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Analysis of transcription factor FOXO in the regulation of stress, aging, and neuromuscular tissue homeostasis in Drosophila melanogaster

Allison Birnbaum

Iowa State University

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Analysis of transcription factor FOXO in the regulation of stress, aging, and neuromuscular tissue homeostasis in Drosophila melanogaster

by

Allison Birnbaum

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics and Genomics

Program of Study Committee:
Hua Bai, Major Professor
Clark Coffman
Maura McGrail
Joshua Selsby
Yanhai Yin

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2020

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DEDICATION

This work is dedicated to all those who have made this dissertation possible through their unwavering support and encouragement. I would especially like to thank my grandmother Helene, whom I wish could have been here to see me graduate. I know you would be proud.
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<td>4EBP</td>
<td>4E-binding protein</td>
</tr>
<tr>
<td>Ac-Tub</td>
<td>Acetylated α-tubulin</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ATMK</td>
<td>Ataxia-telangiectasia mutated kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AVLM</td>
<td>Abdominal ventral longitudinal muscle</td>
</tr>
<tr>
<td>AZ</td>
<td>Active zone</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>DAF-2</td>
<td>Insulin-like growth factor 1 (IGF-1) receptor (C. elegans)</td>
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<tr>
<td>DAF-16</td>
<td>FOXO (C. elegans)</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinase</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box subclass O</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>IIS</td>
<td>Insulin/IGF signaling</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun-N-terminal kinase</td>
</tr>
<tr>
<td>MAP</td>
<td>microtubule-associated protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
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<tr>
<td>MST1/CST-1</td>
<td>mammalian Ste20-like kinase</td>
</tr>
<tr>
<td>MT</td>
<td>microtubule</td>
</tr>
<tr>
<td>mTOR</td>
<td>mechanistic target of rapamycin</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide (NAD) + hydrogen (H)</td>
</tr>
<tr>
<td>NF-kB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NMJ</td>
<td>neuromuscular junction</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PPIN</td>
<td>protein-protein interaction network</td>
</tr>
<tr>
<td>PQ</td>
<td>paraquat</td>
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<tr>
<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RU</td>
<td>RU486 (mifepristone)</td>
</tr>
<tr>
<td>SIRT</td>
<td>Sirtuin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SNARE</td>
<td>soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SSR</td>
<td>Subsynaptic Reticulum</td>
</tr>
<tr>
<td>SV</td>
<td>synaptic vesicle</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>SWItch/Sucrose Non-Fermentable</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless-related integration site</td>
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<tr>
<td>ZEB1/zfh1</td>
<td>Zinc Finger E-Box Binding Homeobox 1</td>
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I would like to thank my committee chair, Dr. Hua Bai, for all his advice and mentorship. I also thank my committee members, Dr. Clark Coffman, Dr. Maura McGrail, Dr. Joshua Selsby, and Dr. Yanhai Yin for their support throughout my graduate studies. You have all provided essential feedback that has pushed this research forward. I would also like to thank Dr. Elizabeth McNeill for her expertise and guidance as I began my exploration into the field of neurobiology. I could not have completed this work without your help.

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ABSTRACT

The transcription factor FOXO is a known regulator of tissue homeostasis and animal lifespan. It was first identified in its ability to promote longevity through the insulin signaling pathway, and has since been implicated in numerous cellular processes. Members of the FOXO protein family control a wide array of cellular functions including metabolism, cell cycle arrest, apoptosis, stress resistance, and aging. In response to various signaling cues, FOXO proteins can localize into the nucleus and interact with DNA to regulate transcription. FOXO is known as a longevity gene, however, how FOXO behavior changes during aging is not well understood. Normal aging involves a progressive decline in cell function and an accumulation of oxidative damage. Additionally, with age comes a reduction in FOXO gene expression, and FOXO protein activity can become dysregulated. To resolve how FOXO activity changes with normal aging, we began with chromatin immunoprecipitation sequencing (ChIP-seq) to compare differences in FOXO chromatin binding between young and old organisms, using Drosophila melanogaster as a model. In Drosophila, there is only one homolog representing the FOXO protein family, known as dFOXO. Through our investigation, we found that the number of dFOXO-bound DNA regions decreases with age, and see a number of these targeted genes undergo changes in expression with aging. Some pathways targeted by FOXO at a young age are Hippo, WNT, and MAPK signaling pathways.

FOXO is also known to mitigate oxidative stress, which is a contributing factor to age-related cellular degeneration. To understand FOXO dynamics in response to oxidative stress, we used Mass Spectrometry to evaluate dFOXO protein interacting networks under control and stress conditions. We observed a change in several dFOXO partners under paraquat-induced stress such as Stonewall, a chromatin modulator, and Hangover, a key transcription factor
regulating neuromuscular junction (NMJ) morphology and neuronal activity. Both FOXO and Hangover show altered NMJ morphologies, and have potentially disrupted vesicle cycling.

Given that FOXO has been linked to homeostatic maintenance of neuronal processes across animal species, we performed genetic analysis to investigate how dFOXO regulate NMJ aging in adult flies. Adult NMJs are known to undergo loss of synaptic homeostasis with aging, which causes functional decline and can lead to neurodegeneration. We profiled adult Drosophila abdominal NMJs and found that loss of function FOXO mutants exhibited morphological profiles similar to those of middle aged wild-type flies. We also observed an abnormal accumulation of late endosomes associated with the NMJ both in aged flies and with knockdown of motor neuron FOXO. Overexpression of FOXO can delay age-dependent late endosome accumulation, suggesting FOXO act as a positive regulator of neuronal homeostasis. We performed a genetic screen and identified pathways such as MAPK that act downstream of FOXO to control NMJ homeostasis during aging.

Collectively, this thesis provides evidence for global changes in FOXO chromatin binding activity under normal aging, highlights FOXO protein network dynamics that occur under oxidative stress, and displays homeostatic regulation of the adult Drosophila neuromuscular junction. These results illustrate how the transcription factor FOXO acts as a modulator of cellular homeostasis, and how FOXO activity is able to promote health and longevity.
CHAPTER 1. GENERAL INTRODUCTION

Senescence is accompanied by a progressive decline in physiological function, leading to impairments of cellular pathways and vulnerability to age-related diseases. With aging comes an accumulation of cellular damage and increase in oxidative stress and inflammation, with a decreased ability to reestablish homeostasis (Lopez-Otin et al 2013). These events are risk factors for the onset of cancers as well as age-related ailments such as neurodegeneration and cardiovascular diseases. However, there are genes which can act in a protective manner against organismal aging. One group that has consistently been labelled as “longevity genes” are the forkhead box subclass O (FOXO) gene family (Martins et al 2016). FOXO’s ability to extend lifespan was first uncovered in C. elegans, where insulin receptor mutants exhibited an increased longevity, which was dependent upon FOXO nuclear activity. This nuclear activation of FOXO also induces increased resistance to stressors (Kenyon et al 1993). Since recognizing the role of FOXO in insulin signaling, it has been discovered that this gene family is involved in the regulation of a number of cellular processes such as energy metabolism, cell cycle arrest, apoptosis, and oxidative stress resistance. FOXOs are conserved across animal species, and have similar cellular behavior among these diverse systems. FOXO proteins can acquire post-translational modifications from a number of cellular pathways, and these modifications dictate FOXO subcellular localization, molecular interactions, and gene expression (Calnan and Brunet 2008). FOXO localization into the nucleus allows for the regulation of transcription. Under increasing levels of reactive oxygen species, FOXOs can activate the expression of cell survival or cell death genes depending on signaling cues and interactions with co-factor proteins. Reactive oxygen species levels increase with aging and can cause oxidative damage to tissues and promote further senescence (Storz 2011). FOXOs act as mediators of redox activity and
cellular homeostasis. By studying FOXO regulated activity and how it changes during organismal aging, we can enhance our knowledge on how FOXO can promote longevity.

The fruit fly is a well-studied invertebrate organism with one gene encoding for FOXO, and a relatively short lifespan. This makes Drosophila melanogaster an excellent model to study systematic changes in FOXO behavior during aging (Puig et al 2003, Martins et al 2016). FOXO still acts as a longevity factor in flies, and nuclear expression of FOXO in certain tissues can promote lifespan extension in response to both insulin and oxidative stress (Wang et al 2003, Hwangbo et al 2004, Giannakou et al 2008). Drosophila FOXO also promotes proteostasis through the upregulation of autophagy pathways (Demontis and Perrimon 2010). Like mammalian systems, fruit flies exhibit both muscular and neuronal functional decline with aging (Mattson and Magnus 2006, Demontis and Perrimon 2010, Wagner et al 2015). Both of these tissues interacted at the neuromuscular junction, and have both shown FOXO function activities that are disrupted during senescence (Demontis and Perrimon 2010, McLaughlin and Broihier 2018). The adult Drosophila neuromuscular junction has only recently begun to be investigated, but has already been reported to undergo mechanistic changes with aging (Wagner et al 2015, Lopez-Arias et al 2017). Therefore, the adult neuromuscular junction can serve as an important model to study the role FOXO plays in tissue homeostasis and aging. By examining FOXO activity, whether it be through DNA targeting, protein interacting networks, or tissue-specific regulation of cellular processes, we can expand our understanding of FOXO function, how FOXO undergoes changes during senescence, and the subsequent effects this has on maintaining cellular homeostasis and tissue integrity.
1.1 Literature Review

FOXO transcription factors

The Forkhead Box protein family is characterized by a conserved DNA-binding domain which allows for transcription factor activity (Kenyon et al 1993, Brunet et al 1999). In this superfamily, proteins contain three alpha-helices and two wing loops, creating the forkhead region, which allows for interaction with DNA (Clark et al 1993, Kaestner et al 2000). The Forkhead box class O (FOXO) subfamily is conserved across animal species. In mammals, the FOXO family has four members, FOXO1, FOXO3, FOXO4, and FOXO6, and all share similar functional activity (Kaestner et al 2000, Hannenhalli and Kaestner 2009). Meanwhile, C. elegans and Drosophila only encode one FOXO protein, which has conserved functional motifs to mammalian FOXO proteins (Lin et al 1997, Junger et al 2003, Puig et al 2003). There is currently one identified consensus DNA-binding motif for FOXO, GTAAA(C/T)A.

Bioinformatics data has uncovered a number of gene promoter regions contain this FOXO DNA binding element, showcasing the potential global effect FOXO can have on cellular regulation (Furuyama et al, 2000, Xuan and Zhang 2005, Alic et al 2011, Bai et al 2013, Webb et al 2016).

In C. elegans, the FoxO ortholog DAF-16 was initially characterized as a part of the insulin signaling pathway (Kenyon et al 1993). Insulin-like receptor mutants mimic dietary restriction and were shown to have increased lifespan through the induction of a developmentally arrested state known as a Dauer stage, which has increased resistance to stressors and enhanced immunity (Albert 1981). This Dauer formation is dependent on a functional DAF-16. Insulin-like growth receptor, DAF-2, and DAF-16 double knockout mutants no longer exhibit extended lifespan (Kenyon et al 1993). Since the discovery of the role DAF-16 plays in insulin signaling, it has been implicated in a number of processes important for regulating cellular homeostasis such as metabolism, cell cycle progression, oxidative stress resistance, apoptosis, autophagy,
tumorigenesis, and DNA repair (Brunet et al 1999, Xie et al 2012, Martins et al 2016). FOXO activity is primarily dependent on its subcellular localization, and can be shuttled into the nucleus, where it may then act as an activator or repressor of transcription through direct DNA-binding activity and through the recruitment of co-factors (Kitamura et al 2002, Calnen and Brunet 2008). There are many genes that have been identified that mediate FOXO cellular activity, as well as many direct transcriptional targets of FOXO that are currently known (Oh et al 2006, van der Vos and Coffer 2008, Alic et al 2011, Riedel et al 2013, Webb and Brunet 2014). For example, the presence of insulin/insulin-like peptides initiates a phosphorylation cascade through PI3K, resulting in Protein Kinase B/Akt kinase phosphorylation and subsequent FOXO phosphorylation and sequestration in the cytoplasm (Brunet et al 1999, Puig et al 2003). When insulin signaling is low or depleted, FOXO is dephosphorylated and transported into the nucleus. However, FOXO also transcriptionally promotes insulin receptor expression, thereby acting as a negative feedback system, modulating its own inhibition (Puig et al 2003). Additionally, FOXO can act as a co-activator or co-repressor of other transcription factors, regulating cellular activity (Calnan and Brunet 2008, van der Vos and Coffer 2008, Kang et al 2017).

FOXO proteins have three Akt phosphorylation sites on three different protein domains. One Akt site regulates protein degradation, while the other two Akt phosphorylation sites are located at domains designated for nuclear export and nuclear localization. FOXO proteins have two other domains including their signature forkhead DNA binding domain, and a glutamate-rich domain for transcription activation (Junger et al 2003). FOXO is shuttled between the nucleus and cytoplasm through an interaction with the protein 14-3-3 (Cahill et al 2001, Obsil et al 2003, Eijkelenboom and Burgering 2013). Growth factors stimulate the binding of 14-3-3 to an Akt
phosphorylation site on FOXO, causing FOXO to be exported out of the nucleus. FOXO6 is constitutively nuclear in mammalian systems and has been shown lack one Akt phosphorylation site, making it incapable of subcellular shuttling. However, it is still able to sense growth factors and have inhibited activity within the nucleus (van der Heide et al 2004).

FOXO regulation is dependent upon post-translational modifications (PMTs), and FOXOs are capable of being phosphorylated, acetylated, methylated, and both mono and polyubiquitinated to regulate function (Calnen and Brunet 2008). FOXO proteins have several serine/threonine and lysine residues that are conserved between species and serve as the sites for PTMs (Barthel et al 2005, Greer and Brunet 2005). These PTMs dictate FOXO behavior and alter their subcellular localization. They can also influence FOXO binding activity, both to DNA and to other protein partners (Yamagata et al 2008, Calnen and Brunet 2008, Webb and Brunet 2014). Under the presence of growth-factors, FOXOs are phosphorylated and sequestered in the cytoplasm, inhibiting transcriptional function. Growth factors that have been shown to influence FOXO behavior include insulin/insulin-like growth factors, epidermal growth factors, and nerve growth factors (Biggs et al 1999, Brunet et al 2001, Rena et al 2002). Meanwhile, cellular stressors can activate pathways that induce the import of FOXO into the nucleus through the addition of new PMTs (Essers et al 2004, Wang et al 2005, Lehtinen et al 2006).

A variety of signaling cascades can cause PTMs on FOXO proteins, prompting nuclear import or export through distinct residue targeting (Glauser and Schlegel 2007). AMPK activity is upregulated under low energy conditions and mediates the FOXO response to dietary restriction, which is often associated with prolonged lifespan (Greer et al 2009). This is accomplished through the phosphorylation and subsequent activation of FOXO, and the transcriptional upregulation of energy maintenance genes (Greer et al 2007a, Greer et al 2007b).
FOXO can also be regulated through several other stress control pathways. Cyclin-dependent kinases (CDKs) monitor cell cycle progression and can control FOXO localization to regulate transcriptional control of apoptotic factors and respond to DNA damage (Huang et al 2006, Yuan et al 2008, Liu et al 2008, Yata and Esashi 2009). In mammals, neuronal cells are shown to have unique CDK1 activity in regard to FOXO regulation (Yuan et al 2008). Both ATM and MAPK proteins have also been shown to activate FOXO in response to DNA damage, and FOXO binding has been identified at promoters of DNA repair genes (Tsai et al 2008, Calnan and Brunet 2008).

Oxidative stress is shown to greatly influence growth-factor inhibition of FOXO (Oh et al 2005, Wang et al 2005). Following increases in reactive oxygen species (ROS), Jun-N-terminal kinase (JNK) mediates phosphorylation of FOXO (Essers et al 2004). This phosphorylation suppresses insulin signaling by decreasing receptor-substrate activity and interrupting FOXO interaction with 14-3-3 protein (Eijkenboom and Burgering 2013). MST1 has also been shown to phosphorylate FOXO and disrupt binding to 14-3-3 under oxidative stress (Lehtinen et al 2006). FOXO proteins can also be mono ubiquitinated or acetylated when reactive oxygen species levels increase, providing opportunities for new protein interactions and new chromatin targets (van der Horst et al 2006, Kitamura et al 2005). The activation of FOXO under oxidative stress overrides growth-factor inhibition and blocks degradation, and this FOXO activation can also promote lifespan extension (Kitamura et al 2005, Kops et al 2002, Essers et al 2004, Brunet et al 2004, Senchuk et al 2018). FOXO serves as a target for numerous pathways that can modify its activity via PTMs. Due to FOXO’s extensive regulatory network, it is involved in the maintenance of numerous homeostatic mechanisms.
FOXO as a homeostatic regulator and longevity factor

Aging involves the progressive decline of physiological integrity, leading to cellular impairments and chronic pathologies (Brooks-Wilson 2013). There are nine described hallmarks of aging which contribute to a functional decline and organismal senescence (Lopez-Otin et al 2013). Dysregulation of nutrient sensing pathways disrupts metabolic homeostasis and is one of the contributing factors to aging and cellular degeneration (Kenyon 2005, Lopez-Otin et al 2013). To maintain metabolic homeostasis, nutrient sensing pathways coordinate with growth factor signaling. In invertebrate systems, growth factor mutants exhibit increased longevity, as well as resistance to stressors and later onsets of chronic diseases (Carter and Brunet 2008). These results can also be seen upon dietary restriction, which is regulated by nutrient-sensing pathways. In particular, the insulin/insulin-like growth factor signaling (IIS) pathway has repeatedly shown an effect on longevity. In some insulin mutants, loss of IIS signaling doubles the lifespan of the organism (Kenyon et al 1993, Tatar et al 2001, Kenyon 2005). This extension in lifespan was found to be dependent on FOXO activity, and FOXO proteins are implicated in increased stress resistance and other longevity mechanisms across animal systems (Kenyon et al 1993, Junger et al 2003, Salih and Brunet 2008, Demontis and Perrimon 2010). FOXO proteins share similar functional domains across animal species, suggesting their impact on stress resistance and longevity are evolutionarily conserved.

FOXO proteins are implicated in regulating expression of autophagy genes, which promotes homeostasis. Autophagy is a process through which the cell can degrade cytoplasmic proteins and organelles to be reused. This process occurs under nutritional stress conditions to balance energy, as well as to remove damaged material and pathogens to promote survival (Glick et al 2010). Overexpression of FOXO3 in muscle tissue causes an increase in autophagosome formation, which envelope damaged materials and fuse with lysosomes for degradation.
(Mammucari et al 2007, Codogno et al 2010). FOXO is also known to promote the expression of a number of autophagy pathway genes (Mammucari et al 2007, Webb and Brunet 2014). In muscle and neuronal tissue, FOXO can promote mitophagy, which is the degradation of damaged mitochondria (Mei et al 2009, Lokireddy et al 2012). FOXO also regulates lipophagy and lipid metabolism in liver tissue, and the reduced activity of these processes with aging may increase triglyceride levels and cause steatosis (Xiong et al 2012, Singh and Cuervo 2012). The inhibition of histone deacetylases increases FOXO activation and promotes autophagy. This also results in suppression of mTOR, a negative regulator of autophagy and lifespan (Vellai et al 2003, Zhang et al 2015).

FOXO has conserved homeostatic effects across species. In the Drosophila heart tissue, activation of FOXO shows a decrease of insulin signaling and prolonged cardiac health. (Wessells et al 2004). In mammals, FOXO also functions as an autophagy promoter in cardiac tissue (Sengupta et al 2009). Activation of autophagy by FOXO in cardiac tissue promotes regression of cardiac hypertrophy and can protect heart tissue from oxidative stress through selective activity in endothelial cells (Abid et al 2005, Ronnebaum and Patterson 2010, Wang et al 2014). The activation of FOXO also reduces toxicity associated with protein accumulation in Alzheimer disease models in mice and C. elegans. (Regitz and Wenzel 2014, Fluteau et al 2015). In muscle tissue, FOXO can prevent protein aggregation formation through activation of downstream target 4EBP/Thor, while loss of either gene results in muscle degeneration and premature aging. This FOXO activity in muscles reduces feeding behavior, resulting in reduced global insulin and promotes proteostasis in other tissues through FOXO-regulated upregulation of the autophagy pathway (Demontis and Perrimon 2010). Additionally, FOXO proteins have
been found to promote stem cell quiescence and rescue stem cell aging through the ability to regulate cell cycle arrest (Kops et al 2002, Miyamoto et al 2007, Artoni et al 2017).

FOXOs are able to function as tumor suppressors, which promotes organismal health. In mammals, activation of FOXO1 inhibits the survival of tumor cells through the upregulation of apoptotic factors (Zhang et al 2011). Loss of FOXO activity is sometimes found in certain cancer cells or serves as a predisposition to cancer development (Paik et al 2007, Wang et al 2014). Additionally, degradation of FOXO by E3 ubiquitin ligases is often seen alongside cellular transformations and eliminates tumor suppression (Greer and Brunet 2005, Huang and Tindall 2007). FOXO overexpression is able to decrease tumor size in PTEN deficient models and can interact with p53 and SMAD in the nucleus to regulate DNA binding. This binding can subsequently induce cell cycle arrest and apoptosis (Nemoto et al 2004, Seoane et al 2004, Greer and Brunet 2005). FOXO also interacts with β-catenin, a key regulator of the WNT signaling pathway that regulates growth and development. Through this interaction, FOXO is able to act as a suppressor of both WNT signaling and tumor growth (Essers et al 2005, Liu et al 2015).

Despite the positive impacts of FOXO activity, overexpression of FOXO can have detrimental effects on the organism. Prolonged FOXO activation results in stalled development and cell death across animal species (Brunet et al 1999, Kramer et al 2003, Puig et al 2003). In diabetic models, overactivation of FOXO can also result in insulin insensitivity, and can contribute to disease pathologies (Nakae et al 2002, Behl et al 2009). FOXO is capable of inducing atrophy in differentiated skeletal and cardiac muscle tissue by enhancing expression of atrogin-1, an E3 ubiquitin ligase that is involved in protein degradation (Sandri et al 2004, Senf et al 2010). This activity can be inhibited through highly coordinated FOXO repression by ZEB1 in mammals, and while the *Drosophila* ortholog zfh1 has not been previously investigated in its
relationship to FOXO, it is involved in the prevention of muscle atrophy (Siles et al 2013, Ninfali et al 2018).

Although there are potential negative effects, FOXO is still perceived as a promoter of health and longevity. FOXO is able to reduce expression of insulin-like peptides in both *C. elegans* and *Drosophila* which extends lifespan (Libina et al 2003, Puig et al 2003 Hwangbo et al 2004, Martins et al 2016). Declines in FOXO expression are seen with aging, contributing to oxidative damage and age-related disease onset (Huang et al 2010, Alverez-Garcia et al 2017). Meanwhile, overexpression of FOXO proteins in certain tissues has been shown to increase lifespan by promoting proteostasis (Giannakou et al 2008, Demontis and Perrimon 2010, Morris et al 2015, Sun et al 2017). Even in humans, variations in FOXO genes are associated with reduced insulin levels and improved healthspan and increased lifespan (Willcox et al 2008, Anselmi et al 2009, Flachsbart et al 2009, Bao et al 2014). Although FOXO proteins function as balancers between stress resistance and cell death, they serve as regulators of cellular health, and it is through this activity that they can modulated lifespan.

**FOXO in oxidative stress**

FOXOs have been well established as mediators of oxidative stress within the cell. Oxidative stress refers to elevated levels of reactive oxygen species (ROS), which are the reactive byproducts of aerobic metabolism. Because of their chemical reactivity, they can cause damage to DNA, lipids, and proteins (Cross et al 1987). Maintenance of intracellular ROS is essential for cellular homeostasis, and loss of this can lead to cell death, cancer, and other age-related diseases (Martins et al 2016). It was first in *C. elegans* where insulin receptor mutants were recognized as having increased lifespan and enhanced stress resistance in a daf-16/FOXO dependent manner (Kenyon et al 1993, Honda et al 1999). It was found that FOXO proteins are, in part, responsible for the upregulation of antioxidant genes, including SOD and catalase, which

FOXOs can be activated by a number of signaling pathways in response to increased levels of ROS (van der Horst and Burgering 2007). The JNK phosphorylation of FOXO activates nuclear localization and promotion of genes involved in antioxidant expression for ROS detoxification and DNA repair. FOXO activation under these circumstances can also lead to the repression of genes involved in cell proliferation, allowing for the removal of potential damage (Storz 2011). MST1 is also able to phosphorylate FOXO under oxidative stress and induce nuclear localization. In *C. elegans*, knockdown of the MST1 ortholog CST-1 accelerates tissue aging and exhibits shortened lifespan through its inability to regulate FOXO (Lehtinen et al 2006). However, in mammalian neurons, MST1 activation of FOXO triggers the expression of apoptotic genes, resulting in neuronal cell death (Yuan et al 2009). While this apoptotic activity may be beneficial in cancer models, it can be detrimental in a healthy organism. Additionally, increases in oxidative stress promote the interaction between β-catenin and FOXO, prompting FOXO upregulation of antioxidant gene MnSOD (Essers et al 2005). Increases in oxidative stress promote the interaction between β-catenin and FOXO, which suppresses WNT signaling (Essers et al 2005, Almeida et al 2007, Hoogeboom et al 2008). In *C. elegans*, β-catenin (bar-1) null mutants were more sensitive to oxidative stress agents than wild-type, exhibiting how this interaction between FOXO and β-catenin is important for FOXO activity under oxidative stress (Essers et al 2005). This interaction between FOXO and β-catenin is also able to promote quiescence in neuronal stem cells (Maiese 2015). Neurons are constantly generating ROS, and dysregulation can lead to neurodegeneration (Klein and Ackerman 2003). FOXO has been shown to have protective effects against oxidative stress in neurodegenerative models (Parker et
al 2012, Koh et al 2012, Tourette et al 2014). This will be discussed in more detail later in this chapter.

FOXO proteins can act as sensors of oxidative stress because they are capable of both promoting stress resistance and inducing cell apoptosis, and the function is dependent on the co-factor (Storz 2011). Acetylation of FOXO by the CREB binding protein (CBP) promotes the expression of apoptosis genes (van der Heide and Smidt 2005, Dansen et al 2009). Histone deacetylases are also known to interact with FOXO and regulate transcription of cell death promoting genes (van der Horst et al 2004, Hori et al 2013). In cases of both caloric restriction and oxidative stress, SIRT1 is known to enter the nucleus to deacetylate FOXO (Kobayashi et al 2005, Wang et al 2007). This can have either positive or negative effects on gene transcription, and in some cases, deacetylation is required for FOXO-mediated transcription activation of autophagy genes (Hariharan et al 2010). In C. elegans, FOXO directly recruits the SWI/SNF remodeling factor, promoting Dauer formation and increased resistance to oxidative stress (Riedel et al 2013). Overall, FOXO functioning as a regulatory element in response to oxidative stress by mediating cellular mechanisms involved in homeostasis and aging.

**Oxidative stress and aging**

Aging serves as a risk factor for the onset of functional impairments and diseases (Lopez-Otin et al 2013). The free radical theory of aging states that the generation of free radical species by mitochondrial metabolism causes cellular damage and deterioration, promoting senescence (Harman 1965). ROS generation and aging have a complex relationship, with both too many and too few ROS being detrimental to the system (Patel et al 2018). Therefore, the balance between ROS and antioxidant activity is crucial for health, cellular integrity, and longevity (Tan et al 2018).
The generation of reactive oxygen species

ROS are primarily generated as a normal part of cellular activity during aerobic respiration. During the mitochondrial electron transport train (ETC), free radicle species are created as by-products of several enzymes during the transfer of electrons and pumping of protons for energy and ATP synthesis (Alfadda and Sallam 2012, Ryu and Zhang et al 2016, Kumari 2018). There are four complexes along the ETC that perform redox reactions, with complexes I, III, and IV utilizing NADH as the substrate, while compounds like succinic acid act as a substrate for complex II (Kumari 2018, Zhao et al 2019). The partial reduction of oxygen by oxidative phosphorylation results in the formation of three reactive oxygen species; superoxide anions ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals ($HO^-$) (Ray et al 2012). These ROS can trigger signaling cascades and activate pathways that can be either damaging or beneficial for cell maintenance depending on the quantity and cellular condition (Paravicini and Touyz 2006, Ishikawa et al 2008, Ray et al 2012). Exogenous sources such as infections can also cause oxidant levels to increase in the cell (Birben et al 2012).

Antioxidants scavenge reactive oxygen species, some through a direct interaction, eliminating potentially damaging effects. ROS levels help promote the expression of endogenous antioxidant genes which protect the cell from harmful stressors, including oxidative stress. These antioxidants serve to counterbalance oxidant levels and can function through enzymatic and non-enzymatic reactions. Superoxide species are scavenged by superoxide dismutase (SOD). There are three forms of SOD that interact with metal ions and localize in different regions of the cell (Birben et al 2012). CuZnSOD/SOD1 can be found in the cytoplasm, while MnSOD/SOD2 is located within the mitochondria. There is also ECSOD/SOD3, which can be found in the extracellular space. The expression of SOD3 is restricted to certain cell types (Zelko et al 2002). Peroxide species are produced from SOD activity, and these are reduced by catalase. $H_2O_2$ can
also be reduced by glutathione peroxidase (Mates et al 1999). Some organelles, such as peroxisomes, are able to both produce and reduce peroxide species (Walton and Pizzitelli 2012). The balance between oxidant and antioxidant species can have a dramatic effect on organismal homeostasis.

**ROS, aging, and age-related diseases**

Excessive oxidative or reductive stress has negative consequences on cellular homeostasis (Perez-Torres et al 2017). Thus, a balance is needed between prooxidants and antioxidants. When imbalances occur, there can be irreversible damage to lipids, proteins, and DNA which cause cells to senesce, dysregulate, or undergo apoptosis (Ray et al 2012). This can be particularly damaging to mitochondrial DNA, as this damage can induce further ROS production while reducing antioxidant expression, causing a positive feedback for mitochondrial dysfunction (Giorgi et al 2018). Mammals with slower aging rates are found to have lower mitochondria DNA oxidative damage in heart and brain tissue when compared to faster aging species, showcasing how mitochondrial function impacts organismal health and lifespan (Barja and Herrero 2000). Meanwhile, reductive stress can inhibit ROS production, leading to reduced cell growth and inflammatory diseases (Perez-Torres et al 2017). ROS is a key part of normal cell signaling pathways, but this tight regulation can be disrupted, leading to oxidative stress which can initiate cellular damage, aging, and the onset of age-related diseases.

In hypoxic conditions, mitochondria produce increased levels of ROS that induce expression of hypoxia-inducible factor 1. This in turn mediates an adaptive response by augmenting oxygen delivery to tissues and normalizing $O_2$ levels. However, this same effect does not pertain to cancer cells (Guzy and Schumacker 2006, Semenza 2008). Exercise also causes an increase in ROS and antioxidant expression, leading to stress tolerance and higher oxidative stress resistance with aging. Exercise-induced resistivity can delay oxidative stress
induced cellular damage, showing how ROS generation can be beneficial under certain circumstances (Meydani and Evans 1993, Radak et al 2008). ROS also play an essential role in the immune system. ROS levels increase in response to pathogen detection, prompting inflammatory responses and phagocyte activation (McCallum and Garsin 2016, Franchina et al 2018). ROS can function as activators of NF-kB and MAP Kinases, which can trigger innate immunity and protective responses (Back et al 2012, Son et al 2013). ROS are also required for cell proliferation and initiation of apoptotic pathways, and are therefore important for proper cell cycle regulation and cell death (Patel et al 2018).

Oxidative stress plays a critical role in the development of age-related diseases, and the reduction of ROS later in life has been shown to improve health span and cellular function (Liguori et al 2018). High ROS levels can induce p53 activation and suppress autophagy, which causes mitochondrial dysfunction, lipid peroxidation, and cell senescence (Lauri et al 2014, Davalli et al 2016). Free radicals are capable of inducing irreversible damage to proteins and organelles, causing increased protein misfolding. Misfolded proteins can aggregate, which prevents their degradation and causes tissue degeneration. This aggregation is an initiating factor of disease in many tissues, and is highly associated with atrophy in muscles and neurons (Levy et al 2019).

Many tissue types are impacted by oxidative stress, and the accretion of oxidative damage during one’s lifetime can result in the onset of age-related diseases. Mitochondrial dysfunction leads in increased ROS levels, and this can contribute significantly to cardiac pathologies such as ischemia and accelerated tissue necrosis (Peoples et al 2019). Malfunctions of antioxidant responses in ocular tissue occur with aging and leads to apoptosis of several different cell types in the eye, contributing to degenerative eye diseases (Buddi et al 2002, Nita
Moreover, diabetes is associated with an increase in free radical production with reduced antioxidant expression, leading to inflammation and vascular complications (De Cristofaro et al 2003, Liguori et al 2018). Many biomarkers of oxidative stress have been identified for numerous age-related diseases, but there is still more information that needs to be uncovered in order to develop therapies to combat the increasing oxidative damage that occurs with aging (Liguori et al 2018).

**The neuromuscular junction**

The neuromuscular junction (NMJ) is a specialized synapse that occurs between muscle tissue and motor neurons and allows for reliable communication between the muscle and the brain for motor function. It is composed of three parts; the presynapse, the postsynapse, and the synaptic cleft (Punga and Ruegg 2012). At the NMJ, the presynapse is the terminal region of the motor neuron axon, and is responsible for the release of neurotransmitters upon excitation. Neurotransmitters are stored in synaptic vesicles that interact with the neuronal plasma membrane for release. Nerve impulses induce calcium channel activity and stimulate synaptic transmission (Katz and Miledi 1970). The NMJ postsynaptic region is within the muscle tissue and contains numerous folds, which increases the surface area for the many embedded receptors that receive neurotransmitters after their secretion. These receptors allow for an influx of sodium ions upon neurotransmitter binding, generating and transmitting the action potential to the muscle (Slater 2017, Omar et al 2020). The synaptic cleft is the space between the neuron terminal and muscle postsynaptic area, and contains an extracellular matrix known as the basal lamina (Patton 2003). It houses proteins to facilitate receptor clustering, secrete laminins, and harbors catabolic enzymes to eliminate prolonged neurotransmitter stimulation on the muscle (Patton 2003, Gonzalez-Freire et al 2014, Omar et al 2020). The neuromuscular junction is
considered part of the peripheral nervous system, or PNS as opposed to the central nervous system, or CNS (Arstikaitis and El-Husseini 2009).

Different species use different neurotransmitter signals. Vertebrate systems utilize acetylcholine as the primary neurotransmitter to induce muscle contraction. This is also used in C. elegans for contraction, but requires GABA release for relaxation. In Drosophila, the NMJ uses glutamate for the synaptic signal (Wu et al 2010). In vertebrate systems, glutamate is more commonly used as a synaptic substrate in the CNS, and postsynaptic regions contain predominantly AMPA and NMDA glutamate receptors (Li et al 2003). However, some reports show these glutamatergic receptors can be located in the postsynaptic regions (muscles) of mammalian NMJs (Mays et al 2009).

NMJs are highly plastic, and have the ability to modify their activity based on neuronal input. Alterations in pre- and postsynaptic machinery can change the number of neurotransmitters released, the number of receptors available, and receptor sensitivity to released signals. The ability to regulate signal release and reception allows for long-term maintenance of synaptic strength. This modulation is also required for establishing learning and memory, and loss of plasticity can result in a number of neurological disorders (Heinbockel 2017). Structural changes occur in response to synaptic plasticity, and are primarily thought to occur during synaptic development (Menon et al 2013). However, there is evidence that structural changes occur post-development in response to neuronal input and with age (Lopez-Arias et al 2017, Sugie et al 2018).

**Structure of motor neurons and the NMJ**

The structural components of motor neurons are important for their functional activity and dictate their growth and appearance. Cytoskeletal elements play an integral part in motor neuron development, growth, guidance, morphology, and function (Kapitein and Hoogenraad
Motor neuron axons may need to travel long distances in order to reach their muscle targets and are exposed to various environments. Motor neurons receive thousands of sensory inputs and contain numerous branches to relay signals (Luo 2002). The cell body, or soma, contains the nucleus and is the center for synthesis and degradation of nearly all neuronal membranes and proteins. There are two types of processes that extend from the cell body known as dendrites and axons (Lodish 2000, Luo 2002). Each motor neuron establishes many distinct dendritic arbors, but will typically only have one axon, which extends throughout the organism and branches out upon contact with the muscle to interact and form the NMJ. Axons cannot synthesize or degrade their own proteins, and thus all components are generated in the cell body and transported along a cytoskeletal network (Lodish 2000).

Motor neurons are highly polarized cells and rely on the asymmetric distribution of molecular components to maintain synaptic function throughout life. This polarity is required for the transport of signals, cellular components, and proteins within the neuronal circuitry and to tissue targets (Jones and Svitkina 2016). This is done through the microtubule (MT) cytoskeleton. The MT cytoskeleton works along with actin to migrate and establish synaptic contacts during neuronal development (Kapitein and Hoogenraad 2011). MTs are key cellular components conserved among cell types, and are involved in cellular processes such as mitosis, cell motility and migration, secretion, and intracellular transport (Nechipurenko and Broihier 2012). The MT cytoskeleton is composed of α- and β-tubulin heterodimers, which polymerize together and form linear filaments. MT polarity arises through having more stable “minus” ends comprised of α-tubulin, and more dynamic “plus” ends made up of β-tubulin (Etienn-Manneville 2010). MTs are highly dynamic and can either polymerize (extend) or depolymerize (truncate). This depolymerization can be rescued and is a normal part of the dynamic instability that allows
for microtubule flexibility (Lasser et al 2018). They can also undergo rapid depolymerization in the form of catastrophe. In motor neurons, the axonal MT network serves as a sort of highway to transport molecules between the soma and the axon terminals (Kapeitein and Hoogenraad 2015).

Transport along the axonal MT network is essential for NMJ establishment and maintenance. Motor proteins kinesin and dynein move along the microtubules and act as delivery systems for vesicles, proteins and other molecules that can act as cargo (Etienne-Manneville 2010). Kinesins and kinesin-related proteins serve as vehicles for anterograde transport, which initiates at the cell body and moves towards the terminal, sending molecules away from the nucleus. Dynein motor proteins are responsible for the reverse process known as retrograde transport, which starts at the terminal and is received at the cell body. This process is important for the degradation of damaged materials. Dynein is activated through the interaction with dynactin, and mutations in either protein can cause embryonic lethality (Holzbaur 2004). These motor proteins utilize neuronal polarity for motility and particularly for fast axonal transport. The loss motor protein function can interrupt transport of essential elements and cause neurodegeneration (Maday et al 2014).

Microtubule bundles are sensitive to their environments and require a certain level of dynamic capability to perform cellular tasks. However, neuronal microtubules also need varying degrees of stability. Many MT filaments do not span the entirety of the axon but are often in segments of varying length, which serve unique purposes. At the plus end, microtubules undergo rapid switches between assembly and disassembly, making them highly labile. Meanwhile, minus ends are highly stable, and avoid dynamic changes in order to maintain structure, polarity, and function (Baas 2013, Baas et al 2016). To regulate these structures are numerous microtubule-associated proteins (MAPS) and post translational modifications that decorate the
microtubules. Tubulin elements that are tyrosinated are highly labile, while detyrosination allows for other PMTs to come in and stabilize the microtubules. Acetylation of α-tubulin acts as a stabilizer, as does polyglutamylation (Song and Brady 2014, Baas et al 2016). MAPs including Tau, Ankyrin, and others can cross-link and stabilize MTs (Baas et al 2016). MAPs are important for maintaining neuronal morphology and protecting against severing proteins that can cause MT catastrophe (Dubey et al 2015). Dysregulation of these MAPs are associated with neurodegenerative diseases, particularly Tau (Baas et al 2016).

**Aging at the neuromuscular junction**

Even in healthy individuals, aging is associated with the gradual impairment of motor neuron function and loss of synaptic contacts from muscle tissue (Manini et al 2013). Under denervation of muscle fibers, there is compensatory sprouting of new motor neuron branches and reinnervation of orphaned muscle tissue that can nearly fully compensate in terms of synaptic strength (Smith and Bodenheimer 1982, Tank et al 2011). This cycling process can fail with aging, and fibers can be left denervated where they experience an overall reduction in muscle mass, leading to muscle atrophy along with neuron cell death (Manini et al 2013, Gonzalez-Freire et al 2017). This is typically a progressive decline and is believed to initiate with changes that occur during senescence at the neuromuscular junction (Punga and Ruegg 2012). With aging comes remodeling of the NMJ, and changes in presynaptic terminal (bouton) morphologies are observed. This can be followed by loss of specific motor units leading to decreased functional activity (Jang and Remmen 2011, Gonzalez Freire et al 2017). Both pre- and postsynaptic changes contribute to age-induced NMJ degeneration.

In the presynaptic compartment, changes in mitochondrial number and function are observed with aging. Mitochondria are important in the axon as they maintain energy and sequester calcium to sustain motor neuron terminal action, particularly during stimulation
Morphological changes to mitochondrial structure with age occurs in many tissues, including muscles and neurons, and can signify dysfunction (Misgeld and Schwarz 2017). This dysfunction also leads to increased levels of nitric oxide and ROS, which can have detrimental effects on aged tissue and limits mitochondrial biogenesis (Navarro and Boveris 2009, Li et al 2013). As previously mentioned, oxidative stress can promote the degeneration of cellular components, particularly as organisms age. Neurons are energy demanding tissues, which results in high ROS generation. Oxidative stress is a major contributing factor to neurodegenerative diseases (Cenini et al 2019). Mitochondria at older synaptic terminals exhibit greater damage due to stress than those at the cell body, and can signal for activation of cell death machinery (Reddy and Beal 2008, Garcia et al 2013). Additionally, redox cross-talk occurs between muscles and neurons across the NMJ, each impacting the functional activity of the other, and knockdown of antioxidant genes in the motor neuron can influence muscle health (Satranatarajan et al 2015, Jackson and McArdle 2016).

With aging comes an increase in inflammation, and this is a risk factor for accelerated decline in strength and mobility as well as a promoter of neuronal death (Ferrucci et al 2005). In vertebrates, Schwann cell aging is associated with the overexpression of IL-6 inflammatory cytokines and reduced axonal regeneration (Saheb-al-Zamani et al 2013). In normal brain aging, there are chronic low-levels of inflammation that induce activation of glia surrounding the neurons. This activation promotes the release of pro-inflammatory cytokines that can act directly onto neurons and impair synaptic plasticity, contributing to dysfunction (Jurgens and Johnson 2012). Microglia activation and subsequent neuroinflammation is a hallmark of age-related neurodegenerative diseases such as Parkinson’s, Huntington’s, and amyotrophic lateral sclerosis (ALS). Neuroinflammation can also initiate motor neuron death (Frakes et al 2014, Komine and
Yamanaka 2015). However, the interaction between glia and motor neuron dysfunction is still not well understood. The functional and structural changes that occur at the neuromuscular junction with aging lead to progressive declines in synaptic transmission and axonal transport and can result in muscle atrophy and neuronal deterioration.

**The Drosophila neuromuscular junction**

In *Drosophila*, the larval neuromuscular junction has been highly stereotyped. Larval pelts contain around 30 muscle fibers with distinct positioning and morphology, and have approximately 38 unique motor neurons that innervate them (Landgraf et al 1997). The larval muscles all have similar physiology and structural gene expression patterning (Keshishian et al 1996). Synapses innervate muscle by the end of embryogenesis and become functional. During the three stages of larval development, the NMJ undergoes extensive remodeling through numerous signaling pathways. This remodeling includes the elongation of branches and a 5-10-fold increase in the addition of new boutons and synaptic transmission sites, known as active zones (Featherstone and Broadie 2000, Nechipurenko and Broihier 2012). On the postsynaptic side, surrounding each bouton terminal is the subsynaptic reticulum (SSR), and is comprised of many labyrinth-like folds and in *Drosophila* contains glutamate receptor clusters. This region is partially dictated by expression of the protein Discs-large, which regulates potassium channel clustering and cell adhesion molecule apparatus (Featherstone and Broadie 2000). Glia also shape the postsynaptic region through the release of WNT which regulates glutamate receptor clustering (Kerr et al 2014). Additionally, MT dynamics within the NMJ have been well characterized. Cytoskeletal patterning and morphology have been well described in the developing NMJ, giving a solid model for examining functional activity (Menon et al 2013). During metamorphosis, there is synaptic remodeling that occurs as new tissues develop. The transition from larval to adult physiology is hallmarked by rewiring and synaptic elimination, or
pruning. This is done in part through ecdysone signaling and requires input from both the presynaptic and postsynaptic regions (Liu et al 2010). This results in adult-specific neuronal circuitry. During this process, innervation also drives muscle development (Hebbar et al 2006). Adult motor neurons also seem to arise from two pools of neurons, those that were in larvae and get re-specified, and those that were dormant but already developed during embryogenesis. During the first 5 days of early adulthood, there is synaptic growth and bouton expansion which occurs in NMJs but does not affect muscle size. Octopamine signaling boutons are also observed at NMJs in the adult, and there is a loss of Fasciclin II expression at glutamatergic synapses, which drives neuron growth during development (Rivlin et al 2004).

The adult *Drosophila* NMJ morphology is far less studied than the larvae, but there are several reports that have done a characterization of the NMJ at specific muscles. One prevalent observation was that the adult NMJ undergoes several molecular and morphological changes with aging. In abdominal muscles, changes occur both with early adult synapse maturation and with aging. Branch length and bouton size are shown to change throughout adulthood and have an inverse relationship with each other. Branch length decreases with age, while boutons show an enlargement of overall area. Bouton number is also reduced during aging in both male and female fruit flies (Wagner et al 2015, Lopez-Arias et al 2017). Additionally, a reduction in the thickness of the SSR was observed with age, and this was found to occur across different adult muscle segments. (Beramendi et al 2007, Wagner et al 2015). This also indicates that aging influences both the pre- and postsynaptic regions. These changes are also seen in vertebrate systems (Prakash and Sieck 1998). In the *Drosophila* postsynapse, aging causes a gradual expansion of glutamate receptor fields, and aged flies were seen to have fewer successful transmissions at higher frequency stimulation, along with reduced climbing ability (Martinez et
al 2007, Wagner et al 2015). Older flies were also found to have reduced endocytic activity at the plasma membrane, which was attributed to dynamin and dynactin dysfunction (Beramendi et al 2007). With age, there were also changes to synaptic vesicle numbers around active zones along with an accumulation of both early and late endosomes in the presynaptic terminals (Wagner et al 2015).

One section of muscles that have been well characterized in adult *Drosophila* are the ventral abdominal muscles. In particular, the A3-A5 muscle segments have been profiled (Hebbar et al 2006, Wagner et al 2015, Lopez-Arias et al 2017). At this region, muscles are paired, and each muscle segment contains 8 to 10 fibers and is innervated by one motor neuron, which then branches out to form synaptic contacts (Hebbar et al 2006). Some groups have reported 10 to 11 terminal branches per muscle, while others have reported 5 to 7. This number remains relatively consistent throughout the life of the fly (Hebbar et al 2006, Wagner et al 2015). These NMJs have also been morphologically characterized during aging, and can be used to further understand mechanisms of synaptic decline during senescence (Wagner et al 2015, Lopez-Arias et al 2017).

**Neuronal endocytosis and rab GTPases**

The synapse releases signal information from the axon terminal in the form of neurotransmitters through a process called exocytosis. These neurotransmitters are stored in synaptic vesicles (SVs), which require supply recovery after their release. Therefore, a process called endocytosis occurs that allows for the recycling of vesicles from the plasma membrane for reuse by the axon terminal. This is a rapidly occurring mechanism that prevents the depletion of SVs, and occurs through several mechanisms, the bulk of which are mediated by Clathrin (Ceccarelli et al 1973, Royle and Lagnado 2003). Calcium influx causes SV fusion with SNARE proteins on the terminal surface, allowing for neurotransmitter release. Clathrin-coated pits then
form to allow vesicle reformation and recycling of SVs (Xie et al 2017). The “kiss and run” model of SV recycling does not involve Clathrin, but may provide faster replenishment of empty vesicles (Fesce et al 1994).

Not all vesicles existing in the axon terminal are designated for synaptic transmission. Specific vesicles are designated for the retrieval of membrane proteins for the initiation of both signaling cascades and protein degradation. These vesicles exhibit functionally similar activity as in other tissues, but are unique to neurons in their capability to traverse entire axons and dendrites. They play a role in axon growth and architecture as well as neuron survival (Cosker and Segal 2014). Endocytic vesicles form near active zones in the presynaptic terminal through clathrin-mediated endocytosis and subsequently compartmentalize (Wucherpfennig et al 2003). Signals from pathways such as bone morphogenic pathway (BMP) and many growth factors are shuttled by retrograde mechanisms to the cell body, where they carry out their cellular activity (Deshpande and Rodal 2015). Errors in the retrograde transport of endocytosed cargo by vesicle dysregulation can lead to neurodegeneration (Verhoeven et al 2003).

The Ras superfamily class of small Rab GTPases are small G proteins that bind guanine triphosphate (GTP) as an “on switch and serve as markers for specific organelles, and can be used to identify vesicles designated for specific functions (Hall 1998, D’Adamo et al 2014). This specificity is critical for neuronal activity. In humans, 24 Rab GTPases have been linked to brain function, and are involved in cargo recognition and vesicle transport (D’Adamo et al 2014). Of particular importance in vesicle endocytosis are Rab5 GTPases. These Rabs mark early/sorting endosomes, which act as a hub for many internalized molecules from the plasma membrane. Synaptic vesicles can also be recycled through early endosomes (Wucherpfennig et al 2003, Grant and Donaldson 2009). The recycling of SVs through early endosomes can be monitored by
a transition of GTP from Rab5 to Rab11, which marks fully formed SVs (Takahashi et al 2012). Another important Rab GTPase in neuronal endocytic trafficking is Rab7. Rab7 GTPases mark late endosomes, and are involved in neurotrophin trafficking and signaling, receptor recycling, and autophagy. Late endosomes are able to recycle receptors to the plasma membrane, transport them to the soma, or can fuse with lysosomes for degradation of endocytosed material. Multivesicular bodies form in sorting endosomes and can envelope damaged cargo, and a switch from the active Rab5 GTP to an active Rab7 GTP hallmarks the formation of late endosomes. Late endosomes are known to predominantly travel retrograde along the axon to the soma for cargo degradation (Guerra and Bucci 2016). Down regulation of Rab7 has also been shown to impact lysosome acidification and impair pH regulation through vacuolar ATPase activation (Bucci et al 2000, Johnson et al 2016). Delays and disruptions of the endocytic pathway are seen with age and neurodegeneration (Boaro et al 1998, Cataldo et al 2000, Wagner et al 2015) Therefore, Rab GTPases are critical for maintaining neuronal homeostasis by regulating processes in endocytosis and exocytosis, promoting autophagy and the degradation of damaged cellular components, and transporting signaling proteins throughout the neuron.

**FOXOs in neuronal homeostasis**

FOXO proteins can regulate diverse processes in neuronal tissue and dictate neuron physiology. FOXO proteins are expressed both during development and in adulthood, indicating they have a functional contribution throughout an organism’s lifespan. In some instances, FOXO activity in the nervous system is similar to its role in other tissues. FOXOs still respond to oxidative stressors, and can maintain neuronal stem cell populations (McLaughlin and Broihier 2018). For neuronal stem cells, similar to other stem cell populations,FOXO expression can prevent proliferation and depletion, and with loss of FOXO there is a rise in ROS levels among these cell types (Paik et al 2009). FOXO is also able to induce neuronal apoptosis under
oxidative stress when phosphorylated by MST1, but can also promote autophagic pathways in neurons and prevent degeneration (Lehtinen et al 2006, Yuan et al 2009, Xu et al 2011, Santo and Paik 2018).

Some FOXO activities are unique to neurons, and can promote youthful nervous system characteristics and maintain homeostatic pathways. FOXOs can act as promoters of autophagy and ROS detoxification in neurodegenerative diseases. In Parkinson’s disease, FOXO overexpression protects against α-Synuclein aggregation in Dopaminergic neurons (Koh et al 2012, Pino et al 2014). FOXO overexpression is also neuroprotective in Huntington’s disease, and is required for the neuroprotective activity of Sirt2 deacetylase (Parker et al 2012, She et al 2018). It is currently unclear if these effects are conducted through nuclear or cytoplasmic FOXO activity. Additionally, FOXOs can block motor neuron toxicity in SOD1 and dynactin mutants, showing potential neuroprotective effects in ALS. This pathway is conserved in vertebrate and invertebrate species (Mojsilovic-Petrovic et al 2009). However, in these degenerative models, ROS can activate p38 MAPK, inhibiting Akt signaling and promoting FOXO-mediated cell death (Davila and Torres-Aleman 2007). This appears to be neuron-specific and does not occur in astrocytes or other surrounding tissues (Davila et al 2016).

In the nervous system, FOXO serves as a regulator of neuronal structural plasticity. The ability to make structural changes in response to environmental cues is essential for maintaining synaptic homeostasis. Though FOXO is expressed during development in both the CNS and PNS, it is in the cytoplasm of most neurons, while motor neurons have predominantly nuclear FOXO localization (Nechipurenko and Broiher 2012, Sears and Broihier 2016). In both mammalian and Drosophila systems, FOXO expression is detected later in the developmental process (Hoekman et al 2006, Nechipurenko et al 2012). In Drosophila larvae, foxo mutants
establish NMJs in the muscle, but fail to show quick response to neuronal stimulation. Mutants do not rapidly bud new boutons in response to stimuli as is seen in wild-type larvae (McLaughlin et al 2017). This is due to excessive microtubule stability and kinesin rigidity present in foxo mutants (Nechipurenko and Broihier 2012, McLaughlin et al 2017). This rigidity is often seen in older neurons (Lu et al 2013). However, constitutively active FOXO disrupts microtubule stability, and causes severe cytoskeletal disruption of the axon at the NMJ (Nechipurenko and Broihier 2012). FOXO also regulates dendritic MT dynamics, and loss of FOXO results in reduced dendrite arborization (Sears and Broihier 2016). In mammals, FOXO is needed to establish neuronal polarity during development, and does so through transcriptionally controlling the expression of regulators of cytoskeletal dynamics and influencing axonal outgrowth (de la Torre-Ubieta et al 2010). In C. elegans, PI3K signaling regulates developmental neurite outgrowth through FOXO (Christensen et al 2011). Ultimately, FOXO’s ability to regulate cytoskeletal dynamics impacts neuronal morphology and its ability to establish successful synaptic contacts.

In adults, FOXO is a regulator of health span and cognitive function. In the brain, pro-inflammatory signals can activate FOXO, counteracting age-dependent degeneration. Loss of FOXO increases neuroinflammation and promotes age-dependent axon degeneration in mammals. This effect was found to be through mTOR complex 1, which can be suppressed by FOXO (Hwang et al 2018). In C. elegans, age-dependent spontaneous aberrant neurite branching was attenuated when DAF-16/FOXO was expressed in the neurons of Daf-16; Daf-2 double mutants. Aberrant neurite branching is promoted by positive insulin signaling, and Daf-16 regulated inhibition is believed to function downstream of the JNK pathway (Tank et al 2011). Certain forms of learning and memory were also preserved with age in C. elegans Daf-2
mutants, and is dependent upon the presence of daf-16. This is also seen in mammalian systems, and is linked specifically to FOXO6 activity (Kim and Webb 2017). These results provide evidence for FOXO-regulated processes that delay aging phenotypes and preserve cognitive function.

Insulin and PI3K signaling are integral in neuronal homeostasis, and have a profound effect on FOXO-mediated neuronal activity. PI3K signaling is implicated in a number of neurological disorders, such as neurofibromatosis. Insulin has also been shown to impair neuron excitability. Defects in motor neuron excitability can come from alterations in activation, so the ability to mediate neurotransmitter release is necessary for maintaining synaptic integrity (Howlett et al 2008). In *Drosophila*, excitability is detected through glutamate release and receptor mediated activity (Bogdanik et al 2004, Howlett et al 2008). Inhibition of FOXO is shown to decrease neuron excitability, while overexpression enhances it. This is coordinated through PI3K and insulin signaling (Howlett et al 2008, Mahoney et al 2016). In adults, prolonged insulin signaling can cause neuropathies and cognitive dysregulation, as can be seen in some patients with type II diabetes (Luchsinger 2012). In fly proboscis, high protein diets caused reduced neurotransmission, while low-calorie diets had the opposite effect. This is believed to be through cytoplasmic retention of inactivated FOXO, as FOXO mutants do not display an excitability response to diet. The reduction of neurotransmission in FOXO mutants can be rescued through Thor/4EBP overexpression, and is linked to the translation of synaptic vesicle fusion machinery proteins that promote exocytosis (Mahoney et al 2016). Genomic studies have also frequently revealed ion channels and neurotransmitter receptors as FOXO targets, suggesting FOXO may regulate neurotransmitter release through a number of mechanisms (Salih

In summary, FOXOs are seen across animal species to regulate processes involved in maintaining homeostasis, which can ultimately cause extensions of lifespan. They are present in many different cell types, and can transcriptionally regulate a variety of cellular pathways. FOXOs are also able to interact with many protein partners, and these partners dictate FOXO activity and transcriptional regulation. Though FOXOs are predominantly associated with insulin signaling, they have regulatory effects in response to ROS, and can promote the mitigation of oxidative stress. ROS-driven stress can accumulate with normal aging, while FOXO activity declines with age. The ramifications of this decline in FOXO function are still not well explored, but evidence links it to the onset of certain age-related diseases and tissue degeneration. This degeneration and disease onset can be seen at the neuromuscular junction, which has unique FOXO-regulated activity. It is important to evaluate how activity of longevity-promoting genes, such as FOXO, change with age, so that we can better understand the mechanisms behind senescence and cellular degeneration.

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CHAPTER 2. AGE-DEPENDENT CHANGES IN TRANSCRIPTION FACTOR FOXO TARGETING IN FEMALE DROSOPHILA MELANOGASTER

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Allison Birnbaum1#, Xiaofen Wu2,3#, Marc Tatar4, Nan Liu2*, Hua Bai1*

1 Department of Genetics, Development, and Cell Biology, Iowa State University, Ames, IA 50011, USA 2 Interdisciplinary Research Center on Biology and Chemistry, Chinese Academy of Sciences, Shanghai, 201210, China 3 University of Chinese Academy of Sciences, Beijing, 100049, China 4 Department of Ecology and Evolutionary Biology, Brown University, Providence, RI, 02912, USA

# Equal contribution of authors. A.B. Performed ChIP-seq and motif analysis, immuno-fluorescence, and gene ontology analysis. X.W. and N.L. designed CRISPR fly line and conducted RNA-seq analysis. A.B. and H.B. wrote the manuscript, editorial suggestions received from M.T.

2.1 Abstract

FOXO transcription factors have long been associated with longevity control and tissue homeostasis. Although the transcriptional regulation of FOXO proteins have been previously characterized (especially in long-lived insulin mutants and under stress conditions), how normal aging impacts the transcriptional activity of FOXO is poorly understood. Here, we conducted a chromatin immunoprecipitation sequencing (ChIP-Seq) analysis in both young and old wild-type fruit flies, *Drosophila melanogaster*, to evaluate the dynamics of FOXO gene targeting during aging. Intriguingly, the number of FOXO-bound genes dramatically decreases with age (from 2617 to 224). Consistent to the reduction of FOXO binding activity, many genes targeted by FOXO in young flies are transcriptionally altered with age, either up-regulated (FOXO-repressing genes) or down-regulated (FOXO-activating genes). In addition, we show that many
FOXO-bound genes in wild-type flies are unique from those in insulin receptor substrate *chico* mutants. Distinct from *chico* mutants, FOXO targets specific cellular processes (e.g., actin cytoskeleton) and signaling pathways (e.g., Hippo, MAPK) in young wild-type flies. FOXO targeting on these pathways decreases with age. Interestingly, FOXO targets in old flies are enriched in cellular processes like chromatin organization and nucleosome assembly. Furthermore, FOXO binding to core histone genes is well maintained at aged flies. Together, our findings provide new insights into dynamic FOXO targeting under normal aging and highlight the diverse and understudied regulatory mechanisms for FOXO transcriptional activity.

### 2.2 Introduction

The process of aging is accompanied by a decline in physiological function and cellular maintenance. It is known that aging dramatically alters gene expression and transcription factor activity (Lopez-Otin et al., 2013). The protein family of Forkhead Box subfamily O transcription factors, or FOXO, has been shown to play an important role in growth, development, stress resistance, and longevity (Greer and Brunet, 2005). FOXO functions downstream of insulin/insulin-like growth factor (insulin/IGF) signaling and is negatively regulated by PI3K-Akt pathway (Brunet et al., 1999). FOXO transcriptionally regulates numerous target genes involving metabolism, cell cycle progression, stress, and apoptosis (Medema et al., 2000; Kitamura et al., 2002; Kops et al., 2002; Martins et al., 2016). Additionally, FOXO proteins were first implemented in lifespan extension in *Caenorhabditis elegans* where insulin-like receptor mutant *daf-2* extends lifespan via FOXO homolog *daf-16* (Kenyon et al., 1993). This lifespan extension through insulin/IGF signaling has been observed across species, from worm to fly to mammal (Kenyon et al., 1993; Tatar et al., 2001; Holzenberger et al., 2003). Studies have found that lifespan extension effects of insulin/IGF deficiency depend on FOXO activity, probably through the transcriptional regulation of key longevity assurance pathways such as xenobiotic...
resistance (Slack et al., 2011; Yamamoto and Tatar, 2011). However, how FOXO elicits this response remains to be fully elucidated.

FOXO activity is not solely dependent on insulin/IGF signaling. FOXO proteins undergo posttranslational modifications in response to other cellular stress signals. Oxidative stress promotes Jun-N-terminal Kinase (JNK)-dependent phosphorylation of mammalian FOXO4 and its nuclear translocation. FOXO proteins can also be activated and phosphorylated by mammalian Sterile 20-like kinase 1 (MST1), to extend lifespan (Essers et al., 2004; Lehtinen et al., 2006; Dansen and Burgering, 2008). In response to DNA damage, cyclin-dependent kinase 2 (CDK2) can phosphorylate and regulate mammalian FOXO1 to delay cell cycle progression and induce apoptosis (Huang and Tindall, 2006). FOXO proteins are also involved in tumor suppression activity and responds to oncogenic stress (Dansen and Burgering, 2008).

Interestingly, two recent chromatin immunoprecipitation-sequencing (ChIP-Seq) studies revealed that FOXO proteins are enriched at the promoters of many target genes in well-fed wild-type C. elegans and Drosophila (Alic et al., 2011; Riedel et al., 2013).

Although insulin/IGF signaling is well-known aging regulators, how insulin/IGF signaling is altered during normal aging remains largely unclear. It is generally believed that insulin/IGF signaling declines with age. This is primarily based on age-dependent decreases in the expression of FOXO target genes (Demontis and Perrimon, 2010; Rera et al., 2012). However, it remains to be determined how aging impacts FOXO transcriptional activity and DNA binding capacity of FOXO transcription factors. Here, we conducted a ChIP-Seq analysis to investigate FOXO binding dynamics under normal aging in Drosophila. Intriguingly, we found that the number of FOXO-bound regions sharply decrease with age. The age-related decrease in FOXO binding is correlated with either the transcriptional activation of FOXO-
repressing genes, or the downregulation of FOXO-activating genes during normal aging. Furthermore, we observed strong FOXO nuclear localization in well-fed wild-type flies, and see a distinct set of FOXO targeted genes between wild-type and insulin mutants. Taken together, our findings provide new evidence linking age-dependent FOXO transcriptional activity to its role in longevity control and tissue maintenance.

2.3 Results

**FOXO exhibits constitutive nuclear localization in young and old adult fat body**

To examine whether *Drosophila* FOXO activity changes with aging, we first performed immunofluorescent staining using a polyclonal antibody against *Drosophila* FOXO to monitor the FOXO nuclear localization in wild-type flies (yw\(^R\)) at two different ages, two-week-old (young flies) and five-week-old (aged flies). Female flies were used in the present study because most of the previous Drosophila aging studies used females and large amount of genomic data are available for female flies. To capture molecular events associated with early onset aging phenotypes, five-week-old flies was used. This is because the epigenetic and transcriptional changes have been previously observed in five-week-old flies (Hall et al., 2017; Peleg, Feller, Ladurner, & Imhof, 2016; Pletcher et al., 2002). Besides, many physiological and functional alterations can been observed at five weeks of age (Demontis & Perrimon, 2010; K. Huang et al., 2019; Wessells, Fitzgerald, Cypser, Tatar, & Bodmer, 2004).

It is known that FOXO functions within adult fat body and muscle to control longevity (Demontis & Perrimon, 2010; Hwangbo, Gershman, Tu, Palmer, & Tatar, 2004), and in nervous system to maintain neuronal morphology and plasticity (McLaughlin, Nechipurenko, Liu, & Broihier, 2016; Sears & Broihier, 2016). The activation of FOXO in these tissues, as indicated by its nuclear localization, is normally observed only in insulin/IGF mutants. Intriguingly, FOXO proteins exhibited constitutive nuclear localization in abdominal fat body tissue of well-
fed wild-type female flies (ywR), where insulin/IGF signaling is presumably active (Figure 2.1A). The constitutive nuclear localization of FOXO was also found in another wild-type line, Oregon R (OreR) (Figure S2.1). FOXO proteins remained nuclear localization during aging, while the colocalization of FOXO with nuclear DAPI staining slightly declined in aged fat body tissue (Figure 2.1A-B). Compared to adult fat body, indirect flight muscles from both two-week-old and five-week-old female flies showed low FOXO nuclear localization (Figure 2.1C, 2.1D, S2.1). In addition, the constitutive nuclear localization of FOXO was also found in adult brain of wild-type female flies (Figure S2.1B). Thus, these results suggest that FOXO could be activated in well-fed wild-type flies to regulate the expression of its target genes, which is consistent with recent ChIP-Seq studies (Alic et al., 2011; Riedel et al., 2013).

**ChIP-Seq analysis reveals age-dependent reduction of FOXO-targeted DNA binding**

To further investigate the FOXO transcriptional activity under normal aging, we performed ChIP-Seq analysis on young (2-week) and aged (5-week) female wild-type flies. Using Illumina high-throughput sequencing, we obtained a total of 261 million reads from FOXO ChIP and input DNA samples at two ages. On average, 90.08% of unique reads were mapped to annotated *Drosophila* reference genome (Figure S2.2, Table S2.1). Intriguingly, our ChIP-Seq analysis revealed that the number of FOXO-bound genomic regions (based on MACS2 peak calling) dramatically decreased with age (Figure 2.2A). There were 9273 peaks identified in young flies (corresponding to 2617 protein coding genes), whereas in aged flies only 1220 peaks (224 genes) were detected (Figure 2.2A, Table S2.1). About 170 genes were shared between two ages. For most of the peaks, a reduction in peak size or a disappearance of peaks was observed in aged flies (Figure 2.2B), while the FOXO binding to a few genomic regions remained unchanged during aging (Figure 2.2C). The reduction of FOXO-bound regions was not due to the decreased quantity of immunoprecipitated genomic DNA (data not shown). In fact, equal amount of ChIP
and input DNA samples were used to generate Illumina sequencing libraries. In addition, a correlation matrix plot showed that the reads from 2-week-old FOXO ChIP samples were most divergent from the input and 5-week-old ChIP samples, further suggesting the differential FOXO-DNA binding activity between young and aged flies (Figure S2.2).

Gene ontology analysis revealed that at young ages, FOXO targeted genes were enriched for pathways like Hippo, WNT, TGF-beta, MAPK, and insulin resistance pathways (Figure 2.2D, Table S2.1:List 10). FOXO was also targeting genes involved in nervous system development, motor neuron stabilization, and regulation of synaptic tissue communication (Table S2.1). Additionally, we found that FOXO bound to the genomic regions containing key autophagy regulators (Atg3, Atg17, Tor, wdb, Pten), which is consistent to previous known functions of FOXO in autophagy and tissue homeostasis (Demontis and Perrimon, 2010). Many Rho and small GTPase proteins, as well as actin cytoskeleton pathways, are also targeted by FOXO at young ages. Many of these FOXO-targeted pathways were absent in aged flies. Instead, processes like nucleosome assembly and chromatin organization were enriched as FOXO-bound targets in aged flies (Figure 2.2D, Table S2.1:List 11). Interestingly, strong FOXO binding was maintained at many core histone genes at old ages (Figure 2.2C, 2.2E).

The age-dependent changes in FOXO binding activity were verified by quantitative PCR (ChIP-qPCR). The FOXO binding to the promoters of two known target genes, insulin receptor InR and adipose triglyceride lipase bmm, were first tested in ChIP-qPCR analysis (Figure 2.2E). FOXO showed similar binding enrichment (6~7 fold) at InR locus between young and old ages (Figure 2.2E). On the other hand, the FOXO binding to bmm promoter slightly decreased with age (Figure 2.2E). We also confirmed that FOXO binding remained unchanged at two histone loci (his1:CG33804 and his2B:CG33908), while the FOXO enrichment at two newly identified
target genes, *Jim* (C2H2 zinc finger transcription factor) and *dlg1* (a key factor for the formation of septate junctions and synaptic junctions), decreased dramatically at old ages (from 80~90-fold to 3~8-fold) (Figure 2.2E). Thus, our ChIP-qPCR analysis confirmed that FOXO binding activity was altered in many target loci during normal aging.

**FOXO-bound genes show age-dependent transcriptional changes**

We next examined whether age-dependent changes in FOXO binding is correlated to age-regulated transcription of FOXO target genes. To do so, we first compared our FOXO ChIP-Seq results to previously published aging transcriptomic analysis on aging *Drosophila* tissues, such as fat body and head tissue. Out of 2447 FOXO target genes (uniquely bound by FOXO at young ages), 408 of them were differentially expressed in aging fat body (172 downregulated, 236 upregulated) (Figure 2.3A, Table S2.1:List 12), while 845 target genes were differentially expressed in aging head tissue (626 downregulated, 219 upregulated) (Figure 2.3C, Table S2.1:List 13). Interestingly, a majority of the FOXO-bound genes showed no age-related transcriptional changes, which is similar to previous studies showing the FOXO binding at the promoters of large number of so-called poised genes (Webb et al., 2013; Webb et al., 2016).

Gene ontology analysis revealed that FOXO target genes differentially expressed in aging fat body were enriched for processes and signaling pathways like chromatin organization, histone modification, hippo signaling, peroxisome, and hormone biosynthesis (Figure 2.3B, Table S2.1:List14). On the other hand, the differentially expressed FOXO targets in aging head tissue were enriched for pathways and processes involving WNT, Hippo, G protein-couple receptor (GPCR), axon guidance, synapse organization, and actin cytoskeleton (Figure 2.3D, Table S2.1:List15).

Although many FOXO-bound target genes exhibited differential expression during aging, it remains unclear whether decreased FOXO-binding activity at old ages contributes to age-
dependent transcriptional changes of these FOXO target genes. To further determine the relationship between FOXO binding and transcriptional changes of FOXO target genes, we performed an RNA-Seq analysis using head tissues dissected from wild-type flies and a foxo null mutants (foxo^c431), a site-specific deletion mutant generated by CRISPR/Cas9 (Figure 2.4A, 2.4B). Out of 2617 FOXO-bound target genes, 101 of them were upregulated in foxo^c431 mutants, while 300 were downregulated in the mutants (Figure 2.4C, Table S2.1:List 16), suggesting that FOXO binding might be important to repress or activate at least a subset of target genes. Based on these data, FOXO target genes can be sorted into three classes, FOXO-repressing (101 genes), FOXO-activating (300 genes), and FOXO-no regulation (1621 genes).

We next asked how reduced FOXO binding during aging impacts the expression of FOXO target genes. To do this, we first constructed new transcriptomic profiles from wild-type head tissue at four different ages, 3d, 15d, 30d, and 45d (Table S2.1:List 17). Interestingly, among three classes of FOXO target genes, FOXO-repressing genes exhibited an increased expression in old flies, whereas FOXO-activating genes were progressively downregulated with age. Expression of FOXO-no regulation genes, on the other hand, did not significantly change during aging (Figure 2.4D). Taken together, these results suggest that age-associated decrease in FOXO binding might contribute directly to the transcriptional alterations of FOXO target genes in old flies.

**FOXO binding differs between wild-type and insulin/IGF mutants**

FOXO binding activity has been primarily studied by evaluating its response to IIS signaling (Murphy, 2006; Alic et al., 2011; Bai et al., 2013; Riedel et al., 2013; Webb et al., 2016). However, our observations on FOXO nuclear localization and DNA binding in well-fed wild-type flies suggest that there might be distinct FOXO transcriptional activity independent of insulin/IGF signaling. To test this possibility, we compared FOXO ChIP-Seq datasets from the
present study (young wild-type) and our previous analysis on insulin receptor substrate *chico* mutants (Bai et al., 2013). Intriguingly, large number of FOXO-bound genes were not shared between wild-type and *chico* mutants. There were 1992 FOXO target genes unique to wild-type, while 1393 genes unique to *chico* mutants (Figure 2.5A, Table S2.1:List 18). Furthermore, distinct FOXO targets between wild-type and *chico* mutants were differentially expressed with age (Figure 2.5B). About 844 age-regulated genes were only bound by FOXO in wild-type flies, while 577 genes unique to *chico* mutants (Table S2.1:List 19). We found that age-regulated FOXO targets unique to *chico* mutants were enriched in metabolic pathway and oxidative-reduction, while those unique to wild-type flies were enriched for chromatin organization, axon guidance, Hippo and MAPK signaling pathways (Figure 2.5C, Table S2.1:List 20-21). When examining each pathway in detail, we noticed that FOXO targets in Hippo and MAPK/EGFR signaling pathways were found in both wild-type and *chico* mutants, although different target genes were apparent between the two conditions (Figure S2.3-S2.4).

To test if distinct FOXO binding activity observed between wild-type flies and insulin/IGF mutants is conserved across species, we reanalyzed the recent *C. elegans Daf-16* ChIP-seq study (Riedel et al., 2013). Interestingly, wild-type worms also showed different Daf-16 binding activity from *daf-2* mutants. There were 2296 genes uniquely bound by Daf-16 to wild-type worms, while 996 were unique to *daf-2* mutants (Figure 2.5D, Table S2.1:List 22). Gene ontology analysis showed that FOXO transcription factors targeted similar pathways in wild-type flies and worms. These pathways were MAPK signaling, cell cycle, FOXO signaling, nervous system development, chromatin remodeling, mTOR signaling, autophagy, and oxidative stress (Figure 2.5E, Table S2.1:List 23). Thus, insulin/IGF-independent FOXO transcriptional activity may be an evolutionarily conserved cellular mechanism.
**Enriched FOXO motifs in wild-type flies**

A signature of FOXO targeting is the 8-nucleotide long canonical binding motif, 5’-TTGTTTAC-3’, which is conserved across species (Furuyama et al., 2000; Bai et al., 2013; Webb et al., 2013). This motif is typically found upstream of the gene coding site in the enhancer or promoter region (Eijkelenboom et al., 2013; Webb et al., 2013). To search for FOXO consensus sequence in the FOXO-bound genomic regions found in young wild-type flies, we conducted motif analysis using the Homer motif finding tool. We used peaks with at least a 2-fold enrichment that were less than 2000 bp in length, and we searched for motifs within 200 bp surrounding the peak region. When insect motif databases were used, we identified only one known motif for Trl (p < 10^{-70}), a GAGA-factor that also found in previous ChIP-Seq data from *C. elegans* (Riedel et al., 2013) (Figure 2.6). Next, we performed Homer de novo motif search and identified a motif for RAP1, a *Saccharomyces cerevisiae* gene that is part of the Myb/SAINT domain family (Figure 2.6), which was also found in a previous Drosophila FOXO ChIP-on-ChIP study (Alic et al., 2011). Using de novo motif search we also found that motifs for transcription factors hb, Adf1, and Aef1 were enriched in FOXO-bound regions. Lastly, when searching against known mammalian motifs, a motif for FOXO1 (with canonical consensus, TGTTTAC) was detected with low significance (p < 10^{-4}). (Figure 2.6). Together, these findings suggest that in wild-type flies FOXO may recognize a unique set of motifs that is different from the canonical consensus sequence.

**2.4 Discussion**

As a key player in longevity control, FOXO transcription factors and their direct targets have been well characterized in many model systems (Alic et al., 2011; Bai et al., 2013; Riedel et al., 2013; Webb et al., 2013). However, whether and how FOXO transcriptional activity changes with age is unclear. In the present study, we performed a ChIP-Seq analysis to examine the
FOXO binding activity during *Drosophila* aging. Intriguingly, genome-wide FOXO-binding activity during *Drosophila* aging. Intriguingly, genome-wide FOXO-binding activity underwent an immense reduction at old ages. Consistently, genes that are negatively regulated by FOXO showed an increased expression with age, whereas the FOXO-activating genes were downregulated in aged flies. Thus, age-associated decrease in FOXO binding is tightly linked to the transcriptional alterations of FOXO target genes at old ages. In addition, we found that FOXO targets distinct sets of genes between wild-type and insulin/IGF mutants across species, suggesting a conserved insulin/IGF-independent transcriptional regulation by FOXO transcription factors.

Changes in transcription factor binding patterns at different stages of life are not exclusive to FOXO. In *C. elegans*, FoxA/PHA-4 exhibits differential binding patterns at different stages of development to regulate organogenesis (Zhong et al., 2010). Similar to FOXO binding pattern, PHA-4 also exhibited binding at poised locations in the genome. The loss of specific FOXO targeting with age observed in the present study could be caused by either altered post-translational modifications of FOXO, or changes in co-transcriptional regulation between FOXO and its partners. It is known that FOXO co-factors play an important role in fine-tuning FOXO transcriptional activity (Essers et al., 2004; Alic et al., 2011; Riedel et al., 2013; Webb et al., 2016). These co-factors include post-translational modifiers and nuclear interacting partners which aid FOXO in recruitment to target binding sites (van der Vos and Coffer, 2008; Daitoku et al., 2011). A previous meta-analysis identified the binding motifs of many of novel transcription factors (EST, NRF and GATA factors) are enriched at FOXO target genes with age-related expression patterns (Webb et al., 2016), which suggests that the interplay between FOXO and these transcription factors may contribute to the altered FOXO transcriptional activity during normal aging. Certain mammalian FOXO co-factors, such as peroxisome proliferator-activated
receptor gamma (PPARγ), and its coactivator (PGC-1α) interact with FOXO and compete for binding with FOXO and β-Catenin (Olmos et al., 2009; Polvani et al., 2012). FOXO acts as a repressor of PPARγ gene transcription, and this repression is lost later in life, suggesting a reduction of FOXO binding at PPARγ locus (Armoni et al., 2006; Polvani et al., 2012). Besides PPARγ and PGC-1α, many other transcription co-regulators and post-translational modifiers have been shown to be involved in transcriptional co-regulation of FOXO target genes, which may play important roles in modulating FOXO transcriptional activity during aging (van der Horst and Burgering, 2007; Daitoku et al., 2011).

Many FOXO-targeted cellular processes (e.g., nervous system development and actin cytoskeleton) and signaling pathways (e.g., Hippo, WNT, TGF-beta, MAPK) are uniquely enriched in young wild-type, but not in chico mutants. Majority of these FOXO targets show age-dependent differential expression patterns. A recent transcriptomic analysis revealed that age-related DAF-16 targets in wild-type C. elegans are distinct from those in DAF-2 mutants, such as MAPK signaling pathway (Li et al., 2019). MAPK signaling is involved in tissue homeostasis with aging (Jiang et al., 2011; Lee and Sun, 2015), and is also enriched among FOXO-bound target genes in wild-type flies. Both the EGFR and JNK cascades of the MAPK signaling pathway are targeted by FOXO. The target genes involved in the EGFR signaling exhibit transcriptional alterations with age in the wild-type fly. In adult Drosophila, EGFR signaling is responsible for maintaining midgut epithelial homeostasis in the adult and has also been shown to regulate cytoskeletal modulation and autophagy (Hazan and Norton, 1998; Jiang et al., 2011; Tan et al., 2016). EGFR regulation of autophagy also impacts glial maintenance and degeneration of the nervous system (Lee and Sun, 2015). Our ChIP-Seq analysis places FOXO as an upstream regulator of MAPK/EGFR pathway to control autophagy and tissue maintenance.
during aging. In addition, our ChIP-Seq analyses identify Hippo pathway as a major FOXO target in wild-type flies. The Hippo pathway was initially characterized for its role in controlling organ size during development, but recently it has shown involvement in autophagy, oxidative stress response, and aging (Udan et al., 2003; Lehtinen et al., 2006; Mao et al., 2015). In adult mice, suppression of Hippo signaling improved cell proliferation and heart tissue regeneration and is a regulator of tissue homeostasis (Heallen et al., 2013). Thus, Hippo signaling may be one of the major FOXO targets in the regulation of cellular homeostatic and longevity.

Our analysis also revealed that FOXO targets chromatin organization and nucleosome assembly processes. This finding suggests that FOXO may be involved in the maintenance of chromatin structure. Recent studies have shown that FOXO recruits SWI/SNF chromatin remodelers to specific target sites to regulate lifespan in *C. elegans* (Riedel et al., 2013). Changes in chromatin structure and overall loss of heterochromatin has long been an indicative measurement of aging (Wood et al., 2010; Larson and Yuan, 2012; Zhang et al., 2015). It is likely that FOXO plays an important role in maintaining chromatin structure and preventing age-related chromatin remodeling. Interestingly, we found that many core histone genes are targeted by FOXO. The binding of FOXO to these histone genes dramatically increases at old ages. It has been shown that the transcripts of histone genes increase during yeast replicative aging, but the levels of core histone proteins (e.g., H3, H2A) dramatically decrease with age. Overexpression of histones or mutation of the histone information regulator (Hir) causes increased lifespan (Feser et al., 2010). How histone genes are transcriptional regulated during aging is unclear. Our findings suggest that FOXO might be one of the molecular mechanisms that contribute to altered histone expression during normal aging.
In summary, using a genome-wide approach we identified dynamic FOXO binding activity during *Drosophila* aging. Our findings further support the important role of FOXO in age-related transcriptional alterations and the regulation of tissue homeostasis and cellular maintenance pathways. Further investigation of the functional significance of the altered FOXO binding with age will be important in understanding how FOXO regulates organismal homeostasis and longevity.

### 2.5 Experimental Procedures

**Fly culture and stocks**

Flies were maintained at 25°C with 12-hour light/dark cycle, 60% humidity on agar-based diet with 0.8% cornmeal, 10% sugar, and 2.5% yeast. *yw* flies (Bai et al., 2013) were used as wild-type for ChIP-Seq. *w^{1118}* (Bloomington #5905) was used as a control genotype for *foxo* mutants in RNA-Seq analysis. Female flies were collected and sorted 1-2 days after eclosion. To age flies, vials contained 25-30 flies were transferred to fresh food every three days.

**CRISPR/Cas9 mutagenesis**

The *foxo* deletion lines were generated through CRISPR/Cas9 mutagenesis as previously described (Ma et al., 2018). Briefly, two sgRNA plasmids targeting FOXO DNA binding domain were injected into fly embryo. To genotyping G0 flies, single fly was homogenized in 50 μl squashing buffer (10 mM Tris buffer (pH 8.5), 25 mM NaCl, 1 mM EDTA, 200 μg/ml Proteinase K), incubated at 37°C for 30 minutes, then followed by inactivation at 95°C for 10 minutes. Screen primers for *foxo* deletion mutants were: F 5’-GGGGCAGATCCCCGCCCAGC-3’, R 5’-GGGCGATTCGAATAGCAGTGC-3’. The virgin females carrying the deletion were backcrossed into *w^{1118}* male flies for five consecutive generations to mitigate background effects.
Transcriptomic analysis (RNA-Seq)

For transcriptomic analysis on the head tissues of aged flies and *foxo* deletion lines (*foxo*^431^), forty heads from female flies were dissected and homogenized in a 1.5 ml tube containing 1 ml of Trizol Reagent (Thermo Fisher Scientific, Waltham, MA, USA. Catalog number: 15596026). Three biological replicates were performed for each age and genotype. Total RNA was extracted following manufacturer instruction. TURBO DNA-free kit was used to remove genomic DNA contamination (Thermo Fisher Scientific, Waltham, MA, USA. Catalog number: AM1907). About 1 μg of total RNA was used for sequencing library preparation. PolyA-tailed RNAs were enriched by NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs (NEB), Ipswich, MA, USA. Catalog number: E7490S). RNA-Seq library was prepared using NEBNext Ultra RNA library Prep Kit for Illumina (NEB, Ipswich, MA, USA. Catalog number: E7420S). The libraries were pooled together in equimolar amounts to a final 2 nM concentration. The normalized libraries were denatured with 0.1 M NaOH (Sigma) and sequenced on the Illumina Miseq or Hiseq 2500 platforms (Single-end, Read length: 100 base pairs) (Illumina, San Diego, CA, USA).

Chromatin immunoprecipitation sequencing (ChIP-Seq)

Chromatin immunoprecipitation (ChIP) protocol was performed and modified from (Bai et al., 2013). Two biological replicates were collected for each age and genotype. About 200 female flies were first anesthetized with FlyNap (Carolina Biological, Burlington, NC, USA. Catalog number: 173010) and ground into a powder in liquid nitrogen. Crosslinking was performed using 1% paraformaldehyde for 20 minutes followed by glycin quenching. The fly homogenate was washed several times with 1X PBS supplemented with protease inhibitors, and incubated briefly with cold cell lysis buffer (5 mM HEPES pH 7.6, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). Chromatin was extracted with nuclear lysis buffer (50 mM HEPES pH 7.6, 10 mM
EDTA, 0.1% Na-deoxycholate, 0.5% N-lauroylsarcosine), and sheared using Branson digital sonifier 250, using 30%, with 30 seconds on, 30 seconds off for 5 cycles. Chromatin immunoprecipitation was carried out using Protein G SureBeads (Bio-Rad, Hercules, CA, USA. Catalog number: 1614023). Pre-cleaned chromatin extracts were incubated with anti-FOXO antibody (Bai et al., 2013) and Protein G SureBeads to precipitate FOXO-DNA complexes.

DNA size selection and library prep were done using NEBNext Ultra II DNA library prep kit and indexed using NEBNext multiplex oligos for Illumina (Primer set 1) (NEB, Ipswich, MA, USA. Catalog number: E7645S, E7335S). DNA from either ChIP or input samples was mixed with AMPure XP beads (Beckman Coulter Inc., Brea, CA, USA. Catalog number: A63881) to select for a final library size ranging from 280bp to 345 bp. Following are the bioanalyzer results from four ChIP samples: Young ChIP DNA_1: 345.7±38.4 bp; Young ChIP DNA_2: 306.3 ±16.4 bp; Aged ChIP DNA_1: 305.8±19.5 bp; Aged ChIP DNA_2: 281.8±14.7 bp. Samples were then diluted to a final concentration of 2 nM for Illumina sequencing on Illumina HiSeq 3000 (Single-end, Read length: 50 base pairs) (Illumina, San Diego, CA, USA).

**Data processing of RNA-Seq and ChIP-Seq**

RNA-Seq reads were first mapped to the reference genome Dm6 with STAR_2.5.3a by default parameter. The read counts for each gene were calculated by HTSeq-0.5.4e. The count files were used as inputs to R package DESeq for normalization. The differential expression genes were computed based on normalized counts from three biological replicates (|log2 foldchange|>1, adj p<0.01).

For ChIP-Seq, raw FASTQ reads were merged using mergePeaks (Homer suite) then uploaded into Galaxy (usegalaxy.org) and checked for quality using FastQC. Files were then run through FASTQ Groomer (https://usegalaxy.org/u/dan/p/fastq) for readability control before mapping reads using Bowtie2 for single-end reads. *D. melanogaster* BDGP Release 6/dm6 was
used as the reference genome. BAM output files were converted to SAM using BAM-to-SAM (http://www.htslib.org/doc/samtools.html) and sorted to generate peak images. Peak calling was performed using MACS2. MACS2 FDR (q-value) was set for a peak detection cutoff of 0.05 and did not build the shifting model. The MFOLD for the model was set from 10-50 to detect fold-enrichment. Peak-calling was set to identify peaks 300 bp in length, and no peaks could exceed 10 Kb in size. After MACS2 peak identification, peak regions were expanded 2 kb (1 kb upstream and 1 kb downstream) and assigned to nearby and overlapping genes using BEDTools/intersect (https://bedtools.readthedocs.io/en/latest/content/bedtools-suite.html) with Dm6.16 genome annotation file (UCSC, Santa Cruz, CA, USA). All non-protein coding identified targets were removed from the data set manually based on annotation symbol.

Venn diagrams

Venn diagram were created using the Bioinformatics and Evolutionary Genomics Venn calculator at Ugent (http://bioinformatics.psb.ugent.be/webtools/Venn/). For cross species comparisons, gene ID’s were converted to fly ID’s using DIOPT (http://www.flyrnai.org/diopt). Genes that were the best possible match for each ortholog were selected for gene list comparison.

Quantitative PCR (qPCR)

Quantitative PCR was run on QuantStudio 3 (ThermoFisher Scientific, Waltham, MA USA) with above ChIP and input library samples. PCR reaction was conducted using PowerUp SYBR Green Master Mix (Life Technologies, CA, USA. Catalog number: 4402953). FOXO binding enrichment was determined based on the fold-change between ChIP samples vs. Input samples. The FOXO binding to Actin5C locus was used as a negative control. Two biological and two technical replicates were performed for each age. Primers are listed in Table S2.

Pathway and gene ontology analysis
Pathway and gene ontology analysis was conducted using Panther (http://www.pantherdb.org/), String (https://string-db.org/) and DAVID (https://david.ncifcrf.gov/). All three methods were used to obtain a more complete picture of shared regulation between datasets. KEGG pathway maps were obtained through KEGG Pathway (http://www.kegg.jp/kegg/pathway.html).

**Motif analysis**

Motif analysis was conducted using Homer’s `findMotifsGenome` script (http://homer.ucsd.edu/homer/ngs/peakMotifs.html) to compare peak regions with Dm6.01 FASTA data from UCSC.

**List of raw datasets used**

ChIP-Seq datasets: GSE62580 (*Drosophila* aging fat body), GSE81100 (*Drosophila* aging head tissue), GSE44686 (*Drosophila chico* heterozygotes FOXO ChIP), GSE15567 (Encode *C. elegans Daf-16* ChIP)

**Immunofluorescent staining**

Flies were anesthetized with FlyNap and dissected in 1X PBS. Fly tissues (muscle or fat body) were then fixed in 4% paraformaldehyde for 20 minutes at room temperature. Tissue was washed in 1X PBST (0.1% Triton X) and blocked with 5% normal goat serum (NGS) for 1 hour at room temperature. Fly tissues were stained with anti-FOXO antibody in 1X PBST at a dilution of 1:1000 for 16 hours at 4°C on a rotator. Tissues were placed in secondary anti-body goat-anti-rabbit conjugate Alexa Fluor 488 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) at a Dilution of 1:250 and kept in the dark at room temperature for 2 hours. The nucleus was stained using SlowFade with DAPI. Images were captured using an epifluorescence-equipped BX51WI microscope (Olympus, Waltham, MA, USA). Image deconvolution was
conducted using CellSens software (Olympus, Waltham, MA, USA), and compiled using ImageJ Fiji.

**Statistical analysis**

GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis and to generate Boxplot. To compare the mean value of treatment groups versus that of control, either student t-test or one-way ANOVA was performed using Dunnett’s test for multiple comparison.

### 2.6 Acknowledgments

We thank Bloomington Drosophila Stock Center for fly stocks. We thank Michael Baker and DNA Facility at Iowa State University (ISU) for help with RNA-Seq analysis, Usha Muppirala and Andrew Severin from ISU Genome Informatics Facility (GIF) for assistance with bioinformatics. We thank Christian Riedel for providing *C. elegans Daf-16* ChIP-Seq data. This work was supported by NIH/NIA R00 AG048016 to HB, AFAR Research Grants for Junior Faculty to HB, and National Program on Key Basic Research Project of China 2016YFA0501900 to NL. The manuscript has been released as a pre-print at bioRxiv (Birnbaum et al., 2018).

### 2.7 Author’s Contributions Statement

Conceived and designed the experiments: AB XW NL HB. Performed the experiments: AB XW. Analyzed the data: AB XW MT NL HB. Wrote the paper: AB XW NL HB. All authors reviewed and approved the manuscript.

### 2.8 Availability of data and materials

The raw data files of sequencing experiments have been deposited in the NCBI Gene Expression Omnibus. The accession number for RNA-Seq data is GSE122470 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE122470).
The accession number for ChIP-Seq data is GSE121102

2.9 Competing Interests

The authors declare that no competing interest exists.

2.10 Figure Legends

**Figure 2.1.** FOXO exhibits constitutive nuclear localization in young and old adult fat body. A) Abdominal fat body of wild-type flies (yw\(^R\)) stained with anti-FOXO at young (2 weeks) and old ages (5 weeks). B) Quantification of Pearson correlation coefficient (R) between FOXO and DAPI staining in fat body tissue. C) FOXO immunostaining in young and old indirect flight muscles of wild-type flies (yw\(^R\)). D) Quantification of Pearson correlation coefficient (R) between FOXO and DAPI in indirect flight muscles. Scale bar: 20 µm. Student t-test (***, p<0.001; ns: not significant).

**Figure 2.2.** FOXO binding activity decreases with age. A) The number of genes targeted by FOXO at young (2 weeks) and old ages (5 weeks). B) Age-dependent FOXO binding at jim locus. C) Age-dependent FOXO binding at his1:CG33804 and his2B:CG33908 loci. D) GO terms for FOXO-targeted pathways uniquely enriched in young or old flies. E) qPCR validation of the FOXO binding enrichment at the selected FOXO targeted genomic loci. FOXO binding at Act5C locus serves as an internal control. The enrichment value is calculated as the fold-change (f. c.) of the FOXO binding (ChIP vs. Input) between FOXO-targeted loci and Act5C locus. Student t-test (***, p<0.001; **, p<0.01; *, p<0.05).

**Figure 2.3.** FOXO target genes show age-dependent transcriptional changes. A) The number of FOXO-bound genes that are differentially expressed in aging fat body. B) Representative biological processes enriched for age-regulated FOXO targets in fat body. C) The number of FOXO-bound genes that are differentially expressed in aging head tissue. D)
Representative biological processes enriched for age-regulated FOXO targets in adult head tissue.

**Figure 2.4. The altered of FOXO binding correlates with age-related transcriptional changes of FOXO targets.** A) The diagram showing foxo locus and the target sites of the guiding RNAs (highlighted in red) used to generate foxo^c431 loss-of-function mutants by CRISPR/Cas9 mutagenesis. PAM: Protospacer adjacent motifs (highlighted in blue). B) Western blots to verify the expression of FOXO proteins in foxo^c431 loss-of-function mutants. β-actin as a loading control. C) The number of FOXO target genes that are differentially expressed between foxo^c431 mutants and wild-type flies. D) Age-dependent transcriptional changes of FOXO target genes. Boxplots represent the mean fold change of genes at Day 15 (d15), Day 30 (d30) and Day 45 (d45), relative to that of Day 3 (d3) in aging head tissue (Student t-test).

**Figure 2.5. FOXO binding differs between wild-type and insulin/IGF mutants.** A) Comparison of FOXO target genes between wild-type and chico mutants. B) Overlap between age-dependent differentially expressed genes (fat body and head) and FOXO-bound targets (wild-type and chico mutants). C) GO terms uniquely enriched in wild-type or chico mutants. D) Daf-16-bound targets genes in wild-type C. elegans and Daf-2 mutants. E) Shared pathways targeted by both fly FOXO and worm Daf-16 in wild-type animals. Enriched C. elegans GO terms are shown.

**Figure 2.6: Lists of motifs that are enriched among FOXO target sites in wild-type flies.** Motifs within 200 bp surrounding the peak region were analyzed using Homer. Three methods were used: motif search against insect motif databases; de novo motif search; motif search against known mammalian motifs.
Supporting information

**Figure S2.1.** A) Abdominal fat body and flight muscle of wild-type flies (OreR) stained with anti-FOXO at young (2 weeks) and old age (5 weeks). Scale bar: 20 µm. B) Brain muscle of wild-type (ywR) stained with anti-FOXO at 2 weeks post-eclosion. Scale bar: 100 µm.

**Figure S2.2.** A) The total number of raw reads and Bowtie alignment percentage for individual ChIP-Seq sample. B) Plot correlation matrix showing the overall correlation among young and old ChIP and input samples.

**Figure S2.3.** FOXO target genes in Hippo signaling pathway. Unique FOXO targets in wild-type flies (ywR) are highlighted in blue. Unique FOXO targets in chico mutants are highlighted in orange. Shared targets are highlighted in green.

**Figure S2.4.** FOXO target genes in MAPK/EGFR signaling pathway. Unique FOXO targets in wild-type flies (ywR) are highlighted in blue. Unique FOXO targets in chico mutants are highlighted in orange. Shared targets are highlighted in green.

**Table S2.1:** Lists of peaks, target genes, and GO terms (See APPENDIX)

**Table S2.2:** Lists of primers used in qPCR analysis

**Table S2.3:** Galaxy Workflow for ChIP-seq analysis

2.11 References


### 2.12 Figures and Tables

![Figure 2.1](image-url)

Figure 2.1
Figure 2.2

A. Venn diagram showing 2w and 5w groups with 2447, 170, and 54 overlaps.

B. 2 weeks and 5 weeks comparison for jim.

C. Comparison of 2 weeks and 5 weeks for his1:CG33804 and his2B:CG33908.

D. Overview of signaling pathways:
- Hippo signaling
- Wnt signaling
- Insulin resistance
- TGF-beta signaling
- MAPK signaling
- Nucleosome assembly

E. Box plots for ChIP/Input (f.c.) of:
- Act5C
- his1:CG33804
- his2B:CG33908
- InR
- bmm
- jim
- dlg1

Significance levels: 
* p < 0.05
** p < 0.01
*** p < 0.001
Figure 2.
Figure 2.4
Figure 2.5
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<td>1012</td>
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Figure 2.6
Figure S2. 1

Figure S2. 2
Figure S2. 3

**Drosophila Hippo Signaling Pathway**

Figure S2. 4

**Drosophila MAPK/EGFR Signaling Pathway**
Table S2. 2 Lists of primers used in qPCR analysis

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<tr>
<td></td>
<td>Reverse</td>
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</tr>
<tr>
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<td>Jim</td>
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<tr>
<td></td>
<td>Reverse</td>
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Table S2. 3 Galaxy Workflow for ChIP-seq analysis

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<td>File to groom</td>
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<tr>
<td><em>select at runtime</em></td>
</tr>
<tr>
<td>Input FASTQ quality scores type</td>
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<tr>
<td><strong>Step 2: Bowtie2</strong></td>
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<td>Is this single or paired library</td>
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- Short read data from your current history
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- Contaminant list
  - *select at runtime*
- Submodule and Limit specifying file
  - *select at runtime*

**Step 4: MACS2 callpeak**
- ChIP-Seq Treatment File
  - *select at runtime*
- ChIP-Seq Control File
  - *select at runtime*
- Are your inputs Paired-end BAM files? | False |
| **Effective genome size** | *D. melanogaster (121,400,000)* |
| **Band width for picking regions to compute fragment size** | 300 |

**Mfold settings:**
- Set lower mfold bound | 10 |
- Set upper mfold bound | 30 |
- Peak detection based on q-value
  - Minimum FDR (q-value) cutoff for peak detection | 0.05 |
- Build Model | Do not build the shifting model (--nomodel) |
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Advanced options
Hide advanced options

2.13 Appendix

The Supplementary Material for Table 2.1 can be found online at:

https://www.frontiersin.org/articles/10.3389/fgene.2019.00312/full#supplementary-material
CHAPTER 3. A PROTEOMIC ANALYSIS OF THE DROSOPHILA FOXO INTERACTOME REVEALS A DYNAMIC CHANGE IN THE FOXO INTERACTING NETWORK IN RESPONSE TO OXIDATIVE STRESS

Modified from a manuscript prepared for G3

Allison Birnbaum¹, Hua Bai¹*

¹ Department of Genetics, Development, and Cell Biology, Iowa State University, Ames, IA

A.B. performed Co-Immunoprecipitation, immuno-fluorescence, and gene ontology analysis. A.B. and H.B. performed Mass Spectrometry, wrote and edited the manuscript.

3.1 Abstract

FOXO transcription factors are able to control a wide array of cellular processes through their ability to regulate transcriptional activity. FOXO proteins can interact with a number of protein partners which work to fine-tune FOXO transcriptional regulation. FOXOs are implemented in insulin signaling to regulate lifespan, but can also respond to oxidative stress to promote cellular pathways such as upregulation of antioxidant gene expression, and initiation of apoptosis of damaged cells, thus acting as agents of organismal homeostasis. While the protein partners that interact with FOXO under insulin signaling have been previously explored, the knowledge of the FOXO interacting network under oxidative stress conditions is less understood. In order to expand our knowledge of FOXO behavior under oxidative stress, we generated a protein-protein interaction network using mass spectrometry under normal and stress conditions to identify partners in FOXO-regulated activity. We observed a dynamic change in the FOXO interacting networks between these two cellular conditions, and confirmed these associations with FOXO using co-immunoprecipitation. Using a survival assay, we identified several FOXO-interacting proteins that regulate oxidative stress resistance. For two transcription factors
identified through our proteomic approach (Stonewall and Hangover), we were able to confirm their co-localization with FOXO in the adult fly brain under control and oxidative stress conditions. Together, our findings show FOXO undergoes a dynamic network change under oxidative stress, and we identified proteins that may interact with FOXO to co-regulate transcription and tissue homeostasis.

3.2 Introduction

The forkhead box subclass O (FOXO) transcription factor family was first identified downstream of the insulin signaling pathway, and is known as a regulator of homeostasis and longevity (Kenyon et al., 1993; Murphy et al., 2003; Giannakou et al., 2004; Hwangbo et al., 2004; Carter and Brunet, 2007; Martins et al., 2016). FOXOs regulate a number of cellular pathways, such as metabolism, cell cycle arrest, apoptosis, redox detoxification, and DNA repair mechanisms (Salih and Brunet, 2008). FOXO proteins are capable of undergoing post-translational modifications in response to cellular cues, such as the presence of insulin, which alters transcriptional activity and DNA-binding properties (Brunet et al., 1999; Matsuzaki et al., 2005; Alic et al., 2011; Bai et al, 2013; Webb et al., 2016).

One of the many cellular processes regulated by FOXO is the ability to respond to oxidative stress. Oxidative stress arises from an abundance of reactive oxygen species (ROS) and is known to impact cellular integrity, and can impair functional processes (Kuether and Arking, 1999; Kops et al., 2002; Essers et al., 2004; Robert Arking, 2006; Lopez-Otin et al., 2013). FOXO is known to protect cells from oxidative stress by activating major antioxidant genes which prevent ROS from accumulating and damaging the cell (Kops et al., 2002; Tan et al., 2008; Akasaki et al., 2014; Klotz et al., 2015). Aging is hallmarked by the accumulation of oxidative damage by intracellular ROS (Harman, 1956; Sohal et al., 2002). In cardiac tissue, ROS can promote hypertrophy and age-related cardiac diseases (Tan et al., 2008; Sugamura and
Keaney, 2011). Additionally, activation of FOXO can prevent declines in cardiac performance with age (Wessells et al., 2004). FOXO also delays protein aggregation in muscles, showing FOXO’s ability to function as a homeostatic regulator (Demontis and Perrimon, 2010).

Increases in insulin signaling and Transforming growth factor β1 (TGFβ1) have been shown to increase ROS levels and induce kidney damage (Liu and Desai, 2015). However, ROS are essential for cellular homeostasis, and a loss of these species can lead to cancers and other age-related diseases (Ungvari et al., 2011; Alfadda and Sallam, 2012; Martins et al., 2016). Long-lived mutants often exhibit the ability to prevent ROS accumulation and resist oxidative damage to organelles and cellular processes (R. Arking et al., 2002; Kenyon, 2005; Niveditha et al., 2017). The ability to balance ROS generation and antioxidant mitigation is key to maintaining intercellular homeostasis. FOXOs are able to respond to ROS to activate antioxidant gene transcription, but they are not the only proteins to do so. Transcription factor Nrf2 also regulates antioxidant expression, and can coordinate with other proteins to respond to stress and localize to specific promoter regions (Kobayashi et al., 2009; Hu et al., 2019). The ability for specific protein complexes to dictate chromatin binding and gene expression under oxidative stress highlights the potential for co-regulation with previously unknown protein partners that are required for transcriptional activation of genes involved in homeostatic pathways.

It is well known that transcription factors function in a network of protein interactions to regulate gene expression, and FOXO is no exception (Stumpf et al., 2008; Vinayagam et al., 2016). Because FOXO activity is regulated by post-translational modifications through proteins such as AKT, JNK, AMPK, and MST1, it is able to interact with a diverse array of protein partners in response to different cellular signals (Essers et al., 2004; M. C. Wang et al., 2005; Lehtinen et al., 2006; Greer et al., 2007; Calnan and Brunet, 2008). The FOXO protein
interaction network helps in FOXO transcriptional regulation and DNA targeting under numerous cellular conditions (van der Vos and Coffer, 2008). This network is able to change in response to signaling cues, and evaluating the interaction between FOXO and its binding partners in different cellular conditions can illuminate the functional activity of FOXO (M. Wang et al., 2013). Moreover, studying the FOXO interacting network can highlight key downstream pathways involved in tissue homeostasis and lifespan extension.

Many FOXO co-factors have been identified and predicted in response to insulin signaling (Vinayagam et al., 2016; Webb et al., 2016). However, the FOXO protein interaction network under oxidative stress has not been well explored. By investigating the dynamics of the FOXO interactome under stress, we can uncover co-regulators of FOXO activity that are involved in FOXO-regulated stress resistance and tissue homeostasis. Here, we implement a proteomic approach to investigate the behavior of sole Drosophila ortholog, dFOXO (hereafter FOXO) in response to oxidative stress. To profile this network, we constructed a FOXO protein-protein interaction network (PPIN) using mass spectrometry under normal and oxidative stress cellular conditions. Our results show FOXO protein partners change upon treatment with Paraquat compared to a basal condition. We observe a change in GO enrichment terms with our paraquat FOXO network, shifting from translation-regulating proteins under untreated conditions to partners implicated in transcription-regulation under stress. Intriguingly, both normal and stressed FOXO PPINs contained chromatin modulating partners. We also observed several transcription factor partners among our dataset, and see co-localization of two of these proteins with FOXO in the brain. Ultimately, our findings support a unique FOXO interacting protein network under increased ROS, and identifies potential transcription factor partners of FOXO that may fine-tune FOXO transcriptional activity.
3.3 Results

FOXO localizes in the nucleus upon treatment with paraquat

FOXO is known to activate under oxidative stress and upregulate genes to mitigate ROS (Kops et al., 2002; Essers et al., 2004; Klotz et al., 2015). To validate FOXO nuclear localization under stress conditions, we challenged S2 cells with 20mM of paraquat (PQ) for 16 hours. Paraquat is a mitochondrial stressor that causes elevated levels of superoxide (R. Arking et al., 2000; M. C. Wang et al., 2003; Hosamani and Muralidhara, 2013). Cells were fixed and stained with an antibody against FOXO. Nuclear regions were stained with DAPI. Colocalization of FOXO and DAPI was used to determine the percent of nuclear FOXO (Figure 3.1A). We detected a significant increase in FOXO nuclear localization under paraquat treatment in vitro (Figure 3.1B). We also see increased nuclear FOXO with other oxidative stressors such as peroxide (Figure S3.1). This nuclear induction of FOXO under paraquat treatment was also observed in Kc167 Drosophila cells (Figure S3.1).

To see if FOXO exhibits the same nuclear localization in vivo, we sought to examine adult Drosophila tissue with known FOXO activity. Expression of FOXO in fat body is known to extend lifespan (Giannakou et al., 2004; Hwangbo et al., 2004). However, there is also high-baseline nuclear FOXO signaling in the fat body of wild-type adult flies (Birnbaum et al 2019). FOXO activity is also known to promote proteostasis in muscle tissue (Demontis and Perrimon, 2010). To examine FOXO behavior under oxidative stress in muscle tissue, we treated young flies (14 days post eclosion) on 5% sucrose with 20mM of PQ for 48 hours. Control flies exhibited low foxo nuclear activity in indirect flight muscle tissue after 48 hours of 5% sucrose treatment. With the addition of 20mM paraquat, we saw an increase in the amount of nuclear FOXO (Figure 3.1C). There was significantly higher nuclear localization of FOXO under
paraquat treatment in the indirect flight muscles (Figure 3.1D). These results show that FOXO translocalizes the nucleus when ROS levels increase in the muscle tissue.

To identify transcriptional targets regulated by FOXO activity under PQ stress, we treated control and hypomorph FOXO mutant flies (Foxo21) with PQ for 48 hours and extracted RNA to evaluate changes in transcriptional activity in whole body tissue. We first examined how PQ feeding impacted expression of the known FOXO target Thor. Thor has been shown to promote proteostasis in a FOXO-dependent manner (Demontis and Perrimon, 2010). Using qPCR, we observed that paraquat feeding caused an upregulation of Thor expression in tissue of paraquat treated control flies (figure 3.1E). This upregulation was not present in FOXO mutant flies, supporting that this mutant line has minimal FOXO activity. As FOXO is known to regulate antioxidant genes in response to oxidative stress we examined known antioxidant genes that may be activating under FOXO transcriptional control in Drosophila. We saw no change in mRNA expression between our normal and paraquat fed control flies for SOD1, SOD2, and Cat gene expression (data not shown). ChIP-seq analysis has revealed glutathione-S-transferase (GstD1) genomic regions were bound by FOXO proteins (Birnbaum et al., 2019). Expression analysis revealed that GstD1 was upregulated under paraquat feeding in control flies, which was diminished in FOXO mutants (Figure 3.1F). These results indicate that Drosophila FOXO upregulates glutathione-s-transferase as a potential antioxidant in response to oxidative stress.

**FOXO interacts with a unique protein network under oxidative stress conditions**

The FOXO protein interaction network allows for fine-tuning of FOXO activity which can involve chromatin binding, transcription regulation, and cytoplasmic retention (Essers et al., 2004; Alic et al., 2011; Riedel et al., 2013; Webb et al., 2016). To identify how the protein interaction network for FOXO changes under oxidative stress, we conducted a proteomic analysis using mass spectrometry to identify proteins associated with immunoprecipitated
FOXO. We transfected KC167 cells with a plasmid containing a flag-tagged FOXO. Negative control samples were transfected with a flag-tagged GFP construct. About 48 hours post transfection, cells were treated with or without 10 mM of PQ for 16 hours before lysis. Proteins were precipitated using an antibody against Flag, and all proteins from 1-250 kDa were subjected to MS/MS analysis. Two biological replicates were used for each condition. We identified and unfiltered raw count of 422 proteins immunoprecipitated with FOXO under normal conditions, and 552 proteins for PQ-treated samples. We used a spectral count cutoff of 5 total detections to eliminate potential false positives for all runs. Proteins that also immunoprecipitated with FLAG-GFP were eliminated from the data set. After filtering, we identified 75 proteins pulled-down with FOXO under normal conditions, and 95 proteins under PQ treatment. There were 21 proteins shared between these two data sets, providing a list of 149 total unique proteins identified (Figure 3.2A, Table 3.1).

Gene ontology enrichment was performed on all three isolated groups to identify represented pathways among each dataset. We found that proteins which appeared only in control samples were functionally enriched for translation activity, ribosome assembly and mRNA surveillance. We also observed enrichment on proteins involved in neurogenesis, endocytosis, cytoskeleton organization, and DNA repair (Figure 3.2B, Table S3.1). These proteins are known to localize to cytosolic ribosome and spliceosome regions (Figure 3.2B). For the proteins uniquely present in PQ-treated samples, we observed enrichment of pathways involving protein import to the nucleus, transcription regulation, and chromatin modulation (Figure 3.2C, Table S3.1). We saw enrichment of DNA repair mechanisms, and observed proteins involved in cellular responses to stress. Proteins involved in apoptosis were also enriched among the PQ subset. These proteins are part of nuclear and microtubule associated
cellular components (Figure 3.2C). Proteins that were shared between both datasets were enriched for pathways involved in neurogenesis, chromatin assembly, and DNA repair. We also observed proteins involved in p53 transcription regulation represented in the overlapping data (Figure 3.2D, Table S3.1). Proteins that were bound to FOXO in both conditions are known to localize to chromatin or were part of chromosome binding complexes (Figure 3.2D). These results show that FOXO interacts with a different set of protein partners under oxidative stress conditions, and these partners may fine-tune FOXO activity to transcriptionally regulate stress resistance genes. This data also supports that FOXO may have nuclear activity under basal conditions that is independent of the response to environmental stressors.

Co-Immunoprecipitation with FOXO and Mass Spectrometry

To investigate the validity of our MS analysis, we performed co-immunoprecipitation (Co-IP) assays for several proteins identified in our results; pzg, Dre4, Ssrp, pep, and stwl. These genes represent several types of prey proteins; control only, PQ only, Overlap, TF, non-TF, and those that showed response to stress. To accomplish this, we generated vectors containing the coding sequence for our five selected prey protein along with an N-terminal HA-tag or Myc-tag. Both tagged bait and prey protein vectors were transfected into KC167 Drosophila cells. For negative controls, Myc-tagged prey proteins were transfected along with a Flag-GFP vector. Dre4 and Ssrp interact as members of a complex and were transfected together. We immunoprecipitated FOXO proteins using an anti-flag antibody and performed western analysis. All five prey proteins were detected by an anti-Myc or anti-HA antibody in our Flag-FOXO pull-down Co-IP samples, but were not strongly found in the Flag-GFP samples (figure 3.3A-D). Input samples collected prior to immunoprecipitation showed bands of the appropriate protein size in both FOXO and GFP transfected samples when membranes were stained with an anti-HA or anti-Myc antibody, showing transfection was successful and tagged proteins were produced.
Using a Co-IP followed by western analysis, we have shown Stwl, pzg, pep, Dre4, and Ssrp proteins are able to interact with FOXO. These results also provide confidence that the proteins detected in our MS analysis are true protein partners of FOXO.

**FOXO protein partners involved in chromatin modulation and transcription regulation respond to oxidative stress treatment**

The ability to defend against oxidative stress can delay the onset of age-related pathologies such as inflammation, neurodegeneration, and cancers (Sykiotis and Bohmann, 2008). FOXO mutant flies have been shown to have reduced survival when challenged with oxidative stress (Junger et al., 2003). We challenged control flies and hypomorphic foxo mutants and confirmed mutants have reduced survival with paraquat treatment (Figure 3.4A). We selected 30 candidate proteins from our Mass Spectrometry results and challenged them with PQ stress to see which proteins respond to oxidative stress. Candidates were selected based on gene ontology for lifespan, response to stress, and associated pathways with known impact on homeostasis. All three of our protein condition groups were represented among candidates. RNAi-inducible fly lines for our 30 candidates were mated with a Daughterless-GAL4 line containing an inducible gene switch, allowing for knockdown of gene expression only during the adult stage. This also allowed lines to serve as their own control for comparison. Progeny were kept on regular food for 3 days post-eclosion, then treated with either 200mM of RU486 to induce dsRNA generation or a vehicle control for 3 days prior to paraquat exposure. To test survival, 6-day post eclosion adult flies were placed on a filter disc containing 5% sucrose solution, 20mM PQ, and 200mM U486 or the vehicle control. 10 flies were placed in each vial. Dead flies were counted every 6 hours. Two control lines were used to ensure RU486 did not interfere with lifespan and resistance to PQ (Figure S3.2). Experiments were run with three biological replicates for each condition.
Overall, we observed 7 lines with altered survival under oxidative stress. Two lines showed reduced survival, while five lines exhibited enhanced survival. The two genes that showed reduced survival were ball and parp (Figure 3.4B, Figure S3.3). These two proteins interacted with FOXO under PQ conditions only. Both genes are involved in chromatin modulation, and parp has been shown to promote survival in response to DNA damage (Ivanovska et al., 2005; Murata et al., 2019). The five genes that showed a significant increase in survival under PQ induced stress were Dre4, Ssrp, Caf1-180, Rump, and Hang (Figure 3.4C& 3.4D, Figure S3.3). Hang and Caf1-180 were bound to FOXO under PQ conditions, while rump was pulled-down under control conditions. Dre4 and Ssrp were bound to FOXO under both control and PQ treatment. Both Dre4 and Ssrp are members of the FACT complex involved in chromatin modulation and heat-shock gene expression (Shimojima et al., 2003; Kwon et al., 2010). This complex interacts with the transcription factor Trl, which also interacts with FOXO and is predicted to share FOXO genomic targets (Shimojima et al., 2003; Birnbaum et al., 2019; Shokri et al., 2019). Another lifespan extension line, Hang encodes for the protein hangover, which has been previously implicated in stress responses to ethanol, heat, and oxidative stress to regulate lifespan (Scholz et al., 2005). Our results provide several proteins that impact lifespan under oxidative stress. These proteins may interact with FOXO to either promote or inhibit response to oxidative stress, thereby acting as potential modulators of FOXO-regulated transcription of homeostatic pathways.

**Transcription factors that precipitate with FOXO regulate stress and Toll/IMD signaling pathways**

As a transcription factor (TF), FOXO is able to bind to genomic sites for transcriptional regulation (Carter and Brunet, 2007; Obsil and Obsilova, 2011). Other TFs can interact with FOXO and bind to promoter regions and co-regulate transcriptional activity (van der Vos and
Coffer, 2008; Alic et al., 2011; Webb et al., 2016). To find potential co-factors that can direct FOXO transcriptional activity, we first needed to identify proteins from our interaction list that are transcription factors. We utilized the Gene Groups tab in Quicksearch on Flybase (Thurmond et al., 2019). By comparing our final list of 149 proteins to the Flybase transcription factor gene group, we identified 10 transcription factors, including FOXO, present in our MS results (Table S3.2). To identify potential co-regulated pathways between FOXO and interacting TFs, we analyzed transcriptomic data from large-scale transcription factor knockdown profiling generated in S2R+ Drosophila cells (GSE81221). PoissonSeq was used on HTseq count files to identify differentially expressed (DE) genes for each transcription factor (N. M. Clark et al., 2019) (Supplementary figures 4 & 5, supplementary table).

Using these criteria, we found 940 protein coding genes to be alternatively regulated upon FOXO knockdown, with 129 genes upregulated under the knockdown, and 811 genes downregulated (0.1 FDR). We performed gene ontology enrichment analysis (GO) using DE genes as defined as having a 1.5-fold change and FDR of 0.1. For FOXO, we saw enrichment of functional categories involved in chromatin modulation and cell fate commitment for upregulated genes. Meanwhile, down-regulated genes were implicated in metabolism, oxidative phosphorylation, transcription initiation and immunity response for downregulated genes (Figure 3.5A). We compared 8 of the co-immunoprecipitated transcription factor DE profiles to our list of FOXO DE genes. GO enrichment on overlapping genes had shared transcriptional regulation of Toll/IMD signaling between FOXO and pzg, Ssrp, TFAM, row and srp (Table S3.2). Stwl appeared only in control samples, and had 129 overlapping genes with FOXO, which were enriched for Toll/IMD signaling as well as metabolic and oxidative phosphorylation pathways (Figure 3.5B, Table S3.2). Hang had 417 shared genes with FOXO, and only appeared in PQ...
samples. Overlapping genes were also functionally enriched for metabolism, oxidative stress and immunity pathways, as well as transcription activation and regulation of other transcription factors (Figure 3.5C, Table S3.2). CG2199 had the most overlap with 539 DE genes shared between the two profiles, and was only detected in PQ treated samples. Surprisingly, CG2199 was the only transcription factor we identified that did not share expression regulation of the Toll/IMD KEGG pathway with FOXO, however enriched processes included immunity response. GO analysis revealed overlapping genes were involved in metabolism and oxidative phosphorylation, as well as transcription, lipid homeostasis, and mitochondrial function (Figure 3.5D, Table S3.2). Overall, we were able to utilize transcriptome data to detect pathways shared between FOXO and identified potential transcription factor co-factors. This data can also provide clues to shared transcriptional targets between FOXO and these partner transcription factors.

**Stonewall colocalizes with FOXO in brain tissue, but is strongly reduced under stress**

Stonewall (stwl) is and Myb-SANT like transcription factor protein that has been linked to the oogenesis and female sterility (K. A. Clark and McKearin, 1996). Stwl has also been shown to regulate apoptosis and cell death in neuronal tissue, act a promoter of heterochromatin, and is transcriptionally upregulated under heat stress conditions (Brun et al., 2006; Yi et al., 2009; Nakayama et al., 2014). In our mass spectrometry analysis, stwl was only detected under control treatment conditions, and interacted with FOXO in our *in vitro* Co-IP analysis (Table 3.1, Figure 3.5A). However, these results do not provide any information about where this interaction is occurring *in vivo*, or how this interaction between FOXO and Stwl is disrupted under oxidative stress. To test for an interaction *in vivo*, we obtained a fly line containing Stwl-GFP. 1-week adult flies were fed 5% sucrose either with or without 20mM PQ for 24 hours. Tissue was extracted from brain, muscle, and fat body. Tissues were labeled with anti-FOXO and anti-GFP antibodies. DAPI was used to stain nuclei (not shown). In control samples, we observed
colocalization of FOXO and stwl in the brain (Figure 3.6A). Under PQ feeding, stwl protein number was significantly reduced, though we still observed colocalization with FOXO (Figure 3.6B). Colocalized between Stwl and FOXO protein was still observed in muscle tissue, while stwl and FOXO colocalization were reduced under paraquat treatment (Figure S3.4A). Fat body tissue showed no co-localization between FOXO and stwl (Figure S3.4B). Given our results, we hypothesize that stwl protein may serve as a repressor of FOXO activity under normal conditions, and it is degraded upon oxidative stress to allow for the activation of FOXO.

**FOXO colocalizes with Hangover in Brain and muscle tissue in the nucleus under oxidative stress**

Hangover (*hang*) encodes for a zinc-finger protein first identified for its role in ethanol tolerance. Mutants also exhibit an altered response to paraquat treatment (Scholz et al., 2005). Hangover protein was bound to FOXO exclusively under PQ conditions, suggesting hangover may play a role in FOXO activity in response to oxidative stress. Hangover has established activity in regulating neuromuscular junction (NMJ) morphology and neuronal activity (Schwenkert et al., 2008; Ruppert et al., 2017). FOXO is also known to affect these processes (Nechipurenko and Broihier, 2012; Santo and Paik, 2018). To investigate the relationship between FOXO and hang in the brain, we treated flies with 5% sucrose with or without 20mM paraquat for 24 hours. We dissected the brains of adult flies expressing a FOXO-GFP. Samples were labeled with anti-Hang (gift from Scholz lab, Germany) and anti-GFP antibodies. DAPI was used to label nuclear sites. Under normal treatment, we observed little colocalization between FOXO and hang staining ($R^2 = 0.070$) we also observed little colocalization between hangover and DAPI ($R^2 = 0.037$) (Figure 3.7A & C). However, with the addition of paraquat feeding, we observed an increase in the amount of colocalization between FOXO and hangover ($R^2 = 0.324$) and between hangover and DAPI ($R^2 = 0.242$) (Figure 3.7B & C). These results
indicate that Hangover associates with FOXO in response to oxidative stress, and this interaction occurs predominantly in the nucleus.

We next sought to find out whether PQ can induce colocalization of FOXO and Hangover in other tissues. As both FOXO and Hangover regulate the NMJ, we used immunofluorescence to label both proteins in the muscle tissue (Schwenkert et al., 2008; Nechipurenko and Broihier, 2012). Using our FOXO-GFP line, we dissected the indirect flight muscles of young adult flies. Surprisingly, we observed numerous hangover protein in the muscle tissue, however we noticed some of these to be just outside of the DAPI staining. FOXO was not well detected under normal conditions. When flies were fed paraquat, we observed increased FOXO nuclear localization, as well as increased hangover colocalization with DAPI (Figure S3.5).

**FOXO and Hangover oppositely modulate NMJ morphology**

FOXO influences the neuromuscular junction by impacting the stability of the axon cytoskeleton through the regulation of the microtubule associated protein (MAP) Futsch (Nechipurenko and Broihier, 2012). This results in increased bouton size and an increased number of Futsch-positive loops during development. Hangover null-mutants exhibit elongated branches and overall smaller boutons in *Drosophila* larvae (Schwenkert et al., 2008). To test if these two genes both impact cytoskeletal stability, wandering L3 larvae from control, FOXO-null, and Hangover-null mutants were dissected and stained with an anti-futsch antibody. Both FOXO and Hang mutants had more futsch-positive loops than wild type larvae (Figure 3.6C). However, loops in Hang mutants contain disrupted futsch staining (Figure 3.6D). These results suggest changes in futsch activity may result in smaller bouton size and extended branch length (Roos et al., 2000).
Studies investigating Hang effect on NMJ structure had been performed in *Drosophila* larvae, however adult morphology was still unknown (Schwenkert et al., 2008) adult flies were placed on normal food and compared to w^{1118} controls. Flies were dissected at 5 days post-eclosion, and NMJ’s were stained with anti-bruchpilot (BRP) and anti-horseradish peroxidase (HRP), labelling bouton active zones and neuronal tissue, respectively. In adults, Hangover mutants also exhibited longer branches and smaller boutons than their wild-type counterparts (Figure S3.5). This phenotype is also opposite what is observed in FOXO mutant adults, which have shorter branches and larger boutons (Figure S3.5). FOXO and Hangover knockout has opposite effect on NMJ morphology, suggesting they may negatively interact under non-stress conditions to control NMJ structure.

### 3.4 Discussion

FOXO has been well established in its ability to interact with numerous protein partners to respond to different environmental conditions (Greer and Brunet, 2005; van der Vos and Coffer, 2008; Vinayagam et al., 2016; Kang et al., 2017; Shokri et al., 2019). However, the dynamics of this network under non-insulin signaling have not been well explored, and only a limited number of interacting proteins have been identified, despite the knowledge that FOXO plays a role in responses to other stressors, particularly oxidative stress (Essers et al., 2004; Akasaki et al., 2014; Martins et al., 2016). In this paper we investigated the alterations in the FOXO protein interacting network between basal and oxidative stress cellular conditions and observed changes in protein networks between the two states. We also identified potential new direct protein partners of FOXO that may control transcriptional activity to promote homeostasis and longevity. Using a proteomic approach, we have uncovered a unique network of proteins interacting with FOXO under oxidative stress that can provide insight into changes in FOXO transcriptional activity upon ROS stimulus.
We were able to observe potential changes in FOXO behavior by examining gene ontology enrichment of our control only, PQ only, and shared protein groups from our Mass Spec analysis. We found control-specific proteins from our list are involved in activation of translation. FOXO is known to act as an inhibitor of mTOR activity, which when activated promotes translation (Hay, 2011). Additionally, cytoplasmic sequestration results in reduced expression of FOXO transcriptional target and known translation inhibitor 4EBP, while mTOR activation can phosphorylate and inactivate 4EBP to promote translation (Puig et al., 2003; Teleman et al., 2005a). The interaction between FOXO and proteins that promote translation may indicate cytoplasmic FOXO has a role in regulating translation, promoting mTOR activity. Among PQ unique proteins in our list, we found enrichment of genes that are involved in the promotion of transcription. FOXO promotes the transcription of antioxidant genes in response to elevated ROS, and can also promote apoptotic genes under prolonged oxidative stress (Kops et al., 2002; Greer et al., 2007; Sengupta et al., 2011; Klotz et al., 2015). Our current list does not provide insight into which pathways are being activated by FOXO at our timepoint. Further investigation into transcriptome profile changes may elucidate the pathways transcriptionally regulated by FOXO under these conditions. We were surprised to find many chromatin modulating proteins associated with FOXO in both treatment conditions. FOXO has been shown to be in the nucleus and have DNA binding activity even under fed, non-stressed conditions (Alic et al., 2011; Riedel et al., 2013; Birnbaum et al., 2019). This finding corresponds to our previous data in which we found heterochromatin promoting genes among FOXO DNA binding targets (Birnbaum et al., 2019). This further indicates that FOXO may have basal nuclear activity to specific genomic locations for chromatin state regulation. We also saw overlapping proteins
were enriched for GO terms involved in DNA repair mechanisms, indicating FOXO may be interacting with these proteins for quick response to DNA damage.

High ROS levels can result in apoptosis and cell death (Greer et al., 2007). FOXO is known to interact with several protein partners under oxidative stress, and these co-factors play an important role in regulating FOXO transcriptional activity (Essers et al., 2004; van der Vos and Coffer, 2008). We performed survival assays for potential protein partners, and identified seven genes that impact organismal response to paraquat induced oxidative stress. Of these 7 genes, 6 of them are known to remodel chromatin in response to cellular conditions (Kelley et al., 1999; Tyler et al., 2001; Tulin et al., 2002; Saunders et al., 2003; Aihara et al., 2004; Scholz et al., 2005). In *C. elegans*, FOXO is known to recruit the SWI/SNF remodeling complex, promoting Dauer formation and promoting longevity and stress resistance (Riedel et al., 2013). Additionally, histone deacetylase Sirt1 interacts with FOXO, regulating FOXO activity and response to oxidative stressors (Brunet et al., 2004). This further implements the role of chromatin remodeling complexes interacting with FOXO under oxidative stress, and supports that these proteins are potential co-factors that can modify FOXO transcriptional regulation and alter chromatin organization.

FOXO’s ability to interact with protein partners allows for the interaction between FOXO and other transcription factors (van der Vos and Coffer, 2008; Alic et al., 2011; Webb and Brunet, 2013). We detected 9 transcription factors from our proteomic data, excluding FOXO. Examination of RNA-seq analysis results revealed altered expression of the Toll/IMD signaling pathway with FOXO-RNAi and with seven other transcription factors from our list. This may be due in part because S2 cell lines are derived from macrophage-like cells (Schneider, 1972). IMD activity has been previously linked to FOXO through FOXO activation of AMP genes under
non-infectious conditions (Becker et al., 2010). Under hypoxic conditions, FOXO is shown to regulate the expression of IMD ligand *Relish* (Nf-kB) (Barretto et al. 2019). FOXO has also been shown to function downstream of Toll signaling in neurons to regulate neuronal activity (McLaughlin et al. 2016). This supports FOXO’s role in regulating, Toll/IMD signaling, and suggests these transcription factors are participants in FOXO regulation of these pathways. To implement the role of each transcription factor in FOXO activity requires further investigation.

Our Mass Spec results provided two transcription factors that may direct FOXO activity. Our analysis revealed Stwl to be a co-factor of FOXO in *Drosophila* cells under control conditions, and we observed Stwl-FOXO co-localization in brain tissue predominantly under control conditions. Stwl regulates cell cycle entry and proliferation, which is opposite to FOXO (Akiyama, 2002; Junger et al., 2003; Brun et al., 2006). Stwl also regulates heterochromatin and euchromatin balance, and contains an MADF protein domain which is also observed in transcription factor alcohol dehydrogenase distal factor 1, *Adf-1* (K. A. Clark and McKearin, 1996; Yi et al., 2009). *Adf-1* is a known regulator of neuronal activity, as is FOXO (DeZazzo et al., 2000; Timmerman et al., 2013; McLaughlin and Broihier, 2018). Stwl has also been shown to regulate expression of the gene *Pumilio*, which can control synaptic plasticity, suggesting Stwl may also function to regulate neuronal activity (Menon et al., 2004; Maines et al., 2007). Further analysis will be needed to determine what genes and pathways Stwl regulates in brain tissue, and which of these are shared with FOXO. Our MS analysis also identified an interaction between FOXO and transcription factor Hang under oxidative stress. Both are shown to regulate neuromuscular junction formation and FasII expression, which is required for NMJ terminal growth (Schwenkert et al., 2008; Nechipurenko and Broihier, 2012). Additionally, Hang is shown to regulate expression of *dnc*, a gene involved in memory function, whose gene region is
also targeted by FOXO (Ruppert et al., 2017; Birnbaum et al., 2019). We hypothesize that FOXO and hangover may antagonize each other during normal conditions, but interact under stress to regulate transcription, however, further investigation of their interaction is required.

In this study we have used a proteomic approach to identify interacting partners of FOXO that may serve as co-factors in FOXO-dependent activity. We have shown that the FOXO protein interacting network changes between basal conditions and paraquat-induced stress, and provide a snapshot of potential pathways co-regulated by FOXO under both cellular environments. We also identify co-factors of FOXO that are always precipitated with FOXO, suggesting constant FOXO regulation of specific pathways. Our Co-IP results have validated several predicted partners, and we have successfully found several genes that alter organismal response to oxidative stress. Additionally, we have identified two transcription factors that co-localize with FOXO in neuronal tissue that may serve as important co-factors in FOXO-mediated activity and maintenance of cellular homeostasis under oxidative stress.

3.5 Experimental Procedures

**Immunoprecipitation and pull-down**

All immunoprecipitation and pulldown experiments were performed in *Drosophila* KC167 cells which have been adapted to serum-free culture media (CCM3) unless otherwise specified. The FOXO coding sequence from the gateway gold collection (LD19191) was cloned into a gateway vector containing an N-terminal Flag-tag using a TOPO/gateway ligation protocol (https://emb.carnegiescience.edu/Drosophila-gateway-vector-collection). An N-terminal Flag-tag was generated for eGFP (DGRC #1072) using the same protocol. For Co-IP analysis, N-terminal tags for HA or Myc were used following the same procedure. Constructs used were as follows; Dre4 (AT29108), pzg (LD15904), pep (LD34072), Ssrp (FI07619), Stwl (LD17962). Plasmid constructs (1 µg) were transfected into around 2 × 10⁶ confluency Kc167 cells using Effectene
reagent (Qiagen, Hilden, Germany). Three days after transfection, cells were harvested and lysed with Peirce IP lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) with proteinase inhibitors (Sigma-Aldrich, St Louis, MO, USA). For pull-down of FOXO protein, total protein isolated were incubated with anti-Flag antibody ((Sigma-Aldrich, St Louis, MO, USA) and SureBeads protein G (Bio-Rad, Hercules, CA, USA). For Co-IP, after pull-down, western blotting was performed to examine protein complex formation. Antibodies for westerns include rabbit anti-HA, mouse anti-Flag (Sigma-Aldrich, St Louis, MO, USA), rabbit anti-Myc and rabbit anti-HA (Cell signaling technologies, Danvers, MA, USA).

**Mass Spectrometry**

After pull-down of Flag-tagged proteins, isolates were briefly run on a western gel (Bio-Rad, Hercules, CA, USA) to generate one band containing protein from 1 to 250 kDa. Gel was incubated with Coomassie blue (Thermo Fisher Scientific, Waltham, MA, USA) and sent to the Taplin Mass Spectrometry Facility (Harvard University, Cambridge, MA, USA) for spectral analysis.

**Fly stocks and husbandry**

Flies were maintained at 25°C, 60% humidity and 12-hour light/dark cycle. Adults were kept on a standard agar-based diet containing 0.8% cornmeal, 10% sugar, and 2.5% yeast. yw^R flies (Bai et al., 2013) were used as wild-type for immunostaining. The strong hypomorph foxo^{21} was used to evaluate the role of FOXO in oxidative stress resistance. Fly stocks used for the oxidative stress survival assay are: BL (Da-GS-Gal4); BL (Attp2); BL (Attp40), BL56884 (aralar1); BL55330 (Ungvari et al.); BL27061 (Bap); BL32478 (caf1-180); BL55202 (CG6686); BL55854 (CG6701); BL38976 (Cindr); BL25879 (Dap160); BL41992 (dhx15); BL34344 (Dre4); BL33942 (Eps-15); BL35181 (fs(1)yb); BL44572 (Gnf1); BL57791 (Hang); BL62513 (Ku80); BL42578 (Larp); BL32873 (Mtp-alpha); BL39009 (Ote); BL57265 (Parp); BL32944
(Pep); BL57793 (pzg); BL25971 (row); BL42604 (Rrp6); BL42665 (rump); BL34933 (Spindly); BL51519 (Ssrp); BL 31081 (Ungvari et al.); BL27057 (woc); BL61359 (XRCC1); BL29347 (zf1). Fly stocks were maintained on food mentioned above. For colocalization immunostaining BL68196 (Stwl), Da-GS-GAL4 (Bai Lab), UAS-FOXO-GFP line (Gift from A. Teleman, Heidelberg University, Heidelberg, Germany (Teleman et al., 2005b)) were used. NMJ visualization and quantification used w¹¹¹⁸, null mutant foxo⁴³¹ (Gifts from N. Liu, Chinese Academy of Sciences, Shanghai, China), and null mutant Hang⁴¹⁰NT (Gift from H. Scholz, University of Cologne, Köln, Germany).

**Oxidative stress survival assay**

For our first survival assay comparing the effect of FOXO on lifespan, ywRX flies and foxo²¹ male flies were used. For our RNAi screening, TRiP males were crossed to Da-GS-GAL4 virgin females and raised on our standard food until 3 days post eclosion. Flies were transferred to food containing 0.8% cornmeal, 10% sugar, and 2.5% yeast and 200mM of Mifepristone or 95% Ethanol vehicle (RU486, Fisher Scientific). After 3 days, flies were transferred to vials with a filter paper containing a mixture of 5% sucrose, 20 mM Paraquat (Methyl Viologen hydrate 98%, Acros Organics, Thermo Fisher Scientific, Waltham, MA, USA), and either 200mM RU486 or vehicle control, based on rearing. This mixture was replaced every 24 hours. 10 male flies were placed in each vial, with 3 biological replicates for each treatment. Dead flies were counted every 6 hours. Survival curves and statistics were calculated by JMP (Cary, NC, USA).

**Immunostaining and imaging**

To examine FOXO nuclear localization under stress conditions, S2 cells were treated with 20mM Paraquat or 2uM H₂O₂ (Sigma-Aldrich, St Louis, MO, USA) or nuclease-free H₂O as a vehicle control for 16 hours. We used 3 biological replicates per condition. Samples were de-identified and percent of nuclear FOXO was manually counted within 5 ROIs (regions of
interest) per sample. ROIs were averaged for each of the biological replicates. Flies (yw^R) were aged 2 weeks as adults on standard food. Muscle, brain, and fat body tissue from 5-10 female flies were dissected in PBS, while Larval and female adult NMJ was dissected in a calcium free media (128mM NaCl, 2mM KCl, 4mM MgCl_2(H_2O)_6, 35.5mM sucrose, 5mM HEPES, 1mM EGTA, H_2O, pH 7.2). All cell and tissue fixations were performed with 4% paraformaldehyde and washed with 1X PBS with 0.1% triton X. All samples were blocked with 5% NGS (Jackson ImmunoResearch, West Grove, PA) for 1 hour, and incubated with primary antibodies at 4°C overnight. Primary antibodies used were rabbit anti-FOXO (Bai et al 2013), mouse anti-GFP (Sigma-Aldrich, St Louis, MO, USA), rabbit anti-Hang (Gift from H. Scholz, Scholz et al 2005), mouse anti-futch (22C10) and mouse anti-BRP (nc82) (DSHB, Iowa City, IA, USA). Samples were washed and incubated with secondary antibodies anti-rabbit-594, anti-mouse-488, and anti-HRP-594 (Jackson ImmunoResearch, West Grove, PA) at room temperature. Samples were stored at 4°C overnight in slowfade Gold with DAPI (Thermo Fisher Scientific, Waltham, MA, USA) before mounting. Images were captured using an epifluorescence-equipped BX51WI microscope (Olympus, Waltham, MA, USA). Image deconvolution was conducted using CellSens software (Olympus, Waltham, MA, USA), and compiled using ImageJ Fiji.

**Quantitative real-time PCR**

14 day post eclosion flies were placed filter paper containing 5% sucrose and 20mM PQ or vehicle control (H2O). Whole bodies of female flies were homogenized in TRIzol (Thermo Fisher). RNA was extracted following company procedure. cDNA was generated using qScript (Quanta) and diluted to a 10uM working solution. Quantitative PCR was run on purified samples (QuantStudio). Cybergreen (Life Technologies, CA, USA) was used for chemical detection. The primers used Enrichment was determined based on the double-delta CT value. *GstD1* (sense 5’- GTACATCGCGAGTTTCACAACA-3’, antisense 5’- GGGTGGACAGCTTCTTGTTCAG-3’),
Thor (sense, 5’ –CCAGGAAGGTTGTCATCTCGG-3’, and antisense 5’ –
CTCGTAGATAAGTTTGGTGCCCTCC-3’), Rpl32 (sense 5’-
AAGAAGCGCACCAAGCACTTCATC- 3’, and antisense 5’ –
TCTGGTGTCGATACCCTTGGGCTT-3’).

Transcription factor transcriptome analysis

HTseq files were obtained from GSE81221 and analyzed using Poissonseq.

Gene ontology

Pathway and gene ontology analysis was conducted using String (https://string-db.org/)
and DAVID (https://david.ncifcrf.gov/).

Statistical analysis

GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used for statistical
analysis and to generate bar graphs and boxplots. To compare the mean value of treatment
groups versus that of control, either student t-test or one-way ANOVA was performed using
Dunnett’s test for multiple comparison.

Venn diagrams

Venn diagram were created using the Bioinformatics and Evolutionary Genomics Venn
calculator at Ugent (http://bioinformatics.psb.ugent.be/webtools/Venn/).

3.6 Acknowledgments

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Hangover antibody and fly lines, and E. McNeill (Iowa State University, Ames, IA) for reagents
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3.7 Competing Interests

The authors declare that no competing interest exists.

3.8 Figure and Table Legends

Figure 3.1. FOXO localizes in the nucleus upon oxidative stress A) Representative image of S2 cells after control and paraquat treatment. Labeled with α-FOXO antibody. Nucleus was stained with DAPI. Control n=5, 20mM PQ n=6. B) Percent between α-FOXO and DAPI for figure A. *P<0.05 C. 14-day ywR flies were treated with Paraquat (PQ) for 24 hours. Fixed tissue was stained with α-FOXO. Nucleus was stained with DAPI. Control n=4, 20mM PQ n=4. D) Colocalization coefficient between α-FOXO and DAPI for figure C. **P<0.01. E) Relative whole-body mRNA expression for Thor between control and PQ treated flies. ywR flies were used as wild-type. F) Relative mRNA expression for GstD1 between control and PQ treated flies. ywR flies were used as wild-type. E-F biological replicates n=3, flies per replicate n=7.

Figure 3.2. Gene ontology enrichment groups are unique among FOXO protein partner groups. A) Venn diagram representation of number of proteins detect from FOXO control MS and FOXO PQ MS. B-D) –Log10(P-value) for gene ontology groups for (B) control, (C) PQ-treated, (D) and overlapping MS results.

Figure 3.3. FOXO protein partners show altered survival under oxidative stress. A) Results display the percentage of flies still alive at each timepoint. Under 20mM paraquat
treatment foxo$^{21}$ mutants show decreased survival compared to control flies. B) Parp knockdown shows reduced survival under PQ-induced oxidative stress compared to controls. C) Ssrp knockdown shows increased survival compared to controls under PQ treatment. D) Hang knockdown shows increased survival under PQ treatment compared to controls.

**Figure 3.4. FOXO transcriptome overlaps with co-precipitated transcription factors.** A) Representative gene ontology enriched processes up- and down-regulated between control and FOXO knockdowns. B-D) Venn diagram and representative GO groups for overlapping genes between transcriptome results for FOXO and (B) Stwl, (C) Hang, and (D) CG2199.

**Figure 3.5. FOXO coimmunoprecipitates with proteins identified by mass spectrometry.** A. Co-IP validation of the interaction between FOXO and stwl in Kc167 cells. B. Co-IP validation of the interaction between FOXO and pep in Kc167 cells. C. Co-IP validation of the interaction between FOXO and pzg in Kc167 cells. D) Co-IP validation of the interaction between FOXO, Ssrp, and Dre4 in Kc167 cells.

**Figure 3.6. Stwl and FOXO protein colocalize in brain tissue under non-stress conditions.** A) Immunofluorescent imaging of Stwl-GFP flies in adult brain tissue. A reduction of stwl protein is observed under PQ stress. Co-labeled with anti-FOXO and anti-GFP. B) Colocalization analysis of FOXO and Stwl under control and PQ-treatment conditions. *P<0.05. control n=2, 20mM PQ n=3.

**Figure 3.7. Functional validation of FOXO and hangover** A) Immunofluorescent imaging of Da-GS-GAL4> UAS-FOXO-GFP flies. Arrows indicate FOXO-Hang colocalization. Co-labeled with anti-Hang and anti-GFP. Nucleus labeled with DAPI. B) Colocalization analysis of FOXO (G), Hangover (R), and DAPI (B) colocalization under control and PQ-treatment
conditions. **C.** Quantification of Futsch-positive loops in control (w1118), foxo$^{C431}$, and HangAE10NT. Control n=5, foxo$^{c431}$ n= 5, Hang$^{AEONT10}$ n= 6. **D)** Visual representation of axon terminals in L3 wandering larvae. Arrows indicate Futsch loops. Scale bar 20μm.

**Table 3.1.** dFOXO interacting candidate proteins identified by mass spectrometry. Protein names are in bold. MS detection group in plain text.

**Supporting information**

**Figure S3.1.** A) Representative image of S2 cells after control and 16-hour peroxide treatment. Labeled with α-FOXO antibody. Nucleus was stained with DAPI. n =5. **B)** Colocalization coefficient between α-FOXO and DAPI for figure A. control n=5, 10mM PQ n=5. C) Colocalization coefficient between α-FOXO and DAPI for KC167 cells treated with 10mM PQ for 16 hours. *P<0.05.

**Figure S3.2.** Mortality assay 5 day old Da-GS-Gal4>UAS-RNAi treated on 5% sucrose with 20mM PQ. Gene name indicates knockdown.

**Figure S3.3.** Volcano plots for RNA-seq files compared to LacZ control. All q values set to 0.05.

**Figure S3.4.** A) Immunofluorescent imaging of Stwl-GFP flies in adult indirect flight muscle tissue under control and PQ stress. Co-labeled with anti-FOXO and anti-GFP. **B)** Immunofluorescent imaging of Stwl-GFP flies in adult fat body tissue under control and PQ stress. Co-labeled with anti-FOXO and anti-GFP. Nucleus stained with DAPI.

**Figure S3.5.** A) Immunofluorescent imaging of Da-GS-GAL4> UAS-FOXO-GFP flies in adult indirect flight muscle tissue under control and PQ stress. Arrows indicate colocalization of Hang and FOXO in nuclei. Co-labeled with anti-Hang and anti-GFP. Nucleus stained with DAPI. **B)** Quantification of bouton area and branch length for 5 day old w$^{1118}$ control flies,
foXO<sup>c431</sup> CRISPR FOXO-null flies, and null mutant Hang<sup>AEl0NT</sup>. *P* < 0.05, **P* < 0.01, ***P* < 0.001.

**Table S3.1:** List of MS Proteins and GO terms (separate file)

**Table S3.2:** List of DE genes and GO terms (separate file)

### 3.9 References


3.10 Figures and Tables

Figure 3.1

**A** FOXO, DAPI, MERGE
- Control
- 20mM PQ

**B** FOXO-DAPI co-localization
- Correlation coefficient (R²)
  - Control
  - 20mM PQ

**C** DAPI, FOXO, MERGE
- Control
- 20mM PQ

**D** Colocalization of FOXO and DAPI in ywR flies
- Treatment

**E** Thor (4EBP)
- relative expression (2⁻¹⁰⁻⁻CΔ)
  - WT
  - foxo²⁻⁻
  - control
  - 20mM PQ

**F** GstD1
- relative expression (2⁻¹⁰⁻⁻CΔ)
  - yw⁴
  - foxo²⁻⁻
Figure 3.2
Figure 3.3
Figure 3.4
Figure 3.5
Figure 3. 6
Figure 3. 7
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B

FOXO-DAPI co-localization

C

Nuclear dFOXO expression in KC167 drosophila cells

Control 100μM H2O2

% of cells with nuclear dFOXO

Treatment

*
Figure S3. 1 continued
Figure S3. 2 continued
Figure S3. 2 continued
Figure S3. 2 continued
Figure S3. 2
Figure S3. 3
Figure S3.5

A

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B

![Graphs showing bouton area (µm²) and branch length (pixels) for different conditions.]

Figure S3.4
CHAPTER 4. FOXO REGULATES NEUROMUSCULAR JUNCTION HOMEOSTASIS DURING DROSOPHILA AGING

Modified from a manuscript submitted to Frontiers in Aging Neuroscience

Allison Birnbaum¹, Kai Chang¹, Elizabeth McNeill², Hua Bai¹

¹ Department of Genetics, Development, and Cell Biology, Iowa State University, Ames, IA 50011, USA
² Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011, USA


4.1 Abstract

The transcription factor foxo is a known regulator of lifespan extension and tissue homeostasis. It has been linked to the maintenance of neuronal processes across many species, and has been shown to promote youthful characteristics by regulating cytoskeletal flexibility and synaptic plasticity at the neuromuscular junction (NMJ). However, the role of foxo in aging neuromuscular junction function has yet to be determined. We profiled adult Drosophila foxo-null mutant abdominal ventral longitudinal muscles and found that young mutants exhibited morphological profiles similar to those of aged wild-type flies, such as larger bouton areas and shorter terminal branches. We also observed changes to the axonal cytoskeleton and an accumulation of late endosomes in foxo null mutants and motor neuron-specific foxo knockdown flies, similar to those of aged wild-types. Motor neuron-specific overexpression of foxo can delay age-dependent changes to NMJ morphology, suggesting foxo is responsible for maintaining NMJ integrity during aging. Through genetic screening, we identify several downstream factors mediated through foxo-regulated NMJ homeostasis, including genes involved in the p38-MAPK pathway. Interestingly, the phosphorylation of p38 was increased in
the motor neuron-specific foxo knockdown flies, suggesting foxo acts as a suppressor of p38/MAPK activation. Our work reveals that foxo is a key regulator for NMJ homeostasis, and it maintains NMJ integrity by repressing MAPK signaling during aging.

4.2 Introduction

Aging involves the progressive functional decline of cellular mechanisms and tissue integrity (Rose 1994; Partridge and Barton 1996; Lopez-Otin et al 2013). In the adult brain, this results in a gradual decline of synaptic contacts to skeletal muscle tissue, resulting in a loss of strength and muscle mass (Hall and Sanes 1993). The neuromuscular junction (NMJ) serves as the synaptic interface between the branched terminals of motor neurons and the skeletal muscle fibers (Punga and Ruegg 2012). The NMJ is highly dynamic in response to cellular signals and stressors, and a dysregulation of molecular processes in both the pre- and post-synaptic regions can lead to the onset of neurodegenerative diseases (Gonzalez-Freire et al 2014; Monani and De Vivo 2014). With aging there is impairment of regulatory systems such as autophagy and redox homeostasis at the NMJ, which can lead to reactive oxygen species (ROS) accumulation followed by organelle damage and cell death (Li et al 2018; Stefanatos and Sanz 2018). Synaptic plasticity, which allows for the maintenance of functional activity to protect against degeneration, can decline during aging, resulting in decreased neuronal responsiveness and synaptic deterioration (Bergado and Anlmaguer 2002; Kempsell and Fieber 2015; Wagner et al 2015).

The Drosophila NMJ synapse utilizes glutamate as the primary neurotransmitter, and has postsynaptic densities that behave similarly to the AMPA-type receptors in the vertebrate central nervous system (Menon et al 2013). The Drosophila neuromuscular junction system has been well stereotyped in larvae, but has only more recently begun to be characterized in the adult organism (Rivlin et al 2004; Hebbar et al 2006; Beramendi et al 2008; Wagner et al 2015;
Lopez-Arias et al 2017). These studies have also discovered a number of morphological changes to the NMJ that occur under normal aging conditions, and can aid in understanding the mechanisms involved in age-dependent neuronal impairment.

The foxo transcription factor family plays an important role in metabolism and development as well as stress resistance and lifespan (Accili and Arden 2004; Greer and Brunet 2005). foxo has long been implemented as a “longevity gene” and as a modulator of protein homeostasis (Kenyon et al 1993, Martins et al 2016). FOXOs can also function as regulators of neuronal homeostasis (Paik et al 2009; Kim and Webb 2017; Schaffner et al 2018). FOXO-mediated pathways permit neuronal plasticity, which have downstream implications on cellular behavior and neuronal morphology (McLaughlin and Broihier 2018). *Drosophila* FOXO (dFOXO, hereafter foxo) promotes cytoskeletal dynamics at the neuromuscular junction during development, where it responds to stimulation to allow rapid structural reorganization. In the *Drosophila* larvae NMJ, loss of foxo results in increased stability of microtubules through the upregulation of kinesin motor protein regulators that also control axon growth (Nechipurenko and Broihier 2012; McLaughlin et al 2016). In mammals, certain foxo isoforms show transcriptional regulation of cytoskeletal polarity genes during development (de la Torre-Ubieta et al 2010). With aging, FOXOs are also implicated in regulating autophagy in neurons under Parkinson’s disease models (Xu et al 2011; Pino et al 2014; Schaffner et al 2018). It has also been shown that aging results in the activation of pro-inflammatory signals and proteotoxic stress in mice brains, and the depletion of neuronal FOXOs causes premature occurrences of these events and induces motor function impairment (Hwang et al 2018). Additionally, loss of foxo impacts synaptic function by delaying neurotransmitter release in both larval and adult drosophila neuromuscular junctions (Howlett et al 2008; Mahoney et al 2016). Aged synapses
also show a reduction in evoked response and neurotransmission (Segal 1982; Porras and Mora 1995; Liu et al 2013). These results indicate that foxo is an important regulator of neuronal function, and that it has influence on functional and structural integrity in regulating the connection between the brain and the muscle tissue.

Our current knowledge of foxo supports the hypothesis that foxo activity promotes youthful NMJ morphology and provides necessary regulation of pathways involved in maintaining synaptic integrity and plasticity. However, how foxo impacts functional regulation of the NMJ during aging still remains to be elucidated. Here, we show that loss of foxo causes morphological alterations to the NMJ in young adults, and shares phenotypic characteristics with those of aging control organisms. We find that knockdown of foxo specifically in the motor neuron results in changes to microtubule morphology and an upregulation of late endocytic vesicles. These events are also observed in middle-aged flies, indicating loss of foxo may lead to a disruption of motor neuron homeostasis. Overexpression of foxo in the motor neuron delays age-related changes to NMJ morphology. Additionally, we identified potential pathways downstream of foxo that may promote aging and decline in synaptic function in the adult motor neurons. These findings provide insight into the role foxo plays in maintaining morphological and synaptic plasticity at the aging neuromuscular junction.

4.3 Results

Loss of foxo causes morphological changes to bouton size and branch length that correspond to aging.

Normal aging causes alterations to the morphology of boutons and branches at the adult Drosophila neuromuscular junction (Beramendi et al 2007, Wagner et al 2015, Lopez-Arias et al 2017). Based on previous literature, we selected three time points post eclosion, 5 days, 25 days, and 40 days to represent young, middle aged, and old adult flies. We visualized the A3
abdominal ventral longitudinal muscle (VLM) segment using immunofluorescence staining. We stained flayed adult abdominal pelts with a monoclonal antibody against bruchpilot (BRP) to mark active zones (AZs) and an antibody against horseradish peroxidase (HRP) to visualize neuronal tissue (Figure 1A). To obtain overall bouton number, we used an antibody against Disc-large (Dlg) to mark post-synaptic regions. Using the two NMJ’s present at each A3, we quantified the six metrics characterized by Wagner et al 2015 and found a significant difference between ages for several metrics, as expected. A significant decrease in branch length was observed with aging, along with a decrease in bouton number (Figure 4.1B, Table 4.1). We also found an expansion of bouton area with age, and this corresponded to an increase in number of AZs per bouton (Figure 4.1C-D, Table 4.1). However, the number of AZs observed in a 100μm² region did not change between ages, which has been shown to remain constant during a normal progression of aging (Table 4.1, Wagner et al 2015).

We next examined the effect loss of foxo had on NMJ morphology in the adult. We used the strong hypomorph foxo²¹ allele and dissected the VLM of 5-day post eclosion animals and characterized the A3 muscle as described previously (Figure 4.1A). We found that foxo mutants had significantly larger bouton areas and shorter branches than their 5-day wild-type counterparts. However, the bouton area and branch length of foxo mutants was not significantly different from those seen in middle age control flies (Figure 4.1B-C, Table 4.1). The average active zone number between young wild-type and foxo mutants was not significantly different, but the foxo-null mutants had more average AZs per bouton compared to controls (Figure 4.1D, Table 4.1, Figure S4.1). When evaluating the raw total number of active zones per bouton, a significant difference was detected between 5-day controls and foxo mutants, but not between 25-day controls and 5-day foxo mutants (Figure S4.1). Additionally, we did not detect a
significant difference in overall AZs per synaptic area in foxo mutants when compared to the three measured control age groups. We did find that foxo mutants on average had more branches and a higher bouton number than wild-type flies (Table 4.1), which may be a compensatory response to reduced neurotransmission under excitatory conditions seen in foxo mutants (Howlett et al 2008, Mahoney et al 2016, McLaughlin and Broihier 2017). We also examined bouton size and branch length in 5-day old flies containing a null foxo<sup>C431</sup> allele generated through CRISPR-Cas9 (Birnbaum et al 2019). We found these null mutants also had enlarged boutons and shorter branch lengths compared to controls at a young age (Figure S4.1). We validated that foxo protein levels were indeed reduced in our mutants (Figure S4.1). These results suggest foxo promotes aging morphologies of boutons and synaptic branches at the neuromuscular junction.

**Loss of foxo results in altered cytoskeleton structure in the adult neuromuscular junction**

Axonal degeneration is a normal consequence of aging, and arises from a number of alterations to cellular and molecular pathways (Salvadores et al 2017). This can result in a withdrawal of axons from their synaptic sites, caused by changes in the underlying cytoskeleton (Manini et al 2013, He et al 2002). Foxo proteins are known to regulate motor neuron microtubule dynamics during development across many species, and can cause a decrease in microtubule stability, allowing for increased plasticity (de la Torre-Ubieta et al 2010, Nechipurenko and Broiheir 2012, McLaughlin et al 2016). However, how foxo effects microtubule dynamics in a post-developmental model has not been examined. We used an antibody against acetylated alpha tubulin (Ac-tub) which marks stable microtubules at our three aging time points. We constructed a 3D model of each A3 segment and assigned all branches containing acetylated a-tubulin one of two characteristics; straight, or undulating. We quantified the percentage of each tubulin morphology found the undulating morphology significantly increases between 5 and 25 days, and between 25 and 40 days (Figure 4.2A, Figure S4.2). In
examining the wild-type flies, we found at the young time point around 70% of branches had a straight tubulin structure. As wild-type flies aged, we saw an increase in sinusoidal bends persisting throughout the branches, creating an undulating pattern (Figure S4.2). This morphology can be associated with axonal retraction (He et al 2002). These results show that there are changes that occur to the axonal microtubule structure with aging.

We next examined our young adult foxo mutants for any disruptions to the axonal microtubule morphology. Foxo mutants exhibited an increased number of undulating microtubules within branches, and had significantly fewer straight microtubule bundles than the control at 5 days (Figure S4.2). When the foxo mutants were compared to the 25-day controls, there was no significant difference detected between the percentage of both undulating and straight microtubule branches (Figure 24.A). The foxo21 line contains a whole body hypomorph, and we could not determine if this change in microtubule morphology was a result of motor neuron foxo activity. Therefore, we used a motor neuron specific Ok6-Gal4 to knock down foxo specifically in the motor neuron. We quantified bouton size between control and foxo-knockdown flies and found that knock-down of foxo in the motor neuron induces an increase in bouton size (figure 24.B). We stained A3 VLMs against Ac-tub, and at one-week post eclosion, and observed nearly a two-fold decrease in the number of straight branches in knockdown flies compared to controls (Ok6-Gal4>ywR) (Figure 24.C, Figure S4.2). We also compared our 1 week foxo knockdown to 25-day controls and found there to be no significant difference between the percentage of undulating and straight microtubules within axonal branches (Figure 24.C, Figure S4.2). Through knocking down foxo in the motor neuron, we have uncovered that foxo motor neuron activity influences bouton size and axonal microtubule morphology. This
undulating pattern is observed at a young age when foxo is downregulated and suggests foxo is involved in maintaining youthful characteristics of the neuronal cytoskeleton.

**Loss of foxo causes impairment of the endocytic pathway in the adult neuromuscular junction**

Aging is also known to cause disruption of endosome-lysosome trafficking which can result in neurodegeneration, and increases in the number of early endosomes and multivesicular bodies (prerequisites for late endosomes) at the synapse (Boaro et al 1998, Nixon et al 2008, Wagner et al 2015, Colacurcipo and Nixon 2016). In *C. elegans*, late endosome formation is suppressed in organisms with delayed aging (Richardson et al 2019). Additionally, late endosomes undergo retrograde transport to degrade cargo, and this transport declines with age (Milde et al 2015, De Vos and Hafezparast 2017). To observe changes in the endocytic degradation pathway, we used an antibody against rab7 to mark late endosomes. We performed immunofluorescent staining at the A3 VLM on 5 day and 25-day adults. To our surprise, we observe *d* rab7 marked punctae around the axon branchpoint site, and noticed a visible increase at this site in middle-aged flies. To quantify the number of rab7 vesicles, we generated circular regions of interest (ROIs) of the same size and used HRP to identify z-slices containing the axon. The slice with the highest punctae number for each ROI was collected and averaged for each specimen. We found the 25-day adult flies had more than 2-fold more Rab7 punctae per ROI associated with the axon branch than the one-week adults, demonstrating there is an increase in the number of late endosomes during aging (Figure 4.2D-E).

We next examined our ok6-gal4 foxo knockdown flies for alterations in late endosome signaling. We used the same method, we quantified neuronal Rab7 punctae. We saw foxo KD flies also had significantly more punctae per ROI than the young controls in nervous system tissue (Figure 4.2 D-E). We tested a second foxo-RNAi line (foxo-RNAi #2) and again observed
significantly more rab7 punctae at the branchpoint than controls (Figure S4.3). Overall, both aging and foxo knockdown result in an increase in the number of late endosomes around the branchpoint of motor neuron, suggesting some cellular disruption in both instances.

**Overexpression of foxo rescues NMJ aging phenotypes**

To test if foxo expression level impacts the late endosome number with aging, we overexpressed foxo in the motor neuron using a UAS system. This was driven by *Ok6-GAL4* to ensure expression specifically in the motor neuron. At one-week post eclosion, no differences were detected in rab7 number between controls and overexpressing foxo flies (Figure 4.3 A-B, C). However, at 25 days, controls had accumulated significantly more late endosomes along the axon compared to overexpression lines (Figure 4.3 A’-B’, C). We next examined how overexpression of foxo impacts bouton morphology with aging, and found controls to have greater average bouton size compared to foxo overexpression at 25 days (Figure 4.3D). To test for the effectiveness of foxo overexpression, we used a daughterless inducible gene switch driver line. To activate the gene switch, we fed flies 200mM of Mifepristone (RU486), and used the whole fly body for RNA extraction. We examined expression of the known foxo target Thor (4EBP), and found a 2-fold increase in expression in RU486 fed flies, indicating this line can sufficiently induce foxo overexpression (Figure S4.3). Altogether, these results indicate that foxo expression can be beneficial to preserve motor neuron morphology and maintain neuronal endocytosis.

**MAPK and Activin signaling can rescue knockdown induced NMJ disruptions**

In order to understand how loss of foxo disrupts NMJ homeostasis, we sought to uncover the pathway that acts downstream of foxo regulation in the motor neuron. To accomplish this, we utilized a combination of genomic and genetic approaches to identify candidate genes. We surveyed foxo ChIP data and compared this with transcriptome data from head tissue of foxo-null mutants and aging controls to select candidates (Birnbaum et al 2019). Only foxo bound
genes from our ChIP-seq that had a fold change 1.5 greater than the input baseline was used for comparison. For transcriptional data, we selected genes with a significant p-value and log$_2$fold of ±0.25, indicating a 1.25 change in expression from both RNA-seq datasets. We identified 207 genes shared between all three data sets (Figure 4.4A, Table S4.1). This gene set was enriched for biological processes involving neurotransmitter secretion and cytoskeletal organization. We also observed enhancement of the Phosphatidylinositol signaling pathway (Table S4.1). We expanded our search to include genes that were bound by foxo and had altered transcription either with loss of foxo or with aging, generating a gene set list of 980 genes. We again found biological processes involving neurotransmission and axonal guidance, however we also found synaptic vesicle endocytosis and Rho signaling to be enriched among this gene set as well. Kegg pathways highly represented among this data include MAPK, WNT, and Hippo. When we examined the 439 genes that were shared between both transcriptome data sets, but were not present in the foxo ChIP analysis, we saw enrichment of oxidation-reduction processes, and immunity regulating pathways (Table S4.1). These results suggest foxo may serve as an indirect regulator in these pathways, or may require specific activation to bind DNA for transcriptional control.

We used our genomic analysis as well as preexisting literature to generate a list of 15 candidates that we hypothesize to function downstream of foxo (Table 4.2). In the foxo-null head transcriptome dataset, we observed upregulation of Activin and MAPK regulating genes. These pathways were also enriched in the foxo ChIP-seq dataset. In the follow-up genetic screening, we opted to use rab7 as a marker due to the ease of rapid quantification. We generated a fly line that containing the Ok6-gal4 driver with a foxo-RNAi to perform a genetic screen to rescue the accumulation of rab7. Our generated fly showed a 27% increase in the number of late endosomes
associated with the axon branchpoint compared to a wild-type (Figure 4.4B). For our assay, we created 13 double knockdown lines containing a knockdown of foxo as well as our candidate genes. We also crossed two overexpression lines with our ok6;foxo-RNAi knockdown. One was for Thor, which is a known foxo target gene and has been shown to rescue foxo mutant neurotransmission defects (Junger et al 2003, Mahoney et al 2016). The other overexpression line we used was for the microtubule associated protein Ankyrin2, which showed a decline in expression in foxo mutants and with aging in our transcriptome data, and was highly enriched in our ChIP-seq (Table 4.2). We used the same method from our previous results to quantify the number of late endocytic vesicles associated with the axon for each cross. Using a student t test, we identified four genes that resulted in a significant reduction in late endosome number compared to the foxo KD line. For our overexpression lines, we saw 28% and 17% less rab7 than the control from Thor and Ankryin2 respectively. We also saw a decline in the number of rab7 vesicles in grk double RNAi flies that was not significant, but was reduced near the baseline. A significant reduction in rab7 marked vesicles was found when we expressed RNAi-mediated knockdown of the JNK transcription factor Kay (Kayak), the TGF-b receptor babo (baboon), and the b variant of p38 (p38b). All three are known to have regulatory effects on axonal transport (Ellis et al 2010, Dreup and Nechiporuk 2013, Gibbs et al 2018). Of the three p38 isoforms, only p38b was able to show this level of reduction. This isoform has also been implicated in axonal transport (Gibbs et al 2018). These results indicate that TGF-b and MAPK signaling are activated upon loss of foxo, and can disrupt normal endocytic activity associated with the motor neuron. After identifying potential rescues of late endosome accumulation in our genetic screen, we tested our positive hits for their ability to rescue other foxo KD induced phenotypes.
Lines that showed a marked reduction in rab7 staining were checked for changes in bouton morphology in the double knockdown lines. All rescued MAPK flies showed a decrease in bouton size, while Thor overexpression in a foxo knockdown exhibited a reduced but non-significant change in average bouton area (Figure 4.4C). To validate that not all lines had smaller bouton areas, we quantified foxo:p38a double knockdown flies, and found no significant difference from foxo knockdown alone. To assess how MAPK and TGF-b impact the cytoskeleton, we performed immunofluorescence against acetylated a-Tubulin for the foxo-RNAi/p38b-RNAi and foxo-RNAi/babo-RNAi double knockdown lines. We observed that foxo-RNAi/p38b-RNAi flies have significantly more straight branches than the foxo-RNAi lines (Figure 4.4D). This was accompanied by reduced splaying along the microtubules (Figure 4.4E). These results support that many of the knockdowns that rescued rab7 accumulation also reduce bouton expansion, and that the b isoform of p38 is likely involved in foxo-mediated NMJ regulation.

We next examined how motor neuron foxo impacts p38 activation. Using our generated ok6-foxoRNAi fly lines, we stained the A3 VLM with antibodies against the phosphorylated state of p38. We observed a significant increase in the intensity of phosphorylated p38 along the motor neuron in the foxo-RNAi flies (figure 4.5A-B). These results show that foxo presence in the motor neuron can suppress MAPK activation.

4.4 Discussion

Foxo is known to act a regulator of synaptic plasticity and microtubule stability (Howlett et al 2008; Nechipurenko and Broiheir 2012). Although we have seen foxo’s regulation at the NMJ during development, it is unclear the role it plays in NMJ maintenance during aging. Foxo promotes youthful characteristics in many different tissues, and has been shown to delay the onset of degenerative properties (Sahil and Brunet 2008, Desmontes and Perrimon 2012). Here
we demonstrate that foxo is a regulator of NMJ homeostasis in post developmental motor neurons. Our data suggests that foxo plays a functional role in maintaining the motor neuron during adulthood, and can promote healthy neuronal tissue when expressed later in life. We have found that foxo contributes to the morphology of synaptic contacts to muscles tissue, and can positively regulate cytoskeletal plasticity and endocytic processes in neuronal tissue. Our results also show that TGF-b and MAPK signaling function downstream of foxo to control these processes and loss of foxo may induce activation of these pathways, resulting in disruption of homeostatic mechanisms and premature cellular aging.

In this paper, we show that loss of foxo causes morphological changes to synaptic structures in young adult female drosophila. While foxo mutants have been previously shown to have larger boutons compared to controls during development, they were not reported to have any changes in branch length or number, nor were any differences reported in bouton number (Nechipurenko and Broihier 2012, McLaughlin et al 2016). We have found this not to be the case in the adult. Our results showed that at 5 days post eclosion, foxo mutant flies had shorter branches compared to control flies. They also had a significantly larger number of branches, which ultimately resulted in a larger bouton number per muscle tissue. We hypothesize that this increase in branching may be due to a compensatory response, as foxo mutants have been shown to have reduced neurotransmission (Howlett et al 2008, Mahoney et al 2016), which may cause a need for more synaptic contacts. Additionally, foxo mutants are shown to have increased microtubule stability due to reduced microtubule sliding (McLaughlin et al 2016). This may inhibit growth to postsynaptic targets, resulting shorter branches. The increase in branch number is contrary to what is observed in Drosophila larval dendrites, where loss of foxo reduces arborization (Sears and Broihier 2016). foxo has also been shown to reduce neurite branching in
aged C. elegans downstream of both JNK and Insulin signaling (Tank et al 2011). However, increases in ROS can induce overgrowth at the Drosophila NMJ, and foxo is known to mitigate oxidative stress through antioxidant transcription activation (Essers et al 2004; Milton et al 2011). This increase may also be the result of ineffective pruning during metamorphosis.

Active zone number is proposed to proportionally increase with bouton size under a healthy aging model (Wagner et al 2015). Although there was no significant difference between average AZ number between controls and foxo mutants, we did observe more AZ’s per bouton in the mutants. Previous studies have shown that accumulation of Brp in axons can limit active zone number at synaptic terminals (Barber et al 2018). Foxo mutant larvae have been shown to have accumulations of Brp in the main axon (McLaughlin et al 2016), but we were unable to capture this in the adult. Based on our current data, we cannot determine whether foxo influences active zone number. Further experimentation using Electron Microscopy of t-bars in presynaptic area could answer this question.

Aging neurons undergo cytoskeletal changes with aging in order to maintain plasticity and synaptic contact to muscle tissue (Mattson and Magnus 2006). Aging can also cause a loss of labile microtubules, while stable elements remain intact (Jones et al 2009). As previously mentioned, foxo has been shown to regulate the axonal cytoskeleton and neuronal plasticity (Nechipuerenko, McLaughlin, Sears and Broihier 2016). During development, foxo mutant larvae have increased futch positive looping and acetylated alpha tubulin is present in the terminal boutons (Nechipurenko and Broihier 2012). Boutons were also shown to have altered Ac-Tub bundling patterns compared to controls (McLaughlin et al 2016). In our adult flies, we observed an increase in sinusoidal Ac-Tub morphologies among presynaptic branches, and this is likely a contributing factor to the truncation of established branches during aging. Dynamic
stretching of axons leads to undulating distortions and mechanical failure along the axon (Tang-Schomer et al 2010). These events have been well studied in injury models, but less so in aging, where regeneration of axonal damage becomes altered (Kleele et al 2014, Hill and Coleman 2016, Geoffroy et al 2016). Taxol, a microtubule stabilizing drug, has been shown to prevent recovery from the undulating phenotype, indicating increased microtubule stability may cause the prevalence of this morphology in foxo mutants and aged controls (Tang-Schomer et al 2010). Additionally, inhibition of dynein has been shown to cause bending in microtubules (Kent et al 2016). Splayed branching in foxo mutants and motor neuron knockdown flies may be an artifact from the altered bundling pattern observed in the larvae (McLaughlin et al 2016). While the cause of this is unknown, all microtubule strands appear to be in the same phase of action, in that they are either all straight or all undulating.

Endocytosis and exocytosis play important roles in maintaining both the pre- and postsynaptic regions. Endosome to lysosome trafficking is affected in aging motor neurons and multivesicular bodies, which are prerequisites for late endosomes, accumulate (Forester et al 2010, Wagner et al 2015). Additionally, disruption of this shuttling is a known symptom of neurodegenerative diseases (Takahasi et al 2002). In our foxo knockdown flies, we found an increase in the number of late endosomes associated with the axon. This was also observed with aging. Late endosomes are involved for the shuttling of damaged cargo to autophagosomes and lysosomes for degradation, which may indicate an increase in autophagy as a protection mechanism against harmful stress (Maiuri and Kroemer 2015; Kaur and Lakkaraju 2018). It has been shown that vesicle axonal transport declines with age as does lysosome acidification (Forester et al 2010, Milde et al 2015, Vagnoni and Bullock 2018). Although foxo has been shown to promote Rab7 protein expression to promote autophagy in cardiomyocytes, this was in
a glucose restricted model and suggests a different mechanism than what we are observing with age (Hariharan et al 2010). Apoptotic neurons can activate glial innate immunity mechanisms, which involved upregulation of endocytic vesicles in glia to eliminate debris. foxo null-mutants have increased apoptotic debris in the brain during development, and loss of foxo expression in larval cortical glia has reduced phagocytosis and enhanced neurodegeneration (McLaughlin et al 2019). It is therefore possible that loss of foxo in the motor neuron results in the exportation of damaged cargo to the glia for elimination. However, more experimentation needs to be done to determine the location and underlying causes for this increase in late endosomes. Meanwhile, overexpression of foxo delays late endosome increase in our adult flies. During development, a constitutively active form of foxo caused severe microtubule destabilization, while wild-type foxo overexpression did not have the same disruption (Nechipurenko and Boihier 2012). This may indicate a role for cytoplasmic foxo in maintaining the integrity of motor neuron.

We first identified likely downstream targets of foxo by examining ChIP-seq and RNA-seq data. When we conducted a genetic screen of our candidates, we found members of MAPK and TGFβ signaling to have profound rescue effects. We observed that knockdown of the activin receptor babo was able to rescue late endosome accumulation. TGFβ signaling has been shown to regulate axon development and Rho GTPase activity in mushroom bodies, and dictates postsynaptic density abundance at the NMJ through motor neuron anterograde signaling (Ng 2008, Kim and O’Connor 2014). TGFβ signaling has also been shown to promote aging in cardiac tissue (Chang et al 2019). We also observed that the MAP kinase p38b isoform, but not p38a or p38c, was able to rescue late endosome upregulation near the control baseline. In ALS mouse models, inhibition of p38 is able to rescue axonal retrograde trafficking (Gibbs et al 2018), and p38b is specifically shown to regulate synaptic morphology in drosophila (Klindinst
et al 2013). Additionally, TGFβ has been shown to activate p38 independently of SMAD and can induce cell apoptosis through this pathway (Yu et al 2002). p38 activation has been associated with increased ROS formation and the promotion of pro-inflammatory cytokines. (Zhao et al 2018) In neuronal tissue, increased ROS and inflammation result in the activation of microglia, with chronic activation being able to induce neuronal damage and apoptosis (Dheen et al 2007; Gwak et al 2017). p38 signaling has also been shown to regulate Rab7 expression in response to interleukin cytokines (Bhattacharya et al 2006), further supporting that the increase in Rab7 observed during aging and under foxo knockdown may be a response to increased inflammation.

Overexpression of the known foxo target gene Thor had fewer rab7 punctae than foxo-RNAi alone, but did not rescue bouton morphology, suggesting this impact on late endosomes may be through a different mechanism. Additionally, some candidates such as liquid facets (lqf) were able to reduce the accumulation of rab7, but had severe thinning of the muscle tissue. This suggests that some genes could decrease MVB formation, but as a detriment to the health of the synapse. In our screen we also targeted rolled (rl), which is the Drosophila ortholog of ERK1/2 (Biggs et al 1994). We expected to see a decrease in late endosome accumulation, but were surprised to find there was no change. We hypothesize that this is due to the potential for two forms of ERK signaling. These two mechanisms of ERK signaling may control opposing processes of synaptic transmission. Rolled hypomorphs show a reduced EJP, similar to what is seen in foxo hypomorphs, suggesting reduction of rolled plays a part in synaptic transmission that may be independent of its role in late endosome shuttling (Wairkar et al 2009). Additionally, blockage of endosome-lysosome fusion will lead to an increase of activated ERK (Ng and Tang 2008), suggesting it may still have functional consequences downstream of foxo activity.

In summary, we have found that loss of foxo in the adult Drosophila results in
morphological changes to synaptic structure that resemble aging morphologies. We also show that motor neuron foxo is required for endocytic homeostasis, and enhanced foxo expression during adulthood can delay aging morphologies. We find evidence to support that foxo may act as a repressor of TGFβ and MAPK signaling, particularly p38 signaling, to maintain youthful characteristics at the NMJ. Identifying the cellular mechanisms regulated by these pathways will provide a greater understanding into how synaptic plasticity is maintained and what homeostatic processes preserve synaptic function during aging.

4.5 Experimental Procedures

Fly stocks and husbandry

The following stocks were used: ywR (Rochele), ywR;+; foxo^{21}, w^{1118}, foxoC431 (Gift from N. Liu, Chinese Academy of Sciences, Shanghai, China), Ok6Gal4 (Gift of E McNeill, Iowa State University), mhcGal4 (Demontis and Perrimon 2010, Cell). Females were collected and sorted 1-2 days after eclosion and placed in vials containing standard CSY food. Fly strains were maintained at 25°C with 12-hour light/dark cycle, and 60% humidity. Flies were transferred to fresh food every three days. Overexpression and TRiP RNAi lines were obtained from the Bloomington stock center unless otherwise specified: UAS-foxo (BDSC_42221), foxo-RNAi #1 (BDSC_32993), foxo-RNAi #2 (BDSC_32427), rl-RNAi (BDSC_34855), khc-73-RNAi (BDSC_36733), lqf-RNAi (BDSC_58130), cpx-RNAi (BDSC_42017), unc-104-RNAi (BDSC_58191), UAS-Ank2GFP (Gift from Ronald Dubreuil, University of Illinois at Chicago), Bsk-RNAi (BDSC_57035), kay-RNAi (BDSC_33379), babo-RNAi (BDSC_25933), p38a-RNAi (BDSC_34744), p38b-RNAi (BDSC_29405), p38c-RNAi (BDSC_64846), grk-RNAi (BDSC_55926), bTub60D-RNAi (BDSC_64856), UAS-Thor (BDSC_9147). Fly lines w^{1118}; ok6-Gal4 and BL32993 (foxo-RNAi) were crossed to double balancer yw; Sp/Cyo; TM2e/TM6BTbHue (Rochele). Markers were used to select carriers. After Ok6 was
combined with the foxo-RNAi, lines were backcrossed for 5 generations before use. Flies containing Ok6 in a yw background were self-crossed to produce a control strain. Line was backcrossed for 5 generations. A daughterless gene switch GAL4 driver was used to validate foxo knockdown and overexpression.

**Immunofluorescence staining**

Flies were subjected to flynap (Carolina, Burlington, NC) and dissected to expose the ventral abdominal muscles (protocol from Wagner et al 2015) in Ca2+ free saline [128mM NaCl, 2mM KCl, 4mM MgCl2(H2O)6, 35.5mM sucrose, 5mM HEPES, 1mM EGTA, H2O, pH 7.2]. Tissue was then fixed in 4% paraformaldehyde for 20 minutes at room temperature. Tissue was washed in 1X PBST (0.1% Triton X) and blocked with 5% NGS (normal goat serum) for 1 hour at room temperature. Samples we incubated overnight at 4°C with 2ug primary antibodies in 5% NGS 1X PBST [ 1X phosphate buffer saline, 0.1% Triton X-100]. Primary antibodies used were as follows: anti-BRP (DSHB nc82), anti-Dlg (DSHB 4F3), anti-Rab7 (DSHB), anti-rab7 (gift from Nakamura lab), anti-Acetylated Tubulin (Sigma Aldrich T7451), anti-phospho-p38 MAPK (CST 9211). Tissues we washed in 1XPBST and incubated 1.5 hours at room temperature in 1XPBST and anti-HRP-594 or HRP-647 (Jackson ImmunoResearch Laboratories Inc, West Grove PA) at a Dilution of 1:200 with secondary antibodies (1:250) and kept in the dark at room temperature for 2 hours. Samples were washed in PBST and mounted in Prolong Diamond (Life technologies). For Fat body analysis, pelts were incubated in slowfade gold with DAPI (life technologies) at 4°C overnight prior to mounting.

**Secondary antibodies**

All secondary antibodies used were from Jackson ImmunoResearch Laboratories Inc and were as follows: 488 donkey anti-mouse, 488 goat anti-rabbit, rabbit anti-HRP-594, donkey anti-HRP-647.
**Imaging analysis and quantification**

Images were captured using an epifluorescence-equipped BX51WI microscope (Olympus, Waltham, MA, USA) and Olympus FV3000 laser scanning confocal (Olympus, Waltham, MA, USA). CellSens software (Olympus, Waltham, MA, USA) was used for deconvolution of stacks. For aging metrics quantifications, maximum Z projections were generated of whole A3 muscle segment. Bouton size and active zone number were quantified in CellSens through a selected region of interest (ROI) aligned to each bouton. Measurements were performed using the “measure and count” module, providing quantification of bouton and active zone number and area. Branch number and branch length were quantified in Fiji (imageJ, Schindelin et al 2012). Acetylated alpha-Tubulin quantification was performed via manual counting using the volume module in Fluoview software. Rab7 quantification was performed in CellSens using a circle we generated a circular ROI (25.75 µm²) to quantify number of punctae. Z-slices quantified were limited to those containing HRP staining at selected regions to avoid non-neuronal associated punctae. ROIs were checked prior to quantification to ensure muscle associated punctae were removed. Intensities for pp38, pERK, and pJNK were conducted using Fiji (NIH) by measuring the sum fluorescence intensity in ROIs defined by HRP immunoreactivity. HRP fluorescence intensity was measured to ensure no differences between control and mutant NMJs. All images were generated using Fiji.

**Quantitative real-time PCR**

3-day post eclosion flies were placed on food containing less than 0.1% ethanol and 200uM Mifepristone (Cayman Chemical) or no other additive for 3 days. Whole bodies of female flies were homogenized in TRIZol (Thermo Fisher). RNA was extracted following company procedure. cDNA was generated using qScript (Quanta) and diluted to a 10uM working solution. Quantitative PCR was run on purified samples (QuantStudio). Cybergreen (Life...
Technologies, CA, USA) was used for chemical detection. The primers used Enrichment was determined based on the double-delta CT value. Thor (sense, 5’ – CCAGGAAGGTTGTCATCTCGG-3’, and antisense 5’ – CTCGTAGATAAGTTTGGTGCTCC-3’), Rpl32 (sense 5’- AAGAAGCGCACCAGCCTTCATC- 3’, and antisense 5’ – TCTGGTGTGATACCCCTTGGGCTT-3’).

**Video capturing**

Stacks of the entire A3 muscle region were acquired with Olympus FV3000 laser scanning confocal in the Fluoview software (Olympus, Waltham, MA, USA). Videos were recorded of 3D renderings using the movie module under the volume setting.

**Venn diagrams**

Venn comparisons were performed using the Bioinformatics and Evolutionary Genomics Venn calculator at http://bioinformatics.psb.ugent.be/webtools/Venn/

**Statistical analysis**

GraphPad Prism (GraphPad Software) was used for statistical analysis. To compare the mean value of treatment groups versus that of control, either student t-test or one-way ANOVA was performed using Tukey multiple comparison. All genetic screen lines were compared to the generated foxo-RNAi line using a student t-test. The metrics during aging were analyzed by two-way ANOVA, including Tukey multiple comparisons test. Before analysis, outliers were identified using Robust regression and Outlier removal (ROUT) method (Q = 1%).

**4.6 Acknowledgments**

We thank Bloomington Drosophila Stock Center (BDSC) and Norbert Perrimon and others at the Drosophila RNAi Screening center (DRSC) for providing fly lines. We also thank Nan Liu and Xiaofen Wu (Chinese Academy of Sciences, Shanghai, China) for fly lines and
RNA-seq data, and and Ronald Dubreuil (University of Illinois at Chicago, Chicago, IL) for fly lines used in this study. We thank Akira Nakamura (RIKEN Center for Developmental Biology, Kobe, Japan) for antibodies used in this study. The authors acknowledge the assistance of rotation student Sean McLaughin. This work was supported by NIH/NIA R00 AG048016 to HB, AFAR Research Grants for Junior Faculty to HB. This manuscript has been released as a pre-print at bioRxiv (Birnbaum et al 2020).

### 4.7 Competing Interests

The authors declare that no competing interest exists.

### 4.8 Figure and Table Legends

**Figure 4.1. Loss of FOXO results in middle-aged NMJ morphology in the abdominal ventral muscle.** A. Representative images of the Abdominal VLM A3 segment for axon terminals with indicated genotypes and ages colabeled with Anti-BRP and anti-HRP. Bar 20 μM. B. Average branch length. *P<0.05, ** P<0.01. Bouton area for 5-day foxo flies is significantly larger than 5-day controls, but is comparable to 25-day control flies. ** P<0.01. C. Average bouton area ** P<0.01, ***P>0.001, ****P< 0.0001. D. Quantification of changes in active zone number per bouton. *P<0.05, ** P<0.01, ***P<0.001. ywR 5d n=6; 25d n=5; 40d n=6; foxo21 5d n=5. Number of boutons quantified per sample analyzed: ywR 5d n=177, 25d n=75, 40d n=96, foxo21 5d n=100. Number of branches analyzed: ywR 5d n=39, 25d n=39, 40d n=40, foxo21 5d n=39.

**Figure 4.2. Aging and loss of FOXO cause changes in microtubule structure and endocytic activity** A. Quantification of anti-Ac-Tub at A3 VLM at control aging timepoints and in foxo mutant. The mean fraction of straight branches was significantly reduced from the young control for all other columns (P< 0.05). B. Average bouton area for ok6-GAL4>ywR compared to ok6-GAL4>foxo-RNAi #1 (*P< 0.05). C. Quantification of anti-Ac-Tub at A3 VLM at specified
genotypes and ages. The % of straight branches of all other columns was significantly reduced from the young control (P< 0.05). **P< 0.01, ***P< 0.001, n.s.- not significant. E. Representative images of main axon branchpoint with indicated genotypes and ages. Colabeled with Anti-Rab7 and anti-HRP. Nervous system tissue is outlined in white. Surrounding muscle tissue has been removed from the image. Bar 10 μM. Number of animals analyzed for Ac-Tub: ywR 1wk n=3, 25d n=3, 40d n=3, foxo21 1wk n=3, ok6-GAL4>ywR 1 wk=5, ok6-GAL4>ywR 25d = 5, ok6-GAL4> foxo-RNAi #1 1 wk=4. Number of branches analyzed for Ac-Tub; ywR 5d n=38, 25d n=37, 40d n=26, foxo21 5d n= 46, ok6-GAL4>ywR 1 wk=70, ok6-GAL4>ywR 25d =90, ok6-GAL4> foxo-RNAi #1 1 wk=75. Number of animals analyzed for Rab7 quantification; ok6-GAL4>ywR 1 wk = 7, ok6-GAL4>ywR 25d = 6, ok6-GAL4> foxo-RNAi #1 1 wk= 8.

**Figure 4.3. Overexpression of motor neuron FOXO delays aging phenotypes A-B.**
Representative figures of control and FOXO-overexpression at 1 week, (A’-B’) and 25 days. Colabelling with anti-Rab7 and anti-HRP. Scale bar 10μm. C. Rab7 quantification for A3 ROIs for indicated genotypes and ages *P < 0.05. D. Average bouton area for 25-day old FOXO overexpression and control **P<0.1. Number of animals analyzed for Rab7 quantification; ok6-GAL4>ywR 1 wk= 7, ok6-GAL4>ywR 25d = 6, ok6-GAL4> UAS-foxo 1 wk= 4. ok6-GAL4> UAS-foxo 25d = 7. Number of animals analyzed for bouton size: ok6-GAL4>ywR 25d=5, ok6-GAL4> UAS-foxo 25d = 5.

**Figure 4.4: MAPK acts downstream of FOXO to regulate NMJ pathways. A.** Venn diagram overlap of ChIP-seq, head foxo431 RNA-seq, and aged head RNA-seq: For RNA-seq data { -0.15 > log2FC > 0.15, P < 0.05}. {FOXO ChIP FC >1.5, FDR< 0.05}. B. Quantification of Rab7 for double mutant flies. Values are set as a percentage with ok6>ywR as baseline. All
significant values are compared to foxoRNAi (in ok6-GAL4 background) *P<0.05. C. Average bouton size quantification for indicated genotypes. Control in Ok6-GAL4 background. *P<0.05, **P<0.01, ***P< 0.001, ****P< 0.0001, n.s. – not significant. D. Quantification of the mean fraction of anti-Ac-Tub branch morphology. Mean fraction of straight branches is significantly higher in p38bRNAi. P<0.05. E. Representative figures of anti-Ac-Tub staining. Arrows indicate undulating (left figure) and straight (right figure) branches. Scale bar 10μm. Number of animals used for figure 4B in order from left to right: n=22;13;7;4;3;6;10;7;4;6;5;4;6;6;6;3;3. Number of animals used for figure 4C bouton size in order from left to right: n=14;11;4;5;4;5;3;3;2. Number of animals used for figure 4D c-Tub: ok6-GAL4; foxo-RNAi >ywR = 4, ok6-GAL4; foxo-RNAi> babo-RNAi = 6, ok6-GAL4; foxo-RNAi > p38b-RNAi = 5. Number of branches used for figure 4D c-Tub: ok6-GAL4; foxo-RNAi >ywR = 75, ok6-GAL4; foxo-RNAi> babo-RNAi = 93, ok6-GAL4; foxo-RNAi > p38b-RNAi = 80.

**Figure 4.5. Motor neuron FOXO inhibits MAPK activation** A. Representative images of 1-week old fly NMJs. Colabeled with anti-phosphorylated-p38 and anti-HRP. Scale bar10μm. B. Relative intensity of P-p38 at anti-HRP masked areas. Intensity was normalized to anti-HRP intensity. *P<0.05. Number of NMJs used: ok6-GAL4 >ywR 1wk = 9; ok6-GAL4; foxo-RNAi >ywR 1 wk = 10

**Table 4.1. Quantification of NMJ morphological characteristics of aging.** ywR is the control line for foxo21. Average bouton number: 5-day ywR v 5-day foxo21 (n.s.). 5-day ywR v 25-day ywR (n.s.). 5-day ywR v 40-day ywR (P< 0.05). 25-day ywR v 5-day foxo21 (P< 0.05). Branch number: 5-day ywR v 5-day foxo21 (P< 0.05). 5-day ywR v 25-day ywR (n.s.). 5-day ywR v 40-day ywR (n.s.). Bouton number and branch number represent both NMJs present at the A3 abdominal segment.
Table 4.2: Downstream candidate genes from FOXO ChIP-seq and RNA-seq

Supporting information

Figure S4.1. A. Raw counts for bouton area B. branch length C. active zone number per bouton D. Average bouton area and branch length for wild-type (w^{1118}) and foxo^{C431}. *P< 0.05, ** P<0.01, ***P>0.001, ****P<0.0001, n.s. not significant. w^{1118} n=5; foxo^{C431} n=5. E. Averaged bouton area and branch length. F. Raw bouton area and branch length for wild-type (w^{1118}) and foxo^{C431}. *P< 0.05, ** P<0.01, ***P>0.001, ****P< 0.0001, n.s. not significant. w^{1118} n=59; foxo^{C431} n=66. G. F. Western analysis of FOXO protein abundance in control and Foxo^{21} mutant whole-body tissue.

Figure S4.2. A. Representative images of anti-Ac-Tub staining for wildtype (yw^R) at 5 and 25 days of age, and foxo^{21} flies at 5 days of age. B. Representative images of anti-Ac-Tub staining for control (Ok6-Gal4> yw^R) at 5 and 25 days of age, and Ok6-Gal4> foxo-RNAi flies at 5 days of age. C. Quantification of acetylated alpha-tubulin structure. Significance values are compared to young control for each grouping. *P< 0.05, ** P<0.01, ***P>0.001.

Figure S4.3. A. Rab7 quantification at 1 week for ok6>ywR, ok6>foxo-RNAi #1, ok6>foxo-RNAi #2. B. Thor relative expression for whole body tissue. Daughterless-Gal4 activated with 200mM of RU486. C. Quantification of FOXO protein intensity in fat body nuclei after RU486 induced knockdown. * P< 0.05. D. Representative images of fat body tissue for control (S106-GS-Gal4> w^{1118}) and FOXO RNAi (S106-GS-Gal4>BL32993). S106-Gal4 activated with 200mM of Mifepristone (RU). Staining with anti-FOXO and DAPI. Scale Bar 10\mu m, n=5.

Figure S4.4. Representative confocal images from genetic screening. A. anti-rab7 and anti-HRP immunofluorescence for ok6;foxo^{RNAi}> p38b^{RNAi}, ok6;foxo^{RNAi}> r1^{RNAi}, ok6;foxo^{RNAi}> p38a^{RNAi}. B. anti-rab7 and anti-HRP immunofluorescence for control (ok6-gal4> yw^R).
ok6;foxoRNAi > ywR, ok6;foxoRNAi > baboRNAi, C. anti-Ac-Tub and anti-HRP immunofluorescence for ok6;foxoRNAi > p38bRNAi, ok6;foxoRNAi > baboRNAi, ok6;foxoRNAi > ywR.

**Table S4.1.** GO terms for ChIP-Seq and RNA-seq overlap

### 4.9 References


4.10 Figures and Tables

Figure 4.1
Figure 4.2
Figure 4.3
Figure 4. 4
Figure 4. 5

Table 4. 1

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FC = fold change
Figure S4. 1
Figure S4. 2

A

B

C

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Figure S4. 4
CHAPTER 5. GENERAL CONCLUSIONS

In this dissertation, the *Drosophila melanogaster* model system was used to investigate the behavior of transcription factor FOXO under stress and aging to gain further insight into how FOXO coordinates tissue homeostasis, and how this activity is altered during senescence. The second chapter of this thesis was a collaborative project designed to explore how FOXO transcription factor activity changes under normal aging. ChIP-seq analysis was performed on wild-type flies at young and aged timepoints, and a decline in the number of FOXO-bound genes was observed with age. Using transcriptome data, we saw FOXO positively-regulated genes have reduced expression with age, while genes that were negatively controlled by FOXO had increased expression in older flies. These results show that both FOXO chromatin binding decrease with age as does FOXO transcription regulation, and this may contribute to age-related cellular dysregulation. In young flies we saw FOXO binding to the chromatin region of genes involved in growth signaling pathways such as Hippo, MAPK, WNT, and TGFβ. These targets are no longer bound by FOXO in older organisms, and expression of genes in these pathways becomes altered with age. We saw unique FOXO-bound regions when comparing ChIP data from young wild-type flies to insulin mutant flies. This suggests that under nutritional stress, FOXO targets different cellular pathways than under normal feeding. One surprising result was that many FOXO-bound regions in young flies coincide with heterochromatin regions, and we see many FOXO targets that are nucleosome assembly genes. These targets were still bound by FOXO in older flies, suggesting FOXO’s role in regulating chromatin structure does not change with age. FOXO has been previously shown to recruit heterochromatin promoting factors, and this work suggests FOXO may play an active role in regulating chromatin structure (Riedel et al 2013). This work has helped define the decline in FOXO activity under normal aging by
demonstrating the reduction of FOXO gene targeting with age, and can be used to elucidate pathways that become disrupted with age.

The third chapter of this thesis evaluates the dynamics of the FOXO protein-protein interaction network. Oxidative stress is known to increase with aging and activate FOXO (Martins et al 2016). We see an increase in FOXO nuclear localization in certain tissue types when exposed to the oxidative stress agent paraquat. Using a proteomics approach, we characterized FOXO protein interacting networks under control and paraquat-induced oxidative stress conditions. We observed a small set of conserved proteins between the two data sets, but predominantly saw unique networks for each condition. Under control conditions, FOXO-bound proteins were enriched for pathways involved in translation, while proteins bound to FOXO exclusively under oxidative stress conditions were involved in transcription according to gene ontology analysis. These results show there is likely a switch to transcription activation upon stress-induced FOXO nuclear localization. Genes activate by FOXO under oxidative stress have been previously described so this switch to translation promotion is expected (Carter and Brunet 2007). We found proteins that precipitated with FOXO under both conditions were enriched for GO processes involved in chromatin modulation, supporting our previous results from our ChIP-seq data that FOXO is involved in regulating chromatin structure. Overall, these results have helped define the FOXO protein interactions that occur under control and oxidative stress conditions, and provide evidence to better understand FOXO function as a homeostatic regulator in response to ROS.

In the fourth chapter, we examined FOXO tissue specific activity and the effect it has on the regulation of homeostatic processes. Dysregulation during aging at the neuromuscular junction (NMJ) can lead to the onset of neurodegenerative diseases (Gonzalez-Freire et al 2014).
Using the adult *Drosophila* NMJ, we observed FOXO-dependent regulation of this synaptic interface. With aging, NMJ structures undergo morphological changes in response to alterations in synaptic activity (Wagner et al 2015). These characteristics seen in aging were also observed at a young age upon the loss of FOXO. Additionally, during aging there are alterations to the axonal microtubule structure, and knocking down FOXO in the motor neuron recapitulated this phenotype. We also saw an increased number of late endosomes associated with the main axon branchpoint as the flies aged. This accumulation also occurred at a young age under knockdown of motor neuron FOXO. Meanwhile, the overexpression of FOXO in the motor neuron delayed the onset of this accumulation, as well as changes to the synaptic morphology. These results indicate that FOXO can promote youthful characteristics in the motor neuron, and loss of FOXO is disruptive to these processes. Additionally, we found knocking down the b isoform of p38 can rescue foxo knockdown induced disruptions and observed increased phosphorylation of p38 within the motor neuron upon loss of FOXO. These results show FOXO acts as a negative regulator of p38 activity. The activation of p38 may be an indirect response, as p38 can be phosphorylated under increased oxidative stress (Zhao et al 2018). Loss of FOXO has been shown to cause increased ROS levels, and thus may induce p38 activation (Paik et al 2009). FOXO and p38 are also in a feedback loop, with phosphorylated p38 being able to activate FOXO in response to increased ROS (Asada et al 2007, Klotz et al 2015). Phosphorylation of p38 can have detrimental effects on the adult system, and is known to cause increased inflammation, which can lead to degeneration and cell death (Gwak et al 2017, Zhao et al 2018). Additionally, MAPK activation has been shown to increase with aging, and is suppressed in caloric restriction models, while this nutrition stress is known to activate FOXO (Kim et al 2002, Hwangbo et al 2004). Further investigation needs to be done to uncover if loss of motor neuron
FOXO results in elevated ROS and inflammation. Ultimately, this study has found that inhibiting FOXO activity in motor neuron tissue causes the early appearance of aging phenotypes at the neuromuscular junction. Our evidence suggests FOXO acts as an inhibitor of MAPK activation, thereby maintaining integrity of the neuromuscular junction.

Overall, this thesis provides evidence for FOXO-regulated cellular homeostasis during normal aging. We used genomic, proteomic, and tissue-specific investigations to characterize FOXO behavior, and have found FOXO-specific activities that impact age-related processes. FOXO's interaction with DNA is reduced with aging under normal feeding conditions, and corresponds to global transcriptome profile changes. We also identify unique protein interacting networks under distinct cellular conditions. These interactions can be further investigated to understand age-related changes in FOXO activity, and explored to better define FOXO regulation of tissue homeostasis. Finally, we have observed tissue specific changes that occur at the neuromuscular junction in response to FOXO, and cause alterations to synaptic structures and processes that share characteristics seen during aging. Altogether, this research outlines alterations in FOXO behavior, and delivers novel insights into FOXO regulation of homeostatic processes. Future work should focus on comprehending the downstream processes that get disrupted by changes in FOXO activity with normal aging. By uncovering this, we can develop novel therapies to target age-related impairments, and preserve tissue homeostasis. This regulation may be particularly important at the neuromuscular junction, where functional loss has detrimental effects on both neuronal and muscular integrity and can cause significant health consequences. Because of this impact on health, it is important to explore the causes of synaptic functional decline, and how proteins such as FOXO can be used to maintain tissue integrity and cellular homeostasis during aging.
5.1 References


