Sex-related differences in the regulation and function of Forkhead Box O1 in adipose endothelial cells

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Abstract

Previous work in the Haas lab showed that adipose endothelial cells (ECs) display sex-related differences in their functional capabilities and transcriptomic profiles. Intriguingly, female ECs exhibit a sex-distinct dependence on the transcription factor, Forkhead Box O1 (FoxO1), which is also more highly expressed in female ECs. Thus, my thesis investigated potential mechanisms leading to higher FoxO1 levels in female ECs and explored the sex-specific function of FoxO1 in cultured adipose ECs. My results showed that while male and female ECs exhibited similar responses to insulin, female ECs had greater levels of the upstream regulators, MDM2 and p-p38. Subcellular fractionation and immunostaining experiments revealed greater nuclear FoxO1 levels in female ECs under basal level conditions. In addition, gene expression analyses highlighted sex-specific effects of FoxO1 inhibition on the expression of nucleosome organization genes. These results provide insights into sex-related differences in EC biology which is imperative for normal tissue function.

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Contributions statement

The majority of experiments and analyses presented in this thesis were completed by me. I performed the extraction of adipose ECs from the mice alongside another lab member, Alex Pislaru. I conducted all subcellular fractionations, western blots, immunostaining, and qPCR experiments. I cultured and treated all cells used in my experiments, except for specific western blot experiments involving siRNA and FoxO1 inhibitor-treated samples, which were prepared by a previous lab member, Connor Breadmore, for independent experiments. In addition, preparation of the ATAC, ChIP, and RNA-seq samples was completed by Alex and me.

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Abbreviations

AKT- protein kinase B

AR- androgen receptor

ASF1- anti-silencing factor 1

ATAC- assay for transposase accessible chromatin

BAT- brown adipose tissue

CAF-1-chromatin assembly factor-1

CD31- cluster of differentiation 31

CE- cytoplasmic extract

ChIP- chromatin immunoprecipitation

CUT&RUN- cleavage under targets and release using nuclease

DBD- dna-binding domain

DEGs- differentially expressed genes

DMEM- dulbecco's modified eagle medium

DMSO- day, cells were treated with vehicle

DTT- dithiothreitol

EC- endothelial cell

eNOS- endothelial no synthase

ER- estrogen receptor

ERK- extracellular-signal-regulated kinases

FAK- focal adhesion kinase

FBS- fetal bovine serum

FoxO1- forkhead box O1

FRE- forkhead response element

FVB- friend virus B

G6Pase- glucose-6-phosphatase

GADD45- growth arrest and DNA damage-inducible protein

GAPDH- glyceraldehyde-3-phosphate dehydrogenase

GLUT1- glucose transporter 1

GSEA- gene set enrichment analysis

GSK3- glycogen synthase kinase 3

HAEC- human aortic endothelial cells

HFD- high fat diet

HIRA- histone regulator A

HMEC- human microvascular endothelial cells

HOX- homeobox

HR- homologous recombination

HSC- haematopoietic stem cells

HUVECs- human umbilical vein endothelial cells

IGFBP-1- insulin-like growth factor binding protein 1

IRE- insulin response element

IRS- insulin receptor substrate

JNK- jun n-terminal kinase

KD- knockdown

KO- knockout

MAPK- mitogen-activated protein kinases

MDM2- mouse double minute 2

NAP- nucleosome assembly proteins

NE- nuclear extract

NES- nuclear export signal

NLS- nuclear localization signal

NO- nitric oxide

NP-40- nonidet p-40

NT- non-targeting

PAD- peripheral artery disease

PBS- phosphate-buffered saline

PI3K- phosphatidylinositol 3-kinase

PKA- protein kinase A

PKC- protein kinase C

PKI- protein kinase inhibitor

RCC1- regulator of chromosome condensation 1

RIPA- radioimmunoprecipitation assay

ROS- reactive oxygen species

SAT- subcutaneous adipose tissue

SDS- sodium dodecyl sulfate

SEM- standard error of the mean

SOD2- superoxide dismutase 2

SRY- sex-determining region Y

SVF- stromal vascular fraction

TGFβ- transforming growth factor beta

VAT- visceral adipose tissue

VEGF-A- vascular endothelial growth factor A

VEGFR2- vascular endothelial growth factor receptor 2

WAT- white adipose tissue

Chapter 1: Literature Review

1.1 Adipose tissue

Adipose tissue is a complex organ that is largely known for its role in energy storage; yet, it is also essential for maintaining systemic metabolic health through its less recognized thermoregulatory and endocrine functions (Chaurasiya et al., 2024). There are three main types of adipose tissue: white adipose tissue (WAT) for lipid storage and homeostasis, brown adipose tissue (BAT) for thermogenesis and energy expenditure, and beige or brite adipose tissue (composed of both BAT and WAT) (Chaurasiya et al., 2024). WAT is further characterized into two subtypes based on its location in the body: visceral adipose tissue (VAT), which is often represented by the omental depot in humans and by the perigonadal depot in mice, and subcutaneous adipose tissue (SAT), which includes the abdominal, gluteal and femoral depots in humans and the anterior axillary region and inguinal fat pad in mice (Börgeson et al., 2022; White & Tchoukalova, 2014). Although there are important differences between humans and mice in the location and function of their fat depots, results from studies done on mouse and human WAT are largely comparable (Börgeson et al., 2022).

Adipose tissue is comprised of mature adipocytes and a heterogeneous mixture of cells known as the stromal vascular fraction (SVF). Within the SVF are immune cells, fibroblasts, preadipocytes, adipose-derived stem cells, and vascular cells, including endothelial cells (ECs) (Chaurasiya et al., 2024). Notably, unlike other organs, adipose is highly dynamic and undergoes normal expansion and regression during adulthood. This requires equally responsive neovascularization where ECs remodel into new capillary networks through a process called angiogenesis to ensure that enough oxygen and nutrients are supplied to the growing cells (Corvera et al., 2022). However, during the development of obesity where there is chronic caloric surplus, the rapid expansion of adipose tissue

is often accompanied by impaired adipose angiogenesis (Corvera & Gealekman, 2014). This results in inadequate vascularization which contributes to the development of dysfunctional adipose tissue characterized by hypoxia, metabolic dysregulation, and chronic inflammation (Corvera & Gealekman, 2014). Notably, experimental enhancement of adipose angiogenesis with vascular endothelial growth factor A (VEGF-A) in obese mice leads to increased capillary density, smaller adipocytes, and improved metabolic health, highlighting the therapeutic potential of improving adipose angiogenic capacity for treating obesity-associated diseases (Robciuc et al., 2016; Sung et al., 2013). As such, ECs are a critical component of the adipose tissue and play an important role in maintaining adipose function and health.

1.2 ECs in the vasculature

ECs line the interior surface of the blood vessel wall, forming a selective barrier between the blood and surrounding tissues. Beyond their structural role in the vasculature, ECs are key players in the regulation of vascular tone, delivery of oxygen and nutrients, immunity, and tissue homeostasis (Trimm & Red-Horse, 2023). Consequently, the loss of EC functional integrity is implicated in various cardiovascular diseases such as atherosclerosis, peripheral artery disease (PAD), and hypertension (Pasut et al., 2025). Notably, ECs are highly heterogeneous and display organ-specific attributes affecting their structural morphology, paracrine factor secretion, and responses to signalling molecules (Augustin & Koh, 2017). This heterogeneity in their functional phenotypes highlights the importance of studying ECs at a tissue-specific level and understanding the microvasculature that governs the microenvironment within different tissues.

1.3 EC functions

While ECs display diverse functions across the different vascular beds, several functions are shared by ECs that are essential for vascular homeostasis. A key endothelium-derived factor that is critical

for normal function and survival of the endothelium is nitric oxide (NO). NO plays several important physiological roles in maintaining vascular homeostasis including promoting platelet aggregation, mediating angiogenesis, and protecting against apoptosis (Azuma et al., 1986; Dimmeler & Zeiher, 1999; Murohara et al., 1998). NO is also involved in limiting inflammation by preventing the adhesion of neutrophils and expression of monocyte chemoattractant proteins (Azuma et al., 1986; Niu et al., 1994; Zeiher et al., 1995). Another well-established function of NO is its regulation of vascular tone. NO is a potent vasodilator produced from L-arginine and O_2 by endothelial NO synthase (eNOS) in vascular ECs to maintain a basal level of smooth muscle cell relaxation and vasodilation (Palmer et al., 1988; Palmer & Moncada, 1989). eNOS activity is primarily stimulated by shear stress which increases intracellular calcium (Ca^{2+}) levels and promotes protein kinase B (PKB/AKT)-dependent phosphorylation of eNOS (Devika & Jaffar Ali, 2013; Dimmeler et al., 1999; Kuchan & Frangos, 1994; Rubanyi et al., 1986). Once produced by the endothelium, NO diffuses into adjacent vascular smooth muscle cells where it stimulates soluble guanylyl cyclase to catalyze the production of cGMP, which lowers intracellular calcium levels and leads to subsequent vasorelaxation (Craven & DeRubertis, 1978; Rashatwar et al., 1987).

Another important role of ECs is forming a semi-permeable barrier between the bloodstream and surrounding tissues (Majno & Palade, 1961). The EC lining not only acts as a physical conduit for blood flow, but it also regulates the dynamic transportation of various molecules including oxygen, nutrients, and metabolic by-products. ECs are connected to one another via adherens junctions and tight junctions, where the abundance and organization of both types of junctions depends on the specific requirements of the vascular bed (Bazzoni & Dejana, 2004). For example, some organs like the liver require greater permeability to allow for the clearance of toxins and waste from the

bloodstream, while other areas like the blood-brain-barrier in the brain require more tight junctions to form tightly associated ECs to prevent the penetration of harmful substances (Augustin & Koh, 2017; Brightman & Reese, 1969).

In addition, ECs are the key drivers of angiogenesis which is the process of new capillaries forming from pre-existing ones. Angiogenesis ensures that diffusion distances are short enough to allow for adequate transfer of materials and is therefore essential during early development as well as adulthood in contexts like wound healing, adipogenesis, and muscle hypertrophy, where oxygen or nutrient demands are augmented (Christiaens & Lijnen, 2010; Nissen et al., 1998; Olfert et al., 2010). While angiogenesis is critical for maintaining healthy tissue, inadequate or excessive angiogenesis can contribute to the progression of pathophysiological conditions as seen in metabolic diseases like obesity or tumour microenvironments (Christiaens & Lijnen, 2010; Eelen et al., 2020).

1.4 Angiogenesis

Angiogenesis is driven by a gradient of pro-angiogenic stimuli like VEGF-A that gets released by hypoxic or nutrient-deprived cells in the microenvironment and induces a phenotypic switch in quiescent ECs into an activated state (Eelen et al., 2020). VEGF-A binds to VEGF receptor 2 (VEGFR2) on the surface of ECs causing the autophosphorylation of specific tyrosine residues on the receptor that initiates downstream signalling cascades (Abhinand et al., 2016). This includes the activation of numerous signalling proteins including protein kinase C (PKC), AKT, p38 mitogen-activated protein kinase (p38 MAPK), focal adhesion kinase (FAK), and extracellular-signal-regulated kinases (ERK) (Abhinand et al., 2016). The activation and cross-talk of multiple signalling pathways regulate processes required for angiogenesis including relaxation of cell-cell contacts, pericyte detachment, and break down of the basement membrane (Eelen et al., 2018).

During sprout formation, ECs take on distinct phenotypes that play different functional roles. The cell that is exposed to the greatest VEGF-A levels becomes the tip cell, which guides the sprout and migrates towards the growth factor signal. The other ECs take on a stalk cell phenotype and mediate sprout elongation by proliferating behind the tip cell. This specification into tip or stalk cells is highly dynamic and must be tightly coordinated to ensure that well perfused and functional vessels are formed (Eelen et al., 2018). After the new sprouts have joined together via anastomosis, maturation and stabilization of the capillary can occur through the recruitment of mural cells including pericytes and through the deposition of the basement membrane (Jain, 2003). Once vascularization has been restored to the tissue, levels of proangiogenic factors released decline, and ECs return to a quiescent state (Eelen et al., 2020).

1.5 EC metabolism

EC metabolism is tightly linked to the functional capabilities of ECs. Despite having direct access to oxygen from the blood, ECs are primarily glycolytic and do not rely heavily on oxidative phosphorylation (Culic et al., 1997; Krützfeldt et al., 1990). Energy from glycolysis is used to drive energy-demanding homeostatic functions including metabolite transport, maintenance of barrier functions, and glycocalyx deposition (Curry & Adamson, 2012; De Bock, Georgiadou, & Carmeliet, 2013). This reliance on glycolysis over oxidative phosphorylation in ECs serves several benefits. One of the main roles of ECs is facilitating the exchange of oxygen between the bloodstream and surrounding tissues. By generating energy through glycolysis, this improves the availability of oxygen that can be transferred to tissues. Additionally, less oxidative phosphorylation results in lower ROS generated via mitochondrial respiration, helping to minimize EC oxidative stress (De Bock, Georgiadou, & Carmeliet, 2013). On the other hand, ECs lining vessels in oxygen-depleted areas with reduced blood flow, such as in ischemia, would be unable

to generate sufficient ATP if they relied mainly, or solely, on oxidative phosphorylation. Moreover, glycolysis has the potential to generate ATP quickly and in comparable amounts to glucose oxidation when glucose is in surplus (De Bock et al., 2013).

Disruption of EC metabolism is a contributing factor in dysfunctional angiogenesis due to its impairment of ATP and substrate availability. Indeed, inhibition of glycolysis through the depletion of glycolytic regulator PFKFB3 impairs vessel formation through its effects on both EC proliferation and migration (De Bock et al., 2013). Conversely, overexpression of PFKFB3 enhances vessel sprouting and promotes tip cell formation, suggesting that varying levels of glycolysis may modulate the phenotypic switch between stalk and tip cells during sprouting (De Bock et al., 2013; Yetkin-Arik et al., 2019). Importantly, while glycolysis is essential for tip cell specification, it was shown that both glycolysis and mitochondrial respiration are utilized during proliferation of stalk cells (Yetkin-Arik et al., 2019). As such, EC metabolism plays a crucial role in influencing EC phenotype and behaviour.

1.6 Forkhead Box O1

One of the key transcriptional regulators of vascular growth and EC metabolism is Forkhead Box O1 (FoxO1). FoxO1 belongs to the family of FoxO transcription factors that are recognized for their winged-helix or forkhead DNA-binding domain (DBD) (Clark et al., 1993). In mammals, four FoxO isoforms (FoxO1, 3, 4 and 6) have been identified, although FoxO6 is specifically expressed in the brain (Jacobs et al., 2003; Santos et al., 2023). FoxO1 plays a critical role during vascular development, and both global and EC-specific FoxO1-deficiency in mice result in embryonic lethality (Furuyama et al., 2004; Hosaka et al., 2004; Sengupta et al., 2012). In contrast, FoxO3 and FoxO4 are dispensable for normal vascular development (Hosaka et al., 2004). Postnatally, FoxO1 acts as a critical regulator of angiogenesis as it restricts proliferation and lowers

metabolic activity in ECs (Wilhelm et al., 2016). In adults, EC-specific deletion of FoxO1 results in increased EC metabolic activity and improved vascular remodelling by adipose ECs in male mice, positioning it as a key suppressor of EC growth and proliferation (Rudnicki, Abdifarkosh, Nwadozi, et al., 2018).

FoxO1 mediates transcriptional regulation through direct DNA-binding and through associations with other binding partners. FoxO1 acts as an activator or repressor by binding to the DNA at conserved forkhead binding motifs characterized by the 8 base pair sequence, TTGTTTAC (Furuyama et al., 2000). While FoxO1 mainly binds as monomers to the DNA, there is evidence of FoxO1 forming a homodimer at the palindromic DNA sequence DIV2 (J. Li et al., 2021). FoxO1 is also a key downstream mediator of the insulin signalling response through its binding and activation of insulin response elements (IREs) (Guo et al., 1999). Importantly, FoxO proteins interact with multiple binding partners to mediate broader transcriptional regulation in a cell-specific manner (van der Vos & Coffer, 2008). For example, Transforming Growth Factor- beta (TGF-β) induces the formation of a complex between FoxOs and Smad proteins, key mediators of the TGF-β signalling pathway (Seoane et al., 2004). This complex was shown to promote expression of p21 and mechanistically required TGF-β activated Smads for FoxOs to bind to the p21 promoter. In primate endometrial cells, FoxO1 was shown to cooperate with the homeobox (HOX) protein HOXA10 to promote transcription of IGFBP-1 (Kim et al., 2003). Intriguingly, a cooperative interaction between FoxO1 and another HOX protein HOXA5 was also observed in human fibroblast cells to promote IGFBP-1 expression; however, in HepG2 cells, this interaction repressed FoxO1-mediated transcription of IGFBP-1 (Foucher et al., 2002). These contrasting outcomes of FoxO1 interaction with the same binding partner highlights the cell-specific effect of FoxO1 interactions on transcriptional regulation.

1.7 FoxO1 functions

At the transcriptional level, FoxO1 regulates genes involved in cell cycle arrest and DNA repair, apoptosis, oxidative stress, and energy metabolism (Furuyama et al., 2004). FoxO1 upregulates the cyclin-dependent kinase inhibitor, $p27^{Kip1}$, to promote cell cycle arrest. This effect was shown to involve the interaction between JNK and FoxO1, where JNK inhibition prevented both the nuclear import of FoxO1 and the upregulation of FoxO1 target genes including p27 following DNA damage (Ju et al., 2014). FoxO1 also promotes the transcriptional activation of Growth Arrest and DNA Damage-inducible protein, GADD45 (Furukawa-Hibi et al., 2002). GADD45 expression is inducible by oxidative stress and promotes G2 arrest in a FOXO-dependent manner (Furukawa-Hibi et al., 2002). Another role of FoxO1 is regulating apoptosis as evidenced by its upregulation of the proapoptotic protein BIM in response to DNA damage (Ju et al., 2014). Furthermore, FoxO1 is a key transcriptional regulator of genes involved in oxidative stress responses. In cardiomyocytes, FoxO1 forms a complex with YAP that binds to the promoters of Superoxide Dismutase 2 (SOD2) and catalase to promote their transcription in response to oxidative stress (Shao et al., 2014). Other major genes targets of FoxO1 are implicated in energy metabolism, including glucose-6-phosphatase (G6Pase) and insulin-like growth factor binding protein 1 (IGFBP-1) (Barthel et al., 2001; Yeagley et al., 2001).

1.8 FoxO1 as a pioneer factor

Previous research has provided evidence for the ability of FoxO1 to act as a pioneer factor (Hatta & Cirillo, 2007). Pioneer factors are transcription factors uniquely capable of binding to closed, nucleosome-occupied chromatin to promote chromatin remodelling and often the recruitment of other transcription factors (Barral & Zaret, 2024). Using purified recombinant FoxO1 and testing its ability to bind nucleosome particles and chromatin arrays in vitro, FoxO1 was shown to bind to

extended and condensed chromatin and modulate active chromatin states (Hatta & Cirillo, 2007). An assay for transposase accessible chromatin with sequencing (ATAC-seq) experiment on human T cells showed that FOXO1 overexpression led to global changes in chromatin accessibility and that DNA-binding motifs for transcription factors of the FOX family was one of the top-ranked differentially accessible motifs, supporting a role of FOXO1 in regulating local chromatin remodelling (Doan et al., 2024). Additionally, Miyamura et al. (2024) investigated the role of FoxO1 in VEGF-A mediated tip-cell formation and showed that FoxO1 preferentially binds to promoter regions of tip-cell enriched genes in HUVECs. These regions included both open and closed chromatin as evidenced by the overlap of ChIP-seq for FoxO1 with ChIP-seq for histone markers of active enhancers (H3K4me3 and H3K27ac) and repressed chromatin regions (H3K27me3) (Miyamura et al., 2024). In sum, FoxO1 may act as a broader regulator of epigenetic programs in ECs that contribute to sex-differences in endothelial function.

1.9 Regulation and localization of FoxO1

Several pathways are implicated in the regulation of FoxO1 including growth factor signalling, nutrient status, and oxidative stress (Brown & Webb, 2018). Importantly, these pathways modify FoxO1 through post-translational modifications such as phosphorylation, ubiquitination, and acetylation, that can affect FoxO1 localization and DNA binding capability (Link & Fernandez-Marcos, 2017).

One of the most well-established ways by which cells regulate FoxO1 activity is through the insulin signalling pathway. Insulin signalling leads to the activation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) (Alessi et al., 1996). AKT-mediated phosphorylation of FoxO1 at serine-256, serine-319, and threonine-24 residues results in nuclear exclusion and a reduction in FoxO1-dependent gene transcription (Biggs et al., 1999; Brunet et al., 1999; Rena et

al., 1999). This process is mediated by the nuclear export signal (NES) downstream of the DBD and a chaperone protein known as 14-3-3 that interacts with phosphorylated FoxO1 in the nucleus (Zhao et al., 2004). Interestingly, serine-256 phosphorylation independently alters FoxO1-DNA binding activity; however, phosphorylation at all three residues is required for nuclear exclusion (Zhang et al., 2002). On the other hand, FoxO1 import is mediated by the nuclear localization signal (NLS) at the N-terminal end of the DBD, along with elements located at the C-terminal end, as demonstrated by impaired nuclear localization observed after mutating amino acids in the N- and C-terminal regions of the DBD (Zhao et al., 2004). Additionally, phosphorylation at non-AKT sites by other kinases such as c-Jun N-terminal kinase (JNK) and p38 can promote nuclear localization under cellular stress (Asada et al., 2007; Ju et al., 2014).

Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific kinases activated by extracellular stimuli that are key components if signalling pathways influencing FoxO1 activity. One of these kinases is p38 MAPK, which has been shown to become activated downstream of insulin via phosphorylation in bovine aortic ECs and HUVECs (G. Li et al., 2007; Madonna et al., 2004). Activated p38 phosphorylates FoxO1 at multiple residues under cellular stress causing nuclear import and greater transcriptional activity of FoxO1 (Asada et al., 2007; Dong et al., 2015).

Another post-translational modification that regulates FoxO1 is ubiquitination, which targets AKT-phosphorylated FoxO1 in the cytoplasm to the proteasome for degradation (Huang et al., 2005; Matsuzaki et al., 2003). One E3 ubiquitin ligase of interest is mouse double minute 2 (MDM2) which has been shown to ubiquitinate FoxO1 and FoxO3a proteins, promoting their degradation (Fu et al., 2009; Milkiewicz et al., 2011). MDM2 is canonically known for its role as the primary negative regular of p53 tumour suppressor protein by promoting its ubiquitination and degradation (Haupt et al., 1997). In turn, p53 transcriptionally induces MDM2 expression, creating a feedback

loop (X. Wu et al., 1993). Like FoxO1, p53 regulates cell cycle arrest and responses to DNA damage and stress; thus, MDM2 might serve as a general coordinator to suppress the levels of multiple negative growth regulators (Fu et al., 2009).

While multiple pathways have been implicated in the regulation of FoxO1, it is important to note that foundational knowledge of FoxO1 regulation has been derived largely from investigation of immortalized cell lines, such as HEK293 cells (Rena et al., 1999; Zhang et al., 2002; Zhao et al., 2004). Thus, the extent to which regulatory pathways apply to FoxO1 regulation in ECs remains uncertain and underscores the need to explore these mechanisms directly.

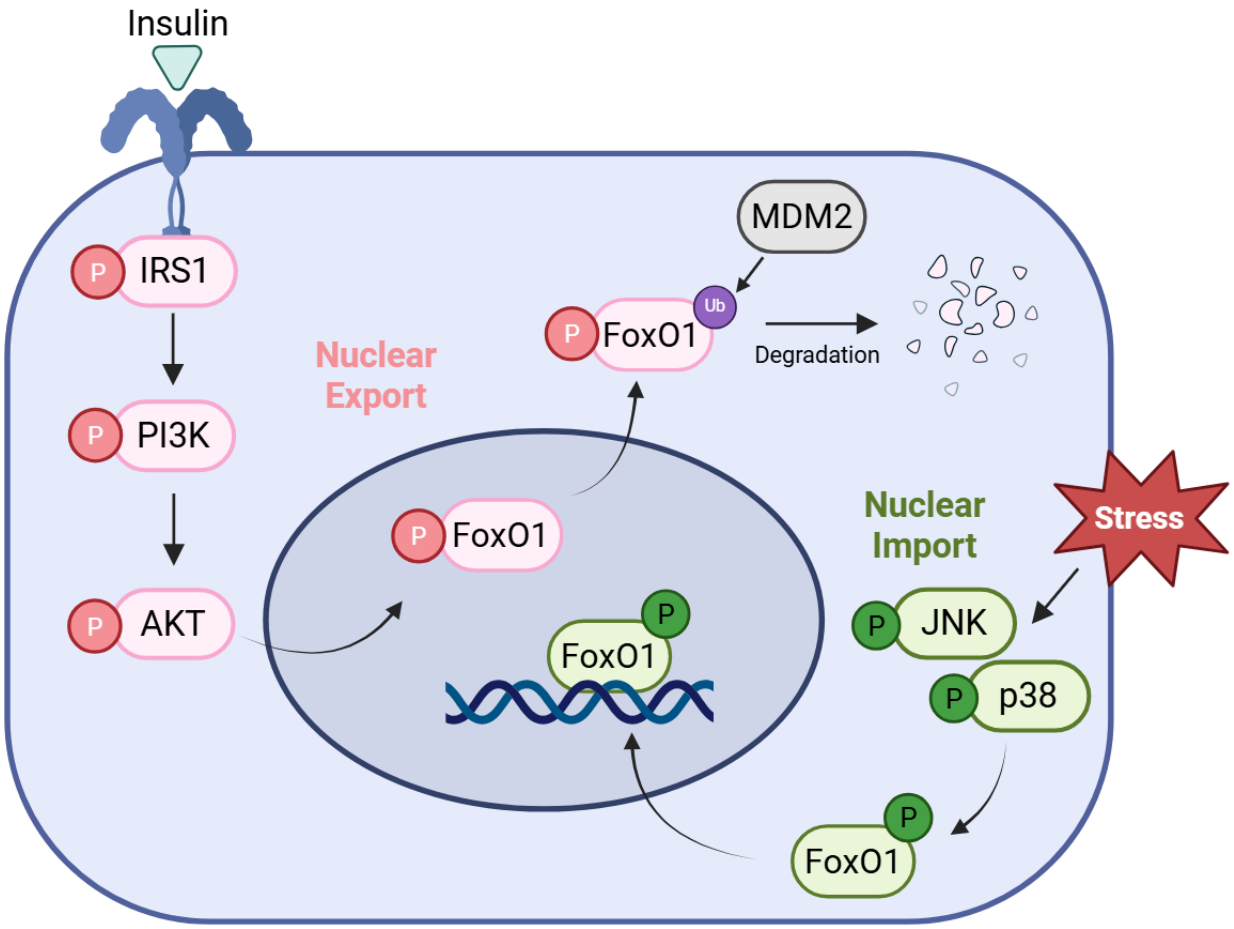

Figure 1.1: Schematic illustrating FoxO1 regulation and subcellular localization. Insulin receptor signalling activates the PI3K/AKT axis through IRS. Activated AKT translocates to the

nucleus where it phosphorylates FoxO1, promoting its nuclear export. In the cytoplasm, AKT-phosphorylated FoxO1 interacts with E3 ubiquitin ligases like MDM2 that ubiquitinate FoxO1 and target it for proteasomal degradation. In contrast, under cellular stress or the presence reactive oxygen species (ROS), JNK and p38 phosphorylate FoxO1 at distinct amino acid residues, promoting the nuclear import and transcriptional activity of FoxO1. Figure was created in BioRender.

1.10 Endothelial-specific FoxO1

In ECs, FoxO1 regulates vessel homeostasis and angiogenesis through its ability to promote EC quiescence and modulate EC metabolic activity. Overexpression of FoxO1 has been shown to keep ECs in a quiescent state by reducing glycolysis, oxidative phosphorylation and overall energy usage (Wilhelm et al., 2016). This is mediated through the suppression of MYC signalling, a potent driver of growth and metabolism, positioning FoxO1 as a key negative regulator of EC proliferation (Wilhelm et al., 2016). On the other hand, EC-specific FoxO1 depletion led to uncoordinated sprouting in the postnatal mouse retina, resulting in hyperplasia and vessel enlargement (Wilhelm et al., 2016). Interestingly, our lab found that EC-specific FoxO1 depletion in adult male mice on HFD improved vascular remodelling in the visceral adipose tissue with increased vascular density and vessel enlargement (Rudnicki et al., 2018). Discrepancies in the magnitude of the effects observed in these studies suggest that the developmental stage and tissue microenvironment contribute greatly to the phenotype caused by EC-specific FoxO1 depletion.

1.11 Sex differences in endothelial cells

Multiple studies have unveiled sex differences in EC transcriptomic profiles. For example, aortic ECs from male and female mice had 1695 differentially expressed genes (DEGs) based on RNA sequencing analysis (Shin et al., 2024). Compared to male ECs, female ECs had a higher expression of genes related to inflammation and apoptosis and lower expression of genes

associated with angiogenesis and oxidative phosphorylation. Another study by Hartman et al. (2020) showed that 14-25% of the EC transcriptome is sex biased. Specifically, in HUVECs isolated from male-female twins, 2,528 DEGs were found between sexes, 114 of which were found on the X or Y chromosome. Genes enriched in the female ECs were involved in endothelial activation pathways such as endothelial to mesenchymal transition, hypoxia and NFB-signaling. On the other hand, male ECs showed enrichment in Myc targets, oxidative phosphorylation and mTOR signalling. These findings suggested the presence of intrinsic sex differences in EC transcriptomes independent from the effect of environment (Hartman et al., 2020). In adult human aortic ECs, genes enriched in female ECs were involved in estrogen responses, while oxidative phosphorylation was enriched in male ECs, consistent with the young ECs (Hartman et al., 2020). Another study showed that female HUVECs had enriched expression for genes related to stress and immune response compared to male HUVECs (Lorenz et al., 2015). Additionally, our lab found that adipose ECs freshly isolated from HFD-fed male and female mice displayed sex-dimorphic transcriptomes, where 1281 genes were differentially expressed between the sexes (Rudnicki et al., 2023). Upregulated genes in males were mainly involved in inflammation and senescence-associated secretory phenotype, while genes upregulated in females were involved in pathways favouring angiogenesis such as proliferation, oxidative phosphorylation, and chromatin remodeling. Intriguingly, FoxO1 was a predicted upstream regulator of DEGs associated with ontologies for chromatin/transcription in female ECs, and FoxO1 mRNA and protein levels were also higher in the females (Rudnicki et al., 2023). Taken together, these studies highlight differences in EC gene expression programs that are context- and sex-dependent.

ECs also show sex differences in their functional properties. Previous work in our lab revealed that female mice on HFD had greater adipose angiogenesis and healthier adipose EC phenotype than

male mice on HFD (Rudnicki et al., 2018). Importantly, these sex differences persist even in cultured cells where isolated adipose ECs from female mice showed greater proliferation in vitro compared to male adipose ECs, suggesting that intrinsic properties contribute to this higher proliferative capacity (Rudnicki et al., 2023). In contrast, female aortic ECs showed decreased angiogenic potential compared to male aortic ECs, supporting that sex differences in ECs may be tissue-specific (Shin et al., 2024). Furthermore, several studies provide evidence of sexual dimorphism in human umbilical vein endothelial cells (HUVECs). Addis et al. (2014) reported that female HUVECs exhibited greater proliferation and migration. Similarly, Lorenz et al. (2015) found that female HUVECs had increased tube formation capacity compared to male cells, as well as higher cell viability after serum starvation. In line with these findings, Boscaro et al. (2020) found that HUVECs from female donors exhibit greater viability, growth rate, and migration compared to male HUVECs. Notably, these studies were done in the absence of sex hormones, suggesting intrinsic sex differences in EC functional capacities.

1.12 Factors contributing to sex differences in ECs

1.12.a Sex chromosomes

Fundamentally, males and females differ in their sex chromosomes (XX or XY) (Miller, 2010). In male cells, the Y chromosome encodes sex-specific genes that are essential for sex determination and development. The most well studied of these genes is sex-determining region Y (SRY), which is involved in the development of male testes and is associated with an increased risk of hypertension (Turner et al., 2009). In female cells, one of the two X chromosomes undergoes random X inactivation, where it is packaged into compacted heterochromatin structure to silence most, but not all, genes (Carrel & Willard, 2005). This process allows for dosage compensation of X-linked gene expression in females and contributes to female resilience in X-linked diseases (Z.

Sun et al., 2022). Notably, about 15% of genes were shown to escape X-inactivation in human fibroblasts, resulting in greater expression of these genes in females (Carrel & Willard, 2005). In mice, the number of X chromosomes within cells was shown to regulate adiposity and metabolism, with XX increasing adiposity relative to XY (X. Chen et al., 2012).

1.12.b Sex hormones

Another primary driver of sex differences in endothelial cells is the effect of sex hormones. Estradiol (E2) is a major form of estrogen, primarily produced by the granulosa cells in female ovaries and the Sertoli cells in the male testis (Boese et al., 2017). While estrogen is classically viewed as the main female sex hormone and testosterone the main male sex hormone, both hormones are present and influence reproductive health and sex characteristics in both sexes. Estrogen binds to two main forms of estrogen receptors (ER) on the plasma membrane, ERα and ERβ. Upon ligand activation, ERα/β form homo- or hetero- dimers that directly bind to DNA in the nucleus and stimulate transcription of target genes (Cowley et al., 1997). In addition, estrogen signalling exerts rapid, non-genomic effects through direct interaction with signalling kinases (Z. Chen et al., 1999; Haynes et al., 2000; Stirone et al., 2005).

E2 is well known to have a vasoprotective role (Epstein et al., 1999). ER engagement promotes vasodilation by upregulating eNOS mRNA and protein expression, while also stimulating the PI3K-AKT axis to stimulate eNOS activity and NO production (Haynes et al., 2000; Hishikawa et al., 1995). Estrogen also promotes vasodilation by the downregulation of the vasoconstrictor ET-1 (Tan et al., 2003). Additionally, multiple studies have demonstrated that E2 promotes EC proliferation. E2 treatment in HUVECs led to increased migration, tube formation capacity, and proliferation, which were blocked by treatment with an E2 antagonist (Morales et al., 1995). ER knockout (KO) in female mice resulted in reduced cerebral capillary density, supporting the role

of estrogen signalling in angiogenesis (Jesmin et al., 2010). This effect occurred through a VEGF/eNOS/AKT- dependent mechanism, where the protein and activation levels of these signalling molecules were decreased in ER KO mice.

Studies on the effects of progesterone, another female sex hormone, on EC function are more limited compared to E2. Progesterone was shown to promote EC migration in HUVECs by activating the actin-binding protein moesin involved in cell motility (Zheng et al., 2012). However, a different group found conflicting results where progesterone had an inhibitory effect on migration of HUVECs (T.-S. Lee et al., 2015). Furthermore, physiological levels of progesterone suppressed EC proliferation through a nuclear-receptor mediated mechanism in multiple EC types including human coronary ECs, bovine aortic ECs, and human endometrial ECs (Vázquez et al., 1999). This inhibitory effect was prevented by treatment with a progesterone receptor antagonist, supporting a progesterone receptor dependent pathway (Vázquez et al., 1999). This finding was corroborated by (Hsu et al., 2008) who found that progesterone inhibits proliferation in HUVECs through a p53-dependent mechanism. From these studies, it becomes clear that the two female sex hormones, E2 and progesterone, exert different effects on EC function.

Testosterone, classically viewed as the male sex hormone, is mainly produced by the testicular Leydig cells and ovarian theca cells (Boese et al., 2017). It can be converted into the more potent form DHT, by the enzyme 5α-reductase (Gloyna & Wilson, 1969). Testosterone may also be converted into E2 by the enzyme aromatase, thus serving as a key intermediate in estrogen synthesis (Simpson et al., 2002; Weisz & Gibbs, 1974).

The effects of testosterone signalling on ECs are less clear than those of estrogen signaling, with many studies reporting conflicting findings. For example, DHT and testosterone were shown to stimulate proliferation in human aortic ECs by inducing the expression of androgen receptor (AR),

VEGF-A, and cyclins (Cai et al., 2011). As well, another study showed that DHT improved angiogenesis in vitro with male HUVECs and in vivo in male mice through an AR-mediated mechanism, but this effect was not seen in the female ECs, partially due to their lower expression of AR (Sieveking et al., 2010). In contrast, one study found that treatment with AR or DHT inhibited EC proliferation in both HUVECs and human microvascular ECs (HMEC-1) (Huo et al., 2019). This was supported by another group who reported that testosterone treatment reduced proliferation and migration in HUVECs, regardless of the ratio of male to female cells in the pools (Gaba et al., 2018). These contradictory results suggest that effects of androgens are highly dependent on the cellular context, and that other regulatory mechanisms likely modulate the response in different experimental models, even within the same cell models (Converse & Thomas, 2021).

1.13 Sex differences in endothelial FoxO1

Our lab reported that female ECs have greater FoxO1 mRNA and protein levels than male ECs (Rudnicki et al., 2023). This finding highlights a paradox where higher FoxO1 levels are associated with greater proliferation in female ECs, despite FoxO1 often being recognized for its role in maintaining EC quiescence (Wilhelm et al., 2016). Furthermore, recent work in our lab has demonstrated the sex-distinct role of FoxO1 in regulating EC biology. It was found that adult female mice with endothelial-specific FoxO1 deletion (EC-FoxO1 KD) fed a HFD had decreased angiogenesis in their adipose tissue, leading to lower capillary density and vascular malformations (Pislaru, 2025). This was accompanied by adipocyte hypertrophy, increased weight gain, and impaired systemic glucose homeostasis. These findings contrast with the positive outcomes previously observed in male mice with EC-FoxO1 KD fed a HFD, which exhibited reduced weight gain, smaller adipocyte size, and increased capillary number (Rudnicki et al., 2018). Furthermore,

the negative impacts of endothelial FoxO1 loss in females persist in culture, as female ECs subjected to sustained inhibition or knockdown of FoxO1 experienced greater mitochondrial dysfunction, DNA damage, and increased senescence, which were not observed in male ECs (Pislaru, 2025). These data suggest that there is a sex-specific dependence on FoxO1 and that, despite being classically viewed as an anti-proliferative factor, FoxO1 is indispensable for maintaining cellular homeostasis in female ECs.

Notably, gene set enrichment analysis (GSEA) of DEGs from mice with EC-FoxO1 KD revealed that genes involved in nucleosome organization were significantly repressed in female adipose ECs but not affected in male ECs. Some genes of interest from this dataset that showed significant downregulation in the female ECs, include *Nap1l3, Nap1l5, Asf1b,* and *H3-3a*. The nucleosome assembly proteins (NAP) family of histone chaperone proteins help assemble and bring nucleosome complexes to the chromatin after DNA replication or DNA repair (Park & Luger, 2006). Specifically, NAP1 facilitates the deposition and transport of H2A/H2B necessary for chromatin assembly (Chang et al., 1997; Mosammaparast et al., 2002). Although NAP1-like proteins are less characterized members of the NAP family and their functions are not completely understood, there is some evidence of their roles in mediating histone assembly and chromatin organization (Park & Luger, 2006). For example, downregulation of NAP1l3 in umbilical cord blood haematopoietic stem cells (HSCs) was shown to impair the maintenance and proliferation of HSCs both in vitro and in vivo, and its ability to bind to histones was demonstrated using sucrose gradient sedimentation and enzyme-linked immunosorbent assay (Heshmati et al., 2018; Shen et al., 2001). Asf1b, another histone chaperone, was shown to be important for S-phase cycle progression and promote cell proliferation through its interaction with H3/H4 histones (Paul et al., 2016; Sanematsu et al., 2006; Schulz & Tyler, 2006). Asf1a is its related isoform that also

associates with H3/H4 complexes. However, Asf1a shows a preferential interaction with the histone chaperone histone regulator A (HIRA) complex in contrast to Asf1b which preferentially interacts with the chromatin assembly factor-1 (CAF-1), illustrating how histone chaperones have specific interactions with binding partners to mediate isoform specific effects (Abascal et al., 2013; Tagami et al., 2004). Finally, *H3-3a* encodes the replication-independent histone variant H3.3 that is expressed throughout the cell cycle (Seal et al., 2022). Intriguingly, in addition to its replication-independent roles during transcription and DNA repair, H3.3 can also play a role replication-dependent role as it was shown that H3.3 and its deposition by ASF1b promote β-cell proliferation (Paul et al., 2016). Overall, by assessing the levels of these nucleosome organization genes in cultured male and female endothelial cells with FoxO1 inhibition, this will provide insights into specific factors that may be contributing to the detrimental impacts of FoxO1 depletion on female ECs.

1.14 The insulin signalling pathway

Beyond its impacts on FoxO1 activity, insulin plays a broader role in cells by regulating cell metabolism and survival. Under fed states, insulin signalling activates two primary signalling pathways: PI3K/AKT and MAPK extracellular signal-regulated kinase (ERK). When insulin binds to its receptor tyrosine kinase on the cell membrane, this leads to the phosphorylation of insulin receptor substrates (IRS proteins) that activate downstream signalling pathways (X. J. Sun et al., 1991). PI3K activation by IRS initiates a signalling cascade that activates AKT (Alessi, 2001). AKT becomes activated via phosphorylation at Thr308 and Ser473, which is mediated by PDK1 (3-phosphoinositide-dependent protein kinase-1) and mTORC (mechanistic Target of Rapamycin Complex), respectively (Alessi et al., 1996, 1997; Sarbassov et al., 2005). Phosphorylation at both residues are required for full activation of AKT (Alessi et al., 1996). Activated AKT is a key

effector of the metabolic effects of insulin signalling as it promotes glucose uptake by stimulating the translocation of the insulin-responsive glucose transporter 4 (GLUT4) to the plasma membrane as well as the synthesis of glucose transporter 1 (GLUT1) (Kohn et al., 1996). AKT also promotes glycogen synthesis through its inhibition of glycogen synthase kinase 3 (GSK3) (Ueki et al., 1998). Another major pathway activated by insulin signalling is the MAPK/ERK pathway. Upon insulin binding, various adaptor proteins are recruited that activate Ras GTPase, which subsequently activates the protein kinase cascade consisting of Raf, MEK, and ERK (Boulton et al., 1991; Pearson et al., 2001). Activated ERK translocates to the nucleus where it phosphorylates Elk-1, c-MYC and other targets, stabilizing their transcriptional activity (Marais et al., 1993; Sears et al., 2000). While AKT and ERK signalling are distinct effectors of insulin signalling, they share overlapping pathways that overall regulate cell metabolism, growth, proliferation, and survival (Mendoza et al., 2011).

1.15 Sex differences in insulin sensitivity

Given the role of insulin signalling in regulating FoxO1, it is important to consider how insulin sensitivity in ECs may affect FoxO1 activity. Current knowledge in the field presents conflicting results surrounding sex differences in insulin sensitivity. For example, one study done on male and female mice revealed greater insulin sensitivity in female perigonadal adipocytes based on higher levels of AKT and ERK phosphorylation after insulin stimulation (Macotela et al., 2009). In contrast, a study done on diabetic male and female Wistar rats showed that insulin sensitivity was lower in females based on whole body parameters, such as blood glucose and serum insulin concentrations. Human studies on insulin sensitivity have largely shown that compared to age-matched males, premenopausal women have higher whole-body insulin sensitivity, as well as higher tissue-specific insulin sensitivity in skeletal muscle and the liver (Goossens et al., 2021).

These conflicting results, along with the absence of studies directly assessing insulin sensitivity in male and female ECs, emphasize the need to explore insulin signalling in adipose ECs.

1.16 Unanswered questions

The findings that female ECs have higher endothelial FoxO1 levels and are affected by FoxO1 depletion differently than males suggest that there may be sex-distinct control of FoxO1 protein (i.e. post-translational modifications or localization) as well as sex-specific functions of endothelial FoxO1. To explore these mechanisms and uncover potential sex differences in FoxO1 regulation, my thesis will investigate pathways that are implicated in FoxO1 regulation, including insulin signalling, MAPK, and E3 ubiquitin ligase MDM2. While the role of insulin in regulating FoxO1 is well-established, conflicting results on sex differences in insulin sensitivity highlight the need to explore whether sex differences in insulin signalling impact FoxO1 levels in adipose ECs. Furthermore, previous work in our lab showed that p38 inhibition increased proliferation in both male and female ECs and that activated p-p38 levels were higher in females (Rezvan, 2020). Therefore, assessing p38 phosphorylation after insulin stimulation in male and female ECs will provide insight into whether sex-differences in p38 activation influence FoxO1 post-translational modifications and subcellular localization. As well, previous work in our lab demonstrated that FoxO1 is ubiquitinated by MDM2 in skeletal muscle ECs . Therefore, evaluating MDM2 levels in male and female ECs may uncover a sex-distinct role of MDM2 in regulating FoxO1. The potential role of FoxO1 as a pioneer factor also raises the question of whether FoxO1 impacts chromatin accessibility in adipose ECs and whether this occurs in a sex-specific manner. My study will explore this question further by analyzing the impact of FoxO1 on chromatin accessibility, as well as its impact on active regulatory regions and gene expression in male and female ECs.

1.17 Hypotheses

I hypothesize that distinct roles of FoxO1 may be caused by differences in upstream regulators, such as insulin signaling, that alter protein localization or degradation. In addition, I hypothesize that FoxO1 has sex-distinct impacts on the epigenome that contribute to differences in gene expression.

1.18 Objectives

Objective 1: Investigate sex differences in upstream regulators of FoxO1 in ECs.

Microvascular ECs from the perigonadal white adipose of male and female mice will be serum starved followed by insulin stimulation. Total protein and phosphorylation levels of AKT will be measured by western blot to assess insulin signalling activity. Since AKT phosphorylation is a key downstream event in the insulin signalling pathway, its activation via phosphorylation reflects the ability of cells to respond to insulin stimulation. In addition, phosphorylated and total p38 MAPK along with total MDM2 protein levels will be measured in male and female ECs to determine whether sex differences in these regulators contribute to differences in FoxO1 protein levels. These data will provide valuable insights into whether FoxO1 regulation in adipose ECs is impacted by sex differences in upstream regulators.

Objective 2: Investigate potential sex differences in FoxO1 sub-cellular localization in adipose ECs under basal or insulin stimulated conditions.

Microvascular ECs isolated from the perigonadal white adipose of male and female mice will be serum starved followed by insulin stimulation. Total protein levels and phosphorylation of FoxO1 (Ser256) in the nuclear and cytoplasmic compartments, as well as the chromatin-associated fraction, will be measured by western blot to reveal potential sex differences in subcellular localization and insulin-dependent post-translational regulation of FoxO1. Immunostaining on

cultured ECs will also be performed to visualize the subcellular localization of pSer256-FoxO1 and FoxO1 in vitro. This information will provide clues about possible explanations for why FoxO1 protein levels are higher in female ECs (Milkiewicz et al., 2011; Rudnicki et al., 2023). For example, it may be that FoxO1 escapes phosphorylation by AKT or gets phosphorylated in the nucleus but is prevented from being exported into the cytoplasm where it can be ubiquitinated and degraded.

Objective 3: Identify sex differences in FoxO1 regulatory regions by assessing genome-wide chromatin accessibility, active regulatory regions, and transcriptomes in cultured adipose ECs.

To explore the transcriptional role of FoxO1 in male and female ECs, an assay for transposase accessible chromatin (ATAC) followed by next-gen sequencing will be performed on cultured adipose ECs treated with or without FoxO1 inhibition to establish FoxO1 regulated genome-wide chromatin accessibility. FoxO1 inhibition with AS1842856 has been shown to prevent transcriptional activity of FoxO1 by directly binding to the active, unphosphorylated form and preventing FoxO1 transactivation (Nagashima et al., 2010). Therefore, FoxO1 inhibition will be used as a tool to examine the impact of loss of FoxO1 transcriptional activity compared to control cells. This experimental design will elucidate if there are any basal or FoxO1-dependent sex differences in the chromatin landscape in adipose ECs. To evaluate the epigenetic modifications that influence chromatin accessibility, chromatin immunoprecipitation (ChIP) sequencing will be performed for H3K27ac. This is used to identify active enhancer and promoter regions, providing insight into which areas of the chromatin are primed for transcription and whether inhibiting FoxO1 will alter these regions. Finally, RNA-seq will be performed to determine which genes are actively being transcribed and their dependence on FoxO1. In sum, these data will provide insight into the function of endothelial FoxO1 and its sex-specific effects on transcriptional regulation.

Chapter 2: Methods

2.1 Adipose endothelial cell isolation and cell culture

Subcutaneous and perigonadal white adipose depots were collected from normal chow FVB mice that were bred in-house at York University. The fat depots were pooled and digested with 0.5% Type I collagenase (#LS004197, Worthington Biochemical, USA) in high-glucose (4.5g/L) DMEM (319-030 CL, Wisent, Canada) for 30 min at 37ºC with shaking. Centrifugation (4000 rpm for 5 min) was used to separate adipocytes from the stromal vascular fraction (SVF). Floating adipocytes and lipid layer were discarded, and the re-suspended SVF was passed through a cell strainer (100µm). ECs were purified using 25µg of biotinylated rat anti-mouse CD31 (#553371, BD Pharmingen™) incubated at 4ºC for 15 min, followed by incubation with 50µL streptavidin-magnetic beads (#557812, BD IMag™) and 25µL of rat anti-mouse VE-cadherin antibody-coated Dynabeads (#138001, BioLegend; #14311D, Thermo Fisher Scientific) at 4ºC for 30 min. ECs were cultured in gelatin-coated T25 flasks with high-glucose (4.5g/L) DMEM (319-030 CL, Wisent, Canada) supplemented with 20% fetal bovine serum (FBS, 090-150, Wisent, Canada), Penicillin-Streptomycin (100 units/mL penicillin and 100 µg/mL of streptomycin, #15140122, Gibco, USA), GlutaMAX™ L-glutamine (2 mM, #35050061, Gibco, USA), and Sodium Pyruvate (1 mM, #11360070, Gibco, USA), until plating for experiments. Male and female ECs were passage-matched for experiments and used between passages 2-5.

2.2 Treatment and whole cell protein extraction of adipose endothelial cells

Adipose ECs from the subcutaneous and perigonadal white adipose of male and female mice were plated into gelatin-coated 6-well plates 24h prior to whole cell protein extraction. For insulin stimulation experiments, each well was serum starved for 2h followed by treatment with insulin

(25µg/mL) for 0-, 10-, or 30-min. For FoxO1 inhibitor experiments, cells were treated with FoxO1 inhibitor (AS1842856, 1µM) for 1 or 3 days, as indicated. For siRNA experiments, cells were transfected with Dharmacon non-targeting siRNA (5nM) or FoxO1 siRNA (5nM) using DharmaFECT transfection reagent for 3 days. For PI3K inhibition experiments, cells were first serum-starved for 2h, then treated with the PI3K inhibitor (LY-294002, 10µM) for 0- or 60- min. For PKA inhibition, cells were first serum-starved for 2h, then treated with the PKA inhibitor, protein kinase inhibitor (PKI) 14-22 amide, myristoylated (10µM; #2546, Tocris), followed by 0- or 30- min insulin treatment (25µg/mL). Whole cell lysates were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer containing 50 mM Tris-Base, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1X phosphatase inhibitor (PhosSTOP™) and 1X protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, Sigma Aldrich, USA).

2.3 Subcellular fractionation

The nuclear and cytoplasmic compartments of adipose ECs were separated using the "Nuclear and Cytoplasmic Extract" protocol by Rockland Immunochemicals (https://www.rockland.com) with modifications. Briefly, cells are collected and lysed in cytoplasmic extract (CE) buffer (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% (v/v) NP-40, 1mM DTT and 1 mM PMSF), followed by centrifugation and wash steps. The nuclear pellet is solubilized with nuclear extract (NE) buffer (20 mM Tris Cl, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM PMSF and 25% (v/v) glycerol), and a final centrifugation at 17,500g for 10 min at 4ºC yields the purified fractions. Before performing subcellular fractionations on ECs, careful optimization of the protocol was carried out using the 10T1/2 cells, an immortalized cell line of mouse embryonic fibroblast cells. Several modifications to the protocol were tested as follows: using either 0.05% (v/v) NP-40 or 0.075% (v/v) NP-40 in the CE buffer (10 mM HEPES, 60 mM KCl, 1 mM EDTA, NP-40, 1mM

DTT and 1 mM PMSF, adjusted to pH 7.6); washing the nuclear pellet with CE-buffer containing or lacking NP-40; and, adding an additional sonication step of the nuclear fraction. To test these modifications, 10T1/2 cells were cultured in high glucose media (10% FBS) and plated 24h before undergoing fractionation. The effectiveness of the protocol was evaluated through western blot of RCC1, GAPDH and H3 as loading controls for the nuclear, cytoplasmic and chromatin-bound fractions, respectively.

The following optimized protocol was employed in all subcellular fractionation experiments with ECs: first, the cell pellet was resuspended with CE buffer containing NP-40 (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% (v/v) NP-40, 1mM DTT and 1 mM PMSF, adjusted to pH 7.6) and centrifuged at 1500rpm for 4 min at 4ºC. This yielded the cytoplasmic fraction in the supernatant, leaving the nuclear pellet behind to be washed with CE buffer containing NP-40 and solubilized with nuclear extract (NE) buffer (20 mM Tris Cl, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM PMSF and 25% (v/v) glycerol, adjusted to pH 8.0). This solution was then subjected to sonication for 5 min (30s on/30s off on high) to release chromatin-bound FoxO1. A final centrifugation of both the cytoplasmic and nuclear fractions at 17,500g for 10 min at 4ºC yielded the purified fractions in the supernatants.

2.4 Western blot analysis

The phosphorylation states of AKT, FoxO1, and p38, along with their total protein levels, were assessed through western blot. The following primary and secondary antibodies were used: anti-Ser473-pAKT (#4058, Cell Signaling), anti-AKT (#9272, Cell Signaling), anti-Ser256-pFoxO1 (#9461, Cell Signaling; ab131339, Abcam), anti-FoxO1 (#2880, Cell Signaling; ab52857, Abcam), anti-Thr180/Tyr182-pp38 (#9211, Cell Signaling), anti-p38 (#9212, Cell Signaling), and goat anti-rabbit IgG peroxidase-conjugated (#111-035-003; Jackson ImmunoResearch). For subcellular

fractionation experiments, the following primary antibodies served as the loading controls: anti-RCC1 (#3589, Cell Signaling) for the nuclear fraction, anti-GAPDH (#5174, Cell Signaling) for the cytoplasmic fraction, anti-TOM20 (#42406, Cell Signaling) as a mitochondrial marker, and anti-H3 (#9717, Cell Signaling) to confirm the extraction of chromatin-bound proteins. Membranes were developed using enhanced chemiluminescence (SuperSignal™ West Pico PLUS Chemiluminescent Substrate ECL; Thermo Fisher Scientific), and images were detected on the Invitrogen iBright 1500 Imaging System (#A44114, Thermo Fisher Scientific) and quantified using densitometry in ImageJ.

2.5 Immunostaining of cultured endothelial cells

Isolated and cultured adipose ECs from the subcutaneous and perigonadal white adipose of male and female mice were plated onto 8-well chamber slides and incubated overnight to allow cells to attach. Each well was serum starved for 3 hours, then treated with insulin (25µg/mL) for 0, 30, or 60 minutes. Cells were then fixed prior to incubation with rabbit anti-FoxO1 (1:75) or rabbit anti-pSer256-FoxO1 (1:75), followed by Cy3-conjugated goat anti-rabbit secondary antibody (1:200). Samples were counterstained with DAPI (1:1000) to visualize nuclei and imaged using a Nikon Eclipse Ti2-E inverted microscope at 40X or 60X magnification, as indicated in the figure legends. Immunofluorescent images were quantified after using the deconvolution function in the NIS-elements software (version 6.10.02). This function improves the signal-to-noise ratio by removing background and reducing blur, which allowed for nuclear regions to be more accurately thresholded during quantification and showed a clearer depiction of individual puncta of FoxO1 signal within the cells. Importantly, deconvolution parameters were kept constant for each image. Nuclear and cytoplasmic signals were quantified in ImageJ by thresholding for the nuclear region with DAPI and calculating mean fluorescence intensity of each region for the cells in view. 4 fields

of view were imaged per biological replicate with 3-7 cells per field of view.

2.6 RT-qPCR

Subcutaneous and visceral adipose ECs were extracted and purified from age-matched male and female FVB mice (3 months old, n=5 per sex). Approximately 25,000 cells (passage 2) were plated onto gelatin-coated 48-well plates. The next day, cells were treated with vehicle (DMSO) or FoxO1 inhibitor (1μM) for 3 days. RNA was extracted using RNeasy Mini Kits (Qiagen Inc.), and RNA quality was assessed using the RNA 6000 Nano chip on the 2100 Bioanalyzer automated electrophoresis system (Agilent Technologies Inc.). RNA was reversed transcribed using M-MLV reverse transcriptase. cDNA was analyzed by real-time quantitative PCR on the Rotor-Gene Q platform (Qiagen Inc.) using TaqMan Master Mix (#ABMG890, Diamed) and Taqman primer sets for *Nap1l3* (Mm01214875_s1), *Nap1l5* (Mm07297729_s1), *H3f3a* (Mm01612808_g1), *Asf1b* (Mm00508243_m1) and *Tbp* (Mm00446973_m1). Expression levels of target genes were calculated relative to the housekeeping gene, *Tbp*, and expressed as $2^{-\Delta Ct}$.

2.7 Statistical Analysis

Results are reported from independent biological replicates. For whole cell extraction, subcellular fractionation, and immunostaining experiments, each independently treated batch of cells was considered one biological replicate. The cells used in these experiments were pooled from multiple mice for each sex. For ATAC, ChIP, and RNA experiments, each set of cells derived from each mouse was considered one biological replicate. Results are presented as mean ± standard error of the mean (SEM). The effects of insulin stimulation and FoxO1 inhibition on male and females ECs were analyzed via two-way ANOVA (Sex × Treatment), followed by Sidak's post hoc tests using Prism 8 (GraphPad software Inc; La Jolla, CA, USA). For all statistical tests, $p < 0.05$ was defined as statistically significant.

Chapter 3: Results

3.1 Male and female adipose ECs exhibit similar responses to insulin signalling

AKT is a key effector of the insulin signalling pathway as it plays an important role in stimulating glucose uptake and glycogen synthesis (Kohn et al., 1996; Ueki et al., 1998). Activation of AKT downstream of insulin signalling involves its phosphorylation at Thr308 and Ser473 by PDK1 and mTORC, respectively (Alessi et al., 1996, 1997; Sarbassov et al., 2005). To compare the insulin signalling responses of male and female serum-starved ECs, I assessed AKT phosphorylation at Ser473 in response to insulin. For both sexes, pSer473-AKT levels were minimal under serum-starved conditions, whereas insulin stimulation at both 10- and 30- min time points promoted AKT phosphorylation (**Fig. 3.1A**). No significant differences in responses were found between males and females (**Fig. 3.1B**).

I next evaluated the AKT-FoxO1 axis by measuring FoxO1 phosphorylation at Ser256 in response to insulin (**Fig. 3.1C**). In line with the activation of AKT, insulin promoted the phosphorylation of FoxO1 in a time-dependent manner, with the greatest levels of FoxO1 phosphorylation being observed at the 30-minute time point. Based on these findings, insulin treatments in subsequent experiments involving FoxO1 were performed for 30 minutes. Importantly, no significant sex differences in pSer256-FoxO1 levels were found between any time points (**Fig. 3.1D**). These results suggest that male and female adipose ECs have similar regulation of FoxO1 by AKT phosphorylation.

Another kinase that is activated downstream of insulin receptor and has been shown to regulate FoxO1 via phosphorylation is p38 MAPK (Asada et al., 2007; G. Li et al., 2007). To explore the acute effect of insulin on p-38 phosphorylation, pThr180/Tyr182-p38 levels were measured in

male and female ECs. In these experiments, insulin had a significant main effect, with increased pThr180/Tyr182-p38 levels upon insulin stimulation (**Fig. 3.1E,F**). As well, female ECs had greater pThr180/Tyr182-p38 levels than males under both control and insulin-stimulated conditions, consistent with previous work in our lab (Rezvan, 2020). Given that p38 can phosphorylate FoxO1 and promote its nuclear localization and transcriptional activity, higher levels of pThr180/Tyr182-p38 in females may contribute to greater FoxO1 nuclear localization in female ECs, despite there being no sex differences in AKT activation or phosphorylation at Ser256. To verify this, it would be interesting to assess whether p38-mediated phosphorylation of FoxO1 increases in line with higher pThr180/Tyr182-p38 levels. However, since there are currently no commercially available antibodies for p38 FoxO1 phosphorylation sites, I was unable to pursue this investigation further.

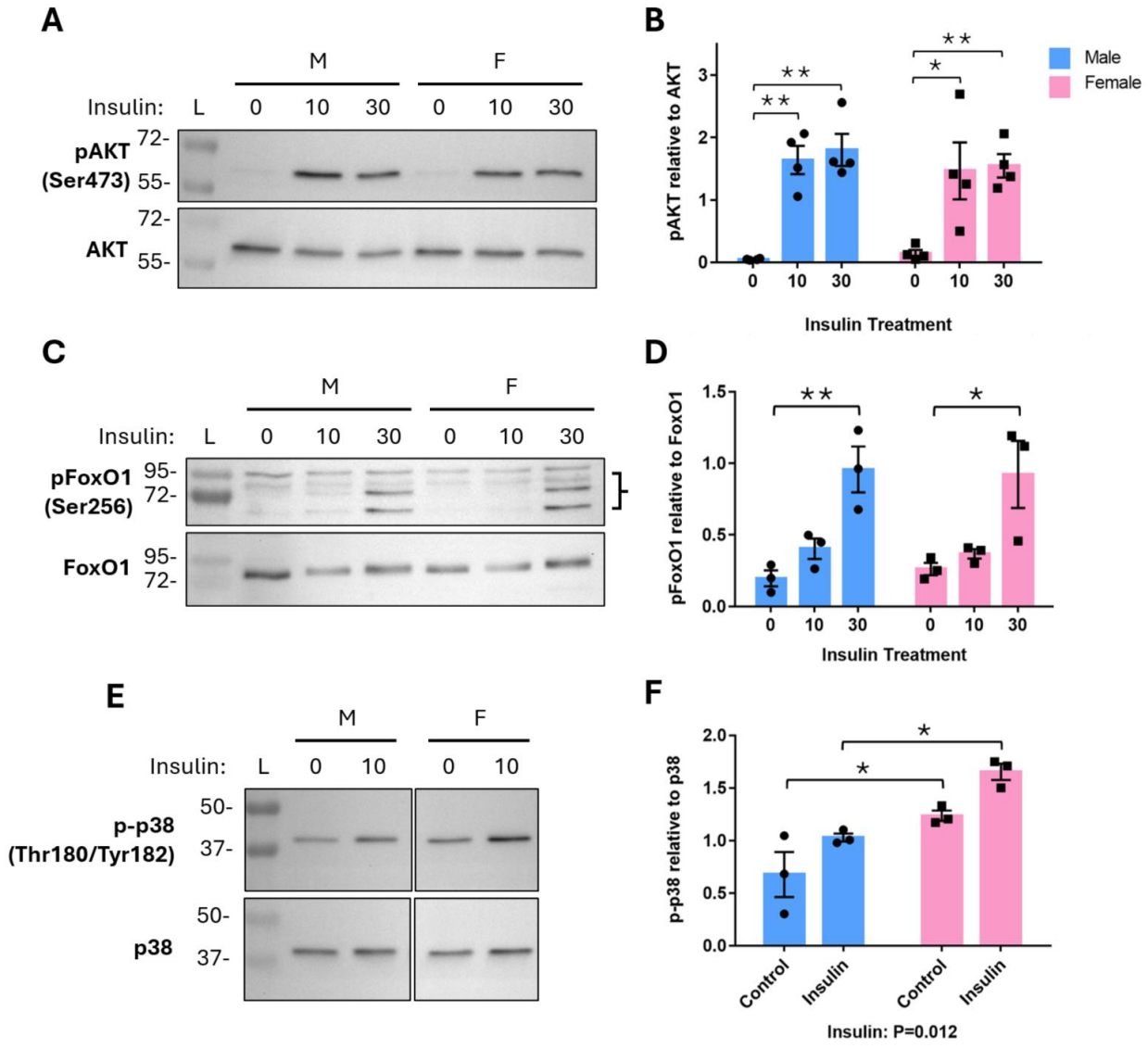

Figure 3.1: Effects of insulin signalling on the phosphorylation of AKT, FoxO1 and p38 MAPK in male and female adipose ECs. Male and female adipose ECs were serum-starved for 2h followed by 0-, 10-, or 30-min of insulin stimulation (25µg/mL) prior to whole cell protein extraction. **A)** Representative western blot of phosphorylated (Ser473) and total AKT levels in male and female adipose ECs. **B)** pAKT levels relative to total AKT, n=4. **C)** Representative western blot of phosphorylated (Ser256) and total FoxO1 levels in male and female adipose ECs. Bracket indicates bands used to quantify pSer256-FoxO1 levels. **D)** pSer256-FoxO1 levels relative to total FoxO1, n=3. **E)** Representative western blot of phosphorylated (Thr180/Tyr182) and total p38 MAPK in male and female adipose ECs. Separation in blots reflects manual exclusion of unrelated samples. Male and female blots for each protein were processed and exposed at the same time. **F)** pThr180/Tyr182-p38 MAPK levels relative to total protein, n=3. In each graph, individual n values are plotted, with bars illustrating the mean ± SEM. Statistical significance was assessed using two-way ANOVA with Sidak's post-hoc test where appropriate. *P < 0.05, **P < 0.01.

3.2 MDM2 levels are higher in female ECs

FoxO1 protein can also be regulated by ubiquitin-mediated degradation (Fu et al., 2009; Milkiewicz et al., 2011). To assess the contribution of the E3 ubiquitin ligase MDM2 on sex differences in FoxO1 regulation, I first measured MDM2 levels in serum-starved male and female ECs. The results revealed that female ECs had approximately 45% higher MDM2 levels compared to male ECs (**Fig. 3.2A,C**). This finding was unexpected since female ECs have greater FoxO1 protein levels than males (Pislaru, 2025), and higher levels of MDM2 should correlate with lower FoxO1 protein given the role of MDM2 in FoxO1 ubiquitination and degradation. With that said, this finding may be consistent with the literature showing that FoxO1 transcriptionally induces MDM2 expression in MCF7 cancer cells (Tomiyasu et al., 2024). Thus, I assessed MDM2 levels in serum-starved ECs with or without short-term (1-day) FoxO1 inhibitor treatment. Consistently, serum-starved female ECs showed greater levels of MDM2 compared to serum-starved male ECs (**Fig. 3.2B,D**). Intriguingly, FoxO1 inhibition resulted in a significant increase in MDM2 levels in the male ECs, while no significant changes were seen in the females. These data go against the idea that FoxO1 promotes MDM2 transcription and rather suggest that MDM2 may not be a direct transcriptional target of FoxO1 in ECs.

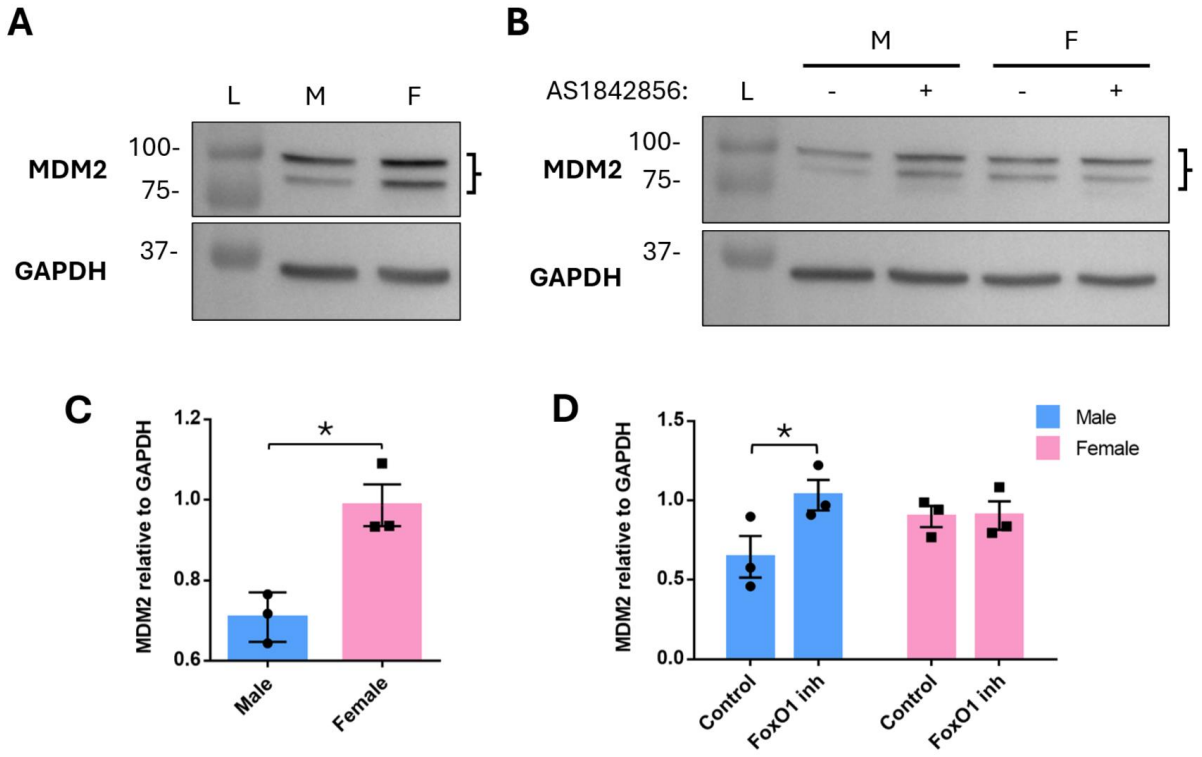

Figure 3.2: MDM2 levels are higher in female ECs and increase in male ECs with pharmacological inhibition of FoxO1. Representative western blots of MDM2 levels in whole cell extracts from male and female adipose ECs **A)** serum-starved (3h) and **B)** serum-starved (2h) with or without FoxO1 inhibitor AS1842856 treatment (1μM, 24h). GAPDH served as a loading control. Brackets indicate bands used to quantify MDM2 levels **C)** MDM2 levels in A) relative to GAPDH levels, n=3. **D)** MDM2 levels in B) relative to GAPDH levels, n=3. Individual n values are plotted, with bars illustrating the mean ± SEM. Statistical significance was assessed by unpaired student t-test or two-way ANOVA with Sidak's post-hoc test where appropriate. *P < 0.05.

3.3 Investigating FoxO1 subcellular localization in male and female ECs

Since FoxO1 is transcriptionally active in the nucleus, its subcellular localization directly affects its functional capabilities. Thus, to assess potential sex differences in the subcellular localization of FoxO1, the following series of experiments investigated FoxO1 localization patterns under serum-starved conditions, where FoxO1 should be localized to the nucleus, and insulin-stimulated conditions, where FoxO1 should be sequestered in the cytoplasm. This was explored through two methodological avenues: subcellular fractionation of cultured ECs and immunostaining on fixed ECs.

3.3.a Optimization of the subcellular fractionation protocol

To optimize the protocol for subcellar fractionation, ensuring clean separation of the nuclear and cytoplasmic compartments, I conducted a series of tests using an immortalized cell line (10T1/2 cells). I first assessed the effects of the concentration of NP-40 (Nonidet P-40) in the cytoplasmic buffer. NP-40 is a mild, non-ionic detergent that disrupts the plasma membrane and allows for the release of cytoplasmic proteins while leaving nuclei intact (Senichkin et al., 2021). After testing the protocol with 0.05% NP-40 in the cytoplasmic extract (CE) buffer, there was evident contamination of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the nuclear fraction (**Fig. 3.3**). GAPDH is often used as housekeeping protein of the cytoplasm as it is an enzyme that catalyzes the conversion of G3P into 1,3-biphosphoglycerate during glycolysis (Tristan et al., 2011). Notably, increasing the concentration of NP-40 to 0.075% lowered GAPDH levels in the nuclear fraction, but not sufficiently. Washing without NP-40 in the CE buffer resulted in cytoplasmic contamination in the nuclear fraction, regardless of concentration. In contrast, the cleanest separation was seen in samples that were washed with CE buffer containing NP-40 in either concentration, which resulted in negligible GAPDH signal in the nuclear fraction, thus

resolving the issue with cytoplasmic contamination. Lastly, to ensure that chromatin-bound proteins would be successfully released into the nuclear fraction, an additional sonication step was tested to see if it would increase the yield of chromatin-bound proteins in the nuclear fraction. Notably, higher levels of histone 3 (H3) were observed in nuclear fractions where sonication was performed. These results suggest that sonication enhances the accessibility of chromatin-bound proteins within the nuclear fraction, which will allow for a more accurate assessment of nuclear FoxO1 levels in adipose ECs. For all the protocols tested, regulator of chromosome condensation 1 (RCC1) was only detected in the nuclear fractions, suggesting no nuclear contamination in the cytoplasmic fraction. Taken together, the protocol using 0.075% NP-40 in the CE buffer, washes with CE buffer containing NP-40, and sonication of the nuclear fraction was chosen for subsequent fractionation experiments with ECs.

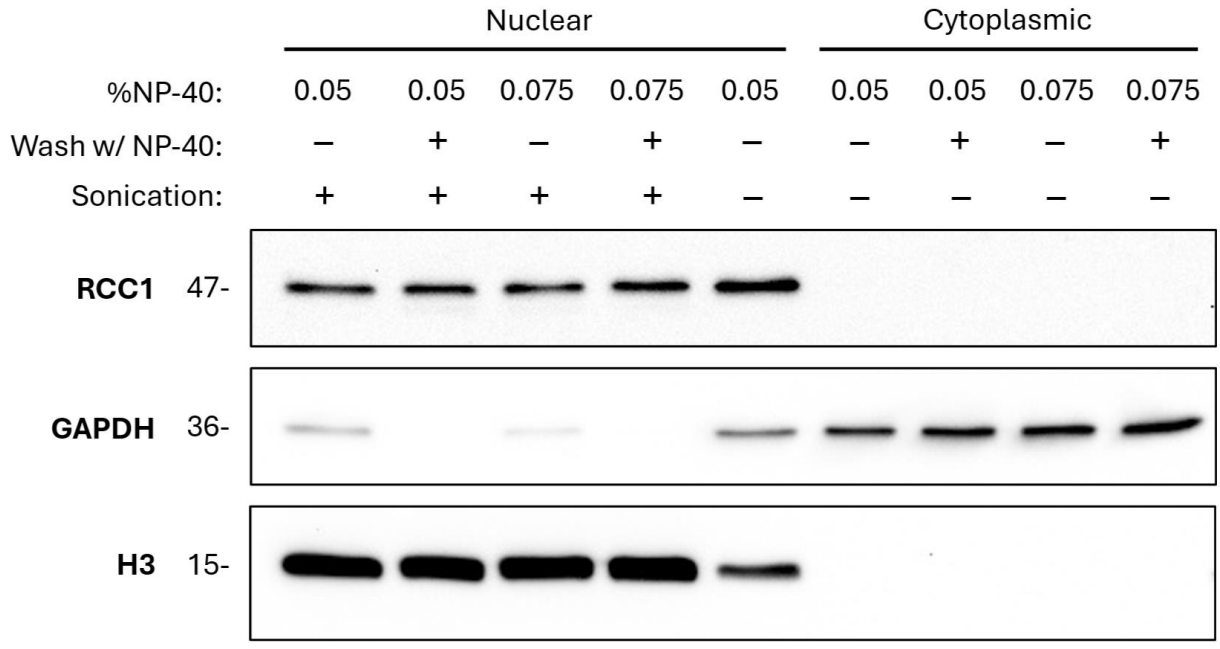

Figure 3.3: Optimization of the protocol for subcellular fractionation with 10T1/2 cells. 10T1/2 cells were plated 24h prior to extraction. Subcellular fractionation was performed on different sets of 10T1/2 cells testing various modifications to the protocol. Modifications involved changing the concentration of NP-40 in the cytoplasmic extract buffer, washing with buffer containing or lacking NP-40, and including or excluding sonication of the nuclear fraction. RCC1

and GAPDH served as loading controls for the nuclear and cytoplasmic compartments, respectively. H3 was measured to assess the presence of chromatin-bound proteins. n=1.

3.3.b FoxO1 subcellular localization in male and female adipose ECs under serum-starved and insulin-stimulated conditions

To assess the subcellular localization and insulin-dependent nuclear exclusion of FoxO1, I measured total FoxO1 levels and phosphorylation of FoxO1 (Ser256) in the nuclear and cytoplasmic compartments of male and female ECs (**Fig. 3.4**). Intriguingly, for both sexes, the signal for pSer256-FoxO1 was more highly pronounced in the nuclear fraction relative to the cytoplasmic fraction, even after 3h of serum starvation. This finding was unexpected since AKT-phosphorylated FoxO1 should be sequestered in the cytoplasm (Biggs et al., 1999; Brunet et al., 1999). Nuclear pSer256-FoxO1 levels increased upon insulin stimulation in the male ECs, but no significant changes in pSer256-FoxO1 levels were observed in female ECs (**Fig. 3.4B**). Intriguingly, cytoplasmic pSer256-FoxO1 levels decreased upon insulin stimulation in female ECs (**Fig. 3.4C**). FoxO1 was present in both the nuclear and cytoplasmic fractions, with more prominent bands observed in the cytoplasmic fraction (**Fig. 3.4D,E,F**). Upon insulin stimulation, nuclear FoxO1 levels significantly decreased in both sexes (**Fig. 3.4D**). Additionally, higher levels of nuclear FoxO1 were observed in female ECs compared to male ECs under serum-starved conditions (**Fig. 3.4D**). No significant changes in cytoplasmic FoxO1 occurred in response to insulin in either sex (**Fig. 3.4E**). When comparing the ratio of nuclear to cytoplasmic FoxO1 levels, it trended to decrease in both sexes; however, only the females showed a near significant reduction (P-value = 0.068) which may indicate greater relative cytoplasmic FoxO1 after insulin stimulation (**Fig. 3.4F**).

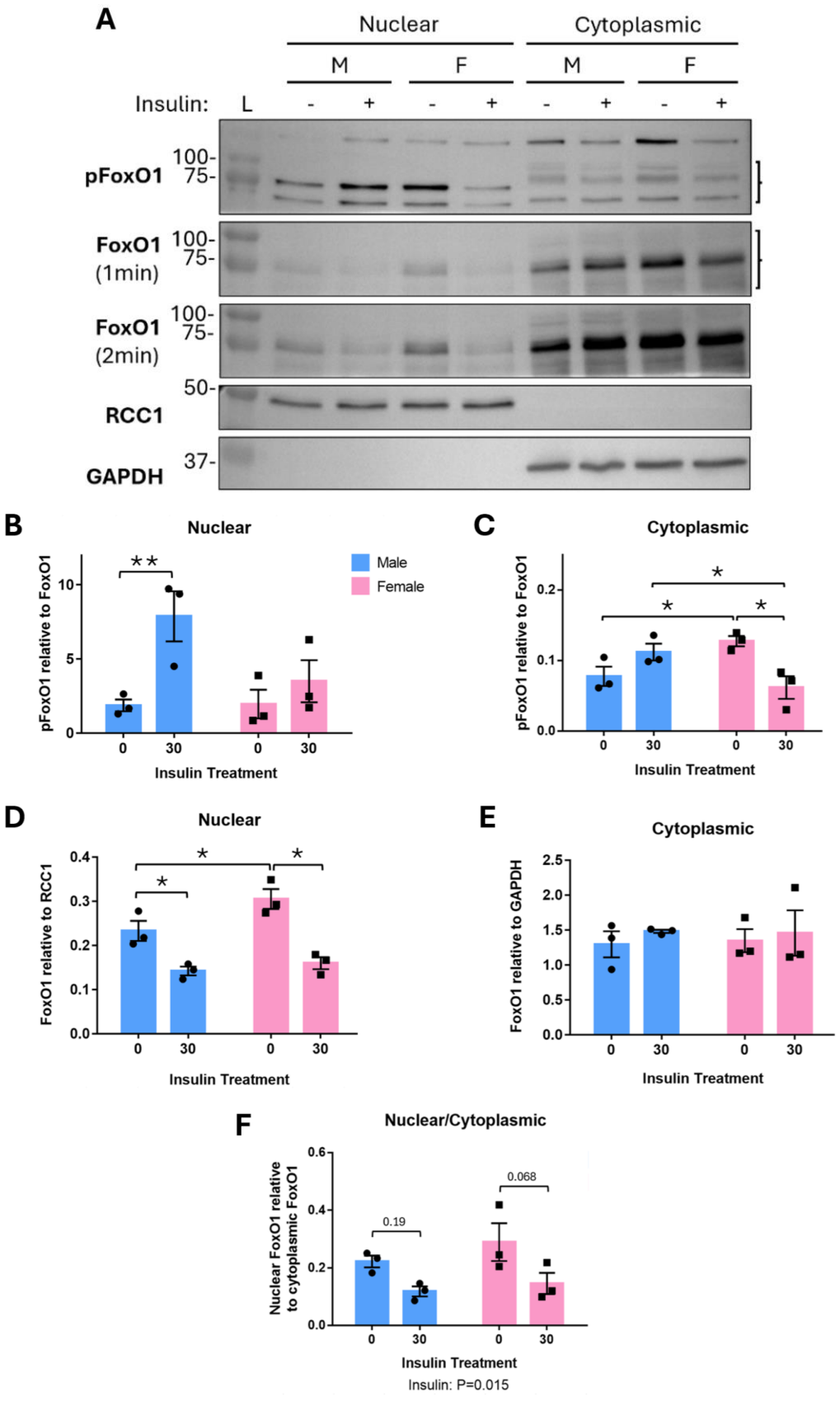
A
Nuclear
Cytoplasmic
M
F
M
F
Insulin:
L
-
+
-
+
-
+
-
+
pFoxO1
100-
75-
FoxO1
(1min)
100-
75-
FoxO1
(2min)
100-
75-
RCC1
50-
GAPDH
37-
B
Nuclear
Male
Female
pFoxO1 relative to FoxO1
Insulin Treatment
C
Cytoplasmic
pFoxO1 relative to FoxO1
Insulin Treatment
D
Nuclear
FoxO1 relative to RCC1
Insulin Treatment
E
Cytoplasmic
FoxO1 relative to GAPDH
Insulin Treatment
F
Nuclear/Cytoplasmic
Nuclear FoxO1 relative to cytoplasmic FoxO1
0.19
0.068
Insulin Treatment
Insulin: P=0.015

Figure 3.4: Effects of insulin on FoxO1 phosphorylation and subcellular localization in male and female adipose ECs. Male and female adipose ECs were serum-starved for 3h followed by 0- or 30-min of insulin treatment (25µg/mL). Representative western blot of phosphorylated (Ser256) and total FoxO1 levels. Times for FoxO1 blots indicate exposure time for better visual representation of nuclear and cytoplasmic FoxO1 levels. RCC1 and GAPDH served as loading controls for the nuclear and cytoplasmic compartments, respectively. Brackets indicate bands used to quantify pSer256-FoxO1 and FoxO1 levels. **B)** and **C)** Quantification of pSer256-FoxO1 levels relative to total FoxO1, n=4. pSer256-FoxO1 and FoxO1 were blotted on separate gels due to issues with membrane stripping and reprobing. Hence, each protein was normalized to its respective loading control prior to representing as a ratio to total FoxO1. **D)** and **E)** Quantification of FoxO1 levels relative to the loading controls for the nuclear and cytoplasmic fractions, respectively, n=4. **F)** Ratio of nuclear/cytoplasmic FoxO1 levels, n=4. For each graph, individual n values are plotted, with bars illustrating the mean ± SEM. Statistical significance was assessed using two-way ANOVA with Sidak's post-hoc test where appropriate. *P < 0.05, **P < 0.01.

3.3.c Presence of mitochondrial Tom20 in the nuclear fraction

The prominent pSer256-FoxO1 signals observed in the nuclear fraction raised the possibility of contamination from a non-nuclear source like the mitochondria, since mitochondria may be tethered to the nucleus through nucleus-associated membrane contact sites (Desai et al., 2020). Furthermore, preliminary findings from a previous lab member indicated that FoxO1 can be localized to the mitochondria of ECs (Nahal, 2024). Thus, to determine whether there may be mitochondrial contamination in the nuclear fraction, membranes were probed for Tom20, an outer mitochondrial membrane protein (**Fig. 3.5**). The results indicated a strong presence of Tom20 in the nuclear fraction but none in the cytoplasmic fraction, suggesting that mitochondria were pelleted with the nuclear fraction. Thus, it is possible that mitochondrial pSer256-FoxO1 contributes to the observed nuclear pSer256-FoxO1 levels.

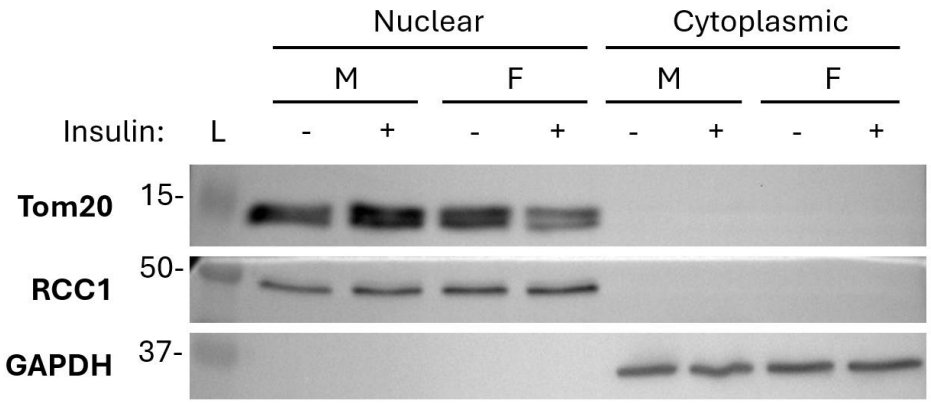

Figure 3.5: Presence of mitochondrial Tom20 in the nuclear fraction. Male and female adipose ECs were serum-starved for 3h followed by 0- or 30-min of insulin treatment. Representative western blot of the mitochondrial protein, Tom20, n=4. RCC1 and GAPDH served as loading controls for the nuclear and cytoplasmic compartments, respectively.

3.3.d Immunostaining of FoxO1 and pSer256-FoxO1 in ECs under serum-starved and insulin-stimulated conditions

To visually assess the distribution of the FoxO1 protein in individual cells, I conducted immunostaining on fixed ECs. This would clarify whether pSer256-FoxO1 was found in the nucleus or was co-localized with the mitochondria. Immunostaining also preserves the spatial context and accounts for cell-cell heterogeneity which is masked when using biochemical subcellular fractionation. Under serum-starved conditions, a clear nuclear FoxO1 signal that overlapped with DAPI was observed in both sexes (**Fig. 3.6A**). After 30- and 60- min of insulin stimulation, FoxO1 signal dispersed more broadly into the cytoplasmic regions, and a distinct nuclear region was no longer evident compared to serum-starved cells. Quantification of the mean fluorescence intensity of FoxO1 signal showed that insulin treatment induced a significant decrease in nuclear FoxO1 in both sexes (**Fig. 3.6C**). These findings support the subcellular fractionation data showing that FoxO1 shuttling patterns in response to insulin stimulation are similar in male and female ECs.

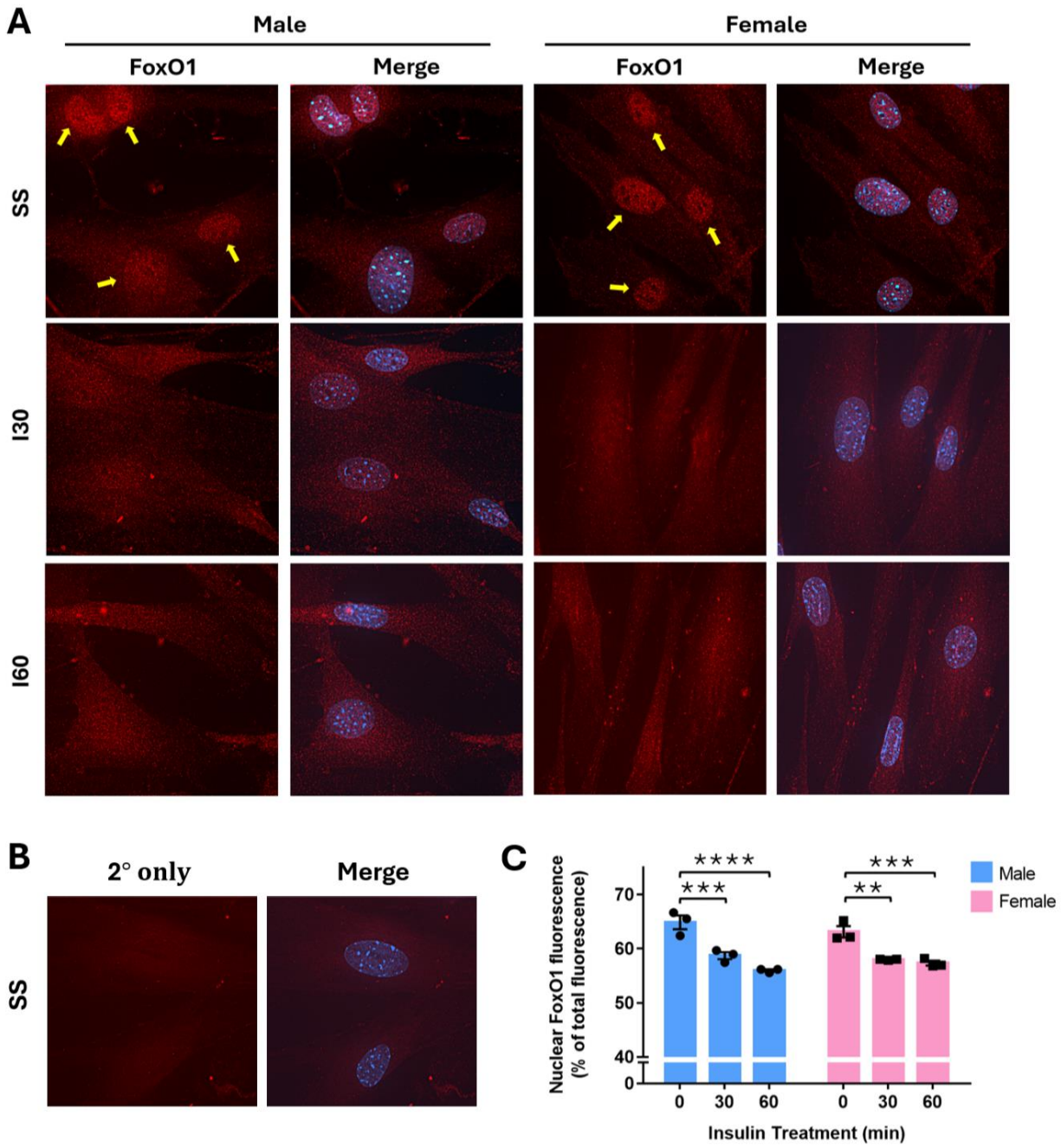

Figure 3.6: Immunostaining of FoxO1 in ECs under serum-starved and insulin-stimulated conditions. Male and female adipose ECs were serum-starved for 3h followed by 0-, 30-, or 60-min of insulin treatment. **A)** Representative images (60X) of FoxO1 subcellular localization. FoxO1 stained with Cy3 (red), nuclei counterstained with DAPI (blue). Yellow arrows indicate nuclear localization of FoxO1 signal. **B)** Secondary only staining with Cy3 (red) of serum-starved female ECs for reference. **C)** FoxO1 mean fluorescence intensity across all nuclei within each field of view expressed as a percent of the sum of the nuclear and cytoplasmic mean FoxO1 fluorescent signals. Values were quantified from 4 fields of view per experiment, with 3-7 cells per field of view, n=3. Individual n values are plotted, with bars illustrating the mean ± SEM. Statistical

significance was assessed using two-way ANOVA with Sidak's post-hoc test. **P < 0.01, ***P < 0.001, ****P<0.0001.

To better understand the subcellular source of pSer256-FoxO1 that was observed in the nuclear fractions of ECs, fixed female ECs were also stained for pSer256-FoxO1 to visualize the subcellular localization of the protein. Under serum starved conditions, a high pSer256-FoxO1 signal was observed in the nuclear regions of the ECs (**Fig. 3.7**). Interestingly, upon insulin stimulation, there was no observable changes in the pSer256-FoxO1 nuclear signal (**Fig. 3.7**). These findings corroborate the results from the subcellular fractionation experiments where high pSer256-FoxO1 nuclear levels were observed in both serum-starved and insulin-treated conditions. They also suggest that mitochondrial contamination in the nuclear fraction is unlikely to explain the strong pSer256-FoxO1 levels observed in the nuclear fraction.

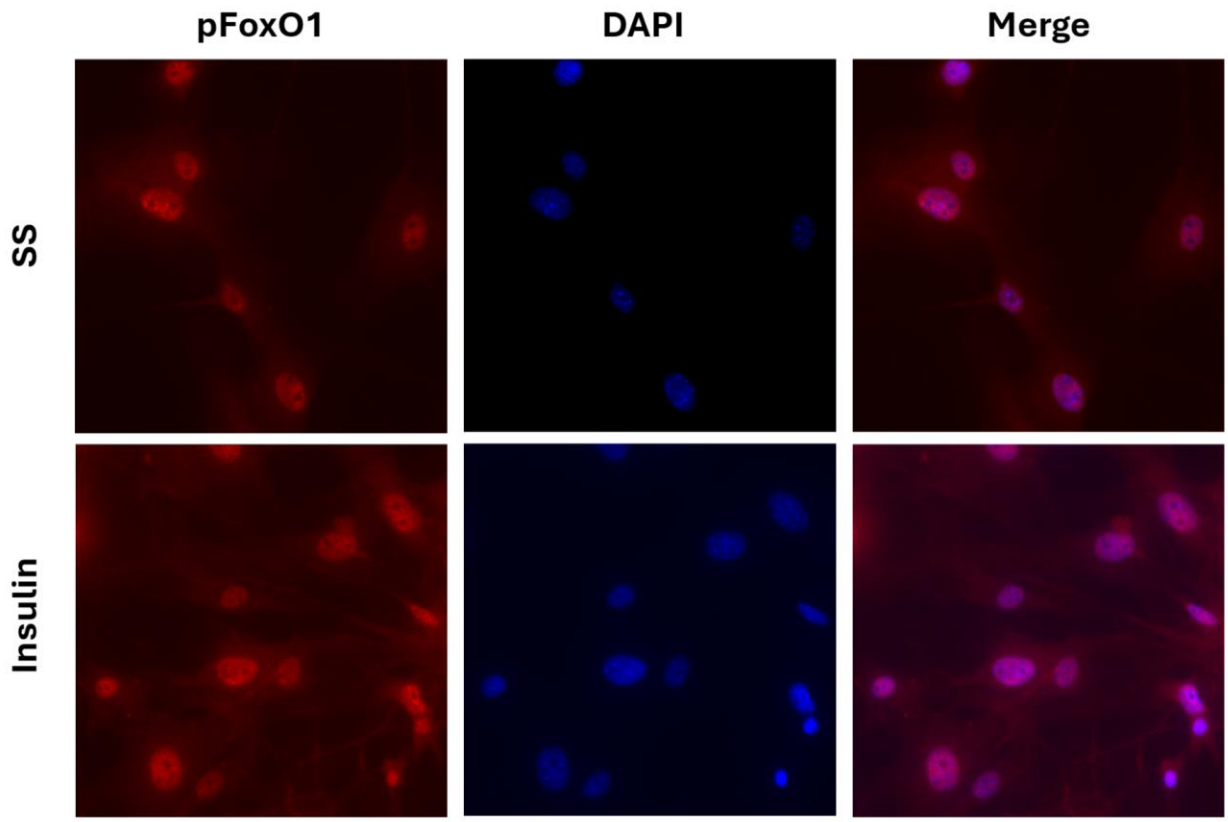

Figure 3.7: Immunostaining of pSer256-FoxO1 in ECs under serum-starved and insulin-stimulated conditions. Female adipose ECs were serum-starved for 3h followed by 0- or 30-min of insulin treatment. Representative immunofluorescent images (40X) of pSer256-FoxO1 subcellular localization. Red: Cy3 for pSer256-FoxO1; Blue: DAPI for cell nuclei. n=1.

3.4 Evaluation of pSer256-FoxO1 patterns in ECs

An intriguing result from both the subcellular fractionations and the immunostaining was the high level of pSer256-FoxO1 observed under serum-starved conditions. It was expected that the lack of serum would diminish signalling pathways that normally induce FoxO1 phosphorylation and inactivation. Thus, further exploration was needed to determine the biological basis of these results. One possible explanation is that kinases other than AKT phosphorylate FoxO1 at Ser256. To test whether PI3K inhibition would abolish pSer256-FoxO1 levels, as would be expected if

AKT was the sole source of pSer256 phosphorylation, I measured pSer256-FoxO1 levels in cells treated with PI3K inhibitor, LY-294002 (10μM). Interestingly, nuclear and cytoplasmic pSer256-FoxO1 levels remained unchanged in both sexes after LY294002 treatment (**Fig. 3.8A**). In addition, the insulin-stimulated increase in pSer473-AKT levels was attenuated by LY294002 treatment in whole cell extracts, verifying the inhibition of PI3K by LY294002 (**Fig. 3.8B**). These results suggested that the observed phosphorylation of FoxO1 was not mediated by AKT. There is compelling evidence that FoxO1 is also a substrate of protein kinase A (PKA), which phosphorylates FoxO1 at all three known AKT phosphorylation sites (Thr24, Ser256, and Ser319) (J.-W. Lee et al., 2011). If PKA is not serum-inactivated like AKT, then it may contribute to the pSer256-FoxO1 levels observed after serum starvation. To assess the potential contribution of PKA in the observed pSer256-FoxO1 levels, female ECs were treated with an inhibitor against PKA, PKI (10μM). As was the case with AKT inhibition, pSer256-FoxO1 levels did not decrease with PKA inhibition in either serum-starved or insulin-treated cells (**Fig. 3.8C**).

Collectively, the findings regarding pSer256-FoxO1 were unexpected as they counter the current understanding of FoxO1 regulation by insulin signalling. This prompted further investigation to evaluate the specificity of the pSer256-FoxO1 antibody (Cell Signaling). To achieve this, pSer256-FoxO1 levels were measured in whole cell extracts from ECs treated with or without FoxO1 siRNA. These extracts were prepared by a lab mate for a separate experiment. If the pSer256-FoxO1 antibody was specific for FoxO1, pSer256-FoxO1 levels were expected to be lower in FoxO1 knockdown (KD) cells. Although pSer256-FoxO1 signals appeared lower in female ECs with FoxO1 KD compared to the non-targeting (NT) siRNA control, pSer256-FoxO1 signals in the male ECs looked similar between NT and FoxO1 KD, despite the decrease in total FoxO1 after FoxO1 KD (**Fig. 3.8D**). However, pSer256-FoxO1 signals from the male ECs were weak overall,

making it difficult to assess differences. To determine whether a different pSer256-FoxO1 antibody would show more consistent results, the pSer256-FoxO1 antibody from Abcam was tested on a different set of male and female control and FoxO1 KD ECs. Under serum starved conditions, a weaker pSer256-FoxO1 signal was observed in the female ECs after FoxO1 KD compared to the NT control; however, the pSer256-FoxO1 signal appeared slightly stronger in the male ECs following FoxO1 KD **(Fig. 3.8E**). Taken together, these results do not show consistent patterns of FoxO1 phosphorylation at Ser256 in response to insulin or FoxO1 KD. Therefore, while the antibody produced detectable signal, the inability to rigorously establish its specificity under my experimental conditions necessitates that the results be interpreted cautiously. Accordingly, I decided not to pursue further analyses regarding pSer256-FoxO1.

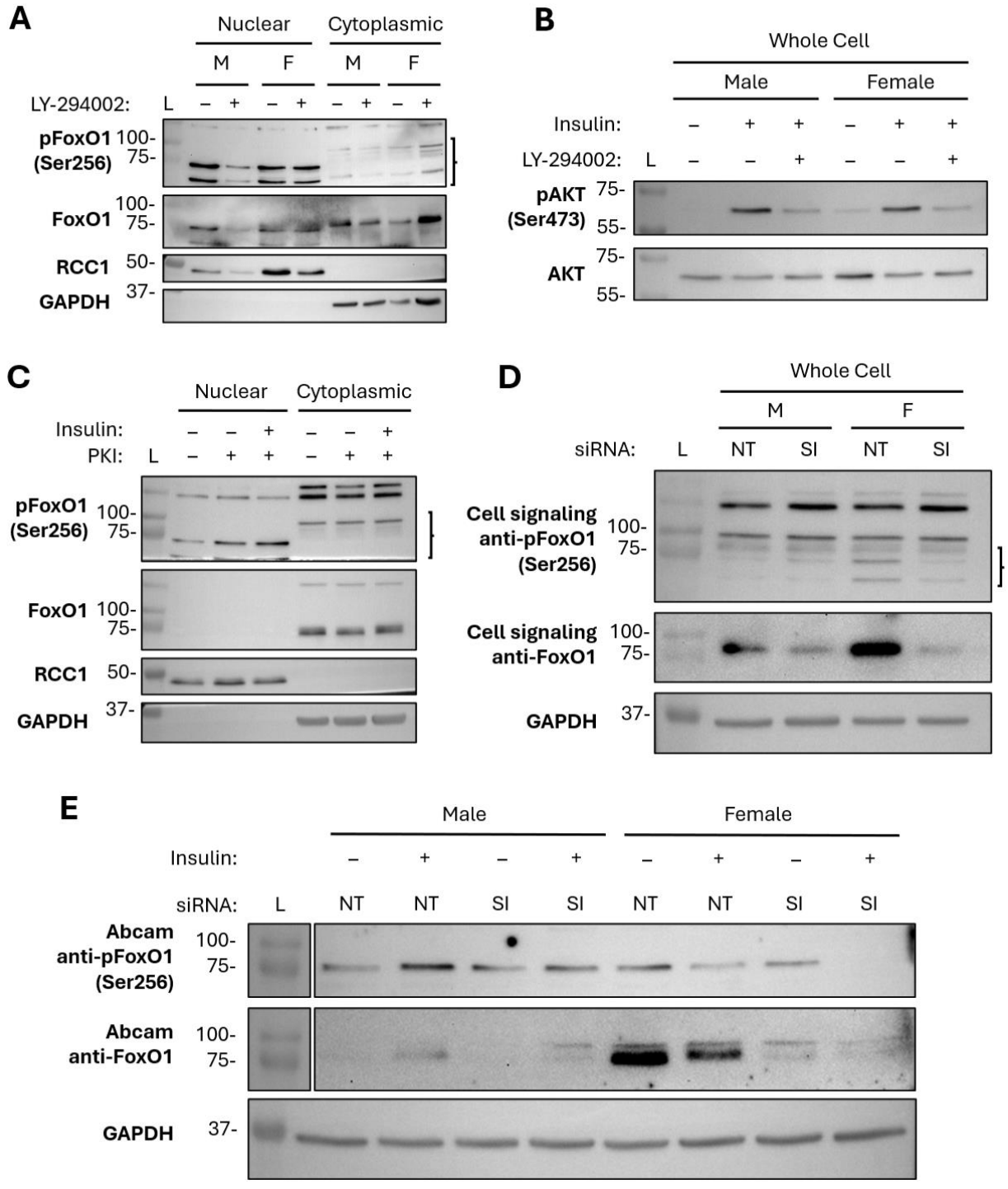

Figure 3.8: Evaluation of pSer256-Foxo1 antibody through kinase inhibition and FoxO1 KD in male and female adipose ECs. A) pSer256-FoxO1 and total FoxO1 in nuclear and cytoplasmic fractions of male and female ECs serum starved for 2h followed by treatment with PI3K inhibitor LY-294002 (10μM) for 0- or 60- min. **B)** pSer473-AKT and total AKT levels in whole cell extracts of male and female ECs serum starved for 2h followed by PI3K inhibitor LY-294002 treatment (10μM) for 0- or 60- min and subsequent 0- or 30- min insulin treatment (25μg/mL). **C)** pSer256-

FoxO1 and total FoxO1 in nuclear and cytoplasmic fractions of male and female ECs serum starved for 2h followed by PKA inhibitor PKI (10μM) for 0- or 60- min and subsequent 0- or 30-min insulin treatment (25μg/mL). **D)** Whole cell extracts from male and female adipose ECs, control or treated with siRNA against FoxO1. Cell signalling antibodies for pSer256-FoxO1 and FoxO1. GAPDH as a loading control. Brackets in A), C) and D) indicate bands used to quantify pSer256-FoxO1 levels. **E)** Whole cell extracts from serum-starved male and female adipose ECs, treated with or without 10-min insulin (25μg/mL), with or without siRNA against FoxO1. Abcam pSer256-FoxO1 and FoxO1 antibodies. GAPDH as a loading control. Ladders were oversaturated and added to blots manually. For all subcellular fractionations, RCC1 and GAPDH served as loading controls for the nuclear and cytoplasmic compartments, respectively.

3.5 Effects of FoxO1 inhibition on nucleosome organization gene expression

After comparing the localization and phosphorylation of FoxO1 between male and female ECs, I next shifted my focus to looking at downstream effects of FoxO1. This investigation was motivated by previous findings from our lab that indicated that female ECs exhibit a more severe phenotype following FoxO1 deletion or inhibition, emphasizing the need to focus on downstream consequences of FoxO1 transcription (Pislaru, 2025). This was achieved using cultured ECs treated with or without FoxO1 inhibitor, AS1842856, which prevents FoxO1 transactivation by directly binding to the active, unphosphorylated form (Nagashima et al., 2010). Thus, FoxO1 inhibitor was used as a tool to examine the impact of loss of FoxO1 transcriptional activity compared to control cells where FoxO1 remained transcriptionally active. Previously, our lab found that genes associated with nucleosome organization were downregulated in adipose ECs in vivo from female mice on HFD diet with endothelial-specific FoxO1 KD compared to control female mice (Pislaru, 2025). Notably, this pathway was only affected in the female adipose ECs. While these effects of genetic FoxO1 KD on chromatin regulatory programs were observed in vivo, we have not tested whether pharmacological FoxO1 inhibition causes the same effects or whether these findings are recapitulated in cultured ECs. Therefore, I assessed the impact of FoxO1

inhibition on mRNA expression levels of select genes involved in nucleosome organization in cultured male and female ECs. These genes were selected from the in vivo dataset as they were significantly downregulated in female ECs after FoxO1 KD, with a more negative fold change compared to male ECs. mRNA levels of the core histone variant gene *H3-3a*, a replication-independent histone protein, were significantly downregulated in female ECs upon FoxO1 inhibition (**Fig. 3.9A**). In contrast, its expression tended to decrease in male ECs with FoxO1 inhibition, but the effect was not statistically significant (**Fig. 3.9A**). The histone chaperone *Asf1b*, which is involved in the deposition of H3/H4 histones, tended to increase with FoxO1 inhibition in male ECs, while its expression in female ECs remained relatively unchanged; correspondingly, male ECs with FoxO1 inhibition expressed significantly higher levels of *Asf1b* than female ECs with FoxO1 inhibition (**Fig. 3.9B**). The histone chaperones, *Nap1l3* and *Nap1l5*, which mediate the deposition of H2A/H2B, showed no significant changes in response to FoxO1 inhibition (**Fig. 3.9C, D**); however, there was a near significant main effect of sex (P-value: 0.063) where female ECs had greater expression than male ECs (**Fig. 3.9C**). Altogether, these results highlight a gene- and sex-specific role of FoxO1 in regulating the expression of genes involved in chromatin regulatory programs.

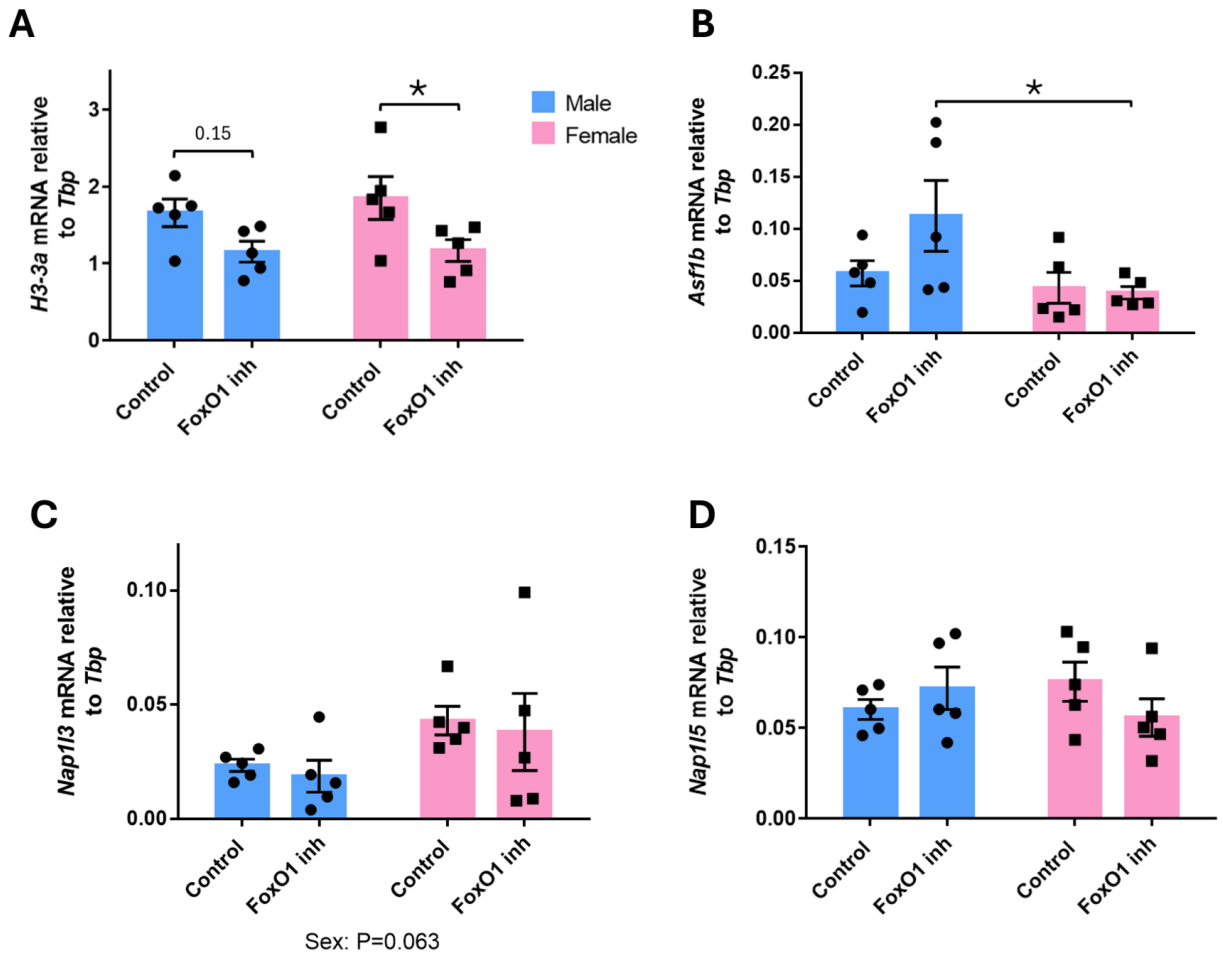

Figure 3.9: FoxO1 inhibition differentially impacts gene expression of nucleosome organization genes in male and female adipose ECs. Male and female ECs were treated with vehicle (DMSO) or FoxO1 inhibitor AS1842856 for 3 days. Gene expression of **A)** H3-3a, **B)** Asf1b, **C)** Nap1l3, and **D)** Nap1l5 were assessed by qPCR. Expression levels are presented relative to the housekeeping gene Tbp. Individual n values are plotted, with bars illustrating the mean ± SEM. Statistical significance was assessed using two-way ANOVA with Sidak's post-hoc test. *P < 0.05.

Chapter 4: Discussion

The overall goal of my thesis was to investigate sex differences in the regulation and function of FoxO1 in adipose ECs isolated from male and female mice. While previous work in the Haas lab revealed higher FoxO1 levels and a more severe impact of FoxO1 loss in female adipose ECs, the underlying explanations for how FoxO1 is regulated and mediates its sex-specific effects in ECs remain to be fully elucidated. My experiments showed that male and female ECs exhibited similar levels of AKT and FoxO1 phosphorylation in response to insulin; however, other upstream regulators of FoxO1 showed sex-related differences, where p38 phosphorylation as well as levels of the E3 ubiquitin ligase MDM2 were higher in female ECs. Assessments of the subcellular localization of FoxO1 revealed similar levels of FoxO1 nuclear export in response to insulin stimulation; however, female ECs had greater nuclear FoxO1 levels than male ECs under basal level conditions, which may enhance the potential for FoxO1 transcriptional activity in female ECs. To assess the downstream functional consequences of FoxO1, gene expression analyses revealed that the core histone variant *H3-3a* was significantly downregulated by FoxO1 inhibition only in female ECs, while expression of the histone chaperone *Asf1b* tended to increase in with FoxO1 inhibition male ECs. These findings indicate a sex-distinct role of FoxO1 in regulating nucleosome organization genes, supporting a sex bias in the functional influence of FoxO1 in female ECs.

4.1 Male and female adipose ECs exhibit similar responses to insulin signalling

Previous work in our lab has shown that female adipose ECs have higher levels of FoxO1 mRNA and protein than male ECs under basal level conditions (Pislaru, 2025; Rudnicki et al., 2023). To investigate the mechanisms underlying this sex difference in endothelial FoxO1, I assessed the

insulin signalling pathway, a well-established regulator of FoxO1 subcellular localization. The insulin/PI3K/AKT axis induces the phosphorylation of FoxO1 by AKT, promoting FoxO1 nuclear exclusion (Biggs et al., 1999; Rena et al., 1999). Based on this pathway, I hypothesized that sex differences in FoxO1 levels in adipose ECs may be influenced by sex-distinct sensitivity to insulin signalling. I found that insulin signalling increased AKT phosphorylation after 10 and 30 minutes, in line with multiple groups who have demonstrated rapid activation of AKT via phosphorylation at Ser473 within minutes of insulin stimulation (Alessi et al., 1996; Andjelkovic et al., 1997; Gray & Coster, 2020). However, no significant sex differences in this response were observed. These findings complement previous work in our lab which showed that ERK phosphorylation, another major pathway activated downstream of insulin signalling, was similar in male and female adipose ECs after 10 minutes of insulin stimulation (Connor Breadmore, Unpublished). Together, these data suggest that insulin sensitivity is similar in male and female ECs. Moreover, downstream phosphorylation of FoxO1 by AKT was also similar in male and female ECs with pSer256-FoxO1 levels increasing significantly after 30 minutes of insulin stimulation. Therefore, the extent to which FoxO1 is regulated by AKT phosphorylation is comparable in male and female ECs.

While AKT activation by insulin has been well established in other cell types, whether the insulin signalling response differs between male and female ECs has not been directly investigated. The present finding that ECs exhibit similar sensitivity to insulin based on downstream AKT phosphorylation levels is intriguing given that multiple studies suggest higher insulin sensitivity in females (Kautzky-Willer et al., 2012; Kuhl et al., 2005; Macotela et al., 2009). With that said, AKT signalling on its own represents an early signalling event that may not accurately predict downstream insulin sensitivity. In addition, insulin sensitivity is normally measured at the whole-body level based on glucose tolerance tests or insulin tolerance tests that assess the rate in which

the body clears blood glucose (Kautzky-Willer et al., 2012; Kuhl et al., 2005). This depends on the body's ability to secrete insulin in response to glucose, and the responsiveness of various metabolic tissues to insulin. Additionally, insulin sensitivity can be measured at a cellular level by assessing multiple cell-type specific indicators. For example, female adipocytes from the perigonadal depot in mice had greater insulin sensitivity than male adipocytes based on measures of AKT and ERK phosphorylation and lipogenesis, since a key action of insulin in adipocytes is promoting lipid synthesis and storage (Macotela et al., 2009). However, no sex differences were observed between adipocytes from the subcutaneous adipose tissue (Macotela et al., 2009). Thus, although insulin sensitivity at the levels of ECs may be similar between males and females, this does not necessarily reflect the insulin sensitivity of the tissue in which the ECs reside.

4.2 Sex differences in upstream regulators of FoxO1

The lack of sex differences in the insulin signalling response between male and female ECs does not explain the higher FoxO1 mRNA and protein levels in female ECs. Therefore, other regulatory mechanisms outside of the PI3K-AKT axis likely contribute to this outcome. Indeed, FoxO1 is modulated by multiple factors beyond insulin signalling, and kinases other than AKT have been shown to phosphorylate FoxO1 to modulate its subcellular localization. One such kinase is p38 MAPK, which is activated by multiple cell stressors including inflammatory cytokines, glucose starvation, and oncogene activation (Han et al., 2020). p38 is distributed throughout the cytoplasmic and nuclear compartments of the cell, and it phosphorylates multiple targets including FoxO1 (Asada et al., 2007; Dong et al., 2015; Yang et al., 2023). This phosphorylation event promotes FoxO1 nuclear import and transcriptional activity. Intriguingly, I found that p38 phosphorylation levels were higher in female ECs, both under serum starvation and insulin-treated conditions. These data align with previous work in our lab which showed that p38 phosphorylation

levels were higher in female adipose ECs cultured in control media (Rezvan, 2020). P38 phosphorylation has previously been shown to correlate with increased nuclear localization of FoxO1 in cardiomyocytes (Puthanveetil et al., 2010). Moreover, inhibition of p38 phosphorylation with SB202190 prevented the nuclear translocation of FoxO1, supporting a role of p38 in the nuclear shuttling of FoxO1 (Puthanveetil et al., 2010). Given the higher levels of p38 phosphorylation in female ECs, this may promote greater nuclear localization of FoxO1, thereby reducing cytoplasmic levels of FoxO1 where it can be degraded. It would be interesting to next assess the level of p38 phosphorylation of FoxO1 and establish whether this regulatory modification on FoxO1 is more abundant in female ECs. While researchers have identified multiple putative p38 phosphorylation sites on FoxO1, differences were found in the phosphorylated amino acid residues in vitro with kinase assays compared to in vivo phosphopeptide mapping and mutagenesis (Asada et al., 2007). More recently, Yang et al. (2023) showed the hepatic p38 stimulates FoxO1 phosphorylation at Ser273. However, currently no commercially available antibodies for p38 phosphorylation sites on FoxO1 have been produced. Thus, the mechanisms by which higher p-p38 levels in female ECs contribute to FoxO1 regulation remain to be fully defined.

FoxO1 levels are also modulated via ubiquitin-mediated degradation, and previous work in our lab demonstrated that FoxO1 is ubiquitinated by the E3 ubiquitin ligase MDM2 in skeletal muscle ECs (Milkiewicz et al., 2011). My results showed that levels of MDM2 were significantly higher in female ECs. This finding was surprising since higher levels of MDM2 were expected to correlate with lower FoxO1 protein, yet female ECs have greater FoxO1 protein levels than males. Interestingly, a recent study on MCF7 cancer cells proposed a reciprocal relationship between MDM2 and FoxO1 where MDM2 mediates FoxO1 degradation while FoxO1 can directly bind to

the promoter of MDM2 to induce its transcription (Milkiewicz et al., 2011; Tomiyasu et al., 2024). To determine whether this mechanism occurs in ECs, I assessed the impact of FoxO1 inhibition on MDM2 levels in serum-starved ECs. Intriguingly, MDM2 levels increased significantly in FoxO1 inhibitor-treated male ECs, but no effect was seen on the female ECs. These data do not directly support a transcriptional activation of MDM2 by FoxO1 in ECs; however, it is possible that this regulatory relationship still occurs, and FoxO1 inhibition may induce a compensatory mechanism or affect other pathways that lead to increased MDM2 protein levels in male ECs. Additionally, while FoxO1 inhibition decreased MDM2 expression in MCF7 cancer cells, it had no effect on MDM2 expression in prostate cancer cell lines 22Rv1 and LNCaP, suggesting that the regulation of MDM2 by FoxO1 is cell-type dependent (Tomiyasu et al., 2024).

Another factor that may affect FoxO1 downstream of MDM2 is the histone methyltransferase EZH2 (catalytic subunit of PRC2). EZH2 has been shown to be a substrate for MDM2 where MDM2 ubiquitinates EZH2, leading to its degradation (Kuser-Abali et al., 2018). Moreover, EZH2 activity has been demonstrated to repress FoxO1 transcription in multiple cancer cell lines through direct binding to the FoxO1 promoter and adding the repressive mark H3K27me3 to histone tails (Ma et al., 2019). Given the role of MDM2 in the degradation of EZH2, higher MDM2 levels may contribute to higher levels of FoxO1 in female ECs by relieving repression on FoxO1 transcription. Further research is required to substantiate this hypothesis regarding the role of EZH2 in the regulation of FoxO1 in ECs.

4.3 FoxO1 subcellular localization patterns in male and female adipose ECs

Despite AKT signalling being similar in male and female ECs, other modes of regulation may modulate FoxO1 activity by affecting its subcellular localization and dictating its accessibility to

DNA. Therefore, it is critical to establish not only total levels of FoxO1 in ECs, but also the distribution of FoxO1 across the nuclear and cytoplasmic cellular compartments to understand its functional capacity. In the present study, subcellular fractionation experiments revealed significantly higher nuclear FoxO1 levels in female ECs under serum-starved conditions. Previous work in our lab showed that female endothelial FoxO1 exhibited enhanced activity based on increased transcriptional activity of forkhead response element (FRE)-luciferase reporter constructs in female ECs compared to male ECs (Pislaru, 2025). Since nuclear localization is required for the ability of FoxO1 to bind and initiate transcription of target genes, higher nuclear FoxO1 levels may contribute to this enhanced FoxO1 activity observed in female ECs.

Conversely, in the cytoplasmic fraction, FoxO1 levels did not significantly increase in either sex with insulin stimulation, despite an expected increase in cytoplasmic levels in line with the decline in nuclear FoxO1 levels. Two possible explanations for these results can be considered. Firstly, the nuclear FoxO1 levels were generally lower than the robust FoxO1 signals in the cytoplasm. Therefore, at a technical level, it may be more difficult to identify small changes occurring to the large pool of FoxO1 in the cytoplasmic fraction compared to the smaller pool in the nuclear fraction. Another reason for this observation could involve the degradation of FoxO1 in the cytoplasm, since phosphorylated FoxO1 gets ubiquitinated by E3 ubiquitin ligases and targeted for proteasomal degradation. Therefore, it is possible that degradation of FoxO1 in the cytoplasm maintains relatively stable cytoplasmic FoxO1 levels despite the impact of insulin signalling. Indeed, insulin-mediated nuclear exclusion of FoxO1 was shown to promote FoxO1 polyubiquitination and subsequent proteasomal degradation in HepG2 cells (Matsuzaki et al., 2003). This process led to reduced FoxO1 protein levels after 4h of insulin stimulation and was dependent on AKT-mediated phosphorylation and cytoplasmic retention of FoxO1. Future

research could explore this mechanism in ECs by using a proteasome inhibitor such as MG132 to determine whether inhibiting proteasomal degradation would allow for the detection of increases in cytoplasmic FoxO1 levels after short-term insulin stimulation.

4.4 pSer256-FoxO1 patterns in ECs

Phosphorylation of FoxO1 by AKT is a well-established mechanism that promotes FoxO1 nuclear export and inactivation (Rena et al., 1999; Zhang et al., 2002; Zhao et al., 2004). Assessments of FoxO1 phosphorylation at Ser256 thus provide a strong indication of the activity status of FoxO1 and an additional layer of regulation on top of subcellular localization. When AKT is activated by insulin signalling and phosphorylates FoxO1 in the nucleus, the chaperone protein 14-3-3 binds to FoxO1, and this complex is exported out of the nucleus into cytoplasm (Biggs et al., 1999). While phosphorylation at all three AKT sites is required for the nuclear export of FoxO1, phosphorylation at Ser256 is necessary for effective phosphorylation at Thr24 and Ser319 (Rena et al., 2001; Zhang et al., 2002). Additionally, phosphorylation at Ser256 reduces DNA binding affinity (Rena et al., 2001). My data revealed prominent pSer256-FoxO1 signals in the nuclear regions of ECs under serum-starved and insulin-treated conditions. Moreover, cytoplasmic pSer256-FoxO1 levels were lower than that of the nuclear fractions, despite greater total FoxO1 levels detected in the cytoplasmic fractions. Based on the regulatory effect of AKT-mediated phosphorylation of FoxO1 in inducing nuclear export, I would have expected to see greater cytoplasmic localization of the pSer256-FoxO1 signal. Given these findings, I considered whether mitochondrial contamination may have contributed to the prominent pSer256-FoxO1 signals observed in the nuclear fraction, since previous work demonstrated that FoxO1 can be localized to the mitochondria in ECs (Nahal, 2024). Indeed, immunoblotting for the mitochondrial membrane protein Tom20 revealed a strong presence of Tom20 in the nuclear fraction, suggesting contamination of mitochondrial proteins.

With that said, immunostaining for pSer256-FoxO1 showed strong signals overlapping with the nuclear staining in female ECs which supported a nuclear source of pSer256-FoxO1 independent of mitochondrial contamination.

The subcellular localization patterns of pSer256-FoxO1 observed in this study were surprising based on current knowledge of FoxO1 regulation by AKT. Normally, under serum-starved conditions, growth factor stimulated activation of the PI3K-AKT axis is reduced, thus preventing downstream phosphorylation of FoxO1. However, I observed high levels of nuclear pSer256-FoxO1 even under serum-starved conditions. Additionally, these levels were higher than that of the cytoplasmic fraction, which goes against the well-established role of Ser256 phosphorylation in promoting cytoplasmic localization. While pSer256-FoxO1 can be present in the nucleus prior to it nuclear export, FoxO1 shuttling normally occurs rapidly within minutes. For example, one study on FoxO1 shuttling dynamics in MCF7 cells showed that stimulation with 2% FBS rapidly reverses the nuclear accumulation of FoxO1 after serum starvation (Lasick et al., 2023). Another study that used real-time imaging of FoxO1-EGFP dynamics in B cells revealed that insulin induces FoxO1 nuclear shuttling within just 10-20 minutes (Meur et al., 2011). Therefore, it remains unclear which mechanisms caused high pSer256-FoxO1 signals to be detected in the nuclei of ECs rather than in the cytoplasm.

Another unexpected finding was how insulin stimulation did not consistently induce FoxO1 phosphorylation. This raised the question of whether AKT or another kinase was responsible for phosphorylating FoxO1 at Ser256. Lee et al. (2011) demonstrated that FoxO1 can be phosphorylated at Ser256 by PKA in vitro in HEK 293 cells and in vivo in HAECs. Unexpectedly, no change in the nuclear pSer256-FoxO1 levels in ECs under serum-starved conditions when treated with inhibitors against PI3K or PKA. These data indicated that the phosphorylation of

FoxO1 occurred independently of PI3K/AKT or PKA activity.

Since the findings regarding pSer256-FoxO1 subcellular localization were not easily explicable, it became necessary to evaluate the efficacy of the pSer256-FoxO1 antibody under my experimental conditions. This set of experiments revealed no consistent decrease in pSer256-Foxo1 levels with FoxO1 KD, despite using widely used pSer256-FoxO1 antibodies from Cell Signaling and Abcam. Based on these discrepancies, I did not have strong confidence in the specificity of the antibody within my experimental context, and assessments of pSer256-FoxO1 subcellular localization revealed here should be interpreted with caution.

4.5 FoxO1 regulation of genes involved in nucleosome organization

DNA is tightly packaged into chromatin with the nucleosome being the basic unit of chromatin (Seal et al., 2022). Controlled nucleosome assembly and disassembly is essential for chromatin function and integrity during remodelling programs including DNA replication, transcription, and DNA repair (Marzluff & Duronio, 2002). Previous transcriptomic data from our lab on adipose ECs from male and female mice with endothelial-specific FoxO1 KD revealed pathways that were underrepresented selectively in female ECs, one of which was the nucleosome organization pathway. Notably, several of these genes were significantly downregulated to a greater magnitude in the female adipose ECs compared to the males. Herein, I provide evidence of a sex-distinct role of FoxO1 in regulating nucleosome organization genes in cultured ECs, providing insight into the regulatory programs that may be contributing to the detrimental effects of FoxO1 depletion on female ECs.

Nucleosome assembly protein 1 like 3 and 5 (Nap1l3 and Nap1l5) are both part of the nucleosome assembly proteins (NAP) family of histone chaperone proteins that interact with H2A/H2B and bring nucleosome complexes to the chromatin after DNA replication or DNA repair (Park & Luger,

2006). Intriguingly, I found that *Nap1l3* expression tended to be higher in control female ECs, although there was no significant impact of FoxO1 inhibition on the expression of either *Nap1l3* or *Nap1l5*. With that said, there was a trend for decreased *Nap1l3* expression in the FoxO1 inhibitor condition except for one sample in the females. This finding would support a sex-distinct role of FoxO1 in regulating histone assembly, with a greater impact of FoxO1 inhibition seen in female ECs compared to the males who showed a less pronounced change in *Nap1l3* expression. It is possible that the expression of these genes is not affected to the same extent that we observed in vivo with FoxO1 KD due to the length of FoxO1 loss. In my study, a 3-day time-point was employed since previous work in our lab demonstrated that female ECs exhibit harmful phenotypic changes within just 3 days of FoxO1 inhibition including decreased proliferation, increased DNA damage, and increased nuclear size as an indicator of senescence (Pislaru, 2025). However, the transcriptomic data for the adipose ECs from mice with EC-FoxO1 KD involved genetic deletion of FoxO1 for an extended period of time (8 weeks) (Pislaru, 2025). This difference may contribute to the discrepancies observed here regarding the effects of FoxO1 on *Nap1l3* and *Nap1l5* expression. Thus, it may be of value for future work to evaluate the expression of these genes at a longer time-point (6 days) of FoxO1 inhibition to potentially establish a more pronounced effect of FoxO1 inhibition as was seen in vivo.

H3-3a expression was significantly downregulated in female ECs with 3-day FoxO1 inhibition. Notably, while the canonical H3.1 and H3.2 variants of H3 are replication-dependent, the H3-3a variant is replication-independent and is expressed throughout the cell cycle (Seal et al., 2022; R. S. Wu et al., 1982). This suggests that changes in *H3-3a* expression are unlikely to be attributable solely to altered proliferation in response to FoxO1 inhibition. Notably, H3.3 plays a critical role in DNA damage repair. It was found that H3.3 is deposited by the histone chaperone histone

regulator A (HIRA) complex at sites of UV-induced DNA damage, which primes the chromatin for reactivation of transcription once DNA repair has been completed (Adam et al., 2013). In addition, H3.3-deficient chicken DT40 cells were hypersensitive to DNA damage (Frey et al., 2014). Moreover, H3.3-depleted HeLa cells exhibited impaired homologous recombination (HR)-dependent DNA repair synthesis, resulting in increased γH2AX foci levels and chromosome breaks after ionizing radiation exposure (Juhász et al., 2018). Therefore, a decrease in *H3-3a* expression with FoxO1 inhibition in female ECs may suggest an impairment in the DNA damage repair response. This is especially compelling considering the increase in γH2AX foci levels our lab observed after 6-day FoxO1 inhibition in female ECs, but not in male ECs (Pislaru, 2025). As such, FoxO1 may contribute to the maintenance DNA repair mechanisms in part through its regulation of H3.3 availability, enhancing resistance to DNA damage and cellular stress in female ECs.

My data showed that *Asf1b* levels increased in male ECs after FoxO1 inhibition and remained relatively unchanged in female ECs. While the expression of its related isoform *Asf1a* remains relatively stable in proliferating and quiescent cells, *Asf1b* expression is tightly linked to cell cycle progression as its mRNA and protein levels decrease upon cell cycle exit, and *Asf1b* depletion impairs proliferation in multiple cancer cell lines (Corpet et al., 2011). Previous work in our lab with cultured ECs revealed that short-term 1-day FoxO1 inhibition causes a burst in proliferation in both male and female ECs (Pislaru, 2025). However, this increase is suppressed after long-term 6-day FoxO1 inhibition. In addition, an anti-proliferative effect of FoxO1 inhibition is seen after just 3 days of FoxO1 inhibition in female ECs, but not in male ECs (Pislaru, 2025). Given that proliferation increases after 1-day FoxO1 inhibition and that *Asf1b* expression more highly expressed in proliferating cells, the higher expression of *Asf1b* in male ECs after 3-day FoxO1

inhibition may be linked to an initial increase in proliferation caused by FoxO1 inhibition. In contrast, female EC proliferation is already reduced by the 3-day time point of FoxO1 inhibition. Therefore, *Asf1b* expression may have increased initially with FoxO1 inhibition and declined as proliferation was suppressed, resulting in similar *Asf1b* expression between control and FoxO1 inhibitor-treated conditions at this 3-day time-point. It would be interesting for future work to assess *Asf1b* mRNA levels in 1-day and 6-day FoxO1 inhibitor-treated ECs to fully capture the dynamics of *Asf1b* expression.

4.6 Significance of Thesis

My thesis provides insights into the sex-specific regulation and function of FoxO1, a key transcription factor regulating EC function. Understanding drivers of sex differences in EC function is a topic that holds considerable implications for understanding cardiovascular health disparities between men and women. Previous work in our lab found that female mice under obesogenic conditions have greater adipose angiogenesis and healthier adipose tissue expansion than male mice (Rudnicki et al., 2018). Further investigation unveiled a sex-distinct dependence of female adipose ECs on FoxO1, establishing FoxO1 as a key contributor to sex differences in adipose vascularization and obesity-driven metabolic dysfunction (Pislaru, 2025). In my thesis, I assessed upstream regulators of FoxO1 to uncover potential mechanisms leading to greater FoxO1 in female ECs. My findings revealed that male and female ECs show similar sensitivity to insulin signalling, a major regulatory pathway governing FoxO1 activity. Notably, while insulin sensitivity has been well-studied at the whole-body level and other cell types, my findings are the first to define insulin sensitivity in male and female ECs at the level of AKT phosphorylation. In addition, my thesis identified sex differences in other regulators of FoxO1, MDM2 and p38, where female ECs had higher levels of both MDM2 and p-p38. Further investigation into specific

regulatory impacts of these proteins on FoxO1 may elucidate sex differences in MDM2-mediated ubiquitination or p-p38 mediated phosphorylation and nuclear localization of FoxO1. Moreover, assessments of subcellular localization of FoxO1 revealed greater nuclear FoxO1 protein levels in female ECs. This difference in localization may support the higher levels of FoxO1 transcriptional activity that were previously observed in female ECs (Pislaru, 2025). Finally, my thesis sets the stage for assessing the impact of FoxO1 activity on epigenetic regulation and transcriptomic profiles. Preliminary findings observed here support a sex-distinct role of FoxO1 in regulating nucleosome organization genes. By investigating the regulation and function of FoxO1 in ECs, my thesis provides insight into the molecular mechanisms that may contribute to observed sex differences in cardiometabolic diseases.

4.7 Limitations

There were a few technical and methodological limitations that may have impacted the results from my experiments. Immunostaining experiments for FoxO1 localization proved to have low FoxO1 signal. This made it difficult to assess absolute intensities due to the higher background noise. In addition, the size of nuclei and cytoplasmic regions varied amongst individual cells in the same condition, which may have introduced confounding factors if comparing absolute intensities of FoxO1 signal. By measuring the nuclear to cytoplasmic ratios, this established a normalization within each field of view, allowing for consistent comparisons across different wells and conditions. Another limitation for the immunostaining was the asynchronous handling of male and female experimental replicates. Specifically, female ECs were plated first due to their faster growth rate compared to the male ECs which were at a lower confluency. However, it was imperative to prioritize maintaining similar confluency when plating for experiments since ECs

exhibit contact-inhibition when they reach full confluence (Lampugnani et al., 2003; Vlodavsky et al., 1978).

My study used ECs isolated from the white adipose tissue of male and female mice. It is important to acknowledge that the results observed here may not be applicable across all ECs, as these cells are highly heterogenous across different vascular beds. Indeed, studies have shown that phenotypic properties of ECs, such as their structural morphology, protein expression, and proliferative capacity, differ based on their origin in the vasculature (Augustin & Koh, 2017; Huxley et al., 2018). In addition, while mouse and humans ECs from the same organ show similarities, there are also species-specific differences, for example, in gene expression or responses to inflammatory factors as observed in brain and lung ECs, respectively (Song et al., 2020; Soroush et al., 2020). Therefore, it would be necessary to verify results obtained in this study before generalizing them to human ECs or other tissue-specific EC populations.

For qPCR experiments, the patterns of gene expression for nucleosome organization genes observed in vitro did not fully match our in vivo data. This may be due to differences in length of FoxO1 loss (3-days in culture versus 8 weeks of FoxO1 loss in vivo), as well as the method of FoxO1 depletion (FoxO1 inhibitor versus genetic FoxO1 deletion). Additionally, experiments done on cultured ECs lack the in vivo microenvironment, and factors such as those from surrounding adipose tissue may induce changes in EC phenotype in vivo that cannot be captured with isolated cultured conditions. With that said, our lab has shown that cultured ECs retain functional properties as observed in vivo. For example, female adipose ECs exhibit greater proliferation and higher FoxO1 mRNA levels both in vitro and in vivo (Pislaru, 2025; Rudnicki et al., 2023). As well, multiple studies with HUVECs cultured in the absence of sex hormones revealed sex differences in proliferation, migration, and tube formation, further supporting sex-intrinsic characteristics that

are maintained in culture (Addis et al., 2014; Boscaro et al., 2020; Lorenz et al., 2015).

It is also important to consider how sex hormones play an important role in the in vivo environment to modulate cell function and responses. Consequently, my experiments which were conducted in culture in the absence of sex hormones do not fully recapitulate in vivo conditions. With regard to this issue, previous work in our lab showed that estradiol treatment on male ECs did not affect FoxO1 protein levels (Pislaru, 2025). In addition, data from gonadectomized male and female mice revealed that loss of circulating sex hormones showed no sex-distinct effect on adipose angiogenesis (Pislaru, 2025). In fact, EC proliferation increased in both sexes, despite VEGF levels remaining unchanged by the gonadectomy (Pislaru, 2025). Overall, although I cannot discount the impact of the lack of sex hormones on ECs, there is compelling evidence that cell-intrinsic properties are retained both in culture and in vivo, despite the absence of sex hormones.

For subcellular fractionation experiments, phosphorylated levels of FoxO1 were normalized to each fraction's loading control rather than total FoxO1 levels. Ideally, it is valuable to normalize protein modifications to the total protein levels to account for differences in total protein availability that may otherwise influence the observed modification levels. This would provide insight into the proportion of modified protein relative to total protein levels in the cell. However, due to the poor nuclear signals for FoxO1 after stripping the membrane, it was necessary to measure total FoxO1 levels on a separate blot from pFoxO1. Thus, normalization of pFoxO1 and FoxO1 were done relative to the loading controls for the nuclear and cytoplasmic fractions, which allowed for more accurate interpretations of pFoxO1 levels, considering the limitation of being unable to consistently detect FoxO1 on the same blot. Another concern was determining which bands to quantify for pSer256-FoxO1 levels. Depending on which antibody was used (Cell signaling or Abcam) or the sample preparation method (whole cell or subcellular fractionation),

multiple bands of different molecular weights could be observed for pSer256-Foxo1, some of which differed from the predicted molecular weight (82kDa). This inconsistency made it difficult to identify which bands were specific for pSer256-FoxO1. In addition, the cell signalling pSer256-FoxO1 antibody is known to cross-react with FoxO4 phosphorylated at Ser193, whose observed molecular weight appears around 65-70kDa. Thus, for whole cell measurements of pSer256-FoxO1 levels, I quantified the bands that showed variation across the conditions and tended to reflect the well-established effects of serum starvation and insulin signalling on FoxO1 phosphorylation. The molecular weights of these bands were used as a reference when deciding which bands to quantify for nuclear-cytoplasmic fractionations. However, the molecular weights of pSer256-FoxO1 bands also varied between the nuclear and cytoplasmic compartments, which may be due to differences in modifications to the protein within each compartment. Thus, I quantified the bands around the predicted molecular weights of FoxO1 and pSer256-FoxO1 (70kDa and 82kDa, respectively), excluding the bands substantially above this range.

The FoxO1 inhibitor AS1842856 has been shown to prevent the ability of FoxO1 to activate transcription (Nagashima et al., 2010); however, to my knowledge, no studies have directly shown that the inhibitor prevents FoxO1 from binding to the DNA. Considering the potential role of FoxO1 as a pioneer factor, it is unclear whether the use of the inhibitor prevents FoxO1 from binding to closed chromatin and stimulating chromatin opening. Therefore, it remains uncertain how this will impact our ability to assess its role as pioneer factor through analyses of the ATAC-, ChIP-, and RNA-seq data. In addition, the use of pharmacological inhibitors may come with off-target effects. For example, AS1842856 was also found to inhibit glycogen synthase kinase-3 (GSK3) in a B-cell acute lymphoblastic leukemia cancer cell line as evidenced by its downregulation in cells treated with AS1842856 (70nM for 24h and 48h) but not in a FoxO1

genetic deletion model (Ketzer et al., 2025). Its direct inhibition of GSK3 was also demonstrated using an in vitro kinase assay that showed inhibition of GSK3 kinase activity with AS1842856 treatment (100nM for 24h) (Ketzer et al., 2025). Another study showed that AS1842856 (10μM) inhibited FOXO3- and FOXO4- dependent reporter gene expression in HEK293 cells (Y.-K. Lee et al., 2021). Based on these studies, I cannot exclude the possibility of off-target effects influencing the observed results from my experiments involving FoxO1 inhibitor. However, several prior observations in our lab support FoxO1-specific effects based on similar results obtained using siRNA against FoxO1. For example, DNA damage and nuclear size were greater in female ECs regardless of whether FoxO1 inhibitor or siRNA were used (Pislaru, 2025). Therefore, this argues against substantial off-target effects of FoxO1 inhibitor at the concentration used in my experiments.

4.8 Future Directions

In my study, I only assessed AKT-mediated phosphorylation of FoxO1 in male and female ECs. However, FoxO1 is regulated by multiple post-translation modifications that affect its functional activity. For example, FoxO1 activity can also be modified through acetylation, where CBP-p300-mediated acetylation of FoxO1 at lysines 245, 248, and 262 reduces FoxO1 DNA binding affinity in vitro and promotes FoxO1 phosphorylation by AKT (Brent et al., 2008; Matsuzaki et al., 2005). Furthermore, the activity of E3 ubiquitin ligases like MDM2 and Skp2 that interact with FoxO1 may lead to differential ubiquitination levels in male and female ECs and affect the rate of proteasomal degradation of FoxO1 (Fu et al., 2009; Huang et al., 2005). A recent post-translational modification that has been implicated in the regulation of FoxO1 is lysine lactylation (Deng et al., 2026). Lactate treatment was shown to promote nuclear translocation of FoxO1 in human aortic ECs, although the exact mechanisms by which FoxO1 nuclear translocation was mediated by

lactylation remain unclear (Deng et al., 2026). Overall, assessing levels of other post-translational modifications will lead to a more comprehensive understanding of how FoxO1 levels and activity are regulated in male and female ECs and in response to various physiological and pathological stimuli.

Our lab previously established a sex-distinct role of FoxO1 in ECs, where FoxO1 loss causes deleterious effects in female ECs that are not observed in male ECs (Pislaru, 2025). To explore molecular mechanisms for the differential impact of FoxO1, an objective that I had initially was to investigate sex differences in FoxO1 gene targets to gain insight into the transcriptional role of FoxO1 in male versus female ECs. This could be achieved through techniques such as ChIP or CUT&RUN (cleave under targets and release using nuclease) that measure protein-DNA interactions; however, we did not have high confidence in the efficacy of the FoxO1 antibody in these types of assays where results are highly dependent on the quality of the antibody. As an alternative, I helped prepare samples for ATAC, ChIP for H3K27ac, and RNA sequencing of control and FoxO1 inhibitor-treated adipose ECs (see Appendix for methods). FoxO1 inhibitor AS1842856 is known to prevent the transcriptional activation of genes by FoxO1 (Nagashima et al., 2010). If FoxO1 inhibitor also prevents FoxO1 binding to DNA, then given the potential pioneer factor function of FoxO1, FoxO1 inhibition may lead to global sex-distinct changes in chromatin accessibility. Furthermore, information from ChIP for H3K27ac will provide an additional layer of knowledge to the ATAC dataset by identifying which regions of open chromatin are actively primed for transcription. If, for example, female ECs experience a greater reduction in H3K27ac marks after FoxO1 inhibition compared to male ECs, then this may suggest a greater contribution of FoxO1 in maintaining active chromatin states in female ECs. Moreover, our lab has not yet conducted RNA-seq on cultured ECs or in ECs where FoxO1 inhibitor was employed

to induce FoxO1 loss. Therefore, these data will provide complementary evidence of the impact of FoxO1 loss in ECs alongside our previous RNA-seq data from freshly isolated adipose ECs with EC-specific genetic deletion of FoxO1 (Pislaru, 2025). In sum, these experiments would allow me to assess the dependence of male and female ECs on FoxO1-mediated transcriptional regulation at the level of chromatin accessibility, active regulatory elements, and gene expression. Although this project is underway and the samples for all three assays have been sent for sequencing, the sequencing results have not yet been received, so I have not been able to conduct analyses to include in my thesis. This will be a future direction that will provide important insights into the functional role of endothelial FoxO1 in shaping male and female sex differences.

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Appendix

6.1 Supplementary methods

6.1.a ATAC sample preparation

Subcutaneous and visceral adipose ECs were extracted and purified from age-matched male and female FVB mice (3 months old, n=5 per sex). Approximately 200,000 cells (passage 2) were seeded onto gelatin-coated 6-well plates. The next day, cells were treated with vehicle (DMSO) or FoxO1 inhibitor (1μM) for 3 days. ATAC-seq library preparation was conducted using the ATAC-Seq Kit from Active Motif (#53150) according to the manufacturer's protocol. Briefly, cells were trypsinized and counted to yield 100,000 cells per reaction. The tagmentation reaction with hyperactive Tn5 transposase was performed for 30 min at 37ºC on a thermocycler with occasional pipetting to mix. After purification, DNA was amplified for 10 cycles using indexed primers and size-selected using SPRI bead solution (1.2X sample volume). A quality control was performed to verify the size distribution and DNA concentration of the prepared libraries prior to sequencing.

6.1.b ChIP sample preparation

ChIP samples were prepared using an established protocol for cultured cells (Schmidt et al., 2009). Subcutaneous and visceral adipose ECs were extracted and purified from age-matched male and female FVB mice (3 months old, n=5 per sex). Approximately 500,000 cells (passage 2) were plated into gelatin-coated T25 flasks. The next day, cells were treated with vehicle (DMSO) or FoxO1 inhibitor (1μM) for 3 days. Cells were fixed in 4ml of fresh 1% formaldehyde solution for 10min at room temperature with gentle rocking. Afterwards, 200uL of 2.5mM glycine was added to quench formaldehyde. Cells were rinsed twice with ice cold PBS containing 1X protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, Sigma Aldrich, USA) and transferred to 15mL canonical tubes for centrifugation for 4 min at 2000 × rcf. Cell pellets were frozen in liquid nitrogen

and stored at -80º C until further processing by the Wilson lab at SickKids.

6.1.c RNA-seq sample preparation

Subcutaneous and visceral adipose ECs were extracted and purified from age-matched male and female FVB mice (3 months old, n=5 per sex). Approximately 25,000 cells (passage 2) were plated onto gelatin-coated 48-well plates. The following day, cells were treated with vehicle (DMSO) or FoxO1 inhibitor (1μM) for 3 days. RNA was extracted using RNeasy Mini Kits (Qiagen Inc.), and RNA quality was assessed using the RNA 6000 Nano chip on the 2100 Bioanalyzer automated electrophoresis system (Agilent Technologies Inc.). RNA library prep and sequencing was conducted at The Centre for Applied Genomics, Toronto.