An analysis of Apc5p/Fob1p interactions in yeast: implications for extended lifespan

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By

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ABSTRACT

Aging is a universal biological phenomenon in all living cells. Questions regarding how cells age are beginning to be answered. Thus, great biological interest and practical importance leading to interventions rest on uncovering the molecular mechanism of aging. This would ultimately delay the aging process while maintaining the physical and mental strengths of youth. The conservation of metabolic and signaling pathways between yeast and humans is remarkably high, leading to the expectation that aging mechanisms are also common across evolutionary bounaries. By utilizing the budding yeast, *Saccharomyces cerevisiae*, one of the best characterized model systems for studying aging, the span in knowledge between yeast and human aging can possibly be bridged.

Evidence is accumulating that a genetic program exists for lifespan determination. Model organisms expressing mutations in single specific genes live longer with increased resistance to stress and cancer development. Mutations that accelerate aging in yeast affect the activity of the APC (Anaphase-Promoting Complex). Our finding that the APC is critical for longevity provides us with a potential central mechanism controlling lifespan determination. The APC is required for mitotic progression and genomic stability in presumably all eukaryotes by targeting regulatory proteins, such as cyclin B (Clb2p in yeast) for degradation. The key feature defining the APC as a central mediator of lifespan is the fact that multiple signaling pathways

regulate APC activity and many of these pathways influence lifespan. For example, Snf1 and PKA have antagonistic effects on the APC and on lifespan. Thus, it is intriguing to speculate that the APC may link these signaling pathways to downstream targets controlling longevity.

Our hypothesis states that the APC targets a protein that reduces lifespan for ubiquitin-dependent degradation. The results from our two-hybrid screen utilizing Apc5p as bait are consistent with this hypothesis, as Fob1p was isolated as an Apc5p binding partner. The *FOB1* gene is located on chromosome IV and the well-known molecular function of *FOB1* is the creation of a unidirectional block in replication of rDNA. Fob1p binds to the rDNA locus and overall stalls progression of the replication fork, which increases rDNA recombination and the production of toxic extrachromosomal rDNA circles (ERCs). The *FOB1* deletion (*fob1*Δ) mutant confers reduced rDNA recombination, and an increased lifespan of more than 50% compared to WT (wild type) cells.

In this study, we expanded on the molecular mechanisms controlling lifespan through a genetic approach, and found that Fob1p was targeted by the APC for degradation in order to prolong lifespan. By utilizing the yeast two-hybrid approach, we confirmed the Apc5p-Fob1p interaction, and determined that the C-terminal half of Fob1p was required for the interaction with Apc5p. BLAST search analysis revealed sequence similarity with the Fob1p C-terminus that was shared with many other proteins

from yeast to humans. We speculate that this shared domain may serve as an APC interaction domain employed across evolutionary boundaries. A genetic interaction analysis revealed the influence of *FOB1* on the APC, and the cell. For example, deletion of *FOB1* increased lifespan in $apc5^{CA}$ and *apc10*Δ mutant cells and partially suppressed the temperature sensitive (*ts*) growth of *apc10*Δ cells. On the other hand, increased *FOB1* expression reduced the lifespan of WT and cells and was toxic to *apc* mutants, particularly the more severe *apc* mutants, *apc10*Δ and *cdc16-1*. Interestingly, overexpression of *SIR2*, which prolongs lifespan and acts antagonistically with Fob1p, was toxic to WT cells, but suppressed $apc5^{CA}$ *ts* defects, especially when *FOB1* was deleted. These observations suggest that accumulation of Fob1p is harmful to yeast cells, especially when the APC is compromised. This notion was borne out when a cell cycle and steady state analysis of Fob1p revealed that Fob1p was an unstable protein, which was stabilized in $apc5^{CA}$ cells. Taken together, the work presented in this thesis supports a model whereby Fob1p is targeted for degradation by the APC in order to prolong lifespan in yeast. In conclusion, the extreme evolutionarily conserved nature of the APC and the Fob1p C-terminal sequence homology observed in human proteins strongly suggests that the mechanism discovered here could be directing human lifespan.

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TO MY FAMILY

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LIST OF ABBREVIATIONS

Δ	defines gene deletion
aa	amino acid
ade	adenine
Amp	ampicilin
APC	Anaphase-Promoting Complex
APS	ammonium persulphate
CA	chromatin assembly
CIP	calf intestinal alkaline phosphatase
CR	caloric restriction
CRL	Cullin-RING Ligase
ddH_2O	double distilled water
DSB	double-stranded DNA break
dsRNA	double-stranded RNA
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ERC	extrachromsome rDNA circle
EtBr	ethidium bromide
FACS	Fluorescence Activated Cell Sorting
GNAT	Gcn5-related N-acetyltransferase

HAT	histone acetyltransferase
HDAC	histone deacetylases
IRESs	internal ribosome entry sites
Kb	kilobase pair
kDa	kilodalton
Leu	leucine
NAD	nicotinamide adenine dinucleotide
OD	optical density
PABP	poly (A) binding protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	polyethylene glycol
RFB	replication fork barrier
RNR	ribonucleotide reductase
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SCF	Skp1/Culin/F-box protein
SD	synthetic dextrose
SDS	sodium dodecyl sulfate

SIR	silent information regulator
TCA	trichloroacetic acid
TEMED	N, N, N', N'-Tetramethylethylenediamine
TPR	tetratrico peptide repeat
Trp	tryptophan
ts	temperature sensitive
Ub	ubiquitin
Ura	uracil
WT	wild type
YPD	yeast extract/peptone/dextrose

CHAPTER ONE: INTRODUCTION AND BACKGROUND

1.1 Chromatin silencing and lifespan in yeast

1.1.1 Chromatin silencing

Chromatin is a complex molecular structure composed of nucleosome repeats in which 147 base pairs of DNA are wrapped around two copies of the four core histones, H2A, H2B, H3 and H4 (LUGER *et al*., 1997; RICHMOND and DAVEY, 2003). The critical components within chromatin that are central to the control of many cellular processes are histones. Chromatin-associated histones are post-translationally modified by a variety of activities and these modifications, including histone acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and ADP-ribosylation (reviewed in EMRE and BERGER, 2006) are responsible for the control of a vast number of cellular activities (Van LEEUWEN and GOTTSCHLING, 2002b; FISCHLE *et al*., 2003). Deacetylation of lysine residues within histone N-terminal tails is believed to play a large role in gene silencing by creating conformational changes within chromatin that renders it resistant to transcription factors (GRUNSTEIN, 1997; EBERHARTER and BECKER, 2002). Silencing of the yeast rDNA locus, in particular, represses rRNA gene repeats (rDNA) recombination, reducing the formation and release of extrachromosomal rDNA circles (ERCs) (KAEBERLEIN *et al*., 1999; KIM *et al*., 1999; PARK *et al*., 1999; LIN *et al*., 2002; ROY and RUNGE, 2000; DEFOSSEZ *et al*.,

2001). As mother cells continue to produce daughter cells they accumulate ERCs, which are not passed onto the daughters (McMURRY and GOTTSCHLING, 2003).

1.1.2 ERCs, a cause of aging in yeast

The accumulation of ERCs in mother cells has been proposed to be a key factor in yeast aging (KENNEDY *et al.*, 1994; SINCLAIR *et al.*, 1997; DEFOSSEZ *et al.*, 2001). In budding yeast, mother cells divide asymmetrically, giving rise to a newly made daughter cell that is smaller than the aging mother cell (KENNEDY *et al.*, 1994). The mother cell adopts phenotypes of aging, including an enlarged size and sterility, and senesces after approximately 20 divisions (BITTERMAN *et al.,* 2002). As mentioned above, aging mother cells accumulate ERCs, but do not pass them to daughter cells until later stage of life (McMURRAY and GOTTSCHLING, 2003). Mutations that slow the generation of these circles, such as that observed in *FOB1* mutants, extend yeast lifespan (KOBAYASHI *et al.,* 1998; DEFOSSEZ *et al.,* 1999). However, mutations to the RAD52 epistasis group (*rad50*Δ*, rad51*Δ*, rad52*Δ; Δ denotes a complete deletion; PARK *et al.,* 1999) that reduce the formation of ERCs surprisingly shorten the lifespan of the mother cells. These RAD52 class mutants, which are defective in DNA repair through homologous recombination, likely, accelerate aging due to spontaneous DNA damage and unrepaired double-strand breaks (DSB; RICHARDSON *et al.,* 2004; LOEILLET *et al.,* 2005). Nevertheless, rDNA instability has not been observed in other organisms, and is evidently an idiosyncratic feature of yeast aging.

1.1.3 Caloric restriction (CR) and aging

Repression of ERC formation is believed to be at the heart of enhanced replicative lifespan afforded by caloric restriction (CR) (LIN *et al.,* 2002). Replicative lifespan is a measure of how many daughter cells a given mother cell will produce (KENNEDY *et al.*, 1994). CR is an experimental method of reducing caloric intake and is correlated with extended lifespan, increased stress resistance and postponement of cancer in organisms ranging from yeast to mammals (reviewed in LONGO and FINCH, 2003; KOUBOVA and GUARENTE, 2003). It has been known for almost 70 years that restricting the food intake of laboratory rats extends their mean and maximum lifespan (McCAY *et al.,* 1935; reviewed in MASORO, 2005). Such lifespan extension has been observed over the years in many other species, including mice, hamsters, dogs, fish, invertebrate animals, and yeast (MASORO, 2002). As well as increasing longevity, CR is reported to cause additional phenotypes, including increased resistance to oxidative stress (ARMENI *et al.*, 1998; MERRY, 2004; DE CABO *et al.,* 2004), enhanced DNA damage repair (GUO *et al.,* 1998; RAO, 2003), decreased levels of oxidatively damaged proteins (YOUNGMAN *et al., 1992;* YU, 1996; SOHOL and WEINDRUCH, 1996), improved glucose homeostasis and insulin sensitivity (MASORO *et al.,* 1989; 1992; KEMNITZ *et al.,* 1994), altered levels of apoptosis (ZHANG and HERMAN, 2002), and delayed onset of a number of age-related diseases (WEINDRUCH and WALFORD, 1988; MATTSON *et al.,* 2002; SAFFREY, 2004; JOLLY, 2005). CR delays the onset and/or slows the progression of most age-associated diseases, including neoplastic diseases, degenerative diseases, and immune diseases (MAEDA *et al.*, 1985;

BRONSON and LIPMAN, 1991; ROE *et al.,* 1995). This action has been viewed as evidence that CR extends lifespan by slowing and/or delaying the aging processes.

In mammalian cells (NEMOTO *et al.*, 2004) and also budding yeast, CR extends lifespan, in part, by increasing the activity of Sir2 (silent information regulator) (KAEBERLEIN *et al.,* 1999; ALBERTO *et al.*, 2003), a member of the conserved sirtuin family of NAD^+-dependent protein deacetylases. These enzymes catalyse a unique reaction in which NAD^+ and acetylated substrate are converted into deacetylated product, nicotinamide, and a novel metabolite O-acetyl ADP-ribose (TANNER *et al.,* 2000; SAUVE *et al.,* 2001; TANNY and MOAZED, 2001; JACKSON and DENU, 2002). In addition, a functional *PNC1* gene is also required for CR-induced replicative life extension. The *PNC1* gene encodes a protein with nicotinamidase activity and CR acts to increase the amount of this enzyme (GHISLAIN *et al.,* 2002; ANDERSON *et al.,* 2003; GALLO *et al.,* 2004). The deacetylase activity of the yeast Sir2p at the rDNA locus involves the generation of nicotinamide, which is an inhibitor of Sir2p deacetylase activity *SIR2* protein. By reducing the level of nicotinamide, Pnc1p increases Sir2p deacetylase activity, and it is this deacetylase activity and subsequent silencing of the rDNA locus that plays a key role in the CR-induced increase in the replicative lifespan of yeast cells (ANDERSON *et al.,* 2003; GALLO *et al.,* 2004).

1.1.4 Chromatin regulation

The role of chromatin as a dynamic and active participant in multiple nuclear

processes was first recognized by its ability to regulate gene expression in eukaryotic cells (reviewed in JENUWEIN and ALLIS, 2001). One way of modulating chromatin structure is by post-translational modification of the histones present in the minimal chromatin unit, the nucleosome. Potential post-translational modifications on the histones include histone acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and ADP-ribosylation (reviewed in EMRE and BERGER, 2006). Most modifications were originally observed on the N-terminal tails of histones, with the exception of ubiquitylation, which occurs on the C-terminal tails of H2A and H2B. The acetylation and deacetylation of histones in nucleosomes play an important role in regulating gene expression and chromatin modification and lead to the "Histone Code Theory" (reviewed in JENUWEIN and ALLIS, 2001).

1.1.4.1 Histone acetyltransferases (HATs)

Histone acetylation has been proposed to play a dual role in the cell. Histone acetylation affects the amino-terminal tails of the histones, where lysine residues can be post-translationally acetylated. Acetylation (with Acetyl-CoA as a donor coenzyme) is carried out by histone acetyltransferases (HATs). First, the covalent addition of acetyl groups to specific lysine residues neutralizes the positive charge of the histone tail, which weakens histone–DNA contacts within the nucleosome and/or histone–histone contacts involved in higher-order chromatin structure. Acetylation of histones also provides an epigenetic marker for gene expression because it blocks association of heterochromatin-stabilizing complexes like *SIR* and it can be recognized by protein

domains, such as bromodomains, present in various components of the transcription machinery (ANNUNZIATO and HANSEN, 2000; FISCHLE *et al.,* 2003). Thus, post-translational modifications of histones do not just change chromatin structure directly; they also modulate interaction of specific proteins with chromatin (JENUWEIN and ALLIS, 2001; NARLIKAR *et al.,* 2002; FISCHLE *et al.,* 2003; VAQUERO *et al.*, 2003). HATs can be grouped into four families in yeast, based on homology:

i. The Gcn5 family members, GNAT (Gcn5-related N-acetyltransferase) which include yeast Gcn5, and human Gcn5/PCAF. Gcn5p is the catalytic subunit of the SAGA transcriptional activation complex (STERNER and BERGER, 2000; ROTH *et al.,* 2001; CARROZZA *et al.,* 2003).

ii. The MYST family of HATs is named after the founding members MOZ, Ybf2/Sas3p, Sas2p and Tip60. Sas3p is part of the NuA3 complex, and the MYST HAT Esa1p, the only essential HAT in yeast, is part of the NuA4 complex (DOYON and COTE, 2004). Apart from their role as specific transcription co-activators, MYST proteins are involved in a wide variety of cell functions such as gene silencing in yeast, dosage compensation in Drosophila and oncogenic transformation leading to specific human diseases such as leukemia (CARROZZA *et al.,* 2003; UTLEY and CÔTÉ, 2003).

iii. The CBP/p300 family, coactivators of different classes of transcription factors, including the tumor suppressor protein p53, act as bridging proteins between inducible transcription factors and the basal transcription apparatus, and as

integrators of diverse signaling pathways. Coactivators of nuclear receptors and associated proteins forming a multicomponent complex have an intrinsic histone acetylase activity in contrast to nuclear receptor and heterodimer Mad-Max corepressors, which recruit histone deacetylase (SHIKAMA and LATHANGUE, 1997; BORGER and DECAPRIO, 2006).

iv. The general transcription factor HATs include the TFIID subunit TAF250, TFIIIC, a general transcription factor in the RNA polymerase III basal machinery, and Nut1p in yeast, a component of the Mediator complex (TABTIANG and HERSKOWITZ, 1998).

1.1.4.2 Histone deacetylases (HDACs)

Histone acetylation is a reversible process and, accordingly, histone deacetylases (HDACs) have been isolated that catalyze this reaction (Table 1.1; PETERSON, 2002). Sir2p, the founding member of the "Sirtuin" family, is a Class III HDAC, which is structurally unrelated to the other two families and has the unusual property of requiring NAD^+ as a cofactor in the deacetylation reaction. HDAC families also include the HDAC I class that resemble yeast Rpd3p, and the Class II HDACs that are similar to yeast Hda1p. The yeast HDACs Hos1p and Hos2p are more similar to Rpd3p, while Hos3p is more closely related to Hda1p. Sir2p, Rpd3 and Hda1p are found to be involved in the extension of the yeast lifespan of mother cells (GUARENTE and KENYON, 2000). Deletion of *SIR2* accelerates the aging process (KAEBERLEIN *et al.,* 1999; TISSENBAUM and GUARENTE, 2001), while *RPD3* knock out increases

Table 1.1: Deacetylase activities in yeast HDACs

Histone deacetylases	**Type of HDACs**
Rpd3p	Class I
Hos2p	Class I
Hos1p	Class II
Hos3p	Class II
Hda1p	Class II
Sir2p	Class III
Hst1p	Class III
Hst2p	Class III
Hst3p	Class III
Hst4p	Class III

in yeast by increasing rDNA silencing (KIM *et al.,* 1999a; CHANG and MIN, 2002). Also, it was found that deletion of both HDA1 and SIR3 showed increased rDNA silencing and extended lifespan (CHANG and MIN, 2002).

1.1.4.2.1 SIR2, a Nicotinamide Adenine Dinucleotide (NAD)-dependent histone deacetylase, promotes longevity in yeast

Members of the evolutionarily conserved Sir2 family include five homologues in yeast (Sir2 and Hst1–4) and seven in humans (SIRT1–7) (FRYE, 1999; 2000), with key roles in cellular processes such as gene expression, apoptosis, metabolism and aging. The Sir2 protein (Sir2p) is of particular interest, as it has been recently linked with coordination of aging mechanisms in yeast, the nematode (*Caenorhabditis elegans*) and mammals (KEABERLEIN and GUARENTE, 1999; FRYE, 1999; 2000; TISSENBAUM and GUARENTE, 2001; CHUA *et al.,* 2005).

Sir2p was first discovered in a screen for regulators of transcription at the silent mating-type loci (MILLER and NASMYTH, 1984; reviewed in GASSER and COCKELL, 2001). Repression at these loci is important for formation of wild type haploid yeast that can mate normally. Sir2p (along with other proteins including Sir1p, Sir3p, Sir4p, and Rap1p) also binds yeast telomeres and helps to repress transcription by deacetylating the N-terminal tails of histones H3 and H4, which help package DNA in the cell (MORETTI *et al.,* 1994; HECHT *et al.,* 1995; BRAUNSTEIN *et al.,* 1996). The removal of acetyl groups from the histone tails causes them to become positively charged, and this is believed to cause a stronger interaction with negatively charged

DNA molecules (FINNIN *et al.,* 1999; BUGGY *et al.,* 2000). Tightly packed DNA/histone complexes contribute to tighter packing of chromatin in this region, which causes transcriptional repression (STRAHL and ALLIS, 2000; WADE, 2001; YOSHIDA *et al.,* 2001). In addition, Sir2p functions in double-stranded DNA break repair and suppression of mitotic recombination in rDNA (RINE *et al.,* 1987; ALBERTO *et al.,* 2003). Therefore, The SIR genes serve several functions, which are crucial for the establishment of 'silent' heterochromatin at telomeres and mating-type (*HM*) loci.

Sir2p is one of the critical components functioning at the rDNA locus (RINE and HERSKOWITZ, 1987). Sir2p was shown to inhibit rDNA recombination, which reduced the generation of ERCs (PETES and BOTSTEIN, 1977; PHILIPPSEN *et al.*, 1978; RUSTSHENKO and SHERMAN, 1994). These ERCs are able to replicate via ARS (Autonomous Replication Sequence) contained within the rDNA repeat, and are preferentially segregated to mother cells during division (Fig. 1.1). The unidirectional replication fork barrier (RFB) in the rDNA is required for the formation of the majority of these ERCs. It was demonstrated that RFB can trigger aging by causing chromosomal breaks, the repair of which results in the generation of ERCs (DEFOSSEZ *et al.*, 1999). Introduction of an ERC into young mother cells shortens life span and accelerates the onset of age-associated sterility (SINCLAIR *et al.,* 1997). Accordingly, a *sir2Δ* mutation results in ERC accumulation and reduced lifespan while increased expression of Sir2p reduces ERC formation and thus extends lifespan in both yeast and worms (KAEBERLEIN *et al.,* 1999; TISSENBAUM and GUARENTE, 2001).

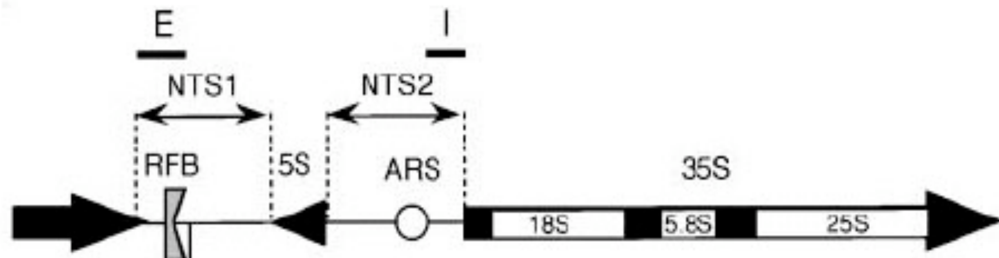


Fig. 1.1 Structure of rDNA repeats in *S. cerevisiae*. There are about 100-200 copies of rDNA units arrayed tandemly in a central position on the chromosome XII. Each repeat of rDNA (9.1 kb) consists of a 35S rRNA coding region (further processed into 5.8S, 18S and 25S RNAs). The 35S and 5S rRNAs are transcribed in directions opposite to each other. The rDNA locus also contains two nontranscribed spacers (NTS), i.e., NTS1 and NTS2. Two DNA elements related to DNA replication, the RFB and ARS are located in NTS1 and NTS2, respectively The genes encoding 35S precursor rRNA and 5S rRNA are indicated as thick arrows, with their directions shown. Adapted from JOHZUKA and HORIUCHI, 2002.

1.2 Fob1 promotes aging by inducing rDNA recombination in yeast

1.2.1 Replication fork barrier (RFB)

Replication fork-blocking sites have been identified in the rDNA from yeast to human cells (ROTHSTEIN *et al.,* 2000). In the yeast *S. cerevisiae,* there are about 100-200 copies of rDNA units arrayed tandemly in a central position on the chromosome XII. Each repeat of rDNA (9.1 kb) consists of a 35S rRNA coding region (further processed into 5.8S, 18S and 25S RNAs; see Fig. 1.1), which 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 nontranscribed spacers (NTS), i.e., NTS1 and NTS2 (SKRYABIN *et al.,* 1984; BREWER and FANGMAN, 1988; LINSKENS and HUBERMAN, 1988). Two DNA elements related to DNA replication, the RFB and *ARS* are located in NTS1 and NTS2, respectively (Fig. 1.1). In the 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. RFB blocks progression of the replication fork in only one direction, which is opposite to that of transcription of 35S rRNA gene, thus ensuring that rDNA is replicated in one direction, the same direction with that of transcription of 35S rRNA and persists even when rDNA is cloned into a plasmid (BREWER *et al.,* 1992). In *E. coli*, it has been demonstrated that RFBs lead to double-strand breaks (DSBs) in the bacterial chromosome (MICHEL *et al.,* 1997; SEIGNEUR *et al.,* 1998). Similarly, RFBs in the

rDNA of yeast elicit the formation of DSBs that must be repaired by the machinery of homologous recombination machinery. ERCs can then be generated by intrachromosomal recombination. It has been shown that the machinery for homologous recombination is required for the generation of ERCs (DEFOSSEZ *et al.*, 1999). Cells have shown a very short lifespan in the absence of this machinery, likely due to the persistence of DSBs (DEFOSSEZ *et al.*, 1999; 2001; PARK, *et al.,* 1999). It was found that ERCs do not form in cells in which double-strand break repair has been eliminated by mutation in genes involved in homologous recombination, such as RAD52 epistasis group genes (PARK, *et al.,* 1999; RICHARDSON *et al.,* 2004; LOEILLET *et al.,* 2005).

1.2.2 *FOB1*, a replication fork block gene in yeast

One of the best-characterized RFBs in eukaryotes is found in rDNA repeats of *S. cerevisiae* (BREWER and FANGMAN, 1988, KOBAYASHI *et al.,* 1998). More recently, a gene (*FOB1*) was isolated whose product (Fob1p), a nucleolar protein required for the RFB, has been shown to influence the aging process in yeast by regulating the abundance of ERC (DEFOSSEZ *et al.,* 1999). The *FOB1* gene is located on chromosome IV and the well-known molecular function of *FOB1* is the creation of a unidirectional block in replication of rDNA (KOBAYASHI and HORIUCHI, 1996). Fob1p binds to the rDNA locus and overall stalls progression of the replication fork, which increases rDNA recombination and ERC accumulation (DEFOSSEZ *et al.,* 1999;

KOBAYASHI and HORIUCHI, 1996). The *FOB1* deletion (*fob1*Δ) mutant confers reduced rDNA recombination, as measured by loss of a marker gene inserted into the rDNA (KOBAYASHI and HORIUCHI, 1996), and an increased lifespan of more than 50% compared to WT cells (DEFOSSEZ *et al.,* 1999). In a *sir2Δ* mutant, deletion of *FOB1* was observed to suppress the *sir2*Δ reduced lifespan phenotype (KAEBERLEIN and GUARENTE, 1999; DEFOSSEZ *et al.,* 1999). Thus, Sir2p and Fob1p play antagonistic roles at the rDNA loci.

The importance of the *FOB1* gene is based on the following relevant findings: *FOB1* is required for rDNA recombination and blocking of the replication fork to prevent collision between DNA replication and rDNA transcription events (KOBAYASHI and HORIUCHI, 1996). In addition, Fob1p is necessary for either contraction or expansion of ribosomal units (KOBAYASHI *et al.,* 1998). Moreover, Fob1p is involved in the control of transcriptional activation occurring at the enhancer region of rDNA (HUANG and MOAZED, 2003). *FOB1* is not evolutionary conserved, however it is noteworthy that a unidirectional replication block in the rDNA is a feature conserved in higher eukaryotes, such as plants, *Xenopus*, and humans (HERNANDEZ *et al.*, 1993; LITTLE *et al.*, 1993; WIESENDANGER *et al.,* 1994). Proteins, such as Sir2p, play an analogous role as Fob1p in these systems. In addition, Fob1p was found to share sequence similarity with retroviral intergrases in a three-dimensional (3-D) model using the catalytic core domains of HIV-1 intergases as template (DLAKIC, 2002). This finding suggests that besides its fork-blocking activity, Fob1p also could act as nuclease.

1.2.3 Fob1p promotes aging in yeast

The short lifespan of a *sir2Δ* mutant reveals a direct failure to repress recombination generated by the Fob1p-mediated replication block in the rDNA (GERSHON and GERSHON, 2000). Accordingly, silencing of the rDNA locus by Sir2p extends lifespan, whereas Fob1p destabilizes the rDNA locus, resulting in reduced lifespan. These observations regarding Fob1p are central to this thesis. Significantly, preliminary work prior to this thesis using the Anaphase-Promoting Complex (APC) subunit Apc5p as bait in a yeast 2-hybrid screen resulted in the isolation of Fob1p. Hence, based on the above findings, I will test the hypothesis that the APC, a ubiquitin protein ligase, targets a lifespan inhibitor (Fob1p) for degradation in order to promote longevity.

1.3 Ubiquitin signaling pathway

1.3.1 Ubiquitin

One of the most complex post-translational modifications to which eukaryotic proteins are subject is the covalent attachment of one protein to another. Ubiquitin is the most familiar of these proteinaceous protein modifiers.

Ubiquitin (Ub), a small molecule of 76 amino acids that can be covalently linked to itself or other proteins, was discovered in the 1970s (SCHLESINGER *et al.*, 1975). The first function attributed to Ub was the proteasome-dependent degradation of short-lived proteins in mammalian cells (CIECHANOVER *et al.*, 1984; FINLEY *et al.*,

1984). Since then, research in this area has exploded and Ub had been associated with nearly every aspect of eukaryotic cell biology (GLICKMAN and CIECHANOVER, 2002; AGUILAR and WENDLAND, 2003; WELCHMAN *et al.*, 2005). Ub is found throughout eukaryotic cells and is highly conserved, with only three amino-acid differences between yeast and humans. This remarkable conservation reflects the importance of Ub's biological functions in eukaryotic cells.

1.3.2. Ubiquitination components

The conjugation of Ub to substrates is completed through three steps involving three different enzymes (Fig. 1.2): a ubiquitin-activating enzyme (E1), multiple ubiquitin-conjugating enzymes (E2), and a growing list of ubiquitin-conjugating enzyme (E3). 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. Additional Ub moieties can be conjugated to Lys48 (HERSHKO and HELLER, 1985; CHAU *et al.,* 1989) or Lys63 (ARNASON and ELLISON, 1994; SPENCE *et al.,* 1995) on the previous Ub to form a polyubiquitin chain.

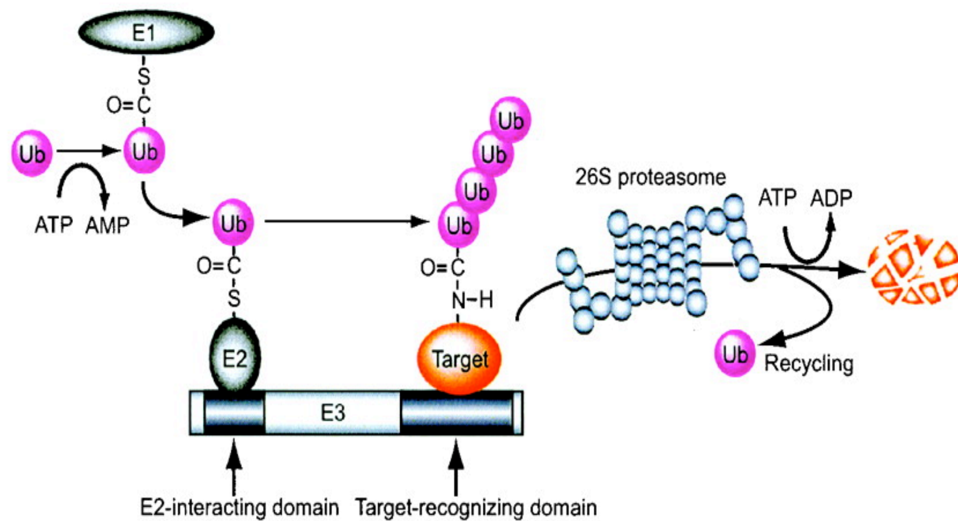


Fig. 1.2 The ubiquitin-proteasome system. E1 enzymes form a thioester bond with ubiquitin (Ub) in an ATP-dependent manner. Ubiquitin is then transferred via an E2 and an E3 (all of which possess both an E2-interacting domain and a target-recognizing domain) to a lysine residue of the target protein, to which it is linked by an isopeptide bond. Polyubiquitylated target proteins are recognized by the S5a subunits of the 26S proteasome and degraded in an ATP-dependent manner. The ubiquitin moieties on the target are removed by deubiquitylating enzymes and recycled. Adapted from HATAKEYAMA and NAKAYAMA, 2003.

1.3.2.1 E1s

During ubiquitination, the first task is to activate the C-terminus of Ub, thus making it capable of conjugating to a substrate. This reaction is catalyzed by an E1 enzyme in two steps. Firstly, 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 (Uba1p; CIECHANOVER *et al.,* 1982; McGRATH *et al.,* 1991), which suggests that the first step in ubiquitination is conserved although there are many ubiquitination substrates.

1.3.2.2 E2s

The next step in the ubiquitination process is the transfer of Ub from the E1 cysteine residue to an E2 cysteine. There is a large family of E2s dedicated to ubiquitination, comprising 11 enzymes in *S. cerevisiae* and many more in higher organisms (PICKART, 2001). Each E2 may serve several E3s and several E2s may serve a single E3. The number of E3s is much larger. Each E3 cooperates with one or a few E2s (CHEN *et al.*, 1993; SOMMER and WOLF, 1997) to recognize specific substrate(s). Different combinations of E2s and E3s lead to the large number and extraordinary diversity of ubiquitination substrates. 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.

1.3.2.3 E3s

The last step in the ubiquitination cascade is the covalent ligation of one or more Ub molecules to the substrate mediated by an E3. There are many E3s in higher organisms, ranging from several hundred to over a thousand (PICKART and EDDINS, 2004). The large number of E3 reflects the breadth of ubiquitination involved in biological functions. Different from E2s, E3s are structurally diverse and may function as single polypeptides or as large multi-subunit protein complexes. Nevertheless, to date, all known E3s belong to only three protein families: Homologous to E6AP Carboxy Terminus (HECT), Really Interesting New Gene (RING), and Ufd2 (Ub fusion degradation protein 2) homology (U-box) proteins (JOHNSON *et al.*, 1995; LIU, 2004; ARDLEY and ROBINSON, 2005).

1.3.2.3.1 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, Cys and His represent zinc binding residues (JOAZEIRO and WEISSMAN, 2000). Every RING motif coordinates two zinc atoms with each atom ligated tetrahedrally by either four cysteines, or three cysteines and a histidine, in a unique cross-brace arrangement. One feature of RING domains is that they can directly bind E2s. The structure of one E3-E2 complex (c-Cbl–UbcH7–ZAP-70 Peptide Complex) revealed that no RING domain side chain comes closer than ~15 Å to the E2 active site cysteine (ZHENG *et al.*, 2000). This observation and other structures (ORLICKY *et al.*, 2003; ZHENG *et al.*, 2002) suggest that RING E3s function in ubiquitination as molecular scaffolds that bring together the substrate lysine and the E2-Ub intermediate. RING E3s can be classified into two types: single-subunit and multi-subunit. Single-subuint RING E3s are a polypeptide containing a RING finger domain (no two or more RING domains protein reported) and other motifs necessary for E3 activity. Multi-subunit RING E3s include the SCF and the APC. The SCF (Skp1/Culin/F-box protein) and APC 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 (Cul 1, 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, Cul1p binds to Skp1p 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 Skp1p, as well as a specific substrate-recognition motif. F-box proteins are highly variable and interchangeable. Thus, Cul1p 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 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).

1.4 The APC promotes mitotic progression, genomic stability and longevity

1.4.1 Anaphase-Promoting Complex (APC)

The eukaryotic cell division cycle involves the replication of chromosomal DNA and equal distribution of DNA to daughter cells in an organized manner. Failure to faithfully duplicate and segregate chromosomes can have dire consequences, such as the

onset of cancer in multicellular organisms. One of the essential regulatory components of chromosome segregation in eukaryotes is a large multi-subunit enzyme termed the APC. The APC, an evolutionarily conserved, multi-subunit complex E3 ubiquitin ligase that is essential for yeast viability, functions as an E3 (ZACHARIAE and NASMYTH, 1999; HARPER *et al.*, 2002). The APC controls progression through mitosis by targeting mitotic inhibitors for degradation by the 26S proteasome (KING *et a.,* 1996; HOYT, 1997), such as cyclin B (Clb2p in yeast), which inhibits cell separation (cytokinesis; HERSHKO, 1999; IRINER, 2002; KRAFT *et al.,* 2006). To initiate anaphase, the APC also targets securin (Pds1p in yeast), an inhibitor of chromosome segregation, and Scc1p/Mcd1p, a protein required for sister chromatid cohesion, for degradation (SUDAKIN *et al.,* 1995; GUACCI *et al.,* 1997; MICHAELIS *et al.*, 1997). In addition, Ase2p, a protein required for elongation of the mitotic spindle during mitosis, is an APC target (UFANO *et al.,* 2004). Defects that alter APC activity are associated with cancer development in humans (PRAY *et al.,* 2002; LIU *et al.,* 2003; WANG *et al.,* 2003; NAKAYAMA and NAKAYAMA, 2006).

1.4.2 APC subunits

Recent studies are now shedding light on the function of individual APC subunits, most of which are essential for yeast viability. Yeast cells lacking APC subunits arrest as large budded cells with the chromosomes aligned along the metaphase plate (ZACHARIAE and NASMYTH, 1999). However, distinct functions for individual

subunits are apparent. In *S. cerevisiae,* the APC contains 13 core subunits, most of which are stably associated throughout the cell cycle (PETERS *et al.*, 1996; GROSSBERGER *et al.*, 1999), and three known different activators (Cdc20p, Cdh1/Hct1p and Ama1p), whose binding to APC is cell cycle regulated (FANG *et al.*, 1998a, b; KALLIO *et al.,* 1998; ZACHARIAE *et al*., 1998a; COOPER *et al.*, 2000).

1.4.2.1 Apc1p, Apc2p, Apc11p, Cdc16p, Cdc27p and Cdc23p

Apc1p has been suggested to play a possible role in the interaction with polyubiquitinated proteins or as a scaffold for the assembly of APC complex (THORNTON *et al*., 2006). Apc1p shares a structural motif with the two large subunits of the 19S cap complex of the 26S proteasome (LUPAS *et al.*, 1997). Apc2p and Apc11p form the catalytic core, responsible for the ubiquitination of target molecules (LEVERSON *et al*., 2000; TANG *et al*., 2001), while Cdc16p, Cdc27p and Apc1p are regulatory subunits, as they are the targets of activating and inhibitory phosphorylation (KOTANI *et al.*, 1998; RUDNER and MURRAY, 2000). Furthermore, Apc1p, the largest subunit of APC, was found in many eukaryotic organisms (STARBORG *et al.,* 1994; YAMASHITA *et al.,* 1996; PETERS *et al.*, 1996; ZACHARIAE, 1996; ZACHARIAE *et al*., 1998b; JORGENSEN *et al.*, 2001). As mentioned above, Apc2p and Apc11p are distant members of the Cullin and RING domain families, respectively (YU, *et al.*, 1998; ZACHARIAE *et al.,* 1998b). Like Cdc16p and Cdc27p, Cdc23p is also an essential protein required for cell cycle progression through mitosis in *S. cerevisiae* (ICHO and WICKNER, 1987; SIKORSKI *et al.*, 1990, 1991, 1993). Cdc23p was suggested to bind

cyclin in the first step toward its ubiquitination and degradation (MEYN *et al.*, 2002).

1.4.2.2 Cdc26p and Apc9p

Cdc26p, unlike Apc9p, which is only found in yeast, has been identified in both yeast (ZACHARIAE *et al.*, 1998b) and vertebrates (GMACHL *et al.*, 2000). But the function of Cdc26p is only required for growth at increased temperatures and Apc9p lacks a phenotype when mutated (ZACHARIAE *et al.*, 1998b; PAGE *et al.*, 2005). Both Cdc26p and Apc9p are unessential APC subunit. Haploid cells containing a deletion of *APC9* are viable at 25° and 37°C. However, Cdc27p was largely absent in precipitates from *apc9Δ* strains. Apc9p might stabilize the interaction of Cdc27p with the APC, which may underscore the requirement for Apc9p for efficient entry into anaphase (ZACHARIAE *et al.*, 1998b).

1.4.2.3 Apc4p and Apc5p

Neither Apc4p nor Apc5p share significant homology to any previously identified proteins (PAGE and HIETER, 1999; CASTRO, 2005). Apart from its association with APC complex, Apc5p binds to human Poly (A) Binding Protein (PABP), a protein involved in many aspects of RNA metabolism and other heavier complexes (KOLOTEVA-LEVINE *et al.,* 2004). As a translational stimulator, PABP is responsible for the activation of 5'-capped 3'-polyadenylated transcripts (GALLIE, 1998; SACHS, 2000). The binding of Apc5p/PABP was shown to inhibit the stimulation of internal ribosome entry sites (IRESs) by PABP (KOLOTEVA-LEVINE *et al.,* 2004), which

suggests a role for Apc5 in the control of gene expression, in addition to its APC function.

1.4.2.4 Apc10

Apc10p is not essential for viability, but encodes a conserved protein and is required for efficient ubiquitination and degradation of mitotic B-type cyclins (GROSSBERGER *et al.,* 1999; AU *et al.,* 2000). A 33-kDa protein called Apc10p or Doc1p was initially identified in a screen that enriched for mutants arrested in M/G1 and killed cells in other cell cycle stages (HWANG and MURRAY, 1997). The *apc10*Δ mutants show, in addition to sterility, *ts* growth with defects in chromosome segregation. Apc10p was found to function as a processivity factor that promotes the building of poly-Ub chains (CARROLL and MORGAN, 2002).

1.4.2.5 APC subcomplex

The cullin subunit Apc2p and its binding partner, the RING finger protein Apc11p, are found in a subcomplex with Apc1p, Apc4p, and Apc5p and are essential for the assembly of multiubiquitin chains from ubiquitin residues donated by E2 enzymes (VODERMAIER *et al.,* 2003). But this complex (Apc1/2/4/5/11p) is not able to bind Cdh1p and to ubiquitinate substrates. Another subcomplex, which consists of all the other APC subunits except Apc2p/Apc11p, was found to recruit Cdh1p via the TPR (Tetratrico Peptide Repeat) subunits Apc3p and Apc7p. However, it is not able to support any ubiquitination reaction.

Apc5p also likely forms a subcomplex with other APC component(s) to ubiquitinate a target protein involved in chromatin assembly (HARKNESS *et al.,* 2002). It was suggested in this study that Apc5p associated with Apc10p to regulate chromatin assembly. This was supported by another study demonstrating that injection of *C. elegans* with *apc-5* or *apc-10* dsRNAs does not result in the expression of the same meiotic phenotypes as other tested APC mutants (DAVIS *et al.,* 2002). Thus, the APC may have additional uncharacterized functions regulated by individual subunits.

1.4.3 Regulation of APC

APC activity is regulated at the protein level by a complex network of interactions (KOTANI *et al.*, 1998; RUDNER and MURRY, 2000; SCHWAB *et al.*, 2001; PASSMORE and BARFORD, 2005).

1.4.3.1 Regulation of APC by two activators, Cdc20 and Cdh1

The APC is regulated by the binding of two conserved activators, Cdc20p and Cdh1p (also known as Hct1p; SCHWAB *et al.*, 1997; 2001; VISINTIN *et al.*, 1997; FANG *et al.*, 1998b; KITAMURA *et al.*, 1998; LORCA *et al.*, 1998). In budding yeast, Cdc20p-dependent APC activity initiates the metaphase to anaphase transition and the series of events that activate the Cdh1p-dependent APC, which induces complete mitotic cyclin destruction (LIM and SURANA, 1996; VISINTIN *et al.*, 1997; SHIRAYAMA *et al.*, 1999). Although phosphorylation of Cdc16p, Cdc23p, and Cdc27p is not essential for viability in budding yeast, this phosphorylation stimulates Cdc20p-dependent APC

activity and Cdc20p binding to the APC *in vivo* (RUDNER and MURRY, 2000).

1.4.3.2 Phosphorylation of APC by Cdc5p and Cdc28p

Studies have indicated APC activity is controlled by MPF-activated Plk (Cdc5p in yeast) in late mitotic progression (KOTANI *et al.*, 1998). In addition to the APC, Cdc5p, a member of a conserved group of protein kinases called the Polo kinases (GLOVER *et al.,* 1996; LANE and NIGG, 1997), is required by yeast cells to complete mitosis. Polo kinases have been implicated in budding yeast (CHARLES *et al.,* 1998; SHIRAYAMA *et al.,* 1998), *Xenopus* (DESCOMBES and NIGG, 1998) and mammalian cells (KOTANI *et al.,* 1998) to function in late mitosis, which activates the cyclin-specific APC activity. In addition, Polo kinases have been shown to be required for cytokinesis and the establishment of bipolar spindles (LLAMAZARES *et al.,* 1991; KITADA *et al.,* 1993; LANE and NIGG, 1997). They have also been shown to phosphorylate a number of mitotic regulatory proteins including CHO-1/mitotic kinesin-like protein 1 (MKLP-1) (LEE *et al.,* 1995), Xcdc25 (KUMAGAI and DUNPHY, 1996), and β-tubulin and microtubule-associated proteins (TAVARES *et al.,* 1996). The phosphorylation of the APC by Cdc5p (Plk in humans) and Cdc28p (MPF in humans) activates the APC (KOTANI *et al.*, 1998; 1999; RUDNER and MURRAY, 2000). Both Cdc5p and Cdc28p phosphorylate the APC subunits Cdc16p and Cdc27p, while Cdc5p also phosphorylates Apc1p (KOTANI *et al.*, 1999; RUDNER and MURRAY, 2000).

1.4.3.3 Phosphorylation of APC by Protein Kinase A (PKA)

Alternatively, another notable example of APC regulation is the negative influence of Protein Kinase A (PKA) signaling on the APC in both yeast and mammalian cells (YAMASHITA *et al*. 1996; KOTANI *et al*. 1998; IRNIGER *et al*. 2000; BOLTE *et al*. 2003). The phosphorylation of APC subunits, such as Cdc27p and Apc1p, by PKA represses APC activity (KOTANI *et al.*, 1998). PKA signaling is induced through glucose signaling (THEVELEIN and DE WINDE, 1999) and recent studies have shown that APC activity is repressed by glucose (IRNIGER *et al.,* 2000; BOLTE *et al.,* 2003). Furthermore, PKA is considered to play an important role in maintaining chromosomal stability in both interphase and metaphase nuclei, through its association with the centrosome, mitotic spindle and microtubules, and cytokinesis (MATYAKHINA *et al.,* 2002). The APC is also required for genome stability (HARTWELL and SMITH, 1985; PALMER *et al.,* 1990; HARKNESS *et al.,* 2002) as chromosome maintenance, chromatin assembly (HARKNESS *et al.,* 2005; HARKNESS, 2005) and histone acetylation (RAMASWAMY *et al.*, 2003) are altered in *apc* mutants. Our lab isolated and described a mutation in the Apc5p APC subunit that rendered cells temperature sensitive (*ts*) at 37°C, predisposed to chromosome loss and chromatin assembly defective *in vitro* (HARKNESS *et al.*, 2002; 2003). These observations suggest that the APC is critical for chromosome maintenance, chromatin metabolism and genomic stability during mitosis.

1.4.3.4 Regulation of APC by other kinases

In addition, the following kinases, Cyclin B/cdk1 and BubR1, are also involved in APC regulation in mammalian cells (YU, 2002; CHAN and YEN, 2003; MORROW *et al.*, 2005). Activation of APC requires phosphorylation by protein kinase Cyclin B/cdk1 (HERSHKO, 1999). BubR1 is an essential component of the spindle checkpoint, which maintains genome stability by phosphorylating Cdc20 to inhibit Cdc20-mediated activation of the APC until all the chromosomes correctly align on the microtubule spindle apparatus via their kinetochores (CHAN *et al.*, 1999; SUDAKIN *et al.*, 2001; TANG *et al.*, 2001; 2004; FANG, 2002). Thus, the APC receives a diverse and complex set of signals that are interpreted to produce the correct response.

1.4.4 The APC is required for prolonged lifespan

Our lab has shown that the APC is required for the longevity of both dividing and non-dividing yeast (HARKNESS *et al.*, 2004). Two routine assays were used to investigate whether the APC influenced aging: replicative and chronological aging assays. Lifespan in dividing cells is measured by scoring the number of daughters a single mother cell produces (replicative assay). This methodology has been employed extensively (KENNEDY *et al.*, 1994; JAZWINSKI, 2004; 2005; PIPER, 2006). In this approach, the finite number of buds produced by a ‘mother’ cell is determined and is designated budding lifespan. After the cells undergo a certain number of buddings they cease to divide. A longer cell cycle (slower bud production) is observed in late buddings

prior to the complete cessation of cell division (MORTIMER and JOHNSTON, 1959). Alternatively, the lifespan of non-dividing cells is measured by counting the number of days a population of cells remains viable after reaching stationary phase (chronological assay; FABRIZIO, 2001), in which the cells are not grown individually on nutrient agar plates but rather whole populations are maintained in liquid medium until cell number reaches a plateau (LONGO, 1999). The cells are then maintained for an additional period on either the expired medium or distilled water (CR; MASORO, 2005; WOLF, 2006). Viability is determined by the ability of individual cells plated from aliquots that are removed periodically from the cultures to form colonies on agar plates. Our lab measured replicative and chronological lifespan in *apc5*^{CA} (chromatin assembly) cells, as *apc5*^{CA} cells suffer genomic instability (HARKNESS *et al.*, 2002). Cells expressing *apc5*^{CA} generated fewer daughters and senesced faster than isogenic WT cells after reaching stationary phase, indicating that the APC is indeed required for normal lifespan. However, Apc5 was recently found in complexes other than the APC in human cells (KOLOTEVA-LEVINE *et al.,* 2004). Therefore, to determine whether *apc5*^{CA}-associated accelerated aging phenotypes reflect a relevant biological role for the APC in aging, three other APC mutants were studied (*apc9Δ, apc10Δ* and *cdc26Δ*). All 3 mutations conferred reduced chronological and replicative lifespan. These observations demonstrated that the APC is a critical factor required for increased longevity (HARKNESS *et al.*, 2004). We predict that the APC targets a protein that accelerates aging for degradation.

1.4.5 The APC physically interacts with Fob1p in a yeast two-hybrid assay

My hypothesis states that the APC targets a protein that reduces lifespan for ubiquitin-dependent degradation. The results from our two-hybrid screen utilizing Apc5p as bait are consistent with this hypothesis, as Fob1p was isolated as an Apc5p binding partner. If binding of Fob1p to the rDNA locus persists in *apc* mutants, cell cycle progression may be blocked. Thus, removal of Fob1p from the rDNA locus may be a prerequisite for cell cycle progression. Since the basic function of the APC is to act as a ubiquitin-protein ligase, I hypothesize that the Apc5p-Fob1p interaction defines Fob1p as a critical target for APC-dependent lifespan determination. This would be an important result as it potentially identifies an APC target that is directly involved in aging in yeast.

1.5 Significance

Aging is a universal biological phenomenon in all living cells. Questions regarding how the cells age are beginning to be answered (reviewed in TROEN, 2003; HELFAND and ROGINA, 2003; SINCLAIR, 2005; VIJG and SUH, 2005). Thus, great biological interest and practical importance leading to interventions rest on uncovering the molecular mechanism of aging. This would ultimately delay the aging process while maintaining the physical and mental strengths of youth.

The conservation of metabolic and signaling pathways between yeast and humans is remarkably high, leading to the expectation that aging mechanisms are also common across evolutionary boundaries (SINCLAIR, 1999; GUARENTE and

KENYON, 2000; LONGO and FINCH, 2003; GOURLAY *et al.,* 2004; HARKNESS, 2006). The biology of aging in humans has always been a topic of interest. Different theories of human aging have been presented, such as the Hormesis Hypothesis of CR (TURTURRO *et al.*, 2000; MATTSON *et al.*, 2002; CALABRESE, 2004; RATTAN, 2004; SINCLAIR, 2005), which states that low intake of calories represents a mildly stressful condition for an organism that requires the organism to induce or activate a survival response to combat the stress. In contrast, the biology of aging in model organisms is relatively well understood. Cells of organisms including yeast, worms, flies, and rodents all display nearly identical survival strategies (SINCLAIR, 1999; GUARENTE and KENYON, 2000; LONGO and FINCH, 2003). One hypothesis is the Xenohormesis Hypothesis (HOWITZ *et al.*, 2003; LAMMING *et al.*, 2004), which states that organisms are conditioned to acquire stress-signaling molecules from other species to allow protection against environmental decline. Furthermore, proposed causes of human aging, such as oxidative damage, genomic instability, and metabolic dysfunction have all been found to affect longevity in these model organisms. It has been demonstrated that some genes regulate lifespan in model organisms have direct correlation with human longevity (SIRT1); model systems have already provided valuable insights into the molecular basis of senescence in humans. For example, activation of the stress pathway mediated by the FOXO proteins, and repressed by the AKT survival pathway, has been shown to promote longevity through studies in worms, flies and yeast (FABRIZIO *et al.*, 2001; GREER and BRENET, 2005; KAEBERLEIN *et al.*, 2005; POWERS *et al.*, 2006).

One of the best characterized model systems for studying aging is the budding yeast, *S. cerevisiae*. Certainly, many of the genes that extend yeast life span have human counterparts. By utilizing this model, the span in knowledge between yeast and human aging can be bridged.

1.6 Rationale and hypothesis

Evidence is accumulating that a genetic program exists for lifespan determination (LONGO and FINCH, 2003; SINCLAIR, 2005). Model organisms expressing mutations in single specific genes live longer with increased resistance to stress and cancer development. Mutations that accelerate aging in yeast affect the activity of the APC. Our finding that the APC is critical for longevity (HARKNESS *et al.,* 2004) provides us with a potential central mechanism controlling lifespan determination. The APC is required for mitotic progression and genomic stability in presumably all eukaryotes by targeting regulatory proteins, such as cyclin B (Clb2p in yeast) for degradation (ZACHARIAE and NASMYTH, 1999; HARPER *et al.,* 2002). The key feature defining the APC as a central mediator of lifespan is the fact that multiple signaling pathways regulate APC activity and many of these pathways influence lifespan. For example, Snf1 and PKA have antagonistic effects on the APC (KOTANI *et al.*, 1998; IRNIGER *et al.*, 2000; HARKNESS *et al.*, 2004) and on lifespan (LIN *et al.*, 2000; 2002; 2003; HARKNESS *et al.*, 2004; HARKNESS, 2006). Thus, it is intriguing to speculate that the APC may link these signaling pathways to downstream targets controlling longevity.

1.7 Objectives of this thesis

I will expand on the molecular mechanisms controlling lifespan through a genetic approach. This thesis will focus on the yeast aging determinant, Fob1p, which acts to accelerate aging (DEFOSSEZ, 1999). The product of the *FOB1* gene is required to elicit a replication block in the rDNA locus; in the *fob1Δ* mutant, the generation of ERCs is decreased and thus extends lifespan in yeast (DEFOSSEZ, 1999). In a yeast two-hybrid screen, designed to identify proteins that interact with APC, using the APC subunit Apc5p as bait, our lab isolated Fob1p. The objective of this thesis is to test whether the APC promotes longevity by targeting Fob1p for degradation in *vivo*. The specific aims of this study are as below:

1. ***Identify the Apc5p interaction domain within Fob1p***

   A two-hybrid approach will be utilized to assess whether subcloned domains of Fob1p interact with Apc5p.

2. ***Assess genetic interactions between FOB1 and APC mutants.***

   We predict that deletion of *FOB1* should overcome mutant phenotypes associated with *apc* mutants.

3. ***Cell cycle and steady state analysis of Fob1p.***

   The purpose of this approach is to determine whether Fob1p is an unstable protein *in vivo* and whether this is APC-dependent.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Yeast genetics

2.1.1 Yeast strains and cell culture

The yeast strains used in this study are listed in Table 2.1. Media used in this study to propagate yeast include YPD (1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose) and SD medium (0.67% Bacto-yeast nitrogen base without amino acids, 2% glucose or galactose, and addition of any necessary auxotrophic supplements at recommended concentrations) (SHERMAN *et al.,* 1983). 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 which were filter sterilized and added into media after autoclaving. To make plates, 2% agar was added to either YPD or SD medium prior to autoclaving. According to the different plasmids, galactose or sucrose was supplemented at 2% in place of glucose. For long term storage, yeast cells were grown on plates (YPD or drop out media) at 30°C. After 2-3 days growth the yeast cells were removed from the plate with a sterile tooth-pick and inoculated into 2 ml liquid culture (YPD or drop out media). Next morning, 1220 µl of

Table 2.1: Yeast strains used in this thesis. All strains are S288c derivatives.

Yeast strains	Yeast genotype	Source/Reference
YTH5	*MATα ade2 his3Δ200 lys2Δ201 ura3-52*	W. NEUPERT
YTH6	*MATa ade2 his3Δ200 lys2Δ201 ura3-52*	W. NEUPERT
YTH225 (PJ69-4A)	*MATa trp1-901 leu2-3 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	E. CRAIG/P. JAMES
YTH1049	*MAT*(?) *cdc16-1 leu2 his3 ura3*	This study; original strain from D. STUART; backcrossed 5 times to YTH5/6
YTH1029	*MATa his3Δ1 Δleu2 Δmet15 Δura3*	Res. Gen. Collection of Yeast Deletion Mutants
YTH1033	As 1029, *apc10::KanMX6*	Res. Gen. Collection of Yeast Deletion Mutants
YTH1037	As 1029, *fob1::KanMX6*	Res. Gen. Collection of Yeast Deletion Mutants
YTH1236	*MAT***a** *ade2 his3Δ200 leu2Δ3,112 lys2Δ201 ura3-52*	HARKNESS *et al.,* 2003
YTH1636	*MAT* (?) *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*(?) *ade2 his3 leu2 ura3 apc10::KanMX6*	This study; a spore from YTH1033 X YTH5; backcrossed 5 times to YTH5/6
YTH3371	*MAT*(?) *ade2 his3 leu2 lys2*(?) *ura3*	This study; a spore from YTH1037 X YTH1637; backcrossed 4 times to YTH5/6
YTH3175	*MAT* (?) *ade2 his3 leu2 ura3 lys2*(?) $apc5^{CA}$-PA*::*$His5^{+}$ *fob::KanMX6*	This study; as YTH3371
YTH3176	*MAT* (?) *ade2 his3 leu2 ura3 lys2*(?) $apc5^{CA}$-PA*::*$His5^{+}$	This study; as YTH3371
YTH3178	*MAT*(?) *ade2 his3 leu2 ura3* $apc5^{CA}$-PA*::*$His5^{+}$ *fob1::KanMX6*	This study; as YTH3371
YTH3179	*MAT*(?) *ade2 his3 leu2 ura3 fob::KanMX6*	This study; as YTH3371
YTH3181	*MAT* (?) *ade2 his3 leu2 ura3 lys2*(?) $apc5^{CA}$-PA*::*$His5^{+}$ *fob1::KanMX6*	This study; as YTH3371
YTH3182	*MAT* (?) *ade2 his3 leu2 ura3 lys2*(?) $apc5^{CA}$-PA*::*$His5^{+}$ *fob1::KanMX6*	This study; as YTH3371

YTH3335	*MAT*(?) *ade2 his3 leu2 ura3 apc10::KanMX6 fob::KanMX6*	This study; a spore from YTH1693 X YTH3179
YTH3608	Congenic with YTH5	This study; a spore from YTH3335 X YTH1236
YTH3611	Congenic with YTH5, *apc10:: KanMX6 fob1::KanMX6*	This study; as YTH3608
YTH3612	Congenic with YTH5, *apc10:: KanMX6 fob1::KanMX6*	This study; as YTH3608

the cells were mixed with 280 µl of 80% glycerol (final 1.5% (v/v)). The cells were then stored at -80°C.

2.1.2 Yeast transformation

Yeast cells were transformed using lab protocols (modified from Current Protocols). A 5 ml culture of *S. cerevisiae* was grown overnight at 30°C 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 freshly prewarmed media, and allowed to grow until an OD_{600} of 1.0 was reached. Yeast cells were then collected by centrifugation at 4000 rpm at 4°C, washed in sterile water, 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 30°C. 5 µl of carrier DNA (single stranded salmon sperm) and 1-5 µl of transforming DNA were added. After vortexing, 300 µl of a PEG4000 solution (5% polyethylene glycol 4000 in LiOAc solution) was added and the contents were mixed by inverting the tube 4-6 times. The transformation mixture was incubated for 20 minutes at 30°C, followed immediately by heat shocking at 42°C 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 using a “hockey stick” in 95% ethanol and incubated at 30°C for 2-4 days.

2.1.3 Yeast plasmid extraction (Smash and Grab)

Yeast plasmid extractions were performed using lab protocol (modified from Current Protocol). Briefly, cells grown on plates were removed with a sterile toothpick and resuspended in 5 ml YPD or appropriate drop out media overnight with gentle shaking at 30°C. Cells from a liquid culture were collected by centrifugation and resuspended into 200 µl SCE (1M sorbital, 0.1 M sodium citrate, and pH 5.8, 0.01 M EDTA) + lyticase (high-yield purification of yeast lytic enzymes; SCOTT and SCHEKMAN, 1980) (0.0033 grams of lyticase, 1 ml SCE). After the cells were incubated for 1 hour at 37°C, 400 µl fresh SDS (sodium dodecyl sulfate)/NaOH lysis buffer (10 N NaOH, 10% SDS and dH2O) was added, and mixed by inverting 5 times. The mixture was then kept on ice for 5 minutes. 300 µl of 3 M NaAC (PH 4.8) was added, mixed and then the mixture was centrifuged for 5 minutes and the aqueous layer was transferred into a new 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 ddH$_2$O. In order to obtain a pure DNA preparation, the precipitated DNA was treated with 5 µl of RNaseA (10 mg/ml stock) at 37°C 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 ddH$_2$O 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 ddH$_2$O.

2.1.4 Sporulation and yeast tetrad dissection

Two haploid strains with opposite mating types were cross-streaked in an X formation on YPD plates to allow diploid formation. After overnight growth at 30°C, 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 ddH_2O, to which 10 µl 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 Geneticin. Double mutants where KanMX6 was used for each deletion were selected by identifying tetrads that segregated 2:2 on Geneticin plates. The plates were incubated for 2-3 days at 30°C.

2.1.5 Spot dilutions

Spot dilution assays were conducted by pipetting 3 µl of cells from samples generated from a 10-fold dilution series onto various media and grown at the temperatures indicated, as previously discussed (HARKNESS *et al.,* 2004).

2.1.6 Lifespan determination

Replicative, or generational, lifespan of the strains tested in this study was based on previously published protocols (KENNEDY *et al.,* 1994; HARKNESS *et al.,* 2004). Briefly, cells from a fresh culture were struck out onto fresh YPD plates and grown overnight at 30°C. Drop out media was used if plasmids were to be maintained. The next day ~30-50 cells containing small buds were micromanipulated 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 scored and discarded. The plates were kept at 30°C during working hours and stored at 4°C to 16°C overnight.

2.1.7 *In vivo* assay of protein interaction using yeast two-hybrid system

Yeast two-hybrid strain YTH225 (PJ69-4A) was transformed simultaneously with different combinations of pGBT-*APC5* and pGAD-*FOB1* constructs (Table 2-2; see Introduction for details). The co-transformed colonies were streaked on SD-TRP-LEU-ADE plates to test for the activation of *ADE2* expression.

2.1.8 Cell cycle dependent stability analysis

2.1.8.1 Arrest and release

Hydoxyurea (HU) was used to arrest the cell cultures in G1. For our first experiments (Fig. 3.11), cells were grown entirely in 10 ml 2% galactose supplemented

Table 2-2 Plasmids and markers

Plasmids	Plasmid marker	Source or reference
pVA3	*TRP1*-2μ 2 hyb.$GAL4_{BD}$	W. NEUPERT
pTD1	*LEU2*-2μ 2 hyb.$GAL4_{AD}$	W. NEUPERT
pGAD424	*LEU2*-2μ 2 hyb.$GAL4_{AD}$	W. NEUPERT
pGBT9	*TRP1*-2μ 2 hyb.$GAL4_{BD}$	W. NEUPERT
pGBD *APC5*	*TRP1*-2μ 2 hyb. $GAL4_{BD}$	T. HARKNESS
pGBD $APC5^{FLS}$	*TRP1*-2μ 2 hyb. $GAL4_{BD}$	T. HARKNESS
pGBD $APC5^{V.P.}$	*TRP1*-2μ 2 hyb. $GAL4_{BD}$	T. HARKNESS
pGAD *FOB1*	*LEU2*-2μ 2 hyb. $GAL4_{AD}$	T. HARKNESS
pGAD *FOB1* D1	*LEU2*-2μ 2 hyb. $GAL4_{AD}$	This study
pGAD *FOB1* D2	*LEU2*-2μ 2 hyb. $GAL4_{AD}$	This study
pGAD *FOB1* D3	*LEU2*-2μ 2 hyb. $GAL4_{AD}$	This study
pGAD *FOB1* D1+2	*LEU2*-2μ 2 hyb. $GAL4_{AD}$	This study
pGAD *FOB1* N-terminal half	*LEU2*-2μ 2 hyb. $GAL4_{AD}$	This study
pGAD *FOB1* C-terminal half	*LEU2*-2μ 2 hyb. $GAL4_{AD}$	This study
YCp50	*CEN-URA3*	W. NEUPERT
YCp50 *FOB1*	*CEN-URA3*	T. KOBAYASHI
YCp50 *APC5*	*CEN-LEU2*	T. HARKNESS
YCp50GAL_{prom}.*CLB2*.HA	*URA3*	D. STUART
YEplac181	*2μ- LEU2*	W. NEUPERT
BG1805	*URA3*	W. XIAO
BG1805 GAL_{prom}. *FOB1*. 6XHis.HA.prot.A	*URA3*	W. XIAO
BG1805 GAL_{prom}. *SIR2*. 6XHis.HA.prot.A	*URA3*	W. XIAO

media at 30°C for 2 days. The cells were then inoculated into 100 ml 2% galactose supplemented media and grown at 30°C for 2-3 days to an OD_{600} of 0.5. Next, 1 ml samples were harvested for protein extract and the remaining yeast cultures were synchronized using overnight incubation in 300 mM HU at RT. Following this, 1 ml sample were taken for protein extract and FACS (Fluorescence Activated Cell Sorting). Yeast cells were then collected by centrifugation at 4000 rpm at 4°C, washed twice in 2% glucose, resuspended in 2% glucose supplemented media to repress expression of *GAL-FOB1-HA* and allow the cells to re-enter the cell cycle. Samples (1 ml) were taken every hour for 7 hours and then again after 25 hours. Protein extracts were prepared from the samples and resolved using SDS-PAGE.

A second arrest and release experiment was performed (Fig. 3.13). For the experiment, the cells were initially grown up in 2% sucrose supplemented media at 30°C to an OD_{600} of 0.5, synchronized using a 6 hour incubation in 300 mM HU and then induced to express Fob1p-HA in a 6 hour incubation in 2% galactose at RT. The cells were then collected by centrifugation, and the HU and galactose were washed away. The cells were resuspended in 2% glucose supplemented media to repress expression of *FOB1-HA* and to allow the cells to re-enter the cell cycle. The cultures were then split, with one half incubated at 30°C and another half at 37°C. Samples were taken before HU and galactose were added, following 6 hours incubation and every hour of release for protein and FACS analysis up to 6 hours.

2.1.8.2 FACS (fluorescence activated cell sorting)

FACS was used to confirm G1 arrest and release into a new round of cell growth. Cells taken from each arrest and release experiment were collected by centrifugation at 4000 rpm for 5 minutes, washed with 1 ml of 50 mM Tris-HCl (pH 8.0), and resuspended in 1 ml of 70% EtOH. The mixture was incubated for 1 hour at RT, centrifuged at 1500 rpm for 30 seconds, and 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 37°C. 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.

2.2 Molecular biology techniques

2.2.1 Bacterial culture and storage

The *Escherichia coli* (*E. coli*) strains DH5α and NM522 were used for bacterial transformations. All plasmids used in this study contained the ampicillin resistance marker gene, *amp*^{R}. 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. Plates were solidified using 2% agar. For short-term storage (2 or 3 months), transformed cells were stored on LB + Amp plates at $4^{o}C$. For long term storage, transformed cells were grown overnight in 2 ml of LB + ampicillin (50 μg/ml), and 1220 μl of the cells were mixed with 280 μl of 80% glycerol. The mixture was then submerged in liquid nitrogen for approximately 10 seconds, and stored in a $-80^{o}C$ freezer.

2.2.2 Preparation of competent cells

For chemical transformation, *E. coli* DH5α was treated as previously described (CHUNG *et al.,* 1989). Cells were grown in LB media to an OD_{600} of 0.4-0.5. The cells were swirled in an ice bath for 10 minutes and diluted 1:1 in ice cold TSS solution (1 X TSS: LB with 10% PEG8000, 5% DMSO, and 50 mM Mg^{2+} ($MgSO_4$ or MgCl, pH 6.5)). The cells were aliquoted, 500 μl/tube, and placed in $-80^{o}C$ for storage.

For electro-transformations, *E. coli* cells were prepared as indicated in the BioRad *E. coli* Pulser manual. Briefly, the cells were incubated in 1 liter of LB medium until an OD_{600} of 0.6 was reached. The culture was collected by centrifugation at 3500 rpm in a Beckman GSA rotor and the pellet was resuspended in 500 ml of 10% sterile glycerol. The centrifugation was repeated 4 times, each time reducing the resuspending volume, with the final volume being 4 ml of cold 10% glycerol. Aliquots of 1 ml were placed into 1.5 ml microcentrifuge tubes and quickly placed in the $-80^{o}C$ freezer for storage.

2.2.3 Bacterial transformation

2.2.3.1 Chemical transformation

Competent *E. coli* cells for chemical transformations were prepared as previously described (CHUNG *et al.,* 1989). Chemical transformation of bacterial was performed using the KCM method (WALHOUT *et al.,* 2000). 1-5 µl of transforming DNA and Distilled water were added to 20 µl of 5 X KCM (0.5 M KCL, 0.15 M $CaCl_2$ and 0.25 M $MgCl_2$) up to a total volume of 100 µl 100 µl of competent cells were added to the tube, mixed up and kept on ice for 20 minutes. The cells were then incubated for 20 minutes at room temperature or heat shocked 5 minutes at 37^oC. After heat shocking, 1 ml of pre-warmed LB or SOC media (2% Bacto-tryptone, 0.5% Yeast extract, 10 mM NaCl, 20 mM $MgCl_2$, 20 mM $MgSO_4$, and 20 mM glucose) was added to the cells, and the cells were incubated at 37^oC for 1 hour with constant shaking. The cells were plated onto LB + Ampicillin (50 µg/ml) plates and incubated at 37^oC overnight.

2.2.3.2 Electro-transformation

Competent *E. coli* cells (NM522) for electroporation were prepared as indicated in the BioRad *E. coli* Pulser manual. Transforming DNA was added to the competent cells to a final concentration no greater than 10% of the final volume. After one minute incubation on ice, the cell mixture was transferred to a pre-chilled 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 microcentrifuge tube. The

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

2.2.4 Plasmid DNA isolation

2.2.4.1 LiCl plasmid Mini-prep

A 5 ml overnight culture of the transformed bacterial cells was centrifuged, the pellet was resuspended fully 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 sat on ice for 5 minutes. 300 µl of 3M 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 fully 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 -80°C. The pellet was then colleted by centrifugation, washed with 500 µl of 70% EtOH, resuspended in 50 µl of 1 X TE and stored at -20°C or -80°C.

2.2.4.2 Large scale DNA isolation (Maxi-prep)

A 5 ml overnight culture of the transformed bacterial cells was subcultured into 500 ml of fresh LB + Amp medium. The culture was grown overnight at 37°C. The cells were harvested by a 15 minute centrifugation at 4000 rpm in a Beckman GSA rotor at 4°C, and resuspended in 100 ml of ice cold STE (0.1 M NaCl, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0). The cells were collected by centrifugation and resuspended in 18 ml of Solution I (50 mM Glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA pH 8.0). Next, 40 ml of freshly prepared solution II (0.2 NaOH, 1% SDS and 88 ml ddH$_2$O) was added and mixed by gently inverting several times and stored at room temperature for 5-10 minutes. 20 ml of ice cold solution III (5 M potassium acetate, 11.5% glacial acetic acid and 28.5% ddH$_2$O) was added, mixed by shaking and stored on ice for 10 minutes. The solution was centrifuged for 15 minutes at 4000 rpm in a 250 ml Corex bottle using a Beckman SS34 rotor. The supernatant was filtered into a 250 ml plastic bottle through four layers of cheesecloth, 0.6 volume of isopropanol was added, and was then incubated for 10 minutes at room temperature. The nucleic acids were recovered by centrifugation at 5000 rpm for 15 minutes at room temperature. The supernatant was decanted carefully to allow the last few drops of supernatant to drain away. The pellet was rinsed with 70% EtOH at room temperature. After draining off the EtOH, the cells were dissolved in 3 ml TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0).

2.2.5 Agarose gel electrophoresis and DNA fragment isolation

For analysis of plasmid and genomic DNA, a 0.8% agarose gel was used. Electrophoresis was performed 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 modified from current protocols. Briefly, DNA was extracted 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 55°C to dissolve the agarose slice. Next, 5 μl of silica powder suspension was added and incubated for 5 minutes at 55°C. After vortexing, the suspension was centrifuged for 5 second and washed 4 times with 500 ul of cold wash buffer. The DNA was collect by centrigation and incubated twice for 5 minutes with 10-20 μl of ddH_2O at 55°C. The eluted DNA was purified by phenol/chloroform (1:1) extraction as described below **(2.2.6).**

2.2.6 Phenol/chloroform extraction of DNA

Phenol/chloroform extraction was performed to remove proteins from nucleic acid samples. The nucleic acid sample was diluted to 200 μl in a 1.5 ml eppendorf tube. RNaseA (5 μl) was added from a stock of 10 mg/ml and incubated for 30 minutes at 37°C. 100 μl of phenol/chloroform mixture (previously mixed 1:1) were added to the tube, and the tube was then inverted several times to mix the phases. After centrifugation

at 13,000 rpm for 3 minutes, the upper layer was transferred to a new tube. To precipitate the DNA, 100 μl of dH_2O, 20 μl of 3 M NaAc (pH 4.8) and 500 μl of EtOH were added and incubated at -80°C for at least 30 minutes. After centrifugation at 13,000 rpm for 10 minutes, the supernatant was discarded. After washing with 70% EtOH, the DNA sample in the tube was dried in a vacuum device at 37°C for 10 minutes and resuspended in 50 μl of ddH_2O.

2.2.7 Construction of plasmids

All plasmids used or constructed in this study are listed in Table 2-2. Plasmid manipulation was performed using enzymes from New England Biolabs as recommended by the manufacturers. Plasmid DNA was linearized with the appropriate restriction enzymes (Table 2-3) and purified using phenol/chloroform extraction method. Alternatively, purified DNA fragments (as an insert) were created by PCR (Polymerase Chain Reaction) using appropriate primers (Table 2-4), and then cloned into the TOPO vector according to the TOPO TA Cloning Kit (Invitrogen). Insert DNA was then combined with linearized pGAD vector DNA (Fig. 2.1) in a 20 μl volume with T4 DNA ligase (1 μl). To increase the probability of concatermeric ligation, which reduces the chance of vector DNA self-ligation, the molar ratio of vector DNA to insert DNA was set to ~1:3. The mixture was incubated at room temperature overnight. This reaction was used to transform *E. coli* competent cells.

Table 2-3 Primers and enzymes used to create clones

plasmids	vectors	inserts	primers	enzymes
pGAD *FOB1* D1	pGAD424	*FOB1*D1	GADFOB1.5'	*Pst* I
			Fob1DOM2.3'	*Bam*H I
pGAD *FOB1* D2		*FOB1*D2	GADFOB1.5'	*Pst* I
			Fob1DOM2.3'	*Blg* II
pGAD *FOB1* D3		*FOB1*D3	Fob1DOM3.5'	*Bam*H I
			GADFOB1.3'	*Sal* I
pGAD *FOB1* D1+2		*FOB1*D1+2	GADFOB1.5'	*Bam*H I
			Fob1DOM2. 3'	*Sal* I
pGAD *FOB1* N-terminal half		*FOB1* N-terminal half	GADFOB1.5'	*Bam*H I
			Fob1N-terminal.3'	*Sal* I
pGAD *FOB1* C-terminal half		*FOB1* C-terminal half	Fob1C-terminal.5'	*Bam*H I
			GADFOB1.3'	*Bgl* II

Table 2-4 PCR primers used to create clones

Primer names	**Primer sequence 5' to 3'**
GADFOB.5'	ATGCTAGGATCCACACGAAACCGCGTTACAATG
GADFOB.3'	ATGCTAGTCGACTTACAATTCCATTGATGTG
Fob1Dom2.3'	ATGCTAAGATCTACATTAGCAAGGGCAAAAG
Fob1Dom3.5'	ATGCTAGGATCCAAGCGGATAATAGCTGTAAC
Fob1N-terminal.3'	ACGTGTCGACAATTGGAACCCTAFCAAATG
Fob1C-terminal.5'	ACGTGGATCCACTTCGTAACATCAAGCATCTTAG

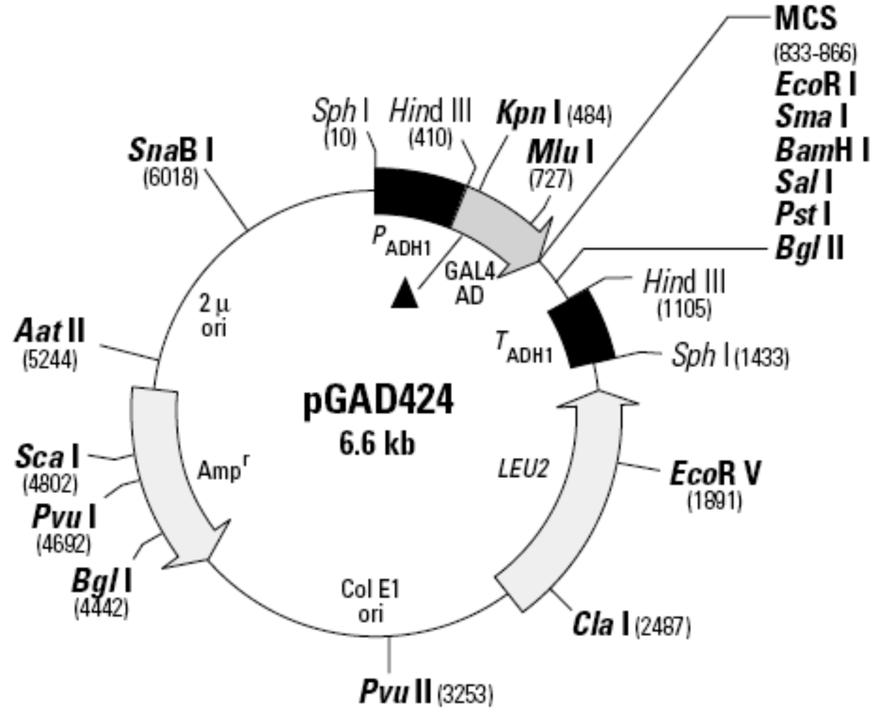


Fig. 2.1 Map of pGAD424 vector (Clontech laboratories, Inc.). This vector generates a hybrid protein that contains the sequences for the GAL4 activation domain (aa 768-881). For the construction of a hybrid protein, the gene encoding the protein of interest is ligated into the MCS in the correct orientation and in the correct reading frame such that a fusion protein is generated. The fusion protein is expressed at high levels in yeast host cells from the constitutive *ADH1* promoter and transcription is terminated by the *ADH1* transcription termination signal. The hybrid protein is targeted to the yeast nucleus by nuclear localization sequences that have been added to the AD sequence from a heterologous source. pGAD424 is a shuttle vector that replicates autonomously in both *E. coli* and *S. cerevisiae*. It carries the amp^R gene (for ampicillin resistance in *E. coli*) and the *LEU2* selectable marker that allows yeast Leu^- auxotrophs carrying pGAD424 to grow on drop out medium lacking Leu.

2.2.8 DNA sequencing

All DNA sequencing was performed at the National Research Council (NRC) Plant Biotechnology Institute (PBI) DNA Technologies Unit using the primers provided (indicate in Table 2-4).

2.2.9 Protein expression and purification

Proteins were extracted according to published lab protocols (HARKNESS *et al.,* 2002). A 10 ml overnight culture of transformed yeast cells was centrifuged at 4000 rpm at 4°C for 5 minutes and resuspended in 1 ml of cold 1 X PBS. The mixture was transferred to eppendorf tube pelleted and resuspended fully in 250 µl of freshly cold solution C (1.85 M NaOH, 7.4% 2-Mercaptoethanol), and 250 µl of 100% TCA (trichloroacetic acid). The mixture was then vortexed rapidly. After incubating on ice for 5 minutes, the cells were centrifuged for 10 minutes at 4°C and washed with cold 1 X PBS. The protein pellet was resuspended in the appropriate amount of solution A (13% SDS and 1 M Tris) and solution B (30% glycerol plus Bromophenol Blue), typically 50 µl each, depending on the pellet size. After boiling for 2 minutes, the proteins were incubated on ice for 2 minutes and sonicated for 3 seconds. Next, the protein lysate was centrifuged for 10 minutes at 4°C. The supernatant was transferred to a new tube and stored at -80°C.

2.2.10 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

Proteins were separated and visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Current Protocols. Gels were prepared with a 15% acrylamide separating gel bed topped with a 4% stacking gel. For one mini-gel (6 cm X 8 cm), the separating and stacking gel mixtures were prepared as follows: 10 ml of separating gel containing 2.3 ml of dH_2O, 5 ml of 30% acrylamide (Sigma), 2.5 ml of 1.5M Tris-HCl (PH 8.8), 100 µl of 10% SDS and 100 µl of 10% APS (Ammonium persulphate); and 2.7 ml of 4% stacking gel with 2.1 ml of dH_2O, 500 µl of 30% acrylamide, 38 µl of 1.0 M Tris-HCl (PH 6.8), 30 µl of 10% SDS and 30 µl of 10% APS. Polymerization was initiated with 4 µl and 3 µl of TEMED (N, N, N', N'-Tetramethylethylenediamine) respectively just before use. The separating gel was poured between plates separated with 1 mm spacers, anchored and sealed in a Bio-Rad mini gel caster, and topped with 1 ml isobutanol for an even level gel surface. After polymerization was complete, isobutanol was drained and the stacking gel was poured over the separating gel. The comb was placed in the stacking gel and the gel was allowed to polymerize (around 20 minutes). Gels were clamped into the electrophoresis apparatus and kept at RT for 20 minutes until the gel was polymerized. Both the top and bottom buffer chambers were filled with 1 X SDS-Page running buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS) and the combs were removed. Samples were kept on ice prior to loading. Gels were run at constant voltage, 150V, for about 1 hour, using bromophenol blue as a running dye. The gel was stained for 60 minutes in Coomassie

stain (0.25% (w/v) Coomassie brilliant blue R250 dissolved in 40% methanol (v/v), 10% (v/v) acetic acid in water). Gels were photographed wet to determine, the equivalency of load for Western blot analysis. Equal samples of protein were then separated again by SDS-PAGE and transferred to a nitrocellulose membrane according to published protocols (Current Protocol) as follow. Briefly, the cassette was assembled by first laying the black side down, then placing the fiber pad, Watman paper, gel, nitrocellulose membrane, Watman paper and the fiber pad in order. The cassette was filled with transblot buffer (1.8% glycine, 0.4% Tris and 400 ml methanol) and transferred under constant voltage, 160V, for about 1 hour, at 4°C. Membranes were stained with Ponceau S staining solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid), to confirm equal load of the proteins and then incubated in 5% PBST blocking milk (PBS, 0.05% Tween (v/v), 5% non-fat milk) for 1 hour at room temperature or overnight at 4°C. Anti-HA high affinity antibodies (Roche Diagnostics GmbH) were diluted 1:1000 in 20 ml PBST milk and the PBST solution was incubated with membranes overnight at 4°C. Membranes were then washed 3 times with 5% PBST blocking milk for 15 minutes each time. The secondary antibody, anti-rat IgG conjugated with horse radish peroxidase (HRP; Sigma), was used at a 1:10000 dilution for 30 minutes at RT. Followed by 3 times washes with 5% PBST blocking milk for 15 minutes each time, and 15 minutes wash with 1X PBST (PBS, 0.05% Tween (v/v)) solution. The Western Lightning Chemiluminescence Reagents (PerkimElmer Life Science) were then utilized for detecting, and the membrane was then exposed to X-ray film.

CHAPTER THREE: RESULTS

3.1 Defining the Fob1p-Apc5p interaction domain in Fob1p

3.1.1 Fob1p-Apc5p yeast two-hybrid interaction

Apc5p is an essential subunit of the APC (ZACHARIAE and NASMYTH 1999; HARPER *et al.,* 2002). A temperature sensitive (*ts*) mutation in the gene encoding this protein was identified in a screen of yeast mutants for those defective for an *in vitro* chromatin assembly assay (HARKNESS *et al.*, 2002). Further work confirmed that the APC was indeed required for chromatin assembly (HARKNESS *et al.*, 2005; ARNASON *et al.*, 2005; HARKNESS, 2005). In order to gain an understanding of the molecular interactions underlying the role of the APC in chromatin assembly, a yeast two-hybrid screen was conducted using *APC5* as bait (HARKNESS, unpublished data). The following describes the results of that screen.

The *APC5* gene was cloned into the two-hybrid bait vector pGBT9. pGBT9 allowed fusion of the *GAL4* DNA binding domain to a gene of interest (all two-hybrid reagents, including the library were generous gifts from ELIZABETH CRAIG; see JAMES *et al.*, 1996 for details). pGBT-*APC5* was transformed into the specially designed two-hybrid yeast strain PJ69-4A (YTH225). The *ADE2* and *HIS3* genes within PJ69-4A were modified by replacing the promoters with the *GAL2* and *GAL1* promoters,

respectively (JAMES *et al.,* 1996). A library of random yeast DNA fragments cloned into the pGAD424 prey vector, which allowed the fusion of a gene to the *GAL4* transcriptional activator domain, was then transformed into the PJ69-4A strain harboring the pGBT-*APC5* construct. This was possible since the pGBT9 plasmid expressed the *TRP1* selectable gene, the pGAD424 plasmid the expressed *LEU2* selectable gene, and PJ69-4A was *trp1*Δ and *leu2*Δ. Thus, transformants were selected on media lacking tryptophan and leucine. From 30 transformations, 1.5 million transformants were obtained that contained pGBT9-*APC5* and a library plasmid. Each plate was then replica-plated to plates lacking tryptophan, leucine and adenine (SD-TRP-LEU-ADE) and tryptophan, leucine and histidine (SD-TRP-LEU-HIS). Since expression of *ADE2* and *HIS3* can only be achieved by recruiting the *GAL* activator domain to the *ADE2* and *HIS3* promoter through interactions with the *GAL* DNA binding domain, growth on the triple drop out plated reflects interaction of a prey molecule with bait. Using *APC5* as bait, the following prey molecules of interest were obtained: *IQG1*, *SRS2*, *PRP2* and *FOB1*. Based on our recent report showing that the APC is required for extended longevity (HARKNESS *et al.,* 2004; HARKNESS, 2006) and that Fob1p promotes yeast aging (DEFOSSEZ *et al.,* 1999), the focus of this thesis was to understand the link between the APC and Fob1p. Considering that the APC targets proteins for degradation by ubiquitination and Fob1p physically interacts with an APC subunit, a logical explanation for these observations would be that the APC targets Fob1p for degradation by ubiquitination, promoting longevity (Fig. 3.1).

The first set of experiments was designed to confirm the two-hybrid interaction

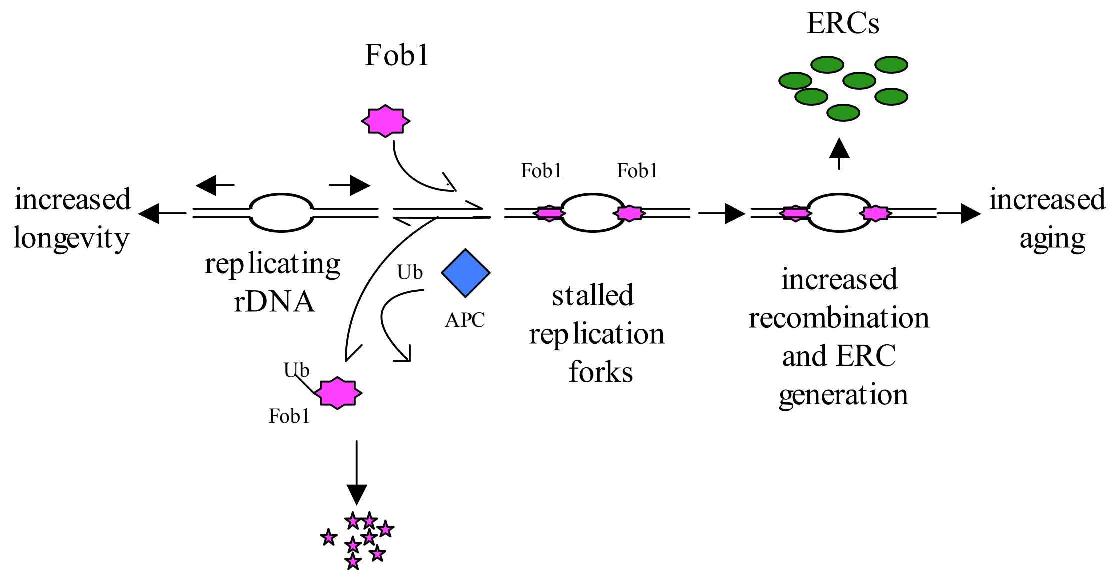


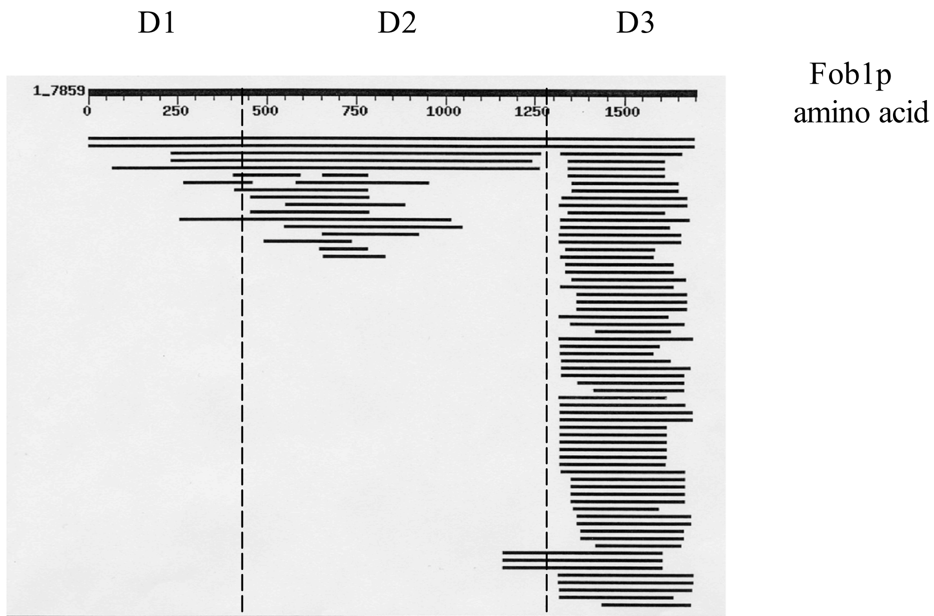
Fig. 3.1 Model depicting the targeting of Fob1p for degradation by the APC as a means to extend lifespan. Fob1p binds to the rDNA locus and overall stalls progression of the replication fork, which increases rDNA recombination, ERCs and furthermore, increases aging in yeast. In this study, APC is believed to target Fob1p for degradation, which increases longevity.

between Fob1p and Apc5p. The yeast strain PJ69-4A (YTH225) was used for all subsequent two-hybrid assays, and all interactions were assessed using SD-TRP-LEU-ADE, as proteins or peptides fused to $Gal4_{BD}$ and $Gal4_{AD}$ will drive the expression of the *ADE2* gene if an interaction occurs, resulting in the growth of this strain on media lacking adenine. The results in Fig. 3.2 show the two-hybrid interaction between Fob1p and Apc5p could indeed be reproduced *in vivo*. Yeast cells transformed with combinations of Apc5p or Fob1p fusions with control vectors and mutants were plated on SD-TRP-LEU and SD-TRP-LEU-ADE media to determine interactions (Fig. 3.2). In addition to the positive control, pVA3/pTD1 (pVA3 and pTD1 encode the Snf1p and Snf4p interacting proteins, respectively), two mutant alleles of *APC5* were used as negative controls in this experiment. The pGBT-$APC5^{FLS}$ allele contains an amino acid substitution at position 43 to generate the FLR to FLS alteration, which results in a *ts* phenotype when expressed as the sole source of *APC5* (HARKNESS, unpublished data). The pGBT-$APC5^{\Delta V\text{-}P}$ mutation was generated by deleting amino acids 40 to 47 (HARKNESS, unpublished data). This allele is not capable of supporting growth when expressed as the sole source of *APC5*. The observation that neither *APC5* mutant allele supports an interaction with Fob1p suggests that this region of Apc5p is required to interact with Fob1p (Fig. 3.2C)

3.1.2 The Apc5p interaction domain is located within the C-terminal half of Fob1p

Yeast two-hybrid assays were used to identify and localize the motif within

A



B

Fob1 Domains

Name	Interaction with Apc5p
full length	+ + +
D1	-
D2	-
D3	-
D1/D2	-
N-terminal half	-
C-terminal half	+

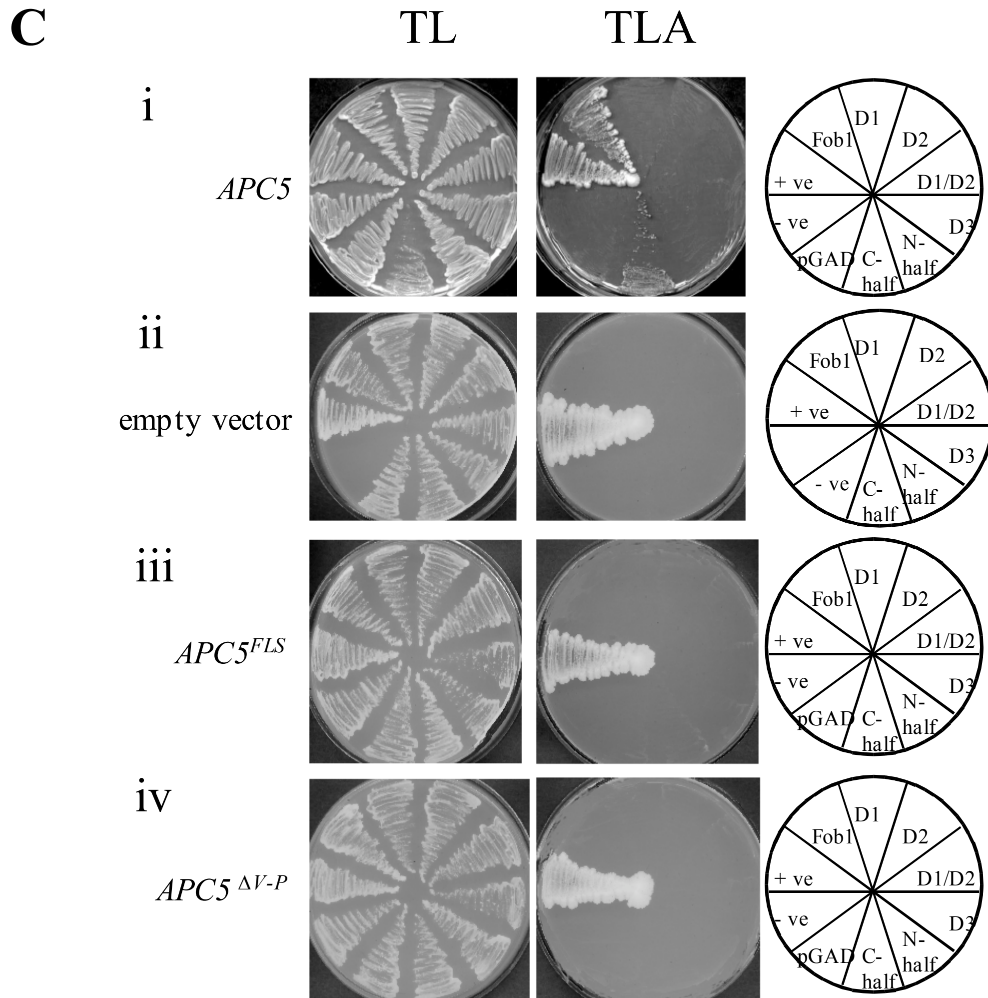


Fig. 3.2 Interactions of pGBT-*APC5* constructs and pGAD-*FOB1* domains in a yeast two-hybrid assay. **(A)** The alignment of sequences from yeast to humans identified in a BLAST search with Fob1p used to define the three Fob1p domains. **(B)** Six subcloned domains of Fob1p that define the sequence motifs identified in **(A). (C)** PJ69-4A was transformed with the different combinations of pGBT and pGAD constructs. All transformants were first selected on SD-TRP-LEU (TL) plates. The same transformants were then struck out on SD-TRP-LEU-ADE (TLA) plates to test for activation of *ADE2* transcription. The transformants which grew on TLA plates indicated an interaction between Fob1p and Apc5p constructs. +ve, pVA3/pTD1 encoding Snf1 and Snf4; -ve, pGBD/pGAD empty vector. Combining the Fob1p constructs with WT pGBT-*APC5* **(i)**, the pGBD empty vector **(ii)**, pGBT-$APC5^{FLS}$ **(iii)** and pGBT-$APC5^{\Delta V\text{-}P}$ **(iv)**. pGBT-$APC5^{FLS}$ and pGBT-$APC5^{\Delta V\text{-}P}$ mutants serve as additional negative controls. The plates were then scanned using an EPSON Perfection 1650 scanner.

Fob1p that is crucial for interaction with Apc5p. Six subcloned domains (D1, D2, D3, D1/D2, N-terminal half and C-terminal half) of Fob1p were chosen according to a BLAST (Basic Local Alignment Search Tool) sequence alignment with Fob1p (Fig. 3. 2A and B). Fob1p is not conserved in higher eukaryotes but motifs within Fob1p share sequence identity with many proteins from yeast to humans (Fig. 3.2A). The sequence motifs within Fob1p were subcloned into the pGAD424 vector to test for interaction with Apc5p. PJ69-4A was transformed with the pGAD424 and pGBT9 constructs, the transformants were selected on SD-TRP-LEU media and then tested on SD-TRP-LEU-ADE. The only Fob1p construct to interact with Apc5p was the C-terminal half domain (Fig. 3.2C panel i). As neither D2 nor D3 interacted with Apc5p, this observation suggests that the region split by D2 and D3 may define the Apc5p binding domain. As noted above for full length Fob1p, the C-terminal half construct did not interact with the Apc5p mutants (Fig. 3.2C, panels: iii and iv).

To gain a greater understanding of this interaction, we analyzed the sequence of the C-terminal half domain of Fob1p using a BLAST search (Fig. 3.3). Fig. 3.3 lists several proteins of interest, found in humans, which share homology with the Fob1p C-terminal half sequence. One notable example is the human Zinc finger protein PEG3. A zinc finger is a protein domain that can bind to DNA, and it consists of two antiparallel β strands and one α helix. One very well explored sub-set of zinc-fingers comprises a pair of cysteine residues in the beta sheets and two histidine residues in the alpha helix which are responsible for binding a zinc ion. As DNA binding proteins, it is feasible that they play a role in gene silencing. This observation describes several

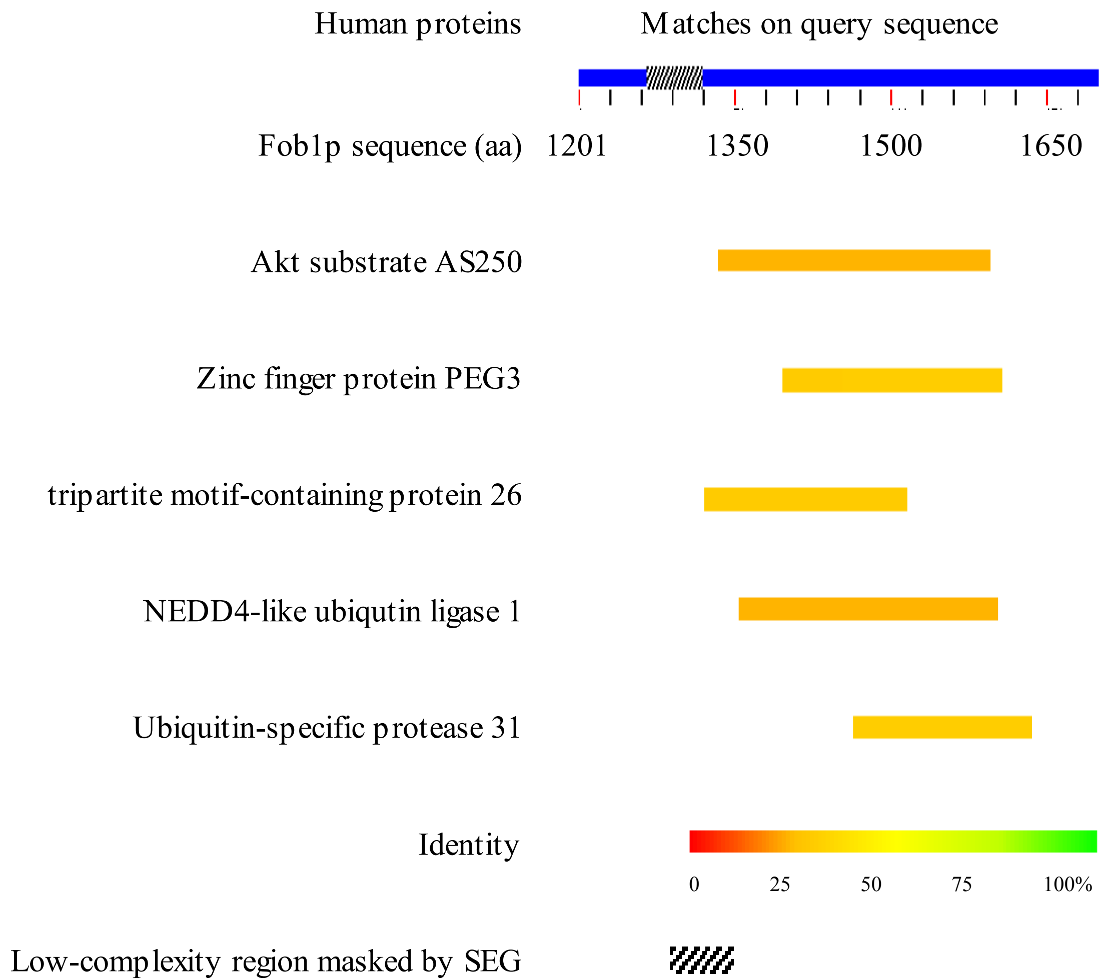


Fig. 3.3 Relevant human proteins sharing homology with C-terminal half of Fob1p, as revealed by BLAST. Akt substrate AS250 contains a predicted GTPase activating protein (GAP) domain. It was found in our lab that the yeast AKT orthologue, Sch9p, inhibits APC activity (WONG and HARKNESS, unpublished). Zinc finger protein PEG3 contains a zinc finger DNA-binding domain. Tripartite motif-containing protein 26 contains a zinc finger domain and a RING finger domain. NEDD4-like ubiqutin ligase 1 is an orthologue of the yeast Rsp5p, an E3 protein. Rsp5p and Apc5p were found to interact in an intracellular pathway regulating chromatin remodeling (HARKNESS *et al.,* 2002; ARNASON *et al.,* 2005). The yeast Ubiquitin-specific protease 31 (USP31) orthologue, Ubp3p associates with Sir2p. Both Ubp3p and Sir2p are essential for gene silencing and interact with the amino terminal segments of histones H3 and H4. SEG is the filtering program for BLAST, which will mask off segments of the query sequence that have low compositional complexity.

important human proteins that may be involved in cell cycle or APC function. The homology search points to the fact that although Fob1p is not evolutionarily conserved, it contains domains that are evolutionarily important and are important for interacting with Apc5p.

3.2 Genetic interaction analysis of *APC5* and *FOB1* mutants

3.2.1 Deletion of *FOB1* in *apc5^{CA}* cells has no effect on growth, but deletion of *FOB1* partially suppresses *apc10*Δ *ts* growth

We hypothesized that if the APC is required for Fob1p turnover, the accumulation of Fob1p in *apc* mutant cells will have a deleterious affect. To test this, we disrupted *FOB1* in *apc* mutant cells with the expectation that removal of *FOB1* from *apc* mutants may alleviate *apc* mutant phenotypes. Thus, single *apc5^{CA}* and *apc10*Δ mutants were crossed with *fob1*Δ cells to generate *apc5^{CA}* *fob1*Δ and *apc10*Δ *fob1*Δ double mutants. The double mutants were compared to WT, *fob1*Δ, *apc10*Δ and *apc5^{CA}* cells with regards to growth on rich media and at various temperatures. It was observed that disruption of *FOB1* had no effect on *apc5^{CA}* (Fig. 3.4A) but partially suppressed the *apc10*Δ growth defect at 34°C (Fig. 3.4B). This suggests that deletion of *FOB1* has differential effect on the *apc* mutants used and may indeed have an impact on APC function.

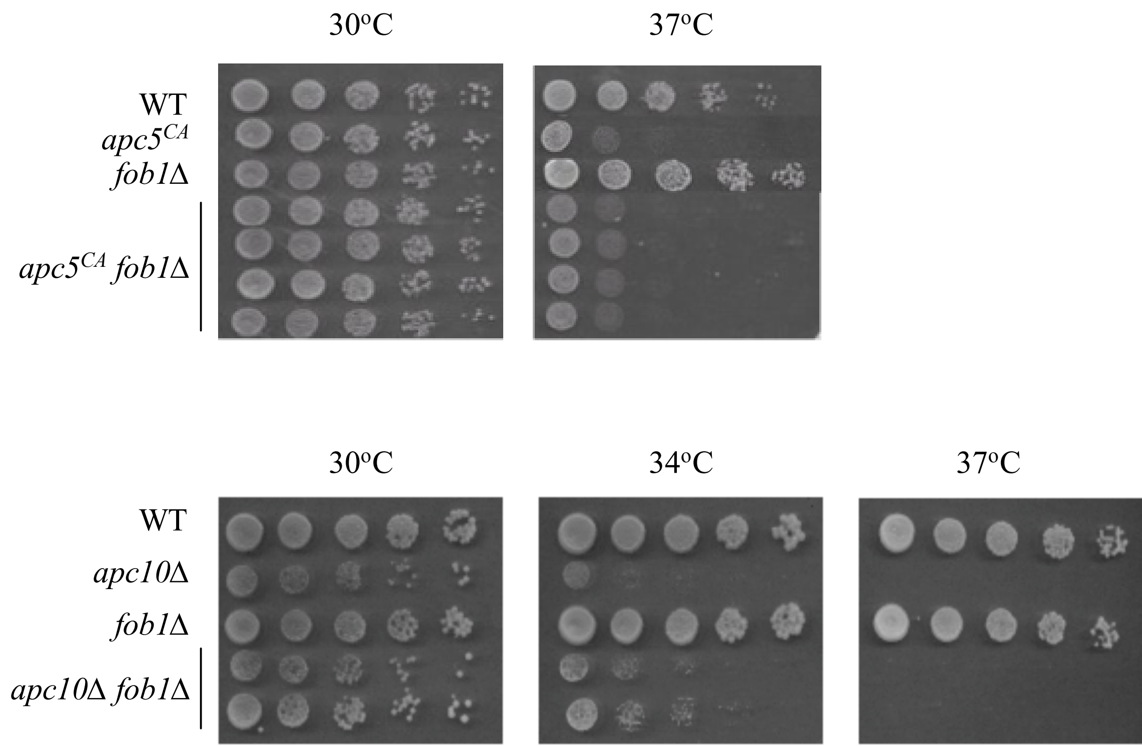


Fig. 3.4 Effects of *FOB1* deletion on *apc5*^{CA} and *apc10*Δ *ts* phenotypes. YTH3371 (WT), YTH3608 (WT), YTH3176 (*apc5*^{CA}), YTH1693 (*apc10*Δ*)*, YTH3179 (*fob1*Δ*)*, YTH3175 (*apc5*^{CA} *fob1*Δ), YTH3178 (*apc5*^{CA} *fob1*Δ), YTH3181 (*apc5*^{CA} *fob1*Δ), YTH3182 (*apc5*^{CA} *fob1*Δ), YTH 3611 (*apc10*Δ *fob1*Δ), and YTH 3612 (*apc10*Δ *fob1*Δ) cells were cultured in liquid YPD at 30°C until they reached log-phase. 10-fold serial dilutions of the cell suspension were then spotted onto YPD plates. The plates were then incubated at 30, 34 or 37°C for 2 days. The plates were scanned using an EPSON Perfection 1650 scanner.

3.2.2 Deletion of *FOB1* in a*pc5*^{CA} and a*pc10*Δ cells extends replicative lifespan

Although deletion of *FOB1* had modest effects on the overall growth rate of only *apc10*Δ cells, it still remained possible that Fob1p expression influenced lifespan in wild type and *apc* mutant cells. Thus, we conducted replicative lifespan assays in cells lacking *FOB1*. We expected that loss of *FOB1* would extend lifespan, as previously shown (DEFOSSEZ *et al.,* 1999). If deletion of *FOB1* suppressed the reduced lifespan observed in *apc* mutants (HARKNESS *et al.,* 2004), this would strongly suggest that Fob1p is a physiologically relevant target of the APC that must be degraded in order to experience maximal longevity. As predicted, deletion of *FOB1* in wild type, *apc5*^{CA} and *apc10*Δ cells extended replicative lifespan (Fig. 3.5). This data suggests that Fob1p is a major obstacle blocking *apc* mutants from experiencing extended longevity. However, it should be noted that deletion of *FOB1* in *apc10*Δ cells does not fully restore lifespan to wild type levels. Therefore, there is likely a Fob1p-independent component to how Apc10p influences longevity in addition to a Fob1p-dependent component. This is also consistent with our earlier report suggesting that Apc5p and Apc10p play redundant roles in promoting longevity (HARKNESS *et al.,* 2004). That is, *apc5*^{CA} *apc10*Δ cells have a shorter lifespan than either single mutant indicating that Apc5p and Apc10p have independent functions in promoting longevity. Lifespan in yeast is now believed to respond to the accumulation of ERCs and the activation of the stress response pathway (KAEBERLEIN *et al.,* 2004). The APC is required for both of these pathways (HARKNESS, 2006). Thus, the *apc10*Δ result likely reflects a role for at least Apc10p in regulating ERC accumulating and in activating the stress response pathway. Taken

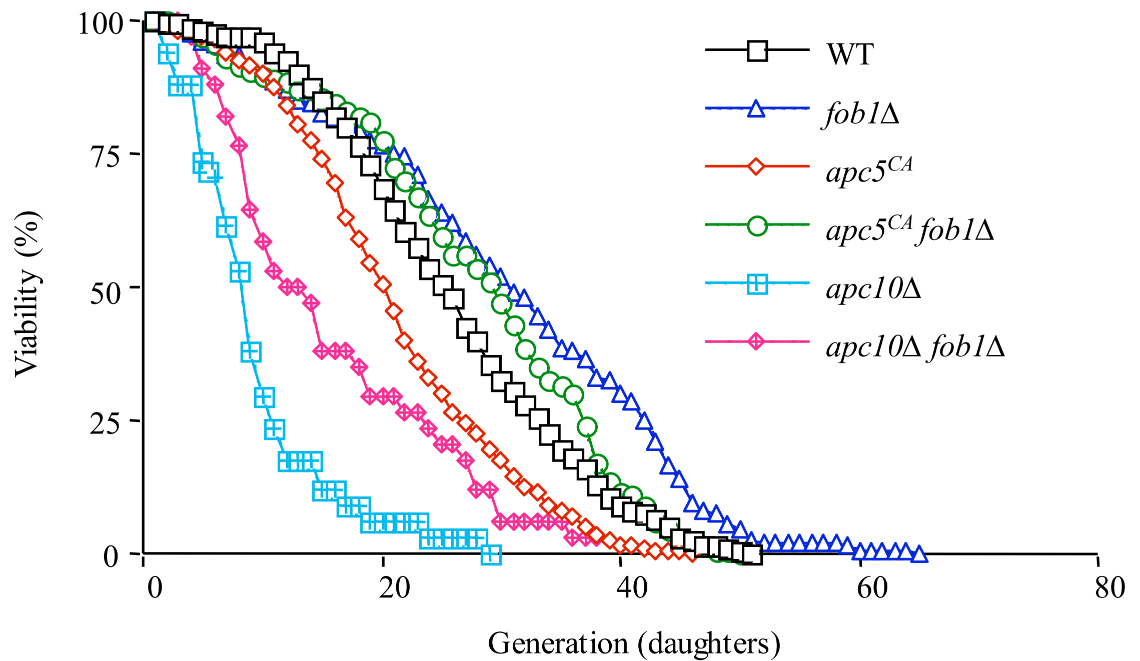


Fig. 3.5 Replicative lifespan of *apc* mutants in the presence and absence of *FOB1*. The wild type curve is the average of 11 separate experiments, the *fob1*Δ and *apc5*^{CA} *fob1*Δ curves are the average of 5 separate experiments, and the *apc5*^{CA} curve is the average of 9 separate experiments. The *apc10*Δ and *apc10*Δ *fob1*Δ were done once. The number of daughters scored for each strain was typically between 30 and 40 for each experiment.

together, these observations support our model that Fob1p is a target of the APC and accumulates in *apc* mutants as a result of compromised APC activity. This ultimately increases the aging process in *apc* mutant cells.

3.2.3 Increased expression of *FOB1* reduces replicative lifespan

To further test our theory that accumulation of Fob1p in *apc* mutants increases the aging process, we expressed *FOB1* in wild type and *apc5*^{CA} cells under the control of the 2µ promoter. Under these conditions, *FOB1* expression is increased as approximately 50 copies of the 2µ plasmid are maintained in cells (ZAKIAN *et al.*, 1979). As predicted, we observed that increased expression of *FOB1* reduces lifespan (Fig. 3.6). Interesting, the lifespan curves of WT and *apc5*^{CA} cells expressing 2µ-*FOB1* is similar, indicating that increased *FOB1* expression is dominant to the *apc5*^{CA} allele.

3.2.4 Overexpression of *FOB1* impairs growth of WT and *apc5*^{CA} cells

The involvement of Fob1p in APC-dependent aging suggests that proper regulation of the rDNA locus, where Fob1p is known to function (KOBAYASHI and HORIUCHI, 1996), may be impaired in *apc5*^{CA} cells. If chromatin in the rDNA locus is misregulated in *apc5*^{CA} cells, then increased expression or increased stability of Fob1p would be predicted to further destabilize the rDNA locus and reduce lifespan to below what is observed when *FOB1* is expressed in wild type cells. It was found that overexpression of Fob1p from the *GAL* promoter, but not increased expression from the 2µ promoter reduced growth of both WT and *apc5*^{CA} cells (Fig. 3.7). The *GAL* promoter

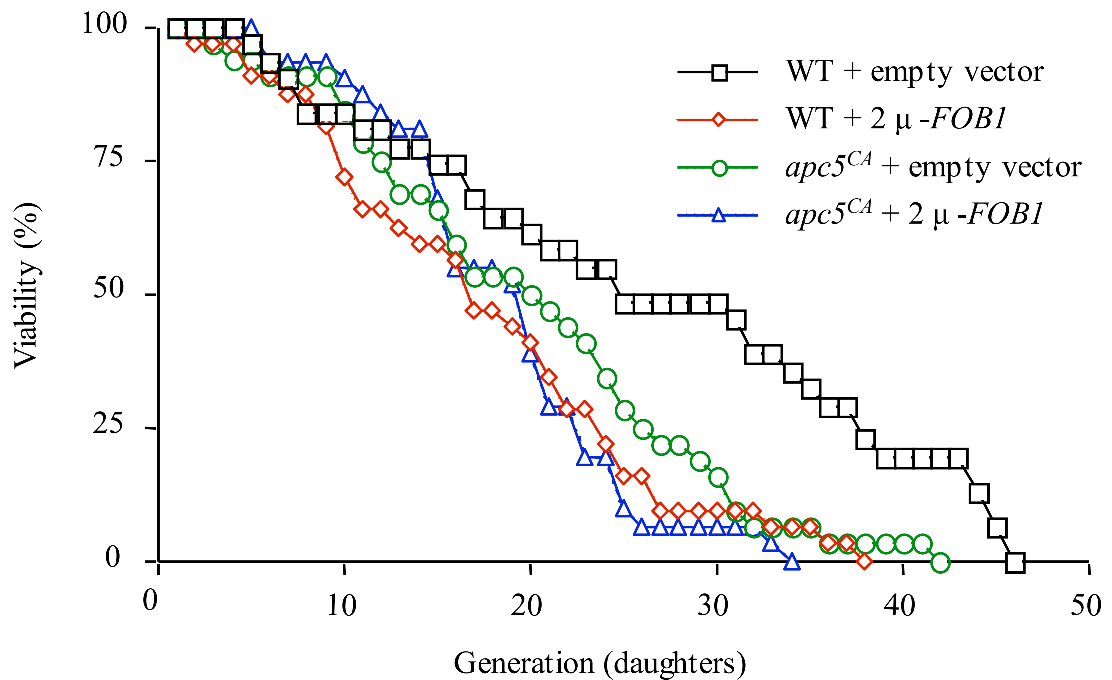


Fig. 3.6 Increased expression of *FOB1* reduces replicative lifespan. The isogenic strains YTH1636 (WT) and YTH1637 (*apc5*^{CA}) were used in this experiment. The strains were transformed with either an empty 2µ vector control or a 2µ vector expressing *FOB1* under the control of its own promoter (a kind gift from TAKEHIKO KOBAYASHI, National Institute for Basic Biology, Okazaki, Japan). The experiment was performed on SD-URA plates to maintain selection of the plasmids. The number of daughter cells scored were WT + empty vector, 31; WT + *FOB1*, 32; *apc5*^{CA} + empty vector, 32; and *apc5*^{CA} + *FOB1*, 32.

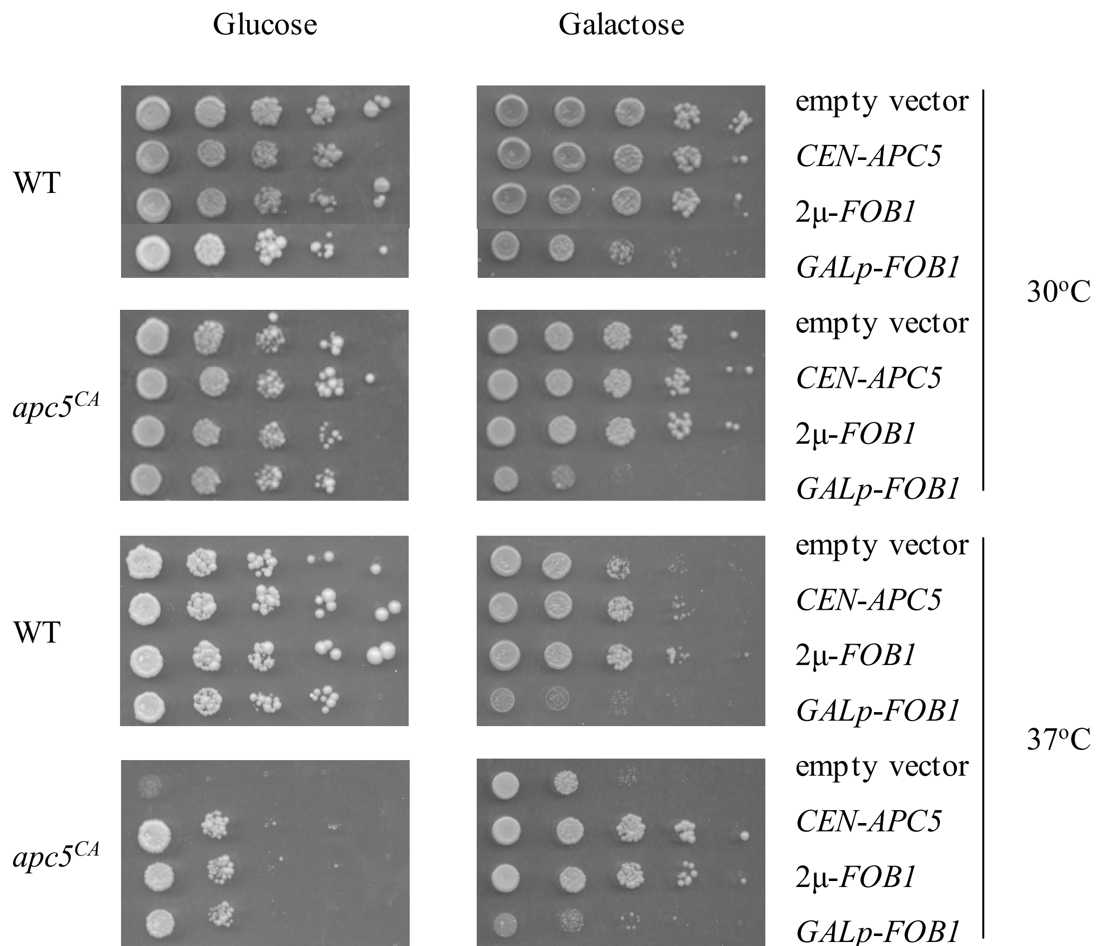


Fig. 3.7 The effect of various levels of *FOB1* expression on cells. *FOB1* was expressed from either a 2μ plasmid under the control of its own promoter or from a 2μ plasmid in which *FOB1* expression was driven by the galactose inducible promoter. Expression from the *GAL* promoter is believed to produce greater quantities of protein than from a 2μ plasmid alone (LAUGHON *et al.,* 1984). YTH1636 (WT) and YTH1637 (*apc5*^{CA}) were transformed with the plasmids shown above, grown overnight in selection media, and then spot diluted onto glucose and galactose supplemented SD-URA plates. The plates were incubated at 30°C and 37°C for 3 to 5 days, and then scanned using an EPSON Perfection 1650 Scanner.

is activated in the presence of galactose and down regulated in the presence of glucose. Thus, the level of *FOB1* expression is lowest from the *GAL* promoter in glucose, higher from the constitutive 2μ plasmid and greatest when the *GAL* promoter is induced with galactose. Interestingly, low level and 2μ expression suppresses the *apc5*^{CA} *ts* defect, even though expression of 2μ-*FOB1* reduces replicative lifespan (Fig. 3.6).

3.2.5 Genetic interaction of *SIR2* and APC

Another key factor functioning at the rDNA locus is Sir2p. In yeast, Sir2p deacetylates histone H3 within the rDNA locus, leading to silencing of the rDNA locus, decreased rDNA recombination, reduced ERC generation and extended lifespan (KAEBERLEIN *et al.,* 1999; TISSENBAUM and GUARENTE, 2001). Fob1p and Sir2p have been shown to act antagonistically at the rDNA locus (Fob1p increases rDNA recombination while Sir2p represses it; JOHZUKA and HORIUCHI, 2002; BENGURIA *et al.,* 2003) and the *fob1*Δ *sir2*Δ mutant has WT lifespan and reduced ERCs (KAEBERLEIN *et al.,* 1999). Furthermore, increased expression of *SIR2* promotes longevity in both yeast and worms (KAEBERLEIN *et al.,* 1999; TISSENBAUM and GUARENTE, 2001). Thus, we predicted that increased *SIR2* expression would benefit the growth of *apc5*^{CA} and *apc5*^{CA} *fob1*Δ mutants. First, overexpression of *GAL-SIR2* in the presence of galactose was toxic at 30°C to all cells tested except the *apc5*^{CA} single mutant (Fig. 3.8). Next, low level expression of *GAL-SIR2* was not toxic to any of the cells tested and suppressed the *ts* growth of *apc5*^{CA} cells. However, in *apc5*^{CA} cells

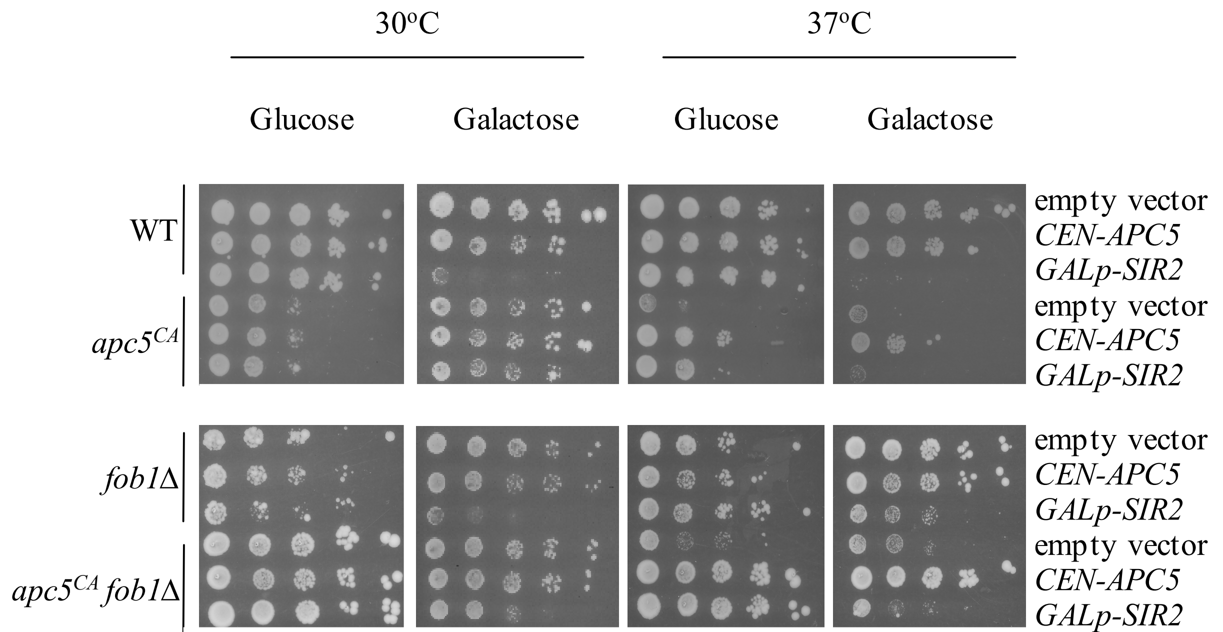


Fig. 3.8 The effect of *SIR2* expression on cells. *SIR2* was expressed from a 2μ plasmid in which *SIR2* expression was driven by the Galactose inducible promoter. YTH3371 (WT), YTH3176 (*apc5*^{CA}), YTH3179 (*fob1*Δ) and YTH3175 (*apc5*^{CA} *fob1*Δ) were transformed with the plasmids shown above, grown overnight in selection media, and then spot diluted onto glucose and galactose supplemented SD-URA plates. The plates were incubated at 30°C and 37°C for 3 to 5 days, and then scanned using an EPSON Perfection 1650 scanner.

lacking *FOB1,* low level expression of *SIR2* completely restored growth to WT levels. Therefore, *SIR2* expression is beneficial to *apc5*^{CA} cells, especially when *FOB1* is deleted. This supports a model where Fob1p and Sir2p have opposite effects on APC function, which ultimately may direct the aging program of the cell (Fig. 3.9).

3.3 Fob1p is degraded in an Apc5p-dependent manner

Our hypothesis focused on the premise that Fob1p serves as a downstream target of the APC (Fig. 3.1). According to this model, Fob1p is targeted for degradation via the APC. Thus, compromised APC function is predicted to allow Fob1p to accumulate in cells, thereby increasing the aging process. The purpose of the next set of experiments was to determine whether Fob1p is indeed an unstable protein *in vivo* and whether Fob1p stability depends on APC activity.

3.3.1 *GAL-FOB1-HA* expressing yeast strain

If Fob1p is targeted by the APC for degradation, then Fob1p is predicted to have increased stability in *apc* mutant cells. To determine whether Fob1p is an unstable protein in yeast we used an arrest/release protocol, which depends on a plasmid expressing an HA tagged *FOB1* gene under the control of the *GAL* promoter (see Fig. 3.7 for details). Fig. 3.10 shows Fob1p-HA is expressed only in galactose supplemental media in WT and *apc5*^{CA} cells. Fob1-HA proteins levels were determined using a Western analysis with antibodies against the HA epitope. A plasmid expressing

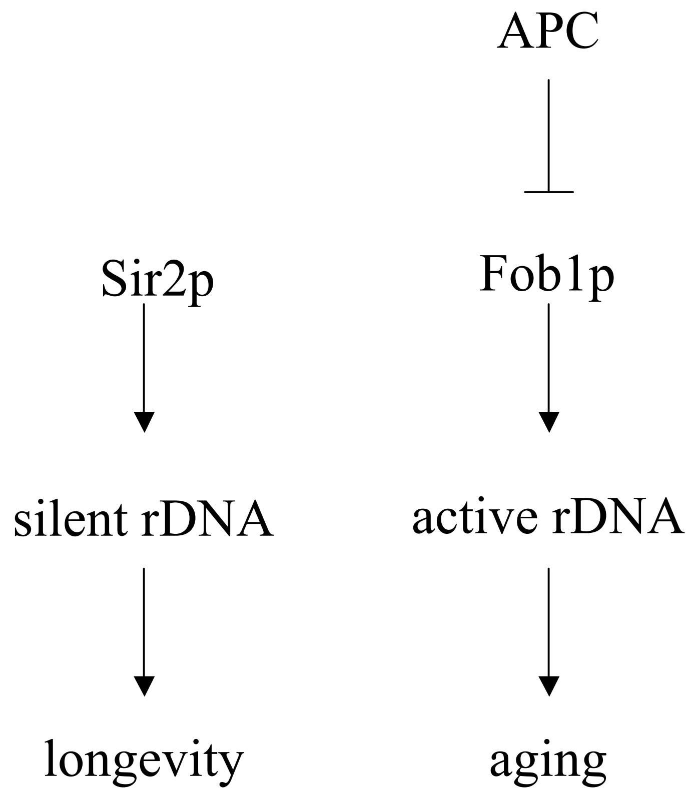


Fig. 3.9 Sir2p and Fob1p have antagonistic effects on rDNA silencing and recombination. Mutations to APC subunits favor Fob1 activity. Increased expression of *SIR2* would therefore increase rDNA silencing and decrease the impact of a defective APC. Increased expression of *SIR2* would have an even greater effect on *apc* mutant cells lacking *FOB1*, as the benefits of Sir2p would no longer be opposed by Fob1p.

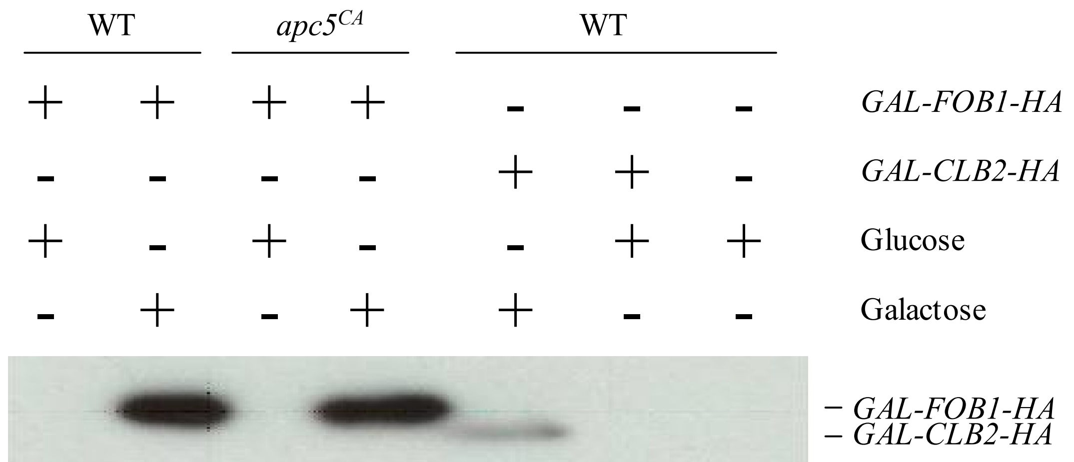


Fig. 3.10 A *GAL-FOB1-HA* plasmid was used as a means to detect Fob1p expression. As shown, Fob1p-HA can be detected in WT and *apc5*^{CA} cells using an antibody against the HA epitope only when grown in the presence of galactose. The positive control, *GAL-CLB2-HA*, was detected under induction of galactose. The negative control, untransformed WT cells (YTH6), does not show a protein band detectable with the HA antibody.

GAL-CLB2-HA under the inducible *GAL* promoter was used as a control. This experiment shows that in unsynchronized steady state cells, overexpressed *GAL-FOB1-HA* is similar in both WT and *apc5*^{CA} cells.

3.3.2 Fob1p is an unstable protein

Since steady state expression of *FOB1* was similar in WT and *apc5*^{CA} cells (Fig. 3.10), we examined cell cycle expression of *FOB1*. Thus, yeast cultures were synchronized in G1 using a 6 hour incubation in 300 mM hydroxyurea (HU). HU is a potent inhibitor of the enzyme ribonucleotide reductase (RNR) and induces a G1 arrest by inhibiting DNA synthesis in a wide variety of cells, including *S. cerevisiae* (SLATER, 1973). HU inhibits DNA synthesis by starving the DNA polymerase at the replication forks for dNTPs (KRAKOFF *et al.,* 1968; KOC *et al.,* 2004). For our first experiments, cells were grown up entirely in galactose to induce *FOB1-HA* expression. Cells were then released into glucose media and turnover of Fob1p-HA was followed every hour. Protein extracts were prepared from the samples taken every hour and resolved using SDS-PAGE. FACS (Fluorescence Activated Cell Sorting) was used to confirm G1 arrest and release into a new round of cell growth. The experiment was initially performed in WT and *apc5*^{CA} cells at 30°C (Fig. 3.11). In WT cells after 4 hours of release, Fob1p levels began to decline, suggesting that Fob1p is turned over (Fig. 3.11A). However in *apc5*^{CA} cells, Fob1p remained stable for at least an additional hour. From our previous results, we observed that overexpression of *FOB1-HA* is toxic to cells (Fig. 3.7).

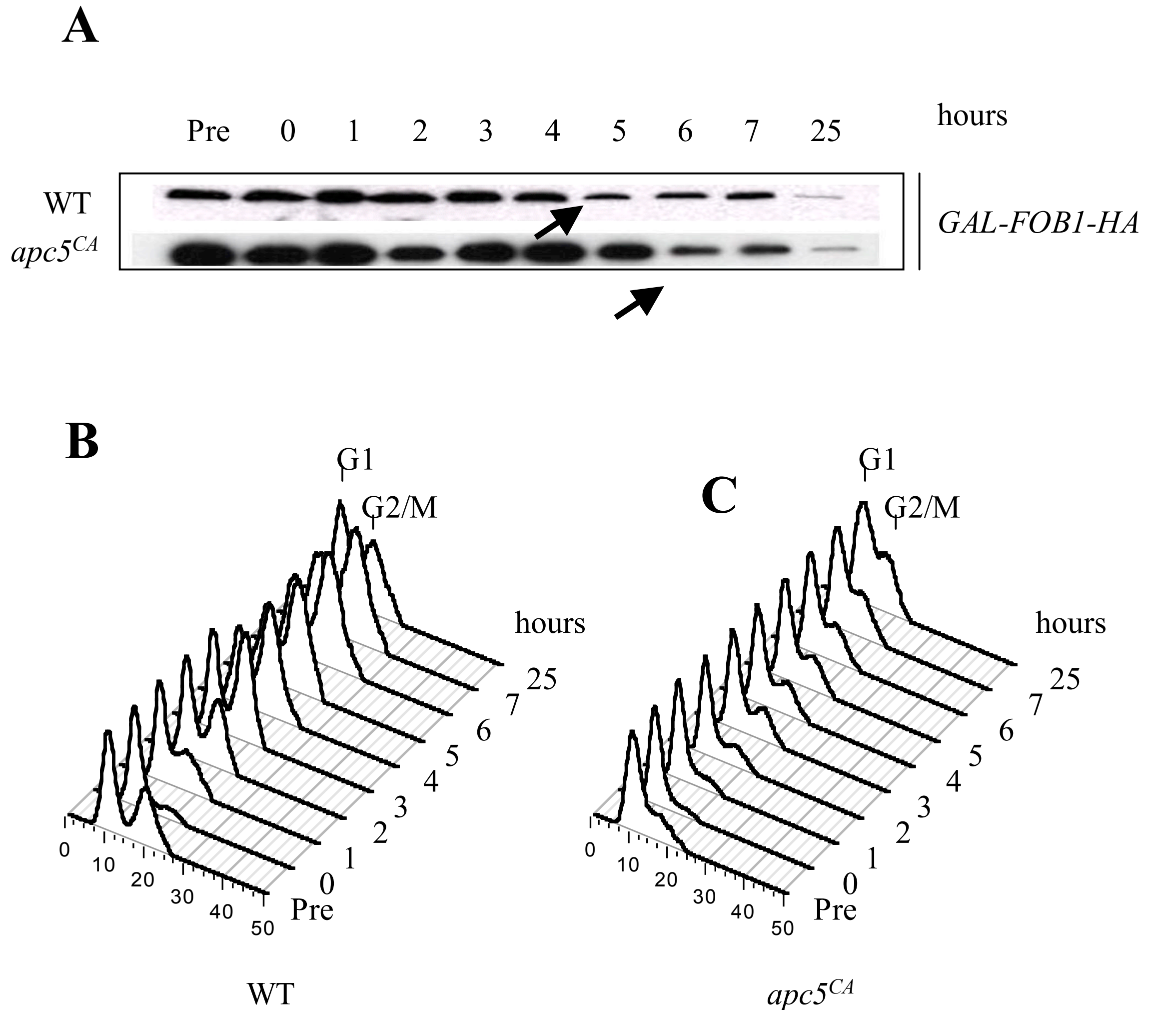


Fig. 3.11 Arrest and release of *GAL-FOB1-HA* expressing cells. **(A)** Cells were grown in galactose, arrested in G1 with 300 mM hydroxyurea (HU) for 6 hours, and then released into glucose at 30°C to induce synchronous re-entry into the cell cycle. Samples were harvested at the times indicated for protein extract preparation and FACS. Fob1p-HA protein levels were monitored by a western analysis with antibodies against the HA epitope. Pre, prior to HU arrest; 0, immediately following release into glucose; 1-25, hours following glucose release. **(B)** FACS profiles of WT cells from experiment in A. G1 refers to cells with unreplicated DNA. G2/M refers to cells with replicated DNA. **(C)** FACS profiles of *apc5^{CA}* cells from experiment in A.

However, WT cells are not as sick when overexpressing *FOB1-HA* as *apc5*^{CA} cells. This was also observed in this experiment (Fig. 3.11B and C). WT cells prior to arrest were actively cycling as shown by G1 and G2/M peaks in the FACS samples. On the other hand, *apc5*^{CA} cells were already arrested in G1 (compare Pre lane in 3.11B with C). This is significant as *apc5*^{CA} cells typically accumulate in G2/M (HARKNESS *et al.,* 2002; 2005; ARNASON *et al.,* 2005). Thus, overexpression of *FOB1-HA* causes *apc5*^{CA} cells to arrest in G1 while having little effect on cell cycle progression in WT cells. Furthermore, release of the WT cells into glucose resulted in cell cycle re-entry. Re-entry into the cell cycle was slow as it took approximately 4 hours to begin degrading Fob1p-HA. In contrast, *apc5*^{CA} cells never re-entered the cell cycle. Therefore, chromic overexpression of *FOB1* in *apc5*^{CA} cells was fatal, and this was not observed to occur in WT cells. This observation is significant, as Fob1p blocks replication through the rDNA and clearly must be removed to allow cell cycle progression. The APC is implicated as Fob1p stability is prolonged in *apc5*^{CA} cells. Moreover, the APC is active in G1, providing a more detailed mechanism of regulation of Fob1p.

3.3.3 Arrest and induction of Fob1p in WT cells

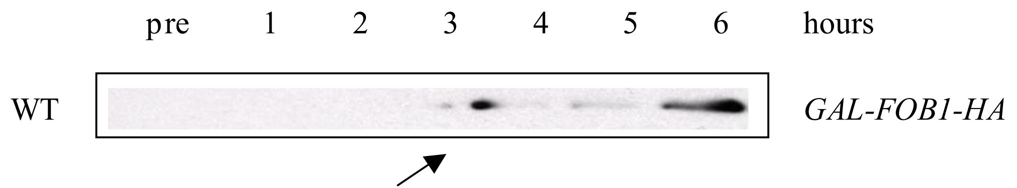
In the previous experiment, the cells were grown in galactose to induce *FOB1-HA* expression, but overexpressed *FOB1* is toxic to cells (Fig. 3.7). Therefore, the experiment was redesigned to first grow the cells in sucrose (to limit expression of *FOB1-HA*), and then add HU and galactose to arrest the cells and induce *FOB1-HA*

expression simultaneously. The cells were grown to an early log optical density (OD_{600} of 0.5), to which HU (300 mM) was added. After 2 hours in HU, galactose was added to 2% for an additional 4 hours. Fob1p was induced and the FACS results confirmed the arrest of the cells in G1 (Fig. 3.12).

3.3.4 Fob1p degradation is delayed in *apc5*^{CA} cells

Upon establishing the arrest and induction protocol (Fig. 3.12), the experimental design was now adapted to include the synchronous release phase. As above, the cells were initially grown in sucrose, HU and galactose were then added to arrest the cells and express Fob1p-HA. The cells were then collected by centrifugation, and the HU and galactose were washed away and 2% glucose supplemented media was added to repress expression of *FOB1-HA* and allow the cells to re-enter the cell cycle. The cultures were then split, with one half incubated at 30°C and another half at 37°C. Samples were taken every hour for protein analysis up to 6 hours. When the experiment was performed with all steps at 30°C (Fig. 3.13A), Fob1p-HA levels steadily decline in WT cells after 1 hour of release up to 5 hours when most of the protein is gone. However, in *apc5*^{CA} cells at 30°C, Fob1p-HA levels remained high, providing evidence that Fob1p is indeed turned over in at least an Apc5p-dependent manner. When the cells were shifted to 37°C, following arrest and expression at 30°C, degradation of Fob1p-HA was again much more rapid in WT cells compared to *apc5*^{CA} cells (Fig. 3.13C and D).

A



B

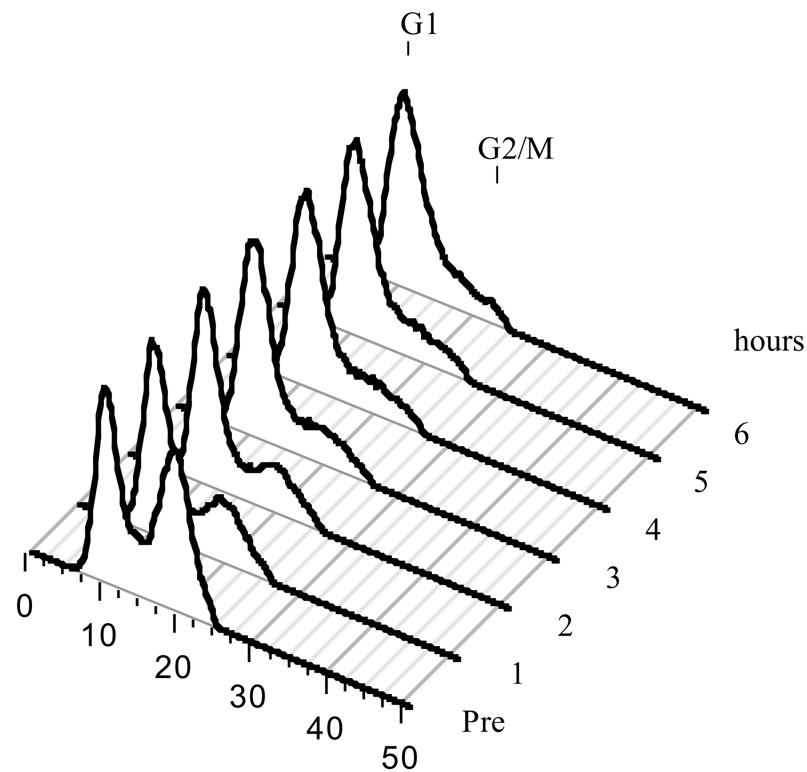


Fig. 3.12 Arrest and expression of *GAL-FOB1-HA* in WT cells. **(A)** Cells were grown overnight in sucrose-supplemented media. HU (300mM) was then added to the cultures. After 2 hours in HU, galactose was added to 2% for an additional 4 hours for a total of 6 hours. **(B)** FACS was used to follow the state of cell cycle arrest. Pre is the 0 time point of HU arrest, 1-6 hours each indicates the time point post HU addition.

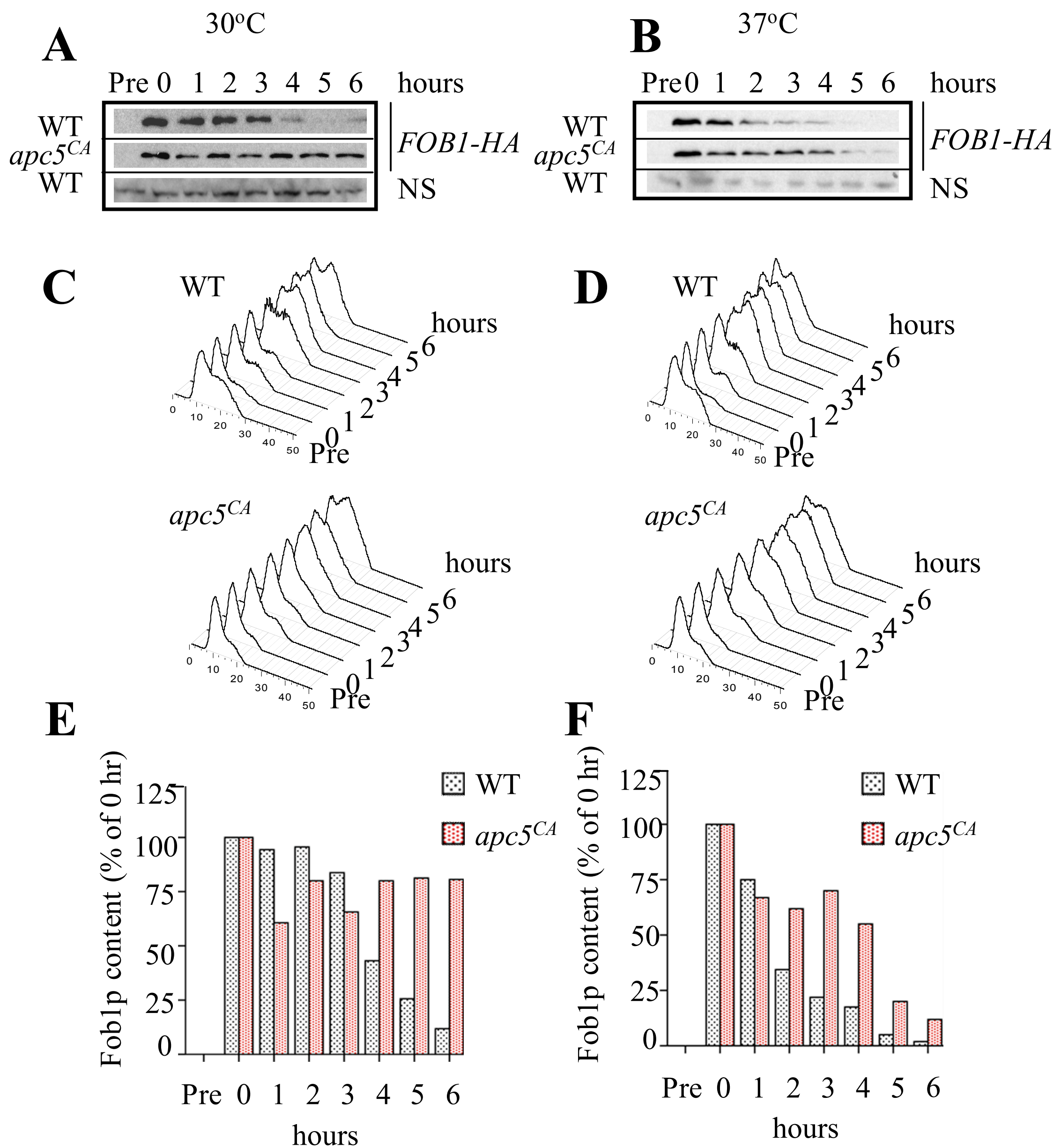


Fig. 3.13 Fob1p degradation is delayed in *apc5^CA* cells. **(A)** Arrest and release of Fob1p-HA in WT and *apc5^CA* at 30°C. Levels of Fob1p-HA continue to decline in WT cells, but remain stable in *apc5^CA* cells. Levels of a NS (non-specific) protein in WT cells are included as a control for protein load. **(B)** Arrest and release of Fob1p-HA in WT and *apc5^CA* at 37°C. Fob1p-HA stability was increased in *apc5^CA* cells compared to WT cells. Levels of a NS (non-specific) protein in WT cells are included as a control for protein load. **(C, D)** FACS was used to follow the state of cell cycle arrest. **(E, F)** The densitometry of Fob1p-HA in WT and *apc5^CA* cells at 30 and 37°C, respectively, was measured using NIH image 1.63. Pre, prior to HU arrest; 0, immediately following release into glucose; 1-6, hours following glucose release. The G1 and G2/M peaks are the same as those depicted in Fig. 3.12.

3.3.5 The severe *apc* mutants, *apc10*Δ and *cdc16-1*, are hypersensitive to even low *FOB1* expression

To examine the effect of *FOB1* on other *apc* mutants, we attempted to repeat the arrest/release experiments in *apc10*Δ *and cdc16-1* cells; however these cells were hypersensitive to any increase in Fob1p expression (Fig. 3.14) and would not grow under any condition, including sucrose and glucose, where very little *FOB1* is presumed to be made, when harboring the *GAL-FOB1-HA* plasmid. *apc10*Δ and *cdc16-1* cells arrest at elevated temperatures at the metaphase transition as large budded cells. Furthermore, deletion of *CDC16* is lethal. Therefore, our observation that even weak expression of *FOB1* is lethal to *apc10*Δ and *cdc16-1* cells supports the notion that the inability to clear Fob1p from the cell blocks cell cycle progression.

Taken together, our data strongly supports a model whereby the *APC* promotes longevity by targeting Fob1p, a known promoter of aging, for degradation (Fig. 3.1)

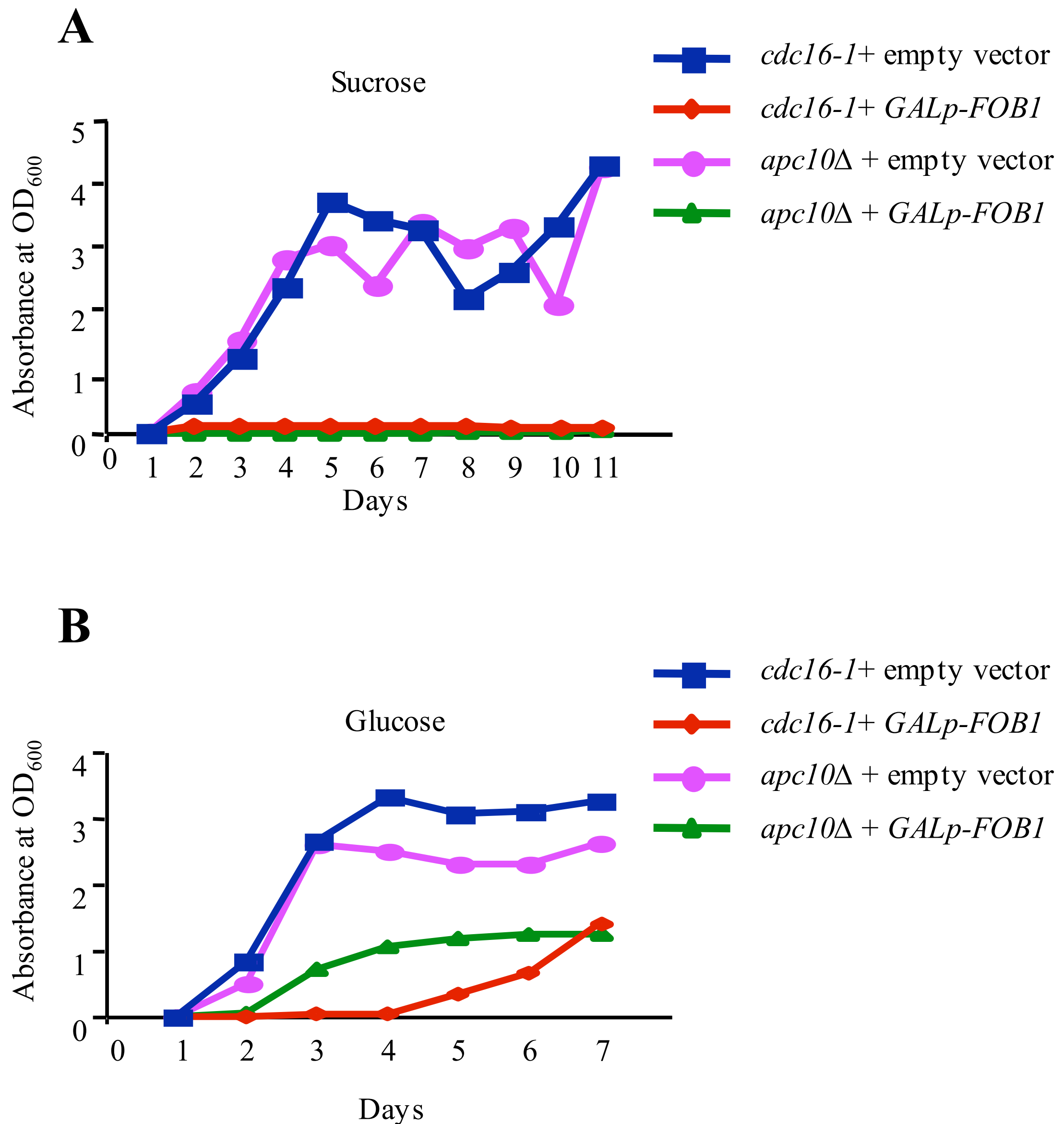


Fig. 3.14 The severe *apc* mutants (*apc10*Δ *and cdc16-1)* are hypersensitive to even low level *FOB1* expression. Cells density OD_{600} was determined every 24 hours for the duration of this experiment. *FOB1* was expressed from a 2μ plasmid in which *FOB1* expression was driven by the galactose promoter. (**A)** Cells were grown in sucrose for 11 days. **(B)** Cells were grown in glucose for 7 days. Glucose represses the *GAL* promoter, whereas sucrose does not. We expect less expression of *GALp-FOB1* when grown in glucose versus sucrose.

CHAPTER FOUR: DISCUSSION

The results described in this study allowed us to make multiple key observations. i) The C-terminus of Fob1p is required for the interaction with Apc5p. However the interaction of the C-terminus of Fob1p and Apc5p is appreciably weaker than the interaction between full length Fob1p and Apc5p. Thus, the 3-D structure of Fob1p may be necessary for its interaction and essential functions. ii) Our genetic studies revealed that deletion of *FOB1* in *apc5*^{CA} cells had no effect on growth, but deletion of *FOB1* partially suppressed *apc10*Δ *ts* growth. iii) Deletion of *FOB1* in *apc5*^{CA} and *apc10*Δ cells extended replicative lifespan, while increased expression of *FOB1* reduced it. iv) Overexpression of *FOB1* impaired growth of WT and *apc5*^{CA} cells. Furthermore, we found that the severe *apc* mutants, *apc10*Δ and *cdc16-1,* are hypersensitive to even low level *FOB1* expression. v) We showed that an additional key factor that functions at the rDNA locus, Sir2p, effected APC function in a manner that is antagonistic to Fob1p. And lastly, vi) our cell cycle and steady state analysis of Fob1p revealed that Fob1p is an unstable protein, which is targeted for degradation in an Apc5p-dependent manner.

4.1 C-terminal half of Fob1p is responsible for interaction with Apc5p *in vivo* in yeast two-hybrid assays

The yeast two-hybrid system has proven invaluable for identifying interactions

between proteins since its inception (FIELDS and SONG, 1989; CHIEN *et al.*, 1991). Yeast two-hybrid analyses provide a method to investigate weak and transient interactions which are difficult with conventional assays. In this study, after the confirmation of an Apc5p-Fob1p interaction in yeast two-hybrid assays, we found that the C-terminal half of Fob1p can interact with Apc5p *in vivo* (Fig. 3.2). However, the interaction between the C-terminal half of Fob1p with Apc5p is appreciably weaker than the interaction between full length Fob1p and Apc5p. This weak interaction has been observed in multiple experiments. This may reflect that these Fob1p domains are weakly expressed or unstable. Alternatively, a full interaction with Apc5p may involve a 3-dimensional motif made up multiple regions of Fob1p. The structure in the Fob1p C-terminal half may have reasonable similarity to the full structure, thus supporting a weak interaction. These possibilities await investigation.

The results (Fig. 3.2B) showed that D2 and D3 of Fob1p cannot interact with Apc5p, which indicated the overlap region between D2 and D3 defines the Apc5p binding domain for Fob1p. As mentioned previously, Fob1p is not conserved in higher eukaryotes, however, motifs within Fob1p, especially the C-terminal half domain of Fob1p shares sequence identity with many proteins from yeast to humans (Fig. 3.2A). This region was analyzed by a BLAST homology search, which identified several human proteins of interest, including Akt substrate AS250, PEG3, tripartite motif-containing protein 26, NEDD4-like ubiquitin ligase 1, and Ubiquitin-specific protease 31. The interest in these proteins is described below.

A 103 amino acid motif in the human Akt substrate AS250, a novel 250 kDa

protein in adipocytes that is a substrate for the insulin-activated protein kinase Akt (GRIDLEY *et al.*, 2006), is 24% identical with amino acids 1338-1620 of Fob1p. The only distinctive domain in AS250 is a predicted GTPase activating protein (GAP) domain at its carboxy terminus (amino acids 1510-1873), which does not overlap with Fob1p related domain (amino acids 757-859). The activation of the protein kinase Akt is involved in the cell survival pathway and in tumor progression. Our recent experiments suggest the yeast AKT orthologue, Sch9p, inhibits APC activity (WONG and HARKNESS, unpublished data). Thus, upon phosphorylation, AS250 may interact with the APC as a means to inhibit APC activity.

A 63 amino acid motif in the human protein PEG3 (paternally expressed gene 3) is 31% identical with amino acids 1398-1593 of Fob1p, and another 89 amino acid motif in ZNF341 (Zinc finger protein 341) is 25% identical with amino acids 1404-1638 of Fob1p. Both PEG3 and ZNF341 contain zinc finger DNA-binding domains. A zinc finger is a protein domain that can bind to DNA. Those proteins contain two antiparallel β strands, and an α helix. One very well explored subset of zinc-fingers comprises a pair of cysteine residues in the beta sheets and two histidine residues in the alpha helix which are responsible for binding a zinc ion. As DNA binding proteins, several scenarios of action can be envisioned. For example, perhaps they play a role in gene activation within the human rDNA locus that is inhibited by APC activity. Or, perhaps, these DNA binding proteins recruit the APC to active region of chromatin that are silenced in part through APC activity.

A 63 amino acid motif in the human tripartite motif-containing protein 26 (Zinc

finger protein 173; Acid finger protein; AFP; RING finger protein 95), is 24% identical with amino acids 1326-1533 of Fob1p. It contains not only a Zinc finger domain, but also a RING finger domain. The RING finger domain can basically be considered a protein interaction domain. RING finger proteins have been implicated in a range of diverse biological processes from transcriptional regulation to targeted proteolysis (BORDEN, 2000). One class of ubiquitin ligases contains catalytic subunits with a RING finger domain. As mentioned previously, Apc11p, the catalytic APC subunit, is one such RING finger protein. Hence, this protein may facilitate an interaction between the APC and a target protein.

A 104 amino acid motif in the human NEDD4-like ubiquitin ligase 1 is 24% identical with amino acids 1353-1623 of Fob1p. Human NEDD4-like ubiquitin ligase 1 is an orthologue of the yeast Rsp5p. Rsp5p and the Nedd4-like E3 belong to the HECT domain-containing family of E3 proteins (HUIBREGTSE *et al.*, 1995; HARVEY and KUMAR, 1999). The HECT domain is critical for ubiquitin ligase activity and contains a conserved cysteine residue that can form a thioester with ubiquitin. Rsp5p and Apc5p were found to interact in an intracellular pathway regulating chromatin remodeling (ARNASON *et al.*, 2005). Therefore, like Fob1p, the NEDD4-like E3 may interact with the APC.

A 64 amino acid motif in the human Ubiquitin-specific protease 31 (USP31), is 31% identical with amino acids 1479-1662 of Fob1p. Human USP31 is an orthologue of the yeast Ubp3p, which is a deubiquitination enzyme and a member of a large family of cysteine proteases that cleave ubiquitin moieties from protein substrates. Ubp3p

associates with Sir2p, both are essential for gene silencing and both interact with the amino terminal segments of histone H3 and H4 (BREW and HUFFAKER, 2002). Thus, USP31 may facilitate APC activities that control genome silencing.

The subset of proteins listed above suggests that the motif expressed in the C-terminal half of Fob1p may serve as an APC interaction domain employed across evolutionary boundaries. This hypothesis could be tested by yeast two-hybrid assays, genetic interaction analyses or *in vivo* coimmunoprecipitation analysis. The implications of these putative interactions would support the significance of the Apc5p-Fob1p interaction, as an evolutionarily conserved design that mediates aging.

4.2 Genetic interaction analysis reveals the influence of *FOB1* on APC

To genetically characterize the interaction of Apc5p with its potential downstream target, Fob1p, a series of genetic analyses were performed. First, the *FOB1* gene was deleted in congenic WT, *apc5*^{CA} and *apc10*Δ cells. The *apc5*^{CA} *fob1*Δ and *apc10*Δ *fob1*Δ double mutants were compared with WT, and the single *apc5*^{CA}, *apc10*Δ and *fob1*Δ mutants with regard to growth rate on different medias and at various temperatures. If accumulation of Fob1p in *apc5*^{CA} cells is at the heart of the *ts* and reduced lifespan phenotypes associated with *apc5*^{CA}, then we expected that these phenotypes would be suppressed when *fob1*Δ and *apc5*^{CA} were combined. To our surprise, the disruption of *FOB1* had no effect on the *apc5*^{CA} growth phenotype (Fig. 3.4). However, deletion of *FOB1* did partially suppress the *apc10*Δ growth phenotype (Fig. 3.4). This is consistent with disruption of *APC10* being more severe than the *apc5*^{CA}

mutation. Thus, perhaps WT levels of Fob1p are more deleterious to *apc10*Δ cells than to *apc5*^{CA}, explaining why deletion of *FOB1* had no effect on the growth of *apc5*^{CA} cells.

Although deletion of *FOB1* did not fully suppress the *apc5*^{CA} or *apc10*Δ growth defects, we continued to investigate whether Fob1p expression influenced the lifespan of WT and *apc* mutant cells. A replicative lifespan analysis revealed that, as we predicted, deletion of *FOB1* extended lifespan in *apc5*^{CA} and *apc10*Δ cells. On the other hand, increased expression of *FOB1* decreased replicative lifespan in *apc5*^{CA} and WT cells (Fig. 3.6). These telling observations are consistent with our hypothesis that deletion of *FOB1* suppresses the reduced lifespan observed in *apc* mutant cells, and that accumulation of Fob1p in *apc* mutants increases the aging process.

We found that extreme overexpression of *FOB1* from the *GAL* promoter in the presence of galactose reduced growth of both WT and *apc5*^{CA} cells. However, low level (from *GAL* promoter in the presence of glucose) and medium level (from the constitutive 2μ plasmid) suppressed the *apc5*^{CA} *ts* defect (Fig. 3.7). Interestingly, even low level expression of *FOB1* was toxic to the severe *apc* mutants, *apc10*Δ and *cdc16-1* (Fig. 3.14). The toxic effects of *FOB1* overexpression is in harmony with our proposal that Fob1p must be targeted for degradation by the APC for normal cell function. This is supported by our finding that *FOB1* overexpression blocks cell cycle progression in G1 in *apc5*^{CA} cells, but not in WT (Fig. 3.11). However, the observation that lower levels of *FOB1* expression suppressed the *apc5*^{CA} growth defect is intriguing (Fig. 3.7). This observation requires future investigation but it could involve Sir2p. Sir2p silences and stabilizes the rDNA locus. Thus, increased *FOB1* expression may stabilize, or optimize,

a Fob1p/Sir2p interaction within rDNA locus that is impaired in *apc5*^{CA} cells.

4.3 Low level expression of *SIR2* and deletion of *FOB1* are required for full suppression of the *apc5*^{CA} growth defect

The Sir2 protein was demonstrated to play a critical role in facilitating the effects of caloric restriction (GUARENTE, and KENYON, 2000; LIN *et al.*, 2000). Sir2p was subsequently shown to be a histone deacetylase required to silence the rDNA locus, which inhibits rDNA recombination (SMITH and BOEKE, 1997; FRITZE *et al.*, 1997; IMAI *et al.*, 2000). Inhibition of rDNA recombination reduced the generation of ERCs, which is significant since the accumulation of ERCs in yeast cells is correlated with accelerated aging (SINCLAIR *et al.*, 1997; DEFOSSEZ *et al.*, 1999). Accordingly, a *sir2*Δ mutation resulted in reduced lifespan and increased expression of Sir2p extended lifespan of both yeast and worms (KAEBERLEIN *et al.*, 1999; TISSENBAUM and GUARENTE, 2001). As already discussed, Fob1p stalls progression of the replication fork through the rDNA locus, and subsequently increases rDNA recombination and ERC accumulation (KOBAYASHI and HORIUCHI, 1996; DEFOSSEZ *et al.*, 1999). Cells lacking the gene encoding Fob1p live longer and the *fob1*Δ mutation suppressed the reduced lifespan observed in *sir2*Δ mutants (KAEBERLEIN *et al.*, 1999; DEFOSSEZ *et al.*, 1999). Thus, silencing of the rDNA locus by Sir2p extends lifespan, whereas Fob1p destabilizes the rDNA locus, resulting in reduced lifespan. The research in this study suggests that in *apc* mutants, Fob1p activity is increased, which would lead to increased rDNA instability. On the other hand, increased expression of *SIR2* would be predicted to

increase rDNA silencing and stability, thus decreasing the impact of a defective *APC*. This is what we observed, as *SIR2* overexpression is toxic to WT and *fob1*Δ cells at all temperatures tested, but not to *apc5*^{CA} cells (Fig. 3.8). Furthermore, weak expression of *SIR2* partially suppresses the *apc5*^{CA} growth defect and completely restores growth of the *apc5*^{CA} *fob1*Δ mutants. The toxic effects of *SIR2* overexpression in WT cells, but not *apc5*^{CA} cells could reflect the inability to replicate through the rDNA locus when *SIR2* activity is too high. In *apc5*^{CA} cells, the effects of higher than normal Fob1p levels require higher than normal levels of *SIR2* activity. This study suggests that Sir2p and Fob1p have antagonistic effects on APC function, and ultimately may direct the aging program of the cell.

4.4 Fob1p, an unstable protein, is targeted for degradation by Apc5p to prolong lifespan in yeast

As suggested by our hypothesis in this study, the APC likely prolongs lifespan by targeting a protein for degradation that normally acts to accelerate aging. Cell cycle dependent stability analyses of Fob1p were therefore performed to determine whether Fob1p is indeed targeted by the APC for degradation. The arrest/release experiments, Western and FACS analyses all lead to the conclusion that Fob1p is an unstable protein. The conclusive link to APC activity was provided when we observed that the degradation of Fob1p is delayed in *apc5*^{CA} cells. This finding is consistent with the model that, if Fob1p is targeted by APC for degradation, then comprised APC function will allow Fob1p to accumulate in cells. Further study is required to confirm

the APC-dependent ubiquitination of Fob1p.

Overall, the results demonstrated in this study that APC targets Fob1p for degradation in order to prolong lifespan in yeast is a novel finding in the literature. Another protein, Geminin, an inhibitor of DNA replication initiation, which is found universally in vertebrates and in *Drosophila* but is absent from yeasts and the nematode *C. elegans*, was shown to be degraded by the APC during mitosis (McGARRY and KIRSCHNER, 1998). Even though Geminin is not the homologue or orthologue of yeast Fob1p, it might be analogous to Fob1p.

The data illustrated in this study allowed us to provide additional support linking the APC to longevity. As formerly discussed, CR has been undoubtedly demonstrated to extend lifespan in virtually all system studied (WEINDRUCH and WALFORD, 1988). In mammalian cells and also budding yeast, CR extends lifespan, partially, by increasing the activity of Sir2p (KAEBERLEIN *et al.,* 1999; ALBERTO *et al.*, 2003). Sir2p and Fob1p were found to act antagonistically at the rDNA locus, where Sir2p represses rDNA recombination, while Fob1p increases it (JOHZUKA and HORIUCHI, 2002; BENGURIA *et al.,* 2003). The suppression of rDNA recombination reduces the production of ERCs, which is linked to aging in yeast. APC, which is essential for prolonged lifespan, is repressed by glucose (KOTANI *et al.,* 1998; IRNIGER *et al.,* 2000), which suggests the possibility that APC activity is induced by CR. Our lab found that the Snf1p kinase, known to be required for longevity (LIN *et al.,* 2003), promoted APC activity by inhibiting the action of the Mig1p transcriptional repressor, which blocked the expression of at least *APC4* and *APC9* (HARKNESS *et al.,* 2004). Snf1p,

the yeast orthologue of the human AMP kinase (WOODS *et al.,* 1994; MOMCILOVIC *et al.,* 2006), is vital when substitute carbon sources are utilized, such as under conditions of CR. Our story comes full circle as deletion of *FOB1* was recently shown to restore normal lifespan to cells harboring a deletion of the *SIP2* gene, which is essential for Snf1p activity (LIN *et al.,* 2003). It was not understood why deletion of *FOB1* would suppress the short lifespan of *sip2*Δ cells, but our work shows that in the absence of functional Snf1p, the APC is not fully active (HARKNESS *et al.,* 2004). Therefore, Fob1p would be expected to accumulate in *sip2*Δ cells (Fig. 3.15).

The results established in this study provide a possible mechanism linking the APC not only to longevity, but possibly, through Sir2p and Snf1p, to CR. The mechanistic action of CR in higher eukaryotes is presently a matter of hot debate (SINCLAIR, 2005). Our ability to link APC function to Sir2p in this study and to Snf1p in a prior study, suggests a solid foundation upon which to examine the role of the APC in human aging. Thus, the molecular mechanisms regulating longevity and CR in yeast likely utilize highly conserved proteins, such as Sir2p, Snf1p and the APC (Fig. 3.15).

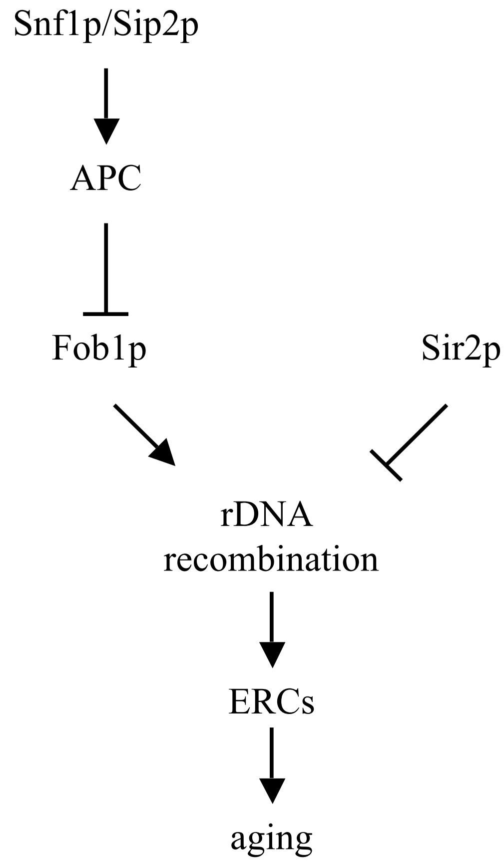


Fig. 3.15 Snf1p activated APC suppresses the effect of aging by targeting Fob1p for degradation. Sir2p and Fob1p were found to act antagonistically at the rDNA locus, where Sir2p represses rDNA recombination, while Fob1p increases it. The APC is not fully activated in the absent of Snf1p, which explains Fob1p accumulates in *sip2*Δ cells. Adapted from HARKNESS, 2006.

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