Effects of Tripeptide IRW (Ile-Arg-Trp) on metabolism, mitochondrial biogenesis, and lifespan extension

by

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ABSRACT

Bioactive tripeptide IRW (Ile-Arg-Trp) was initially identified as an ACE (angiotensin-converting enzyme) inhibitory peptide from egg protein ovotransferrin. The pharmacological spectrum of IRW extends towards metabolic boosting and anti-diabetic properties as well. The overall objectives of this thesis were to understand the anti-aging effects of IRW via the study of its impact on the NAD axis, mitochondrial biogenesis, and lifespan extension.

Firstly, treatment of muscle (L6) cells with IRW increased intracellular NAMPT protein levels and boosted NAD^+. Both immunoprecipitation and recombinant NAMPT assays indicated the possible NAMPT activating ability of IRW. Similarly, IRW increased NAMPT mRNA and protein levels in liver and muscle tissues of C57BL/6J mice fed a high-fat diet (HFD). A significant increased level of circulating NAD^+ was also observed following IRW treatment. Dosing of *Drosophila melanogaster* with IRW elevated both D-NAAM (fly NAMPT) and NAD^+ *in vivo*. However, IRW treatment did not boost NAMPT levels in SIRT1 KO cells, indicating a possible SIRT1-dependency for the pharmacological effect. Overall, these data indicate that IRW is a novel small peptide booster of the NAMPT pool.

Next, IRW was also investigated for its impact on mitochondrial biogenesis. Results showed that IRW activates mitochondrial biogenesis resulting in increased mitochondrial DNA, ATP surge, improved metabolic and microbiome function. IRW activated PGC1α, the master regulator of mitochondrial biogenesis, in multiple cell lines and tissues of C57BL/6J HFD mice. It also increased mitochondrial DNA in muscles of aged *Drosophila* fed with IRW for a week. The CRISPR-Cas9 experiments elucidated the underlying mechanism as FAM120B dependent, a

constitutive activator of peroxisome proliferator activated receptor gamma (PPARγ). To the best of our knowledge, IRW is the first bioactive peptide to induce mitochondrial biogenesis *in vitro* and *in vivo*.

Finally, the ability of IRW to extend lifespan was evaluated using the *Drosophila* model *in vivo*. Different fly lines, such as *w*, *wy*, and W*dah* were fed regular fly medium supplemented with IRW. IRW treatment at 50 and 100 μM concentrations prolonged the median life span of white mutant (*w*) by 5.1 and 12.08% (respectively) and of yellow mutant (*wy*) by 12.1 and 22.9% (respectively). Likewise, midlife IRW feeding in W*dah* flies improved lifespan significantly as well. Also, IRW treatment at these concentrations significantly improved the histone markers in flies and activated the expression of multiple gene pathways involved in sirtuins (SIRT1), antioxidant defense (SOD2), autophagy (ATG7), and insulin signaling (dInR). Together, our study identifies the first bioactive peptide with the ability to extend lifespan *in vivo* and suggests an important prospective role of IRW intake for healthy aging in humans.

Overall, this research demonstrated the potential of IRW as an anti-aging functional food due to its role in promoting NAD^+ levels and mitochondrial biogenesis leading to extension in lifespan.

PREFACE

This thesis contains original research work conducted by Khushwant Singh Bhullar and has been written according to the guidelines for a paper format thesis of the Faculty of Graduate Studies and Research at the University of Alberta, Canada. The overall concept of this research work was developed under the guidance of my supervisor Dr. Jianping Wu and the research was funded by The Natural Science and Engineering Research Council of Canada (NSERC). The animal experimental protocol was approved by the Animal Care and Use Committee of the University of Alberta (Protocol# 00001402) in accordance with guidelines issued by the Canadian Council on Animal Care.

The thesis has been divided into of six chapters: Chapter 1 provides a general introduction on the context and the objectives of the thesis; Chapter 2 is a literature review on several topics relevant to this thesis, including NAD^+ metabolism, mitochondria, and their biogenesis, NAD+ and mitochondrial convergence in aging, peptides modulating the NAD axis and mitochondria and current therapies, and the potential use of bioactive peptides in mitigation of aging; Chapter 3 contains the work on *in vitro* and *in vivo* ability of IRW to boost NAMPT and NAD levels, which has been published as Bhullar *et al* 2021, "Tripeptide IRW Upregulates NAMPT Protein Levels in Cells and Obese C57BL/6J Mice" in *Journal of Agricultural and Food Chemistry, 69(5), pp.1555-1566);* Chapter 4 explores the ability of IRW to initiate mitochondrial biogenesis and underlying mechanisms of IRW-stimulated mitochondrial biogenesis; Chapter 5 investigates the effects and mechanisms of IRW on lifespan extension in *Drosophila in vivo*; and Chapter 6 gives concluding remarks and discusses future research directions.

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Dedications

Father Kirpal (the Merciful) has ordered thus: Whatever a child wants, he shall be given.

(Guru Arjan Dev Ji)

Dedicated to Biji Surinder Kaur Ji, at whose lotus feet

the writer imbibed sweet elixir of

Holy Naam-The Word

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LIST OF ABBREVIATIONS USED

12S	Mitochondrially encoded 12S ribosomal RNA
ACE	Angiotensin converting enzyme
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ATG7	Autophagy related 7
ATP	Adenosine triphosphate
ATP5a	Mitochondrial membrane ATP synthase (F(1)F(0) ATP synthase or Complex V)
BMI	Body Mass Index
Cas9	CRISPR-associated protein 9
CAT	Catalase
CD38	Cluster of differentiation 3
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
COX	Cytochrome c oxidase
CR	Caloric restriction
CRISPR	Clustered regularly interspaced short palindromic repeats
Cyt B	Cytochrome b
DBC1	Deleted in breast cancer 1
dInR	Drosophila insulin receptor
DMEM	Dulbecco's Modified Eagle's Medium

DNA	Deoxyribonucleic acid
D-NAAM	Drosophila Nicotinamidase
eNOS	Endothelial nitric oxide synthase
FAM120B	Family with Sequence Similarity 120B
FOXO3a	Forkhead box O3
gRNAs	Guide RNAs
HFD	High-fat diet
HFD	High fat diet
ILPs	Insulin-like peptides
IRW	Ile-Arg-Trp
KAT2A	Histone Acetyltransferase KAT2A
KO	Knockout
LFD	Low fat diet
MEF2A	Myocyte Enhancer Factor 2A
mRNA	Messenger RNA
MT-ATP	Mitochondrially encoded ATP synthase membrane subunit
mTOR	Mechanistic target of rapamycin
NAD	Nicotinamide Adenine Dinucleotide
NAM	nicotinamide
NAMPT	Nicotinamide phosphoribosyltransferase
ND	NADH (Nicotinamide Adenine Dinucleotide) Dehydrogenase
NMN	nicotinamide mononucleotide
NMRK2	Nicotinamide Riboside Kinase 2

NO	Nitric oxide
NQO1	NAD(P)H Quinone Dehydrogenase 1
NR	nicotinamide riboside
NRF1	Nuclear respiratory factor 1
NRF2	Nuclear factor erythroid 2-related factor 2
NSDHL	NAD(P) Dependent Steroid Dehydrogenase-Like
OXPHOS	Oxidative phosphorylation
PARP	poly (ADP-ribose) polymerase
PGC1α	Peroxisome proliferator activated receptor gamma coactivator 1 alpha
PNC1	pyrazinamidase/nicotinamidase 1
PPARγ	Peroxisome proliferator-activated receptor gamma
RHEB	Ras homolog enriched in brain
RNA	Ribonucleic acid
RT	Reverse Transcriptase
SD	Standard Deviation
SIRT1	silent mating type information regulation 2 homolog 1
SOD2	Superoxide dismutase 2, mitochondrial
STACs	Sirtuin-activating compound
T2D	Type 2 diabetes
TFAM	Transcription factor A, mitochondrial
TIMM	Mitochondrial import inner membrane translocase subunit
TOMM	Mitochondrial import receptor subunit
µM	Micromolar (10^{-6} mol/L)

CHAPTER 1 – General Introduction and Objectives

1.1 General Introduction

Aging, characterized by a progressive loss of physiological integrity is the leading risk factor for many diseases including metabolic disorders, vision loss, cancer, immune dysfunction, etc. (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013). Among hallmarks of aging, NAD^+ decline and mitochondrial dysfunction are the key hallmarks of the aging process (Campisi, 2013). Levels of NAD^+ are reduced during aging and in associated metabolic disorders. This decline during aging leads to worsening of metabolic disorders, mitochondrial dysfunction, and oxidative damage (Chini, Tarragó, & Chini, 2017; Gomes et al., 2013). Owing to the convergence of NAD^+ and mitochondria in metabolic function, the NAD^+ decline is further exacerbated by mitochondrial dysfunction (Stein & Imai, 2012). Mitochondria, well known as "the powerhouse" of the cell, reflecting their vital role as the primary bioenergetic source remarkably influence aging and related decline in cellular functions (Natarajan et al., 2020). The quality and quantity of mitochondria are crucial to maintaining energy production and redox homeostasis, however, this balance is disrupted during the aging process (Sreedhar, Aguilera-Aguirre, & Singh, 2020). Further, the reduced efficiency of mitochondrial bioenergetics and NAD^+ homeostasis with aging may also accompany the reduced biogenesis of mitochondria (Rodgers, Lerin, Haas, Gygi, Spiegelman, & Puigserver, 2005; Stein et al., 2012). Currently, there is a strong research effort to identify bioactive molecules with the ability to boost NAD^+ levels and improve mitochondrial dynamics.

Both natural bioactives and synthetic molecules have been developed to boost NAD and mitochondrial function and mitigate the aging and metabolic decline. Plant polyphenols such as

butein, piceatannol, isoliquiritigenin, piceatannol, resveratrol along with synthetic polyphenols such as SRT1720, SRT2104, 1,4-DHP derivative, and UBCS039 have been tested for their anti-aging activity (Dai, Sinclair, Ellis, & Steegborn, 2018). One group of natural bioactives which have received recent attention for anti-aging activity are bioactive peptides (Bhullar & Wu, 2020). Bioactive peptides containing 2 to 20 amino acids are well known to extend a beneficial effect on human health beyond their known nutritional value (Kitts & Weiler, 2003). Multiple food derived bioactive peptides improve metabolic function and mitigate mitochondrial dysfunction, the two hallmarks of aging and associated morbidities (Kitts et al., 2003). On the metabolic front, bioactive peptides exhibit hypocholesterolemic activity (Pak, Koo, Kasymova, & Kwon, 2005), activate the AMPK pathway (Lammi, Zanoni, & Arnoldi, 2015), lower hyperglycemia (Zambrowicz et al., 2015), and extend cardioprotection (Majumder & Wu, 2010, 2011). Likewise, bioactive peptides lower oxidative stress (Rizzello, Lorusso, Russo, Pinto, Marzani, & Gobbetti, 2017) and activate antioxidant enzymes (He, Wang, Yang, Wang, Ju, & Yuan, 2019; You, Zhao, Regenstein, & Ren, 2011). Therefore, exploring the bioactive peptides with the ability to improve mitochondrial function and NAD^+ levels is a rationale research approach.

One particularly promising peptide is IRW (Ile-Arg-Trp), initially identified as an inhibitor of angiotensin converting enzyme (ACE) from egg white protein ovotransferrin (Majumder et al., 2011). IRW is also a robust antioxidant peptide with the ability to ease oxidative stress *in vivo* (Wu, 2020). The unique feature of IRW to increase endothelial nitric oxide synthase (eNOS) supports its vasodilatory properties (Majumder et al., 2013). With its ability to activate the NO pathway, a critical initiator of mitochondrial biogenesis, IRW can be explored for its ability to activate mitochondrial biogenesis (Nisoli et al., 2003). Given the critical role of NAD+ in the

sphere of IRW's biological activity (Koch et al., 2018), we hypothesized that IRW can increase NAMPT pool and consequently improve NAD^+ levels.

1.2 Objectives and Hypothesis

Given the above background, we hypothesized that egg white ovotransferrin derived tripeptide IRW can promote metabolic and mitochondrial health through NAD boosting and an increase in mitochondrial number. The overall objectives of this research are to understand the impact of IRW on the NAD^+/NAMPT axis and to explore its potential application as an activator of mitochondrial biogenesis. The specific objectives of the research are:

1) To investigate the *in vitro* and *in vivo* potential of IRW on NAD^+/NAMPT axis

2) To investigate the *in vitro* and *in vivo* effects of IRW on mitochondrial biogenesis

3) To study the mechanisms involved in the IRW-stimulated mitochondrial biogenesis; and

4) To study the *in vivo* efficacy of IRW on lifespan in *Drosophila melanogaster*.

1.3 Chapter Format

There are six chapters in the thesis and the brief description of each chapter are given as follows:

Chapter 1 gives a brief introduction on metabolism and mitochondria and the growing interests in developing bioactives to promote overall metabolic and mitochondrial health. Following with the general introduction, the thesis objectives and hypothesis are descried, and the chapter format is introduced.

Chapter 2 provides a literature review on the current knowledge of the NAD^+ pathway and its regulation, metabolic and aging relevance of NAD^+, therapeutic NAD^+ boosting, mitochondria and mitochondrial biogenesis, small molecule for aging, bioactive peptides, and their nutraceutical use. In the end, the perspectives on developing IRW and its potential use as anti-aging treatment are summarized.

Chapter 3 reports the *in vitro* and *in vivo* effect of IRW on stimulating NAD and NAMPT activity. Immortalized rat skeletal (L6) myoblast cells were used to investigate effects of IRW on NAMPT protein and NAD^+ pools. The effects of IRW on *in vivo* levels of NAD and NAMPT were analyzed using C57BL/6J mice fed a high fat diet (HFD) in combination with IRW. The effects on these metabolic markers were characterized by immunoprecipitation assay, immunoblotting, ELISA, and qPCR analysis. Objective 1 is addressed in this chapter.

Chapter 4 elucidates the ability of IRW to initiate mitochondrial biogenesis *in vitro* and *in vivo*. Multiple cells lines including A7R5, L6, 293T were used while *in vivo* levels of mitochondrial biogenesis were analyzed using C57BL/6J mice fed a high fat diet (HFD) in combination with IRW. Western blots accompanied by biochemical techniques such as ELISA, flowcytometry, microscopy and qPCR were used to study the activation of signaling pathways, including PGC1α and related pathways. Microbiome analysis was conducted to study gut microbiota changes induced by IRW. CRISPR-Cas9 and click chemistry were used to elucidate and validate the peptide target pathways *in vitro* in 293T cells. Objectives 2 and 3 are addressed in this chapter.

Chapter 5 reports the *in vivo* effects of ovotransferrin on lifespan extension in *Drosophila melanogaster*. *Drosophila* species such as *w*, *wy*, and W^{dah} flies were used to access the impact of IRW on lifespan (days), key histone markers, and genes associated with lifespan extension.

Western blots accompanied by qPCR were used to study the activation of signaling pathways associated with lifespan extension. Objective 4 is addressed in this chapter.

Chapter 6 presents a general summary on the key findings and the significance of this study. The recommended future studies are outlined according to the limitations and challenges of this research.

1.4 References

Bhullar, K. S., & Wu, J. (2020). Dietary peptides in aging: Evidence and prospects. *Food Science and Human Wellness, 9*(1), 1-7.

Campisi, J. (2013). Aging, cellular senescence, and cancer. *Annual review of physiology, 75*, 685-705.

Chini, C. C., Tarragó, M. G., & Chini, E. N. (2017). NAD and the aging process: Role in life, death and everything in between. *Molecular and cellular endocrinology, 455*, 62-74.

Dai, H., Sinclair, D. A., Ellis, J. L., & Steegborn, C. (2018). Sirtuin activators and inhibitors: promises, achievements, and challenges. *Pharmacology & therapeutics, 188*, 140-154.

Gomes, A. P., Price, N. L., Ling, A. J., Moslehi, J. J., Montgomery, M. K., Rajman, L., . . . Hubbard, B. P. (2013). Declining NAD+ induces a pseudohypoxic state disrupting nuclear-mitochondrial communication during aging. *Cell, 155*(7), 1624-1638.

He, R., Wang, Y., Yang, Y., Wang, Z., Ju, X., & Yuan, J. (2019). Rapeseed protein-derived ACE inhibitory peptides LY, RALP and GHS show antioxidant and anti-inflammatory effects on spontaneously hypertensive rats. *Journal of Functional Foods, 55*, 211-219.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current pharmaceutical design, 9*(16), 1309-1323.

Koch, C., Fitch, A., Simon, M., Qin, S., Leo, N., Methe, B., . . . Morris, A. (2018). Alterations in Tryptophan Metabolism and Nicotinamide Adenine Dinucleotide (NAD) Synthesis in Individuals with Pulmonary Arterial Hypertension. In *A63. RIDE THE LIGHTNING: BASIC AND TRANSLATIONAL STUDIES IN PULMONARY HYPERTENSION* (pp. A2104-A2104): American Thoracic Society.

Lammi, C., Zanoni, C., & Arnoldi, A. (2015). Three peptides from soy glycinin modulate glucose metabolism in human hepatic HepG2 cells. *International journal of molecular sciences, 16*(11), 27362-27370.

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., & Kroemer, G. (2013). The hallmarks of aging. *Cell, 153*(6), 1194-1217.

Majumder, K., Chakrabarti, S., Morton, J. S., Panahi, S., Kaufman, S., Davidge, S. T., & Wu, J. (2013). Egg-derived tri-peptide IRW exerts antihypertensive effects in spontaneously hypertensive rats. *PloS one, 8*(11), e82829.

Majumder, K., & Wu, J. (2010). A new approach for identification of novel antihypertensive peptides from egg proteins by QSAR and bioinformatics. *Food Research International, 43*(5), 1371-1378.

Majumder, K., & Wu, J. (2011). Purification and characterisation of angiotensin I converting enzyme (ACE) inhibitory peptides derived from enzymatic hydrolysate of ovotransferrin. *Food Chemistry, 126*(4), 1614-1619.

Natarajan, V., Chawla, R., Mah, T., Vivekanandan, R., Tan, S. Y., Sato, P. Y., & Mallilankaraman, K. (2020). Mitochondrial dysfunction in age-related metabolic disorders. *Proteomics, 20*(5-6), 1800404.

Nisoli, E., Clementi, E., Paolucci, C., Cozzi, V., Tonello, C., Sciorati, C., . . . Moncada, S. (2003). Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science, 299*(5608), 896-899.

Pak, V., Koo, M., Kasymova, T., & Kwon, D. (2005). Isolation and identification of peptides from soy 11S-globulin with hypocholesterolemic activity. *Chemistry of natural compounds, 41*(6), 710-714.

Rizzello, C. G., Lorusso, A., Russo, V., Pinto, D., Marzani, B., & Gobbetti, M. (2017). Improving the antioxidant properties of quinoa flour through fermentation with selected autochthonous lactic acid bacteria. *International journal of food microbiology, 241*, 252-261.

Rodgers, J. T., Lerin, C., Haas, W., Gygi, S. P., Spiegelman, B. M., & Puigserver, P. (2005). Nutrient control of glucose homeostasis through a complex of PGC-1α and SIRT1. *Nature, 434*(7029), 113-118.

Sreedhar, A., Aguilera-Aguirre, L., & Singh, K. K. (2020). Mitochondria in skin health, aging, and disease. *Cell death & disease, 11*(6), 1-14.

Stein, L. R., & Imai, S.-i. (2012). The dynamic regulation of NAD metabolism in mitochondria. *Trends in Endocrinology & Metabolism, 23*(9), 420-428.

Wu, J. (2020). A Novel Angiotensin Converting Enzyme 2 (ACE2) Activating Peptide: A Reflection of 10 Years of Research on a Small Peptide Ile-Arg-Trp (IRW). *Journal of Agricultural and Food Chemistry, 68*(49), 14402-14408.

You, L., Zhao, M., Regenstein, J. M., & Ren, J. (2011). In vitro antioxidant activity and in vivo anti-fatigue effect of loach (Misgurnus anguillicaudatus) peptides prepared by papain digestion. *Food Chemistry, 124*(1), 188-194.

Zambrowicz, A., Pokora, M., Setner, B., Dąbrowska, A., Szołtysik, M., Babij, K., . . . Chrzanowska, J. (2015). Multifunctional peptides derived from an egg yolk protein hydrolysate: isolation and characterization. *Amino acids, 47*(2), 369-380.

CHAPTER 2 - Literature Review

2.1 Nicotinamide adenine dinucleotide

Nicotinamide adenine dinucleotide (NAD^+), an essential coenzyme for redox reactions, was first described in 1906 as a factor that could enhance the fermentation rate in yeast (Harden & Young, 1906). Later, it was reported to be involved in hydrogen transfer in redox reaction (Warburg & Christian, 1936). Chemically, as a vital redox carrier, NAD^+ receives hydride (the anion of hydrogen H^-) from metabolic processes including glycolysis, the TCA cycle, and fatty acid oxidation (FAO) to form NADH, a central hydride donor involved in ATP synthesis (Liana Roberts Stein & Imai, 2012). Apart from energy metabolism, NAD^+ presents itself as a co-substrate for various enzymes including sirtuins, PARPs, and NADases (CD157, CD38, and SARM1) (Chambon, Weill, & Mandel, 1963; Frye, 1999; Imai, Armstrong, Kaeberlein, & Guarente, 2000; Landry et al., 2000). NAD^+ is highly compartmentalized and independently regulated in its main subcellular pools *viz.* the cytoplasm, mitochondria, and nucleus (Cambronne & Kraus, 2020). Therefore, the dynamic NAD^+ impacts an assortment of cellular processes and pathophysiological conditions, and post-synthesis modification of fundamental biomolecules, including DNA, RNA, and proteins (Cahová, Winz, Höfer, Nübel, & Jäschke, 2015; Y. G. Chen, Kowtoniuk, Agarwal, Shen, & Liu, 2009; Walters, Matheny, Mizoue, Rao, Muhlrad, & Parker, 2017).

NAD^+ biosynthesis takes place *de novo* from tryptophan via the kynurenine pathway (KP) or from vitamin precursors, such as nicotinic acid (NA), via the Preiss–Handler pathway or via the salvage pathway (Figure 2.1). The *de novo* generation of NAD^+ from dietary tryptophan (W) by

the KP is initialized by the rate-limiting enzyme indoleamine 2,3-dioxygenase (IDO) or the rate-limiting enzyme tryptophan 2,3-dioxygenase (TDO) (Katsyuba et al., 2018). The downstream product of this cycle, α-amino-β-carboxymuconate ε-semialdehyde (ACMS), can spontaneously condense to quinolinic acid (QA) (Badawy, 2017). QA can be transformed by quinolinate phosphoribosyltransferase (QPRT) into nicotinamide mononucleotide (NAMN), at which point it converges with the Preiss–Handler pathway (Youn et al., 2016). The Preiss-Handler pathway can convert dietary nicotinic acid (NA) absorbed via SLC5A8 or SLC22A13 transporters to nicotinamide mononucleotide (NAMN) by nicotinic acid phosphoribosyltransferase (NAPRT). The NAMN is subsequently transformed into nicotinic acid adenine dinucleotide (NAAD) by nicotinamide mononucleotide adenylyltransferases (NMNAT1, NMNAT2, and NMNAT3) (Marletta, Massarotti, Orsomando, Magni, Rizzi, & Garavaglia, 2015). The NAAD is finally amidated to NAD^+ by NAD synthase (NADSYN) using glutamine as nitrogen donor (Brazill, Li, Zhu, & Zhai, 2017). The third pathway i.e., the salvage pathway is most often used by the cell to maintain the cellular NAD^+ levels via recycling from nicotinamide (NAM), nicotinamide riboside (NR), and nicotinamide mononucleotide (NMN) (Braidy et al., 2019). The precursor NR changes to NMN and is ultimately adenylylated by NMNAT to yield NAD^+(Zhou et al., 2002).

Subcellular distribution of NAD^+ exists in both the oxidized NAD(P)+ and the reduced NAD(P)H. The NAD^+ concentration in cell lines including HEK293T, U2OS, and HeLa ranges from 40 to 70 μM (Cantó, Menzies, & Auwerx, 2015; Sallin, Reymond, Gondrand, Raith, Koch, & Johnsson, 2018). Detailed analysis of U2OS cells revealed a concentration of ~70 μM for cytoplasmic NAD^+, ~110 μM for nuclear NAD^+, and ~90 μM for mitochondrial NAD+, respectively (Sallin et al., 2018). The comparable diminution of NAD^+ in the cytoplasm endorses a probable exchange of NAD^+ between these cellular compartments, while a higher concentration

of mitochondrial NAD^+ suggests its segregation from the cytosolic and nuclear NAD^+ pools (Houtkooper, Cantó, Wanders, & Auwerx, 2010). Further, some evidence indicates that mammalian mitochondria are competent in transporting in NAD^+ as well as its precursors, such as NMN and NR (Cantó et al., 2012; Davila et al., 2018; Nikiforov, Dölle, Niere, & Ziegler, 2011). Further, via recycling the NAD^+, the NAD^+ pool in each cellular compartment is maintained independently (Berger, Lau, Dahlmann, & Ziegler, 2005).

2.1.1 NAMPT and its link to NAD^+ biology

Nicotinamide phosphoribosyltransferase (NAMPT) was originally proposed to be a cytokine that was a cofactor for B-cell maturation and its bacterial and murine homologues were later found as enzymes involved in NAD biosynthesis (Martin, Shea, & Mulks, 2001; Rongvaux et al., 2002; Samal, Sun, Stearns, Xie, Suggs, & McNiece, 1994). As discussed in the section above, NAD^+ can be synthesized *de novo* from tryptophan or NAD precursors such as NAM, NA, NMN, and NR. Among these NAM, the predominant NAD^+ precursor in mammals is catalyzed to NMN, thereby catalysing the rate-limiting step in the NAD^+ salvage pathway from NAM (Imai, 2009). Next, the NMN is converted into NAD^+ by nicotinamide/nicotinic acid mononucleotide adenylyltransferases 1-3 (NMNAT1-3)(Cantó et al., 2015; Imai, 2009). The ubiquitous expression of NAMPT in nearly all organs, tissues, and cells indicates its pleiotropic functions in human physiology (Friebe et al., 2011). As a vital regulator of the intracellular NAD pool, NAMPT directly influences the activity of NAD-dependent enzymes, such as sirtuins and PARPs, thus, regulating both cellular metabolism and mitochondrial biogenesis (Bowlby, Thomas, D'Agostino Jr, & Kridel, 2012; Koltai et al., 2010; Pillai, Isbatan, Imai, & Gupta, 2005).

2.1.2 Metabolic and aging relevance of NAD^+

Levels of NAD^+ are reduced in a variety of metabolic disorders and during aging. The NAD^+ levels are correlated with insulin resistance as metabolic stress (diabetes and IR) significantly weakens the role of NAMPT-regulated NAD^+ biosynthesis in metabolic organs (Yoshino, Mills, Yoon, & Imai, 2011). IR and diabetes are indicators of critical whole-body abnormal metabolism with close relation to obesity (Yach, Stuckler, & Brownell, 2006; S. Yamaguchi & Yoshino, 2017). Further, a sharp decline of NAD^+ levels in cells is observed in many tissues with obesity, including the skeletal muscles, liver, and adipose tissue (Yoshino et al., 2011). Likewise, NAMPT, the NAD^+ synthesis plays an important role in the pathogenesis of metabolic complications. Obese individuals have higher levels of NAMPT in visceral fat and serum but lower levels of NAMPT in subcutaneous fat tissue and skeletal muscles (Berndt et al., 2005; Chang, Chang, Lin, Shin, & Lee, 2011; Terra et al., 2012). Based on these findings, the effect of NAMPT on metabolism depends on its enzymatic activity and the target tissue. A boost in NAD^+ levels by enhancing NR in mouse tissues and mammalian cells activate SIRT1 and SIRT3, which ultimately leads to increased oxidative metabolism and prevents metabolic abnormalities induced by HFD (Cantó et al., 2012). The *in vivo* knockout (KO) of NAMPT in mice leads to IR which manifests as increased plasma free fatty acid content, and both were normalized by administering NMN (Stromsdorfer et al., 2016). The therapeutic potential of NMN was based on the recovery of NAD^+ levels and enhancement of energy expenditure *in vivo* (Stromsdorfer et al., 2016; Yoshino et al., 2011). Likewise, lowering of CD38, a NADase, in obese mice improves glucose intolerance with HFD, which could be further improved by supplementation of NR (Camacho-Pereira et al., 2016). Interestingly, adding the amino acid leucine to a HFD can also increase the expression of NAMPT and SIRT1 and elevate the level of NAD^+ in cells leading to deacetylation of PGC1α, thus, promoting mitochondrial biogenesis (Li, Xu, Lee, He, & Xie, 2012). Similar to metabolism, NAD^+

and its metabolites govern mechanisms related to aging decline. The decrease in NAD^+ during the aging process leads to oxidative damage, metabolic disorder and its worsening, mitochondrial dysfunction, and impacts lifespan via cell signaling pathways such as NFκB, PGC1α, and HIF1α, by sirtuins and PARP (Fang et al., 2017). Initial studies showed that NAD^+ levels depleted in aging worms which shortened the worm lifespan, an observation also made in the mice and rats (Braidy, Guillemin, Mansour, Chan-Ling, Poljak, & Grant, 2011; Fang et al., 2017). Similarly, in humans, the plasma levels of NAD^+ and its metabolites, $NADP^+$ and NAAD also decline during aging (Clement, Wong, Poljak, Sachdev, & Braidy, 2019). Levels of NAMPT expression are also severely impeded in different tissues of aging mice including liver, skeletal muscle, WAT, and pancreas (Liana R Stein & Imai, 2014; Yoshino et al., 2011). This pan-species decline in the NAD^+ levels is attributed to enhanced NAD^+ consumption in addition to reduced biosynthesis. For example, CD38-null aged mice maintain the normal NAD^+ levels and ensuing mitochondrial respiration and metabolic functions (S. Johnson & Imai, 2018). Additionally, the genetic and pharmacological replenishment of NAD^+ improves the age-related biological function and increases lifespan in worms and mice (Mouchiroud et al., 2013; H. Zhang et al., 2016). Similarly, improved expression of NAMPT in aging human smooth muscle cells prolonged lifespan while supplementation of NAD^+ precursors such as NR and NMN extended mice lifespan (de Picciotto et al., 2016; Gomes et al., 2013). Further, additional research shows that NAD^+ dependent sirtuins can prolong the lifespan of multiple organisms including yeast, worms, flies, and mice (Kanfi et al., 2012; Satoh et al., 2013). It is vital to note that aging-related NAD^+ decline is proposed to be a cause of mitochondria dysfunction as well (Gomes et al., 2013). NAD^+ supplementation triggers dual activation of mitochondrial unfolded protein response (UPRmt) and antioxidant defense leading to anti-aging activity and lifespan extension (Mouchiroud et al., 2013). The NAD+ decline

and abnormal initiation of NAD^+ consuming PARPs inhibits the SIRT1 activity and prompts mitochondrial dysfunction (Gomes et al., 2013; Scheibye-Knudsen et al., 2014). In summary, NAD^+ is emerging as a central metabolite in metabolism and aging research, and its decline and replenishment are becoming established features of metabolism and aging research.

2.1.3 Therapeutic NAD^+ boosting

Apart from pre-clinical testing in cells and mice, several clinical trials have investigated NAD^+ precursors, especially NR, to study their metabolism and aging impact (Pfluger, Herranz, Velasco-Miguel, Serrano, & Tschöp, 2008; Radenkovic & Verdin, 2020). Overall, compared to preclinical studies, clinical studies are less advanced but have demonstrated that NMN and NR administration in humans is safe (Martens et al., 2018). Noteworthy, there are more phase I clinical trials using NR than those using NMN and have been summarized in Table 1. On a positive note, multiple clinical trials showed that interim NR administration has some beneficial effects in healthy elderly individuals, obese individuals, and patients with ALS (de la Rubia et al., 2019; Dollerup et al., 2018; Dollerup et al., 2019; Martens et al., 2018). Currently there are 80 clinical trials investigating the therapeutic boosting of NAD^+ in various disease models (www.clinicaltrials.gov as accessed on May 19, 2021). However, additional clinical studies are needed to determine the proper dose and time of administration of NAD^+ or its precursor(s), long-term toxicological impact, racial and genetic diversity of participants, to better address the successful translation of the NAD^+ boosting strategy.

2.2 Mitochondria and their resurgence

Mitochondria are classically placed at the heart of eukaryotic cell metabolism. However, the emerging picture of mitochondria can be summed up as that of 'multi-facet' and 'cross-talking' structural organelles whose behavior is responsive to cellular needs. Until the 1950s, mitochondria were solely studied for their metabolic pathways, and by the 1970s their role was mainly settled to ATP generation and metabolites for macromolecule synthesis (Chandel, 2018). However, in the 1990s, seminal papers showed their role in apoptosis (X. Liu, Kim, Yang, Jemmerson, & Wang, 1996) as well in free radical(s) mediated cellular adaptation (Furukawa et al., 2017). Following this, mitochondria have been studied extensively for their versatile activities in cellular and pathological conditions (Nunnari & Suomalainen, 2012). Thus, it is now clear that mitochondria are indeed more than ATP synthesizers and function as critical signaling organelles deciding the fate of cell and organ systems (Figure 2.2).

2.2.1 Mitochondrial Biogenesis

Mitochondrial biogenesis is an intricate and adaptive response process. It requires coordinated transcription of mitochondrial genes in the nucleus, replication of the mtDNA, as well as the synthesis, and import of proteins to the existing mitochondrial reticulum (Pagliarini et al., 2008). The coordination of the two cellular genomes is achieved by nucleus-encoded mitochondrial transcription factors such as TFAM, nuclear respiratory factor (NRF)-1, peroxisome proliferator-activated receptors (PPARs), and PGC-1α, a transcriptional coactivator of NRF-1, TFAM and PPAR-γ (Kashyap et al., 2005; Nisoli et al., 2003). Following the synthesis of nuclear-encoded proteins, they are imported to mitochondria via the translocase of the outer membrane (TOM)-complex (Dolezal, Likic, Tachezy, & Lithgow, 2006). Any imbalance in the coordination of these genomes (and their protein expression) leads to proteotoxic stress and subsequent activation of

mitochondrial turnover mechanisms (Matsuda et al., 2010). Also, pathological, or age-related changes in mitochondrial biogenesis lead(s) to perturbed mitochondrial and cell function.

As stated earlier, mitochondrial biogenesis is a complex process and involves multiple factors (Figure 2.3). One of the crucial factors is NRF1, identified initially as a transcription factor binding to cytochrome c promoter (Virbasius, Virbasius, & Scarpulla, 1993). Its binding sites are evolutionarily conserved in promoters of many mitochondrial genes and also regulate expression of TFAM, and thereby coordinates the increase in mitochondrial biogenesis (Picca & Lezza, 2015). NRF1 also affects the expression of mitochondrial subunit(s) such as COXIV, and PGC-1α indirectly via the transcription factor MEF2A (Ramachandran, Yu, & Gulick, 2008). Interestingly, NRF1 also directly controls the expressions of ten nuclear-encoded mitochondrial COX subunits and three mitochondrial-encoded COX subunits indirectly via TFAM (Dhar, Ongwijitwat, & Wong-Riley, 2008). Further, NRF-1 null animals and cells, lacking a vital member of mitochondrial biogenesis cascade, exhibit reduced mtDNA content support its role in mitochondria biogenesis (Huo & Scarpulla, 2001). Although NRF-1 is obligatory for mitochondrial biogenesis, yet its sole expression is insufficient to drive this complex cellular mechanism. Three other players include PGC1α, TFAM and TOM machinery, which move the process of mitochondrial biogenesis towards a successful end. PGC-1α has emerged as a prominent regulator of mitochondrial biogenesis, thus becoming a critical metabolic node. Identified through its functional interaction with PPARγ, the expression of PGC-1α expression is finely tuned to reflect cellular energy needs, as it increases during adaptive thermogenesis, fasting and muscle exercise (Huo et al., 2001). Following this initial discovery, PGC-1α was found to activate NRF1, indicating its central role in mitochondrial biogenesis (Wu et al., 1999). Apart from the energy sensing mechanisms, PGC-1α is also activated by p38 MAPK, whose parallel activation during exercise or by overexpression

augments the expression of PGC-1α (M. Zhao et al., 1999). AMP-activated protein kinase (AMPK) is a crucial sensor of the energy status of the cell and is activated in muscle during exercise (Jäger, Handschin, Pierre, & Spiegelman, 2007). Similar to p38 MAPK, PGC-1α activity increases in muscle cells following treatment with AMPK activators, indicating crosstalk with two energy sensors (Jäger et al., 2007). TFAM also plays a vital role in mitochondrial biogenesis as it is considered as a necessary initiation of human mitochondrial transcription (Shi et al., 2012). TFAM is involved in many functions: mtDNA transcription, mtDNA maintenance, and replication (Shi et al., 2012; Uittenbogaard & Chiaramello, 2014). It is interesting to note that TFAM is considered the histone-like protein of mtDNA as a high number of bound TFAM molecules (~1000 proteins/mtDNA genome) is reported in mammalian cell lines (Chacinska et al., 2010). This histone-like feature of TFAM is proposed to help in both mtDNA replication and mtDNA packaging for biogenesis. Finally, the import of 99% of nucleus-encoded mitochondrial precursor proteins is vital for making new mitochondria. Most mitochondrial proteins are synthesized on cytosolic ribosomes and imported through the TOM complex (Shiota et al., 2015). Following passage through the TOM channel into intermembrane space, the imported proteins are used by different sorting machineries for various purposes (Harbauer et al., 2014; Shiota et al., 2015). The small TOM proteins Tom5, Tom6, and Tom7 regulate the assembly of the TOM complex and further stabilizes it (Harbauer et al., 2014). Among these small TOM proteins, TOM6 has emerged as an essential regulator of mitochondrial biogenesis. In cells, phosphorylation of the cytosolic precursor of Tom6 by Cdk1 leads to an enhanced import of Tom6 into mitochondria (Gorman et al., 2016). On the mitochondrial surface, increased Tom6 promotes assembly of the protein import channel Tom40 and import of fusion proteins, thus stimulating a direct means for increasing mitochondrial biogenesis (Gorman et al., 2016).

Research into mitochondrial physiology has been instrumental for understanding the association between dysfunctional mitochondria and general health, and disease. An isolated factor seldom causes mitochondrial dysfunction in common diseases such as obesity, hypertension, and diabetes; instead, they result from a cumulative impact of polygenic influences. Among these, impaired fitness, loss of metabolic and mitochondrial function, are underlying factors for mitochondrial dysfunction, preceding or accompanying a disease state. Hence, the multiscale regulatory networks that govern mitochondrial biogenesis present themselves as drug targets for the treatment of mitochondrial dysfunction and related disease state(s). Theoretically, there are two strategies to regulate mitochondrial bioenergetics. The first targets upstream regulators (i.e., energy and nutrient sensors like PGC-1α, mTOR, and AMPK), the second targets downstream effector pathways that respond to these regulators (i.e., transcription factors, cofactors, transporters, and nuclear receptors such as NRF1, TFAM, TOM, and COX subunits), and the cumulative effect of intervention leads to mitochondrial biogenesis.

2.2.2 Mitochondrial dysfunction and metabolism

Mitochondrial dysfunction contributes to this oxidative stress which plays a major role in the pathogenesis of metabolic syndrome, insulin resistance, and T2DM (Furukawa et al., 2017; Henriksen, Diamond-Stanic, & Marchionne, 2011). The increase of electron supply to the electron transport chain (ETC) of mitochondria as result of free radicals that arise from the nutrient excess in cells which leads to a compensatory increase in mitochondrial fatty acid oxidation, NADH, and FADH2 production from the tricarboxylic acid cycle (Wensveen, Valentić, Šestan, Turk Wensveen, & Polić, 2015). Further, amplified oxidative stress in adipocytes leads to a vicious cycle of additional deterioration in mitochondrial function by damaging ETC components and other mitochondrial constituents ultimately leading to aggravated metabolic dysfunction

(Galloway et al., 2012; Kowaltowski & Vercesi, 1999). Similar to adipocytes, mitochondria dysfunction contributes to IR in skeletal muscles as well (Montgomery & Turner, 2015; Pagel-Langenickel, Bao, Pang, & Sack, 2010). Interestingly, at the initial stage of the metabolic disease, the increased availability of free fatty acids leads to activation of the PGC1α pathway but their excess subsequently excess leads to accumulation of toxic lipids, pancreatic beta cell failure, and insulin resistance (Erion & Shulman, 2010; Ma, Zhao, & Turk, 2012; Montgomery et al., 2015). This oxidative stress can be attributed to increased metabolic demand on pancreatic β-cells due to hyperglycemia (Ma et al., 2012; Mulder & Ling, 2009). Overall, IR cells exhibit diminished mitochondrial energy production, decreased mitochondrial biogenesis, and increased vulnerability to oxidative stress (Burkart et al., 2016; Rius-Pérez, Torres-Cuevas, Millán, Ortega, & Pérez, 2020). Overall, there are two strategies that present themselves to alleviate mitochondrial function 1) enhancing metabolic capacity via NAD^+ boosting and 2) enhancing mitochondrial biogenesis. These two targets can successfully help alleviate different features associated (as discussed above) with metabolic function and help improve metabolic function.

2.3 Aging and Mitochondria

Aging, a gradual functional decline, as an idea and a scientific endeavor has riveted both scientists and philosophers throughout history. Multiple philosophers such as Plato (428–347 BC) and Giacomo Leopardi (1798–1837) argued about aging from their philosophical understandings either as a stage of supreme philosophical understanding or as an evil stage of disability (Baars, 2012). Aging is frequently characterized as the gradually advancing functional decline of organisms with time, possibly triggered by the simultaneous deterioration of various interconnected cellular functions (Nussey, Froy, Lemaitre, Gaillard, & Austad, 2013). In its broadest sense, aging

indicates changes that occur during an organisms' lifespan, while specific changes leading to increased risk of disease are termed senescence (McHugh & Gil, 2018). Senescence is, therefore, the gradual decline of bodily functions over time ultimately leading to decreased healthspan, fertility, and increased risk of disease or mortality (McHugh et al., 2018). Scientific literature indicates multiple widespread theories as to why aging takes place. These include the free radical theory of aging, changes in immunological functions, telomere shortening, and the presence of senescence genes in the DNA (Effros, 2005; Harman, 1993; Kruk, Rampino, & Bohr, 1995). However, gerontologists propose the likelihood that there may not be a single universal cause of aging. For scientific argument, most of these theories, if not all, can, however, be classified into two categories: damage theories and program hypotheses and combined theories, which contains certain elements of both groups. Of our interest, the mitochondrial damage theory highlights a prevailing idea of cellular oxidative damage (Harman, 1981). Around 2-3% of the oxygen taken up in cells is chemically reduced by the addition of single electrons, however, partial reduction of oxygen can produce an array of ROS (F. B. Johnson, Sinclair, & Guarente, 1999). The electron transport chain in the mitochondria, NADPH oxidase, and the 5-lipoxygenase are the three major sources of ROS which increase the susceptibility of mitochondrial DNA (mtDNA) to oxidative damage (Angelova & Abramov, 2018). Mitochondrial preservation is, hence, vital to safeguard cellular homeostasis and impaired mitochondrial maintenance has been described as a shared hallmark of human aging (Haas, 2019).

Since the postulation of the free radical theory of aging and its refinement to the mitochondrial theory of aging, mitochondria have been put in the limelight of aging research (Alexeyev, 2009). Both theories suggest that the cellular deterioration seen with increasing age is ROS driven, and the focal point of mitochondrial aging extends to mtDNA mutations as well.

Research evidence shows that both mtDNA point mutations and deletions accumulate with age, however, it is still unclear if these mutations are causal or just correlate with aging (Kennedy, Salk, Schmitt, & Loeb, 2013). For instance, mice with a wild-type nuclear genome with inherited maternal mtDNA mutations show signs of premature aging such as hair greying and loss, changed curvature of the spine, reduced body size, and premature death (Ross et al., 2013). Although multiple mtDNA mutations occur, most pathogenic mtDNA mutations have a threshold level of 60%-90% which is required to be exceeded to cause oxidative phosphorylation (OXPHOS) dysfunction leading to severe cellular dysfunction and apoptotic cell death (Stewart & Chinnery, 2015). However, cells can alternatively trigger activation of anti-aging mechanisms such as mitochondrial biogenesis to increase the mitochondrial number and overall OXPHOS capacity, allowing the cellular energy supply and cellular homeostasis to be preserved (Kauppila, Kauppila, & Larsson, 2017). This presents mitochondrial rejuvenation via biogenesis as an anti-aging mechanism. The enhanced mitochondrial biogenesis, though in itself energy requiring, is often seen as a very attractive strategy to boost cellular function, and it can even be used as a potential mitochondrial anti-aging therapy (Viscomi, Bottani, & Zeviani, 2015). The efficacy of mitochondrial biogenesis is indicated by a study of patients with a mitochondrial disease which found that unaffected carriers had higher mitochondrial mass and higher mtDNA copy number in comparison with affected individuals (Giordano et al., 2014). This suggests that an increase in mitochondrial number and mt.DNA can have protective effects. Muscle-specific overexpression of PGC1α can improve mitochondrial phenotypes and can also alleviate premature aging phenotypes of a long-lived mouse (Dillon et al., 2012; Viscomi et al., 2011). This evidence, although limited, warrants investigation of small molecule activators of mitochondrial biogenesis for anti-aging therapy.

2.3.3 NAD^+ and Mitochondrial convergence in aging

NAD^+ and mitochondria are the vital drivers of aging and related pathophysiologies. Aging associated variations occur in most tissues and organs. Several studies have shown that declining NAD^+ levels and mitochondrial function are profoundly implicated in aging-associated changes (Yaku, Okabe, & Nakagawa, 2018a). The decline in mitochondrial function and NAD^+ metabolism provoke and intensify the pathology of various aging-associated diseases, including obesity, diabetes, and neurodegenerative diseases (Camacho-Pereira et al., 2016; Yaku et al., 2018a). The age related decline in NAD^+ via NADase CD38 depends on the mitochondrial SIRT3 dependent mechanism (Camacho-Pereira et al., 2016). Mounting evidence suggests NAD and NAMPT levels decline with age in various regions of the brain (L.-Y. Liu et al., 2012; Zhu, Lu, Lee, Ugurbil, & Chen, 2015). A similar trend is observed in mitochondrial number as well (Swerdlow, 2011). Like the brain, numerous findings indicate a significant reduction in NAD^+ levels and mitochondrial number in skeletal muscle with aging (Camacho-Pereira et al., 2016; Frederick et al., 2016; Konopka & Nair, 2013). Multiple studies have also reported a reduction in NAD^+ levels and mitochondrial levels in metabolic organs including the liver and adipose tissues (Yaku, Okabe, & Nakagawa, 2018b). Overall, multiple lines of evidence suggest that NAD^+ and mitochondrial number decline with age, and their boost with dietary activators can help alleviate characteristic features of aging.

2.3.4 Small molecules for aging

Aging is the leading risk aspect for chronic diseases and disability with a significant socioeconomic impact and exceptional health care expenditures (Steptoe & Zaninotto, 2020). The difficulty and challenges in the implementation of calorically restricted diets in humans have led to the search for pharmacological agents that can mitigate characteristics of aging and mediate the

extension of life and healthspan. Moreover, it seems unlikely that people would be prone to deal with low-calorie dietetic interventions (Archer, Hand, & Blair, 2013; Banfield, Liu, Davis, Chang, & Frazier-Wood, 2016). However, Okinawan and Mediterranean diets, both rich in vitamins, minerals, and phytochemicals act via a hormetic-manner activating several stress-response pathways (Martel et al., 2019; Martucci et al., 2017). Anti-aging pharmacological agents either stimulate processes that deteriorate with aging (autophagy, mitochondrial dysfunction) or inhibit key processes that accelerate aging (telomere attrition, DNA instability, oxidative stress). The discovery of the role of SIRT proteins as anti-aging agents has prompted researchers towards the development of SIRT activators (Bonkowski & Sinclair, 2016). Plant polyphenols such as butein, piceatannol, isoliquiritigenin, and resveratrol along with SRT1720, SRT2104, 1,4 DHP derivative, and UBCS039 have been identified as SIRT activators with anti-aging activity (Figure 2.4) (Dai, Sinclair, Ellis, & Steegborn, 2018). Likewise, telomerase(s) have become another target of intervention to slow the deleterious effects of the aging process. Telomerase activators such as synthetic androgen danzol, TA-65, and AGS-499 have been evaluated for successful anti-aging activity (Eitan, Tichon, Gazit, Gitler, Slavin, & Priel, 2012; Harley, Liu, Flom, & Raffaele, 2013). An emerging line of anti-aging intervention that would deserve more detailed research includes non-coding RNAs (Olivieri et al., 2017; Sousa-Franco, Rebelo, da Rocha, & Bernardes de Jesus, 2019). Overall, looking at aging demographics and resultant morbidities, there exists a tremendous scientific potential and economic attraction for the development of anti-aging therapies.

2.4 Bioactive peptides

Bioactive peptides exhibit a wide range of biological activities, including antihypertensive, antimicrobial, antioxidant, glucose and immune-modulatory, etc. (Daroit & Brandelli, 2021).

These bioactive peptides are obtained from both plant and animal sources, including those synthesized by microorganisms, and those obtained by enzymatic proteolysis (Mada, Ugwu, & Abarshi, 2019). Peptides in biological systems such as glutathione (an antioxidant peptide), enkephalins (analgesic peptides), oxytocin (uterine contraction), defensins (antimicrobial activity) along with insulin and glucagon are well studied for their biological activities (Hamley, 2017). Food proteins and peptides have been conventionally recognized for their nutritional function as an essential source of amino acids. For example, casein-derived phospho-peptides increase bone calcification in children with vitamin D independent rickets leading to the discovery of similar bioactive peptides with the ability to modulate beneficial physiological responses in humans (Lorenzo et al., 2018). Owing to such pharmacological significance, research on bioactive peptides is continuously expanding and herein the biological activities of selected plant and animal derived peptides are concisely presented. However, before reviewing the biological activities, it is vital to take into consideration that the majority of bioactive peptides are extracted using endoproteinases. Presuming that these bioactive peptides might be orally administered, its vulnerability to gastrointestinal and brush border-associated gastric enzymes is a pertinent issue (Giromini, Cheli, Rebucci, & Baldi, 2019). Only the bioactive peptides resistant to these physiological processes will potentially maintain their activities *in vivo*; therefore, we solely focus on peptides with the capacity to exert beneficial effects *in vivo*.

Plant proteins such as soy, wheat, rice, corn, and sunflower proteins are a rich source of bioactive proteins and peptides. The *in vivo* antihypertensive effects of bioactive peptides in spontaneously hypertensive rats (SHR) have been demonstrated by the peptides RGQVIYVL, LPRL, YADLVE, LRLESF, HLNVVHEN, and PGSGCAGTDL isolated from quinoa bran albumin and a mung bean protein (Sonklin, Alashi, Laohakunjit, Kerdchoechuen, & Aluko, 2020;

Zheng et al., 2019). Soybean derived peptide VHVV improved neuronal survival while the di-peptide GR promoted neurogenesis *in vivo* (Ju et al., 2019; Shimizu et al., 2018). Similar to these soybean derived peptides, the PPKNW peptide also exhibited neuroprotective activity by reducing the accumulation of the β-amyloid plaque in the brain of APP/PS1 mice (M. Wang et al., 2019). Next, rapeseed protein-derived peptides (LY, RALP, and GHS) exert antihypertensive effects in SHR through the modulation of RAAS enzymes (He et al., 2019). Similarly, *in vivo* assessment of VIKP peptide, designed following in silico evaluations of amaranth 11S globulin, demonstrated its ability to lower systolic blood pressure by decreasing plasma ACE activity in SHRs (Suárez, Aphalo, Rinaldi, Añón, & Quiroga, 2020). Apart from anti-hypertensive properties, YWDHNNPQIR, from rapeseed protein ameliorates renal fibrosis in obese IR C57BL/6 mice (M. Zhang et al., 2018). Its derived fragment DHNNPQIR ameliorated liver dysfunction by modulating insulin resistance, cell cycle, and oxidative stress (Q. Zhao et al., 2018). Interestingly, this peptide also helped to treat pulmonary fibrosis in mice via blockade of the TGF-β/MAPK signaling pathway (D. Wang et al., 2019). Similarly, bioactive peptides from potato protein hydrolysates such as DIKTNKPVIF exhibited anti-hepatosteatosis activity in aging mice and antihypertensive effects in SHR (Dumeus et al., 2018; Marthandam Asokan, Wang, Su, & Lin, 2019). Another potato protein derived peptide IF attenuated cardiac hypertrophy and renal oxidative damage linked with hypertension in SHR (C. Y. Huang, Nithiyanantham, Liao, & Lin, 2020; Tsai et al., 2020). Similar to food sources, animal protein derived peptides exhibit vital *in vivo* biological activities as well. Particularly, dairy proteins have been widely studied as a source of multifunctional bioactive peptides such as IPP and VPP for their antihypertensive activity (N. Yamaguchi, Kawaguchi, & Yamamoto, 2009). Similarly, the reduction in hypertensive activity is observed after oral administration of the casein-derived peptic peptides RYLGY and AYFYPEL

in vivo (Sánchez-Rivera, Ferreira Santos, Sevilla, Montero, Recio, & Miralles, 2019). Other casein-derived peptides YLGYLEQLLR, YLG, and YLGYL exert anxiolytic effects in different mice model studies (Benoit et al., 2020; Mizushige, Uchida, & Ohinata, 2020; Nagai, Mizushige, Matsumura, Inoue, & Ohinata*, 2019). While other casein derived peptides such as LLY, RELEELNVPGEIVESLSSSEESITR, and TKLTEEEKNR exhibited a spectrum of pharmacological effects as antioxidant, anabolic, and anticoagulant properties (G. Liu et al., 2018; H. Liu, Tu, Cheng, Xu, Xu, & Du, 2019; Sowmya et al., 2018; Sun et al., 2018). Similar to milk proteins, peptides released through the hydrolysis of egg and meat proteins have also been recently related to diverse biological activities. Numerous antioxidant and ACE-I inhibitory peptides have been described as generated by proteolysis of meat proteins (Xing, Liu, Cao, Zhang, & Guanghong, 2019). Likewise, peptide rich egg hydrolysate and egg-derived peptides (IQW and IRW) have shown antioxidant and anti-hypertensive properties (Liao, Jahandideh, Fan, Son, & Wu, 2018). Peptides obtained from other animal sources such as oyster hydrolysate, IVVPK and YA, exhibit cytoprotective and ACE-inhibitory, and anti-inflammatory activities (Bang, Jin, & Choung, 2020; Siregar et al., 2020). Similarly, fish derived peptide LSGYGP, lowered blood pressure while the tripeptide OGE displayed anti-thrombotic activity in rats in rats *in vivo* (J. Chen et al., 2020; Song, Tian, & Li, 2020). Overall, numerous peptides produced by the hydrolysis of dietary food and animal proteins are associated to a wide range of pharmacological activities. These proteins such as milk, meat, cereal, legume proteins, mushrooms, algae, fish, and poultry are thoroughly studied, and diverse bioactive peptides from these sources have been well characterized.

2.4.1 Peptides modulating the NAD axis and mitochondria

Among known peptides, only two food derived bioactives, IF and DIKTNKPVIF (Figure 2.4), have exhibited ability to protect the cardiac tissue by activating mitochondrial biogenesis in SHRs (Lin et al., 2020). The only other strong evidence comes from any peptide group is natriuretic peptides, peptide hormones, which initiate mitochondrial biogenesis via activation of PGC-1α and its downstream effectors (Shen & Matsui, 2019). As antioxidant peptides have the ability to increase the antioxidant enzymes and eNOS activity *in vivo*, therefore, they have strong potential of increasing mitochondrial biogenesis (Miyashita et al., 2009). However, a research gap lies in the investigation of these peptides to activate mitochondrial biogenesis. As mitochondrial pharmacology is an emerging discipline, the role of food derived antioxidant peptides holds great promise and potential for new therapeutic approaches with implications for activation of mitochondrial biogenesis. IRW, owing to its antioxidant and pharmacological spectrum holds strong potential in boosting mitochondrial health and mitigation of aging. Further, IRW is a robust antioxidant peptide with the ability to alleviate oxidative stress *in vivo* (Majumder, Chakrabarti, Davidge, & Wu, 2013; Majumder, Chakrabarti, Morton, et al., 2013). Its ability to counter complex vasculature and hypertension pathologies is now well established (Majumder, Liang, Chen, Guan, Davidge, & Wu, 2015). With its ability to activate the NO pathway, a critical initiator of mitochondrial biogenesis, it certainly holds promise for activation of either upstream or downstream regulators of mitochondrial biogenesis (Nisoli et al., 2003).

2.4.2 IRW: The story so far

IRW, along with the other two tri-peptides, IQW and LKP, was discovered as novel ACE inhibitory peptide through an integrated *in silico* digestion and quantitative structure and activity relationship (QSAR) prediction (Majumder & Wu, 2010). Later, IRW emerged as a promising pharmacological peptide, particularly, as an inhibitor of angiotensin converting enzyme (ACE)

from ovotransferrin in 2011 (Majumder & Wu, 2011). IRW presents a unique pharmacological spectrum with anti-hypertensive, insulin sensitization, and bone anabolic properties. The anti-hypertensive properties of IRW form the basis of pharmacological exploration. As an *in vivo* ACE inhibitory peptide, IRW administration significantly abridged blood pressure in hypertensive rats at a daily dose of 15 mg/kg body weight (Majumder et al., 2015). Paradoxical with the classic RAS system, IRW administration had minimal impact on plasma ACE but significantly reduced levels of Ang II (Majumder, Chakrabarti, Morton, et al., 2013). Using a transcriptome analysis, IRW treatment significantly up-regulated the expression of angiotensin converting enzyme-2 (ACE2), which cleaves the carboxyl-terminal phenylalanine of vasoconstrictive Ang II to form vasodilatory Ang (1-7) (Majumder et al., 2015; Patel, Velkoska, Freeman, Wai, Lancefield, & Burrell, 2014). This unique feature of ACE2 rationalizes the Ang II reduction following IRW treatment in hypertensive rats despite unaltered levels of ACE (Majumder, Chakrabarti, Morton, et al., 2013; Majumder et al., 2015). Further, the use of MasR antagonist A779 abolished the anti-hypertensive effect of IRW, confirming the underlying role of the ACE2/Ang(1-7))/MasR axis (Liao, Fan, Davidge, & Wu, 2019). Importantly, the unique feature of IRW to increase endothelial nitric oxide synthase (eNOS) supports its vasodilatory properties (Majumder, Chakrabarti, Morton, et al., 2013). Next, as an insulin sensitizer, IRW helps alleviate insulin resistance, a pathological feature central to metabolic syndrome (Zeyda & Stulnig, 2009). IRW triggers Akt phosphorylation both in cells and animals, indicating an improved insulin sensitivity (Liao et al., 2019; Son, Chan, & Wu, 2018). Further, our upcoming reports on IRW will describe in detail the mechanisms underlying the ability of IRW to modulate metabolic syndrome-related obesity and insulin resistance. As obesity and metabolic syndrome are coupled with lower bone mineral density, the impact of IRW on bone health was found to be advantageous (Gower & Casazza, 2013).

Mechanistically, IRW promoted osteoblast proliferation, differentiation, and mineralization, through PI3K-Akt stimulus and downstream increase of RUNX2 (Runt-related transcription factor 2) (Shang, Bhullar, Hubbard, & Wu, 2019). Similar to this observation, IRW exhibited the ability to impede LPS-induced osteoclastogenesis in part via its anti-inflammatory activity and possible suppression of NF-κB nuclear translocation and ERK1/2 phosphorylation (W. Huang, Chakrabarti, Majumder, Jiang, Davidge, & Wu, 2010). We have recently completed the *in vivo* study assessing the impact of IRW on bone health and the results will be reported shortly.

2.5 Current aging related drug market

As the world population is aging and an increasing number of consumers are observing food as a medicine, and as a consequence, dietary supplements and tailored nutrition stand out as one of the fastest growing healthcare categories. The currently available therapies focusing on mitochondrial boosting or aging can be categorized to 1) dietary therapy, 2) vitamin and supplement therapy, and pipeline therapies such as NV556, KL1333, and others. Among these, supplement and vitamin intake remain the chief approach to improve health status, mitigate aging, and boost mitochondrial health. Current studies estimate the global dietary supplements market size at USD 140.3 billion in 2020 and it is projected to grow at a compound annual growth rate of 8.6% from 2021 to 2028 (Arenas-Jal, Suñé-Negre, Pérez-Lozano, & García-Montoya, 2020). The sales of vitamins and supplements in Canada ranged between 800 million dollars per quarter of 2020, suggesting a yearly sale of more than 3 billion dollars (Nwosu & Ubaoji, 2020). Currently, exercise therapy has been demonstrated to be beneficial for mitochondrial diseases as it directly targets both upstream and downstream effectors of mitochondrial biogenesis (Nunnari et al., 2012). Among small molecules, there are a few treatment options for mitochondrial dysfunction and depleted mitochondrial

biogenesis (Gorman et al., 2016). We can broadly divide these treatment options into "disease-tailored" and "non-tailored strategies", with the latter acting on common pathways, thus in theory more relevant to different mitochondrial diseases. The "disease-tailored" therapies such as EPI-743, idebenone, KH176, MTP-131 mainly target antioxidant mechanisms and cardiolipin stabilization (Hirano, Emmanuele, & Quinzii, 2018). Only one drug candidate, Bezafibrate, a pan-PPAR activator is being tested (NCT02398201) for mitochondrial biogenesis (Hirano et al., 2018). Also, most of the "non-tailored strategies" targeting the activation of mitochondrial biogenesis are in the preclinical or early clinical stage (Hirano et al., 2018). This strategy has incorporated multiple vitamins and cofactors in patients with mitochondrial disorders, however, these therapies have not been proven to be effective. The dietary supplements increase respiratory chain flux (CoQ10, riboflavin), serve as antioxidants (e.g., CoQ10, idebenone, α-lipoic acid, vitamin C and E), and/or act as cofactors (e.g., riboflavin, thiamine), or function as mitochondrial substrates (L-carnitine) (Gorman et al., 2016; Lorenzo et al., 2018; Nunnari et al., 2012). Moreover, translating these preclinical studies to bedside remains a challenge as well-controlled clinical trials of high quality are necessary to define the efficacy of potential therapies towards mitochondrial biogenesis. Clearly, there are important unmet needs for therapeutic options in the treatment and prevention of mitochondrial and related metabolic diseases.

2.6 Anticipated significance of the work

As a potent antioxidant, IRW has been well studied in our lab for antioxidant and vasodilatory activity. To our knowledge, our proposed study is the first to investigate the effect of a short peptide on NAD stimulus and mitochondrial biogenesis. Given the lack of such molecules and huge research gap, there is increasing interest in developing safe molecules for mitigation of aging.

Outcomes of this research will contribute to a growing body of knowledge on the biological function of short peptides, in particular, their mito-stimulatory activity, which would help establish the future application of IRW as an anabolic agent for the prevention and mitigation of aging diseases. As mitochondrial and metabolic disorders in total cost billions of dollars in healthcare expenditures; the development of IRW as a potential alternative for the prevention and reduction of mitochondrial diseases may lead to significant economic and social benefits. Elucidating the signaling pathways underlying the proposed activity of IRW through cell and *in vivo* studies can help us gain further insight into its mechanisms of action and, therefore, support its application in clinical settings. Last but not least, this study may also provide the egg industry a novel approach to diversify and add value to the use of eggs via the development of nutraceuticals.

2.7 References

Alexeyev, M. F. (2009). Is there more to aging than mitochondrial DNA and reactive oxygen species? *The FEBS journal, 276*(20), 5768-5787.

Angelova, P. R., & Abramov, A. Y. (2018). Role of mitochondrial ROS in the brain: from physiology to neurodegeneration. *FEBS letters, 592*(5), 692-702.

Archer, E., Hand, G. A., & Blair, S. N. (2013). Validity of US nutritional surveillance: National Health and Nutrition Examination Survey caloric energy intake data, 1971–2010. *PloS one, 8*(10), e76632.

Arenas-Jal, M., Suñé-Negre, J., Pérez-Lozano, P., & García-Montoya, E. (2020). Trends in the food and sports nutrition industry: A review. *Critical reviews in food science and nutrition, 60*(14), 2405-2421.

Baars, J. (2012). *Aging and the Art of Living*: JHU Press.

Badawy, A. A. (2017). Kynurenine pathway of tryptophan metabolism: regulatory and functional aspects. *International Journal of Tryptophan Research, 10*, 1178646917691938.

Banfield, E. C., Liu, Y., Davis, J. S., Chang, S., & Frazier-Wood, A. C. (2016). Poor adherence to US dietary guidelines for children and adolescents in the national health and nutrition examination survey population. *Journal of the Academy of Nutrition and Dietetics, 116*(1), 21-27.

Bang, J. S., Jin, Y. J., & Choung, S.-Y. (2020). Low molecular polypeptide from oyster hydrolysate recovers photoaging in skh-1 hairless mice. *Toxicology and applied pharmacology, 386*, 114844.

Benoit, S., Chaumontet, C., Schwarz, J., Cakir-Kiefer, C., Boulier, A., Tomé, D., & Miclo, L. (2020). Anxiolytic Activity and Brain Modulation Pattern of the α-Casozepine-Derived Pentapeptide YLGYL in Mice. *Nutrients, 12*(5), 1497.

Berger, F., Lau, C., Dahlmann, M., & Ziegler, M. (2005). Subcellular compartmentation and differential catalytic properties of the three human nicotinamide mononucleotide adenylyltransferase isoforms. *Journal of Biological Chemistry, 280*(43), 36334-36341.

Berndt, J., Klöting, N., Kralisch, S., Kovacs, P., Fasshauer, M., Schön, M. R., . . . Blüher, M. (2005). Plasma visfatin concentrations and fat depot–specific mRNA expression in humans. *Diabetes, 54*(10), 2911-2916.

Bonkowski, M. S., & Sinclair, D. A. (2016). Slowing ageing by design: the rise of NAD+ and sirtuin-activating compounds. *Nature reviews Molecular cell biology, 17*(11), 679.

Bowlby, S. C., Thomas, M. J., D'Agostino Jr, R. B., & Kridel, S. J. (2012). Nicotinamide phosphoribosyl transferase (Nampt) is required for de novo lipogenesis in tumor cells. *PloS one, 7*(6), e40195.

Braidy, N., Berg, J., Clement, J., Khorshidi, F., Poljak, A., Jayasena, T., . . . Sachdev, P. (2019). Role of nicotinamide adenine dinucleotide and related precursors as therapeutic targets for age-related degenerative diseases: rationale, biochemistry, pharmacokinetics, and outcomes. *Antioxidants & redox signaling, 30*(2), 251-294.

Braidy, N., Guillemin, G. J., Mansour, H., Chan-Ling, T., Poljak, A., & Grant, R. (2011). Age related changes in NAD+ metabolism oxidative stress and Sirt1 activity in wistar rats. *PloS one, 6*(4), e19194.

Brazill, J. M., Li, C., Zhu, Y., & Zhai, R. G. (2017). NMNAT: It's an NAD+ synthase… It'sa chaperone… It'sa neuroprotector. *Current opinion in genetics & development, 44*, 156-162.

Burkart, A. M., Tan, K., Warren, L., Iovino, S., Hughes, K. J., Kahn, C. R., & Patti, M.-E. (2016). Insulin resistance in human iPS cells reduces mitochondrial size and function. *Scientific reports, 6*(1), 1-12.

Cahová, H., Winz, M.-L., Höfer, K., Nübel, G., & Jäschke, A. (2015). NAD captureSeq indicates NAD as a bacterial cap for a subset of regulatory RNAs. *Nature, 519*(7543), 374-377.

Camacho-Pereira, J., Tarragó, M. G., Chini, C. C., Nin, V., Escande, C., Warner, G. M., . . . Galina, A. (2016). CD38 dictates age-related NAD decline and mitochondrial dysfunction through an SIRT3-dependent mechanism. *Cell metabolism, 23*(6), 1127-1139.

Cambronne, X. A., & Kraus, W. L. (2020). Location, Location, Location: Compartmentalization of NAD+ Synthesis and Functions in Mammalian Cells. *Trends in biochemical sciences*.

Cantó, C., Houtkooper, R. H., Pirinen, E., Youn, D. Y., Oosterveer, M. H., Cen, Y., . . . Cettour-Rose, P. (2012). The NAD+ precursor nicotinamide riboside enhances oxidative metabolism and protects against high-fat diet-induced obesity. *Cell metabolism, 15*(6), 838-847.

Cantó, C., Menzies, K. J., & Auwerx, J. (2015). NAD+ metabolism and the control of energy homeostasis: a balancing act between mitochondria and the nucleus. *Cell metabolism, 22*(1), 31-53.

Chacinska, A., van der Laan, M., Mehnert, C. S., Guiard, B., Mick, D. U., Hutu, D. P., . . . Pfanner, N. (2010). Distinct forms of mitochondrial TOM-TIM supercomplexes define signal-dependent states of preprotein sorting. *Molecular and cellular biology, 30*(1), 307-318.

Chambon, P., Weill, J., & Mandel, P. (1963). Nicotinamide mononucleotide activation of a new DNA-dependent polyadenylic acid synthesizing nuclear enzyme. *Biochemical and biophysical research communications, 11*(1), 39-43.

Chandel, N. S. (2018). Mitochondria: back to the future. *Nature reviews Molecular cell biology, 19*(2), 76-76.

Chang, Y. H., Chang, D. M., Lin, K. C., Shin, S. J., & Lee, Y. J. (2011). Visfatin in overweight/obesity, type 2 diabetes mellitus, insulin resistance, metabolic syndrome and cardiovascular diseases: a meta-analysis and systemic review. *Diabetes/metabolism research and reviews, 27*(6), 515-527.

Chen, J., Ryu, B., Zhang, Y., Liang, P., Li, C., Zhou, C., . . . Qian, Z. J. (2020). Comparison of an angiotensin-I-converting enzyme inhibitory peptide from tilapia (Oreochromis niloticus) with captopril: inhibition kinetics, in vivo effect, simulated gastrointestinal digestion and a molecular docking study. *Journal of the Science of Food and Agriculture, 100*(1), 315-324.

Chen, Y. G., Kowtoniuk, W. E., Agarwal, I., Shen, Y., & Liu, D. R. (2009). LC/MS analysis of cellular RNA reveals NAD-linked RNA. *Nature chemical biology, 5*(12), 879-881.

Clement, J., Wong, M., Poljak, A., Sachdev, P., & Braidy, N. (2019). The plasma NAD+ metabolome is dysregulated in "normal" aging. *Rejuvenation research, 22*(2), 121-130.

Dai, H., Sinclair, D. A., Ellis, J. L., & Steegborn, C. (2018). Sirtuin activators and inhibitors: promises, achievements, and challenges. *Pharmacology & therapeutics, 188*, 140-154.

Daroit, D. J., & Brandelli, A. (2021). In vivo bioactivities of food protein-derived peptides–A current review. *Current Opinion in Food Science*.

Davila, A., Liu, L., Chellappa, K., Redpath, P., Nakamaru-Ogiso, E., Paolella, L. M., . . . Baur, J. A. (2018). Nicotinamide adenine dinucleotide is transported into mammalian mitochondria. *Elife, 7*, e33246.

de la Rubia, J. E., Drehmer, E., Platero, J. L., Benlloch, M., Caplliure-Llopis, J., Villaron-Casales, C., . . . Carrera, S. (2019). Efficacy and tolerability of EH301 for amyotrophic lateral sclerosis: a randomized, double-blind, placebo-controlled human pilot study. *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration, 20*(1-2), 115-122.

de Picciotto, N. E., Gano, L. B., Johnson, L. C., Martens, C. R., Sindler, A. L., Mills, K. F., . . . Seals, D. R. (2016). Nicotinamide mononucleotide supplementation reverses vascular dysfunction and oxidative stress with aging in mice. *Aging cell, 15*(3), 522-530.

Dhar, S. S., Ongwijitwat, S., & Wong-Riley, M. T. (2008). Nuclear respiratory factor 1 regulates all ten nuclear-encoded subunits of cytochrome c oxidase in neurons. *Journal of Biological Chemistry, 283*(6), 3120-3129.

Dillon, L. M., Williams, S. L., Hida, A., Peacock, J. D., Prolla, T. A., Lincoln, J., & Moraes, C. T. (2012). Increased mitochondrial biogenesis in muscle improves aging phenotypes in the mtDNA mutator mouse. *Human molecular genetics, 21*(10), 2288-2297.

Dolezal, P., Likic, V., Tachezy, J., & Lithgow, T. (2006). Evolution of the molecular machines for protein import into mitochondria. *Science, 313*(5785), 314-318.

Dollerup, O. L., Christensen, B., Svart, M., Schmidt, M. S., Sulek, K., Ringgaard, S., . . . Treebak, J. T. (2018). A randomized placebo-controlled clinical trial of nicotinamide riboside in obese men: safety, insulin-sensitivity, and lipid-mobilizing effects. *The American journal of clinical nutrition, 108*(2), 343-353.

Dollerup, O. L., Trammell, S. A., Hartmann, B., Holst, J. J., Christensen, B., Møller, N., . . . Jessen, N. (2019). Effects of nicotinamide riboside on endocrine pancreatic function and incretin hormones in nondiabetic men with obesity. *The Journal of Clinical Endocrinology & Metabolism, 104*(11), 5703-5714.

Dumeus, S., Shibu, M. A., Lin, W.-T., Wang, M.-F., Lai, C.-H., Shen, C.-Y., . . . Huang, C.-Y. (2018). Bioactive peptide improves diet-induced hepatic fat deposition and hepatocyte proinflammatory response in SAMP8 ageing mice. *Cellular Physiology and Biochemistry, 48*(5), 1942-1952.

Effros, R. B. (2005). Roy Walford and the immunologic theory of aging. *Immunity & Ageing, 2*(1), 1-3.

Eitan, E., Tichon, A., Gazit, A., Gitler, D., Slavin, S., & Priel, E. (2012). Novel telomerase-increasing compound in mouse brain delays the onset of amyotrophic lateral sclerosis. *EMBO molecular medicine, 4*(4), 313-329.

Erion, D. M., & Shulman, G. I. (2010). Diacylglycerol-mediated insulin resistance. *Nature medicine, 16*(4), 400-402.

Fang, E. F., Lautrup, S., Hou, Y., Demarest, T. G., Croteau, D. L., Mattson, M. P., & Bohr, V. A. (2017). NAD+ in aging: molecular mechanisms and translational implications. *Trends in molecular medicine, 23*(10), 899-916.

Frederick, D. W., Loro, E., Liu, L., Davila Jr, A., Chellappa, K., Silverman, I. M., . . . Davis, J. G. (2016). Loss of NAD homeostasis leads to progressive and reversible degeneration of skeletal muscle. *Cell metabolism, 24*(2), 269-282.

Friebe, D., Neef, M., Kratzsch, J., Erbs, S., Dittrich, K., Garten, A., . . . Stumvoll, M. (2011). Leucocytes are a major source of circulating nicotinamide phosphoribosyltransferase

(NAMPT)/pre-B cell colony (PBEF)/visfatin linking obesity and inflammation in humans. *Diabetologia, 54*(5), 1200-1211.

Frye, R. A. (1999). Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. *Biochemical and biophysical research communications, 260*(1), 273-279.

Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., . . . Shimomura, I. (2017). Increased oxidative stress in obesity and its impact on metabolic syndrome. *The Journal of clinical investigation, 114*(12), 1752-1761.

Galloway, C. A., Lee, H., Nejjar, S., Jhun, B. S., Yu, T., Hsu, W., & Yoon, Y. (2012). Transgenic control of mitochondrial fission induces mitochondrial uncoupling and relieves diabetic oxidative stress. *Diabetes, 61*(8), 2093-2104.

Giordano, C., Iommarini, L., Giordano, L., Maresca, A., Pisano, A., Valentino, M. L., . . . Roberti, M. (2014). Efficient mitochondrial biogenesis drives incomplete penetrance in Leber's hereditary optic neuropathy. *Brain, 137*(2), 335-353.

Giromini, C., Cheli, F., Rebucci, R., & Baldi, A. (2019). Invited review: Dairy proteins and bioactive peptides: Modeling digestion and the intestinal barrier. *Journal of dairy science, 102*(2), 929-942.

Gomes, A. P., Price, N. L., Ling, A. J., Moslehi, J. J., Montgomery, M. K., Rajman, L., . . . Hubbard, B. P. (2013). Declining NAD+ induces a pseudohypoxic state disrupting nuclear-mitochondrial communication during aging. *Cell, 155*(7), 1624-1638.

Gorman, G. S., Chinnery, P. F., DiMauro, S., Hirano, M., Koga, Y., McFarland, R., . . . Turnbull, D. M. (2016). Mitochondrial diseases. *Nature reviews Disease primers, 2*(1), 1-22.

Gower, B. A., & Casazza, K. (2013). Divergent effects of obesity on bone health. *Journal of clinical densitometry, 16*(4), 450-454.

Haas, R. H. (2019). Mitochondrial dysfunction in aging and diseases of aging. Multidisciplinary Digital Publishing Institute.

Hamley, I. W. (2017). Small bioactive peptides for biomaterials design and therapeutics. *Chemical reviews, 117*(24), 14015-14041.

Harbauer, A. B., Opalińska, M., Gerbeth, C., Herman, J. S., Rao, S., Schönfisch, B., . . . Meisinger, C. (2014). Cell cycle–dependent regulation of mitochondrial preprotein translocase. *Science, 346*(6213), 1109-1113.

Harden, A., & Young, W. J. (1906). The alcoholic ferment of yeast-juice. Part II.—The coferment of yeast-juice. *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character, 78*(526), 369-375.

Harley, C. B., Liu, W., Flom, P. L., & Raffaele, J. M. (2013). A natural product telomerase activator as part of a health maintenance program: metabolic and cardiovascular response. *Rejuvenation research, 16*(5), 386-395.

Harman, D. (1981). The aging process. *Proceedings of the National Academy of Sciences, 78*(11), 7124-7128.

Harman, D. (1993). Free radical involvement in aging. *Drugs & aging, 3*(1), 60-80.

He, R., Yang, Y.-J., Wang, Z., Xing, C.-r., Yuan, J., Wang, L.-F., . . . Ju, X.-R. (2019). Rapeseed protein-derived peptides, LY, RALP, and GHS, modulates key enzymes and intermediate products of renin–angiotensin system pathway in spontaneously hypertensive rat. *NPJ science of food, 3*(1), 1-6.

Henriksen, E. J., Diamond-Stanic, M. K., & Marchionne, E. M. (2011). Oxidative stress and the etiology of insulin resistance and type 2 diabetes. *Free Radical Biology and Medicine, 51*(5), 993-999.

Hirano, M., Emmanuele, V., & Quinzii, C. M. (2018). Emerging therapies for mitochondrial diseases. *Essays in biochemistry, 62*(3), 467-481.

Houtkooper, R. H., Cantó, C., Wanders, R. J., & Auwerx, J. (2010). The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. *Endocrine reviews, 31*(2), 194-223.

Huang, C. Y., Nithiyanantham, S., Liao, J. Y., & Lin, W. T. (2020). Bioactive peptides attenuate cardiac hypertrophy and fibrosis in spontaneously hypertensive rat hearts. *Journal of food and drug analysis, 28*(1), 94-102.

Huang, W., Chakrabarti, S., Majumder, K., Jiang, Y., Davidge, S. T., & Wu, J. (2010). Egg-derived peptide IRW inhibits TNF-α-induced inflammatory response and oxidative stress in endothelial cells. *Journal of Agricultural and Food Chemistry, 58*(20), 10840-10846.

Huo, L., & Scarpulla, R. C. (2001). Mitochondrial DNA instability and peri-implantation lethality associated with targeted disruption of nuclear respiratory factor 1 in mice. *Molecular and cellular biology, 21*(2), 644-654.

Imai, S.-i. (2009). Nicotinamide phosphoribosyltransferase (Nampt): a link between NAD biology, metabolism, and diseases. *Current pharmaceutical design, 15*(1), 20-28.

Imai, S.-I., Armstrong, C. M., Kaeberlein, M., & Guarente, L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature, 403*(6771), 795-800.

Jäger, S., Handschin, C., Pierre, J. S.-., & Spiegelman, B. M. (2007). AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α. *Proceedings of the National Academy of Sciences, 104*(29), 12017-12022.

Johnson, F. B., Sinclair, D. A., & Guarente, L. (1999). Molecular biology of aging. *Cell, 96*(2), 291-302.

Johnson, S., & Imai, S. i. (2018). NAD+ biosynthesis, aging, and disease. *F1000Research, 7*.

Ju, D.-T., Kuo, W.-W., Ho, T.-J., Chang, R.-L., Lin, W.-T., Day, C. H., . . . Huang, C.-Y. (2019). Bioactive peptide vhvv upregulates the long-term memory-related biomarkers in adult spontaneously hypertensive rats. *International journal of molecular sciences, 20*(12), 3069.

Kanfi, Y., Naiman, S., Amir, G., Peshti, V., Zinman, G., Nahum, L., . . . Cohen, H. Y. (2012). The sirtuin SIRT6 regulates lifespan in male mice. *Nature, 483*(7388), 218-221.

Kashyap, S. R., Roman, L. J., Lamont, J., Masters, B. S. S., Bajaj, M., Suraamornkul, S., . . . Liu, Y. (2005). Insulin resistance is associated with impaired nitric oxide synthase activity in skeletal muscle of type 2 diabetic subjects. *The Journal of Clinical Endocrinology & Metabolism, 90*(2), 1100-1105.

Katsyuba, E., Mottis, A., Zietak, M., De Franco, F., van der Velpen, V., Gariani, K., . . . Liscio, P. (2018). De novo NAD+ synthesis enhances mitochondrial function and improves health. *Nature, 563*(7731), 354-359.

Kauppila, T. E., Kauppila, J. H., & Larsson, N.-G. (2017). Mammalian mitochondria and aging: an update. *Cell metabolism, 25*(1), 57-71.

Kennedy, S. R., Salk, J. J., Schmitt, M. W., & Loeb, L. A. (2013). Ultra-sensitive sequencing reveals an age-related increase in somatic mitochondrial mutations that are inconsistent with oxidative damage. *PLoS Genet, 9*(9), e1003794.

Koltai, E., Szabo, Z., Atalay, M., Boldogh, I., Naito, H., Goto, S., . . . Radak, Z. (2010). Exercise alters SIRT1, SIRT6, NAD and NAMPT levels in skeletal muscle of aged rats. *Mechanisms of ageing and development, 131*(1), 21-28.

Konopka, A. R., & Nair, K. S. (2013). Mitochondrial and skeletal muscle health with advancing age. *Molecular and cellular endocrinology, 379*(1-2), 19-29.

Kowaltowski, A. J., & Vercesi, A. E. (1999). Mitochondrial damage induced by conditions of oxidative stress. *Free Radical Biology and Medicine, 26*(3-4), 463-471.

Kruk, P. A., Rampino, N. J., & Bohr, V. A. (1995). DNA damage and repair in telomeres: relation to aging. *Proceedings of the National Academy of Sciences, 92*(1), 258-262.

Landry, J., Sutton, A., Tafrov, S. T., Heller, R. C., Stebbins, J., Pillus, L., & Sternglanz, R. (2000). The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proceedings of the National Academy of Sciences, 97*(11), 5807-5811.

Li, H., Xu, M., Lee, J., He, C., & Xie, Z. (2012). Leucine supplementation increases SIRT1 expression and prevents mitochondrial dysfunction and metabolic disorders in high-fat diet-induced obese mice. *American Journal of Physiology-Endocrinology and Metabolism, 303*(10), E1234-E1244.

Liao, W., Fan, H., Davidge, S. T., & Wu, J. (2019). Egg white–derived antihypertensive peptide IRW (Ile-Arg-Trp) reduces blood pressure in spontaneously hypertensive rats via the ACE2/ang (1-7)/mas receptor Axis. *Molecular nutrition & food research, 63*(9), 1900063.

Liao, W., Jahandideh, F., Fan, H., Son, M., & Wu, J. (2018). Egg protein-derived bioactive peptides: Preparation, efficacy, and absorption. *Advances in food and nutrition research, 85*, 1-58.

Lin, W. T., Nithiyanantham, S., Hsieh, D. J. Y., Chen, R. J., Day, C. H., Liao, J. Y., . . . Huang, C. Y. (2020). Bioactive peptides attenuate cardiac apoptosis in spontaneously hypertensive rat hearts through activation of autophagy and mitochondrial biogenesis pathway. *Environmental toxicology, 35*(7), 804-810.

Liu, G., Sun, S., Guo, B., Miao, B., Luo, Z., Xia, Z., . . . Tang, J. (2018). Bioactive peptide isolated from casein phosphopeptides promotes calcium uptake in vitro and in vivo. *Food & function, 9*(4), 2251-2260.

Liu, H., Tu, M., Cheng, S., Xu, Z., Xu, X., & Du, M. (2019). Anticoagulant Decapeptide Interacts with Thrombin at the Active Site and Exosite-I. *Journal of Agricultural and Food Chemistry, 68*(1), 176-184.

Liu, L.-Y., Wang, F., Zhang, X.-Y., Huang, P., Lu, Y.-B., Wei, E.-Q., & Zhang, W.-P. (2012). Nicotinamide phosphoribosyltransferase may be involved in age-related brain diseases. *PloS one, 7*(10), e44933.

Liu, X., Kim, C. N., Yang, J., Jemmerson, R., & Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell, 86*(1), 147-157.

Lorenzo, J. M., Munekata, P. E., Gomez, B., Barba, F. J., Mora, L., Perez-Santaescolastica, C., & Toldra, F. (2018). Bioactive peptides as natural antioxidants in food products–A review. *Trends in food science & technology, 79*, 136-147.

Ma, Z. A., Zhao, Z., & Turk, J. (2012). Mitochondrial dysfunction and β-cell failure in type 2 diabetes mellitus. *Experimental diabetes research, 2012*.

Mada, S. B., Ugwu, C. P., & Abarshi, M. M. (2019). Health promoting effects of food-derived bioactive peptides: A review. *International Journal of Peptide Research and Therapeutics*, 1-18.

Majumder, K., Chakrabarti, S., Davidge, S. T., & Wu, J. (2013). Structure and activity study of egg protein ovotransferrin derived peptides (IRW and IQW) on endothelial inflammatory response and oxidative stress. *Journal of Agricultural and Food Chemistry, 61*(9), 2120-2129.

Majumder, K., Chakrabarti, S., Morton, J. S., Panahi, S., Kaufman, S., Davidge, S. T., & Wu, J. (2013). Egg-derived tri-peptide IRW exerts antihypertensive effects in spontaneously hypertensive rats. *PloS one, 8*(11), e82829.

Majumder, K., Liang, G., Chen, Y., Guan, L., Davidge, S. T., & Wu, J. (2015). Egg ovotransferrin-derived ACE inhibitory peptide IRW increases ACE2 but decreases proinflammatory genes expression in mesenteric artery of spontaneously hypertensive rats. *Molecular nutrition & food research, 59*(9), 1735-1744.

Majumder, K., & Wu, J. (2010). A new approach for identification of novel antihypertensive peptides from egg proteins by QSAR and bioinformatics. *Food Research International, 43*(5), 1371-1378.

Majumder, K., & Wu, J. (2011). Purification and characterisation of angiotensin I converting enzyme (ACE) inhibitory peptides derived from enzymatic hydrolysate of ovotransferrin. *Food Chemistry, 126*(4), 1614-1619.

Marletta, A. S., Massarotti, A., Orsomando, G., Magni, G., Rizzi, M., & Garavaglia, S. (2015). Crystal structure of human nicotinic acid phosphoribosyltransferase. *FEBS Open Bio, 5*, 419-428.

Martel, J., Ojcius, D. M., Ko, Y.-F., Ke, P.-Y., Wu, C.-Y., Peng, H.-H., & Young, J. D. (2019). Hormetic effects of phytochemicals on health and longevity. *Trends in Endocrinology & Metabolism, 30*(6), 335-346.

Martens, C. R., Denman, B. A., Mazzo, M. R., Armstrong, M. L., Reisdorph, N., McQueen, M. B., . . . Seals, D. R. (2018). Chronic nicotinamide riboside supplementation is well-tolerated and elevates NAD+ in healthy middle-aged and older adults. *Nature communications, 9*(1), 1-11.

Marthandam Asokan, S., Wang, T., Su, W.-T., & Lin, W.-T. (2019). Antidiabetic effects of a short peptide of potato protein hydrolysate in STZ-induced diabetic mice. *Nutrients, 11*(4), 779.

Martin, P. R., Shea, R. J., & Mulks, M. H. (2001). Identification of a Plasmid-Encoded Gene fromHaemophilus ducreyi Which Confers NAD Independence. *Journal of Bacteriology, 183*(4), 1168-1174.

Martucci, M., Ostan, R., Biondi, F., Bellavista, E., Fabbri, C., Bertarelli, C., . . . Santoro, A. (2017). Mediterranean diet and inflammaging within the hormesis paradigm. *Nutrition reviews, 75*(6), 442-455.

Matsuda, N., Sato, S., Shiba, K., Okatsu, K., Saisho, K., Gautier, C. A., . . . Sato, F. (2010). PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. *Journal of Cell Biology, 189*(2), 211-221.

McHugh, D., & Gil, J. (2018). Senescence and aging: Causes, consequences, and therapeutic avenues. *Journal of Cell Biology, 217*(1), 65-77.

Miyashita, K., Itoh, H., Tsujimoto, H., Tamura, N., Fukunaga, Y., Sone, M., . . . Sonoyama, T. (2009). Natriuretic peptides/cGMP/cGMP-dependent protein kinase cascades promote muscle mitochondrial biogenesis and prevent obesity. *Diabetes, 58*(12), 2880-2892.

Mizushige, T., Uchida, T., & Ohinata, K. (2020). Dipeptide tyrosyl-leucine exhibits antidepressant-like activity in mice. *Scientific reports, 10*(1), 1-10.

Montgomery, M. K., & Turner, N. (2015). Mitochondrial dysfunction and insulin resistance: an update. *Endocrine connections, 4*(1), R1-R15.

Mouchiroud, L., Houtkooper, R. H., Moullan, N., Katsyuba, E., Ryu, D., Cantó, C., . . . Schoonjans, K. (2013). The NAD+/sirtuin pathway modulates longevity through activation of mitochondrial UPR and FOXO signaling. *Cell, 154*(2), 430-441.

Mulder, H., & Ling, C. (2009). Mitochondrial dysfunction in pancreatic β-cells in type 2 diabetes. *Molecular and cellular endocrinology, 297*(1-2), 34-40.

Nagai, A., Mizushige, T., Matsumura, S., Inoue, K., & Ohinata*, K. (2019). Orally administered milk-derived tripeptide improved cognitive decline in mice fed a high-fat diet. *The FASEB Journal, 33*(12), 14095-14102.

Nikiforov, A., Dölle, C., Niere, M., & Ziegler, M. (2011). Pathways and subcellular compartmentation of NAD biosynthesis in human cells: from entry of extracellular precursors to mitochondrial NAD generation. *Journal of Biological Chemistry, 286*(24), 21767-21778.

Nisoli, E., Clementi, E., Paolucci, C., Cozzi, V., Tonello, C., Sciorati, C., . . . Moncada, S. (2003). Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science, 299*(5608), 896-899.

Nunnari, J., & Suomalainen, A. (2012). Mitochondria: in sickness and in health. *Cell, 148*(6), 1145-1159.

Nussey, D. H., Froy, H., Lemaitre, J.-F., Gaillard, J.-M., & Austad, S. N. (2013). Senescence in natural populations of animals: widespread evidence and its implications for bio-gerontology. *Ageing research reviews, 12*(1), 214-225.

Nwosu, O. K., & Ubaoji, K. I. (2020). Nutraceuticals: History, Classification and Market Demand. In *Functional Foods and Nutraceuticals* (pp. 13-22): Springer.

Olivieri, F., Capri, M., Bonafè, M., Morsiani, C., Jung, H. J., Spazzafumo, L., . . . Suh, Y. (2017). Circulating miRNAs and miRNA shuttles as biomarkers: perspective trajectories of healthy and unhealthy aging. *Mechanisms of ageing and development, 165*, 162-170.

Pagel-Langenickel, I., Bao, J., Pang, L., & Sack, M. N. (2010). The role of mitochondria in the pathophysiology of skeletal muscle insulin resistance. *Endocrine reviews, 31*(1), 25-51.

Pagliarini, D. J., Calvo, S. E., Chang, B., Sheth, S. A., Vafai, S. B., Ong, S.-E., . . . Chen, W. K. (2008). A mitochondrial protein compendium elucidates complex I disease biology. *Cell, 134*(1), 112-123.

Patel, S. K., Velkoska, E., Freeman, M., Wai, B., Lancefield, T. F., & Burrell, L. M. (2014). From gene to protein—experimental and clinical studies of ACE2 in blood pressure control and arterial hypertension. *Frontiers in physiology, 5*, 227.

Pfluger, P. T., Herranz, D., Velasco-Miguel, S., Serrano, M., & Tschöp, M. H. (2008). Sirt1 protects against high-fat diet-induced metabolic damage. *Proceedings of the National Academy of Sciences, 105*(28), 9793-9798.

Picca, A., & Lezza, A. M. S. (2015). Regulation of mitochondrial biogenesis through TFAM–mitochondrial DNA interactions: useful insights from aging and calorie restriction studies. *Mitochondrion, 25*, 67-75.

Pillai, J. B., Isbatan, A., Imai, S.-i., & Gupta, M. P. (2005). Poly (ADP-ribose) polymerase-1-dependent cardiac myocyte cell death during heart failure is mediated by NAD+ depletion and reduced Sir2α deacetylase activity. *Journal of Biological Chemistry, 280*(52), 43121-43130.

Radenkovic, D., & Verdin, E. (2020). Clinical evidence for targeting NAD therapeutically. *Pharmaceuticals, 13*(9), 247.

Ramachandran, B., Yu, G., & Gulick, T. (2008). Nuclear respiratory factor 1 controls myocyte enhancer factor 2A transcription to provide a mechanism for coordinate expression of respiratory chain subunits. *Journal of Biological Chemistry, 283*(18), 11935-11946.

Rius-Pérez, S., Torres-Cuevas, I., Millán, I., Ortega, Á. L., & Pérez, S. (2020). PGC-1α, Inflammation, and oxidative stress: An integrative view in metabolism. *Oxidative medicine and cellular longevity, 2020*.

Rongvaux, A., Shea, R. J., Mulks, M. H., Gigot, D., Urbain, J., Leo, O., & Andris, F. (2002). Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. *European journal of immunology, 32*(11), 3225-3234.

Ross, J. M., Stewart, J. B., Hagström, E., Brené, S., Mourier, A., Coppotelli, G., . . . Olson, L. (2013). Germline mitochondrial DNA mutations aggravate ageing and can impair brain development. *Nature, 501*(7467), 412-415.

Sallin, O., Reymond, L., Gondrand, C., Raith, F., Koch, B., & Johnsson, K. (2018). Semisynthetic biosensors for mapping cellular concentrations of nicotinamide adenine dinucleotides. *Elife, 7*, e32638.

Samal, B., Sun, Y., Stearns, G., Xie, C., Suggs, S., & McNiece, I. (1994). Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. *Molecular and cellular biology, 14*(2), 1431-1437.

Sánchez-Rivera, L., Ferreira Santos, P., Sevilla, M. A., Montero, M. J., Recio, I., & Miralles, B. (2019). Implication of Opioid Receptors in the Antihypertensive Effect of a Bovine Casein Hydrolysate and αs1-Casein-Derived Peptides. *Journal of Agricultural and Food Chemistry, 68*(7), 1877-1883.

Satoh, A., Brace, C. S., Rensing, N., Cliften, P., Wozniak, D. F., Herzog, E. D., . . . Imai, S.-i. (2013). Sirt1 extends life span and delays aging in mice through the regulation of Nk2 homeobox 1 in the DMH and LH. *Cell metabolism, 18*(3), 416-430.

Scheibye-Knudsen, M., Mitchell, S. J., Fang, E. F., Iyama, T., Ward, T., Wang, J., . . . Hasan-Olive, M. M. (2014). A high-fat diet and NAD+ activate Sirt1 to rescue premature aging in cockayne syndrome. *Cell metabolism, 20*(5), 840-855.

Shang, N., Bhullar, K. S., Hubbard, B. P., & Wu, J. (2019). Tripeptide IRW initiates differentiation in osteoblasts via the RUNX2 pathway. *Biochimica et Biophysica Acta (BBA)-General Subjects, 1863*(6), 1138-1146.

Shen, W., & Matsui, T. (2019). Intestinal absorption of small peptides: A review. *International Journal of Food Science & Technology, 54*(6), 1942-1948.

Shi, Y., Dierckx, A., Wanrooij, P. H., Wanrooij, S., Larsson, N.-G., Wilhelmsson, L. M., . . . Gustafsson, C. M. (2012). Mammalian transcription factor A is a core component of the mitochondrial transcription machinery. *Proceedings of the National Academy of Sciences, 109*(41), 16510-16515.

Shimizu, A., Mitani, T., Tanaka, S., Fujii, H., Maebuchi, M., Amiya, Y., . . . Katayama, S. (2018). Soybean-Derived Glycine–Arginine Dipeptide Administration Promotes Neurotrophic Factor Expression in the Mouse Brain. *Journal of Agricultural and Food Chemistry, 66*(30), 7935-7941.

Shiota, T., Imai, K., Qiu, J., Hewitt, V. L., Tan, K., Shen, H.-H., . . . Kamiya, M. (2015). Molecular architecture of the active mitochondrial protein gate. *Science, 349*(6255), 1544-1548.

Siregar, A. S., Nyiramana, M. M., Kim, E.-J., Shin, E.-J., Woo, M. S., Kim, J.-M., . . . Kim, H. J. (2020). Dipeptide YA is Responsible for the Positive Effect of Oyster Hydrolysates on Alcohol Metabolism in Single Ethanol Binge Rodent Models. *Marine Drugs, 18*(10), 512.

Son, M., Chan, C. B., & Wu, J. (2018). Egg white ovotransferrin-derived ACE inhibitory peptide ameliorates angiotensin II-stimulated insulin resistance in skeletal muscle cells. *Molecular nutrition & food research, 62*(4), 1700602.

Song, H., Tian, Q., & Li, B. (2020). Novel Hyp-Gly-containing antiplatelet peptides from collagen hydrolysate after simulated gastrointestinal digestion and intestinal absorption. *Food & function, 11*(6), 5553-5564.

Sonklin, C., Alashi, M. A., Laohakunjit, N., Kerdchoechuen, O., & Aluko, R. E. (2020). Identification of antihypertensive peptides from mung bean protein hydrolysate and their effects in spontaneously hypertensive rats. *Journal of Functional Foods, 64*, 103635.

Sousa-Franco, A., Rebelo, K., da Rocha, S. T., & Bernardes de Jesus, B. (2019). LncRNAs regulating stemness in aging. *Aging cell, 18*(1), e12870.

Sowmya, K., Mala, D., Bhat, M. I., Kumar, N., Bajaj, R. K., Kapila, S., & Kapila, R. (2018). Bio-accessible milk casein derived tripeptide (LLY) mediates overlapping anti-inflammatory

and anti-oxidative effects under cellular (Caco-2) and in vivo milieu. *The Journal of nutritional biochemistry, 62*, 167-180.

Stein, L. R., & Imai, S.-i. (2012). The dynamic regulation of NAD metabolism in mitochondria. *Trends in Endocrinology & Metabolism, 23*(9), 420-428.

Stein, L. R., & Imai, S. i. (2014). Specific ablation of Nampt in adult neural stem cells recapitulates their functional defects during aging. *The EMBO journal, 33*(12), 1321-1340.

Steptoe, A., & Zaninotto, P. (2020). Lower socioeconomic status and the acceleration of aging: An outcome-wide analysis. *Proceedings of the National Academy of Sciences, 117*(26), 14911-14917.

Stewart, J. B., & Chinnery, P. F. (2015). The dynamics of mitochondrial DNA heteroplasmy: implications for human health and disease. *Nature Reviews Genetics, 16*(9), 530-542.

Stromsdorfer, K. L., Yamaguchi, S., Yoon, M. J., Moseley, A. C., Franczyk, M. P., Kelly, S. C., . . . Yoshino, J. (2016). NAMPT-mediated NAD+ biosynthesis in adipocytes regulates adipose tissue function and multi-organ insulin sensitivity in mice. *Cell reports, 16*(7), 1851-1860.

Suárez, S., Aphalo, P., Rinaldi, G., Añón, M. C., & Quiroga, A. (2020). Effect of amaranth proteins on the RAS system. In vitro, in vivo and ex vivo assays. *Food Chemistry, 308*, 125601.

Sun, S., Liu, F., Liu, G., Miao, J., Xiao, H., Xiao, J., . . . Cao, Y. (2018). Effects of casein phosphopeptides on calcium absorption and metabolism bioactivity in vitro and in vivo. *Food & function, 9*(10), 5220-5229.

Swerdlow, R. H. (2011). Brain aging, Alzheimer's disease, and mitochondria. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1812*(12), 1630-1639.

Terra, X., Auguet, T., Quesada, I., Aguilar, C., Luna, A. M., Hernandez, M., . . . Lucas, A. (2012). Increased levels and adipose tissue expression of visfatin in morbidly obese women: the relationship with pro-inflammatory cytokines. *Clinical endocrinology, 77*(5), 691-698.

Tsai, B. C.-K., Hsieh, D. J.-Y., Lin, W.-T., Tamilselvi, S., Day, C. H., Ho, T.-J., . . . Huang, C.-Y. (2020). Functional potato bioactive peptide intensifies Nrf2-dependent antioxidant defense against renal damage in hypertensive rats. *Food Research International, 129*, 108862.

Uittenbogaard, M., & Chiaramello, A. (2014). Mitochondrial biogenesis: a therapeutic target for neurodevelopmental disorders and neurodegenerative diseases. *Current pharmaceutical design, 20*(35), 5574-5593.

Virbasius, C.-m. A., Virbasius, J. V., & Scarpulla, R. C. (1993). NRF-1, an activator involved in nuclear-mitochondrial interactions, utilizes a new DNA-binding domain conserved in a family of developmental regulators. *Genes & development, 7*(12a), 2431-2445.

Viscomi, C., Bottani, E., Civiletto, G., Cerutti, R., Moggio, M., Fagiolari, G., . . . Zeviani, M. (2011). In vivo correction of COX deficiency by activation of the AMPK/PGC-1α axis. *Cell metabolism, 14*(1), 80-90.

Viscomi, C., Bottani, E., & Zeviani, M. (2015). Emerging concepts in the therapy of mitochondrial disease. *Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1847*(6-7), 544-557.

Walters, R. W., Matheny, T., Mizoue, L. S., Rao, B. S., Muhlrad, D., & Parker, R. (2017). Identification of NAD+ capped mRNAs in Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences, 114*(3), 480-485.

Wang, D., Yan, Z., Bu, L., An, C., Deng, B., Zhang, J., . . . Zhang, B. (2019). Protective effect of peptide DR8 on bleomycin-induced pulmonary fibrosis by regulating the TGF-β/MAPK

signaling pathway and oxidative stress. *Toxicology and applied pharmacology, 382*, 114703.

Wang, M., Amakye, W. K., Guo, L., Gong, C., Zhao, Y., Yao, M., & Ren, J. (2019). Walnut-derived peptide PW5 ameliorates cognitive impairments and alters gut microbiota in APP/PS1 transgenic mice. *Molecular nutrition & food research, 63*(18), 1900326.

Warburg, O., & Christian, W. (1936). Pyridin, der wasserstoffübertragende Bestandteil von Gärungsfermenten. *Helvetica Chimica Acta, 19*(1), E79-E88.

Wensveen, F. M., Valentić, S., Šestan, M., Turk Wensveen, T., & Polić, B. (2015). The "Big Bang" in obese fat: Events initiating obesity-induced adipose tissue inflammation. *European journal of immunology, 45*(9), 2446-2456.

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., . . . Scarpulla, R. C. (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell, 98*(1), 115-124.

Xing, L., Liu, R., Cao, S., Zhang, W., & Guanghong, Z. (2019). Meat protein based bioactive peptides and their potential functional activity: a review. *International Journal of Food Science & Technology, 54*(6), 1956-1966.

Yach, D., Stuckler, D., & Brownell, K. D. (2006). Epidemiologic and economic consequences of the global epidemics of obesity and diabetes. *Nature medicine, 12*(1), 62-66.

Yaku, K., Okabe, K., & Nakagawa, T. (2018a). NAD metabolism: Implications in aging and longevity. *Ageing research reviews, 47*, 1-17.

Yaku, K., Okabe, K., & Nakagawa, T. (2018b). Simultaneous measurement of NAD metabolome in aged mice tissue using liquid chromatography tandem-mass spectrometry. *Biomedical Chromatography, 32*(6), e4205.

Yamaguchi, N., Kawaguchi, K., & Yamamoto, N. (2009). Study of the mechanism of antihypertensive peptides VPP and IPP in spontaneously hypertensive rats by DNA microarray analysis. *European Journal of Pharmacology, 620*(1-3), 71-77.

Yamaguchi, S., & Yoshino, J. (2017). Adipose tissue NAD+ biology in obesity and insulin resistance: From mechanism to therapy. *BioEssays, 39*(5), 1600227.

Yoshino, J., Mills, K. F., Yoon, M. J., & Imai, S.-i. (2011). Nicotinamide mononucleotide, a key NAD+ intermediate, treats the pathophysiology of diet-and age-induced diabetes in mice. *Cell metabolism, 14*(4), 528-536.

Youn, H.-S., Kim, T. G., Kim, M.-K., Kang, G. B., Kang, J. Y., Lee, J.-G., . . . Im, Y. J. (2016). Structural insights into the quaternary catalytic mechanism of hexameric human quinolinate phosphoribosyltransferase, a key enzyme in de novo NAD biosynthesis. *Scientific reports, 6*(1), 1-14.

Zeyda, M., & Stulnig, T. M. (2009). Obesity, inflammation, and insulin resistance–a mini-review. *Gerontology, 55*(4), 379-386.

Zhang, H., Ryu, D., Wu, Y., Gariani, K., Wang, X., Luan, P., . . . Aebersold, R. (2016). NAD+ repletion improves mitochondrial and stem cell function and enhances life span in mice. *Science, 352*(6292), 1436-1443.

Zhang, M., Yan, Z., Bu, L., An, C., Wang, D., Liu, X., . . . Xie, J. (2018). Rapeseed protein-derived antioxidant peptide RAP alleviates renal fibrosis through MAPK/NF-κB signaling pathways in diabetic nephropathy. *Drug design, development and therapy, 12*, 1255.

Zhao, M., New, L., Kravchenko, V. V., Kato, Y., Gram, H., Di Padova, F., . . . Han, J. (1999). Regulation of the MEF2 family of transcription factors by p38. *Molecular and cellular biology, 19*(1), 21-30.

Zhao, Q., Xu, H., Hong, S., Song, N., Xie, J., Yan, Z., . . . Jiang, X. (2018). Rapeseed protein-derived antioxidant peptide RAP ameliorates nonalcoholic steatohepatitis and related metabolic disorders in mice. *Molecular pharmaceutics, 16*(1), 371-381.

Zheng, Y., Wang, X., Zhuang, Y., Li, Y., Tian, H., Shi, P., & Li, G. (2019). Isolation of novel ACE-inhibitory and antioxidant peptides from quinoa bran albumin assisted with an in silico approach: Characterization, in vivo antihypertension, and molecular docking. *Molecules, 24*(24), 4562.

Zhou, T., Kurnasov, O., Tomchick, D. R., Binns, D. D., Grishin, N. V., Marquez, V. E., . . . Zhang, H. (2002). Structure of human nicotinamide/nicotinic acid mononucleotide adenylyltransferase: basis for the dual substrate specificity and activation of the oncolytic agent tiazofurin. *Journal of Biological Chemistry, 277*(15), 13148-13154.

Zhu, X.-H., Lu, M., Lee, B.-Y., Ugurbil, K., & Chen, W. (2015). In vivo NAD assay reveals the intracellular NAD contents and redox state in healthy human brain and their age dependences. *Proceedings of the National Academy of Sciences, 112*(9), 2876-2881.

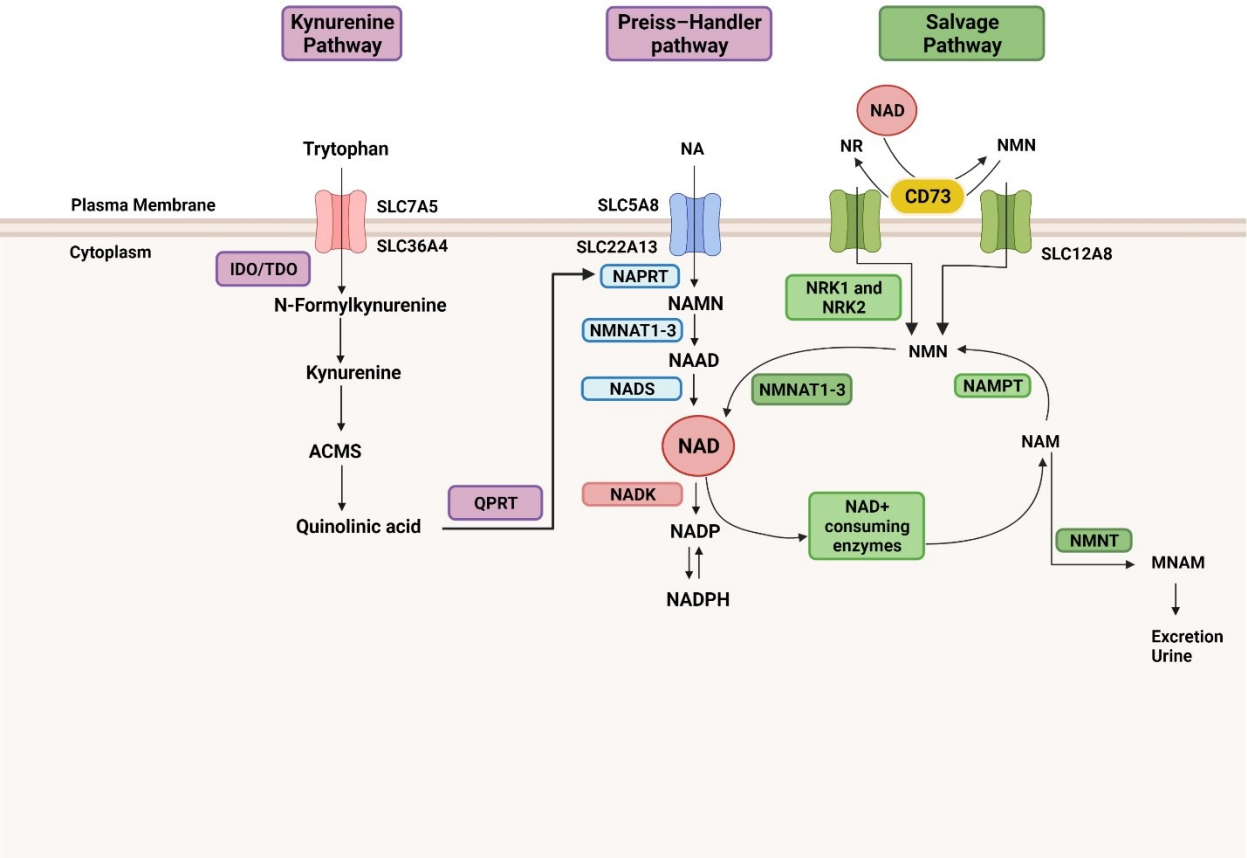


Figure 2.1. **Overview of NAD$^+$ metabolism**. NAD$^+$ levels are maintained by three independent biosynthetic pathways. The kynurenine pathway (or de novo synthesis pathway) uses the dietary amino acid tryptophan to generate NAD$^+$. Tryptophan enters the cell via the transporters SLC7A5 and SLC36A4. Within the cell, tryptophan is converted to N-formylkynurenine by the rate-limiting enzyme indoleamine 2,3-dioxygenase (IDO) or the rate-limiting enzyme tryptophan 2,3-dioxygenase (TDO). N-Formylkynurenine is transformed into l-kynurenine, which is further converted to 3-hydroxykynurenine (3-HK) by kynurenine 3-monooxygenase (KMO) and to 3-hydroxyanthranilic acid (3-HAA) by tryptophan 2,3-dioxygenase (KYNU). The next step is performed by 3-hydroxyanthranilic acid oxygenase (3HAO) to generate α-amino-β-carboxymuconate ε-semialdehyde (ACMS). This compound can spontaneously condense and rearrange into quinolinic acid, which is transformed by quinolinate phosphoribosyltransferase (QPRT) into nicotinamide mononucleotide (NAMN), at which point it converges with the Preiss–Handler pathway. The Preiss–Handler pathway uses dietary nicotinic acid (NA), which enters the cell via SLC5A8 or SLC22A13 transporters, and the enzyme nicotinic acid phosphoribosyltransferase (NAPRT) to generate NAMN, which is then transformed into nicotinic acid adenine dinucleotide (NAAD) by nicotinamide mononucleotide adenylyltransferases (NMNAT1, NMNAT2 and NMNAT3). The process is completed by the transformation of NAAD into NAD+ by NAD+ synthetase (NADS). The NAD$^+$ salvage pathway recycles the nicotinamide

(NAM) generated as a by-product of the enzymatic activities of NAD^+-consuming enzymes (sirtuins, poly(ADP-ribose) polymerases (PARPs) and the NAD+ glycohydrolase and cyclic ADP-ribose synthases CD38, CD157 and SARM1). Initially, the intracellular nicotinamide phosphoribosyltransferase (iNAMPT) recycles NAM into nicotinamide mononucleotide (NMN), which is then converted into NAD+ via the different NMNATs. NAM can be alternatively methylated by the enzyme nicotinamide N-methyltransferase (NNMT) and secreted via the urine. In the extracellular space, NAM is generated as a by-product of the ectoenzymes CD38 and CD157 and can be converted to NMN by extracellular NAMPT (eNAMPT). NMN is then dephosphorylated by CD73 to nicotinamide riboside (NR), which is transported into the cell via an unknown nucleoside transporter (question mark). NMN can be imported into the cell via an NMN-specific transporter (SLC12A8 in the small intestine). Intracellularly, NR forms NMN via nicotinamide riboside kinases 1 and 2 (NRK1 and NRK2). NMN is then converted to NAD^+ by NMNAT1, NMNAT2 and NMNAT3.

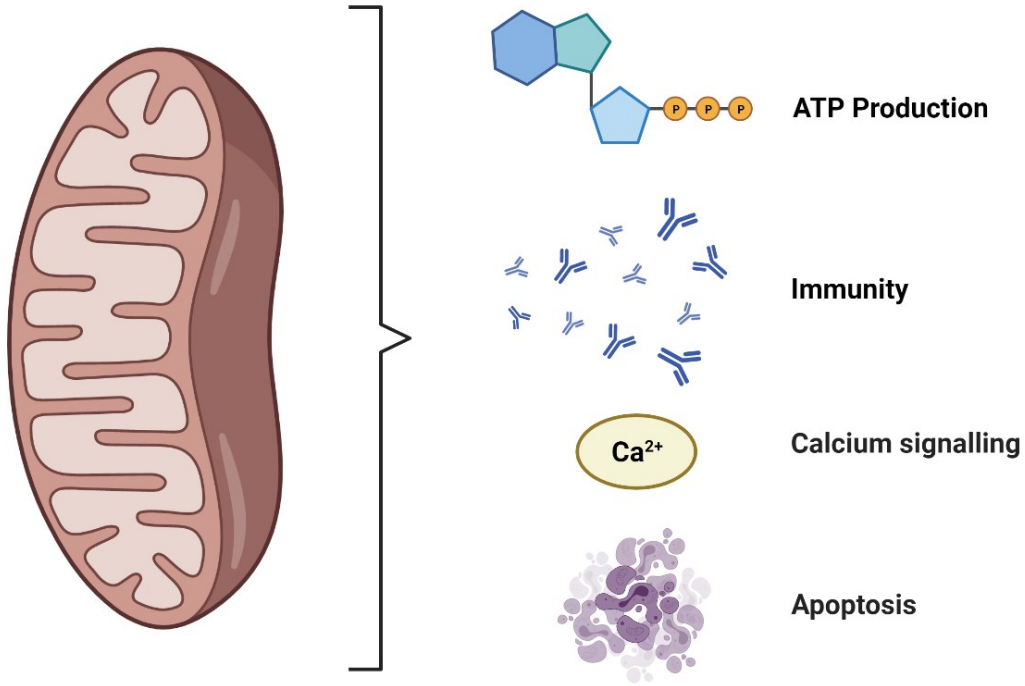


Figure 2.2. **Key functions of Mitochondria.** Traditionally referred to as the powerhouses of cells, mitochondria play a vital role in ATP production, innate immunity, calcium homeostasis, and apoptosis, programmed cell death.

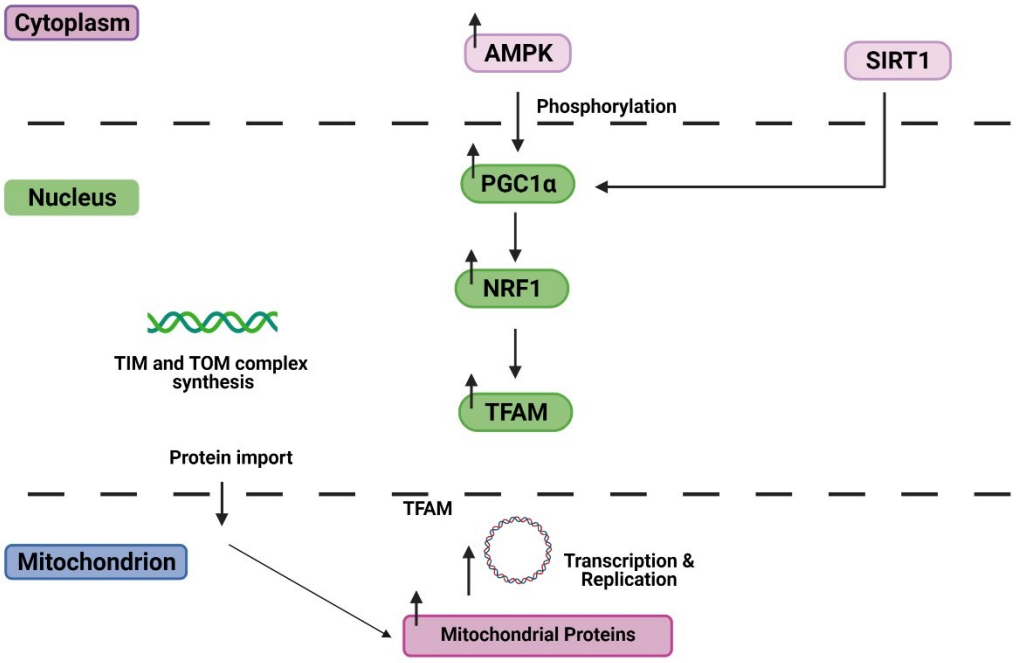


Figure 2.3. **Overview of mitochondrial biogenesis**. PGC1α activation plays a vital role in mitochondrial biogenesis via downstream activation of NRF1 and TFAM. This process is co-ordinated by both nuclear and mitochondrial genomes (nDNA and mtDNA) to enhance the mitochondrial proteins content. AMPK, 5' adenosine monophosphate activated protein kinase; NRF1, the nuclear respiratory factor; PGC1α, the peroxisome proliferator-activated receptor-γ coactivator-1α; SIRT1, the silent information regulator-1; TFAM, the transcription factor α; TIM, the translocase of the inner membrane; TOM, The translocase of the outer membrane.

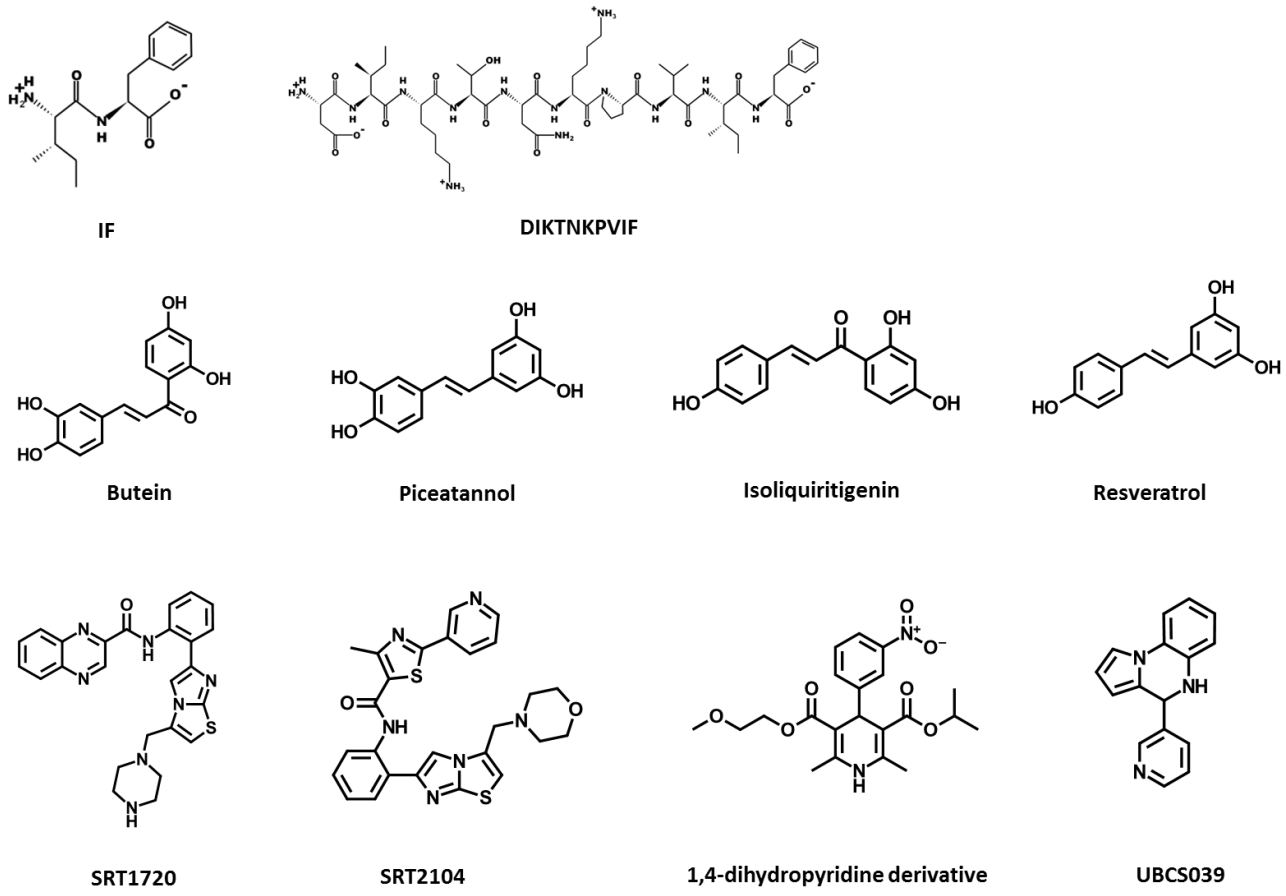


Figure 2.4. Key peptides and small molecules with anti-aging activity.

Table 2.1: Selected human clinical trials focusing on NAD pathway

NAD^+ precursor	Description	Dose and Duration	Outcome	NCT/UMIN no.
NMN	Study of efficacy against insulin sensitivity and β-cell functions in elderly women	Long-term oral NMN administration: 250mg daily for 8 weeks	Not completed	NCT03151239
NMN	Study of pharmacokinetics, safety, and efficacy with regard to glucose metabolism in healthy volunteers	Long-term oral NMN administration for 8 weeks. Dose is not described	No Results Posted	UMIN000030609
NMN	Study of pharmacokinetics, safety, and efficacy with regard to blood pressure and physical endurance in healthy volunteers	Long term oral NMN administration; 300mg daily for 60 days	No Results Posted	NCT04228640
NR	Study of safety and efficacy with regard to physical activities in elderly people	Crossover of placebo for 6 weeks and oral NR 500mg twice daily for 6 weeks	No Results Posted	NCT02921659
NR	Study of efficacy with regard to bone, skeletal muscle and metabolic functions in ageing	1,000mg NR daily in a regimen of 500mg every 12hours for 4.5 months	No Results Posted	NCT03818802
NR	Study of cognitive performance in subjective cognitive decline and mild cognitive impairment in ageing	Long-term oral NR administration: 1,200mg for 8 weeks	No Results Posted	NCT04078178
NAM	Study of efficacy with regard to the severity of Parkinson disease symptoms	Long-term administration of NAM: 200mg daily for 18 month	Recruiting	NCT03808961

CHAPTER 3 - Tripeptide IRW upregulates NAMPT protein levels in cells and obese C57BL/6J mice

Abstract: Nicotinamide adenine dinucleotide (NAD^+) plays a vital role in cellular processes that govern human health and disease. Nicotinamide phosphoribosyltransferase (NAMPT) is a rate-limiting enzyme in NAD^+ biosynthesis. Thus, boosting NAD^+ level via an increase in NAMPT levels is an attractive approach for countering the effects of aging and metabolic disease. This study aimed to establish IRW (Ile-Arg-Trp), a small tripeptide derived from ovotransferrin, as a booster of NAMPT levels. Treatment of muscle (L6) cells with IRW increased intracellular NAMPT protein levels (2.2 fold, $p<0.05$) and boosted NAD^+ levels ($p<0.01$). Both immunoprecipitation and recombinant NAMPT assays indicated possible NAMPT activating ability of IRW ($p<0.01$). Similarly, IRW increased NAMPT mRNA and protein levels in liver (2.6 fold, $p<0.01$) and muscle tissues (2.3 fold, $p<0.05$) of C57BL/6J mice fed a high fat diet (HFD). A significant increased level of circulating NAD^+ was also observed following IRW treatment (4.7 fold, $p<0.0001$). Dosing of *Drosophila melanogaster* with IRW elevated both D-NAAM (fly NAMPT) and NAD^+ *in vivo* ($p<0.05$). However, IRW treatment did not boost NAMPT levels in SIRT1 KO cells, indicating a possible SIRT1-dependency for the pharmacological effect. Overall, these data indicate that IRW is a novel small peptide booster of NAMPT pool.

3.1 Introduction

In recent years, remarkable efforts have been undertaken to identify drug targets for combating aging and the metabolic decline associated with aging (Frederick et al., 2016; Warburg & Christian, 1936). One pathway that has shown therapeutic promise is the NAD^+ (nicotinamide adenine dinucleotide) biosynthesis pathway, which plays a vital role in diverse cellular processes that govern human health and disease (Frederick et al., 2016). NAD^+ plays critical roles in the TCA cycle, glycolysis, and mitochondrial oxidative phosphorylation (Imai, 2010; Krebs & Veech, 1969; Warburg et al., 1936). Recent research has uncovered roles for this metabolic cofactor in fundamental cellular processes such as cell signaling, DNA repair, aging biomarkers like silent mating type information regulation 2 homolog 1 (SIRT1), adapter protein $p66^{SHC}$, and Forkhead box O3 (FOXO3a), and epigenetics (Fang et al., 2016; Frederick et al., 2016; Pérez et al., 2018). While the reason that NAD^+ declines with age remains elusive, its diminution leads to aging related reduced blood flow, slower metabolism, and also abrogates our ability to exercise and receive its benefits (Das et al., 2018). The NAD^+/SIRT1 axis related $p66^{SHC}$ also plays a vital role in metabolic outcomes as $p66^{SHC-/-}$ mice exhibits leaner phenotype and an increased metabolic rate (Pérez et al., 2018). Therefore, NAD^+ replenishment is a successful way of diminishing the side effects of aging and slowing the age-related metabolic decline (J. Li et al., 2017). Along with direct NAD^+ supplementation, cell-permeable NAD^+ precursor molecules, including niacin, nicotinamide mononucleotide (NMN), nicotinamide riboside (NR), and nicotinamide (NAM) have been shown to provide therapeutic benefit in several therapeutic contexts (Fang et al.; Pillai et al., 2010; Rajman, Chwalek, & Sinclair, 2018). Likewise, NADase inhibitors that impede NAD-degrading enzymes, including CD38 inhibitors such as quercetin, and luteolin have shown promising clinical results (Rajman et al., 2018). An alternative strategy for boosting NAD^+ levels

that has received less attention is the possible induction of the rate-limiting enzyme in NAD^+ synthesis, nicotinamide phosphoribosyltransferase (NAMPT).

NAMPT, previously known as visfatin or PBEF, is a rate limiting enzyme that regulates NAD^+ biosynthesis in cells (Imai; Yang et al., 2007). It is present in both intracellular and extracellular forms and its expression is believed to control the body's response to stress, exercise, nutrient status, and circadian rhythms (Frydelund-Larsen, Akerstrom, Nielsen, Keller, Keller, & Pedersen, 2007; Ramsey et al., 2009; Revollo et al., 2007; Yang et al., 2007). NAMPT is highly conserved, even in invertebrates, where its homolog D-NAAM (*Drosophila* nicotinamidase), performs a similar role to the mammalian NAMPT (Balan et al., 2008). There is ample evidence that NAMPT plays a key role in metabolic disorders. For example, the levels of both NAMPT and NAD^+ are significantly reduced in obesity and high-fat diet (HFD) feeding in animals (Balan et al., 2008; Cantó et al., 2012; Mercader, Granados, Caimari, Oliver, Bonet, & Palou, 2008; Yoshino, Mills, Yoon, & Imai, 2011). Furthermore, knockout of NAMPT in mice leads to severe IR and triggers a rapid decline in metabolic health (Stromsdorfer et al., 2016). Thus, boosting the systemic NAD^+ levels with NAMPT activators has been proposed as an attractive pharmacological approach for countering the effects of aging and metabolic disease. Yet, only a handful of NAMPT activators, such as P7C3-A20, have been identified to date (Wang et al., 2014; Zhang et al., 2011). Apart from the pharmacological approach, regular exercise also increases NAD^+ biosynthesis by activation or increase in levels of NAMPT (Koltai et al., 2010). Despite their ability to boost metabolism, food bioactives such as food-derived peptides remain largely unexplored for their ability to boost NAD^+ via NAMPT increase (Udenigwe & Rouvinen-Watt, 2015).

IRW (Ile-Arg-Trp), a small bioactive tripeptide, was initially identified as an ACE (angiotensin converting enzyme) inhibitory peptide from egg protein ovotransferrin (Majumder,

Chakrabarti, Morton, et al., 2013). ACE is a target for controlling high blood pressure. The blood pressure lowering activity of IRW was associated with its ability to improve vasodilation and eNOS levels, via activation of ACE2, a homolog of ACE but with an opposite effect (Majumder, Chakrabarti, Morton, et al., 2013). In addition, this peptide was also shown to activate the insulin signalling pathway (PI3K/Akt activation) in muscle cells (Udenigwe et al., 2015). Given the critical role of NAD^+ in the sphere of IRW's biological activity,(Majumder, Chakrabarti, Morton, et al., 2013) we hypothesized that IRW can increase the NAMPT pool and consequently improve NAD^+ levels. Therefore, the objective of the study was to determine ovotransferrin derived IRW as a novel booster of NAMPT levels. Here, we show that IRW treatment in cell and animal model studies boosts NAD^+ biosynthesis through induction of NAMPT protein levels.

3.2 Materials and methods

3.2.1 Chemical and reagents

Tripeptide IRW was synthesized by GenScript (Piscataway, NJ, U.S.A). Peptide sequence and purity (99.8%) were validated by HPLC-MS/MS. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, 0.25% trypsin-EDTA, TRIzol, and phosphate buffer saline were purchased from Gibco/Invitrogen (Carlsbad, CA, U.S.A). Primers were obtained from Gibco/Invitrogen (Carlsbad, CA, U.S.A) while the reverse transcriptase (RT) system kit was obtained from Applied Biosystems (Foster City, CA, U.S.A). NAMPT inhibitor FK866 and activator P7C3-A20 were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A). Antibodies reactive to NAMPT (ab236874), $P66^{SHC}$ (ab33770), FOXO3a (ab23683) and CD38 (ab216343) were obtained from Abcam Inc (Toronto, ON, Canada). Total OXPHOS Rodent WB Antibody Cocktail (ab110413) and NAMPT Activity Assay Kit (ab221819) were also obtained

from Abcam Inc (Toronto, ON, Canada). The SIRT1 (D739) and DBC1 (5857) antibodies were obtained from Cell Signaling Technology (Danvers, MA, U.S.A). Anti-rabbit IgG, HRP-linked antibody from New England Biolabs. Goat anti-rabbit IRDye 680RD secondary antibody and donkey anti-mouse 800CW secondary antibody was obtained from Licor Biosciences (Lincoln, NE, U.S.A). Goat anti-rabbit IgG(H+L) secondary antibody AlexaFluor546 and rabbit anti-mouse IgG(H+L) secondary antibody AlexaFluor594 was purchased from Molecular Probes (Waltham, MA, U.S.A). All other chemicals and reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A).

3.2.2 Human Tissue studies

Matched pairs of normal and obese snap frozen, RNAlater preserved, muscle tissues were obtained from Proteogenex (Inglewood, CA, USA) after being prospectively collected at multiple hospital institutions with institutional review and board approval. All the experimental procedures associated with the human samples were conducted in appropriate and designated BSL II labs. The obtained human muscle samples were verified for their negative serological status (HIV, HEPA, HEPB, HEPC, and other microbial contaminants).

3.2.4 Cell culture

L6 cells (CRL-1458™) were grown and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotics in an atmosphere of 5% CO_2 at 37°C. Likewise, other supplementary cells lines including 293T (CRL-11268™), MDA-MB-231 (ATCC® HTB-26™) and HeLa (CCL-2™) were grown in (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotics in an atmosphere of 5% CO_2 at 37°C according to ATCC's instructions. Cells were grown in the indicated media and were growth-treated at ~80% confluency for 24 h in media containing 0.1% FBS. Low serum levels were

maintained during treatment to prevent apoptosis that accompanies complete serum deprivation of the cultured cells. The cells were treated with IRW (25 μM or 50 μM), or medium containing vehicle (ddH_2O) for 24 h. Following the treatment, cell lysates were collected using RIPA buffer.

3.2.5 Animal model study

Male C57BL/6J mice (6 weeks) were obtained from the Charles River Breeding Laboratories (St. Constant, QC, Canada) and maintained on a 12 h light/dark cycle with free access to food and water for 2 weeks. The ND (TD.0110675: Rodent Diet With 10.4 kcal% Fat), HFD (TD.06415: Rodent Diet With 45 kcal% Fat) and Casein (CA.160030) were obtained from Envigo Teklad Diets (Madison, WI, USA). All the experimental procedures followed the University of Alberta Guide for the Care and Use of Laboratory Animals, and the animal protocol was approved by the animal ethics committee at the University of Alberta. After one week of adaptation, mice were divided into three groups (8 mice per group): normal diet group (ND) fed with low fat diet (10.4% kcal from fat) for 14 weeks, high fat diet group (HFD) fed with HFD (45% kcal from fat) for 14 weeks, the treatment group (IRW45) first fed with high fat diet for 6 weeks followed by IRW (45 mg/kg BW) + high fat diet for another 8 weeks. All mice had ad libitum access to food and water. At the end of the experiment, mice were fasted for 6 h before being sacrificed by CO_2 asphyxiation. The organ tissues were weighed and recorded during the sacrifice. All of the tissues and serum samples were snap-frozen and stored at -80°C until further analysis.

3.2.6 *Drosophila melanogaster* study

To examine the effects of IRW on D-NAAM levels (NAMPT homolog), *yellow white* (*yw*) mutant female flies were used in the current study. All the virgin *yw* flies were reared, and the feeding trials were conducted in a humidified, temperature-controlled incubator at 25°C and 65% humidity on a 12-h light:12-h dark cycle. The standard fly food (ND: Normal diet) included 10%

sugar/yeast: 2% agar, 10% sucrose, 10% autolyzed yeast powder, 3% Nipagin, 0.3% propionic acid. The HFD group contained additional 30% coconut oil (as the fat source). IRW was dissolved in nuclease-free water and added to the molten media at ~50°C, stirred vigorously and then agitated constantly while being poured in plastic tubes. The final concentration of IRW in the food was 25 and 50 μM. Flies were kept in 30 ml plastic vials containing 4-5 ml of food/IRW^{+} media. For the no-drug control, the equivalent carrier was added (nuclease-free water). There were ~150 flies in each treatment group. The flies were transferred to vials containing fresh medium/treatment every 2 days for a period of 10 days. Following the completion of the study, whole-body protein or RNA material was obtained for further analysis.

3.2.7 Western Blot

Cells were grown in 24-well tissue culture plates until they reached ~80% confluency. They were then treated with 50 μM and 25 μM IRW for 24 h. After incubation, the culture medium was removed, and the cells were lysed in boiling Laemmle's buffer containing 50 μM dithiothreitol (DTT) and 0.2% Triton-X-100. These cell lysates were run on SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with primary antibodies according to the recommended concentration(s). After incubating with the secondary antibodies, protein bands were detected using a Licor Odyssey BioImager (Licor Biosciences, Lincoln, NB, USA) and quantified by densitometry using Image Studio Lite 5.2 software.

3.2.8 CRISPR-Cas9 guided Knockout cells

Guide RNAs (gRNAs) targeting human SIRT1 were prepared using Alt-R CRISPR-Cas9 tracrRNA, ATTO 550 and Alt-R CRISPR-Cas9 crRNA (IDT, Coralville, IA, USA) as previously described(Cromwell et al., 2018). The oligonucleotide sequences included SIRT1 crRNA: CUGAAUAUACCUCAGCGCCA, SIRT1 Sequencing Primer Fwd

TTTTCACACTTCCCTCCTTCAT, SIRT1 Sequencing Primer Rev: TCCTTGCTCTATCGAGTTCACA. The KO cells were treated with IRW (50 μM) or vehicle for 24 hours.

3.2.9 RT-PCR study

Total RNA was isolated from cell cultures, mouse tissues and flies by extraction with TRIzol reagent according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA using the reverse transcriptase (RT) system kit. All of the qPCR experiments and analyses were conducted using the MIQE guidelines for qPCR (Bustin et al., 2009).

3.2.10 NAD^+ measurement

NAD^+ was measured using the NAD colorimetric assay kit (ab65348). For cell samples, the L6 cells were treated with various compounds for 24 h and protein was extracted using RIPA buffer (ab156034). For animal tissues, plasma, liver, and muscle samples were homogenized in RIPA buffer and then centrifuged (15,000 x g for 15 min at 4°C) to obtain supernatant. This supernatant was used to quantify NAD^+ at OD 450 nm according to the manufacturer's instructions. The amount of NAD^+ was measured as pmol/μL.

3.2.11 RNA sequencing

Total RNA (500 ng) was used for the preparation of RNAseq libraries with the NEBNext Ultra II Directional RNA Library Prep Kit from Illumina (NEB). The RNA integrity number (RIN) for all samples used in RNAseq was >8, ensuring the high-quality selection of physiological RNAs and not degradation products. The mRNAs were enriched with oligo-dTs complementary to the poly-A tail, attached to paramagnetic beads and then chemically fragmented. Next, mRNA fragments were reverse-transcribed, and second-strand cDNA synthesis was performed. Double-stranded cDNAs were A-tailed to enable adapter ligation, and finally, libraries were indexed by PC (15 PCR

cycles). Libraries were sequenced on a NextSeq 500 instrument (Illumina), following a paired-end 150 cycle protocol. Samples were quantified with Kallisto,(Bray, Pimentel, Melsted, & Pachter, 2016) with 100 bootstraps and bias correction. Differential expression analyses were conducted with DESeq2 (Love, Huber, & Anders, 2014). Transcripts were considered differentially expressed when they had a corrected p-Value < 0.05. Deregulated transcripts were annotated using the BioMart database from Ensembl.

3.2.12 Docking study

Docking of IRW was performed using Smina (Koes, Baumgartner, & Camacho, 2013) and the 'cross-docking' strategy (Wingert & Camacho, 2018; Wingert, Oerlemans, & Camacho, 2018). The top-ranked models of IRW tripeptide were predicted by Smina to bind in the same pocket as the known ligand FK866.

3.2.13 NAMPT immunoprecipitation and activity assay

NAMPT immunoprecipitation and activity were performed using the NAMPT Activity Assay Kit (ab221819) according to the manufacturer's instructions. The NAMPT activity was measured using the two-step activity assay for immunoprecipitated cell lysates as described in the protocol booklet (Step 14). The enzyme-based activity was finally calculated based on conversion of WST-1 to WST-1 formazan after performing necessary steps (two-step method; ab221819) at OD 450 nm on a microplate reader. The data were expressed as fold change (activity, *w.r.t* vehicle) for NAMPT immunoprecipitation study and NAMPT activity (w.r.t WST-1 formazan formation) for enzyme-based activity.

3.2.14 Cell fractionation study

Cell fractionation was performed using the cell fractionation kit obtained from Cell Signaling Technology (Danvers, MA, U.S.A) and performed according to the manufacturer's instructions.

3.2.15 Statistical analysis

A completely randomized design (CRD) was used to perform all the experiments conducted in this study where the treatments are assigned completely at random to cells and animals so that each experimental unit has the same chance of receiving any the treatment option(s). All data are presented as mean ± standard deviation (SD) of minimum three independent experiments. All statistical analyses were performed using GraphPad Prism software version 5.02 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± SD and analyzed by unpaired Student's t-test (Figure 3.1E; Figure 3.4 A-C; Figure 3.7 A,C), or one-way ANOVA followed by Dunnett's test (vs HFD). $p<0.05$ was considered significant.

3.3 Results

3.3.1 Effect of aging and obesity on NAMPT, SIRT1, and $p66^{SHC}$

Given the important role of NAD^+ and NAMPT in metabolism (Figure 3.1A), we characterized their levels in muscle tissues of normal and obese patients with significant aging patterns. In addition, we examined two markers, SIRT1 and $p66^{SHC}$, based on their close relationship with cellular NAD^+ levels (Imai, 2010; Nemoto et al., 2006). NAMPT expression significantly decreased in the muscle tissues of obese patients compared to the healthy individuals (Figure 3.1B), revealing a possible correlative link between the expression of this gene and obesity. Interestingly, protein levels of SIRT1 in obese patients were decreased, while the levels of $p66^{SHC}$, a key enzyme involved in negative regulation of oxidative homeostasis and lifespan extension, were increased (Figure 3.1 C, D). These data confirm previous reports which show a correlation between increased oxidative stress via SIRT1 and $p66^{SHC}$ modulation and decreased NAMPT expression (Imai, 2010; Nemoto et al., 2006). Consistent with the decrease in NAMPT expression,

we found that levels of NAD^+ in muscle tissues of obese individuals were significantly reduced as well (Figure 3.1E). Interestingly, when the NAMPT levels were compared between the same muscle tissues of young (40 or less) and older individuals (40 or more), independently of their body mass index (BMI) status, there was no significant difference between the two groups (Figure S3.7). These data indicate the vital role of obesity in lowering the NAMPT levels among human subjects.

3.3.2 IRW treatment increases NAMPT expression in cultured cells

After observing the effects of obesity and aging on levels of NAMPT, SIRT1, and $p66^{SHC}$, it was determined if IRW could alter the expression of these proteins in cultured cells or HFD mice. Our initial investigation was performed using L6 cells, a rat skeletal muscle cell line. Following IRW treatment (50 μM), L6 cells showed a significant increase in protein levels of NAMPT *in vitro* ($p<0.05$) (Figure 3.2A). However, no increase was observed at the lower concentration of IRW (25 μM). IRW treatment also did not significantly increase SIRT1 at 25 and 50 μM treatment levels *in vitro* ($p=0.06$) (Figure 3.2B). Further, immunoprecipitation analysis using endogenous NAMPT immunoprecipitated from cell extracts showed that NAMPT activity was significantly increased after IRW treatment (50 μM) ($p<0.01$) (Figure 3.2C). Similarly, NAMPT activity assay using recombinant human NAMPT protein showed significantly increased NAMPT activity, based on WST-1 formazan reaction *in vitro*, following IRW treatment at 25 μM ($p<0.05$) and 50 μM ($p<0.01$) (Figure 3.2D). However, to confirm the increase in NAMT activity by IRW, additional substrate-based *in vitro* enzyme kinetics using the Michaelis-Menten equation are required.

3.3.3 IRW treatment increases NAMPT and related biomarkers in C57BL/6J mice

Next, we observed the impact of IRW feeding (45mg/kg BW) on NAMPT levels and closely related biomarkers in C57BL/6J HFD mice. Results showed that NAMPT levels were significantly

increased in both muscle ($p<0.05$) and liver tissues ($p<0.001$) of C57BL/6J mice by an average of 4- and 6-folds respectively (Figure 3.3 A,E). The levels of FOXO3a levels, a NAMPT regulator, were also increased in both muscle ($p<0.01$) and liver tissues ($p<0.001$) as well as *in vivo* (Figure 3.3 B,E). However, the levels of SIRT1 were increased solely in liver tissues ($p<0.001$) (Figure 3.3 G) while the levels of SIRT1 remained unchanged in muscle tissues *in vivo* (Figure. 3.3 C). Likewise, levels of oxidative stress gene $p66^{SHC}$ were reduced in both muscle and liver tissues following IRW feeding (45mg/kg BW) *in vivo* (Figure S3.4 B,C). These results show the activation of the NAMPT/FOXO3a/SIRT1 axis towards a possible increase in NAD^+ in cells and in mice.

3.3.4 IRW treatment increases mRNA levels of NAMPT and NAD^+ expression in cultured cells and in C57BL/6J mice

In addition to decreasing NAMPT levels, high-fat diet feeding is known to cause a subsequent decrease in NAD^+ levels (Balan et al., 2008; Cantó et al., 2012; Mercader et al., 2008; Yoshino et al., 2011). Thus, we tested whether treatment with IRW counteracted these changes and, further, if so, whether mRNA levels of NAMPT accompanied such changes. Gene expression analysis of L6 muscle cells indicated that NAMPT mRNA levels numbers were significantly ($p<0.01$) altered by treatment with 50 μM IRW (Figure 3.4A), consistent with the observed induction of the protein (Figure 3.2A). However, the increase in NAMPT mRNA levels persists but was not statistically significant in 293T cells (Figure S3.2C). Treatment with IRW in HFD-fed mice increased levels of RNA expression levels in both the muscle (Figure 3.4B) and the liver (Figure 3.4C). IRW also improved NAD^+ content and mitigated the effects of FK866 mediated NAMPT inhibition in cultured cells ($p<0.01$) (Figure 3.4D). Treatment with IRW noticeably increased NAD^+ levels in both muscle ($p<0.05$) and liver tissues ($p<0.01$) of HFD-fed C57BL/6J obese mice (Figure 3.4E, F). Finally, the circulating levels of NAD^+ levels were significantly improved ($p<0.001$) after

treatment with IRW *in vivo*, indicating an overall improvement in the metabolic status of HFD mice (Figure 3.4G).

3.3.5 IRW increases expression of NAMPT and related NAD^+ synthesis pathway genes

We next sought to understand the mechanisms by which IRW increases NAMPT protein levels and NAD^+ production and to investigate its relationship to other metabolism specific genes. Based on the above results, we expected that IRW treatment would increase the levels of genes associated with the NAD^+ pathway. To do this, we conducted whole-genome transcriptome analysis. Consistent with the changes observed in L6 cells and HFD fed C57BL/6J mice, IRW treatment increased NAMPT gene expression in the liver of C57BL/6J mice (Figure 3.5A), suggesting that its protein induction was mediated by an increase in mRNA transcription. This finding aligns with strong increase in NAMPT protein levels in liver tissues of mice (Figure 3.3E). Interestingly, several other genes related to NAD^+ biosynthesis in the liver and muscle tissues, including NAD(P)H Quinone Dehydrogenase 1 (NQO1), NAD(P) Dependent Steroid Dehydrogenase-Like (NSDHL), Isocitrate Dehydrogenase (NADP), Mitochondrial (IDH2) in the liver (Figure 3.5B-D), and Nicotinamide Riboside Kinase 2 (NMRK2) in the muscle (Figure 3.5E) were increased by IRW treatment at the mRNA level. NQO1 plays multiple roles in cellular adaptation to stress and exhibits therapeutic potential via the ability to generate NAD^+ and stimulate sirtuin and poly (ADP-ribose) polymerase (PARP) activities. This is particularly crucial and has gained robust attention concerning its potential in the treatment of the metabolic syndrome (Ross & Siegel, 2017). Likewise, modulation of NSDHL and IDH2 by IRW are suggestive of an improvement in the metabolic status of HFD fed C57BL/6J mice treated with IRW. Further, the fact that NMRK2 is increased (which is capable of boosting NAD^+ levels), aligns with the increased levels of NAD^+ in skeletal muscle. This finding may explain an increase in NAMPT levels in muscle tissues of

IRW treated C57BL/6J mice as NMRK2 is the most predominantly expressed NAD^+ biosynthesis gene in muscle tissues (Fletcher et al., 2017).

3.3.6 IRW modulates D-NAAM expression in *yw Drosophila melanogaster*

In agreement with the data from mammalian cells and tissues, we found that treatment of *yw Drosophila melanogaster* with IRW peptide resulted in a minor increase in the levels of SIRT1, and D-NAAM, concomitant with an induction of NAD^+ levels (Figure 3.6A-C). Additionally, $p66^{SHC}$ levels were reduced by IRW treatment (25 μM and 50 μM) in HFD group (Figure S3.4 D,E). However, an increase in FOXO3a levels and a decline in $p66^{SHC}$ levels in the ND group was negligible (Figure 3.6A, S3.4 D,E). When coupled with the earlier data indicating increased NAMPT expression in cultured cells and C57BL/6J mice, these results provide strong evidence that IRW supplementation can boost D-NAAM/NAMPT levels, and consequently NAD^+ *in vivo*.

3.3.7 IRW stimulated NAMPT increase is dependent on SIRT1

SIRT1 is an NAD^+ dependent protein deacetylase and is an essential regulator in energy metabolism (Koltai et al., 2010). We stimulated both wild type (WT) and SIRT1 knock out (KO) 293T cells with 50 μM IRW for 24 h. At first, we observed that SIRT1 KO cells had lower NAD^+ levels compared to WT cells (Figure 3.7A). This indicates a key role of SIRT1 in NAD^+ metabolism and associated metabolic function. Secondly, we confirmed by western blot analysis that basal expression of NAMPT is dependent on SIRT1 (Figure 3.7B). Results showed that SIRT1 KO cells had lower levels of NAMPT compared to the WT cells, indicating a direct relation between NAMPT and SIRT1. Further, IRW treatment was unable to increase NAMPT protein and mRNA levels in SIRT1 KO 293T cells (Figure 3.7 B,C). We observed that IRW treatment was unable to increase the NAMPT levels in the SIRT1 KO cells as observed in WT cells, indicating SIRT1 dependency in IRW's mechanism of action.

3.3.8 IRW interaction with NAMPT

Binding of IRW to the NAMPT active site was identified by molecular docking approaches (Figure 8). Top ranked models of IRW tripeptide were predicted by smina to bind in the same pocket as the NAMPT ligand FK866 (Figure 3.8 A,B). The top pose identified by smina appears to be stabilized by a large number of hydrogen bonds made by both backbone and side-chain residues. In particular, the arginine side chain of IRW is in position to make hydrogen bonds with the Asp184 backbone as well as Asn182 and Glu376 side chains (Figure 3.8 A). Other hydrogen bonds are between the C-terminal oxygen atoms of IRW and Lys189 and Val350, as well as one between the backbone of arginine, a crystal water (HOH861) present in co-crystal PDB 4KFN (Figure 3.8 A). This differs from FK866 which interacts primarily through hydrophobic interactions, making only a single hydrogen bond with Ser275 (Figure 3.8 B). Therefore, the data indicate that in an intracellular milieu where IRW increases the NAMPT pool to produce more NAD^+, the interaction of IRW with active sites of NAMPT may promote NAD^+ accumulation.

3.4 Discussion

NAD^+ is a cofactor required for over 500 enzymatic reactions and plays a key role in human health and disease (Ansari & Raghava, 2010). Compromised NAD^+ status is considered a key hallmark of metabolic dysfunction and age-related disease (Frederick et al., 2016; Imai, 2010; Warburg et al., 1936). Moreover, there is evidence that diminished NAD^+ levels can accelerate the onset of age-related disease, while pharmacological interventions that stimulate NAD^+ can delay such progression (Fang et al., 2016). The present research outlines a new approach for boosting levels of this important metabolite and could have therapeutic benefits (Revollo et al., 2007). Skeletal muscle is one of the primary tissues acceding to NAD^+ dysregulation associated with obesity

(Blüher, 2019). Therefore, human skeletal muscle samples were chosen to compare changes in NAMPT and related biomarkers *ex vivo*. This data indicated the depletion of both NAMPT and SIRT1 in obese human skeletal muscle tissues, providing evidence that metabolic dysfunction diminishes NAD^+ biosynthesis. To our surprise, $p66^{SHC}$ was also severely affected by an increase in BMI of patients. This showed altered and pathological redox stress in the skeletal muscle of obese patients. The results from human tissue samples strengthened our approach that stimulation of NAMPT, upstream of NAD^+ biogenesis, may prove to be effective in delaying and treating a variety of metabolic disorders. Therefore, improvement of metabolic disorders is associated with NAMPT replenishment, (Fletcher et al., 2017), and we hypothesized that owing to its ability to modulate metabolic and hypertension pathways, (Fletcher et al., 2017) IRW can increase NAMPT levels.

This work represents the first identification of a natural peptide-based booster for NAMPT pool and contributes to the limited collection of compounds targeting NAMPT for the purposes of boosting NAD^+ biosynthesis. The availability of a small peptide of this node allows the identification of lead molecules in yeast, fly, or mammalian model systems. Such a small molecule will also be useful to dissect the NAD^+ based drug discovery in detail and suggests that other members of this pathway could be a druggable node for short peptides. Importantly, we show that this peptide not only works in cell culture but also in mice, as initially evidenced by our studies which established the ability of IRW to increase NAMPT levels in cultured cells, without exhibiting toxicity to cells (Figure S3.1). The higher NAD^+ and NAMPT production in the liver of HFD fed C57BL/6J mice treated with IRW may be attributed to the organ's direct role in NAMPT/NAD^+ pathway (de Boer, Bahr, Boker, Manns, & Tietge, 2009; Ramsey et al., 2009). The bioavailability prerequisite for further exploration appeared to be met, given the ability of

IRW to elevate NAMPT and NAD^+ *in vivo* at a moderate dose (45 mg/kg BW). Interestingly, we also found that not all cell types responded equally to IRW treatment. Apart from L6 cells, Hela and MDAMB231 cells also showed an increase in NAMPT levels after IRW treatment (Figure S3.2 A,B). However, IRW showed a much weaker effect in 293T cells. The smaller increases of NAMPT mRNA in 293T cells portend that sensitivity to IRW treatment is not uniform across different cell types (Figure S3.2 C). This may be due to the fact that human NAD^+ metabolism varies extensively across cell types,(Fang et al., 2016; Imai, 2010; Krebs et al., 1969; Warburg et al., 1936) thus also reflected in our study. Furthermore, we also found that levels of other key intracellular enzymes that consume NAD^+, such as SIRT1, FOXO3a, and $p66^{SHC}$ were altered by IRW treatment, while negative regulators of NAD^+ (NDAase) such as the CD38 and DBC1 remained unchanged (Figure S3.3 A-F). We showed that the effects on NAMPT protein induction are mediated at the mRNA level (Figure 3.4 A-C, Figure 3.5A) and that knockout of SIRT1 may have some ability to perturb this induction (Figure 3.7 B-C). Future work will need to be performed to examine the precise mechanism(s) elucidating how IRW is able to transcriptionally induce NAMPT, and whether or not the induction of physiologically related factors, such as SIRT1 and FOXO3a, are directly or indirectly related, or independent from NAMPT induction (Araki, Sasaki, & Milbrandt, 2004).

Treatment of cultured cells with resveratrol and other sirtuin activators has been shown to activate PGC1α and prevent diseases commonly associated with metabolic dysfunction and aging (Lagouge et al., 2006). In line with this, we observed that IRW increases the levels of antioxidant components of oxidative phosphorylation, indicating that the beneficial effects of IRW may be in part due to PGC1α mediated increase in mitochondrial biogenesis and a shift toward a more oxidative state of the cell (Figure S3.5 A-C). Therefore, we next aim to understand if and how

IRW can mimic resveratrol and caloric restriction, protect against a high-fat diet, and improve mitochondrial function and lifespan, possibly by activating PGC1α axis. This future direction further corroborates the striking parallels between IRW and resveratrol, and increased SIRT1 levels observed in mice liver. Further, to better understand the ability of IRW to increase and stabilize NAMPT in cells, we co-treated cells with IRW and FK866. IRW managed to preserve the fall in NAMPT and NAD^+ levels. These results were concordant with the modulation of $p66^{SHC}$ (Figure S3.4 A-E), which regulates the cellular response to oxidative stress and life span (Migliaccio et al., 1999). Also, the knockout of $p66^{SHC}$ is associated with increased NAD^+ pool and favorable environment for Sirtuin activity (Pérez et al., 2018). Therefore, the $p66^{SHC}$ reduction by IRW further establishes a relationship between improved metabolic outcomes with reduced oxidative stress and diminished DNA damage.

Our findings are similar to the latest discovery of SBI-797812, a small molecule activator of NAMPT (Gardell et al., 2019). However, the mode of interaction of IRW was different from the SBI-797812, which is an active-site targeted ligand. Similar to IRW, P7C3-A20 also binds directly to NAMPT and facilitates the replenishment of NAD^+ levels in doxorubicin-treated U2OS cells, but not in normal cells at the basal levels at the dose of 3 μM (Wang et al., 2014). P7C3-A20 also re-shapes gut microbiota towards alleviation of obesity and exhibits cognitive benefits by repairing the blood brain barrier *in vivo* (Hua et al., 2020; Vázquez-Rosa et al., 2020). We also conducted an experiment to compare the protein levels of NAMPT following treatment with IRW and P7C3-A20 in L6 cells (Figure 3.8C). Interestingly, our results showed a statistically insignificant increase (average 2-fold increase; $p=0.4461$) in NAMPT levels following P7C3-A20 treatment (3 μM) in L6 cells (Figure 3.8C). This observation is in contradiction to previous reports on P7C3-A20 indicating an increase in NAMPT expression at the tested concentration (3 μM)

(Wang et al., 2014). These observations of P7C3-A20 treatment in L6 cells (Figure 3.8C) might be attributed to cell type as the original paper used U2OS and H2122 (both cancer cells) instead of normal cells. However, IRW extended the NAMPT increase in both normal (L6 cells) (Figure 3.2) and cancer cells (Hela and MDAMB231) (Figure S3.2 A,B).

Apart from cultured mammalian cells and mouse model studies, IRW had a potent effect on the D-NAAM and NAD^+ levels in *Drosophila* flies warranting its further investigation in metabolic and mitochondrial studies. Using 293T SIRT1 knockout cells, we were able to test whether the pharmacological effect of IRW treatment on NAMPT levels is dependent upon SIRT1. Following treatment with IRW, WT cells exhibited NAMPT increase while SIRT1 KO cells exhibited no improvement in NAMPT synthesis at both mRNA and protein levels. This data indicates that IRW improved NAMPT levels in a manner that is to a certain extent dependent upon SIRT1. It is also worth noting that the basal NAMPT levels are reduced in SIRT1 KO cells indicating the cross-dependency of both metabolic nodes (Figure 3.7B). Therefore, we favor a possible model of the mechanism whereby IRW first interacts SIRT1, which leads to subsequent increase in levels of NAMPT (Figure 3.9). However, extensive subsequent experimentation is required to support and establish this idea.

Previous studies on IRW have shown that it has robust antioxidant activity, antihypertensive activity, NO-mediated vasodilation activity, and anti-inflammatory activity (*in vivo*) (W.-Y. Huang, Majumder, & Wu, 2010; W. Huang, Chakrabarti, Majumder, Jiang, Davidge, & Wu, 2010; Liao, Fan, Davidge, & Wu, 2019; Majumder, Chakrabarti, Davidge, & Wu, 2013; Majumder, Chakrabarti, Morton, et al., 2013; Majumder, Liang, Chen, Guan, Davidge, & Wu, 2015). It is now recognized that NAMPT plays a key role in hypertension (SA Capettini, Montecucco, Mach, Stergiopulos, AS Santos, & F da Silva, 2012). Therefore, it is also tempting

to speculate that the induction of NAMPT levels may underlie many of the therapeutic benefits of IRW treatment, (Shang, Bhullar, Hubbard, & Wu, 2019) especially in the case of cardiovascular disease(s). The well studied renin–angiotensin system regulation of IRW might be associated with underlying NAMPT and NAD^+ surge (W.-Y. Huang et al., 2010; W. Huang et al., 2010; Liao et al., 2019; Majumder, Chakrabarti, Davidge, et al., 2013; Majumder, Chakrabarti, Morton, et al., 2013; Majumder et al., 2015; SA Capettini et al., 2012). Molecular studies with IRW also helped us to study its impact on NAD^+ consuming SIRT1 axis, indicating a systemic improvement in the metabolic status of HFD fed C57BL/6J mice. Besides, IRW supplementation in flies improved D-NAAM levels and increased NAD^+ levels to reinforce the robust *in vivo* effect of the peptide. The therapeutic utility of IRW although is in line with NAD^+ boosters such as NR or NMN. Further, augmented NAMPT levels by IRW holds an advantage over NAD^+ precursors via activation of SIRT1 and a possible upturn in DNA repair and slowing of the aging clock (Y. Li, He, He, Li, & Lindgren, 2013; Y. Li et al., 2011). The ability of IRW to catalytically promote NAD^+ synthesis in different cell organelles (Figure S3.6 A, B) possibly via increased NAMPT activity or increased NAMPT pool (Figure 3.2 C,D) along with its ability to work via the SIRT1 axis are two discriminating features that are likely to be pharmacologically beneficial. Future work can investigate this hypothesis. In addition to elucidating the mechanism through which IRW transcriptionally induces NAMPT and related metabolic factors, it will be interesting to investigate which chemical properties of IRW are necessary for its effectiveness. For example, does the tryptophan in the peptide mediate the majority of the effects, or if the full peptide sequence is necessary; are alternative sequences resembling IRW equally or more effective at boosting NAMPT levels. However, our pilot study in this regard did not show a discriminating result, so, a detailed study is needed to fully comprehend the structure-function relationship (Figure S3.6 C,

D). Building on this improved peptide architecture, bioavailability, structure-function, and pharmacokinetic studies can be performed in mice to improve formulations. Ultimately, IRW, the first small peptide booster of NAMPT levels *in vivo*, and can be further explored for the treatment of diseases mediated by low levels of NAMPT and NAD^+.

3.5 Conclusions

Dysregulation of NAD^+ has been implicated in a wide number of diseases (Frederick et al., 2016; Imai, 2010). Several molecules capable of boosting NAD^+ levels in the biosynthetic pathway have now been discovered and shown to benefit numerous disease conditions and even extend lifespan (Fang et al., 2016). As the rate-limiting enzyme in the synthesis of NAD^+, NAMPT activation or increase in pool levels represents an alternative approach to boost NAD^+ levels. There are only two known synthetic activators, SBI-797812 and P7C3-A20, and the latter is in dispute; our own test also failed to show the ability of P7C3-A20 to increase protein levels of NAMPT. In this study, we found for the first time IRW is a small dietary peptide activator of NAMPT. Furthermore, we showed that IRW treatment boosts the level of this metabolite in both cells and *in vivo*. A role of food on health is well established; our finding can lead to the development of a food derived therapeutic intervention for the mitigation of ageing-related chronic diseases. This small peptide can also serve as a lead compound for the rational design of novel druggable components for therapeutical uses.

3.6 References

Ansari, H. R., & Raghava, G. P. (2010). Identification of NAD interacting residues in proteins. *BMC bioinformatics, 11*(1), 160.

Araki, T., Sasaki, Y., & Milbrandt, J. (2004). Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science, 305*(5686), 1010-1013.

Balan, V., Miller, G. S., Kaplun, L., Balan, K., Chong, Z.-Z., Li, F., . . . Freeman, D. C. (2008). Life span extension and neuronal cell protection by Drosophila nicotinamidase. *Journal of Biological Chemistry, 283*(41), 27810-27819.

Blüher, M. (2019). Obesity: global epidemiology and pathogenesis. Nature Reviews Endocrinology, 288e298.

Bray, N. L., Pimentel, H., Melsted, P., & Pachter, L. (2016). Near-optimal probabilistic RNA-seq quantification. *Nature biotechnology, 34*(5), 525-527.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., . . . Shipley, G. L. (2009). The MIQE Guidelines: M inimum I nformation for Publication of Q uantitative Real-Time PCR E xperiments. Oxford University Press.

Cantó, C., Houtkooper, R. H., Pirinen, E., Youn, D. Y., Oosterveer, M. H., Cen, Y., . . . Cettour-Rose, P. (2012). The NAD+ precursor nicotinamide riboside enhances oxidative metabolism and protects against high-fat diet-induced obesity. *Cell metabolism, 15*(6), 838-847.

Cromwell, C. R., Sung, K., Park, J., Krysler, A. R., Jovel, J., Kim, S. K., & Hubbard, B. P. (2018). Incorporation of bridged nucleic acids into CRISPR RNAs improves Cas9 endonuclease specificity. *Nature communications, 9*(1), 1-11.

Das, A., Huang, G. X., Bonkowski, M. S., Longchamp, A., Li, C., Schultz, M. B., . . . Lu, Y. (2018). Impairment of an endothelial NAD+-H2S signaling network is a reversible cause of vascular aging. *Cell, 173*(1), 74-89. e20.

de Boer, J. F., Bahr, M. J., Boker, K. H., Manns, M. P., & Tietge, U. J. (2009). Plasma levels of PBEF/Nampt/visfatin are decreased in patients with liver cirrhosis. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 296*(2), G196-G201.

Fang, E. F., Kassahun, H., Croteau, D. L., Scheibye-Knudsen, M., Marosi, K., Lu, H., . . . Wilson, M. A. (2016). NAD+ replenishment improves lifespan and healthspan in ataxia telangiectasia models via mitophagy and DNA repair. *Cell metabolism, 24*(4), 566-581.

Fletcher, R. S., Ratajczak, J., Doig, C. L., Oakey, L. A., Callingham, R., Xavier, G. D. S., . . . Migaud, M. E. (2017). Nicotinamide riboside kinases display redundancy in mediating nicotinamide mononucleotide and nicotinamide riboside metabolism in skeletal muscle cells. *Molecular metabolism, 6*(8), 819-832.

Frederick, D. W., Loro, E., Liu, L., Davila Jr, A., Chellappa, K., Silverman, I. M., . . . Davis, J. G. (2016). Loss of NAD homeostasis leads to progressive and reversible degeneration of skeletal muscle. *Cell metabolism, 24*(2), 269-282.

Frydelund-Larsen, L., Akerstrom, T., Nielsen, S., Keller, P., Keller, C., & Pedersen, B. K. (2007). Visfatin mRNA expression in human subcutaneous adipose tissue is regulated by exercise. *American Journal of Physiology-Endocrinology and Metabolism, 292*(1), E24-E31.

Gardell, S. J., Hopf, M., Khan, A., Dispagna, M., Sessions, E. H., Falter, R., . . . Petucci, C. (2019). Boosting NAD+ with a small molecule that activates NAMPT. *Nature communications, 10*(1), 1-12.

Hua, X., Sun, D. Y., Zhang, W. J., Fu, J. T., Tong, J., Sun, S. J., . . . Wang, S. N. (2020). P7C3-A20 alleviates fatty liver by shaping gut microbiota and inducing FGF21/FGF1, via the AMP-activated protein kinase/CREB regulated transcription coactivator 2 pathway. *British Journal of Pharmacology*.

Huang, W.-Y., Majumder, K., & Wu, J. (2010). Oxygen radical absorbance capacity of peptides from egg white protein ovotransferrin and their interaction with phytochemicals. *Food Chemistry, 123*(3), 635-641.

Huang, W., Chakrabarti, S., Majumder, K., Jiang, Y., Davidge, S. T., & Wu, J. (2010). Egg-derived peptide IRW inhibits TNF-α-induced inflammatory response and oxidative stress in endothelial cells. *Journal of Agricultural and Food Chemistry, 58*(20), 10840-10846.

Imai, S.-i. (2010). A possibility of nutriceuticals as an anti-aging intervention: activation of sirtuins by promoting mammalian NAD biosynthesis. *Pharmacological research, 62*(1), 42-47.

Koes, D. R., Baumgartner, M. P., & Camacho, C. J. (2013). Lessons learned in empirical scoring with smina from the CSAR 2011 benchmarking exercise. *Journal of chemical information and modeling, 53*(8), 1893-1904.

Koltai, E., Szabo, Z., Atalay, M., Boldogh, I., Naito, H., Goto, S., . . . Radak, Z. (2010). Exercise alters SIRT1, SIRT6, NAD and NAMPT levels in skeletal muscle of aged rats. *Mechanisms of ageing and development, 131*(1), 21-28.

Krebs, H., & Veech, R. (1969). Equilibrium relations between pyridine nucleotides and adenine nucleotides and their roles in the regulation of metabolic processes. *Advances in enzyme regulation, 7*, 397-413.

Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., . . . Elliott, P. (2006). Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1α. *Cell, 127*(6), 1109-1122.

Li, J., Bonkowski, M. S., Moniot, S., Zhang, D., Hubbard, B. P., Ling, A. J., . . . Gorbunova, V. (2017). A conserved NAD+ binding pocket that regulates protein-protein interactions during aging. *Science, 355*(6331), 1312-1317.

Li, Y., He, J., He, X., Li, Y., & Lindgren, U. (2013). Nampt expression increases during osteogenic differentiation of multi-and omnipotent progenitors. *Biochemical and Biophysical Research Communications, 434*(1), 117-123.

Li, Y., He, X., Li, Y., He, J., Anderstam, B., Andersson, G., & Lindgren, U. (2011). Nicotinamide phosphoribosyltransferase (Nampt) affects the lineage fate determination of mesenchymal stem cells: a possible cause for reduced osteogenesis and increased adipogenesis in older individuals. *Journal of Bone and Mineral Research, 26*(11), 2656-2664.

Liao, W., Fan, H., Davidge, S. T., & Wu, J. (2019). Egg White–Derived Antihypertensive Peptide IRW (Ile-Arg-Trp) Reduces Blood Pressure in Spontaneously Hypertensive Rats via the ACE2/Ang (1-7)/Mas Receptor Axis. *Molecular nutrition & food research, 63*(9), 1900063.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology, 15*(12), 550.

Majumder, K., Chakrabarti, S., Davidge, S. T., & Wu, J. (2013). Structure and activity study of egg protein ovotransferrin derived peptides (IRW and IQW) on endothelial inflammatory response and oxidative stress. *Journal of agricultural and food chemistry, 61*(9), 2120-2129.

Majumder, K., Chakrabarti, S., Morton, J. S., Panahi, S., Kaufman, S., Davidge, S. T., & Wu, J. (2013). Egg-derived tri-peptide IRW exerts antihypertensive effects in spontaneously hypertensive rats. *PloS one, 8*(11), e82829.

Majumder, K., Liang, G., Chen, Y., Guan, L., Davidge, S. T., & Wu, J. (2015). Egg ovotransferrin-derived ACE inhibitory peptide IRW increases ACE2 but decreases proinflammatory genes expression in mesenteric artery of spontaneously hypertensive rats. *Molecular nutrition & food research, 59*(9), 1735-1744.

Mercader, J., Granados, N., Caimari, A., Oliver, P., Bonet, M., & Palou, A. (2008). Retinol-binding protein 4 and nicotinamide phosphoribosyltransferase/visfatin in rat obesity models. *Hormone and metabolic research, 40*(07), 467-472.

Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P. P., . . . Pelicci, P. G. (1999). The p66 shc adaptor protein controls oxidative stress response and life span in mammals. *Nature, 402*(6759), 309-313.

Nemoto, S., Combs, C. A., French, S., Ahn, B.-H., Fergusson, M. M., Balaban, R. S., & Finkel, T. (2006). The mammalian longevity-associated gene product p66shc regulates mitochondrial metabolism. *Journal of Biological Chemistry, 281*(15), 10555-10560.

Pérez, H., Finocchietto, P. V., Alippe, Y., Rebagliati, I., Elguero, M. E., Villalba, N., . . . Carreras, M. C. (2018). p66Shc inactivation modifies RNS production, regulates Sirt3 activity, and improves mitochondrial homeostasis, delaying the aging process in mouse brain. *Oxidative medicine and cellular longevity, 2018*.

Pillai, V. B., Sundaresan, N. R., Kim, G., Gupta, M., Rajamohan, S. B., Pillai, J. B., . . . Gupta, M. P. (2010). Exogenous NAD blocks cardiac hypertrophic response via activation of the

SIRT3-LKB1-AMP-activated kinase pathway. *Journal of Biological Chemistry, 285*(5), 3133-3144.

Rajman, L., Chwalek, K., & Sinclair, D. A. (2018). Therapeutic potential of NAD-boosting molecules: the in vivo evidence. *Cell metabolism, 27*(3), 529-547.

Ramsey, K. M., Yoshino, J., Brace, C. S., Abrassart, D., Kobayashi, Y., Marcheva, B., . . . Lee, C. (2009). Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. *Science, 324*(5927), 651-654.

Revollo, J. R., Körner, A., Mills, K. F., Satoh, A., Wang, T., Garten, A., . . . Townsend, R. R. (2007). Nampt/PBEF/visfatin regulates insulin secretion in β cells as a systemic NAD biosynthetic enzyme. *Cell metabolism, 6*(5), 363-375.

Ross, D., & Siegel, D. (2017). Functions of NQO1 in cellular protection and CoQ10 metabolism and its potential role as a redox sensitive molecular switch. *Frontiers in physiology, 8*, 595.

SA Capettini, L., Montecucco, F., Mach, F., Stergiopulos, N., AS Santos, R., & F da Silva, R. (2012). Role of renin-angiotensin system in inflammation, immunity and aging. *Current pharmaceutical design, 18*(7), 963-970.

Shang, N., Bhullar, K. S., Hubbard, B. P., & Wu, J. (2019). Tripeptide IRW initiates differentiation in osteoblasts via the RUNX2 pathway. *Biochimica et Biophysica Acta (BBA)-General Subjects, 1863*(6), 1138-1146.

Stromsdorfer, K. L., Yamaguchi, S., Yoon, M. J., Moseley, A. C., Franczyk, M. P., Kelly, S. C., . . . Yoshino, J. (2016). NAMPT-mediated NAD+ biosynthesis in adipocytes regulates adipose tissue function and multi-organ insulin sensitivity in mice. *Cell reports, 16*(7), 1851-1860.

Udenigwe, C. C., & Rouvinen-Watt, K. (2015). The role of food peptides in lipid metabolism during dyslipidemia and associated health conditions. *International journal of molecular sciences, 16*(5), 9303-9313.

Vázquez-Rosa, E., Shin, M.-K., Dhar, M., Chaubey, K., Cintrón-Pérez, C. J., Tang, X., . . . Barker, S. (2020). P7C3-A20 treatment one year after TBI in mice repairs the blood–brain barrier, arrests chronic neurodegeneration, and restores cognition. *Proceedings of the National Academy of Sciences, 117*(44), 27667-27675.

Wang, G., Han, T., Nijhawan, D., Theodoropoulos, P., Naidoo, J., Yadavalli, S., . . . McKnight, S. L. (2014). P7C3 neuroprotective chemicals function by activating the rate-limiting enzyme in NAD salvage. *Cell, 158*(6), 1324-1334.

Warburg, O., & Christian, W. (1936). Pyridine, the hydrogen transfusing component of fermentative enzymes. *Helv Chim Acta, 19*, 79-88.

Wingert, B. M., & Camacho, C. J. (2018). Improving small molecule virtual screening strategies for the next generation of therapeutics. *Current opinion in chemical biology, 44*, 87-92.

Wingert, B. M., Oerlemans, R., & Camacho, C. J. (2018). Optimal affinity ranking for automated virtual screening validated in prospective D3R grand challenges. *Journal of computer-aided molecular design, 32*(1), 287-297.

Yang, H., Yang, T., Baur, J. A., Perez, E., Matsui, T., Carmona, J. J., . . . Rosenzweig, A. (2007). Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival. *Cell, 130*(6), 1095-1107.

Yoshino, J., Mills, K. F., Yoon, M. J., & Imai, S.-i. (2011). Nicotinamide mononucleotide, a key NAD+ intermediate, treats the pathophysiology of diet-and age-induced diabetes in mice. *Cell metabolism, 14*(4), 528-536.

Zhang, R.-Y., Qin, Y., Lv, X.-Q., Wang, P., Xu, T.-Y., Zhang, L., & Miao, C.-Y. (2011). A fluorometric assay for high-throughput screening targeting nicotinamide phosphoribosyltransferase. *Analytical biochemistry, 412*(1), 18-25.

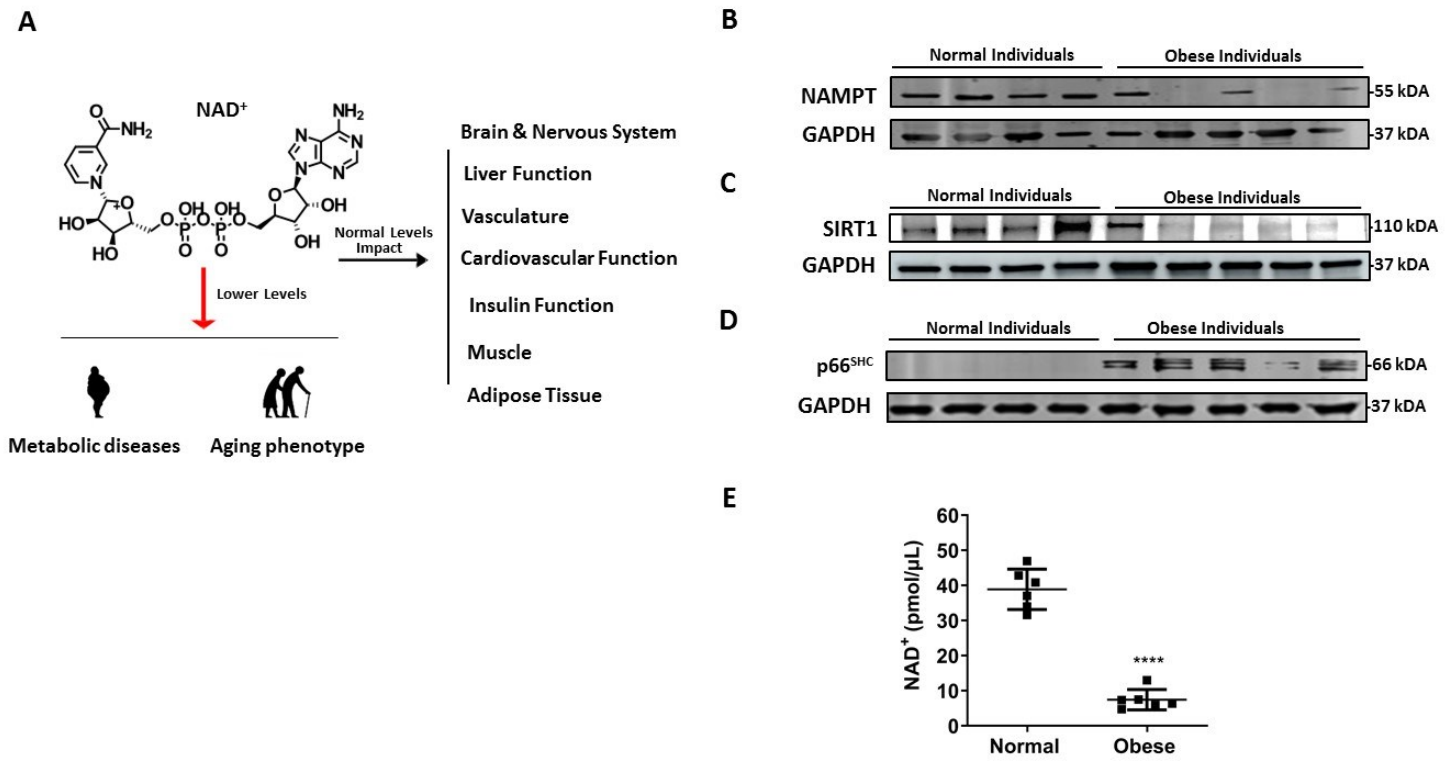


Figure 3.1 NAD$^+$ and NAMPT as key indicators of human health and aging. Physiological effects of NAD$^+$ as a key metabolic molecule (A) that controls metabolic function and aging mechanisms, and changes in physiological levels of (B) NAMPT (C) SIRT1 (D) p66^{SHC} and (E) NAD$^+$ levels in muscle tissues (n=4-5) of obese individuals (BMI:32.5-41.5), compared to normal individuals (BMI: 21.1-24.7). The age of the normal BMI individuals was 48, 35, 44, and 49 (average 44 years old) while the obese individuals were 40, 72, 80, 69, and 42 years old (average 66 years old).

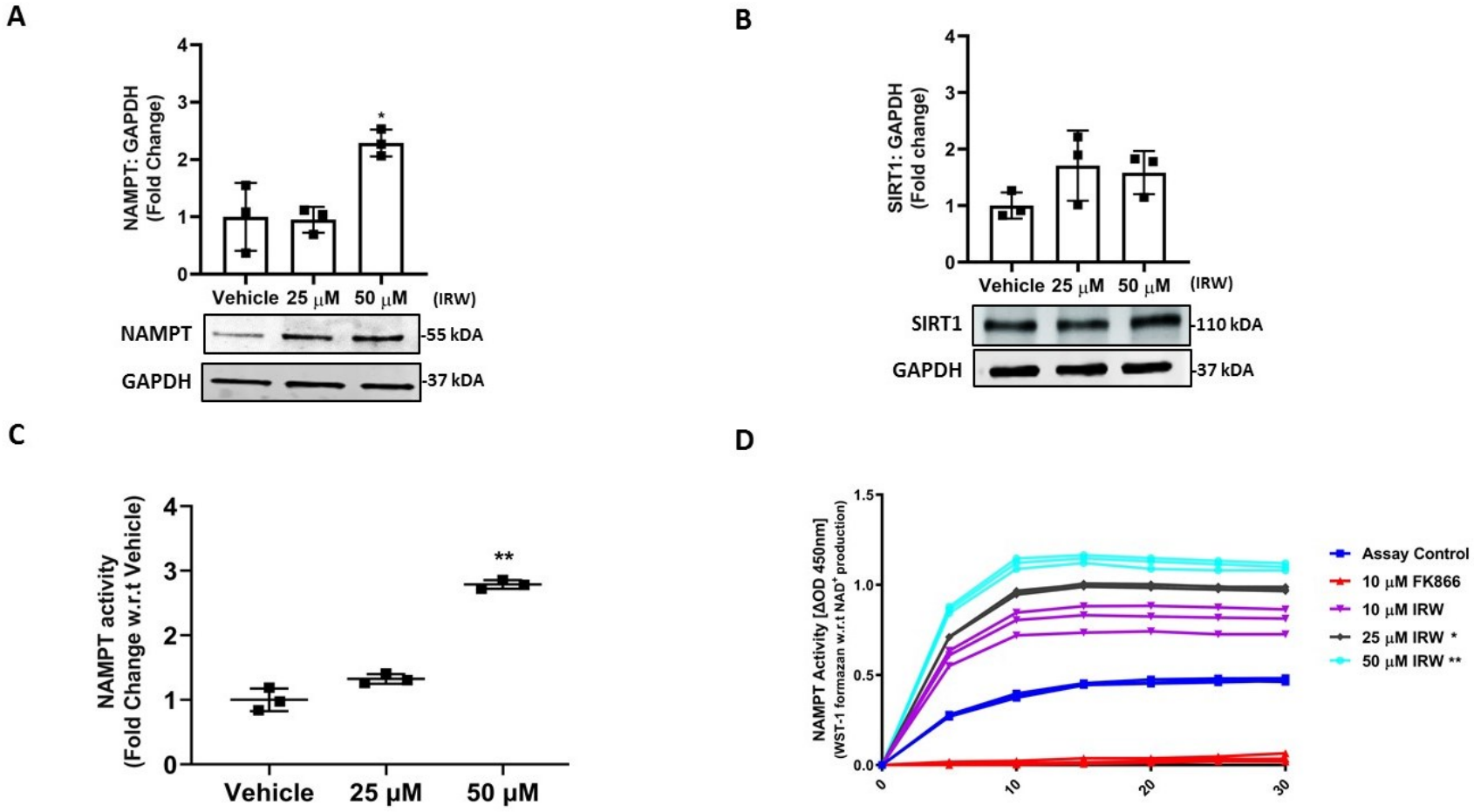


Figure 3.2 IRW modulates the expression of NAMPT in L6 cells. (A) NAMPT protein level (B) SIRT-1 protein level (C) NAMPT immunoprecipitation in L6 cells and (D) NAMPT activity using recombinant NAMPT protein. The L6 cells (n=3) were incubated with indicated concentrations of IRW for 24 h analyzed by western blot using GAPDH as housekeeping control. The concentration of FK866 in NAMPT activity assay was 10 μM. Significant differences were determined by one-way ANOVA. $^{*}p<0.05$, $^{**}p<0.01$.

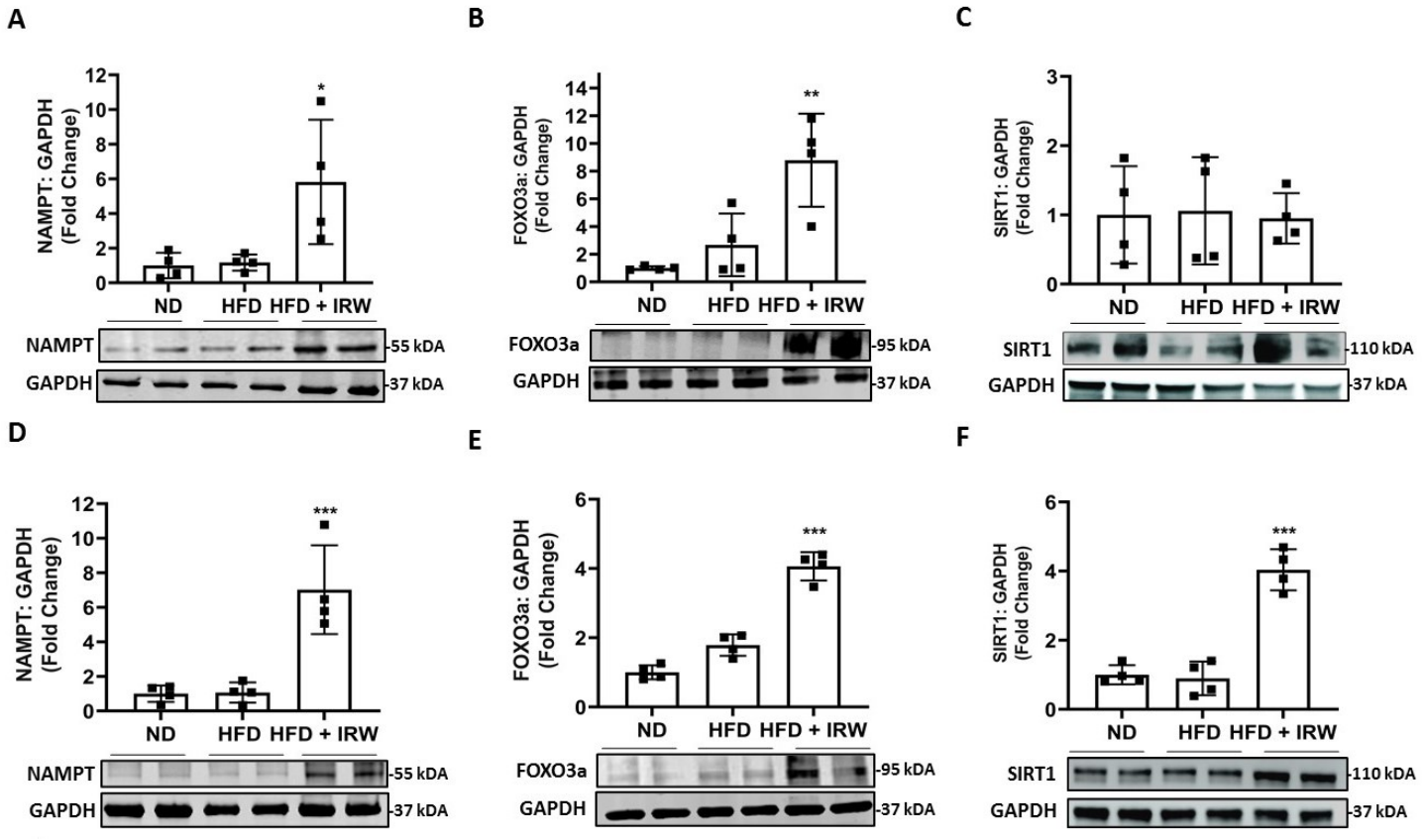


Figure 3.3 IRW modulates the expression of NAMPT and related metabolic markers in C57BL/6J mice tissues. (A) NAMPT-muscle (B) FOXO3a-Muscle (C) SIRT1-Muscle (D) NAMPT-Liver (E) FOXO3a-Liver (F) SIRT1-Liver tissues of C57BL/6J mice fed a high-fat diet fed a high-fat diet to develop obesity, mild hyperglycemia, dyslipidemia, and impaired glucose tolerance. The C57BL/6J mice were divided into three groups (n=6) (1) control diet group (ND): receiving regular diet (10.4.% fat) for 14 weeks 2) HFD group: receiving HFD (45% fat) for 14 weeks 3) Treatment group (HFD+IRW): receiving HFD (40% fat) for 14 weeks with the incorporation of IRW (45mg/kg BW) during the last 8 weeks. Significant differences were determined by one-way ANOVA followed by Dunnett's post-hoc comparison test (w.r.t HFD). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

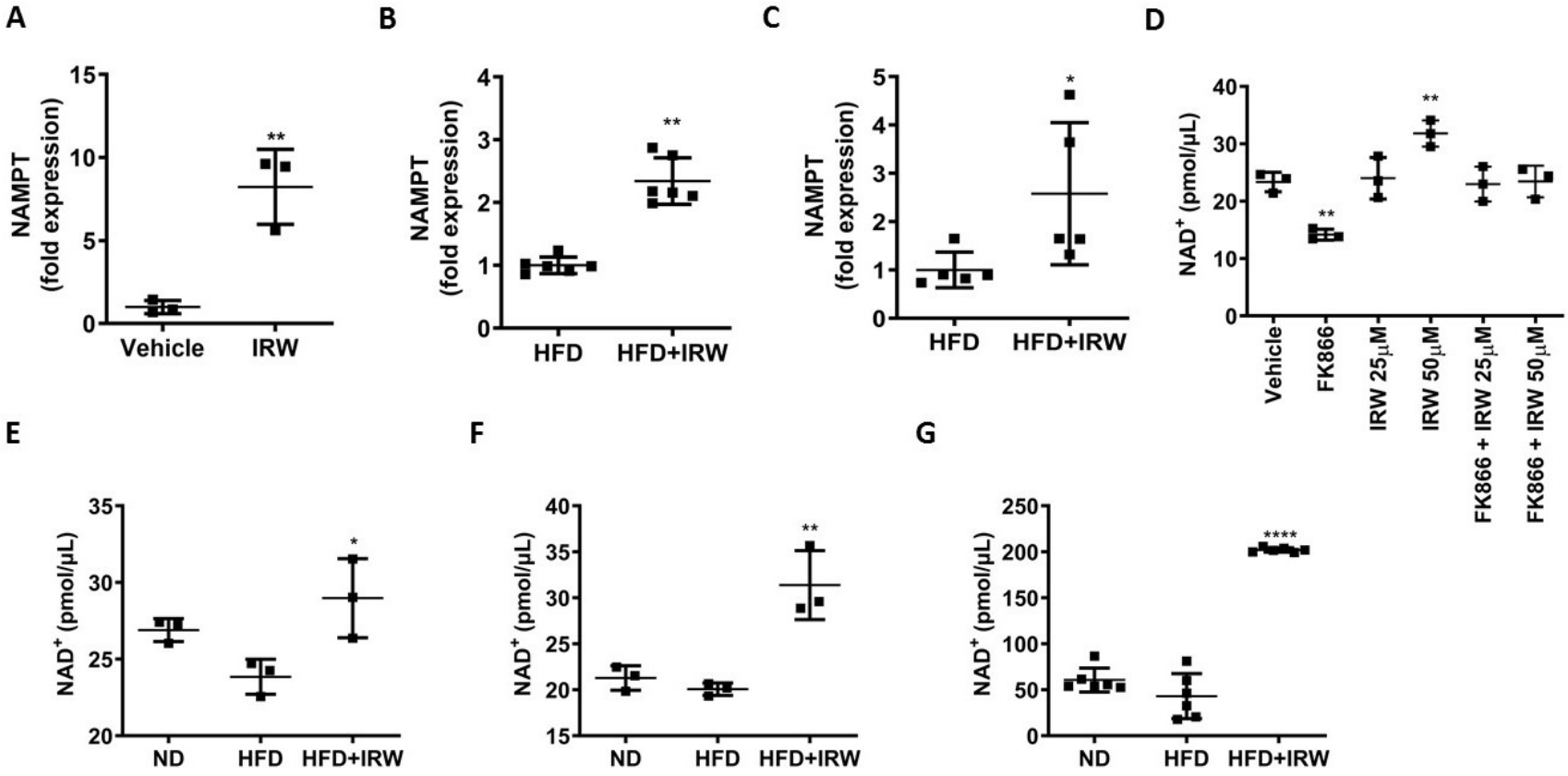


Figure 3.4 IRW increases NAMPT mRNA levels and NAD$^+$ expression in L6 cells and C57BL/6J mice tissues. NAMPT mRNA expression was quantified by qPCR and normalized to GAPDH in (A) L6 cells (n=3). The cells were incubated with IRW (50 µM) for 24 h and qPCR was performed B) Muscle and C) Liver tissues of C57BL/6J mice (n=6) fed a high-fat diet to develop obesity, mild hyperglycemia, dyslipidemia, and impaired glucose tolerance show rise in NAMPT levels (w.r.t HFD). IRW improved NAD$^+$ levels in (D) L6 cells (E) Muscle (F) Liver tissues and (G) Plasma of HFD fed C57BL/6J mice. The C57BL/6J mice were divided into three groups (n=6) (1) control diet group (ND): receiving regular diet (10.4% fat) for 14 weeks 2) HFD group: receiving HFD (45% fat) for 14 weeks 3) Treatment group (HFD+IRW): receiving HFD (40% fat) for 14 weeks with the incorporation of IRW (45mg/kg BW) during the last 8 weeks. Significant differences were determined by a two-tailed Student's t-test (Fig. 4A-C) and one-way ANOVA followed by Dunnett's post-hoc comparison test (w.r.t HFD) (Fig. 3E-G). $^{*}p<0.05$, $^{**}p<0.01$, $^{****}p<0.0001$.

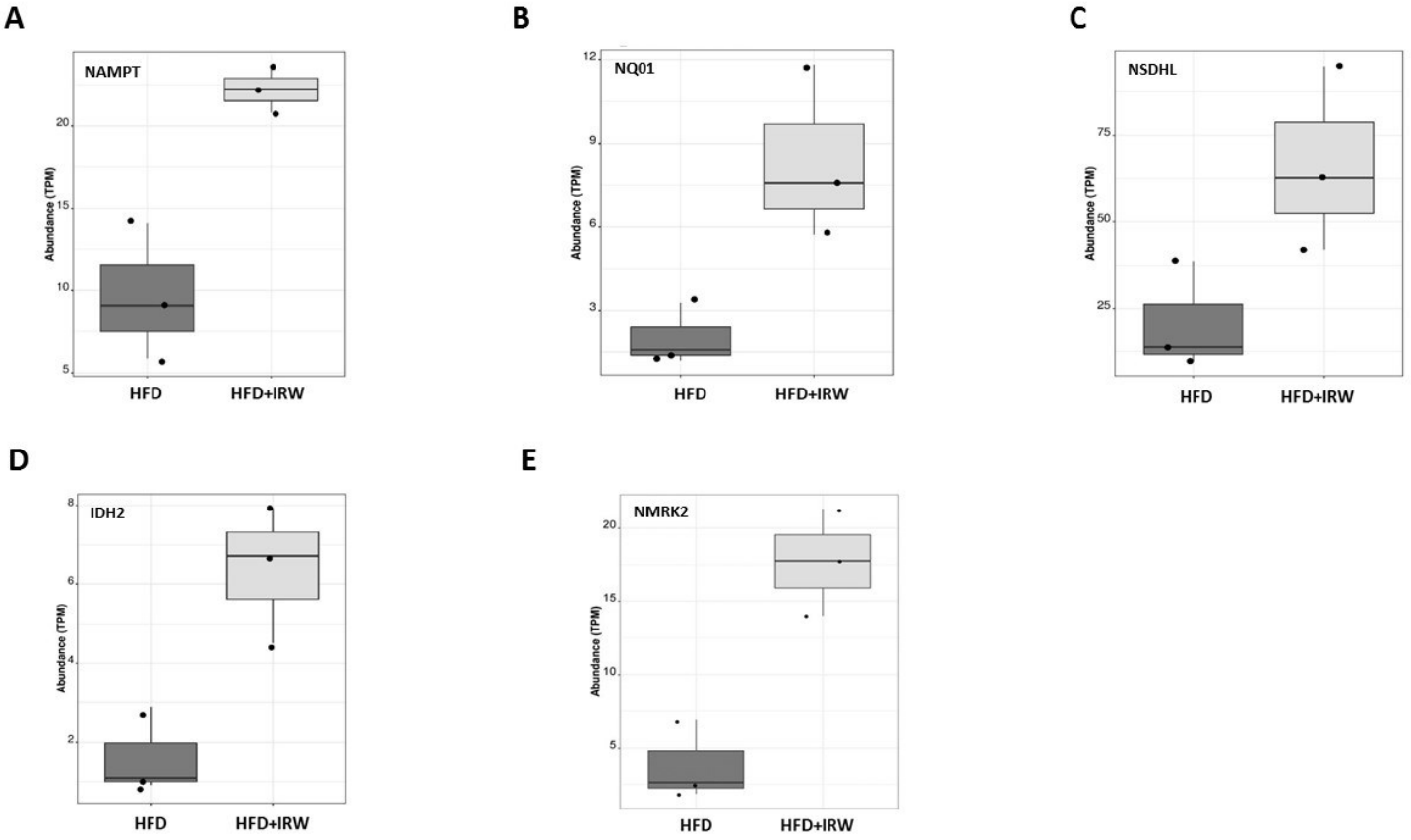


Figure 3.5 IRW increases the expression of mRNA transcripts of NAMPT and related genes in C57BL/6J mice. Data from RNA sequencing of C57BL/6J mice A-D) Liver and E) Muscle tissues, respectively. The C57BL/6J mice were divided into two groups (n=3) (1) HFD group: fed HFD (45% fat) for 14 weeks (2) Treatment group (HFD+IRW): fed HFD (45% fat) for 14 weeks with the incorporation of IRW (45 mg/kg BW) during last 8 weeks. NAMPT (nicotinamide phosphoribosyltransferase (NAMPT), NQ01 (NAD(P)H:quinone oxidoreductase), NSDHL (NAD(P)H steroid dehydrogenase-like protein), IDH2 (Isocitrate dehydrogenase (NADP) follow mitochondrial), NMRK2 (Nicotinamide Riboside Kinase 2) and TPM: transcripts per million. The RNA-seq analysis was conducted using RStudio.

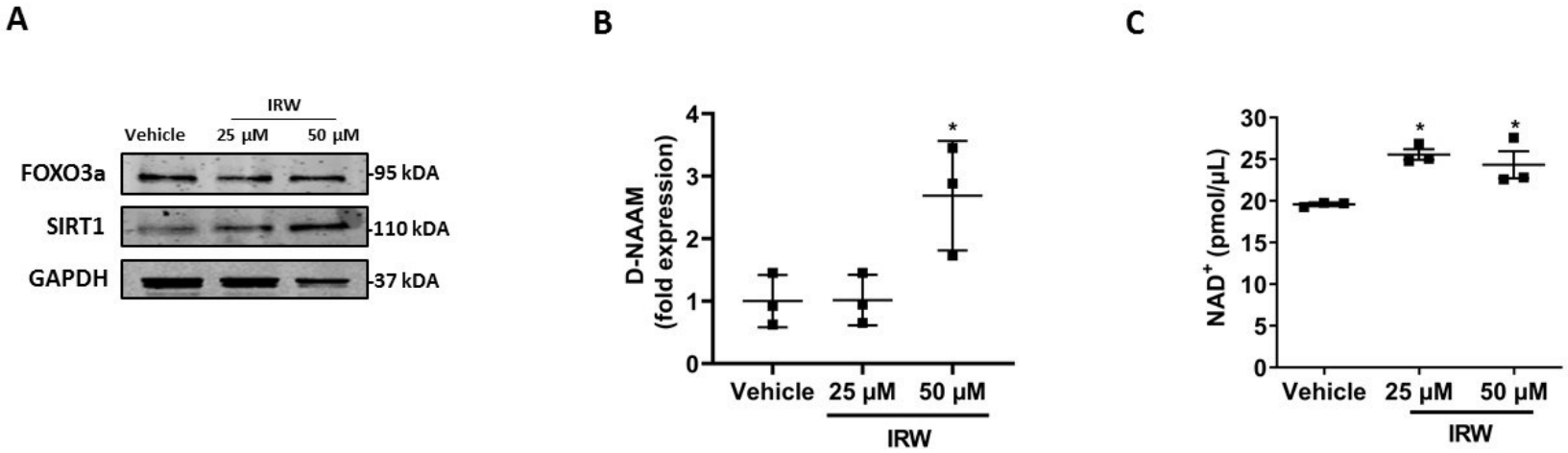


Figure 3.6 IRW modulates the expression of D-NAAM in *yw* Drosophila Melanogaster. (A) Dose-response effect of IRW-induced FOXO3a and SIRT1 expression in *yw* flies (B) Effect of IRW treatment on D-NAAM and (C) total NAD+ levels of *yw* Drosophila Melanogaster. The *yw* flies were divided into two groups (n=30, per group) 1) Vehicle: receiving regular diet for 10 days 2) IRW group 1: receiving low IRW dose (25 μM) supplemented in the regular diet for 10 days and IRW group 2: receiving high IRW dose (50 μM) supplemented in regular diet for 10 days. Flies were fed with food containing vehicle or indicated concentrations of IRW for 10 days and whole-body RNA was extracted using TRIzol reagent, respectively. Significant differences were determined by one-way ANOVA. $^{*}p<0.05$.

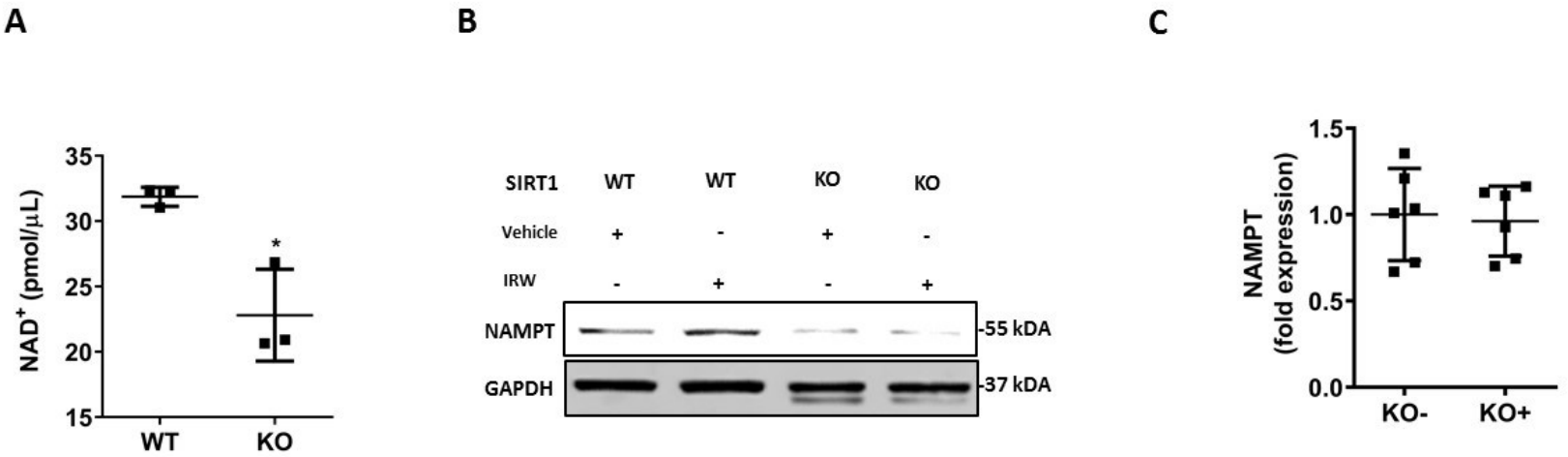


Figure 3.7 IRW stimulated NAMPT increase is SIRT1 dependent. (A) SIRT1 KO 293T cells exhibit lower NAD^+ levels compared to the WT cells (n=3) (B) IRW-mediated increase in NAMPT levels is dependent on SIRT1 in 293T cells. IRW (50 µM) induced rapid increase in NAMPT pool in WT cells is not mimicked in SIRT1 KO cells. (C) SIRT1 KO 293T cells (n=6) treated with IRW (50 µM) did not exhibit an increase in NAMPT expression, β-actin was used as control. Significant differences were determined by a two-tailed Student's t-test. $^*p<0.05$.

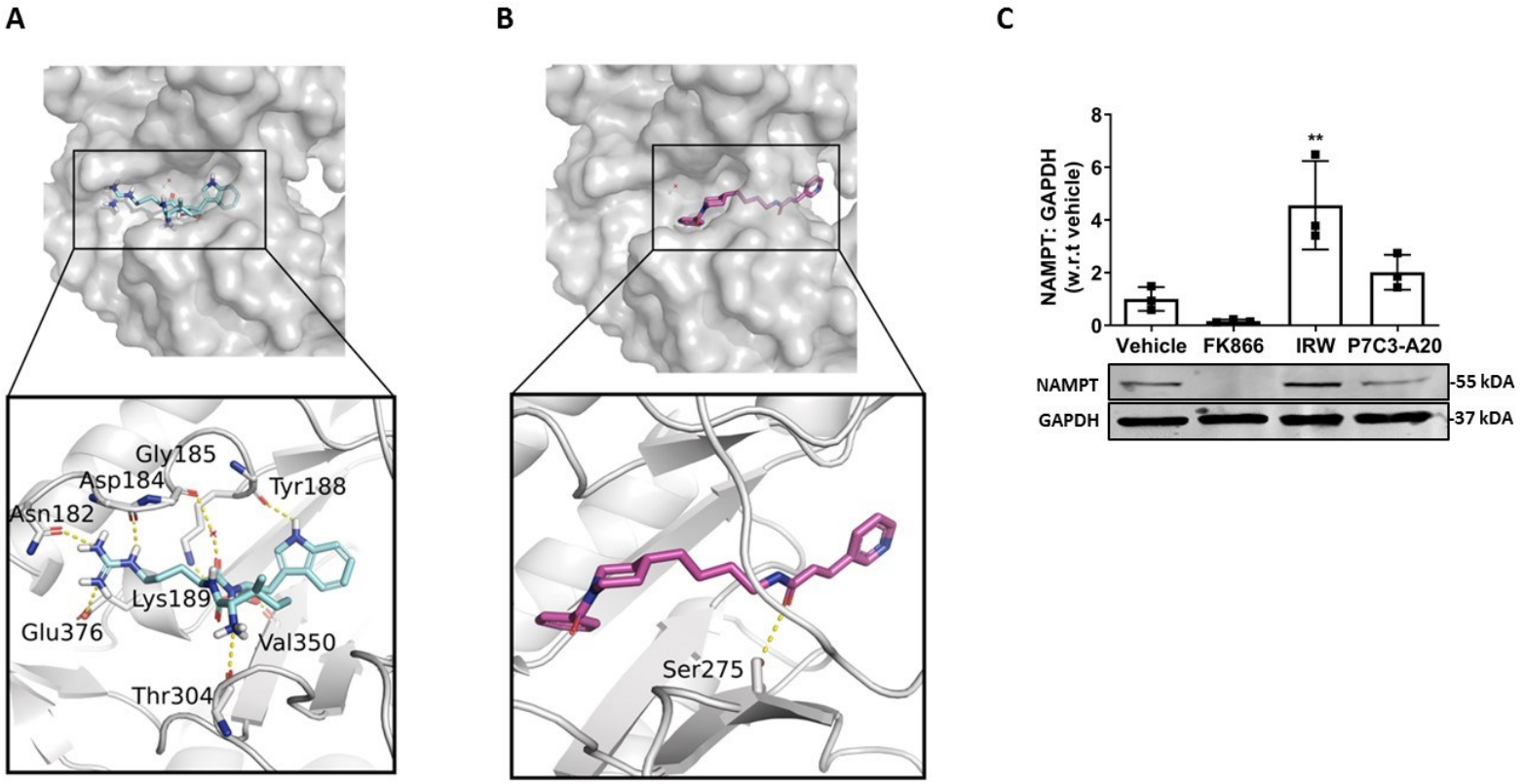


Figure 3.8 Molecular docking and NAMPT increase comparison. Interaction of (A) IRW (B) FK866 with the active sites of NAMPT and (C) Comparative NAMPT levels of IRW (50 µM) with FK866 (10 µM, NAMPT inhibitor) and P7C3-A20 (3 µM, NAMPT activator) treatment in L6 cells. Significant differences were determined by one-way ANOVA. $^{**}p<0.01$.

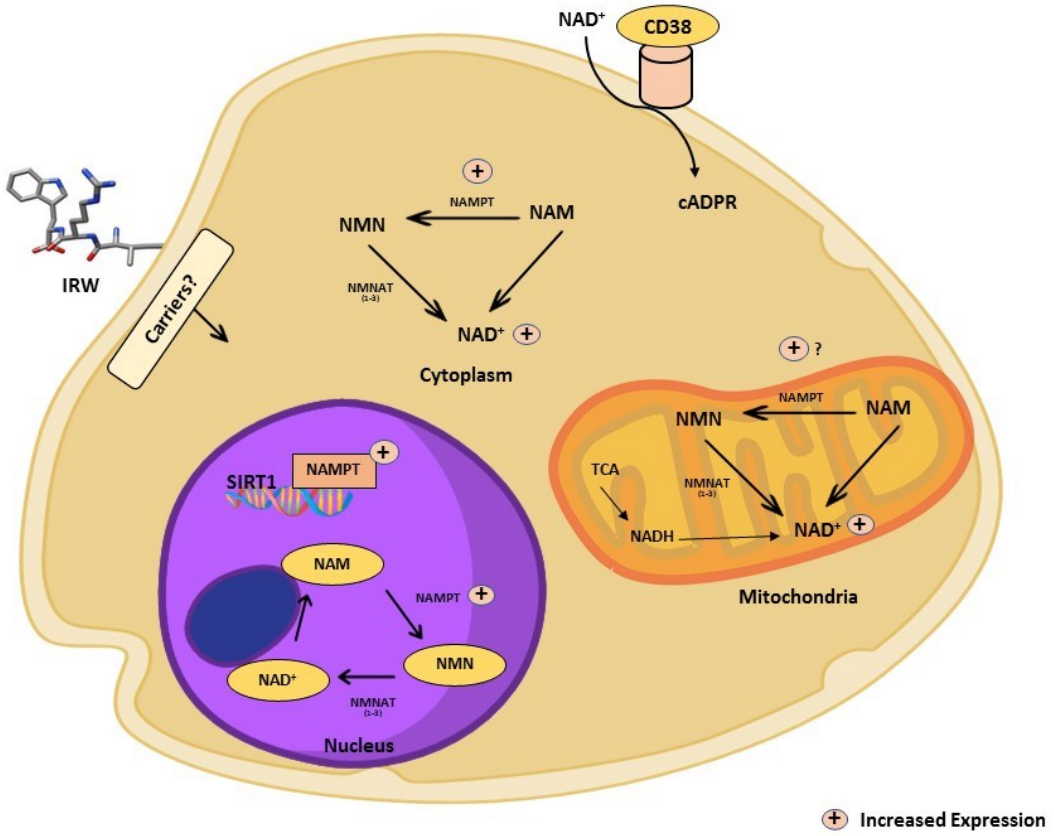
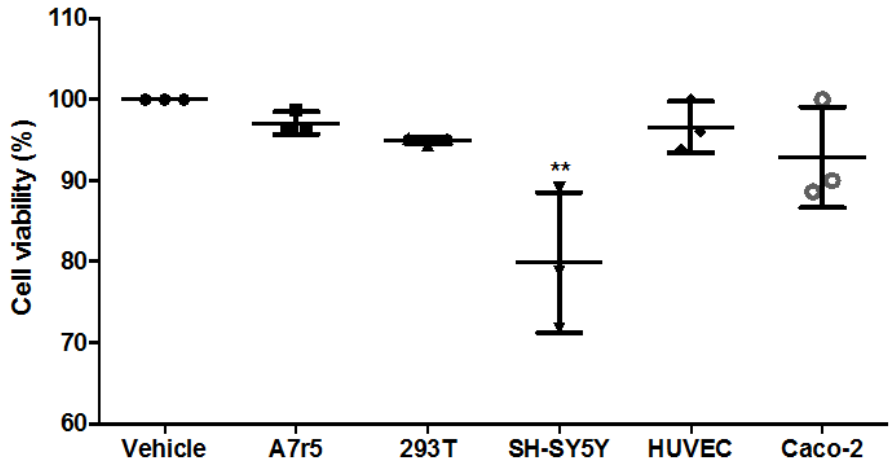
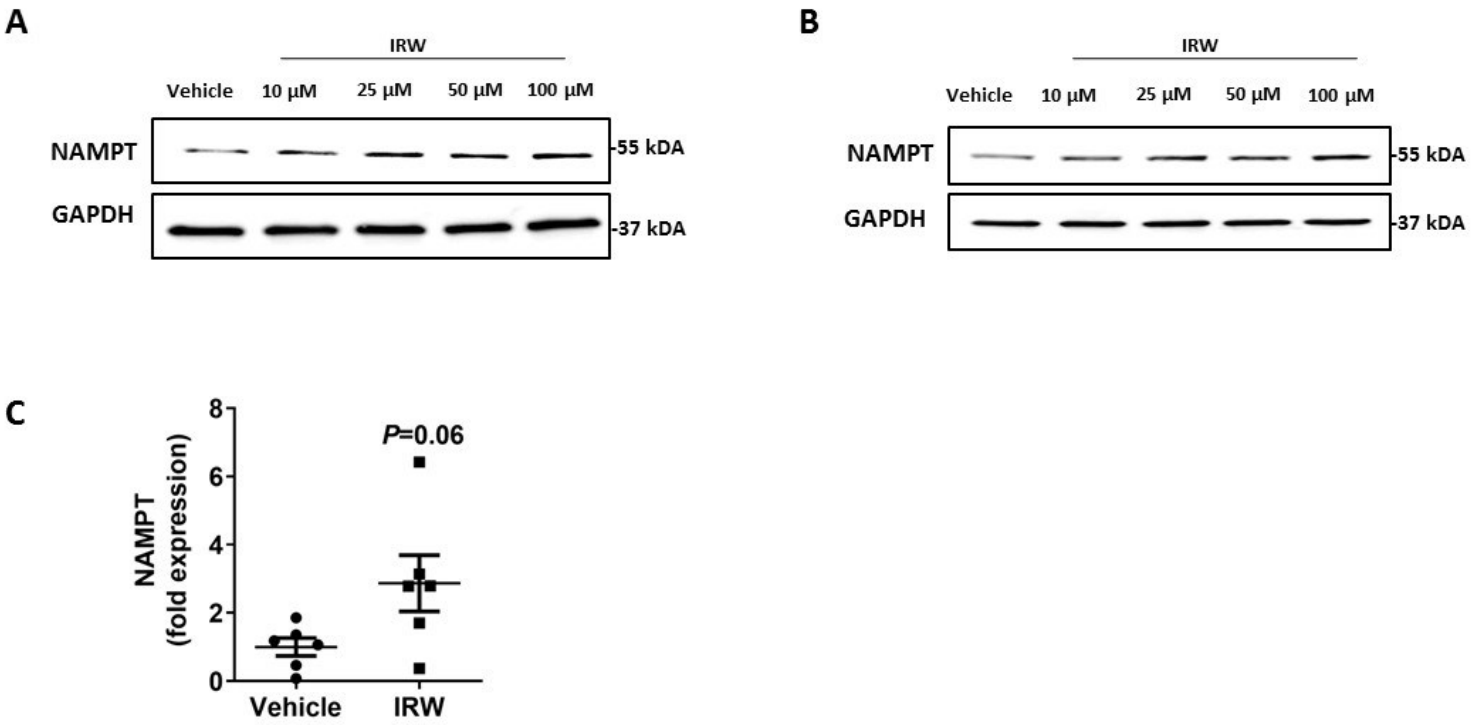


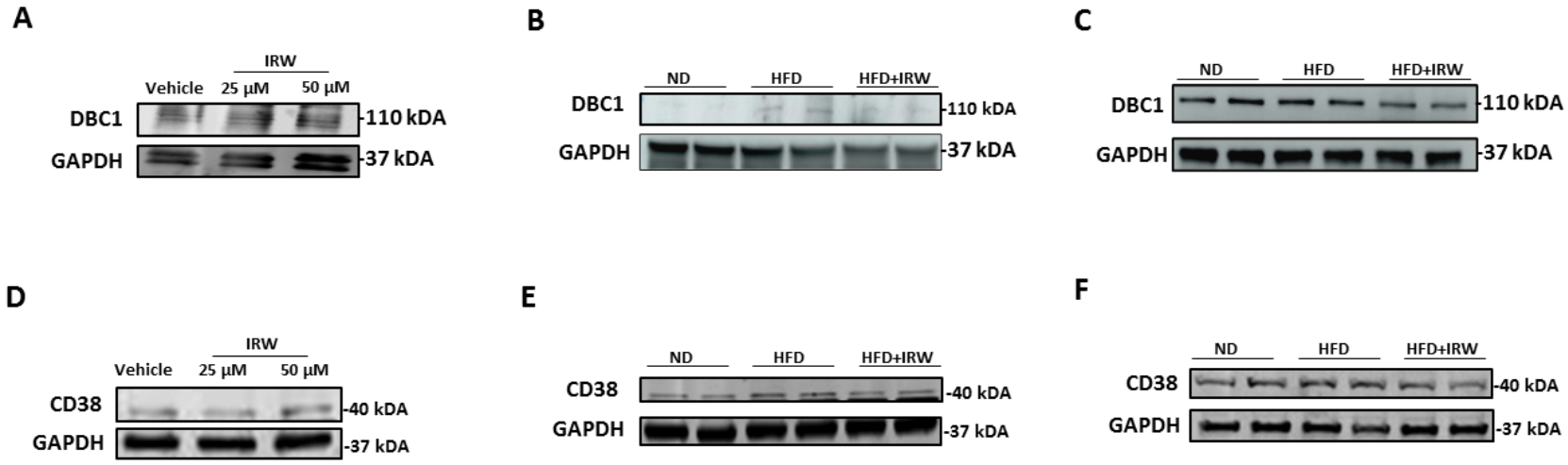
Figure 3.9 Moderate dose of IRW increases NAMPT levels.



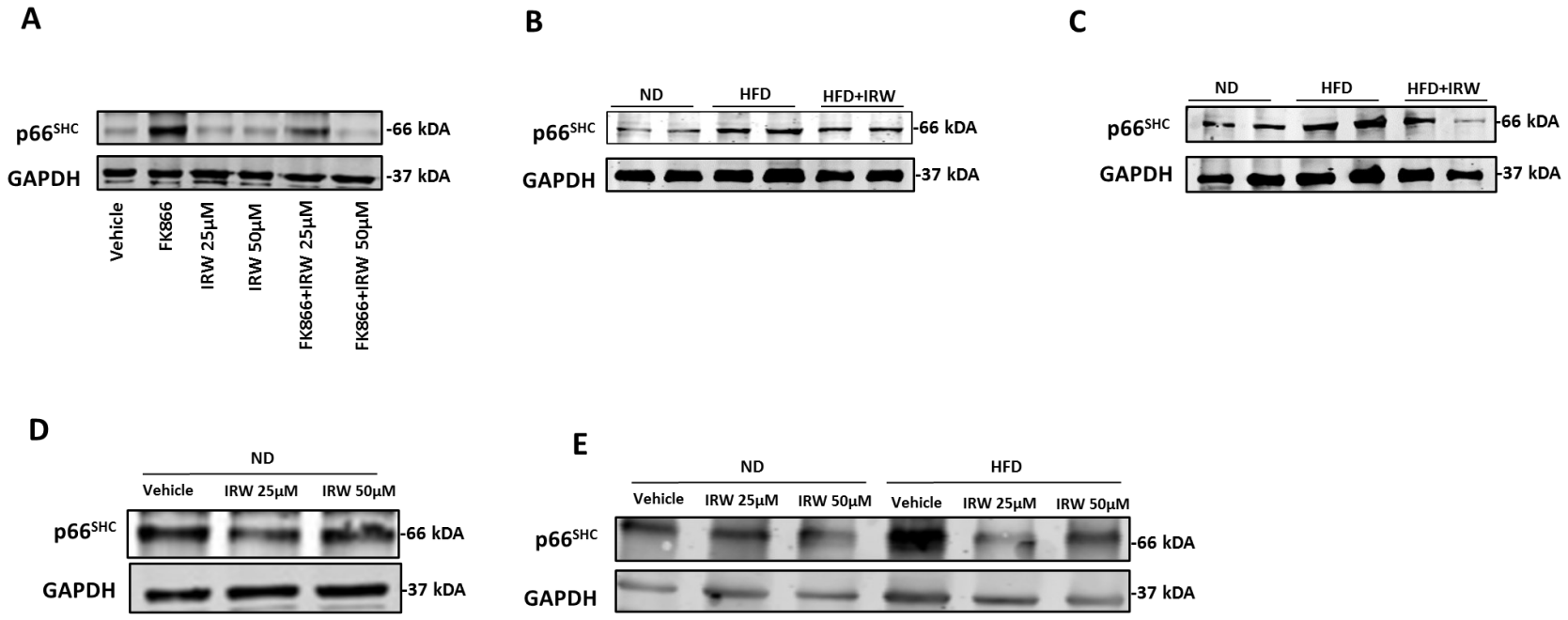
Supplementary Figure 3.1 Impact of IRW on cellular viability of different cells. Cell viability of different mammalian cells pre-treated with 50 µM of IRW for 24h using MTT assay. Results are the mean ± S.E.M of experiments performed in triplicates.



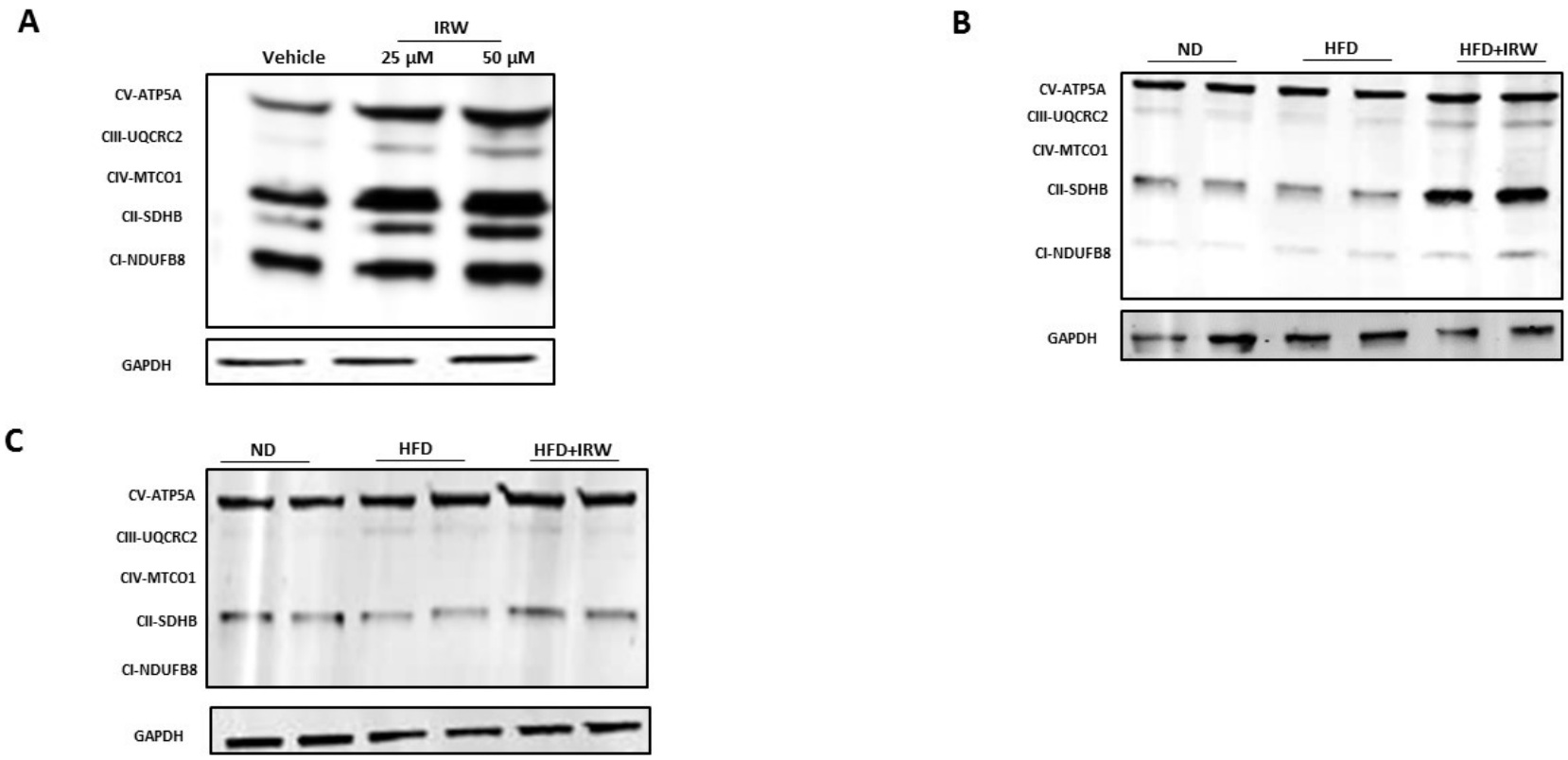
Supplementary Figure 3.2 Impact of IRW on NAMPT in different mammalian cell lines. (A, B) Dose-dependent response of IRW-induced NAMPT expression in Hela and MDAMB231 cells. Cells were incubated with indicated concentrations of IRW for 24 h and analyzed by western blot (C) NAMPT mRNA expression in 293T cells was quantified by qPCR and normalized to β-actin



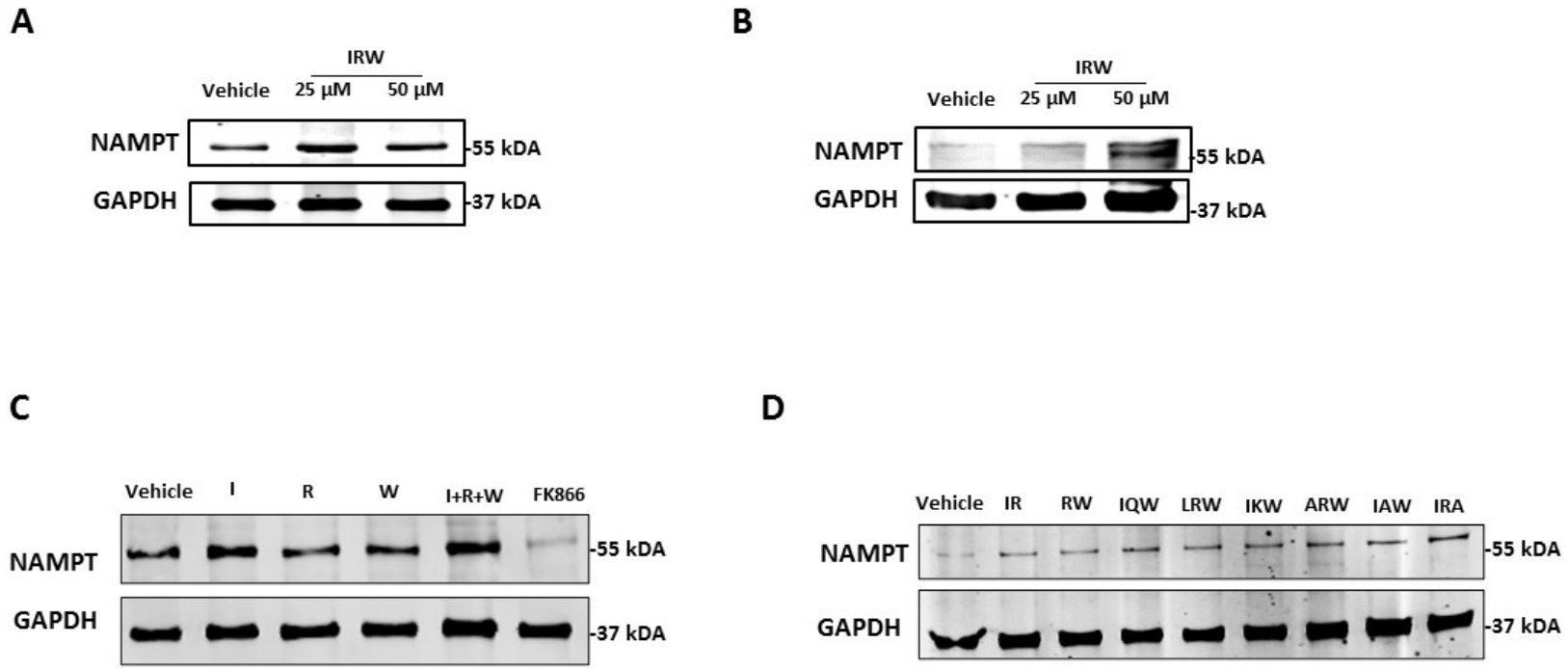
Supplementary Figure 3.3 Impact of IRW on DBC1 and CD38. (A) IRW-induced no impact of DBC1 in L6 cells. The cells were incubated with indicated concentrations of IRW for 24 h and analyzed by western blot B) Muscle and C) Liver tissue of C57BL/6J mice exhibited marginal changes in DBC1 levels. IRW had no impact on NDAase CD38 in (D) L6 cells (E) Muscle (F) Liver tissues of C57BL/6J mice fed a high-fat diet. The C57BL/6J mice were divided into three groups (n=6, per group) (1) control group (ND): receiving regular diet (9% fat) for 14 weeks 2) HFD group: receiving HFD (40% fat) for 14 weeks 3) Treatment group (HFD+IRW): receiving HFD (40% fat) for 14 weeks with incorporation of IRW (45mg/kg BW) during the last 8 weeks.



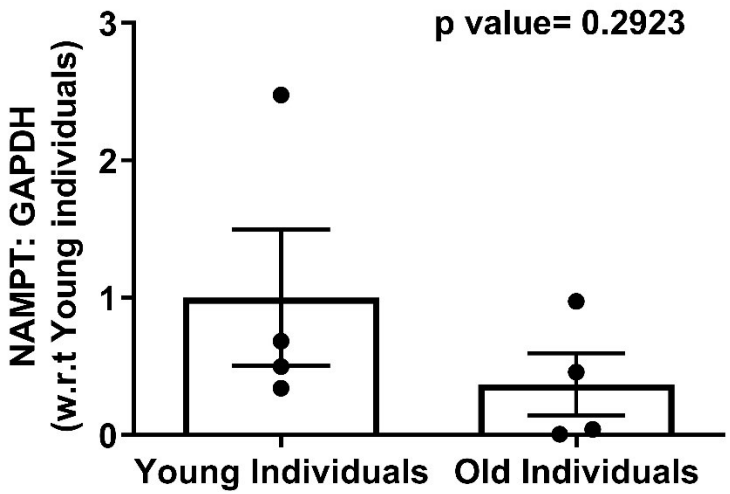
Supplementary Figure 3.4 IRW lowers the protein levels of p66^{SHC} in cells, mice tissues, and flies (A) L6 cells (B) Muscle (C) Liver tissues of C57BL/6J mice fed a high-fat diet and (D-E) *yw Drosophila Melanogaster*. The C57BL/6J mice were divided into three groups (n=6) (1) control diet group (ND): receiving regular diet (9% fat) for 14 weeks 2) HFD group: receiving HFD (40% fat) for 14 weeks 3) Treatment group (HFD+IRW): receiving HFD (40% fat) for 14 weeks with the incorporation of IRW (45mg/kg BW) during the last 8 weeks. The *yw* flies were divided into two groups (n=30, per group) 1) Vehicle: receiving regular diet for 10 days 2) IRW group 1: receiving low IRW dose (25 µM) supplemented in the regular diet for 10 days and IRW group 2: receiving high IRW dose (50 µM) supplemented in regular diet for 10 days. Flies were fed with ND or HFD food containing indicated concentrations of IRW for 10 days and whole-body protein and RNA were extracted using RIPA buffer, respectively. ND: Normal diet, HFD: High fat diet.



Supplementary Figure 3.5 Impact of IRW on OXPHOS complexes in mitochondria. (A) IRW-induced increase in 5 OXPHOS complexes of L6 cells. The cells were incubated with indicated concentrations of IRW for 24 h and analyzed by western blot B) Muscle and C) Liver tissue of C57BL/6J mice exhibited a marginal increase in OXPHOS complexes. The C57BL/6J mice were divided into three groups (n=6, per group) (1) control group (ND): receiving regular diet (9% fat) for 14 weeks 2) HFD group: receiving HFD (40% fat) for 14 weeks 3) Treatment group (HFD+IRW): receiving HFD (40% fat) for 14 weeks with incorporation of IRW (45mg/kg BW) during the last 8 weeks.



Supplementary Figure 3.6 Increase in NAMPT levels in different cellular compartments and structure-function relationship (A) Nucleus and (B) Mitochondrial following treatment with IRW; and the impact of (C) amino acids (D) analogous peptides on NAMPT levels. The cells were incubated with indicated concentrations of IRW for 24 h, the cell compartments were separated and analyzed by western blot. GAPDH was used as the control.



Supplementary Figure 3.7 Comparative analysis of NAMPT levels in muscle tissues of younger and older individuals. The values are based on human muscle NAMPT blot shown in Figure 1B.

Table 3.1: List of the primers used in the study

Gene	Forward primer	Reverse primer
D-NAAM	TTGTCATCGAAGATTCAAACGGA	GGCGGTCATCACTGTCCTTG
Mouse NAMPT	ACCAGCGGGGAACTTTGTTA	ACGTCCTGCTCGATGTTCAG
Rat NAMPT	AGGGGCATCTGCTCATTTGG	CTCTGCCGCTGGAACAGAAT
Human NAMPT	CTTCGGTTCTGGTGGAGGTT	AATCGGCCCTTTTTGGACCT
Mouse GAPDH	AAGAGGGATGCTGCCCTTAC	TACGGCCAAATCCGTTCACA
Rat GAPDH	TGATTCTACCCACGGCAAGTT	TGATGGGTTTCCCATTGATGA
Human β-actin	TCGTGCGTGACATTAAGGAG	GTCAGGCAGCTCGTAGCTCT

CHAPTER 4 - Tripeptide IRW is an activator of Mitochondrial Biogenesis

Abstract: Mitochondria are vital cell organelle that generate ATP and carry out diverse functions for cellular energy metabolism. Recently, the importance of mitochondria in cellular functions has been revisited and is now associated with immune, reproductive, endocrine, and other physiological functions. Mitochondrial biogenesis is proposed to be abridged in response to disease states and can be modulated with nutritional and environmental stimuli. Therefore, the activation of mitochondrial biogenesis in wake of metabolic and other physiological stresses presents itself as a potential medicinal intervention. In this study, we show that IRW, a tripeptide obtained from ovotransferrin, activates mitochondrial biogenesis resulting in increased mitochondrial DNA, ATP surge, improved metabolic and microbiome function. IRW activates PGC1α, the master regulator of mitochondrial biogenesis, in cells and multiple tissues of C57BL/6J HFD mice. It also increased mitochondrial DNA in muscles of aged *Drosophila* fed with IRW for a week. The underlying mechanism was dependent on FAM120B, a constitutive activator of peroxisome proliferator activated receptor gamma (PPARγ).

4.1 Introduction

Arising from α-proteobacterium engulfed by a eukaryotic progenitor, mitochondria are evolutionarily preserved organelles that perform a myriad of diverse, yet highly interconnected cellular functions (Lane & Martin, 2010; Tovar et al., 2003). Until the 1950s, mitochondria were solely studied for the metabolic pathways they participate in, and by the 1970s their role was mainly settled to ATP generation and metabolites for macromolecule synthesis (N. S. Chandel, 2018). However, in the 1990s, seminal papers showed its role in apoptosis (X. Liu, Kim, Yang, Jemmerson, & Wang, 1996) as well as in redox homeostasis (N. Chandel, Maltepe, Goldwasser, Mathieu, Simon, & Schumacker, 1998). Following this, mitochondria have been studied extensively for their versatile activities in cellular and pathological conditions (Nunnari & Suomalainen, 2012). Thus, it is now clear that mitochondria are indeed more than ATP synthesizers and function as critical signaling organelles influencing the fate of cell and organ systems. Therefore, the slowdown of mitochondrial (mt.) turnover is implicated in a spectrum of human diseases spanning from neonatal disorders, obesity, and metabolic syndrome(s) to age-onset neurodegenerative diseases (Lane et al., 2010; Schmidt, Pfanner, & Meisinger, 2010). In healthy cells, normal mitochondrial function is maintained by three pathways *viz*. mitochondrial biogenesis, fusion-fission cycles, and mitochondrial quality control (Uittenbogaard & Chiaramello, 2014). Induced mitochondrial biogenesis represents a unique therapeutic strategy to counter mitochondrial dysfunction or age-related loss owing to critical role of mitochondria in these processes (Nunnari et al., 2012).

Mitochondrial biogenesis is an intricate and adaptive response process. It requires coordinated transcription of mt. genes in the nucleus, replication of the mtDNA, as well as the synthesis, and import of proteins to existing mitochondria (Pagliarini et al., 2008). The

coordination of the two cellular genomes is achieved by nucleus-encoded mitochondrial transcription factors such as TFAM, NRF1, and PGC1α, a transcriptional coactivator of NRF1, TFAM, and PPARγ (Kashyap et al., 2005; Nisoli et al., 2003). Among these factors, PGC1α is a principal coordinator of mitochondrial biogenesis (Besse-Patin, Jeromson, Levesque-Damphousse, Secco, Laplante, & Estall, 2019). PGC1α has emerged as a prominent regulator of mitochondrial biogenesis, thus becoming a critical metabolic node. Another crucial mitochondrial biogenesis factor, NRF1, regulates expression of TFAM, and thereby coordinates the increase in mitochondrial number (Picca & Lezza, 2015). It also affects the expression of few mt. factors such as COX4, and PGC1α indirectly via transcription factor MEF2A (Ramachandran, Yu, & Gulick, 2008). Interestingly, NRF1 also directly controls the expressions of ten nuclear-encoded mitochondrial COX subunits and three mitochondrial-encoded COX subunits via TFAM (Dhar, Ongwijitwat, & Wong-Riley, 2008). Although NRF1 is essential for mitochondrial biogenesis, yet its sole expression is insufficient to drive this complex cellular mechanism. Two other key players include TFAM and TOMM machinery, which move the process of mitochondrial biogenesis towards a successful end. It is interesting to note that TFAM is considered the histone-like protein of mtDNA as a high number of bound TFAM molecules (~1000 proteins/mtDNA genome) is reported in mammalian cell lines (Kukat, Wurm, Spåhr, Falkenberg, Larsson, & Jakobs, 2011). This histone-like property of TFAM is proposed to help in both mtDNA replication and mtDNA packaging for biogenesis. Finally, the import of 99% of nucleus-encoded mitochondrial precursor proteins is vital for making new mitochondria. Most mitochondrial proteins are synthesized on cytosolic ribosomes and imported through the TOMM complex (Chacinska et al., 2010). Among these small TOMM proteins, TOMM 20 and TOMM6 have emerged as essential regulators of mitochondrial biogenesis (Harbauer et al., 2014; Pfanner & Meijer, 1997). This plethora of

molecular determinants of mitochondrial physiology ensures an isolated factor seldom causes mitochondrial dysfunction in common diseases such as obesity, hypertension, and diabetes; instead, they result from a cumulative impact of polygenic influences. Consequently, we look at the mitochondrial physiology as a whole for solutions. Hence, the regulatory networks that govern mitochondrial biogenesis constitute attractive drug targets for the treatment of mitochondrial dysfunction and related diseases.

Artificial induction of mitochondrial biogenesis is an exciting therapeutic approach as it promises to counter a myriad of mitochondrial and metabolic pathologies and improve their prognosis. Pharmacological and/or nutritional modulation of mitochondrial biogenesis are appealing approaches for maintaining and improving mitochondrial homeostasis. Several lifestyles and nutritional approaches stimulate mitochondrial biogenesis, such as caloric restriction (CR) and the dietary intake of bioactive compounds (e.g., polyphenols). Exercise therapy has also been demonstrated to be beneficial for mitochondrial diseases as it directly targets both upstream and downstream effectors of mitochondrial biogenesis, including PGC1α (Nunnari et al., 2012). Since real-life pertinence of caloric restriction is barely sustainable in the long term, alternative approaches that can mirror CR are of pharmacological interest. Only one drug candidate, Bezafibrate, a pan-PPAR activator is being tested (NCT02398201) for induction of mitochondrial biogenesis. Similar to polyphenols, antioxidant peptides can reduce the formation of oxidation products along with the induction of antioxidant enzymes *in vivo* (Lorenzo et al., 2018). However, the ability of such peptides to activate mitochondrial biogenesis remains to be assessed in detail. To the best of our knowledge, a single study has been published reporting on the proficiency of natriuretic peptides and peptide hormones to initiate mitochondrial biogenesis via activation of PGC1α and its downstream effectors (Miyashita et al., 2009). As mitochondrial pharmacology is

an emerging discipline, the role of food derived antioxidant peptides holds great promise for new therapeutic approaches for activation of mitochondrial biogenesis. We have recently reported on the ability of IRW, an ovotransferrin derived short peptide, to increase the levels of antioxidant components of oxidative phosphorylation (OXPHOS) and NAMPT *in vivo* (Chapter 3), both related to the activated PGC1α axis (Bhullar et al., 2021). Because IRW also activates the NO pathway, a critical initiator of mitochondrial biogenesis, we sought to investigate the ability of IRW to initiate mitochondrial biogenesis in cells, flies, and mice, as well as to identify the underlying cellular mechanisms.

4.2 Materials and methods

4.2.1 Chemicals and Reagents

Peptides used in this study IRW, IQW, IKW, IR, RW, and GGG with a minimum purity of 99% were obtained from GenScript (Piscataway, NJ, USA). Synthesized peptides were dissolved in nuclease free water at a stock concentration of 100 mM, aliquoted, and stored at −80 °C until further usage. The amino acids I, R, and W (reagent grade, ≥98% purity) were obtained from Sigma-Aldrich Canada Co. (Oakville, ON, Canada). Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Eagle's Minimum Essential Medium (EMEM), F-12K Medium (Kaighn's Modification of Ham's F-12 Medium), Trypsin-EDTA Solution, 1X, Penicillin-Streptomycin Solution, Dimethylsulfoxide (DMSO), and other supplies associated with cell culture were obtained from life technologies (Thermo Scientific, Mississauga, ON, Canada). Antibodies including PGC1α (ab54481), FAM120B (ab118589), PPARγ (ab178860), mTOR (ab134903), Tubulin (ab7291), TOMM20 (ab186735), TFAM (ab119684), COX4 (ab110272),

NRF1 (ab175932), GST1 (ab224622), SIRT1 (ab189494), KAT2A (ab217876), MnSOD (ab74231), HSP90 (ab13492) were obtained from Abcam, Inc. (Toronto, ON, Canada). The SirT3 5490 antibody (NEB) was provided by Dr. Basil Hubbard's lab, University of Alberta, Canada.

4.2.2 Human tissue study

Matched pairs of normal and obese snapfrozen, RNAlater-preserved, muscle tissues were obtained from Proteogenex (Inglewood, CA) after being prospectively collected at multiple hospitals with institutional review and ethics board approval. All of the experimental procedures associated with the human samples were conducted in appropriate and designated BSL II labs. The obtained human muscle samples were verified for their negative serological status (HIV, HEPA, HEPB, HEPC, and other microbial contaminants).

4.2.3 Cell culture

All the cell lines used in this study viz. A7R5 (ATCC® CRL-1444™), 293T (ATCC® ACS-4500™), L6 (ATCC® CRL-1458™), Caco-2 (ATCC® HTB-37™), SH-SY5Y (ATCC® CRL-2266™), HA-VSMC (ATCC® CRL-1999™), and HUV-EC-C (HUVEC) (ATCC® CRL-1730™) were obtained from ATCC and cultured according to the supplier's instructions (ATCC, Baltimore, MD, USA). The cells were grown in the media as per ATCC's instructions and were cultured until ~80% confluency for 24 h in media containing 0.1% FBS. Low serum levels were maintained during IRW or other peptide(s) or amino acid addition. The cells were treated with IRW (25 or 50 μM), mock peptides (IQW, IKQ, IR, RW) or amino acids (I, R, or W), or medium containing vehicle (ddH2O) for 8 or 24 h. Following the treatment, cell lysates were collected using RIPA buffer.

4.2.4 RNA extraction and qPCR

Total RNA was isolated from cells, animal tissues, and Wdah flies with TRIzol reagent. RNA was quantified using a Nano Drop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and its integrity assessed by running it on an agarose gel. Next, the cDNA was synthesized from 1 µg of total RNA using a reverse transcriptase (RT) system kit (Thermo Scientific, Mississauga, ON, Canada). All of the qPCR experiments and analyses were conducted using the MIQE guidelines for qPCR (Bustin et al., 2009).

4.2.5 Western blots

Protein was extracted from cells and tissues using RIPA buffer, and western blot was performed according to the methodology described in our recent report on IRW (Bhullar et al., 2021).

4.2.6 Other assays

The ATP content was measured using ATP assay kit (Colorimetric/Fluorometric) (ab83355) and citrate synthase activity was measured using citrate synthase activity assay kit (ab119692), obtained from Abcam, Inc. (Toronto, ON, Canada). Mitochondrial biogenesis was measured using MitoBiogenesis™ In-Cell ELISA Kit (ab110217/MS643), obtained from Abcam, Inc. (Toronto, ON, Canada). Oxygen consumption was measured using Oxygen Consumption Rate Assay Kit (Item No. 600800), purchased from Cayman Chemical (Ann Arbor, MI, USA).

4.2.7 Flow Cytometry

The mitochondrial content was measured using the MitoTracker™ Green FM (Invitrogen™, M7514). The selection of MitoTracker™ Green FM is based on its ability to be essentially nonfluorescent in aqueous solutions and exhibit fluorescence once it accumulates in the lipid environment of mitochondria. Therefore, the false-positive results are negligible, enabling a

correct count of mitochondria via bright green, fluorescein-like fluorescence. Briefly, cells were cultured and treated as indicated, trypsinized, centrifuged at 400 rpm, and resuspended in buffer made from PBS and FBS (3:1) with MitoTracker™ Green FM at a concentration between 100-400 nM. After incubating cells with dye for 15-30 min at 37°C, mitochondria were analyzed using the BD FACSCanto™ (BD FACS Canto II) flow cytometry cell analyzer (BD Biosciences, San Jose, CAL, USA). The FlowJo software was used for the analysis of flow cytometry data (Tree Star, Inc. OR, USA).

4.2.8 Fluorescence microscopy

All fluorescence imaging experiments were performed on a Zeiss Colibri Fluorescence Microscope (Carl Zeiss Canada Ltd., Toronto, Canada). Cells were grown at a density of 100 cells per well on chamber slides Nunc® Lab-Tek® Chamber Slide™ system (Millipore-Sigma, C7182). Cells were treated as described earlier and mitochondria were imaged using MitoTracker™ Green FM (Invitrogen™, M7514). The filters for excitation: 490 nm and emission: 516 nm were used.

4.2.9 Transmission electron microscopy (TEM)

Cells were treated with vehicle or peptides for 24 h and fixed using TEM fixing solution: 2% PFA +2.5% GTA in 0.1M phosphate buffer. All experiments for TEM were conducted in 100 mm cell culture dishes and a minimum of $4x10^6$ cells were collected for each sample (n=4). After trypsinizing and fixing the cells, cells were kept at 4°C till further analysis. For the development of EM images, the cell pellet(es) was rinsed with 0.1M phosphate buffer (10 min x 3), postfix was conducted with 1% OsO4/0.1M phosphate buffer for 1 h. The pellets were rinsed with H_2O (10 min, 3X) and stained with 1% uranyl acetate/H2O overnight. Samples were rinsed with H_2O, (10 min, 3X) and stained with 1% lead nitrate/aspartate solution 30 min at 60°C water bath. The samples were then washed with H_2O (10 min, 3X). Next, samples were dehydrated with 30%,

50%, 70% and 95% ethanol (10 min) and 100% ethanol (10 min, 3X). Next, a graded infiltration procedure was performed using: 25% Spurr's (2 h); 50% Spurr's (2 h); 75% Spurr's (4 h) and 100% Spurr's (24 h with 3-4 changes). Following graded infiltration, the specimen was transferred into a flat mold with fresh Spurr's resin, polymerized at 70°C oven 20-24 hrs, and a minimum of 8 images were collected for each treatment group using a Hitachi H-7650 TEM Microscope (Hitachi, Dallas, TX, U.S.A.).

4.2.10 HFD Animal Study

Male C57BL/6J mice (6 weeks) were obtained from the Charles River Breeding Laboratories (St. Constant, QC, Canada) and maintained on a 12 h light/dark cycle with free access to food and water for 2 weeks. The low-fat diet (LFD; TD.0110675: Rodent Diet With 10.4 kcal% fat), high-fat diet (HFD; TD.06415: Rodent Diet With 45 kcal% fat), and casein (CA.160030) were obtained from Envigo Teklad Diets (Madison, WI). All of the experimental procedures followed the University of Alberta Guide for the Care and Use of Laboratory Animals, and the animal protocol was approved by the animal ethics committee at the University of Alberta. After 1 week of adaptation, the mice were divided into three groups (8 mice per group): LFD group fed LFD diet for 14 weeks, HFD group fed with HFD for 14 weeks, the treatment group (IRW group) first fed with HFD for 6 weeks followed by IRW (45 mg/kg BW) + high-fat diet for another 8 weeks. All mice had ad libitum access to food and water. At the end of the experiment, the mice were fasted for 6 h before being sacrificed by CO2 asphyxiation. The organ tissues were weighed and recorded during the sacrifice. All of the tissues and serum samples were snap-frozen and stored at −80 °C until further analysis.

4.2.11 Hypertensive Animal Study

For second animal study, male SHRs (spontaneously hypertensive rats; 16–17 weeks old) were obtained from Charles River (Senneville, QC, Canada) were randomly assigned into three groups, untreated (vehicle, n=5) and IRW (3 and 15 mg/kg body weight, n = 5); after 18 days of treatment, the rats were sacrificed, and their tissue were collected snap-frozen and stored at −80 °C until further analysis. The experimental procedures were approved by the University of Alberta Animal Welfare Committee (Protocol # 611/09/10/D) in accordance with the guidelines issued by the Canada Council on Animal Care and also adhered to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

4.2.12 RNA sequencing

RNA sequencing was conducted according to conditions and methodology described in Chapter 3.

4.2.13 Microbiota analysis

Genomic DNA was extracted from stool using the FastDNA Spin kit (MP Biomedicals) and quantified on a ND-1000 NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Samples were diluted to 50 ng/mL and then quantified with PicoGreen. qPCR reactions contained 8 µL H2O, 10 µL Fast SYBR Green Master Mix, 1 µL 10 µM forward and reverse primers, and 2 µL target DNA. PCR cycling conditions were 5 min at 50°C, 5 min at 95°C (15 sec at 95°C, 1 min at 60°C) (40 cycles), followed by a melting curve step progressing from 60°C to 95°C for 12 min. qPCR was performed in MicroAmp 96-well optical plates with the 7900HT instrument (Qiagen Inc, Toronto, Canada). Results were analyzed with SDS 2.3 software. Nonspecific amplification was determined using melting curves and visualizing products on a

QIAxcel (Qiagen Inc, Toronto, Canada) and target DNA copy number was determined by comparison with standard curves from the purified PCR product.

4.2.14 *Drosophila melanogaster* studies

To examine the impact of IRW on mitochondrial biogenesis in flies, W^{dah} flies were fed the tripeptide IRW (50 μM) for 1 week (from day 28 to day 35). Following the treatment, flies were dissected and incubated as shown previously (Aparicio, Rana, & Walker, 2019). Briefly, flies were dissected at day 35 after one week of IRW peptide feeding. Dissected flies were incubated with the following primary antibodies: mouse- anti-atp5a 1:250 (15H4C4, abcam) and mouse-anti-dsDNA 1;250 (ab27156, Abcam). Images were taken using Zeiss LSM880 confocal microscope (Carl Zeiss Microscopy, NY, U.S.A) and analyzed using the ImageJ software to measure mitochondrial size and mitochondrial DNA.

4.2.15 Peptide target identification

HOOK™ Activated Agarose (Amine Reactive, Cat.786-066) was used to conjugate IRW to make agarose conjugated IRW complex (G-Biosciences, St. Louis, MO, U.S.A). This complex was incubated with A7R5 cells, and the protein targets pulled down (extracted) following incubation were run on a 6% gel. Next, a in-gel trypsin digestion was performed on the samples. Briefly, excised gel bands were destained twice in 100 mM ammonium bicarbonate/acetonitrile (50:50). The samples were reduced (10 mm BME in 100 mm bicarbonate) and alkylated (55 mM iodoacetamide in 100 mm bicarbonate). After dehydration trypsin (6 ng/ul, Promega Sequencing grade) was added to just cover the gel pieces and the digestion was allowed to proceed overnight (~16 h) at room temperature. Tryptic peptides were first extracted from the gel using 97% water/2% acetonitrile/1% formic acid followed by a second extraction using 50% of the first

extraction buffer and 50% acetonitrile. Fractions containing tryptic peptides were resolved and ionized by using nanoflow HPLC (Easy-nLC II, Thermo Scientific, Mississauga, ON, Canada) coupled to an LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Scientific, Mississauga, ON, Canada). Nanoflow chromatography and electrospray ionization were accomplished by using a PicoFrit fused silica capillary column (ProteoPepII, C18) with 100µm inner diameter (300Å, 5µm, New Objective). Peptide mixtures were injected onto the column at a flow rate of 3000 nL/min and resolved at 500 nL/min using a 60-minute linear gradient from 0 to 45% v/v aqueous ACN in 0.2% v/v formic acid. The mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey Orbitrap spectra using external mass calibration, with a resolution of 30 000 and *m*/*z* range of 400–2000. The fourteen most intense multiply charged ions were sequentially fragmented by using collision induced dissociation, and spectra of their fragments were recorded in the linear ion trap; after two fragmentations all precursors selected for dissociation were dynamically excluded for 60 s. Data was processed using Proteome Discoverer 1.4 (Thermo Scientific, Mississauga, ON, Canada) and a reviewed Uniprot (uniprot.org) rattus proteome database was searched using SEQUEST (Thermo Scientific, Mississauga, ON, Canada). Search parameters included a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.8 Da. Peptides were searched with carbamidomethyl cysteine as a static modification and oxidized methionine and deamidated glutamine and asparagine as dynamic modifications.

4.2.16 CRISPR-Cas9 KO cells

Guide RNAs (gRNAs) designed to target FAM120B were annealed using equimolar amounts of Alt-R CRISPR-Cas9 tracrRNA, ATTO 550 (IDT) and Alt-R CRISPR-Cas9 crRNA (IDT) as previously described (Cromwell et al., 2018). Ribonucleoprotein complexes (RNP) were created

by mixing equimolar quantities of Cas9 (NEB) and the previously assembled gRNA. This was transfected into HEK293Ts cells using Lipofectamine CRISPRMAX (Thermofisher) according to manufacturer's instructions. After 24 h of incubation at 37˚C, 5% CO_2, cells were sorted based on the ATTO 550 fluorescent marker on the tracrRNA on a BD FACS Aria III instrument (Flow Cytometry Core, University of Alberta). Positive cells were single-sorted onto a 96-well plate and grown for 2-4 weeks. Once density of cells reached ~50%, they were moved to a 48-well plate, and then a 24-well plate. Once the cells on the 24-well plate reached ~80% confluence, they were split 50/50, with half being transferred to a 6-well plate and half spun down for DNA extraction. DNA was extracted from the cells using QuickExtract™ DNA Extraction Solution (Lucigen) following manufacturer's instructions. Briefly, 500 µL of cells were spun down at 300xg for 5 mins. The pellet was washed with PBS pH 7.4 (Gibco) and then resuspended in 100 µL of QuickExtract™ (Lucigen). This solution was added to a thermocycler and the following protocol was run as: 65˚C for 10mins, 68˚C for 10mins, and 98˚C for 3mins. The resulting DNA was used directly as a template for PCR reactions amplifying the Cas9 target site. These PCR reactions were purified with QIAquick PCR Purification Kit (Qiagen) and analyzed by Sanger sequencing to confirm the formation of gene-disrupting indels following Cas9 cleavage. Clone populations with demonstrated indel formation were further confirmed for protein knock-out by Western blot. The oligonucleotide sequences 5'→3' used in the assay were as follows, FAM120B crRNAv1: rArArG rArGrC rCrArG rArArA rUrArC rArGrG rUrUrG rUrUrU rUrArG rArGrC rUrArU, FAM120B Sequencing Primer F: CGT ACG TAT GCC AGC CCT TT, and FAM120B Sequencing Primer R: TTA GTC AAG GCC AGA GCA GC.

4.2.17 T7 endonuclease assay

A bulk sample of all FACs sorted positive cells was kept determining the efficiency of the gRNA, as previously described (Cromwell et al., 2018). Briefly, 48 hours after FACs sorting, gDNA was extracted from the cell population, as described above. PCR reactions targeting the regions surrounding the Cas9 target site were preformed and purified with QIAquick PCR Purification Kit (Qiagen). 200 ng of the resulting PCR product(s) were denatured and reannealed using the following thermocycling program: 95˚C for 5 mins, ramp -2˚C/sec to 85˚C for 1 sec, ramp -0.1˚C/sec to 25˚C for 1 sec. 1 μL of T7 Endonuclease I (NEB) was added to the reaction and incubated at 37˚C for 15 mins. The resulting DNA product was analyzed on a 1.5% TAE agarose gel to determine indel (%). This was calculated using densitometry (Image J) using the following formula: indel (%) = $100 \times (1-(1-\text{fractioncut})0.5)$.

4.2.18 Statistical analysis

A completely randomized design (CRD) was used to perform all the experiments conducted in this study where the treatments are assigned completely at random to cells and animals so that each experimental unit has the same chance of receiving any the treatment option(s). All statistical analyses were performed using GraphPad Prism software version 5.02 (GraphPad Software, San Diego, CA). Data are presented as mean ± standard error of mean deviation (SEM) and analyzed by unpaired Student's t-test (two groups), or one-way analysis of variance (ANOVA) followed by Dunnett's test (w.r.t HFD). $p < 0.05$ was considered significant.

4.3 Results

4.3.1 IRW initiates mitochondrial biogenesis in mammalian cells

PGC1α and TFAM are vital factors involved in the maintenance of mitochondrial homeostasis. Our immunoblotting results showed a significant depletion in the protein pools of both PGC1α and TFAM in muscle tissues of obese individuals (body mass index (BMI): 32.5−41.5), compared to normal individuals (BMI: 21.1−24.7) (Figure 4.1A). This suggests the obesity-related depletion of mitochondrial number and the need for its stimulus via activation of PGC1α and TFAM using pharmacological agents. IRW, an antioxidant tripeptide with minimal impact on mitochondrial potential (50 μM, Figure 4.1B; Figure S4.1 A&B) (was assessed for its ability to initiate mitochondrial biogenesis via activation of the PGC1α axis. Our results showed that 24 h of IRW treatment (50 μM) significantly increased the mRNA levels of PGC1α in A7R5 cells ($p<0.001$) (Figure 4.1 C). Likewise, we observed an increase in mitochondrial DNA content as demonstrated by significantly increased mtDNA:nDNA ratio using 12S ($p<0.01$) and ND1 ($p<0.001$) as the mitochondrial markers (Figure 4.1 D&E). A similar increase in mRNA levels of PGC1α and mitochondrial content was observed in 293T and L6 cells (Figure S4.1 C&D; F-H). However, no increase in mRNA levels of PGC1α or increase in mitochondrial nucleic acid ratio was seen in Caco2 cells (Figure S4.1 E,I). These experiments helped us to identify the appropriate concentration (50 μM) and time (24h) of IRW treatment for further experiments, which was in line with our recent findings on IRW (Bhullar et al., 2021). Next, we observed if the IRW triggered an increase in PGC1α mRNA content translated to enhanced protein expression. Our results showed that treatment of A7R5 cells with IRW (50 μM) for 24h increased the protein levels of key cellular markers of mitochondrial biogenesis including PGC1α, PPARγ, TFAM, NRF1, COX4, and TOMM20 (Figure 4.1 F&G). However, no impact was observed on levels of sirtuins (SIRT1 and SIRT3) (Figure 4.1H). An analogous increase in protein levels of PGC1α, TFAM, COX4, and NRF1 was observed in 293T cells, L6, and SH-SY5Y cells, but not in Caco2 cells (Figure S4.1 J-

M). Interestingly, IRW (50 μM) treatment increased the expression of histone mark H4K8ac, a dynamic histone modification related to anti-aging activity and lifespan extension (Peleg et al., 2016). In line with this, we observed an increase in protein levels of KAT2A/GCN5, a paradigmatic histone acetyltransferase (Figure 4.1I). The increased mitochondrial activity was accompanied by an increase in ATP and citric acid synthase levels in different cell types, indicating an overall improvement in the mitochondrial number and function (Figure 4.1 J,K). However, no increase in the oxygen consumption levels was observed in multiple cell types tested (Supp Figure S4.1 N). Next, to confirm the activation of mitochondrial biogenesis, we employed the MitoBiogenesis™ In-Cell ELISA kit, designed to measure drug-induced effects on mitochondrial biogenesis. Our results showed a significant increase in mitochondrial biogenesis across different cell lines except in Caco2 cells *in vitro* ($p<0.05$). Further, the unique tri-peptide structure of IRW was associated with an increase in activation of mitochondrial biogenesis as its analog IWQ or IKW (Figure 4.1 M), or constituent amino acids were unable to activate either TFAM or PGC1α in cells.

4.3.2 IRW increases mitochondrial number and density

Next, we investigated whether the ability of IRW to increase mRNA and protein levels of key biomarkers of mitochondrial biogenesis translates to an actual increase in the mitochondrial number. Flow cytometry analysis using MitoTracker Green dye showed that IRW treatment (50 μM) significantly increased the total number of mitochondria in different cell types (Figure 4.2 A-C). There was a strong increase in mitochondria number in A7R5 (Figure 4.2A), 293T (Figure 4.2B), and L6 cells (Figure 4.2C). However, in line with our previous observations, no increase in mitochondrial number was observed in Caco2 cells (Figure 4.2D). These results were confirmed by fluorescent microscopy analysis, indicating increased mitochondrial number/density (Figure

4.2 E-H). Finally, the electron microscopy analysis confirmed the visible increase in mitochondrial number following IRW treatment (50 μM) in both A7R5 and 293T cells (Figure 4.2 I,J). This set of experiments provided strong evidence in favor of the ability of IRW to increase the mitochondrial number in mice *in vivo*.

4.3.3 IRW increases multiple factors related with mitochondrial biogenesis

Next, RNA sequencing analysis was conducted to study the detailed impact of IRW on multiple pathways associated with mitochondrial biogenesis in A7R5 and 293T cells. IRW treatment prompted massive transcriptional changes related to mitochondrial biogenesis pathways. In A7R5 cells, IRW treatment (50 μM) increased the expression TIMM and TOMM complex including TIMM 13, TIMM 22, TOMM7, and TOMM40L (Figure 4.3A), all involved in the import of nuclear-encoded mitochondrial preproteins into mitochondrial matrix for the successful formation of new mitochondria. This was confirmed by an increase in the expression of mRNAs of multiple mitochondrial complexes such as MT-ATP6, MT-ATP8, MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND5, MT-ND6, and COX complexes (COX 4l1, COX7A2, and COX7C) (Figure 4.3A). Similar to A7R5 cells, IRW treatment (50 μM) in 293T cells also increased the expression of TOMM complex members such as TOMM5 and TOMM6, and TIMM complex members as well including TIMM8 and 13 (Figure 4.3B). However, the impact of IRW treatment on mitochondrial complex members was limited to MT-ATP6, MT-ND3, and COX7B in 293T cells (Figure 4.3B). Contrarily, transcription of MT-ND5 and MT-ND2 decreased in 293T cells following IRW treatment (50 μM). However, there was a surge in nuclear sirtuin mRNA (SIRT 2, 6, and 7) in 293T cells (Figure 4.3B). Other genes involved in mitochondrial biogenesis modulated by IRW treatment (50 μM) in A7R5 and 293T cells are shown in the lowermost panels of Figure 4.3 (Figure 4.3 A,B).

4.3.4 IRW increases mRNA copy number of mitochondrial genes

Following RNA sequencing, qPCR was used to validate the findings of transcriptomics in A7R5 cells (Figure 4.4). IRW treatment (50 μM) significantly increased the mRNA copy number of the genes in mitochondrial DNA. Among the protein-coding genes of mtDNA, ATP synthase-related ATP06 ($p<0.01$) and ATP08 ($p<0.0001$) were significantly increased following IRW treatment (Figure 4.4 A,B). Similarly, cytochrome c oxidase encoding CO-01 ($p<0.01$), CO-02 ($p<0.001$), and CO-03 ($p<0.01$) were significantly increased following IRW treatment in A7R5 cells (Figure 4.4 C-E). However, no significant increase in Cyt-B mRNA was observed (Figure 4.4 F). Interestingly, mRNA of all NADH dehydrogenase related genes ND1-6 was significantly boosted after IRW treatment (50 μM) in A7R5 cells (Figure 4.4 G-K). These results further confirm our earlier findings on the ability of IRW to increase mtDNA content.

4.3.5 IRW increases various mitochondrial factors in tissues of C57BL/6J HFD mice

RNAseq results in several mice organs showed that IRW feeding (45 mg/kg BW) modulated multiple factors associated with mitochondrial biogenesis (Figure 4.5). These included changes in key transcription factors such as PGC1α, TFAM, and NRF1 (Figure 4.5A). Expression of a series of mitochondrially encoded genes was somewhat increased in multiple tissues such as the aorta, muscle, and kidney, but was minimal in the liver *in vivo*, where expression of such complex of genes was notably lower (Figure 4.5B). In line with results from cell culture, we observed an increase in the multiple members of the TIMM and TOMM complex in different tissues of IRW fed mice (Figure 4.5C). A prominent increase in COX16 subunit was observed in the aorta and kidney, and to a lesser extent in liver and muscle (Figure 4.5D). However, unlike cell cultures RNAseq results, there were minimal changes in sirtuins in different tissues (Figure 4.5E, Figure 4.2B). Other key factors associated with mitochondrial biogenesis are shown in subsequent panels

(Figure 4.5 F,G). qPCR was used to validate the findings from RNAseq analysis. Next, we selected kidney tissues for validation of key biomarkers related to mitochondrial biogenesis. Results showed a significant increase in mRNA levels of PGC1α ($p<0.05$), ATP06 ($p<0.01$), CO-1 ($p<0.05$), CO-3 ($p<0.05$), Cyt B ($p<0.05$) in IRW fed C57BL/6J HFD group (Figure 4.6 A-F). Moreover, I also found a significant increase in ND3 ($p<0.01$), ND4 ($p<0.05$), ND6 ($p<0.05$), and TIMM8 ($p<0.05$) mRNA levels following IRW feeding (45 mg/kg BW) in kidney tissues of C57BL/6J HFD mice (Figure 4.6 H-K). However, no increase was observed in ATP08 and ND2 transcription in kidney tissues of IRW fed C57BL/6J HFD mice (IRW group) (Figure 4.5 C,G). Similarly, we found increased mRNA expression of ND3 and ND6 subunits in the muscle ($p<0.01$) and TOMM 34 in the aorta ($p<0.001$) tissues of C57BL/6J HFD mice *in vivo* (Figure 4.6 L-N). These results from RNAseq and qPCR confirmed a strong modulation of transcriptome by IRW feeding (45 mg/kg BW) in different tissues of C57BL/6J HFD mice.

4.3.6 IRW increases protein levels of multiple factors related with mitochondrial biogenesis in tissues of C57BL/6J mice

The protein expression of mitochondrial biogenesis biomarkers in tissues of C57BL/6J HFD mice (Figure 4.7 F,L) was also studied. In kidney tissues from C57BL/6J HFD mice fed with IRW (45 mg/kg BW), compared to the HFD group, we observed a significant increase ($p<0.05$) in the protein levels of COX4, NRF1, PGC1α and TOMM20 (Figure 4.7 A-E), but not of TFAM (Figure. 4.7 C). In muscle, COX4 and TOMM20 were upregulated (Figure. 4.7 G,K), but contrary to our expectations, no statistical increase was observed in protein levels of NRF1, TFAM, and PGC1α (Figure 4.7 H-J). Yet, there was a statistically significant increase in both ATP and citric acid synthase activity in muscle tissues of C57BL/6J HFD mice (Figure S4.2 B,C). Compared to the HFD group, a robust increase in COX4 (Figure. 4.7M), but not PGCα (Figure. 4.7N; Figure S4.2

A), levels in the plasma of IRW fed C57BL/6J HFD mice. This suggests an increase in free mitochondria in plasma of IRW fed C57BL/6J HFD mice, in line with recent findings on free mitochondria in plasma (Al Amir Dache et al., 2020; Stephens et al., 2020). Like HFD mice, SHR fed IRW-treated (15 mg/kg BW) mice also showed a strong increase in COX4, NRF1, TFAM, TOMM20, and PGC1α in aorta tissue (Figure S4.2 D,E).

4.3.7 IRW induces changes in microbiota and intestinal markers of mitochondrial biogenesis in C57BL/6J HFD mice

Experiments were done to determine whether the gut microbiota was modulated by IRW in C57BL/6J HFD mice (HFD *vs* IRW group) and whether such changes were related to initiation of mitochondrial biogenesis, culminating in increased mitochondrial number. Principal coordinate analysis (PCoA) from Bray-Curtis dissimilarities showed marked differences in microbiome composition, indicating the total microbial shifts across the treatment groups (Figure 4.8 A). The number of observed OTUs (operational taxonomic units) and the Shannon diversity index also indicated changes in the total microbiome in IRW fed C57BL/6J HFD mice *in vivo* (Figure 4.8 B,C). The 16S analysis of microbiome showed modulation of 89 key families, genera and species of microbiota associated with metabolic and mitochondrial function, indicating a strong modulatory impact of IRW feeding (45 mg/kg BW) in mice *in vivo* (Figure 4.8D; Figure S4.3). The members of microbiota modulated by IRW included *Mogibacteriaceae*, *Lachnospiraceae*, *Erysipelotrichaceae*, *Bacteroides*, *Sutterella*, *Roseburia*, *Paraprevotella*, *Akkermansia muciniphila*, *Bacteroides caccae*, *and Bacteroides ovatus* (Figure 4.8D). Likewise, IRW feeding significantly altered multiple secondary metabolites produced by microbiota in C57BL/6J HFD mice *in vivo* (Figure 4.8E). These included CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase, leader peptidase (prepilin peptidase)/N-methyltransferase, ATP-

dependent DNA helicase PcrA, penicillin-binding protein 1A, 3-hydroxybutyryl-CoA epimerase, DNA ligase (ATP), leucyl/phenylalanyl-tRNA--protein transferase, tryptophan-rich sensory protein, Trp repressor binding protein, NAD(P)H-quinone oxidoreductase subunit 5, hemoglobin, catalase/peroxidase, cytochrome c oxidase subunit I, cytochrome c oxidase subunit II, transposase, proline dehydrogenase, superoxide dismutase, Fe-Mn family, phosphatidylserine synthase, membrane fusion protein, HSP20 family protein, methylenetetrahydrofolate reductase (NADPH) (Figure 4.8E). We next analyzed intestinal tissue to observe changes associated with mitochondrial biogenesis. Our results showed a significant increase in TFAM and COX4 protein levels in the intestine of IRW fed C57BL/6J HFD mice in vivo ($p<0.01$) (Figure 4.8 F-I). However, IRW feeding (45 mg/kg BW) did not increase intestinal levels of PGC1α levels in vivo (Figure 4.8 G,I). These results suggest a correlation between gut microbial changes and the activation of mitochondrial biogenesis in IRW fed C57BL/6J HFD mice in vivo (Figure 4.8A-I; Figure S4.3).

4.3.8 IRW increases mitochondrial DNA content in flies

We also studied the role if IRW was able of increase mitochondrial biogenesis in *Drosophila.* Immunofluorescence staining of *Drosophila* fly muscles shows that IRW treatment (From day 28 to day 35) increases mitochondrial DNA content (Figure 4.9A). These results are consistent with our findings indicating an increase in mt. DNA in mammalian cells and mice (Figure 4.1 C-E, Figure 4.3, and Figure 4.6). However, there was no major change in the size of mitochondria (Figure 4.9B), indicating the absence of mitofusion/fission changes following treatment of IRW over 1 week. These results are similar to cell and mice RNA sequencing, which also indicated no increase in mitofusion (MFN2 or OPA1) or mitofission (DRP1) related genes (Figure 4.2 and Figure 4.5).

4.3.9 Ability of IRW to induce mitochondrial biogenesis depends on FAM120B

Finally, using biochemical techniques, we identified the central target of IRW involved in the activation of mitochondrial biogenesis. HOOK™ activated agarose (amine reactive) was used to conjugate IRW to make an intermediate Schiff base complex and finally agarose conjugated IRW complex (Figure 4.10A). This complex was incubated with A7R5 cells and agarose beads were used to pull down the IRW interacting targets (Figure S4.4 A). The proteomics analysis showed that IRW interacted with FAM120B (Figure 4.10 B,C). Other key targets included glutathione S-transferase-1 (GST-1) and heat shock protein 90 (HSP90) (Figure S4.4 B-F). Treatment of A7R5 cells with IRW (50 μM) also showed increased levels of FAM120B (Figure 4.10D). Likewise, in aorta tissue of IRW fed (high:45 mg/kg BW) C57BL/6J HFD mice *in vivo* (Figure 4.10D). Following identification of FAM120B, the CRISPR-Cas9 technique was used to make FAM120B KO cell line (Figure 4.10E) and verified using T7 Endonuclease assay (Figure 4.10F). The CRISPR-Cas9 method was used to generate 6 KO colonies, which were grown, and target KO was confirmed using immunoblotting (Figure 4.10G). Among these, two cell lines ΔC2 and ΔC5 were used to check the FAM120B dependent ability of IRW to initiate mitochondrial biogenesis. Our results showed that in FAM120B KO cells, the ability of IRW to initiate mitochondrial biogenesis was completely abolished (Figure 4.10G). Compared to WT cells, IRW was unable to increase the expression of PGC1α, NRF1, COX4, TFAM, and TOMM complex in FAM120B KO cells (Figure 4.10G). This novel click chemistry and CRISPR-Cas9 guided finding showed that IRW interacts with FAM120B, a PPARγ co-activator, leading to subsequent activation of PGC1α and its downstream targets: NRF1, COX4, TFAM, and TOMM complex, all together culminating in mitochondrial biogenesis.

4.4 Discussion

Mitochondria are vital regulators of cellular function and metabolism. We have recently identified a metabolism essential enzyme, NAMPT, as a potential target of IRW both *in vitro* and *in vivo* (Bhullar et al., 2021). In the current study, we investigated the ability of IRW to initiate mitochondrial biogenesis and observed a vivid increase in mitochondrial number in both cells and *in vivo* (Figure 4.1-8). The biogenesis of mitochondria is dependent on multiple and intricately related factors, resulting in ATP and NAD surge (Figure 4.1J, Supplementary Figure 9B) (Bhullar et al., 2021; Fontecha-Barriuso et al., 2020). After treating cells with IRW, a surge in mRNA and protein levels of PGC1α, and its downstream targets such as NRF1, TFAM, and TOM complex members was observed (Figure 4.1-4.8). The ability of IRW to initiate mitochondrial biogenesis also extended to mice and W^{dah} flies *in vivo* (Figure 4.4-9). Using click chemistry and CRISPR-Cas9 technology, we elucidated that the effect of IRW is exerted in a FAM120B dependent manner (Figure 4.10). Overall, IRW is capable of activating mitochondrial biogenesis both *in vitro* and *in vivo*, via FAM120B mediated initiation of the PGC1α pathway.

Mitochondria are at the converging center of many cellular processes such as ATP production via OXPHOS, Ca^{2+} signaling, heme synthesis, and amino acid metabolism, and additionally maintain metabolic nodes, such as NAD, ATP, AMP, etc. (Jones, Yao, Vicencio, Karkucinska-Wieckowska, & Szabadkai, 2012). More than 2000 proteins are presented in the mitochondria, but the vast majority of them are imported into the mitochondria, indicating an anterograde transport process (Schmidt et al., 2010). To preserve themselves and their host cell, mitochondria must maintain a turnover balance between biogenesis and mitophagy. Mitochondrial biogenesis is a complicated process, and requires the production, import, and fusion of proteins to the existing mitochondria, as well as replication of mtDNA. Mechanistically, a key player in this nucleus-to-mitochondria anterograde signaling is PGC1α, the master regulator of mitochondrial

biogenesis (Fontecha-Barriuso et al., 2020). As a cardinal transcriptional regulator during mitochondrial biogenesis, PGC1α activates NRF1, which then triggers transcription of TFAM leading to upregulation of mtDNA replication (Wu et al., 1999). Besides this PGC1α axis, mitochondrial biogenesis also depends on the import of more than 1,000 nuclear proteins. Thus, mitochondria employ the TOM complex, their central entry gate for virtually all nuclear-encoded mitochondrial proteins to make more mitochondria (Neupert & Herrmann, 2007). These signals converge on mitochondrial biogenesis by regulating PGC1α activity and its cellular associates. In the present study, we confirm the surge in the PGC1α/NRF1/TFAM pathway accompanied by an activated cytosolic faced TOM complex, culminating in mitochondrial biogenesis after IRW treatment.

During aging and disease conditions such as obesity, hypertension, and neurological diseases, etc., mitochondria decline in number. In the past two decades, there has been a substantial effort to determine whether pharmacological and/or nutritional approaches can initiate mitochondrial biogenesis. Multiple prior reports indicated that multiple bioactive compounds such as polyphenols and some peptides can influence mitochondrial biogenesis in cells and *in vivo*. Our findings indicate that IRW mimics the ability of polyphenols like quercetin, curcumin, resveratrol, ellagitannin, and epigallocatechin-3-gallate (EGCG) (Chung, Manganiello, & Dyck, 2012; Davinelli, De Stefani, De Vivo, & Scapagnini, 2020). Particularly, IRW can mimic resveratrol's ability to increase transcriptional activity of PGC1α *in vivo* (Figure 4.5-4.7) (Baur et al., 2006). Hydroxytyrosol, a polyphenolic compound found in olives accelerated the PGC1α activity in skeletal muscles of rats (Feng et al., 2011). Likewise, curcumin, a well-known polyphenolic compound upregulates the PGC1α axis in skeletal muscle and cerebral cortex of rats (Hamidie, Yamada, Ishizawa, Saito, & Masuda, 2015; L. Liu et al., 2014). Similar to others, myricetin

enhances mitochondrial activity by activating PGC1α (Jung et al., 2017). Interestingly, in subjects with type 2 diabetes the administration of epicatechin-rich cocoa stimulates mitochondrial biogenesis via NO, PGC1α, and TFAM in skeletal muscle (Taub et al., 2012). This closely resembles the pattern of transcriptional initiated by IRW, warranting clinical exploration of IRW. As molecular induction of mitochondrial biogenesis envisages survival in critically ill patients, IRW may improve mitochondrial functional capacity in the face of acute clinical stressors (Carré et al., 2010). Further, it is also possible that IRW supplementation may mimic exercise mediated mitochondrial biogenesis in working muscles (Cartee, Hepple, Bamman, & Zierath, 2016). The ability of IRW to initiate mitochondrial biogenesis holds strong promise for neurological diseases as impaired mitochondrial biogenesis is a feature of neurodegenerative conditions including Alzheimer's disease (AD) and Parkinson's disease (Corona & Duchen, 2015; W. Qin et al., 2009). As ~20% of AD cases in developed countries could be prevented by enhancing physical activity, which indicates massive disruption of mitochondrial homeostasis and the accumulation of mtDNA damage (Picard & Turnbull, 2013). Based on this, we propose that IRW must be explored for improvement in mitochondrial content and function towards mitigation of neurological disorders.

The pharmacological activity of IRW resembles CR, defined as a cutback in caloric intake without malnutrition, which is a viable strategy to initiate mitochondrial biogenesis (Nisoli et al., 2005). This closely resembles the pattern of transcriptional changes prompted by IRW and warrants clinical exploration of IRW as a potential therapeutic for treatment of diseases associated with mitochondrial abnormalities. As molecular induction of mitochondrial biogenesis may improve survival in critically ill patients, IRW has the potential to improve mitochondrial functional capacity in the face of acute clinical stressors (Klingenspor, 2003). Interestingly, we did not observe any effect of IRW on the sirtuin axis (especially SIRT1) in cells or *in vivo*. This

differentiates IRW from resveratrol as the latter's ability to initiate mitochondrial biogenesis depends on SIRT1 (Bhullar & Hubbard, 2015). Even though IRW does increase SIRT1 in liver tissues of C57BL/6J mice fed HFD (Chapter 3), but we didn't observe an increase in mitochondrial biogenesis in the liver (Bhullar et al., 2021). This indicates SIRT1 independent activation of mitochondrial biogenesis by IRW both in cells and *in vivo*. Multiple basic science findings indicate that SIRT1 promotes a deacetylation mediated increase of PGC1α, along with TFAM and OXPHOS (Gerhart-Hines et al., 2007). Massive disruption of mitochondrial homeostasis and the accumulation of mtDNA damage has been reported for AD (Mootha et al., 2003).

Pharmacological agents (like IRW) with the ability to boost mitochondrial abundance have potential to help in clinical remediation of diseases like T2 diabetes, obesity, metabolic syndrome, neurodegenerative disorders, and renal diseases. Apart from the natural bioactive compounds such as polyphenols, various synthetic mitochondrial therapies are being developed to improve mitochondrial function and number. The AMPK activator AICAR acts as an AMP analog and stimulates mitochondrial biogenesis and improves OXPHOS *in vivo* (Kukidome et al., 2006; Narkar et al., 2008). Interestingly, IRW mimics downstream AICAR activity, since NAD, OXPHOS, and mt.number increase. The direct impact of IRW on AMPK in muscle and other tissues will be addressed in upcoming reports on IRW. The ability of IRW to improve mitochondrial function is also similar to SIRT1 activators such SRT1720, SRT1460, SRT2104, and SRT237, despite its SIRT1 independent mechanisms (Milne et al., 2007). Similarly, G protein-coupled receptors such as formoterol and rimonabant (Tedesco et al., 2008; Wills et al., 2012), PPARγ agonists (thiazolidinediones) (Pardo, Enguix, Lasheras, Feliu, Kralli, & Villena, 2011), and cGMP modulators fall in the area of IRW's mitochondrial pharmacology (Nisoli et al., 2004). Similar to IRW, natriuretic peptides like atrial natriuretic peptide (ANP) and brain natriuretic

peptide (BNP) improve mitochondrial function via cGMP-dependent signaling (Miyashita et al., 2009).

The development of pharmacological candidates to induce mitochondrial biogenesis demands making molecules that (a) directly amass and bind targets within mitochondria (b) modulate signaling outside and inside mitochondria that ultimately improve mitochondrial function/number (c) alter other interrelated subsystems of physiological paradigms which support changes in mitochondrial physiology. Changes in the category (b) have been discussed above, following this, we investigated if IRW can modulate supportive mechanisms (category c) such as microbiota, a factor apparently involved in mitochondrial biogenesis (Vezza, Abad-Jiménez, Marti-Cabrera, Rocha, & Víctor, 2020). We found that IRW microbiota modulates key microbial families associated with mitochondrial biogenesis. IRW lowers levels of obesity related microbiota families *Mogibacteriaceae*, *Lachnospiraceae*, and *Erysipelotrichaceae*, genus Bacteroides, and species like *Sutterella, Akkermansia muciniphila, Bacteroides caccae, and Bacteroides ovatus* (Panasevich et al., 2016; J. Qin et al., 2012). These results are similar to gut microbiota mediated metabolic effects of metformin to boost metabolism and trigger mitochondrial biogenesis (Karnewar et al., 2018; Owen, Doran, & Halestrap, 2000). Also, this vast spectrum of microbiota modulation by IRW indicates that regardless of their distinct roles, mitochondria and microbiota bacteria share many common physiological features. This modulation of microbiota in parallel with mitochondrial biogenesis supports the endosymbiotic theory of the bacterial origin of mitochondria (Degli Esposti et al., 2014). It is previously known that the gut microbiota can directly modulate mitochondrial DNA (Saint-Georges-Chaumet, Attaf, Pelletier, & Edeas, 2015). Results of the current research highlight the need of further study of the interplay between

mitochondria and microbiota, and how such relationships could be exploited for the treatment of metabolic disorders such as obesity and diabetes.

Finally, by using click chemistry and CRISPR-Cas9 mutagenesis we elucidated the underlying mechanism, whereby IRW impacts mitochondrial biogenesis. Namely, the protein FAM120B, a coactivator of PPARγ (Li, Kang, & Wang, 2007) was found to interact with IRW and this lead to a downstream cascade that culminates in increased mitochondrial biogenesis via activation of PGC1α. As PPARγ is closely related to the PGC1α pathway, the interaction of IRW with FAM120B supports the role of IRW on the activation of mitochondrial biogenesis. An interaction between FAM120B and PGC1α was not reported in the initial characterization studies (Chidambaram et al., 2020; Li et al., 2007). We propose that PGC1α upregulation by FAM120B occurs via PPARγ. It is, IRW acts as a ligand of FAM120B thus promoting PPARγ/PGC1α transactivation. The specific mechanism whereby IRW mediates FAM120B-PPARγ-PGC1α interaction warrants further investigation (Figure 4.10I).

4.5 Conclusions

IRW, a tripeptide obtained from ovotransferrin, activates the PGC1α pathway resulting in increased mitochondrial biogenesis, ATP surge, and improved mitochondrial function. IRW activated PGC1α signaling cascades to increase the mRNA and protein levels of multiple players involved in mitochondrial biogenesis in C57BL/6J HFD mice and flies. IRW rescued the HFD fed mice from oxidative and mitochondrial stress by promoting mitochondrial biogenesis in multiple tissues. Interestingly, IRW also increased mitochondria abundance in plasma. The underlying mechanism of mitochondria biogenesis activation by IRW seems to rely on the interaction of IRW

with FAM120B, a constitutive coactivator of PPARγ, resulting in downstream activation of the PGC1α axis. Our findings open the exciting possibility of using IRW in clinical trials for the treatment of metabolic diseases associated with mitochondrial malfunctioning.

4.6 References

Al Amir Dache, Z., Otandault, A., Tanos, R., Pastor, B., Meddeb, R., Sanchez, C., . . . Grange, T. (2020). Blood contains circulating cell-free respiratory competent mitochondria. *The FASEB Journal, 34*(3), 3616-3630.

Aparicio, R., Rana, A., & Walker, D. W. (2019). Upregulation of the autophagy adaptor p62/SQSTM1 prolongs health and lifespan in middle-aged Drosophila. *Cell reports, 28*(4), 1029-1040. e1025.

Baur, J. A., Pearson, K. J., Price, N. L., Jamieson, H. A., Lerin, C., Kalra, A., . . . Lewis, K. (2006). Resveratrol improves health and survival of mice on a high-calorie diet. *Nature, 444*(7117), 337-342.

Besse-Patin, A., Jeromson, S., Levesque-Damphousse, P., Secco, B., Laplante, M., & Estall, J. L. (2019). PGC1A regulates the IRS1: IRS2 ratio during fasting to influence hepatic metabolism downstream of insulin. *Proceedings of the National Academy of Sciences, 116*(10), 4285-4290.

Bhullar, K. S., & Hubbard, B. P. (2015). Lifespan and healthspan extension by resveratrol. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1852*(6), 1209-1218.

Bhullar, K. S., Son, M., Kerek, E., Cromwell, C. R., Wingert, B. M., Wu, K., . . . Wu, J. (2021). Tripeptide IRW Upregulates NAMPT Protein Levels in Cells and Obese C57BL/6J Mice. *Journal of Agricultural and Food Chemistry*.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., . . . Shipley, G. L. (2009). The MIQE Guidelines: M inimum I nformation for Publication of Q uantitative Real-Time PCR E xperiments. Oxford University Press.

Carré, J. E., Orban, J.-C., Re, L., Felsmann, K., Iffert, W., Bauer, M., . . . Breen, P. (2010). Survival in critical illness is associated with early activation of mitochondrial biogenesis. *American journal of respiratory and critical care medicine, 182*(6), 745-751.

Cartee, G. D., Hepple, R. T., Bamman, M. M., & Zierath, J. R. (2016). Exercise promotes healthy aging of skeletal muscle. *Cell metabolism, 23*(6), 1034-1047.

Chacinska, A., van der Laan, M., Mehnert, C. S., Guiard, B., Mick, D. U., Hutu, D. P., . . . Pfanner, N. (2010). Distinct forms of mitochondrial TOM-TIM supercomplexes define signal-dependent states of preprotein sorting. *Molecular and cellular biology, 30*(1), 307-318.

Chandel, N., Maltepe, E., Goldwasser, E., Mathieu, C., Simon, M., & Schumacker, P. (1998). Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proceedings of the National Academy of Sciences, 95*(20), 11715-11720.

Chandel, N. S. (2018). Mitochondria: back to the future. *Nature reviews Molecular cell biology, 19*(2), 76-76.

Chidambaram, S. B., Bhat, A., Ray, B., Sugumar, M., Muthukumar, S. P., Manivasagam, T., . . . Sakharkar, M. K. (2020). Cocoa beans improve mitochondrial biogenesis via PPARγ/PGC1α dependent signalling pathway in MPP+ intoxicated human neuroblastoma cells (SH-SY5Y). *Nutritional neuroscience, 23*(6), 471-480.

Chung, J. H., Manganiello, V., & Dyck, J. R. (2012). Resveratrol as a calorie restriction mimetic: therapeutic implications. *Trends in cell biology, 22*(10), 546-554.

Corona, J. C., & Duchen, M. R. (2015). PPARγ and PGC-1α as therapeutic targets in Parkinson's. *Neurochemical research, 40*(2), 308-316.

Davinelli, S., De Stefani, D., De Vivo, I., & Scapagnini, G. (2020). Polyphenols as caloric restriction mimetics regulating mitochondrial biogenesis and mitophagy. *Trends in Endocrinology & Metabolism, 31*(7), 536-550.

Degli Esposti, M., Chouaia, B., Comandatore, F., Crotti, E., Sassera, D., Lievens, P. M.-J., . . . Bandi, C. (2014). Evolution of mitochondria reconstructed from the energy metabolism of living bacteria. *PloS one, 9*(5), e96566.

Dhar, S. S., Ongwijitwat, S., & Wong-Riley, M. T. (2008). Nuclear respiratory factor 1 regulates all ten nuclear-encoded subunits of cytochrome c oxidase in neurons. *Journal of Biological Chemistry, 283*(6), 3120-3129.

Feng, Z., Bai, L., Yan, J., Li, Y., Shen, W., Wang, Y., . . . Chen, Y. (2011). Mitochondrial dynamic remodeling in strenuous exercise-induced muscle and mitochondrial dysfunction: regulatory effects of hydroxytyrosol. *Free Radical Biology and Medicine, 50*(10), 1437-1446.

Fontecha-Barriuso, M., Martin-Sanchez, D., Martinez-Moreno, J. M., Monsalve, M., Ramos, A. M., Sanchez-Niño, M. D., . . . Sanz, A. B. (2020). The role of PGC-1α and mitochondrial biogenesis in kidney diseases. *Biomolecules, 10*(2), 347.

Gerhart-Hines, Z., Rodgers, J. T., Bare, O., Lerin, C., Kim, S. H., Mostoslavsky, R., . . . Puigserver, P. (2007). Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1α. *The EMBO journal, 26*(7), 1913-1923.

Hamidie, R. D. R., Yamada, T., Ishizawa, R., Saito, Y., & Masuda, K. (2015). Curcumin treatment enhances the effect of exercise on mitochondrial biogenesis in skeletal muscle by increasing cAMP levels. *Metabolism, 64*(10), 1334-1347.

Harbauer, A. B., Opalińska, M., Gerbeth, C., Herman, J. S., Rao, S., Schönfisch, B., . . . Meisinger, C. (2014). Cell cycle–dependent regulation of mitochondrial preprotein translocase. *Science, 346*(6213), 1109-1113.

Jones, A. W., Yao, Z., Vicencio, J. M., Karkucinska-Wieckowska, A., & Szabadkai, G. (2012). PGC-1 family coactivators and cell fate: roles in cancer, neurodegeneration, cardiovascular disease and retrograde mitochondria–nucleus signalling. *Mitochondrion, 12*(1), 86-99.

Jung, H.-Y., Lee, D., Ryu, H. G., Choi, B.-H., Go, Y., Lee, N., . . . Kim, S.-H. (2017). Myricetin improves endurance capacity and mitochondrial density by activating SIRT1 and PGC-1α. *Scientific reports, 7*(1), 1-10.

Karnewar, S., Neeli, P. K., Panuganti, D., Kotagiri, S., Mallappa, S., Jain, N., . . . Kotamraju, S. (2018). Metformin regulates mitochondrial biogenesis and senescence through AMPK mediated H3K79 methylation: Relevance in age-associated vascular dysfunction. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1864*(4), 1115-1128.

Kashyap, S. R., Roman, L. J., Lamont, J., Masters, B. S. S., Bajaj, M., Suraamornkul, S., . . . Liu, Y. (2005). Insulin resistance is associated with impaired nitric oxide synthase activity in skeletal muscle of type 2 diabetic subjects. *The Journal of Clinical Endocrinology & Metabolism, 90*(2), 1100-1105.

Klingenspor, M. (2003). Cold-induced recruitment of brown adipose tissue thermogenesis. *Experimental physiology, 88*(1), 141-148.

Kukat, C., Wurm, C. A., Spåhr, H., Falkenberg, M., Larsson, N.-G., & Jakobs, S. (2011). Super-resolution microscopy reveals that mammalian mitochondrial nucleoids have a uniform size and frequently contain a single copy of mtDNA. *Proceedings of the National Academy of Sciences, 108*(33), 13534-13539.

Kukidome, D., Nishikawa, T., Sonoda, K., Imoto, K., Fujisawa, K., Yano, M., . . . Araki, E. (2006). Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. *Diabetes, 55*(1), 120-127.

Lane, N., & Martin, W. (2010). The energetics of genome complexity. *Nature, 467*(7318), 929-934.

Li, D., Kang, Q., & Wang, D.-M. (2007). Constitutive coactivator of peroxisome proliferator-activated receptor (PPARγ), a novel coactivator of PPARγ that promotes adipogenesis. *Molecular Endocrinology, 21*(10), 2320-2333.

Liu, L., Zhang, W., Wang, L., Li, Y., Tan, B., Lu, X., . . . Mu, J. (2014). Curcumin prevents cerebral ischemia reperfusion injury via increase of mitochondrial biogenesis. *Neurochemical research, 39*(7), 1322-1331.

Liu, X., Kim, C. N., Yang, J., Jemmerson, R., & Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell, 86*(1), 147-157.

Lorenzo, J. M., Munekata, P. E., Gomez, B., Barba, F. J., Mora, L., Perez-Santaescolastica, C., & Toldra, F. (2018). Bioactive peptides as natural antioxidants in food products–A review. *Trends in food science & technology, 79*, 136-147.

Milne, J. C., Lambert, P. D., Schenk, S., Carney, D. P., Smith, J. J., Gagne, D. J., . . . Vu, C. B. (2007). Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature, 450*(7170), 712-716.

Miyashita, K., Itoh, H., Tsujimoto, H., Tamura, N., Fukunaga, Y., Sone, M., . . . Sonoyama, T. (2009). Natriuretic peptides/cGMP/cGMP-dependent protein kinase cascades promote muscle mitochondrial biogenesis and prevent obesity. *Diabetes, 58*(12), 2880-2892.

Mootha, V. K., Bunkenborg, J., Olsen, J. V., Hjerrild, M., Wisniewski, J. R., Stahl, E., . . . Kamal, M. (2003). Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria. *Cell, 115*(5), 629-640.

Narkar, V. A., Downes, M., Ruth, T. Y., Embler, E., Wang, Y.-X., Banayo, E., . . . Juguilon, H. (2008). AMPK and PPARδ agonists are exercise mimetics. *Cell, 134*(3), 405-415.

Neupert, W., & Herrmann, J. M. (2007). Translocation of proteins into mitochondria. *Annu. Rev. Biochem., 76*, 723-749.

Nisoli, E., Clementi, E., Paolucci, C., Cozzi, V., Tonello, C., Sciorati, C., . . . Moncada, S. (2003). Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science, 299*(5608), 896-899.

Nisoli, E., Falcone, S., Tonello, C., Cozzi, V., Palomba, L., Fiorani, M., . . . Francolini, M. (2004). Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. *Proceedings of the National Academy of Sciences, 101*(47), 16507-16512.

Nisoli, E., Tonello, C., Cardile, A., Cozzi, V., Bracale, R., Tedesco, L., . . . Clementi, E. (2005). Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. *Science, 310*(5746), 314-317.

Nunnari, J., & Suomalainen, A. (2012). Mitochondria: in sickness and in health. *Cell, 148*(6), 1145-1159.

Owen, M. R., Doran, E., & Halestrap, A. P. (2000). Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochemical Journal, 348*(3), 607-614.

Pagliarini, D. J., Calvo, S. E., Chang, B., Sheth, S. A., Vafai, S. B., Ong, S.-E., . . . Chen, W. K. (2008). A mitochondrial protein compendium elucidates complex I disease biology. *Cell, 134*(1), 112-123.

Panasevich, M. R., Morris, E. M., Chintapalli, S. V., Wankhade, U. D., Shankar, K., Britton, S. L., . . . Rector, R. S. (2016). Gut microbiota are linked to increased susceptibility to hepatic steatosis in low-aerobic-capacity rats fed an acute high-fat diet. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 311*(1), G166-G179.

Pardo, R., Enguix, N., Lasheras, J., Feliu, J. E., Kralli, A., & Villena, J. A. (2011). Rosiglitazone-induced mitochondrial biogenesis in white adipose tissue is independent of peroxisome proliferator-activated receptor γ coactivator-1α. *PloS one, 6*(11), e26989.

Peleg, S., Feller, C., Forne, I., Schiller, E., Sévin, D. C., Schauer, T., . . . Klima, C. (2016). Life span extension by targeting a link between metabolism and histone acetylation in Drosophila. *EMBO reports, 17*(3), 455-469.

Pfanner, N., & Meijer, M. (1997). Mitochondrial biogenesis: the Tom and Tim machine. *Current Biology, 7*(2), R100-R103.

Picard, M., & Turnbull, D. M. (2013). Linking the metabolic state and mitochondrial DNA in chronic disease, health, and aging. *Diabetes, 62*(3), 672-678.

Picca, A., & Lezza, A. M. S. (2015). Regulation of mitochondrial biogenesis through TFAM–mitochondrial DNA interactions: useful insights from aging and calorie restriction studies. *Mitochondrion, 25*, 67-75.

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., . . . Shen, D. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature, 490*(7418), 55-60.

Qin, W., Haroutunian, V., Katsel, P., Cardozo, C. P., Ho, L., Buxbaum, J. D., & Pasinetti, G. M. (2009). PGC-1α expression decreases in the Alzheimer disease brain as a function of dementia. *Archives of neurology, 66*(3), 352-361.

Ramachandran, B., Yu, G., & Gulick, T. (2008). Nuclear respiratory factor 1 controls myocyte enhancer factor 2A transcription to provide a mechanism for coordinate expression of respiratory chain subunits. *Journal of Biological Chemistry, 283*(18), 11935-11946.

Saint-Georges-Chaumet, Y., Attaf, D., Pelletier, E., & Edeas, M. (2015). Targeting microbiota-mitochondria inter-talk: Microbiota control mitochondria metabolism. *Cellular and Molecular Biology, 61*(4), 121-124.

Schmidt, O., Pfanner, N., & Meisinger, C. (2010). Mitochondrial protein import: from proteomics to functional mechanisms. *Nature reviews Molecular cell biology, 11*(9), 655-667.

Stephens, O. R., Grant, D., Frimel, M., Wanner, N., Yin, M., Willard, B., . . . Asosingh, K. (2020). Characterization and origins of cell-free mitochondria in healthy murine and human blood. *Mitochondrion, 54*, 102-112.

Taub, P. R., Ramirez-Sanchez, I., Ciaraldi, T. P., Perkins, G., Murphy, A. N., Naviaux, R., . . . Ceballos, G. (2012). Alterations in skeletal muscle indicators of mitochondrial structure and biogenesis in patients with type 2 diabetes and heart failure: effects of epicatechin rich cocoa. *Clinical and translational science, 5*(1), 43-47.

Tedesco, L., Valerio, A., Cervino, C., Cardile, A., Pagano, C., Vettor, R., . . . Lutz, B. (2008). Cannabinoid type 1 receptor blockade promotes mitochondrial biogenesis through endothelial nitric oxide synthase expression in white adipocytes. *Diabetes, 57*(8), 2028-2036.

Tovar, J., León-Avila, G., Sánchez, L. B., Sutak, R., Tachezy, J., Van Der Giezen, M., . . . Lucocq, J. M. (2003). Mitochondrial remnant organelles of Giardia function in iron-sulphur protein maturation. *Nature, 426*(6963), 172-176.

Uittenbogaard, M., & Chiaramello, A. (2014). Mitochondrial biogenesis: a therapeutic target for neurodevelopmental disorders and neurodegenerative diseases. *Current pharmaceutical design, 20*(35), 5574-5593.

Vezza, T., Abad-Jiménez, Z., Marti-Cabrera, M., Rocha, M., & Víctor, V. M. (2020). Microbiota-mitochondria inter-talk: A potential therapeutic strategy in obesity and type 2 diabetes. *Antioxidants, 9*(9), 848.

Wills, L. P., Trager, R. E., Beeson, G. C., Lindsey, C. C., Peterson, Y. K., Beeson, C. C., & Schnellmann, R. G. (2012). The β2-adrenoceptor agonist formoterol stimulates mitochondrial biogenesis. *Journal of Pharmacology and Experimental Therapeutics, 342*(1), 106-118.

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., . . . Scarpulla, R. C. (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell, 98*(1), 115-124.

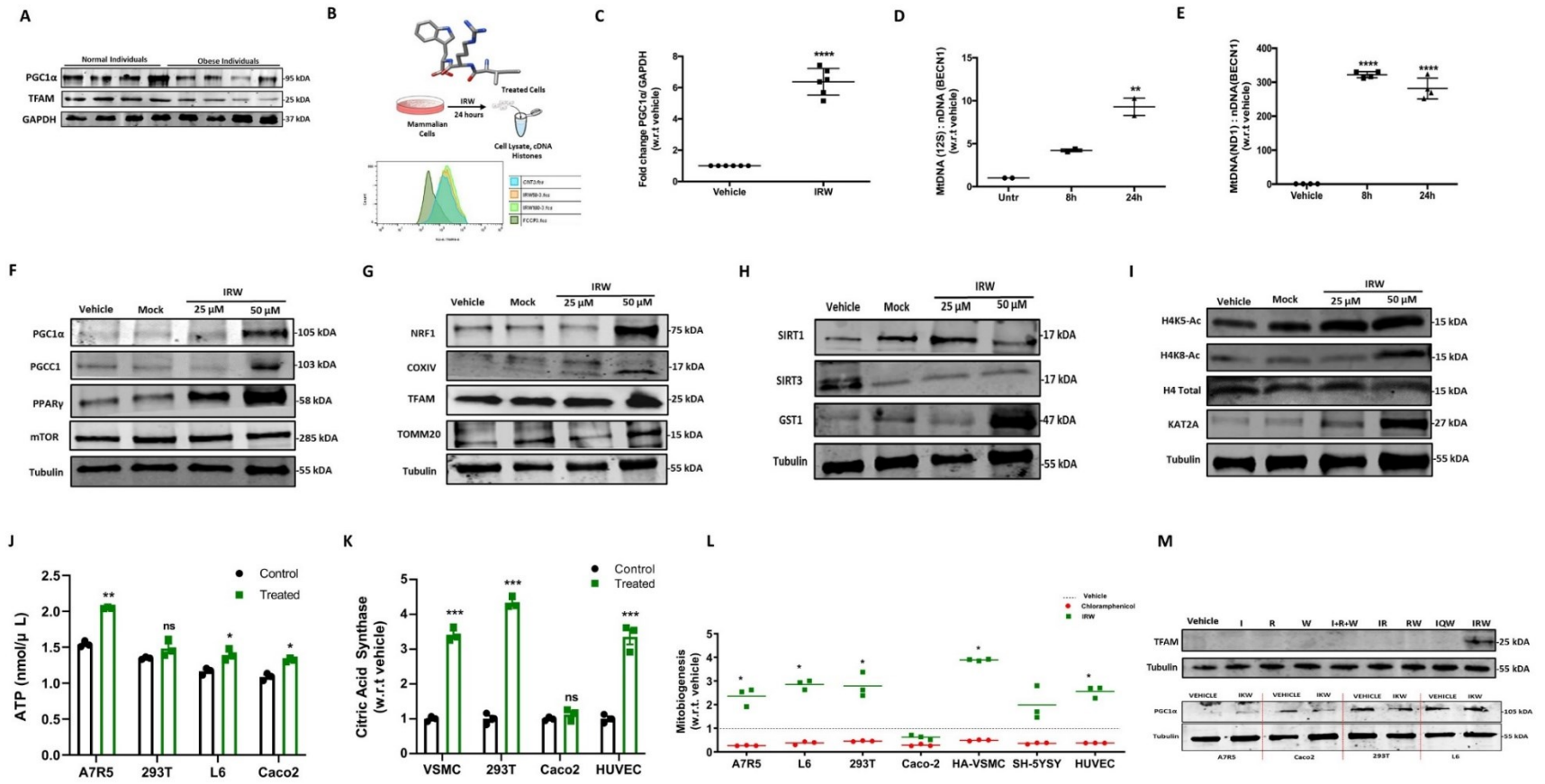


Figure 4.1 Tripeptide IRW increases markers of mitochondrial biogenesis in mammalian cells. (A) loss of PGC1α in obese individuals. IRW and its impact on (B) mitochondrial potential (C) mRNA levels of PGC1α in A7R5 cells (D-E) mitochondrial DNA content in A7R5 cells (F-I) protein expression of key markers of mitochondrial biogenesis (J) ATP and (K) citric acid synthase levels in different mammalian cell lines (L) overall mitobiogenesis as indicated by the increase in mitochondrial DNA in different cell lines (M) impact of constituent amino acids and peptides (IQW and IKW) on markers of mitochondrial biogenesis. Cells were treated with vehicle, mock (GGG, IQW, IKW), IRW (25 or 50 μM), constituent amino acids or dipeptides (50 μM) for 8 or 24 h, and protein or RNA was extracted using RIPA buffer or Trizol reagent. The experiments were performed in a minimum of triplicates and significant differences were determined by t-test or one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001.

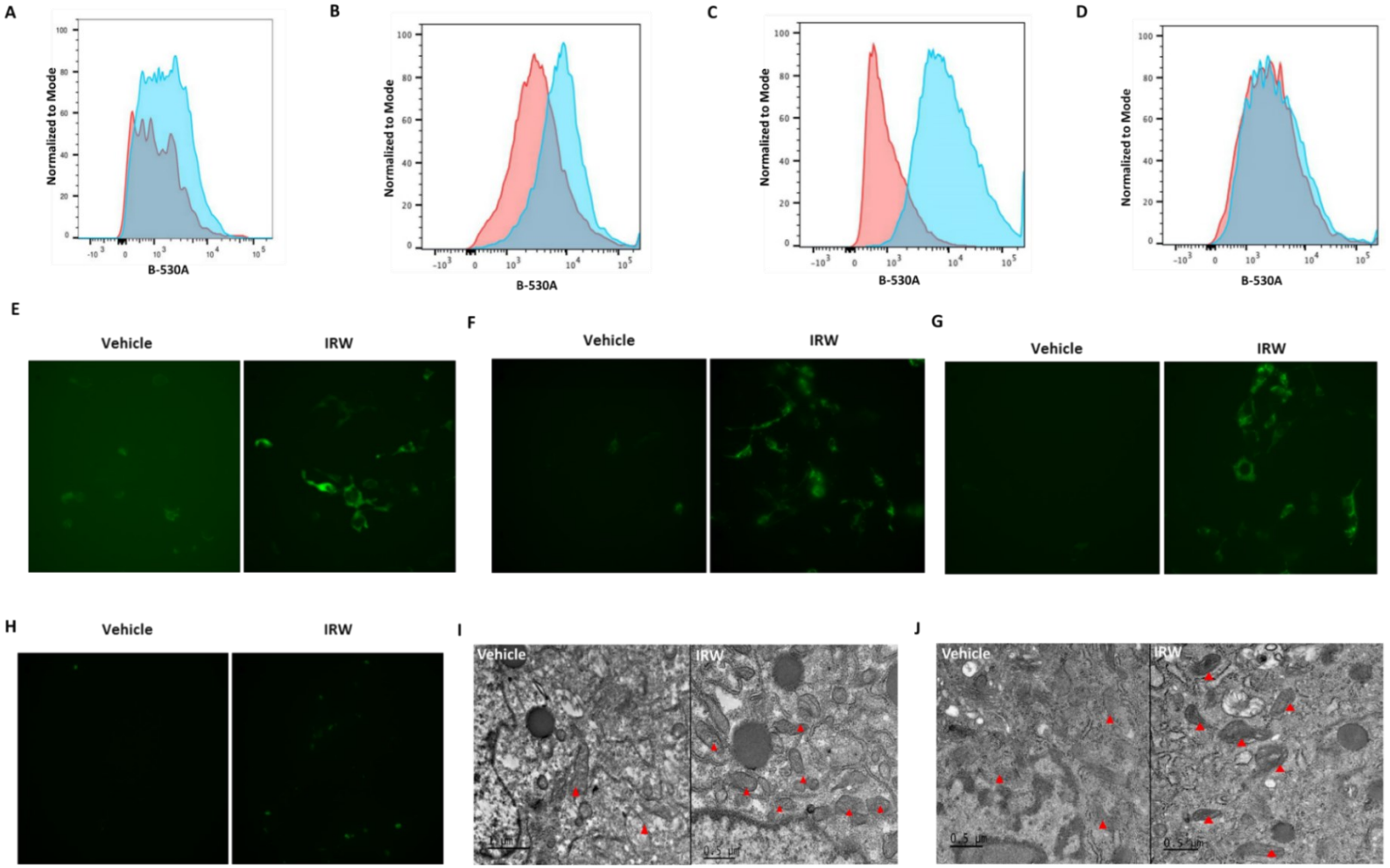


Figure 4.2 Tripeptide IRW increases mitochondrial mass and number in mammalian cells. Impact of IRW on (A-D) total number of mitochondria as measured by flowcytometry using MitoTracker Green in A7R5, 293T cells, L6, and Caco2 cells respectively (E-H) increased density (total number of mitochondria) as measured by fluorescent microscopy using MitoTracker Green in A7R5, 293T cells, L6, and Caco2 cells, respectively. (I-J) The electron micrographs indicating changes in mitochondrial number in A7R5 and 293T cells, respectively. The red triangles point towards the mitochondria. All cells in these experiments were treated with vehicle or IRW (50 µM) for 24 hours, imaged according to the protocols described in the methodology. The experiments were performed at least six times.

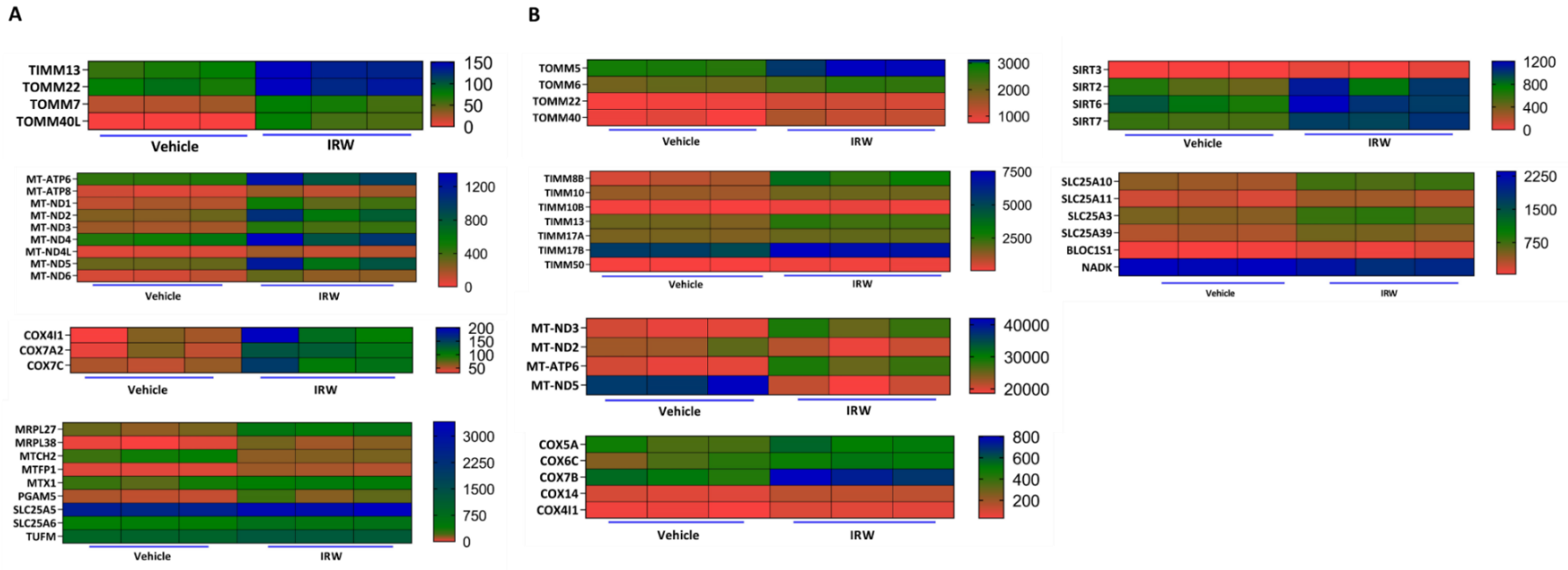


Figure 4.3 Heatmaps showing the effect of IRW on gene expression of various mitochondrial factors in (A) A7R5 cells and (B) 293T cells. The colored legends represent transcripts per million, red indicate low gene expression while green and blue indicate high gene expression. Cells were pre-treated with vehicle (ddH_2O), and 50 µM of IRW for 24 h and RNA was extracted using TRIzol Reagent. The sequencing was conducted as described in the methods.

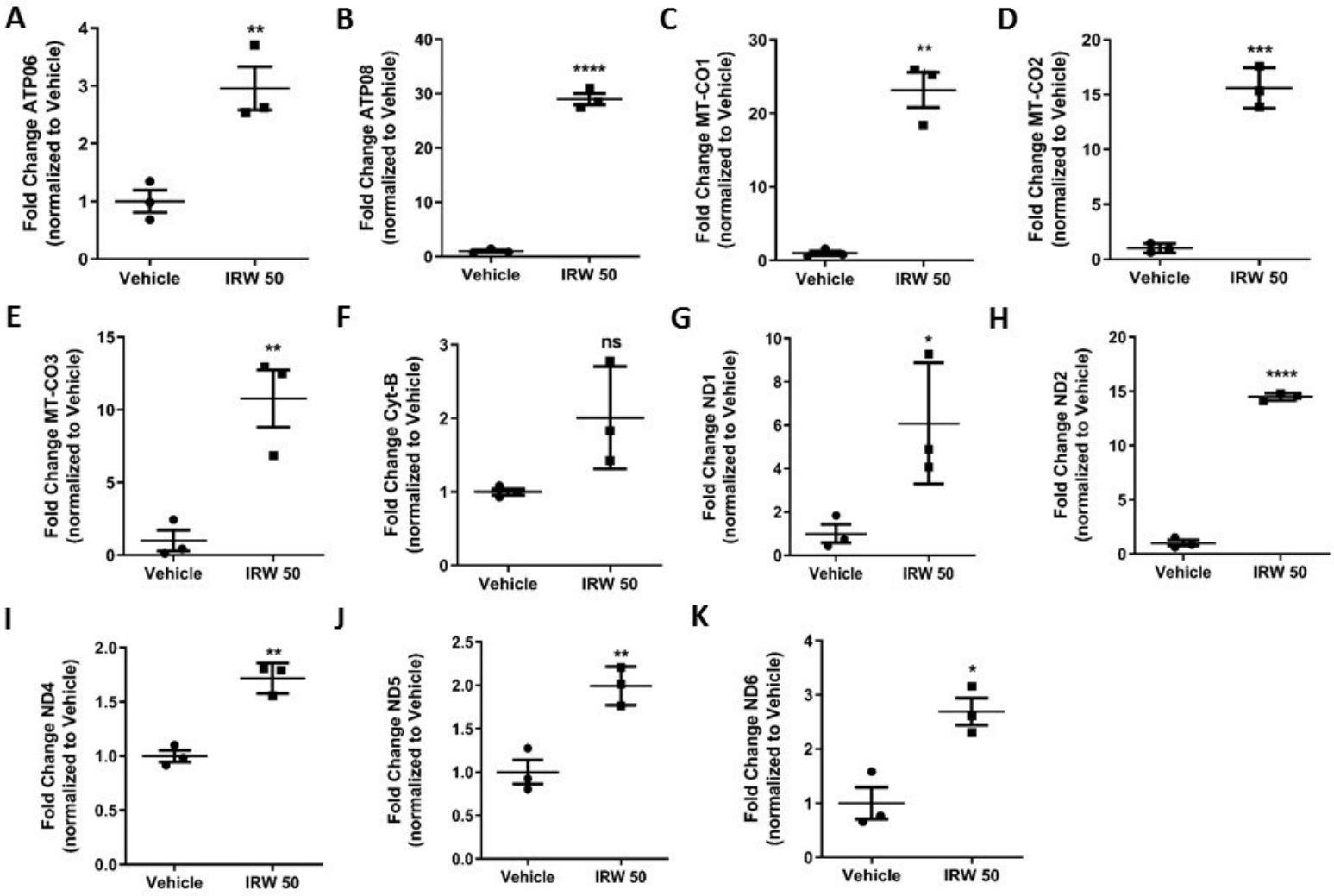


Figure 4.4 Validation of RNA-seq findings using RT-PCR indicating increase in mRNA levels of mitochondrial biogenesis in aorta smooth muscle (A7r5) cells. (A) MT-ATP06 (B) MT-ATP08 (C) MT-CO1 (D) MT-CO2 (E) MT-Cyt-B (F) MT-CO3 (G) MT-ND1 (H) MT-ND2 (I) MT-ND4 (J) MT-ND5 (K) MT-ND6. Cells were pre-treated with vehicle (ddH_2O) or 50 μM of IRW for 24 h and qPCR was performed as described in methodology in triplicates. Significant differences were determined by a t-test. *$p<0.05$, **$p<0.01$, ****$p<0.0001$.

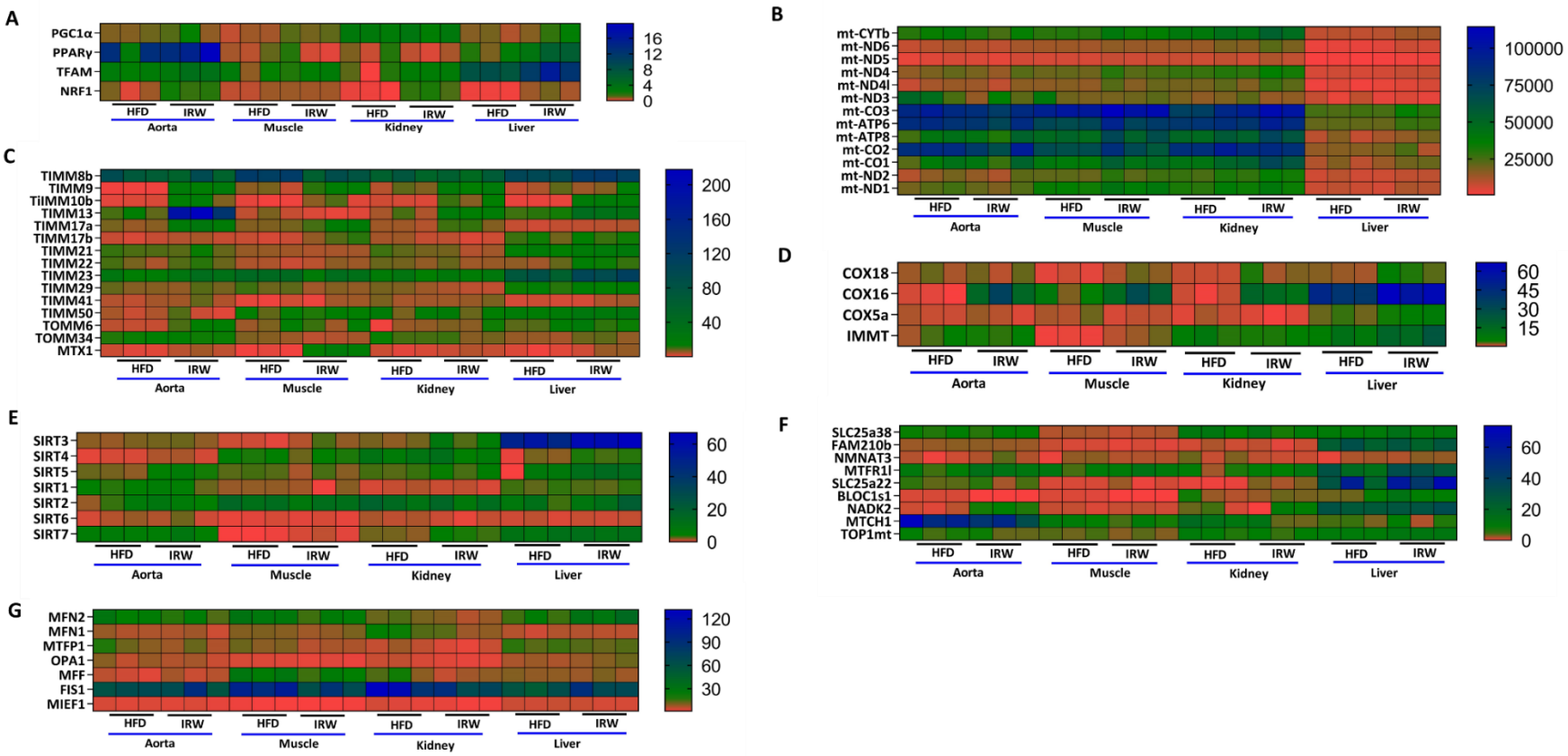


Figure 4.5 Effect of IRW on various mitochondrial factors in Aorta, Kidney, Muscle, and Liver tissues of HFD-fed C57BL/6J mice. Impact of IRW supplementation on (A) Regulators of mitochondrial biogenesis (B) Mitochondrial DNA (C) Mitochondrial protein import (D) Mitochondrial subunits (E) Sirtuins (F) Mitochondrial metabolism (G) Mitochondrial fusion and fission Mitochondrial biomarkers in tissues of C57BL/6J mice. The colored legends represent transcripts per million. C57BL/6J mice were fed a high-fat diet to develop obesity, mild hyperglycemia, and impaired glucose tolerance. The C57BL/6J mice were divided into three groups (1) LFD group: receiving regular diet (10.4% fat) for 14 weeks 2) HFD group: receiving HFD (40% fat) for 14 weeks 3) Treatment group (IRW): receiving HFD (40% fat) for 14 weeks with incorporation of IRW (45mg/kg BW) during the last 8 weeks.

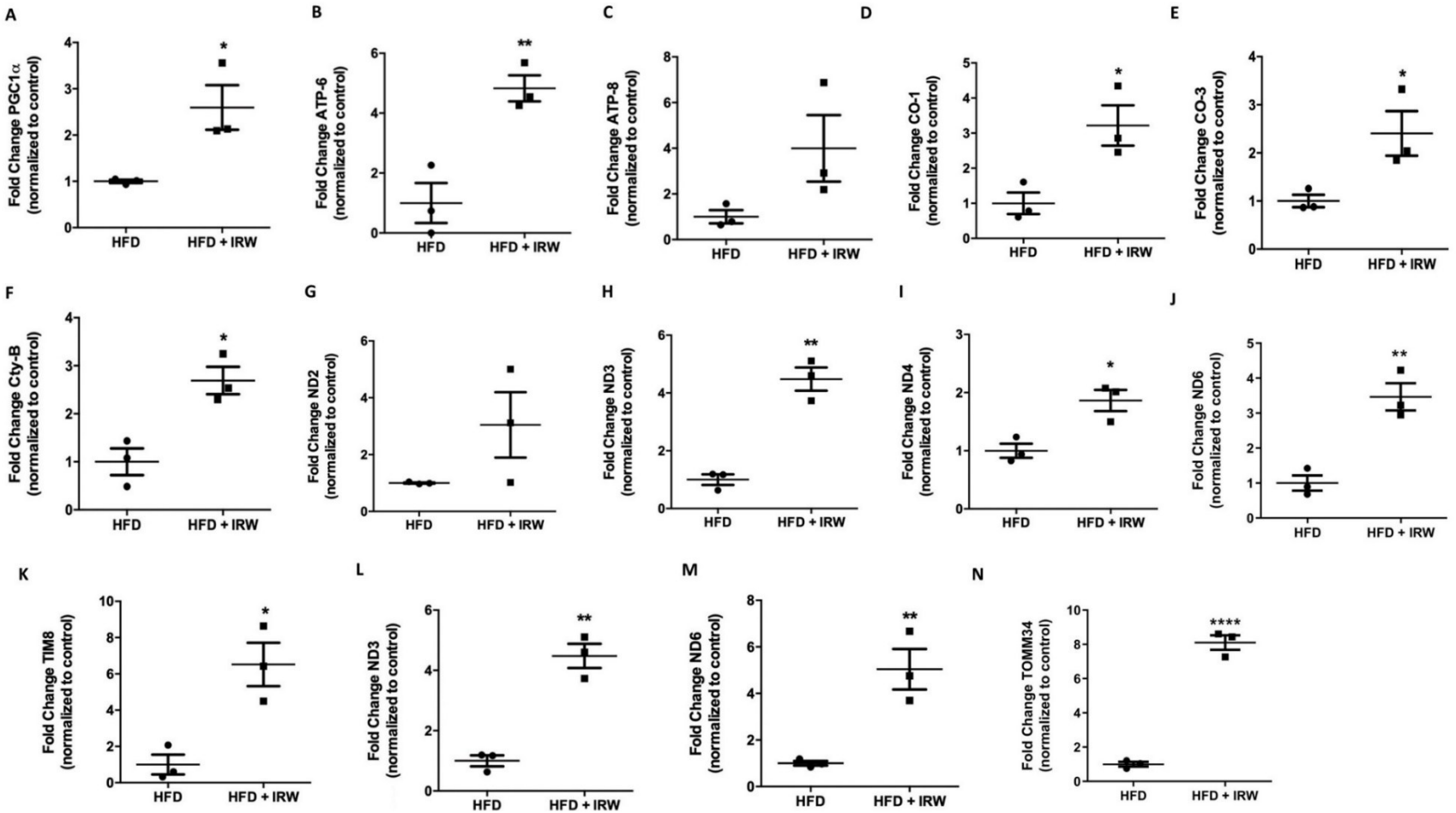


Figure 4.6 Validation of RNA-seq findings using RT-PCR indicating increase in mRNA levels of genes related to mitochondrial biogenesis in Kidney, Muscle, and Aorta tissues of HFD-fed C57BL/6J mice (A) PGC1α -Kidney (B) ATP6-Kidney (C) ATP8-Kidney (D) CO1-Kidney (E) CO3-Kidney (F) CtyB-Kidney (G) ND2-Kidney (H) ND3-Kidney (I) ND4-Kidney (J) ND6-Kidney (K) TIM8-Kidney (L) ND3-Muscle (M) ND6-Muscle (N) TOM34-Aorta. C57BL/6J mice fed a high-fat diet to develop obesity, mild hyperglycemia, and impaired glucose tolerance. The C57BL/6J mice were divided into three groups (1) LFD group: receiving regular diet (10.4% fat) for 14 weeks 2) HFD group: receiving HFD (40% fat) for 14 weeks 3) Treatment group (IRW): receiving HFD (40% fat) for 14 weeks with incorporation of IRW (45mg/kg BW) during the last 8 weeks. qPCR was performed as described in methodology Significant differences were determined by a one-way ANOVA. *p<0.05, **p<0.01, ****p<0.0001.

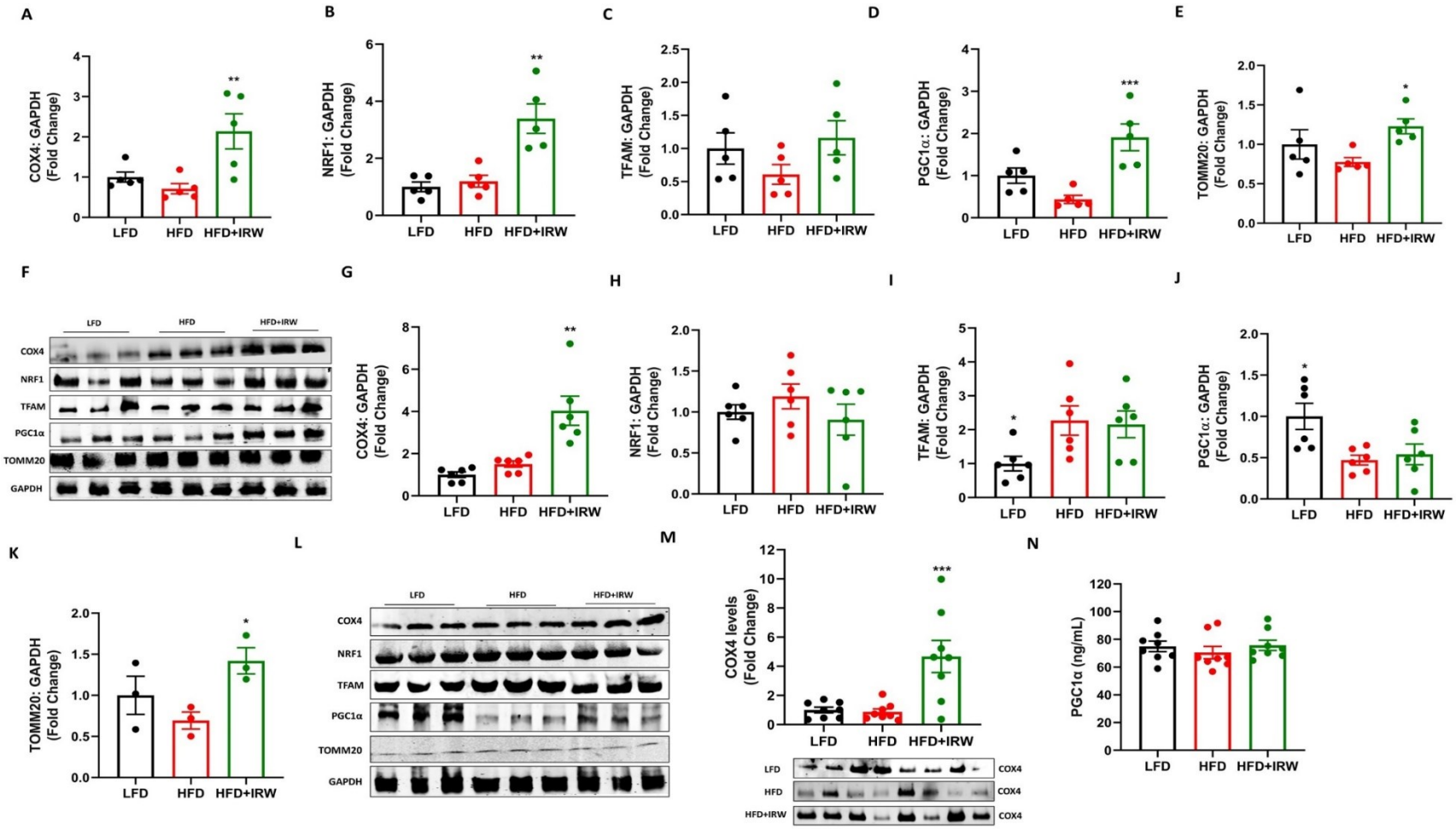


Figure 4.7 Tripeptide IRW increases markers of mitochondrial biogenesis in kidney, muscle, and plasma of HFD-fed mice. Impact of IRW supplementation on (A) COX4-Kidney (B) NRF1-Kidney (C) TFAM-Kidney (D) PGC1α-Kidney (E) TOMM20-Kidney (F) representative blots (G) COX4-Muscle (H) NRF1-Muscle (I) TFAM-Muscle (J) PGC1α-Muscle (K) TOMM20-Muscle (L) representative blots (M) COX4-Plasma (N) PGC1α-Plasma. C57BL/6J mice fed a high-fat diet to develop obesity, mild hyperglycemia, and impaired glucose tolerance. The C57BL/6J mice were divided into three groups (1) LFD group: receiving regular diet (10.4% fat)

for 14 weeks 2) HFD group: receiving HFD (40% fat) for 14 weeks 3) Treatment group (IRW): receiving HFD (40% fat) for 14 weeks with incorporation of IRW (45mg/kg BW) during the last 8 weeks. Significant differences were determined by a one-way ANOVA. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

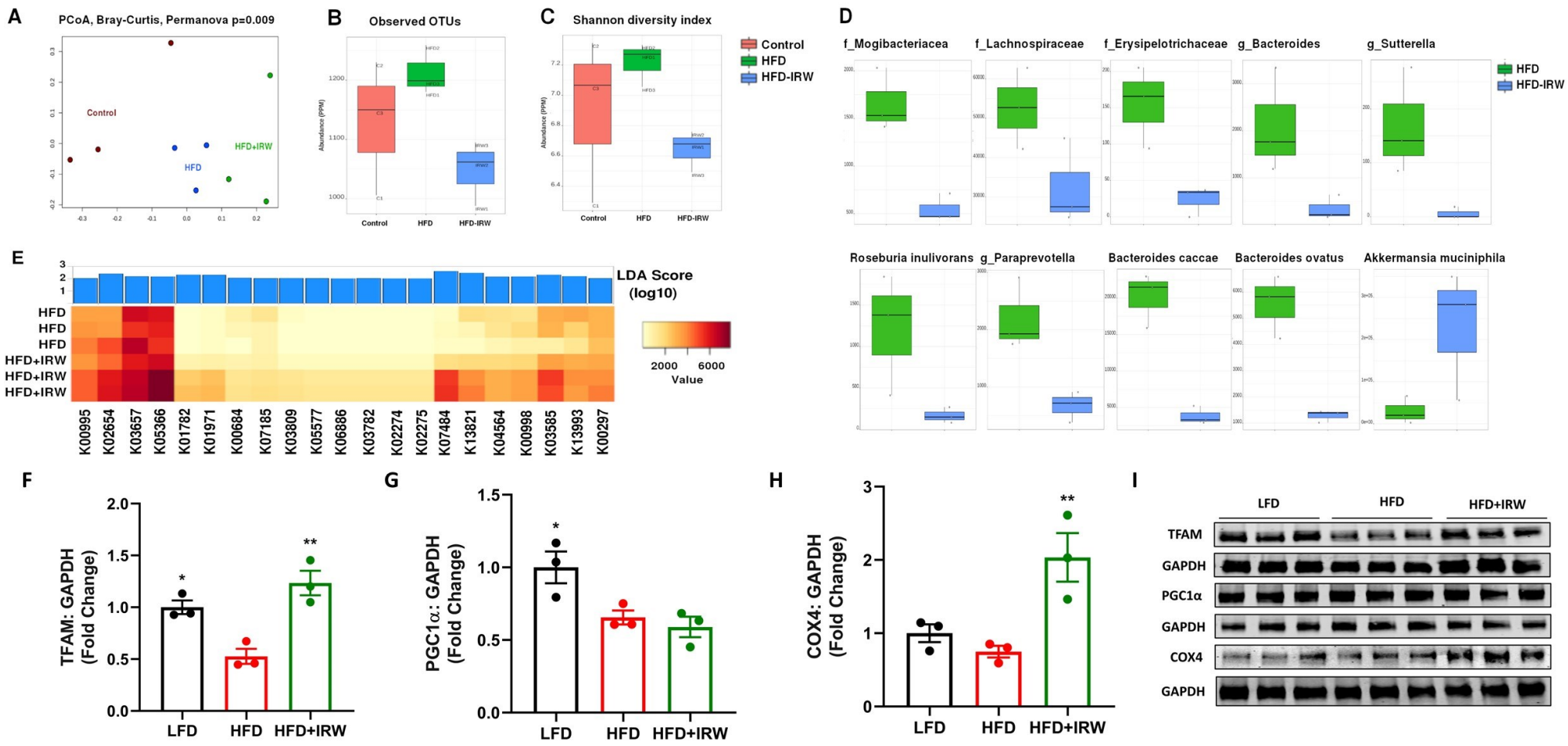


Figure 4.8 Tripeptide IRW induces changes in microbiota and intestinal markers of mitochondrial biogenesis in HFD-fed mice. (A) Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity shows strong changes in sequence composition indicating total microbiota shift across the treatment groups (B) OTUs (operational taxonomic units) and (C) the Shannon diversity index also indicated changes in the total microbiome following IRW treatment (D) Modulation of key families, genus and species of microbiota

associated with metabolic and mitochondrial function (E) Changes in metabolic profile of microbiota (F) Intestinal TFAM (G) Intestinal PGC1α (H) Intestinal COX4 of (I) C57BL/6J mice fed a high-fat diet to develop obesity, mild hyperglycemia and impaired glucose tolerance. The C57BL/6J mice were divided into three groups (1) LFD group: receiving regular diet (10.4% fat) for 14 weeks 2) HFD group: receiving HFD (40% fat) for 14 weeks 3) Treatment group (IRW): receiving HFD (40% fat) for 14 weeks with incorporation of IRW (45mg/kg BW) during the last 8 weeks. K00995: CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase, K02654: leader peptidase (prepilin peptidase)/N-methyltransferase, K03657: ATP-dependent DNA helicase PcrA, K05366: penicillin-binding protein 1A, K01782: 3-hydroxybutyryl-CoA epimerase, K01971: DNA ligase (ATP), K00684: leucyl/phenylalanyl-tRNA--protein transferase, K07185: tryptophan-rich sensory protein, K03809: Trp repressor binding protein, K05577: NAD(P)H-quinone oxidoreductase subunit 5, K06886: hemoglobin,K03782:catalase/peroxidase, K02274: cytochrome c oxidase subunit I, K02275: cytochrome c oxidase subunit II, K07484: transposase, K13821: proline dehydrogenase, K04564: superoxide dismutase, Fe-Mn family, K00998: phosphatidylserine synthase, K03585: membrane fusion protein, K13993: HSP20 family protein, K00297: methylenetetrahydrofolate reductase (NADPH). Significant differences were determined by a one-way ANOVA. *$p<0.05$, **$p<0.001$.

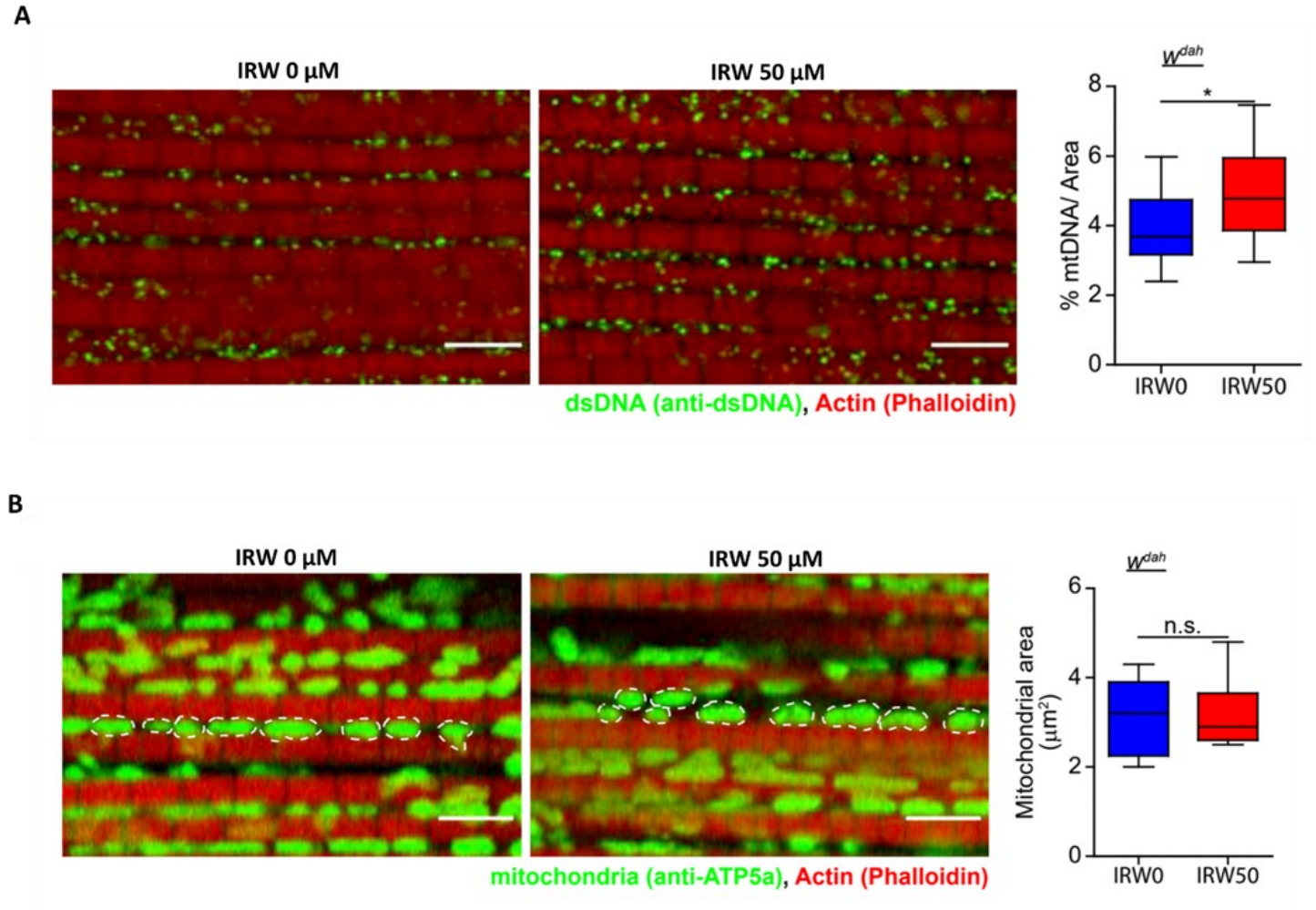


Figure 4.9 Impact of IRW supplementation on (A) mitochondrial DNA content and (B) mitochondrial size. Briefly, W^{dah} flies were dissected at day 35 after one week of IRW feeding (50 µM). Dissected flies were incubated with mouse-anti-dsDNA 1;250 (for mtDNA) and mouse- anti-atp5a 1:250 (for mitochondrial area). Images were taken using Zeiss LSM880 confocal microscope and analyzed using the ImageJ software to measure mitochondrial DNA and mitochondrial size.

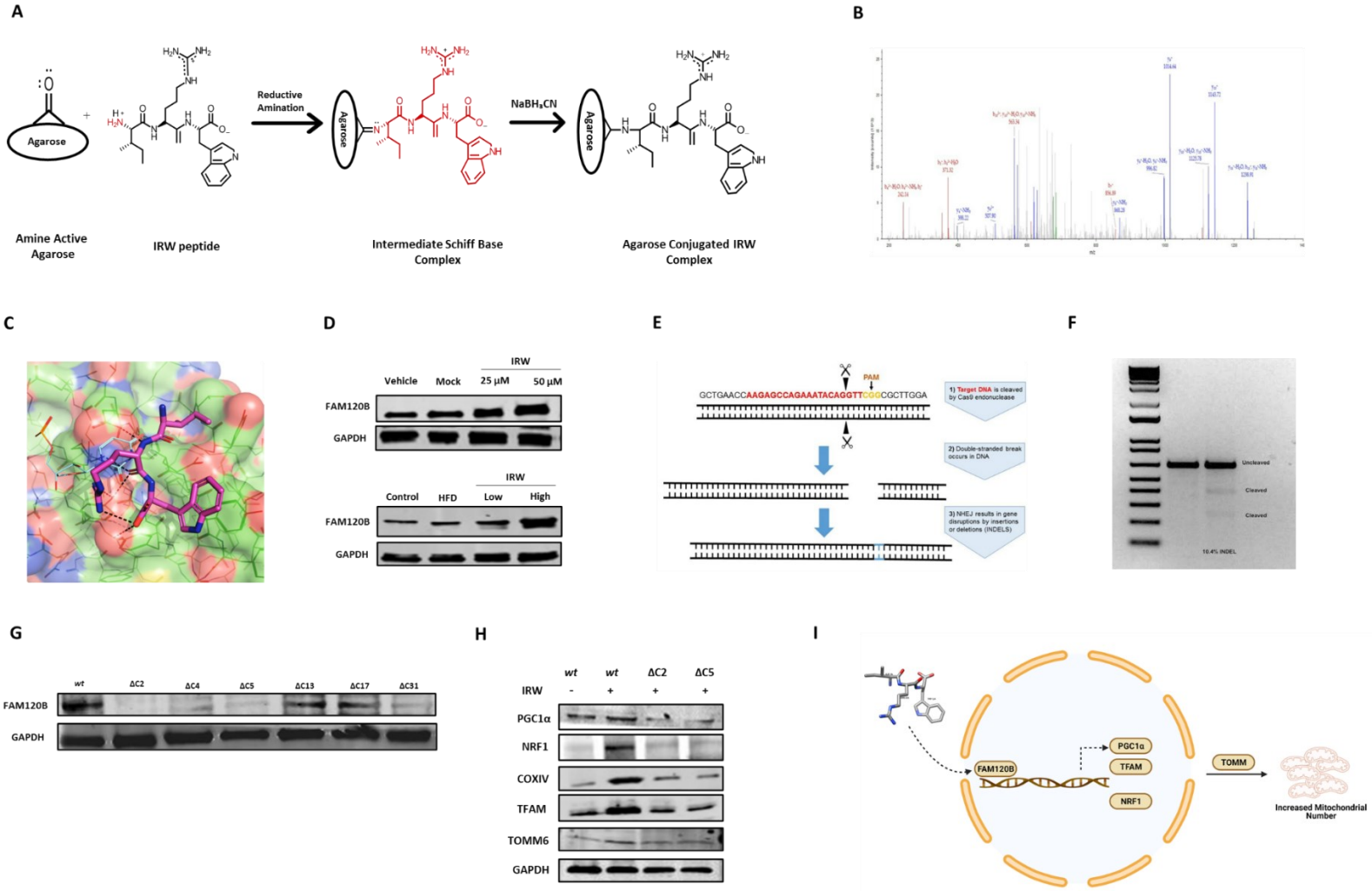
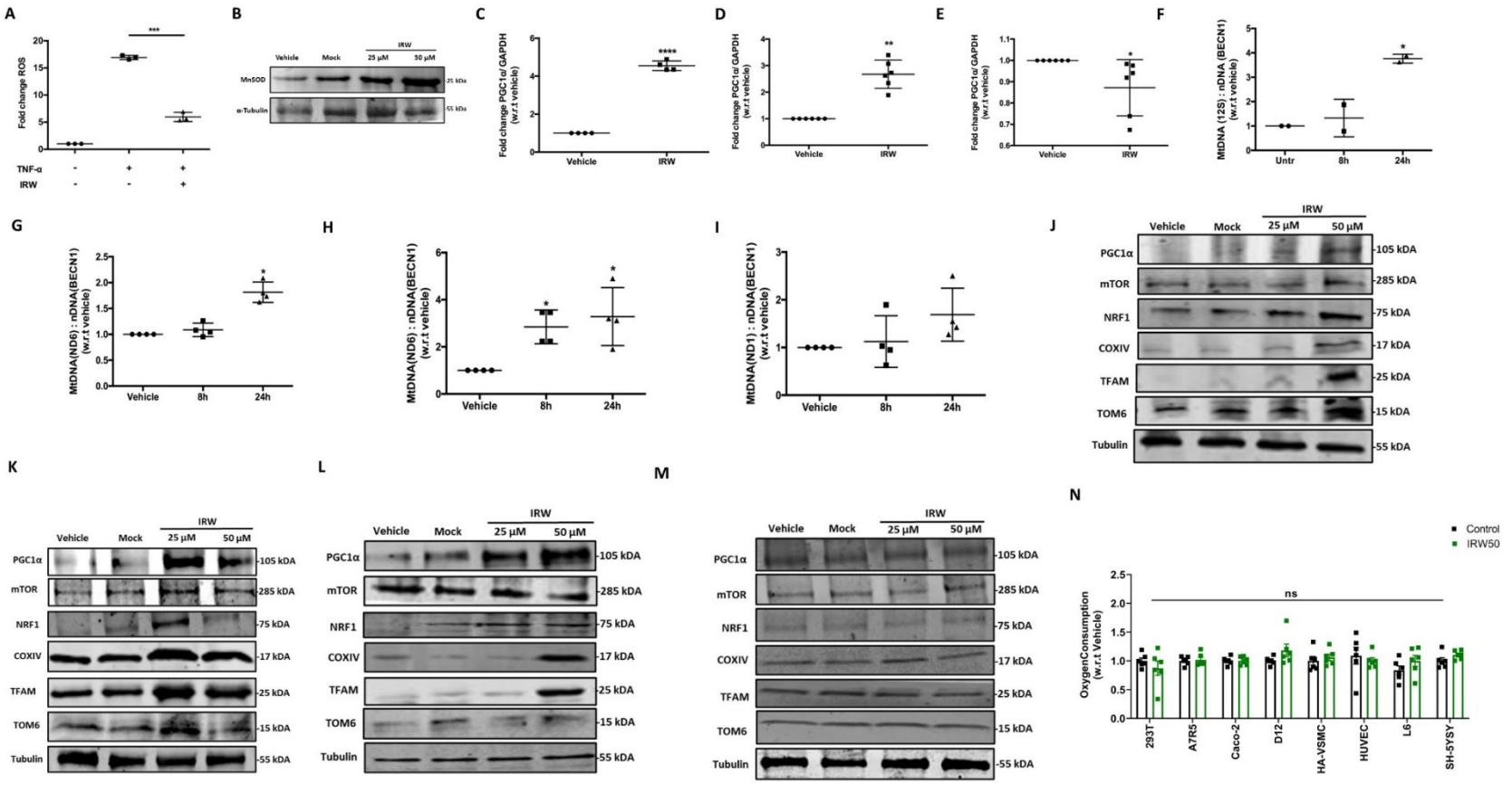
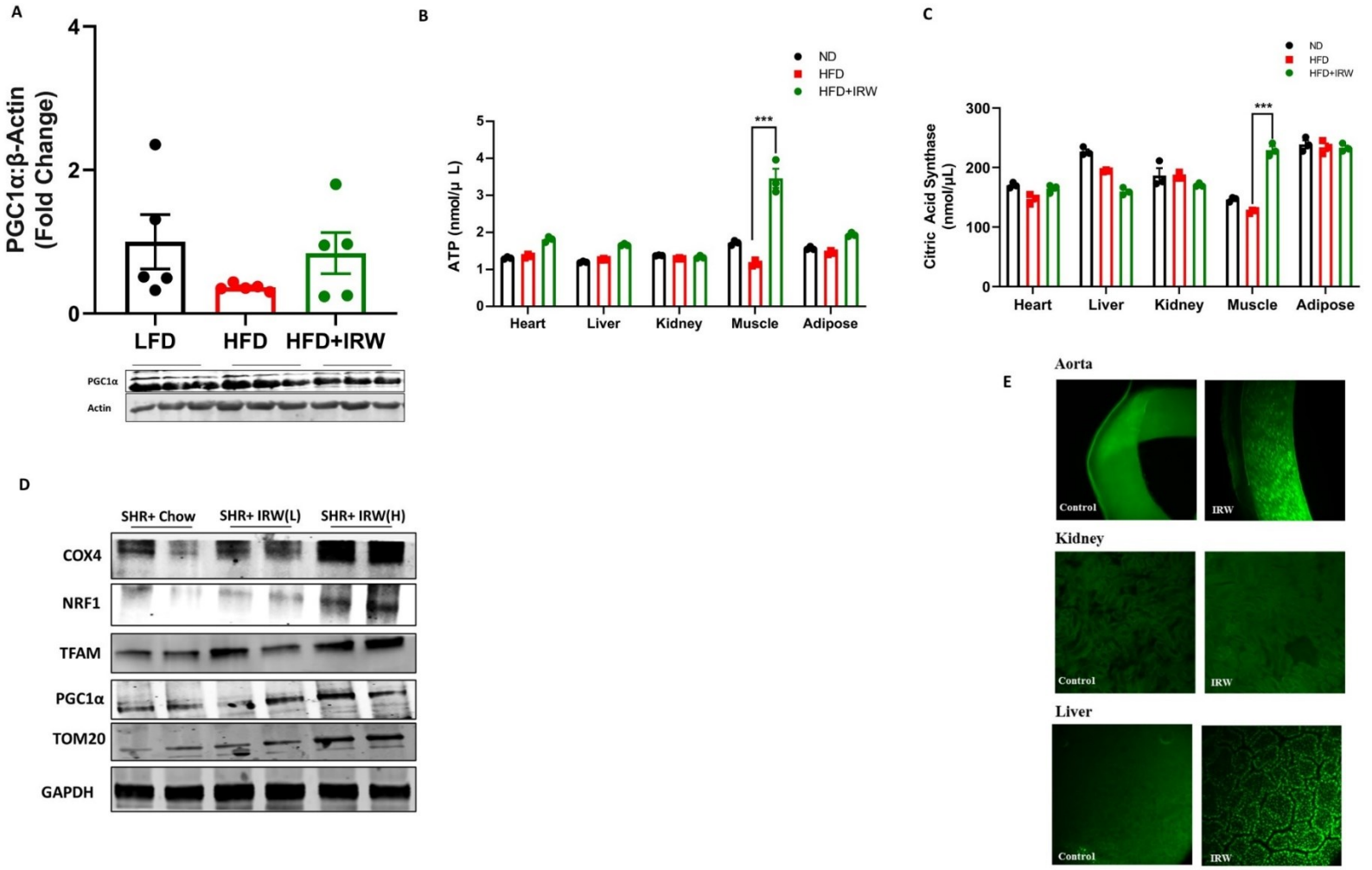


Figure 4.10 Identification of target for IRW's pharmacological action. (A) Chemical conjugation of peptide IRW with amine active agarose beads for target identification (B) MS spectra showing the binding site FAM120B (C) Docking study showing possible binding

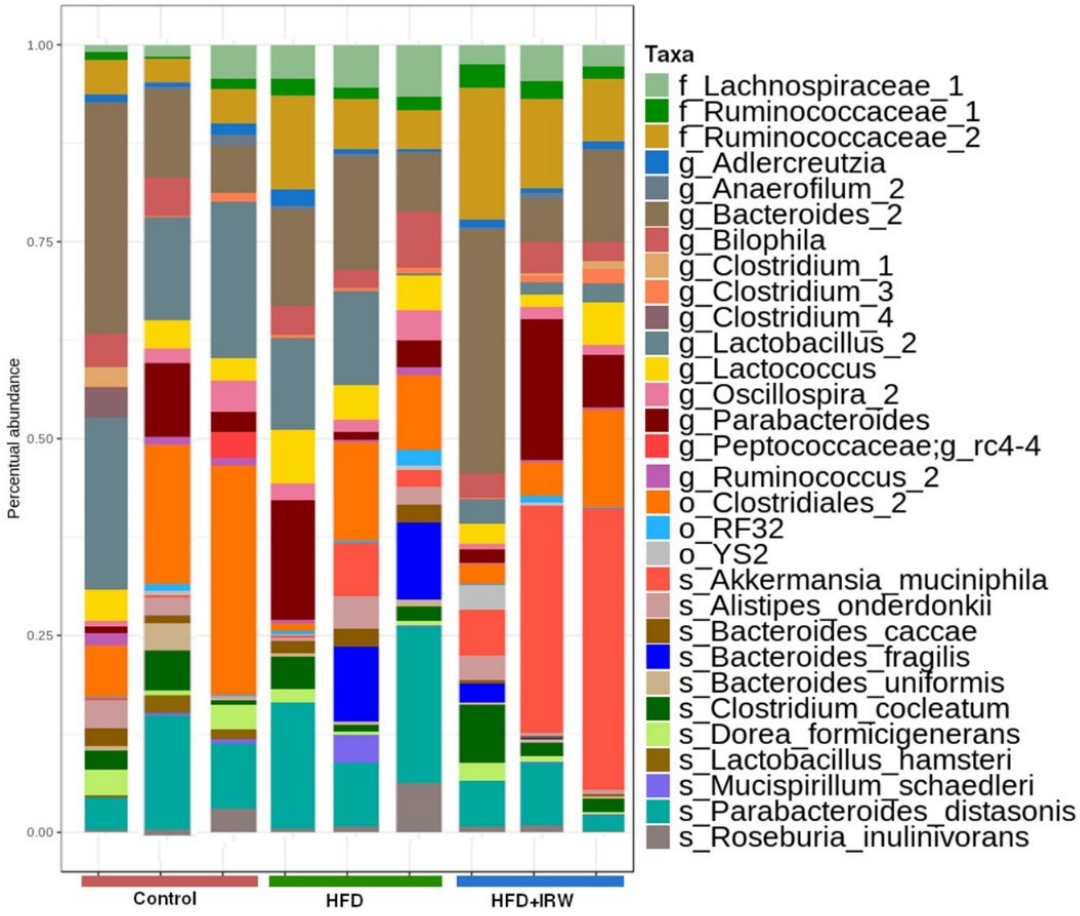
mode of IRW with FAM120B (D) A representative western blot data showing changes in FAM120B expression in A7R5 cells following treatment with vehicle (ddH2O), mock (GGG, 50 μM) and 25 μM or 50 μM of IRW for 24h and changes in FAM120B expression in Aorta following IRW administration (3 and 15mg/kg BW; low and high dose) in C57BL/6J mice (n=8) fed on high fat diet (HFD) for 14 weeks (E) Cleavage of the target DNA results in a double stranded break. This is repaired by error-prone NHEJ (non-homologous end joining) which results in gene disruption (F) T7E1 Assay Results showing Indel percentage of FACS (fluorescence-activated cell sorted) HEK293T CRISPR/Cas9 genetically engineered cells. Mismatched PCR product is cleaved by T7E1 (G) Confirmation using Western blots and (H) the effect of IRW on biomarkers of mitochondrial biogenesis was diminished in FAM120B KO 293T cells and (I) summarized.



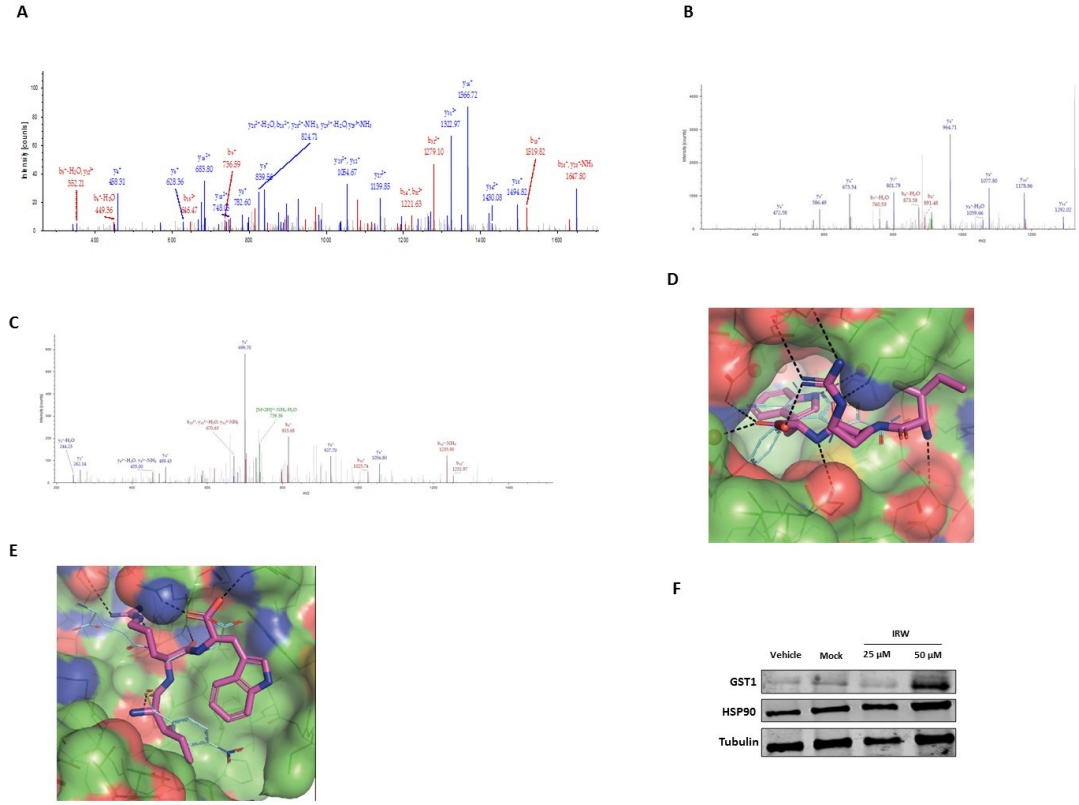
Supplementary Figure 4.1 Tripeptide IRW increases markers of antioxidant activity and mitochondrial biogenesis in mammalian cells. Impact of IRW treatment on (A) TNFα induced ROS (B) mitochondrial SOD2 (C-E) mRNA levels of PGC1α in 293T cells, L6, and Caco2 cells (G-I) relative mitochondrial DNA in 293T cells, L6, and Caco2 cells (J-M) protein expression of key markers of mitochondrial biogenesis in 293T cells, L6, SH-SY5Y, and Caco2 cells (N) oxygen consumption levels in different mammalian cell lines. Cells were treated with vehicle, mock (IQW) or IRW (25 or 50 μM) for 8 or 24 hours, and protein or RNA was extracted using RIPA buffer or Trizol reagent. The experiments were performed in a minimum of triplicates and significant differences were determined by t-test or one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001.



Supplementary Figure 4.2 Impact of IRW supplementation on PGC1α pathway. IRW supplementation increases (A) PGC1α levels in adipose tissues of C57BL/6J mice fed a high-fat diet (B-C) ATP and citric acid synthase levels n various tissues of C57BL/6J mice fed a high-fat diet (D) Markers of mitochondrial biogenesis in aorta tissue of hypertensive rats and (D) PGC1α levels in Aorta, kidney, and liver tissues of hypertensive rats. Significant differences were determined by a one-way ANOVA. ***p<0.001



Supplementary Figure 4.3 The detailed list of modulated groups of microbiota by IRW in C57BL/6J mice fed a high-fat diet to develop obesity, mild hyperglycemia, and impaired glucose tolerance. The C57BL/6J mice were divided into three groups (1) LFD group: receiving regular diet (10.4% fat) for 14 weeks 2) HFD group: receiving HFD (40% fat) for 14 weeks 3) Treatment group (IRW): receiving HFD (40% fat) for 14 weeks with incorporation of IRW (45mg/kg BW) during the last 8 weeks.



Supplementary Figure 4.4 Identification of secondary targets for IRW binding **A**. Complete spectrum showing binding of IRW to its cellular targets **B**. glutathione S-transferase-1 (GST-1) **C**. heat shock protein 90 (HSP90) **D.** Model of IRW (magenta) peptide bound to the GST1 (green) active based on a co-crystal structure (PDB ID: 1GLQ) of GST-1 **E.** Model of IRW (magenta) peptide bound to the HSP90 (green) active based on a co-crystal structure (PDB ID: 1GLQ) of GST-1 **F**. A representative western blot data showing changes in different examined cellular targets in A7R5 cells. Cells were treated with vehicle (ddH_2O), mock (GGG, 50 µM) and 25 µM or 50 µM of IRW for 24h and protein was extracted using RIPA buffer.

CHAPTER 5 - Tripeptide IRW extends lifespan of *Drosophila melanogaster*

Abstract: Bioactive peptides are being rapidly identified and evaluated for their anti-aging activities. We evaluated the ability of IRW to extend lifespan using the Drosophila model *in vivo*. Different fly lines, such as *w*, *wy*, and W^{dah} were fed regular fly medium supplemented with IRW (at a concentration of 50 or 100 μM dissolved in ddH2O). IRW treatment at 50 and 100 μM concentrations prolonged the median life span of white mutant (*w*) by 5.1 and 12.08% (respectively) and of yellow mutant (*wy*) by 12.1 and 22.9% (respectively). Likewise, midlife IRW feeding in W^{dah} flies improved lifespan significantly as well. Also, IRW treatment at these concentrations significantly improved the histone markers in flies and activated the expression of multiple gene pathways involved in sirtuins (SIRT1), antioxidant defense (SOD2), autophagy (ATG7), and insulin signaling (dInR). Together, our study identifies the first bioactive peptide with the ability to extend lifespan *in vivo* and suggests an important prospective role of IRW intake for healthy aging in humans.

5.1 Introduction

Aging can be defined as the drop in physiological function over time that leads to reduced fertility, declined healthspan, and ensuing death (Liguori et al., 2018; Melzer, Pilling, & Ferrucci, 2020). Since ancient times, humanity has undertaken a quest to uncover an elixir adept at extending lifespan and impeding aging (Bhullar & Hubbard, 2015). For the last several decades, biologists have dedicated numerous efforts to understanding the processes that regulate lifespan. These scientific endeavors advance gained unprecedented scientific credibility as our expanded understanding of molecular genetics brought us closer to realizing this goal (Melzer et al., 2020). The early experiments in 1939 showing the ability of caloric restriction in mice and rats to increase lifespan laid the foundation of experimental gerontology and aging research (McCay, Maynard, Sperling, & Barnes, 1939). Currently, numerous dietary and pharmacological interventions have shown the capacity to extend lifespan and prevent age-related health decline (Bhullar et al., 2015; Melzer et al., 2020). These observations and pharmacological discoveries have opened avenues to slow aging and improve healthspan, the dual concepts which describe together the length of healthy life and the fraction of total lifespan free from disease.

Based on the idea of heritable traits of radically varying lifespans, biologists showed genetic and reproductive basis lifespan determination in *Drosophila* species (Hyde, 1913; Rose & Charlesworth, 1980). This discovery revolutionized aging research and now hundreds of genes have been identified in *Caenorhabditis elegans* (889 genes), *Drosophila* (202 genes), mice (136 genes), and yeast (911 genes) (Available at https://genomics.senescence.info). This indicates an intricate network of coalescing intracellular signaling pathways and higher-order processes (Kapahi et al., 2010). Multiple pathways such as insulin/IGF-1 like signaling (Nässel & Broeck, 2016), mechanistic target of rapamycin (mTOR) (Kapahi, Zid, Harper, Koslover, Sapin, & Benzer,

2004), AMP-activated protein kinase (AMPK) (Ulgherait, Rana, Rera, Graniel, & Walker, 2014) along sirtuins and mitochondrial antioxidants (Melzer et al., 2020). These cellular lifespan mechanisms are conserved across taxa, including mammals. Targeting these conserved aging pathways could, therefore, extend lifespan and improve healthspan. This theory remains to be tested in well-planned clinical trials but is strongly supported by several lines of preclinical evidence (Bhullar et al., 2015; Martin-Montalvo et al., 2013; Morselli et al., 2009; J. G. Wood et al., 2004).

One of the key goals of aging research is the identification of the molecules that can boost healthspan and contribute to active lifespan longevity. In recent times, the promising direction of research in this aspect is the identification of such substances among the natural bioactive and synthetic molecules. Various small molecule(s) such as metformin, rapamycin, resveratrol, spermidine, and withanolides have shown the ability to prolong lifespan in multiple model organisms (Harrison et al., 2009; Koval, Zemskaya, Aliper, Zhavoronkov, & Moskalev, 2021; Martin-Montalvo et al., 2013; Morselli et al., 2009; J. G. Wood et al., 2004). Further, on the cellular level, the increased expression of PNC1 (pyrazinamidase/nicotinamidase 1), the NAD^+ rate-limiting enzyme, leads to lifespan extension in *Saccharomyces cerevisiae* (Anderson, Bitterman, Wood, Medvedik, & Sinclair, 2003). The mammalian functional equivalent of PNC1 called NAMPT is also involved in lifespan extension (Yang, Lavu, & Sinclair, 2006). Interestingly, in *Drosophila*, overexpression of the NAMPT homolog D-NAAM extends the mean lifespan by 30% (Balan et al., 2008). Further, administration of NAD^+ or its precursor(s) and NAD^+ activators also extend lifespan in different organisms (Bhullar et al., 2015; Fang et al., 2016; Hashimoto, Horikawa, Nomura, & Sakamoto, 2010; Lee, Caudal, Abell, Gowda, & Tian, 2019). In our recent findings, we showed that IRW (Ile-Arg-Trp), a small tripeptide derived from ovotransferrin,

elevated NAMPT, D-NAAM (fly NAMPT), and NAD^+ *in vivo* (Bhullar et al., 2021). Therefore, we hypothesize that IRW, owing to its ability to boost NAMPT/NAD^+ axis can extend the *Drosophila* lifespan. *Drosophila* as a short-lived invertebrate is an extremely effective tool for studying aspects of ageing and lifespan extension. The use of *Drosophila* as a model lifespan organism since 1913 has lead to identification of various anti-aging molecules (Hyde, 1913; Piper & Partridge, 2018). Further, owing to its high genetic similarity (~77%) with human aging genes and the ease of generating large populations, we selected *Drosophila* lines to study the impact of IRW on lifespan extension. Therefore, the overall goal of our study was to determine whether IRW feeding resulted in positive impacts on the *Drosophila* lifespan. We found that IRW feeding improves the whole lifespan and mid-lifespan extends lifespan in *Drosophila* species. Feeding of IRW activated multiple lifespan-related pathways and extended *Drosophila* lifespan. To the best of our knowledge, this study is the first evidence showing the ability of a bioactive peptide to extend lifespan *in vivo*.

5.2 Materials and methods

5.2.1 Flies

For the first set of experiments, *Drosophila melanogaster* of the white (*w*) and yellow stock (*wy*), obtained and were cultured for two generations on normal strength 1.0 sucrose-yeast (SY) medium {(agar (20 g/L), sucrose (100 g/L), and yeast (100 g/L)c as described previously (Magwere, Chapman, & Partridge, 2004). Nipagin (33 g/L) and propionic acid (3 ml/L) were added as antifungal and antibacterial agents. Following the hatching of flies, the sexes were then sorted into female and male groups using CO_2 anesthesia. Thirsty flies were allocated to each vial with fly

food, and the total number of flies in each treatment group was 150. Flies were maintained throughout the experiment at 25°C and 60% humidity on a 12:12 hour light: dark cycle in controlled-temperature incubators and were taken out briefly once every 3 days for the transfer to fresh food and the scoring of deaths. For assay control of caloric restriction, the fly food was modified as {(agar (15 g/L), sucrose (60 g/L), and yeast (60 g/L)}. Next, W^{dah} flies were reared as described previously (Aparicio, Rana, & Walker, 2019). Briefly, flies were reared in vials containing cornmeal medium (1% agar, 3% yeast, 1.9% sucrose, 3.8% dextrose, 9.1% cornmeal, 1.1% acid mix, and 1.5% methylparaben, all concentrations given in wt/vol). Flies were collected under light nitrogen-induced anesthesia and housed at a density of 30 female flies per vial. All flies were kept in a humidified, temperature-controlled incubator with 12 h on/off light cycle at 25°C. RU486 was dissolved in ethanol and administered in the media while preparing food. Flies were flipped to fresh vial every 2-3 days and scored for death.

5.2.2 Life span assay

Tripeptide IRW was fed to *w*, *wy*, and W^{dah} at different concentrations as shown in Figure 5.1 and 5.2. For the midlife lifespan analysis, W^{dah} flies were fed the tripeptide IRW (50 μM) for 1 week (from day 28 to day 35).

5.2.3 Histone extraction and Western blot

Following completion of experiments, fly histones were extracted using histone extraction kit (ab113476) obtained from Abcam Inc. (Abcam Inc, Toronto, Canada). After extraction, the pH of histones was neutralized using NaoH (1M) solution. The western blot was conducted as described in our recent report (Bhullar et al., 2021). The antibodies Acetyl-Histone H3 (Lys56) mouse mAb (PTM-162), Acetyl-Histone H4 (Lys8) mouse mAb (PTM-164), Tri-methyl-Histone H3 (Lys36)

rabbit pAb (PTM-625), Di-methyl-Histone H4 (Lys20) rabbit pAb (PTM-635), Histone H3 rabbit Ab (PTM-1002), Anti-histone H4 rabbit pAb (PTM-1004) were obtained from PTM Bio LLC (Chicago, IL, USA).

5.2.4 RNA extraction and qPCR

Total RNA was isolated from *Drosophila* with TRIzol reagent. RNA was quantified using a Nano Drop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and its integrity assessed by running it on an agarose gel. The cDNA was synthesized from 1 µg of total RNA using a reverse transcriptase (RT) system kit (Thermo Scientific, Mississauga, ON, Canada). All of the qPCR experiments and analyses were conducted using the MIQE guidelines for qPCR (Bustin et al., 2009).

5.2.5 Statistical Analysis

A completely randomized design (CRD) was used to perform all the experiments conducted in this study where the treatments are assigned completely at random to groups of flies so that each experimental unit has the same chance of receiving any the treatment option(s). Data expressed as mean ± SEM of n=150 flies per group in triplicates. Data analysis was conducted by one-way ANOVA followed by Bonferroni's post-hoc test. * $p<0.05$ and ** $p<0.01$ versus vehicle.

5.3 Results

5.3.1 IRW increases life span of *Drosophila melanogaster* mutant white (*w*) and yellow (*yw*) flies

The effect of IRW at concentrations of 50 and 100 µM on the life span of female *w, wy,* and W^{dah} *D. melanogaster* was studied. IRW at 50 and 100 µM concentrations significantly

increased the median life span in *w* flies by 5.1% ($p<0.01$) and 12.08% ($p<0.01$) respectively (Figure 5.1A). Likewise, there was a significant increase in the lifespan of *wy* flies following IRW feeding at 50 μM (12%; $p<0.01$) and 100 μM (23%; $p<0.001$) concentrations (Figure 5.1B). While we also observed a significant increase in lifespan of *wy* flies compared to *w* flies at 100 μM concentration (Figure 5.1B). Next, we studied if these benefits of IRW extended to W^{dah} flies. The 50 and 100 μM of IRW treatment had no significant impact on studied life span parameters in W^{dah} flies. We also then tested the lower concentrations of IRW (1, 10, and 25 μM), which exhibited no effect on lifespan parameters in W^{dah} flies (Figure 5.1C). Contrary to our expectations, the lower concentration of IRW decreased the median survival in W^{dah} flies, an observation similar to our pilot studies in *w* and *wy* flies as well. However, the trend from whole life feeding of IRW showed that there was modulation in mid-life survival of W^{dah} flies following IRW treatment (Figure 5.1C). Therefore, we next explored the impact of IRW on mid-life survival in W^{dah} flies.

5.3.2 IRW increases midlife life span of *Drosophila melanogaster* mutant W^{dah} flies

The effect of IRW at 10, 25, 50, 100, and 200 μM concentrations on the lifespan of after feeding starting day 28 onwards till the end of the lifespan was observed (Figure 5.2A). The results showed that 50 μM IRW significantly increased the survival of W^{dah} flies by 6.03% ($p<0.05$) following mid-life feeding (Figure 5.2A). Next, based on these findings, we further explored the lifespan extension by feeding 50 μM IRW for 2 weeks during their midlife lifespan (Day 28-Day 42). Results showed that 50 μM IRW feeding strongly increased the survival of flies ($p<0.001$) compared to the vehicle. However, 100 μM IRW feeding for 2 weeks failed to increase the lifespan while 200 μM managed to significantly improve lifespan ($p<0.05$). These results indicate the ability of IRW to improve midlife healthspan and survival in W^{dah} flies.

5.3.3 IRW modulates critical histone lifespan markers in *Drosophila melanogaster*

Following the evaluation of the lifespan, we evaluated changes in histone markers in flies following IRW treatment. Based on the lifespan modulation, histone changes were evaluated at 10 and 20 day period(s) for *w* and *wy* flies, and at 28, 35, and 42 day time period for W^{dah} flies. IRW treatment (50 μM) triggered an increase in H3K8AC and H3K56AC histone markers at 10 and 20 day period(s) in *w* flies (Figure 5.3 A-C). In *w* flies, IRW treatment increased H3K8AC by ~8 fold after 10 days of treatment while this increase declined to ~5 fold after 20 days of treatment (Figure 5.3A). Likewise, IRW treatment increased H3K56AC by ~10 fold after 10 days of treatment while this increase declined to the levels of the vehicle after 20 days of treatment (Figure 5.3B). IRW did not activate any histone marker tested for methylation (H4K29ME2 and H3K36ME3) in *w* flies (Fig S12 A-C). Similar to *w* flies, *wy* flies also exhibited increased H3K8AC markers at both 10 and 20-day markers (Figure 5.3D). Interestingly, *wy* flies exhibited an increase in H3K36ME3 marker after 10 days of IRW treatment, which declined over the next day period (Figure S5.1 E). However, no increase was observed in H3K56AC and H4K20ME2 histone markers in *wy* flies (Figure S5.1 D-F). Next, we checked the changes in the selected markers for W^{dah} flies. Our results showed that treatment with 50 μM IRW increased H3K8AC expression at the day 35 and 42, one and two weeks after the IRW treatment initiation (Figure 5.4A). However, the other two histone markers, H3K56AC, and H3K36ME3 exhibited no significant change after 2 weeks of mid-life IRW feeding in W^{dah} flies (Figure 5.4 B-D).

5.3.4 IRW activates key pathways related to lifespan extension in *Drosophila melanogaster*

Lifespan modulation involves multiple signaling pathways converging in adaptation and survival. We analyzed changes in the expression of genes involved in lifespan extension (sirtuins and spargel), antioxidant defense (NRF2 pathway), autophagy (ATG7), and insulin signaling (dINR,

PI3K and RHEB). The time points were consistent in the previous experiments above. Firstly, in the *w Drosophila* flies, there was a significant increase in SIRT1, SIRT4, and SIRT6 levels after 20 days of 50 μM IRW feeding (Figure 5.5A-C) ($p<0.05$). However, there were no increased levels of spargel mRNA after 10 or 20 days of 50 μM IRW feeding (Figure 5.5 D). Next, we observed a strong increase in antioxidant enzyme SOD2 ($p<0.001$) after 20 days of IRW feeding (compared to the vehicle) (Figure 5.5 F). However, no statistically significant increase was observed in NRF2 and CAT levels after IRW treatment (Figure 5.5 E,G). Interestingly, we observed a significant decline in autophagy indicator, ATG7, after 10 days of IRW treatment while its levels increased significantly after the 20th day of treatment (Figure 5.5 H). Next, there was a strong increase in the levels of *Drosophila* insulin receptor, dINR, and its downstream target RHEB after 20 days of IRW treatment in the *w Drosophila* flies *in vivo* (Figure 5.5 I, K). However, despite activation of dINR, IRW treatment failed to modulate levels of dINR dependent PI3K levels in the *w* flies (Figure 5.5J). In the case of *wy Drosophila* flies, there was a strong increase in SIRT1 ($p<0.01$), SIRT4 ($p<0.05$), and SIRT6 ($p<0.05$) levels after 10 days of 50 μM IRW feeding (Figure 5.6 A-C) ($p<0.05$). Unlike *w* flies, there was a significant increase in levels of spargel mRNA after 10 days of 50 μM IRW feeding in *wy* flies (Figure 5.6 D). Likewise, activation of the NRF2 pathway along with its downstream effectors SOD2 and CAT was observed after 10 days of IRW treatment (Figure 5.6 E-G). Interestingly, we observed a significant increase in autophagy indicator, ATG7, after 10 days of IRW treatment in *wy* flies (Figure 5.6 H). Next, we observed a marginal but statistically insignificant increase in levels of dINR ($p= 0.0569$) after 10 days of IRW feeding accompanied by a significant increase in downstream PI3K and RHEB levels after 10 days of IRW treatment in *wy* flies (Figure 5.6 I-K). Overall, we observed increased lifespan biomarkers in *w* flies after 20 days and 10 days in *wy* flies after IRW treatment *in vivo* (Figure 5.5 H). Finally,

in the W^{dah} flies, similar to *w* and *wy* flies, there was a significant increase in levels of SIRT1 ($p<0.05$) and SIRT6 ($p<0.05$) after 2 weeks of IRW treatment (Figure 5.7 A,C). However, no increase in levels of SIRT4 and spargel was observed in W^{dah} flies (Figure 5.7 B,D). Likewise, compared to the starting of IRW treatment, a statistically significant increase was observed in NRF2 ($p<0.05$) and its downstream SOD2 ($p<0.05$) and CAT ($p<0.01$) enzymes after 2 weeks of IRW treatment (Figure 5.7 E-G). In line with these observations, ATG7 levels increased significantly after 2 weeks, compared to day 1 of the treatment (Figure 5.7 H). Remarkably, the levels of dINR were significantly increased after 1 and 2 weeks of IRW feeding ($p<0.05$; $p<0.01$) accompanied by a significant increase in downstream PI3K levels after 1 and 2 weeks of IRW treatment in W^{dah} flies ($p<0.01$) (Figure 5.7 I,J). Similar to other biomarkers, RHEB levels in W^{dah} flies, compared to the beginning of treatment, were significantly increased after 2 weeks of IRW treatment ($p<0.05$) (Figure 5.7 K). A major weakness in our observation of W^{dah} flies was the absence of significant changes, compared to relative days as the changes were solely significant after 2 weeks, compared to day 0 of the treatment cycle (Figure 5.7A-K).

5.4 Discussion

In this study, we tested the effects of IRW on the lifespan in *Drosophila* flies. IRW did enhance the lifespan in *Drosophila*, but in some trials caused no change in lifespan in *Drosophila* flies both wild-type W^{dah} and *w and wy* mutant populations as well. How might IRW extend lifespan in *Drosophila*? Based on our findings, this question can be addressed in four ways. Firstly, during their natural lifespan, *Drosophila* are proposed to eat dietary materials and microbes from fermenting/rotting fruit (Spieth, 1974). Therefore, they have likely evolved to produce antimicrobial molecules for their survival against the possibility of bacterial infections. Hence, the

production of antimicrobial molecules and their resultant physiological response form the basis of *Drosophila* survival and lifespan maintenance. Ovotransferrin, as a key component of egg white, is involved in the protection of yolk and embryo against microbial attackers by a mixture of antimicrobial components (Hincke, Nys, Gautron, Mann, Rodriguez-Navarro, & McKee, 2012). Extensive research has shown that ovotransferrin and its derivative peptides are well known for their strong antimicrobial activity against a broad spectrum of bacteria and other microbes via chelation of iron (Ma, Guo, Fu, & Jin, 2020; Moon et al., 2013). Therefore, intake of IRW, an antimicrobial and antioxidant peptide derived from ovotransferrin might activate the survival and lifespan boosting pathways, evolutionarily developed in flies as a response to external microbes. Interestingly, iron chelation, a property of ovotransferrin, has been shown to significantly extend lifespan in *C. elegans*, however, the iron chelation ability of ovotransferrin-derived IRW remains unexplored (Schiavi et al., 2015).

Secondly, the ability of IRW to extend lifespan can be linked to its ability to modulate key changes in histones. It is now well understood that organisms react to various nutrients and bioactives, which eventually converge onto chromatin structure towards the regulation of multiple signaling pathways involved in the specific cellular response. Our data showed a strong modulation of H4K8AC, H3K56AC, and H3K36ME3 histone markers (Figure 5.3, 5.4). H4K8AC is a vital histone marker linking aging as it's level declines in midlife flies in comparison to young flies (Peleg et al., 2016). An increase in H4K8AC in *w*, *wy*, and W^{dah} flies demonstrates a strong ability of IRW to reverse this vital aging histone marker. This indicates a vital epigenetic modification induced by IRW *in vivo*. Similar to H4K8AC, H3K56AC, is involved in lifespan modulation and survival. Depletion (or mutation) of H3K56AC has been associated with the reduced lifespan of *S. cerevisiae* (Feser et al., 2010; Ngubo, Reid, & Patterton, 2019). Its increase

in aging flies after IRW feeding confirms the epigenetic modulation by the tested peptide. Moreover, acetylation of H3K56 occurs globally on recently synthesized histones and at specific promoters during S-phase leading to DNA repair (Chen et al., 2008). The increased activity of H3K56AC by IRW indicates DNA repair and possibly the synthesis of new histones in *w* flies. However, the ability of IRW to increase the H3K56AC marker in *wy* and W^{dah} flies was strong but statistically insignificant. Next, the increase in the H3K36ME3 histone marker is another vital finding as this trimethylated histone marker is strongly associated with aging. Its decline during the aging process triggers cryptic transcription and shorter lifespan, observed in a variety of organisms such as S. *cerevisiae*, *D.* melanogaster, and *C. elegans* (Sen et al., 2015; S. H. Wood et al., 2015). Therefore, lifespan extension and improvement in survival of flies by IRW can be attributed in part to H3K36ME3 acceleration and restoration of transcriptional fidelity (Pu et al., 2015). It will be interesting in the future to explore the modulation of methyltransferase *met-1*, the controller of global H3K36ME3 marks, by IRW *in vivo*. Also, IRW was unable to modulate H3K36ME3 along with other studied markers in *w* and W^{dah} flies indicating the contribution of genetic factors in IRW guided modulation of various epigenetic marks (Figure S5.1). These diversity in changes of histones markets highlight molecular mechanisms underlying the pharmacological activity of IRW.

Thirdly, the extension in lifespan by IRW can be explained by the activation of multiple pathways such as sirtuins, antioxidant response, insulin, and autophagy initiation. Activation of sirtuins, including SIRT1, SIRT4, and SIRT6 extends lifespan in *Drosophila* and mammals (Kanfi et al., 2012; Rogina & Helfand, 2004; J. G. Wood et al., 2018). Sirtuins directly impact AMP-activated protein kinase (AMPK), insulin pathways, target of rapamycin (TOR), and forkhead box O (FOXO). Interestingly, *w* flies exhibited an increase in sirtuins after 20 days while *wy* flies after

10 days of IRW treatment. These results are similar to resveratrol, a polyphenol found in the skin of red grapes, a vital sirtuin-activating compound (STACs) (Howitz et al., 2003). Similarly, two other STACs (SRT1720 and SRT2104) also extend lifespan and improves the healthspan of mice fed a standard diet (Mercken et al., 2014; Mitchell et al., 2014). To our surprise, IRW treatment in flies activated the pathways such as AMPK, TOR or FOXO (Figure S5.2). However, the levels of spargel, the PGC1α was increased solely in *wy* flies, but not in *w* or W^{dah} flies, indicating variation in mitochondrial response of flies. Likewise, activation of lifespan boosting antioxidant NRF2 pathway, and its downstream targets varied between *w* and *wy* flies. This variation in sirtuin and antioxidant response of flies, especially in w and *wy* flies is reflected in their lifespan extension at 100 μM treatment levels as well. The W^{dah} flies also showed an increase in nuclear sirtuins (SIRT1 and SIRT6) but in the mitochondrial sirtuin SIRT4. The increase in midlife feeding and resultant lifespan extension can be related to this surge in sirtuins. This boost in sirtuin was accompanied by lifespan modulation NRF2 pathway as well indicating intricate involvement of longevity and survival mechanisms (Sykiotis & Bohmann, 2008). It is also to vital note that the increase in autophagy relate to ATG7 gene, crucial for stress survival and normal lifespan maintenance in *Drosophila* (Juhász, Érdi, Sass, & Neufeld, 2007). Finally, our findings showing activation of the dInR by IRW indicate its ability to mimic insulin, leading to activation of both the PI3K and RHEB (Slack et al., 2010). However, these results are contradictory to literature as downregulation of dInR is associated with an increase in *Drosophila* lifespan (Shen et al., 2013). In *Drosophila*, the dInR/dPI3K pathway is also considered to regulate body size and life span (Puig, Marr, Ruhf, & Tjian, 2003). The dInR upregulation inhibits hunger stimuli, therefore discriminative food selection of high quality food, such as peptide rich (IRW boosted) food (Lingo, Zhao, & Shen, 2007). IRW may act as an insulin mimetic that promotes growth and proliferation by activating

downstream dPI3K (Puig et al., 2003). Theoretically, we propose that in *Drosophila*, high levels of insulin-like peptides (DILPs) activate dInR, which negatively influence this pathway. On the contrary, IRW may inhibit the activity or availability of DILPs and activate dInR transcription. Thus, IRW feeding might sensitize the pathway due to low levels of available DILPs. Therefore, once the dInR is re-activated, the growth and survival of *Drosophila* via the dInR pathway could thus be balanced or even up regulated. This indicates a unique mechanism, facilitating the increase in the lifespan. However, this hypothesis needs to be tested using detailed *in vivo* experiments. Finally, the ability of IRW to increase NAD^+ both in *Drosophila* can be another contributing factor to lifespan extension (Bhullar et al., 2021). The upregulation of the NAD salvage pathway, which recycles NAD^+ from NAM, can extend lifespan and mimic calorie restriction in yeast (Anderson et al., 2003; Yang et al., 2006). Similar to IRW, NAD^+ booster nicotinamide riboside supplementation in mice resulted in a significant increase in longevity (Zhang et al., 2016). Fourthly, changing the dietary amino acid balance in a diet impacts reproduction and lifespan in *Drosophila* flies. Interestingly, isoleucine (I), arginine (R), and tryptophan (W) have been found to influence egg laying/reproduction in *Drosophila* (Piper et al., 2017). As lifespan is inversely related to reproduction (Partridge, Green, & Fowler, 1987), the feeding of IRW containing survival booster amino acids to non-reproducing flies may have contributed to lifespan extension as well. Moreover, flies fed medium without I exhibit lifespan shortened by 30-70%, therefore, incorporation of additional I in fly diet can (possibly) improve the lifespan (Piper et al., 2014). Further, R is also quintessential for the growth of *Drosophila* and its removal from the diet leads to death (Hinton, 1956). Therefore, the addition of R-containing peptide (IRW) might support better survival and extend lifespan. Overall, these results indicate activation and convergence of multiple signaling pathways towards lifespan extension in flies.

5.5 Conclusions

This research reveals that chronic IRW exposure lengthens the lifespan of *Drosophila* flies. The effects of IRW resembled some extent the effects of caloric restriction, even though fly food had more amino acid content. While it is clear from our previous study (Chapter 3) that IRW modulates oxidative phosphorylation (Bhullar et al., 2021), we find that long-term intake of IRW *in vivo*, includes increased antioxidant defenses and enzymes, possibly leading to lower mitochondrial damage. Our recent study has shown that the dose of IRW associated with these lifespan effects was well tolerated in mice (Bhullar et al., 2021). Further clinical studies are necessary to establish the effects of chronic IRW intake exposure towards health and aging in humans. In conclusion, our results support the model that links histone marks, antioxidant, and sirtuin pathways in an evolutionarily conserved pathway, with IRW as an activator of the pathway. The significant and intermittent effect of IRW on lifespan in *Drosophila* warrants its further investigation as a geroprotective molecule.

5.6 References

Anderson, R. M., Bitterman, K. J., Wood, J. G., Medvedik, O., & Sinclair, D. A. (2003). Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae. *Nature, 423*(6936), 181-185.

Aparicio, R., Rana, A., & Walker, D. W. (2019). Upregulation of the autophagy adaptor p62/SQSTM1 prolongs health and lifespan in middle-aged Drosophila. *Cell reports, 28*(4), 1029-1040. e1025.

Balan, V., Miller, G. S., Kaplun, L., Balan, K., Chong, Z.-Z., Li, F., . . . Freeman, D. C. (2008). Life span extension and neuronal cell protection by Drosophila nicotinamidase. *Journal of Biological Chemistry, 283*(41), 27810-27819.

Bhullar, K. S., & Hubbard, B. P. (2015). Lifespan and healthspan extension by resveratrol. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1852*(6), 1209-1218.

Bhullar, K. S., Son, M., Kerek, E., Cromwell, C. R., Wingert, B. M., Wu, K., . . . Wu, J. (2021). Tripeptide IRW Upregulates NAMPT Protein Levels in Cells and Obese C57BL/6J Mice. *Journal of Agricultural and Food Chemistry, 69*(5), 1555-1566.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., . . . Shipley, G. L. (2009). The MIQE Guidelines: M inimum I nformation for Publication of Q uantitative Real-Time PCR E xperiments. Oxford University Press.

Chen, C.-C., Carson, J. J., Feser, J., Tamburini, B., Zabaronick, S., Linger, J., & Tyler, J. K. (2008). Acetylated lysine 56 on histone H3 drives chromatin assembly after repair and signals for the completion of repair. *Cell, 134*(2), 231-243.

Fang, E. F., Kassahun, H., Croteau, D. L., Scheibye-Knudsen, M., Marosi, K., Lu, H., . . . Wilson, M. A. (2016). NAD+ replenishment improves lifespan and healthspan in ataxia telangiectasia models via mitophagy and DNA repair. *Cell metabolism, 24*(4), 566-581.

Feser, J., Truong, D., Das, C., Carson, J. J., Kieft, J., Harkness, T., & Tyler, J. K. (2010). Elevated histone expression promotes life span extension. *Molecular cell, 39*(5), 724-735.

Harrison, D. E., Strong, R., Sharp, Z. D., Nelson, J. F., Astle, C. M., Flurkey, K., . . . Carter, C. S. (2009). Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature, 460*(7253), 392-395.

Hashimoto, T., Horikawa, M., Nomura, T., & Sakamoto, K. (2010). Nicotinamide adenine dinucleotide extends the lifespan of Caenorhabditis elegans mediated by sir-2.1 and daf-16. *Biogerontology, 11*(1), 31-43.

Hincke, M. T., Nys, Y., Gautron, J., Mann, K., Rodriguez-Navarro, A. B., & McKee, M. D. (2012). The eggshell: structure, composition and mineralization. *Front Biosci, 17*(1), 1266-1280.

Hinton, T. (1956). The effects of arginine, ornithine and citrulline on the growth of Drosophila. *Archives of biochemistry and biophysics, 62*(1), 78-85.

Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., . . . Zhang, L.-L. (2003). Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature, 425*(6954), 191-196.

Hyde, R. R. (1913). Inheritance of the length of life in Drosophila ampelophila. *Proceedings of the Indiana Academy of Science* (Vol. 23, pp. 113-124).

Juhász, G., Érdi, B., Sass, M., & Neufeld, T. P. (2007). Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila. *Genes & development, 21*(23), 3061-3066.

Kanfi, Y., Naiman, S., Amir, G., Peshti, V., Zinman, G., Nahum, L., . . . Cohen, H. Y. (2012). The sirtuin SIRT6 regulates lifespan in male mice. *Nature, 483*(7388), 218-221.

Kapahi, P., Chen, D., Rogers, A. N., Katewa, S. D., Li, P. W.-L., Thomas, E. L., & Kockel, L. (2010). With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. *Cell metabolism, 11*(6), 453-465.

Kapahi, P., Zid, B. M., Harper, T., Koslover, D., Sapin, V., & Benzer, S. (2004). Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. *Current Biology, 14*(10), 885-890.

Koval, L., Zemskaya, N., Aliper, A., Zhavoronkov, A., & Moskalev, A. (2021). Evaluation of the geroprotective effects of withaferin A in Drosophila melanogaster. *Aging (Albany NY), 13*(2), 1817.

Lee, C. F., Caudal, A., Abell, L., Gowda, G. N., & Tian, R. (2019). Targeting NAD+ metabolism as interventions for mitochondrial disease. *Scientific reports, 9*(1), 1-10.

Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., . . . Bonaduce, D. (2018). Oxidative stress, aging, and diseases. *Clinical interventions in aging, 13*, 757.

Lingo, P. R., Zhao, Z., & Shen, P. (2007). Co-regulation of cold-resistant food acquisition by insulin-and neuropeptide Y-like systems in Drosophila melanogaster. *Neuroscience, 148*(2), 371-374.

Ma, B., Guo, Y., Fu, X., & Jin, Y. (2020). Identification and antimicrobial mechanisms of a novel peptide derived from egg white ovotransferrin hydrolysates. *LWT, 131*, 109720.

Magwere, T., Chapman, T., & Partridge, L. (2004). Sex differences in the effect of dietary restriction on life span and mortality rates in female and male Drosophila melanogaster.

The Journals of Gerontology Series A: Biological Sciences and Medical Sciences, 59(1), B3-B9.

Martin-Montalvo, A., Mercken, E. M., Mitchell, S. J., Palacios, H. H., Mote, P. L., Scheibye-Knudsen, M., . . . Blouin, M.-J. (2013). Metformin improves healthspan and lifespan in mice. *Nature communications, 4*(1), 1-9.

McCay, C. M., Maynard, L., Sperling, G., & Barnes, L. L. (1939). Retarded growth, life span, ultimate body size and age changes in the albino rat after feeding diets restricted in calories: four figures. *The Journal of nutrition, 18*(1), 1-13.

Melzer, D., Pilling, L. C., & Ferrucci, L. (2020). The genetics of human ageing. *Nature Reviews Genetics, 21*(2), 88-101.

Mercken, E. M., Mitchell, S. J., Martin-Montalvo, A., Minor, R. K., Almeida, M., Gomes, A. P., . . . Zhang, Y. (2014). SRT 2104 extends survival of male mice on a standard diet and preserves bone and muscle mass. *Aging cell, 13*(5), 787-796.

Mitchell, S. J., Martin-Montalvo, A., Mercken, E. M., Palacios, H. H., Ward, T. M., Abulwerdi, G., . . . Sinclair, D. A. (2014). The SIRT1 activator SRT1720 extends lifespan and improves health of mice fed a standard diet. *Cell reports, 6*(5), 836-843.

Moon, S., Lee, J., Lee, Y., Chang, K., Paik, J., Ahn, D., & Paik, H. (2013). Screening for cytotoxic activity of ovotransferrin and its enzyme hydrolysates. *Poultry science, 92*(2), 424-434.

Morselli, E., Galluzzi, L., Kepp, O., Criollo, A., Maiuri, M. C., Tavernarakis, N., . . . Kroemer, G. (2009). Autophagy mediates pharmacological lifespan extension by spermidine and resveratrol. *Aging (Albany NY), 1*(12), 961.

Nässel, D. R., & Broeck, J. V. (2016). Insulin/IGF signaling in Drosophila and other insects: factors that regulate production, release and post-release action of the insulin-like peptides. *Cellular and Molecular Life Sciences, 73*(2), 271-290.

Ngubo, M., Reid, J., & Patterton, H.-G. (2019). Structural clusters of histone H3 and H4 residues regulate chronological lifespan in Saccharomyces cerevisiae. *bioRxiv*.

Partridge, L., Green, A., & Fowler, K. (1987). Effects of egg-production and of exposure to males on female survival in Drosophila melanogaster. *Journal of Insect Physiology, 33*(10), 745-749.

Peleg, S., Feller, C., Forne, I., Schiller, E., Sévin, D. C., Schauer, T., . . . Klima, C. (2016). Life span extension by targeting a link between metabolism and histone acetylation in Drosophila. *EMBO reports, 17*(3), 455-469.

Piper, M. D., Blanc, E., Leitão-Gonçalves, R., Yang, M., He, X., Linford, N. J., . . . Niemeyer, C. (2014). A holidic medium for Drosophila melanogaster. *Nature methods, 11*(1), 100-105.

Piper, M. D., & Partridge, L. (2018). Drosophila as a model for ageing. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1864*(9), 2707-2717.

Piper, M. D., Soultoukis, G. A., Blanc, E., Mesaros, A., Herbert, S. L., Juricic, P., . . . Yang, M. (2017). Matching dietary amino acid balance to the in silico-translated exome optimizes growth and reproduction without cost to lifespan. *Cell metabolism, 25*(3), 610-621.

Pu, M., Ni, Z., Wang, M., Wang, X., Wood, J. G., Helfand, S. L., . . . Lee, S. S. (2015). Trimethylation of Lys36 on H3 restricts gene expression change during aging and impacts life span. *Genes & development, 29*(7), 718-731.

Puig, O., Marr, M. T., Ruhf, M. L., & Tjian, R. (2003). Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. *Genes & development, 17*(16), 2006-2020.

Rogina, B., & Helfand, S. L. (2004). Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proceedings of the National Academy of Sciences, 101*(45), 15998-16003.

Rose, M., & Charlesworth, B. (1980). A test of evolutionary theories of senescence. *Nature, 287*(5778), 141-142.

Schiavi, A., Maglioni, S., Palikaras, K., Shaik, A., Strappazzon, F., Brinkmann, V., . . . Braeckman, B. P. (2015). Iron-starvation-induced mitophagy mediates lifespan extension upon mitochondrial stress in C. elegans. *Current Biology, 25*(14), 1810-1822.

Sen, P., Dang, W., Donahue, G., Dai, J., Dorsey, J., Cao, X., . . . Lee, J. Y. (2015). H3K36 methylation promotes longevity by enhancing transcriptional fidelity. *Genes & development, 29*(13), 1362-1376.

Shen, L.-R., Xiao, F., Yuan, P., Chen, Y., Gao, Q.-K., Parnell, L. D., . . . Lai, C.-Q. (2013). Curcumin-supplemented diets increase superoxide dismutase activity and mean lifespan in Drosophila. *Age, 35*(4), 1133-1142.

Slack, C., Werz, C., Wieser, D., Alic, N., Foley, A., Stocker, H., . . . Partridge, L. (2010). Regulation of lifespan, metabolism, and stress responses by the Drosophila SH2B protein, Lnk. *PLoS Genet, 6*(3), e1000881.

Spieth, H. T. (1974). Courtship behavior in Drosophila. *Annual review of entomology, 19*(1), 385-405.

Sykiotis, G. P., & Bohmann, D. (2008). Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila. *Developmental cell, 14*(1), 76-85.

Ulgherait, M., Rana, A., Rera, M., Graniel, J., & Walker, D. W. (2014). AMPK modulates tissue and organismal aging in a non-cell-autonomous manner. *Cell reports, 8*(6), 1767-1780.

Wood, J. G., Rogina, B., Lavu, S., Howitz, K., Helfand, S. L., Tatar, M., & Sinclair, D. (2004). Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature, 430*(7000), 686-689.

Wood, J. G., Schwer, B., Wickremesinghe, P. C., Hartnett, D. A., Burhenn, L., Garcia, M., . . . Helfand, S. L. (2018). Sirt4 is a mitochondrial regulator of metabolism and lifespan in Drosophila melanogaster. *Proceedings of the National Academy of Sciences, 115*(7), 1564-1569.

Wood, S. H., van Dam, S., Craig, T., Tacutu, R., O'Toole, A., Merry, B. J., & de Magalhães, J. P. (2015). Transcriptome analysis in calorie-restricted rats implicates epigenetic and post-translational mechanisms in neuroprotection and aging. *Genome biology, 16*(1), 1-18.

Yang, H., Lavu, S., & Sinclair, D. A. (2006). Nampt/PBEF/Visfatin: a regulator of mammalian health and longevity? *Experimental gerontology, 41*(8), 718-726.

Zhang, H., Ryu, D., Wu, Y., Gariani, K., Wang, X., Luan, P., . . . Aebersold, R. (2016). NAD+ repletion improves mitochondrial and stem cell function and enhances life span in mice. *Science, 352*(6292), 1436-1443.

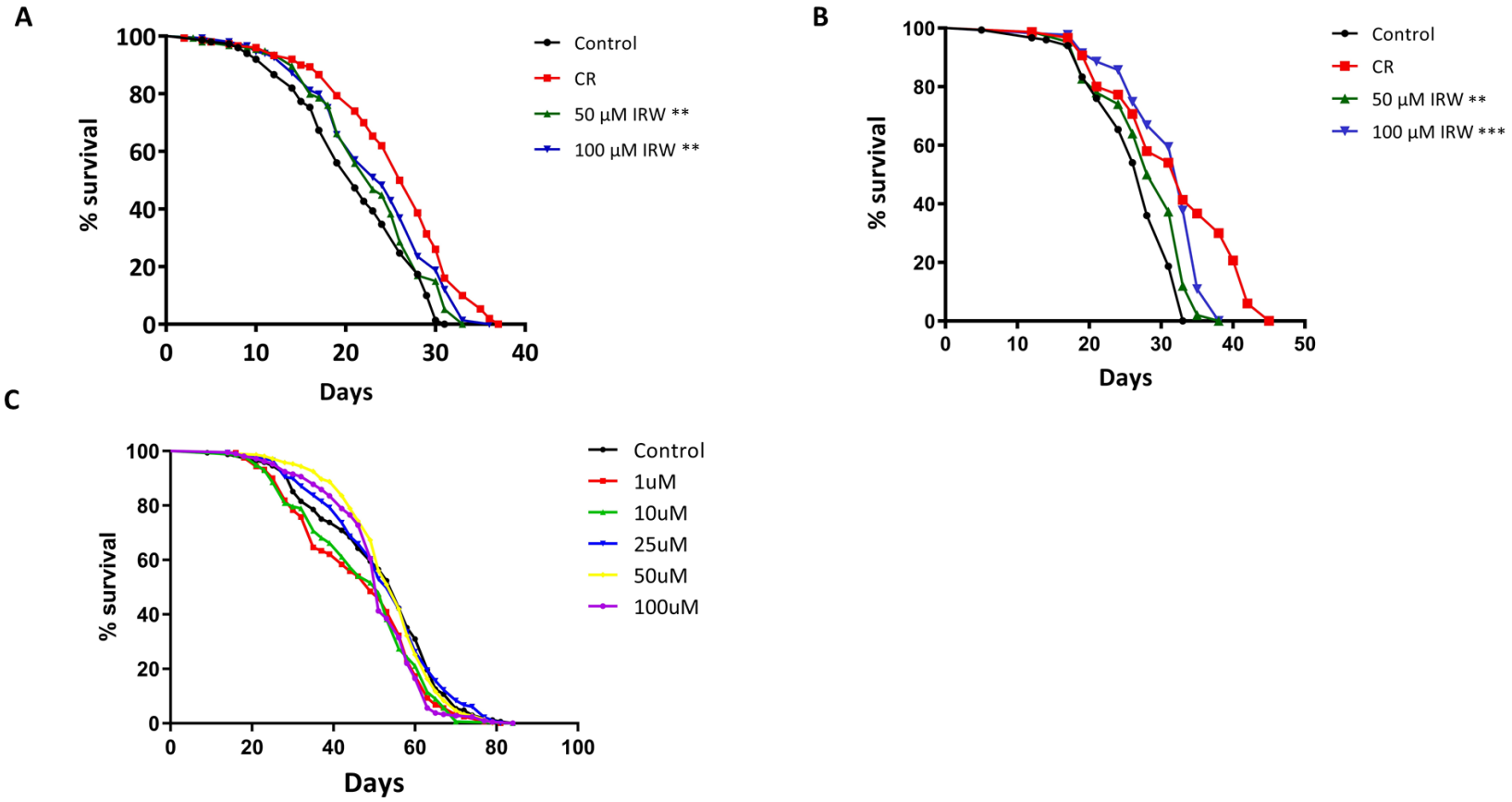


Figure 5.1 Supplementation of IRW increases lifespan in *w* and yw flies but not in *Wdah* flies after whole-life feeding. The white mutant(w) flies (A), the yellow mutant flies (wy) (B) and the *Wdah* flies (C) were fed different dose of IRW for their whole life and overall lifespan was measured.

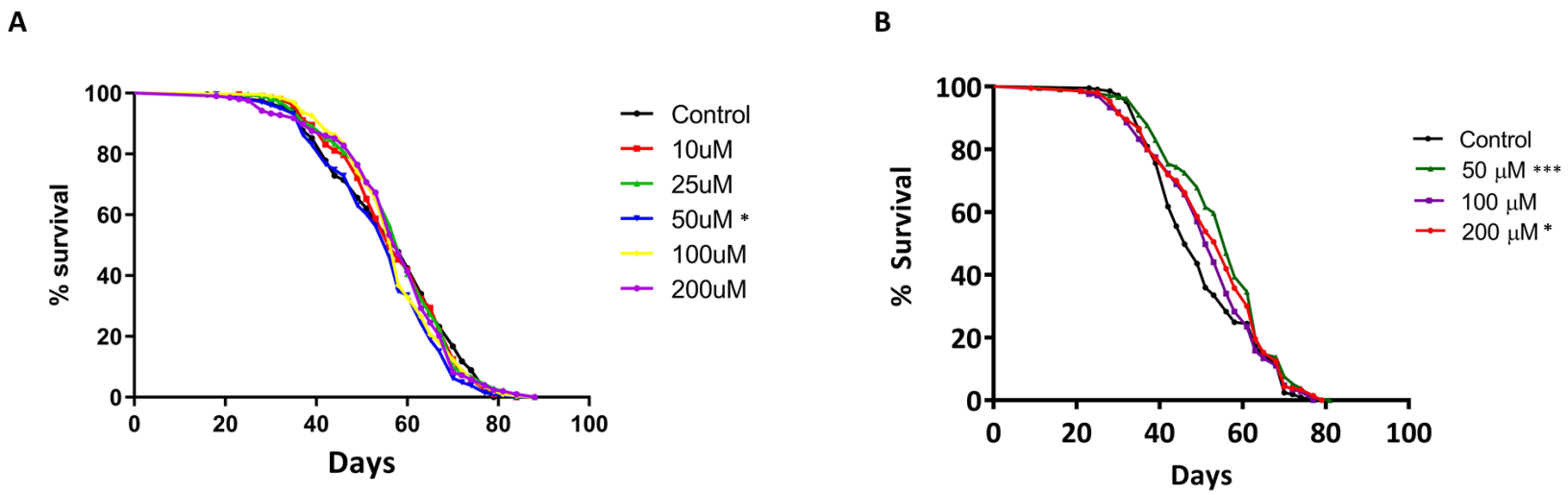


Figure 5.2 Supplementation of IRW increases midlife lifespan in *Wdah* flies. The flies were fed different dose of IRW for their whole midlife (A) starting day 28 onwards till the end of the lifespan or 50 µM IRW for 2 weeks during their midlife lifespan (Day 28-Day42) and lifespan was measured. The selection of time points for midlife IRW feeding was based on overall lifespan extension data in *Wdah* flies.

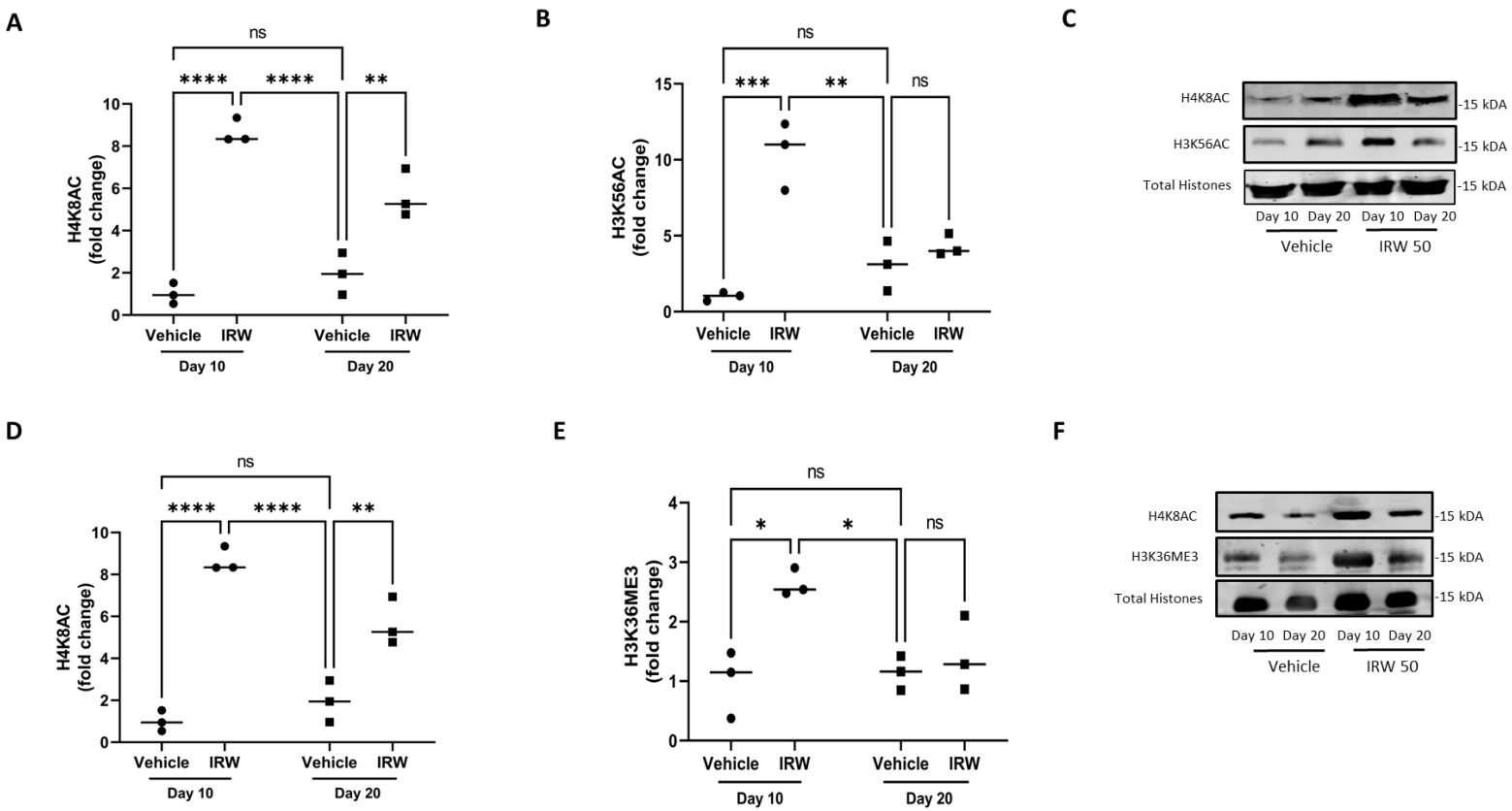


Figure 5.3 Supplementation of IRW changes the histone pattern in Drosophila melanogaster *w* white and *yw* yellow mutant flies. The flies were fed different dosage of IRW for their entire lifespan (10, 25, 50, and 100 μM) and total proteins were extracted during different time points (Day 0, 10, 20, and 30). Based on lifespan extension data, histone analysis was conducted for 50 μM in *w* mutant flies and 100 μM for *yw* mutant flies at Day 10 and Day 20.

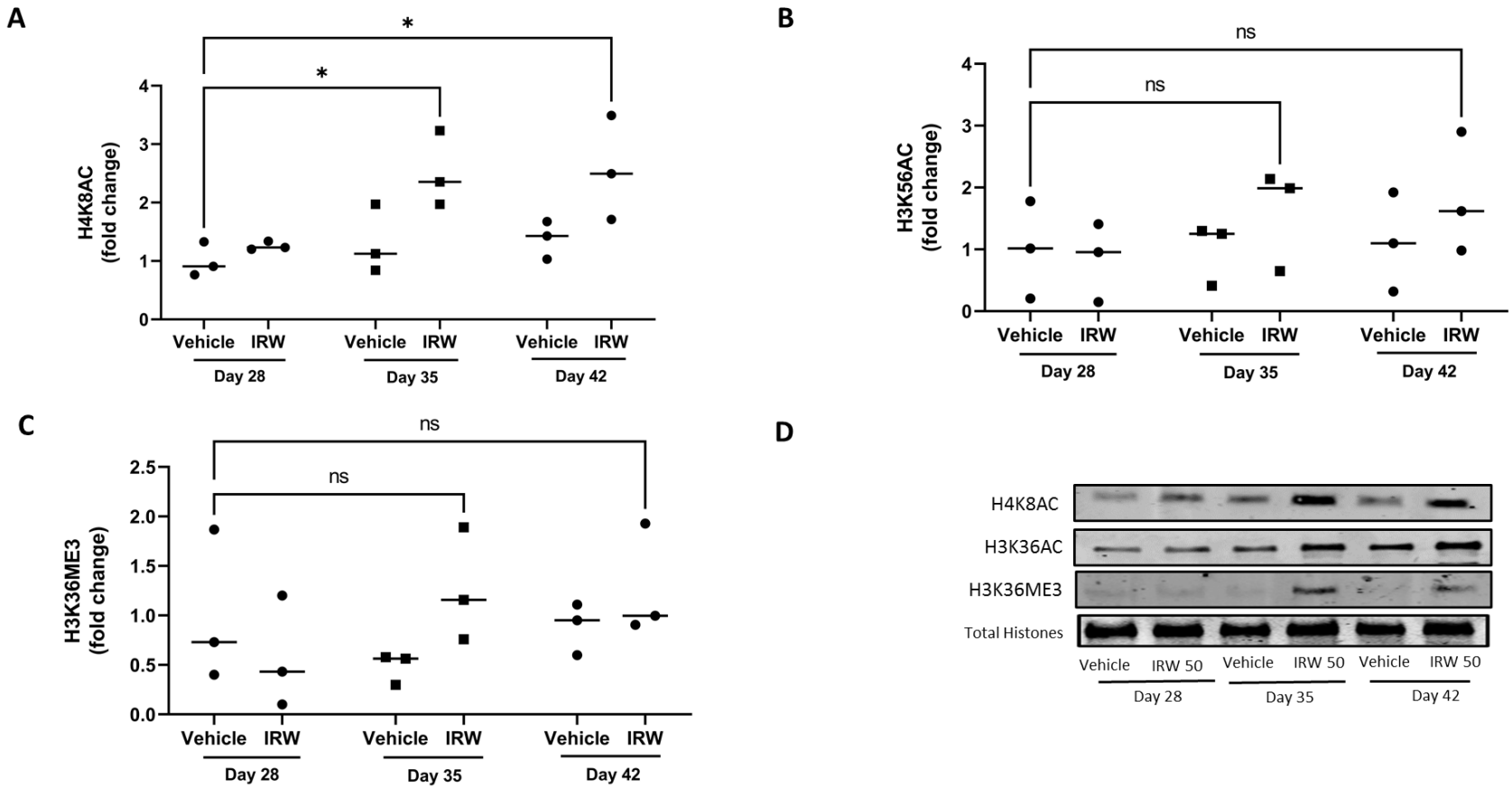


Figure 5.4 Supplementation of IRW changes the histone pattern in Drosophila melanogaster *Wdah* flies. The flies were fed 50 µM IRW for 2 weeks during their midlife lifespan (Day 28- Day42) and histones were extracted. The selection of time points for IRW feeding was based on lifespan extension data in *Wdah flies*.

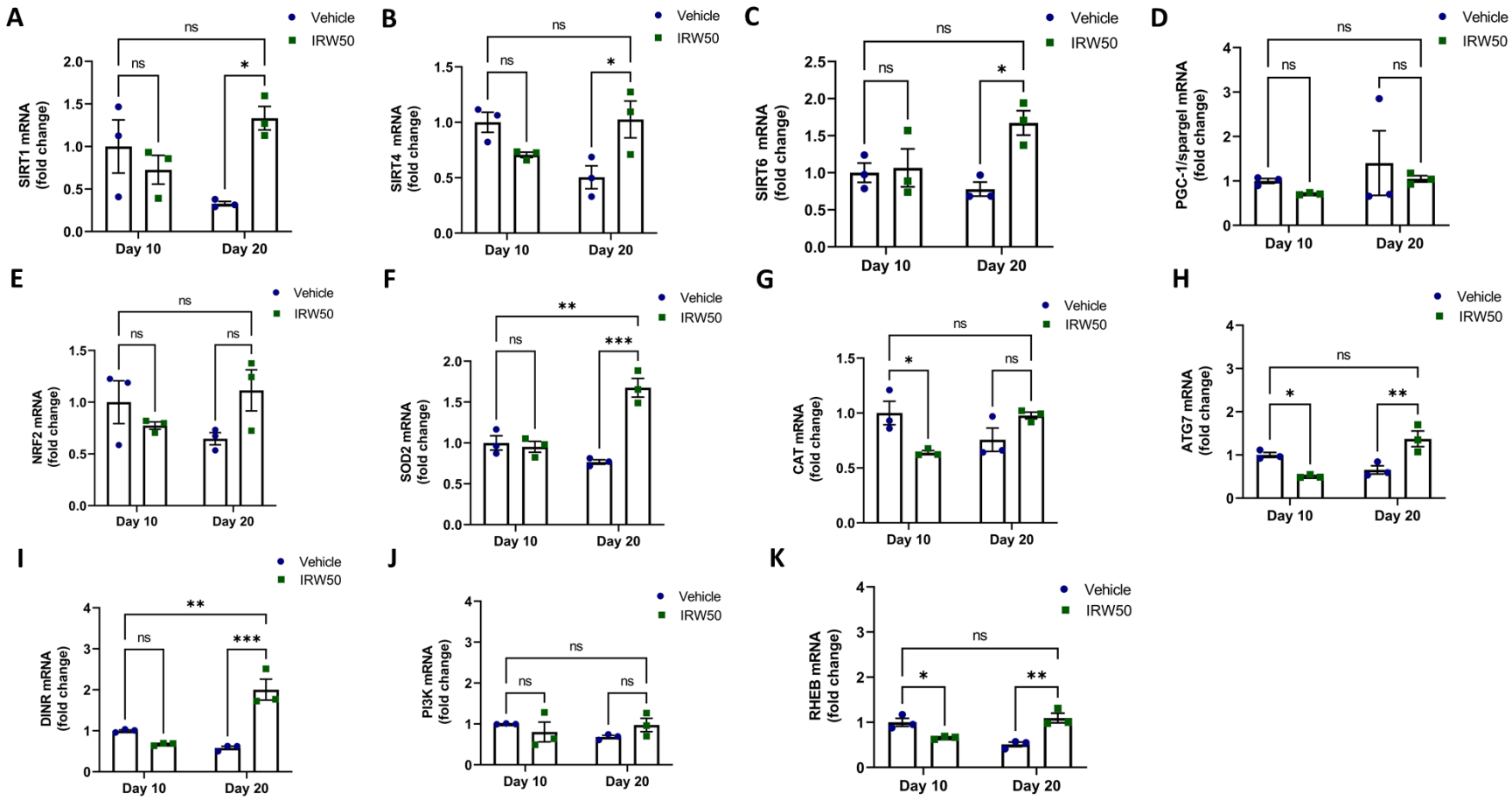


Figure 5.5 Supplementation of IRW modulates multiple genes associated with lifespan in white mutant (*w*) flies. The flies were fed 50 µM IRW for their whole lifespan duration and RNA was extracted and cDNA was synthesized using a commercial kit. The selection of time points for IRW feeding was based on lifespan extension data in *W* flies.

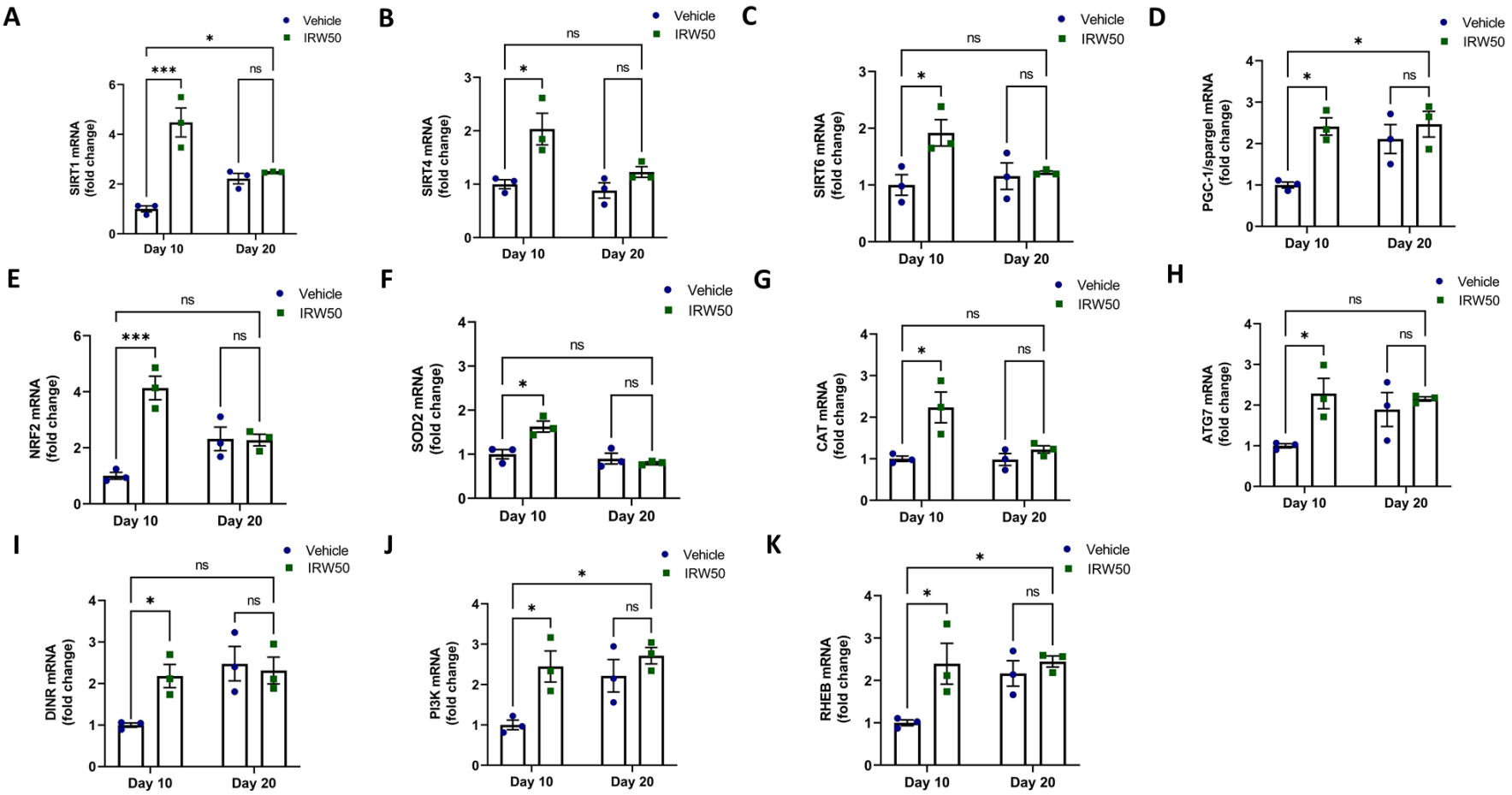


Figure 5.6 Supplementation of IRW modulates multiple genes associated with lifespan in yellow mutant (*yw*) flies. The flies were fed 50 μM IRW for 2 weeks during their midlife lifespan (Day 28-Day42) and histones were extracted. The selection of time points for IRW feeding was based on lifespan extension data in *Wdah flies*.

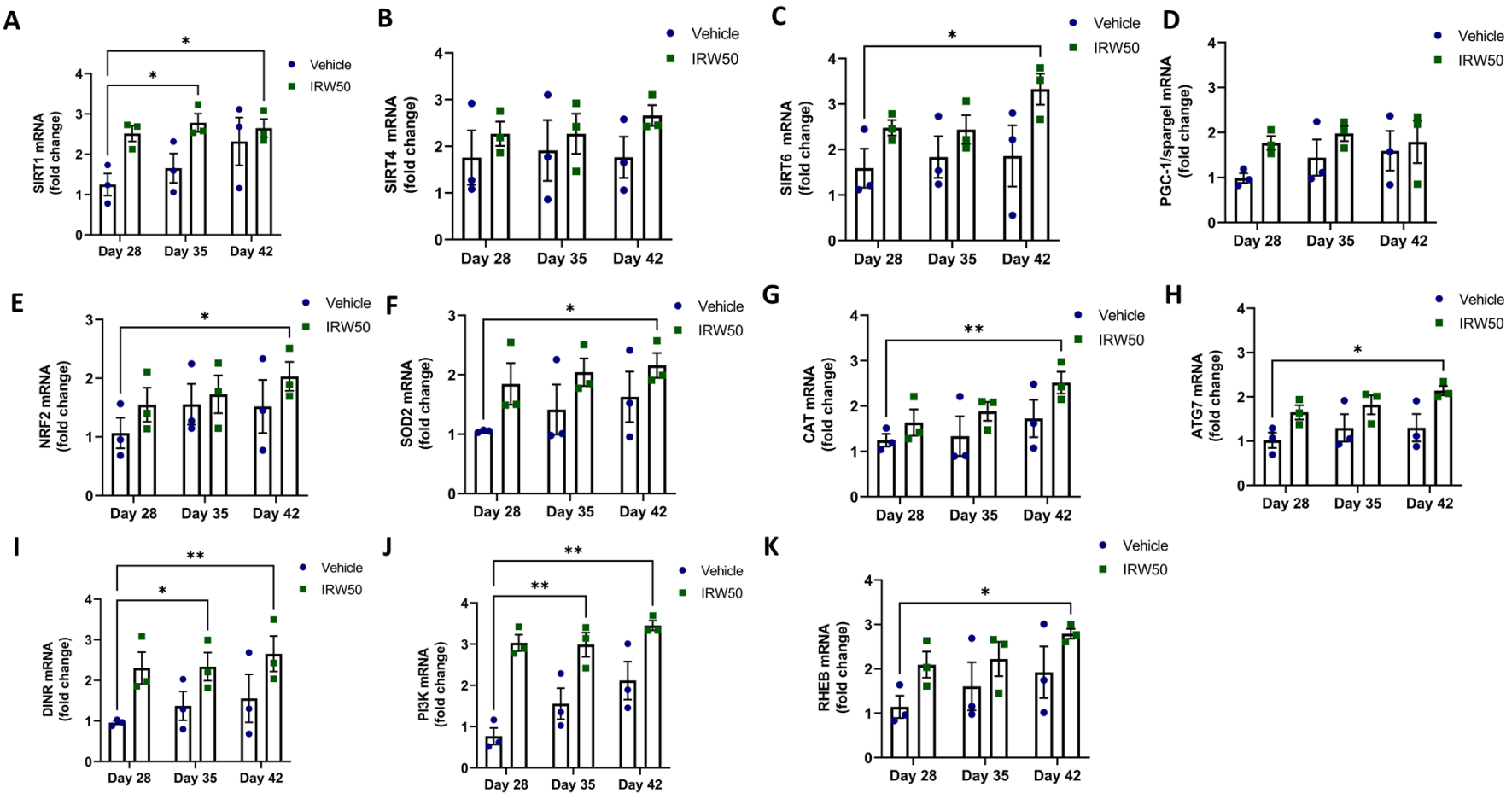


Figure 5.7 Supplementation of IRW modulates multiple genes associated with lifespan in *W^{dah}* flies. The flies were fed 50 μM IRW for 2 weeks during their midlife lifespan (Day 28-Day42) and histones were extracted. The selection of time points for IRW feeding was based on lifespan extension data in *Wdah flies*.

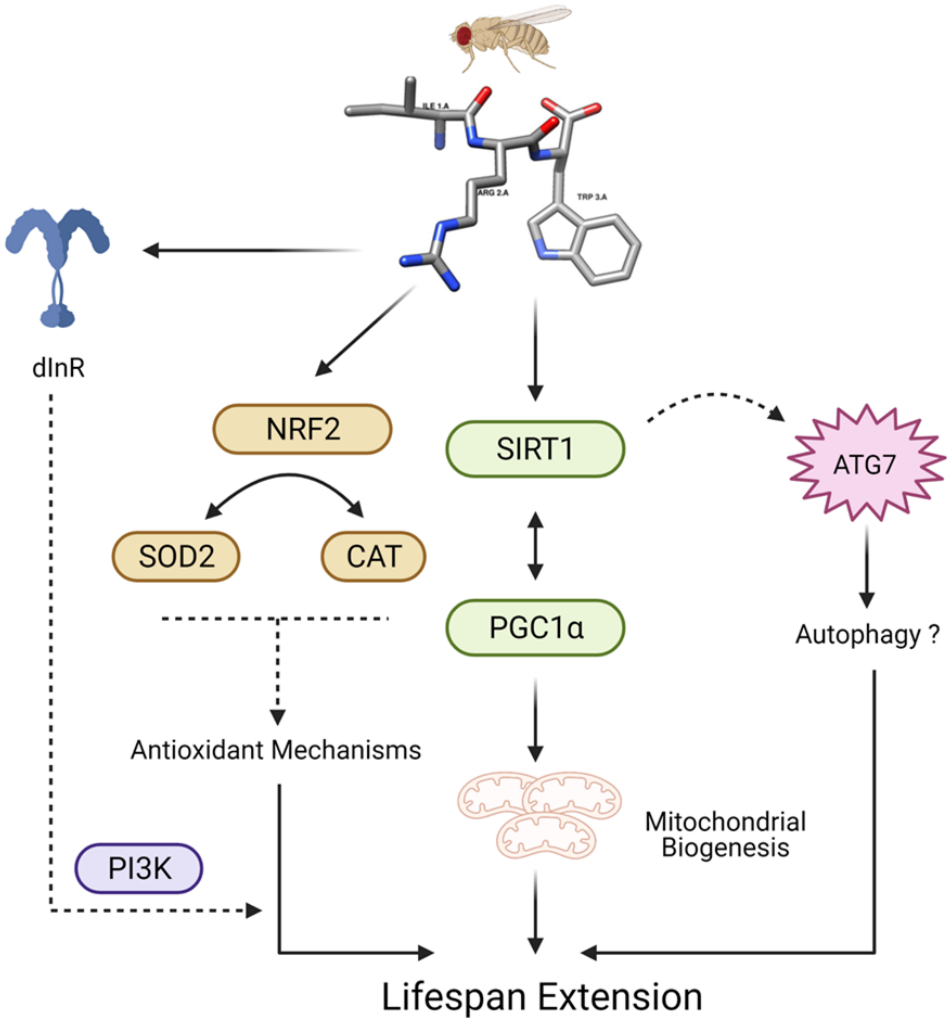
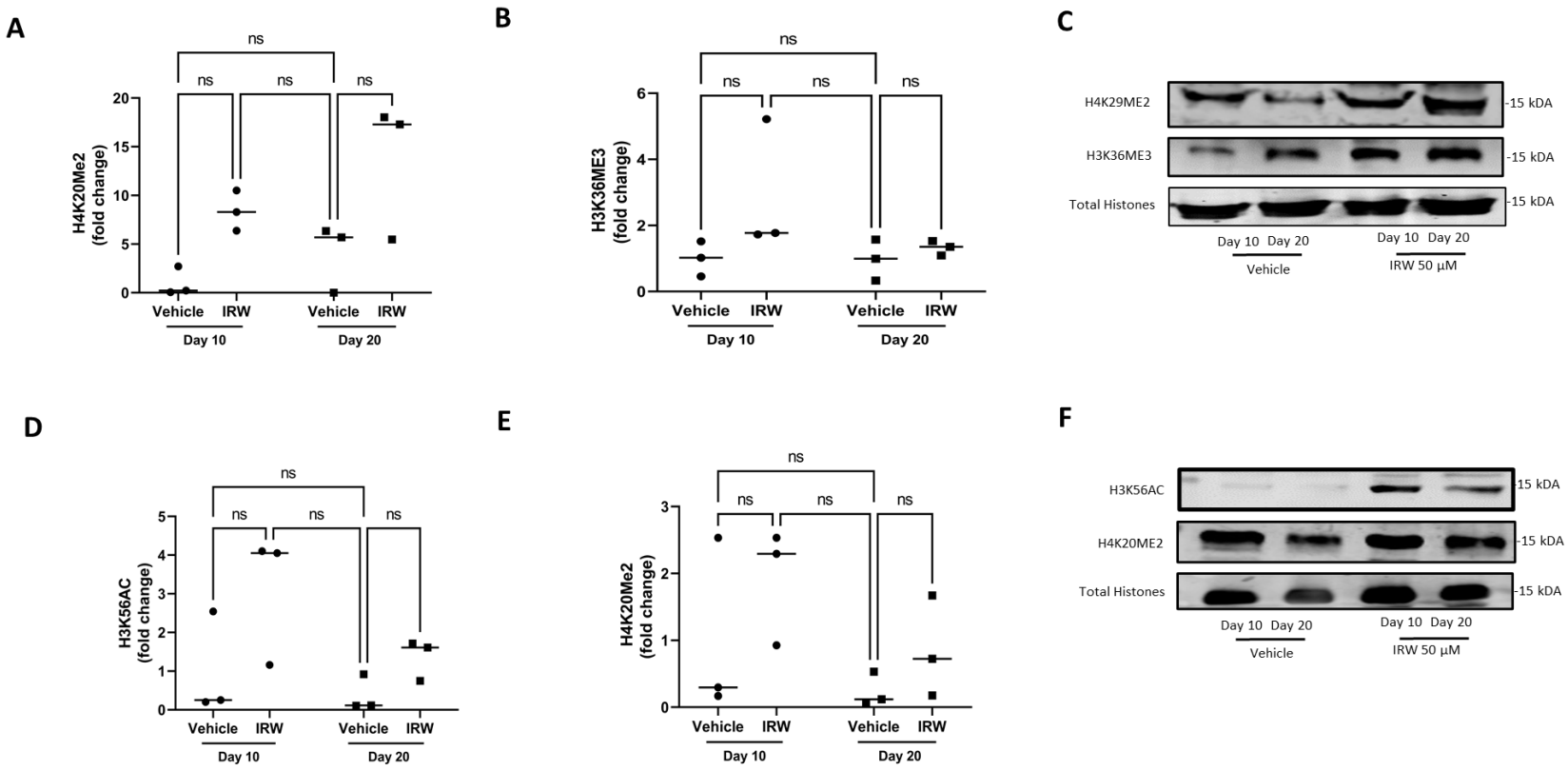
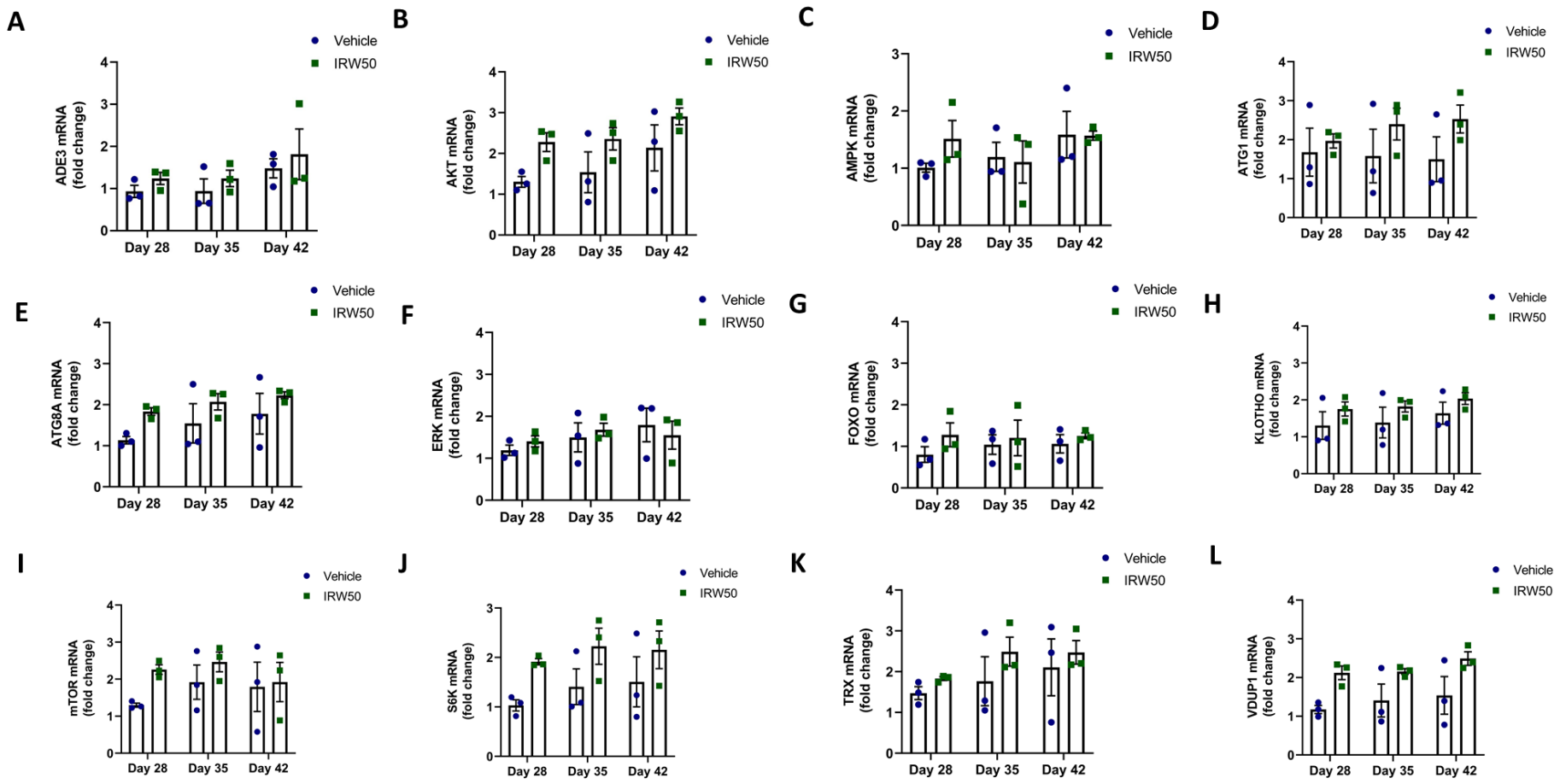


Figure 5.8 A proposed signal transduction pathway leading to lifespan extension in *Drosophila Melanogaster*.



Supplementary Figure 5.1 Impact of IRW supplementation on various histone pattern in Drosophila melanogaster *w* white and *yw* yellow mutant flies. The flies were fed different dosage of IRW for their entire lifespan (10, 25, 50, and 100 µM) and total proteins were extracted during different time points (Day 0, 10, 20, and 30). Based on lifespan extension data, histone analysis was conducted for 50 µM in *w* mutant flies and 100 µM for *yw* mutant flies at Day 10 and Day 20.



Supplementary Figure 5.2 Impact of IRW supplementation on various multiple genes associated with lifespan in *Wdah* flies. The flies were fed 50 μM IRW for 2 weeks during their midlife lifespan (Day 28-Day42) and histones were extracted. The selection of time points for IRW feeding was based on lifespan extension data in *Wdah flies*.

CHAPTER 6 - General Summary and Discussion

6.1 Key Findings of the Present Research

The overall purposes of this research were to understand the regulatory roles of IRW in metabolic and mitochondrial activity and to explore its potential application as a bioactive peptide. The key outcomes of each study are listed below:

1) The first objective to investigate the in vitro and in vivo potential of IRW on NAD^+/NAMPT axis activation (Chapter 3)

The NAD^+ (nicotinamide adenine dinucleotide) biosynthesis pathway plays a vital role in diverse cellular processes that govern human health and disease (Frederick et al., 2016). Research reports indicate a decline of NAD^+ with age and metabolic stress, leading to reduced blood flow, diminished mitochondrial activity, and slower metabolism (Das et al., 2018; Li et al., 2017). Hence, NAD^+ replenishment is an effective way of diminishing the side effects of aging and slowing the age-related metabolic decline (Li et al., 2017; Shi et al., 2017). Apart from direct supplementation of NAD or its precursors, NAMPT upregulation presents itself as an alternative therapeutic strategy. Given the critical role of NAD^+ in the sphere of IRW's biological activity such as anti-hypertensive and metabolic boosting ability, along with the presence of tryptophan, we hypothesized that IRW can increase NAMPT levels and consequently improve NAD^+ levels. The treatment of muscle cells (L6) with IRW (50 μM) increased levels of NAMPT, shown by both immunoblotting and immunoprecipitation in L6 cells. A similar effect was seen *in vivo* in muscle and liver tissues of C57BL/6J mice fed IRW (45 mg/kg BW). In line with our hypothesis, IRW increased the NAMPT mRNA levels and NAD^+ expression in both L6 cells and C57BL/6j HFD mice muscle and liver tissues *in vivo*. Likewise, IRW also increased the expression of D-NAAM in *yw D. melanogaster* establishing the ability of IRW as a NAMPT activator across species. Finally, using CRISPR-Cas9 KO cells, we found that IRW stimulated NAMPT increase in a

SIRT1-dependent manner. We also conducted an experiment to compare the protein levels of NAMPT following treatment with IRW and P7C3-A20 in L6 cells. Interestingly, our results showed a statistically significant increase in NAMPT levels following IRW treatment compared to P7C3-A20 treatment in L6 cells.

2) The second objective was to study the in vitro and in vivo effects of IRW on mitochondrial biogenesis (Chapter 4)

Evolving from α-proteobacterium symbiosis, mitochondria are vital cell organelles that perform a myriad of distinct and interconnected cellular functions (Chandel, 2021). The decline in mitochondrial number and function is implicated in a spectrum of human diseases spanning from pediatric disorders and metabolic syndrome to age-onset neurological diseases (Johannsen & Ravussin, 2009). Therefore, artificial induction of mitochondrial biogenesis is a rationale therapeutic approach to counter mitochondrial decline associated ailments (Picard, Wallace, & Burelle, 2016). As IRW increases the levels of antioxidant components of oxidative phosphorylation (OXPHOS) and activates the NO pathway, a critical initiator of mitochondrial biogenesis, we next investigated the ability of IRW to initiate mitochondrial biogenesis in cells, flies, and mice. Briefly, we found that IRW initiates expression of PGC1α in multiple cell lines along with associated indicators of mitochondrial biogenesis such as TFAM, NRF1, TOM, and COX4; accompanied by a surge in cellular ATP levels as well. The PGC1α surge was accompanied by a tangible increase in the number of mitochondria, as shown by flow cytometry, fluorescence, and electron microscopy in different cell lines. The results obtained from cellular experiments were replicated in different tissues of C57BL/6j HFD mice at mRNA and protein levels *in vivo*. Interestingly, notable microbiome changes were induced by IRW *in vivo* in C57BL/6j HFD mice which possibly aided in mitochondrial biogenesis in the mice GI tract as well. Finally, the notable

increase in mitochondrial number in muscles of W^{dah} flies confirmed the ability of IRW to trigger mitochondrial biogenesis across multiple biological systems.

3) The third objective was to study the mechanisms involved in the IRW-stimulated mitochondrial biogenesis (Chapter 4)

Next, by employing click chemistry and CRISPR-Cas9 gene editing we elucidated the underlying mechanism, whereby IRW induces mitochondrial biogenesis. We identified a cellular target, namely FAM120B, a coactivator of PPARγ, which interacts with IRW. This leads to a downstream cascade that concludes in increased mitochondrial biogenesis via activation of PGC1α. As PGC1α activation and PPARγ pathway are closed related, the interaction of IRW with FAM120B endorses IRW driven mitochondrial biogenesis.

4) The fourth objective was to the study the in vivo efficacy of IRW on lifespan in Drosophila melanogaster (Chapter 5)

Mitochondria play a vital role in controlling the lifespan of organisms as ROS originating from mitochondria directly modulate the aging process (Harman, 1956). This theory termed the free radical theory of aging has now evolved as the mitochondrial theory of aging and overexpression of mitochondrial antioxidants has shown to extend the lifespan of transgenic animals (Mitsui et al., 2002; Parkes, Elia, Dickinson, Hilliker, Phillips, & Boulianne, 1998). Further, the enhanced expression of PNC1 (pyrazinamidase/nicotinamidase 1), the NAD^+ rate-limiting enzyme, leads to lifespan extension in *Saccharomyces cerevisiae* (Anderson, Bitterman, Wood, Medvedik, & Sinclair, 2003). The mammalian functional equivalent of PNC1 called NAMPT also impacts the lifespan (Yang, Lavu, & Sinclair, 2006). Likewise, overexpression of the *Drosophila* NAMPT homolog, D-NAAM, expands the mean lifespan by 30% (Balan et al., 2008). Additionally, feeding

of NAD^+ or its precursor(s) and NAD^+ activators also improve lifespan in different organisms (Bhullar & Hubbard, 2015; Fang et al., 2016; T. Hashimoto, Horikawa, Nomura, & Sakamoto, 2010; Lee, Caudal, Abell, Gowda, & Tian, 2019). Based on the findings above, we proposed that IRW owing to its ability to boost mitochondrial number, D-NAAM (fly NAMPT), and NAD^+ *in vivo* (Bhullar et al., 2021) might extend the *Drosophila* lifespan. Our results showed that IRW treatment (50 and 100 μM) prolonged the median life span of white mutant (*w*) and yellow mutant (*wy*) flies. Likewise, midlife IRW feeding in W^{dah} flies improved lifespan significantly as well. Next, IRW treatment at the tested concentrations radically improved the age-related histone markers in flies and subsequently improved the expression of SIRT1, SOD2, ATG7, and dInR, all intimately involved in lifespan extension (Chen, Zhou, Ge, & Wang, 2020; Giannakou & Partridge, 2007; Y. Hashimoto, Ookuma, & Nishida, 2009; Liling, Roska, Arfiansyah, Maryam, & Nainu, 2021).

6.2 Significance of This Research

The exact reasons why we age are poorly understood, however, NAD^+ metabolism and mitochondria dictate key physiological changes associated with aging. To deal with the challenge of age-related changes, various molecules are being explored. This research has shown the ability of IRW as a small dietary peptide activator of NAMPT. Currently, there are only two known synthetic NAMPT activators, SBI-797812 and P7C3-A20, and the latter is in debate; our test also failed to show the ability of P7C3-A20 to increase protein levels of NAMPT. Based on these results, IRW has emerged as the first small peptide booster of NAMPT levels *in vivo* and can be further explored for the treatment of diseases mediated by low levels of NAMPT and NAD+. Next, our findings identified IRW as an activator of mitochondrial biogenesis, with a close pharmacological resemblance to caloric resistance. Our research established IRW as the first

peptide which initiated the PGC1α pathway at basal levels in cells and rescued the HFD fed mice from oxidative and mitochondrial stress by promoting mitochondrial biogenesis. Further, to the best of our knowledge, IRW is the first bioactive to increase mitochondria abundance in plasma *in vivo*. Based on its ability to boost metabolism and mitochondrial number, our study showed the first bioactive peptide with the ability to extend lifespan *in vivo* and suggests an important prospective role of IRW intake for healthy aging in humans.

In conclusion, this thesis demonstrated the potential use of IRW as a functional food and/or nutraceutical for boosting metabolism, mitochondrial function, and lifespan extension via NAD^+ increase, mitochondrial biogenesis, and related anti-aging mechanisms.

6.3 Future Research Directions

It should be noted that there are various limitations in the research findings reported in this thesis. Firstly, we used only established cell lines for the in vitro studies (e.g., A7R5, L6, and 293T, etc.); validation of these effects of IRW should be made with human primary cells as well, such as primary smooth muscle cells. Additionally, the metabolism of IRW after oral administration and the resultant metabolites remain elusive. Thirdly, the NAMPT activation lacked detailed enzymatic analysis using enzyme kinetics. Next, the animal study animal was focused on mild obesity, and the impact of IRW on aging and obese mice was missed. Thus, it is difficult to precisely monitor the impact of IRW on aged and obese mice. As the IRW was mixed into the diet and could be taken ad libitum, the precise intake of IRW is not known and is estimated, but not accurate. Likewise, an extra group of mice with a normal diet and IRW incorporated could help understand the in vivo basal impact of IRW on mitochondrial biogenesis and NAMPT surge. Finally, in this study, IRW exhibited lifespan extension in a few normal (non-mutant) flies only with preserved physiological and metabolic function; however, no direct evidence indicated the ability of IRW to

prolong lifespan in physiologically challenged mutant flies. Also, I acknowledge that while drawing wholistic conclusions, not all parameters were, or could be, controlled for example, the impact of asphyxiation on animals while measuring mitochondrial parameters, number of limited replications and lack of detailed animal studies. Based on the key findings and the limitations of the research, the recommended future studies are outlined below:

1. Use of primary cells can help understand the pharmacological impact of IRW in a detailed manner. Primary cells, isolated directly from fresh tissue, display distinct physiology compared to the immortalized cell lines. Apart from the phenotypic differences, immortalized cell lines can lose physiological properties from modifications induced by cell passage. Although in this study, all cell lines were used between passage 3-9, yet the use of primary cells can help to further understand the beneficial impact of IRW *in vitro*.

2. Although detailed research on the pharmacological impact of IRW has been conducted, yet metabolism of IRW is not fully understood. As a tripeptide, oral administration of IRW will go through changes in the gastrointestinal tract and may be digested into a dipeptide and an amino acid. The bioavailability of IRW and the remaining intact or broken IRW after digestion should be studied to estimate the effective dosage of IRW to exert beneficial effects *in vivo*. As a pig's GI tract bears a close resemblance to human physiology, a piglet model might provide insights into the metabolism of ovotransferrin. Further, it remains to be investigated if the digested metabolites of IRW are responsible for the physiological activity after oral administration. Therefore, a comparative study between the parent molecule and digested metabolites should be conducted in the future as well.

3. NAMPT enzyme kinetics can further help us understand the IRW's ability to activate this enzyme. Briefly, enzyme kinetics analysis can help us determine the speed at which an enzyme-

catalyzed chemical reaction proceeds. As the speed of the reaction depends on the amount of the enzyme and substrate used in the reaction. Therefore, in presence of IRW, kinetic analysis can help understand the V_{max} and K_M for IRW.

4. Next, it will be interesting to observe the impact of IRW on NAD^+/NAMPT axis and mitochondrial biogenesis in a mouse model of aging. Various mouse models of aging are available and can aid drug discovery to counter provide aging process. Multiple aging models such as $Wrn^{-/-}$, $Wrn^{-/-}Terc^{-/-}$, $PolgA^{mut/mut}$ can be used to investigate anti-aging mechanisms of IRW. Interestingly, these mouse models exhibit insulin resistance, elevated triglycerides, weak bone health, and lifespan depletion, all key features of human aging (Kõks, Dogan, Tuna, González-Navarro, Potter, & Vandenbroucke, 2016).

5. Measurement of mitochondrial number in vivo is a challenge. Currently, there is no direct protocol to measure it in live animals, except for plasma. However, NMR and optical spectroscopy tools can be employed to measure both phosphorylation and oxidation sides of mitochondrial energetics. ^{13}C NMR can be used to quantify mitochondrial respiration by measuring flux through the TCA cycle (Befroy, Petersen, Rothman, & Shulman, 2009). Optical spectroscopy can be used to measure oxygenation of skeletal muscle oxygen store as a function of mitochondrial health. This can display key new insights into changes in mitochondrial capacity and quality following treatment with IRW.

6. *Drosophila melanogaster* has a long scientific history of drug discovery in research on mitochondria, metabolism, and aging. Cellular pathways, particularly related to insulin, mTOR, Nrf2, DNAAM, SIRT1, and AMPK can all modify *Drosophila* longevity and impact its metabolic and mitochondrial health (Piper & Partridge, 2018). Therefore, the use of mutant flies devoid of normal workings of these key pathways can help elucidate the underlying pathways which help

IRW`s pharmacological efficacy. Also, in line with mice microbiome study, fly gut function, a key to fly longevity, the impact of IRW should be assessed on the fly microbiome in future studies as well.

6.4 References

Anderson, R. M., Bitterman, K. J., Wood, J. G., Medvedik, O., & Sinclair, D. A. (2003). Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae. *Nature, 423*(6936), 181-185.

Balan, V., Miller, G. S., Kaplun, L., Balan, K., Chong, Z.-Z., Li, F., . . . Freeman, D. C. (2008). Life span extension and neuronal cell protection by Drosophila nicotinamidase. *Journal of Biological Chemistry, 283*(41), 27810-27819.

Befroy, D. E., Petersen, K. F., Rothman, D. L., & Shulman, G. I. (2009). Assessment of in vivo mitochondrial metabolism by magnetic resonance spectroscopy. *Methods in enzymology, 457*, 373-393.

Bhullar, K. S., & Hubbard, B. P. (2015). Lifespan and healthspan extension by resveratrol. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1852*(6), 1209-1218.

Bhullar, K. S., Son, M., Kerek, E., Cromwell, C. R., Wingert, B. M., Wu, K., . . . Wu, J. (2021). Tripeptide IRW Upregulates NAMPT Protein Levels in Cells and Obese C57BL/6J Mice. *Journal of Agricultural and Food Chemistry, 69*(5), 1555-1566.

Chandel, N. S. (2021). Mitochondria. *Cold Spring Harbor Perspectives in Biology, 13*(3), a040543.

Chen, C., Zhou, M., Ge, Y., & Wang, X. (2020). SIRT1 and aging related signaling pathways. *Mechanisms of ageing and development, 187*, 111215.

Das, A., Huang, G. X., Bonkowski, M. S., Longchamp, A., Li, C., Schultz, M. B., . . . Lu, Y. (2018). Impairment of an endothelial NAD+-H2S signaling network is a reversible cause of vascular aging. *Cell, 173*(1), 74-89. e20.

Fang, E. F., Kassahun, H., Croteau, D. L., Scheibye-Knudsen, M., Marosi, K., Lu, H., . . . Wilson, M. A. (2016). NAD+ replenishment improves lifespan and healthspan in ataxia telangiectasia models via mitophagy and DNA repair. *Cell metabolism, 24*(4), 566-581.

Frederick, D. W., Loro, E., Liu, L., Davila Jr, A., Chellappa, K., Silverman, I. M., . . . Davis, J. G. (2016). Loss of NAD homeostasis leads to progressive and reversible degeneration of skeletal muscle. *Cell metabolism, 24*(2), 269-282.

Giannakou, M. E., & Partridge, L. (2007). Role of insulin-like signalling in Drosophila lifespan. *Trends in biochemical sciences, 32*(4), 180-188.

Harman, E. (1956). Protein oxidation in aging and age-related diseases. *Gerontology, 11*, 298-300.

Hashimoto, T., Horikawa, M., Nomura, T., & Sakamoto, K. (2010). Nicotinamide adenine dinucleotide extends the lifespan of Caenorhabditis elegans mediated by sir-2.1 and daf-16. *Biogerontology, 11*(1), 31-43.

Hashimoto, Y., Ookuma, S., & Nishida, E. (2009). Lifespan extension by suppression of autophagy genes in Caenorhabditis elegans. *Genes to Cells, 14*(6), 717-726.

Johannsen, D. L., & Ravussin, E. (2009). The role of mitochondria in health and disease. *Current opinion in pharmacology, 9*(6), 780-786.

Kõks, S., Dogan, S., Tuna, B. G., González-Navarro, H., Potter, P., & Vandenbroucke, R. E. (2016). Mouse models of ageing and their relevance to disease. *Mechanisms of ageing and development, 160*, 41-53.

Lee, C. F., Caudal, A., Abell, L., Gowda, G. N., & Tian, R. (2019). Targeting NAD+ metabolism as interventions for mitochondrial disease. *Scientific reports, 9*(1), 1-10.

Li, J., Bonkowski, M. S., Moniot, S., Zhang, D., Hubbard, B. P., Ling, A. J., . . . Gorbunova, V. (2017). A conserved NAD+ binding pocket that regulates protein-protein interactions during aging. *Science, 355*(6331), 1312-1317.

Liling, S., Roska, T. P., Arfiansyah, R., Maryam, F., & Nainu, F. (2021). Pharmacological Effect of Muntingia calabura Leaves on the Expression of sod1 and sod2 in Drosophila.

Mitsui, A., Hamuro, J., Nakamura, H., Kondo, N., Hirabayashi, Y., Ishizaki-Koizumi, S., . . . Yodoi, J. (2002). Overexpression of human thioredoxin in transgenic mice controls oxidative stress and life span. *Antioxidants and Redox Signaling, 4*(4), 693-696.

Parkes, T. L., Elia, A. J., Dickinson, D., Hilliker, A. J., Phillips, J. P., & Boulianne, G. L. (1998). Extension of Drosophila lifespan by overexpression of human SOD1 in motorneurons. *Nature genetics, 19*(2), 171-174.

Picard, M., Wallace, D. C., & Burelle, Y. (2016). The rise of mitochondria in medicine. *Mitochondrion, 30*, 105-116.

Piper, M. D., & Partridge, L. (2018). Drosophila as a model for ageing. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1864*(9), 2707-2717.

Shi, H., Enriquez, A., Rapadas, M., Martin, E. M., Wang, R., Moreau, J., . . . Hughes, J. N. (2017). NAD deficiency, congenital malformations, and niacin supplementation. *New England Journal of Medicine, 377*(6), 544-552.

Yang, H., Lavu, S., & Sinclair, D. A. (2006). Nampt/PBEF/Visfatin: a regulator of mammalian health and longevity? *Experimental gerontology, 41*(8), 718-726.

BIBLIOGRAPHY

Al Amir Dache, Z., Otandault, A., Tanos, R., Pastor, B., Meddeb, R., Sanchez, C., Grange, T. (2020). Blood contains circulating cell-free respiratory competent mitochondria. *The FASEB Journal, 34*(3), 3616-3630.

Alexeyev, M. F. (2009). Is there more to aging than mitochondrial DNA and reactive oxygen species? *The FEBS journal, 276*(20), 5768-5787.

Anderson, R. M., Bitterman, K. J., Wood, J. G., Medvedik, O., & Sinclair, D. A. (2003). Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae. *Nature, 423*(6936), 181-185.

Angelova, P. R., & Abramov, A. Y. (2018). Role of mitochondrial ROS in the brain: from physiology to neurodegeneration. *FEBS letters, 592*(5), 692-702.

Ansari, H. R., & Raghava, G. P. (2010). Identification of NAD interacting residues in proteins. *BMC bioinformatics, 11*(1), 160.

Aparicio, R., Rana, A., & Walker, D. W. (2019). Upregulation of the autophagy adaptor p62/SQSTM1 prolongs health and lifespan in middle-aged Drosophila. *Cell reports, 28*(4), 1029-1040. e1025.

Araki, T., Sasaki, Y., & Milbrandt, J. (2004). Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science, 305*(5686), 1010-1013.

Archer, E., Hand, G. A., & Blair, S. N. (2013). Validity of US nutritional surveillance: National Health and Nutrition Examination Survey caloric energy intake data, 1971–2010. *PloS one, 8*(10), e76632.

Arenas-Jal, M., Suñé-Negre, J., Pérez-Lozano, P., & García-Montoya, E. (2020). Trends in the food and sports nutrition industry: A review. *Critical reviews in food science and nutrition, 60*(14), 2405-2421.

Baars, J. (2012). *Aging and the Art of Living*: JHU Press.

Badawy, A. A. (2017). Kynurenine pathway of tryptophan metabolism: regulatory and functional aspects. *International Journal of Tryptophan Research, 10*, 1178646917691938.

Balan, V., Miller, G. S., Kaplun, L., Balan, K., Chong, Z.-Z., Li, F., Freeman, D. C. (2008). Life span extension and neuronal cell protection by Drosophila nicotinamidase. *Journal of Biological Chemistry, 283*(41), 27810-27819.

Banfield, E. C., Liu, Y., Davis, J. S., Chang, S., & Frazier-Wood, A. C. (2016). Poor adherence to US dietary guidelines for children and adolescents in the national health and nutrition examination survey population. *Journal of the Academy of Nutrition and Dietetics, 116*(1), 21-27.

Bang, J. S., Jin, Y. J., & Choung, S.-Y. (2020). Low molecular polypeptide from oyster hydrolysate recovers photoaging in skh-1 hairless mice. *Toxicology and applied pharmacology, 386*, 114844.

Baur, J. A., Pearson, K. J., Price, N. L., Jamieson, H. A., Lerin, C., Kalra, A., Lewis, K. (2006). Resveratrol improves health and survival of mice on a high-calorie diet. *Nature, 444*(7117), 337-342.

Befroy, D. E., Petersen, K. F., Rothman, D. L., & Shulman, G. I. (2009). Assessment of in vivo mitochondrial metabolism by magnetic resonance spectroscopy. *Methods in enzymology, 457*, 373-393.

Benoit, S., Chaumontet, C., Schwarz, J., Cakir-Kiefer, C., Boulier, A., Tomé, D., & Miclo, L. (2020). Anxiolytic Activity and Brain Modulation Pattern of the α-Casozepine-Derived Pentapeptide YLGYL in Mice. *Nutrients, 12*(5), 1497.

Berger, F., Lau, C., Dahlmann, M., & Ziegler, M. (2005). Subcellular compartmentation and differential catalytic properties of the three human nicotinamide mononucleotide adenylyltransferase isoforms. *Journal of Biological Chemistry, 280*(43), 36334-36341.

Berndt, J., Klöting, N., Kralisch, S., Kovacs, P., Fasshauer, M., Schön, M. R., Blüher, M. (2005). Plasma visfatin concentrations and fat depot–specific mRNA expression in humans. *Diabetes, 54*(10), 2911-2916.

Besse-Patin, A., Jeromson, S., Levesque-Damphousse, P., Secco, B., Laplante, M., & Estall, J. L. (2019). PGC1A regulates the IRS1: IRS2 ratio during fasting to influence hepatic metabolism downstream of insulin. *Proceedings of the National Academy of Sciences, 116*(10), 4285-4290.

Bhullar, K. S., & Hubbard, B. P. (2015). Lifespan and healthspan extension by resveratrol. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1852*(6), 1209-1218.

Bhullar, K. S., & Wu, J. (2020). Dietary peptides in aging: Evidence and prospects. *Food Science and Human Wellness, 9*(1), 1-7.

Bhullar, K. S., Son, M., Kerek, E., Cromwell, C. R., Wingert, B. M., Wu, K., Wu, J. (2021). Tripeptide IRW Upregulates NAMPT Protein Levels in Cells and Obese C57BL/6J Mice.

Blüher, M. (2019). Obesity: global epidemiology and pathogenesis. Nature Reviews Endocrinology, 288e298.

Bonkowski, M. S., & Sinclair, D. A. (2016). Slowing ageing by design: the rise of NAD+ and sirtuin-activating compounds. *Nature reviews Molecular cell biology, 17*(11), 679.

Bowlby, S. C., Thomas, M. J., D'Agostino Jr, R. B., & Kridel, S. J. (2012). Nicotinamide phosphoribosyl transferase (Nampt) is required for de novo lipogenesis in tumor cells. *PloS one, 7*(6), e40195.

Braidy, N., Berg, J., Clement, J., Khorshidi, F., Poljak, A., Jayasena, T., Sachdev, P. (2019). Role of nicotinamide adenine dinucleotide and related precursors as therapeutic targets for age-related degenerative diseases: rationale, biochemistry, pharmacokinetics, and outcomes. *Antioxidants & redox signaling, 30*(2), 251-294.

Braidy, N., Guillemin, G. J., Mansour, H., Chan-Ling, T., Poljak, A., & Grant, R. (2011). Age related changes in NAD+ metabolism oxidative stress and Sirt1 activity in wistar rats. *PloS one, 6*(4), e19194.

Bray, N. L., Pimentel, H., Melsted, P., & Pachter, L. (2016). Near-optimal probabilistic RNA-seq quantification. *Nature biotechnology, 34*(5), 525-527.

Brazill, J. M., Li, C., Zhu, Y., & Zhai, R. G. (2017). NMNAT: It's an NAD+ synthase… It'sa chaperone… It'sa neuroprotector. *Current opinion in genetics & development, 44*, 156-162.

Burkart, A. M., Tan, K., Warren, L., Iovino, S., Hughes, K. J., Kahn, C. R., & Patti, M.-E. (2016). Insulin resistance in human iPS cells reduces mitochondrial size and function. *Scientific reports, 6*(1), 1-12.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Shipley, G. L. (2009). The MIQE Guidelines: M inimum I nformation for Publication of Q uantitative Real-Time PCR E xperiments. Oxford University Press.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Shipley, G. L. (2009). The MIQE Guidelines: M inimum I nformation for Publication of Q uantitative Real-Time PCR E xperiments. Oxford University Press.

Cahová, H., Winz, M.-L., Höfer, K., Nübel, G., & Jäschke, A. (2015). NAD captureSeq indicates NAD as a bacterial cap for a subset of regulatory RNAs. *Nature, 519*(7543), 374-377.

Camacho-Pereira, J., Tarragó, M. G., Chini, C. C., Nin, V., Escande, C., Warner, G. M., Galina, A. (2016). CD38 dictates age-related NAD decline and mitochondrial dysfunction through an SIRT3-dependent mechanism. *Cell metabolism, 23*(6), 1127-1139.

Cambronne, X. A., & Kraus, W. L. (2020). Location, Location, Location: Compartmentalization of NAD+ Synthesis and Functions in Mammalian Cells. *Trends in biochemical sciences*.

Campisi, J. (2013). Aging, cellular senescence, and cancer. *Annual review of physiology, 75*, 685-705.

Cantó, C., Houtkooper, R. H., Pirinen, E., Youn, D. Y., Oosterveer, M. H., Cen, Y., Cettour-Rose, P. (2012). The NAD+ precursor nicotinamide riboside enhances oxidative metabolism and protects against high-fat diet-induced obesity. *Cell metabolism, 15*(6), 838-847.

Cantó, C., Menzies, K. J., & Auwerx, J. (2015). NAD+ metabolism and the control of energy homeostasis: a balancing act between mitochondria and the nucleus. *Cell metabolism, 22*(1), 31-53.

Carré, J. E., Orban, J.-C., Re, L., Felsmann, K., Iffert, W., Bauer, M., Breen, P. (2010). Survival in critical illness is associated with early activation of mitochondrial biogenesis. *American journal of respiratory and critical care medicine, 182*(6), 745-751.

Cartee, G. D., Hepple, R. T., Bamman, M. M., & Zierath, J. R. (2016). Exercise promotes healthy aging of skeletal muscle. *Cell metabolism, 23*(6), 1034-1047.

Chacinska, A., van der Laan, M., Mehnert, C. S., Guiard, B., Mick, D. U., Hutu, D. P., Pfanner, N. (2010). Distinct forms of mitochondrial TOM-TIM supercomplexes define signal-dependent states of preprotein sorting. *Molecular and cellular biology, 30*(1), 307-318.

Chambon, P., Weill, J., & Mandel, P. (1963). Nicotinamide mononucleotide activation of a new DNA-dependent polyadenylic acid synthesizing nuclear enzyme. *Biochemical and biophysical research communications, 11*(1), 39-43.

Chandel, N. S. (2018). Mitochondria: back to the future. *Nature reviews Molecular cell biology, 19*(2), 76-76.

Chandel, N. S. (2021). Mitochondria. *Cold Spring Harbor Perspectives in Biology, 13*(3), a040543.

Chandel, N., Maltepe, E., Goldwasser, E., Mathieu, C., Simon, M., & Schumacker, P. (1998). Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proceedings of the National Academy of Sciences, 95*(20), 11715-11720.

Chang, Y. H., Chang, D. M., Lin, K. C., Shin, S. J., & Lee, Y. J. (2011). Visfatin in overweight/obesity, type 2 diabetes mellitus, insulin resistance, metabolic syndrome and cardiovascular diseases: a meta-analysis and systemic review. *Diabetes/metabolism research and reviews, 27*(6), 515-527.

Chen, C., Zhou, M., Ge, Y., & Wang, X. (2020). SIRT1 and aging related signaling pathways. *Mechanisms of ageing and development, 187*, 111215.

Chen, C.-C., Carson, J. J., Feser, J., Tamburini, B., Zabaronick, S., Linger, J., & Tyler, J. K. (2008). Acetylated lysine 56 on histone H3 drives chromatin assembly after repair and signals for the completion of repair. *Cell, 134*(2), 231-243.

Chen, J., Ryu, B., Zhang, Y., Liang, P., Li, C., Zhou, C., Qian, Z. J. (2020). Comparison of an angiotensin-I-converting enzyme inhibitory peptide from tilapia (Oreochromis niloticus) with captopril: inhibition kinetics, in vivo effect, simulated gastrointestinal digestion and a molecular docking study. *Journal of the Science of Food and Agriculture, 100*(1), 315-324.

Chen, Y. G., Kowtoniuk, W. E., Agarwal, I., Shen, Y., & Liu, D. R. (2009). LC/MS analysis of cellular RNA reveals NAD-linked RNA. *Nature chemical biology, 5*(12), 879-881.

Chidambaram, S. B., Bhat, A., Ray, B., Sugumar, M., Muthukumar, S. P., Manivasagam, TSakharkar, M. K. (2020). Cocoa beans improve mitochondrial biogenesis via PPARγ/PGC1α dependent signalling pathway in MPP+ intoxicated human neuroblastoma cells (SH-SY5Y). *Nutritional neuroscience, 23*(6), 471-480.

Chini, C. C., Tarragó, M. G., & Chini, E. N. (2017). NAD and the aging process: Role in life, death and everything in between. *Molecular and cellular endocrinology, 455*, 62-74.

Chung, J. H., Manganiello, V., & Dyck, J. R. (2012). Resveratrol as a calorie restriction mimetic: therapeutic implications. *Trends in cell biology, 22*(10), 546-554.

Clement, J., Wong, M., Poljak, A., Sachdev, P., & Braidy, N. (2019). The plasma NAD+ metabolome is dysregulated in "normal" aging. *Rejuvenation research, 22*(2), 121-130.

Corona, J. C., & Duchen, M. R. (2015). PPARγ and PGC-1α as therapeutic targets in Parkinson's. *Neurochemical research, 40*(2), 308-316.

Cromwell, C. R., Sung, K., Park, J., Krysler, A. R., Jovel, J., Kim, S. K., & Hubbard, B. P. (2018). Incorporation of bridged nucleic acids into CRISPR RNAs improves Cas9 endonuclease specificity. *Nature communications, 9*(1), 1-11.

Dai, H., Sinclair, D. A., Ellis, J. L., & Steegborn, C. (2018). Sirtuin activators and inhibitors: promises, achievements, and challenges. *Pharmacology & therapeutics, 188*, 140-154.

Daroit, D. J., & Brandelli, A. (2021). In vivo bioactivities of food protein-derived peptides–A current review. *Current Opinion in Food Science*.

Das, A., Huang, G. X., Bonkowski, M. S., Longchamp, A., Li, C., Schultz, M. B., Lu, Y. (2018). Impairment of an endothelial NAD+-H2S signaling network is a reversible cause of vascular aging. *Cell, 173*(1), 74-89. e20.

Davila, A., Liu, L., Chellappa, K., Redpath, P., Nakamaru-Ogiso, E., Paolella, L. M., Baur, J. A. (2018). Nicotinamide adenine dinucleotide is transported into mammalian mitochondria. *Elife, 7*, e33246.

Davinelli, S., De Stefani, D., De Vivo, I., & Scapagnini, G. (2020). Polyphenols as caloric restriction mimetics regulating mitochondrial biogenesis and mitophagy. *Trends in Endocrinology & Metabolism, 31*(7), 536-550.

de Boer, J. F., Bahr, M. J., Boker, K. H., Manns, M. P., & Tietge, U. J. (2009). Plasma levels of PBEF/Nampt/visfatin are decreased in patients with liver cirrhosis. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 296*(2), G196-G201.

de la Rubia, J. E., Drehmer, E., Platero, J. L., Benlloch, M., Caplliure-Llopis, J., Villaron-Casales, C., Carrera, S. (2019). Efficacy and tolerability of EH301 for amyotrophic lateral sclerosis: a randomized, double-blind, placebo-controlled human pilot study. *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration, 20*(1-2), 115-122.

de Picciotto, N. E., Gano, L. B., Johnson, L. C., Martens, C. R., Sindler, A. L., Mills, K. F., Seals, D. R. (2016). Nicotinamide mononucleotide supplementation reverses vascular dysfunction and oxidative stress with aging in mice. *Aging cell, 15*(3), 522-530.

Degli Esposti, M., Chouaia, B., Comandatore, F., Crotti, E., Sassera, D., Lievens, P. M.-J., Bandi, C. (2014). Evolution of mitochondria reconstructed from the energy metabolism of living bacteria. *PloS one, 9*(5), e96566.

Dhar, S. S., Ongwijitwat, S., & Wong-Riley, M. T. (2008). Nuclear respiratory factor 1 regulates all ten nuclear-encoded subunits of cytochrome c oxidase in neurons. *Journal of Biological Chemistry, 283*(6), 3120-3129.

Dillon, L. M., Williams, S. L., Hida, A., Peacock, J. D., Prolla, T. A., Lincoln, J., & Moraes, C. T. (2012). Increased mitochondrial biogenesis in muscle improves aging phenotypes in the mtDNA mutator mouse. *Human molecular genetics, 21*(10), 2288-2297.

Dolezal, P., Likic, V., Tachezy, J., & Lithgow, T. (2006). Evolution of the molecular machines for protein import into mitochondria. *Science, 313*(5785), 314-318.

Dollerup, O. L., Christensen, B., Svart, M., Schmidt, M. S., Sulek, K., Ringgaard, S., Treebak, J. T. (2018). A randomized placebo-controlled clinical trial of nicotinamide riboside in obese men: safety, insulin-sensitivity, and lipid-mobilizing effects. *The American journal of clinical nutrition, 108*(2), 343-353.

Dollerup, O. L., Trammell, S. A., Hartmann, B., Holst, J. J., Christensen, B., Møller, N., Jessen, N. (2019). Effects of nicotinamide riboside on endocrine pancreatic function and incretin hormones in nondiabetic men with obesity. *The Journal of Clinical Endocrinology & Metabolism, 104*(11), 5703-5714.

Dumeus, S., Shibu, M. A., Lin, W.-T., Wang, M.-F., Lai, C.-H., Shen, C.-Y., Huang, C.-Y. (2018). Bioactive peptide improves diet-induced hepatic fat deposition and hepatocyte proinflammatory response in SAMP8 ageing mice. *Cellular Physiology and Biochemistry, 48*(5), 1942-1952.

Effros, R. B. (2005). Roy Walford and the immunologic theory of aging. *Immunity & Ageing, 2*(1), 1-3.

Eitan, E., Tichon, A., Gazit, A., Gitler, D., Slavin, S., & Priel, E. (2012). Novel telomerase-increasing compound in mouse brain delays the onset of amyotrophic lateral sclerosis. *EMBO molecular medicine, 4*(4), 313-329.

Erion, D. M., & Shulman, G. I. (2010). Diacylglycerol-mediated insulin resistance. *Nature medicine, 16*(4), 400-402.

Fang, E. F., Kassahun, H., Croteau, D. L., Scheibye-Knudsen, M., Marosi, K., Lu, H., Wilson, M. A. (2016). NAD+ replenishment improves lifespan and healthspan in ataxia telangiectasia models via mitophagy and DNA repair. *Cell metabolism, 24*(4), 566-581.

Fang, E. F., Lautrup, S., Hou, Y., Demarest, T. G., Croteau, D. L., Mattson, M. P., & Bohr, V. A. (2017). NAD+ in aging: molecular mechanisms and translational implications. *Trends in molecular medicine, 23*(10), 899-916.

Feng, Z., Bai, L., Yan, J., Li, Y., Shen, W., Wang, Y., Chen, Y. (2011). Mitochondrial dynamic remodeling in strenuous exercise-induced muscle and mitochondrial dysfunction: regulatory effects of hydroxytyrosol. *Free Radical Biology and Medicine, 50*(10), 1437-1446.

Feser, J., Truong, D., Das, C., Carson, J. J., Kieft, J., Harkness, T., & Tyler, J. K. (2010). Elevated histone expression promotes life span extension. *Molecular cell, 39*(5), 724-735.

Fletcher, R. S., Ratajczak, J., Doig, C. L., Oakey, L. A., Callingham, R., Xavier, G. D. S., Migaud, M. E. (2017). Nicotinamide riboside kinases display redundancy in mediating nicotinamide mononucleotide and nicotinamide riboside metabolism in skeletal muscle cells. *Molecular metabolism, 6*(8), 819-832.

Fontecha-Barriuso, M., Martin-Sanchez, D., Martinez-Moreno, J. M., Monsalve, M., Ramos, A. M., Sanchez-Niño, M. D., Sanz, A. B. (2020). The role of PGC-1α and mitochondrial biogenesis in kidney diseases. *Biomolecules, 10*(2), 347.

Frederick, D. W., Loro, E., Liu, L., Davila Jr, A., Chellappa, K., Silverman, I. M., Davis, J. G. (2016). Loss of NAD homeostasis leads to progressive and reversible degeneration of skeletal muscle. *Cell metabolism, 24*(2), 269-282.

Friebe, D., Neef, M., Kratzsch, J., Erbs, S., Dittrich, K., Garten, A., Stumvoll, M. (2011). Leucocytes are a major source of circulating nicotinamide phosphoribosyltransferase (NAMPT)/pre-B cell colony (PBEF)/visfatin linking obesity and inflammation in humans. *Diabetologia, 54*(5), 1200-1211.

Frydelund-Larsen, L., Akerstrom, T., Nielsen, S., Keller, P., Keller, C., & Pedersen, B. K. (2007). Visfatin mRNA expression in human subcutaneous adipose tissue is regulated by exercise. *American Journal of Physiology-Endocrinology and Metabolism, 292*(1), E24-E31.

Frye, R. A. (1999). Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. *Biochemical and biophysical research communications, 260*(1), 273-279.

Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Shimomura, I. (2017). Increased oxidative stress in obesity and its impact on metabolic syndrome. *The Journal of clinical investigation, 114*(12), 1752-1761.

Galloway, C. A., Lee, H., Nejjar, S., Jhun, B. S., Yu, T., Hsu, W., & Yoon, Y. (2012). Transgenic control of mitochondrial fission induces mitochondrial uncoupling and relieves diabetic oxidative stress. *Diabetes, 61*(8), 2093-2104.

Gardell, S. J., Hopf, M., Khan, A., Dispagna, M., Sessions, E. H., Falter, R., Petucci, C. (2019). Boosting NAD+ with a small molecule that activates NAMPT. *Nature communications, 10*(1), 1-12.

Gerhart-Hines, Z., Rodgers, J. T., Bare, O., Lerin, C., Kim, S. H., Mostoslavsky, R., Puigserver, P. (2007). Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1α. *The EMBO journal, 26*(7), 1913-1923.

Giannakou, M. E., & Partridge, L. (2007). Role of insulin-like signalling in Drosophila lifespan. *Trends in biochemical sciences, 32*(4), 180-188.

Giordano, C., Iommarini, L., Giordano, L., Maresca, A., Pisano, A., Valentino, M. L., Roberti, M. (2014). Efficient mitochondrial biogenesis drives incomplete penetrance in Leber's hereditary optic neuropathy. *Brain, 137*(2), 335-353.

Giromini, C., Cheli, F., Rebucci, R., & Baldi, A. (2019). Invited review: Dairy proteins and bioactive peptides: Modeling digestion and the intestinal barrier. *Journal of dairy science, 102*(2), 929-942.

Gomes, A. P., Price, N. L., Ling, A. J., Moslehi, J. J., Montgomery, M. K., Rajman, L., B. P. (2013). Declining NAD+ induces a pseudohypoxic state disrupting nuclear-mitochondrial communication during aging. *Cell, 155*(7), 1624-1638.

Gorman, G. S., Chinnery, P. F., DiMauro, S., Hirano, M., Koga, Y., McFarland, R., Turnbull, D. M. (2016). Mitochondrial diseases. *Nature reviews Disease primers, 2*(1), 1-22.

Gower, B. A., & Casazza, K. (2013). Divergent effects of obesity on bone health. *Journal of clinical densitometry, 16*(4), 450-454.

Haas, R. H. (2019). Mitochondrial dysfunction in aging and diseases of aging. Multidisciplinary Digital Publishing Institute.

Hamidie, R. D. R., Yamada, T., Ishizawa, R., Saito, Y., & Masuda, K. (2015). Curcumin treatment enhances the effect of exercise on mitochondrial biogenesis in skeletal muscle by increasing cAMP levels. *Metabolism, 64*(10), 1334-1347.

Hamley, I. W. (2017). Small bioactive peptides for biomaterials design and therapeutics. *Chemical reviews, 117*(24), 14015-14041.

Harbauer, A. B., Opalińska, M., Gerbeth, C., Herman, J. S., Rao, S., Schönfisch, B., Meisinger, C. (2014). Cell cycle–dependent regulation of mitochondrial preprotein translocase. *Science, 346*(6213), 1109-1113.

Harden, A., & Young, W. J. (1906). The alcoholic ferment of yeast-juice. Part II.—The coferment of yeast-juice. *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character, 78*(526), 369-375.

Harley, C. B., Liu, W., Flom, P. L., & Raffaele, J. M. (2013). A natural product telomerase activator as part of a health maintenance program: metabolic and cardiovascular response. *Rejuvenation research, 16*(5), 386-395.

Harman, D. (1981). The aging process. *Proceedings of the National Academy of Sciences, 78*(11), 7124-7128.

Harman, D. (1993). Free radical involvement in aging. *Drugs & aging, 3*(1), 60-80.

Harman, E. (1956). Protein oxidation in aging and age-related diseases. *Gerontology, 11*, 298-300.

Harrison, D. E., Strong, R., Sharp, Z. D., Nelson, J. F., Astle, C. M., Flurkey, K., Carter, C. S. (2009). Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature, 460*(7253), 392-395.

Hashimoto, T., Horikawa, M., Nomura, T., & Sakamoto, K. (2010). Nicotinamide adenine dinucleotide extends the lifespan of Caenorhabditis elegans mediated by sir-2.1 and daf-16. *Biogerontology, 11*(1), 31-43.

Hashimoto, Y., Ookuma, S., & Nishida, E. (2009). Lifespan extension by suppression of autophagy genes in Caenorhabditis elegans. *Genes to Cells, 14*(6), 717-726.

He, R., Wang, Y., Yang, Y., Wang, Z., Ju, X., & Yuan, J. (2019). Rapeseed protein-derived ACE inhibitory peptides LY, RALP and GHS show antioxidant and anti-inflammatory effects on spontaneously hypertensive rats. *Journal of Functional Foods, 55*, 211-219.

He, R., Yang, Y.-J., Wang, Z., Xing, C.-r., Yuan, J., Wang, L.-F., Ju, X.-R. (2019). Rapeseed protein-derived peptides, LY, RALP, and GHS, modulates key enzymes and intermediate products of renin–angiotensin system pathway in spontaneously hypertensive rat. *NPJ science of food, 3*(1), 1-6.

Henriksen, E. J., Diamond-Stanic, M. K., & Marchionne, E. M. (2011). Oxidative stress and the etiology of insulin resistance and type 2 diabetes. *Free Radical Biology and Medicine, 51*(5), 993-999.

Hincke, M. T., Nys, Y., Gautron, J., Mann, K., Rodriguez-Navarro, A. B., & McKee, M. D. (2012). The eggshell: structure, composition and mineralization. *Front Biosci, 17*(1), 1266-1280.

Hinton, T. (1956). The effects of arginine, ornithine and citrulline on the growth of Drosophila. *Archives of biochemistry and biophysics, 62*(1), 78-85.

Hirano, M., Emmanuele, V., & Quinzii, C. M. (2018). Emerging therapies for mitochondrial diseases. *Essays in biochemistry, 62*(3), 467-481.

Houtkooper, R. H., Cantó, C., Wanders, R. J., & Auwerx, J. (2010). The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. *Endocrine reviews, 31*(2), 194-223.

Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., Zhang, L.-L. (2003). Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature, 425*(6954), 191-196.

Hua, X., Sun, D. Y., Zhang, W. J., Fu, J. T., Tong, J., Sun, S. J., Wang, S. N. (2020). P7C3-A20 alleviates fatty liver by shaping gut microbiota and inducing FGF21/FGF1, via the AMP-activated protein kinase/CREB regulated transcription coactivator 2 pathway. *British Journal of Pharmacology*.

Huang, C. Y., Nithiyanantham, S., Liao, J. Y., & Lin, W. T. (2020). Bioactive peptides attenuate cardiac hypertrophy and fibrosis in spontaneously hypertensive rat hearts. *Journal of food and drug analysis, 28*(1), 94-102.

Huang, W., Chakrabarti, S., Majumder, K., Jiang, Y., Davidge, S. T., & Wu, J. (2010). Egg-derived peptide IRW inhibits TNF-α-induced inflammatory response and oxidative stress in endothelial cells. *Journal of Agricultural and Food Chemistry, 58*(20), 10840-10846.

Huang, W.-Y., Majumder, K., & Wu, J. (2010). Oxygen radical absorbance capacity of peptides from egg white protein ovotransferrin and their interaction with phytochemicals. *Food Chemistry, 123*(3), 635-641.

Huo, L., & Scarpulla, R. C. (2001). Mitochondrial DNA instability and peri-implantation lethality associated with targeted disruption of nuclear respiratory factor 1 in mice. *Molecular and cellular biology, 21*(2), 644-654.

Hyde, R. R. (1913). Inheritance of the length of life in Drosophila ampelophila. *Proceedings of the Indiana Academy of Science* (Vol. 23, pp. 113-124).

Imai, S.-i. (2009). Nicotinamide phosphoribosyltransferase (Nampt): a link between NAD biology, metabolism, and diseases. *Current pharmaceutical design, 15*(1), 20-28.

Imai, S.-i. (2010). A possibility of nutriceuticals as an anti-aging intervention: activation of sirtuins by promoting mammalian NAD biosynthesis. *Pharmacological research, 62*(1), 42-47.

Imai, S.-I., Armstrong, C. M., Kaeberlein, M., & Guarente, L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature, 403*(6771), 795-800.

Jäger, S., Handschin, C., Pierre, J. S.-., & Spiegelman, B. M. (2007). AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α. *Proceedings of the National Academy of Sciences, 104*(29), 12017-12022.

Johannsen, D. L., & Ravussin, E. (2009). The role of mitochondria in health and disease. *Current opinion in pharmacology, 9*(6), 780-786.

Johnson, F. B., Sinclair, D. A., & Guarente, L. (1999). Molecular biology of aging. *Cell, 96*(2), 291-302.

Johnson, S., & Imai, S. i. (2018). NAD+ biosynthesis, aging, and disease. *F1000Research, 7*.

Jones, A. W., Yao, Z., Vicencio, J. M., Karkucinska-Wieckowska, A., & Szabadkai, G. (2012). PGC-1 family coactivators and cell fate: roles in cancer, neurodegeneration, cardiovascular disease and retrograde mitochondria–nucleus signalling. *Mitochondrion, 12*(1), 86-99.

Ju, D.-T., Kuo, W.-W., Ho, T.-J., Chang, R.-L., Lin, W.-T., Day, C. H., Huang, C.-Y. (2019). Bioactive peptide vhvv upregulates the long-term memory-related biomarkers in adult spontaneously hypertensive rats. *International journal of molecular sciences, 20*(12), 3069.

Juhász, G., Érdi, B., Sass, M., & Neufeld, T. P. (2007). Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila. *Genes & development, 21*(23), 3061-3066.

Jung, H.-Y., Lee, D., Ryu, H. G., Choi, B.-H., Go, Y., Lee, N., Kim, S.-H. (2017). Myricetin improves endurance capacity and mitochondrial density by activating SIRT1 and PGC-1α. *Scientific reports, 7*(1), 1-10.

Kanfi, Y., Naiman, S., Amir, G., Peshti, V., Zinman, G., Nahum, L., Cohen, H. Y. (2012). The sirtuin SIRT6 regulates lifespan in male mice. *Nature, 483*(7388), 218-221.

Kapahi, P., Chen, D., Rogers, A. N., Katewa, S. D., Li, P. W.-L., Thomas, E. L., & Kockel, L. (2010). With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. *Cell metabolism, 11*(6), 453-465.

Kapahi, P., Zid, B. M., Harper, T., Koslover, D., Sapin, V., & Benzer, S. (2004). Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. *Current Biology, 14*(10), 885-890.

Karnewar, S., Neeli, P. K., Panuganti, D., Kotagiri, S., Mallappa, S., Jain, N., Kotamraju, S. (2018). Metformin regulates mitochondrial biogenesis and senescence through AMPK mediated H3K79 methylation: Relevance in age-associated vascular dysfunction. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1864*(4), 1115-1128.

Kashyap, S. R., Roman, L. J., Lamont, J., Masters, B. S. S., Bajaj, M., Suraamornkul, S., Liu, Y. (2005). Insulin resistance is associated with impaired nitric oxide synthase activity in skeletal muscle of type 2 diabetic subjects. *The Journal of Clinical Endocrinology & Metabolism, 90*(2), 1100-1105.

Katsyuba, E., Mottis, A., Zietak, M., De Franco, F., van der Velpen, V., Gariani, K., Liscio, P. (2018). De novo NAD+ synthesis enhances mitochondrial function and improves health. *Nature, 563*(7731), 354-359.

Kauppila, T. E., Kauppila, J. H., & Larsson, N.-G. (2017). Mammalian mitochondria and aging: an update. *Cell metabolism, 25*(1), 57-71.

Kennedy, S. R., Salk, J. J., Schmitt, M. W., & Loeb, L. A. (2013). Ultra-sensitive sequencing reveals an age-related increase in somatic mitochondrial mutations that are inconsistent with oxidative damage. *PLoS Genet, 9*(9), e1003794.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current pharmaceutical design, 9*(16), 1309-1323.

Klingenspor, M. (2003). Cold-induced recruitment of brown adipose tissue thermogenesis. *Experimental physiology, 88*(1), 141-148.

Koch, C., Fitch, A., Simon, M., Qin, S., Leo, N., Methe, B., Morris, A. (2018). Alterations in Tryptophan Metabolism and Nicotinamide Adenine Dinucleotide (NAD) Synthesis in Individuals with Pulmonary Arterial Hypertension. In *A63. RIDE THE LIGHTNING: BASIC AND TRANSLATIONAL STUDIES IN PULMONARY HYPERTENSION* (pp. A2104-A2104): American Thoracic Society.

Koes, D. R., Baumgartner, M. P., & Camacho, C. J. (2013). Lessons learned in empirical scoring with smina from the CSAR 2011 benchmarking exercise. *Journal of chemical information and modeling, 53*(8), 1893-1904.

Kõks, S., Dogan, S., Tuna, B. G., González-Navarro, H., Potter, P., & Vandenbroucke, R. E. (2016). Mouse models of ageing and their relevance to disease. *Mechanisms of ageing and development, 160*, 41-53.

Koltai, E., Szabo, Z., Atalay, M., Boldogh, I., Naito, H., Goto, S., Radak, Z. (2010). Exercise alters SIRT1, SIRT6, NAD and NAMPT levels in skeletal muscle of aged rats. *Mechanisms of ageing and development, 131*(1), 21-28.

Konopka, A. R., & Nair, K. S. (2013). Mitochondrial and skeletal muscle health with advancing age. *Molecular and cellular endocrinology, 379*(1-2), 19-29.

Koval, L., Zemskaya, N., Aliper, A., Zhavoronkov, A., & Moskalev, A. (2021). Evaluation of the geroprotective effects of withaferin A in Drosophila melanogaster. *Aging (Albany NY), 13*(2), 1817.

Kowaltowski, A. J., & Vercesi, A. E. (1999). Mitochondrial damage induced by conditions of oxidative stress. *Free Radical Biology and Medicine, 26*(3-4), 463-471.

Krebs, H., & Veech, R. (1969). Equilibrium relations between pyridine nucleotides and adenine nucleotides and their roles in the regulation of metabolic processes. *Advances in enzyme regulation, 7*, 397-413.

Kruk, P. A., Rampino, N. J., & Bohr, V. A. (1995). DNA damage and repair in telomeres: relation to aging. *Proceedings of the National Academy of Sciences, 92*(1), 258-262.

Kukat, C., Wurm, C. A., Spåhr, H., Falkenberg, M., Larsson, N.-G., & Jakobs, S. (2011). Super-resolution microscopy reveals that mammalian mitochondrial nucleoids have a uniform size and frequently contain a single copy of mtDNA. *Proceedings of the National Academy of Sciences, 108*(33), 13534-13539.

Kukidome, D., Nishikawa, T., Sonoda, K., Imoto, K., Fujisawa, K., Yano, M., Araki, E. (2006). Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. *Diabetes, 55*(1), 120-127.

Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Elliott, P. (2006). Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1α. *Cell, 127*(6), 1109-1122.

Lammi, C., Zanoni, C., & Arnoldi, A. (2015). Three peptides from soy glycinin modulate glucose metabolism in human hepatic HepG2 cells. *International journal of molecular sciences, 16*(11), 27362-27370.

Landry, J., Sutton, A., Tafrov, S. T., Heller, R. C., Stebbins, J., Pillus, L., & Sternglanz, R. (2000). The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proceedings of the National Academy of Sciences, 97*(11), 5807-5811.

Lane, N., & Martin, W. (2010). The energetics of genome complexity. *Nature, 467*(7318), 929-934.

Lee, C. F., Caudal, A., Abell, L., Gowda, G. N., & Tian, R. (2019). Targeting NAD+ metabolism as interventions for mitochondrial disease. *Scientific reports, 9*(1), 1-10.

Li, D., Kang, Q., & Wang, D.-M. (2007). Constitutive coactivator of peroxisome proliferator-activated receptor (PPARγ), a novel coactivator of PPARγ that promotes adipogenesis. *Molecular Endocrinology, 21*(10), 2320-2333.

Li, H., Xu, M., Lee, J., He, C., & Xie, Z. (2012). Leucine supplementation increases SIRT1 expression and prevents mitochondrial dysfunction and metabolic disorders in high-fat diet-induced obese mice. *American Journal of Physiology-Endocrinology and Metabolism, 303*(10), E1234-E1244.

Li, J., Bonkowski, M. S., Moniot, S., Zhang, D., Hubbard, B. P., Ling, A. J., Gorbunova, V. (2017). A conserved NAD+ binding pocket that regulates protein-protein interactions during aging. *Science, 355*(6331), 1312-1317.

Li, Y., He, J., He, X., Li, Y., & Lindgren, U. (2013). Nampt expression increases during osteogenic differentiation of multi-and omnipotent progenitors. *Biochemical and Biophysical Research Communications, 434*(1), 117-123.

Li, Y., He, X., Li, Y., He, J., Anderstam, B., Andersson, G., & Lindgren, U. (2011). Nicotinamide phosphoribosyltransferase (Nampt) affects the lineage fate determination of mesenchymal stem cells: a possible cause for reduced osteogenesis and increased adipogenesis in older individuals. *Journal of Bone and Mineral Research, 26*(11), 2656-2664.

Liao, W., Fan, H., Davidge, S. T., & Wu, J. (2019). Egg white–derived antihypertensive peptide IRW (Ile-Arg-Trp) reduces blood pressure in spontaneously hypertensive rats via the ACE2/ang (1-7)/mas receptor Axis. *Molecular nutrition & food research, 63*(9), 1900063.

Liao, W., Jahandideh, F., Fan, H., Son, M., & Wu, J. (2018). Egg protein-derived bioactive peptides: Preparation, efficacy, and absorption. *Advances in food and nutrition research, 85*, 1-58.

Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., Bonaduce, D. (2018). Oxidative stress, aging, and diseases. *Clinical interventions in aging, 13*, 757.

Liling, S., Roska, T. P., Arfiansyah, R., Maryam, F., & Nainu, F. (2021). Pharmacological Effect of Muntingia calabura Leaves on the Expression of sod1 and sod2 in Drosophila.

Lin, W. T., Nithiyanantham, S., Hsieh, D. J. Y., Chen, R. J., Day, C. H., Liao, J. Y., Huang, C. Y. (2020). Bioactive peptides attenuate cardiac apoptosis in spontaneously hypertensive rat hearts through activation of autophagy and mitochondrial biogenesis pathway. *Environmental toxicology, 35*(7), 804-810.

Lingo, P. R., Zhao, Z., & Shen, P. (2007). Co-regulation of cold-resistant food acquisition by insulin-and neuropeptide Y-like systems in Drosophila melanogaster. *Neuroscience, 148*(2), 371-374.

Liu, G., Sun, S., Guo, B., Miao, B., Luo, Z., Xia, Z., Tang, J. (2018). Bioactive peptide isolated from casein phosphopeptides promotes calcium uptake in vitro and in vivo. *Food & function, 9*(4), 2251-2260.

Liu, H., Tu, M., Cheng, S., Xu, Z., Xu, X., & Du, M. (2019). Anticoagulant Decapeptide Interacts with Thrombin at the Active Site and Exosite-I. *Journal of Agricultural and Food Chemistry, 68*(1), 176-184.

Liu, L., Zhang, W., Wang, L., Li, Y., Tan, B., Lu, X., Mu, J. (2014). Curcumin prevents cerebral ischemia reperfusion injury via increase of mitochondrial biogenesis. *Neurochemical research, 39*(7), 1322-1331.

Liu, L.-Y., Wang, F., Zhang, X.-Y., Huang, P., Lu, Y.-B., Wei, E.-Q., & Zhang, W.-P. (2012). Nicotinamide phosphoribosyltransferase may be involved in age-related brain diseases. *PloS one, 7*(10), e44933.

Liu, X., Kim, C. N., Yang, J., Jemmerson, R., & Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell, 86*(1), 147-157.

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., & Kroemer, G. (2013). The hallmarks of aging. *Cell, 153*(6), 1194-1217.

Lorenzo, J. M., Munekata, P. E., Gomez, B., Barba, F. J., Mora, L., Perez-Santaescolastica, C., & Toldra, F. (2018). Bioactive peptides as natural antioxidants in food products–A review. *Trends in food science & technology, 79*, 136-147.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology, 15*(12), 550.

Ma, B., Guo, Y., Fu, X., & Jin, Y. (2020). Identification and antimicrobial mechanisms of a novel peptide derived from egg white ovotransferrin hydrolysates. *LWT, 131*, 109720.

Ma, Z. A., Zhao, Z., & Turk, J. (2012). Mitochondrial dysfunction and β-cell failure in type 2 diabetes mellitus. *Experimental diabetes research, 2012*.

Mada, S. B., Ugwu, C. P., & Abarshi, M. M. (2019). Health promoting effects of food-derived bioactive peptides: A review. *International Journal of Peptide Research and Therapeutics*, 1-18.

Magwere, T., Chapman, T., & Partridge, L. (2004). Sex differences in the effect of dietary restriction on life span and mortality rates in female and male Drosophila melanogaster. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences, 59*(1), B3-B9.

Majumder, K., & Wu, J. (2010). A new approach for identification of novel antihypertensive peptides from egg proteins by QSAR and bioinformatics. *Food Research International, 43*(5), 1371-1378.

Majumder, K., & Wu, J. (2011). Purification and characterisation of angiotensin I converting enzyme (ACE) inhibitory peptides derived from enzymatic hydrolysate of ovotransferrin. *Food Chemistry, 126*(4), 1614-1619.

Majumder, K., Chakrabarti, S., Davidge, S. T., & Wu, J. (2013). Structure and activity study of egg protein ovotransferrin derived peptides (IRW and IQW) on endothelial inflammatory response and oxidative stress. *Journal of agricultural and food chemistry, 61*(9), 2120-2129.

Majumder, K., Chakrabarti, S., Morton, J. S., Panahi, S., Kaufman, S., Davidge, S. T., & Wu, J. (2013). Egg-derived tri-peptide IRW exerts antihypertensive effects in spontaneously hypertensive rats. *PloS one, 8*(11), e82829.

Majumder, K., Liang, G., Chen, Y., Guan, L., Davidge, S. T., & Wu, J. (2015). Egg ovotransferrin-derived ACE inhibitory peptide IRW increases ACE2 but decreases proinflammatory genes expression in mesenteric artery of spontaneously hypertensive rats. *Molecular nutrition & food research, 59*(9), 1735-1744.

Majumder, K., Liang, G., Chen, Y., Guan, L., Davidge, S. T., & Wu, J. (2015). Egg ovotransferrin-derived ACE inhibitory peptide IRW increases ACE2 but decreases proinflammatory genes expression in mesenteric artery of spontaneously hypertensive rats. *Molecular nutrition & food research, 59*(9), 1735-1744.

Marletta, A. S., Massarotti, A., Orsomando, G., Magni, G., Rizzi, M., & Garavaglia, S. (2015). Crystal structure of human nicotinic acid phosphoribosyltransferase. *FEBS Open Bio, 5*, 419-428.

Martel, J., Ojcius, D. M., Ko, Y.-F., Ke, P.-Y., Wu, C.-Y., Peng, H.-H., & Young, J. D. (2019). Hormetic effects of phytochemicals on health and longevity. *Trends in Endocrinology & Metabolism, 30*(6), 335-346.

Martens, C. R., Denman, B. A., Mazzo, M. R., Armstrong, M. L., Reisdorph, N., McQueen, M. B., Seals, D. R. (2018). Chronic nicotinamide riboside supplementation is well-tolerated and elevates NAD+ in healthy middle-aged and older adults. *Nature communications, 9*(1), 1-11.

Marthandam Asokan, S., Wang, T., Su, W.-T., & Lin, W.-T. (2019). Antidiabetic effects of a short peptide of potato protein hydrolysate in STZ-induced diabetic mice. *Nutrients, 11*(4), 779.

Martin, P. R., Shea, R. J., & Mulks, M. H. (2001). Identification of a Plasmid-Encoded Gene fromHaemophilus ducreyi Which Confers NAD Independence. *Journal of Bacteriology, 183*(4), 1168-1174.

Martin-Montalvo, A., Mercken, E. M., Mitchell, S. J., Palacios, H. H., Mote, P. L., Scheibye-Knudsen, M., Blouin, M.-J. (2013). Metformin improves healthspan and lifespan in mice. *Nature communications, 4*(1), 1-9.

Martucci, M., Ostan, R., Biondi, F., Bellavista, E., Fabbri, C., Bertarelli, C., Santoro, A. (2017). Mediterranean diet and inflammaging within the hormesis paradigm. *Nutrition reviews, 75*(6), 442-455.

Matsuda, N., Sato, S., Shiba, K., Okatsu, K., Saisho, K., Gautier, C. A., Sato, F. (2010). PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. *Journal of Cell Biology, 189*(2), 211-221.

McCay, C. M., Maynard, L., Sperling, G., & Barnes, L. L. (1939). Retarded growth, life span, ultimate body size and age changes in the albino rat after feeding diets restricted in calories: four figures. *The Journal of nutrition, 18*(1), 1-13.

McHugh, D., & Gil, J. (2018). Senescence and aging: Causes, consequences, and therapeutic avenues. *Journal of Cell Biology, 217*(1), 65-77.

Melzer, D., Pilling, L. C., & Ferrucci, L. (2020). The genetics of human ageing. *Nature Reviews Genetics, 21*(2), 88-101.

Mercader, J., Granados, N., Caimari, A., Oliver, P., Bonet, M., & Palou, A. (2008). Retinol-binding protein 4 and nicotinamide phosphoribosyltransferase/visfatin in rat obesity models. *Hormone and metabolic research, 40*(07), 467-472.

Mercken, E. M., Mitchell, S. J., Martin-Montalvo, A., Minor, R. K., Almeida, M., Gomes, A. P., Zhang, Y. (2014). SRT 2104 extends survival of male mice on a standard diet and preserves bone and muscle mass. *Aging cell, 13*(5), 787-796.

Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P. P., Pelicci, P. G. (1999). The p66 shc adaptor protein controls oxidative stress response and life span in mammals. *Nature, 402*(6759), 309-313.

Milne, J. C., Lambert, P. D., Schenk, S., Carney, D. P., Smith, J. J., Gagne, D. J., Vu, C. B. (2007). Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature, 450*(7170), 712-716.

Mitchell, S. J., Martin-Montalvo, A., Mercken, E. M., Palacios, H. H., Ward, T. M., Abulwerdi, G., Sinclair, D. A. (2014). The SIRT1 activator SRT1720 extends lifespan and improves health of mice fed a standard diet. *Cell reports, 6*(5), 836-843.

Mitsui, A., Hamuro, J., Nakamura, H., Kondo, N., Hirabayashi, Y., Ishizaki-Koizumi, S., Yodoi, J. (2002). Overexpression of human thioredoxin in transgenic mice controls oxidative stress and life span. *Antioxidants and Redox Signaling, 4*(4), 693-696.

Miyashita, K., Itoh, H., Tsujimoto, H., Tamura, N., Fukunaga, Y., Sone, M., Sonoyama, T. (2009). Natriuretic peptides/cGMP/cGMP-dependent protein kinase cascades promote muscle mitochondrial biogenesis and prevent obesity. *Diabetes, 58*(12), 2880-2892.

Mizushige, T., Uchida, T., & Ohinata, K. (2020). Dipeptide tyrosyl-leucine exhibits antidepressant-like activity in mice. *Scientific reports, 10*(1), 1-10.

Montgomery, M. K., & Turner, N. (2015). Mitochondrial dysfunction and insulin resistance: an update. *Endocrine connections, 4*(1), R1-R15.

Moon, S., Lee, J., Lee, Y., Chang, K., Paik, J., Ahn, D., & Paik, H. (2013). Screening for cytotoxic activity of ovotransferrin and its enzyme hydrolysates. *Poultry science, 92*(2), 424-434.

Mootha, V. K., Bunkenborg, J., Olsen, J. V., Hjerrild, M., Wisniewski, J. R., Stahl, E., Kamal, M. (2003). Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria. *Cell, 115*(5), 629-640.

Morselli, E., Galluzzi, L., Kepp, O., Criollo, A., Maiuri, M. C., Tavernarakis, N., Kroemer, G. (2009). Autophagy mediates pharmacological lifespan extension by spermidine and resveratrol. *Aging (Albany NY), 1*(12), 961.

Mouchiroud, L., Houtkooper, R. H., Moullan, N., Katsyuba, E., Ryu, D., Cantó, C., Schoonjans, K. (2013). The NAD+/sirtuin pathway modulates longevity through activation of mitochondrial UPR and FOXO signaling. *Cell, 154*(2), 430-441.

Mulder, H., & Ling, C. (2009). Mitochondrial dysfunction in pancreatic β-cells in type 2 diabetes. *Molecular and cellular endocrinology, 297*(1-2), 34-40.

Nagai, A., Mizushige, T., Matsumura, S., Inoue, K., & Ohinata*, K. (2019). Orally administered milk-derived tripeptide improved cognitive decline in mice fed a high-fat diet. *The FASEB Journal, 33*(12), 14095-14102.

Narkar, V. A., Downes, M., Ruth, T. Y., Embler, E., Wang, Y.-X., Banayo, E., Juguilon, H. (2008). AMPK and PPARδ agonists are exercise mimetics. *Cell, 134*(3), 405-415.

Nässel, D. R., & Broeck, J. V. (2016). Insulin/IGF signaling in Drosophila and other insects: factors that regulate production, release and post-release action of the insulin-like peptides. *Cellular and Molecular Life Sciences, 73*(2), 271-290.

Natarajan, V., Chawla, R., Mah, T., Vivekanandan, R., Tan, S. Y., Sato, P. Y., & Mallilankaraman, K. (2020). Mitochondrial dysfunction in age-related metabolic disorders. *Proteomics, 20*(5-6), 1800404.

Nemoto, S., Combs, C. A., French, S., Ahn, B.-H., Fergusson, M. M., Balaban, R. S., & Finkel, T. (2006). The mammalian longevity-associated gene product p66shc regulates mitochondrial metabolism. *Journal of Biological Chemistry, 281*(15), 10555-10560.

Neupert, W., & Herrmann, J. M. (2007). Translocation of proteins into mitochondria. *Annu. Rev. Biochem., 76*, 723-749.

Ngubo, M., Reid, J., & Patterton, H.-G. (2019). Structural clusters of histone H3 and H4 residues regulate chronological lifespan in Saccharomyces cerevisiae. *bioRxiv*.

Nikiforov, A., Dölle, C., Niere, M., & Ziegler, M. (2011). Pathways and subcellular compartmentation of NAD biosynthesis in human cells: from entry of extracellular precursors to mitochondrial NAD generation. *Journal of Biological Chemistry, 286*(24), 21767-21778.

Nisoli, E., Clementi, E., Paolucci, C., Cozzi, V., Tonello, C., Sciorati, C., Moncada, S. (2003). Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science, 299*(5608), 896-899.

Nisoli, E., Falcone, S., Tonello, C., Cozzi, V., Palomba, L., Fiorani, M., Francolini, M. (2004). Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. *Proceedings of the National Academy of Sciences, 101*(47), 16507-16512.

Nisoli, E., Tonello, C., Cardile, A., Cozzi, V., Bracale, R., Tedesco, L., Clementi, E. (2005). Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. *Science, 310*(5746), 314-317.

Nunnari, J., & Suomalainen, A. (2012). Mitochondria: in sickness and in health. *Cell, 148*(6), 1145-1159.

Nussey, D. H., Froy, H., Lemaitre, J.-F., Gaillard, J.-M., & Austad, S. N. (2013). Senescence in natural populations of animals: widespread evidence and its implications for bio-gerontology. *Ageing research reviews, 12*(1), 214-225.

Nwosu, O. K., & Ubaoji, K. I. (2020). Nutraceuticals: History, Classification and Market Demand. In *Functional Foods and Nutraceuticals* (pp. 13-22): Springer.

Olivieri, F., Capri, M., Bonafè, M., Morsiani, C., Jung, H. J., Spazzafumo, L., Suh, Y. (2017). Circulating miRNAs and miRNA shuttles as biomarkers: perspective trajectories of healthy and unhealthy aging. *Mechanisms of ageing and development, 165*, 162-170.

Owen, M. R., Doran, E., & Halestrap, A. P. (2000). Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochemical Journal, 348*(3), 607-614.

Pagel-Langenickel, I., Bao, J., Pang, L., & Sack, M. N. (2010). The role of mitochondria in the pathophysiology of skeletal muscle insulin resistance. *Endocrine reviews, 31*(1), 25-51.

Pagliarini, D. J., Calvo, S. E., Chang, B., Sheth, S. A., Vafai, S. B., Ong, S.-E., Chen, W. K. (2008). A mitochondrial protein compendium elucidates complex I disease biology. *Cell, 134*(1), 112-123.

Pak, V., Koo, M., Kasymova, T., & Kwon, D. (2005). Isolation and identification of peptides from soy 11S-globulin with hypocholesterolemic activity. *Chemistry of natural compounds, 41*(6), 710-714.

Panasevich, M. R., Morris, E. M., Chintapalli, S. V., Wankhade, U. D., Shankar, K., Britton, S. L., Rector, R. S. (2016). Gut microbiota are linked to increased susceptibility to hepatic steatosis in low-aerobic-capacity rats fed an acute high-fat diet. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 311*(1), G166-G179.

Pardo, R., Enguix, N., Lasheras, J., Feliu, J. E., Kralli, A., & Villena, J. A. (2011). Rosiglitazone-induced mitochondrial biogenesis in white adipose tissue is independent of peroxisome proliferator-activated receptor γ coactivator-1α. *PloS one, 6*(11), e26989.

Parkes, T. L., Elia, A. J., Dickinson, D., Hilliker, A. J., Phillips, J. P., & Boulianne, G. L. (1998). Extension of Drosophila lifespan by overexpression of human SOD1 in motorneurons. *Nature genetics, 19*(2), 171-174.

Partridge, L., Green, A., & Fowler, K. (1987). Effects of egg-production and of exposure to males on female survival in Drosophila melanogaster. *Journal of Insect Physiology, 33*(10), 745-749.

Patel, S. K., Velkoska, E., Freeman, M., Wai, B., Lancefield, T. F., & Burrell, L. M. (2014). From gene to protein—experimental and clinical studies of ACE2 in blood pressure control and arterial hypertension. *Frontiers in physiology, 5*, 227.

Peleg, S., Feller, C., Forne, I., Schiller, E., Sévin, D. C., Schauer, T., Klima, C. (2016). Life span extension by targeting a link between metabolism and histone acetylation in Drosophila. *EMBO reports, 17*(3), 455-469.

Pérez, H., Finocchietto, P. V., Alippe, Y., Rebagliati, I., Elguero, M. E., Villalba, N., Carreras, M. C. (2018). p66Shc inactivation modifies RNS production, regulates Sirt3 activity, and improves mitochondrial homeostasis, delaying the aging process in mouse brain. *Oxidative medicine and cellular longevity, 2018*.

Pfanner, N., & Meijer, M. (1997). Mitochondrial biogenesis: the Tom and Tim machine. *Current Biology, 7*(2), R100-R103.

Pfluger, P. T., Herranz, D., Velasco-Miguel, S., Serrano, M., & Tschöp, M. H. (2008). Sirt1 protects against high-fat diet-induced metabolic damage. *Proceedings of the National Academy of Sciences, 105*(28), 9793-9798.

Picard, M., & Turnbull, D. M. (2013). Linking the metabolic state and mitochondrial DNA in chronic disease, health, and aging. *Diabetes, 62*(3), 672-678.

Picard, M., Wallace, D. C., & Burelle, Y. (2016). The rise of mitochondria in medicine. *Mitochondrion, 30*, 105-116.

Picca, A., & Lezza, A. M. S. (2015). Regulation of mitochondrial biogenesis through TFAM–mitochondrial DNA interactions: useful insights from aging and calorie restriction studies. *Mitochondrion, 25*, 67-75.

Pillai, J. B., Isbatan, A., Imai, S.-i., & Gupta, M. P. (2005). Poly (ADP-ribose) polymerase-1-dependent cardiac myocyte cell death during heart failure is mediated by NAD+ depletion and reduced Sir2α deacetylase activity. *Journal of Biological Chemistry, 280*(52), 43121-43130.

Pillai, V. B., Sundaresan, N. R., Kim, G., Gupta, M., Rajamohan, S. B., Pillai, J. B., Gupta, M. P. (2010). Exogenous NAD blocks cardiac hypertrophic response via activation of the SIRT3-LKB1-AMP-activated kinase pathway. *Journal of Biological Chemistry, 285*(5), 3133-3144.

Piper, M. D., & Partridge, L. (2018). Drosophila as a model for ageing. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1864*(9), 2707-2717.

Piper, M. D., Blanc, E., Leitão-Gonçalves, R., Yang, M., He, X., Linford, N. J., Niemeyer, C. (2014). A holidic medium for Drosophila melanogaster. *Nature methods, 11*(1), 100-105.

Piper, M. D., Soultoukis, G. A., Blanc, E., Mesaros, A., Herbert, S. L., Juricic, P., Yang, M. (2017). Matching dietary amino acid balance to the in silico-translated exome optimizes growth and reproduction without cost to lifespan. *Cell metabolism, 25*(3), 610-621.

Pu, M., Ni, Z., Wang, M., Wang, X., Wood, J. G., Helfand, S. L., Lee, S. S. (2015). Trimethylation of Lys36 on H3 restricts gene expression change during aging and impacts life span. *Genes & development, 29*(7), 718-731.

Puig, O., Marr, M. T., Ruhf, M. L., & Tjian, R. (2003). Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. *Genes & development, 17*(16), 2006-2020.

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., Shen, D. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature, 490*(7418), 55-60.

Qin, W., Haroutunian, V., Katsel, P., Cardozo, C. P., Ho, L., Buxbaum, J. D., & Pasinetti, G. M. (2009). PGC-1α expression decreases in the Alzheimer disease brain as a function of dementia. *Archives of neurology, 66*(3), 352-361.

Rajman, L., Chwalek, K., & Sinclair, D. A. (2018). Therapeutic potential of NAD-boosting molecules: the in vivo evidence. *Cell metabolism, 27*(3), 529-547.

Ramachandran, B., Yu, G., & Gulick, T. (2008). Nuclear respiratory factor 1 controls myocyte enhancer factor 2A transcription to provide a mechanism for coordinate expression of respiratory chain subunits. *Journal of Biological Chemistry, 283*(18), 11935-11946.

Ramsey, K. M., Yoshino, J., Brace, C. S., Abrassart, D., Kobayashi, Y., Marcheva, B., Lee, C. (2009). Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. *Science, 324*(5927), 651-654.

Revollo, J. R., Körner, A., Mills, K. F., Satoh, A., Wang, T., Garten, A., Townsend, R. R. (2007). Nampt/PBEF/visfatin regulates insulin secretion in β cells as a systemic NAD biosynthetic enzyme. *Cell metabolism, 6*(5), 363-375.

Rius-Pérez, S., Torres-Cuevas, I., Millán, I., Ortega, Á. L., & Pérez, S. (2020). PGC-1α, Inflammation, and oxidative stress: An integrative view in metabolism. *Oxidative medicine and cellular longevity, 2020*.

Rizzello, C. G., Lorusso, A., Russo, V., Pinto, D., Marzani, B., & Gobbetti, M. (2017). Improving the antioxidant properties of quinoa flour through fermentation with selected autochthonous lactic acid bacteria. *International journal of food microbiology, 241*, 252-261.

Rodgers, J. T., Lerin, C., Haas, W., Gygi, S. P., Spiegelman, B. M., & Puigserver, P. (2005). Nutrient control of glucose homeostasis through a complex of PGC-1α and SIRT1. *Nature, 434*(7029), 113-118.

Rogina, B., & Helfand, S. L. (2004). Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proceedings of the National Academy of Sciences, 101*(45), 15998-16003.

Rongvaux, A., Shea, R. J., Mulks, M. H., Gigot, D., Urbain, J., Leo, O., & Andris, F. (2002). Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. *European journal of immunology, 32*(11), 3225-3234.

Rose, M., & Charlesworth, B. (1980). A test of evolutionary theories of senescence. *Nature, 287*(5778), 141-142.

Ross, D., & Siegel, D. (2017). Functions of NQO1 in cellular protection and CoQ10 metabolism and its potential role as a redox sensitive molecular switch. *Frontiers in physiology, 8*, 595.

Ross, J. M., Stewart, J. B., Hagström, E., Brené, S., Mourier, A., Coppotelli, G., Olson, L. (2013). Germline mitochondrial DNA mutations aggravate ageing and can impair brain development. *Nature, 501*(7467), 412-415.

SA Capettini, L., Montecucco, F., Mach, F., Stergiopulos, N., AS Santos, R., & F da Silva, R. (2012). Role of renin-angiotensin system in inflammation, immunity and aging. *Current pharmaceutical design, 18*(7), 963-970.

Saint-Georges-Chaumet, Y., Attaf, D., Pelletier, E., & Edeas, M. (2015). Targeting microbiota-mitochondria inter-talk: Microbiota control mitochondria metabolism. *Cellular and Molecular Biology, 61*(4), 121-124.

Sallin, O., Reymond, L., Gondrand, C., Raith, F., Koch, B., & Johnsson, K. (2018). Semisynthetic biosensors for mapping cellular concentrations of nicotinamide adenine dinucleotides. *Elife, 7*, e32638.

Samal, B., Sun, Y., Stearns, G., Xie, C., Suggs, S., & McNiece, I. (1994). Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. *Molecular and cellular biology, 14*(2), 1431-1437.

Sánchez-Rivera, L., Ferreira Santos, P., Sevilla, M. A., Montero, M. J., Recio, I., & Miralles, B. (2019). Implication of Opioid Receptors in the Antihypertensive Effect of a Bovine Casein Hydrolysate and αs1-Casein-Derived Peptides. *Journal of Agricultural and Food Chemistry, 68*(7), 1877-1883.

Satoh, A., Brace, C. S., Rensing, N., Cliften, P., Wozniak, D. F., Herzog, E. D., Imai, S.-i. (2013). Sirt1 extends life span and delays aging in mice through the regulation of Nk2 homeobox 1 in the DMH and LH. *Cell metabolism, 18*(3), 416-430.

Scheibye-Knudsen, M., Mitchell, S. J., Fang, E. F., Iyama, T., Ward, T., Wang, J., Hasan-Olive, M. M. (2014). A high-fat diet and NAD+ activate Sirt1 to rescue premature aging in cockayne syndrome. *Cell metabolism, 20*(5), 840-855.

Schiavi, A., Maglioni, S., Palikaras, K., Shaik, A., Strappazzon, F., Brinkmann, V., Braeckman, B. P. (2015). Iron-starvation-induced mitophagy mediates lifespan extension upon mitochondrial stress in C. elegans. *Current Biology, 25*(14), 1810-1822.

Schmidt, O., Pfanner, N., & Meisinger, C. (2010). Mitochondrial protein import: from proteomics to functional mechanisms. *Nature reviews Molecular cell biology, 11*(9), 655-667.

Sen, P., Dang, W., Donahue, G., Dai, J., Dorsey, J., Cao, X., Lee, J. Y. (2015). H3K36 methylation promotes longevity by enhancing transcriptional fidelity. *Genes & development, 29*(13), 1362-1376.

Shang, N., Bhullar, K. S., Hubbard, B. P., & Wu, J. (2019). Tripeptide IRW initiates differentiation in osteoblasts via the RUNX2 pathway. *Biochimica et Biophysica Acta (BBA)-General Subjects, 1863*(6), 1138-1146.

Shen, L.-R., Xiao, F., Yuan, P., Chen, Y., Gao, Q.-K., Parnell, L. D., Lai, C.-Q. (2013). Curcumin-supplemented diets increase superoxide dismutase activity and mean lifespan in Drosophila. *Age, 35*(4), 1133-1142.

Shen, W., & Matsui, T. (2019). Intestinal absorption of small peptides: A review. *International Journal of Food Science & Technology, 54*(6), 1942-1948.

Shi, H., Enriquez, A., Rapadas, M., Martin, E. M., Wang, R., Moreau, J., Hughes, J. N. (2017). NAD deficiency, congenital malformations, and niacin supplementation. *New England Journal of Medicine, 377*(6), 544-552.

Shi, Y., Dierckx, A., Wanrooij, P. H., Wanrooij, S., Larsson, N.-G., Wilhelmsson, L. M., Gustafsson, C. M. (2012). Mammalian transcription factor A is a core component of the mitochondrial transcription machinery. *Proceedings of the National Academy of Sciences, 109*(41), 16510-16515.

Shimizu, A., Mitani, T., Tanaka, S., Fujii, H., Maebuchi, M., Amiya, Y., Katayama, S. (2018). Soybean-Derived Glycine–Arginine Dipeptide Administration Promotes Neurotrophic Factor Expression in the Mouse Brain. *Journal of Agricultural and Food Chemistry, 66*(30), 7935-7941.

Shiota, T., Imai, K., Qiu, J., Hewitt, V. L., Tan, K., Shen, H.-H., Kamiya, M. (2015). Molecular architecture of the active mitochondrial protein gate. *Science, 349*(6255), 1544-1548.

Siregar, A. S., Nyiramana, M. M., Kim, E.-J., Shin, E.-J., Woo, M. S., Kim, J.-M., Kim, H. J. (2020). Dipeptide YA is Responsible for the Positive Effect of Oyster Hydrolysates on Alcohol Metabolism in Single Ethanol Binge Rodent Models. *Marine Drugs, 18*(10), 512.

Slack, C., Werz, C., Wieser, D., Alic, N., Foley, A., Stocker, H., Partridge, L. (2010). Regulation of lifespan, metabolism, and stress responses by the Drosophila SH2B protein, Lnk. *PLoS Genet, 6*(3), e1000881.

Son, M., Chan, C. B., & Wu, J. (2018). Egg white ovotransferrin-derived ACE inhibitory peptide ameliorates angiotensin II-stimulated insulin resistance in skeletal muscle cells. *Molecular nutrition & food research, 62*(4), 1700602.

Song, H., Tian, Q., & Li, B. (2020). Novel Hyp-Gly-containing antiplatelet peptides from collagen hydrolysate after simulated gastrointestinal digestion and intestinal absorption. *Food & function, 11*(6), 5553-5564.

Sonklin, C., Alashi, M. A., Laohakunjit, N., Kerdchoechuen, O., & Aluko, R. E. (2020). Identification of antihypertensive peptides from mung bean protein hydrolysate and their effects in spontaneously hypertensive rats. *Journal of Functional Foods, 64*, 103635.

Sousa-Franco, A., Rebelo, K., da Rocha, S. T., & Bernardes de Jesus, B. (2019). LncRNAs regulating stemness in aging. *Aging cell, 18*(1), e12870.

Sowmya, K., Mala, D., Bhat, M. I., Kumar, N., Bajaj, R. K., Kapila, S., & Kapila, R. (2018). Bio-accessible milk casein derived tripeptide (LLY) mediates overlapping anti-inflammatory and anti-oxidative effects under cellular (Caco-2) and in vivo milieu. *The Journal of nutritional biochemistry, 62*, 167-180.

Spieth, H. T. (1974). Courtship behavior in Drosophila. *Annual review of entomology, 19*(1), 385-405.

Sreedhar, A., Aguilera-Aguirre, L., & Singh, K. K. (2020). Mitochondria in skin health, aging, and disease. *Cell death & disease, 11*(6), 1-14.

Stein, L. R., & Imai, S. i. (2014). Specific ablation of Nampt in adult neural stem cells recapitulates their functional defects during aging. *The EMBO journal, 33*(12), 1321-1340.

Stein, L. R., & Imai, S.-i. (2012). The dynamic regulation of NAD metabolism in mitochondria. *Trends in Endocrinology & Metabolism, 23*(9), 420-428.

Stephens, O. R., Grant, D., Frimel, M., Wanner, N., Yin, M., Willard, B., Asosingh, K. (2020). Characterization and origins of cell-free mitochondria in healthy murine and human blood. *Mitochondrion, 54*, 102-112.

Steptoe, A., & Zaninotto, P. (2020). Lower socioeconomic status and the acceleration of aging: An outcome-wide analysis. *Proceedings of the National Academy of Sciences, 117*(26), 14911-14917.

Stewart, J. B., & Chinnery, P. F. (2015). The dynamics of mitochondrial DNA heteroplasmy: implications for human health and disease. *Nature Reviews Genetics, 16*(9), 530-542.

Stromsdorfer, K. L., Yamaguchi, S., Yoon, M. J., Moseley, A. C., Franczyk, M. P., Kelly, S. C., Yoshino, J. (2016). NAMPT-mediated NAD+ biosynthesis in adipocytes regulates adipose tissue function and multi-organ insulin sensitivity in mice. *Cell reports, 16*(7), 1851-1860.

Suárez, S., Aphalo, P., Rinaldi, G., Añón, M. C., & Quiroga, A. (2020). Effect of amaranth proteins on the RAS system. In vitro, in vivo and ex vivo assays. *Food Chemistry, 308*, 125601.

Sun, S., Liu, F., Liu, G., Miao, J., Xiao, H., Xiao, J., Cao, Y. (2018). Effects of casein phosphopeptides on calcium absorption and metabolism bioactivity in vitro and in vivo. *Food & function, 9*(10), 5220-5229.

Swerdlow, R. H. (2011). Brain aging, Alzheimer's disease, and mitochondria. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1812*(12), 1630-1639.

Sykiotis, G. P., & Bohmann, D. (2008). Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila. *Developmental cell, 14*(1), 76-85.

Taub, P. R., Ramirez-Sanchez, I., Ciaraldi, T. P., Perkins, G., Murphy, A. N., Naviaux, R., Ceballos, G. (2012). Alterations in skeletal muscle indicators of mitochondrial structure and biogenesis in patients with type 2 diabetes and heart failure: effects of epicatechin rich cocoa. *Clinical and translational science, 5*(1), 43-47.

Tedesco, L., Valerio, A., Cervino, C., Cardile, A., Pagano, C., Vettor, R., Lutz, B. (2008). Cannabinoid type 1 receptor blockade promotes mitochondrial biogenesis through endothelial nitric oxide synthase expression in white adipocytes. *Diabetes, 57*(8), 2028-2036.

Terra, X., Auguet, T., Quesada, I., Aguilar, C., Luna, A. M., Hernandez, M., Lucas, A. (2012). Increased levels and adipose tissue expression of visfatin in morbidly obese women: the relationship with pro-inflammatory cytokines. *Clinical endocrinology, 77*(5), 691-698.

Tovar, J., León-Avila, G., Sánchez, L. B., Sutak, R., Tachezy, J., Van Der Giezen, M., Lucocq, J. M. (2003). Mitochondrial remnant organelles of Giardia function in iron-sulphur protein maturation. *Nature, 426*(6963), 172-176.

Tsai, B. C.-K., Hsieh, D. J.-Y., Lin, W.-T., Tamilselvi, S., Day, C. H., Ho, T.-J., Huang, C.-Y. (2020). Functional potato bioactive peptide intensifies Nrf2-dependent antioxidant defense against renal damage in hypertensive rats. *Food Research International, 129*, 108862.

Udenigwe, C. C., & Rouvinen-Watt, K. (2015). The role of food peptides in lipid metabolism during dyslipidemia and associated health conditions. *International journal of molecular sciences, 16*(5), 9303-9313.

Uittenbogaard, M., & Chiaramello, A. (2014). Mitochondrial biogenesis: a therapeutic target for neurodevelopmental disorders and neurodegenerative diseases. *Current pharmaceutical design, 20*(35), 5574-5593.

Ulgherait, M., Rana, A., Rera, M., Graniel, J., & Walker, D. W. (2014). AMPK modulates tissue and organismal aging in a non-cell-autonomous manner. *Cell reports, 8*(6), 1767-1780.

Vázquez-Rosa, E., Shin, M.-K., Dhar, M., Chaubey, K., Cintrón-Pérez, C. J., Tang, X., Barker, S. (2020). P7C3-A20 treatment one year after TBI in mice repairs the blood–brain barrier, arrests chronic neurodegeneration, and restores cognition. *Proceedings of the National Academy of Sciences, 117*(44), 27667-27675.

Vezza, T., Abad-Jiménez, Z., Marti-Cabrera, M., Rocha, M., & Víctor, V. M. (2020). Microbiota-mitochondria inter-talk: A potential therapeutic strategy in obesity and type 2 diabetes. *Antioxidants, 9*(9), 848.

Virbasius, C.-m. A., Virbasius, J. V., & Scarpulla, R. C. (1993). NRF-1, an activator involved in nuclear-mitochondrial interactions, utilizes a new DNA-binding domain conserved in a family of developmental regulators. *Genes & development, 7*(12a), 2431-2445.

Viscomi, C., Bottani, E., & Zeviani, M. (2015). Emerging concepts in the therapy of mitochondrial disease. *Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1847*(6-7), 544-557.

Viscomi, C., Bottani, E., Civiletto, G., Cerutti, R., Moggio, M., Fagiolari, G., Zeviani, M. (2011). In vivo correction of COX deficiency by activation of the AMPK/PGC-1α axis. *Cell metabolism, 14*(1), 80-90.

Walters, R. W., Matheny, T., Mizoue, L. S., Rao, B. S., Muhlrad, D., & Parker, R. (2017). Identification of NAD+ capped mRNAs in Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences, 114*(3), 480-485.

Wang, D., Yan, Z., Bu, L., An, C., Deng, B., Zhang, J., Zhang, B. (2019). Protective effect of peptide DR8 on bleomycin-induced pulmonary fibrosis by regulating the TGF-β/MAPK signaling pathway and oxidative stress. *Toxicology and applied pharmacology, 382*, 114703.

Wang, G., Han, T., Nijhawan, D., Theodoropoulos, P., Naidoo, J., Yadavalli, S., McKnight, S. L. (2014). P7C3 neuroprotective chemicals function by activating the rate-limiting enzyme in NAD salvage. *Cell, 158*(6), 1324-1334.

Wang, M., Amakye, W. K., Guo, L., Gong, C., Zhao, Y., Yao, M., & Ren, J. (2019). Walnut-derived peptide PW5 ameliorates cognitive impairments and alters gut microbiota in APP/PS1 transgenic mice. *Molecular nutrition & food research, 63*(18), 1900326.

Warburg, O., & Christian, W. (1936). Pyridin, der wasserstoffübertragende Bestandteil von Gärungsfermenten. *Helvetica Chimica Acta, 19*(1), E79-E88.

Wensveen, F. M., Valentić, S., Šestan, M., Turk Wensveen, T., & Polić, B. (2015). The "Big Bang" in obese fat: Events initiating obesity-induced adipose tissue inflammation. *European journal of immunology, 45*(9), 2446-2456.

Wills, L. P., Trager, R. E., Beeson, G. C., Lindsey, C. C., Peterson, Y. K., Beeson, C. C., & Schnellmann, R. G. (2012). The β2-adrenoceptor agonist formoterol stimulates mitochondrial biogenesis. *Journal of Pharmacology and Experimental Therapeutics, 342*(1), 106-118.

Wingert, B. M., & Camacho, C. J. (2018). Improving small molecule virtual screening strategies for the next generation of therapeutics. *Current opinion in chemical biology, 44*, 87-92.

Wingert, B. M., Oerlemans, R., & Camacho, C. J. (2018). Optimal affinity ranking for automated virtual screening validated in prospective D3R grand challenges. *Journal of computer-aided molecular design, 32*(1), 287-297.

Wood, J. G., Rogina, B., Lavu, S., Howitz, K., Helfand, S. L., Tatar, M., & Sinclair, D. (2004). Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature, 430*(7000), 686-689.

Wood, J. G., Schwer, B., Wickremesinghe, P. C., Hartnett, D. A., Burhenn, L., Garcia, M., Helfand, S. L. (2018). Sirt4 is a mitochondrial regulator of metabolism and lifespan in Drosophila melanogaster. *Proceedings of the National Academy of Sciences, 115*(7), 1564-1569.

Wood, S. H., van Dam, S., Craig, T., Tacutu, R., O'Toole, A., Merry, B. J., & de Magalhães, J. P. (2015). Transcriptome analysis in calorie-restricted rats implicates epigenetic and post-translational mechanisms in neuroprotection and aging. *Genome biology, 16*(1), 1-18.

Wu, J. (2020). A Novel Angiotensin Converting Enzyme 2 (ACE2) Activating Peptide: A Reflection of 10 Years of Research on a Small Peptide Ile-Arg-Trp (IRW). *Journal of Agricultural and Food Chemistry, 68*(49), 14402-14408.

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Scarpulla, R. C. (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell, 98*(1), 115-124.

Xing, L., Liu, R., Cao, S., Zhang, W., & Guanghong, Z. (2019). Meat protein based bioactive peptides and their potential functional activity: a review. *International Journal of Food Science & Technology, 54*(6), 1956-1966.

Yach, D., Stuckler, D., & Brownell, K. D. (2006). Epidemiologic and economic consequences of the global epidemics of obesity and diabetes. *Nature medicine, 12*(1), 62-66.

Yaku, K., Okabe, K., & Nakagawa, T. (2018a). NAD metabolism: Implications in aging and longevity. *Ageing research reviews, 47*, 1-17.

Yaku, K., Okabe, K., & Nakagawa, T. (2018b). Simultaneous measurement of NAD metabolome in aged mice tissue using liquid chromatography tandem-mass spectrometry. *Biomedical Chromatography, 32*(6), e4205.

Yamaguchi, N., Kawaguchi, K., & Yamamoto, N. (2009). Study of the mechanism of antihypertensive peptides VPP and IPP in spontaneously hypertensive rats by DNA microarray analysis. *European Journal of Pharmacology, 620*(1-3), 71-77.

Yamaguchi, S., & Yoshino, J. (2017). Adipose tissue NAD+ biology in obesity and insulin resistance: From mechanism to therapy. *BioEssays, 39*(5), 1600227.

Yang, H., Lavu, S., & Sinclair, D. A. (2006). Nampt/PBEF/Visfatin: a regulator of mammalian health and longevity? *Experimental gerontology, 41*(8), 718-726.

Yang, H., Yang, T., Baur, J. A., Perez, E., Matsui, T., Carmona, J. J., Rosenzweig, A. (2007). Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival. *Cell, 130*(6), 1095-1107.

Yoshino, J., Mills, K. F., Yoon, M. J., & Imai, S.-i. (2011). Nicotinamide mononucleotide, a key NAD+ intermediate, treats the pathophysiology of diet-and age-induced diabetes in mice. *Cell metabolism, 14*(4), 528-536.

You, L., Zhao, M., Regenstein, J. M., & Ren, J. (2011). In vitro antioxidant activity and in vivo anti-fatigue effect of loach (Misgurnus anguillicaudatus) peptides prepared by papain digestion. *Food Chemistry, 124*(1), 188-194.

Youn, H.-S., Kim, T. G., Kim, M.-K., Kang, G. B., Kang, J. Y., Lee, J.-G., Im, Y. J. (2016). Structural insights into the quaternary catalytic mechanism of hexameric human quinolinate phosphoribosyltransferase, a key enzyme in de novo NAD biosynthesis. *Scientific reports, 6*(1), 1-14.

Zambrowicz, A., Pokora, M., Setner, B., Dąbrowska, A., Szołtysik, M., Babij, K., Chrzanowska, J. (2015). Multifunctional peptides derived from an egg yolk protein hydrolysate: isolation and characterization. *Amino acids, 47*(2), 369-380.

Zeyda, M., & Stulnig, T. M. (2009). Obesity, inflammation, and insulin resistance–a mini-review. *Gerontology, 55*(4), 379-386.

Zhang, H., Ryu, D., Wu, Y., Gariani, K., Wang, X., Luan, P., Aebersold, R. (2016). NAD+ repletion improves mitochondrial and stem cell function and enhances life span in mice. *Science, 352*(6292), 1436-1443.

Zhang, M., Yan, Z., Bu, L., An, C., Wang, D., Liu, X., Xie, J. (2018). Rapeseed protein-derived antioxidant peptide RAP alleviates renal fibrosis through MAPK/NF-κB signaling pathways in diabetic nephropathy. *Drug design, development and therapy, 12*, 1255.

Zhang, R.-Y., Qin, Y., Lv, X.-Q., Wang, P., Xu, T.-Y., Zhang, L., & Miao, C.-Y. (2011). A fluorometric assay for high-throughput screening targeting nicotinamide phosphoribosyltransferase. *Analytical biochemistry, 412*(1), 18-25.

Zhao, M., New, L., Kravchenko, V. V., Kato, Y., Gram, H., Di Padova, F., Han, J. (1999). Regulation of the MEF2 family of transcription factors by p38. *Molecular and cellular biology, 19*(1), 21-30.

Zhao, Q., Xu, H., Hong, S., Song, N., Xie, J., Yan, Z., Jiang, X. (2018). Rapeseed protein-derived antioxidant peptide RAP ameliorates nonalcoholic steatohepatitis and related metabolic disorders in mice. *Molecular pharmaceutics, 16*(1), 371-381.

Zheng, Y., Wang, X., Zhuang, Y., Li, Y., Tian, H., Shi, P., & Li, G. (2019). Isolation of novel ACE-inhibitory and antioxidant peptides from quinoa bran albumin assisted with an in silico

approach: Characterization, in vivo antihypertension, and molecular docking. *Molecules, 24*(24), 4562.

Zhou, T., Kurnasov, O., Tomchick, D. R., Binns, D. D., Grishin, N. V., Marquez, V. E., Zhang, H. (2002). Structure of human nicotinamide/nicotinic acid mononucleotide adenylyltransferase: basis for the dual substrate specificity and activation of the oncolytic agent tiazofurin. *Journal of Biological Chemistry, 277*(15), 13148-13154.

Zhu, X.-H., Lu, M., Lee, B.-Y., Ugurbil, K., & Chen, W. (2015). In vivo NAD assay reveals the intracellular NAD contents and redox state in healthy human brain and their age dependences. *Proceedings of the National Academy of Sciences, 112*(9), 2876-2881.