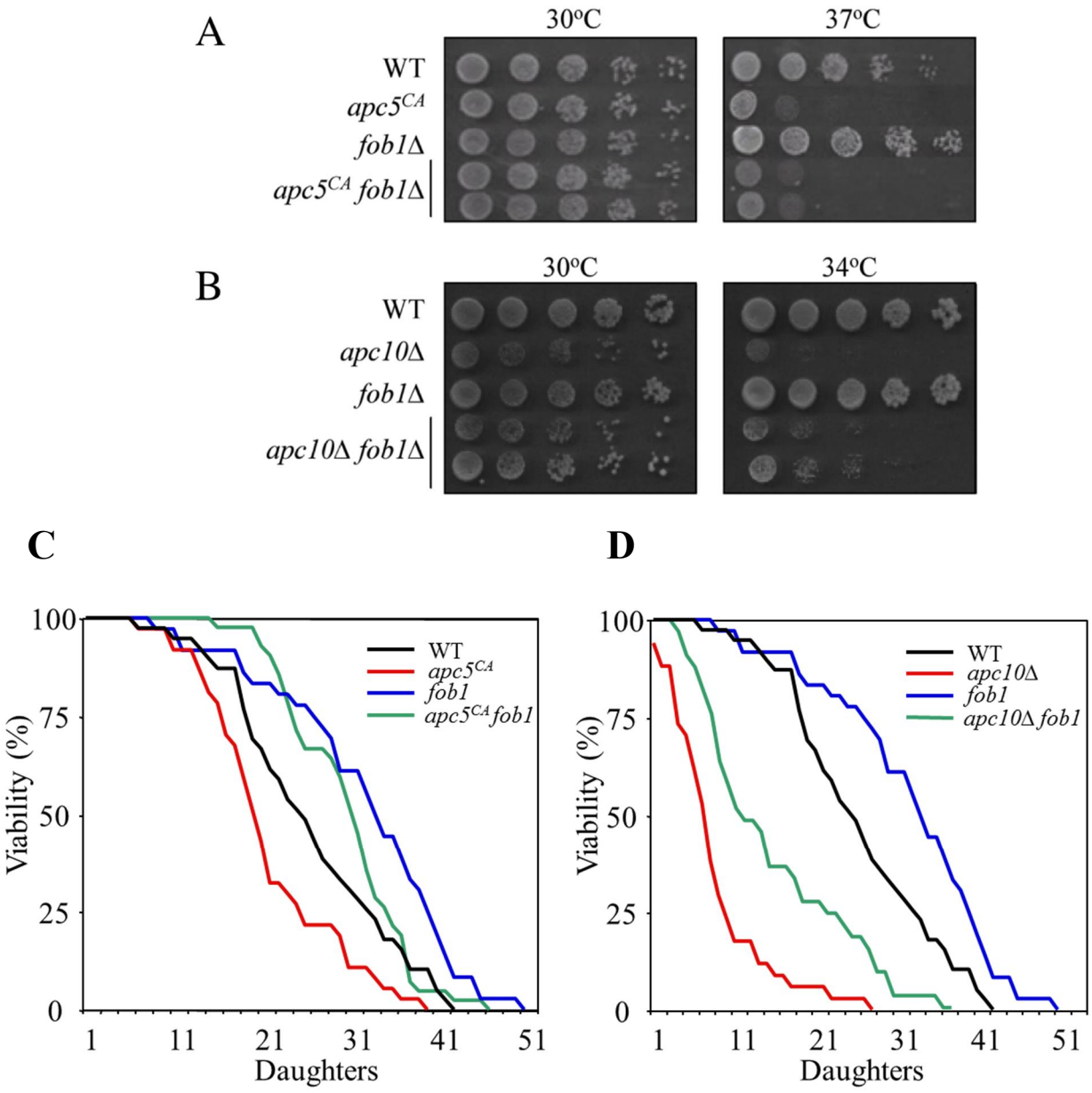


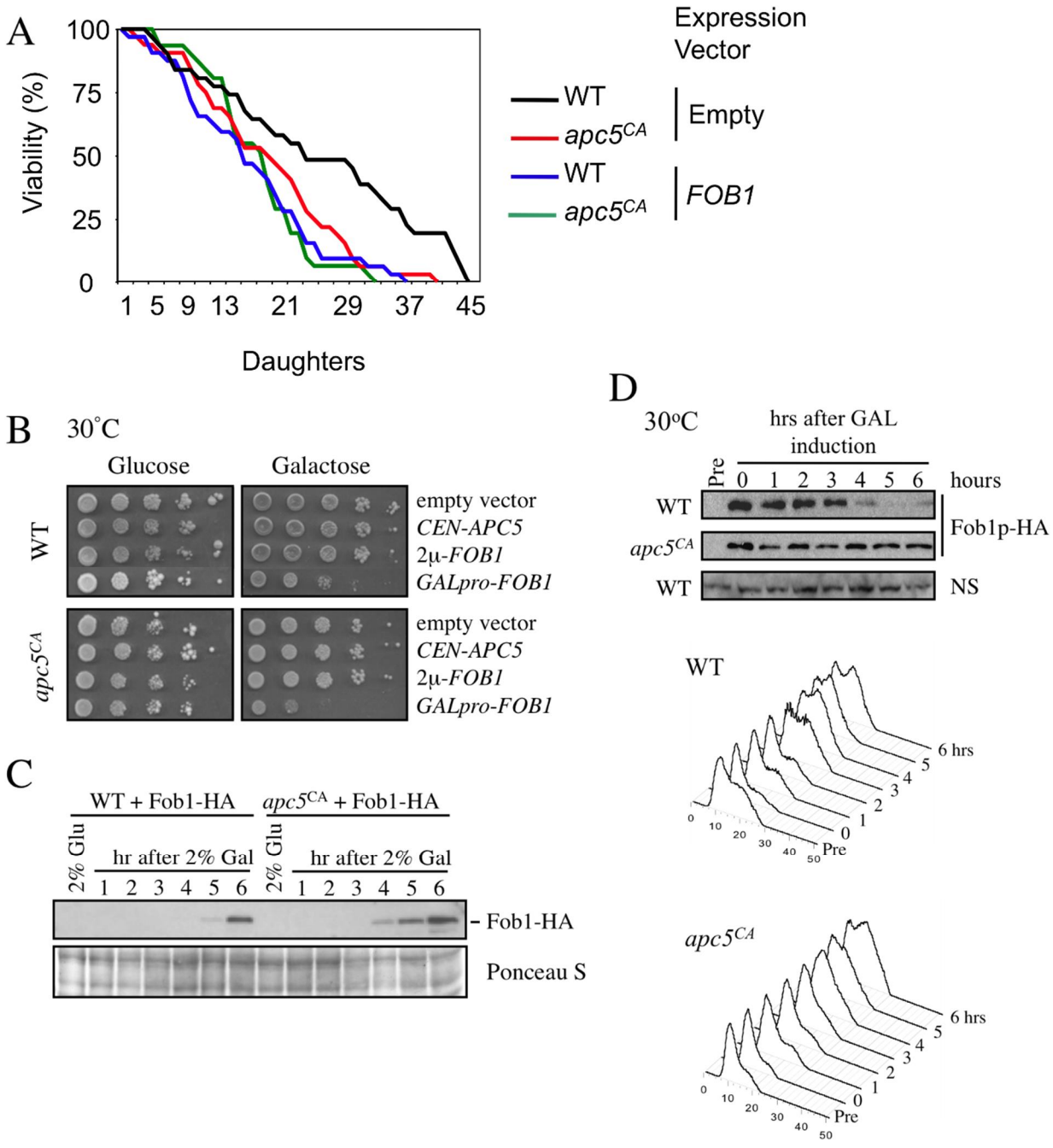
Figure 6.4. Deletion of *FOB1* in APC mutants has little impact on growth, but a positive influence of APC mutant replicative lifespan

(A) WT, *apc5*^{CA}, *fob1Δ,* and *apc5*^{CA} *fob1Δ* cells were grown to an OD_{600} of 0.5 and then 10-fold serially diluted and spotted onto YPD plates and incubated at 30ºC and 37ºC for 3 days.

(B) WT, *apc10Δ, fob1Δ* and *apc10Δ fob1Δ* cells were treated as above.

(C) Replicative lifespan for WT, *apc5*^{CA}, *fob1Δ,* and *apc5*^{CA} *fob1Δ* cells. The number of mother cells used for RLS determination were 39, 37, 36 and 42, respectively.

(D) Replicative lifespan for WT, *apc10Δ, fob1Δ* and *apc10Δ fob1Δ* cells. The number of mother cells used for RLS determination were 39, 34, 36 and 34, respectively. Data generated by Jing Chen, Chen, 2006 M.Sc. Thesis.


A
100
75
50
25
0
Viability (%)
1 5 9 13 21 29 37 45
Daughters
Expression Vector
WT
apc5CA
WT
apc5CA
Empty
FOB1
B
30˚C
Glucose
Galactose
WT
apc5CA
empty vector
CEN-APC5
2μ-FOB1
GALpro-FOB1
empty vector
CEN-APC5
2μ-FOB1
GALpro-FOB1
C
WT + Fob1-HA
apc5CA + Fob1-HA
2% Glu
hr after 2% Gal
1 2 3 4 5 6
2% Glu
hr after 2% Gal
1 2 3 4 5 6
Fob1-HA
Ponceau S
D
30ºC
hrs after GAL induction
Pre
0 1 2 3 4 5 6
hours
WT
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WT
Fob1p-HA
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0 10 20 30 40 50
apc5CA
6 hrs
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4
3
2
1
0
Pre
0 10 20 30 40 50

Figure 6.5. Increased *FOB1* expression reduces replicative lifespan while overexpression is toxic

(A) RLS determination for WT and *apc5*^{CA} cells harboring 2u-*FOB1* or YCp50 empty vector. The number of mother cells used for WT + YCp50, WT + 2u-*FOB1*, *apc5* ^{CA} + YCp50, and *apc5*^{CA} + 2u-*FOB1* were 31, 32, 32 and 31 respectively. Data generated by Dr. Troy Harkness

(B) WT and *apc5*^{CA} cells harboring YCp50 empty vector, *CEN-APC5*, 2u-*FOB1* or *GAL*$_{pro}$-*FOB1-HA* were serially diluted and spotted onto 2% glucose or 2% galactose-supplemented SD media. Plates were incubated at 30ºC, 3 days for glucose and 5 days for galactose plates.

(C) WT and *apc5*^{CA} cells expressing *GAL*$_{pro}$-*FOB1-HA* were grown to early log phase, then induced with 2% galactose. Samples were taken every hour during the induction period and analyzed by Western blotting with antibodies against HA. The Ponceau S stained blot is shown for load control.

(D) The cells used above were released into fresh 2% glucose media after the 6 hour galactose induction and allowed to re-enter the cell cycle. Every hour for 6 hours samples were removed for FACS and Western analyses. FACS data is a measure of cellular DNA content where the first peak represent cells in G1 and the second peak G2. Chen, 2006 M.Sc. Thesis

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