MAMMALIAN HCF-1 IS A CONSERVED CO-REGULATOR OF THE LONGEVITY-PROMOTING FOXO TRANSCRIPTION FACTORS, AND IS REQUIRED FOR PANCREATIC BETA CELL FUNCTION

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MAMMALIAN HCF-1 IS A CONSERVED CO-REGULATOR OF THE LONGEVITY-PROMOTING FOXO TRANSCRIPTION FACTORS, AND IS REQUIRED FOR PANCREATIC BETA CELL FUNCTION

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As the world's elderly population grows, and the incidence of age-related diseases increases, knowledge of the factors that affect longevity will be essential to develop therapies that counter the effects of aging. Aging is a highly complex phenomenon, but many genetic and environmental lifespan determinants have been characterized with the use of model organisms. Importantly, many of these longevity determinants function in human lifespan modulation. In the nematode *C. elegans*, the transcriptional regulator HCF-1 represses the insulin-responsive transcription factor DAF-16 to affect aging and stress responses. Whether mammalian HCF-1 homologs function in a conserved manner to regulate the mammalian DAF-16 homologs, known as FoxO transcription factors, has not yet been determined.

My studies indicate that HCF-1 proteins interact with FoxO transcription factors, and regulate the transcriptional targets of FoxO in mammalian cells. Furthermore, while the DAF-16 co-regulator SIR-2.1 acts upstream of *C. elegans* HCF-1 to modulate lifespan, the mammalian homolog of SIR-2.1, SIRT1, targets mammalian HCF-1 for deacetylation. These findings highlight the well-conserved nature of the interaction between FoxO transcription factors and the FoxO co-

regulators HCF-1 and SIRT1, and implicate HCF-1 as a novel longevity determinant in mammals.

FoxO transcription factors are critical regulators of multiple physiological processes, including the maintenance of glucose homeostasis. In the pancreas, FoxO1 regulates β-cell function through repression of the transcription factor Pdx1. My analyses indicate that HCF-1 is required for survival, proliferation and glucose-stimulated insulin secretion in the INS-1 β-cell line. Inactivation of HCF-1 leads to reduced *Pdx1* gene transcription and resultant decreases in insulin gene transcription and cellular insulin content. However, while HCF-1 and FoxO1 physically interact in β-cells, FoxO1 localization to the *Pdx1* promoter is not increased by HCF-1 depletion, suggesting that HCF-1 promotes transcription of *Pdx1* through a FoxO1-independent mechanism. Rather, I find that HCF-1 associates with the transcription factor E2F1, which is also known to affect *Pdx1* expression. Both HCF-1 and E2F1 localize to the promoter of the *Pdx1* gene, suggesting that HCF-1, in association with E2F1, directly promotes *Pdx1* gene expression. Thus, my results implicate HCF-1 as a critical modulator of both mammalian glucose homoestasis and longevity.

BIOGRAPHICAL SKETCH

Terri Iwata grew up in the rural town of Wahiawa, Hawaii where her earliest education came at the hands of her older sister and two older brothers. Indeed, she has her sister to thank for acquiring early skills in mathematics and reading, and her brothers to thank for instilling in her a strong work ethic. While she did not find many opportunities to leave her small town, her voracious reading exposed her to places she yearned to experience herself someday. With the encouragement of her teachers, who recognized and nurtured her love of learning, she set her sights on leaving home and experiencing the world beyond Hawaii's shores. She finally got her chance when she was accepted to Stanford University, and entered a world that felt especially made for her, a place where curiosity could be shared and indulged, and where she filled her head with new ideas, and her heart with lasting friendships. During her junior year, Terri had the exciting opportunity of studying abroad with Stanford's Overseas Studies Program in Kyoto, Japan. During that time she completed an internship at the Kyoto University Primate Research Institute, where she was first exposed to, and inspired by, the practice of lab animal medicine. Upon returning to the United States, Terri decided to pursue a career as a lab animal veterinarian. In 2004, Terri graduated from Stanford University with a BS in Biological Sciences, with Distinction, and a Minor in Political Science, and was also honored to be elected to Phi Beta Kappa. After working in a small animal clinic in Hawaii for one year, Terri returned to the Mainland to begin her veterinary studies at Cornell University. During her first summer as a vet student, Terri decided to gain

valuable experience in basic science research by participating in the Veterinary Investigator Program, where she worked in the lab of Dr. Mark Roberson. Under the guidance of graduate students Li Han, Patty Clark and Dr. Stuart Bliss, Terri quickly became immersed in the world of reproductive endocrinology, and discovered her love of conducting experiments and making novel discoveries. With the encouragement of Dr. Roberson, Terri applied, and was accepted, into the Dual DVM/PhD Degree program at Cornell. She chose to do her research in the lab of Dr. Siu Sylvia Lee, studying mammalian factors implicated in lifespan determination. Upon completion of her PhD, Terri will continue her veterinary studies by starting her clinical rotations at the Cornell University Hospital for Animals. She expects to complete her Doctor of Veterinary Medicine degree in 2013, after eight long (and cold) years in Ithaca, NY. She then hopes to enter a lab animal medicine residency program and continue her journey to becoming a lab animal veterinarian and independent research scientist.

To my grandfather, Kinji Mihashi,

And to my best friend, Steve

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I thank my friends and family who have helped me persevere with their love and encouragement. Thank you to Deanna Rodriguez, Annie Wang, Annie Li and Futaba Elkins for their moral support. Thank you, also, to Jennifer Elliot, for being my constant friend. I am fortunate to have a large and loving family in Hawaii, who support my every decision, including: my parents, Motoko and Melvin; my siblings, Cindy, Tad and Burt; their spouses, Gordon, Noriko and Monika; and my nephews, Akira, Casey and Nathan. I am equally fortunate to have a kind and caring family here on the Mainland, with my in-laws, Helen and Richard, and Karen, Matt and Caleb.

Finally, I would like to thank my husband and best friend, Steve, for the incredible love and support he has given me. I try my best to deserve it.

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LIST OF ABBREVIATIONS

AcK	acetyl-lysine
AKT	v-akt murine thymoma viral oncogene
AMPK	5'-adenosine monophosphate-activated protein kinase
C/EBP	CCAAT/enhancer binding protein
ChIP	Chromatin immunoprecipitation
DAF-2	Abnormal dauer formation 2
DAF-16	Abnormal dauer formation 16
DBE	DAF-16 binding element
Dox	Doxycycline
DNA	Deoxyribonucleic acid
ESC	Embryonic stem cell
Fn3	Fibronectin repeat type 3
FoxO	Forkhead box, Class O, transcription factor
Gadd45a	Growth arrest and DNA damage 45a
GFP	Green fluorescent protein
H3K4	Histone 3 lysine 4
HA	hemagglutinin
IIS	Insulin/IGF-1 signaling
IRS-2	Insulin receptor substrate 2
HCF-1	Host cell factor 1
HCF-2	Host cell factor 2
Hr	Hour
IGFBP1	Insulin-like growth factor binding protein 1
IGF-1	Insulin-like growth factor 1

IP	Immunoprecipitation
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MLL	Mixed lineage leukemia
NAD	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
OGT	*O*-linked-β-*N*-acetylglucosaminyl transferase
PDK1	3-phosphoinositide-dependent protein kinase 1
Pdx1	Pancreatic duodenal homeobox 1
PGC-1α	Peroxisome proliferator-activated receptor-γ co-activator 1alpha
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PPARγ	Peroxisome proliferator-activated receptor gamma
RNAi	RNA interference
TCF	T-cell factor
shRNA	short hairpin RNA
siRNA	small interfering RNA
SIR-2.1	Silent information regulator 2.1
VP-16	Virion protein 1

CHAPTER 1

INTRODUCTION

The Centers for Disease Control and Prevention estimates that by 2030, the number of U.S. adults aged 65 or older will more than double to over 70 million individuals. Aging is a major risk factor for the development of many diseases, including osteoporosis, cancer, cardiovascular disease, and neurodegenerative disorders. Thus, identifying and characterizing the factors leading to functional decline with age is a major priority in developing therapies that can prevent or delay the onset of disease. Aging is recognized to be a highly complex process, involving interactions between an organism's genetic background and environment. Therefore, to facilitate the identification of factors involved in the determination of lifespan and healthspan, many of the studies aimed at elucidating the biology of aging have utilized the genetic model organism *Caenorhabditis elegans*. As a result of these efforts, numerous genetic factors that influence the aging process have been discovered, and importantly, many of these genes have subsequently been demonstrated to affect aging in mammals. The example of the insulin/IGF-1 signaling (IIS) pathway serves to highlight the enormous contribution made by research first initiated by investigating aging in *C. elegans*.

In *C. elegans*, mutations that disrupt the IIS pathway extend lifespan up to twice that of wildtype animals (Kenyon et al., 1993). As a consequence of reduced insulin signaling, the forkhead box, class O (FoxO) transcription factor DAF-16 is activated, thereby allowing for the expression of a multitude of lifespan extending

genes. Subsequent studies revealed that IIS plays a similar role in determining the lifespan of both flies and mice (Tatar et al., 2001; Bluher et al., 2003; Holzenberger et al., 2003). These findings lent support to the idea that, due to the evolutionarily conserved nature of longevity-affecting genes, studies in relatively simple organisms like *C. elegans* could provide valuable insight into the aging process of humans. As further evidence in support of this premise, recent studies have shown significant association between longevity and polymorphisms within genes encoding the human insulin receptor (Kojima et al., 2004), the IGF-1 receptor (Suh et al., 2008), the protein kinase AKT (Pawlikowska et al., 2009), and both FoxO1 and FoxO3 (Willcox et al., 2008; Anselmi et al., 2009; Flachsbart et al., 2009; Li et al., 2009; Pawlikowska et al., 2009).

The field of aging has experienced incredible growth with the identification of many novel factors involved in *C. elegans* lifespan determination. The transcriptional co-regulator Host Cell Factor 1 (HCF-1) was identified as a longevity determinant, and novel co-repressor of DAF-16, the major longevity-promoting factor downstream of the IIS pathway (Li et al., 2008a). HCF-1, like DAF-16/FoxO, is evolutionarily conserved between worms and humans, suggesting that HCF-1 represents a novel human longevity factor as well. Better understanding the function and regulation of HCF-1 in mammals will thus provide a valuable first step in elucidating the specific roles putative longevity factors play in complex organisms, and will shed light on the complex interactions between various factors that control the aging process.

1.1 Host Cell Factor 1

Since the discovery of FoxO transcription factors as key longevity modulators, a multitude of studies have sought to describe in fine detail the regulation of these transcription factors. Investigations over the past decade have revealed an elegant network of interactions governing the activity of FoxO proteins. The relationship between multiple extracellular signals, intracellular components and nuclear factors provide inputs that modulate FoxO transcriptional activity in a highly specific manner (Calnan and Brunet, 2008; Landis and Murphy, 2010; Daitoku et al., 2011). Of the nuclear co-factors of DAF-16 in *C. elegans*, HCF-1 represents the first nuclear co-repressor of DAF-16 to be identified.

C. elegans HCF-1 was first identified in a genome-wide RNAi screen as a novel longevity determinant (Hamilton et al., 2005). Knockdown or mutation of *hcf-1* leads to robust and significant lifespan extension as well as increased oxidative and heavy-metal stress resistance, these phenotypes being dependent on *daf-16* (Li et al., 2008a). HCF-1 associates with DAF-16 in the nucleus and in doing so, prevents DAF-16 localization to a subset of its target genes' promoters (Li et al., 2008a). Further investigation revealed that *C. elegans hcf-1* acts downstream of the *C. elegans* sirtuin *sir-2.1* to affect both stress resistance and lifespan (Rizki et al., 2011). HCF-1 and SIR-2.1 physically associate and share in the regulation of a large number of DAF-16 target genes important for aging and stress response (Rizki et al., 2011).

Notably, HCF-1, like FoxO and insulin/IGF1 signaling, is highly conserved across species. In mammals, two homologs of *C. elegans* HCF-1 are present, HCF-1

and HCF-2. While mammalian HCF-1's role as a transcriptional regulator has been characterized, no clear function of mammalian HCF-2 has been determined.

Human HCF-1 was first identified as a cellular protein which interacts with Oct-1 and the herpes simplex virus protein VP16 to form a transcriptional complex responsible for viral immediate early gene activation (Gerster and Roeder, 1988; Kristie and Sharp, 1993). Subsequent studies showed that HCF-1 is required for multiple aspects of cell cycle progression (Goto et al., 1997; Wilson et al., 1997; Julien and Herr, 2003, 2004), DNA damage induced apoptosis (Tyagi and Herr, 2009) and embryonic stem cell (ESC) pluripotency (Dejosez et al., 2008; Dejosez et al., 2010). HCF-1 functions as a transcriptional regulator for a diverse array of transcription factors (Luciano and Wilson, 2000; Piluso et al., 2002; Luciano and Wilson, 2003; Tyagi et al., 2007; Vercauteren et al., 2008; Mazars et al., 2010). In addition, HCF-1 influences gene expression by recruiting chromatin remodeling factors, such as the Sin3 histone deacetylase and the Set1/MLL1 histone methyltransferases (Wysocka et al., 2003; Yokoyama et al., 2004) to target gene promoters. These two functions of HCF-1, co-regulation of transcription factors and recruitment of chromatin modifiers, are integrated and play a critical role in major cellular processes. During the G1-S phase transition of the cell cycle, HCF-1 associates with E2F1 and recruits the histone methyltransferases MLL and Set1 to E2F1 target gene promoters, thereby promoting H3K4 trimethylation and activation of gene expression at these promoters (Tyagi et al., 2007). However, during the early G1 phase of the cell cycle, HCF-1 associates with the repressive E2F4 transcription factor and recruits the histone deacetylase Sin3a, causing repression of E2F4-target genes

(Tyagi et al., 2007). Similarly, HCF-1 associates with E2F1 and promotes E2F1 target gene activation after DNA damage by recruiting the MLL/Set1 histone methyltransferase complexes to the promoters of E2F1 target genes (Tyagi and Herr, 2009). Thus, HCF-1 regulates cellular functions by selectively associating with specific transcription factors and chromatin modifiers under particular cellular contexts.

To further illustrate HCF-1's role as a transcriptional co-regulator, in pluripotent embryonic stem cells, HCF-1 binds the zinc-finger transcriptional regulator Ronin to coordinate regulation of genes important for maintaining embryonic stem cell (ESC) pluripotency and self-renewal capacity (Dejosez et al., 2008; Dejosez et al., 2010). Overexpression of Ronin allows ESCs to continue to self-renew in an undifferentiated state under conditions that normally promote ESC differentiation (Dejosez et al., 2008; Dejosez et al., 2010). However, this phenotype is abolished when Ronin is no longer capable of binding HCF-1, indicating that HCF-1 is essential for Ronin's ability to maintain ESC pluripotency (Dejosez et al., 2010). Consistent with this observation, HCF-1 and Ronin occupy the promoters of many shared genes, including genes involved in transcription initiation, mTOR signaling and oxidative phosphorylation (Dejosez et al., 2010). Precisely how Ronin and HCF-1 affect gene expression in ESCs is still unclear.

C. elegans HCF-1, mammalian HCF-1 and mammalian HCF-2 share structural homology in that all contain two highly conserved protein interaction domains common to all HCF family members: the N-terminal Kelch domain and the C-terminal fibronectin 3 repeat domain (Fn3) (Figure 1.1) (Liu et al., 1999; Lee and Herr, 2001).

However, in addition to these two domains, mammalian HCF-1 contains unique structural motifs, including an N-terminal basic region, a centrally-located repeat region, and a C-terminal acidic region (Figure 1.1). The repeat region contains proteolytic cleavage sites necessary for the processing of full length HCF-1 into corresponding N-terminal and C-terminal polypeptides (Kristie and Sharp, 1993; Wilson et al., 1995). These N-terminal and C-terminal fragments associate via non-covalent interactions (Wilson et al., 2000). Interestingly, these separate fragments of HCF-1 have unique functions with respect to regulating the cell cycle. The N-terminal subunit of HCF-1 is required for G1-S phase progression, while the C-terminal fragment of HCF-1 is involved in M-phase progression (Goto et al., 1997; Julien and Herr, 2003, 2004). Furthermore, the full length non-cleaved HCF-1 protein possesses functions distinct from those of the HCF-1 fragments (Vogel and Kristie, 2006). The mechanism regulating the proteolysis of HCF-1 was recently discovered to be dependent on the *O*-linked-β-*N*-acetylglucosaminyl transferase (OGT) protein which modifies the HCF-1 N-terminal subunit and directly cleaves the full length HCF-1 protein at its proteolytic cleavage sites (Capotosti et al., 2011; Daou et al., 2011). Besides *O*-GlcNAcylation, little is known about the post-translational modifications of HCF-1. *C. elegans* HCF-1 undergoes phosphorylation (Wysocka et al., 2001) and mammalian HCF-1 contains acetylated lysine residues (Choudhary et al., 2009). However, the proteins that mediate these post-translational modifications and what their consequences may be still remains unknown.

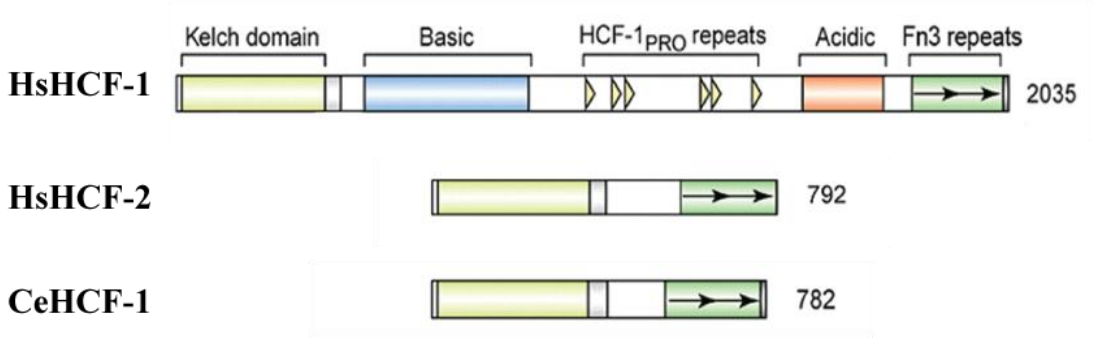


Figure 1.1 Conservation of HCF-1 protein family members

Mammalian HCF-1 (HsHCF-1) and HCF-2 (HsHCF-2), and *C. elegans* HCF-1 (CeHCF-1) share conserved Kelch and Fibronectin 3 (Fn3) repeat domains. The mammalian HCF-1 protein contains unique motifs, including a central proteolytic processing domain ($HCF\text{-}1_{PRO}$) which is cleaved to generate multiple independent N-terminal and C-terminal polypeptides which then associate non-covalently. Figure modified from Wysocka and Herr, 2003.

Despite the striking structural differences between mammalian and *C. elegans* HCF-1, and the overall structural similarity between mammalian HCF-2 and *C. elegans* HCF-1 **(**Figure 1.1**),** *C. elegans* HCF-1 exhibits stronger functional homology to human HCF-1 than to human HCF-2. While human HCF-1 and HCF-2, along with *C. elegans* HCF-1 are able to stabilize the VP16 complex, only human HCF-1 and *C. elegans* HCF-1 are able to promote VP-16 transcriptional activity (Liu et al., 1999; Lee and Herr, 2001). Furthermore, loss of *hcf-1* mimics the cell proliferation and histone phosphorylation defects found in mammalian cells lacking functional HCF-1 (Lee et al., 2007). These observations suggest that mammalian HCF-1 may share with *C. elegans* HCF-1 a functional role in determining lifespan as well.

1.2 Forkhead box, Class O (FoxO) transcription factors

The forkhead box, class O (FoxO) transcription factors play critical roles in longevity, metabolism, stress response, cell proliferation and apoptosis across species (Arden, 2008). FoxO family members are highly conserved, from *C. elegans* to mammals, and are characterized by a conserved DNA-binding domain which recognizes a consensus binding sequence known as the DAF-16 Binding Element (Furuyama et al., 2000). *C. elegans* and *Drosophila* contain a single FoxO gene, *daf-16* and *dFoxO*, respectively. In contrast, mammals have four FoxO family members, *FoxO1, FoxO3, FoxO4* and *FoxO6*. As mediators of extensive cellular and physiological processes, FoxO proteins receive inputs from a variety of environmental signals and are subjected to a high degree of regulatory control. In turn, FoxO

proteins activate or repress a broad range of genes to achieve the appropriate cellular response to environmental conditions.

1.2.1 Conserved regulation of FoxO transcription factors

A number of extracellular signals converge upon the FoxO transcription factors to modulate their activity. The insulin/IGF-1 signaling (IIS) cascade represents one of the major inputs by which FoxO factors are regulated (Figure 1.2). Genetic studies using *C. elegans* provided early evidence that DAF-16/FoxO factors are key downstream effectors of IIS (Lin et al., 1997; Ogg et al., 1997). Upon binding of insulin/IGF-1 peptide ligands, the insulin/IGF-1 receptor is activated, autophosphorylated and phosphorylates cytoplasmic target proteins (Kloet and Burgering, 2011). Phosphorylation of the insulin receptor substrate (IRS) proteins by the insulin receptor activates the highly conserved phosphoinositide 3-kinase (PI3K) cascade. PI3K activation produces 3'phosphorylated phosphoinositides, which serve, in turn, to activate the protein kinase AKT/PKB, via phosphorylation by phosphoinositide-dependent protein kinase 1 (PDK1). Activated AKT/PKB then phosphorylates FoxO. Phosphorylation of FoxO prevents its nuclear translocation, thus rendering it inactive, whereas reduction in insulin signaling results in dephosphorylation of FoxO and activation of its transcriptional activity. The IIS pathway is an extremely well-conserved mechanism of FoxO regulation, functioning in *C. elegans* and *Drosophila* as well as in mammals (Figure 1.2).

Besides insulin/IGF-1 signaling, FoxO activity is regulated via phosphorylation by additional highly conserved kinases. The stress-activated Jun-N-

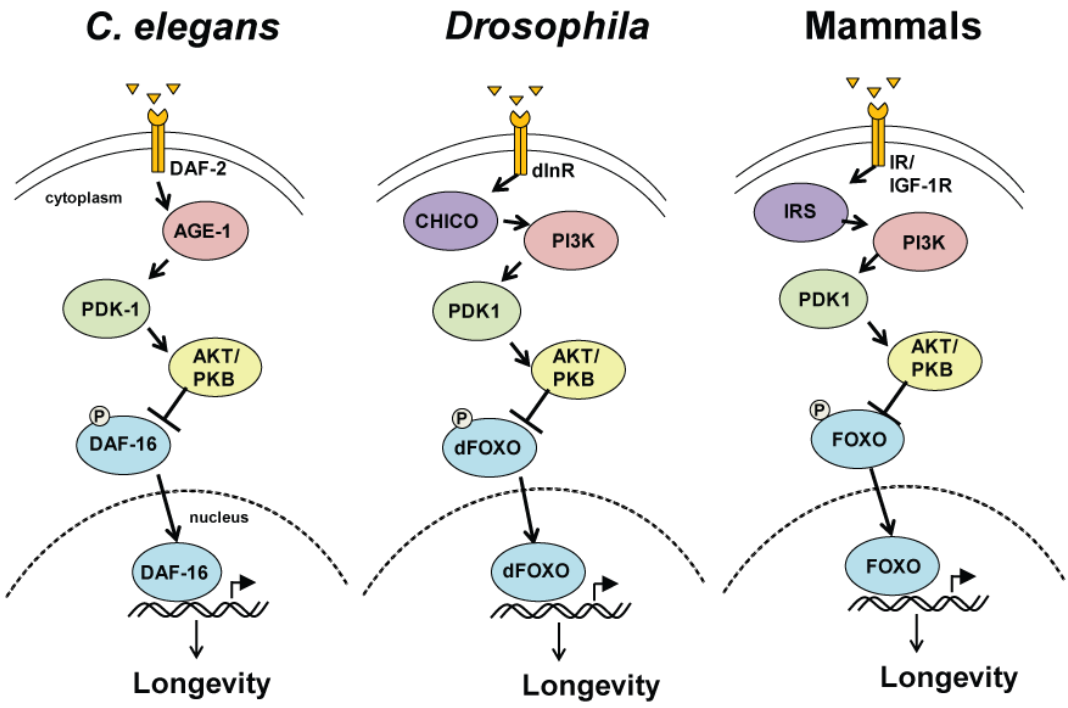


Figure 1.2 The conserved insulin/IGF-1 signaling pathway affects longevity.

The insulin/IGF-1 signaling pathway affects lifespan of diverse organisms. In *C. elegans*, inactivation of *daf-2*, *age-1*, *pdk-1*, or *akt-1/akt-2* results in lifespan extension that is dependent on *daf-16*. In *Drosophila*, mutation of the insulin receptor (dInR), or of the insulin receptor substrate, known as Chico, extends lifespan, while overexpression of dFOXO extends lifespan. And in mice, mutation of the insulin receptor (IR), IGF-1 receptor (IGF-1R), or insulin receptor substrate (IRS) extends lifespan.

terminal kinase (JNK) phosphorylates FoxO and promotes FoxO nuclear translocation in *C. elegans, Drosophila* and mammalian cells (Essers et al., 2004; Oh et al., 2005; Wang et al., 2005). In *Drosophila* and *C. elegans*, this activation of FoxO by JNK promotes lifespan extension (Oh et al., 2005; Wang et al., 2005). FoxO factors are also modulated by the conserved MST1 protein kinases. Mammalian MST-1 phosphorylates FoxO in response to oxidative stress, and thereby promotes FoxO nuclear translocation and cell death, while in *C. elegans,* the MST1 ortholog CST-1 promotes longevity through activation of DAF-16/FoxO (Lehtinen et al., 2006). Finally, the energy responsive AMP-activated protein kinase (AMPK) phosphorylates DAF-16/FoxO, in both *C. elegans* and mammalian cells (Greer et al., 2007a; Greer et al., 2007b). In mammalian cells, phosphorylation of FoxO by AMPK in response to nutrient deprivation activates FoxO transcriptional activity (Greer et al., 2007b), while in *C. elegans*, one method of lifespan-extension mediated by dietary restriction is dependent on AMPK activation of DAF-16/FoxO (Greer et al., 2007a).

Conserved regulation of FoxO transcription factors extends to nuclear FoxO co-factors as well. β-catenin acts as a transcriptional co-activator downstream of the Wnt signaling pathway to regulate T-cell factor (TCF) mediated transcription. In *C. elegans*, the β-catenin homolog *bar-1* physically associates with DAF-16 and promotes activation of DAF-16/FoxO target genes (Essers et al., 2005), while in mammalian cells, β-catenin binds to FoxO and enhances FoxO transcriptional activity (Essers et al., 2005; Hoogeboom et al., 2008). Finally, the conserved sirtuin family of protein deacetylases/ADP-ribosyltransferases represents nuclear co-factors of DAF-

16/FoxO which modulate DAF-16/FoxO in multiple species in a highly coordinated fashion, and will be described in greater detail in Chapter 1.2.3.

1.2.2 Cellular and tissue-specific functions of mammalian FoxO proteins

Given the central and highly conserved role that FoxO proteins play in the aging process, many studies have attempted to clarify the specific celluar and tissue-specific functions of mammalian FoxOs. Of the four mammalian FoxO proteins, FoxO1, FoxO3 and FoxO4 are similarly regulated by AKT-mediated phosphorylation and nucleo-cytosolic shuttling downstream of insulin/IGF-1 signaling (Brunet et al., 1999; Guo et al., 1999; Kops et al., 1999; Nakae et al., 1999; Rena et al., 1999). In contrast, FoxO6 is expressed predominantly in the nucleus, although it remains sensitive to the effects of AKT-mediated transcriptional repression (Jacobs et al., 2003; van der Heide et al., 2005). Due to the highly conserved nature of their DNA-binding domain, all FoxO proteins exhibit similar binding specificity to their consensus DAF-16 binding element, or DBE, consisting of the sequence TTGTTTAC (Furuyama et al., 2000) . In addition, mammalian FoxO proteins also recognize the insulin responsive sequence, consisting of the sequence TT(G/A)TTTC (Brunet et al., 1999). The shared DNA binding sequence of FoxO proteins allows these transcription factors to share in regulating their target genes, although some specificity of function is believed to be conferred by their somewhat varied expression patterns (Salih and Brunet, 2008).

As previously discussed, FoxO transcription factors receive regulatory input from multiple signaling pathways, and thus, are able to modulate cellular responses to

varying environmental conditions. FoxO target genes are involved in regulating many critical cellular processes (Figure 1.3). Activation of FoxO transcription factors, for instance due to reduced growth factor signaling or increased cellular stress conditions, leads to FoxO-mediated upregulation of genes which lead to cell cycle arrest (Ho et al., 2008). FoxO proteins upregulate expression of the genes encoding the cyclin-dependent kinase inhibitors, *p21* and *p27,* and the retinoblastoma protein family member, *p130,* thus promoting G1/S phase arrest (Ho et al., 2008). FoxO factors also upregulate the growth and DNA-damage response gene *Gadd45a,* and the cyclin gene c*yclin G2,* to promote G2/M phase cell cycle arrest (Ho et al., 2008). FoxO transcription factor activation can also lead to apoptosis. FoxO proteins upregulate the transcriptional expression of multiple pro-apoptotic Bcl-2 family members, such as the pro-apoptotic members *Bim* and *Puma* (van der Vos and Coffer, 2011). The FoxO transcriptional target genes *FasL* and *TRAIL* also promote apoptosis by encoding ligands of cellular death receptors (van der Vos and Coffer, 2011). As a result of their effects on cell cycle arrest and apoptosis, FoxO transcription factors play key roles in tumor suppression (Zhang et al., 2011a). FoxO transcription factors are also central actors in regulating energy homeostasis. In response to changes in nutrient availability, FoxO transcription factors regulate expression of genes involved in altering metabolism. For example, FoxO promotes expression of the *phosphoenolpyrvate carboxykinase* and *glucose-6 phophatase* genes to promote hepatic gluconeogenesis, and the *IGFBP1* gene to regulate IGF-1 bioavailability and insulin sensitivity (Gross et al., 2008). This brief introduction highlights just a handful of the FoxO transcriptional targets identified, and serves to illustrate the key finding

that FoxO transcription factors act as master regulators of a multitude of cellular processes, including, but not limited to, cellular proliferation, apoptosis, metabolism, autophagy, stress resistance and cell differentiation (van der Vos and Coffer, 2011).

While many FoxO target genes have been identified through cell culture studies, mouse models have corroborated the critical role that FoxO factors play in regulating cellular processes in multiple tissue types. Evidence from mouse models in which FoxO function has been perturbed supports the idea that FoxOs have both redundant and non-redundant functions *in vivo*. *FoxO1* was the first of the mammalian FoxOs to be knocked out in mice (Nakae et al., 2002). *FoxO1* null embryos die *in utero* at embryonic day 11 due to cardiovascular developmental defects (Furuyama et al., 2004; Hosaka et al., 2004). In contrast, *FoxO3* and *FoxO4* homozygous knockout mice are viable. *FoxO3* null mice, while viable, are characterized by female age-dependent infertility resulting from premature ovarian follicle depletion (Castrillon et al., 2003; Hosaka et al., 2004). *FoxO4* knockout mice are phenotypically normal (Hosaka et al., 2004). These results indicate that FoxO1 plays a unique role in the development of the cardiovascular system (Hosaka et al., 2004). Interestingly, the first study looking at mice with conditional triple *FoxO1*, *FoxO3,* and *FoxO4* knocked out in adulthood showed that these mice exhibit age-dependent overproliferation of endothelial cells resulting in the development of hemangiomas, and ultimately death (Paik et al., 2007), indicating that FoxOs do share redundant functions as this phenotype was not seen in single FoxO mutants. With the development of additional tissue-specific single, double and triple *FoxO1*, *FoxO3*, and *FoxO4* knockout mice, we have discovered much about the redundancy of FoxO

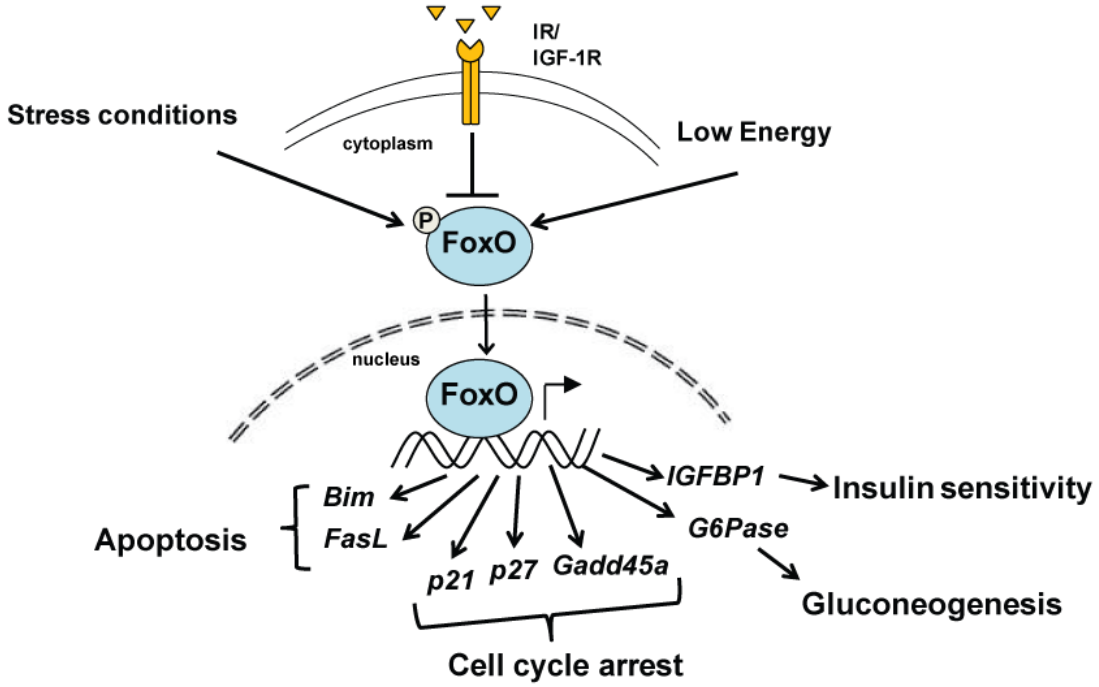


Figure 1.3 FoxO factors modulate expression of genes involved in diverse cellular processes.

Mammalian FoxO transcription factors are regulated by multiple extracellular signals. When activated, FoxO proteins modulate the expression of genes involved in diverse cellular processes, allowing cells to respond to varying environmental stimuli.

factors *in vivo*. FoxO1, FoxO3 and FoxO4 are all critically involved in maintaining the hematopoietic stem cell and neural stem cell pools (Miyamoto et al., 2007; Tothova et al., 2007; Paik et al., 2009), in regulating hepatic lipid metabolism and glucose production (Tao et al., 2011; Zhang et al., 2012), and in promoting cardiomyocyte survival in response to oxidative stress (Sengupta et al., 2011). Similarly, both FoxO1 and FoxO3 function in the immune system by inhibiting T-cell hyperactivation and promoting regulatory T-cell development (Lin et al., 2004; Kerdiles et al., 2010), and regulating B-cell proliferation and differentiation (Dengler et al., 2008; Hinman et al., 2009).

1.2.2.1 The role of FoxO in the regulation of metabolism

As downstream effectors of insulin-signaling, FoxOs play critical roles in regulating whole-body energy homeostasis. Of the mammalian FoxO proteins, FoxO1 has been most widely studied for its role in affecting metabolism. In addition to the mentioned role of FoxO1 in promoting hepatic glucose production and lipid metabolism, FoxO1 is also involved in regulating skeletal muscle, adipose tissue and pancreatic β-cell function. In skeletal muscle, FoxO1 is responsible for inhibiting skeletal muscle differentiation, promoting skeletal muscle atrophy, and mediating the switch between carbohydrate and fatty acid oxidation under hypoglycemic conditions (Furuyama et al., 2003; Kamei et al., 2004; Sandri et al., 2004; Bastie et al., 2005).

FoxO1 also plays a role in inhibiting adipocyte differentiation (Nakae et al., 2003). Preadipocytes subjected to specific adipogenic stimuli are committed to undergo differentiation into mature adipocytes (Cristancho and Lazar, 2011). The

differentiation program is induced by a transcriptional cascade that promotes the expression of genes associated with mature, functional adipocytes. Of the many transcription factors involved in adipogenesis, peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein (C/EBP) –α, -β, and –δ are the principle factors driving adipogenesis (Cristancho and Lazar, 2011). C/EBPβ and C/EBPδ expression is increased early after adipogenic stimulation, and they in turn transactivate the *PPARγ* and C/*EBPα* genes. PPARγ and C/EBPα auto- and trans-regulate their own expression, further upregulating the transcription of their target genes during terminal differentiation of mature adipoctyes (Cristancho and Lazar, 2011). FoxO1 inhibits differentiation of adipocytes by repressing the expression and transcriptional activity of PPARγ (Dowell et al., 2003; Armoni et al., 2006; Fan et al., 2009). In addition, FoxO1 also upregulates the cell cycle inhibitor p21, thus inhibiting the progression of the cell cycle which is necessary for early initiation of adipogenesis (Nakae et al., 2003).

The role of FoxO1 in pancreatic β-cells is multi-faceted. Pancreatic β-cells respond to increased circulating glucose by secreting insulin, which promotes the cellular uptake and storage of glucose, thus maintaining tight control of serum glucose levels. Failure of pancreatic β-cell function is a key feature of diabetes (DeFronzo and Abdul-Ghani, 2011). β-cell function is determined both by pancreatic β-cell mass and by the ability of β-cells to appropriately secrete insulin in response to elevated glucose levels. The insulin/IGF-1 signaling pathway critically regulates insulin secretion and β-cell proliferation, and as such, disruption of this signaling pathway leads to the development of diabetes (Withers et al., 1998; Kulkarni et al., 1999; Kubota et al.,

2000; Kulkarni et al., 2002; Xuan et al., 2002). Mice lacking insulin receptor substrate 2 (IRS-2) develop diabetes due to peripheral insulin resistance and failure of β-cell proliferation (Withers et al., 1998; Kubota et al., 2000). *IRS-2* knockout mice also exhibit reduced expression of the pancreatic duodenal homeobox 1 (Pdx1) transcription factor (Kitamura et al., 2002), which is essential for pancreatic development, β-cell differentiation and maintenance of β-cell function (Jonsson et al., 1994; Offield et al., 1996; Ahlgren et al., 1998; Brissova et al., 2002; Johnson et al., 2003). Haploinsufficiency of *FoxO1* in *IRS2* knockout mice restores β-cell proliferation and *Pdx1* expression, and prevents the development of diabetes (Kitamura et al., 2002). Similarly, mice with heterozygous deletion of the insulin receptor also develop diabetes, exhibit reduced β-cell mass and reduced *Pdx1* expression, and these phenotypes are suppressed by haploinsufficiency of *FoxO1* (Nakae et al., 2002). And in mice with β-cell-specific abalation of the *PDK1* gene, which encodes a kinase acting downstream of the insulin/IGF-1 pathway, diabetes develops due to reduced β-cell mass (Hashimoto et al., 2006). Haploinsufficiency of *FoxO1* in β-cell-specific *PDK1* knockout mice restores β-cell mass and glucose homeostasis (Hashimoto et al., 2006). These results suggest that the development of diabetes in mice with impaired insulin-signaling is largely due to FoxO1 activation. In β-cells, FoxO1 inhibits *Pdx1* expression by opposing FOXA2-mediated transcription of the *Pdx1* gene (Kitamura et al., 2002). FoxO1 also represses Pdx1 activity by affecting Pdx1 nuclear translocation (Kitamura et al., 2002; Kawamori et al., 2006). Importantly, transgenic expression of *Pdx1* restores β-cell mass and function in

diabetic *IRS-2* knockout mice (Kushner et al., 2002) similar to the effect of FoxO1 haploinsufficiency.

In addition to inactivated insulin/IGF-1 signaling, pancreatic β-cell dysfunction can also result from exposure to a variety of stress-inducing stimuli (Evans et al., 2002). Activation of FoxO1 in β-cells appears to mediate many of the stress-responses that lead to β-cell failure. Exposure of β-cells to elevated fatty acids increases JNK-FoxO1 signaling leading to apoptosis. By decreasing FoxO1 activity, β-cells are protected from fatty-acid induced apoptosis (Martinez et al., 2008). Similarly, the inflammatory cytokine prostaglandin E_2 (PGE_2) activates JNK-FoxO1, leading to β-cell dysfunction as a result of FoxO1-mediated Pdx1 cytoplasmic translocation (Meng et al., 2009). Glucocorticoid exposure results in β-cell failure, as well, by activating FoxO1, leading to inhibition of Pdx1 (Zhang et al., 2009b). Thus, multiple lines of evidence suggest that inappropriate activation of FoxO1, either through cellular stress signaling pathways or through compromised insulin/IGF-1 signaling, results in the repression of Pdx1 and consequent β-cell dysfunction.

However, in contrast to the findings mentioned above, oxidative stress also promotes FoxO1 transactivation of the insulin gene transcription factors *MafA* and *NeuroD*, suggesting that FoxO1 may prevent oxidative stress induced β-cell failure (Kitamura et al., 2005). It appears, then, that FoxO1 may play multiple roles in β-cells based on specific cellular signals.

More recently, the effects of FoxO1 on pancreatic β-cell function *in vivo* have been examined using mouse models with tissue-specific FoxO1 activation or ablation (Kim et al., 2012; Kobayashi et al., 2012). Mice with expression of a constitutively

active form of FoxO1 in the hypothalamus and pancreas exhibited impaired glucose tolerance due, in part, to decreased glucose-stimulated insulin secretion (Kim et al., 2012). While previous studies indicated that FoxO1 activation in β-cells results in reduced cell proliferation, transgenic hypothalamic- and pancreas-specific FoxO1 knockin mice had significantly increased number of islet cells, despite a significant decrease in *Pdx1* mRNA levels, suggesting that there are compensatory mechanisms allowing for β-cell proliferation in the face of FoxO1-mediated reduction in Pdx1 levels (Kim et al., 2012). Consistent with the decrease in *Pdx1* expression, islets of FoxO1 transgenic mice also contained less insulin (Kim et al., 2012).

In contrast to the transgenic FoxO1 knockin mice, mice with pancreas- specific ablation of FoxO1 showed improved glucose tolerance and increased islet cell mass while on a high fat high sucrose diet (Kobayashi et al., 2012). However, β-cell specific ablation of FoxO1 did not improve the glucose tolerance or islet mass of mice on a high fat high sucrose diet (Kobayashi et al., 2012). These phenotypes were correlated with *Pdx1* expression levels as pancreas-specific FoxO1 knockout mice showed elevated *Pdx1* levels whereas β-cell specific FoxO1 knockout mice showed no change in *Pdx1* expression. The apparent differences between the pancreas-specific and β-cell specific FoxO1 knockout mice might be explained by the different temporal and spatial expression of the systems used to generate deletion of *FoxO1* in these animals (Kobayashi et al., 2012). Furthermore, when pancreas-specific or β-cell specific FoxO1 knockout mice were crossed with diabetic *db/db* mice lacking the leptin receptor, glucose intolerance increased and glucose-stimulated insulin secretion decreased (Kobayashi et al., 2012). These observations run counter to the previous

findings in which haploinsufficiency of *FoxO1* restored glucose homeostasis in multiple diabetic mouse models (Kitamura et al., 2002; Nakae et al., 2002; Hashimoto et al., 2006). The role of FoxO1 in regulating pancreatic β-cell function thus appears quite complex. Elucidating the mechanisms underlying the differential effects of FoxO1 in pancreatic β-cells will be critical for the future development of novel therapeutics aimed at treating diabetes.

1.2.3 Sirtuins as conserved FoxO regulators

SIRT1/SIR-2.1 are members of the evolutionarily conserved sirtuin nicotinamide adenine dinucleotide (NAD^+)-dependent family of protein deacetylases/ADP-ribosyltransferases. Silent Information Regulator 2 (*SIR2*), the founding member of this family, was first identified in the budding yeast *Saccharomyces cerevisiae* as being involved in establishing transcriptional silencing at the mating-type loci (Ivy et al., 1986; Rine and Herskowitz, 1987). Later, it was revealed that overexpression of *SIR2* extends yeast replicative lifespan, whereas *SIR2* deletion shortens lifespan (Kaeberlein et al., 1999). This phenotype is associated with SIR2's role in suppressing the formation of detrimental extrachromosal ribosomal DNA circles (ERC) resulting from homologous recombination at these loci (Kaeberlein et al., 1999). Investigation of *sir-2.1*, the *C. elegans* homolog of yeast *SIR2*, demonstrated that overexpression of *sir-2.1* could extend lifespan in this species as well (Tissenbaum and Guarente, 2001; Viswanathan et al., 2005; Berdichevsky et al., 2006). Furthermore, overexpression of *dSIR2* in *Drosophila* also extends lifespan, highlighting the conserved nature of sirtuin function in lifespan determination (Rogina

and Helfand, 2004). In *C. elegans*, SIR-2.1 functions as a co-activator of DAF-16/FoxO by associating with DAF-16/FoxO in the nucleus in a stress- and 14-3-3 dependent manner (Berdichevsky et al., 2006). Association of SIR-2.1 with DAF-16/FoxO enhances DAF-16/FoxO transcriptional activity, thus promoting lifespan extension and stress-resistance (Berdichevsky et al., 2006).

In mammals, seven members of the sirtuin family are present, SIRT1-7, each containing a highly conserved NAD^+-binding domain and catalytic domain (Haigis and Sinclair, 2010). However, these mammalian sirtuins exhibit differential subcellular localization patterns, and enzymatic activities (Table 1.1). In particular, SIRT1 and SIRT2 exhibit both nuclear and cytoplasmic localization, whereas SIRT3, SIRT4 and SIRT5 reside in mitochondria, and SIRT6 is nuclear while SIRT7 is found in the nucleolus (Imai and Guarente, 2010). Of the sirtuins, yeast Sir2, and mammalian SIRT1, SIRT2, SIRT3, SIRT5, SIRT6 and SIRT7 have shown NAD^+-dependent deacetylase activity (Imai et al., 2000; Onyango et al., 2002; North et al., 2003; North et al., 2005), although SIRT5, SIRT6 and SIRT7 have very little deacetylase activity relative to SIRT1-3 (North et al., 2005). SIRT4 and SIRT6 function as ADP-ribosyltransferases (Liszt et al., 2005; Haigis et al., 2006; Michishita et al., 2008) and recently, SIRT5 was shown to function as a demalanoylase and desuccinylase (Du et al., 2011) which could explain the relatively weak or absent deacetylase activity exhibited by these sirtuins. A growing number of sirtuin target proteins have been identified, and the functional importance of sirtuin-mediated enzymatic modification of these targets is an area of much interest (Haigis and Sinclair, 2010; Imai and Guarente, 2010).

Table 1.1 Enzymatic activity and localization of mammalian sirtuins.

	Enzymatic activity	Localization
Sirt1	**Deacetylase**	**Nucleus, Cytoplasm**
Sirt2	**Deacetylase**	**Cytoplasm, Nucleus**
Sirt3	**Deacetylase**	**Mitochondria**
Sirt4	**ADP-ribosyltransferase**	**Mitochondria**
Sirt5	**Deacetylase, Demalanoylase, Desuccinylase**	**Mitochondria**
Sirt6	**Deacetylase, ADP-ribosyltransferase**	**Nucleus**
Sirt7	**Deacetylase**	**Nucleolus**

Modified from Satoh, et al., 2011

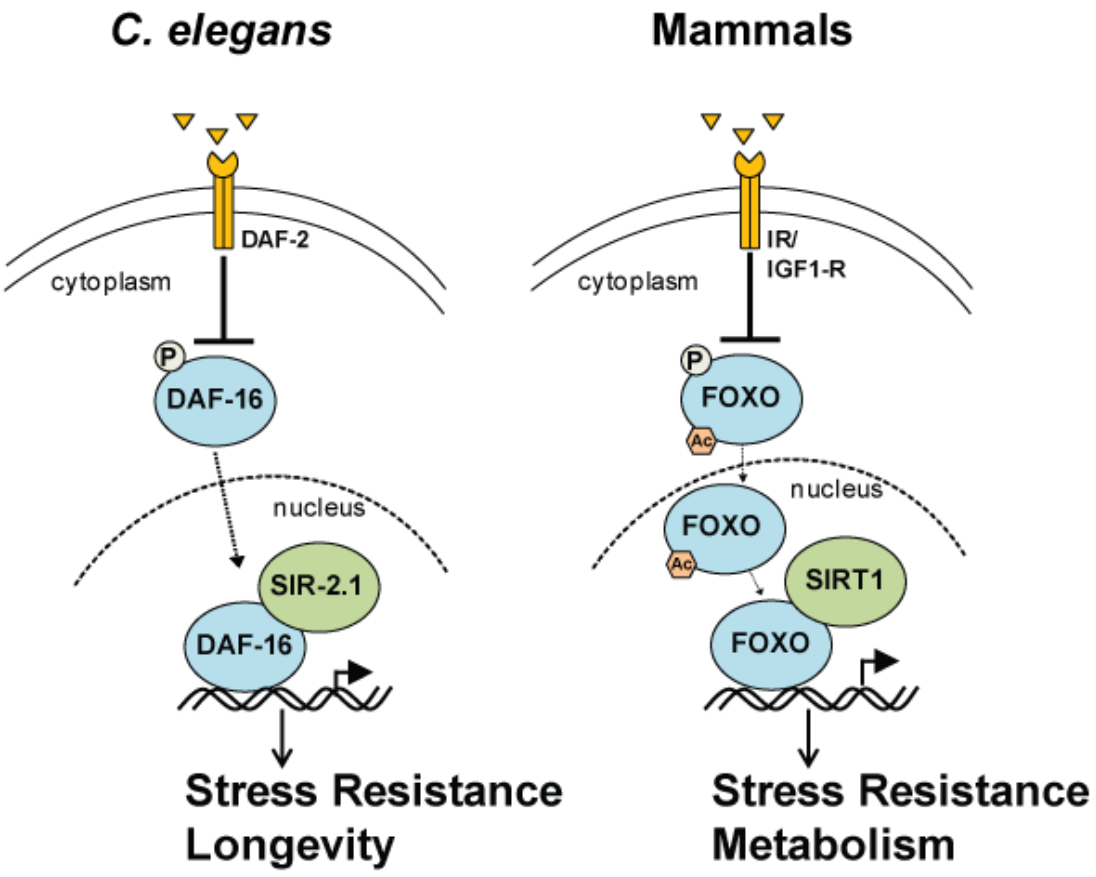


Figure 1.4 Sirtuins modulate DAF-16/FoxO transcription factor activity.

C. elegans SIR-2.1 and mammalian SIRT1, members of the nicotinamide adenine dinucleotide (NAD^+)-dependent family of protein deacetylases/ADP-ribosyltransferases, form a protein complex with DAF-16/FoxO transcription factors, and affect DAF-16/FoxO transcriptional activity. In *C. elegans*, SIR-2.1 activates DAF-16-mediated transcription of genes responsible for promoting stress resistance and longevity. Mammalian SIRT1 directly deacetylates FoxO proteins, thus increasing FoxO DNA binding ability, and modulating FoxO transcriptional activity to affect cellular stress resistance and metabolism.

Of the seven sirtuins, SIRT1 has been most extensively studied both *in vitro* and *in vivo.* After the discovery that SIR-2.1 functions in *C. elegans* to extend lifespan through activation of DAF-16, multiple groups showed that mammalian FoxO proteins are also targets of SIRT1 (Figure 1.4) (Brunet et al., 2004; Daitoku et al., 2004; Motta et al., 2004; van der Horst et al., 2004). SIRT1 directly deacetylates FoxO in a stress-dependent manner, thereby altering FoxO transcriptional activity by either repression or activation in a gene- and context-specific manner (Brunet et al., 2004; Motta et al., 2004). To explain the differential effects of SIRT1 in modulating FoxO activity, it has been postulated that SIRT1 acts to modify FoxO transcriptional responses to favor expression of cell cycle arrest genes while inhibiting apoptotic gene expression (Brunet et al., 2004). What is known about FoxO acetylation is that it appears to inhibit the DNA-binding capability of FoxO (Matsuzaki et al., 2005; Tsai et al., 2007) while also increasing AKT-mediated phosphorylation of FoxO (Matsuzaki et al., 2005). SIRT1 may have multiple effects on FoxO-mediated transcription by either increasing FoxO DNA binding capacity and promoting FoxO nuclear translocation, or by deacetylating histones around FoxO target genes, thereby inhibiting FoxO transactivation (Calnan and Brunet, 2008). In addition to direct effects of SIRT1 on FoxO, there may also be indirect regulation of FoxO through SIRT1's effects on FoxO co-factors. Like FoxO, the transcriptional regulator peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α) is deacetylated by SIRT1 resulting in increased activation of PGC-1α (Nemoto et al., 2005; Rodgers et al., 2005). PGC-1α itself is an important co-activator for FoxO1 in regulating hepatic gluconeogenesis (Puigserver et al., 2003; Housley et al., 2009). Therefore, it

is possible that SIRT1 may modulate FoxO activity through deacetylating PGC-1α and/or other FoxO co-factors.

More recently, the predominantly cytoplasmic sirtuin SIRT2 has also emerged as a critical FoxO deacetylase (Jing et al., 2007; Wang et al., 2007a; Wang and Tong, 2009; Zhao et al., 2010). In 3T3-L1 adipocytes, SIRT2 associates with, and deacetylates FoxO1, thereby causing increased FoxO1 nuclear localization and resulting in FoxO1 inhibition of adipocyte differentiation (Jing et al., 2007; Wang and Tong, 2009). SIRT2 also deacetylates FoxO1 thus activating oxidative stress responses and preventing FoxO1-mediated autophagy (Wang et al., 2007a; Zhao et al., 2010). These findings highlight the diverse modes of FoxO regulation through deacetylation by sirtuins, and the complexity exhibited by the mammalian sirtuin-FoxO regulatory interaction.

Given the conserved role of sirtuins in promoting longevity, many studies have examined the *in vivo* functions of SIRT1 in mammals. SIRT1 null mice on an inbred genetic background have increased perinatal lethality and developmental defects, and those that survive to adulthood are small and sterile (McBurney et al., 2003). Due to these complications, many studies of SIRT1 function *in vivo* have utilized transgenic mice. In particular, SIRT1's role in the regulation of metabolism has been extensively analyzed. The interest in the metabolic functions of SIRT1 stem from the findings that caloric restriction, a highly effective method of lifespan extension in diverse organisms, is dependent on SIR2 homologs in yeast (Lin et al., 2000) and *Drosophila* (Rogina and Helfand, 2004). While the exact role of sirtuins in mediating the longevity effects of caloric restriction continue to be debated (Haigis and Sinclair,

2010), SIRT1 clearly plays a role in regulating glucose homeostasis. Transgenic mice overexpressing SIRT1 are leaner, with increased metabolic rate, and improved glucose tolerance relative to wildtype mice (Bordone et al., 2007). Another strain of transgenic SIRT1 mice did not have increased metabolic rate or lower body weight, but did show improved glucose tolerance and were resistant to developing diabetes under conditions that promote insulin resistance (Banks et al., 2008). In yet another study, transgenic SIRT1 mice had increased metabolic rates compared to control mice when fed a high fat diet, and were also protected from developing high fat diet-induced hepatic steatosis and glucose intolerance (Pfluger et al., 2008). Furthermore, mice overexpressing SIRT1 specifically in pancreatic β-cells exhibit enhanced insulin secretion in response to glucose, and thus impoved glucose tolerance (Moynihan et al., 2005) while SIRT1 homozygous knockout mice have reduced circulating insulin levels (Bordone et al., 2006). Finally, while wildtype mice respond to a calorie-restricted diet by increasing physical activity, SIRT1 null mice do not (Chen et al., 2005; Boily et al., 2008), and SIRT1 null mice also do not have an increased lifespan in response to caloric restriction (Boily et al., 2008), indicating that mammalian SIRT1 is required for these phenotypes. While these and other studies illustrate the beneficial effect of SIRT1 in regulating glucose homeostasis and the metabolic response to dietary manipulations, there are conflicting reports of the role that SIRT1 plays in specific tissues (Haigis and Sinclair, 2010). Liver-specific SIRT1 knockout mice have been reported to exhibit both increased and decreased high fat diet-induced fatty liver disease (Chen et al., 2008a; Purushotham et al., 2009; Wang et al., 2010). The reason for this discrepancy is unclear. However, more recent studies looking at hepatic

overexpression of SIRT1 showed that these mice were protected from glucose intolerance and hepatic steatosis under conditions that promote insulin resistance (Li et al., 2011). Thus, the overall conclusion from these studies is that SIRT1 promotes glucose homeostasis and ameliorates the effects of dietary and genetic conditions that promote insulin resistance.

In addition to roles in metabolism, SIRT1 also protects against the development of age-related diseases, such as cancer (Firestein et al., 2008; Oberdoerffer et al., 2008; Wang et al., 2008b; Herranz et al., 2010), neurodegeneration (Araki et al., 2004; Qin et al., 2006; Kim et al., 2007; Donmez et al., 2010) and cardiovascular disease (Alcendor et al., 2007; Zhang et al., 2008). However, despite the beneficial effects of SIRT1 on metabolism and age-related pathologies, overexpression of SIRT1 does not appear to extend mammalian lifespan (Herranz et al., 2010), although SIRT1 null mice do live shorter than wildtype mice (Boily et al., 2008; Li et al., 2008b). Further studies of SIRT1 mouse models are needed to determine whether SIRT1 affects longevity in mammals, and whether any of the beneficial effects of SIRT1 are mediated by FoxO. Thus far, the evidence supports a role for SIRT1 in promoting metabolic adaptation to adverse conditions, and preventing the onset of age-related diseases.

1.3 HCF proteins as potential conserved regulators of FoxO transcription factors in mammals.

Given that HCF proteins are highly conserved between *C. elegans* and mammals, and that *C. elegans* HCF-1 acts as a transcriptional repressor of DAF-16, I

hypothesize that HCF-1 and/or HCF-2 function as co-repressors of mammalian FoxO transcription factors. Furthermore, as SIR-2.1 acts upstream of *C. elegans* HCF-1 in modulating DAF-16 activitiy, I hypothesize that mammalian HCF proteins interact with mammalian sirtuins to co-regulate FoxO transcriptional activity. Thus, by regulating the activity of FoxO factors, which are critically involved in modulating expression of diverse cellular target genes, mammalian HCF proteins are likely to be required for appropriate control of FoxO-mediated processes, including apoptosis, cellular proliferation, and metabolism.

CHAPTER 2

HCF-1 AND SIRT1, THE MAMMALIAN HOMOLOGS OF TWO EVOLUTIONARILY CONSERVED LONGEVITY DETERMINANTS, COLLABORATE TO REGULATE FOXO[1]

2.1 ABSTRACT

The conserved DAF-16/FoxO transcription factors and SIR-2.1/SIRT1 deacetylases are critical for diverse biological processes, particularly longevity and stress response, and complex regulation of DAF-16/FoxO by SIR-2.1/SIRT1 is central to appropriate biological outcomes. *Caenorhabditis elegans* Host Cell Factor 1 (HCF-1) is a longevity determinant previously shown to act as a co-repressor of DAF-16. Genetic analyses have shown that *hcf-1* acts downstream of *sir-2.1* to influence lifespan and oxidative stress response in *C. elegans*, while gene expression profiling has revealed a striking 80% overlap between the DAF-16 target genes responsive to *hcf-1* mutation and *sir-2.1* overexpression. Analogous to its role in regulating DAF-16/SIR-2.1 target genes in *C. elegans*, mammalian HCF-1 and HCF-2 also repress the expression of FoxO/SIRT1 target genes. Protein-protein association studies demonstrate that SIRT1 and HCF-1/HCF-2 form protein complexes with mammalian FoxOs, highlighting the conservation of this regulatory relationship. Furthermore, SIRT1 acts to promote the deacetylation of HCF-1, but not HCF-2, at lysine residues located in a region unique to mammalian HCF-1. Our findings uncover a conserved interaction between the key longevity determinants SIR-2.1/SIRT1 and HCF-1, and provide new insights into the complex regulation of FoxO proteins.

[1] Chapter 2 is modified from Rizki G, Iwata TN, Li J, Riedel CG, Picard CL, Jan M, Murphy CT, and Lee SS, *PLoS Genetics* (2011).

2.2 INTRODUCTION

The Insulin/Insulin-like Growth Factor-1(IGF-1) signaling (IIS) cascade is one of the most highly conserved and best characterized longevity pathways in eukaryotes. When stimulated, the insulin/IGF-1-like receptors initiate a kinase cascade that leads to the phosphorylation, and cytoplasmic retention of the main downstream effectors, Forkhead box, Class O (FoxO) transcription factors. Reduction in IIS signaling leads to the dephosphorylation of FoxO, allowing nuclear translocation and transcriptional activation of FoxO (Burgering and Kops, 2002; Kenyon, 2010). The *C. elegans* FoxO ortholog DAF-16, as well as the *Drosophila*, mouse, and human FoxO transcription factors are all critical for longevity, metabolism, and stress response (Kenyon et al., 1993; Lin et al., 1997; Giannakou et al., 2004; Hwangbo et al., 2004; Arden, 2008; Kappeler et al., 2008; Wang et al., 2008a; Willcox et al., 2008; Li et al., 2009; Yuan et al., 2009), suggesting that the mechanisms underlying FoxOs' ability to affect physiology are highly conserved across species. Indeed, much of our understanding of FoxO regulation comes from studies done on *C. elegans* DAF-16. When activated, DAF-16/FoxO selectively regulates the transcription of a large number of genes which cumulatively act to elevate stress resistance, alter metabolic and developmental responses, improve immunity, and extend lifespan (Lee et al., 2003; McElwee et al., 2003; Murphy et al., 2003; Halaschek-Wiener et al., 2005). To integrate many different environmental stimuli and coordinate proper transcriptional responses, DAF-16/FoxO activity must be tightly controlled. DAF-16/FoxO activity is known to be regulated by post-translational modifications, nuclear/cytoplasmic translocation and association with transcriptional co-regulators. Little is known about the interplay

between DAF-16/FoxO and its nuclear regulators and how these multiple factors coordinately act on DAF-16/FoxO to ensure proper transcriptional outcomes.

Host Cell Factor-1 (HCF-1) belongs to a family of highly conserved HCF proteins and acts as a nuclear co-repressor of DAF-16 (Lee and Herr, 2001; Li et al., 2008a). Inactivating *hcf-1* robustly extends lifespan and confers oxidative stress resistance in a *daf-16*-dependent manner in *C. elegans*. In the nucleus, HCF-1 associates with DAF-16 and limits its access to a subset of target gene promoters (Li et al., 2008a). *C. elegans* HCF-1 shares high structural homology with two mammalian counterparts, HCF-1 and HCF-2 (Lee and Herr, 2001). Although mammalian HCF-1 has been studied extensively, HCF-2 functions remain largely unknown. Mammalian HCF-1 was originally identified as a binding partner of the Herpes Simplex Virus VP16 transcription factor (Gerster and Roeder, 1988). Apart from VP16, HCF-1 has been shown to associate with a number of transcription factors to stimulate or repress their transactivation properties (Lu et al., 1997; Gunther et al., 2000; Lu and Misra, 2000; Piluso et al., 2002; Wysocka et al., 2003; Tyagi et al., 2007). HCF-1 is an important regulator of cellular proliferation as it promotes progression through multiple phases of the cell cycle via assembling transcriptional complexes to modulate E2F transcription factor activities (Julien and Herr, 2004; Tyagi et al., 2007). Whether mammalian HCF proteins function as conserved FoxO regulators has yet to be determined.

SIR-2.1, the *C. elegans* homolog of the yeast NAD+-dependent protein deacetylase Sir2p, is an important DAF-16 co-factor. SIR-2.1 is thought to activate DAF-16 in conferring longevity as well as stress resistance (Tissenbaum and

Guarente, 2001; Berdichevsky et al., 2006; Wang et al., 2006). Heat stress stimulates the physical association of SIR-2.1 with DAF-16 via the scaffolding protein 14-3-3, which promotes the transactivation of DAF-16 (Berdichevsky et al., 2006; Wang et al., 2006). Overexpression of Sir2 homologs in worms, yeast and flies extends lifespan (Kaeberlein et al., 1999; Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004; Berdichevsky et al., 2006), emphasizing the evolutionarily conserved role of Sir2 in longevity determination. In mammals, SIRT1 associates with and directly deacetylates FoxO1, 3, and 4 in a stress-dependent manner (Brunet et al., 2004; Daitoku et al., 2004; Motta et al., 2004; Yang et al., 2005). This high degree of conservation suggests that other factors involved in SIR-2.1/DAF-16 interactions in *C. elegans* may be functionally conserved in mammals as well.

Importantly, we have shown that *C. elegans hcf-1* acts downstream of *sir-2.1* to regulate *daf-16,* and thereby modulate lifespan and oxidative stress response in C. elegans (Rizki et al., 2011). In addition, we showed that HCF-1 and SIR-2.1 regulate a common subset of DAF-16 target genes important for ensuring longevity and stress response (Rizki et al., 2011). In this study, we sought to examine whether the two conserved DAF-16/FoxO nuclear regulators, HCF-1 and SIRT1 interact in a conserved manner in mammals. We demonstrated that both mammalian HCF-1 and HCF-2 affect the expression of several SIRT1/FoxO transcriptional targets, and both HCF-1 and HCF-2 physically associate with FoxO3 and SIRT1. Furthermore, we find that SIRT1 acts to specifically target HCF-1, and not HCF-2, for deacetylation at a number of lysine residues located within the basic region of the HCF-1 protein. Our findings uncover a new regulatory mechanism between the critical longevity determinants

DAF-16/FoxO and SIR-2.1/SIRT1, and implicate an important role of HCF-1 in aging and age-related diseases in diverse organisms.

2.3 RESULTS

2.3.1 Mammalian HCF-1 and HCF-2 regulate the expression of a subset of FoxO target genes.

In *C. elegans,* HCF-1 functions downstream of SIR-2.1 to repress the activity of DAF-16 (Li et al., 2008a; Rizki et al., 2011). HCF-1 belongs to a highly conserved family of proteins (Julien and Herr, 2003; Wysocka and Herr, 2003; Tyagi et al., 2007). In mammals, two homologs of HCF-1 are present: HCF-1 and HCF-2. Mammalian HCF-1 plays a role in transcriptional regulation and cell cycle progression. The functions of HCF-2 remain unknown. SIRT1, the mammalian homolog of SIR-2.1, is known to interact with and deacetylate the DAF-16 homolog FoxO3 and thereby affects FoxO3 transcriptional activity (Brunet et al., 2004; Motta et al., 2004). Given that HCF-1, DAF-16 and SIR-2.1 are highly conserved between *C. elegans* and mammals, we tested whether mammalian homologs of HCF-1 could affect the transcription of FoxO- and SIRT1- co-regulated target genes. Since mammalian HCF-1 is required for proper cell cycle progression, we employed a transient knockdown approach by transfecting siRNA duplexes targeting the *HCF-1* gene into INS-1 rat insulinoma cells. The *HCF-1* siRNA effectively reduced *HCF*-1 mRNA and HCF-1 protein levels (Figure 2.1A-B), and is specific as it did not affect the expression of *HCF-2* mRNA (Figure 2.1A). We examined the expression of *p27*, *Bim*, *Gadd45a*, and *IGFBP1*, four FoxO target genes that are affected by SIRT1

deacetylation of FoxO (Brunet et al., 2004; Motta et al., 2004). Depletion of *HCF-1* resulted in a significant increase in the level of *Bim*, *Gadd45a*, and *IGFBP1* transcripts, but did not affect *p27* expression (Figure 2.2A). We observed similar results using a second *HCF-1*-targeting siRNA duplex (Figure 2.2A). We next assessed whether HCF-2 could also affect FoxO target gene expression. Similar to *HCF-1* knockdown, cells treated with *HCF-2* siRNA exhibited increased expression of *Gadd45a* and no change in *p27* (Figure 2.2B). However, unlike the case with HCF-1, cells depleted of *HCF-2* did not show any significant changes in *Bim*, or *IGFBP1* transcripts (Figure 2.2B). Our data reveal that HCF proteins negatively regulate the expression of a subset of FoxO and SIRT1 transcriptional target genes. Furthermore, HCF-1 appears to play a more substantial role in regulating FoxO target genes relative to HCF-2. The observation that HCF-1 and HCF-2 have specific effects on a subset of FoxO targets tested is also consistent with our findings in *C. elegans* suggesting HCF-1 to be a specificity factor for DAF-16/FoxO.

2.3.2 Mammalian HCF-1 and HCF-2 physically associate with FoxO3 and SIRT1.

In *C. elegans*, HCF-1 is able to physically associate with both DAF-16 and SIR-2.1. We therefore hypothesized that mammalian HCF-1 may also be involved in physical associations with FoxO3 and SIRT1. To examine the physical interactions between these proteins, we transfected HEK293T cells with plasmids encoding either Flag-tagged FoxO3 or Flag-tagged SIRT1. We then performed co-immunoprecipitation experiments with these cell lysates by using Flag-antibody

Figure 2.1 Specific knockdown of *HCF-1* and *HCF-2* by siRNA.

INS-1 cells transfected with *HCF-1* (A,B) or *HCF-2* (C) siRNA were analyzed by RT-qPCR and Western blotting. (A) Two *HCF-1* targeting siRNAs were used to knockdown *HCF*-1. siHCF-1 #1 treatment resulted in a moderate increase in *HCF-2* expression. *HCF-2* was not affected by siHCF-1 #2. (B) *HCF-1* siRNA substantially reduced HCF-1 protein levels. ** indicates a non-specific band. HCF-1 is known to be proteolytically processed and is detected as multiple bands on SDS-PAGE. (C) Knockdown of *HCF-2* did not affect *HCF-1* expression. Values are normalized to the level of *β-actin*. The mean normalized mRNA level for each gene in sicontrol treated cells was set to 1. The data represented are pooled from three independent experiments and are represented as mean +/- SEM. * denotes a *p*-value <0.05.

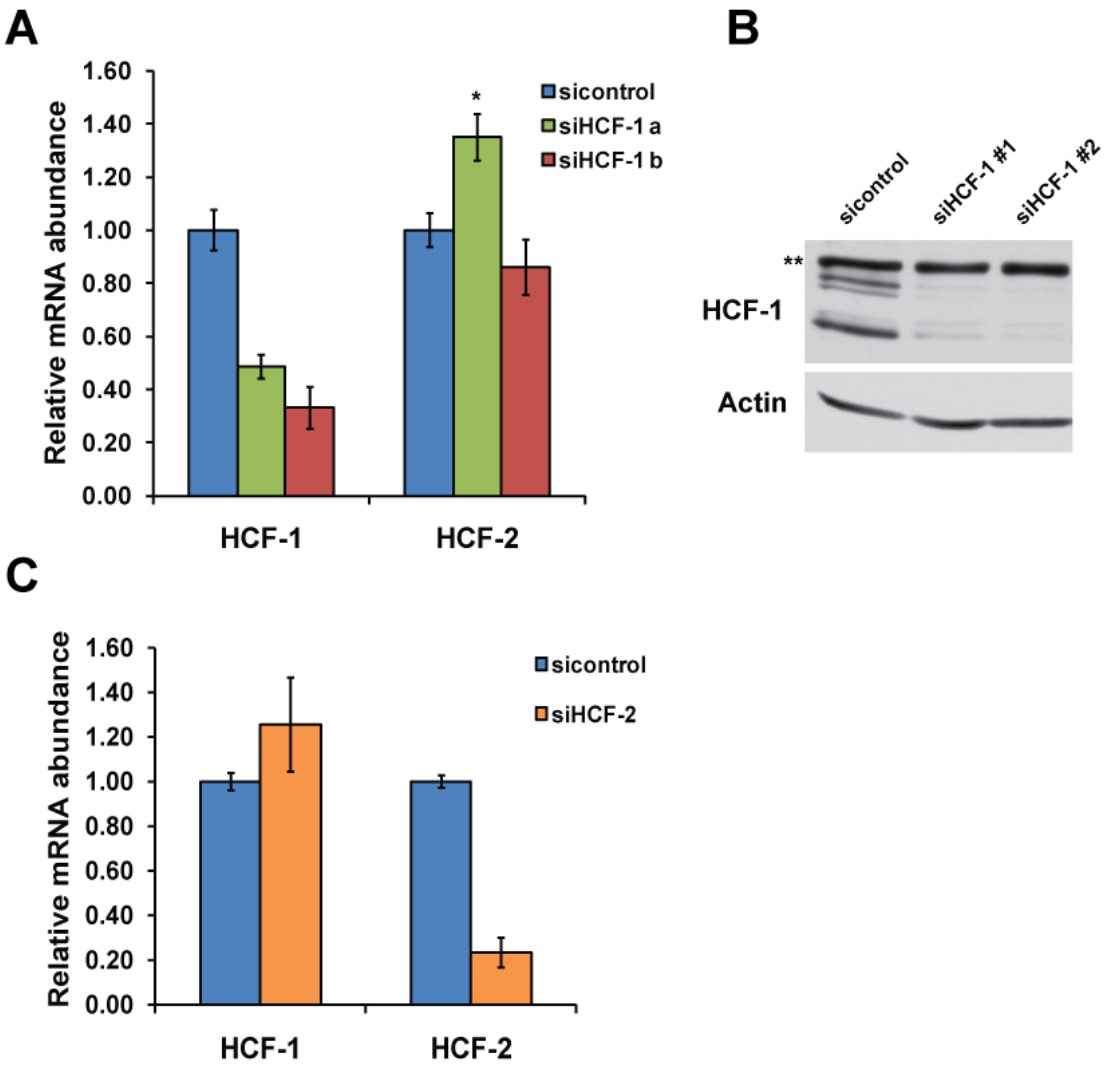
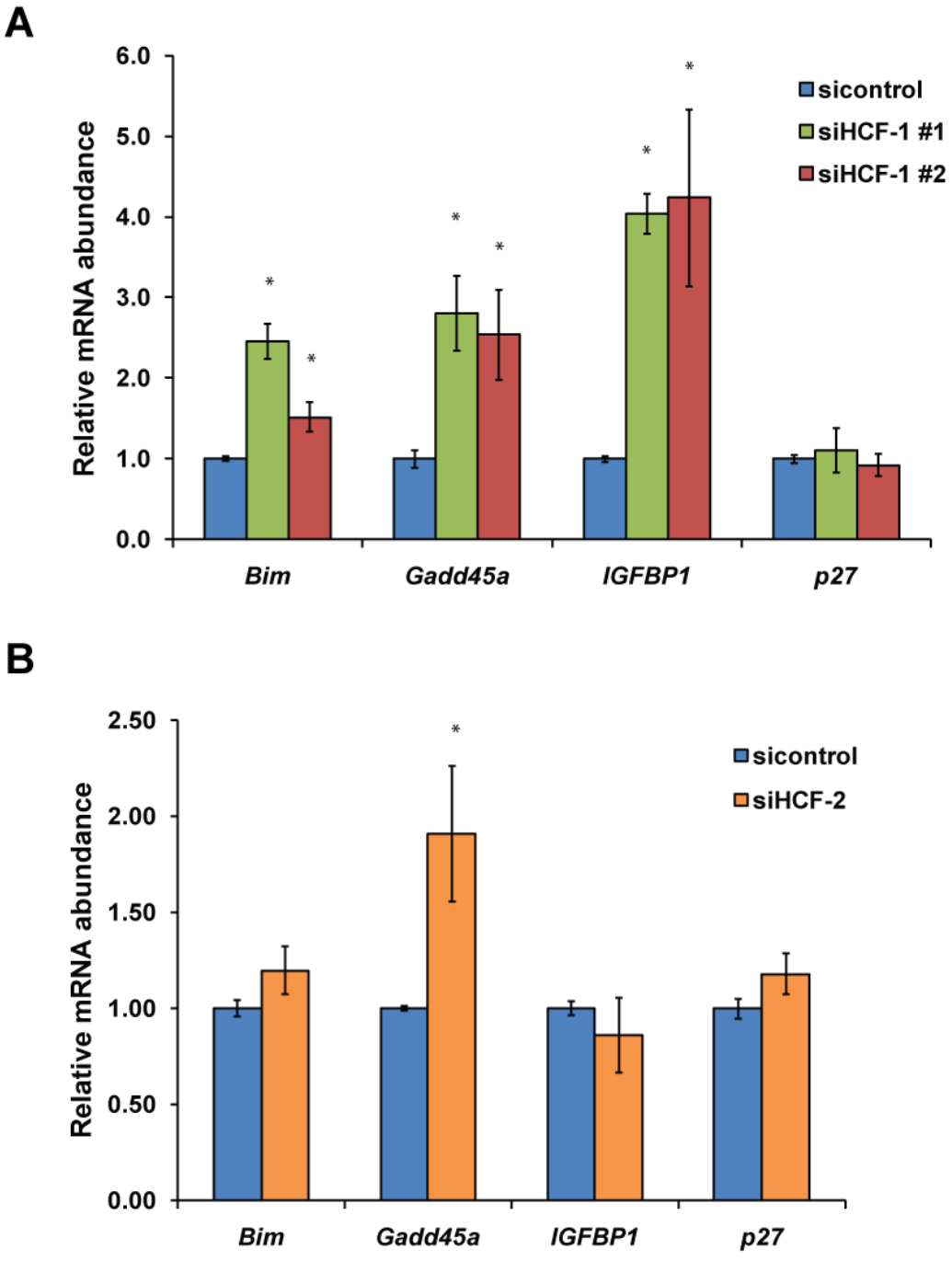

A
Relative mRNA abundance
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
sicontrol
siHCF-1 a
siHCF-1 b
HCF-1
HCF-2
B
sicontrol
siHCF-1 #1
siHCF-1 #2
**
HCF-1
Actin
C
Relative mRNA abundance
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
sicontrol
siHCF-2
HCF-1
HCF-2

Figure 2.2 Mammalian HCF-1 and HCF-2 regulate the expression of FoxO target genes.

INS-1 cells treated with *HCF-1* (A) or *HCF-2* (B) siRNA compared to control siRNA. mRNA levels of *Bim, Gadd45a, p27,*and *IGFBP1* were quantified using RT-qPCR and normalized to the level of *β-actin*. The mean normalized RNA level for each gene in sicontrol treated cells was set to 1. The data represented are pooled from three independent experiments and are represented as mean +/- SEM. * denotes a *p*-value <0.05 relative to sicontrol.


A
Relative mRNA abundance
6.0
5.0
4.0
3.0
2.0
1.0
0.0
sicontrol
siHCF-1 #1
siHCF-1 #2
Bim
Gadd45a
IGFBP1
p27
B
Relative mRNA abundance
2.50
2.00
1.50
1.00
0.50
0.00
sicontrol
siHCF-2
Bim
Gadd45a
IGFBP1
p27

conjugated agarose beads. Both FoxO3 and SIRT1 were found to interact with the endogenous mammalian HCF-1 protein (Figure 2.3A-B). We also tested whether the closely related HCF-2 protein could also physically interact with FoxO3 and SIRT1. Like HCF-1, HCF-2 was also present in a protein complex with FoxO3 and SIRT1 (Figure 2.3C-D). These results indicate that the physical associations between HCF-1 family members, DAF-16/FoxO and SIR-2.1/SIRT1 are highly conserved between *C. elegans* and mammals.

2.3.3 SIRT1 promotes HCF-1 deacetylation.

Since SIRT1 is a protein deacetylase and is well known to modulate substrate activity by deacetylating its targets, we assessed whether SIRT1 affects HCF-1 or HCF-2 acetylation status. We immunoprecipitated HCF-1 or HCF-2 from HEK293T cells overexpressing HA-tagged versions of these proteins, and then probed for acetylation using a pan-acetyl-lysine antibody. Under normal culture conditions, we detected a low level of HCF-1 and HCF-2 acetylation. Treatment of the cells with nicotinamide, a known SIRT1 inhibitor, dramatically increased the levels of HCF-1 acetylation (Figure 2.4A). However, unlike HCF-1, HCF-2 was not affected by nicotinamide treatment (Figure 2.4A). To further confirm that the increased acetylation was due to SIRT1 inhibition, we transfected HEK293T cells with plasmids encoding the HA-HCF-1 protein as well as short-hairpin RNA targeting the human *SIRT1* gene (Figure 2.4B). Immunoblotting revealed that in cells transfected with the *SIRT1* shRNA, SIRT1 expression was reduced compared to the control shRNA-

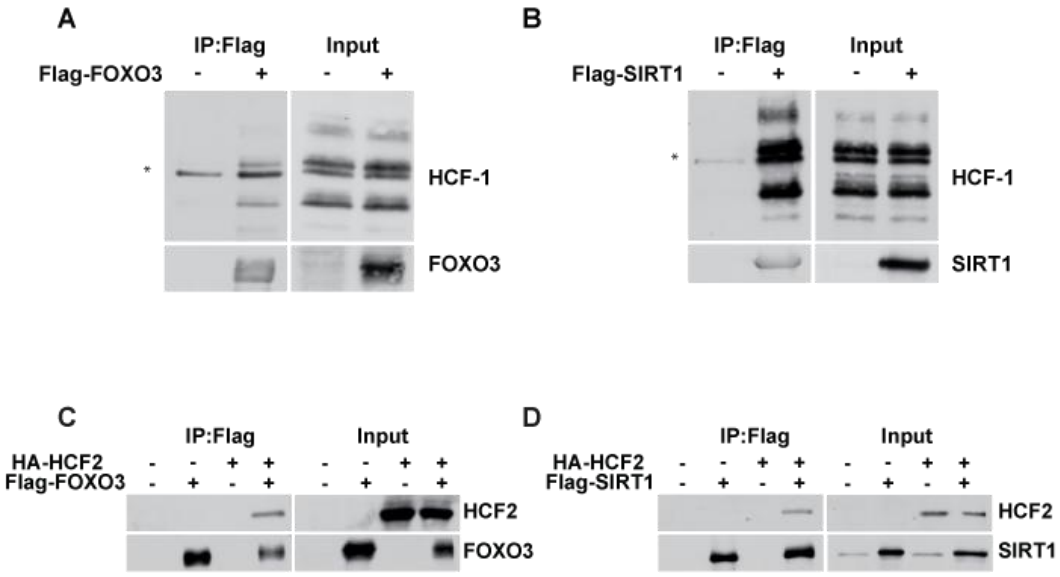


Figure 2.3 Mammalian HCF-1 and HCF-2 physically associate with FoxO3 and SIRT1.

(A, B) HEK293T cells were transfected with plasmids encoding Flag-FoxO3 (A) or Flag-SIRT1 (B). Cell lysates were collected 48 hours later and incubated with anti-Flag-conjugated agarose beads. Immunoprecipitated protein complexes were analyzed by western blot using anti-HCF-1, anti-FoxO3 or anti-SIRT1 antibodies. * denotes a non-specific band. (C, D) HEK293T cells were co-transfected with plasmids encoding HA-HCF2 and either Flag-FoxO3 (A) or Flag-SIRT1 (B). Cell lysates were collected 48 hours later and incubated with either anti-Flag- or anti-HA-conjugated agarose beads. Immunoprecipitated protein complexes were analyzed by western blot using anti-HCF-2, anti-FoxO3 or anti-SIRT1 antibodies.

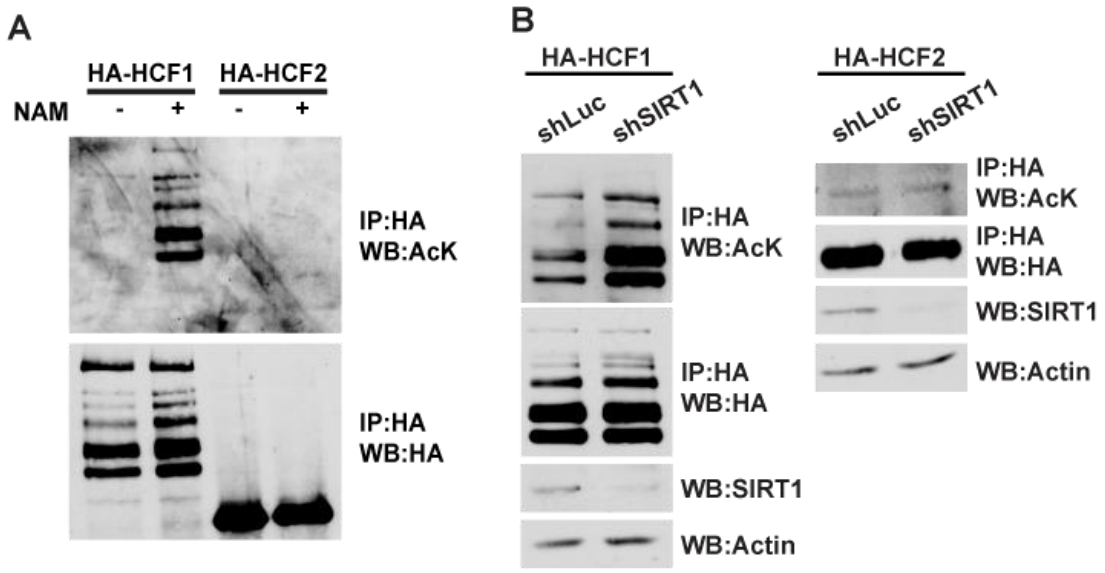


Figure 2.4 SIRT1 promotes deacetylation of HCF-1, but not HCF-2.

(A) HEK293T cells overexpressing HA-HCF1 or HA-HCF2 were treated with 10mM nicotinamide (NAM) for 6 hours. HCF-1 or HCF-2 was immunoprecipitated using anti-HA-conjugated agarose beads and analyzed by western blot using an anti-acetyl-lysine antibody. (B) HEK293T cells were co-transfected with HA-HCF1 (left) or HA-HCF2 (right) and a shRNA plasmid targeting either the control *luciferase* gene or *SIRT1*. HCF-1 or HCF-2 was immunoprecipitated and analyzed as in (A). Knockdown of SIRT1 was confirmed by immunoblotting with anti-SIRT1 antibody.

transfected cells. In cells with decreased levels of SIRT1 protein, HCF-1 acetylation was increased (Figure 2.4B, left panel), indicating that SIRT1 is responsible for HCF-1 deacetylation. By contrast, HCF-2 acetylation was not affected by SIRT1 knockdown (Figure 2.4B, right panel). Thus, our results show that SIRT1 specifically targets HCF-1, and not HCF-2, for deacetylation.

2.3.4 The basic region of mammalian HCF-1 undergoes deacetylation in response to increased sirtuin activity.

We next sought to determine whether a specific domain of the HCF-1 protein is targeted for deacetylation by sirtuins. We utilized a series of human HCF-1 truncation mutant expression constructs that have previously been used to dissect the functions of specific HCF-1 domains (Wilson et al., 1997) (Figure 2.5A). We assessed whether each truncated form of HCF-1 was acetylated, and if so, whether the acetylation status of that fragment was affected by the sirtuin inhibitor nicotinamide. The HCF-1 domains we analyzed included the full-length protein (FL); a region containing the N-terminal Kelch domain (N380, residues 1-380); a region including the Kelch domain as well as the basic region (N1011, residues 1-1011); and a C-terminal fragment including the Fibronectin 3 repeat domain (C600, residues 1436-2035). Each fragment also included an N-terminal HA epitope tag. We transfected each expression plasmid into HEK293T cells, and then treated the cells with nicotinamide for 6 hours. After immunoprecipitation of the fragments, we performed western blot analysis to detect acetylation levels. As previously observed, full length HCF-1 was acetylated and this acetylation was robustly increased in the presence of

nicotinamide. The N380 fragment of HCF-1 including only the Kelch domain, however, showed no acetylation, with or without nicotinamide. In contrast, the region of HCF-1 including both the Kelch domain and the basic region, N1011, was clearly acetylated, and acetylation was dramatically increased with nicotinamide treatment, similar to that of the full-length HCF-1 protein. The C-terminal fragment of HCF-1 (C600) was also clearly acetylated, but showed little change with nicotinamide treatment. Thus, we conclude that the region of HCF-1 sensitive to sirtuin-mediated deacetylation involves the basic region of HCF-1.

2.3.5 Lysine 813 of HCF-1 is deacetylated by sirtuins.

Recent proteomic analysis has identified mammalian HCF-1 to be acetylated at a number of lysine residues (Choudhary et al., 2009). Of the five lysines identified to be acetylated, two reside within the basic region; lysine 813 (K813) and lysine 836 (K836) (Choudhary et al., 2009). To determine if one or both of these sites was sensitive to sirtuin-mediated deacetylation, we mutated each of these residues to either arginine, which conserves the charged nature of lysine, but is unable to be acetylated, or to glutamine, which is non-charged and mimics the acetylated form of lysine. We then repeated our analysis of the nicotinamide sensitivity of these mutated HCF-1 proteins. Interestingly, mutation of K813 to either arginine or glutamine reduced, but did not abolish, the acetylation induced by nicotinamide (Figure 2.6). By contrast, mutation of K836 to either arginine or glutamine did not reduce the acetylation levels

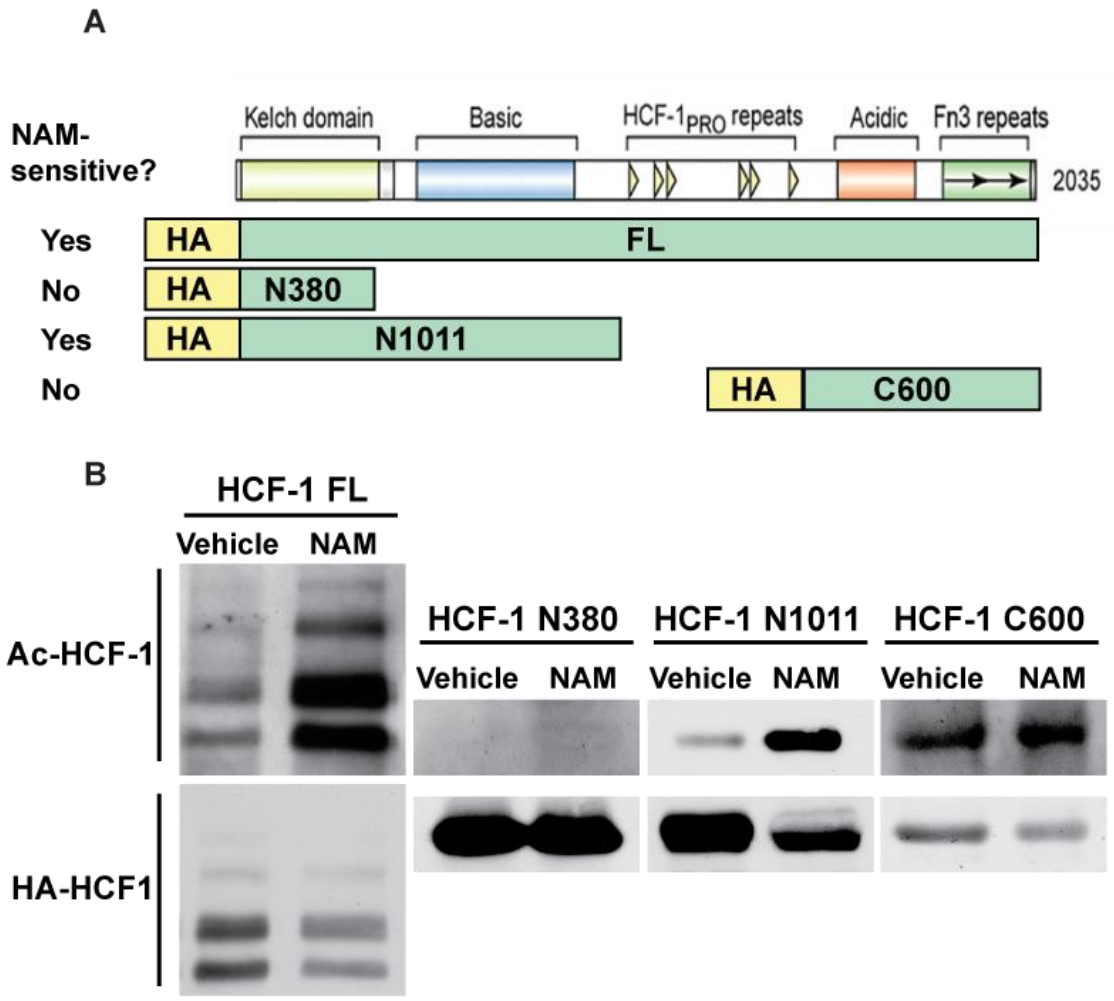
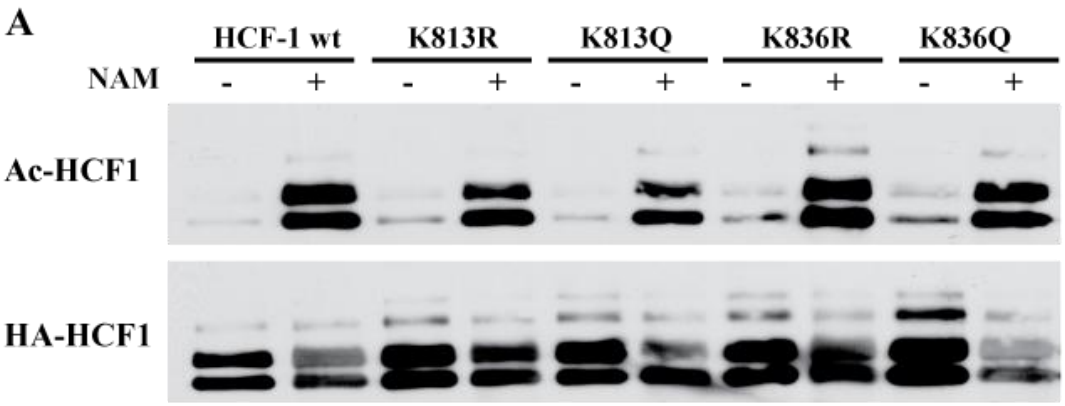


Figure 2.5 The basic region of HCF-1 is targeted for deacetylation by sirtuins.

(A) Diagram of HCF-1 deletion mutants used to map the region of HCF-1 targeted for deacetylation by sirtuins, as determined by nicotinamide (NAM) sensitivity. FL = full length HCF-1; N380 = HCF-1 residues 1-380; N1011 = HCF-1 residues 1-1011; C600 = HCF-1 residues 1436-2035. Full length and deletion mutants were tagged with the HA epitope at the N-terminus. (B) HCF-1 truncation mutants were overexpressed in HEK293T cells. Cells were treated with 10mM NAM for 6 hours. HCF-1 was immunoprecipitated using anti-HA-conjugated agarose beads and analyzed by western blot using an anti-acetyl-lysine antibody.



B

NAM-induced acetylation relative to wildtype HCF1	
HCF1 wt	1.00
K813R	0.44
K813Q	0.33
K836R	1.24
K836Q	0.99

Figure 2.6 Sirtuins target lysine 813 of HCF-1 for deacetylation.

(A) Wildtype (wt) and mutant HCF-1 proteins in which lysine (K) 813 or 836 were mutated to arginine (R) or glutamine (Q) were overexpressed in HEK293T cells. Cells were treated with nicotinamide (NAM). HCF-1 was immunoprecipitated using anti-HA-conjugated agarose beads and analyzed by western blot using an anti-acetyl-lysine antibody. (B) Quantification of the relative fold change in NAM-induced acetylation of wt and mutant forms of HCF-1, as quantified from the blot shown in (A).

of this HCF-1 mutant, and in fact, mutation of K836 to arginine slightly increased nicotinamide-induced acetylation. These results indicate that K813 represents one of the lysines deacetylated by sirtuins, and indicates that additional, as yet to be identified, lysines are also targeted for deacetylation by sirtuins.

2.4 DISCUSSION

The highly conserved FoxO transcription factors are master regulators of diverse biological processes (van der Horst and Burgering, 2007) and as such, their transcriptional activities are tightly controlled (Essers et al., 2005; Berdichevsky et al., 2006; Berman and Kenyon, 2006; Lehtinen et al., 2006; Wolff et al., 2006; Li et al., 2008a). Although a number of different transcriptional co-factors of DAF-16/FoxO have been identified, little is known about how they functionally interact to fine-tune DAF-16/FoxO activity. In this study, we expanded upon our previous identification of the nuclear DAF-16 co-repressor HCF-1, and find that mammalian HCF-1 acts as an integral conserved component of the regulatory network involving SIR-2.1/SIRT1 and DAF-16/FoxO. We have shown that, as in *C. elegans*, mammalian HCF-1 likely functions downstream of SIRT1 to regulate a distinct subset of FoxO target genes.

Considering the high conservation of DAF-16/FoxO-related pathways, it is not surprising that the regulatory relationship among HCF-1, SIR-2.1 and DAF-16 we uncovered previously in worms turns out to be conserved in mammals. Our findings in mammalian cells are nevertheless very exciting as they implicate the HCF proteins to be key components linking FoxO and SIRT1, two critical master regulators of physiology in mammals. Our results indicate that while both mammalian HCF-1 and

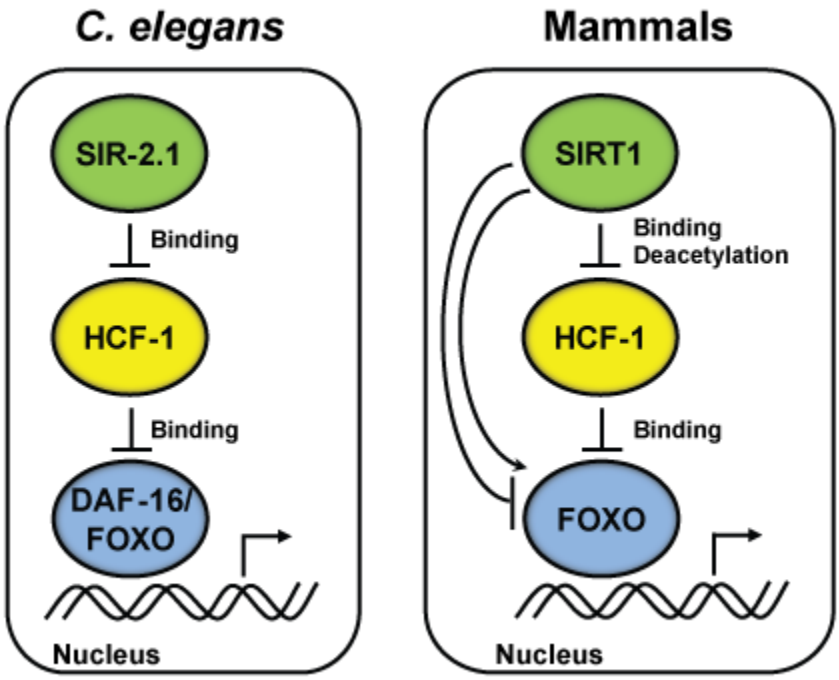


Figure 2.7 Conserved regulation of FoxO by HCF-1 and SIRT1

In *C. elegans*, HCF-1 forms a complex with SIR-2.1, and together they regulate the transcriptional activity of DAF-16/FoxO. We find that this functional relationship is conserved in mammals, where SIRT1 is able to bind and deacetylate HCF-1 while HCF-1 is able to bind FoxO, and affect FoxO transcriptional target gene expression. Our results highlight HCF proteins to be key components of the regulatory network linking SIR-2.1/SIRT1 and DAF-16/FoxO in diverse organisms.

HCF-2 are able to interact with SIRT1 and FoxO3, HCF-1 has a greater effect on FoxO target gene expression. Interestingly, while both mammalian HCF-1 and HCF-2 as well as *C. elegans* HCF-1 are able to support the formation of the Herpes Simplex Virus VP16-transcriptional complex, only mammalian and *C. elegans* HCF-1 are able to promote VP16 transcriptional activity (Lee and Herr, 2001). Thus, it appears that the evolutionarily conserved functions of HCF proteins are retained in mammalian HCF-1. Alternatively, HCF-1 and HCF-2 likely have tissue-specific functions and are regulated differently under different cellular contexts.

While our data indicate that parallel regulatory mechanisms are shared between *C. elegans* and mammalian HCF-1, they also suggest the modes of regulation between HCF-1, SIRT1, and FoxO in mammals are likely more complex than what is observed in *C. elegans*. We note that in the case of the mammalian FoxO target genes *Bim* and *IGFBP1*, HCF-1 and SIRT1 appear to affect FoxO target gene expression in a similar manner (Figure 2.2A and (Brunet et al., 2004; Motta et al., 2004)), and thus would appear to act in concert rather than antagonistically. On the other hand, HCF-1 and SIRT1 appear to have antagonistic effects on the FoxO target gene *Gadd45a*. It is important to keep in mind that in mammals, SIRT1 regulation of FoxO transcription factors is complex; in some instances SIRT1 acts as a repressor and in other cases as an activator of FoxO (Brunet et al., 2004; Motta et al., 2004), while in *C. elegans* the predominant role of SIR-2.1 is as an activator of DAF-16. It is likely that in mammals, the interplay between SIRT1 and HCF-1 results in collaborative as well as antagonistic effects on FoxO transcriptional activity in a gene- and context-dependent manner. Future genome-wide studies examining the effects of HCF-1 on

FoxO/SIRT1-regulated gene expression will provide further insights into the relationship between HCF-1 and SIRT1.

In mammals, SIRT1 directly deacetylates FoxO proteins and modifies their transcriptional activities. Our data indicate that SIRT1, and likely other sirtuins, facilitate the deacetylation of HCF-1 as well. Interestingly, the region of HCF-1 which we found to be deacetylated in response to sirtuins is not conserved between *C. elegans* and mammals (Figure 1.1). Consistent with this finding, we are unable to detect deacetylation of *C. elegans* HCF-1 by SIR-2.1 (G. Rizki, unpublished data). These results suggest that HCF-1 has evolved to include additional regulatory modules that can be acted upon by its co-evolving modifiers.

Mammalian SIRT1 is also able to deacetylate and activate the FoxO co-activator PGC1α (Puigserver et al., 2003; Nemoto et al., 2005; Rodgers et al., 2005). It seems likely, then, that SIRT1 affects multiple FoxO responses by deacetylating FoxO and specific FoxO co-regulators to achieve activation and/or repression of the appropriate target genes. Furthermore, while our analyses indicate that SIRT1 promotes HCF-1 deacetylation, we note that the increased HCF-1 acetylation observed with nicotinamide treatment was dramatically greater than that observed with SIRT1 knockdown (Figure 2.4) suggesting that other sirtuins in addition to SIRT1 function to modify HCF-1. It will be interesting in the future to determine which, if any, other sirtuins modulate HCF-1 acetylation, and whether this action is due to direct deacetylation of HCF-1 by sirtuins, or indirectly through other acetyltransferases/deacetylases.

Besides being acetylated, HCF-1 is known to be post-translationally modified by phosphorylation and *O*-GlcNAcylation (Wysocka et al., 2001; Wang et al., 2007b). These various post-translational modifications may be dynamically regulated by each other, allowing for specific control of HCF-1 function in response to environmental cues. Interestingly, recent analyses indicate that the *O*-GlcNAc transferase (OGT) catalyzes the addition of *O*-GlcNAc moieties on the mammalian HCF-1 protein at multiple serine and threonine residues located in and adjacent to the basic region of HCF-1 (Capotosti et al., 2011). OGT binding to the basic region appears to enhance its interaction with HCF-1, thus promoting its ability to proteolytically cleave the full length HCF-1 protein at the proteolytic processing domain (Capotosti et al., 2011). Our results point to the intriguing possibility that the basic region represents a major site of post-translational modification of HCF-1, and suggests possible cross-talk between acetylation and *O*-GlcNAcylation in determining functional outcomes of HCF-1. Our analyses also indicate that lysine 813 of human HCF-1 is one, but not the only, lysine targeted for sirtuin-mediated deacetylation. Future studies aimed at identifiying the other lysines targeted by sirtuins will facilitate examination of the functional outcomes resulting from acetylation/deacetylation of HCF-1.

As our study suggests, regulation of DAF-16/FoxO activity likely entails a well-coordinated response by SIR-2.1/SIRT1 and HCF-1 that is achieved through highly specific interactions with additional co-factors and/or distinctive protein modifications. We have also shown that 14-3-3 proteins likely physically associate with HCF-1 in *C. elegans* (Rizki et al., 2011). Previous studies in *C. elegans* indicate that 14-3-3 proteins act as bridging molecules that bring SIR-2.1 and DAF-16 into a

protein complex in the nucleus (Berdichevsky et al., 2006; Wang et al., 2006). Whether 14-3-3 proteins are also involved in the regulation of FoxO by SIRT1 and HCF in mammals remain to be investigated.

In conclusion, our findings establish a novel link between two evolutionarily conserved DAF-16/FoxO regulators. This study expands our understanding of the complex role that nuclear factors play in determining the specificity of DAF-16/FoxO activity. These results further implicate HCF-1 as a novel factor that may affect mammalian aging and age-related pathologies through interactions with SIRT1 and FoxO.

2.5 MATERIALS AND METHODS

2.5.1 Plasmids, shRNA and siRNA for mammalian cells

Flag-FoxO3 and Flag-SIRT1 were obtained from Addgene and have been described previously (Brunet et al., 2004). The HCF-1 deletion mutants were kindly provided by W. Herr (Wilson et al., 1997). The plasmids encoding HA-HCF-1 and HA-HCF-2 were generated by cloning the human HCF-1 and HCF-2 cDNA into the vector pCMV-HA (Clontech). PCR mutagenesis was utilized to generate the HA-HCF-1 K813R, K813Q, K836R and K836Q mutants. The plasmid encoding the short-hairpin RNA targeting the human *SIRT1* gene was generously provided by W.L. Kraus (Zhang et al., 2009a). The plasmid encoding shRNA targeting the firefly *luciferase* gene was generously provided by L. Qi (Cornell University). siRNA duplexes directed against rat *HCF-1* and *HCF-2* were purchased from Dharmacon and targeted the following sequences: siHCF-1 #1: 5'-GGAAGAGACTGAAGGCAAA-3';

siHCF-1 #2: 5'-AGAACAACATTCCGAGGTA-3'; siHCF-2: 5'-GGGAATGGTTGAATATGGA-3'. Non-targeting control siRNA was also from Dharmacon. Cells were collected 48 hours post-transfection, or treated for an additional 6 hours with nicotinamide (10mM, Sigma).

2.5.2 Cell culture and Transfection

HEK293T cells were maintained in DMEM containing 4.5g/L glucose and 10% bovine calf serum and were transfected with the indicated plasmids using calcium phosphate. INS-1 cells were maintained in RPMI-1640 medium containing 11.1mM glucose, 10% fetal bovine serum, 1mM pyruvate, 10mM HEPES, and 50μM 2-mercaptoethanol. INS-1 cells were transfected with siRNA at a concentration of 10nM using Lipofectamine RNAiMax (Invitrogen). siRNA transfections were performed twice, 24 hours apart, and cells were collected 24 hours after the second transfection.

2.5.3 Reverse-transcription coupled quantitative PCR (RT-qPCR):

RNA was isolated from mammalian cells using Trizol reagent and was reverse-transcribed using Superscript III First-Strand kit (Invitrogen). cDNAs were analyzed by quantitative-PCR using the SYBR Green system on a Roche LightCycler 480 real time PCR machine and quantified relative to a standard curve. *β-actin* was used as an internal control. The following primers were used: *β-actin* forward: 5'-CTAAGGCCAACCGTGAAAAG-3'; *β-actin* reverse: 5'-AACACAGCCTGGATGGCTAC-3'; *HCF-1* forward: 5'-

GCTGGAAAAGCTCCTGTCAC-3’; *HCF-1* reverse: 5’-CACTCATCTGTGGGTTGCTG-3’; *HCF-2* forward: 5’-TTGAAAGCAGAGCAATGGTG-3’; *HCF-2* reverse: 5’-AGTCGGGTACGTCTGCATTT-3’; *Bim* forward: 5’-GCCCCTACCTCCCTACAGAC-3’; *Bim* reverse: 5’-CAGGTTCCTCCTGAGACTGC-3’; *p27* forward: 5’-GTGGACCAAATGCCTGACTC-3’; *p27* reverse: 5’-TTCTGTTCTGTTGGCCCTTT-3’; *Gadd45a* forward: 5’-GCAGAGCTGTTGCTACTGGA-3’; *Gadd45a* reverse: 5’-TGTGATGAATGTGGGTTCGT-3’; *IGFBP1* forward: 5’-CTGCCAAACTGCAACAAGAA-3’; *IGFBP1* reverse: 5’-TTCCCACTCCATGGGTAGAC-3’

2.5.4 Immunoblotting and Immunoprecipitations

For co-immunoprecipitation experiments, HEK293T cells were transfected with the indicated plasmids. 48 hours after transfection, cells were lysed in lysis buffer (50mM Tris-HCl pH 8.0, 100mM NaCl, 2mM EDTA, 1% TritonX-100, 10mM NaF, 1mM sodium orthovanadate, 1mM PMSF, 10mM nicotinamide, 1mM trichostatin A, and Roche complete protease inhibitor cocktail). Cell extracts were incubated with either Flag- or HA-conjugated agarose beads (Sigma) overnight at 4°C. Beads were washed five times in lysis buffer and eluted by boiling in SDS sample buffer. Immunoprecipitates were analyzed by western blotting using the following

antibodies: anti-HA (Covance), anti-FoxO3 (Upstate), anti-SIRT1 (gift from W.L. Kraus), anti-HCF-1 (Bethyl Labs).

2.5.5 Acetylation of HCF-1 and HCF-2

HA-tagged HCF1 was expressed and immunoprecipitated from HEK293T cells as described above. Acetylation was determined by western blotting for acetylated-lysine residues using an anti-acetyl-lysine antibody (Cell Signaling).

2.6 ACKNOWLEDGEMENTS

We thank W. Herr, W.L. Kraus and L. Qi for providing valuable reagents; G. Rizki, J. Li, C. Riedel, C. Picard, M. Jan and C. T. Murphy for contributions to the published manuscript (Rizki et al., 2011); and members of the Lee, Kraus, and Qi labs for helpful discussions.

CHAPTER 3

THE TRANSCRIPTIONAL CO-REGULATOR HCF-1 PROMOTES *PDX1* EXPRESSION, AND IS REQUIRED FOR INS-1 β-CELL SURVIVAL, PROLIFERATION AND GLUCOSE-STIMULATED INSULIN SECRETION[2]

3.1 ABSTRACT

The transcriptional co-activator HCF-1 plays critical roles in promoting cell cycle progression in multiple mammalian cell types, but its role in modulating pancreatic β-cell function has not been investigated. We examined the phenotypes of INS-1 pancreatic β-cells in which HCF-1 was depleted by siRNA. Reducing HCF-1 expression resulted in decreased INS-1 β-cell proliferation, increased INS-1 β-cell death, and reduced glucose-stimulated insulin secretion. HCF-1 is a known co-activator of the E2F1 transcription factor, and loss of E2F1 results in pancreatic β-cell dysfunction and reduced expression of the pancreatic β-cell transcription factor Pdx1. E2F1 and HCF-1 form a protein complex in INS-1 β-cells, and HCF-1 promotes *Pdx1*, and Pdx1 targets', gene expression. Chromatin immunoprecipitation experiments revealed that HCF-1 and E2F1 co-localize to the *Pdx1* promoter. These results indicate that HCF-1 represents a novel transcriptional regulator of *Pdx1* and is critically important for maintaining pancreatic β-cell function.

[2] I, together with SS Lee, conceived the experiments and analyzed the data contained in Chapter 3. I performed all of the experiments with the exception of the co-immunoprecipitation experiment (Figure 3.4) which was done by M Mierzejewski.

3.2 INTRODUCTION

Diabetes develops due to a deficiency in circulating insulin caused by pancreatic β-cell destruction and/or impaired β-cell function. In type 1 diabetes, pancreatic β-cells are selectively destroyed resulting in reduced β-cell mass, while in type 2 diabetes, loss of insulin-secretory ability as well as β-cell apoptosis lead to defects in glucose homeostasis (Mathis et al., 2001; DeFronzo and Abdul-Ghani, 2011). Understanding the factors responsible for maintaining β-cell mass and β-cell function is, therefore, a key step in developing therapeutics to prevent the development of diabetes.

The transcriptional regulator host cell factor-1 (HCF-1) plays critical roles in cell cycle progression (Wilson et al., 1997; Julien and Herr, 2003), DNA-damage induced apoptosis (Tyagi and Herr, 2009) and stem cell pluripotency (Dejosez et al., 2008). HCF-1 largely functions as a scaffolding protein assembling appropriate transcriptional complexes at target gene promoters and bridging interactions between transcription factors and chromatin remodeling factors (Wysocka et al., 2003; Yokoyama et al., 2004; Tyagi et al., 2007; Tyagi and Herr, 2009). To date, no studies have examined whether HCF-1 may be involved in modulating β-cell function.

Recently, HCF-1 was implicated to function as a novel repressor of FoxO transcription factors in mammals (Rizki et al., 2011). FoxO proteins are major downstream effectors of insulin/IGF-1 signaling and are known to affect pancreatic β-cell function. Genetic disruption of the insulin/IGF-1 signaling pathway leads to β-cell failure and the development of diabetes (Withers et al., 1998; Kulkarni et al., 1999; Kubota et al., 2000; Kulkarni et al., 2002; Xuan et al., 2002; Hashimoto et al.,

2006). Haploinsufficiency of FoxO1 restores β-cell proliferation and islet cell mass, and prevents the development of diabetes in both *Pdk1* and *IRS2* mutant mice (Kitamura et al., 2002; Hashimoto et al., 2006), indicating that the diabetic phenotype of these mice is largely due to FoxO1 activation. In β-cells, FoxO1 inhibits the expression of the pancreatic duodenal and homeobox 1 (Pdx1) gene, a critical transcription factor, essential for pancreatic development, β-cell differentiation and maintenance of β-cell function (Jonsson et al., 1994; Offield et al., 1996; Ahlgren et al., 1998; Brissova et al., 2002; Johnson et al., 2003). FoxO1 opposes FoxA2-mediated transcription of the *Pdx1* gene (Kitamura et al., 2002) and also represses Pdx1 activity by affecting Pdx1 nuclear translocation (Kitamura et al., 2002; Kawamori et al., 2006). Importantly, transgenic expression of *Pdx1* restores β-cell mass and function in diabetic *IRS-2* knockout mice (Kushner et al., 2002), similar to the effect of FoxO1 haploinsufficiency (Kitamura et al., 2002).

In addition to regulating FoxO transcription factors, HCF-1 acts as a co-activator of the cell cycle regulator E2F1, thus promoting G1-to-S phase progression (Tyagi et al., 2007). Recent studies also implicate E2F1 in the maintenance of pancreatic β-cell function. E2F1 null mice exhibit decreased pancreatic islet cell proliferation, impaired glucose-stimulated insulin secretion and reduced *Pdx1* expression (Fajas et al., 2004). E2F1 also acts as a transcriptional activator of the *Kir6.2* gene, which encodes a subunit of the ATP-sensitive potassium channel responsible for coupling membrane depolarization and insulin release to glucose metabolism (Annicotte et al., 2009).

Thus, we wondered whether HCF-1, which is known to interact with both FoxO and E2F1 transcription factors, may also be involved in regulating pancreatic β-cell function. In this study, we analyzed the function of HCF-1 in the INS-1 pancreatic β-cell line. Our analyses identified HCF-1 as a critical factor essential for β-cell survival and glucose-stimulated insulin secretion, and a co-activator of the *Pdx1* gene.

3.3 RESULTS

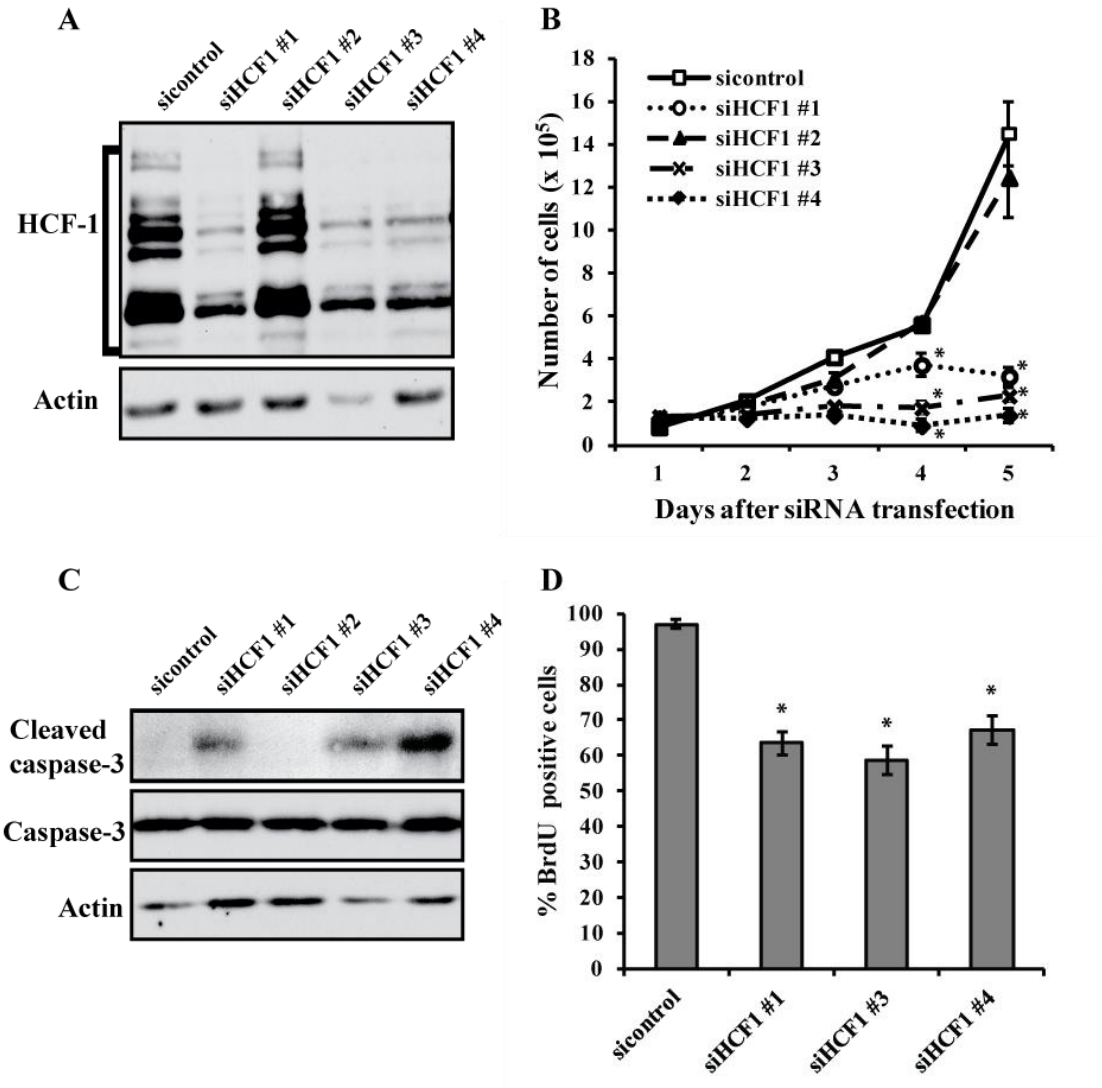
3.3.1 Depletion of HCF-1 increases apoptosis and reduces INS-1 β-cell proliferation

Previous studies have indicated the requirement for HCF-1 in cell cycle progression (Wilson et al., 1997; Julien and Herr, 2003). However, a role for HCF-1 specifically in pancreatic β-cells has not been established. We thus characterized the growth of INS-1 β-cells in which HCF-1 levels were reduced by siRNA. HCF-1 siRNA targeting four distinct regions (si#1, #2, #3, #4) of the *HCF-1* mRNA were transfected into INS-1 cells and cell numbers monitored over time. Of the four *HCF-1* siRNAs utilized, three exhibited effective knockdown of HCF-1 protein levels (si#1, #3, #4) (Figure 3.1A). Four days after siRNA transfection, these three *HCF-1* siRNA treated cell populations exhibited significantly less growth compared to control siRNA treated cells (Figure 3.1B). The single *HCF-1* siRNA (si#2) which did not reduce HCF-1 protein levels correspondingly did not produce a reduction in cell growth. The results indicate that knockdown of HCF-1 correlates with a reduction in β-cell population over time.

The reduced number of cells observed after *HCF-1* siRNA treatment could result from either increased apoptosis and/or reduced cell proliferation. To test whether apoptosis was increased in the *HCF-1* siRNA-treated cells, we examined caspase-3 cleavage. We observed elevated levels of cleaved caspase-3 in the cell lines treated with the same *HCF-1* siRNA duplexes that produced reductions in cell growth (si#1, #3, #4), while the single *HCF-1* siRNA (si#2) which did not affect HCF-1 protein levels or cell growth showed no activation of caspase-3 (Figure 3.1C). Thus, loss of HCF-1 reduces β-cell populations due to increased apoptosis. These results are consistent with our previous finding that knockdown of HCF-1 results in significant upregulation of the pro-apoptotic FoxO target gene, *Bim* (Rizki et al., 2011). We next tested whether HCF-1 is required for cell proliferation by analyzing BrdU incorporation in β-cells depleted of HCF-1. Using the three *HCF-1* siRNAs (si#1, #3, #4) which showed effective knockdown, we observed that *HCF-1* siRNA-treated cells had significantly less BrdU incorporated into their DNA (Figure 3.1D), indicating a failure in cell cycle progression, as has previously been shown in other mammalian cell types (Julien and Herr, 2003). Whether the cells that failed to progress through S phase are undergoing apoptosis or are in cell cycle arrest awaits further analysis. Our results suggest that HCF-1 is required for INS-1 β-cell growth by promoting both cell survival and cell proliferation.

Figure 3.1 HCF-1 is required for proliferation and survival of INS-1 β-cells.

(A) Western blot analysis of HCF-1 protein levels from INS-1 cells transfected twice with control or four different *HCF-1* siRNAs. (B) Cell growth curves of INS-1 cells treated with control or *HCF-1* siRNA. X-axis depicts days after the first siRNA transfection. Experiment was done twice with similar results. Data shown are from one representative experiment and expressed as mean +/- SEM for each time point. *denotes a p-value <0.05 relative to sicontrol (Student's t-test). (C) Western blot analysis showing caspase-3 activation in cells from (B), at four days after the first siRNA transfection. (D) BrdU incorporation assay of cells treated with *HCF-1* siRNA, three days after the first siRNA transfection. Experiment was performed twice with similar results. Data shown are from one representative experiment, and are expressed as the mean and 95% confidence interval. *denotes a p-value <0.001 relative to sicontrol (Chi-squared test). At least 400 cells per condition were counted.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B
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siHCF1 #3
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Number of cells (x 10^5)
Days after siRNA transfection
C
sicontrol
siHCF1 #1
siHCF1 #2
siHCF1 #3
siHCF1 #4
Cleaved caspase-3
Caspase-3
Actin
D
% BrdU positive cells
sicontrol
siHCF1 #1
siHCF1 #3
siHCF1 #4

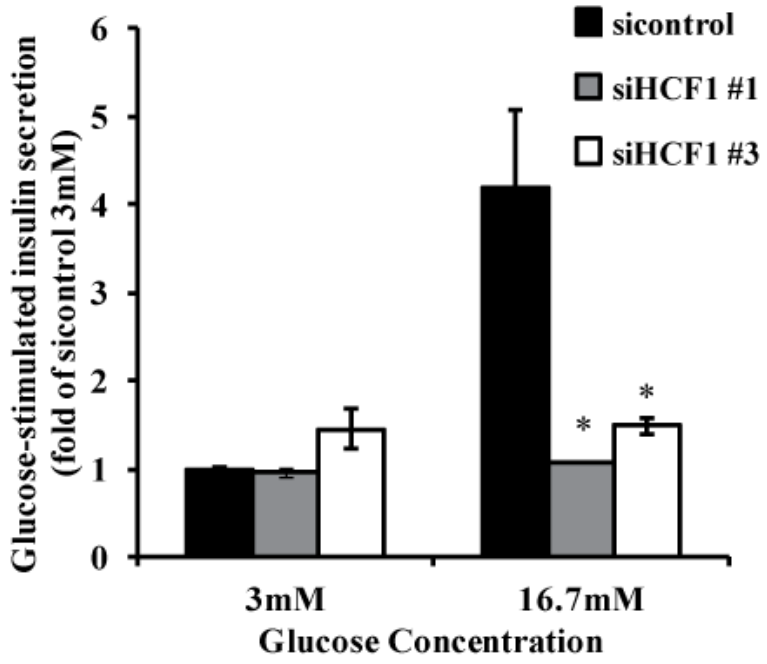
3.3.2 HCF-1 is required for glucose-stimulated insulin secretion in INS-1 β-cells

Given that HCF-1 is essential for survival and proliferation of INS-1 β-cells, we were interested in testing whether HCF-1 also affects these cells' functional ability to secrete insulin in response to glucose. INS-1 β-cells transfected with control siRNA exhibited a robust insulin secretion response when stimulated with high (16.7 mM) vs low (3mM) glucose, as determined by ELISA analysis. *HCF-1* siRNA-treated cells (si#1 and si#3), by contrast, did not increase insulin secretion in response to high glucose, indicating that HCF-1 is required for glucose-stimulated insulin secretion in the INS-1 β-cell model (Figure 3.2A). We then examined whether the reduced insulin secretion phenotype might arise from an overall reduction in insulin content in these cells. Indeed, analysis of the intracellular insulin content showed that HCF-1 knockdown cells have reduced levels of insulin (Figure 3.2B), indicating that the reduction in secreted insulin may be due to the overall decrease in the cellular insulin pool. However, since the reduction in intracellular insulin levels appeared modest, additional defects associated with insulin secretion likely also contribute to the greatly impaired ability of the *HCF-1* siRNA-treated cells to produce extracellular insulin in response to high glucose. To test whether overexpression of HCF-1 could result in increased insulin secretion, we took advantage of an inducible lentiviral overexpression vector to produce stable cell lines which overexpress HCF-1 in response to doxycycline (Figure 3.3A). However, we did not observe an enhanced insulin secretion phenotype in response to HCF-1 overexpression (Figure 3.3B). This result implies that, while HCF-1 is essential for appropriate insulin biosynthesis and secretion in response to glucose, the levels of HCF-1 in these cells is not limiting.

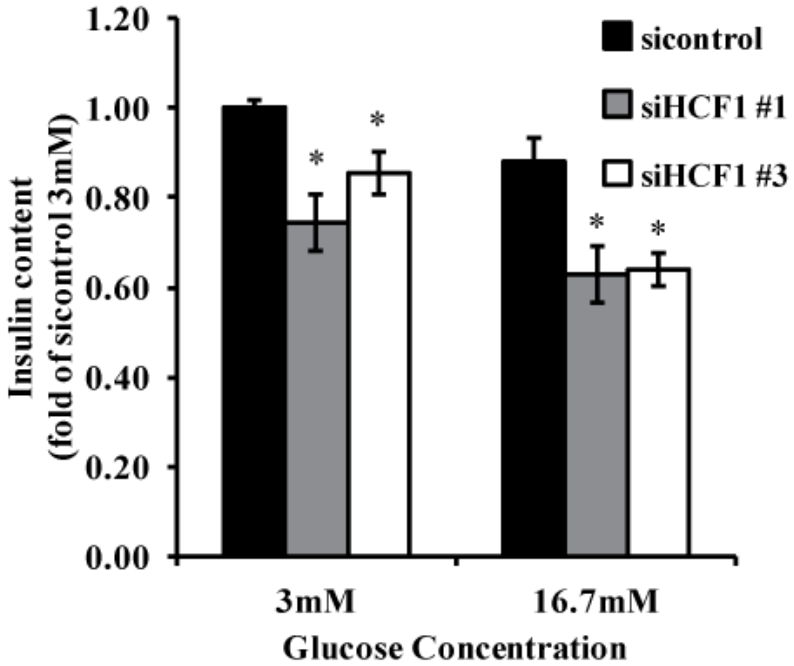
Figure 3.2 HCF-1 is required for insulin biosynthesis and secretion.

(A) Glucose-stimulated insulin secretion analysis of cells treated with *HCF-1* siRNA. Insulin secretion determined by insulin ELISA and normalized to total cellular protein. Results shown are pooled from three independent experiments, and represent mean +/- SEM. * denotes a p-value <0.05 relative to sicontrol (Student's t-test). (B) Cellular insulin content analysis of cells treated with *HCF-1* siRNA. Insulin content determined by insulin ELISA and normalized to total cellular protein. Results shown are pooled from three independent experiments, and represent mean +/- SEM. * denotes a p-value <0.05 relative to sicontrol (Student's t-test).

A


sicontrol
siHCF1 #1
siHCF1 #3
Glucose-stimulated insulin secretion (fold of sicontrol 3mM)
0
1
2
3
4
5
6
*
*
3mM
16.7mM
Glucose Concentration

B


sicontrol
siHCF1 #1
siHCF1 #3
Insulin content (fold of sicontrol 3mM)
0.00
0.20
0.40
0.60
0.80
1.00
1.20
*
*
*
*
3mM
16.7mM
Glucose Concentration

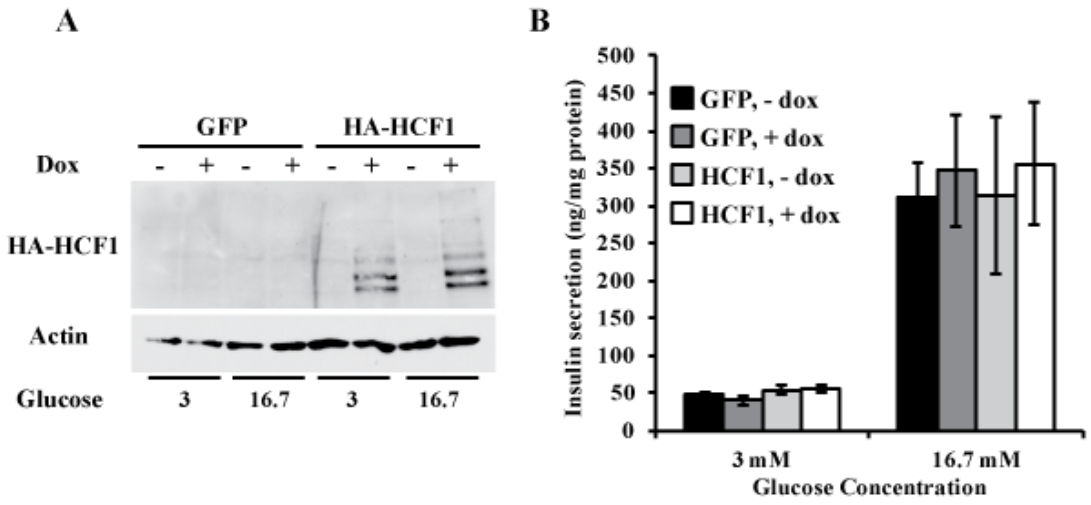


Figure 3.3 HCF-1 overexpression does not increase insulin secretion.

(A) Western blot analysis of HA-tagged HCF-1 in the inducible HCF-1 overexpression cell line. Cells were treated with vehicle or 200ng/ml of doxycycline for 24 hours. (B) Glucose-stimulated insulin secretion analysis of INS-1 cells with induced overexpression of GFP or HA-HCF1. Results shown are pooled from four independent experiments, and represent the mean +/- SEM.

3.3.3 HCF-1 physically interacts with FoxO1 and E2F1

C. elegans HCF1 and mammalian HCF-1 proteins are able to physically interact with FoxO transcription factors and repress FoxO transcriptional activity (Li et al., 2008a; Rizki et al., 2011). HCF-1 also acts as a co-activator of the E2F1 transcription factor (Tyagi et al., 2007). Loss of E2F1 (Fajas et al., 2004; Annicotte et al., 2009) or activation of FoxO1 (Kitamura et al., 2002; Hashimoto et al., 2006; Buteau et al., 2007) results in reduced β-cell proliferation and reduced glucose-stimulated insulin secretion. Therefore, we wondered whether HCF-1 affects β-cell phenotypes through interactions with FoxO1 or E2F1. We thus assessed whether HCF-1 physically associates with either FoxO1 or E2F1 in INS-1 cells. We induced overexpression of a doxycycline-inducible HA-tagged HCF-1 protein, and immunoprecipitated HCF-1 using HA-antibody conjugated beads. We were able to clearly detect both endogenous FoxO1, as well as endogenous E2F1, immunoprecipitating with HCF-1 (Figure 3.4). Therefore, in INS-1 β-cells HCF-1 is found in protein complexes with both FoxO1 and E2F1, suggesting that HCF-1 acts with FoxO1 and/or E2F1 to regulate β-cell function.

3.3.4 Pdx1 expression is reduced in HCF-1 knockdown cells

Both E2F1 and FoxO1 affect the expression of the β-cell transcription factor Pdx1, which is itself critical for the development and function of pancreatic β-cells. In mature β-cells, Pdx1 promotes insulin gene transcription and insulin secretion (Ohlsson et al., 1993; Brissova et al., 2002) and is required for β-cell survival (Johnson et al., 2003; Johnson et al., 2006) and β-cell proliferation in response to

systemic insulin resistance (Kulkarni et al., 2004). E2F1 inactivation reduces Pdx1 expression (Fajas et al., 2004), whereas FoxO1 activation represses Pdx1 expression (Kitamura et al., 2002; Buteau et al., 2007). As HCF-1 knockdown phenocopies Pdx1 inactivation, we wondered whether HCF-1 may in fact cooperate with E2F1 or FoxO1 to regulate expression of Pdx1. We therefore tested whether *Pdx-1* mRNA and protein levels were altered in cells with reduced HCF-1 levels. Indeed, we found that *HCF-1* siRNA-treated cells showed significant reductions in levels of Pdx1 protein (Figure 3.5A) and mRNA transcripts (Figure 3.5B). In addition, the Pdx1 target genes *Ins1* and *Ins2* also showed significantly reduced expression (Figure 3.5B), correlating well with our observation of reduced intracellular insulin levels in cells depleted of HCF-1 (Figure 3.2B). These results suggest that loss of HCF-1 leads to diminished Pdx1 activity through reductions in Pdx1 expression, which likely contribute to reduce proliferation, survival, and insulin secretion in β-cells.

3.3.5 HCF-1 and E2F1 are enriched at the *Pdx1* promoter

A well known function of HCF-1 is to regulate gene expression by assembling chromatin modifiers and transcription factors into appropriate complexes at target gene promoters (Wysocka et al., 2003; Tyagi et al., 2007). During the G1-to-S phase transition, HCF-1 associates with E2F1 at E2F1 target gene promoters, and recruits the MLL family of histone methyltransferases to promote expression of E2F1 target genes (Tyagi et al., 2007; Tyagi and Herr, 2009). HCF-1-mediated regulation of transcriptional activity can also occur through interactions with proteins away from target gene promoters. *C. elegans* HCF-1 represses the transcriptional activity of the

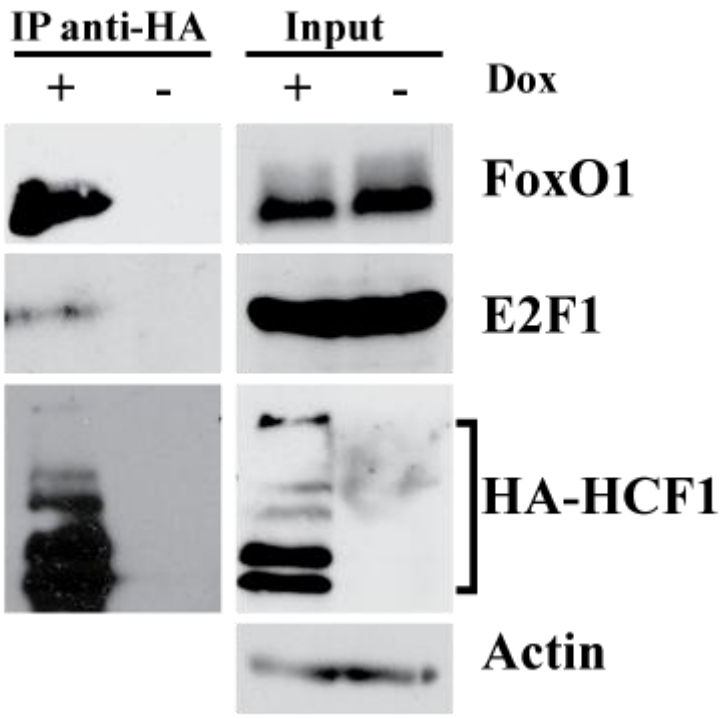
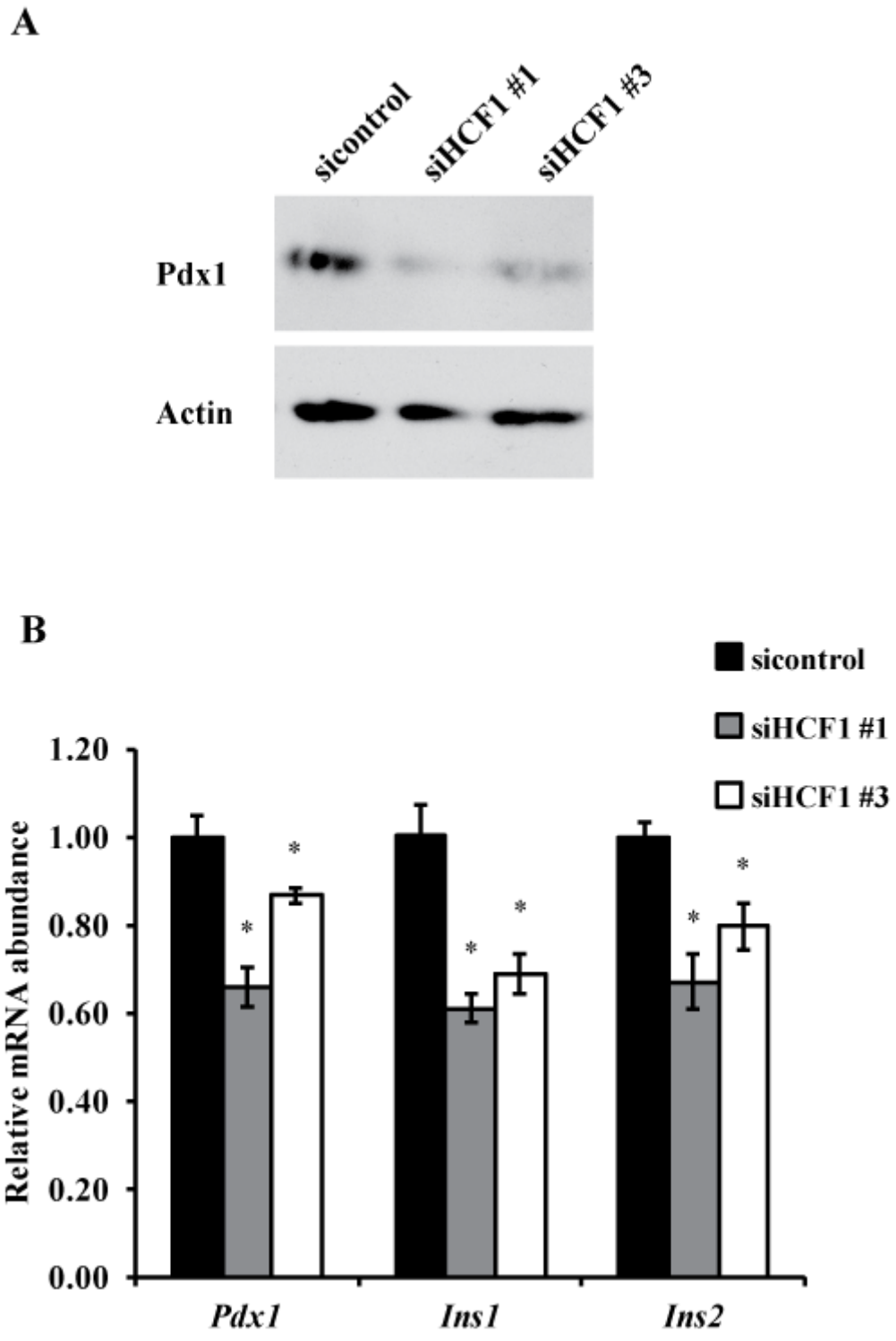


Figure 3.4 HCF-1 physically interacts with FoxO1 and E2F1.

INS-1 cells with stable integration of pInducer20-HA-HCF1 were treated with 1ug/ml doxycycline for 24 hours to induce expression of HA-HCF1. HA-HCF1 was immunoprecipitated with anti-HA-conjugated agarose beads, and analyzed by western blot with the indicated antibodies.

Figure 3.5 HCF-1 regulates expression of Pdx-1.

(A) Western blot analysis of Pdx-1 protein levels in HCF-1 knockdown cells. (B) RT-qPCR analysis of *Pdx1*, *Ins1* and *Ins2* transcript levels in HCF-1 knockdown cells. The mean normalized RNA level for each gene in sicontrol treated cells was set to 1. The data shown are pooled from three independent experiments and are represented as mean +/- SEM. * denotes a p-value <0.05 relative to cells treated with sicontrol siRNA (Student's t-test).


A
sicontrol
siHCF1 #1
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Pdx1
Actin
B
sicontrol
siHCF1 #1
siHCF1 #3
Relative mRNA abundance
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
Pdx1
Ins1
Ins2

C. elegans FoxO homolog by binding to it, thus preventing it from localizing to the promoters of its target genes (Li et al., 2008a). Given that HCF-1 forms a complex with both FoxO1 and E2F1 in β-cells (Figure 3.4), it is possible that HCF-1 affects the ability of either E2F1 or FoxO1 transcription factors to regulate expression of *Pdx1*. We therefore performed chromatin immunoprecipitation (ChIP) experiments to assess whether HCF-1 represses FoxO1 localization to the *Pdx1* promoter, or, alternatively, whether HCF-1 binds to the *Pdx1* promoter in association with E2F1.

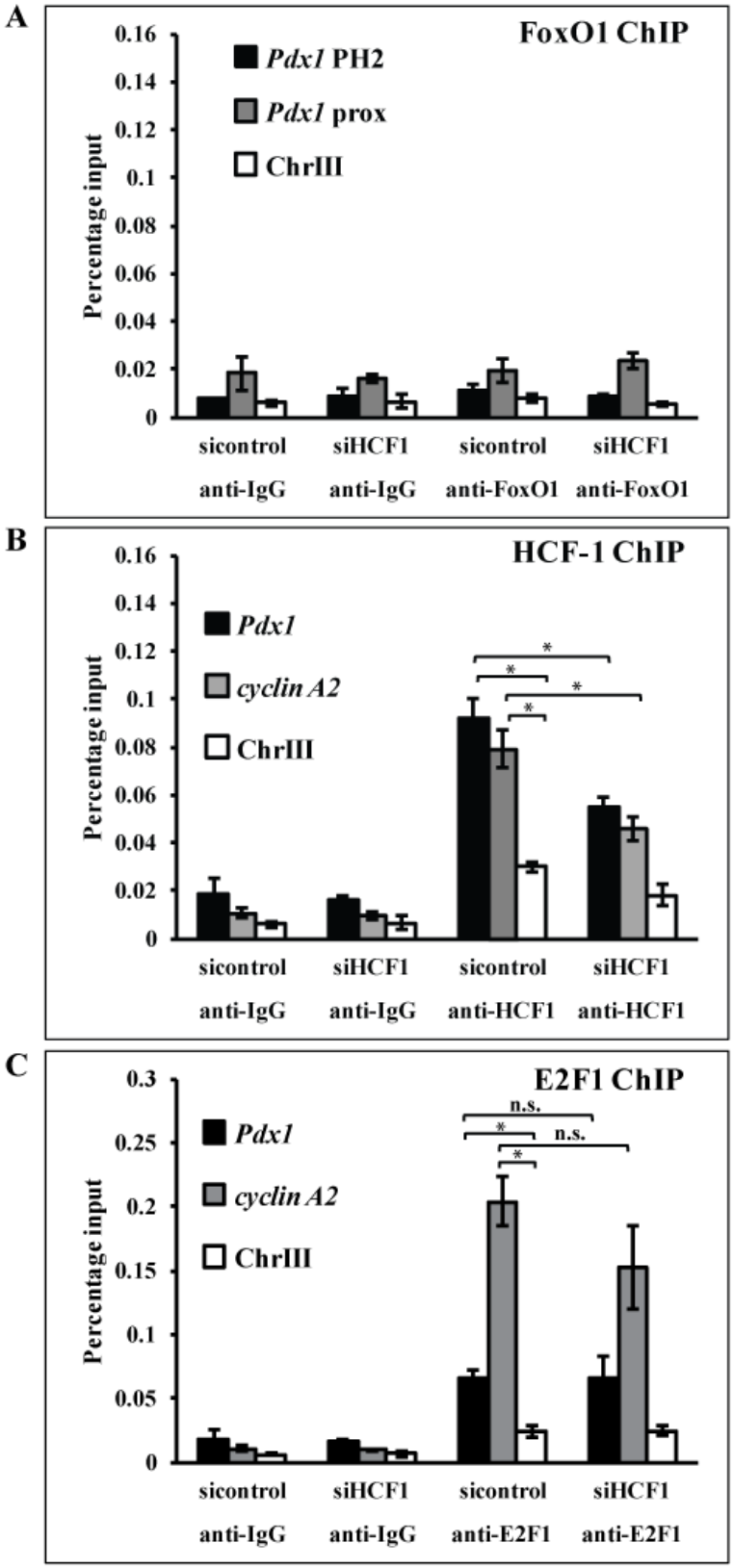
FoxO1 has been shown to compete with FoxA2 for binding to the consensus forkhead binding sequence located in the highly conserved Pdx1 homology 2 (PH2) region of the mouse *Pdx1* promoter (Kitamura et al., 2002). Examination of the proximal promoter region of the rat *Pdx1* promoter revealed an additional consensus forkhead binding sequence located 186 bp upstream of the transcription start site. We performed ChIP analysis to determine FoxO1 enrichment at both of these locations, with or without HCF-1 knockdown. As a negative control, we examined FoxO1 localization to a non-coding region of chromosome 3 which lacks FoxO binding sites. We did not detect enrichment of FoxO1 at any of these regions, either under basal or HCF-1 knockdown conditions, as compared to the isotype-matched IgG control (Figure 3.6A). These results indicate that FoxO1 binding is not increased by depletion of HCF-1. Therefore, HCF-1 likely does not inhibit FoxO1 transcriptional repression of *Pdx1*.

Next we examined whether HCF-1 and E2F1might be enriched at the *Pdx1* promoter. While E2F1 localization to the *Pdx1* promoter has not been previously examined, E2F1 is known to bind sites predominantly located in the proximal

promoter of its target genes, and near to CpG islands (Bieda et al., 2006). The proximal promoter of the rat *Pdx1* gene contains a highly conserved CpG island (Park et al., 2008) as well as a sequence (TTCGCGG) located 168 bp upstream of the transcription start site resembling the consensus E2F1 binding element (TTTXGCGC) (Tao et al., 1997). We therefore wondered whether an HCF-1/E2F1 complex might localize to the *Pdx1* promoter, thus promoting *Pdx1* expression. Interestingly, we found that HCF-1 enrichment at the *Pdx1* promoter was significantly higher than at the non-coding region of chromosome 3, and comparable to that observed at the known HCF-1/E2F1 target gene *cyclin A2* (Tyagi et al., 2007) (Figure 3.6B). *HCF-1* siRNA treatment reduced HCF-1 occupancy at both the *Pdx1* and *cyclin A2* promoters, indicating the specificity of HCF-1 binding at these promoters (Figure 3.6B). Examination of E2F1 promoter localization also revealed significant enrichment of E2F1 at both the *cyclin A2* promoter as well as at the *Pdx1* promoter, indicating that HCF-1 and E2F1 are both localized to the proximal promoter of *Pdx1* (Figure 3.6C). E2F1 enrichment, in contrast to HCF-1, was not significantly affected by HCF-1 knockdown (Figure 3.6C), consistent with previous studies indicating that HCF-1 is recruited by E2F1 to E2F1 target gene promoters (Tyagi et al., 2007). These results indicate that HCF-1 and E2F1 may indeed cooperate to promote *Pdx1* gene regulation. HCF-1 might generally promote E2F1 transcriptional activity in pancreatic β-cells. Consistent with this possibility, we also observe reduced expression of the E2F1 transcriptional target gene *Kir6.2* in HCF-1 knockdown cells (Figure 3.7).

Figure 3.6 HCF-1 occupies the *Pdx1* promoter

INS-1 cells treated with *HCF-1* siRNA or control siRNA were subjected to chromatin immunoprecipitation assays using antibodies directed against (A) HCF-1, (B) E2F1, (C) FoxO1 or isotype-matched rabbit IgG. Immunoprecipitated DNA was quantified using qPCR and calculated as percent of input DNA. Results shown are pooled from three independent experiments and represent the mean +/- SEM. FoxO1 was not enriched at either the PH2 region (*Pdx1* PH2) or at the proximal promoter region of *Pdx1* (*Pdx1* prox) relative to the IgG control. HCF-1 and E2F1 enrichment at the Pdx1 and cyclin A2 promoters was significantly greater than at the negative control region (chrIII) (* denotes a p-value <0.05, Student's t-test). Knockdown of HCF-1 reduced HCF-1 enrichment at the *Pdx1* and *cyclin A2* promoters whereas E2F1 enrichment was not affected by HCF-1 knockdown (n.s. = not significant). *Cyclin A2* is a known E2F1 target and was included as a control.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Pdx1 PH2
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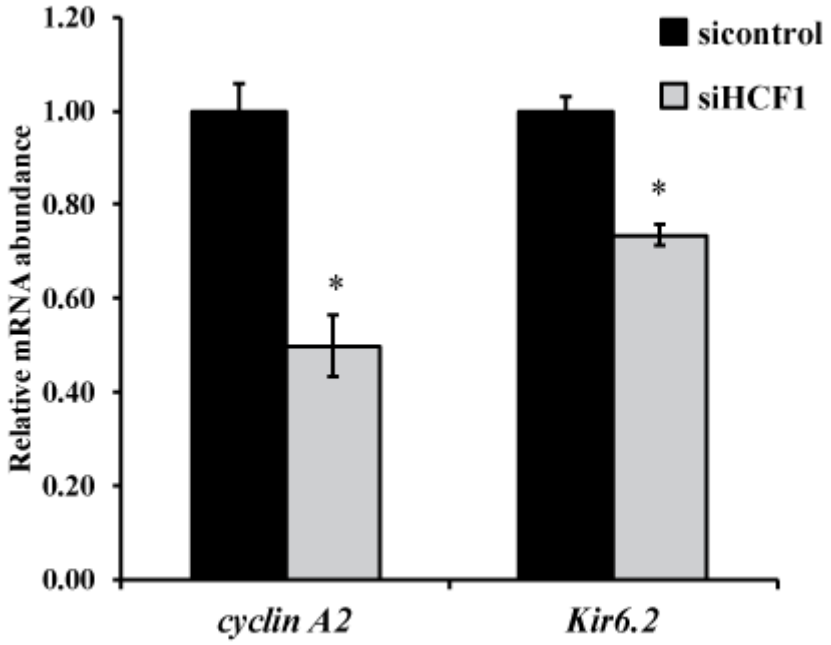


Figure 3.7 HCF-1 knockdown reduces expression of E2F1 target genes.

RT-qPCR analysis of *Kir6.2* and *cyclin A2* transcript levels in cells treated with *HCF-1* siRNA #1. The mean normalized RNA level for each gene in sicontrol treated cells was set to 1. The data shown are pooled from three independent experiments and are represented as mean +/- SEM. * denotes a p-value <0.05 relative to sicontrol (Student's t-test).

3.4 DISCUSSION

Here we have shown that the transcriptional co-regulator HCF-1 is required for survival, proliferation and glucose-stimulated insulin secretion in the INS-1 β-cell line. Previous studies have highlighted the importance of HCF-1 in regulating cell cycle progression (Wilson et al., 1997; Julien and Herr, 2003; Tyagi et al., 2007). We were, therefore, not surprised to observe reduced proliferation in INS-1 β-cells depleted of HCF-1. In addition to decreased cell proliferation, we also observe increased cell death resulting from HCF-1 knockdown. This is consistent with our previous finding that HCF-1 represses expression of the pro-apoptotic factor Bim (Rizki et al., 2011) which itself promotes pancreatic β-cell death (McKenzie et al., 2009; Santin et al., 2011). Thus, it is likely that loss of HCF-1 *in vivo* will result in reduced β-cell mass, a key feature of diabetes (Donath and Halban, 2004).

We have also identified the β-cell transcription factor Pdx1 as a transcriptional target of HCF-1. Pdx1 is required for maintenance of β-cell function by promoting insulin gene transcription and secretion (Ohlsson et al., 1993; Brissova et al., 2002), β-cell survival (Johnson et al., 2003; Johnson et al., 2006) and β-cell proliferation (Kulkarni et al., 2004). Loss of *Pdx1* expression results in reduced β-cell mass, reduced pancreatic insulin content, and reduced glucose-stimulated insulin secretion (Ahlgren et al., 1998; Brissova et al., 2002), which are phenotypes we observe with reduced HCF-1 expression. Importantly, human *PDX1* mutations are associated with the development of diabetes (Stoffers et al., 1997; Stoffers et al., 1998; Macfarlane et al., 1999). Thus, as a co-activator of *Pdx1*, and as a repressor of *Bim* gene expression,

HCF-1 represents a novel β-cell factor implicated in affecting diabetes development and progression.

We have previously identified HCF-1 as a transcriptional co-repressor of FoxO transcription factors (Salih and Brunet, 2008; Rizki et al., 2011). As a major downstream effector of insulin-signaling, FoxO1 prevents β-cell proliferation under reduced insulin-signaling conditions by repressing transcription of *Pdx1* (Kitamura et al., 2002). Consistent with a role for HCF-1 as a transcriptional co-repressor of FoxO1, we observe physical interactions between HCF-1 and FoxO1, and decreased levels of *Pdx1* when HCF-1 is depleted. However, as we did not observe increased occupancy of the *Pdx1* promoter by FoxO1 when HCF-1 is depleted, mammalian HCF-1 likely modulates *Pdx1* expression through a FoxO1-independent mechanism. Future studies examining global FoxO chromatin localization in the absence of HCF-1 will be critical for determining which specific subset of FoxO target genes may be co-repressed by HCF-1.

A well-established role of HCF-1 is to promote E2F1-mediated transcription to affect cell proliferation (Tyagi et al., 2007). Recent studies have highlighted a role for E2F1 in pancreatic β-cell function *in vivo*. E2F1 null mice have reduced expression of *Pdx1*, reduced pancreatic β-cell mass and reduced pancreatic insulin content (Fajas et al., 2004). E2F1 also regulates insulin secretion through promoting the expression of the K_{ATP} channel, *Kir6.2*, involved in glucose-stimulated insulin secretion (Annicotte et al., 2009). Our data suggest HCF-1 likely modulates pancreatic β-cell function by regulating the expression of multiple critical E2F1 target genes involved in distinct aspects of β-cell function. Future global analysis to identify the transcriptional targets

commonly regulated by HCF-1 and E2F1 will reveal the key downstream mediators likely contributing to the common phenotypes observed in HCF-1- and E2F1-deficient β-cells.

In conclusion, we have identified a role for transcriptional co-regulator HCF-1 as an essential regulator of *Pdx1* expression, with consequences to β-cell growth, survival and glucose-stimulated insulin secretion. Thus, HCF-1 represents a novel target for future therapies aimed at treating and preventing the progression of diabetes.

3.5 MATERIALS AND METHODS

3.5.1 Cell culture, siRNA transfection and immunoblotting

INS-1 cells were maintained and transfected with siRNA as previously described, with some modifications (Rizki et al., 2011). In brief, INS-1 cells were transfected twice, one day apart, with 5nM siRNA using Lipofectamine RNAiMax per the manufacturer's protocol. siRNA duplexes directed against rat *HCF-1* were purchased from Dharmacon and targeted the following sequences: ; siHCF-1 #1: 5'-AGAACAACATTCCGAGGTA-3'; siHCF-1 #2: 5'-GCTTATAAATTTCGAGTTG-3; siHCF-1 #3 5'-CGGCAAGATTATCGAGTAC-3'; siHCF-1 #4: 5'-GGAAGAGACTGAAGGCAAA-3'. Non-targeting control siRNA was also from Dharmacon. Preparation of cell lysates and immunoblotting were performed as described previously (Rizki et al., 2011). The following antibodies were used: HCF-1 (Bethyl Labs), β-actin (Millipore), Pdx1, total caspase-3, cleaved caspase-3, FoxO1 (Cell signaling), E2F1 (Santa Cruz) and HA (Covance).

3.5.2 Cell growth curve and caspase-3 cleavage analysis

10^5 cells were seeded in each well of a 6-well plate at the time of the first siRNA transfection. 3 wells per condition were counted using the trypan blue exclusion method. Cells were lysed and western blot analysis was done using total caspase-3 and cleaved caspase-3 antibody.

3.5.3 BrdU incorporation assay

10^5 cells were seeded in each well of a 6-well plate containing a glass coverslip at the time of the first siRNA transfection. Three days after the first siRNA transfection, cells were incubated in media containing 10 μM BrdU (Sigma). The following day, cells were fixed and stained with anti-HCF-1 and anti-BrdU antibody (Julien and Herr, 2003). Cells were fixed in 2% paraformaldehyde, permeabilized with 0.5% TritonX-100, and blocked for 20 minutes in 3% BSA. Coverslips were incubated with anti-HCF-1 antibody (Bethyl Labs) for 1 hour, washed and incubated with cy3-conjugated anti-rabbit IgG for 30 minutes, fixed and DNA denatured with 4N HCl for 20 minutes, and incubated with FITC-conjugated anti-BrdU (BD Biosciences) antibody. Nuclei were stained with DAPI. Coverslips were mounted on glass slides using Vectashield (Vector Labs). Coverslips were examined and pictures obtained on a Leica DM 5000B fluorescent microscope. At least 400 cells were counted per siRNA treatment.

3.5.4 Reverse-transcription coupled quantitative PCR (RT-qPCR):

RNA was isolated from INS-1 cells treated with the indicated siRNAs using Trizol reagent and was reverse-transcribed using Superscript III First-Strand kit (Invitrogen). cDNAs were analyzed by quantitative-PCR using the SYBR Green system on a Roche LightCycler 480 real time PCR machine and quantified relative to a standard curve. *β-actin* was used as an internal control. Primer sequences can be found in Table 3.1.

3.5.5 Glucose-stimulated insulin secretion and intracellular insulin content analysis

10^6 INS-1 cells were sequentially transfected with sicontrol or *HCF-1* siRNA in 10 cm plates. One day after the second siRNA transfection, cells were trypsinized and seeded at 10^6 cells per well in 24-well plates in RPMI medium containing 5mM glucose. The following day, cells were washed once with warm KRB buffer (119mM NaCl, 4.74mM KCl, 2.54mM Ca Cl_2, 1.19mM $MgSO_4$, 1.19mM KH_2PO_4, 25mM $NaHCO_3$, 10mM HEPES and 0.2% fatty acid free BSA) and incubated for one hour in 0.5mL KRB buffer at 37°C. Cells were then incubated in 0.5mL of KRB buffer containing either 3mM or 16.7mM glucose for 1 hour at at 37°C. Supernatant was collected and insulin measured using a rat/mouse insulin ELISA kit (Millipore). Cells were lysed in cell lysis buffer or incubated with acifidied ethanol (75% ethanol, 1.5% HCl) overnight at -20°C to measure intracellular insulin content. Insulin measurements were normalized to cellular protein content as determined by Bradford assay. In the case of HCF-1 overexpression, cells with stable integration of

pInducer20-HA-HCF1 were treated with 200ng/ml doxycycline for 24 hours before being subjected to the glucose-stimulated insulin secretion assay.

3.5.6 Co-Immunoprecipitation Assay

INS-1 cells were infected and selected for stable integration of pInducer20-HA-HCF1, as previously described (Meerbrey et al., 2011). Stable cells were treated with 1ug/ml of doxycycline for 24 hours to induce expression of HA-HCF1. Cells were collected, and lysed in buffer containing 50mM Tris-HCl pH 8.0, 100mM NaCl, 2mM EDTA, 1% TritonX-100, 10mM NaF, 1mM sodium orthovanadate, 1mM PMSF, 10mM nicotinamide, 1mM trichostatin A, and protease inhibitors. Cell extracts were incubated HA-conjugated agarose beads (Sigma) overnight at 4°C. Beads were washed five times in lysis buffer and eluted with HA-peptide (Sigma). Immunoprecipitates were analyzed by western blotting using the following antibodies: anti-HA (Covance), anti-FoxO1 (Cell Signaling), anti-E2F1 (Santa Cruz).

3.5.7 Chromatin Immunoprecipitation (ChIP) Assays

1.5 x 10^6 INS-1 cells were transfected with siRNA in 10cm plates as described above. Two days after the second siRNA transfection, cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature. Glycine was added to a final concentration of 125mM for 5 minutes. Cells were washed with cold PBS and lysed in buffer containing 25mM HEPES, 1.5mM MgCl2, 10mM KCl, 0.5% NP40, 1mM DTT and protease inhibitors. Nuclei were pelleted and lysed in buffer containing 50mM HEPES, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium

deoxycholate, and protease inhibitors, and sonicated using a Diogenode Bioruptor. Chromatin was immunoprecipitated overnight using antibodies against HCF-1 (Bethyl labs A301-399A), E2F1 (Santa Cruz, C-20) or FoxO1 (Santa Cruz, H-128) and recovered with protein A agarose resin (Thermo Scientific). The protein A resin was washed, immunoprecipitated complexes eluted, and crosslinks reversed. DNA was purified using Qiagen PCR purification kit, and assayed by quantitative PCR. Primer sequences can be found in Table 3.1.

3.5.8 Data Analysis

Data are represented as mean +/- SEM and analyzed by the Student's t-test, except for the BrdU incorporation assay, which is represented as mean and 95% confidence interval and analyzed by Chi-squared test. A p-value of less than 0.05 was considered to be statistically significant.

3.6 ACKNOWLEDGEMENTS

We thank L. Qi for providing the INS-1 cells; S. Elledge for the pInducer lentiviral vector; members of the Lee lab and Cornell AIMS group for helpful comments and suggestions.

Table 3.1-PCR primers

RT-qPCR Primers	**Sequence**
β-actin forward	CTAAGGCCAACCGTGAAAAG
β-actin reverse	AACACAGCCTGGATGGCTAC
HCF-1 forward	GCTGGAAAAGCTCCTGTCAC
HCF-1 reverse	CACTCATCTGTGGGTTGCTG
Pdx1 forward	GAGAATCAGGCTCCCAACTG
Pdx1 reverse	GGGACCGCTCAAGTTTGTAA
Ins1 forward	TACAATCATAGACCATCAGCAAGC
Ins1 reverse	ACCAGGTGAGGACCACAAAG
Ins2 forward	GAAGTGGAGGACCCACAAGT
Ins2 reverse	CAGTGCCAAGGTCTGAAGGT
Kir6.2 forward	CCTCCTATCTGGCTGACGAG
Kir6.2 reverse	GTGGGCACTTTAACGGTGTT

ChIP Primers	**Sequence**
(Pdx1) PH2 forward	TCCCTCAAGTTTTCGCTCAG
(Pdx1) PH2 reverse	CTGGGCTTTGCAAAAAGAAA
Pdx1 (prox) forward	GCTCATTAGGAGCGGTTTTG
Pdx1 (prox) reverse	GCTGGCGAGAGACAGAGAAG
cyclin A2 forward	CCCTCATTGGTCCATTTCAA
cyclin A2 reverse	GCCGGCTGTTCTTGTAGTTC
ChrIII forward	GCTCATTGGCTGAACACTCA
ChrIII reverse	AGGCCCCTGTCAAACTACCT

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

The utility of genetic model organisms in helping to demystify complex biological phenomena is clear. As a result of experiments done in a simple roundworm, our understanding of the aging process has broadened tremendously. The discoveries made in *C. elegans* are extremely relevant to humans as we observe highly conserved mechanisms of longevity determination between worms and mammals. Most importantly, these findings have promoted the development of therapeutics aimed at preventing or delaying the onset of age-related diseases in people (Yang and Hung, 2009; Blum et al., 2011). My research, which stems from the finding that HCF-1 acts in *C. elegans* longevity determination, shows that mammalian HCF-1 plays critical roles in regulating cellular proliferation, surivival and metabolism and is involved in a regulatory network involving the sirtuin SIRT1 and the FoxO transcription factors. These interactions are highly conserved between *C. elegans* and mammals (Rizki et al., 2011), again highlighting the importance of HCF-1 as an evolutionarily conserved modulator of putative longevity determinants.

Many studies have focused on the various cytosolic signaling pathways that respond to extracellular signals and converge on FoxO to modulate cellular responses. However, relatively few studies have examined in depth the nuclear regulation of FoxO transcriptional activity. My results are the first to implicate mammalian HCF proteins as conserved nuclear co-repressors of FoxO, with consequences to FoxO

target genes involved in apoptosis, cell cycle arrest and metabolism. These findings point to a novel nuclear FoxO/HCF-1 regulatory complex responsible for determining cellular fates, with direct implications to mammalian cancer biology, metabolism and aging.

By identifying HCF-1 as a novel target of sirtuin-mediated deacetylation, I have also shed light on a novel actor likely to be integral in mediating sirtuin-dependent cellular processes, particularly those sensitive to energy availability. Given their dependence on cellular NAD^+ levels, sirtuins are sensitive to cellular redox status and energy status. NAD^+ acts as a co-factor for glycolytic redox reactions, the Krebs cycle, the catabolism of macromolecules, as well as a number of other cellular processes. Thus the pool of NAD^+ available for sirtuin-mediated enzymatic reactions is limited (Imai and Guarente, 2010). NAD^+ biosynthesis involves the enzyme nicotinamide phosphoribosyltransferase (NAMPT), which is regulated by ATP levels through AMP-kinase. Thus, under low energy conditions, AMPK activation leads to increased NAD^+ levels and increased SIRT1 activity (Imai and Guarente, 2010), mirroring the effect of AMPK on FoxO activation, reviewed in Chapter 1. The likely outcome of SIRT1 activation under low energy conditions would be increased HCF-1 deacetylation. My results suggest, then, that SIRT1 deacetylation of HCF-1 may lead to inactivation of HCF-1, further enhancing FoxO activity under low energy states.

Furthermore, the effect of SIRT1-mediated deacetylation of HCF-1 may extend to other processes regulated by HCF-1, independent of FoxO. As HCF-1 is known to be involved in cell proliferation, it is tempting to think that HCF-1 may act as a nutrient sensor, linking cellular energy levels to cellular proliferation. Under low

energy states, HCF-1 may be inhibited by SIRT1-mediated deacetylation. Conversely, as glucose stimulates the production of ATP, elevated glucose concentrations would inhibit SIRT1-mediated deacetylation of HCF-1, resulting in more acetylated HCF-1, which might then act to promote the transcription of genes involved in cell growth. In support of this hypothesis, I observe that increasing concentrations of glucose increases HCF-1 acetylation (Figure AIII). Future studies directly assessing the role that acetylation plays in modulating HCF-1 activity will be critical to further our understanding of how HCF-1 regulates key cellular processes.

My analyses have identified not only that sirtuins promote deacetylation of HCF-1, but that they do so by targeting lysines found within the basic region of HCF-1. Interestingly, HCF-1 proteolysis is regulated by the glucose-sensitive *O*-linked-β-*N*-acetylglucosaminyl transferase (OGT) protein (Capotosti et al., 2011; Daou et al., 2011). OGT modifies HCF-1 by the addition of *O*-GlcNac moieties on residues located in and around the basic region (Wang et al., 2007b; Capotosti et al., 2011). Given that post-translational modifications may interact to direct the functional activity of target proteins, it is possible that *O*-GlcNacylation and deacetylation of HCF-1 crosstalk to regulate HCF-1 proteolysis and activity. Future studies will help elucidate how post-translational modifications of HCF-1 may alter HCF-1 function in response to specific environmental signals.

In the context of pancreatic β-cells, my work is the first to show that HCF-1 is required for β-cell proliferation, survival and glucose-stimulated insulin secretion. I have found that HCF-1, likely in cooperation with E2F1, promotes the expression of Pdx1, a critical transcription factor with major roles in pancreatic β-cell

differentiation, growth, survival, insulin synthesis and glucose-stimulated insulin secretion. These findings clearly implicate HCF-1 as a novel factor involved in maintenance of pancreatic β-cell function with direct implications to diabetes development and progression.

As discussed, HCF-1 may be critically involved in responding to energy availability by promoting cell survival and cell growth. Given the dependence of insulin secretion on glucose concentrations, and given the likely role that HCF-1 plays in responding to cellular energy levels through SIRT1-mediated deacetylation, this leads to the intriguing possibility that SIRT1 interactions with HCF-1 may alter glucose-mediated insulin secretion from pancreatic β-cells. SIRT1 is already known to be involved in pancreatic insulin secretion (Moynihan et al., 2005; Bordone et al., 2006), suggesting that SIRT1 and HCF-1 may interact to affect pancreatic β-cell function as well. Future studies looking directly at HCF-1 acetylation in the context of insulin secretion will thus be of great interest. So too will studies examining the *in vivo* consequences of altered HCF-1 activity on pancreatic β-cell function.

In addition to a role in pancreatic β-cells, my preliminary experiments in pre-adipocytes and mouse embryonic stem cell lines, discussed in Appendix I and Appendix II, suggest that HCF-1 and HCF-2 play important roles in these cell types as well. A more extensive analysis of cells, and possibly mice, with altered activities of HCF-1 and HCF-2 will provide valuable insight into the cell- and tissue-specific functions of these factors.

Ultimately, as IIS and FoxO factors are believed to affect longevity in mammals, it will be of great interest to perform lifespan analyses with mice in which

HCF levels have been altered. Given that HCF-1 is critical for promoting cell cycle proliferation and glucose stimulated insulin secretion in cell culture systems, it is likely that whole body ablation of HCF-1 will result in non-viable and/or diabetic animals that are unlikely to live prolonged lives. However, in mammals as well as in invertebrates, the IIS pathway and FoxO transcription factors appear to act in specific tissues, most notably adipose and neuronal tissues, to confer lifespan extension (Bluher et al., 2003; Libina et al., 2003; Giannakou et al., 2004; Broughton et al., 2005; Taguchi et al., 2007). This suggests, then, that altering HCF-1 function in brain and adipose tissue alone may be sufficient to promote longevity.

In conclusion, my studies are the first to a) establish a role for HCF-1 in repressing mammalian FoxO activity, b) identify HCF-1 as a novel target deacetylated by sirtuins, and c) provide evidence for HCF-1's essential role in regulating pancreatic β-cell function and glucose homeostasis. With the development of additional reagents to study the *in vitro* and *in vivo* functions of mammalian HCF-1, we will be able to dissect in detail the molecular mechanisms that govern the activity of this essential transcriptional regulator, and gain insight into possible methods of targeting HCF-1 to prevent metabolic and age-associated diseases, and to prolong healthy lives.

APPENDIX I

INVESTIGATING THE ROLE OF HCF-1 AND HCF-2 IN ADIPOCYTE DIFFERENTIATION

In addition to roles in pancreatic β-cells, FoxO1 also regulates the differentiation of adipocytes (Nakae et al., 2003). As we have shown that mammalian HCF-1 and HCF-2 functionally and physically interact with FoxO proteins (Rizki et al., 2011), we sought to determine whether HCF-1 may also be involved in the regulation of adipocyte differentiation.

During adipogenesis, a cascade of transcriptional activation mediated by the transcription factors peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs) leads to the differentiation of fibroblast-like preadipocytes into lipid-filled adipocytes (Lefterova and Lazar, 2009). An early increase in the expression of C/EBPβ and C/EBPδ during adiopcyte differentiation drives the induction of *PPARγ* and C/*EBPα* (Cristancho and Lazar, 2011). PPARγ expression is further upregulated by auto-regulation of its own promoter, and through continued transcriptional activation by C/EBPα and C/EBPδ (Cristancho and Lazar, 2011). FoxO1 inhibits adipocyte differentiation by repressing the transcription of the *PPARγ* gene (Armoni et al., 2006), as well as by inhibiting the transcriptional activity of PPARγ itself (Dowell et al., 2003; Fan et al., 2009). Expression of constitutively active FoxO1 prevents adipogenesis (Nakae et al., 2003), whereas haploinsufficiency of FoxO1 results in increased adipose PPARγ activity (Kim et al., 2009). We

hypothesized that HCF proteins repress FoxO1 in adipocytes, thereby acting to promote adipogenesis.

To determine whether HCF proteins are involved in regulating adipocyte differentiation, we utilized the 3T3-L1 cell model of adipogenesis (Green and Kehinde, 1975). During adipocyte differentiation, we observe upregulation of both *HCF-1* and *HCF-2* expression (Figure AI.1). Using retroviral-mediated shRNA to knockdown *HCF-1* or *HCF-2*, we found that reducing HCF-1 resulted in decreased expression of *PPARγ* (Figure AI.2A), whereas HCF-2 knockdown results in increased *PPARγ* expression (Figure AI.2A). These observations suggest that HCF-1 may indeed play a role in repressing FoxO1 as reducing HCF-1 expression may lead to increased activation of FoxO1 and subsequent downregulation of the FoxO1 target, *PPARγ*. In contrast, HCF-2 knockdown results in upregulation of *PPARγ*, suggesting that HCF-1 and HCF-2 play divergent roles in adipocytes. Future analysis of HCF-1 and HCF-2 in adipocytes will help to better define the role of HCF proteins in adipogenesis.

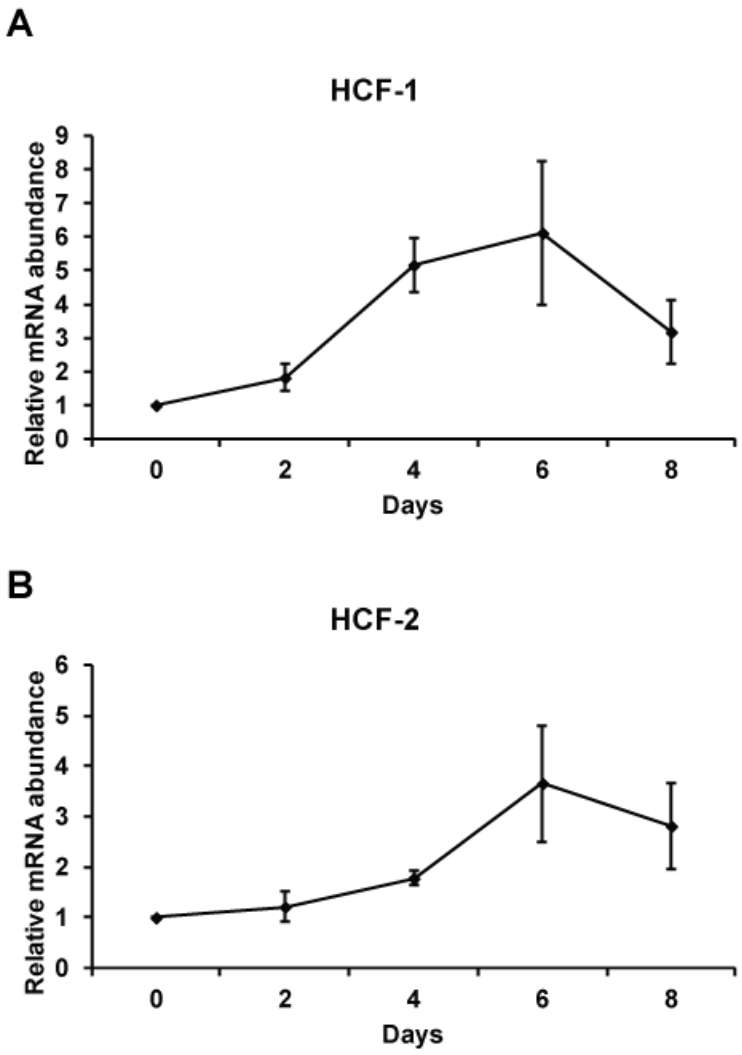
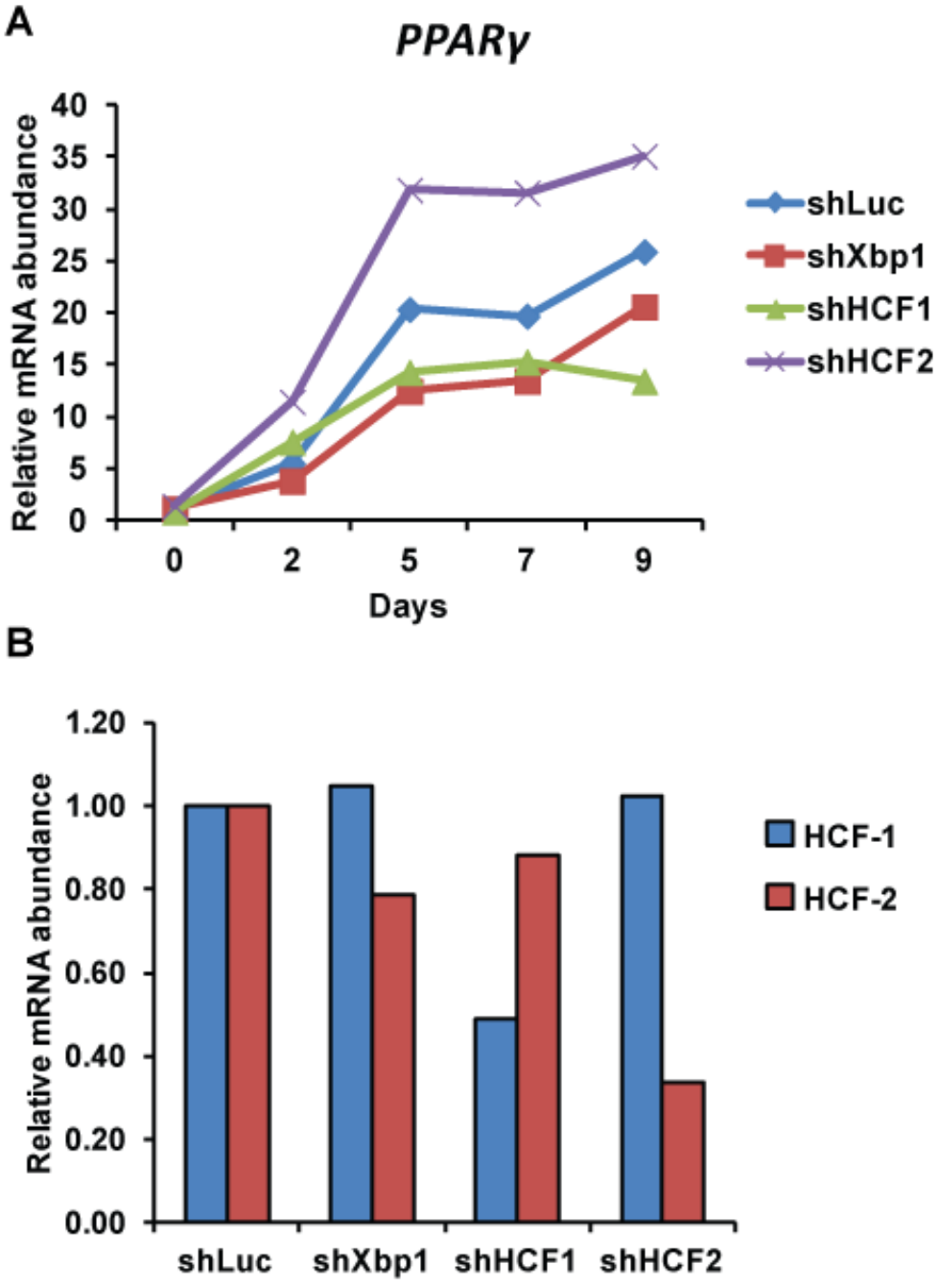


Figure AI.1 *HCF-1* and *HCF-2* expression is upregulated during adipogenesis. 3T3-L1 preadipocytes were induced to differentiate with a cocktail of 3-isobutyl-1-methylxanthine, dexamethasone and insulin on day 0. mRNA levels of *HCF-1* (A) and *HCF-2* (B) were analyzed at the indicated timepoints. Values are normalized to the level of the ribosomal protein transcript, *L32*. The mean normalized mRNA level for each gene at day 0 was set to 1. The data represented are pooled from three independent experiments.

Figure AI.2 HCF-1 and HCF-2 regulate expression of the FoxO1 target gene, *PPARγ*, in a divergent manner.

3T3-L1 preadipocytes were infected with retroviral shRNA vectors targeting the *luciferase*, *Xbp1*, *HCF-1* or *HCF-2* genes. shLuc served as a negative control, while shXbp1 served as a positive control for reduced *PPARγ* gene expression (Sha et al., 2009). (A) mRNA levels of *PPARγ* over the course of adipocyte differentiation. (B) mRNA levels of *HCF*-1 and *HCF*-2 in the indicated cell lines. Values are normalized to the level of the ribosomal protein transcript, *L32*. The mean normalized mRNA level for each gene at day 0 was set to 1. Experiment was conducted at least three times with similar results. Data shown are from one representative experiment.


A
PPARγ
Relative mRNA abundance
40
35
30
25
20
15
10
5
0
0
2
5
7
9
Days
shLuc
shXbp1
shHCF1
shHCF2
B
Relative mRNA abundance
1.20
1.00
0.80
0.60
0.40
0.20
0.00
shLuc
shXbp1
shHCF1
shHCF2
HCF-1
HCF-2

APPENDIX II

EXAMINATION OF HCF-1 AND HCF-2 IN MOUSE EMBRYONIC STEM CELLS

Recent studies in mammalian systems suggest that aging is influenced by stem cells, and the functional decline in stem cells with age can contribute to pathological disease conditions (Sharpless and DePinho, 2007; Rossi et al., 2008). For instance, hematopoietic stem cells (HSCs), the best-characterized of adult stem cells, are known to undergo age-dependent changes. A decline in HSC function leads to disease conditions that manifest in older individuals, such as immunodeficiency, neoplasia, and anemia (Sharpless and DePinho, 2007). Other stem cell compartments are similarly affected by aging. One hypothesis for this decline in stem cell function is that reactive oxygen species (ROS)-induced damage can impair stem cell self-renewal (Rossi et al., 2008). Support for this theory comes from studies indicating that FoxO proteins function to maintain the hematopoietic stem cell, as well as the neural stem cell pool by affecting ROS levels (Miyamoto et al., 2007; Tothova et al., 2007; Paik et al., 2009). A conditional FoxO1/3/4-null mouse model exhibited defects in HSC quiescence and self-renewal, and increased levels of endogenous ROS (Tothova et al., 2007). Mice with a homozygous deletion of the *FoxO3* gene also showed elevated ROS levels in HSC, and defects in the maintenance of the quiescent state (Miyamoto et al., 2007). In neural stem cells, combined loss of FoxO1, FoxO3 and FoxO4 resulted in increased ROS levels, decreased cell proliferation, and hence a reduced

neural stem cell pool (Paik et al., 2009). SIRT1 has also been implicated to be essential for hematopoietic stem cell differentiation (Ou et al., 2011; Matsui et al., 2012) suggesting that HCF-1, which interacts with both FoxO and SIRT1, may also be involved in maintaining adult stem cell function.

In addition, both FoxO and SIRT1 play critical roles in embryonic stem cells (ESCs). ESCs exhibit the key properties of self-renewal capacity and the potential to differentiate into any cell type in the body. Thus, pluripotent ESCs are an attractive potential agent for the treatment of age-dependent diseases. FoxO1 functions to maintain pluripotency of human and mouse embryonic stem cells by promoting the expression of the pluripotency factors *OCT4* and *SOX2* (Zhang et al., 2011b). Recent studies indicate that SIRT1 is a regulator of ESC differentiation and responses to oxidative stress. SIRT1, in contrast to FoxO1, regulates p53 activity in ESC to inhibit differentiation and induce apoptosis under increased ROS conditions (Han et al., 2008). These studies imply that factors involved in lifespan modulation also play a role in both adult and embryonic stem cell function. Given the importance of stem cell function in tissue homeostasis and implications for therapies targeting stem cells, identification of additional factors involved in stem cell maintenance is of great interest.

Recently, HCF-1 has also been implicated in ESC regulation through a novel stem cell factor, Ronin. Ronin is required to maintain ESC pluripotency, and Ronin associates with HCF-1 and other chromatin modifiers in ESCs (Dejosez et al., 2008). Furthermore, HCF-1 and Ronin occupy the promoters of many shared genes in ESCs (Dejosez et al., 2010). Precisely how Ronin interacts with HCF-1 to affect

pluripotency of ESCs is still unclear. HCF-1 is well-known to be critical for the progression of multiple phases of the cell cycle (Julien and Herr, 2003, 2004; Tyagi et al., 2007). HCF-1 can also recruit chromatin modifiers to target gene promoters of specific E2F transcription factors that regulate cell cycle progression (Tyagi et al., 2007). E2F1 is also associated with increased expression of genes specifically upregulated in pluripotent embryonic stem cells, and is important for maintaining the rapid cell cycling of ESCs (Stead et al., 2002; Chen et al., 2008b). Thus, HCF-1 may be involved in regulating ESC function through interactions with FoxO1, SIRT1 and/or E2F1.

To assess whether HCF-1 proteins may be involved in regulating ESC pluripotency and self-renewal, we first analyzed the expression of HCF-1 and HCF-2 in ESCs during differentiation. Mouse ESCs can be maintained in a pluripotent and self-renewing state when grown in culture media containing the cytokine leukemia inhibitory factor (LIF). Removal of LIF, and/or treatment with retinoic acid, promotes differentiation of mouse ESCs. Consistent with a role for HCF-1 in regulating ESC self-renewal and differentiation I find that HCF-1 is expressed in undifferentiatied ESCs, and expression of HCF-1 is reduced during retinoic-acid and LIF-withdrawal induced differentiation (Figure AII.1A-B). In contrast, *HCF-2* mRNA levels appear relatively stable over the differentiation time course (Figure AII.1C). This suggests that HCF-1 plays an important role in undifferentiated ESCs, and that loss of HCF-1 expression may promote differentiation of ESCs.

We next analyzed the phenotype of mouse ESCs in which expression of *HCF-1* or *HCF-2* was reduced. We electroporated mouse ESCs with plasmids encoding

shRNA targeting either HCF-1 or HCF-2, and selected for stable expression of the shRNA. We then induced differentiation of the ESCs by removing leukemia inhibitory factor, and performed RT-qPCR to assess the expression level of various pluripotency factors. *HCF-1* expression was reduced about by 50% in shHCF1-expressing ESCs at the initiation of LIF-withdrawal compared to ESCs expressing a negative control shRNA (Fig. AII.2A), whereas *HCF-2* expression was reduced by about 10-fold throughout the time course of LIF-withdrawal (Fig. AII.2B). Interestingly, cells depleted of HCF-2 exhibited reduced levels of the pluripotency factors *Nanog*, *Oct4*, and *Sox2* at two and four days after LIF-withdrawal (Fig. AII.2C-E). In contrast, *HCF-1* knockdown cells expressed elevated levels of *Nanog*, but no consistent changes to *Oct4* or *Sox2* (Fig. AII.2C-E). To test whether cells with reduced *HCF*-1 or *HCF*-2 levels were more differentiated than the control cells, we measured alkaline phosphatase activity in these ESCs. Alkaline phosphatase is highly expressed in undifferentiated ESCs, and can be used as a marker for the differentiated versus undifferentiated state. Despite the changes we see in gene expression, there was no significant change in the level of alkaline phosphatase activity in cells expressing shRNA against either *HCF-1* or *HCF-2* as compared to the negative control cells. However, we did not measure alkaline phosphatase activity during LIF-withdrawal, which likely accounts for this discrepancy as changes in pluripotency genes' expression were not apparent in our *HCF*-1 and *HCF*-2 knockdown cell lines until differentiation had been initiated. While these results hint at the possibility that HCF-1 and HCF-2 may be involved in ESC differentiation, these experiments were

peformed only once, necessitating further analysis to determine what, if any, role HCF-1 or HCF-2 play in ESC self-renewal and differentiation.

Figure AII.1 HCF-1 expression is reduced during mouse embryonic stem cell differentiation.

(A) Western blot of HCF-1 protein levels in mouse embryonic stem cells (mESCs) kept in the undifferentiated state with the presence of leukemia inhibitory factor (+ LIF), or induced to differentiate by the withdrawal of LIF and the addition of retinoic acid, (-LIF, +RA). (B) mRNA levels of *HCF-1* in undifferentiated or differentiating mESCs as quantified by RT-qPCR. (C) mRNA levels of *HCF-2* in undifferentiated or differentiating mESCs as quantified by RT-qPCR. Results shown are pooled from two independent experiments, and represented as mean +/- SD.

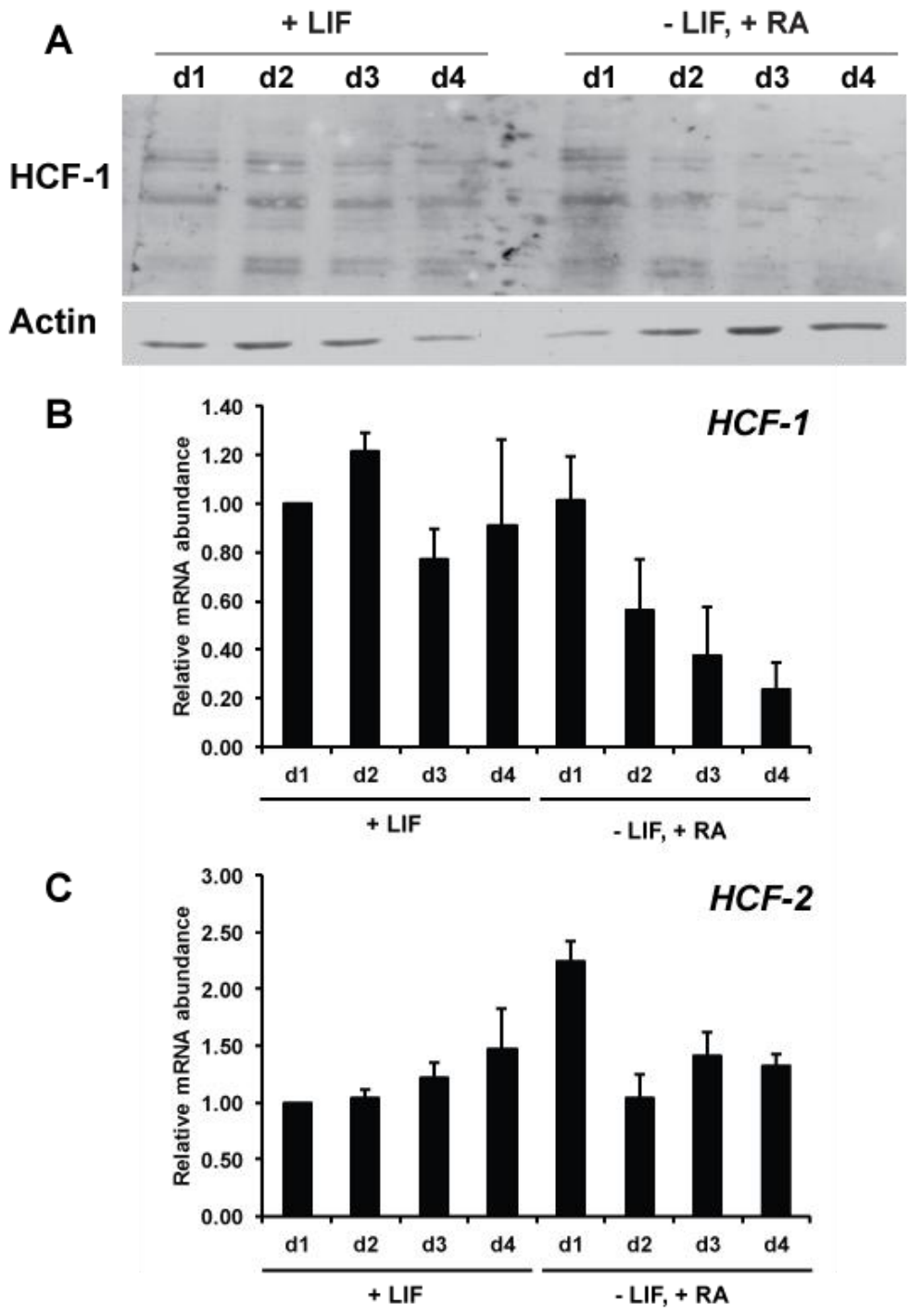
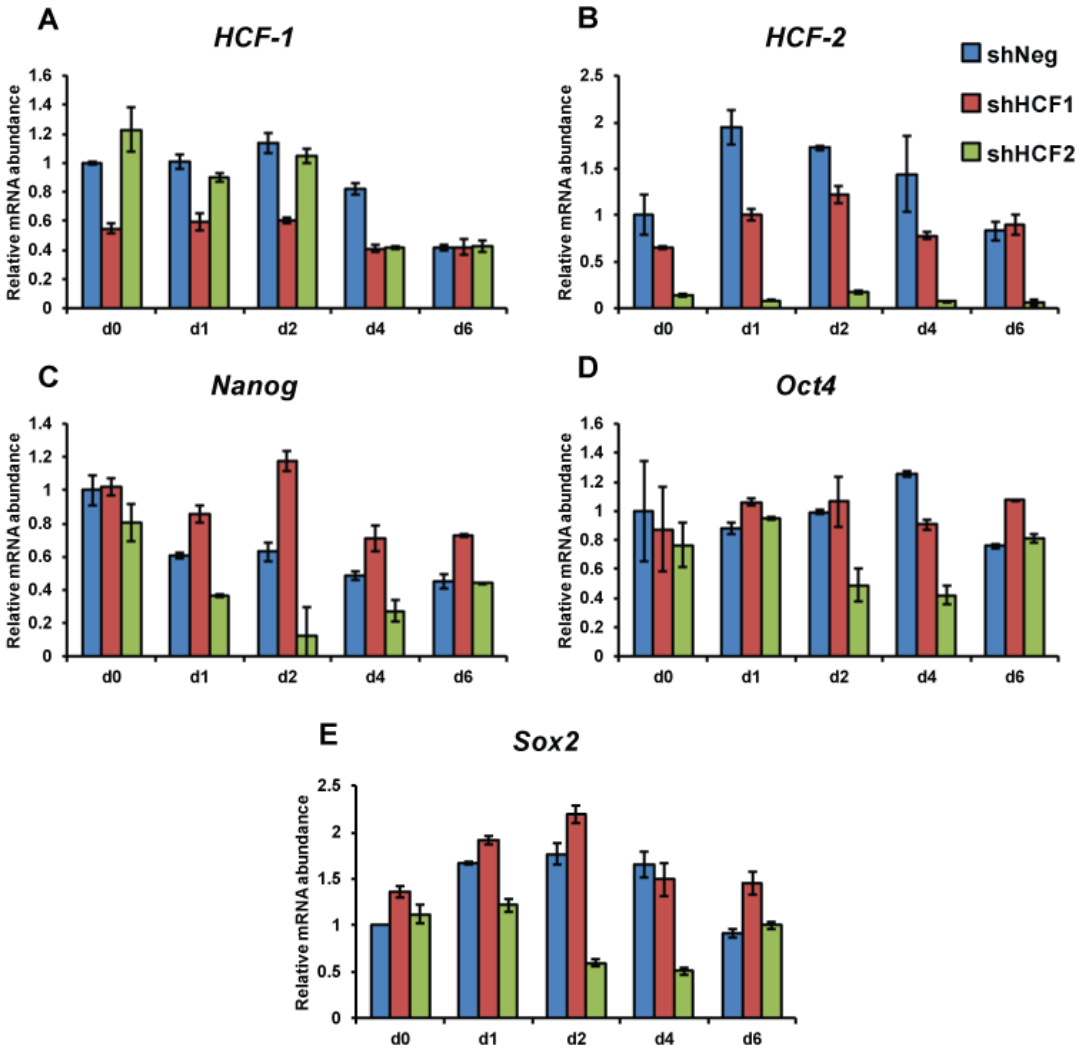

A
+ LIF
- LIF, + RA
d1
d2
d3
d4
d1
d2
d3
d4
HCF-1
Actin
B
HCF-1
Relative mRNA abundance
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
d1
d2
d3
d4
d1
d2
d3
d4
+ LIF
- LIF, + RA
C
HCF-2
Relative mRNA abundance
3.00
2.50
2.00
1.50
1.00
0.50
0.00
d1
d2
d3
d4
d1
d2
d3
d4
+ LIF
- LIF, + RA

Figure AII.2 HCF-1 and HCF-2 may regulate the expression of ESC pluripotency genes.

Mouse ESCs were selected for stable expression of shRNA targeting *HCF-1* or *HCF-2*. Leukemia inhibitory factor (LIF) was removed from the culture media to induce differentiation. RT-qPCR was used to analyze the level of gene expression on the indicated day after LIF withdrawal. mRNA values for each gene are normalized to the level of *β-actin*, and the mean normalized mRNA level day 0 in shNeg cell lines was set to 1. Results shown are mean +/- SD. Experiment was performed once.


A
HCF-1
Relative mRNA abundance
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
d0
d1
d2
d4
d6
B
HCF-2
shNeg
shHCF1
shHCF2
Relative mRNA abundance
2.5
2
1.5
1
0.5
0
d0
d1
d2
d4
d6
C
Nanog
Relative mRNA abundance
1.4
1.2
1
0.8
0.6
0.4
0.2
0
d0
d1
d2
d4
d6
D
Oct4
Relative mRNA abundance
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
d0
d1
d2
d4
d6
E
Sox2
Relative mRNA abundance
2.5
2
1.5
1
0.5
0
d0
d1
d2
d4
d6

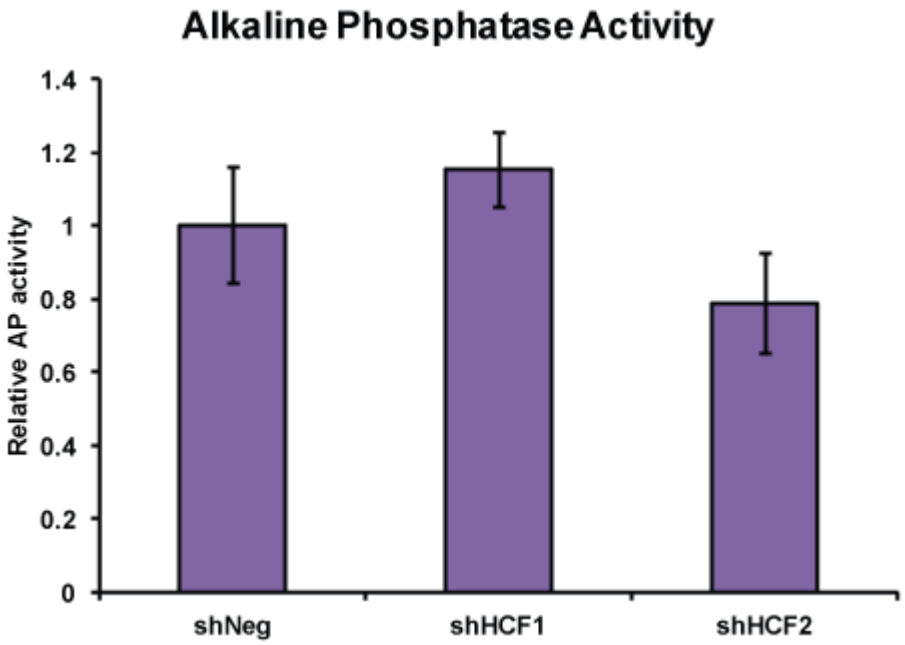


Figure AII.3 Alkaline phosphatase activity in HCF-1 and HCF-2 knockdown ESCs.

Alkaline phosphatase activity was measured in mouse ESCs selected for stable expression of shRNA targeting *HCF-1* or *HCF-2* using the StemTAG Alkaline Phosphatase Activity Assay Kit from Cell Biolabs, Inc. Values are normalized to total protein content, and shown as mean +/- SD. Experiment was performed once.

APPENDIX III

HCF-1 ACETYLATION IS INCREASED UNDER HIGH GLUCOSE CONDITIONS

As a NAD^+-dependent enzyme, SIRT1 activity is known to be regulated by nutrient and energy status (Imai and Guarente, 2010). NAD^+ is an essential cofactor for glycolytic redox reactions and other cellular metabolic processes, which results in a limited pool of NAD^+ available for sirtuin-mediated enzymatic reactions (Imai and Guarente, 2010). NAD^+ biosynthesis is regulated by the AMP-activated protein kinase (AMPK) (Fulco et al., 2008; Canto et al., 2009). Under low glucose conditions, AMPK activation leads to increased NAD^+ levels and hence increased SIRT1 activity (Fulco et al., 2008). Therefore, we wondered whether energy availability might affect SIRT1-mediated deacetylation of HCF-1.

We overexpressed HA-tagged HCF-1 in HEK293T cells, and treated the cells with varying glucose concentrations. HA-HCF-1 was immunoprecipitated from the cell lysate and acetylation determined by western blotting. Interestingly, we observed that increasing glucose concentrations result in increased levels of HCF-1 acetylation (Figure AIII). Thus, we conclude that HCF-1 deacetylation by SIRT1 is likely sensitive to cellular energy status.

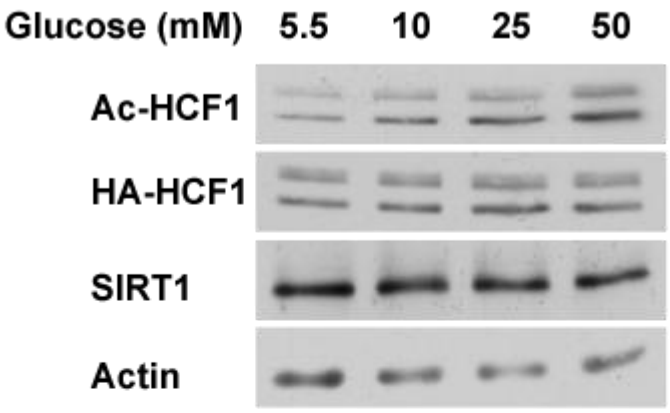
Given that HCF-1 is required for glucose-stimulated insulin secretion in INS-1 β-cells, it is tempting to think that HCF-1 may act as a glucose sensor, and that by being acetylated or deacetylated, HCF-1 function may be responsive to cellular energy

availability. Future studies examining the functional consequences of HCF-1 acetylation will help elucidate how energy status may affect HCF-1-dependent cellular processes.

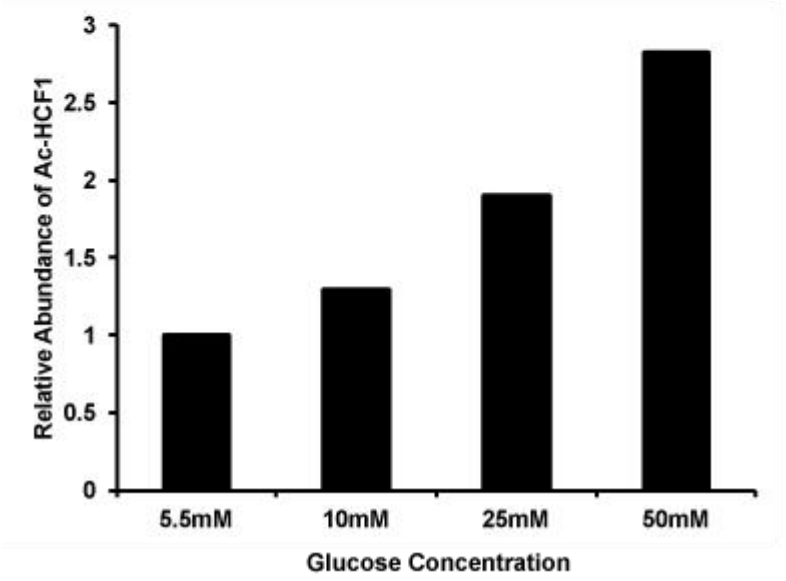
Figure AIII HCF-1 acetylation is sensitive to glucose concentrations.

(A) HA-tagged HCF-1 was immunoprecipitated from cells treated with the indicated glucose concentrations for 6 hours. Acetylated HCF-1 was detected using a pan-acetyllysine antibody. Whole cell lysate was subjected to immunoblotting for SIRT1 and β-actin. (B) Ac-HCF1 levels were normalized to total HA-HCF-1 levels and quantified using a Biorad ChemiDoc imaging system. The normalized level of Ac-HCF1 was set to 1 in the 5.5mM glucose condition.

A


Glucose (mM) 5.5 10 25 50
Ac-HCF1
HA-HCF1
SIRT1
Actin

B


Relative Abundance of Ac-HCF1
3
2.5
2
1.5
1
0.5
0
5.5mM
10mM
25mM
50mM
Glucose Concentration

APPENDIX IV

MAMMALIAN HCF-2 IS UPREGULATED BY SERUM STARVATION BUT IS NOT REQUIRED FOR CELL CYCLE ARREST OR PROGRESSION

Given that HCF-1 is required for multiple aspects of cell cycle progression (Goto et al., 1997; Wilson et al., 1997; Julien and Herr, 2003, 2004), we were curious to test whether the closely related HCF-2 protein might be involved in cell cycle regulation as well. We have observed that mouse NIH3T3 fibroblast cells treated with serum depletion and the PI3-kinase inhibitor LY294002 exhibit elevated levels of HCF-2 mRNA transcripts (Figure AIV.1A). LY294002 treatment alone induces *HCF-2* expression by about 50% (Figure AIV.1A) while serum starvation alone was able to induce *HCF-2* expression ~3-fold. (Figure AIV.1A). The combination of serum depletion and LY294002 treatment is additive, suggesting that these are two independent pathways mediating *HCF*-2 upregulation. We then tested whether *HCF-2* upregulation is a general stress response by examining HCF-2 mRNA levels after oxidative stress (H_2O_2) or UV treatment (Figure AIV.1B-C). However, UV treatment only mildly induced *HCF-2* expression (~15% after 12 hours), while H_2O_2 reduced *HCF-2* expression, indicating that serum depletion specifically promotes the robust upregulation of *HCF-2*.

As serum depletion promotes G1 cell cycle arrest (Pardee, 1974; Campisi et al., 1984), we wondered whether HCF-2 might be involved in either promoting cell cycle arrest or in reiniating the cell cycle after serum stimulation. To test this, we

generated stable NIH3T3 cell lines in which shRNA targeting the *HCF-2* gene was constitutively expressed. *HCF*-2 transcripts were reduced by ~70% in the cells expressing the HCF-2 shRNA relative to control cells expressing shRNA targeting the *luciferase* gene (Figure AIV.2A). We then subjected these cells to serum depletion (0.5% calf serum) and assessed their DNA content by propidium iodide staining and flow cytometry analysis. The shLuc and shHCF2 cells behaved similarly, with both cell lines exhibiting progressively increased percentage of cells with G1 DNA content (Figure AIV.2B). This result suggests that HCF-2 is not required for cell cycle arrest in response to serum deprivation. We next assessed whether cells depleted of *HCF-2* exhibited any defects in re-entering the cell cycle after being arrested by serum depletion. Cells growing in serum-containing culture media ("+Serum", 10% calf serum) were first subjected to serum deprivation (0.5% calf serum) for 48 hours ("-Serum 48h") to induce G1 arrest (Figure AIV.2C). Media containing 10% calf serum was then added back to the cells and cells were collected at the indicated timepoints. Flow cytometric analysis of cellular DNA content again revealed that after 48 hours of serum deprivation, cells were predominantly in the G1 phase of the cell cycle. After serum stimulation, both the shLuc and shHCF2 cell lines were able to re-enter the cell cycle, as seen by the increase in G2/M-phase and S-phase cell populations (Figure AIV.2C). These results indicate that reducing *HCF-2* expression does not impair the ability of cells to either exit or re-enter the cell cycle. One caveat of these experiments is that although we have reduced expression of *HCF-2* in our stable cell lines, there may still be sufficient HCF-2 protein remaining in these cells to carry out the protein's cellular functions. In addition, as there are no commercial antibodies available that

can detect endogenous HCF-2 protein, we are unable to assess the extent of HCF-2 protein reduction in our shHCF2 cell lines. Future studies utilizing HCF-2 knockout cells will be useful in determining whether HCF-2 plays a role in regulating cell cycle events.

Figure AIV.1 *HCF-2* is upregulated by serum starvation, but not by UV or oxidative stress.

(A) NIH3T3 cells were treated with vehicle, serum starvation ("SS"), 20μM LY294002 ("LY"), or a combination of serum starvation and LY294002. Cells were collected after 12 hours. (B) NIH3T3 cells were irradiated with $20J/m^2$ of UV-C light. Cells were collected at the indicated timepoints after exposure. (C) NIH3T3 cells were treated with 1mM H_2O_2 for the indicated number of hours. *HCF-2* transcripts were measured by RT-qPCR, and normalized to the level of *β-actin*. All results are from one representative experiment and are shown as mean +/- S.D.

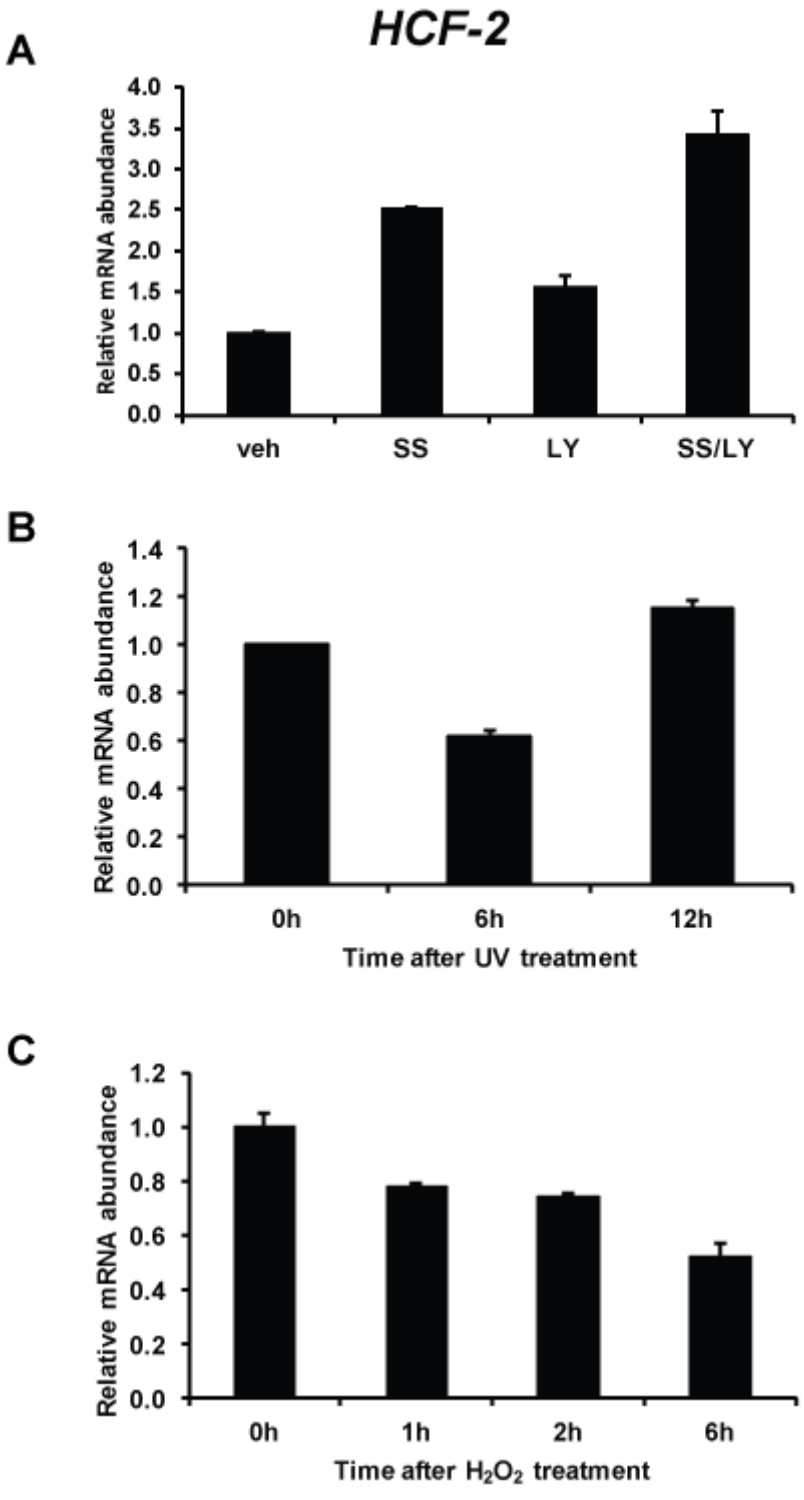
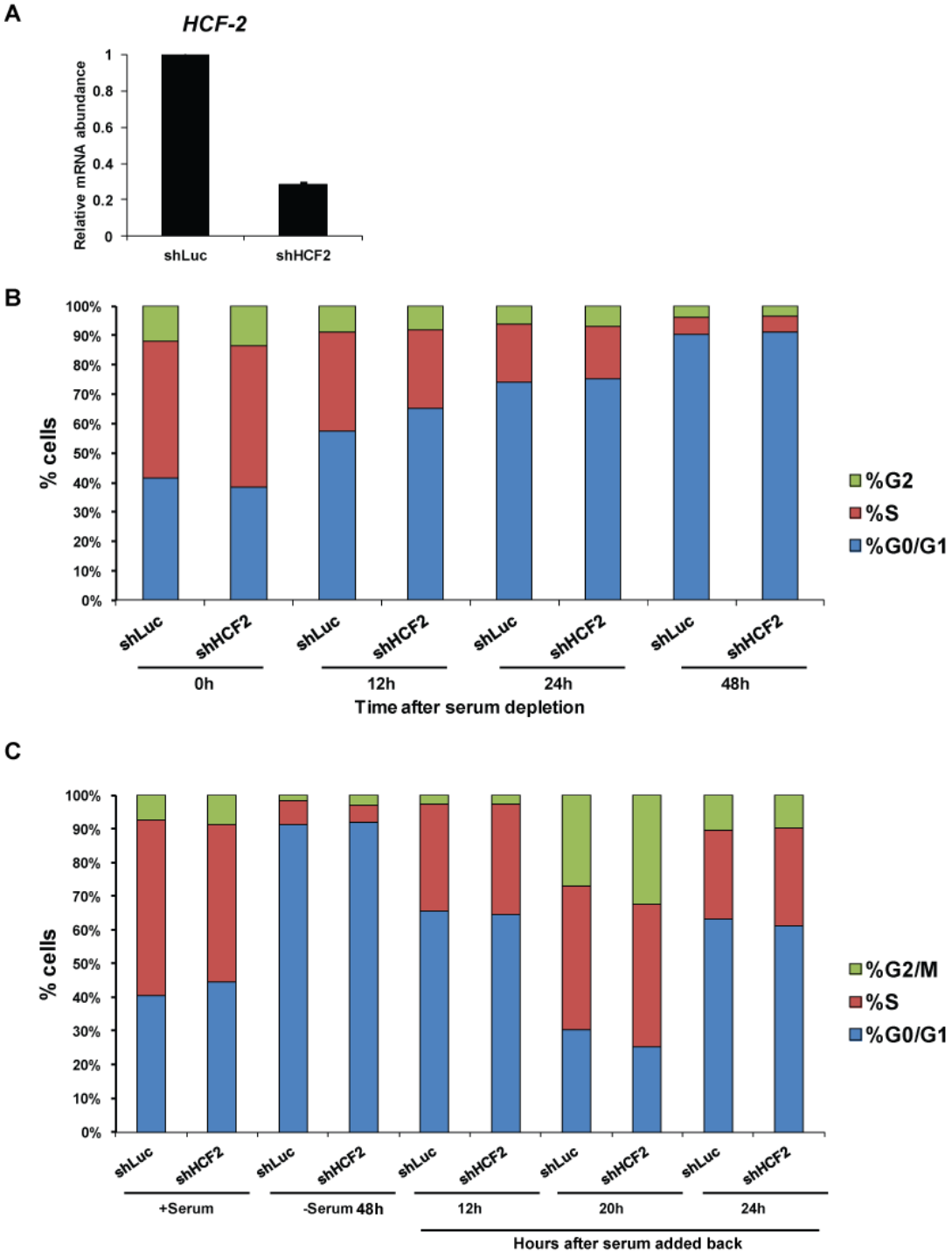

A
HCF-2
Relative mRNA abundance
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
veh
SS
LY
SS/LY
B
Relative mRNA abundance
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0h
6h
12h
Time after UV treatment
C
Relative mRNA abundance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0h
1h
2h
6h
Time after H2O2 treatment

Figure AIV.2 HCF-2 is not required for cell cycle arrest or cell cycle progression

(A) *HCF-2* mRNA levels in NIH3T3 cells stably expression shRNA targeting the control *luciferase* gene (shLuc) or the *HCF-2* gene (shHCF2). (B) Stable shRNA cell lines were subjected to serum deprivation (0.5% calf serum) for the indicated time points. Percentage of cells in each phase of the cell cycle was determined by propidium iodide staining and flow cytometry. Data were analyzed with FlowJo software. Experiment was performed once. (C) Stable shRNA cell lines were subjected to serum deprivation (0.5% calf serum) for 48 hours and then re-stimulated with 10% calf serum. Cells were collected before and after serum deprivation, and at the indicated time points after re-stimulation with 10% calf serum. DNA content was analyzed as in (B). Experiment was performed once.


A
HCF-2
Relative mRNA abundance
1
0.8
0.6
0.4
0.2
0
shLuc
shHCF2
B
% cells
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
shLuc
shHCF2
0h
12h
24h
48h
Time after serum depletion
%G2
%S
%G0/G1
C
% cells
+Serum
-Serum 48h
12h
20h
24h
Hours after serum added back
%G2/M
%S
%G0/G1

APPENDIX V

C. ELEGANS HCF-1 MODULATES LIFESPAN UPSTREAM OF HSF-1 AND SMK-1, AND IN PARALLEL TO THE GERMLINE SIGNALING PATHWAY[3]

While *C. elegans* DAF-16 represents a major downstream target of HCF-1 in longevity determination, we wondered if other pathways and nuclear factors may also interact with HCF-1 to modulate lifespan. In parallel to the insulin/IGF-1 signaling pathway, signals originating in the *C. elegans* germline converge on DAF-16 to affect lifespan (Hsin and Kenyon, 1999). Worms in which the germline precursor cells are ablated (Hsin and Kenyon, 1999), or in which genetic manipulation prevents germ cell proliferation (Arantes-Oliveira et al., 2002) exhibit a robust extension of lifespan, which is dependent on *daf-16.* Germline defective mutant worms also require *kri-1*, which encodes an ankyrin repeat protein, to exhibit lifespan extension (Berman and Kenyon, 2006).

In addition to *daf-16,* a number of other nuclear factors are known to impact *C. elegans* longevity. HSF-1 is the *C. elegans* homolog of heat shock factor (HSF) protein, a transcription factor responsible for promoting cellular stress resistance. In addition to transcriptional upregulation of chaperone proteins in response to proteotoxic stress, HSF-1 is an essential regulator of developmental processes (Akerfelt et al., 2010). Furthermore, HSF-1 also plays an important role in longevity

[3] The analysis of *glp-1 (e2141);hcf-1(pk924)* lifespan was published in Li J., Ebata A., Dong Y., Rizki, G. Iwata T., and Lee SS, *PLoS Biology* (2008). The lifespan analysis of *smk-1* and *hsf-1* RNAi treated *hcf-1(pk924)* mutant worms have also been performed by Ji Li (*smk-1*), Gizem Rizki (*smk-1*) and Elliot Kahen (*smk*-1 and hsf-*1*) with similar results.

determination. In *C. elegans*, inactivation of *hsf-1* shortens lifespan, whereas overexpression of *hsf-1* results in exended lifespan and increased stress resistance (Garigan et al., 2002; Hsu et al., 2003). Interestingly, depletion of *hsf-1* shortens the prolonged lifespan of IIS mutant worms, but not that of *daf-16* mutant worms, while overexpression of *hsf-1* requires *daf-16* to significantly extend lifespan (Hsu et al., 2003; Morley and Morimoto, 2004). These results, along with the finding that DAF-16 and HSF-1 regulate a shared subset of target genes (Hsu et al., 2003), indicate that DAF-16 and HSF-1 work cooperatively to promote longevity and proteotoxic stress resistance.

C. elegans SMK-1 encodes the worm homolog of suppressor of MEK null (SMEK), first identified in *Dictyostelium* as a downstream component of the mitogen activpated protein kinase pathway (Mendoza et al., 2005). SMK-1 acts as a co-activator of DAF-16 and affects longevity, immune, UV, and oxidative stress responses of *C. elegans* (Wolff et al., 2006).

To determine whether HCF-1 may influence longevity through interactions with the germline signaling pathway, SMK-1 or HSF-1, we performed genetic epistasis analysis. Our results show that HCF-1 acts in parallel to the germline signaling pathway to affect lifespan. In contrast, we find that *hsf-1* and *smk-1* are both required for the lifespan extension phenotype of *hcf-1* mutant worms. These results suggest that HCF-1 affects longevity of *C. elegans* through interactions with multiple nuclear factors.

RESULTS

***hcf-1* acts in parallel to the germline signaling pathway, but upstream of *kri-1*, to modulate lifespan.**

hcf-1 mutant worms exhibit reduced brood size and increased embryonic lethality (Lee et al., 2007; Li et al., 2008a). As germline proliferation defects are known to promote longevity, we tested whether the extended lifespan of *hcf-1* mutant worms could be related to their brood size phenotype. To do this, we tested the epistatic relationship between *hcf-1* mutant worms, and worms lacking functional *glp-1* or *kri-1*. *glp-1* encodes a LIN-12/Notch family receptor that is required for germline proliferation, and, when inactivated, leads to significant *daf-16*-dependent lifespan extension (Arantes-Oliveira et al., 2002). *kri-1* encodes the homolog of human KRIT1, an ankyrin repeat protein which is required for *glp-1* mutant lifespan extension (Berman and Kenyon, 2006). We generated double mutant worms harboring a putative null mutation of *hcf-1* and a temperature-sensitive mutation of *glp-1*. At the non-permissive temperature, *glp-1(e2141)* worms lack a germline and are long-lived (Arantes-Oliveira et al., 2002). We reasoned that if *hcf-1* mutation extends lifespan through effects on germline proliferation, worms lacking both *hcf-1* and *glp-1* will not exhibit any further increase in lifespan extension beyond that of the single mutant worms. In fact, we found that the double mutant worms did indeed live significantly longer than either the single *glp-1* or *hcf-1* mutant worms (Figure AV.1A), indicating that *hcf-1* affects *C. elegans* lifespan independently of its effects on brood size. Interestingly, however, we did find that mutation or knockdown of *kri-1* in the *hcf-1* mutant background did suppress the lifespan extension phenotype of *hcf-1* mutant worms, suggesting that *hcf-1* acts upstream of *kri-1* in lifespan modulation.

kri-1 is required for the lifespan extending effects of the *glp*-1 mutation (Berman and Kenyon, 2006) (Figure AV.IB) and for DAF-16 nuclear localization seen in *glp-1*, but not IIS, mutant worms (Berman and Kenyon, 2006). *kri-1* and also mediates the upregulation of lipid hydrolysis seen in both *glp-1* and IIS mutant worms (Wang et al., 2008a). *hcf-1* mutation does not affect DAF-16 nuclear localization (Li et al., 2008a), but does affect lipid and fatty acid metabolism (Rizki et al., 2011). Therefore, it is possible that *kri-1* mediates the lipid metabolism effects of *hcf-1* inactivation. Future analysis of the role that lipid metabolism plays in *hcf-1* mutant lifespan will help to clarify the importance of altered fat metabolism in promoting longevity.

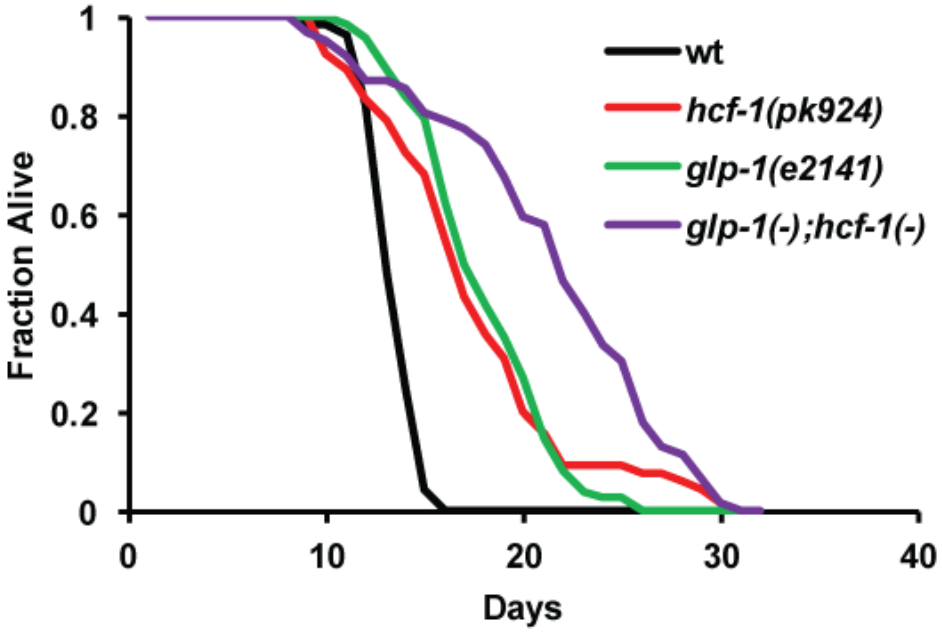
hsf-1 and _smk-1_ are both required for _hcf-1_ mutation to extend lifespan.

We next assessed whether *hcf-1* interacts with other nuclear factors to affect lifespan in *C. elegans*. We treated *hcf-1* mutant worms with RNAi targeting *hsf-1* or *smk-1* and assessed the effect of the RNAi on worm lifespan. As a positive control, we included *daf-16* RNAi, which suppresses the long-lived phenotype of *hcf-1* mutant worms (Li et al., 2008a) (Figure AV.2A). We found that knockdown of either *hsf-1* or *smk-1* suppressed the lifespan extension conferred by *hcf-1* mutation (Figure AV.2B-C). These results indicate that, in addition to DAF-16, HSF-1 and SMK-1 act as essential nuclear factors mediating the effects of HCF-1 on *C. elegans* lifespan. As both HSF-1 and SMK-1 interact with DAF-16 to affect longevity, it will be of great interest to determine whether HCF-1 may coordinate the interactions between these multiple factors, and by what mechanism it is able to do so.

Figure AV.1 *hcf-1* acts in parallel to the germline signaling pathway, but upstream of *kri-1*, to modulate lifespan.

(A) Lifespans of adult populations of wildtype (wt), *hcf-1(pk924)*, *glp-1(e2141)*, and *hcf-1(pk924);glp-1(e2141)* mutant worms grown at 25°C. (B) Lifespans of adult populations of wt, *hcf-1(pk924)*, *glp-1(e2141)*, *kri-1(ok1251)* and the indicated double mutant worms grown at 25°C. Each of the lifespan experiments was performed at least twice with similar results. Data from one representative experiment is shown. Qualitative data and statistical analysis is included in Table AV.1.

A


Fraction Alive
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
Days
wt
hcf-1(pk924)
glp-1(e2141)
glp-1(-);hcf-1(-)

B

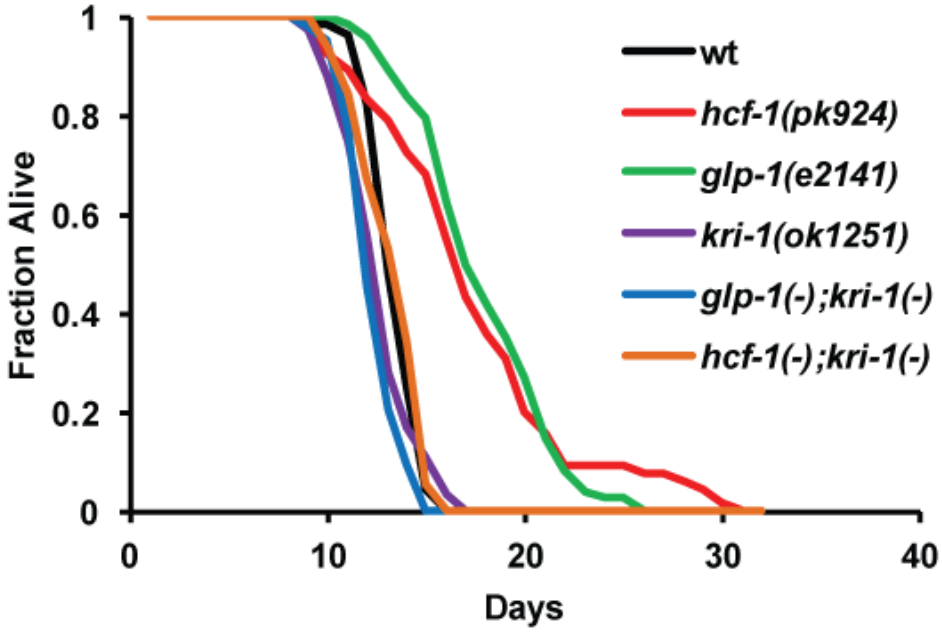
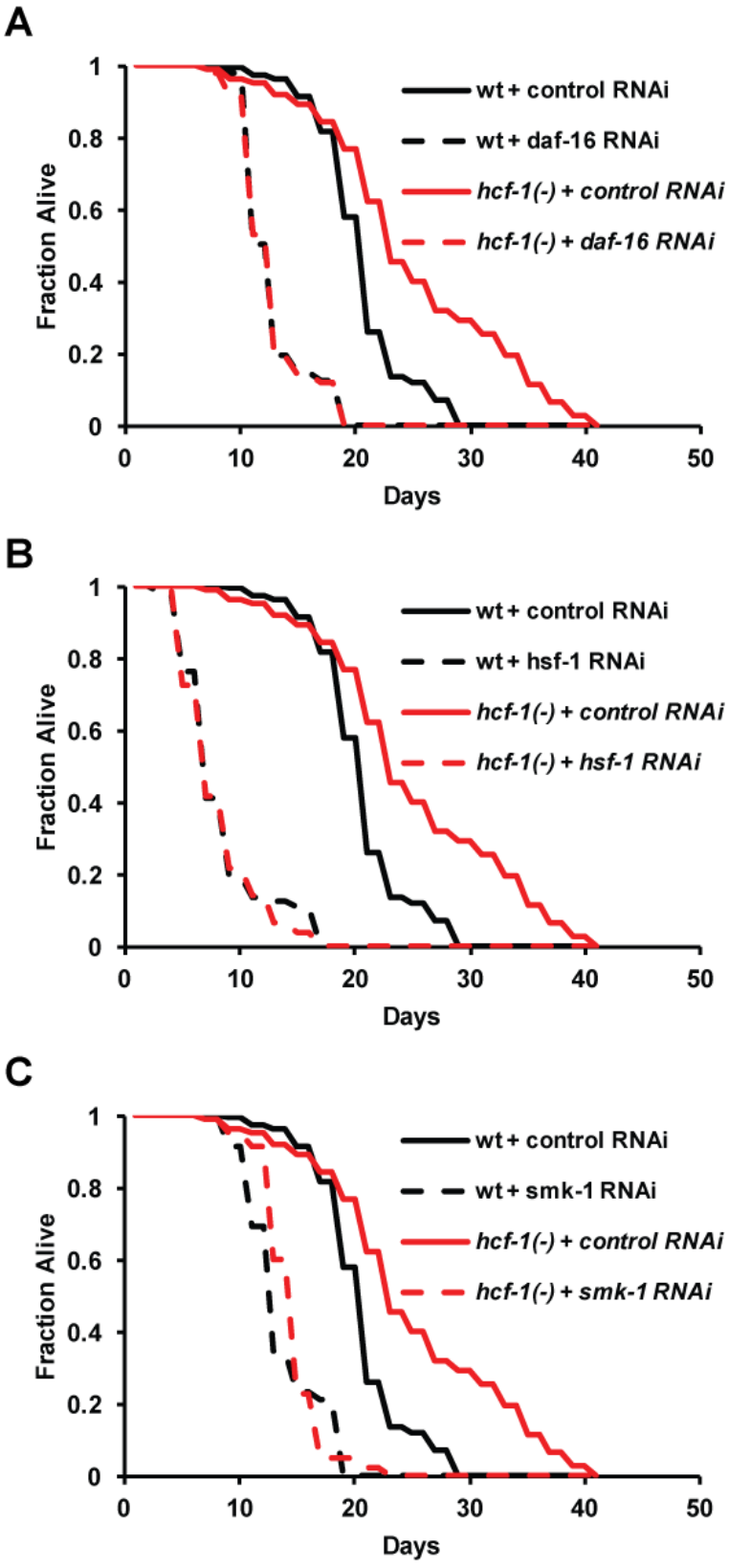

Fraction Alive
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
Days
wt
hcf-1(pk924)
glp-1(e2141)
kri-1(ok1251)
glp-1(-);kri-1(-)
hcf-1(-);kri-1(-)

Figure AV.2 *hsf-1* and *smk-1* are both required for *hcf-1* mutation to extend lifespan.

Lifespans of adult populations of wildtype (wt) and *hcf-1(pk924)* mutant worms treated with *daf-16* RNAi (A), *hsf-1* RNAi (B) or *smk-1* RNAi (C). Data are from one experiment. Qualitative data and statistical analysis is included in Table AV.2.


A
Fraction Alive
Days
wt + control RNAi
wt + daf-16 RNAi
hcf-1(-) + control RNAi
hcf-1(-) + daf-16 RNAi
B
Fraction Alive
Days
wt + control RNAi
wt + hsf-1 RNAi
hcf-1(-) + control RNAi
hcf-1(-) + hsf-1 RNAi
C
Fraction Alive
Days
wt + control RNAi
wt + smk-1 RNAi
hcf-1(-) + control RNAi
hcf-1(-) + smk-1 RNAi

Strain	Mean survival (Days ± SEM)	Total N	p-value vs wt	p-value vs hcf-1(pk924)	% of wt lifespan	% of hcf-1 (pk924) lifespan
wildtype	13.57 ± 0.19	56	NA	<0.001	NA	77%
hcf-1(pk924)	17.53 ± 0.63	66	<0.001	NA	129%	NA
glp-1(e2141)	17.96 ± 0.4	75	<0.001	0.952	132%	102%
kri-1(ok1251)	12.75 ± 0.22	75	<0.05	<0.001	94%	73%
hcf-1(pk924);kri-1(ok1251)	13.39 ± 0.2	70	0.970	<0.001	99%	76%
glp-1(e2141);hcf-1(pk924)	21.4 ± 0.74	62	<0.001	<0.001	157%	122%
glp-1(e2141);kri-1(ok1251)	12.45 ± 0.2	44	<0.001	<0.001	92%	71%

Data are representative of at least two independent experiments. Statistical analyses were done using SPSS software, with Kaplan Meier analysis and log-rank test to determine significance. p-values <0.05 are

Table AV.1 ***hcf-1*** **acts in parallel to the germline signaling pathway to modulate lifespan.**

Strain + RNAi	Mean survival (Days ± SEM)	Total N	p-value vs wt + control RNAi	p-value vs hcf-1(pk924) + control RNAi	% of wt + control RNAi	% effect of hcf-1(pk924) vs wt + RNAi
wt + control	20.94 ± 0.27	324	NA	<0.001	NA	
wt + daf-16	12.88 ± 0.18	240	<0.001	<0.001	62%	
wt + hsf-1	8.45 ± 0.25	220	<0.001	<0.001	40%	
wt + smk-1	13.37 ± 0.25	104	<0.001	<0.001	64%	
hcf-1(pk924) + control	25.16 ± 0.8	103	<0.001	NA	120%	20%
hcf-1(pk924) + daf-16	12.74 ± 0.3	97	<0.001	<0.001	61%	-1%
hcf-1(pk924) + hsf-1	8.22 ± 0.29	123	<0.001	<0.001	39%	-3%
hcf-1(pk924) + smk-1	14.57 ± 0.27	83	<0.001	<0.001	70%	9%

Data shown are from one experiment. Similar results were obtained by Ji Li, Gizem Rizki and Elliot Kahen (unpublished). Statistical analyses were done using SPSS software with Kaplan Meier analysis and log-rank test to determine significance. p-values <0.05 are considered significant.

Table AV.2 *hcf-1* acts upstream of *hsf-1* and *smk-1* to modulate lifespan.

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