THE FORKHEAD BOX TRANSCRIPTION FACTORS, *FKH1* AND *FKH2*, ALONG WITH THE ANAPHASE-PROMOTING COMPLEX REGULATE *SACCHAROMYCES CEREVISIAE* LIFESPAN

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ABSTRACT

Forkhead box (Fox) transcription factors have a conserved function in regulating lifespan and onset of age related disease in organisms from worms to mammals. Key functions in this process are the regulation of the cell cycle, oxidative stress response, and apoptosis. A complex post-translational code from nutrient, growth factor, and stress induced signals regulates Fox activity, target specificity, stability, and subcellular localization; however, many of the Fox mechanisms and targets responsible for regulating lifespan remain elusive. The budding yeast, *Saccharomyces cerevisiae*, is a powerful model for unravelling the genetic mechanism and pathways. Yeast encodes four Fox transcription factors, Fkh1, Fkh2, Fhl1 and Hcm1, and their roles in aging are only recently being examined. In this study, we utilized the chronological lifespan and oxidative stress assays, to explore evolutionary conservation of lifespan regulation in two of the yeast Fox orthologs, *FKH1* and *FKH2*. We observed that deletion of both *FKH* genes in *S. cerevisiae*, impedes normal lifespan and stress resistance. Furthermore, *fkh1*Δ *fkh2*Δ cells were found to be non-responsive to caloric restriction, an intervention that extends lifespan from yeast to mammals. Conversely, increased expression of the *FKHs* leads to extended lifespan and improved stress resistance. Additionally, we show the Anaphase-Promoting Complex (APC) genetically interacts with the *FKHs*, likely functioning in a linear pathway under normal conditions, as *fkh1*Δ *fkh2*Δ post-mitotic survival defect is epistatic to that observed in $apc5^{CA}$ mutants. However, under stress conditions, post-mitotic survival is dramatically impaired in $apc5^{CA}$ *fkh1*Δ *fkh2*Δ beyond either $apc5^{CA}$ or *fkh1*Δ *fkh2*Δ. Finally, we observed that both the *FKHs* and APC genetically interact with nutrient-responsive lifespan-regulating kinase encoding genes *SCH9* and *TOR1*. This study establishes that the yeast *FKHs* play a role as regulators of lifespan in yeast and identifies the APC as a novel component of this mechanism. We speculate this involves combined regulation of stress response, genomic stability, and cell cycle.

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LIST OF ABBREVIATIONS

Δ	gene deletion
°C	degrees Celsius
4E-BP	eIF4E binding protein
AGC	Protein kinase A, G, and C family
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
APC	Anaphase-Promoting Complex
ATP	adenosine triphosphate
bp	base pairs
C. elegans	*Caenorhabditis elegans*
CA	chromatin assembly defective
cAMP	cyclic adenosine monophosphate
Cdc	Cell division cycle
Cdh	Cdc20 homolog
CDK	Cyclin dependent kinase
CDKI	CDK inhibitor
CFU	colony forming unit
ChIP	Chromatin IP
Clb	Cyclin B
CLS	chronological lifespan (assay)
CoIP	co-immunoprecipitation
CR	caloric restriction
D. melanogaster	*Drosophila melanogaster*
DM	depleted media
DNA	deoxyribonucleic acid
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin-protein ligase
eIF	eukaryotic initiation factor
EDTA	ethylenediaminetetraacetic acid
FBD	forkhead box DNA biding domain
FHA	forkhead associated domain
Fhl	Forkhead-like
Fkh	Forkhead homolog
Fox	Forkhead box containing
FoxA	Fox subfamily A
FoxM	Fox subfamily M
FoxO	Fox subfamily O

G1	gap 1 of the cell cycle
G2	gap 2 of the cell cycle
G418	geneticin; antibiotic agent similar to kanamycin
Gal	galactose
Gis	Gig1-2 suppressor
Glc	glucose (dextrose)
GTP	guanine triphosphate
H_2O_2	hydrogen peroxide
HAT	Histone (protein) acetyltransferase
Hcm	High-copy suppressor of calmodulin
HDAC	Histone (protein) deacetylase
Hog	High osmolarity glycerol response
IGF	Insulin-like growth factor
InsR	Insulin receptor
IP	immunoprecipitation
JNK	c-Jun N-terminal kinase
KanMX	kanamycin/G418 resistance selective marker
M	mitosis/chromosome segregation
MAPK	Mitogen activated protein kinase
Mcm	Minichromosome maintenance
MnSOD	Manganese SOD
mRNA	messenger RNA
Msn	Multicopy suppressor of SNF1 mutation
MST	Mammalian ortholog of ste20
NaOH	sodium hydroxide
Ndd	Nuclear division defective
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBST	phosphate buffered saline with Tween-20
PCR	polymerase chain reaction
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PKA	Protein kinase A
PP2A	Protein phosphatase 2 A
Prom	promoter
Ras	Rat sarcoma/homologous to RAS proto-oncogene
RING	really interesting new gene E3 domain
RLS	replicative lifespan (assay)
RNA	ribonucleic acid
ROS	reactive oxygen species

rpm	rotations per minute
rDNA	ribosomal DNA
rRNA	ribosomal RNA
S	S phase of the cell cycle; DNA replication
S6K	ribosomal protein S6 kinase
S. cerevisiae	*Saccharomyces cerevisiae*
SAGA	Spt-Ada-Gcn5 acetyltransferase complex
SCF	Skp/Cullin/F-box
SD	synthetically defined media
SDC	SD with complete supplementation of amino/nucleic acids
SD^{Ura-}	SD without uracil
SDS	sodium dodecyl sulfate
Sir	Silent information regulator
Sirtuin	Sir2 orthologs
Skp	Suppressor of kinetochore protein mutant
Snf	Sucrose non-fermenting
SOD	Superoxide dismutase
TAE	Tris-base, glacial acetic acid, EDTA
TAP	tandem affinity purification
Tap	Two A phosphatase associated protein
TCA	trichloroacetic acid
Tor	Target of rapamycin
TORC	Tor complex
tRNA	transfer RNA
WT	wild-type
YPD	yeast extract, peptone, dextrous media

1 Introduction[1]

1.1 Aging

Throughout history humans have sought to understand the causes and conditions for aging and organismal senescence. Studies as early as the 1930s demonstrated that lifespan and health span of laboratory animals can be lengthened by environment interventions, such as caloric restriction [CR; (McCay and Crowell, 1934)], suggesting aging includes a modifiable genetic/molecular component, rather than existing purely as an inevitable and passive accumulation of damage. Relatively recently, genetic and biochemical studies have provided insight into the complex mechanisms regulating these processes. Cell health is regulated by nutrient, stress, and growth factor signaling pathways controlling growth, repair, survival, division, arrest, and replacement. Our current understanding also suggests the health of an organism is directly related to the fitness of its organs, tissues, cells, and ultimately its sub-cellular constituents. Genomic stability (the ability of the cell to resist mutation, chromatin irregularities, etc.) and protein maintenance/turnover are dominant forces in preventing cellular/tissue senescence or uncontrolled growth and tumour formation. Our understanding of processes that increase or decrease cellular health span is enhanced through the identification of gene products influencing the incidence of tissue degeneration and age-related diseases, such as Alzheimer's, diabetes, and cancer (Jia et al., 2012; Kloet and Burgering, 2011; Postnikoff and Harkness, 2012; Salminen and Kaarniranta, 2012; Stünkel and Campbell, 2011; Ziv and Hu, 2011).

Recent studies have shed more light on the complexity of CR as a lifespan altering mechanism. In rhesus monkeys, CR induces conflicting results on survival (the ultimate lifespan of the organism) and health span (the development of age related decrease in fitness). When compared to an *ad libitum* laboratory diet, a 30% reduction of caloric intake both extends lifespan and reduces the rates of age related diseases, such as cancer and diabetes (Bodkin et al., 2003; Colman et al., 2009). However, in a study featuring a more nutritionally complete diet, CR increased health indicators, but not survival (Mattison et al., 2012), suggesting that source and

[1] Portions of this chapter have been published Postnikoff and Harkness (2012).

type of calories may have a significant impact on CR as a lifespan altering intervention for humans. Additionally, emerging evidence in model organisms further reinforces this idea. Using alternate carbon sources, rather than glucose, can extend yeast lifespan (Ashrafi et al., 1999; MacLean et al., 2001; Wu et al., 2013). Dietary composition, including the ratio of macronutrients, affect life and health spans in metazoan (multicellular animal) models as well (Fanson et al., 2009; Le Couteur et al., 2014; Lee et al., 2008; Murtagh-Mark et al., 1995; Simpson and Raubenheimer, 2007; Solon-Biet et al., 2014). Emerging evidence suggests diets high in protein may elevate systemic growth factor signaling molecules resulting in accelerated aging and disease development in mice and humans (Levine et al., 2014; Solon-Biet et al., 2014). Furthermore, pharmacological inhibition of the amino acid responsive kinase TOR (Target of rapamycin) by treatment with rapamycin increases lifespan in models from yeast to mice (Bjedov et al., 2010; Harrison et al., 2009; Miller et al., 2011; Powers et al., 2006). These results highlight the importance of elucidating mechanisms behind both growth factor and amino acid/nitrogen regulated cellular processes in the understanding of human disease.

1.2 *Saccharomyces cerevisiae*

The budding yeast *Saccharomyces cerevisiae* is utilized as an efficient and effective model organism for the elucidation of molecular and genetic mechanisms within eukaryotic cells due to evolutionary conservation of many genes and protein sequences/structures, as well as cellular mechanisms present throughout eukaryotic heterotrophs. Particularly attractive characteristics are yeast's ability to be maintained in a haploid state and an efficient DNA homologous recombination mechanism, facilitating ease of mutation. Furthermore, the approximately 6400 genes in the yeast genome contain one or no introns, further simplifying the molecular environment by the absence of alternative splice variants. These features make yeast an attractive model for unravelling and linking many conserved eukaryotic genetic, molecular, and cellular processes involved in a complex multivariable process, including aging.

1.2.1 Lifespan Analysis

Two assays are conventionally used for studying the lifespan of yeast cells: the replicative lifespan assay (RLS) and the chronological lifespan assay (CLS). RLS, a measure of the replicative capacity of individual cells, also referred to as mitotic lifespan, exploits the

asymmetrical cell division of *S. cerevisiae*; typically, the smaller budding daughter cells are removed from the mother utilizing a dissecting microscope fitted with a micro-manipulating needle (Kennedy et al., 1994; Longo et al., 2012; Mortimer and Johnston, 1959; Postnikoff and Harkness, 2014). Scoring the total number of daughters an individual cell produces is considered a model for stem cell survival and regenerative capacity. Alternatively, CLS measures post-mitotic longevity by determining the length of time a culture of quiescent cells remains metabolically (and mitotically) viable, often achieved through the analysis of colony forming units (CFUs) present in a fixed volume of stock culture plated over a span of weeks to months (Longo and Fabrizio, 2012; Longo et al., 2012; Postnikoff and Harkness, 2014). The cellular processes in this assay are considered analogous to survival of post-mitotic cell populations in higher organisms (Longo et al., 2012). Also, using mitotic re-entry as a biomarker for survival, it could be argued that CLS is analogous to the functional lifespan of quiescent stem cell populations.

Proof of principal of the value of *S. cerevisiae* assays as models for metazoan lifespan analysis is multifold. First, manipulation of environmental conditions and genes discovered in metazoans to regulate aging and the development of age related diseases directly affect yeast lifespan assays. For example, the RAS (Rat sarcoma) family of GTPases are involved in growth factor signal transduction and oncogenic transformation of many tumours (Bos, 1989; Rajalingam et al., 2007). Mice that are deficient in RasGrf1 (Ras activating guanine nucleotide exchange factor) have increased lifespans (Borras et al., 2011). $Ras2^{val19}$, a dominant active form of yeast Ras2, decreases both CLS and RLS, while disruption of Ras2 signaling increases CLS (Fabrizio et al., 2003; Hlavata et al., 2003; Pichova et al., 1997). Additionally, *ras1*Δ extends RLS, while *RAS2* overexpression can increase stress recovery and RLS through a mechanism independent of the down-stream effector Protein kinase A [PKA; (Shama et al., 1998; Sun et al., 1994)].

Secondly, the Anaphase-Promoting Complex (APC) was discovered to affect mouse aging and the regulation of both RLS and CLS in yeast (Baker et al., 2005; Baker et al., 2004; D'Arcy et al., 2010; Harkness et al., 2004; Lara-Gonzalez et al., 2011; Postnikoff et al., 2012). The APC is a cell cycle regulating ubiquitin-protein ligase (E3), characterized by its role in commencing sister-chromatid separation at metaphase-anaphase transition in mitosis (M) and its continued role in

mitotic exit through to the end of Gap 1 (G1) and entry into DNA replication [S; (McLean et al., 2011; Thornton and Toczyski, 2006)]. Mechanisms of APC-dependent lifespan likely involves maintenance of genomic stability during sister chromatid separation at the metaphase/anaphase transition, as well as through G1 entry/maintenance and the concurrent regulation of stress responses (D'Arcy et al., 2010; Harkness et al., 2004; Harkness, 2006; Lara-Gonzalez et al., 2011; Postnikoff et al., 2012; Searle et al., 2004; Simpson-Lavy et al., 2009).

A third line of evidence supporting the value of yeast as a model organism for studying aging is the direct discovery of highly conserved novel lifespan regulating mechanisms in yeast, with subsequent identification in metazoans. Two examples are the Sir2 (Silent information regulator) and TOR pathways. Sir2, and its orthologs the Sirtuins, are a family of nicotinamide adenine dinucleotide (NAD+)-dependent histone (protein) deacetylases (HDAC) that respond to stress and the energy state within the cell, resulting in lifespan extension in multiple model organisms (Boily et al., 2008; Chen et al., 2005; Donmez and Guarente, 2010; Fabrizio et al., 2005; Guarente, 2013a; Kaeberlein et al., 1999; Li et al., 2008c; Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001; Wood et al., 2004). TOR is a PI3K (Phosphatidylinositol 3-kinase)-related serine/threonine protein kinase that regulates cell growth, proliferation, motility, survival, protein synthesis, and transcription (Loewith and Hall, 2011). The TOR complexes were first characterized in yeast, followed closely by mammalian cells (Brown et al., 1994; Cafferkey et al., 1993; Chiu et al., 1994; Heitman et al., 1991; Kunz et al., 1993; Sabatini et al., 1994; Sabers et al., 1995), and subsequently linked to metabolic regulation/calorie sensing and aging in yeast, worms, flies and mice (Barbet et al., 1996; Bjedov et al., 2010; Hara et al., 1998; Harrison et al., 2009; Jia et al., 2004; Kapahi and Zid, 2004; Kapahi et al., 2004; Rohde et al., 2001; Schmelzle and Hall, 2000; Thomas and Hall, 1997; Vellai et al., 2003; Wanke et al., 2008).

1.3 Growth Signaling Pathways

1.3.1 Insulin/Insulin-like Growth Factor Signaling

Coincidental to CR being an environmental intervention to senescence, genetic screens in *Caenorhabditis elegans* identified the insulin signaling pathway as the first, and now best characterized, genetically encoded regulator of aging (Kenyon, 2011; Kenyon et al., 1993; Lin et

al., 1997). The evolutionarily conserved metazoan insulin-signaling pathway is critical to numerous cellular processes (Bartke, 2011; Haigis and Yankner, 2010; Maki, 2010; Shaw, 2011). Perhaps most important is the decisive role it plays in cellular, and whole organism, survival. Overactive insulin-signaling leads to increased survival and growth of cells that would otherwise be conditionally cell-cycle arrested, with concurrent increased initiation of intracellular damage repair or programmed cell death mechanisms (Zhang et al., 2011). Insulin-signaling activates cell cycle progression mechanisms, while suppressing stress responses, such as pro-apoptotic and DNA repair pathways, increasing the proliferative capacity and oncogenic potential of cells in a sugar rich environment. Due to a possible increase in survival of individual damaged cells under these conditions, this scenario increases the probability of premature organism death due to cancer. On the other hand, reduced insulin-signaling relieves repression of stress response mechanisms, including cell cycle arrest and DNA repair pathways, increasing cell maintenance capacity and whole organism survival. A clear link between diabetes, cancer, other age related diseases, and the insulin-signaling pathway has been established (Ben Sahra et al., 2010; Slawson et al., 2010; Wysocki and Wierusz-Wysocka, 2010), highlighting the importance of further investigation of the precise activity of these processes.

Genetic screens in the nematode *C. elegans* initially identified AGE-1 (further characterized as the gene encoding the catalytic subunit of PI3K), and subsequently other members of the insulin-signaling pathway, as regulators of the aging process (Kenyon, 2011; Kenyon et al., 1993). Specifically, decreased activity of the evolutionarily conserved InsR (Insulin receptor)/PI3K/PDK1 (Phosphoinositol-dependent kinase)/AKT (Protein kinase B) pathway has been observed to increase longevity in a variety of model organisms, including worms, flies and mice, while over-activity has been observed in many cancer cells [Figure 1.1; (Kloet and Burgering, 2011; Speakman and Mitchell, 2011; Tatar et al., 2003)]. In this study we will focus on two major opposing downstream effectors of the insulin signaling/AKT pathway: 1) the phosphorylation of the stress response forkhead box transcription factor (Fox) O family, including Daf-16 (Dauer formation) in *C. elegans*, which leads to dissociation from DNA, nuclear export/cytoplasmic sequestration and degradation, inhibiting FoxO-dependent cellular protection (Kenyon et al., 1993; Lin et al., 1997; Ogg et al., 1997; Tatar et al., 2003); 2) the activation of TOR signaling, which increases anabolic processes and build-up of toxic by-

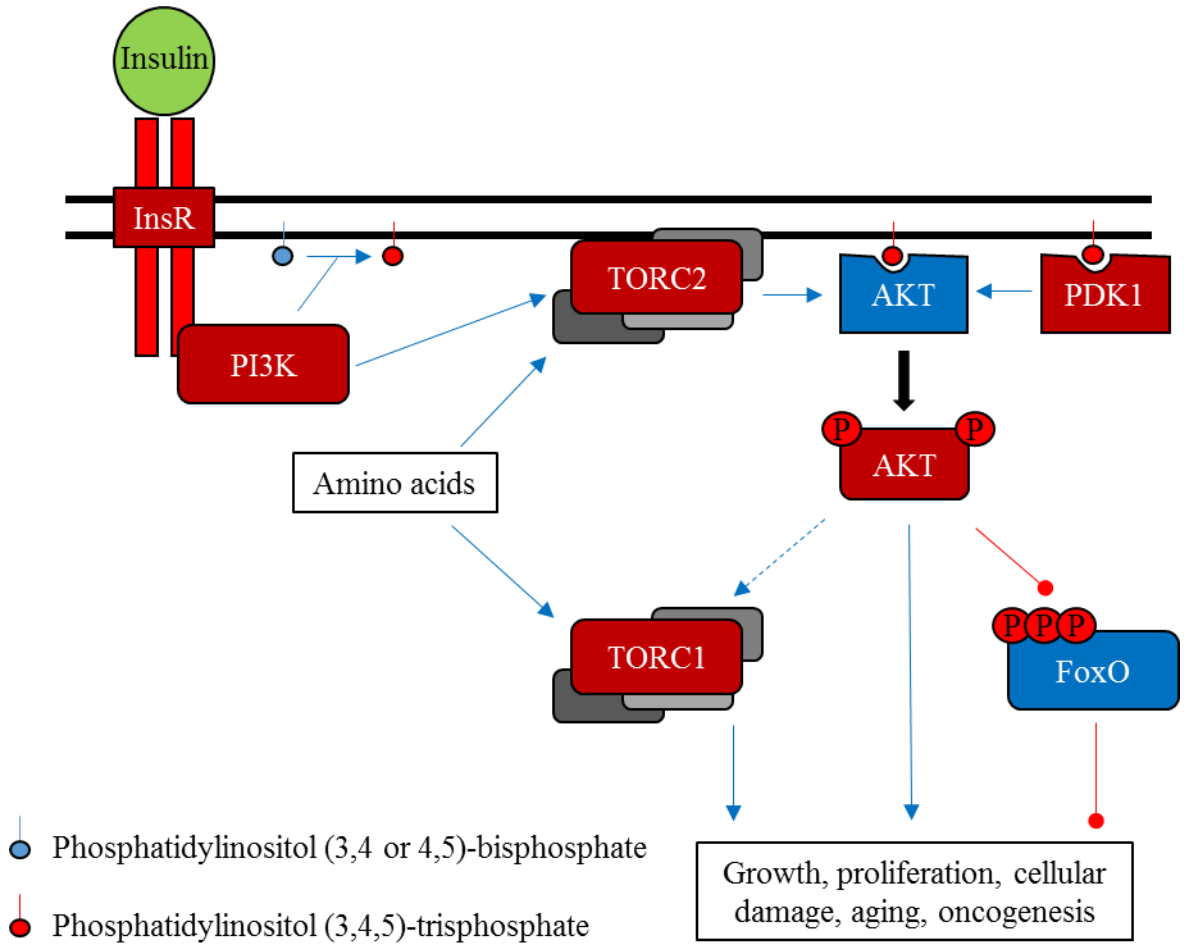

Figure 1.1 Metazoan insulin signaling pathway
Insulin/insulin receptor binding (as well as other growth factors binding to their respective receptors) leads to the conversion of phosphatidylinositol bisphosphate to triphosphate and the activation of the TORC2. AKT and PDK1 associate with the altered lipids, leading to AKT's phosphorylation and activation. Activated AKT represses the stress responsive FoxOs, removes inhibition of TORC1, represses apoptotic mechanisms and activates cell cycle regulators leading to cell growth, proliferation, and survival. (Bhaskar and Hay, 2007; Brunet et al., 1999; Greer and Brunet, 2008; Hay, 2005; Mora et al., 2004; Zhang et al., 2011)

products associated with a high metabolic state, while inhibiting catabolic processes, such as autophagy, preventing degradation of damaged cellular components (Hay, 2011; Loewith and Hall, 2011).

1.3.2 TOR Signaling

The TOR kinase integrates nutrient, amino acid, and growth factor cues to serve as a central regulator of eukaryotic growth and proliferation (Takahara and Maeda, 2013). Highly conserved from yeast to humans, TOR proteins were identified (and named) from studies of the growth inhibiting properties of the bacterial anti-fungal compound rapamycin (Cafferkey et al., 1993; Heitman et al., 1991). TOR proteins associate with two complexes, the rapamycin sensitive TORC1 (in mammals consisting of mTOR, Raptor, mLST8, PRAS40 and DEPTOR; Table 1.1) and rapamycin insensitive TORC2 (mTOR, Rictor, mSIN, mLST8, PRR/Protor and DEPTOR), both of which are essential for cell survival (Cybulski and Hall, 2009; Loewith and Hall, 2011; Loewith et al., 2002). The TORCs function with PDK1 in the activation of serine/threonine Protein kinase A, G and C family (AGC) members, such as AKT, the S6K (ribosomal protein S6 kinase) family, Protein kinase C and the SGK [Serum glucocorticoid kinases; (Jacinto and Lorberg, 2008; Mora et al., 2004; Su and Jacinto, 2011)]. TORC1 activity influences ribosome biogenesis and global rate of transcription and translation, increasing other anabolic processes, such as glycogen and lipid biosynthesis, while inhibiting catabolic processes, including autophagy (De Virgilio and Loewith, 2006b; Loewith and Hall, 2011; Loewith et al., 2002; Wullschleger et al., 2006; Xiao and Grove, 2009). TORC1 is activated by amino acid responsive GTPases, while it is repressed by the FoxOs and AMPK (AMP responsive kinase), the latter monitors AMP:ATP ratios and is activated in response to starvation [Figure 1.2; (Bai et al., 2007; Bolster et al., 2002; Chen et al., 2010; Greer et al., 2007b; Hardie et al., 1998; Hay, 2011; Kemp et al., 1999; Khatri et al., 2010; Kimura et al., 2003; Wessells et al., 2009)]. The TORC1 system primarily functions through S6K, eukaryotic translation initiation factors (eIF,), and specific PP2A (Protein phosphatase 2 A) complexes associated with Tap42 [Two A phosphatase Associated Protein; (Berset et al., 1998; Bodenmiller et al., 2010; Cherkasova and Hinnebusch, 2003; Cosentino et al., 2000; Di Como and Arndt, 1996; Hara et al., 1998; Huber et al., 2009; Jordens et al., 2006; Soulard et al., 2010; Urban et al., 2007; Zheng and Jiang, 2005)]. TORC2 primarily integrates growth factor signals to promote stress responses necessary for cell survival

Table 1.1 Evolutionary conservation of the TORC1 and TORC2 complexes

Mammals	*S. cerevisiae*	Essential in *S. cerevisiae*?	Potential function
TORC1			
mTOR	Tor1 or Tor2	No/Yes	Protein kinase, Scaffold
Raptor	Kog1	Yes	Substrate recognition
mLST8	Lst8	Yes	Stabilize kinase domain
Not identified	Tco89	No	Regulatory complex
PRAS40	Not identified	-	
DEPTOR	Not identified	-	
TORC2			
mTOR	Tor2	Yes	Protein kinase, Scaffold
mSIN1	Avo1	Yes	Localize complex to plasma membrane
Not identified	Avo2	No	Unknown function
Rictor	Tsc11	No	Scaffold
mLST8	Lst8	Yes	Stabilize kinase domain
PRR5/Protor	Bit61/Bit2	No	Unknown function
DEPTOR	Not identified	-	

Data obtained from (Cybulski and Hall, 2009; Loewith and Hall, 2011)

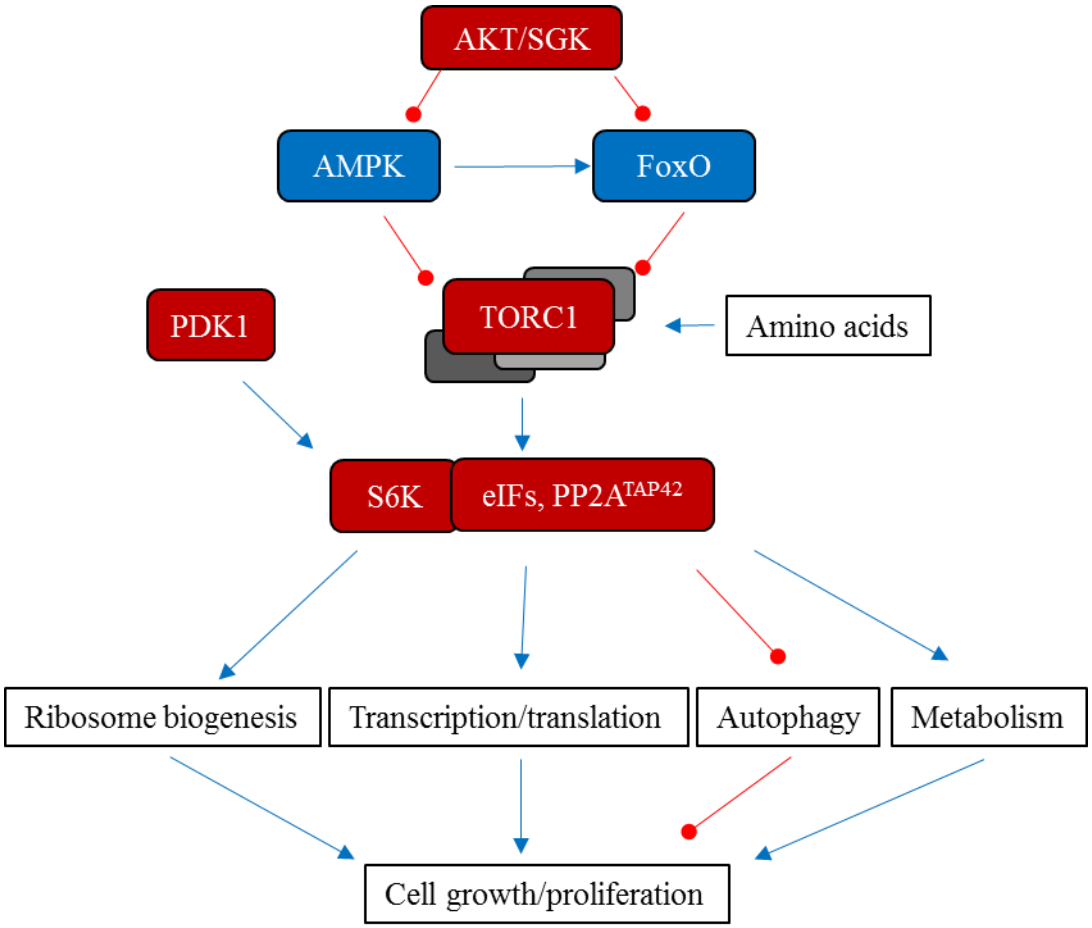

Figure 1.2 TORC1 signaling pathway
TORC1 is directly activated by amino acid levels and indirectly through insulin/glucose signaling. TORC1 up regulates anabolic processes such as ribosome, protein and lipid biosynthesis, while repressing catabolic processes such as autophagy, ultimately stimulating cell growth and proliferation. TORC1 does this through the phosphorylation of S6K and eukaryotic initiation factors, as well as the assembly of the PP2A^{TAP42} complex. Information obtained from (Hands et al., 2009; Jacinto and Lorberg, 2008; Loewith et al., 2002; Takahara and Maeda, 2013; Wullschleger et al., 2006).

and actin cytoskeleton organization (Cybulski and Hall, 2009). Importantly, TORC2 mediates cross-talk between the insulin/IGF signaling pathway and TOR signaling by phosphorylating AKT and SGK, both of which repress FoxO function [Figure 1.3; (Brunet et al., 1999; Brunet et al., 2001; Sarbassov et al., 2005)].

1.3.3 Yeast nutrient signaling

Many nutrient signaling mechanisms are highly conserved between yeast and higher eukaryotes, including the Ras/cAMP/PKA pathway, the Snf1 (Sucrose non-fermenting; AMPK ortholog) pathway, and both TORC pathways, with modifications (Zaman et al., 2008). For example *S. cerevisiae* has two paralogous *TOR* genes, *TOR1* and *TOR2*; comparatively a single Tor kinase domain is found in other organisms, such as the mammalian mTOR; however, the TORC1 and TORC2 complexes are highly conserved in structure and function (Table 1.1), with the exception that both Tor1 and Tor2 associate with the rapamycin sensitive TORC1, while only Tor2 associates with TORC2 (Loewith et al., 2002; Reinke et al., 2004; Wedaman et al., 2003; Wullschleger et al., 2006). Another variation is that glucose depletion regulates the yeast Snf1 kinase complex, rather than AMP:ATP ratios, as is the case for the mammalian AMPK complex (Hong et al., 2003; Mitchelhill et al., 1994; Nath et al., 2003; Wilson et al., 1996; Woods et al., 1994). A major difference from mammals is that yeast lacks direct conservation of insulin and other higher eukaryotic growth factor receptors, instead responding directly to environmental conditions; for example, yeast directly respond to environmental glucose levels by increasing glycolysis, growth and proliferation rates, while concurrently repressing stress responses [Figure 1.4; (Barbieri et al., 2003; Fabrizio et al., 2001; Thevelein and de Winde, 1999)]. This occurs primarily through glucose responsive G-protein receptors activating the Ras/PKA pathway, as well as activation of Sch9, a fungal kinase identified as a high-copy suppressor of Ras/PKA pathway defects (Liu et al., 2005; Thevelein and de Winde, 1999; Toda et al., 1988).

Sch9, a fungal member of the AGC kinases, is a potential ortholog of both AKT and S6K kinases (Fabrizio et al., 2001; Toda et al., 1988; Urban et al., 2007). Activation of Sch9 is initiated at phytosphingosine residues on the plasma membrane by phosphorylation by the yeast PDK1 orthologs, Pkh1/2, in a glucose and serine dependent manner (Liu et al., 2005; Mirisola et al., 2014; Voordeckers et al., 2011). Decreases in Sch9 signaling lead to increased lifespan, linked in

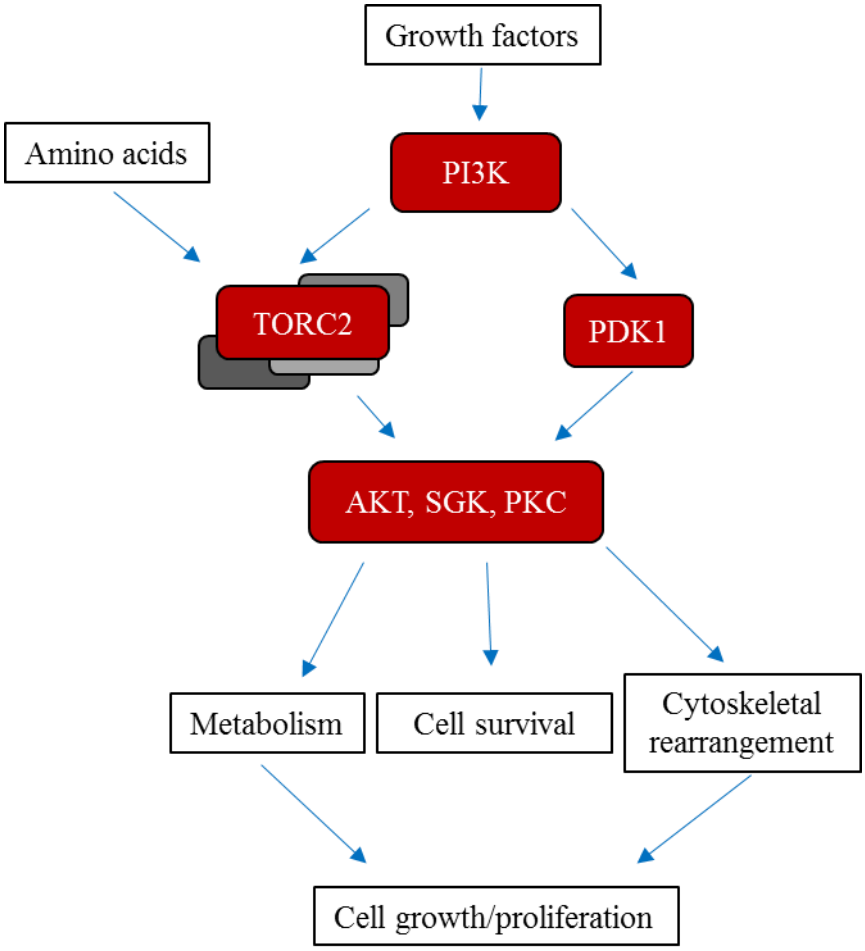

Figure 1.3 TORC2 signaling pathway
TORC2 along with PDK1 phosphorylate AGC kinases resulting in the up regulation of metabolic processes (such as lipid biosynthesis) and cytoskeletal rearrangements through growth factor and nutrient signaling. TORC2 signaling ultimately results in cell growth, proliferation and survival. Information from (Bhaskar and Hay, 2007; Cybulski and Hall, 2009; Loewith et al., 2002; Su and Jacinto, 2011; Takahara and Maeda, 2013).

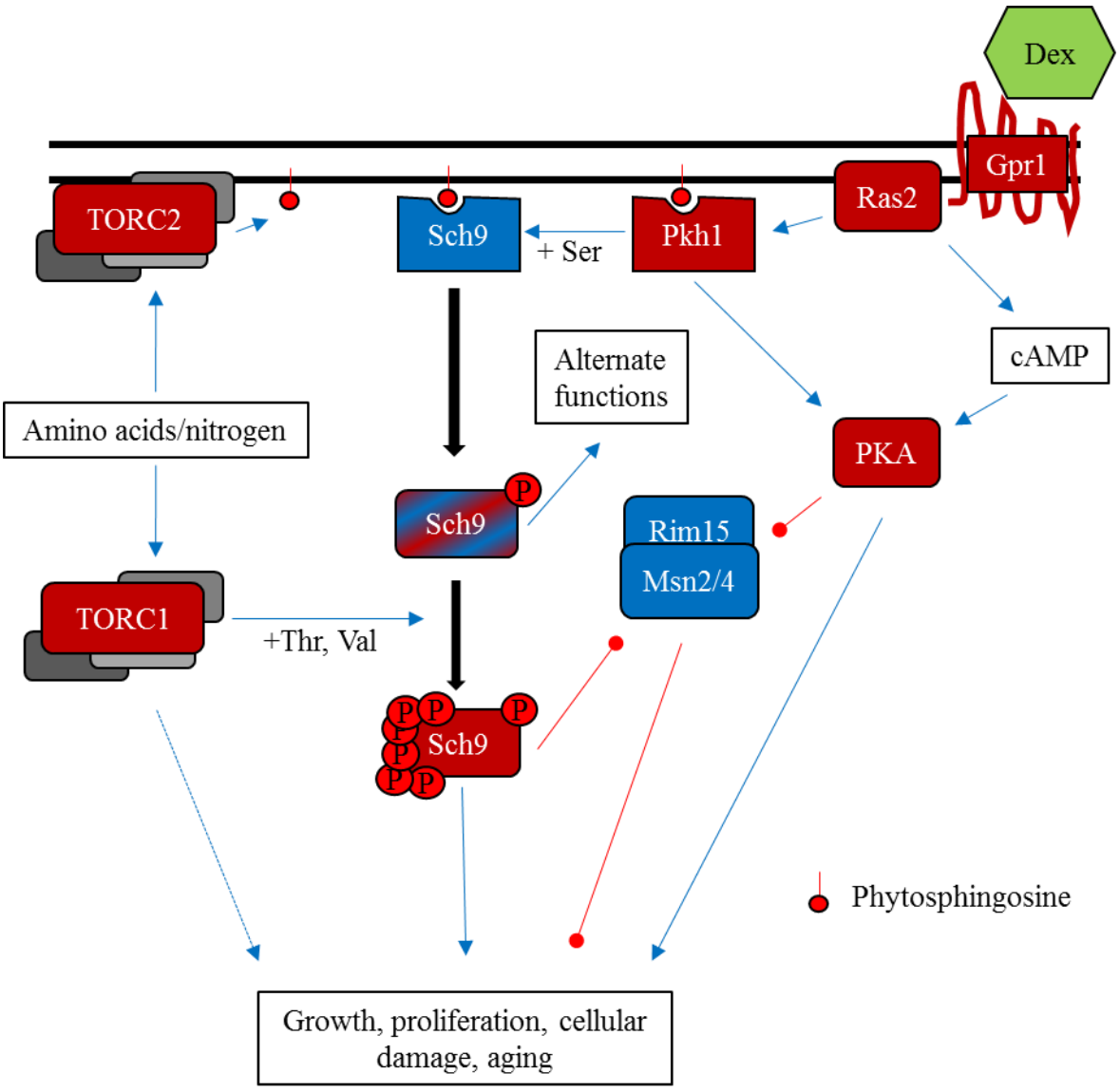

Figure 1.4 Yeast nutrient signaling pathway
Phytosphingosine bound Sch9 is activated by Pkh1 in the presence of glucose and the amino acid serine, followed by further activation by TORC1 phosphorylation in the presence of valine and threonine. Sch9 along with the parallel glucose activated PKA pathway control cell size, growth, proliferation and stress resistance. (Kaeberlein et al., 2005; Liu et al., 2005; Mirisola et al., 2014; Smets et al., 2008; Thevelein and de Winde, 1999; Toda et al., 1988; Urban et al., 2007; Voordeckers et al., 2011; Wei et al., 2008; Wei et al., 2009)

part to its regulation of non-Fox stress responsive transcription factors Msn2, Msn4 and Gis1through a mechanism analogous to the AKT repression of the FoxOs (Wei et al., 2008). However, under certain conditions Sch9 functions as the yeast S6K ortholog: secondary activation of Sch9 occurs in a TORC1 (and not TORC2) dependent manner, leading to the involvement of Sch9 in ribosome biogenesis and transcription/translation regulation (Mirisola et al., 2014; Urban et al., 2007). Moreover, CLS studies show epistatic responses in cells with *TOR1* and/or *SCH9* disruptions (Wei et al., 2009). Finally, characterization of gene expression patterns in cells with combinations of Sch9 and TORC1 disruptions delineated multiple genetic interactions between these two kinases including epistatic (linear mechanisms; S6K-like), additive (parallel), and opposite (antagonistic) mechanisms (Smets et al., 2008). This suggests Sch9 may retain evolutionarily conserved functions that are distributed between S6K and other nutrient responsive AGC kinases in metazoans.

1.4 Forkhead box transcription factors

Fox transcription factors have been identified in a wide range of species from yeast to humans. They are characterized by a common 110-amino acid winged-helix DNA-binding domain, known as the forkhead box (Kaufmann and Knöchel, 1996; Kaufmann et al., 1995; Lai et al., 1991; Weigel and Jackle, 1990; Weigel et al., 1989). All Fox proteins bind to the consensus core nucleotide sequence A/C AA C/T A due to the highly conserved nature of the Fox DNA-binding domain (Lalmansingh et al., 2012). Fox proteins are documented throughout fungal and animal species, while plant Fox proteins have yet to be identified, suggesting they originated in the animal/fungal ancestor after the evolutionary split of autotrophs and heterotrophs (Baldauf, 1999). Furthermore, analyses of fungal Fox genes found they are equally related to all animal Fox genes, suggesting only one proto-Fox gene was present at the divergence of animals and fungus (Baldauf, 1999; Mazet et al., 2003). Phylogenetic and comparative analyses have identified 50 Fox genes in humans that are grouped into 19 subclasses (A to S) based on the relationships between paralogous genes, as well as between vertebrate and invertebrate genes (Hannenhalli and Kaestner, 2009; Jackson et al., 2010; Kaufmann and Knöchel, 1996; Mazet et al., 2003; Murakami et al., 2010).

The Foxs are involved in the regulation of growth, development, metabolism, immunity, and tissue remodelling, many having temporal and spatial tissue/cell type specificity. They accomplish this primarily through transcriptional regulation of genes involved in cell cycle, metabolism, and a myriad of stress responses, including detoxification, DNA repair, apoptosis, autophagy, and cell cycle arrest (Calnan and Brunet, 2008; Fu and Tindall, 2008; Greer and Brunet, 2008; Ho et al., 2008; Katoh and Katoh, 2004; Lalmansingh et al., 2012; Laoukili et al., 2007; Tran et al., 2003; Wijchers et al., 2006; Zhang et al., 2011). Dysregulation of Fox genes has been linked to cancers, metabolic syndromes, autoimmune disorders, and a wide spectrum of developmental defects (Fu and Tindall, 2008; Greer and Brunet, 2008; Hannenhalli and Kaestner, 2009; Kloet and Burgering, 2011; Laoukili et al., 2007; Lehmann et al., 2003; Myatt and Lam, 2007). The identification of the FoxOs as major lifespan determining regulators of the insulin-signaling pathway has resulted in them becoming the best studied Fox family; subsequently, other Fox families have also been identified as potential regulators of aging, such as FoxM and FoxA (Anders et al., 2011; Laoukili et al., 2005; Laoukili et al., 2007; Panowski et al., 2007; Zeng et al., 2009), suggesting that increasing our understanding of general Fox biology may elucidate novel mechanisms of cellular longevity. This study focuses primarily on the functional similarities/relationships between the vertebrate FoxO and FoxM families with *S. cerevisiae FKH1* (Forkhead homolog) and *FKH2* genes.

1.4.1 FoxOs

The FoxO family is highly evolutionarily conserved, with members found in diverse metazoan lineages including mammals, flies, and nematodes (Lin et al., 2001; Mazet et al., 2003; Moskalev et al., 2011; Willcox et al., 2008; Yamamoto and Tatar, 2011). These transcription factors serve in longevity determination and tumor suppression through regulation of cell cycle and stress responses (Katayama et al., 2008; Kloet and Burgering, 2011; Kops et al., 2002a; Nogueira et al., 2008; Stahl et al., 2002; Tran et al., 2002). The FoxOs integrate signals from energy, growth factors, and stress signaling cascades to activate and repress transcription of genes involved in cell differentiation, metabolism, cell-cycle arrest and progression, apoptosis, autophagy, DNA-damage repair, and scavenging reactive oxygen species (ROS)/cell detoxification through the expression of genes such as the SODs (Superoxide dismutases), all of which may contribute to FoxO-dependent lifespan extension (Brunet et al., 2004; Cai and Xia, 2008; Demontis and

Perrimon, 2010; Katayama et al., 2008; Khatri et al., 2010; Kops et al., 2002a; Kops et al., 2002b; Lehtinen et al., 2006; Luo et al., 2007; Martínez-Gac et al., 2004; Miyamoto et al., 2007; Nogueira et al., 2008; Pinkston-Gosse and Kenyon, 2007; Puig et al., 2003; Schmidt et al., 2002; Seoane et al., 2004; Sunters et al., 2006; Tran et al., 2002; Zhang et al., 2011). FoxO proteins have been shown to interact with multiple cofactors that mediate their activity through posttranslational modifications, including phosphorylation, ubiquitination, methylation, glycosylation, and acetylation. These molecular variances regulate FoxO transcription factor efficiency, localization, stability and association with cofactors (Calnan and Brunet, 2008; Hay, 2011; Kops et al., 2002a; Tzivion et al., 2011; Zhang et al., 2011). Thus, a dynamic and complex molecular network controls FoxO protein function, yet specific lifespan regulating downstream targets remain speculative.

The best characterized FoxO posttranslational modification is phosphorylation. As mentioned previously, insulin signaling, via AKT, phosphorylates multiple sites on the FoxOs, leading to conformational changes to the FoxO protein structure, exposing a nuclear export signal, increasing binding to 14-3-3 protein chaperones, and increasing the binding efficiency of the SCF^{SKP2} complex (Skp/ Cullin/SKP2 F-box containing) E3. Consequently, the FoxOs undergoes nuclear export, cytoplasmic sequestration, and proteasomal degradation (Calnan and Brunet, 2008; Honda and Honda, 1999; Huang et al., 2005; Kloet and Burgering, 2011; Lin et al., 2001; Tzivion et al., 2011). Phosphorylation of the FoxOs through ROS, DNA damage, and hypoxic/starvation signaling pathways, such as the stress response mitogen activated protein kinases (MAPK) c-Jun N-terminal kinase (JNK) and p38, as well as the MAPK related kinase Mammalian ortholog of Sterile 20 (MST) 1, results in their nuclear shuttling and transcription factor activation, trumping AKT derived inhibition (Cai and Xia, 2008; Lehtinen et al., 2006; Sunters et al., 2006; Valis et al., 2011). Similarly, phosphorylation by AMPK, a kinase involved in sensing cellular energy states, results in FoxO transcription factor activation and gene target specificity (Greer et al., 2007a; Greer et al., 2007b; Peserico et al., 2013). Understanding how the combinations of posttranslational modifications lead to specific cellular outcomes could be invaluable for the development of therapies targeting aging and age related diseases, such as cancer, neurodegenerative disorders and diabetes.

1.4.2 FoxM

FoxM family members are expressed in proliferating cells, where they regulate growth, cell cycle, DNA repair, and stress resistance genes (Dai et al., 2013; Laoukili et al., 2005; Monteiro et al., 2013; Park et al., 2009; Wang et al., 2005). The regulation of mitotic entry, spindle checkpoint and mitotic exit genes, such as Cyclin B, Aurora kinase, Polo-like kinase, CENP, etc. allows for appropriate progression through mitosis, controlling chromosome separation, while preventing potential catastrophic damage to the chromatin/genome (Laoukili et al., 2005; Wang et al., 2005; Wonsey and Follettie, 2005). Furthermore, FoxMs, like the FoxOs, regulate free radical scavengers, such as MnSOD (Manganese SOD), increasing cellular protection in proliferating cells (Park et al., 2009). FoxM down regulation has been observed in both progeric cells and aged cells, while growth hormone activation and induction of FoxM nuclear-translocation increases regenerative capacity in aged mice (Krupczak-Hollis et al., 2003; Laoukili et al., 2007; Ly et al., 2000; Wang et al., 2001), indicating a role in preventing organismal senescence. However, human FoxM over expression is involved in cancer progression and malignancy, where it is involved in increasing proliferation rates and inducing angiogenesis, while protecting the oncogenic cells from ROS-induced apoptosis (Alvarez-Fernandez and Medema, 2013; Dai et al., 2013; Kwok et al., 2010; Laoukili et al., 2007; Madureira et al., 2006; Monteiro et al., 2013; Petrovic et al., 2010; Wang et al., 2008; Wang et al., 2010; Wilson et al., 2011; Zeng et al., 2009). This indicates that understanding the regulation of FoxM functions may increase our understanding of both dividing cell longevity and tissue regeneration, as well as cancer development/treatment.

1.4.3 Yeast forkhead box transcription factors

The *S. cerevisiae* genome encodes four Fox genes, *FHL1* (Forkhead-like), *HCM1* (High-copy suppressor of calmodulin), *FKH1* and *FKH2*, which are involved in cell cycle regulation and protein synthesis (Hermann-Le Denmat et al., 1994; Hollenhorst et al., 2000; Hollenhorst et al., 2001; Knott et al., 2012; Kumar et al., 2000; Martin et al., 2004; Ostrow et al., 2014; Pic et al., 2000; Pramila et al., 2006; Zhu et al., 2000). Based on sequence analysis, each of the fungal Fox proteins is thought to be equally distant to all metazoan Fox proteins, suggesting a single Fox gene was present at the evolutionary divergence of the fungal/animal lineages (Mazet et al., 2003). This suggests that conserved Fox functions may be differentially distributed amongst

fungal and metazoan Fox families. Specific yeast Fox gene functions have been documented. Fhl1 is involved in ribosome biogenesis in a TORC1/Sch9-dependent manner, positively and negatively regulating RNA polymerase II activity at ribosomal subunit encoding genes linking nutrient signaling to rRNA processing and ribosome biogenesis (Hermann-Le Denmat et al., 1994; Martin et al., 2004; Rudra et al., 2005; Schawalder et al., 2004; Xiao and Grove, 2009). *HCM1* is expressed in a cell cycle dependent manner where it controls genes involved in late S phase to early mitotic progression, including *FKH1* and *FKH2* (Pramila et al., 2006). Hcm1 localization and activity has been linked to nutrient signaling through both high nutrient (through Tor1/Sch9) and starvation (through Sir2 and Snf1) responses, where it regulates cell cycle progression and stress response (Rodriguez-Colman et al., 2010; Rodriguez-Colman et al., 2013). Finally, Fkh1 and Fkh2 regulate genes involved in mitotic entry/progression/exit, DNA replication initiation, and stress responses (Knott et al., 2012; Ostrow et al., 2014; Postnikoff and Harkness, 2012; Postnikoff et al., 2012; Shapira et al., 2004; Zhu et al., 2000). Initial investigations found that the deletion of any individual yeast Fox gene has little to no effect on CLS (Wei et al., 2008); however, recent findings suggest Fkh1 and Fkh2 regulate RLS, and along with Hcm1, CLS (Garay et al., 2014; Postnikoff et al., 2012). Here we will focus on the role of Fkh1/2 in the regulation of lifespan and stress responses.

1.4.3.1 Fkh1 and Fkh2

Fkh1 and Fkh2 are thought to be paralogs arising from a relatively recent gene duplication event. Expressed in late S phase/early gap 2 (G2), they regulate cell cycle progression, specifically mitotic entry, progression and exit, G1 maintenance and S phase initiation. As mentioned earlier, they also regulate stress responses, including cell cycle arrest in response to oxidative stress (Hollenhorst et al., 2000; Hollenhorst et al., 2001; Knott et al., 2012; Koranda et al., 2000; Ostrow et al., 2014; Pic et al., 2000; Postnikoff and Harkness, 2012; Postnikoff et al., 2012; Pramila et al., 2006; Shapira et al., 2004; Sherriff et al., 2007; Zhu et al., 2000). The deletion of individual *FKH* genes gives rise to subtle opposing phenotypes (Hollenhorst et al., 2000; Kumar et al., 2000; Pic et al., 2000; Zhu et al., 2000). For example, deletion of *FKH1* is associated with slight increases in Clb2 (Cyclin B) levels and *CLB2* gene cluster expression and a decrease in genomic silencing at established Fkh binding sites, such as within the mating type locus, while the deletion of *FKH2* has the opposite effects (Hollenhorst et al., 2000). However, the Fkhs also

share redundant functions as deletion of both genes significantly alters growth, cell morphology, and gene transcription phenotypes (Hollenhorst et al., 2000; Shapira et al., 2004; Zhu et al., 2000). For example, deletion of both *FKHs* deregulates *CLB2* expression, increasing background expression throughout the cell cycle, while removing both the general repression throughout G1-S-G2 and peak expression at mitotic entry. These responses are not observed when a single *FKH* is deleted (Hollenhorst et al., 2000; Hollenhorst et al., 2001; Zhu et al., 2000).

1.4.3.1.1 Fkh1/2 in stress response

The majority of FoxO-like stress responses (example *SOD* gene expression) in yeast have been attributed to other non-Fox transcription factors, such as Msn2 (Multicopy suppressor of *SNF1* mutation) and Msn4 (Fabrizio et al., 2003; Fabrizio et al., 2004b; Fabrizio et al., 2001; Wei et al., 2008); however, the Fkhs have also been observed to regulate stress responses. Microarray analysis of *FKH* deletion strains show altered stress resistance gene expression, while the deletion of the *FKHs* disrupts oxidative stress-induced cell cycle arrest (Shapira et al., 2004; Zhu et al., 2000). Additionally, the Fkhs, and known co-activators such as Mcm1 (Minichromosome maintenance) and Ndd1 (Nuclear division defective), translocate from the nucleus to the cytoplasm under acute hypoxic stress, while strains lacking *FKH2* show decreased colony size when grown under anaerobic conditions (Dastidar et al., 2012; Samanfar et al., 2013), indicating a role for the Fkhs in responding and adapting to environmental stresses, such as growth in low oxygen.

Yeast cellular processes may be regulated via multi-subunit complexes, the net composition of which determines the overall expression. The Fkhs potentially co-regulate many gene loci. A large scale chromatin immuno-precipitation (ChIP) study supports this idea as they found Fkh1 is associated with the promoter sequences of 3302 genes and Fkh2 precipitates with the promoters of 775 genes of the approximately 6400 genes identified in the yeast genome (Venters et al., 2011). While an in depth review of these potential transcriptional targets is beyond the scope of this thesis, a few genes are noteworthy. For example, both lists include the free radical scavenging metallo-SOD genes *SOD1* and *SOD2*, as well as other genes involved in ROS scavenging. Also at least one of the Fkhs binds to genes involved in cell cycle regulation including CDKs [*CDC28* and *PHO85* (Phosphate metabolism)], cyclins [*CLB1*, *CLB2*, *CLB3*,

CLB4, *CLB5*, *CLB6*, *CLN1*, *CLG1* (Cyclin-like gene), *PCL1* (Pho85 cyclin), *PCL2*, *PCL5*, *PCL7*, and *PCL9*], the CDKI *SIC1*, the CDKI-like protein *SPL1* (Suppressor of Plc1 deletion), and APC subunits/co-activators [*APC1*, *APC9*, *APC11*, *CDC20*, *CDC26*, *CDH1* (*CDC20* homolog), and *MND2* (Meiotic nuclear divisions)] to name a few. Additionally, genes involved in the nutrient/stress response mechanisms are also potential targets; these include but aren't limited to nutrient response G protein couple receptors involved in nutrient signaling [*GPR1* (G-Protein coupled Receptor) and *GPA2* (G protein alpha subunit)], members of Ras/PKA signaling [*RAS1*, *RAS2*, *TPK1* (Takashi's protein kinase; catalytic subunit of yeast PKA), *TPK2*, and *BCY1* (Bypass of cyclic-AMP requirement; PKA regulatory subunit)], TOR signaling [*LST8* (Lethal with sec thirteen), *TCO89* (Tor complex one), and *TAP42*], and stress response kinases such as the Msn2/4 regulator RIM15 (Regulator of *IME2*), *SNF1*, and the stress responsive MAPK *HOG1* (High osmolarity glycerol response). Also regulated are genes involved in telomere length and integrity; of note is *TEL2* (Telomere maintenance), a gene involved not only in telomere length and maintenance, but is also in the stability/biogenesis of TORC1 (Runge and Zakian, 1996; Stirling et al., 2011). Last, some genes involved in yeast programmed cell death/apoptosis regulation are potential targets. This includes several mammalian orthologs including *BXI1* (Bax inhibitor), a BCL-2 ortholog with both pro- and anti-apoptotic functions (Buttner et al., 2011; Cebulski et al., 2011). This is of particular interest as the FoxOs control the expression of BCL-2 family members, both the pro-apoptotic Bim and BNIP3, as well as the anti-apoptotic Bcl-XL (Dijkers et al., 2002; Dijkers et al., 2000; Gilley et al., 2003; Stahl et al., 2002; Tang et al., 2002). The Fkhs interact with various transcriptional activators, repressors, kinases and chromatin modifiers, including, but not limited to, the stress, starvation, and lifespan regulating HDAC, Sir2 and the SAGA (Spt-Ada-Gcn5 Acetyltransferase) complex, a key histone (protein) acetyl-transferase (HAT) complex in yeast (Guarente, 2013b; Linke et al., 2013; Venters et al., 2011), indicating that they may play a role in both the activation and repression of diverse cellular processes. We are only beginning to understand the capacity of the Fkhs to influence a multitude of cellular processes through altering the composition and activity of many transcriptional regulation complexes.

1.4.3.1.2 **Fkh1/2 in cell cycle regulation**

The *FKHs* are expressed in a cell cycle dependent manner in late S/early G2, in part through regulation by the Fox protein Hcm1 (Pramila et al., 2006). During the Gap phases, the Fkhs recruit chromatin remodeling proteins such as Sir2, Isw1 (Imitation switch subfamily) and Isw2 to repress mitotic specific gene expression (Linke et al., 2013; Sherriff et al., 2007). The yeast Fkh1 and Fkh2 regulate the transcription of gene clusters required for cell-cycle progression, such as the *SIC1*, *CLN2* (Cyclin), and *CLB2* gene clusters (Zhu et al., 2000). Under standard *in vivo* conditions, Mcm1 and Fkh2 occupy most mitotic regulating promoters; increases in Cdc28/Cdk1 (Cell-division cycle/Cyclin-dependent kinase)-cyclin and Cdc5 activity during mitotic entry facilitates the binding of the co-activator Ndd1 to Mcm1-Fkh2 complexes (Hollenhorst et al., 2001; Koranda et al., 2000; Loy et al., 1999). Thus, Fkhs act to inhibit the expression of G2/M progression genes until the appropriate growth signals regulating the binding of Ndd1 to Fkh2 are present. At this point there is a switch to mitotic progression via the up-regulation of Clb2 and Cdc5 (a polo-like kinase), which further activates the Fkh2/Ndd1 complex, increasing the expression of the *CLB2* gene cluster in a feed forward amplification (Reynolds et al., 2003). The expression of other genes, such as the rest of the Clb2 gene cluster is similarly regulated: this includes the APC sub-units/activators and the G1 transcription factors Swi5 (Switching deficient) and Ace2 (Activator of *CUP1* expression), as well as the Histone gene cluster (Zhu et al., 2000). In cells were *FKH2* is deleted, Fkh1 regulates this periodic increase of gene expression in a yet to be determined Mcm1/Ndd1-independent manner (Reynolds et al., 2003).

The Fkh1 and Fkh2 regulated *CLN2* and *CLB2* gene clusters include targets and regulators of the APC (Ko et al., 2007; Qiao et al., 2010; Sari et al., 2007; Zhu et al., 2000). The APC is a highly conserved E3 that primarily controls progression through mitosis and G1. The observation that the Fkh proteins control transcription of many genes required for APC function suggests that the APC may be a critical downstream target of the Fkhs (discussed in more detail below). The Fkh proteins also control the transcription of the histone gene cluster (Zhu et al., 2000). Interestingly, the yeast APC is required for histone protein expression and post-translational modification, which may be a shared function with the Fkh proteins, as deletion of *FKH1* and *FKH2* in a

mutant strain with a chromatin assembly deficient allele of the APC5 gene (*apc5*^{CA}) further impairs histone protein levels (Postnikoff et al., 2012; Turner et al., 2010).

In late mitosis, Fkh-regulated transcription factors, Swi5 and Ace2, activate the expression of the *SIC1* gene cluster, which regulates M/G1 progression genes, including the yeast CDK inhibitor (CDKI) Sic1 (Substrate inhibitor of cyclin-dependent protein kinase). Phosphatase activity and proteasomal degradation of the mitotic program inactivate the Mcm1-Fkh2-Ndd1 complex, degrading Ndd1 in the process (Wittenberg and Reed, 2005). Additionally, besides repressing expression of the *CLB2* gene cluster, the Fkhs also bind to and repress many Swi5 targeted promoters, further slowing early G1 progression (Voth et al., 2007). Simultaneously, the Fkhs are involved in the expression of tRNAs, potentially speeding growth in the Gap phases (Venters et al., 2011). Recently, the Fkhs were discovered to play a role in the initiation of DNA synthesis; Fkh1, and to a lesser extent Fkh2, bind to and regulate early replication origin firing in the initiation of S phase (Knott et al., 2012; Ostrow et al., 2014).

1.4.4 Evolutionary conservation of Fkh1/2: FoxO or FoxM?

FKH1 and *FKH2* are considered FoxM orthologs due to their regulation of the *CLB2* gene cluster, the timing of their expression during the cell cycle, and their phosphorylation activation by CDK-cyclin B complexes and polo-like kinases (Carlsson and Mahlapuu, 2002; Katoh and Katoh, 2004; Laoukili et al., 2007; Pramila et al., 2006). Additionally, the non-forkhead box transcription factors Msn2/4 and Gis1 (Gig1-2 suppressor) are considered the functional analogs of the FoxOs due to their role in the expression of stress response and Gap 0 (G0; quiescence/senescence) genes (Fabrizio et al., 2003; Fabrizio et al., 2004b; Fabrizio et al., 2001; Wei et al., 2008). However, building evidence challenges that paradigm. Specifically, Fkh1 and Fkh2 have demonstrated functional orthology to both FoxM and FoxOs based on three lines of evidence: phylogenetic analysis of the Foxs; the shared regulation of the cell cycle; their role in the response and regulation of environmental changes/stresses.

Sequence analysis suggests that all fungal and animal Fox proteins evolved from a single gene, which subsequently gave rise to the diversity of Foxs identified (Hannenhalli and Kaestner, 2009; Mazet et al., 2003; Wang et al., 2009b). The highly conserved nature of the forkhead box

DNA binding domain suggests that paralogous Fox proteins may have shared overlapping functions within an organism, while conservation of genetic code would determine conserved functions between organisms. This would indicate that retained locus regulation by Fox proteins between yeast and humans may be regulated by multiple Fox paralogs within each respective species. Furthermore, the FoxM family has only been identified in deuterostome lineages suggesting that they are the result of a gene duplication event well after the animal/fungus split, likely from the FoxO lineage, due to sequence analysis (Jackson et al., 2010; Mazet et al., 2003). Therefore, retained mechanistic and genetic functions between the yeast Fkh1/2 and FoxM are likely also shared in part with other mammalian Fox proteins, especially the FoxO family.

Next, the regulation of cell cycle is shared by Fkh1/2, FoxM and FoxO families (Alvarez et al., 2001; Hollenhorst et al., 2000; Wijchers et al., 2006; Zhu et al., 2000). Specifically, the FoxO proteins repress mitotic progression gene expression in both G2 and G1, a feature shared with the Fkhs, when coupled with chromatin remodelling cofactors such as Sir2 and Isw1/2 (Kops et al., 2002b; Linke et al., 2013; Schmidt et al., 2002; Sherriff et al., 2007). On the other hand, FoxMs, the yeast Fkhs, and to a lesser extent FoxOs activate the expression of mitotic progression genes as well as play a role in the transition from G1 to S phase [Figure 1.5; (Alvarez et al., 2001; Hollenhorst et al., 2000; Hollenhorst et al., 2001; Knott et al., 2012; Kumar et al., 2000; Laoukili et al., 2005; Laoukili et al., 2007; Marlow et al., 2012; Murakami et al., 2010; Ostrow et al., 2014; Venters et al., 2011; Wang et al., 2005; Wang et al., 2008; Zhu et al., 2000)]. The Fkhs repress inappropriate *CLB2* gene cluster expression throughout the cell cycle until mitotic specific activating signals and co-factor binding switches Fkh function and activates gene cluster's expression (Hollenhorst et al., 2000; Koranda et al., 2000; Pic et al., 2000; Reynolds et al., 2003; Sherriff et al., 2007; Zhu et al., 2000). This is counteracted by the degradation of activating kinases and co-factors, as well as potential Fkh-dephosphorylation as a part of mitotic exit, at which time they resume their cell cycle repressive role, which is similar to the FoxOs (Loy et al., 1999; Sherriff et al., 2007). Additionally, the Fkhs indirectly regulate the expression of the *SIC1* gene cluster controlling Cdc28-cyclin interactions in G1 entrance (Zhu et al., 2000). Furthermore, the Fkhs indirectly and directly regulate the progression through G1 into S phase: indirectly through the expression of *SWI5* and *ACE2*, transcription factors that regulate the expression of genes involved in cell cycle progression (Pic et al., 2000; Zhu et al., 2000); and

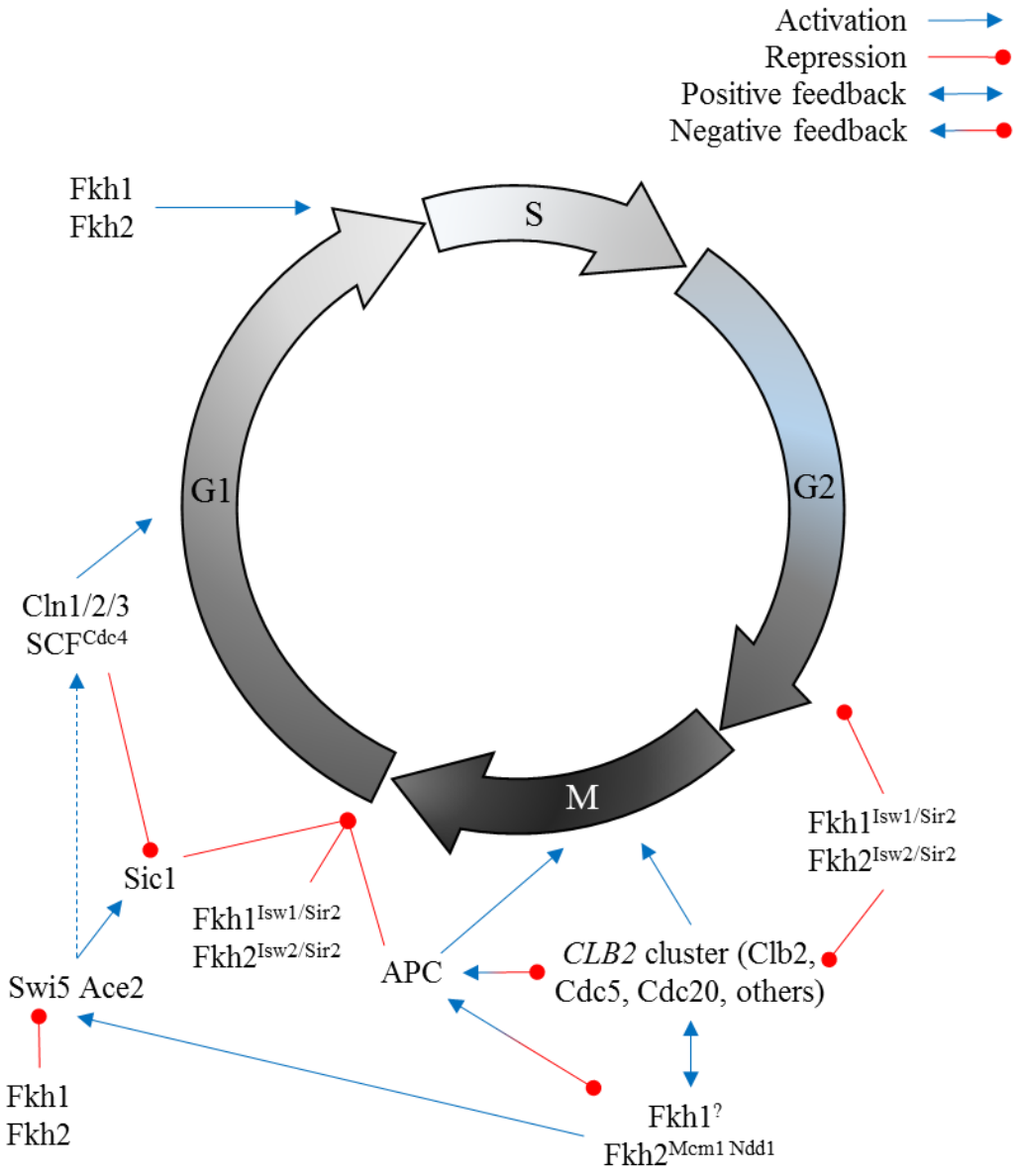

Figure 1.5 Fkh1 and Fkh2 co-regulate the cell cycle in *S. cerevisiae*
The Fkhs (predominantly Fkh2) recruit HATs, HDACs and other transcriptional machinery, resulting in changes to activation and repression of the *CLB2* (mitotic progression) and *SIC1* (G1 entry) gene clusters. Additionally, the Fkhs (especially Fkh1) regulate origin of replication licensing initiating transition into S phase. Dashed line represents indirect regulation. Model based on data from (Hollenhorst et al., 2000; Knott et al., 2012; Linke et al., 2013; Ostrow et al., 2014; Sherriff et al., 2007; Voth et al., 2007; Zhu et al., 2000).

directly through the regulation of cell growth and origin of replication firing (Knott et al., 2012; Ostrow et al., 2014; Venters et al., 2011). In mammals, the FoxOs are characterized by their repressive role of cell proliferation in G1 and G2 through the expression of CDKIs and the repression of cyclins [Figure 1.6; (Gomis et al., 2006; Ho et al., 2008; Katayama et al., 2008; Martínez-Gac et al., 2004; Medema et al., 2000; Schmidt et al., 2002; Seoane et al., 2004; Takano et al., 2007)]. They have also been found to conditionally increase the expression of genes, such as cyclin B and polo-like kinase, important for G2 and M phase progression, as well as increase the expression of G1 progression cyclin A in some tumour cell lines (Alvarez et al., 2001; Marlow et al., 2012). The Fkhs have analogous function through the recruitment of both activators and repressors of transcription to genes involved in G2-M progression, as well as through the indirect regulation of *SIC1* (Hollenhorst et al., 2000; Kumar et al., 2000; Linke et al., 2013; Sherriff et al., 2007; Zhu et al., 2000). FoxMs function in cell cycle progression, both from G2-M and G1-S (Laoukili et al., 2005; Wang et al., 2005; Wang et al., 2002a; Wang et al., 2002b; Yoshida et al., 2007). The former is a function thought to be predominately regulated by Fkh2 under standard conditions, while the latter is likely regulated by Fkh1 and other yeast transcription factors [Figure 1.5; (Ho et al., 2008; Ostrow et al., 2014)].

Finally, FoxM, FoxO and Fkh1/2 are all involved in cellular responses to toxic stress. The FoxOs activate the expression of genes required for apoptosis and stress resistance, while repressing genes involved in cell cycle progression (Delpuech et al., 2007; Fu and Tindall, 2008; Zhang et al., 2011). The FoxMs similarly increase stress resistance gene expression, but also maintain expression of cell cycle progression genes rather than those that induce apoptosis (Li et al., 2008b; Monteiro et al., 2013; Park et al., 2009; Tan et al., 2007; Wang et al., 2002b; Wang et al., 2001). A similar pattern was observed in microarray analysis of asynchronous *fkh1*Δ *fkh2*Δ cells where many stress response genes were down regulated (Zhu et al., 2000). Additionally, *FKH1* or *FKH2* are required for the induction of ROS-induced cell cycle arrest (Shapira et al., 2004), a function more commonly associated with FoxOs than the FoxMs.

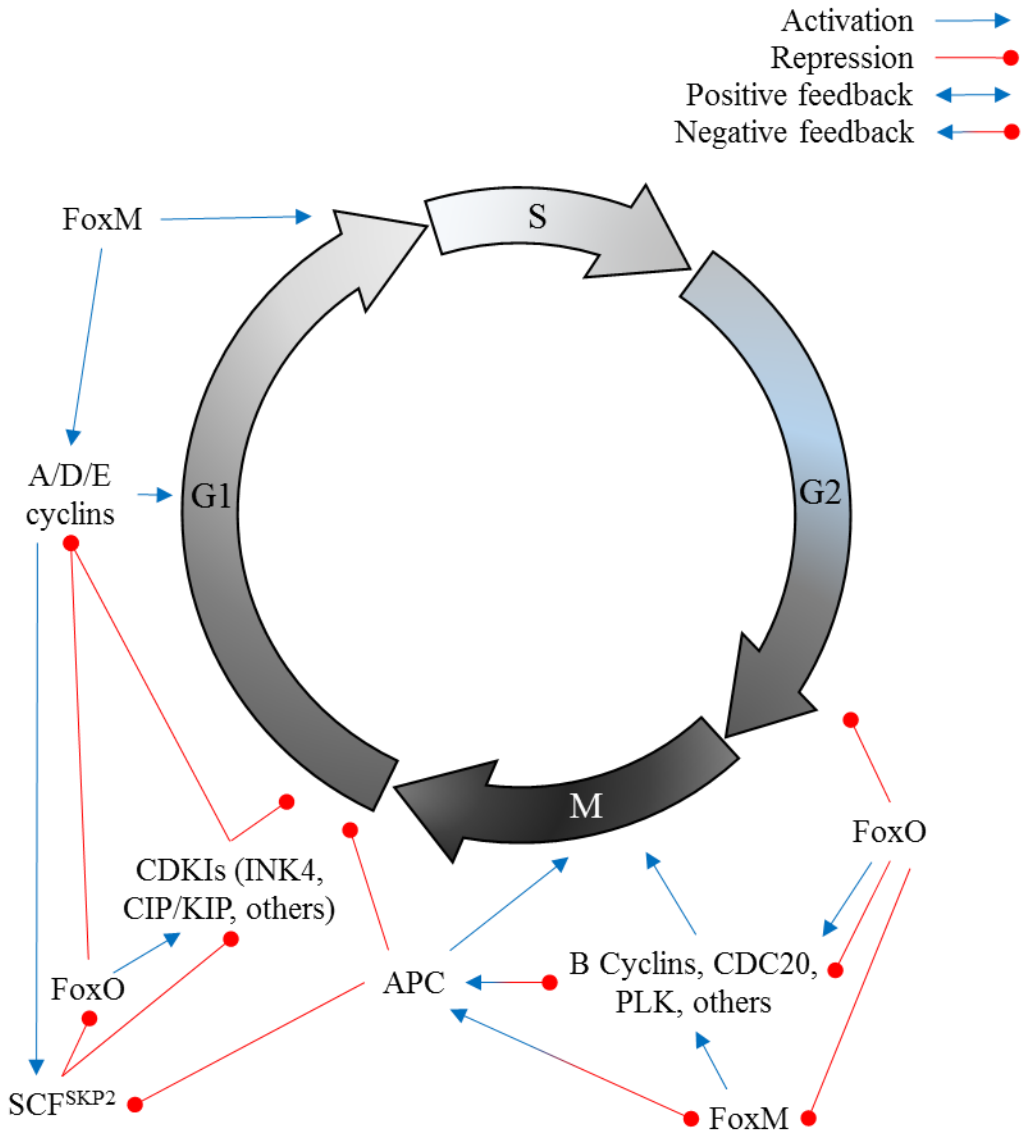

Figure 1.6 FoxOs and FoxMs co-regulate the cell cycle in mammals
The FoxOs inhibit transition from G2 to M by increasing G2 cyclin expression while inhibiting mitotic cyclins and related genes; however, the FoxOs can increase the expression of mitotic cyclins and related genes in certain conditions. In G1 the FoxOs inhibit the cyclins involved in G1-S transition while increasing the expression of CDKIs. FoxM is involved in progression through both G2-M and G1-S by increasing expression of the appropriate cyclins. Model modified from (Ho et al., 2008) with additional information from (Gao et al., 2009; Lin et al., 2009; van Leuken et al., 2008; Wang et al., 2005; Wang et al., 2008).

1.5 Anaphase-Promoting Complex

The APC is an E3 that regulates passage through mitosis and the cell cycle by selectively targeting cyclins, mitotic kinases, F-box proteins, and proteins involved in chromatin adhesion/separation for proteasomal degradation, resulting in appropriately timed sister-chromatid separation at metaphase-anaphase transition, as well as mitotic exit and early G1 (Barford, 2011; McLean et al., 2011). The addition of the small peptide ubiquitin is a posttranslational modification often involved in protein localization; most often, the formation of poly-ubiquitin chains target the modified substrate to the proteasome where it is degraded. A ubiquitin-activating enzyme (E1) attaches ubiquitin onto ubiquitin-conjugating enzymes (E2). E3s bring E2s and target substrates together, catalysing ubiquitin transfer either directly from the E2 to the substrate in the case of really interesting new gene (RING) domain containing E3s or transferring the ubiquitin from the E2 to the E3 before attaching it to the substrate in the case of homology to E6AP C terminus (HECT) domain E3s (Berndsen and Wolberger, 2014; Nandi et al., 2006). The highly evolutionary conserved APC is a RING E3 comprised of subunits that can be classified in three general regions: a cullin RING zinc-finger domain catalytic cluster, similar in structure to the SCF, comprised of Apc11, Apc2, and Apc10; a regulatory cluster including Apc3/Cdc27, Apc6/Cdc16, Apc7, Apc8/Cdc23, and Cdc26; and structural and scaffolding subunits that connect the regulatory and catalytic groups, as well as increase the complex's stability, which includes Apc1, Apc4, Apc5, and Apc13 [Table 1.2; Figure 1.7; (Barford, 2011; da Fonseca et al., 2011; McLean et al., 2011; Schreiber et al., 2011; Thornton et al., 2006; Thornton and Toczyski, 2006)]. APC activity and substrate specificity throughout the mitotic cell cycle is regulated by association of specific co-activators to the regulatory subunits: Cdc20 for progression from metaphase through anaphase, and Cdh1 during mitotic exit and G1 maintenance. Besides its role in cell cycle regulation, the APC is associated with genomic stability through roles in chromatin assembly, histone modifications, and spindle checkpoint regulation (Charles et al., 1998; Garcia-Higuera et al., 2008; Harkness et al., 2002; Harkness et al., 2004; Islam et al., 2011; Kotani et al., 1998; McLean et al., 2011; Menzel et al., 2013; Qiao et al., 2010; Thornton and Toczyski, 2006; Turner et al., 2010).

Table 1.2 Conservation of APC subunits in *S. cerevisiae* and mammals

Mammals	*S. cerevisiae*	Essential in *S. cerevisiae*?	Function
Core subunits			
Apc1	Apc1	Yes	Scaffold
Apc2	Apc2	Yes	E2 binding
Apc3	Cdc27	Yes	Protein binding
Apc4	Apc4	Yes	Scaffold
Apc5	Apc5	Yes	Scaffold
Apc6	Cdc16	Yes	Subunit binding
Apc7	Not identified	-	
Apc8	Cdc23	Yes	Subunit binding
Not identified	Apc9	No	Complex stabilization
Apc10	Apc10	No	Substrate recognition
Apc11	Apc11	Yes	E2 binding/Catalytic subunit
Cdc26	Cdc26	No	Role in heat shock
Apc13	Swm1	No	Complex stabilization/Role in meiosis
Not identified	Mnd2	No	Role in meiosis
Apc16	Not identified	-	
Co-activators			
Cdc20/Fzy/ p55CDC	Cdc20	Yes	Activation and substrate specificity in mitosis and meiosis
Cdh1/Fzr1	Cdh1	No	Activation and substrate specificity in G1, mitosis and meiosis

Information obtained and modified from (McLean et al., 2011; Thornton and Toczyski, 2006).

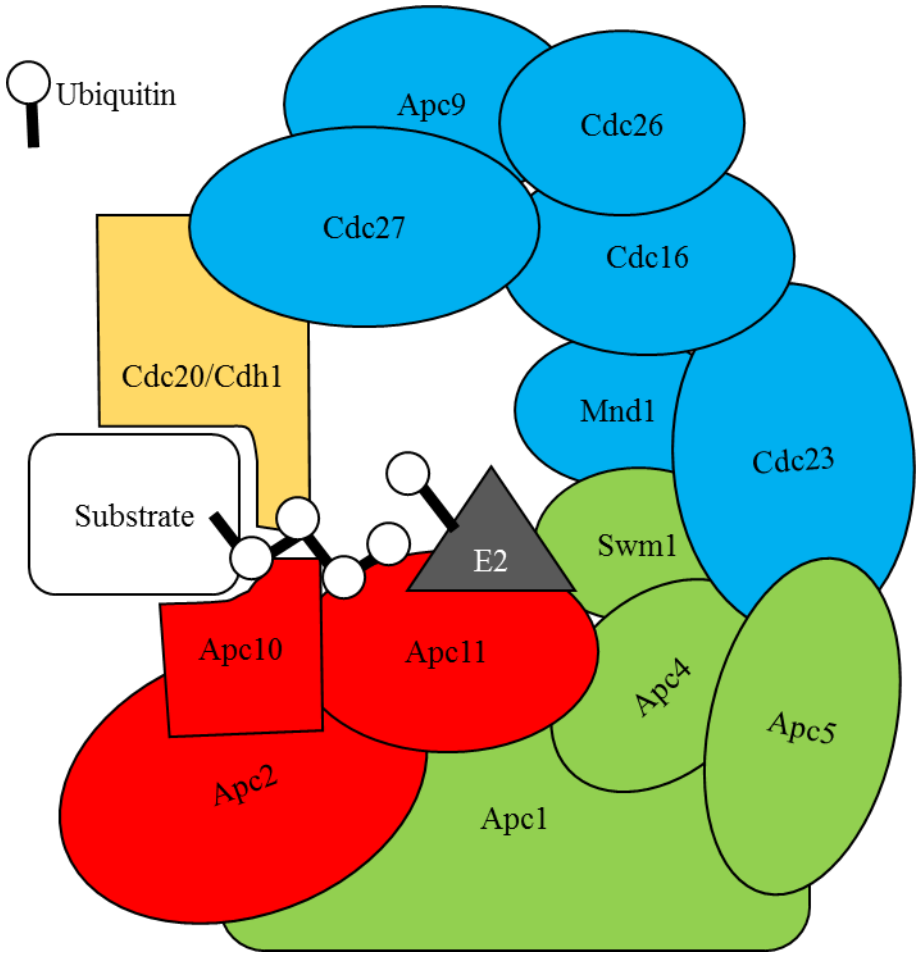

Figure 1.7 Schematic of the *S. cerevisiae* Anaphase Promoting Complex
Red subunits indicate catalytic proteins, green indicates structural subunits, blue indicates subunits in regulatory region/unknown function, and yellow indicates the location of one of the co-activators Cdc20 or Cdh1, which associate with Cdc27 and Apc10. Target substrate is recognized by Apc10, while Apc11 catalyzes ubiquitin transfer from the E2 to the target. Information obtained and modified from (Schreiber et al., 2011; Thornton et al., 2006; Thornton and Toczyski, 2006). For more detailed EM structures of *S. cerevisiae* and human APCs see (Buschhorn et al., 2011; da Fonseca et al., 2011; Schreiber et al., 2011).

1.5.1 APC^{Cdc20}: the regulation of mitosis

The APC is kept dormant during S and G2 through the sequestration of the activating subunits (Cdc20 and Cdh1), potentially by inhibitory phosphorylation by CDK/cyclin complexes and PKA (Bolte et al., 2003; Kotani et al., 1998; Searle et al., 2004). Upon the cell reaching critical size and protein content in G2, polo-like kinases (Cdc5 in *S. cerevisiae*) are activated by removal of inhibitory phosphorylation by way of TORC1 activated $PP2A^{Tap42}$ (Nakashima et al., 2008). Polo-like kinase activation leads to the release Cdc20, freeing it to bind to the rest of the APC (Kotani et al., 1998). Further activation by the polo-like kinases, and CDK/mitotic-cyclin complexes and proper spindle formation, removes inhibition by the Bub/Mad spindle checkpoint proteins leading to the full activation of the APC^{Cdc20} (Fang et al., 1998; Hardwick et al., 2000; Shah et al., 2004; Tang et al., 2001). This leads to the release of cyclin specific phosphatases (such as Cdc14 in *S. cerevisiae*), the activation of APC^{Cdh1} complexes, and the targeting of the mitotic cyclins, polo-like kinases, Cdc20, and securins for proteasome-dependent degradation, resulting in the initiation of chromosome segregation and cytokinesis (Barford, 2011; Harper et al., 2002; Passmore, 2004; Visintin et al., 1998). However, the specific role of each of the co-activators at metaphase/anaphase transition *in vivo* remain unclear, as both APC^{Cdc20} and APC^{Cdh1} are capable of the ubiquitination of the major APC substrates (Castro et al., 2005; Thornton and Toczyski, 2003).

1.5.2 APC^{Cdh1}: mitotic exit, G1 maintenance and beyond

APC^{Cdh1} continues to target mitotic specific activators for proteasomal degradation, including those mentioned earlier, such as mitotic cyclins, polo-like kinases, and Cdc20, altering the protein landscape through ubiquitin mediated degradation, resetting the recently divided daughter cells to a G1 state. In G1, in mammalian cells, stability of the FoxO and FoxM proteins is controlled antagonistically by the SCF and APC ubiquitin-protein ligase/E3 complexes, respectively (Figure 1.8). The APC targets FoxM1 for degradation, while indirectly stabilizing the FoxOs during G1 (Laoukili et al., 2008; Park et al., 2008). The SCF^{SKP2} complex targets phosphorylated FoxO proteins and CDKIs for degradation (Huang et al., 2005; Huang and Tindall, 2011; Wang et al., 2005).The APC potentially controls this process through targeting nuclear SKP2 for proteasomal degradation (van Leuken et al., 2008). Degradation of SKP2 by the APC^{CDH1} blocks SCF^{SKP2} function, thereby protecting nuclear FoxO from degradation and

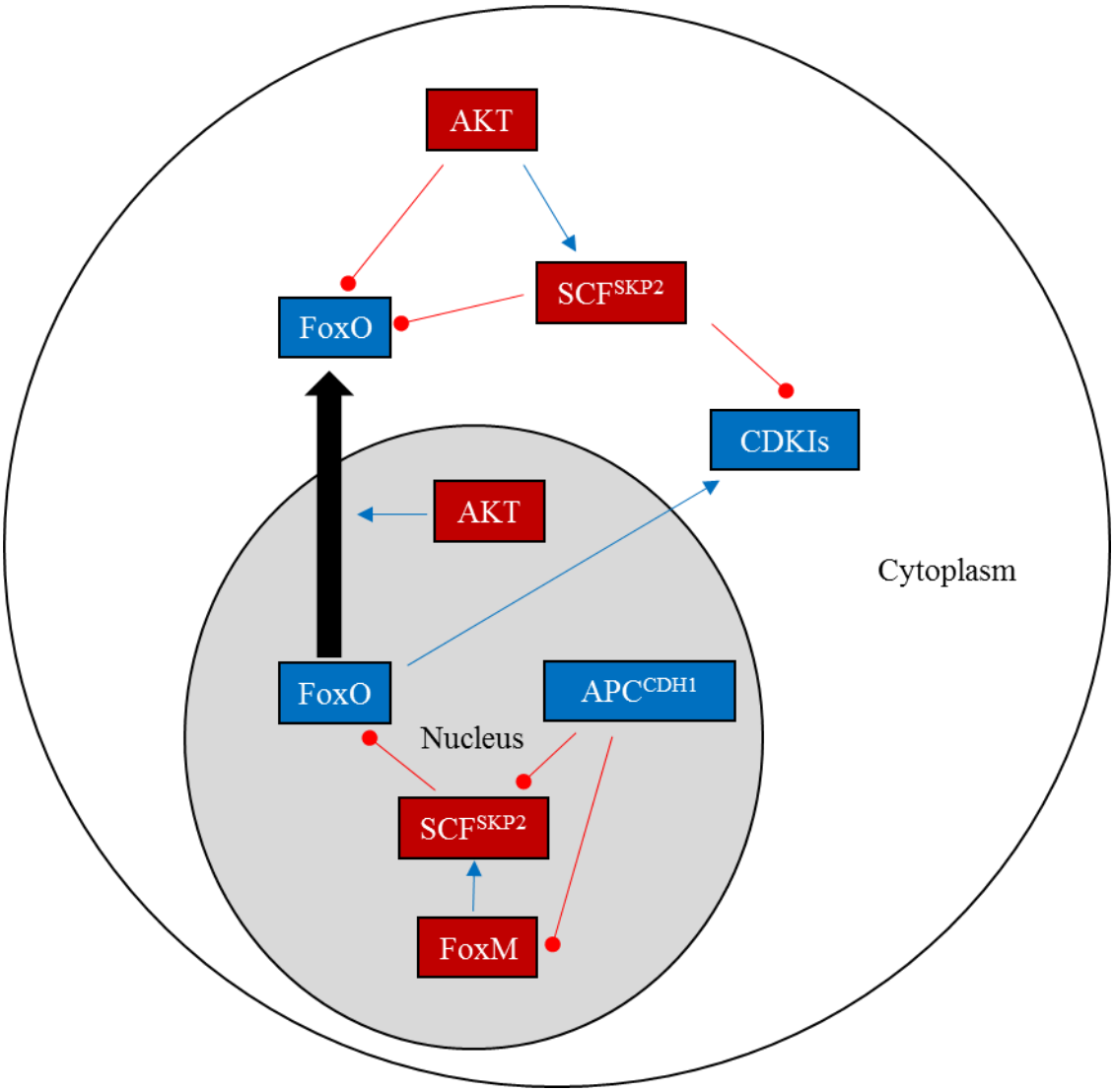

Figure 1.8 Insulin signaling and the APC control Fox protein abundance in G1
The FoxOs, through CDKIs, lead to cell cycle arrest in early G1, a process mediated by the APC^{CDH1} degradation of nuclear FoxM and SKP2. Insulin signaling, through AKT, mediates FoxO and CDKI localization and degradation, in part through the activation and protection of the SCF^{SKP2} complex. See text for details.

allowing the FoxOs to function in quiescence and tumor suppressor activity. On the other hand, AKT phosphorylates FoxO proteins, both leading to their nuclear export and increasing the efficiency of SCF^{SKP2}-dependent ubiquitination and degradation, while AKT also phosphorylates and activates SKP2 (Gao et al., 2009; Lin et al., 2009). AKT phosphorylation of SKP2 is believed to signal cytosolic localization, thereby protecting SKP2 from nuclear APC-dependent ubiquitination and degradation. However, these mechanisms may be cell type dependent, as a subsequent study was unable to reproduce these findings (Bashir et al., 2010). In yeast, besides degrading mitotic progression proteins, the APC^{Cdh1} potentially degrades Ndd1 (Loy et al., 1999). As mentioned earlier this switches Fkh2 function from activation to repression at the *CLB2* cluster, analogous to the degradation of FoxM and stabilization of FoxOs in mammals. Furthermore, preliminary data indicate APC and SCF defects may stabilize Fkh1 and Fkh2 under some conditions (Malo and Harkness, unpublished), while SCF^{Cdc4} targets Sic1 for degradation (Feldman et al., 1997; Verma et al., 1997), suggesting that ubiquitination of the Fox protein/CDKI network of G1 control may be conserved from yeast to humans. In summary, a highly conserved signaling pathway exists, where the APC and growth factor signaling compete in the regulation of the SCF, with the winner perhaps deciding the cell fate between Fox-mediated quiescence, repair or death versus growth, division and survival.

1.5.3 APC: a potential Fox-dependent regulator of longevity

Transcription of mammalian mitotic genes, including APC components, is regulated by FoxM1, as well as conditionally by the FoxOs (Alvarez et al., 2001; Laoukili et al., 2005; Takano et al., 2007; Wang et al., 2005). Murine APC regulation is also implicated in aging, as mutations to BubR1, a component of the mitotic spindle checkpoint, lead to premature aging defects and progeric phenotypes (Baker et al., 2004). BubR1 phosphorylates and inhibits premature APC^{Cdc20} activity (Baker et al., 2004; D'Arcy et al., 2010; Lara-Gonzalez et al., 2011). Inappropriate activation of APC^{Cdc20} results in the loss of nuclear lamina structure and transcriptional derepression leading to progeria, while premature activation of anaphase may result in chromatin number aberrations potentially leading to cancer. Cultured murine cells lacking Cdh1 have inefficient proliferation due to defects in both mitotic exit and S phase entry, as well as the accumulation of aberrancies in the numbers and structures of chromatin. Furthermore, heterozygous Cdh1 knock-out mice show increased rates of spontaneous tumour formation

(Garcia-Higuera et al., 2008). Additionally, the APC^{Cdh1} appears to play a role in the stabilization of at least FoxO1 through the degradation of Skp2, an F box protein of the SCF ubiquitin-protein ligase complex responsible for FoxO1 degradation. The APC's protection of the FoxOs is inhibited by insulin signaling (Gao et al., 2009; Huang et al., 2005). Furthermore, the APC^{Cdh1} targets FoxM1 for degradation and the absence APC^{Cdh1}-dependent degradation of FoxM1 can lead to tumour formation and cell cycle abnormalities (Laoukili et al., 2008; Park et al., 2008; Tang et al., 2008; Wang et al., 2008).

Similar to what has been observed in mammals, the APC has been demonstrated as critical to genomic stability and longevity in yeast (Baker et al., 2004; Harkness et al., 2002; Harkness et al., 2004; Hartwell and Smith, 1985; Li et al., 2008a; Palmer et al., 1990; Postnikoff and Harkness, 2012). Additionally, Fkh1 and Fkh2 regulate the *CLB2* gene cluster for progression through mitosis, which includes genes required for APC activity (*APC1*, *CDC5*, *CLB2*, and *CDC20*), as well as APC targets [*CLB2*, *CDC5*, *CDC20* and *IQG1*; (Barford, 2011; Harper et al., 2002; Ko et al., 2007; Zhu et al., 2000)]. Yeast APC may play a role in switching the activity of at least Fkh2, by potentially degrading the transcription co-activator, Ndd1, allowing for the binding of repressors Isw2 or Sir2 (Linke et al., 2013; Sherriff et al., 2007). This would switch Fkh2 function from FoxM to FoxO-like activity at those sites upon G1 entry. Mutations to individual APC subunits have been found to decrease RLS and CLS, while over-expression of *APC10* increases at least RLS (Harkness et al., 2004; Menzel et al., 2013; Postnikoff et al., 2012). Additionally, *cdh1*Δ results in decreased stress resistance, in part due to the stability/activity of Clb2 and Hsl1 (Histone synthetic lethal), which in turn inhibit stress response MAPK pathways such as Hog1 (Simpson-Lavy et al., 2009). Finally, the APC regulates genomic stability through the regulation of histone modifications, levels and deposition, as well as chromatin dynamics, processes that have been linked to aging phenotypes in yeast (Feser et al., 2010; Harkness et al., 2002; Harkness et al., 2004; Menzel et al., 2013; Postnikoff et al., 2012; Turner et al., 2010). These data strongly implicate an interactive role between the APC and Fox proteins in the co-regulation of an evolutionarily conserved pathway controlling genomic stability, tumour suppression, and aging from yeast to mammals.

2 Hypothesis, Aims and Rationale

2.1 Hypothesis

The central hypothesis of this study is that the yeast forkhead box transcription factors, Fkh1 and Fkh2, and the Anaphase-Promoting Complex/Cyclosome (APC) co-regulate chronological lifespan, likely through the regulation of cell cycle and stress resistance mechanisms. Furthermore, that this lifespan regulation is controlled by the nutrient responsive kinases Sch9 and Tor1.

2.2 Specific aims

1) To examine evolutionary conservation of known metazoan Fox gene functions by the *S. cerevisiae* forkhead box containing transcription factors Fkh1 and Fkh2.

2) To observe the involvement of nutrient and stress response kinases in the regulation of *FKH*-dependent lifespan.

3) To characterize the role of the Anaphase-Promoting Complex/Cyclosme in Fkh and Sch9/Tor1 dependent lifespan and stress resistance regulation.

2.3 Rationale

Many eukaryotic signaling mechanisms are conserved between fungal and animal systems, especially regarding responses to extracellular nutrient availability and stressing agents, as well as intracellular energy levels and damage. These signals regulate cell growth, division, repair, nutrient scavenging, and cell death through mechanisms that are at least partially conserved between lineages (De Virgilio and Loewith, 2006a; Hardie et al., 1998; Hay, 2011; Mitchelhill et al., 1994; Takahara and Maeda, 2013; Zaman et al., 2008). The nutrient/stress responses regulating FoxO and FoxM forkhead box transcription factor families play fundamental roles in decreasing metazoan age-related health decline and cancer progression (Berdichevsky and Guarente, 2006; Burgering, 2008; Calnan and Brunet, 2008; Greer and Brunet, 2008; Laoukili et al., 2005; Monsalve and Olmos, 2011; Wilson et al., 2011; Wonsey and Follettie, 2005; Zhao et al., 2011). The budding yeast *S. cerevisiae* is a powerful model organism for elucidating genetic

and biochemical mechanisms involved in these processes. The yeast forkhead box containing proteins, Fkh1 and Fkh2, are potentially orthologous to those of metazoans as they regulate gene expression involved in the progression from G2 to M and M to G1 phase of the cell cycle, as well as stress response genes, similar to the FoxM/FoxO families of higher eukaryotes (Alvarez et al., 2001; Hollenhorst et al., 2000; Laoukili et al., 2005; Postnikoff and Harkness, 2012; Takano et al., 2007; Wang et al., 2005; Wijchers et al., 2006; Zhu et al., 2000). However, it has yet to be determined if these pathways are regulated by nutrient/stress response signals and whether the Fkhs regulate longevity and stress response mechanisms in a manner similar to metazoan Fox proteins. Additionally, downstream targets and interacting partners specifically involved in lifespan regulation are ill defined. The ability to genetically and biochemically manipulate yeast should enable the discovery of novel forkhead lifespan determining factors. Supporting the idea that *FKH1* and *FKH2* function at least partially orthologous to metazoan FoxOs are microarray data suggesting the Fkhs redundantly regulate expression of cell cycle progression genes as well as stress resistance genes and that the *FKHs* are required for hydrogen peroxide cell cycle arrest, the regulation of which is implicated in the aging process (Honda and Honda, 1999; Kops et al., 2002a; Shapira et al., 2004; Zhu et al., 2000).

Should the yeast Fox genes be responsible for lifespan extension, the identification of both co-regulators and gene targets is paramount for establishing evolutionary conservation and the relevance of yeast as a model for eukaryotic aging. Many Fox activity modifying enzymes characterized in higher eukaryotes have structurally/functionally conserved orthologs in the budding yeast. After establishing Fkh1 and Fkh2 as regulators of lifespan, in subsequent sections we will focus on the relationship of the Fkhs with nutrient signaling mechanisms through Sch9 and Tor1 kinases, the yeast orthologs of AKT/S6K and mTOR respectively, as these signaling mechanisms control FoxO-dependent lifespan extension in higher eukaryotes (Brunet et al., 1999; Hay, 2011; Kenyon, 2011). Establishment of a relationship between the Fkhs and nutrient/growth factor/stress signaling orthologs strengthens our understanding of conservation between the FoxO-dependent lifespan of higher eukaryotes and Fkh-dependent lifespan in yeast.

Conservation of Fox-dependent lifespan regulation in yeast realizes the potential of utilizing the molecular/genetic power of yeast as a model organism to identify novel regulatory targets for

study in higher eukaryotes. The best characterized Fkh role is transcriptional regulation of genes encoding proteins involved in mitotic progression and exit, many of which are activators, targets or sub-units of the APC (Charles et al., 1998; Ko et al., 2007; Zhu et al., 2000). Furthermore, in higher eukaryotes, the APC is indirectly involved in the stabilization of the FoxOs and directly involved in the degradation of the FoxMs in G1 (Bashir et al., 2010; Gao et al., 2009; Huang et al., 2005; Huang and Tindall, 2011; Laoukili et al., 2008; Park et al., 2008), both of which may be required for both lifespan extension and tumour suppression. The highly conserved APC is implicated in longevity regulation in both yeast and mammals (Baker et al., 2005; Baker et al., 2004; Harkness et al., 2004; Harkness, 2006; Kim and Kao, 2005; Li et al., 2008a; Menzel et al., 2013; Postnikoff et al., 2012), making it an ideal novel target for investigation of shared Fkh/Fox-dependent lifespan extension. In the final section, we explore genetic interactions between the APC, the Fkhs, and Sch9/Tor1 signaling.

As well as providing insight into basic yeast biology, orthologous regulation and function of the Fkhs with higher eukaryotic mechanisms would establish budding yeast as a model for understanding the function, evolutionary history, interactions, and regulation of the forkhead box transcription factors. Moreover, it would support the utilisation of yeast genetics to determine novel targets and co-regulators involved in Fox-dependent mammalian lifespan regulation. Depending on the degree of conservation of molecular interactions and post-translation modification regulation between the Fkhs and the mammalian Foxs, yeast could be utilized as a platform for rapid drug screening of responses in specific forkhead functions such as cell death regulation, glucose tolerance induction, cell cycle arrest or entry, DNA repair mechanisms, regulation of autophagy, etc. reducing costs and development time of targeted pro-longevity, pro-health, anti-tumour and anti-diabetes pharmaceuticals.

3 Materials and Methods

3.1 Yeast genetics

3.1.1 Yeast strains and cell culture

All yeast strains used in this study were haploid and are listed in Table 3.1. Nomenclature used in this thesis identifies the specific gene affected within the strain. For example, *apc5*^{CA} refers to a specific mutation within the *APC5* gene coding sequence that results in a chromatin assembly defective (CA) phenotype (Harkness et al., 2002). Deletion of an entire gene is indicated by "Δ" following the gene name, example *apc10*Δ refers to the deletion of the entire *APC10* gene. Yeast cells were cultured at 30˚C in rich medium [YPD - 1% yeast extract (VWR, CA9000-726), 2% peptone (VWR, CA07224-1000), 2% glucose (Glc; dextrose)] or in synthetically defined (SD) medium [0.67% yeast nitrogen base without amino acids (VWR, CA99501-686), 2% Glc or galactose (Gal), plus supplementation of necessary amino acids at recommended concentrations]. Omission of specific amino acids provided selection pressure for the maintenance of transformed plasmids. Selection of the KanMX marker utilized 0.2 mg/ml G418 (Geneticin, Gibco, #11811) added to the YPD. To make solid media, 2% agar was added to the liquid medium prior to autoclaving. Molten agar was cooled to approximately 55-60˚C before pouring into petri dishes (VWR, 25384-302). For long term storage, cells were grown to log phase in YPD, suspended in 1.5% glycerol and stored at -80˚C.

3.1.2 Yeast transformation

Cultures were inoculated into 5 ml of YPD and grown at 30˚C overnight. Overnight cultures were diluted to an optical density at 600 nm (OD_{600}) 0.5 in 5 ml of fresh YPD and allowed to double in density (approximately 2 h). Cells were washed in sterile water, resuspended in 0.5 ml of 100 mM LiAc solution [0.1 M lithium acetate, 10 mM Tris-HCl pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA)] and incubated at 30˚C for 15 min. The LiAc solution was removed and 5 µl of denatured salmon sperm DNA and 3-5 µl of transforming DNA [either a plasmid (Table 3.2) or amplified product from the polymerase chain reaction (PCR; 3.2.2)] were added. For a list of plasmids used in this study see Table 3.2. The solution was thoroughly mixed with 300 µl polyethylene glycol (PEG) solution [40% PEG (3500), 100 mM LiAc, 10 mM Tris-HCl pH 8.0, 1 mM EDTA]. Transformations were incubated at 30˚C for 20 min followed by

Table 3.1 List of strains used in this study

Strain	Genotype	Source
YTH5	*MATα ade2 his3Δ200 lys2Δ201 ura3-52*	Harkness *et al* 2002
YTH457	*MATα ade2 his3Δ200 lys2Δ201 ura3-52 apc5^{CA}*	Harkness *et al* 2002
YTH1235	*MAT*a *ade2 his3Δ200 leu2 lys2Δ201 ura3-52*	Harkness *et al* 2002
YTH1637	*MATα ade2 his3Δ200 lys2Δ201 ura3-52 apc5^{CA}*-PA::*His5*	Harkness *et al* 2004
YTH1693	*MAT*(?) *his3 leu2 lys2*(?) Δ*met15 ura3 apc10*Δ::*kanMX6*	Harkness *et al* 2005
YTH2427	*MAT*(?) *ade2 his3 leu2 lys2*(?) *met15*(?) *ura3 fkh1*Δ::*kanMX6*	Postnikoff *et al.* 2012
YTH2431	*MAT*(?) *ade2 his3 leu2 lys2*(?) *met15*(?) *ura3 fkh1*Δ::*kanMX6 apc5^{CA}*-PA::*His5*	Postnikoff *et al.* 2012
YTH2444	*MAT*(?) *ade2 his3 leu2 lys2*(?) *met15*(?) *ura3 fkh2*Δ::*kanMX6*	Postnikoff *et al.* 2012
YTH2449	*MAT*(?) *ade2 his3 leu2 lys2*(?) *met15*(?) *ura3 fkh2*Δ::*kanMX6 apc5^{CA}*-PA::*His5*	Postnikoff *et al.* 2012
YTH2579	*MAT*(?) *ade2 his3 leu2 lys2*(?) *met15*(?) *ura3 fkh1*Δ::*kanMX6 fkh2*Δ::*kanMX6*	Postnikoff *et al.* 2012
YTH2581	*MAT*(?) *ade2 his3 leu2 lys2*(?) *met15*(?) *ura3 fkh1*Δ::*kanMX6 fkh2*Δ::*kanMX6 apc5^{CA}*-PA::*His5*	Postnikoff *et al.* 2012
YTH3124	*MAT*(?) *his3 leu2 lys2*(?) Δ*met15 ura3 apc10*Δ::*kanMX6 fkh1*Δ::*kanMX6*	Postnikoff *et al.* 2012
YTH3346	*MAT*(?) *his3 leu2 lys2*(?) Δ*met15 ura3 apc10*Δ::*kanMX6 fkh2*Δ::*kanMX6*	Postnikoff *et al.* 2012
YTH3409	*MAT*(?) *his3 leu2 lys2*(?) Δ*met15 ura3 apc10*Δ::*kanMX6 fkh1*Δ::*kanMX6 fkh2*Δ::*kanMX6*	Postnikoff *et al.* 2012
YTH3501	*MAT*(?) *ade2 his3 leu2 lys2*(?) *met15*(?) *ura3 sch9*Δ::*URA*	This study
YTH3503	*MAT*(?) *ade2 his3 leu2 lys2*(?) *met15*(?) *ura3 tor1*Δ::*kanMX6 apc5^{CA}*-PA::*His5*	This study
YTH3504	*MAT*(?) *ade2 his3 leu2 lys2*(?) *met15*(?) *ura3 sch9*Δ::*URA tor1*Δ::*kanMX6*	This study
YTH3505	*MAT*(?) *ade2 his3 leu2 lys2*(?) *met15*(?) *ura3 sch9*Δ::*URA3 apc5^{CA}*-PA::*His5*	This study
YTH3506	*MAT*(?) *ade2 his3 leu2 lys2*(?) *met15*(?) *ura3 sch9*Δ::*URA3 tor1*Δ::*kanMX6 apc5^{CA}*-PA::*His5*	This study
YTH3743	*MAT*(?) *his3 leu2*(?) *lys2*(?) Δ*met15 ura3*	This study
YTH3744	*MAT*(?) *his3 leu2*(?) *lys2*(?) Δ*met15 ura3 sch9*Δ::*URA3 tor1*Δ::*kanMX6*	This study
YTH3745	*MAT*(?) *his3 leu2*(?) *lys2*(?) Δ*met15 ura3 sch9*Δ::*URA3 tor1*Δ::*kanMX6*	This study
YTH3747	*MAT*(?) *his3 leu2*(?) *lys2*(?) Δ*met15 ura3 sch9*Δ::*URA3 tor1*Δ::*kanMX6*	This study

YTH3760	*MAT*(?) *his3 leu2*(?) *lys2*(?) Δ*met15 ura3 fkh1*Δ::*kanMX6 fkh2*Δ::*kanMX6*	This study
YTH3762	*MAT*(?) *his3 leu2*(?) *lys2*(?) Δ*met15 ura3 sch9*Δ::*URA3 fkh1*Δ::*kanMX6 fkh2*Δ::*kanMX6*	This study
YTH3764	*MAT*(?) *his3 leu2*(?) *lys2*(?) Δ*met15 ura3 tor1*Δ::*kanMX6 fkh1*Δ::*kanMX6 fkh2*Δ::*kanMX6*	This study
YTH3766	*MAT*(?) *his3 leu2*(?) *lys2*(?) Δ*met15 ura3 sch9*Δ::*URA3 tor1*Δ::*kanMX6 fkh1*Δ::*kanMX6 fkh2*Δ::*kanMX6*	This study
YTH3926	as YTH1235, but *FKH1-TAP::HIS3*	Postnikoff *et al.* 2012
YTH3929	as YTH1235, but *FKH2-TAP::HIS3*	Postnikoff *et al.* 2012
YTH3935	as YTH3501, but *FKH1-TAP::HIS3*	This study
YTH3937	as YTH3501, but *FKH2-TAP::HIS3*	This study
YTH3943	as YTH3504, but *FKH1-TAP::HIS3*	This study
YTH3944	as YTH3504, but *FKH2-TAP::HIS3*	This study
YTH4265	as YTH3929, but *LEU2::GAL1/10$_{prom}$-FKH2-TAP::HIS3*	Postnikoff *et al.* 2012
YTH4269	*MAT*a *ade2 his3*Δ*200 leu2 lys2*Δ*201 ura3-52*	Postnikoff *et al.* 2012
YTH4315	*MAT*a *his3*Δ*1* Δ*leu2* Δ*met15* Δ*ura3 FKH1-GFP::HIS3*	Open Biosystems
YTH4316	*MAT*a *his3*Δ*1* Δ*leu2* Δ*met15* Δ*ura3 FKH2-GFP::HIS3*	Open Biosystems
YTH4501	*MAT*(?) *ade2 his3*Δ*200 leu2 lys2*Δ*201 ura3-52 LEU2::GAL1/10$_{prom}$-FKH1-TAP::HIS3*	This study
YTH4502	*MAT*(?) *ade2 his3*Δ*200 leu2 lys2*Δ*201 ura3-52 LEU2::GAL1/10$_{prom}$-FKH2-TAP::HIS3*	This study
YTH4503	*MAT*(?) *ade2 his3*Δ*200 leu2 lys2*Δ*201 ura3-52 LEU2::GAL1/10$_{prom}$-FKH1-TAP::HIS3 LEU2::GAL1/10$_{prom}$-FKH2-TAP::HIS3*	This study
YTH4516	as YTH3926, but *LEU2::GAL1/10$_{prom}$-FKH1-TAP::HIS3*	Postnikoff *et al.* 2012
YTH4726	*MAT*a *ade2 his3*Δ*200 leu2 lys2*Δ*201 ura3-52 hog1*Δ::*kanMX6*	This study
YTH4772	as YTH4501, but *hog1*Δ::*kanMX6*	This study
YTH4773	as YTH4502, but *hog1*Δ::*kanMX6*	This study
YTH4774	as YTH4503, but *hog1*Δ::*kanMX6*	This study
#70	*MAT*(?) *ade2 his3*Δ*200 lys2*Δ*201 ura3-52 snf1*Δ*::kanMX6*	Terra Arnason
#220	*MAT*(?) *his3 leu2*(?) *lys2*(?) Δ*met15*(?) *ura3 snf1*Δ*::kanMX6 fkh1*Δ::*kanMX6 fkh2*Δ::*kanMX6*	Terra Arnason

Table 3.2 List of plasmids used in this study

Plasmid	Vector	Insert	Source
YCp50	*CEN-URA3*		M. Ellison
YEplac181-Gal	*CEN-LEU2*	*GAL1/10*$_{Prom}$	W. Neupert
BG1805-*FKH1*	2μ-URA3	*GAL1prom-FKH1-HA*	W. Xiao
BG1805-*FKH2*	2μ-URA3	*GAL1prom-FKH2-HA*	W. Xiao

heat shock at 42˚C for 15 min. Following the incubations, cells were washed in 100 µl of 1 M sorbitol and resuspended in 100 µl of 1 M sorbitol. The cell/sorbitol solution was spread on selective agar media, allowed to dry and incubated at 30˚C until colonies were observed: typically two to five days. In the case of KanMX marker selection, following the 42˚C incubation cells were washed in 100 µl of 1 M sorbitol, resuspended in 1 ml YPD and incubated 8-12 h to allow for cell recovery and expression of the KanMX construct before plating on G418 containing media.

3.1.3 New strain formation

3.1.3.1 Genetic crossing

Mutants were repeatedly backcrossed with a wild type S288c background strain until multiple isolates displayed identical phenotypes (typically 5 or 6 crosses were needed). Haploid strains of opposite mating type were combined on solid YPD medium and allowed to conjugate for two days to form diploid cells. Diploids were then transferred to a sporulation plate (1% potassium acetate, 0.1% yeast extract, 0.05% Glc, 2% agarose, 5 ml adenine) and incubated at room temperature. The formation of tetrads (usually taking one to two weeks) was determined by light microscopy. Tetrads appeared as four small spore cells encapsulated by a spore coat, often forming a regular tetrahedron. Sporulated tetrads were suspended in 100 µl of lyticase solution (distilled water with 0.5 mg/ml lyticase). Twenty-five µl of the tetrad/lyticase solution was applied to a YPD plate and allowed to dry for 20 to 30 min. Once dry, individual tetrads were separated into single cells using a micromanipulator (Singer MSM) and incubated at 30˚C to form colonies. The colonies were struck onto selective media to determine desired genotypes. Two mutations with the same selection marker were identified by 2:2 segregation of spore genotypes from a single tetrad. Alternate phenotypes were used to determine triple and quadruple mutants with the same selective marker, such as flocculence for *fkh1*Δ::KanMX *fkh2*Δ::KanMX double mutants or temperature sensitivity for strains containing *tor1*Δ::KanMX.

3.1.3.2 Transformation and homologous recombination

Mutant DNA sequences were amplified by PCR from genomic DNA extracts (3.2.1) or plasmids. To accomplish this, primers were designed with the 3’ sequence of 18-20 nucleotides to prime from the selectable marker with a 5’ sequence of 50-60 nucletides homologous with the site of

integration/recombination. PCR products were transformed into yeast cells (3.1.2), allowing for genomic integration by homologous recombination (Figure 3.1).

3.1.4 Spot dilutions

For spot dilution assays, 3 µl of cells from a 10-fold dilution series were pipetted onto various solid media and grown at the temperatures indicated. The starting spot generally contained $3x10^4$ cells.

3.1.5 Chronological lifespan assay

Chronological lifespan assays were performed as previously described, with modifications (Fabrizio and Longo, 2003, 2007; Harkness et al., 2004; Postnikoff and Harkness, 2014; Postnikoff et al., 2012). Overnight SD complete media (SDC; SD media supplemented with all amino acids) cultures were diluted to OD_{600} 0.5 in fresh SDC with a flask to culture volume ratio of 5:1, and were incubated at 200 RPM at 30ºC. Each day the same volume of culture was diluted 1:1000 in water, with 10 µl of the diluted sample plated to evaluate CFUs as a measure of viability. When the CFU counts peaked, this was deemed "stationary phase" and denoted as Day 1. Every two days, CFU counts were determined and compared to Day 1. Cultures were maintained in the nutrient depleted medium (DM) in which they grew. For severe caloric restriction (SCR) experiments, once stationary phase was reached in SDC (Day1), cultures were washed and re-suspended in sterile distilled water, with washes of equal volume of water every two to four days to remove metabolites produced by the cells. For overexpression experiments, Gal was added to appropriate cultures upon reaching Day 1, to final concentration of 0.05%. Mild CR was examined by growing cultures in 0.5% Glc with subsequent maintenance in the resulting DM.

3.1.6 Oxidative stress resistance assay

Cultures were grown in 2% YPD or SDC at 30ºC for five days and then diluted to OD_{600} 1 in culture specific DM. Each of these cultures were divided in half and 100 mM H_2O_2 (EMD Chemicals) was added to one of the samples. Both samples were incubated at 30ºC for 1 h. Viability was determined by plating 10 µl of culture diluted 1:1000 in water onto 2% YPD and comparing the CFU growth of the H_2O_2-treated culture to that of the non-treated control.

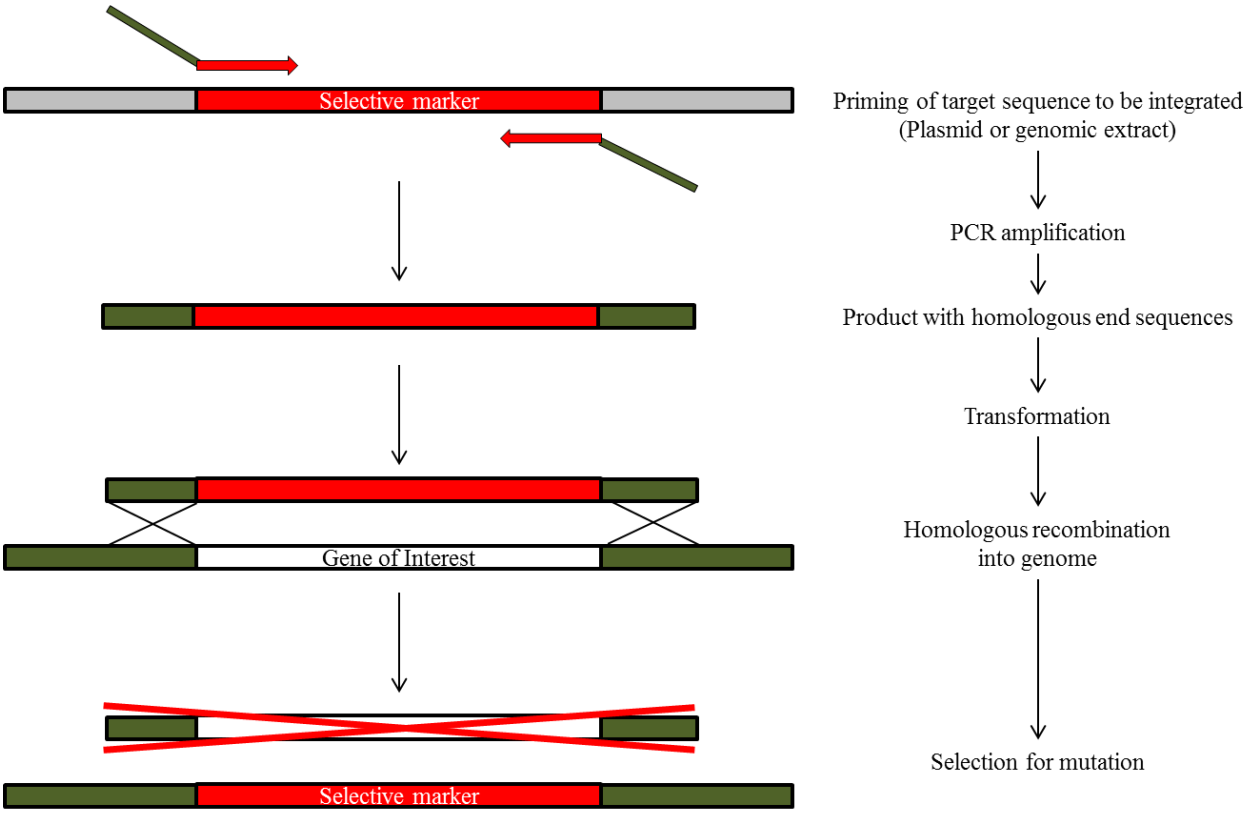

Figure 3.1 Schematic of genomic mutation through homologous recombination
Mutant DNA sequences/selectable markers are amplified by PCR, using primers with the 3' sequence of 18-20 nucleotides to prime from the selectable marker with a 5' sequence of 50-60 nucleotides homologous with the site of integration/recombination. These products were transformed into yeast cells, allowing for genomic integration by homologous recombination. This is followed by selection for stable integration.

3.1.7 Fluorescence microscopy

Samples from CLS cultures were washed and mounted in PBS or Ultracruz mounting medium (Santa Cruz Biotechnology sc-24941) and imaged using 100x oil immersion with an Olympus BX51 fluorescent microscope. Images were captured using an INFINITY 3-1UM camera and analyzed with Infinity Analyse software version 5.0.3 (Lumenera).

3.1.7.1 Annexin 5 staining

Exposed phosphatidylserine, a marker of apoptosis, was detected in samples from day 1 CLS cultures though conjugation with highly fluorescent FITC-coupled annexin V. Yeast cells were washed in 1 X PBS buffer (pH 6.8), followed by incubation in 1 X PBS buffer with 5.5% glusulase (*Boehringer Mannheim*) and 15 U/ml lyticase (*Sigma Chemical Co.*) for 2 h at room temperature to digest the cell wall without compromising the integrity of the plasma membrane. The cells were then washed in PBS, and resuspended in binding buffer/sorbitol. Two µl annexin-FITC (CLONETECH Laboratories, Inc.) and 2 µl propidium iodide (500 µg/ml; PI) were added to the 36 µl cell suspension and incubated for 20 min at room temperature. The cells were washed and suspended in PBS and applied to a microscope slide. Cells were scored for no staining (healthy), annexin V only (cells undergoing apoptosis as detected by phosphatidylserine translocation to external surface of cell membrane), and cells stained with nuclear PI (dead/dying cells with disrupted membrane integrity with an undetermined cause of death).

3.2 Molecular biology techniques

3.2.1 Yeast genomic DNA extraction (Smash and Grab)

Cells were pelleted in a 2 ml bead beating tube, suspended with 100 µl glass beads in 150 µl breaking buffer [2% triton X-100, 1% Sodium dodecyl sulfate (SDS), 100 mM NaCl, 10 mM Tris-HCL pH 8.0, 1 mM EDTA] and 150 µl phenol:chloroform:isoamyl alcohol (25:24:1), and beat with a bead beater (Mini-Beadbeater, BIOSPEC Products, INC.) for three min. The lysate was centrifuged for 10 min at 14000 rpm with the upper aqueous layer transferred to a fresh tube. One-tenth the volume transferred of 3M NaAc pH 4.8 and 2.5 times the volume 95% ethanol was added. The tube was put on ice for 5 min to precipitate genomic DNA, which was pelleted at 4˚C at 14000 rpm for 10 min, washed with 70% ethanol, and resuspended in 40 µl sterile distilled water.

3.2.2 PCR

DNA fragments for transformation were amplified using PCR from plasmids, or genomic yeast DNA obtained by Smash and Grab (3.2.1), as the template. Primers were designed to include 250-500 base pairs of homology upstream and downstream, if the final product was used for homologous recombination. For example, we used this approach to move *hog1*Δ::*KanMX6* into the *FKH* over-expression strains. See Table 3.3 for primer sequences. A typical PCR reaction mixture contained 0.2 mM of each deoxyribonucleotide triphosphate (dNTP; Bio Basic Inc., #D0056), 1 ng of forward and reverse primers, 0.5 ng of genomic DNA, 1X PCR Buffer (Sigma, #P2317), 0.5 µl *Taq* Polymerase (New England Biolabs, #M0267), and distilled water, to a final volume of 50 µl. Amplification was carried out in a thermocycler (Eppendorf Mastercycler) with a standard PCR protocol: 98˚C for four min, followed by 30 repeats of 1 min denaturing at 98˚C, 1 min annealing at 52˚C, and 2 min of elongation at 72˚C. A final 5 min incubation at 72˚C was followed by indefinite 4˚C storage. Volumes of reagents, temperatures and times were adjusted to optimize reactions as necessary.

3.2.3 Agarose gel electrophoresis

Analysis of plasmid and genomic DNA was performed using a 1% agarose gel containing 0.5 µg/ml ethidium bromide for visualization under ultra-violet light. The gel was immersed in 1 X TAE (24% Tris-base, 5.7% glacial acetic acid, 10% EDTA pH 8.0). Samples were run parallel to 5 µl of a DNA ladder (Fermentas, #SM0313).

3.2.4 TCA protein extraction

One ml of OD_{600} 1 culture was centrifuged for 3 min at 4000 rpm and the supernatant removed for protein analysis. The cells were suspended in 250 µl of freshly made, ice cold solution C (1.85 M NaOH, 7.4% 2-Mercaptoethanol) plus 250 µl of 100% trichloroacetic acid (TCA; VWR, CATX1045), incubated on ice for 10 min and centrifugated for 10 min at 4˚C. Pellets were washed in 1 ml of sterile distilled water and resuspended in 25 µl solution A (13% SDS and 1 M Tris) and 25 µl solution B (30% glycerol plus a small amount of Bromophenol Blue). Samples were stored at -80˚C and prepared for Western analysis as needed.

Table 3.3 List of primer sequences used in this study

Name*	Sequence	Function
Fkh1 GAL up	5'GATCCTGGGCTGTATACGAACTATACTTACCACTAAATTTTTGTTCCCTACTGGTAACAGACATTGGATGGACGCAAAGAAG	Amplify the *LEU2* marker coding region of YEplac181-Gal vector for insertion 5' of genomic *FKH1* coding region
Fkh1 GAL down	5'AATAATAGTGTGTAAATTGTGCGTTCAATTAGCAAAGAAAGCTTGGAGAGACACAGTAATAATAATAACGAGAACACACAGGG	Amplify the GAL_{PROM} marker coding region of YEplac181-Gal vector for insertion 5' of genomic *FKH1* coding region
Fkh2 GAL up	5'GTGCTGCCATAATTTGTTTGAGTCATATTTAGTCGTTCGTTCATCTCGTTAAAATTGCTGCTGGACATTGGATGGACGCAAAGAAG	Amplify the *LEU2* marker coding region of YEplac181-Gal vector for insertion 5' of genomic *FKH2* coding region
Fkh2 GAL down	5'GATACACATAAATATTGGTGTGCTCCCTCCGTTTCCTTTATTGAAACTTTATCAATGCGCAAGAATAACGAGAACACACACAGGG	Amplify the GAL_{PROM} marker coding region of YEplac181-Gal vector for insertion 5' of genomic *FKH2* coding region
Universal GAL up	5'AAAAAATTGTTAATATACCTCTATACTTTAACGTCTTAAGCAAGGATTTTCTTAAC	Amplify the GAL_{PROM} marker coding region of YEplac181-Gal vector for insertion before any gene
Universal GAL down	5'CTGTCGCCGAAGAAGTTAAGAAAATCCTTGCTTAAGACGTTAAAGTATAGAGG	Amplify the *LEU2* marker coding region of YEplac181-Gal vector for insertion before any gene
HOG1 500 bp upstream FWD	5' TTGGTAGCCCTTCATTACGG	Amplify the genomic *HOG1* coding region for transfer of the *hog1*Δ::*KanMX6* construct
HOG1 500 bp downstream REV	5' AGTTTCTGCCACAGTCCGTG	Amplify the genomic *HOG1* coding region for transfer of the *hog1*Δ::*KanMX6* construct
FKH1+500TAP	5' ACCCGAAGGTAGTATTTC	Amplify the +/- 500 bp of genomic *FKH1* stop codon for transfer of 3' (c-terminal) epitope tags
FKH1-500TAP	5' GCACTGAGTTCTTCGAGATC	Amplify the +/- 500 bp of genomic *FKH1* stop codon for transfer of 3' (c-terminal) epitope tags
FKH2+500TAP	5' ACACCAGAAAGAGGAAGC	Amplify the +/- 500 bp of genomic *FKH2* stop codon for transfer of 3' (c-terminal) epitope tags
FKH2-500TAP	5' GGAAAATCACCCACTTGG	Amplify the +/- 500 bp of genomic *FKH2* stop codon for transfer of 3' (c-terminal) epitope tags

*Primer names as in Harkness lab stocks; up(stream), FWD, and + represent forward primers, while down(stream), REV, and - represent reverse primers.

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

Yeast whole cell protein extraction was performed as previously described (3.2.4). Samples were resolved on a 10% acrylamide SDS-PAGE gel and transferred to nitrocellulose membrane (PALL) at 400 mAmps for 1 h. The membranes were blocked in 5% non-fat milk (Biorad) and phosphate buffered saline with Tween-20 (PBST) overnight at 4°C and incubated with primary antibody [rabbit anti-TAP antibody (Open Biosystems) at a dilution of 1:1000 and mouse monoclonal anti-GAPDH (Sigma) at a dilution of 1:20,000] in 5% non-fat milk and PBST for 1.5 h at room temperature or overnight at 4ºC. Membranes were washed three times in PBST for 15 min and incubated in 1:10,000 dilution of secondary antibody in 5% non-fat milk and PBST for 1 h at room temperature. Membranes were washed three times in PBST for 15 min, processed with Enhanced Chemiluminescence reagent (PerkinElmer), and exposed to Kodak film.

4 *FKH1* and *FKH2* regulate lifespan in yeast[2]

4.1 Introduction

The Fox family of transcription factors has been demonstrated to regulate metazoan cellular growth, development, and lifespan, as well as affecting cancer development (Katoh and Katoh, 2004; Lehmann et al., 2003; Wijchers et al., 2006). One well characterized sub-family, the FoxOs, have been shown to regulate processes involved in the homeostasis of metazoan cells and tissues with the net outcome of lifespan extension and tumor suppression (Calnan and Brunet, 2008; Fu and Tindall, 2008; Hay, 2011; Monsalve and Olmos, 2011; Tran et al., 2003; Zhang et al., 2011); however, other Foxs have been implicated in exacerbating aging and the onset of age related diseases such as cancer and diabetes (Laoukili et al., 2007; Lehmann et al., 2003; Wijchers et al., 2006). The development of these conditions involve numerous cellular processes, especially activation and repression of the cell growth and proliferation, as well as regulating stress response pathways, such as free radical scavenging, DNA damage repair, increased genomic stability, autophagy, and apoptotic mechanisms (Greer and Brunet, 2008; Tran et al., 2003; Zhang et al., 2011). The regulation of these processes involves a complex system of post translational modifications and cofactor binding (Boccitto and Kalb, 2011; Calnan and Brunet, 2008; Daitoku et al., 2011; Huang and Tindall, 2011; Martínez-Gac et al., 2004; Tzivion et al., 2011). A challenge to the study of fundamental Fox function is the presence of 50 identified human Fox genes in 19 sub-families, each with potential alternative splice variants that share the highly conserved forkhead box DNA binding domain (Katoh and Katoh, 2004; Myatt and Lam, 2007; Wijchers et al., 2006). This suggests potential competition for DNA binding sites and allows for differential regulation of similar gene clusters under specific conditions.

The budding yeast *S. cerevisiae* is a powerful tool for elucidating genetic and molecular mechanisms of cellular processes; however, the four yeast Fox genes have been found to not individually regulate chronological lifespan (Wei et al., 2008). Two of these, Fkh1 and Fkh2, are phenotypically redundant in function in M/G1 progression/arrest, similar to the lifespan regulating FoxOs, as both are involved in cell cycle arrest in response to hydrogen peroxide

[2] The majority of this chapter has been published in Postnikoff et al. (2012)

(Linke et al., 2013; Shapira et al., 2004). These characteristics led us to initiate an examination of the conserved role of Fkh1 and Fkh2 as redundant functional orthologs of the pro-life/health span Fox proteins of higher eukaryotes, i.e. the FoxOs. Should yeast Fox proteins demonstrate homologous functions with those of higher eukaryotes, then yeast may be utilized to characterize the fundamentals of Fox regulatory mechanisms. This chapter provides evidence that the Fkhs are redundant positive determinants in yeast lifespan. It also provides evidence supporting evolutionary conservation of function between the Fkhs and the FoxOs with regards to caloric restriction response, oxidative stress resistance, cell cycle arrest, and apoptosis.

4.2 Methods

To investigate the genetics of *FKH1* and *FKH2*, both deletion and Gal inducible expression mutants were examined in actively dividing cells by spot dilutions (3.1.4), in stationary phase using CLS (3.1.5) and oxidative stress resistance (3.1.6) assays maintained in standard (depleted 2% Glc media), as well as lifespan and stress resistance increasing conditions, including CR (depleted 0.5% Glc media) and severe CR (SCR; maintained in water upon entry to stationary phase). Fkh-TAP (c-terminal epitope tag fusion protein) expressing cell lysates were used to determine Fkh abundance by western analysis using anti-TAP antibodies (Open Biosystems). Cellular localization of the Fkhs was determined by fluorescent microscopy of Fkh-c terminal GFP (green fluorescent protein) fusion protein localization (3.1.7). Finally, apoptosis was observed in Fkh1 over-expressing cells, through the observation of phosphatidylserine translocation as determined by fluorescent microscopy of cells co-stained with Annexin V and PI (3.1.7.1).

4.3 Results

4.3.1 *FKH1* and *FKH2* redundantly regulate chronological lifespan

Past studies found deletion of individual yeast Fox genes (*FKH1*, *FKH2*, *HCM1* and *FHL1*) did alter CLS (Wei et al., 2008). However, *FKH1* and *FKH2* have been shown to be phenotypically redundant, as at least one of them is required for proper M/G1 progression and cell cycle arrest in response to hydrogen peroxide (Hollenhorst et al., 2000; Koranda et al., 2000; Shapira et al., 2004; Zhu et al., 2000). The Harkness lab has shown that *fkh1*Δ or *fkh2*Δ individual mutants have no effect on replicative lifespan, while the *fkh1*Δ *fkh2*Δ double mutant was unable to be

assayed due to cell wall abnormalities (Postnikoff et al., 2012). To further examine the relationship of the *FKHs* with lifespan, we investigated the CLS of strains containing combinations of *fkh1*Δ and/or *fkh2*Δ gene disruptions. Congruent with the literature, little to no difference in the CLSs of *fkh1*Δ, *fkh2*Δ, and wild type were observed. However, the *fkh1*Δ *fkh2*Δ double mutant had a reduced CLS compared to wild type when the post-mitotic cultures were maintained in either DM or SCR conditions (Figures 4.1 and 4.2 A). In DM the average lifespan (50 % survival) of *fkh2*Δ was 1.29 times greater than wild type and *fkh1*Δ (Figure 4.1 A); however, this is not significant, due to overlapping standard error of the mean. The lifespan of *fkh1*Δ *fkh2*Δ was approximately half that of the wild type, suggesting genetic redundancy of the *FKHs* in the regulation of yeast lifespan. This redundancy became more apparent when the cultures were maintained in SCR, as wild type and either single mutant had the same lifespans, while *fkh1*Δ *fkh2*Δ had an average lifespan 0.25 times that of wild type and the single mutants (Figure 4.1 B). When we directly compared the CLS of wild type and *fkh1*Δ *fkh2*Δ strains maintained in DM to SCR conditions we observed *fkh1*Δ *fkh2*Δ strains did not respond to SCR, while the wild type benefited from a two to three time increase in lifespan (Figure 4.2 A). A similar trend was observed in mild CR conditions. A single exploratory CLS was performed on cultures grown in 0.5% SDC and maintained in the resulting DM. The 50% survival of the 0.5% DM *fkh1*Δ *fkh2*Δ was extended compared to 2% DM, while by 10% the survival was mildly decreased (Figure 4.2 B). These data suggest that *FKHs* may be involved in CR and other stress-induced lifespan regulatory mechanisms.

4.3.2 Increased *FKH* expression extends chronological lifespan

Short-lived mutant strains are often met with skepticism as to whether the genes are *bona fide* lifespan regulators or merely ones that cause a premature death due to a disease state similar to developmental defects. In contrast, studies showing elongated survival are respected as directly relevant, such as the increased expression of the FoxO/Daf-16 transcription factors increasing lifespan in multiple model organisms (Giannakou et al., 2004; Henderson and Johnson, 2001; Hwangbo et al., 2004). To address this concern and further investigate the role of the *FKHs* in the regulation of lifespan, we controlled gene expression by integrating the *GAL1*/*GAL10* Gal inducible promoter upstream of the genomic *FKH1-TAP* or *FKH2-TAP* coding region

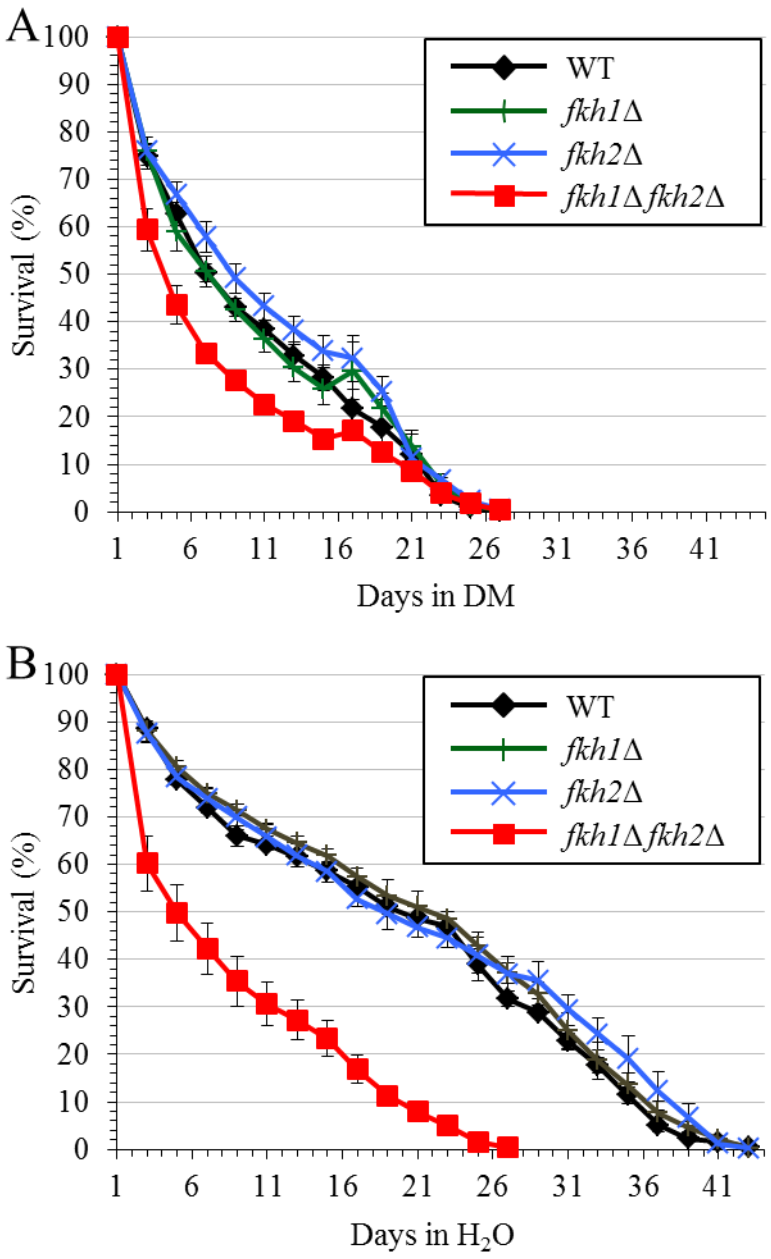

Figure 4.1 The redundant *FKHs* are required for chronological lifespan extension
Cultures were grown in 2% Glc SDC. Upon entry into stationary phase the cultures were maintained in DM (A) or water (B). Error bars represent +/- standard error of the mean of n=3.

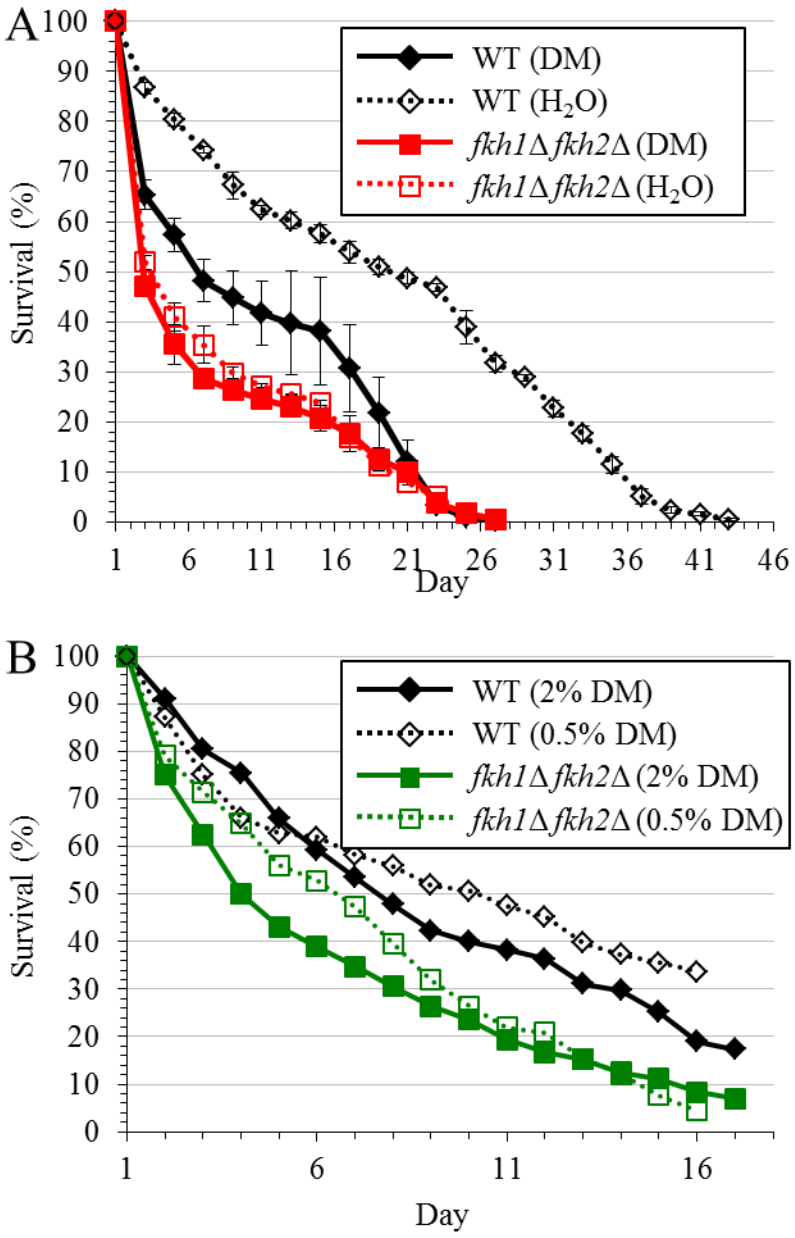

Figure 4.2 The *FKHs* are required for caloric restriction dependent lifespan
A) Chronological lifespan comparing wild type (black) to strains containing *fkh1*Δ *fkh2*Δ alleles (red) grown in 2% Glc SDC and maintained in DM (filled markers) or water (empty markers). Error bars represent +/- standard error of the mean of at least n=3. B) Chronological lifespan comparing wild type (black) to strains containing *fkh1*Δ *fkh2*Δ alleles (green) grown in 2% (filled markers) or 0.5% (empty markers) Glc SDC followed by maintenance in DM throughout stationary phase.

(*GAL$_{PROM}$-FKH1*, *GAL$_{PROM}$-FKH2*; Figure 4.3 A). To accomplish this two sequences of the YEplac181-Gal plasmid were amplified for each of the *FKHs*: 1) the *LEU2* selective marker gene using primers Fkh# GAL up and Universal GAL; and 2) the *GAL1*/*GAL10* promoter using primers Universal GAL up and Fkh# GAL down primers. The 5' sequence of the *LEU2* product and the 3' sequence of the *GAL1*/*GAL10* promoter product contained homology to genomic sequence immediately upstream of the respective *FKH* coding sequence, displacing the native promoter. The 3' sequence of the *LEU2* product and the 5' sequence of the *GAL1*/*GAL10* promoter product were homologous to one another, due to conserved sequence designed into the 'Universal GAL' promoters. For integration and the presence of the selective marker three recombinations were required, one combining the two products and the other two between the genome and the ends of the newly formed larger fragment. Strains were tested for protein levels after Gal induction of the integrated promoters (Figure 4.3 B and C). Once expression control was verified, strains with altered *FKH1* and *FKH2* expression were crossed to produce a strain with both *FKH* genes under the Gal inducible promoter (*GAL$_{PROM}$-FKH1/2*). Again, Gal inducible protein levels were confirmed by western analysis (Figure 4.3 D). Although neither Fkh1 nor Fkh2 was observable by western blot analysis in samples derived from cultures maintained in Glc, the strains showed wild type morphology rather than *fkh1*Δ *fkh2*Δ abnormal phenotypes, such as flocculence (Figure 4.3; data not shown). This suggests low level *FKH* expression from the *GAL* promoter, even in the absence of Gal stimulus as previously observed in our lab using a similar system (Turner et al., 2010).

We tested the beneficial effects of the Fkhs on post-mitotic cells by performing the CLS assay with the *GAL$_{PROM}$-FKH*s in DM with and without the addition of 0.05% Gal at stationary phase Day 1. A low concentration of Gal was used to prevent re-entry into the cell cycle. The cultures containing the Gal inducible promoter showed a trend toward extended CLS under non-inducing conditions (no Gal added), especially with regards to maximal lifespan; for example, *GAL$_{PROM}$-FKH1* had about 1.2 times the lifespan of wild type at 50% survival and this increased to 2 times wild type lifespan at 10% survival (Figure 4.4 A). The addition of 0.05% Gal extended the mean CLS of all of the strains, with wild type CLS increasing 1.33 times, *GAL$_{PROM}$-FKH1* 1.5 times, *GAL$_{PROM}$-FKH2* three times, and *GAL$_{PROM}$-FKH1/2* 2.33 times relative to non-induced

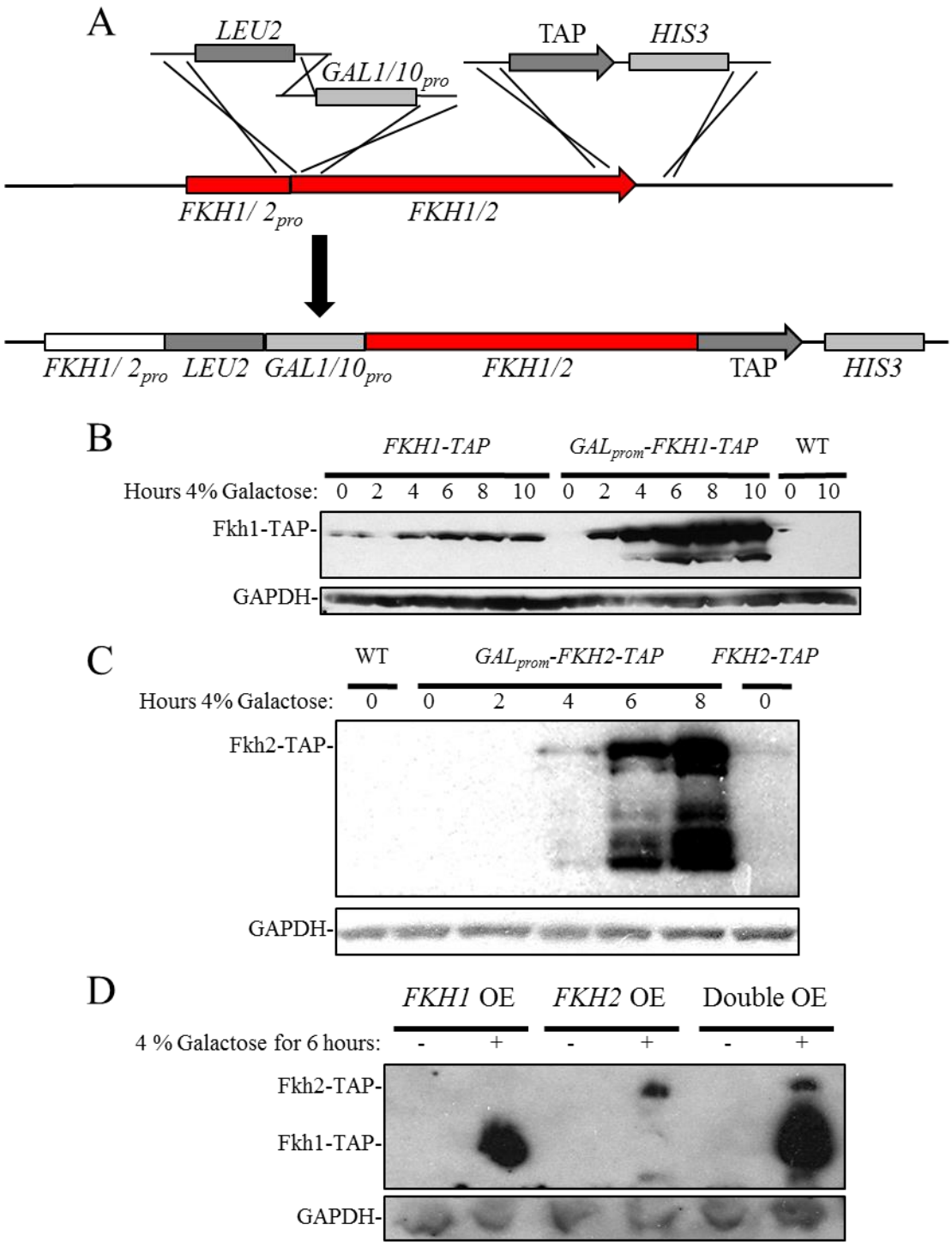

Figure 4.3 Development of constructs to endogenously control the expression of *FKH1* and *FKH2*
A) The model demonstrating the strategy of integrating the *GAL1*/10 promoter to control *FKH* expression. B-D) Western analysis to confirm protein abundance control in (B) *GALpromFKH1-TAP*, (C) *GALpromFKH2TAP*, and (D) the double over expression mutant (OE; *GALpromFKH1-TAP GALpromFKH2-TAP*), which was generated through crossing.

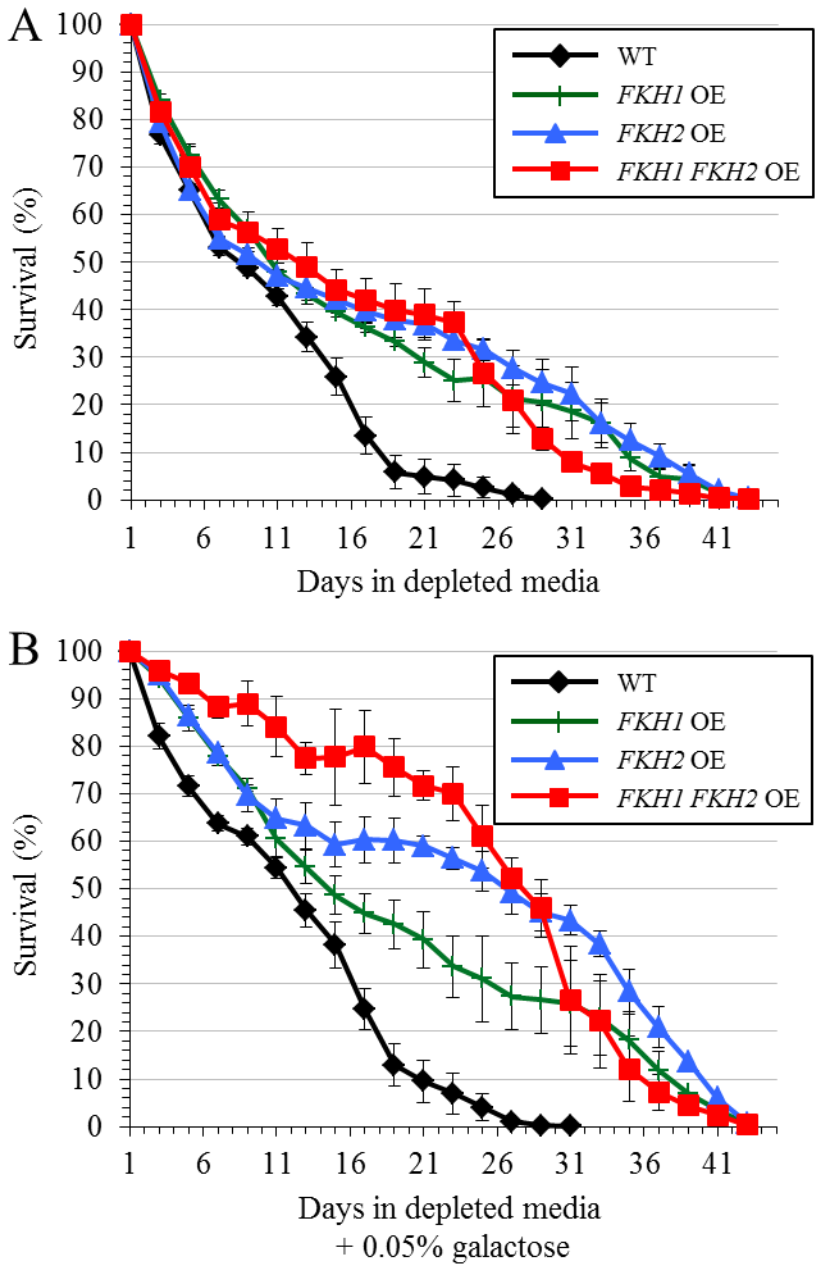

Figure 4.4 Over-expression of the *FKHs* extends chronological lifespan
Cultures were grown in 2% Glc SDC. Upon entry into stationary phase the cultures were maintained in DM (A) or 0.05% Gal (w/v) to induce *FKH* expression (B). Error bars represent +/- standard error of the mean of n=3.

conditions (Figure 4.4 A and B). However, maximal lifespan extension was not altered between inducing and non-inducing conditions. This could be due to the de-repression of alternate carbon source promoters under prolonged stationary phase conditions, resulting in late lifespan increases in Fkh levels. If this was the case, it would still suggest that increased Fkh levels in aged populations confers beneficial lifespan regulatory effects. Overall, these data indicate the activation of the promoter led to striking increases in lifespan, suggesting increased Fkh levels may extend CLS.

4.3.3 The presence of *FKHs* in post-mitotic cells

The Fkhs are expressed in actively dividing cells corresponding to cell cycle progression, specifically expressed in S phase and regulate progression through mitosis (Hollenhorst et al., 2000; Kumar et al., 2000; Pramila et al., 2006; Tu et al., 2005; Zhu et al., 2000). Our data presented in this chapter so far provide indirect evidence of a role for the *FKHs* in post-mitotic lifespan regulation, but not of the presence or activity of the Fkh proteins under standard conditions. For example, the observations presented in Section 4.3.1 suggest the deletion of both genes affects post-mitotic lifespan; however, this could be an artefact of mitotic growth phase dysfunction that decreases cellular fitness later in life, such as genomic instability from improper chromatin metabolism or segregation, or cell wall malformations in actively dividing cells that decrease the population's fitness, rather than a specific post-mitotic role in lifespan extension. Section 4.3.2 provides evidence that induced expression of the *FKHs* in post-mitotic cells results in increased lifespan. However, these findings do not inform us of the presence of the Fkhs in post-mitotic cells under the control of their native promoters.

To address the above concerns, we examined the presence of the Fkhs in post-mitotic cells throughout the CLS assay. Protein lysates were prepared following Day 1 and 5 stationary phase from cultures expressing *FKH1-TAP* or *FKH2-TAP*. The cultures were grown to stationary phase in 2% Glc SDC and maintained in the DM or in sterile distilled water. Fkh1-TAP, detected by western analysis, was present in both Day 1 and 5 post-mitotic cultures (Figure 4.5). Expression of Fkh2-TAP was inconsistent and often undetectable (data not shown). Likely this was due to Fkh2 being expressed at low levels in cycling cells (Rodriguez-Colman et al., 2010), and protein synthesis and specific protein abundance decreasing during transition to stationary phase

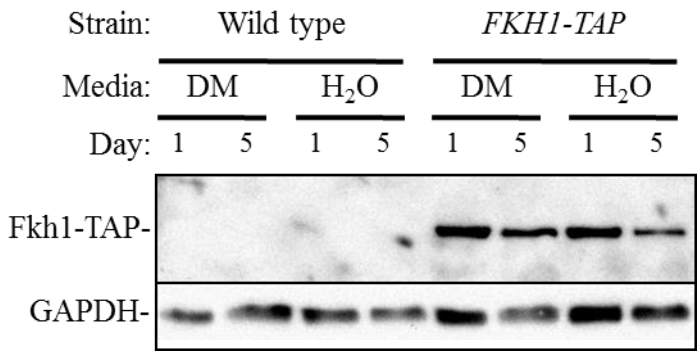

Figure 4.5 Stationary phase Fkh1 protein concentration is not altered by maintenance in water
Cultures were grown in 2% Glc SDC. Upon entry into stationary phase the cultures were maintained in DM or water. Western analysis of Fkh1 abundance at stationary phase day 1 and 5 in both DM and water relative to GAPDH.

(Bataille et al., 1991; Boucherie, 1985). Similar Fkh1-TAP protein levels were observed in cultures maintained in DM and water suggesting the role of the Fkhs in SCR lifespan extension seen in 4.3.2 is likely not linked to increased protein abundance, but to a change in their activity. The FoxO family of transcription factors is highly regulated by post-translational modifications, many of which affect nuclear import/export in response to environmental conditions. Specifically, phosphorylation induced by nutrient signaling results in FoxO nuclear export, while oxidative radical stress and DNA damage can induce nuclear import [reviewed in (Tran et al., 2003; Van Der Heide et al., 2004; Vogt et al., 2005)].

To investigate whether sub-cellular localization of the Fkhs is responsible for increased CLS due to SCR, as inferred in Section 4.3.1, we utilized strains expressing genomic *FKH1-GFP* or *FKH2-GFP* chimeric constructs (Open Biosystems). For CLS analysis, the cultures were grown in 2% Glc SDC and maintained in either DM or water (Figure 4.6 A). Subcellular localization of the Fkhs was observed under 100x fluorescent microscopy. In Day 5 stationary phase cultures maintained in DM, nuclear GFP fluorescence was observed in many cells (Figure 4.6 B). When Fkh1-GFP and Fkh2-GFP were monitored throughout CLS, we observed a larger proportion of the Fkh protein remained nuclear in water compared to DM. For example, Day 13 stationary phase cells appeared healthier, with a larger proportion of nuclear Fkh2-GFP when maintained in water (Figure 4.6 C). The percentage of cells harbouring nuclear Fkh1-GFP or nuclear Fkh2-GPF was consistently greater when the cells were maintained in H_2O relative to DM (Figure 4.6 D). These data suggest that the Fkh-dependent SCR lifespan extending effect may be due to their increased nuclear localization under these conditions.

The data presented to this point indicate: 1) Fkh proteins are present in post-mitotic chronologically aging cells under SCR and show increased nuclear localization (Sections 4.3.1 and 4.3.3); 2) induced overexpression of the *FKHs* in post-mitotic cells increases lifespan (4.3.2). Based on this evidence, we predicted induced *FKH* expression maintained in SCR conditions would yield a further increase to lifespan extension. To test this, CLS was performed on wild type and GAL_{PROM}-*FKH1/2* cultures grown in 2% Glc SDC, and in DM or water with 0.05% Gal added upon entry to stationary phase. Although a general reduction of lifespan was observed

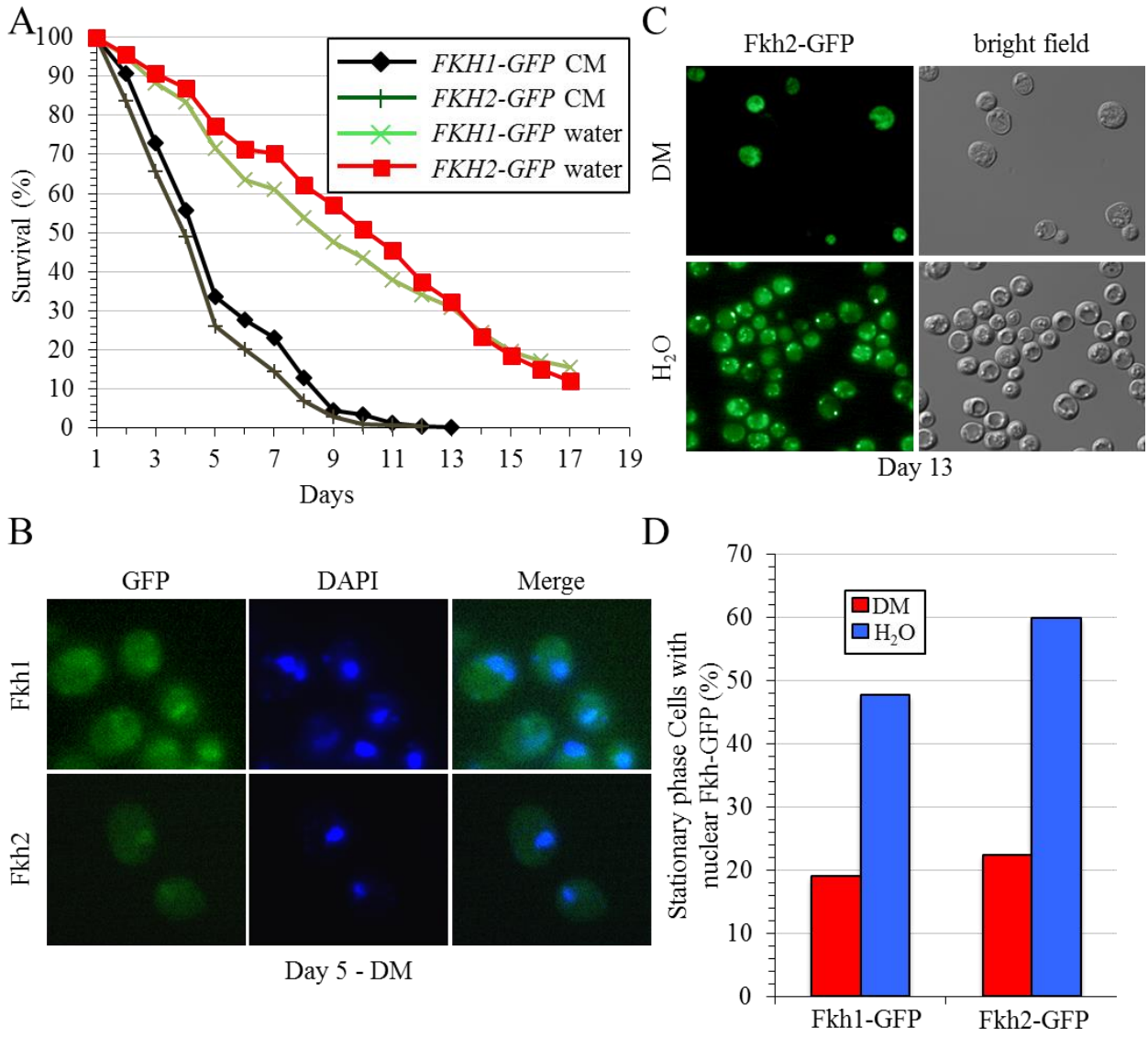

Figure 4.6 Nuclear localization of Fkh-GFP corresponds with severe caloric restriction lifespan extension

Cultures were grown in 2% Glc SDC. Upon entry into stationary phase the cultures were maintained in DM or water. A) Chronological lifespan comparing DM and water. B) Fkh1-GFP and Fkh2-GFP localization compared to DAPI from stationary phase day 5 cultures. C) comparison of a standard field of view of Fkh2-GFP localization in cultures maintained in DM or water at stationary phase day 13. D) Percentage of cells with nuclear localized Fkh-GFP. Average of data collected from 5-10 random fields of view (~55-500 cells) per day counted every other day for first 13 days of chronological lifespan.

compared to the previous CLS data (likely due to changes in water distillation and media autoclaving procedures after the transition to the new laboratory), the relational data within this experiment conforms to this theory: an approximate 1.5 fold increase in lifespan was observed in both *GAL$_{PROM}$-FKH1/2* maintained in DM (induced *FKH1*/2 expression) and wild type maintained in water (SCR) when compared to the wild type control maintained in DM (Figure 4.7); furthermore, the CLS of *GAL$_{PROM}$-FKH1/2* maintained in water (combination of induced *FKH1*/2 expression and SCR) was about 3 times that of wild type control, indicating an additive effect. These data support the model that both the increased presence and environmental activation of the Fkhs act synergistically to increase CLS.

4.3.4 The *FKHs* regulate post-mitotic oxidative stress resistance

Although Fkh1 and Fkh2 have not previously been shown to control yeast longevity, they have been linked to oxidative stress responses (Shapira et al., 2004), an evolutionarily conserved mechanism proposed for lifespan extension (Fabrizio et al., 2005; Pijl, 2012; Tang, 2011). To further examine the role of the *FKHs* in oxidative stress resistance, we treated cultures with 100 mM H_2O_2 for 1 h and compared the CFU from treated and untreated samples as a measure of survival. Logarithmically growing cultures in 2% YPD had less than 1% survival in both wild type and *fkh1*Δ *fkh2*Δ (data not shown). In Day 5 stationary phase cultures, maintained in depleted YPD, the survival rates were: wild type 53.7 +/- 1.7 %; *fkh1*Δ 42.2 +/- 0.3 %; *fkh2*Δ 48.5 +/- 1.4 %; *fkh1*Δ *fkh2*Δ 32.9 +/- 1.5 % (Figure 4.8 A). This suggests additive mechanisms for each *FKH* in protecting cells from H_2O_2-induced cell death. A post H_2O_2 treatment spot dilution assay comparing relative growth potential (rather than survival of individual CFUs) demonstrated that stationary phase cells exhibit increased stress resistance compared to mitotically active cells and confirmed that the combined *fkh1*Δ *fkh2*Δ alleles diminishes this effect (Figure 4.8 B). To address whether the Fkhs' role in longevity is a manifestation of their involvement in stress resistance in post-mitotic cells, wild type and *fkh1*Δ *fkh2*Δ cultures were grown in SDC, maintained to Day 5 stationary phase in either water or DM, and treated with 100 mM H_2O_2. Wild type cultures exhibited increased resistance to H_2O_2 when maintained in water compared to DM. However, this effect was nullified in *fkh1*Δ *fkh2*Δ cultures (Figure 4.8 C). Finally, to examine if *FKH* induction increased oxidative stress resistance, *GAL$_{PROM}$-FKH* cultures were grown in SDC, maintained to Day 5 stationary phase in DM with or without 0.05%

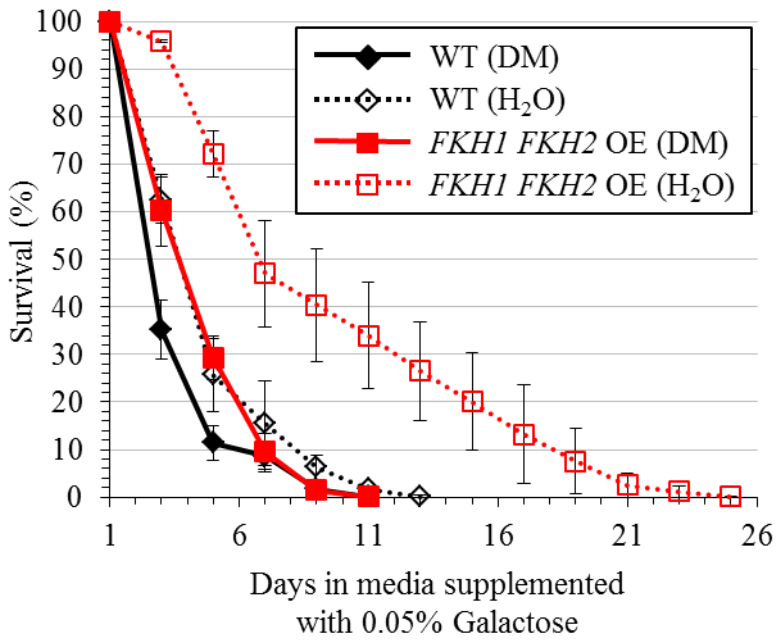

Figure 4.7 Caloric restriction further extends lifespan of cells over-expressing the *FKHs*
Cultures were grown in 2% Glc SDC. Upon entry into stationary phase the cultures were maintained in DM or water, all of which included 0.05% Gal (w/v) to induce *FKH* expression. Error bars represent +/- standard error of the mean of n=3.

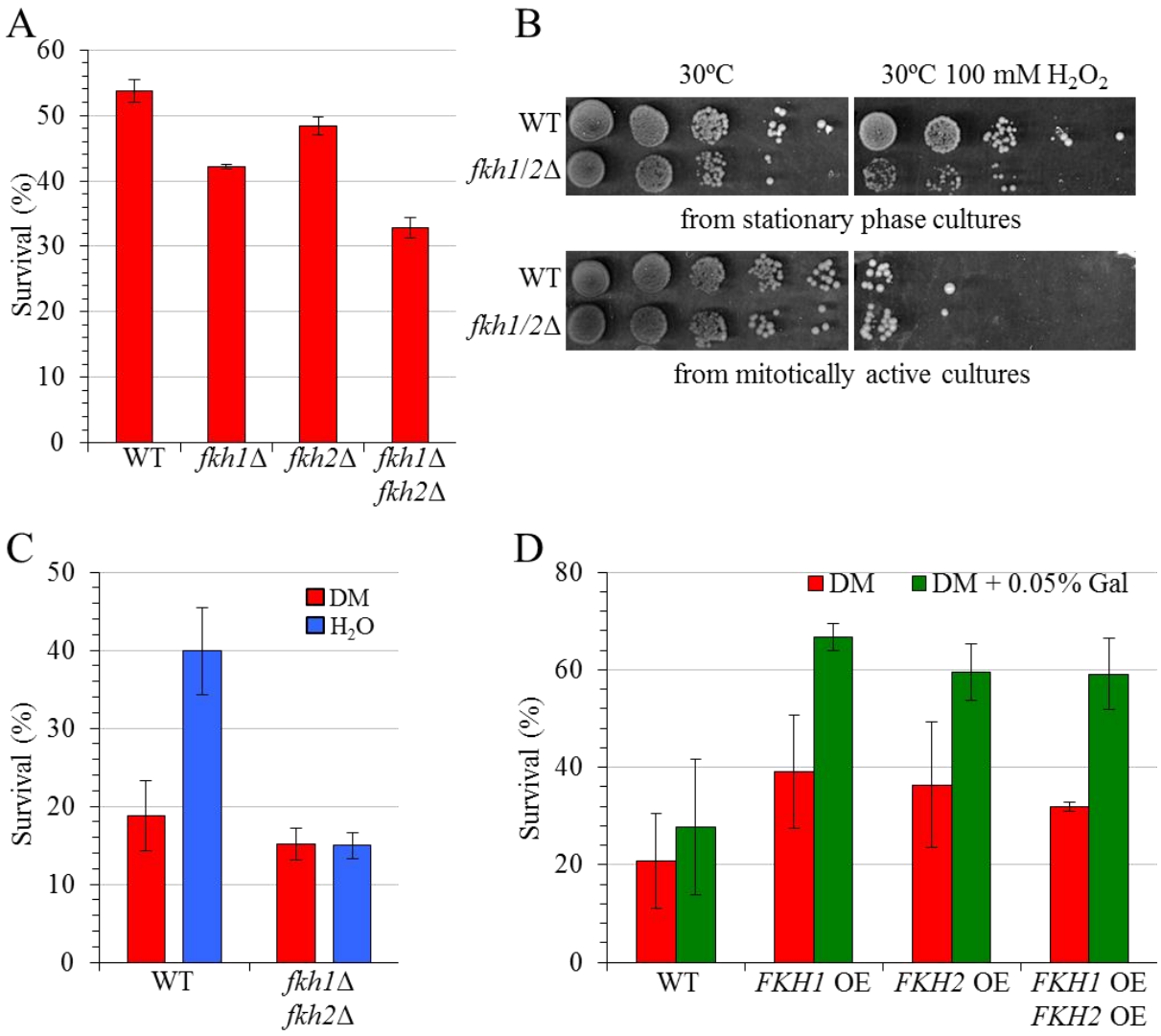

Figure 4.8 The *FKHs* regulate hydrogen peroxide stress resistance in stationary phase cells
Cultures were grown in 2% YPD until day 5 stationary phase, treated with 100 mM H_2O_2 for 1 h, and plated to determine survival of colony forming units (A) or serial diluted for comparative growth potential (in both logarithmically growing and stationary phase cultures; B). C) Cultures were grown in 2% Glc SDC. Upon entry into stationary phase the cultures were maintained in DM or water. 5 days after entry to stationary phase cultures were treated with 100 mM H_2O_2 for 1 h and assayed for colony forming unit survival. D) Cultures were grown in 2% Glc SDC. Upon entry into stationary phase the cultures were maintained in DM with or without the addition of 0.05% Gal to induce *FKH* over-expression. 5 days after entry to stationary phase cultures were treated with 100 mM H_2O_2 for 1 h and assayed for colony forming unit survival. Error bars represent +/- standard error of the mean of at least n=3.

Gal, and assayed for CFU survival after treatment with 100 mM H_2O_2. Gal induction led to an increase in survival in all strains: wild type 1.33 fold; *GAL$_{PROM}$-FKH1* 1.71 fold; *GAL$_{PROM}$-FKH2* 1.63 fold; *GAL$_{PROM}$-FKH1/2* 1.85 fold (Figure 4.8 D). Furthermore, under Gal induction conditions all three *GAL$_{PROM}$-FKH* strains survived about two times that of wild type. These data suggest that the Fkh proteins are involved in post-mitotic oxidative stress resistance with comparative phenotypes trends to those seen in CLS.

4.3.5 The *FKHs* may regulate apoptosis when highly over-expressed

When investigating the *FKH* over-expression constructs (Figure 4.3) and in previous experiments using plasmids containing a Gal inducible *FKH1* construct, we observed cells over-expressing *FKH*s (grown using 2% Gal containing media) grew slowly, if at all (Fig 4.9 A). Specifically, cells over-expressing *FKH1* had a 100 fold decrease in growth, with colonies taking much longer to form than wild type. Cells over-expressing *FKH2* alone or both *FKH1* and *FKH2* together had greater than 10,000 fold growth defect, potentially being unable to grow at all. These data suggest either an inhibition of growth through cell cycle arrest and/or cell death.

If this growth defect were due solely to cell cycle arrest, we hypothesized that *FKH* induction upon entry into stationary phase would increase CLS, both through preventing inappropriate cell cycle re-entry and increasing stress resistance mechanisms. To test this, wild type cells containing either an empty vector or a Gal inducible *FKH1* vector were grown in 2% Glc SD lacking uracil (SD^{Ura-}) to maintain the plasmid. Upon entry into stationary phase, cultures were split in half with one group receiving 2% Gal, while the other was maintained in DM. The plating was performed on SD^{Ura-} plates to select for surviving cells containing plasmids, thus only cells able to express the *FKH1* construct would be observed. The addition of 2% Gal increased the lifespan of cultures containing the empty vector, increasing the 50% survival from Day 11 to Day 19 (Figure 4.9 B). This increase could have been due to a hormetic affect caused by metabolic changes induced through Gal (a less efficient carbon source) and/or re-initiation of cells into the cell cycle in the stock culture. However, the addition of Gal to cultures with the *GAL$_{PROM}$-FKH1* decreased the 50% survival to approximately Day 2 (Figure 4.9 B). Possible explanations for the decrease lifespan include: cell death, cell cycle arrest, or plasmid loss. To address cell cycle arrest, plates were incubated for extended lengths (from 3 days to 7 days) to

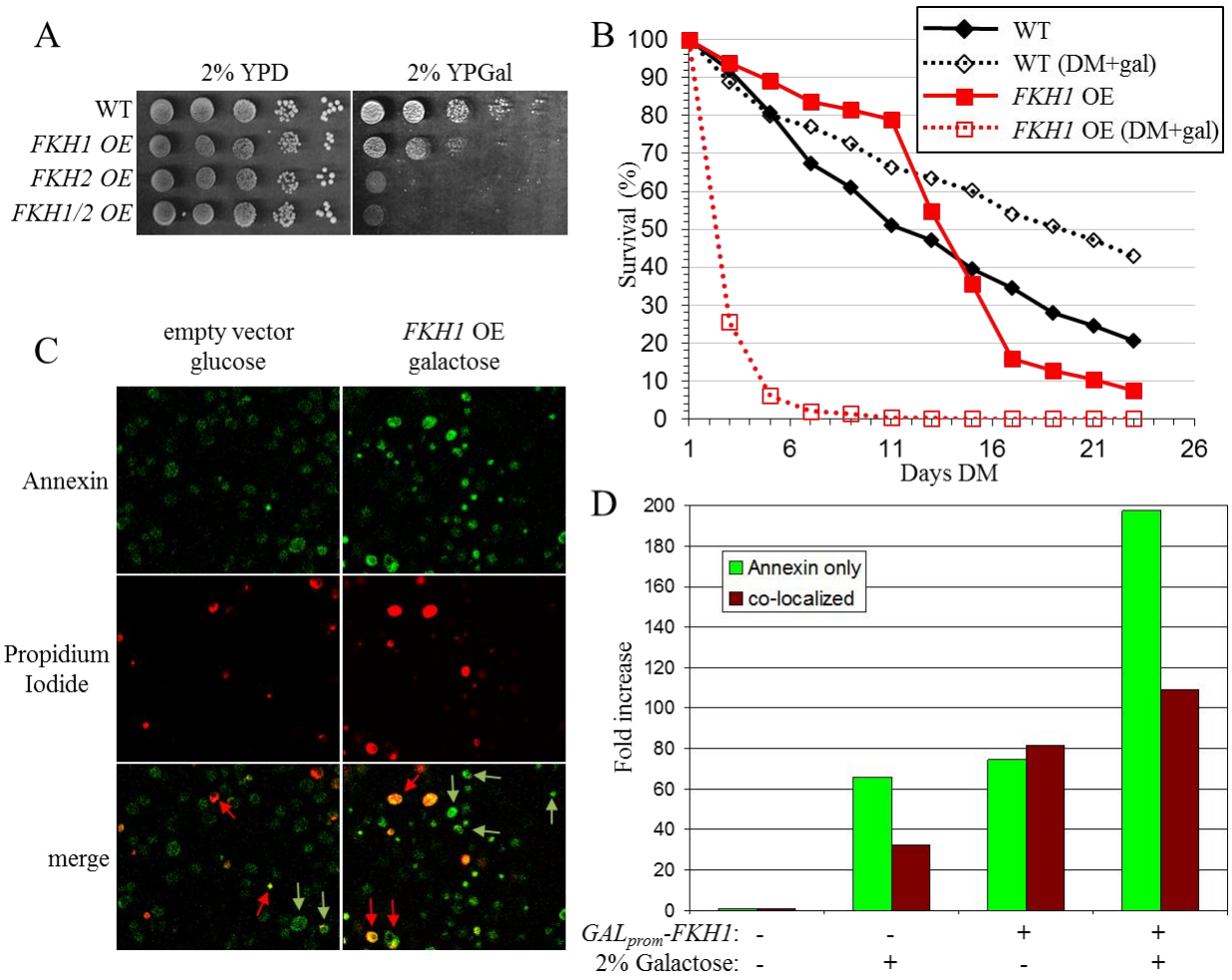

Figure 4.9 High levels of *FKH* expression causes cell cycle arrest and/or apoptosis
A) serial diluted FKH OE strains plated on Glc or Gal containing media. B) Cultures of wild type cell containing either YCp50 empty vector (WT) or a Gal inducible *FKH1*-HA expression vector (*FKH1* OE) were grown in 2% Glc SD^{Ura-}. Upon entry into stationary phase, the cultures were maintained in DM with or without 2% Gal to induce *FKH1* expression. n=2. C) Cells from 24 h after Gal induction in the lifespan represented in B, co-stained with Annexin V and propidium iodide (PI) with green arrows pointing to sample Annexin only (apoptotic) cells and red arrows to PI stained (dead) cells. D) Relative proportion of cells stained with Annexin only (apoptotic cells) and co-stained with Annexin and PI or PI only (dead cells).

assess if colonies grew slower or needed more recovery time before re-initiation into the cell cycle, with no change to survival. To control for the potential plasmid loss, genomically integrated *GAL$_{PROM}$-FKH* constructs were used (Section 4.3.2). Examination of CLS of these mutants, in the presence of 2% Gal, also showed a decrease in lifespan (data not shown) suggesting plasmid loss was not responsible for the previous results.

In higher eukaryotes, under certain conditions, the FoxOs regulate initiation of apoptosis, which is critical to their role as tumour suppressors (Fu and Tindall, 2008; Zhang et al., 2011). Yeast has been shown to undergo an apoptosis-like programmed cell death, with many cellular changes conserved with those documented in higher eukaryote apoptosis (Carmona-Gutierrez et al., 2010). To determine whether cells over-expressing *FKH1* were initiating programmed cell death, we examined cells co-stained with annexin V and PI from Day 2 stationary phase cultures, treated the same as the CLS presented in Figure 4.9 B, using a fluorescent microscope (Figure 4.9 C). Annexin V bound to a fluorescent marker is a membrane impermeable probe that binds to phosphatidylserine, which translocates from the intracellular to extracellular leaflet of the plasma membrane during apoptosis. Co-staining with PI, a membrane impermeable fluorescent DNA intercalating agent, distinguishes between cells with translocated phosphatidylserine (undergoing apoptosis; only annexin detected) and those with a disrupted plasma membrane (already dead; not necessarily from apoptosis; both annexin and PI detected). We found a 70 fold increase in cells presenting annexin in both empty vector 2% Gal and *GAL$_{PROM}$-FKH1* DM cultures, while cells from the *GAL$_{PROM}$-FKH1* 2% Gal culture had a 200 fold increase, when compared to empty vector DM control culture (Figure 4.9 D, green columns). A greater proportion of cells also showed plasma membrane disruptions, indicated by annexin/PI co-fluorescence, in cultures with empty vector 2% Gal (30 fold), *GAL$_{PROM}$-FKH1* DM (80 fold) and *GAL$_{PROM}$-FKH1* 2% Gal (110 fold) compared to empty vector DM control culture (Figure 4.9 D, red columns). These data suggest that, under certain conditions, increasing expression of *FKH1,* and potentially *FKH2,* can initiate programmed cell death.

4.4 Discussion

Data presented in this chapter suggest an evolutionarily conserved role for the *S. cerevisiae* forkhead transcription factors Fkh1 and Fkh2 in lifespan determination and stress response,

characteristics associated with the metazoan FoxO family, conserved from worms to mice (Bjedov et al., 2010; Kenyon, 2011; Kloet and Burgering, 2011) and implicated in human lifespan through the identification of specific FoxO3A alleles enriched in many centenarian populations (Willcox et al., 2008). Specifically, we found the two yeast *FKH* genes redundantly regulate CLS, post-mitotic oxidative stress resistance, and potentially apoptosis. This aligns with the literature, as the *FKHs* have been shown to have genetic redundancy, with the deletion of both genes necessary to alter growth, cell morphology, and gene transcription phenotypes (Hollenhorst et al., 2000; Shapira et al., 2004; Sherriff et al., 2007; Voth et al., 2007; Zhu et al., 2000). We also found induction of *FKH1* or *FKH2* expression increased stress resistance and CLS. Therefore, the Fkhs appear to have a dual function in cell cycle progression and in stress response. Microarray analysis reported in the literature supports *FKH* function in these roles, as comparison between wild type and *fkh1*Δ *fkh2*Δ cells arrested in G1 primarily identified changes a series of genes involved in cell cycle progression, whereas the comparisons of transcript profiles in asynchronous cells identified many stress response genes (Zhu et al., 2000). These observations demonstrate evolutionary conservation of function between the yeast Fkhs and higher eukaryote FoxO transcription factors, where they respond to stress and extend cellular lifespan.

The initial focus of our work was to determine whether the conserved yeast Fkh proteins were involved in longevity, as displayed by metazoan FoxOs. Our work demonstrates that the Fkh proteins are involved in both RLS (performed by T. Harkness) and CLS extension (Postnikoff et al., 2012). Moreover, our work also demonstrates that the Fkh proteins are necessary for extended lifespan in response to SCR. Recently, the Rim15 stress responsive transcription factor was identified as a major mediator of SCR lifespan extension (Wei et al., 2008). They found that the deletion of *RIM15* blocked extended lifespan in *ras2*Δ, *sch9*Δ and *tor1*Δ strains, indicating that the phenomenon of SCR funnels through Rim15. Interestingly, although deletion of *RIM15* in the extremely long-lived *ras2*Δ *sch9*Δ mutant reduced lifespan under normal conditions, this strain could still respond to SCR, suggesting other factors compensate for the absence of Rim15 (Wei et al., 2008). Our data suggest Fkh1/Fkh2 may regulate this compensatory pathway.

Much of the literature on *FKH1* and *FKH2* focuses on progression of cell cycle through mitosis and into G1, classifying them as FoxM1 orthologs (Carlsson and Mahlapuu, 2002; Katoh and Katoh, 2004; Laoukili et al., 2005; Laoukili et al., 2007; Zhu et al., 2000). It has been proposed that the yeast Fox Hcm1 functions as a FoxO ortholog (Rodriguez-Colman et al., 2010; Rodriguez-Colman et al., 2013). Hcm1 is involved in the regulation of cell cycle progression from S to G2 phase, with *FKH1* and *FKH2* among its transcriptional targets (Pramila et al., 2006). Hcm1 has recently been found to regulate stress response, metabolic changes, and G2 cell cycle arrest via stress response proteins, such as Sir2 and Snf1, as well as growth signals from Sch9/Tor1 kinases (Rodriguez-Colman et al., 2010; Rodriguez-Colman et al., 2013), features common to the FoxOs. If the Fkhs function exclusively as FoxM orthologs, our data raise a case for the further investigation of FoxM1 as a direct therapeutic target in preventing cancer and aging. However, the classification of Fkh1 and Fkh2 in the FoxM subfamily may be hasty, as phylogenetic analyses of Fox genes have found that the yeast forkhead box transcription factors are equally divergent from those in higher eukaryotes, suggesting a single Fox gene at the divergence of animals and fungi (Mazet et al., 2003; Postnikoff and Harkness, 2012; Wang et al., 2009b). Furthermore, due to sequence similarities, the FoxM subfamily likely diverged from the FoxOs during deuterostome evolution, as FoxM family members have yet to be characterized in invertebrate protostome model systems (Mazet et al., 2003; Wang et al., 2009b). In this light, it could be hypothesized that the original ancestral Fox functioned in integrating metabolic and stress signals to regulate growth and cell cycle progression, versus cell cycle arrest, stress resistance and programmed cell death, the tight regulation of which is critical for individual and colonial growth and survival. Duplications and evolutionary divergence in yeast appears to have separated the regulation of these functions temporally throughout the cell cycle, with Hcm1 acting primarily through late S phase, and Fkh1 and Fkh2 primarily during M phase. In metazoans, Fox divergence is more complex, with expression and function being regulated developmentally and in a cell-type and tissue-specific manner (Lehmann et al., 2003; Wijchers et al., 2006). The ubiquitously expressed FoxO and FoxM subfamilies are delineated by function rather than cell cycle stage, with the FoxOs primarily regulating cell cycle arrest, stress resistance and programmed cell death, while FoxMs regulate growth and cell cycle progression. The evolutionarily conserved functions highlight the logic in investigating the yeast Fox proteins to identify novel targets in the regulation of age and developmental disease in higher eukaryotes.

5 Extra and intra cellular signaling regulate *FKH* dependent lifespan

5.1 Introduction

Growth factors such as insulin initiate kinase phosphorylation cascades, activating energetically taxing cellular processes such as ribosome production, cell growth and cell division (Figure 5.1). Insulin and insulin like growth factors, as well as other growth factors, lead to the phosphorylation of AKT kinases through PI3K/PDK1 and TORC2. AKT represses AMPK and TSC (Tuberous sclerosis proteins), which in turn are responsible for the repression of both the TORC1 activator Rheb and TORC1 formation itself (Bhaskar and Hay, 2007; Hay, 2005, 2011). Amino acid activated TORC1 increases ribosome production and mRNA translation through the direct activation of S6K and indirectly through activation of eIFs (Hay, 2011; Loewith and Hall, 2011), as well as other processes, such as cell cycle regulation/progression, through the regulation of certain PP2A complexes (Di Como and Arndt, 1996; Jordens et al., 2006; Loewith and Hall, 2011; Nakashima et al., 2008; Zheng and Jiang, 2005). Some PP2A activity interferes with CDK/cyclin phosphorylation, slowing cell division and resulting in anabolic cell growth (Alvarez-Fernandez et al., 2011); however, in yeast, TORC1-dependent PP2A complex formation initiates mitosis through the removal of inhibitory phosphate groups from the polo-like kinase, Cdc5 (Nakashima et al., 2008). CDK/cyclins and polo-like kinases regulate FoxM1 and Fkh1/2 positive feedback functions in mitotic initiation (Alvarez-Fernandez et al., 2011; Barford, 2011; Harper et al., 2002; Murakami et al., 2010). Finally, active S6K inhibits insulin signaling through the internalization and degradation of plasma-membrane bound components of the insulin signaling pathway (Haruta et al., 2000; Shah et al., 2004; Tremblay et al., 2005; Zhang et al., 2008); furthermore, PP2A dephosphorylates AKT at PDK1 specific sites (Padmanabhan et al., 2009), potentially forming a negative feedback circuit whereby TORC1 inactivates insulin signaling. This mechanism likely evolved to regulate nutrient sharing and homeostasis between cells in multicellular organisms; starving cells express receptors to increase nutrient uptake while decreasing anabolic growth, whereas, fed cells limit nutrient uptake and increase growth.

FoxO regulation and function are central to the core of the insulin/TOR feedback loop (Figure 5.1). FoxOs regulate both AKT and TORC1 processes. Firstly, the FoxOs can be phosphorylated on three residues by the AKT kinases. Phosphorylation results in FoxO inactivation through

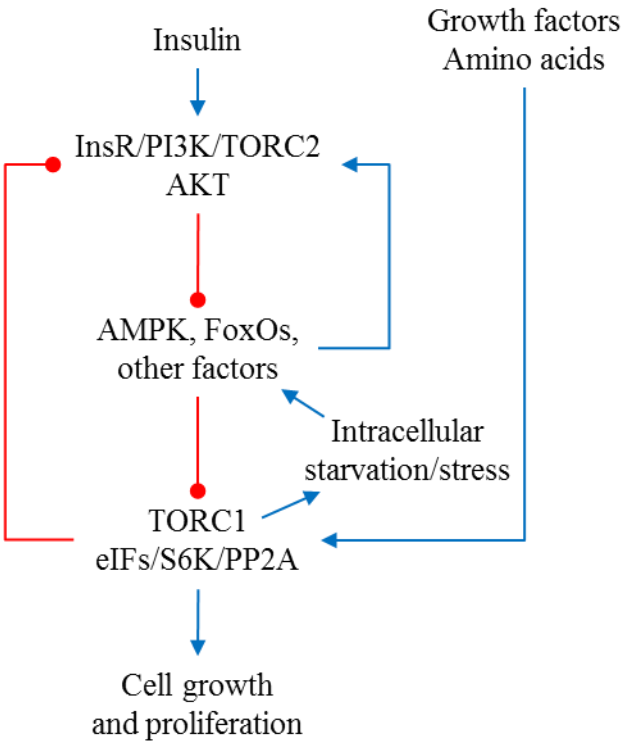

Figure 5.1 Feedback regulation of insulin/TOR signaling pathways in higher eukaryotes
Insulin signaling activates a series of negative feedback mechanisms that regulate cell growth and division. For details refer to the text of Section 5.1.

dissociation from the DNA, nuclear export/cytosolic sequestering by 14-3-3 chaperones, and SCF^{SKP2}-specific degradation (Calnan and Brunet, 2008; Fu and Tindall, 2008; Huang et al., 2005; Huang and Tindall, 2011; Tran et al., 2003; Tzivion et al., 2011). Additionally, FoxO activity potentiates the insulin signaling pathway through the increased expression of the insulin receptor and its direct substrates (Chandarlapaty et al., 2011; Guo et al., 2006; Marr et al., 2007; Matsumoto et al., 2006; Puig et al., 2003). Secondly, when active, FoxO subfamily members have been shown to repress TORC1 function across metazoan evolutionary lines. This is achieved through transcriptional control, in part through the up-regulation of repressors of activators and targets of TORC1, such as TSC, AMPK activating Sestrins, the Rheb inhibitor Bnip3, and eIF inhibitors (Chen et al., 2010; Demontis and Perrimon, 2010; Hay, 2011; Khatri et al., 2010; Nogueira et al., 2008; Tettweiler et al., 2005; Wessells et al., 2009), with simultaneous down-regulation of the TORC1 regulatory sub-unit Raptor (Jia et al., 2004). Opposed to phosphorylation by AKT, the FoxOs are phosphorylated by stress response kinases, such as oxidative stress and DNA damage activated MAPKs and related kinases (i.e. JNK, p38 and Mst1) and the starvation sensing AMPK, resulting in FoxO stabilization, nuclear localization and activation (Cai and Xia, 2008; Choi et al., 2009; Greer and Brunet, 2008; Greer et al., 2007a; Greer et al., 2007b; Ho et al., 2012; Lehtinen et al., 2006; Sunters et al., 2006; Valis et al., 2011). Free radical production and resulting cell damage, as well as intracellular nutrient depletion, are FoxO activating stresses, and by-products of the energy intensive TORC1 regulated anabolic growth, suggesting another loop in the regulatory feedback mechanisms of growth factor signaling.

In *S. cerevisiae,* orthologous genes/proteins have been identified for many components of the growth factor and stress signaling pathways; however, the specifics of these mechanisms require further investigation. The deletion of genes encoding growth factor response kinases greatly increase both RLS and CLS in yeast (Fabrizio et al., 2004b; Kaeberlein et al., 2005; Wei et al., 2008; Wei et al., 2009). Of note is the serine/threonine specific protein kinase Sch9, which shows homology in sequence, regulation, and function to metazoan AKT and S6K kinase families, as it is activated by both TORC1 and PDK1 orthologs (Liu et al., 2005; Mora et al., 2004; Urban et al., 2007; Voordeckers et al., 2011). Yeast encodes two TOR orthologs, Tor1 and Tor2, of which Tor2 is essential and has been found associated with TORC2 and to a lesser

extent TORC1; while Tor1 is non-essential, it has only been found associated with TORC1 and to be directly involved in activation of Sch9 (De Virgilio and Loewith, 2006b; Loewith et al., 2002; Urban et al., 2007; Wullschleger et al., 2006). Tor1/Sch9 signaling represses the stress resistance activating transcription factors Msn2, Msn4, and Gis1, which are considered to be primary CLS regulating targets of this mechanism (De Virgilio and Loewith, 2006a; Fabrizio et al., 2004b; Fabrizio et al., 2001; Wei et al., 2008). However, strains lacking Msn2/Msn4 or Gis1 still resulted in a CR-induced increase in CLS, indicating other factors are involved in this mechanism (Wei et al., 2008), potentially Fkh1/2. In Chapter 4 we introduced the *FKHs* as regulators of caloric restriction/stress mediated lifespan, here we investigate genetic interactions between nutrient response kinases Sch9 and Tor1, as well as Snf1, the yeast AMPK kinase catalytic alpha subunit ortholog, and Hog1, a stress activated MAPK and potential p38/JNK/Mst1 ortholog, with Fkh1 and Fkh2.

5.2 Methods

To investigate genetic interactions between *SCH9, TOR1*, *FKH1*, and *FKH2* in actively dividing cells, spot dilutions (3.1.4) and western analyses (3.2.5; using Fkh-TAP constructs and anti-TAP antibodies) were used, in the presence and absence of stress conditions. Chronological lifespan (3.1.5) and oxidative stress resistance (3.1.6) assays were performed to examine genetic interactions in stationary phase cells under standard and stressed conditions.

5.3 Results

5.3.1 *SCH9, TOR1,* and *FKH1/2* genetically interact to regulate growth and lifespan

Metazoan FoxO-dependent lifespan is in part due to feedback regulation with TOR2C/AKT and TOR1C/S6K signaling pathways, controlling cell growth, cell cycle, stress resistance, and DNA repair homeostasis in response to nutrient availability (Greer and Brunet, 2008; Hay, 2011; Zhang et al., 2011). We have identified the yeast forkhead box transcription factors Fkh1 and Fkh2 as regulators of some forms of stress resistance and lifespan regulation (See Chapter 4). Tor1 and Sch9 (orthologs of mTOR and AKT/S6K respectively) have also been shown to regulate yeast lifespan growth and stress resistance (Fabrizio et al., 2004b; Fabrizio et al., 2001; Kaeberlein et al., 2005; Wei et al., 2008; Wei et al., 2009). Another link between the Fkhs and nutrient signaling is that *fkh1*Δ *fkh2*Δ strains exhibit pseudo-hyphal growth (Hollenhorst et al.,

2000; Zhu et al., 2000). Hyphal and pseudo-hyphal growth involve elongation of cells to locate favourable growth conditions and are associated with glucose, amino-acid, and oxygen starvation controlled in part by TORC and Sch9 signaling (Cullen and Sprague, 2000; Cutler et al., 2001; Kuchin et al., 2003; Stichternoth et al., 2011). However, potential Tor1/Sch9 signaling and Fkh1/Fkh2 interactions have yet to be determined.

To examine genetic interactions between these two mechanisms, logarithmically growing cells with combinations of *fkh1*Δ, *fkh2*Δ, *sch9*Δ and *tor1*Δ were spotted, followed by growth at permissive and restrictive temperatures (Figure 5.2). Strains with the following gene disruptions had associated phenotypes: *fkh1*Δ *fkh2*Δ strains (*fkh1*Δ *fkh2*Δ, *sch9*Δ *fkh1*Δ *fkh2*Δ, *tor1*Δ *fkh1*Δ *fkh2*Δ and *sch9*Δ *tor1*Δ *fkh1*Δ *fkh2*Δ) were flocculent and pseudo-hyphal (visualized by microscopy; data not shown); s*ch9*Δ strains (*sch9*Δ, *sch9*Δ *fkh1*Δ *fkh2*Δ, *sch9*Δ *tor1*Δ, and *sch9*Δ *tor1*Δ *fkh1*Δ *fkh2*Δ) were slow growing, with smaller cell and colony size and showed slower accumulation of adenine biosynthetic metabolite intermediates, indirectly observed through the colonies remaining white; *tor1*Δ strains (*tor1*Δ, *tor1*Δ *fkh1*Δ *fkh2*Δ, *sch9*Δ *tor1*Δ, and *sch9*Δ *tor1*Δ *fkh1*Δ *fkh2*Δ) were temperature sensitive. The temperature sensitivity of *sch9*Δ *tor1*Δ was greater than either single mutant, indicating these factors likely regulate parallel pathways. The quadruple mutant appeared to be less temperature sensitive than *sch9*Δ *tor1*Δ. The Fkhs may be partially responsible for *sch9*Δ *tor1*Δ slow growth and temperature sensitivity. Perhaps nutrient/growth factor signaling through separate Sch9 and Tor1 signaling mechanisms repress Fkh-dependant cell cycle arrest/apoptotic functions, as described earlier in Section 4.3.5, while activating Fkh-dependent cell cycle progression.

Next we examined genetic interactions in post-mitotic cultures utilizing CLS analysis. In cultures maintained in DM, *fkh1*Δ *fkh2*Δ strains had a lifespan half that of wild type at 50% survival (Day 4 and 7 respectively; Figure 5.3) congruent with the data presented in Chapter 4. The *sch9*Δ mutant strain (Day 26) had a 3.7 fold lifespan extension (Figure 5.3 A). The *sch9*Δ *fkh1*Δ *fkh2*Δ strain had two 50% survivals (Day 20 and 26) due to an apparent re-growth between Days 23 and 25. The trend observed prior to re-growth suggests that *SCH9* functions at least partially separate from the *FKHs*, with the triple having 0.77 times the lifespan of *sch9*Δ. However, post re-growth both *sch9*Δ and the triple mutants showed very similar curves suggesting *sch9*Δ may

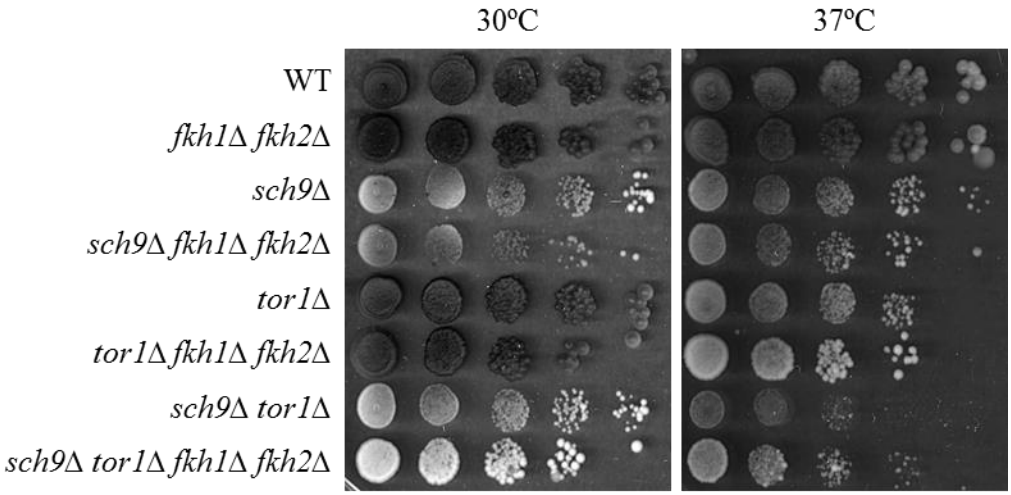

Figure 5.2 The redundant *FKHs* do not interact with *SCH9* or *TOR1* to regulate logarithmic growth

Tenfold serial dilutions with the starting spot having approximately 30,000 cells plated and grown on 2% YPD at 30°C and 37°C of strains with *fkh1*Δ and *fkh2*Δ alleles with *sch9*Δ or *tor1*Δ.

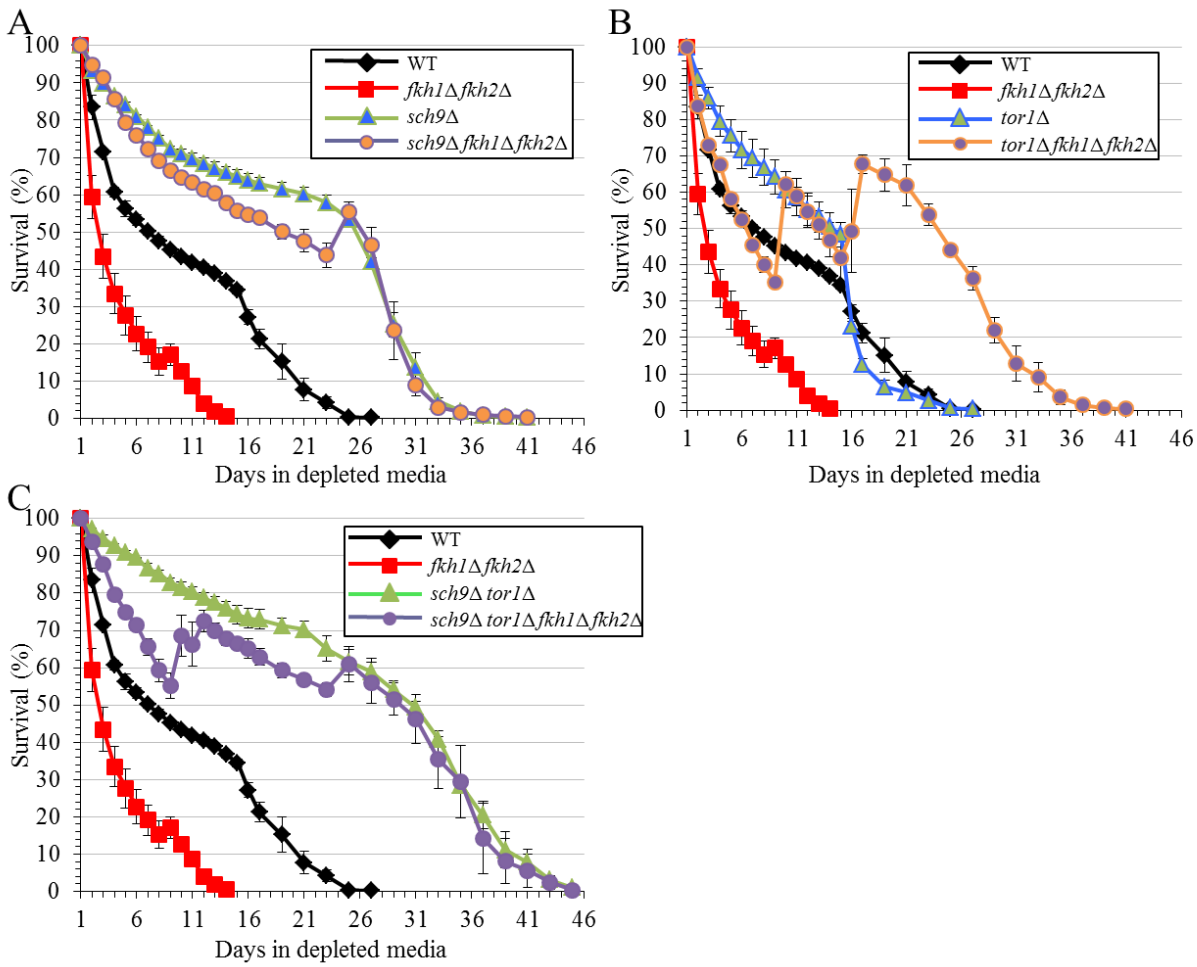

Figure 5.3 The *sch9*Δ and *tor1*Δ alleles are epistatic to *fkh*Δ alleles under standard conditions of chronological lifespan

Cultures were grown in 2% Glc SDC. Upon entry into stationary phase the cultures were maintained in DM. Error bars represent +/- standard error of the mean of n=3.

be epistatic to *fkh1*Δ *fkh2*Δ lifespan (refer to Section 5.4 for further discussion of regrowth phenotypes in CLS). These data suggest a genetic interaction between the two mechanisms that may be more complex than direct epistasis. The simplest explanation for the partial epistasis of *sch9*Δ lifespan extension over the short-lived *fkh1*Δ *fkh2*Δ phenotype is that in these conditions the Fkhs primary lifespan altering function is to repress Sch9 signaling. In conjunction with the observation that *fkh1*Δ *fkh2*Δ CLS did not respond to SCR (Figure 4.2) it would suggest that Fkh repression of Sch9 function may be nutrient/stress dependent.

Strains with the *tor1*Δ allele had a 50% survival at 14 days, twice that of wild type (Figure 5.2B); however, after 50% survival, the *tor1*Δ cultures died more rapidly reaching 10% survival by day 17 compared to day 21 in wild type. Furthermore, in some CLS experiments, *tor1*Δ strains showed drastically reduced lifespan, with several of our *tor1*Δ glycerol stocks consistently assaying with decreased lifespan (data not shown). Reasons for this anomalous decrease in survival remain unclear; however, *tor1*Δ has been found to both increase and decrease sensitivity

to various stresses [Figure 5.2/Section 5.3.3 this study; (Corcoles-Saez et al., 2012; Delaney et al., 2013; Ding et al., 2013; Dziedzic and Caplan, 2011; Kapitzky et al., 2010; North et al., 2012; Sinha et al., 2008; Wang et al., 2009a; Wei et al., 2009)], suggesting Tor1 may decrease survival under some environmental conditions, while increase it in others. Consistent with the idea that TOR may be involved in both pro and anti-aging mechanisms, chronic rapamycin induced TORC1 inactivation in mice can extend median and maximal lifespan (Harrison et al., 2009; Miller et al., 2011), but also can lead to immunosuppression, diabetes-like metabolic changes and tissue specific pathologies in mice and humans (Blagosklonny, 2012; Lamming et al., 2013). Alternatively, *tor1*Δ strains may generate spontaneous suppressors at a high rate that has yet to be documented in the literature. Because of these concerns, we were careful to test the *tor1*Δ strains by CLS for increased lifespan prior to further use of that strain. The *tor1*Δ *fkh1*Δ *fkh2*Δ strain had two occurrences of re-growth (between Days 10-11 and 15-17) making three distinctive curves: the first conforms to the rate of the *fkh1*Δ *fkh2*Δ curve, with a 50% survival of 6.5 days similar to wild type; the second follows that of *tor1*Δ strain with a 50% survival at Day 14; the third is similar to the *sch9*Δ curve (Figure 5.3 A) reaching the final 50% survival at day

24. These data suggest a strong genetic interaction; however, the mechanisms involved in this co-regulation are unclear.

We found that *sch9*Δ *tor1*Δ lifespan (50% at day 30; <1% by Day 45; Figure 5.3 C) was extended beyond either *sch9*Δ (50% at Day 26; <1% by Day 41) or *tor1*Δ (50% at Day 14; <1% by Day 26) alone, indicating a partial redundancy in *SCH9* and *TOR1* lifespan regulatory mechanisms. Past studies indicate Sch9 is a direct target of Tor1 phosphorylation (Urban et al., 2007) and the genes regulate lifespan in a linear function, with *sch9*Δ phenotype being epistatic to *tor1*Δ (Wei et al., 2008; Wei et al., 2009). The inconsistency of our results with the published literature may be due the use of a different genetic background and amino acid composition in the SDC media used. These changes could favour alternative pathways uncoupling Sch9 and Tor1 signaling, such as a case of Sch9 activation by phosphorylation by the mammalian PI3K yeast orthologs Pkh1, -2, and -3 or through Ras/PKA signaling (Liu et al., 2005; Voordeckers et al., 2011). The *sch9*Δ *tor1*Δ *fkh1*Δ *fkh2*Δ mutant had decreased fluctuating survival for the first 23 days indicating partial dependence on the Fkhs in *sch9*Δ *tor1*Δ lifespan extension; this was followed by a death curve consistent with the *sch9*Δ *tor1*Δ mutant, proceeding from day 25. A possible interpretation of these data is that a primary function of the Fkhs in lifespan regulation in these conditions may be the repression of Sch9 and Tor1 signaling pathways. This may be similar to metazoan FoxO repression of TORC1 and S6K signaling.

5.3.2 Stress conditions alter the nature of the energy signaling/forkhead interaction

Initial experiments, performed in SCR conditions, prior to the establishment of a standard CLS protocol, resulted in different genetic interactions from those presented in the previous section. In these earlier experiments, the relationship between single and double mutants had similar trends to those observed in Figure 5.3. Compared to wild type, *fkh1*Δ *fkh2*Δ had a reduced lifespan, while *sch9*Δ or *tor1*Δ had extended CLS, which was further extended in the *sch9*Δ *tor1*Δ double mutant (Figure 5.4 A-C). Under these conditions, the triple and quadruple mutants had initial lifespan curves similar to *fkh1*Δ *fkh2*Δ; with the exception that the triple mutants experienced regrowth between 9 and 11 days, while the quadruple mutant did not. This epistatis of the *fkh1*Δ *fkh2*Δ phenotype in the quadruple mutant suggests that parallel Sch9 and Tor1 regulated pathways inhibit the CLS; this relationship between Tor1/Sch9 is similar to metazoan

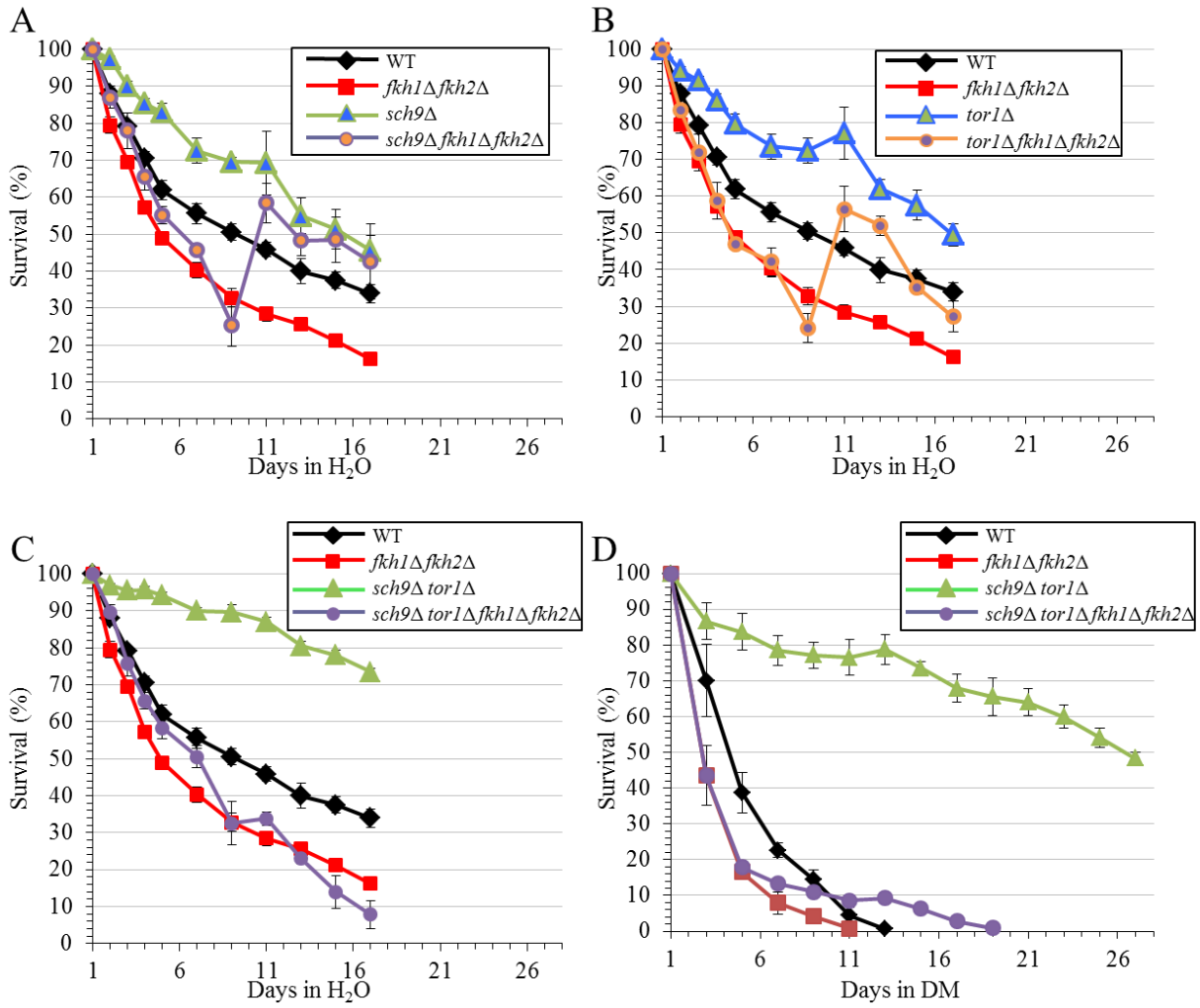

Figure 5.4 The *fkh*Δs are epistatic to *sch9*Δ and *tor1*Δ under stressed chronological lifespan conditions (maintained in falcon tubes)

A-C) Cultures were grown in 2% YPD. Upon entry into stationary phase the cultures were maintained in water. Error bars represent +/- standard error of the mean of n=3. D) Cultures were grown in 2% Glc SDC. Upon entry into stationary phase the cultures were maintained in DM. Error bars represent +/- standard error of the mean of n=3.

AKT/FoxO genetic interactions, with the Fkhs predominantly responsible for the lifespan extension seen in nutrient signaling disruptions.

Between running the experiments that resulted in Figure 5.3 and 5.4 A-C, four variables were changed in the CLS protocol: 1) data from Figure 5.3 were generated in DM, while those from Figure 5.4 A-C were generated in SCR conditions; 2) nutritionally rich YPD was switched to SDC; 3) in Figure 5.4 A-C, Day 1 was arbitrarily set to 3 days after initial culture inoculation, compared to cultures being set back to an equal OD_{600} from seeding overnight cultures and peak survival (100%; Day1) determined for each strain (most strains took 2 days, while strains with *sch9*Δ and/or *fkh1*Δ *fkh2*Δ took 3-4 days); and 4) plastic 50 ml falcon tubes were switched to glass Erlenmeyer flasks. First, preliminary replication of the *sch9*Δ, *tor1*Δ, *fkh1*Δ, and *fkh2*Δ experiments with maintenance in SCR conditions showed similar trends to those presented in Figure 5.3 (data not shown). Next, we tested media and growth vessel composition differences (YPD versus SDC; glass versus plastic Erlenmeyer flasks) utilizing the more accurate up to date protocol. In all combinations the genetic relationships were similar to those presented in Figure 5.3 with *sch9*Δ *tor1*Δ lifespan extension phenotype mostly epistatic to the *fkh1*Δ *fkh2*Δ (data not shown). This suggests SDC versus YPD nutrient composition, SCR, and potential exogenous chemicals from the plastic of the flasks were not the stresses responsible for the change in epistasis.

Finally, the experiment was replicated using the standard protocol (grown in SDC, set back at 1:5 culture to flask volume in SDC, maintained in DM, assayed for peak survival, etc.) with the exception of using falcon tubes (the final altered parameter). Under these conditions the quadruple mutant had a lifespan similar to the *fkh1*Δ *fkh2*Δ mutant, replicating the relationship observed in Figure 5.4 C (Figure 5.4 D). The use of Erlenmeyer flasks, rather than falcon tubes, may allow for more turbidity in the CLS culture and a greater culture/air surface area, facilitating a homogeneous suspension of cells, nutrients, and metabolites, as well as greater oxygenation of the culture; on the other hand, in Falcon tubes, the cells came out of suspension and the surface of the media was less disturbed. We hypothesize this may have resulted in localized changes to nutrient availability, excreted metabolite accumulation, or decreased oxygen, especially deeper in the pellet. The Fkhs have been found to change localization in response to hypoxic conditions

(Dastidar et al., 2012), while at least Fkh2 regulates growth/survival in low oxygen conditions (Samanfar et al., 2013). These data suggest that under potential low oxygen, stressed conditions, the Fkhs become necessary for the major lifespan extension conferred by the disruption of nutrient signaling through Sch9 and Tor1.

Next, we assessed the effects of oxidative free radical stress by growing yeast cultures with combinations of *sch9*Δ, *tor1*Δ, and *fkh1*Δ *fkh2*Δ disruptions to Day 5 stationary phase and treating them with 100 mM H_2O_2 for 1 h at 30°C. Cultures of *sch9*Δ (89.4 +/- 0.9%), *tor1*Δ (82.0 +/- 3.9%), or *sch9*Δ *tor1*Δ (91.0 +/- 1.2%) were twice as stress resistant as wild type (43.9 +/- 1.6%; Figure 5.5). Survival of *sch9*Δ *fkh1*Δ *fkh2*Δ (40.4 +/- 2.3%) and *tor1*Δ *fkh1*Δ *fkh2*Δ (36.2 +/- 2.3%) cells was similar to that of wild type, while both *fkh1*Δ *fkh2*Δ (5.2 +/- 0.5%) and *sch9*Δ *tor1*Δ *fkh1*Δ *fkh2*Δ (10.2 +/- 1.7%) had greatly reduced survival. This relationship is congruent with the trends in the lifespan data presented in Figure 5.4, strengthening the argument of the necessity of the Fkhs to respond to major post-mitotic stresses, a mechanism that is inhibited by Sch9 and Tor1 signaling.

5.3.3 Stress and nutrient response regulation of the forkheads

Metazoan FoxO activity, localization and stability is regulated through AKT dependent phosphorylation (Kenyon, 2011; Tzivion et al., 2011; Zhang et al., 2011). To elucidate potential conservation of a similar molecular mechanism through Sch9 and Tor1 interaction with Fkhs, we examined Fkh protein levels in asynchronous logarithmically growing *sch9*Δ, and *sch9*Δ *tor1*Δ cultures. As mentioned previously, some strains containing *tor1*Δ developed a short lived phenotype; included in these were the *tor1*Δ *FKH1-TAP* and *tor1*Δ *FKH2-TAP* strains (data not shown), potentially due to secondary mutations. Therefore, these strains were not assessed for Fkh-TAP levels. If the AKT/FoxO interaction holds true, we expect under increasing Glc concentrations Fkh protein levels may decrease and a larger phosphorylated Fkh band may be observed, while in *sch9*Δ or *sch9*Δ *tor1*Δ mutants any Glc responsive changes would be diminished. A non-specific 60 kDa band was detected in all samples, including wild type, which does not encode the TAP epitope tag; this band was used for load comparison (Figure 5.6). Fkh1-TAP (~75 kDa band) levels decreased in 6% Glc in otherwise wild type cells, while in *sch9*Δ cells the reverse was true, as Fkh1-TAP levels increased in 6% Glc. In *sch9*Δ *tor1*Δ cells Fkh1-

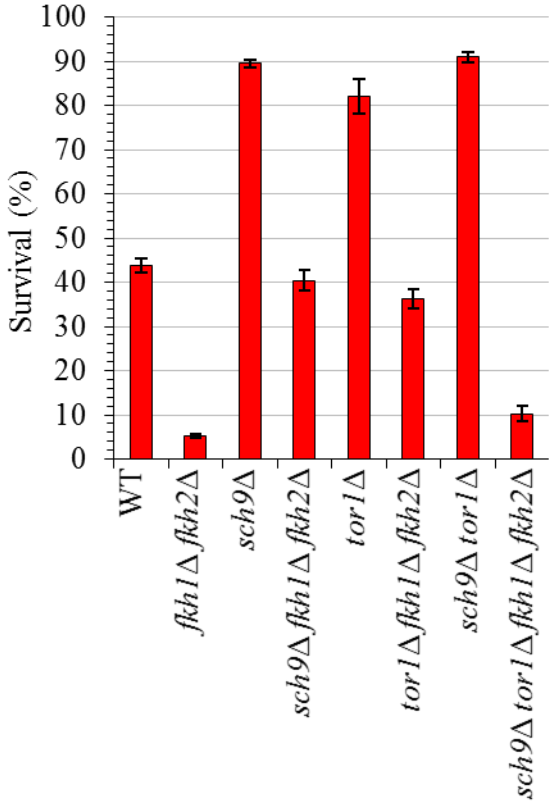

Figure 5.5 The *FKHs* increase oxidative stress resistance in cells lacking Sch9 or Tor1
Cultures were grown in 2% YPD to day 5 stationary phase, treated with 100 mM H_2O_2 for 1 h, and plated to determine survival of colony forming units. Error bars represent +/- standard error of the mean of at least n=3.

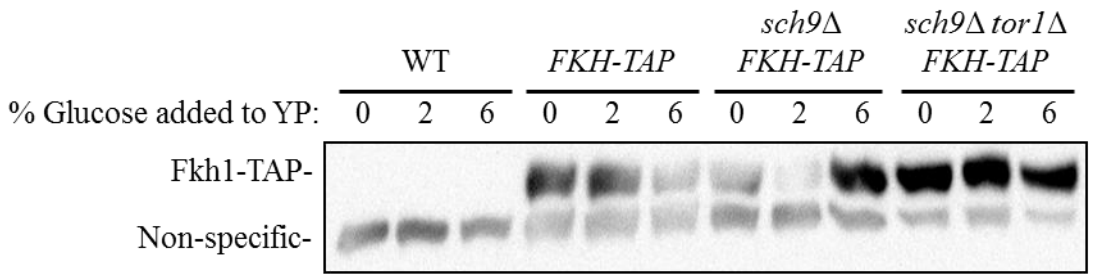

Figure 5.6 Sch9 and Tor1 regulate Fkh1-TAP levels
Pre-diauxic shift overnight cultures were set back to OD_{600} 1 in their own partially depleted media, 0%, 2% or 6% Glc were added, followed by incubation at 30°C for 1 h. Proteins were extracted by the TCA method and analyzed by western blotting.

TAP protein levels were high compared to the other two backgrounds and did not fluctuate with variance in Glc levels. A follow up experiment confirmed these results and examined the relationship of Fkh1-TAP levels with *tor1*Δ and using tubulin as a loading control. Fkh1-TAP were the same in wild type and *tor1*Δ cells, elevated in *sch9*Δ and more so in *sch9*Δ *tor1*Δ cells (M. Malo and T. Harkness; unpublished). In preliminary experiments, Fkh2-TAP levels did not appear to greatly fluctuate with Glc concentration or in *sch9*Δ and *sch9*Δ *tor1*Δ cells (data not shown). These data suggest that nutrient activated Sch9 and Tor1 signaling may be responsible for regulating at least Fkh1 protein abundance in these conditions, either through gene expression or, more likely, protein stability, due to the rapidity of change.

Low nutrient and stress conditions appear to be integral for *FKH*-regulated cellular survival. Although Fkh stress resistance could passively arise from not being repressed by nutrient signaling kinases, it may also include active mediation through stress responsive kinases. In metazoans, AMPK/p38/MST1/JNK stress activated kinase signaling mechanisms phosphorylate FoxO proteins facilitating their nuclear import, DNA binding and stress response gene expression specificity in conditions including nutrient and energy depletion, DNA damage, toxic stress, and membrane damage (Cai and Xia, 2008; Choi et al., 2009; Greer et al., 2007a; Greer et al., 2007b; Ho et al., 2012; Lehtinen et al., 2006; Sunters et al., 2006; Vogt et al., 2005; Zhang et al., 2011). To examine the potential conservation of these regulatory mechanisms we performed CLS assay on strains with deletions of *SNF1*, the gene encoding the yeast AMPK catalytic subunit ortholog, and the *FKHs.* Strains with *snf1*Δ, *fkh1*Δ *fkh2*Δ, and *snf1*Δ *fkh1*Δ *fkh2*Δ deletions had similar lifespans, reaching 50% survival after approximately 4 days and maximal lifespan at 11-13 days, compared to the 7 and 27 days of wild type (Figure 5.7 A). This suggests that the *FKHs* and *SNF1* may act through the same pathway to regulate lifespan, and suggests further experimentation is warranted to determine the mechanism of this interaction.

Next we tested the necessity of the yeast stress responsive activated MAPK (i.e. JNK/Mst1) ortholog encoding gene, *HOG1*, in *FKH* over-expression dependent stress resistance by knocking out the *HOG1* gene in the *FKH* over-expressing strains developed in Section 4.3.2. *hog1*Δ cultures with *FKH1*- and/or *FKH2*-over-expression to stationary phase in 2% YPD, at

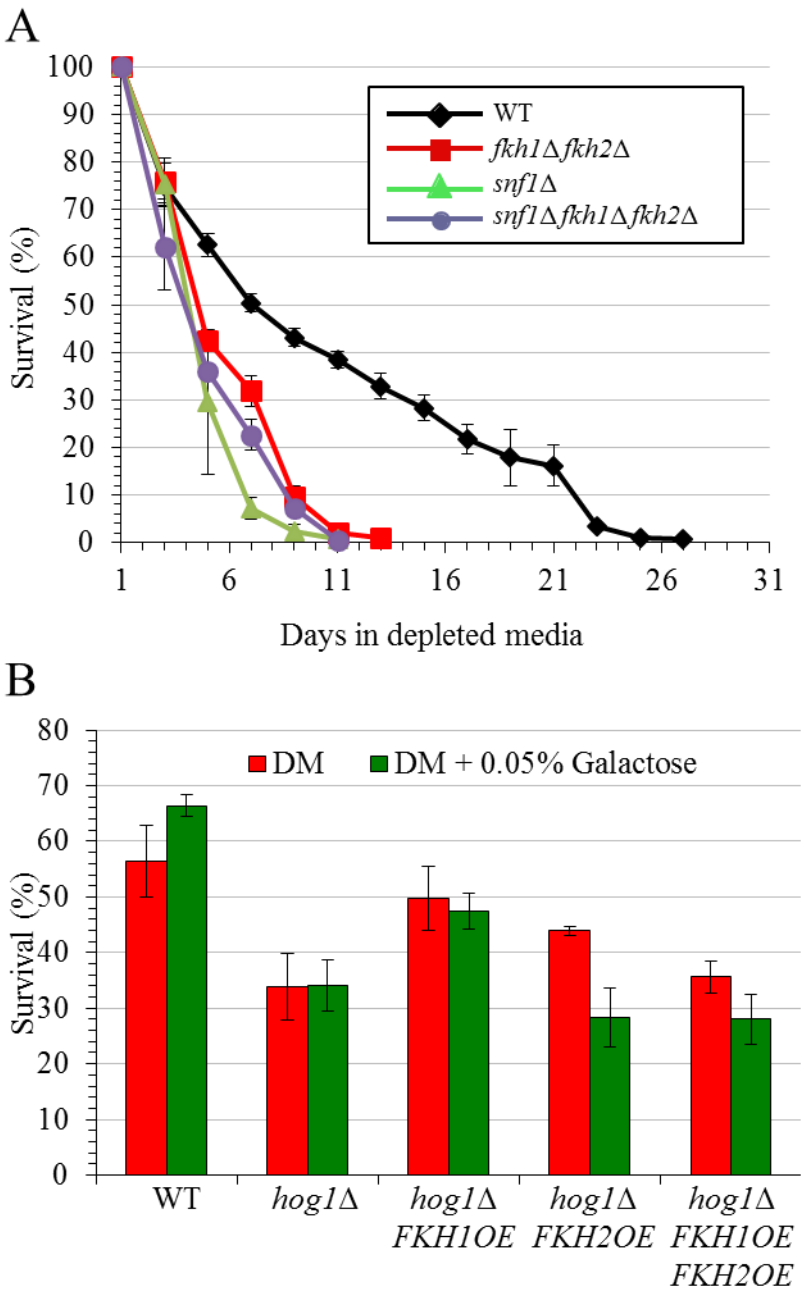

Figure 5.7 Stress response kinases Snf1 and Hog1 may promote Fkh function
A) CLS cultures were grown in 2% Glc SDC. Upon entry into stationary phase the cultures were maintained in DM. Error bars represent +/- standard error of the mean of n=3. B) Cultures were grown in 2% YPD. Upon entry into stationary phase the cultures were maintained in DM with or without the addition of 0.05% Gal to induce *FKH* expression. 5 days after entry to stationary phase cultures were treated with 100 mM H_2O_2 for 1 h and assayed for colony forming unit survival. Error bars represent +/- standard error of the mean of at least n=3.

which point cultures were split and 0.05% Gal was added to one half. At day 5 stationary phase, the cultures were split again and half were treated with 100 mM H_2O_2 for 1 h at 30°C and assayed for survival. The survival of *hog1*Δ (33.9 +/- 6.0% glc/34.1 +/- 4.6% gal) was reduced compared to wild type (56.4 +/- 6.5% glc/66.4 +/- 2.0% gal; Figure 5.7 B). The deregulation of normal *FKH* gene expression partially recovered *hog1*Δ in Glc conditions. Unlike the increased stress resistance observed in Gal induced cultures presented in Figure 4 D; the addition of Gal had no effect or decreased survival in all strains with *hog1*Δ (Figure 5.7 B). These data, taken with the decrease in expression of the *FKHs* under the regulation of the *GAL1/10* promoter versus the endogenous promoter in Glc observed in Figures 4.3 B and C, suggest that in *hog1*Δ strains increased Fkh levels may be deleterious to cellular survival in these stress conditions. Alternatively, Hog1 may facilitate stress resistance conferred by the Fkhs.

5.4 Discussion

In this chapter we present data suggesting genetic interactions between *SCH9* and *TOR1* regulated signaling with *FKH* dependent lifespan and stress resistance regulation. In standard CLS conditions, the lifespan extending effects of *sch9*Δ or *tor1*Δ are epistatic to the *fkh1*Δ *fkh2*Δ, extending lifespan beyond that of wild type; this relationship switches with the *FKHs* becoming necessary for *sch9*Δ *tor1*Δ survival in non-standard (potentially hypoxic) culture conditions such as in the presence of hydrogen peroxide. Furthermore, the *FKHs* genetically interact with the stress activated kinase encoding genes *HOG1* and *SNF1*, suggesting the Fkhs function in mechanisms switching cellular processes between growth and stress resistance.

Our data indicate redundancy in lifespan regulation and separate growth phenotypes (i.e. colony colour and size, cell size, temperature sensitivity) between *sch9*Δ and *tor1*Δ, while genetic interactions between *fkhs* with *tor1*Δ differ from *sch9*Δ, suggesting they may function in separate pathways. Contrary to our findings, previous experiments indicate Sch9 is a direct target of $TORC1^{Tor1}$ (Urban et al., 2007), and form a linear pathway in the regulation of CLS (Wei et al., 2009). Urban *et al.* present data that Sch9 is phosphorylated by TORC1, resulting in Sch9 regulating ribosome biogenesis and translation initiation, thus identifying Sch9 as an S6K ortholog (Urban et al., 2007). Wei *et al* found *tor1*Δ and *sch9*Δ are epistatic in Msn2/4-dependent lifespan regulation (Wei et al., 2009). However, gene expression profile analysis of

wild type and *sch9*Δ, in the presence or absence of rapamycin treatment, identified gene clusters regulated by Sch9 through TORC1-dependent and -independent mechanisms (Smets et al., 2008). Under favourable pro-growth conditions, TORC1 and Sch9 activate the expression of genes involved in translation, through linear and parallel mechanisms, congruent with the findings of Urban *et al.* (Urban et al., 2007). On the other hand, when TORC1 is inactive, Sch9 increases the expression of genes involved in stress resistance and respiration. Additionally, Sch9 and TORC1 show independent and additive control of nitrogen starvation induced genes (Smets et al., 2008) and have parallel functions in the repression of autophagy (Kamada et al., 2010; Yorimitsu et al., 2007). In this light, the differences in *tor1*Δ/*sch9*Δ CLS results observed between this study and the literature could be attributed to media/growth conditions (i.e. supplemented amino acid concentrations) and/or the genetic background of the yeast strains, resulting in a switch between linear and parallel Sch9 and TORC1 pathways regulating survival (Wei et al., 2009).

A complication with CLS data is the presence of regrowth or gasping observed in strains featuring combinations of both *fkh*Δs with at least one of *sch9*Δ or *tor1*Δ. Gasping is thought to arise from a better adapted subpopulation utilizing nutrients released from dead and dying cells or the efficient utilization of ethanol, and perhaps acetic acid, for oxidative phosphorylation, initiating growth and repopulation of the stock culture (Fabrizio et al., 2004a; Fabrizio et al., 2005; Fabrizio and Longo, 2003, 2007). Maintenance in water with frequent replacement is often used to avoid this artefact; however, we observed regrowth phenotypes even under this regiment in preliminary testing (data not shown). The greatest regrowth phenotypes were associated with *tor1*Δ, which under certain conditions increases autophagy and glycogen accumulation (Kamada et al., 2010; Wilson et al., 2002). This suggests intracellular stored energy or autophagy may be responsible for providing the nutrients for regrowth. The specific combinations of mutant alleles giving rise to regrowth could result from the inclusion of *fkh*Δ-induced increases in mutation rate from genomic instability associated with decreased histone and chromatin maintenance, as well as derepression of Clb2 and potentially other cell cycle responsive genes (Postnikoff et al., 2012). These circumstances would be in combination with increased stress response activated by Msn2/4 in response to *sch9*Δ and/or *tor1*Δ (Fabrizio et al., 2004b; Wei et al., 2008). In this case, the adaptive regrowth might be analogous to tumour formation rather than healthy lifespan, in

which case the initial trajectories of the survival curves and not the maximal lifespan should be considered relevant to this study. Reinterpreting Figure 5.3 in this light, ignoring the regrowth and any curve beyond, *fkh1*Δ *fkh2*Δ may be epistatic to *tor1*Δ in standard CLS conditions, while *sch9*Δ is partially epistatic to *fkh1*Δ *fkh2*Δ lifespan. When interpreted this way, these data suggest a linear mechanism between Tor1 and the Fkhs to regulate longevity, while in these conditions Sch9 may be functioning through a separate mechanism (Figure 5.8 A). However, if the regrowth and maximal lifespans are considered, the Fkhs appear to be repressors of separate redundant Tor1 and Sch9 pathways (Figure 5.8 B). Additionally, *tor1*Δ *fkh1*Δ *fkh2*Δ has a lifespan greater than *tor1*Δ and similar to *sch9*Δ. Inactivation of TORC1 was found to cause a Sch9-dependent increase of stress response (Smets et al., 2008), suggesting a potential mechanism for the extended *tor1*Δ *fkh1*Δ *fkh2*Δ CLS.

Furthermore, in the stress conditions documented in Figure 5.4, both Sch9 and Tor1 may be redundantly functioning through the repression of the Fkhs (Figure 5.8 C). In the oxidative stress analysis (Figure 5.5), the triple mutants showed intermediate survival between *fkh1*Δ *fkh2*Δ/*tor1*Δ *sch9*Δ *fkh1*Δ *fkh2*Δ and *sch9*Δ/*tor1*Δ/*sch9*Δ *tor1*Δ strains. This further suggests a role for Sch9 or Tor1 in stress adaptation/recovery and cell growth/survival, similar to the one described in CLS gasping phenotypes. Alternatively, Sch9 increases the expression of stress resistance and respiration genes in TORC1-unfavourable conditions (Smets et al., 2008); interpreting this in light of our data, either Sch9 or TORC1 in the absence of the other and the Fkhs may increase a stress response pathway. In this scenario, nitrogen or carbohydrate starvation may lead to increased stress resistance and respiration, actively facilitating a cell's ability to adapt to changing environments. This activation of a potentially adaptive stress response in the absence of the *FKHs* could be at least partially responsible for regrowth phenotypes observed in the CLS assays.

The mechanisms of the genetic interactions between the *FKHs* and *SCH9*/*TOR1* are likely two-fold. First, the Fkhs may function to alter the expression of Sch9 and Tor1, their regulators, or their lifespan related targets. For example, the Fkhs may repress *SCH9*, *TOR1*, or the loci of genes encoding proteins downstream of TORC1/Sch9 or increase the expression of upstream repressors during G0 for cell cycle arrest/cellular maintenance, perhaps in a mechanism

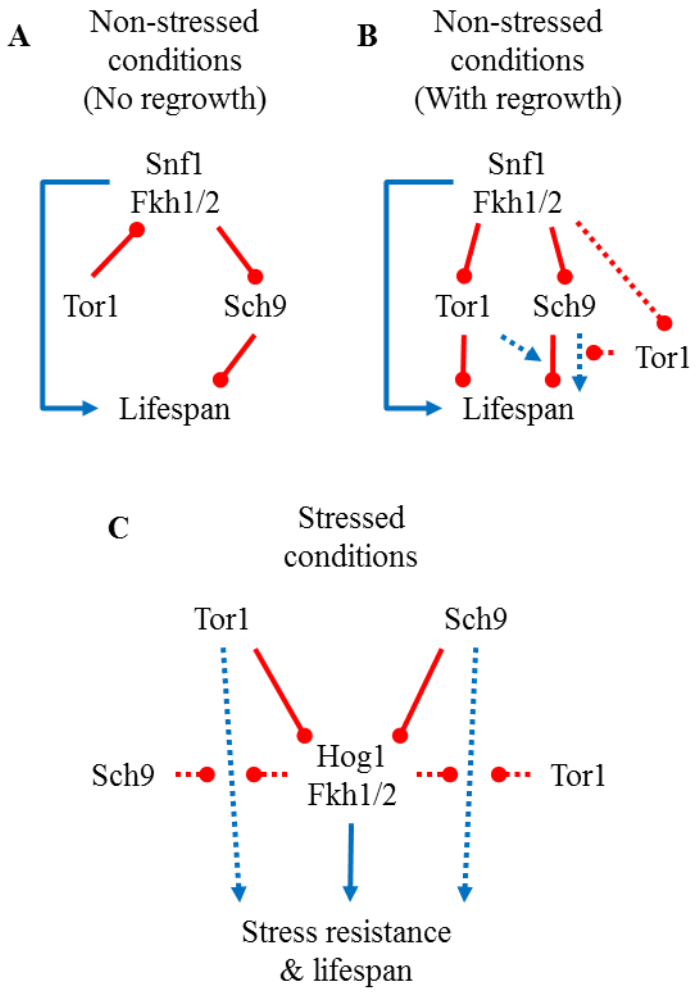

Figure 5.8 Models of Fkh/Sch9/Tor1 signaling genetic interactions
A) In non-stressed conditions the Fkhs are involved in the control of redundant Sch9 and Tor1 lifespan shortening pathways, although this interaction varies if regrowth is not considered. B) This relationship changes if complete lifespan curves (including regrowth) are taken into considered (from Figure 5.3 in Section 5.3.1). C) In stressed conditions, redundant *SCH9* and *TOR1* pathways repress *FKH*- dependent lifespan and stress resistance; however, deletion of *SCH9* or *TOR1* partially recover *fkh*Δ lifespan and stress resistance defects. Dashed lines indicate speculation from the triple mutant data and information for (Smets et al., 2008).

analogous to metazoan FoxO control of mTORC1 through Raptor, E4-BP, Sestrin, and TSC expression (Chen et al., 2010; Demontis and Perrimon, 2010; Hay, 2011; Jia et al., 2004; Khatri et al., 2010; Nogueira et al., 2008; Tettweiler et al., 2005; Wessells et al., 2009). The deletion of the *FKHs* may increase the expression of components of the TORC1 pathway, accelerating aging in otherwise wild type cells, while *sch9*Δ or *tor1*Δ strains have increased lifespan regardless of *FKHs* in standard conditions. Although possible, direct Fkh regulation of *SCH9* or TORC1 subunit genes is unlikely, as neither Fkh was found associated with these genes promoters in a genome wide ChIP assay (Venters et al., 2011), nor were Fkh binding consensus sequences found associated with the upstream promoter regions of these genes in a manual search utilizing the *Saccharomyces* Genomic Database.

Conversely, Sch9 and Tor1 signaling may alter Fkh function, likely through: 1) Fkh dissociation from DNA, nuclear export, and/or degradation; 2) changing Fkh binding location or altering co-factor association. In favour of option one, Fkh-TAP data suggest that at least Fkh1 protein levels can be altered in a Glc dependant manner through Sch9 and Tor1. After one hour in high Glc concentrations, Fkh1 levels drop in wild type cells, but not in *sch9*Δ or *sch9*Δ *tor1*Δ strains. Although we have not ruled out changes in *FKH1* expression, we interpret these levels as indicative of altered protein stability, perhaps in a manner similar to AKT/SCF^{SKP2} degradation of the FoxOs (Gao et al., 2009; Huang et al., 2005; Huang and Tindall, 2011; Lin et al., 2009). Supporting the second option, TORC1 indirectly activates Cdc5 in an Sch9-independent manner (Nakashima et al., 2008), while Cdc5 phosphorylation facilitates Fkh2-Ndd1 association (Darieva et al., 2006), switching Fkh2 function from repressing to activating the expression of the *CLB2* gene cluster (Darieva et al., 2006; Wittenberg and Reed, 2005), which consequently can result in decreased stress resistance (Simpson-Lavy et al., 2009).

6 The APC is involved in *FKH/SCH9* dependent lifespan regulation[3]

6.1 Introduction

Fkh1/2 regulate the *CLB2* gene cluster (Zhu et al., 2000), which includes genes required for APC activity, as well as APC targets for degradation (Barford, 2011; Harper et al., 2002; Ko et al., 2007). The APC is a highly conserved multi-subunit E3 that promotes mitotic progression and G1 maintenance by targeting cell cycle regulators, such as the securin Pds1 and the B type cyclin Clb2 in yeast, for proteasome-dependent degradation (Barford, 2011; Harper et al., 2002; Passmore, 2004). The APC has been demonstrated to be critical for regulating genomic stability and longevity in yeast and higher eukaryotic organisms (Baker et al., 2004; Harkness et al., 2002; Harkness et al., 2004; Hartwell and Smith, 1985; Li et al., 2008a; Palmer et al., 1990). In yeast, mutation to individual APC subunits decreases RLS and CLS, while over-expression of *APC10* increases RLS (Harkness et al., 2004). Consistent with this, the yeast APC plays a role in stress response, possibly by targeting proteins that promote growth, rather than defense mechanisms, for degradation (Harkness et al., 2002; Simpson-Lavy et al., 2009; Turner et al., 2010). In mice, mutations to the APC regulator BubR1, a component of the spindle checkpoint, lead to premature aging (Baker et al., 2005; Baker et al., 2004). One protein targeted for degradation by the mammalian APC is FoxM1 with inappropriate FoxM1 stability and activity involved in oncogenesis (Laoukili et al., 2008; Park et al., 2008). Furthermore, mammalian APC^{CDH1} indirectly stabilizes FoxO during G1 by targeting components of the SCF^{SKP2} for degradation. SCF^{SKP2} is the E3 responsible for identifying FoxO for proteasomal degradation (Gao et al., 2009; Huang et al., 2005; Huang and Tindall, 2011). This chapter examines the genetic interactions between pathways regulating nutrient signaling or *FKH1/2* with the APC, with regards to lifespan and stress resistance in yeast.

6.2 Methods

Spot dilutions were used to investigate genetic interaction between the APC with *SCH9* and *TOR1*, or *FKH1* and *FKH2* in actively dividing cells (3.1.4) Elevated temperature was used as a stress condition to analyse temperature sensitive growth phenotype interactions. Oxidative stress

[3] Parts of this chapter have been published Postnikoff et al. (2012)

resistance assays (3.1.6) were performed to assess the combined effects of these genes on post-mitotic free radical stress resistance. Finally, CLS assays (3.1.5) were performed to examine genetic interactions in stationary phase cells under standard and SCR conditions as well as in the presence of H_2O_2 as an alternative stress.

6.3 Results

6.3.1 *FKH1* and *FKH2* interact with APC subunit encoding genes

Integral to the power of yeast as a model organism is the capacity to elucidate genetic mechanisms for cellular processes. One of the goals of this study was to identify potential evolutionarily conserved lifespan regulating targets of the forkhead box transcription factors. Fkh1/2 regulate the *CLB2* gene cluster (Zhu et al., 2000), which encode proteins required for APC function including several APC subunits (Barford, 2011; Harper et al., 2002; Ko et al., 2007), implicating the APC as a candidate for co-regulation of Fkh-dependent processes. To elucidate potential interactions between the *FKHs* and the APC, we examined colony growth of cells over-expressing the *FKHs* or *fkh*Δ in combination with mutations to APC subunits. The deletion of one or both *FKHs* had no effect on growth at elevated temperatures in an otherwise wild type background, while mutations to *APC5* or *APC10* decreased growth phenotypes at higher temperatures (Figure 6.1 A and B). In backgrounds containing either *apc10*Δ or $apc5^{CA}$, the deletion of *FKH1* partially restored the temperature sensitive defect, while the deletion of *FKH2* had no effect on $apc5^{CA}$ growth and enhanced *apc10*Δ growth defects at elevated temperatures. This is not surprising, as opposing phenotypes for *fkh1*Δ and *fkh2*Δ have previously been described (Hollenhorst et al., 2000; Hollenhorst et al., 2001; Pic et al., 2000; Zhu et al., 2000). Furthermore, the deletion of both *FKH* genes in $apc5^{CA}$ or *apc10*Δ mutant cells exacerbated the temperature sensitive phenotype (Figures 6.1 A and B). The permissive temperature of 30°C was often found to be restrictive for the *apc10*Δ *fkh1*Δ *fkh2*Δ mutant, requiring incubation at room temperature (data not shown).

To further investigate the role of the *FKHs* in APC deficient backgrounds, we transformed wild type and $apc5^{CA}$ cells with the YCp50 empty vector or one containing either GAL_{PROM}-*FKH1* or -*FKH2*. When spotted on 2% Glc SD^{Ura-} followed by incubation at 30°C, the presence of the GAL_{PROM}-*FKHs* altered growth rate/colony size, however, total growth was unaltered. On 2%

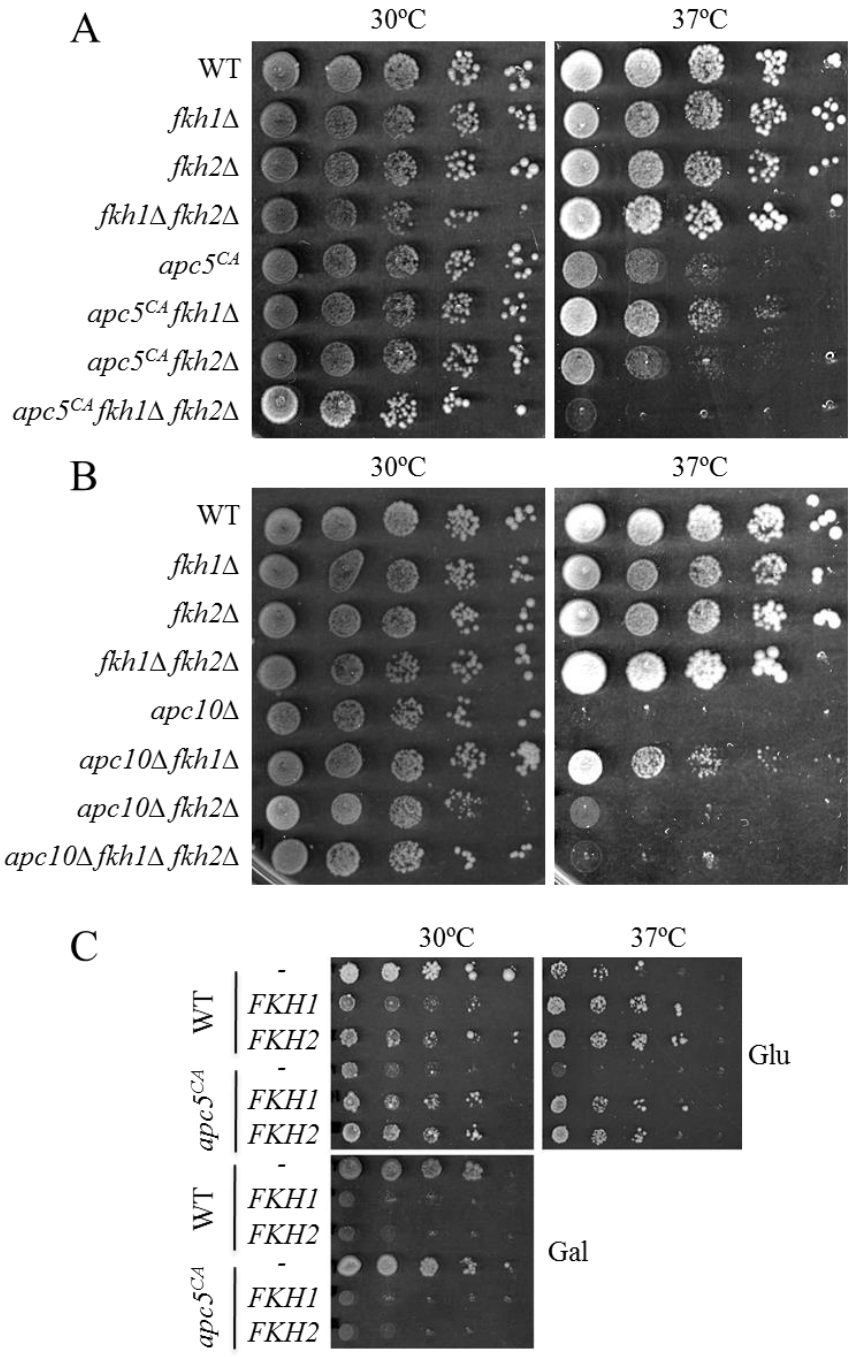

Figure 6.1 The *FKHs* interact with the APC to regulate logarithmic growth
Tenfold serial dilutions with the starting spot having approximately 30,000 cells plated and grown on 2% YPD at 30°C and 37°C of strains with *fkh1*Δ or *fkh2*Δ in combination with (A) *apc5*^{CA} or (B) *apc10*Δ. C) Serial dilutions of wild type and *apc5*^{CA} with either an empty vector, Gal inducible *FKH1* expression vector, or Gal inducible *FKH2* expression vector plated on 2% Glc or Gal SD^{Ura-} at 30°C and 37°C.

Gal SD^{Ura-} plates, the presence of either *GAL$_{PROM}$-FKH* reduced growth in both wild type and *acp5^{CA}* backgrounds (Figure 6.1 C), similar to what was previously described in Section 4.3.5. On 2% Glc SD^{Ura-}, both YCp50 empty vector controls were temperature sensitive at 37°C compared to 30°C: with *apc5^{CA}*-YCp50 having a more severe phenotype than wild type-YCp50. The presence of either *GAL$_{PROM}$-FKH* plasmid completely recovered these growth defects on 2% Glc SD^{Ura-} plates at 37°C in both backgrounds. We suspect increased Fkh activity, due to high copy number of the gene with a transcriptionally leaky promoter, can overcome the combined stresses induced from plasmid dependency, SD nutrient conditions and elevated temperature, as well as completely recovering the *apc5^{CA}* growth defect at elevated temperatures.

6.3.2 Deletion of the *FKHs* reverses defective APC CLS

Forkhead box transcription factors and the APC have been shown to regulate lifespan in many organisms (Baker et al., 2005; Baker et al., 2004; Giannakou et al., 2004; Harkness et al., 2002; Harkness et al., 2004; Lin et al., 1997; Postnikoff et al., 2012). In yeast, the Fkhs regulate gene expression of APC activators/subunits/targets (Zhu et al., 2000) and genetically interact under permissive growth conditions (Section 6.3.1). In mammalian cells, the pro-cell cycle oncogene, FoxM1, regulates APC activators/targets for mitotic progression (Korver et al., 1997; Laoukili et al., 2005; Laoukili et al., 2007; Wang et al., 2005) and then becomes a target of the APC in G1, during unfavourable growth conditions (Laoukili et al., 2008). Conversely, the tumour-suppressing FoxOs may be involved in both the expression and repression of APC activators (Alvarez et al., 2001; Takano et al., 2007). The APC indirectly stabilizes at least FoxO1, through the ubiquitin-dependent degradation of the SCF F-box protein Skp2, an E3 complex responsible for FoxO1 ubiquitination and subsequent proteasomal degradation (Gao et al., 2009; Huang et al., 2005).

To investigate the potential interaction between *FKH* and APC-dependent lifespan regulation, we performed CLS assays on strains combining *fkh1*Δ, *fkh2*Δ and *apc5^{CA}* alleles. When grown in SDC maintained in DM, *apc5^{CA}* mean lifespan (50% survival) was 0.33 times that of wild type (Figure 6.2 A). The deletion of *FKH1* or *FKH2* in the *apc5^{CA}* background recovered survival defects to wild type levels (Figure 6.2 A and B). Finally, lifespan of the *fkh1*Δ *fkh2*Δ *apc5^{CA}* strain was comparable to the *fkh1*Δ *fkh2*Δ strain (Figure 6.2 C). These data suggest that in

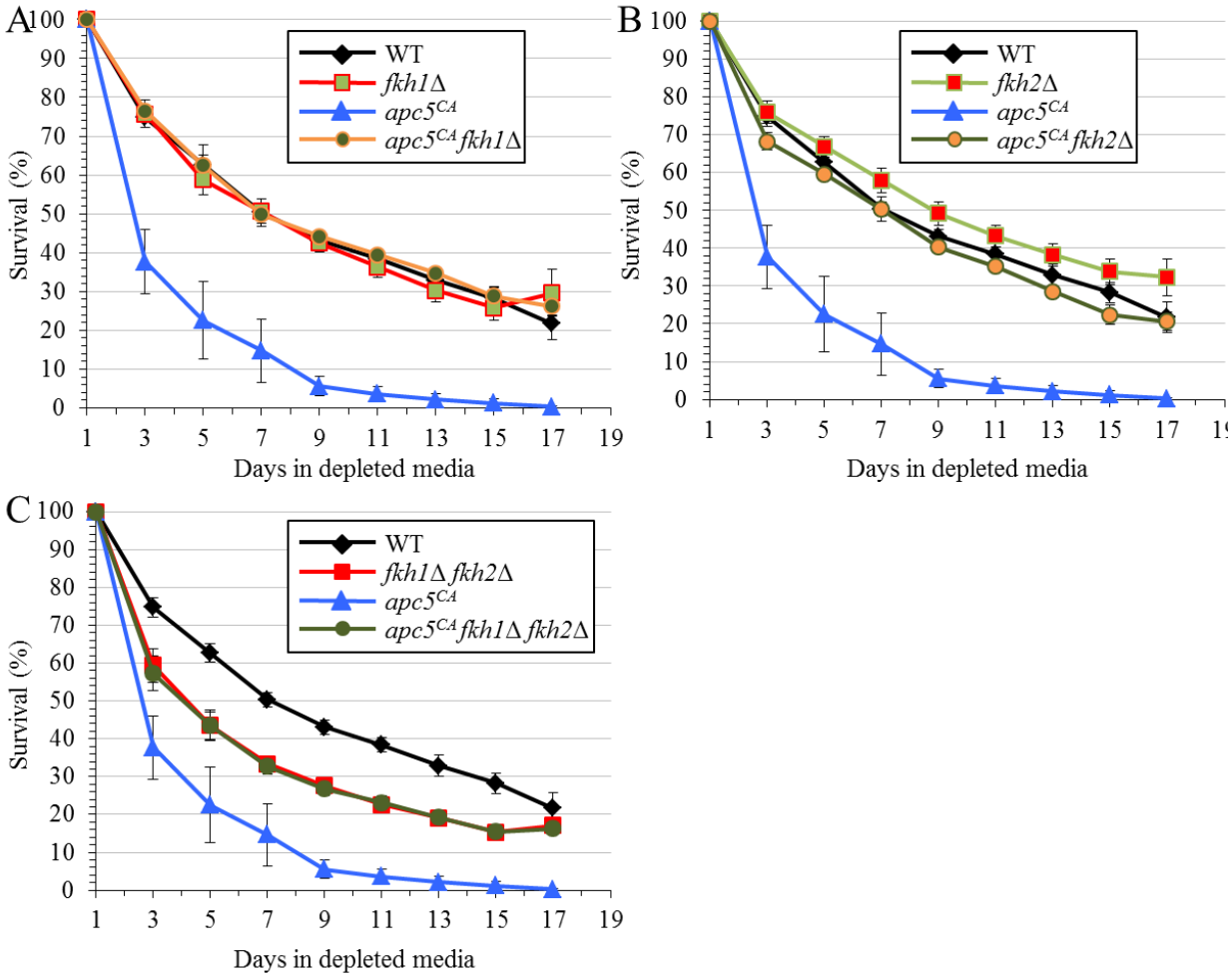

Figure 6.2 The redundant *FKHs* interact with *APC5* to regulate chronological lifespan
Cultures were grown in 2% Glc SDC. Upon entry into stationary phase the cultures were maintained in DM. Error bars represent +/- standard error of the mean of n=3.

unstressed DM conditions, the *fkh* phenotype is epistatic to that of *apc5*^{CA}, indicating that these genes regulate longevity through the same mechanism.

6.3.3 Deletion of both *FKHs* exacerbate APC mutations under stress conditions

Next we examined CLS of strains grown in SDC and maintained in water. SRC conditions extended the lifespan and 50% survival of *apc5*^{CA}, *fkh1*Δ *apc5*^{CA}, and *fkh2*Δ *apc5*^{CA} strains compared to DM (Figures 6.2 and 6.3). The CLS of the *fkh1*Δ *fkh2*Δ strain remained approximately the same, with the lifespan curve conforming to that of DM conditions. The *fkh1*Δ *fkh2*Δ *apc5*^{CA} strain's 50% lifespan remained approximately the same; however, the maximal lifespan (less than 5% survival) decreased by half when maintained in SCR conditions (Figures 6.2 C and 6.3 C). In the literature, SCR is considered a mild protective stress, with the combination of CR and osmotic stress hormetically activating major stress resistance mechanisms, while simultaneously removing the buildup of metabolic toxins such as acetic acid (Burtner et al., 2009; Fabrizio and Longo, 2003, 2007; Longo and Fabrizio, 2012; Wei et al., 2008). The decrease in the triple mutant's survival may be due to a stronger inhibition of stress resistance mechanisms resulting in mild hormetic stress becoming lethal. To test the theory of *FKH* and APC co-regulation of stress resistance throughout lifespan, CLS assays were performed with the addition of 25 mM H_2O_2 upon entry into stationary phase. The 50% survival of all strains tested was less than three days in the presence of H_2O_2. However, if the lifespan is compared at 1% survival and beyond, the trends appeared the same as those under SCR conditions with: wild type having the greatest lifespan; *fkh1*Δ *fkh2*Δ or *apc5*^{CA} strains having a similar, slightly reduced lifespan; *fkh1*Δ *fkh2*Δ *apc5*^{CA} having the most impaired lifespan (Figure 6.4 A).

A similar trend was observed in the survival of stationary Day 5 cultures (grown in YPD and maintained in DM) treated with 100 mM H_2O_2. Mutation to *FKH1* (42.2+/-0.3 %), *FKH2* (48.5+/-1.4 %), both (39.2+/-1.5 %), or *APC5* (27.6+/-1.1 %) reduced survival relative to wild type (53.7+/-1.7 %; Figure 4.4 B). The deletion of a single *FKH* partially recovered *apc5*^{CA} H_2O_2 resistance defects as seen in data for *fkh1*Δ *apc5*^{CA} (35.4+/-2.0 %) and *fkh2*Δ *apc5*^{CA} (36.5+/-1.7 %) strains. However, *fkh1*Δ *fkh2*Δ *apc5*^{CA} had a severe reduction in survival (3.5+/-0.9 %). We also tested combinations of *fkh1*Δ, *fkh2*Δ and *apc10*Δ mutant strains resistance to 100 mM H_2O_2

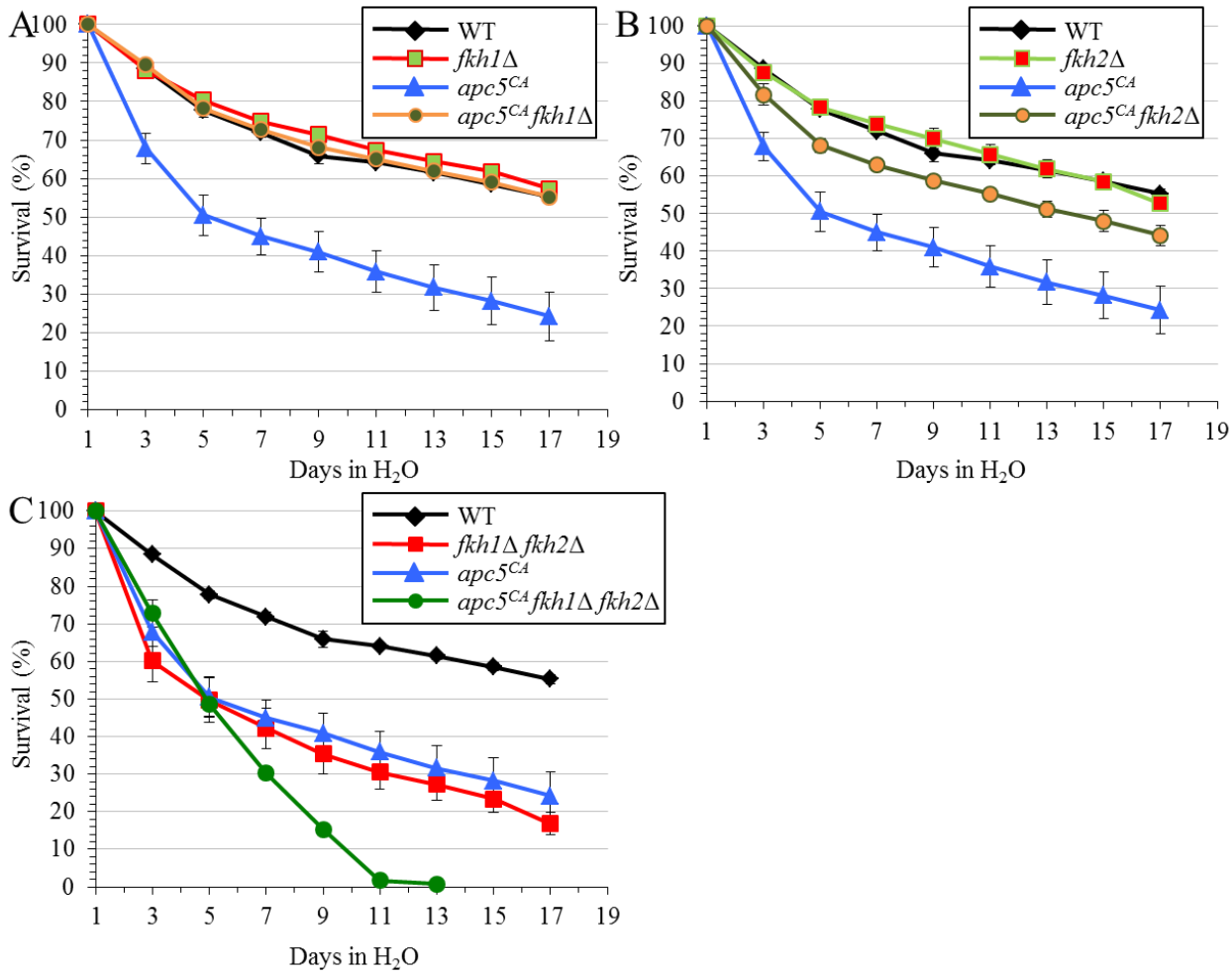

Figure 6.3 The *FKHs* and *APC5* synergistically regulate chronological lifespan under severe caloric restriction

Cultures were grown in 2% Glc SDC. Upon entry into stationary phase the cultures were maintained in water with washing and replacement every 2 days. Error bars represent +/- standard error of the mean of n=3.

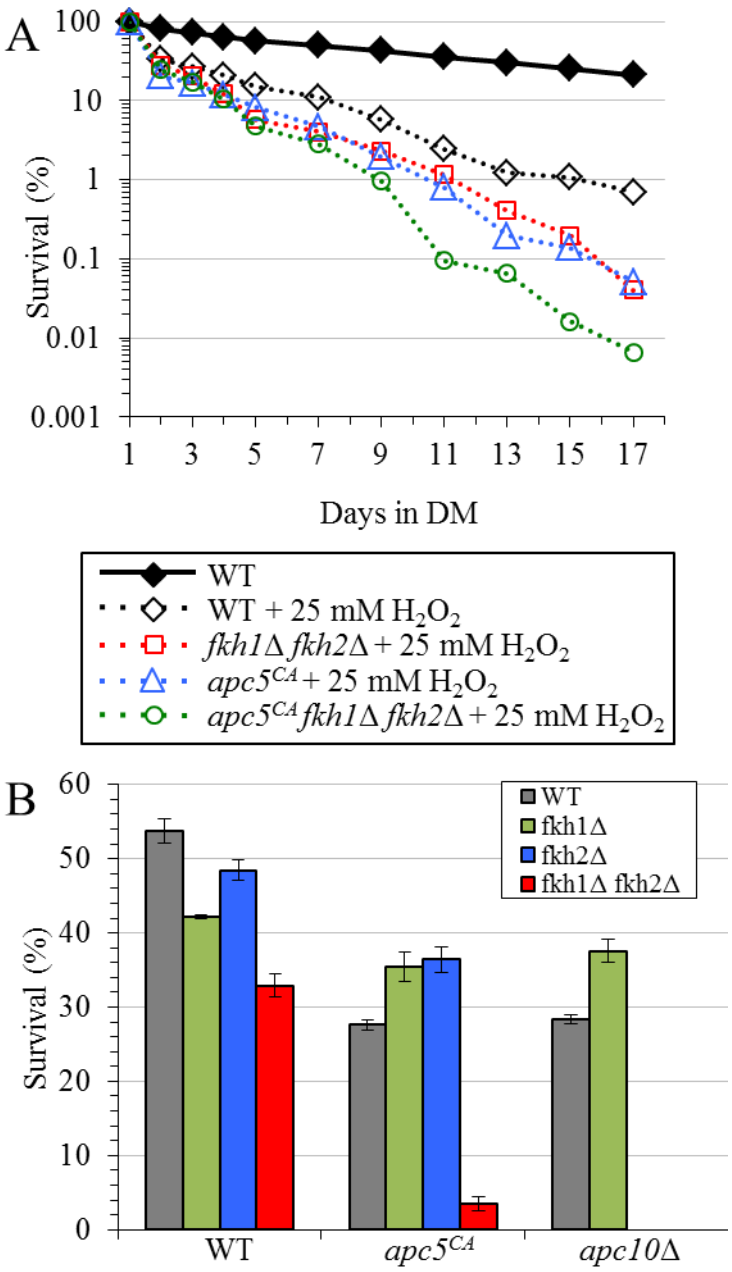

Figure 6.4 The *FKHs* function in a redundant manner with the APC to respond to H_2O_2 stress
A) Cultures were grown in 2% Glc SDC. Upon entry into stationary phase the cultures were maintained in DM with or without the addition of 25 mM H_2O_2. B) Cultures were grown in 2% YPD and maintained in DM. At stationary phase day 5 cultures were treated with 100 mM H_2O_2. Error bars represent +/- standard error of the mean of n=3.

to examine if the results were *APC5* specific or general to the APC (Figure 6.4 B). The *apc10*Δ strain had a survival of 28.3+/-0.7%, which was partially recovered in combination with the *fkh1*Δ allele (37.6+/-1.6 %). Interestingly, in *fkh2*Δ *apc10*Δ and *fkh1*Δ *fkh2*Δ *apc10*Δ strains no colonies were observed when treated in three separate replicates (a complete kill), suggesting a severe decrease in oxidative stress resistance with these mutant combinations.

6.3.4 The APC interacts directly with *SCH9* and partially with *TOR1*

The *FKHs* co-regulate lifespan with S*CH9/TOR1* (Chapter 5) and the APC (this chapter), with the interactions changing in stressed conditions. To further characterize this lifespan regulatory network, we assessed genetic relationships between *SCH9*, *TOR1* and the APC subunits. First, we examined the genetic interactions of combinations of $apc5^{CA}$, *sch9*Δ, and *tor1*Δ in logarithmically growing cells, when spotted and grown at permissive (30°C) and restrictive (37°C) temperatures. The phenotype of *sch9*Δ was epistatic to that of $apc5^{CA}$, recovering the $apc5^{CA}$ temperature sensitive growth defect (Figure 6.5), suggesting that Sch9 and APC are in a linear genetic pathway. The severity of the *tor1*Δ temperature sensitivity prevented assessment of genetic interactions between *tor1*Δ with $apc5^{CA}$ or *sch9*Δ; alternatively, neither *tor1*Δ nor $apc5^{CA}$ temperature sensitive phenotypes were recovered in combination, suggesting that they function in separate pathways. Similar phenotypic interactions were observed in the combinations of *sch9*Δ, *tor1*Δ, and *apc10*Δ alleles, suggesting this is a general interaction between the APC and *SCH9* (data not shown).

Next, we examined the interactions of Sch9 and Tor1 signaling and the APC with regards to CLS. In cultures grown in SDC and maintained in DM, the *sch9*Δ phenotype was epistatic to the severely decreased $apc5^{CA}$ CLS (Figure 6.6 A), similar to the interaction observed in Figure 6.5. Deletion of *TOR1* partially recovered the $apc5^{CA}$ phenotype, proportional to the CLS extension of *tor1*Δ to wild type (Figure 6.6 B). This indicates that *APC5* and *TOR1* function in parallel lifespan regulating mechanisms. The 50% survival of the *sch9*Δ *tor1*Δ $apc5^{CA}$ strain was the same as *sch9*Δ and *sch9*Δ $apc5^{CA}$ strains, which were decreased relative to the CLS of *sch9*Δ *tor1*Δ. Maximal lifespan of the triple mutant was similar to *sch9*Δ *tor1*Δ (Figure 6.6 C). These data strengthen the argument that *SCH9* and the APC function in a linear mechanism that is parallel to *TOR1*. Since the genetic relationship of the *FKHs* with both the APC and *SCH9*/*TOR1*

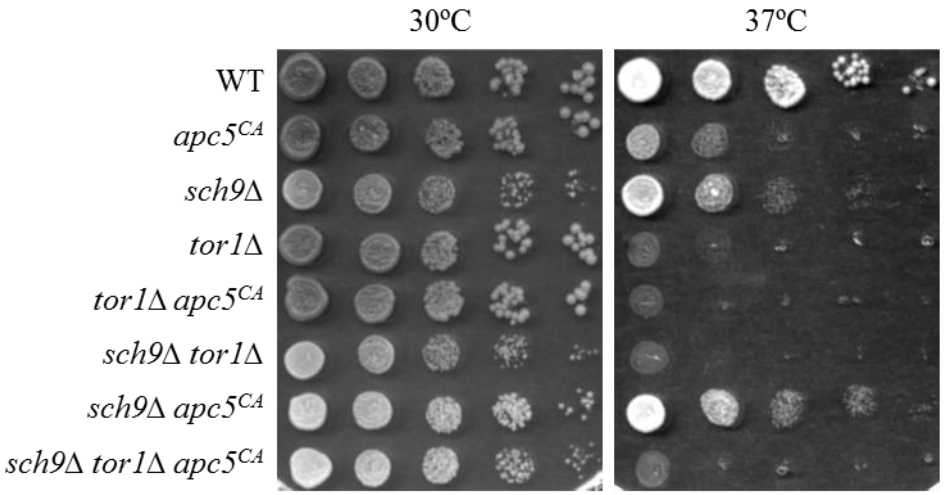

Figure 6.5 The *sch9*Δ allele interacts with *apc5*^{CA} to regulate logarithmic growth
Tenfold serial dilutions with the starting spot having approximately 30,000 cells plated and grown on 2% YPD at 30°C and 37°C.

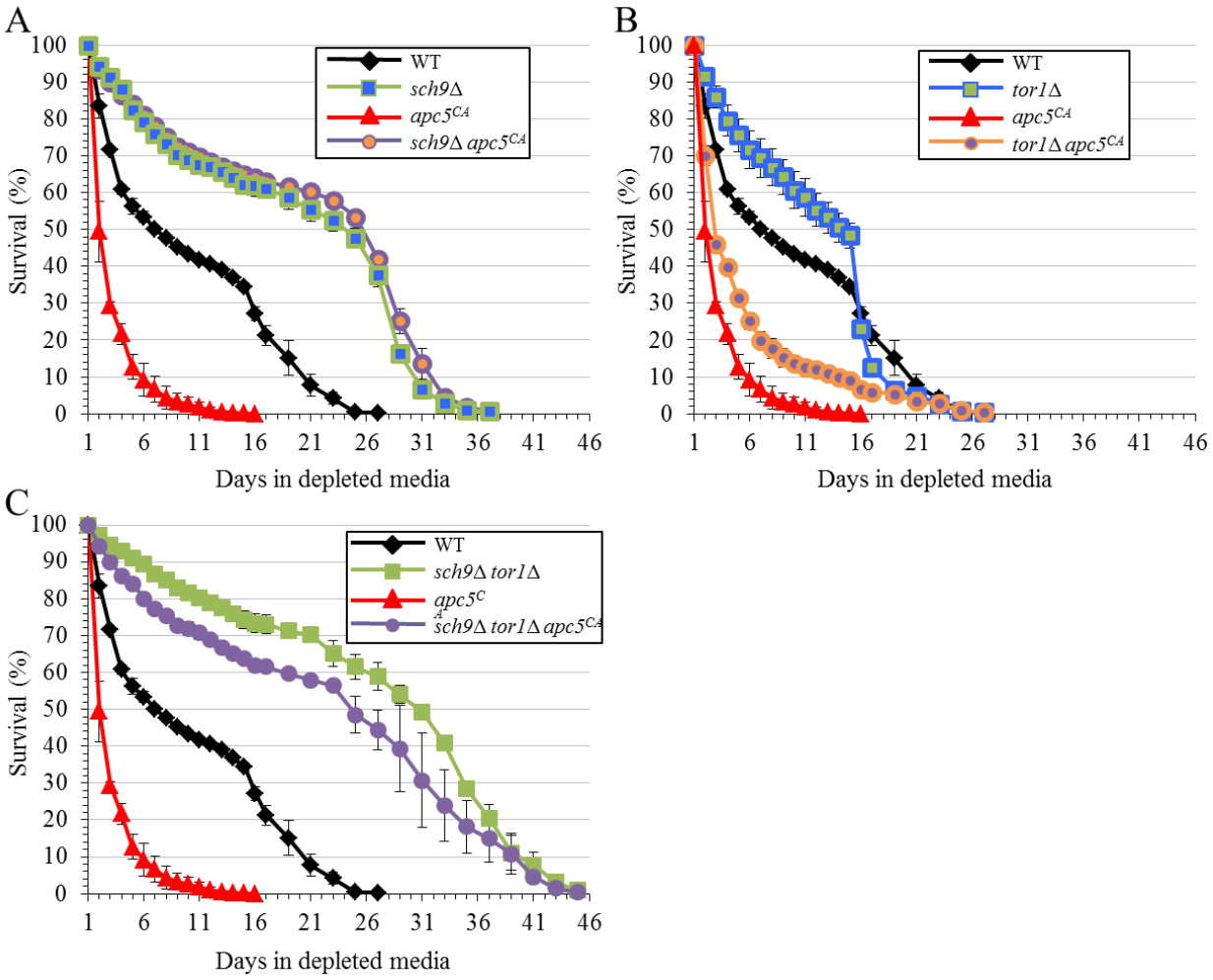

Figure 6.6 *SCH9* and *TOR1* genetically interact with *APC5* under standard conditions of chronological lifespan

Cultures were grown in 2% Glc SDC. Upon entry into stationary phase the cultures were maintained in DM. Error bars represent +/- standard error of the mean of n=3.

signaling changes under stress conditions, we replicated the CLS under the combined potential hypoxic stress/SCR conditions described Section 5.3.3 (cultured in falcon tubes in 2% Glc YPD and maintained in SCR). The relationships between combinations of *sch9*Δ, *tor1*Δ and *apc5*^{CA} were the same as previously described (Figure 6.7 A-C), suggesting these interaction are less conditional than those with the *FKHs*.

To further characterize genetic interactions under stressed conditions, we examined the effects of oxidative free radical stress on yeast cultures with combinations of *sch9*Δ, *tor1*Δ, or *apc5*^{CA} mutations at Day 5 stationary phase, treating them with 100 mM H_2O_2 for 1 h at 30°C. Similar to what we previously reported (Figures 5.5 and 6.4 B), the *apc5*^{CA} allele reduced survival (22.4 +/- 1.2%) compared to wild type (53.3 +/- 1.3%), whereas *sch9*Δ (81.0 +/- 2.5%), *tor1*Δ (91.2 +/- 1.3%), and *sch9*Δ *tor1*Δ (95.3 +/- 1.4%) showed increased survival (Figure 6.8). Consistent with the relational trends observed in the lifespans, *sch9*Δ *apc5*^{CA} (85.5 +/-1.9%) and *sch9*Δ *tor1*Δ *apc5*^{CA} (94.6 +/- 1.1%) showed similar survival to the respective backgrounds without the *apc5*^{CA} allele, while the *tor1*Δ *apc5*^{CA} strain had a survival (57.2 +/- 2.4%) intermediate to either allele alone. These data support a linear interaction between *SCH9* and the APC, while strengthening the argument of Chapter 5 for a parallel role in *SCH9* and *TOR1* regulation of lifespan and stress resistance.

6.4 Discussion

Our work demonstrates that the APC may serve as a downstream Fkh1/Fkh2 target that co-regulates Fox-dependent longevity. The APC and Fkhs interact genetically and functionally to ensure normal yeast lifespan, as well as responding to SCR and stress in non-dividing cells. As mentioned previously, increased expression of *FKH1* or *FKH2* increases both RLS and CLS in yeast [Section 4.3.2; (Postnikoff et al., 2012)]. As expected from factors with redundant function, deletion of both *FKH1* and *FKH2* was required to reduce yeast CLS. Disruption of both *FKH1* and *FKH2* in cells harbouring a temperature sensitive allele of the gene encoding the APC subunit *APC5*, exhibited a similar CLS as *fkh1*Δ *fkh2*Δ cells, indicating that *fkh1*Δ *fkh2*Δ CLS is epistatic to *apc5*^{CA} CLS in DM control conditions; however, the CLS of *apc5*^{CA} was shorter than those containing the *fkh1*Δ *fkh2*Δ disruptions, suggesting that in the absence of stress the presence of both Fkhs is deleterious to lifespan in APC defective mutants. This could occur

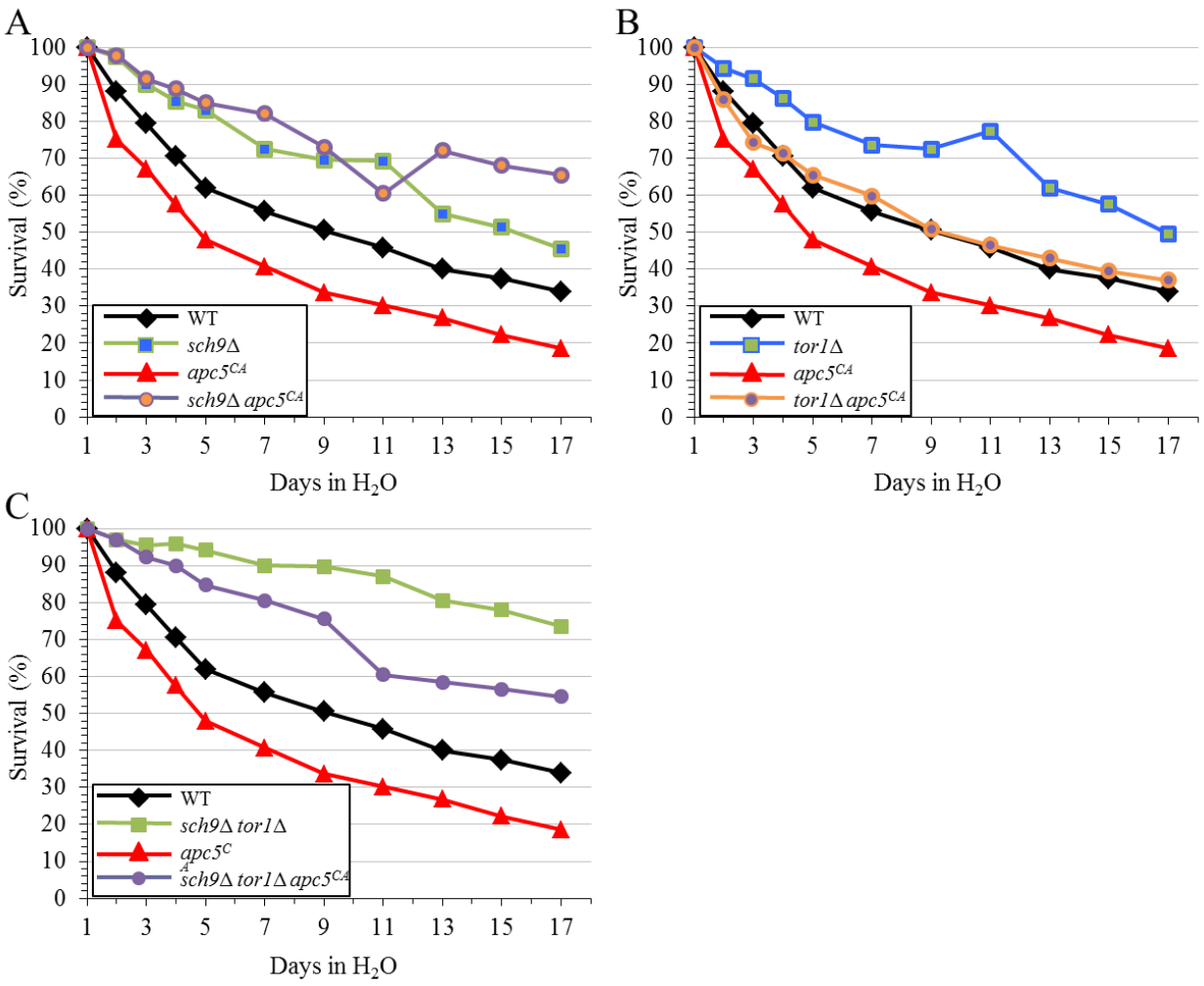

Figure 6.7 *SCH9* and *TOR1* genetically interact with *APC5* under stress conditions (SCR and falcon tube induced) of chronological lifespan

Cultures were grown in 2% YPD. Upon entry into stationary phase the cultures were switched to and maintained in water. Error bars represent +/- standard error of the mean of n=3.

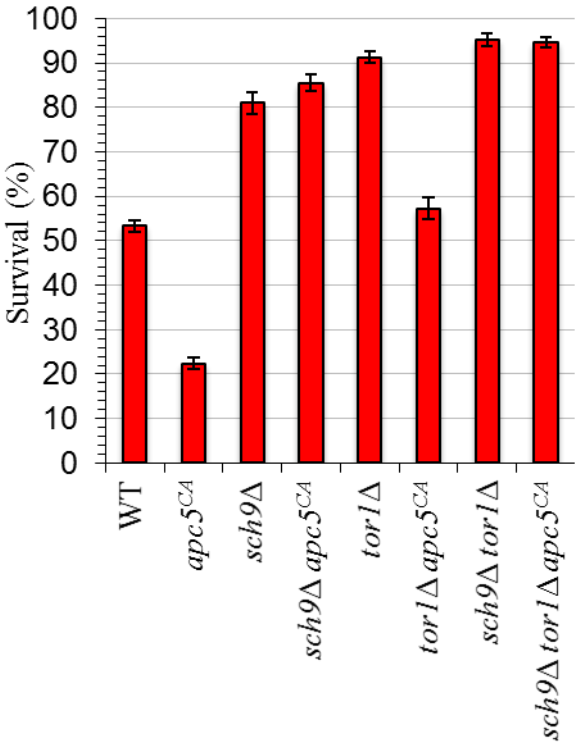

Figure 6.8 Apc5 interacts differently with Sch9 and Tor1 to regulate H_2O_2 stress resistance
Cultures were grown in 2% YPD to day 5 stationary phase, treated with 100 mM H_2O_2 for 1 h, and plated to determine survival of colony forming units. Error bars represent +/- standard error of the mean of at least n=3.

through APC-dependent degradation of known growth-promoting co-activators of the Fkhs, such as Clb2, Cdc5, Ndd1, etc. (Charles et al., 1998; Loy et al., 1999; Simpson-Lavy et al., 2009), switching Fkh function from FoxM like cell-cycle progression to FoxO like maintenance of quiescence in the post-diauxic state (Figure 6.9 A).

Under stressed conditions, such as maintenance in SCR or exposure to either oxidative or heat stress, *apc5*^{CA} *fkh1*Δ *fkh2*Δ cells showed a decreased growth capacity, an increased stress sensitivity, and a reduced CLS. The CLS of *fkh1*Δ *fkh2*Δ cells did not increase in SCR, while the triple mutant exhibited a dramatically shortened lifespan. This indicates the Fkhs and the APC may work together in redundant/parallel mechanisms to ensure prolonged stress resistance and longevity (Figure 6.9 B). This may be coordinated through Fkh transcriptional up-regulation of stress response genes, in tandem with chromatin assembly and histone modification known to be functions of the APC (Arnason et al., 2005; Harkness et al., 2005; Harkness et al., 2002; Islam et al., 2011; Turner et al., 2010). Additionally, the APC and Fkh1/2 may redundantly affect the same stress resistance altering targets through different mechanisms. For example, the presence of Clb2, a B-type cyclin involved in mitotic cell cycle progression, has been found to decrease stress resistance (Simpson-Lavy et al., 2009). Clb2 protein levels are co-regulated by the APC and Fkh1/2 through different mechanisms: 1) Fkhs repress transcription of *CLB2* throughout most of the cell cycle, from mitotic exit to late G2, while up-regulating its transcription during mitotic entry in the presence of Cdc5 (a polo-like kinase), Clb2 (and possibly other B type cyclins), and Fkh2 co-activator Ndd1 (Hollenhorst et al., 2000; Hollenhorst et al., 2001; Koranda et al., 2000; Loy et al., 1999; Pic et al., 2000; Zhu et al., 2000); 2) the APC^{CDH1} poly-ubiquitinates B-type cyclins, the polo-like kinase, and possibly Ndd1, targeting them for proteasomal degradation during mitotic exit/G1 entry (Loy et al., 1999; McLean et al., 2011; Qiao et al., 2010). In higher eukaryotes, co-regulation of the *CLB2* gene cluster orthologs by the Fox proteins is conserved. Specifically, the Fox family has been shown to activate and repress genes promoting mitotic progression and G1 arrest under certain conditions, with FoxOs primarily acting in an anti-cell cycle manner, while FoxMs appear to be pro-cell cycle (Alvarez et al., 2001; Kops et al., 2002a; Kops et al., 2002b; Laoukili et al., 2005; Laoukili et al., 2007; Schmidt et al., 2002; Takano et al., 2007; Wang et al., 2005; Wijchers et al., 2006; Wonsey and Follettie, 2005). Furthermore, we found that Clb2, and H2B, H3 and H4 histone levels were

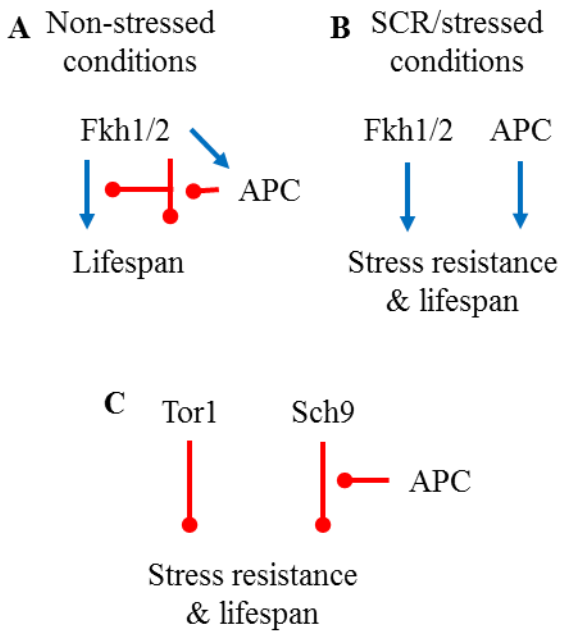

Figure 6.9 Models of APC/*FKH* and APC/*SCH9*/*TOR1* interactions
A) Under standard non stressed conditions *FKH1/2* genetically interact with the APC (Section 6.3.2). The epistatic nature of this interaction suggest the *FKHs* may be responsible for the decreased lifespan observed in APC defective mutants. The APC may be responsible for switching Fkh function to decreasing the transcription of pro-aging/cell cycle genes that increase cellular stress, such as *CLB2* (Linke et al., 2013; Loy et al., 1999; Simpson-Lavy et al., 2009; Zhu et al., 2000). B) Under stress conditions, including SCR, *fkh* and *apc* phenotypes are cumulative, indicating their involvement in parallel lifespan altering mechanisms (Sections 6.3.1 and 6.3.3). C) The data in Section 6.3.4 suggest that *TOR1* and the APC function in parallel antagonistic pathways; while the APC and *SCH9* show epistasis, indicating a linear antagonistic mechanism. Tor1 and Sch9 function in both linear and parallel mechanisms to one another (Smets et al., 2008). Taken in this context, our data may indicate for the APC in the direct regulation of a TORC1-independent lifespan regulating function of Sch9 signaling.

abnormal in strains with *apc5*^{CA} and/or *fkh1*Δ *fkh2*Δ disruptions (Postnikoff et al., 2012), suggesting these factors may co-regulate many processes, not just cell cycle. The recovery of APC defective phenotypes by the deletion of the individual *FKHs* genes suggests the Fkh proteins themselves may be targets of APC regulation. At least one Fox, FoxM1, is a target of the APC for proteasomal degradation (Laoukili et al., 2008), and the stability of FoxO proteins is indirectly regulated by the APC in higher eukaryotes (Gao et al., 2009; Huang et al., 2005). Interestingly, an evolutionarily conserved potential APC binding/targeting motif is present in the forkhead box domain. Preliminary investigations suggest that Fkh stability may indeed be regulated by both the APC and SCF, similar to the Foxs of higher eukaryotes, as Fkh stability and cycling appear to be modified in APC and SCF mutant backgrounds (Malo and Harkness, unpublished). The data presented in this chapter, in the context of the literature, suggest that Fox proteins and the APC co-regulate lifespan by regulating genomic stability, executing precise progression through the cell cycle, and affecting levels of stress modifying genes such as the B type cyclins, in organisms from yeast to mice (Laoukili et al., 2005; Linke et al., 2013; Sherriff et al., 2007; Simpson-Lavy et al., 2009; Thornton and Toczyski, 2003; Wonsey and Follettie, 2005; Zhu et al., 2000).

Next, we show strong genetic interactions between the APC and *SCH9*, in the regulation of growth, oxidative stress and lifespan. Interactions between the APC and Sch9/Tor1 signaling are different than their interaction with the Fkhs. *SCH9* and *TOR1* appear to interact with the *FKHs* in a separate, redundant manner that changes with environmental conditions, as discussed in Chapter 5. On the other hand, *sch9*Δ and APC subunit mutants show strong epistatic interactions, while the APC has an antagonistic interaction with *tor1*Δ suggesting they function in separate pathways. This delineates parallel Tor1 and APC/Sch9 signaling pathways (Figure 6.9 C). A possible explanation for the mechanisms of the APC/Sch9 interaction is that Sch9, or other members of a linear Sch9-dependent signaling pathway involved in growth and stress resistance, are targeted for proteasomal degradation by the APC. In this scenario, mutation to the APC may increase Sch9 signal stability, decreasing survival and growth in stress conditions.

In the presence of TORC1 activating conditions, Sch9 and TORC1 co-repress Msn2/4-dependent stress response gene expression, while when treated with rapamycin (TORC1 repressive

conditions), the presence Sch9 may enhance stress response gene expression (Smets et al., 2008). Taken with this finding, a potential role for the APC may be in specifically targeting TORC1 activated Sch9 for degradation, thereby slowing growth and increasing stress resistance. With the disruption of *SCH9* bypassing the need for the APC to remove the stress and lifespan repressive subpopulation of Sch9, relieving this stress regardless of the condition of the APC. Another possibility is that Sch9 functions in conjunction with the growth factor/RAS/cyclic-AMP/PKA pathway, which inactivates APC function through phosphorylation (Bolte et al., 2003; Kotani et al., 1998; Searle et al., 2004). If Sch9 is involved in the phosphorylative inactivation of the APC, the removal of this inhibitory signal in *sch9*Δ strains could relieve mutant subunit stress, increasing alternate beneficial APC functions, such as Clb2 degradation.

The interaction between *TOR1* and the APC could be two fold; TORC1 could be repressing APC function through Sch9 signaling, while simultaneously activating normal APC function through Cdc5 phosphorylation via Tap42/PP2A activation (Charles et al., 1998; Nakashima et al., 2008; Song and Lee, 2001). Furthermore, disruption of TORC1 function decreases protein synthesis while increasing autophagy, both of which could relieve toxic accumulation of APC targets (Loewith and Hall, 2011). TORC1/Sch9 could also function to increase the abundance of critical APC targets by increasing their expression or activities, speeding up cell cycle/repressing checkpoints, and reducing time for the APC to function. Cells with APC mutations may be unable to function rapidly enough, allowing for the accumulation of stress response repressing targets such as Clb2 (Simpson-Lavy et al., 2009). This could be analogous to AKT activation of SCF^{SKP2} and CDK/G1-cyclin complex formation leading to the inactivation of FoxO/APC^{CDH1} quiescence and stress resistance, a mechanism found to be inappropriately regulated in many cancers in higher eukaryotes (Bashir et al., 2010; Huang et al., 2005; Huang and Tindall, 2011; Lin et al., 2009; Nakayama and Nakayama, 2006). The inactivation of TORC1/Sch9 signaling through sch9Δ may decrease cell growth to a point where defective APC can keep up with the rate of cell cycle progression. Finally, the APC may function in a linear pathway with TORC1. Since TORC1 can be comprised of Tor1 or Tor2 (Loewith et al., 2002), *tor1*Δ cells may only have reduced TORC1 (and Sch9) function, leading to a partial recovery of APC-deficiency phenotypes, while complete disruption of the downstream effector Sch9 leads to full recovery.

These interactions may be able to be teased out more accurately with further testing using rapamycin rather than *tor1*Δ.

7 General Discussion

Data presented in this study expand our knowledge of the *S. cerevisiae* Fox encoding genes *FKH1* and *FKH2*, establishing their role in regulating post-mitotic stress responses and lifespan. Additionally, this regulation acts in conjunction with nutrient signaling (*SCH9/TOR1*), stress signaling (*HOG1/SNF1*) and cell cycle (APC) genes. Specifically, we found:

- The *FKHs* redundantly regulate post-mitotic lifespan
    - Deletion of both *FKH1* and *FKH2* results in shortened CLS (Figure 4.1)
    - Increased expression of the either or both *FKHs* extended CLS (Figure 4.4)
    - SCR fails to extend lifespan in *fkh1*Δ *fkh2*Δ strains (Figure 4.2)
- The *FKHs* regulate stress responses
    - Deletion of the *FKHs* decreases post-mitotic oxidative stress resistance, while increased *FKH* expression increases this resistance (Figure 4.8)
    - Greatly increased *FKH* expression leads to cell cycle arrest and/or apoptosis (Figure 4.9)
- The *FKHs* genetically co-regulate post-mitotic lifespan and oxidative stress resistance with nutrient (*SCH9/TOR1*) and stress (*SNF1 and HOG1*) signaling (Figure 5.8)
    - The nature of the genetic interactions between the *FKHs*, *SCH9* and *TOR1* was condition dependent. Under normal conditions *sch9*Δ and *tor1*Δ phenotypes were dominant to *fkh1*Δ *fkh2*Δ (Figure 5.3), while in stressed conditions *fkh1*Δ *fkh2*Δ phenotype became dominant (Figures 5.4 and 5.5)
    - *SNF1* and the *FKHs* co-regulate CLS (Figure 5.7 A)
    - *HOG1* is required for increased *FKH* expression to increase oxidative stress resistance (Figure 5.7 B)
- The *FKHs* and APC subunits co-regulate post-mitotic survival (Figure 6.9 A and B)
    - The nature of these interactions was condition dependent. Under standard conditions the *FKHs* and APC function together to maintain normal CLS (Figure 6.2), while in stressed conditions they appear to function in primarily in a separate additive manner (Figures 6.1, 6.3 and 6.4)
- The APC and *SCH9* function in a linear genetic mechanism, while the APC and TOR1 appear to function through parallel mechanisms in the regulation of growth, post-mitotic

lifespan and stress resistance in all growth conditions examined (Figures 6.5, 6.6, 6.7, 6.8 and 6.9 C)

Here I discuss possible mechanisms and evolutionary conservation for forkhead box proteins, as well as propose directions for future research.

7.1 Evolutionary role of the Forkhead box transcription factors in lifespan regulation

Metazoan Fox gene function is involved in many developmental defects, as well as the regulation of aging and cancer (Lehmann et al., 2003; Myatt and Lam, 2007). This is likely through Fox proteins serving as transcription regulators of cell cycle and stress responses, such as the FoxO control of G2-M and G1-S cell cycle checkpoints and progression, as well as regulation of stress resistance, DNA repair, and apoptotic processes (Brunet et al., 1999; Dijkers et al., 2002; Kops et al., 2002b; Medema et al., 2000; Nemoto and Finkel, 2002; Tran et al., 2002). The FoxO subfamily contains the best characterized Fox proteins, spurred on by the early identification of their role in longevity regulation (Lin et al., 1997; Ogg et al., 1997). Emerging evidence suggests other Fox proteins regulate similar processes, such as the exclusive vertebrate FoxM subfamily, which is also involved in cell-cycle regulation, aging, and cancer (Korver et al., 1997; Laoukili et al., 2007; Mazet et al., 2003; Pandit et al., 2009; Petrovic et al., 2010; Tang et al., 2008; Wang et al., 2010). In this study and (Postnikoff and Harkness, 2012), we characterized the mitotic regulating yeast FoxM ortholog genes, *FKH1* and *FKH2*, in their ability to regulate post-mitotic longevity, hydrogen peroxide stress resistance and potentially apoptosis, in a manner similar to the FoxOs.

7.1.1 Common regulation

Pro-growth signaling cascades likely lead to increased activation of cell cycle regulators, inducing rapid proliferation in favourable conditions. Selection pressure would favour cells able to outcompete other cells in utilizing resources, and these would propagate specific genetic codes. In the absence of pro-growth signals or the presence of quorum (colony size/culture density) and stress sensing signaling, populations of cells would be selected for the ability to fine tune growth/proliferation and adapt to their ever-changing environment. Key proteins integrate external environmental signals to the intracellular molecular environment. The Fox proteins and

the APC may serve within this cellular capacity for dynamic responses, through controlling gene expression (activation and repression) and specifying protein stability, respectively.

Aging and age related diseases arise from the deregulation of major cellular processes, such as cell cycle control, metabolism, and programmed cell death, leading to tumor formation or tissue damage/destruction, without replacement. The mechanisms leading to the breakdown of cellular processes often involve stress-induced molecular damage, such as from reactive oxygen species. These result from improper repair, removal and replacement of damaged cellular components, and detoxification, as well as normal cell maintenance. The regulation of many of these processes have been found in part to be controlled by the Fox transcription factors. For example, Fox proteins conserved from yeast to mammals regulate cell growth and cell cycle. In stress conditions, the Fkhs and FoxOs induce cycle arrest (Katayama et al., 2008; Shapira et al., 2004; Tran et al., 2002). On the other hand, in pro-growth conditions cell cycle progression is controlled by the Fkhs, FoxM, and FoxOs, in part through the regulation of B type cyclins (Alvarez et al., 2001; Hollenhorst et al., 2000; Laoukili et al., 2005; Pic et al., 2000; Postnikoff and Harkness, 2012; Postnikoff et al., 2012; Tang et al., 2008; Wang et al., 2005; Wittenberg and Reed, 2005). We demonstrated that the yeast Fkhs also protect cells against hydrogen peroxide free radical damage (Postnikoff et al., 2012), a feature associated with FoxO-dependent increased lifespan (Kops et al., 2002a; Murphy et al., 2003; Tettweiler et al., 2005). We also provided preliminary evidence that under certain conditions the Fkhs regulate apoptosis (Section 4.3.5); similarly, the FoxOs conditionally regulate apoptosis (Brunet et al., 1999; Cai and Xia, 2008; Dijkers et al., 2000; Fu and Tindall, 2008; Lehtinen et al., 2006; Luo et al., 2007; Shinoda et al., 2004; Zhang et al., 2011). Furthermore, we identified novel genetic interactions between the *FKHs* and four signaling pathways: TORC1, Sch9, Snf1, and Hog1 (Figures 7.1 and 7.2), orthologous to regulators of metazoan FoxOs (Calnan and Brunet, 2008; Huang and Tindall, 2011; Kloet and Burgering, 2011; Vogt et al., 2005). Finally, we speculate that the yeast Fkhs may function as transcriptional switches integrating extra- and intracellular signals to influence many cellular processes. Besides the roles previously identified and those discussed in this study, the Fkhs may regulate other cellular processes associated with metazoan Fox dependent longevity, such as genomic stability, protein homeostasis, organelle maintenance, and metabolism (Calnan and Brunet, 2008; Greer and Brunet, 2008; Wijchers et al., 2006).

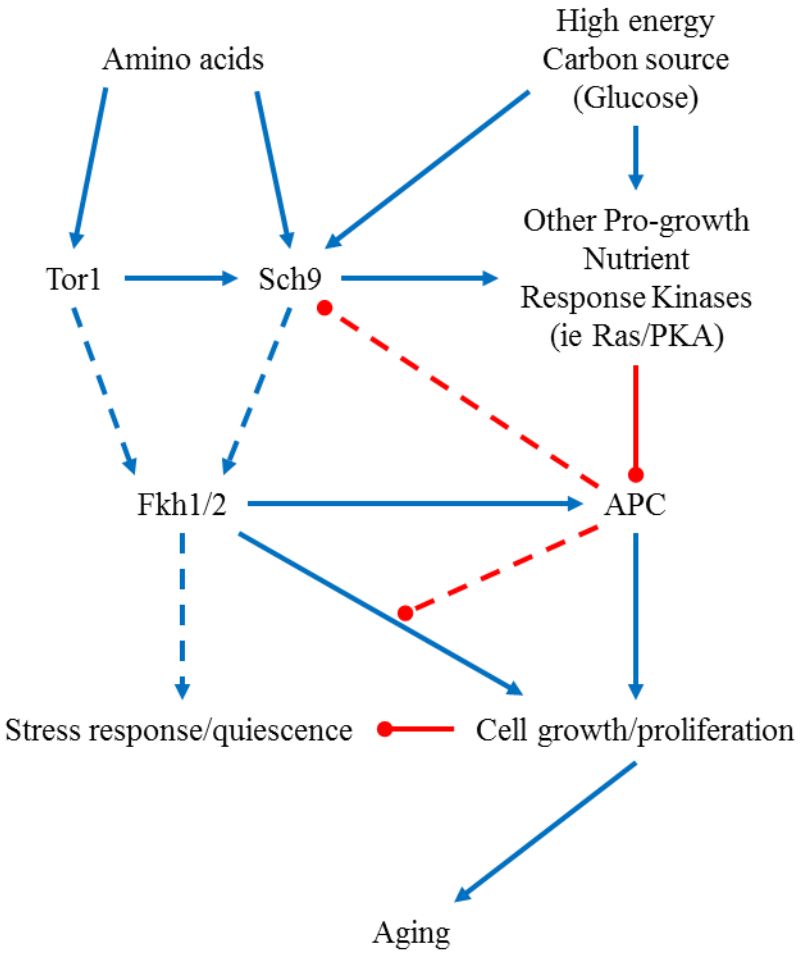

Figure 7.1 Nutrient signaling regulates Fkh and APC function
Under normal growth conditions nutrient signaling may regulate the Fkhs' and APC's function to promote proper cellular growth and cell cycling. This process would likely be in constant flux with stress pathways such as those presented in Figure 7.2; this switch may occur from nutrient depletion or damage and the accumulation of toxins, such as reactive oxygen species, as a by-product of energy consumption. Dashed lines represent genetic interactions suggested by data from this study. Additional data from (Bolte et al., 2003; Castro et al., 2005; Hollenhorst et al., 2000; Kotani et al., 1998; Kumar et al., 2000; Mirisola et al., 2014; Ostrow et al., 2014; Urban et al., 2007; Zhu et al., 2000).

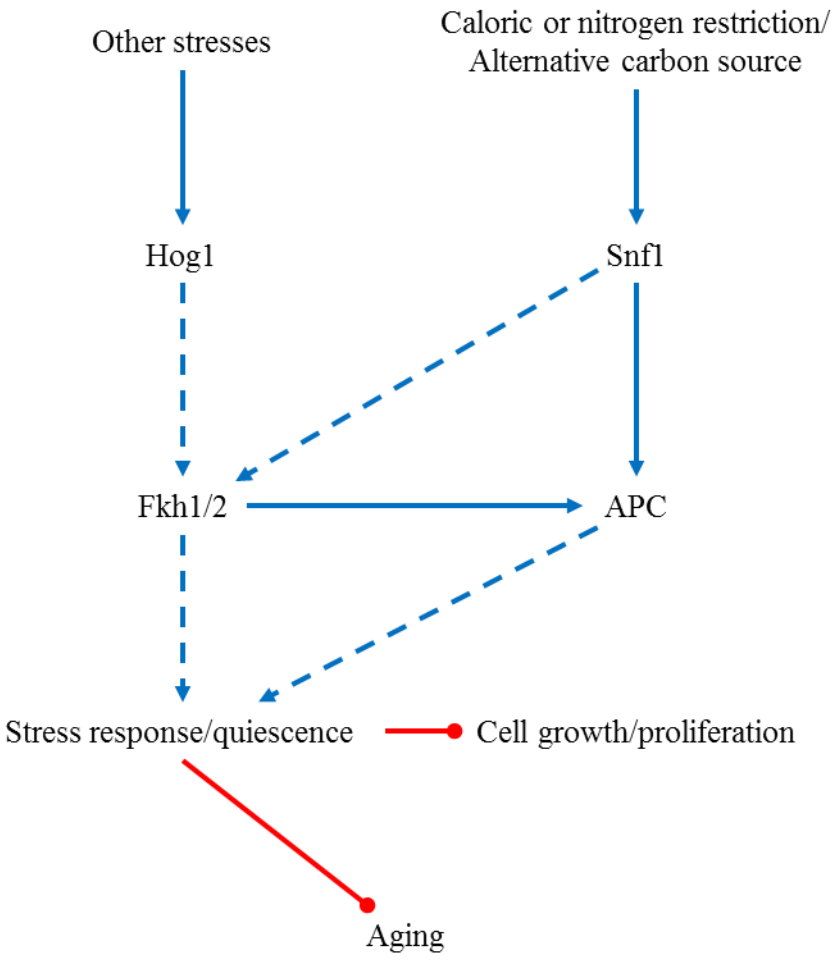

Figure 7.2 Stress signaling regulates Fkh and APC function
Under stressed conditions, Fkh and APC function may be switched to promote cell cycle and growth arrest, as well as stress responses, leading to a delay in aging phenotypes. These processes would likely be involved in detoxification of the cell, repair of damaged cellular components or programmed cell death should the cell be beyond salvation. Dashed lines represent genetic interactions suggested by data from this study. Additional data from (Fabrizio et al., 2004b; Fabrizio et al., 2001; Harkness et al., 2004; Westfall et al., 2004; Wilson et al., 1996; Zhu et al., 2000).

7.1.1.1 Cell cycle and growth regulation

Proper control of the segregation of cellular contents between mother and daughter cells is a fundamental aspect of life. Disruptions to this process can lead to cell death without replacement or over proliferation, both of which decrease the evolutionary fitness of genetically similar cellular populations, which can be observed in the form of dystrophies and cancers in higher eukaryotes. In yeast to humans, Fox proteins function in the feedback control of cell cycle regulation (Figures 1.5 and 1.6). In fact, one of the best characterized functions of the Fkhs is the regulation of the *CLB2* (mitotic progression) and *SIC1* (G1 entry) gene clusters (Zhu et al., 2000). However, here we observed a major role in post-mitotic longevity and stress resistance, which are partly co-regulated with the APC. The ability to maintain genomic integrity in cycling cells could play a role in lifespan extension. Defects in the APC, the Fkhs, and FoxM may lead to improper mitotic progression and chromatin instability (Baker et al., 2004; Castro et al., 2005; Fang et al., 1998; Hollenhorst et al., 2000; Kumar et al., 2000; Laoukili et al., 2005; Searle et al., 2004; Wang et al., 2005; Wonsey and Follettie, 2005; Zhu et al., 2000), potentially accumulating deleterious mutations prior to entry into stationary phase. Another key function of the APC and the Fkhs is G1 entry, where they have a role in the inactivation of mitotic progression proteins. During entry into G1, the Fkh2 co-activator Ndd1 is degraded, likely in an APC-dependent manner, changing Fkh2 to serve in the repression of its bound loci (Loy et al., 1999; Sherriff et al., 2007). Fkh1 functions in a similar mechanism, though with unknown co-factors (Reynolds et al., 2003). Furthermore, the Fkhs bind to the promoters of genes involved in G1 progression and interfere with Swi5 dependent activation of these loci (Voth et al., 2007). APC^{Cdh1} targets mitotic progression proteins such as cyclins, polo-like kinases, and potentially transcriptional activators, including FoxM1 and Ndd1, for proteasomal degradation (Charles et al., 1998; Laoukili et al., 2008; Loy et al., 1999; Park et al., 2008; Simpson-Lavy et al., 2009), resetting the cell cycle. If conditions for growth are unfavourable, the combination of these factors likely is involved in the transition into and out of G0, both of which could affect results of CLS assays.

Evidence suggests a complex co-regulation of stress resistance and lifespan processes by the APC and the Fkhs. This likely involves the regulation of the B-type cyclins; for example, the abnormal presence of Clb2 in S and G2 results in decreased stress resistance (Simpson-Lavy et al., 2009). The mutation of *APC5* or the deletion of both *FKHs* result in partial Clb2 protein

stability in G1 arrested cells (Menzel et al., 2013; Postnikoff et al., 2012). The APC targets Clb2 for proteasomal degradation (Charles et al., 1998; Simpson-Lavy et al., 2009; Thornton and Toczyski, 2003), while the Fkhs are likely involved through the regulation of APC subunits and activators, the direct regulation of the *CLB2* locus, and the indirect regulation of the Clb2 competitive inhibitor Sic1 (Zhu et al., 2000). Furthermore, in backgrounds lacking both *FKH* genes, low level Clb2 expression is observed throughout the cell cycle, as opposed to periodic expression in mitosis and throughout the rest of the cell cycle observed in the presence of either Fkh (Hollenhorst et al., 2000; Kumar et al., 2000; Postnikoff et al., 2012; Reynolds et al., 2003). This suggests the Fkhs are redundantly involved in the regulation of both Clb2 transcriptional repression and degradation.

Another lifespan altering process likely co-regulated through the Fkhs and APC is histone metabolism/chromatin dynamics. Chromatin states and histone over expression increase lifespan (Dang et al., 2009; Feser et al., 2010; Greer et al., 2010; Han and Brunet, 2012). The Histone gene cluster was identified as a transcriptionally regulated target of the Fkhs (Zhu et al., 2000). In congruence, we found that the abundance of the core histones H2B, H3 and H4 is decreased in mutant strains combining *fkh*Δ and $apc5^{CA}$ (Postnikoff et al., 2012), potentially increasing chromatin instability and decreasing lifespan. The *FKH*s may be involved in this process through their effects on expression of the APC and histones, while recruiting chromatin modifying protein localization throughout the genome (Linke et al., 2013; Ostrow et al., 2014; Postnikoff et al., 2012; Sherriff et al., 2007; Venters et al., 2011; Zhu et al., 2000), while the APC may regulate chromatin assembly via interactions with histone modifying Gcn5/SAGA complex (Islam et al., 2011; Turner et al., 2010).

In Chapters 4 and 6 we described a shift in relationships between the *FKHs*, the APC and SCR. In DM conditions, the state of the Fkhs may be in flux between cell cycle activating and repressing complexes, which could lead to inappropriate growth in nutrient poor conditions, cell damage and aging. Mild over expression of the *FKHs* may lead to a stoichiometric shift, with inactive transcription-inhibiting Fkhs outcompeting the modified active Fkh proteins (such as those bound to Ndd1) for DNA binding sites or increased DNA-unbound Fkhs diluting co-activators, such as Ndd1 away from the DNA. Either way this could change the balance of the

DNA-bound Fkhs to a cell cycle repressive/quiescent maintenance role. Similarly, the APC may be involved in the inactivation of pro-cell cycle (and aging) Fkh complexes, targeting both deleterious Fkh co-activators (Ndd1, Clb2, Cdc5, etc.) and targets (such as Clb2, Cdc5) for degradation (Loy et al., 1999; Wittenberg and Reed, 2005).

In SCR conditions we observed a Fkh dependent increase in lifespan regulation and shift in *FKH*/APC genetic interactions. Sir2, a HDAC that may be involved in CR mechanisms, binds to both Fkh1 and Fkh2 at the *CLB2* locus, actively repressing the latter's expression (Linke et al., 2013). In SCR conditions, the Fkhs could preferentially form repressive complexes, potentially with Sir2, over complexes with co-activators, inhibiting partial cell cycle progression in post-diauxic cells. In this case, the APC would have a diminished role in switching off pro-cell cycle/aging Fkh gene expression complexes, but may still regulate lifespan by the degradation (and further repression) of these pro-aging factors, hence the lack of epistasis between $apc5^{CA}$ and *fkh1*Δ *fkh2*Δ phenotypes. Furthermore, in *fkh1*Δ *fkh2*Δ cells, these active repressive complexes may not form, resulting in increased inappropriate background levels of potential anti-quiescent gene expression, such as Clb2; similar to the increased Clb2 levels in cycling *fkh1*Δ *fkh2*Δ cells (Postnikoff et al., 2012). This lack of repressive complex formation could nullify the Fkh-dependent beneficial effects of CR on lifespan extension. Additionally, disruption to the APC in *fkh1*Δ *fkh2*Δ cells would potentially allow for the further accumulation/activation of factors, like Clb2, resulting in a synergistic decrease in lifespan. Specific Fkh co-regulators in these processes remain speculative as Ndd1 is degraded in mitotic exit (Loy et al., 1999), and *sir2*Δ has been found to both increase and decrease CLS (Casatta et al., 2013; Fabrizio et al., 2005; Feser et al., 2010; Orozco et al., 2012, 2013), indicating these may not be the soul regulator involved in switching Fkh function in this manner.

Nutrient signaling likely affects this process in numerous ways. TORC1 may regulate cell cycle in part by co-enhancing general transcription and translation through Sch9 and eIFs, increasing the abundance of cell cycle regulators, as well as increasing cellular components/size, both of which are prerequisites for cell cycling (Kunz et al., 1993; Schmelzle and Hall, 2000; Thomas and Hall, 1997; Toda et al., 1988). The yeast Fox protein, Fhl1, is involved in the coordination of TORC1/Sch9-dependent expression of rDNA and ribosomal protein genes (Martin et al., 2004);

however, some *fhl*Δ phenotypes suggest another mechanism in this regulation (Hall et al., 2006; Rudra et al., 2005). Fkh1 may fulfill this role as it contains also contains the Fox and forkhead associated domains and binds fairly ubiquitously throughout the genome (Venters et al., 2011). Additionally, at least Fkh1 has been found binding upstream of and regulating tRNA encoding genes (Venters et al., 2011), suggesting a further role in the regulation of general translation. TORC1 also regulates proliferation through $PP2A^{Tap42}$, which activates mitotic progression through the activation of the polo-like kinase Cdc5 (Cherkasova and Hinnebusch, 2003; Di Como and Arndt, 1996; Nakashima et al., 2008), while perhaps competitively inhibiting the formation of PP2A-complexes involved in destabilizing CDK-cyclin interactions. These events may lead to the activation of mitosis-regulating function of the Fkhs and APC.

7.1.1.2 Stress responses

Although genetic proliferation and survival is arguably the ultimate goal of the biological machine, this is kept in check by limited resources, as well as ever changing intra and extracellular environmental conditions. Environment signaling pathways interact to fine tune optimal homeostatic conditions for cell proliferation and survival. It is unclear which influences longevity more: increased stress response or decreased growth (Hands et al., 2009); however, both interventions are likely integral to longevity. Malignant and aggressive cancers often arise from a combination of unregulated growth and adaptive stress responses, such as the ability to survive in hypoxic-like environments (Davies et al., 2014). In contrast, extended inhibition of growth and division, combined with excessive stress responses, such as programmed cell death and senescence, can lead to degenerative and inflammatory diseases.

The Fkhs and APC appear to be involved in integrating cellular responses to growth and stress conditions, altering the molecular landscape to accommodate a balance between growth/proliferation and stress responses (Charles et al., 1998; Harkness et al., 2002; Harkness et al., 2004; Hollenhorst et al., 2000; Hollenhorst et al., 2001; Kotani et al., 1998; Pic et al., 2000; Postnikoff and Harkness, 2012; Postnikoff et al., 2012; Qiao et al., 2010; Turnell et al., 2005; Zhu et al., 2000). Utilization of proliferative regulators in stress signaling likely allows for immediate responses to changing conditions, with minimal superfluous noise and wasted resources that separate factors would likely involve. The Fkhs serve as efficient regulators of

many processes due to their presence at the promoters of numerous coding sequences, as well as their association with both transcriptional activators and repressors, such as SAGA, Ndd1, Sir2 and Isw1/2 (Koranda et al., 2000; Linke et al., 2013; Sherriff et al., 2007; Venters et al., 2011).

The *FKHs* genetically interact with stress response kinases *SNF1* and *HOG1* (Figure7.2). Although the nature of these interactions is not clear, Snf1 and Hog1 are involved in regulating starvation, oxidative, and osmotic stresses (Alepuz et al., 1997; Bertram et al., 2002; Cullen and Sprague, 2000; Kuchin et al., 2003; Thompson-Jaeger et al., 1991). The Fkhs bind to the promoter region of both genes (Venters et al., 2011), and FoxM regulates JNK's (Hog1; yeast orthologs in parentheses) expression in metazoans (Wang et al., 2008). Simultaneously, AMPK (Snf1) and p38/JNK (Hog1) phosphorylate the FoxOs, increasing their activity (Cai and Xia, 2008; Greer et al., 2007a; Greer et al., 2007b; Ho et al., 2012; Peserico et al., 2013; Salminen and Kaarniranta, 2012; Sunters et al., 2006). Snf1 also interacts with the APC to regulate yeast longevity (Harkness et al., 2004), strengthening its role in Fkh/APC lifespan regulation. This may be conserved in metazoans, as AMPK phosphorylates the APC (Banko et al., 2011), possibly altering APC function to inhibit cell cycle in energy poor conditions.

Forkhead box transcription factors have been proposed to regulate lifespan in part through their ability to counter cell stress and damage from ROS. In *C. elegans,* DAF-16 mediates this damage through the regulation of anti-oxidants such as SODs, metallothioneins, and heat-shock proteins (Barsyte et al., 2001; Honda and Honda, 1999; Walker et al., 2001). In yeast, orthologous anti-oxidant proteins were found to affect CLS (Longo et al., 1996), while ChIP analysis found Fkh1, and in some cases Fkh2, binds to the manganese superoxide encoding genes, *SOD1* and *SOD2,* as well as genes encoding glutathione-dependent oxidoreductases, the copper metallothionein regulating transcription factor gene *CUP2,* and numerous heat shock factors and proteins (Venters et al., 2011), indicating a potential role for the Fkhs as regulators of their expression. In conjunction with our observations that the Fkhs partially regulate free radical stress resistance, these binding assays support an evolutionarily conserved role of Fox proteins in the regulation of oxygen, metal and free radical toxicity, potentially contributing to cellular protection and lifespan extension.

7.2 Future directions

The major body of this study investigates genetic interactions of yeast forkhead box transcription factors with highly evolutionarily conserved lifespan regulating kinases. Follow up investigations could include all four yeast Fox proteins (Fkh1, Fkh2, Fhl1 and Hcm1) as all have high levels of homology in the forkhead box DNA binding domain. Additionally, Fkh1, Fkh2, and Fhl1 share the conserved forkhead associated protein-protein binding domain, suggesting the potential for redundant regulation and DNA site/protein binding competition between these four Fox proteins. Furthermore, Fhl1 is directly involved in regulation of the expression of ribosomal components through TORC1/Sch9 (Martin et al., 2004; Rudra et al., 2005) and mRNA translation may be a key lifespan regulating process (Hands et al., 2009; Syntichaki et al., 2007a, b). Hcm1 also has a role in Sch9/TORC1/Snf1/Sir2 regulated oxidative stress resistance and cell cycle arrest (Rodriguez-Colman et al., 2010; Rodriguez-Colman et al., 2013). The global nature of Fkh1 binding (Venters et al., 2011) may warrant investigation into potential Fox control of diverse cellular processes such as genomic stability, DNA repair, telomere maintenance, chromatin remodeling, protein homeostasis, organelle maintenance, programmed cell death, cell cycle regulation, stress resistances, and metabolism.

Besides Sch9, Snf1, Hog1, and TORC1, many other known FoxO and lifespan regulators have potential yeast orthologs, such as the sirtuins (Sir2; yeast orthologs in parentheses), the Ras/PKA pathway (Ras1, Ras2, Tpk1-3, and Bcy1), SCF^{SKP2} (SCF^{Cdc4}), PTEN (Tep1), MST1 (Ste20), JNK/MAPK p38 (Ssk2/22), 14-3-3 chaperones (Bmh1/2), and PRMT1 (Hmt1, Hsl7, or Rmt2) that could be investigated for conserved regulation. As well, the role of non-conserved yeast-specific lifespan regulating factors, such as Msn2/4, Gis1, Rim15, and Fob1 could be explored. Since many seemingly complex genetic interactions are implicated, it would of great interest to define the molecular mechanisms of these genetic interactions, as well as linking specific mechanisms to the regulation of cellular outputs. For example, we have identified conditions that implicate the Fkhs in stress resistance, longevity, cell cycle arrest, and apoptosis. To understand the role of the Fkhs in these processes, numerous techniques could be employed. Fkh protein levels and localization could be explored in various conditions via western analysis and fluorescent protein hybrids. Protein complexes involved in these processes could be identified using the Fkhs as bait, followed by pull downs and mass spectrophotometry to identify bound

proteins. This technique could also potentially identify post translational modifications to the Fkhs themselves. If found associated with DNA, ChIP could be utilized to identify direct targets of Fkh regulation, either at a specific level, or genome wide, such as presented in Venters, *et al* (2011). Furthermore, conditional Fkh-dependent transcript level changes (such as in the lifespan extending conditions with the *FKH* over-expression strains) could be analysed through quantitative PCR, RNA sequencing, or microarrays to ascertain potential novel lifespan regulating targets of the Foxs. The data generated could identify and correlate specific Fox regulated cellular functions, to transcriptional targets, bound co-factors, and posttranslational modifications. This would greatly advance our understanding of the roles of Foxs as regulators of lifespan in response to specific biological conditions. Additionally, further investigations are warranted into lifespan regulating targets of the APC, as well as distinguishing the roles of mitotic APC^{Cdc20} and G1 entry APC^{Cdh1} in longevity regulation.

7.3 Conclusions

This study supports a model where *S. cerevisiae FKH1* and *FKH2* function is evolutionarily conserved with higher eukaryotic FoxO proteins with regards to lifespan, oxidative stress resistance, and perhaps apoptosis. Extrapolation suggests predictable cell regulation responsibilities for proteins containing the highly conserved Forkhead box and Forkhead associated protein domains. We show that the *FKHs* are required for increased stress resistance and survival in response to SCR. Importantly, we present data that implicates Fkh and APC dependent lifespan and stress resistance are in the same genetic pathway, and in opposition to parallel nutrient and growth signaling kinase pathways featuring Sch9 and Tor1. Furthermore, we provide evidence that the Fkhs are co-promoting these processes with stress response kinases Hog1 and Snf1. These data indicate that the interaction between nutrition, growth, and stress signaling with Fox proteins and the APC is evolutionarily conserved from yeast to mammals. Although further investigation is warranted to tease out the cellular and biochemical mechanisms in yeast, our findings support the utilization of yeast as a model to understand the fundamental mechanisms, regulators, binding partners, and targets of Fox proteins, with respect to longevity and age related diseases.

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