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Elucidating The Role Of Type I Interferon Signaling In Adipocyte Biology

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Abstract

Brown and beige adipose tissues represent promising therapeutic targets for combating the rapidly growing obesity pandemic. The goal of this thesis work was to better understand the signaling pathways that affect brown and beige adipose function. First, we investigated pathways regulated by PRDM16, a critical transcription factor for brown and beige fat development. We found that PRDM16 represses type I Interferon (IFN) responses in both preadipocytes and mature adipocytes to promote thermogenic and mitochondrial function. Type I IFN signaling is a critical antiviral pathway, which was previously unexplored in the context of brown fat biology. We found that brown adipocyte character and mitochondrial function were disrupted by ectopic IFN signaling and that increased PRDM16 expression could reverse these negative effects. Additionally, we showed that PRDM16 is required to protect brown fat function from type I IFN signaling in vivo. Utilizing multiple transcriptional assays, we determined that PRDM16 blocks IFN regulatory factor 1 (IRF1)-mediated activation of ISGs by competitively binding ISG promoter regions. Adipose inflammation has been implicated in the progression of obesity and insulin resistance. Next, we aimed to determine the role of type I IFN in diet-induced obesity. We found that ISGs are activated in multiple tissues of mice early in the course of high-fat diet (HFD) feeding. Blocking IFN responses using an IFN alpha receptor knockout mouse led to protection from diet-induced obesity and insulin resistance. Additionally, HFD-induced type I IFN decreased energy expenditure, potentially due to mitochondrial dysfunction in the subcutaneous adipose depot. Together this work demonstrates for the first time the negative effects of type I IFN in adipose tissue that may contribute to obesity and insulin resistance.

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ELUCIDATING THE ROLE OF TYPE I INTERFERON SIGNALING IN ADIPOCYTE BIOLOGY

I dedicate this thesis to my husband, Owen Coyle, my parents Harry Kissig Jr. and Lynn Kissig, and my sister, Brienne Flagg. Thank you for always supporting me and my goals. I could not have done it without you!

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ABSTRACT

ELUCIDATING THE ROLE OF TYPE I INTERFERON SIGNALING IN ADIPOCYTE BIOLOGY

Megan E.K. Coyle

Patrick Seale, Ph.D.

Brown and beige adipose tissues represent promising therapeutic targets for combating the rapidly growing obesity pandemic. The goal of this thesis work was to better understand the signaling pathways that affect brown and beige adipose function. First, we investigated pathways regulated by PRDM16, a critical transcription factor for brown and beige fat development. We found that PRDM16 represses type I Interferon (IFN) responses in both preadipocytes and mature adipocytes to promote thermogenic and mitochondrial function. Type I IFN signaling is a critical antiviral pathway, which was previously unexplored in the context of brown fat biology. We found that brown adipocyte character and mitochondrial function were disrupted by ectopic IFN signaling and that increased PRDM16 expression could reverse these negative effects. Additionally, we showed that PRDM16 is required to protect brown fat function from type I IFN signaling *in vivo*. Utilizing multiple transcriptional assays, we determined that PRDM16 blocks IFN regulatory factor 1 (IRF1)-mediated activation of ISGs by competitively binding ISG promoter regions. Adipose inflammation has been implicated in the progression of obesity and insulin resistance. Next, we aimed to determine the role of type I IFN in diet-induced obesity. We found that ISGs are activated in multiple tissues of mice early in the course of high-fat diet (HFD) feeding. Blocking IFN responses using an IFN alpha receptor knockout mouse led to protection from diet-induced obesity and insulin resistance. Additionally, HFD-induced type I IFN decreased energy expenditure, potentially due to mitochondrial dysfunction in the subcutaneous adipose depot. Together this work demonstrates for the first time the negative effects of type I IFN in adipose tissue that may contribute to obesity and insulin resistance.

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CHAPTER 1 : Introduction

I. Obesity and comorbidities

Over 1.9 billion people around the world are now obese or over weight, which is more than double the number of people in 1980 (Finucane et al., 2011). The occurrence of obesity is growing at a disturbing rate and this is considered by many to be a pandemic (Swinburn et al., 2011). According to the World Health Organization, in developed nations the rate of mortality for obese individuals is greater than lean individuals, establishing obesity as a major health risk. This higher mortality rate is caused predominantly by obesity-associated comorbidities, including non-alcoholic fatty liver disease (NAFLD) and steatosis (NASH), cardiovascular disease, type 2 diabetes, and cancer. The correlation of obesity to these diseases is well established, however, direct mechanisms demonstrating how obesity causes these health risks remain unclear.

The relationship between obesity and type 2 diabetes is well recognized and the most extensively studied among the obesity-associated comorbidities. Type 2 diabetes develops when the body can no longer appropriately respond to insulin (American Diabetes, 2012). The stage preceding type 2 diabetes, in which peripheral tissues can no longer utilize glucose due to dysfunctional insulin signaling, is called insulin resistance. In the case of nutritional excess, insulin resistance is initially protective, but over time leads to type 2 diabetes (American Diabetes, 2012). Signaling pathways in multiple tissues, including muscle, liver, and adipose, have been implicated in the development of whole-body insulin resistance (Petersen & Shulman, 2006). The importance of adipose function in the prevention of insulin resistance is demonstrated in models of lipodystrophy, where insulin resistant is prevalent (Garg, 2000; Kim et al., 2000). Adipose is in fact a multifaceted tissue with roles in energy balance, endocrine signaling, inflammatory signaling, and thermoregulation (Pellegrinelli et al., 2016), presenting an intriguing area of study for the prevention and treatment of obesity and insulin resistance.

II. Three types of adipose tissue and their function

White adipose tissue

White adipose tissue (WAT) is composed of adipocytes containing large unilocular lipid droplets. The primary function of this tissue is to store excess calories in the form of triglycerides to be accessible when nourishment is unavailable. Evolutionarily, this is a protective mechanism against starvation. However, most humans are sedentary with an abundance of nutrients available and this leads to obesity (Gesta et al., 2007). In obese individuals, adipose has to store continuously more lipid causing adipose expansion (Pellegrinelli et al., 2016). Adipose can expand through increasing adipocyte size (hypertrophy) or proliferation (hyperplasia) (Jeffery et al., 2015; Wang et al., 2013a). One complication of obesity occurs when adipose tissue reaches its capacity to expand (Freedland, 2004; Kim et al., 2014; Strissel et al., 2007; van Beek et al., 2015), at which point lipid begins to accumulate in other tissues (Ravussin & Smith, 2002). Ectopic lipid accumulation in non-adipogenic tissues, like liver and muscle, has been associated with insulin resistance (Goodpaster et al., 1997; Kelley et al., 1991; Ryysy et al., 2000).

There are two primary types of WAT in mammals: visceral adipose (VAT) and subcutaneous adipose (SAT). VAT is located intra-abdominally and surrounds internal organs, while SAT is located under the skin. These depots both store lipid, but seem to function differently (Ibrahim, 2010) and differentiate at different development time points (Wang et al., 2013a). In certain studies, increased visceral adiposity has been shown to be associated of insulin resistance (Banerji et al., 1997; Lemieux et al., 1996; Pouliot et al., 1992). VAT also has been shown to have more inflammation and immune cell infiltration (Weisberg et al., 2003; Xu et al., 2003). For these reasons, VAT is termed “unhealthy” adipose.

While long thought to be an inert tissue, the important endocrine properties of white adipose are now well established. Adipocyte-secreted proteins, termed “adipokines”, have multiple functions in energy homeostasis. For example, leptin secretion from WAT is critical for

signaling energy availability to the brain and leptin deficiency leads to obesity in mice and humans (Pan et al., 2014; Zhang et al., 1994). Adiponectin, another adipokine produced by adipocytes, is a major regulator of insulin sensitization (Ahima & Lazar, 2008; Hu et al., 1996). Additional adipokines have roles in adipogenesis, fatty acid oxidation, glucose uptake, energy expenditure, and inflammation (Ahima & Lazar, 2008), demonstrating the critical role of adipose tissue in human physiology.

Brown and beige adipose tissue

It has long been known that brown adipose tissue (BAT) is present in small mammals and infant humans and is located in the interscapular region (Fig 1.1) (Cannon & Nedergaard, 2004). Importantly, BAT is both developmentally and functionally distinct from WAT. Brown adipocytes arise from the same *Myf5*$^{+}$ precursor as muscle (Seale et al., 2008; Timmons et al., 2007), while white adipocyte precursors are predominantly *Myf5*$^{-}$. Morphologically, BAT is characterized as containing a high number of mitochondria and small multilocular lipid-containing adipocytes (Cannon & Nedergaard, 2004). While WAT functions to store energy, BAT utilizes lipids to generate heat through the protein Uncoupling protein 1 (UCP1) (Klingenberg et al., 1999), a process termed non-shivering or adaptive thermogenesis. Upon cold exposure, the sympathetic nervous system (SNS) releases norepinephrine, which binds to the β_3-adrenergic receptors on brown adipocytes. This leads to lipolysis and transport of free fatty acids (FFA) into the mitochondria where they bind UCP1, activating the protein and causing a proton leak across the membrane and uncoupling the electron transport chain (Fedorenko et al., 2012; Nicholls, 2006). The inefficiency of this process leads to heat generation (Fig 1.2) (Nicholls, 2006), making BAT an important evolutionary adaptation to protect against hypothermia.

Within WAT resides a third distinct type of adipocyte, called a "brown-in-white" (brite) or "beige" adipocyte (Cousin et al., 1992; Petrovic et al., 2010). These cells are not classic brown adipocytes as they are not derived from *Myf5*$^{+}$ precursors (Petrovic et al., 2010; Seale et al., 2008). However, upon cold exposure, these cells express UCP1 and have higher mitochondria

content (Cousin et al., 1992), making them functionally similar to brown adipocytes (Fig 1.2). In addition to cold, the peroxisome proliferator-activating receptor gamma (PPARγ)-ligands, thiazolidinediones (TZDs), are potent activators of beige adipogenesis (Petrovic et al., 2010; Tiraby et al., 2003). Converting white adipose into a more "brown-like" adipose is an exciting concept, but it is debated whether beige adipocytes arise from differentiation of precursors or spontaneous trans-differentiation of mature white adipocytes. Lineage-tracing studies in mice have found that precursors within WAT will differentiate into UCP1-expressing cells during cold exposure (Wang et al., 2013a). It has also been found that mature adipocytes can be activated to express UCP1 (Himms-Hagen et al., 2000; Vitali et al., 2012). Further work in this area is needed to conclude how beige adipose develops.

Factors in Beige and Brown Adipogenesis

Adipose	Developmental lineage	Location (mice)	Location (humans)	Activators
Brown	Myf5+, PDGFRα+, EBF2+	Interscapular, cervical, axillary, perirenal	Neck, interscapular	Cold, thiazolidinediones, FGF21, natriuretic peptides, BMP7/8b, orexin
Beige	Myf5-, PDGFRα+, EBF2+	Subcutaneous WAT	Supraclavicular	Cold, thiazolidinediones, FGF21, natriuretic peptides, Irisin

Figure 1.1 Factors in Beige and Brown Adipogenesis

Brown and beige adipocytes develop from distinct precursor pools. In mice, brown fat is predominantly found in the interscapular, cervical, axillary, and perirenal depots, whereas beige fat is most prominent in subcutaneous white fat depots. In humans, the delineation between brown and beige adipose is less well defined; however, depots of brown adipose have been identified in the neck and interscapular regions, whereas beige adipose has been found in the supraclavicular region. In addition to cold exposure, there are multiple synthetic activators of brown and beige adipocytes. Adapted from Kissig et al, 2016.

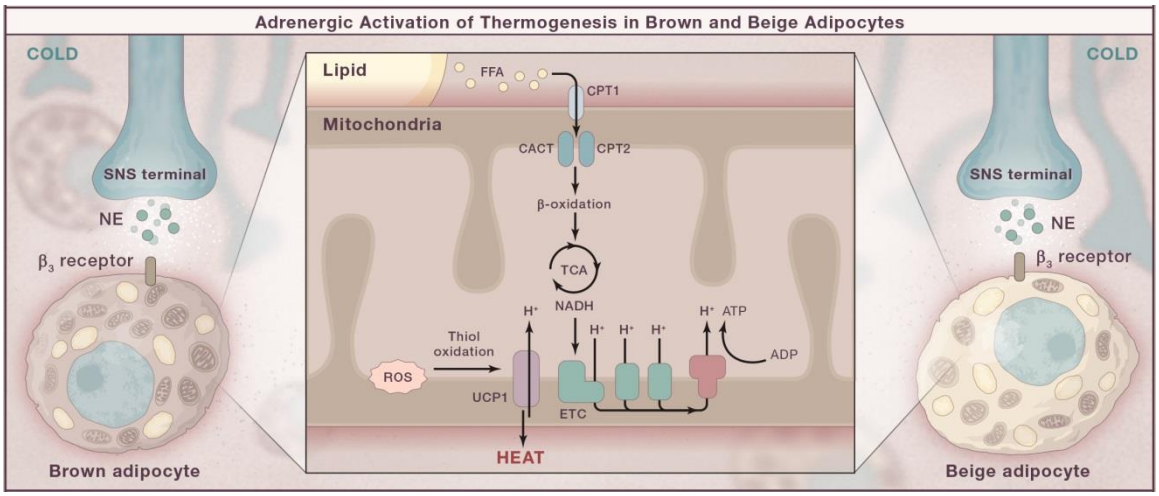

Figure 1.2 Adrenergic Activation of Thermogenesis in Brown and Beige Adipocytes

Cold exposure elicits sympathetic neurons to secrete norepinephrine (NE), which binds to beta-adrenergic receptors on brown and beige fat cells. This triggers a signaling cascade leading to an increase in thermogenic gene expression and lipolysis. Mitochondria oxidize the released free fatty acids (FFAs). Further, UCP1 binds FFAs which activates its function to catalyze the leak of protons across the inner mitochondrial membrane, resulting in the production of heat rather than ATP from the oxidation of available substrates. Adapted from Kissig et al, 2016.

Investigation into brown adipose has increased dramatically since it was discovered that adult humans have active brown adipose tissue (Cypess et al., 2009). While the interscapular brown adipose found in infants and small children is undetectable in adults (Lean, 1989), utilization of Fluorodeoxyglucose - Positron Emission Topography (FDG-PET) scans combined with molecular analysis found that adults have *Ucp1*-expresing brown adipose in their neck and supraclavicular region (Cypess et al., 2009) (Fig 1.1). Interestingly, upon cold exposure both older and obese individuals have less activated brown fat (Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). In many mouse studies, it has been shown that inducing brown and beige adipose increases energy, reduces weight gain, and improves insulin sensitivity (Auffret et al., 2012; Bostrom et al., 2012; Cederberg et al., 2001; Collins et al., 1997; Feldmann et al., 2009; Guerra et al., 1998; Seale et al., 2011). Humans who were cold-exposed for two hours daily for six weeks had more activated brown adipose and lower fat mass with no other changes in diet or exercise (Yoneshiro et al., 2013). This work suggests activating brown adipose in humans could be a promising therapeutic for combating obesity. It is unclear whether adult

humans have classic brown fat or inducible beige adipose. Unbiased studies comparing human supraclavicular brown adipose and brown and beige adipose from mice showed a more similar gene signature between human brown and mouse beige adipose (Sharp et al., 2012; Wu et al., 2012). Together this work emphasizes how critical investigating the activation and regulation of brown and beige adipose is for potential obesity therapies.

III. Transcriptional regulation of brown and beige adipose

Activators

Brown and beige adipogenesis is tightly controlled by numerous transcription factors that are critical for both the development and activation of these tissues. As in white adipose (Rosen et al., 1999), PPARγ and CCAAT/enhancer-binding protein (C/EBP) factors are required for the differentiation of brown and beige adipocytes (Karamanlidis et al., 2007; Tai et al., 1996). These factors are required to transcriptionally activate thermogenic gene expression, such as *Ucp1* (Kelly et al., 1998; Sears et al., 1996; Siersbaek et al., 2012). In order to determine factors specific to brown adipose tissue development, the Spiegelman lab conducted an unbiased comparison of genes in white and brown adipose from mice. This study revealed the transcription factor PRD1-BF1-RIZ1 homologous-domain-containing protein 16 (PRDM16) as a critical factor for brown adipose function and UCP1 expression (Seale et al., 2007). PRDM16 regulates brown and white gene expression through multiple mechanisms [discussed below]. Another transcription factor important for the development of brown adipose is Early B cell factor 2 (EBF2) which was found to have binding sites near PPARγ-binding at brown genes such as *Ucp1* and *Prdm16* (Rajakumari et al., 2013). EBF2 is more highly expressed in brown adipose compared to white adipose and genetic loss of function in mice leads to defective BAT development (Rajakumari et al., 2013). While PRDM16 expression increases through differentiation (Seale et al., 2007), EBF2 is a marker for committed brown and beige precursors (Wang et al., 2014). Further studies are

necessary to determine the function and developmental regulation of EBF2 in adipocytes precursors.

Certain factors are specifically required for thermogenic activation of mature adipocytes. PPARγ-coactivator 1α (PGC1α) is highly expressed in BAT, but its expression is dispensable for BAT development (Leone et al., 2005; Lin et al., 2004). PGC1α functions to increase the transcriptional activity of PPARγ at the UCP1 promoter and is required for the progression of mitochondrial biogenesis upon cold-exposure (Puigserver et al., 1998; Tiraby et al., 2003; Uldry et al., 2006) (Fig 1.3). Sympathetic stimulation of brown adipocytes leads to a swift activation of signaling pathways leading to the transcriptional induction of thermogenic genes (Collins, 2011). Primarily this is mediated by cyclic AMP (cAMP) and protein kinase A (PKA) (Cummings et al., 1996). PKA phosphorylates CRE-binding protein (CREB) and p38-mitogen activated protein kinase (p38/MAPK) which synergistically activate *Ucp1* (Xue et al., 2005). CREB binds the *Ucp1* promoter to transcriptionally activate its expression (Yubero et al., 1998), while p38/MAPK phosphorylates PGC1α to then induce expression of *Ucp1* and other brown fat-selective genes (Cao et al., 2004) (Fig1.3).

Repressors

While many would like to utilize “browning” of white adipose as a potential therapy for obesity, there are multiple mechanisms repressing the brown fat program in WAT. Retinoblastoma (Rb) factors pRB and p107 block brown fat activation by repressing transcription of *Pgc1α* (Scime et al., 2005). The deletion of p107 in mice leads to the accumulation of multilocular, UCP1-expressing cells within WAT (Scime et al., 2005). Alternatively, the browning of WAT is accompanied by decreased Rb expression (Hansen et al., 2004). Another repressor of brown fat-selective gene expression is receptor-interacting protein 140 (RIP140), which interacts with PGC1α to suppress its activation of brown fat genes (Christian et al., 2005; Hallberg et al., 2008). TWIST1, a helix-loop-helix transcription factor, also binds to PGC1α and blocks activation of its target genes (Pan et al., 2009). Forkhead transcription factor O1 (FOXO1) has a similar role

in white and brown adipocytes whereby it blocks differentiation by blocking PPARγ and C/EBPβ activity (Nakae et al., 2008; Nakae et al., 2003).

More recently, work from the Spiegelman and Gupta labs has established Zinc finger protein 423 (ZFP423) as a critical lineage determination factor for white adipocytes. ZFP423 is expressed in committed adipocyte precursors (Gupta et al., 2010) and is repressed by paralog ZFP521 to promote osteoblast commitment (Addison et al., 2014; Kang et al., 2012). When Zfp423 is inactivated in precursors, differentiation of white adipose tissue is disrupted (Shao et al., 2017); however, when Zfp423 is deleted from mature adipocytes, it leads to a switch from white to beige phenotype (Shao et al., 2016). Zfp423 blocks beige fat development by blocking EBF2 transcriptional activity (Shao et al., 2016). Zfp423 represents an intriguing target in promoting beige adipogenesis in white adipose depots of humans.

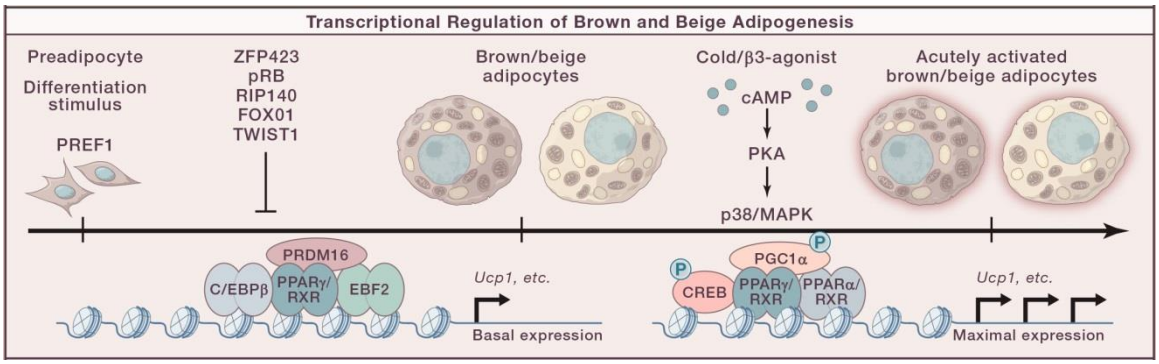

Figure 1.3 Transcriptional Regulation of Brown and Beige Adipogenesis

Brown and beige adipocyte differentiation from precursor cells is regulated by an overlapping set of both pan-adipogenic and brown fat-specific transcription factors. Activators include peroxisome proliferator-activated receptor gamma (PPARγ), early B cell factor 2 (EBF2), PR domain-containing protein 16 (PRDM16), and C/EBPβ. Conversely, many transcription factors, including ZFP423, FOXO1, TWIST1, p107, LXRa, pRB, and RIP140, repress brown-fat-selective genes either directly or by repressing activators such as PPARγ coactivator-1α (PGC1α). Adapted from Kissig et al, 2016.

IV. PRDM16: diverse regulatory roles in adipose and other tissues

PRDM16 Structure

PRDM16 was first described as being located near a chromosomal breakpoint in human acute myeloid leukemia (Nishikata et al., 2003). The full-length PRDM16 consists of an N-terminal PR domain, the primary characteristic defining the PRDM family of proteins, which is similar to SET domains of histone lysine methyltransferases (Hohenauer & Moore, 2012). Additionally, it contains two clusters of C2H2-type zinc fingers: zinc finger 1 (ZF1), containing seven zinc fingers at the N-terminal region, and ZF2, with three zinc fingers at the C-terminal region. These ZFs are thought to mediate many DNA and protein interactions of PRDM16 (Ishibashi & Seale, 2015) (Fig 1.4A). While PRDM16 is predicted to bind DNA (Seale et al., 2007), thus far all PRDM16 functions have been attributable to binding of cofactors. Other domains include a proximal regulatory region (PRR) and C-terminal activation domains (AD), which are not well defined (Ishibashi & Seale, 2015).

PRDM16 function in brown and beige adipose

The most well defined role for PRDM16 is in regulating brown and beige adipose. PRDM16 is expressed in multiple tissues, but the highest levels are in BAT (Seale et al., 2007). It was initially shown that *in vitro* knockdown of PRDM16 in brown adipocytes reduced brown fat-selective gene expression including *Ucp1* and mitochondrial genes (Seale et al., 2007). Further investigation revealed PRDM16-expression induces brown-like fat development in a variety of mesodermal cell types, including white preadipocytes, skeletal myoblasts and fibroblasts (Kajimura et al., 2009; Seale et al., 2008; Seale et al., 2007). Notably, PRDM16 was the first factor demonstrated to delineate brown adipocyte versus myoblast development (Seale et al., 2008).

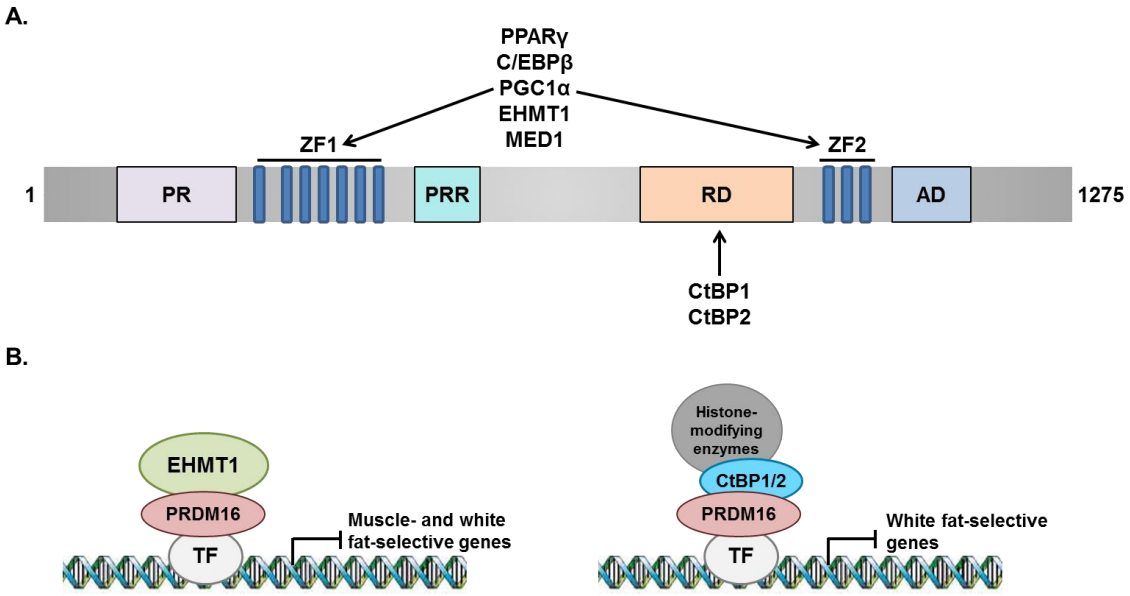

Figure 1.4 PRDM16 Structure and Cofactors

A. Schematic of PRDM16 protein structure. Domains include PR domain, zinc-fingers 1 and 2 (ZF1/ZF2), proximal regulatory region (PRR), repression domain (RD), activation domain (AD). ZF1 and ZF2 are critical for cofactor binding. In addition, the RD region contains binding sites for CtBP1 and CtBP2.

B. Schematic of PRDM16 repressive mechanisms. PRDM16 binds EHMT1 to repress muscle and white fat-selective genes. Additionally, PRDM16 represses white fat-selective genes by binding to CtBP1/2.

Adapted from Ishibashi & Seale, 2015 and Chi & Cohen, 2015.

PRDM16 employs multiple mechanisms to promote brown /beige adipocyte differentiation. Initially it was found that PRDM16 activates the transcription of brown fat-specific genes, like *Ucp1*, through binding other known activators of brown fat function, including PPARγ, PGC1α, and C/EBP-β (Kajimura et al., 2009; Seale et al., 2008; Seale et al., 2007) (Fig 1.3). More recently, a novel mechanism has been described where PRDM16 promotes chromatin looping by recruiting the Mediator complex to super enhancers at brown fat-selective genes (Harms et al., 2015; Iida et al., 2015). This mechanism demonstrates the multifaceted transcriptional functions of PRDM16.

PRDM16 acts not only as a transcriptional activator, but also as a repressor of other lineage-specific gene programs. PRDM16 represses white adipocyte-specific genes, such as

Resistin, through interactions with C-terminal-binding proteins (CtBPs) (Kajimura et al., 2008). Additionally, muscle-specific genes are downregulated by PRDM16 through binding with euchromatic histone-lysine N-methyltransferase (EHMT1) (Ohno et al., 2013) (Fig 1.4B). The brown fat-specific deletion of EHMT1 leads to a loss of brown fat characteristics and an increase in myogenic gene expression (Ohno et al., 2013). The N-terminal PR domain of PRDM16 may also be required for repression of myogenic genes during adipogenesis through methylation of Histone H3K9 (Li et al., 2015). Together these studies have shown that PRDM16 has multiple mechanisms of regulation that work in concert to promote brown fat development and activity.

Ectopic PRDM16 expression in white adipocytes is sufficient to promote browning (Seale et al., 2007). This was replicated *in vivo* using an adipocyte-specific Prdm16-overexpressing transgenic mouse line, which displayed extensive browning of the subcutaneous depot (Seale et al., 2011). The increased beige fat content led to increased energy expenditure and protection against DIO (Seale et al., 2011). Not only is PRDM16 sufficient, but it is also required for beige adipose activation by cold and TZDs (Cohen et al., 2014; Ohno et al., 2012). TZD treatment of white adipocytes was shown to stabilize the PRDM16 protein, promoting *Ucp1* expression (Ohno et al., 2012). Sirt1 deacetylation of PPARγ, which mimics TZD treatment, enhances binding to PRDM16 leading to brown fat gene activation (Qiang et al., 2012). Both of these mechanisms indicate the importance of PRDM16 expression in browning of white adipocytes. The adipose-specific (*Adiponectin-driven* Cre) depletion of PRDM16 established the requirement of PRDM16 for cold-induced browning of white adipose *in vivo* (Cohen et al., 2014). This model also revealed a role for PRDM16 in maintaining subcutaneous adipose in an anti-inflammatory state, although the mechanism of this effect remains unclear.

While the requirement for PRDM16 in brown adipocyte differentiation is established in culture, brown fat-specific (*Myf5*-driven Cre) PRDM16-deficiency demonstrated that PRDM16 is largely dispensable for BAT embryonic development due to compensation by another PRDM family member, PRDM3. However, as mice age PRDM16 is required for the maintenance of brown fat-selective and mitochondrial genes and overall function of the tissue (Harms et al.,

2014). Interestingly, the adipose-specific loss of function mice displayed PRDM16 deletion from brown adipose as well as white, but there was no observable defect in the BAT (Cohen et al., 2014). These mice were not observed in advanced age and so it is possible a loss of brown fat character would occur over time. Another potential explanation for this discrepancy is that *Adiponectin* is expressed much later in the differentiation of a brown adipocyte than *Myf5*, which would indicate PRDM16 may have important regulatory functions in brown fat precursors.

PRDM16 function in other tissues

Whole body PRDM16-deletion is embryonically lethal and multiple defects including cleft palate, altered craniofacial development, and impaired cardiac development are observed in these embryos (Bjork et al., 2010). PRDM16 is also critically required in the development and maintenance of certain stem cells, including hematopoietic stem cells (HSCs) and neural stem cells (Aguilo et al., 2011; Chuikov et al., 2010). In these populations, loss of PRDM16 expression increases levels of reactive oxygen species (ROS) and leads to cell death (Chuikov et al., 2010). Similarly, PRDM16 ablation in astrocytoma cells leads to mitochondrial dysfunction and apoptosis (Lei et al., 2016). PRDM16 also promotes mitochondrial function and reduces endoplasmic reticulum stress in HSCs by inducing the expression of the mitochondrial fusion protein Mitofusin 2 (Luchsinger et al., 2016). Overall, these studies establish a role for PRDM16 in stem cell maintenance, where it functions primarily to protect mitochondria and cells from stress. While mitochondrial dysfunction is apparent in PRDM16-deficient BAT (Harms et al., 2014), the role of PRDM16 in mitochondrial maintenance is not clear. Additionally, it is not known whether PRDM16 functions to protect brown/beige adipose from cellular stresses.

V. Adipose tissue inflammation

Toll-like receptors

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) activated by various ligands presented by pathogens, such as lipopolysaccharide (LPS). Ligand-binding leads to cytokine secretion and immune cell response, a critical first step in the innate immune response (Takeda & Akira, 2001). Notably, many TLR family members are expressed on mouse and human adipocytes and when stimulated elicit the secretion of pro-inflammatory factors (Bes-Houtmann et al., 2007; Kopp et al., 2009; Lin et al., 2000). Previously it was unknown how the inflammatory signals were initiated within adipose, but it was found that FFAs released from adipose tissue mimic TLR4 ligands causing an induction of an immune response (Shi et al., 2006; Song et al., 2006). Coordinately, TLRs themselves are upregulated in visceral adipose tissue in diet-induced or genetic models of obesity (Kim et al., 2012). Activation of TLR4 leads to insulin resistance in adipocytes (Song et al., 2006) and TLR4 deficiency in mice provides protection against DIO-associated insulin resistance (Shi et al., 2006). TLR4 is expressed on other tissues including muscle (Reyna et al., 2008) and in initial studies TLR4 was deleted in the whole body, leaving the question of which tissue was directly responsible for improving insulin signaling. Saberi et al. demonstrated that mice reconstituted with TLR4-deficient HSCs were protected against diet-induced insulin resistance (Saberi et al., 2009). However, the contribution of adipose TLR4 and its downstream signaling pathways to the development of whole body insulin resistance still remains to be elucidated.

TNFα and IL6

One of the first inflammatory factors shown to be induced in adipose with obesity was tumor necrosis factor alpha (TNFα). In the early 1990s, increased expression of TNFα was shown in multiple mouse models of obesity (Hotamisligil et al., 1993). Obese humans also have increased adipose TNFα (Hotamisligil et al., 1995; Kern et al., 1995). In both humans and mice TNFα expression corresponds with development of insulin resistance (Hotamisligil et al., 1995;

Hotamisligil et al., 1993; Kern et al., 1995). Rodents lacking functional TNFα or its receptors are more insulin sensitive and glucose tolerant (Cheung et al., 1998; Uysal et al., 1997; Ventre et al., 1997). It is believed that TNFα induces insulin resistance in adipocytes by decreasing the expression of glucose transporter 4 and insulin substrate receptor 1 (Stephens et al., 1997; Stephens & Pekala, 1992). Macrophages also produce more TNFα in DIO which is associated with insulin resistance (De Taeye et al., 2007; Yamakawa et al., 1995). While the connection between TNFα expression, obesity-induced inflammation, and insulin resistance is strong, pharmaceutical inhibitors of TNFα function have not improved insulin sensitivity in obese patients (Ofei et al., 1996; Paquot et al., 2000), suggesting it is not the primary effector.

Interleukin (IL6) is also increased in obese subjects and higher levels correlates with an increased likelihood of developing insulin resistance (Pradhan et al., 2001; Roytblat et al., 2000; Vozarova et al., 2001). Interestingly, weight loss can reduce IL6 levels (Bastard et al., 2000) demonstrating a strong correlation between IL6 and adiposity in humans (Carey et al., 2004).While ectopic treatment of IL6 disrupts insulin signaling (Klover et al., 2003), knockdown of IL6 does not rescue insulin sensitivity in obese mice (Wallenius et al., 2002). These discrepancies indicate further *in vivo* study on the role of IL6 in obesity-induced insulin resistance is required.

IFNγ

Interferon gamma (IFNγ) is a type II IFN and is a critical mediator of adaptive immunity (Schroder et al., 2004). IFNγ is secreted from T helper 1 cells (Th1) and CD8+ T cells to activate other immune cells to mount a defense against pathogens (Schroder et al., 2004). Notably, IFNγ is also secreted from immune cells of the adipose tissue in DIO mouse models (Rocha et al., 2008; Wensveen et al., 2015). While results on weight gain varied, three independent groups showed that ablating IFNγ signaling in mice improves diet-induced insulin resistance (O'Rourke et al., 2012; Rocha et al., 2008; Wong et al., 2011). IFNγ has multiple effects that may contribute to the development of insulin resistance: 1) IFNγ signaling in DIO leads to increased inflammatory cell infiltration and cytokine expression in adipose causing systemic inflammation (O'Rourke et

al., 2012; Zhang et al., 2011) and 2) IFNγ can act directly on adipocytes to disrupt insulin signaling, lipid storage, and differentiation (McGillicuddy et al., 2009). The contribution of IFNγ-mediated disruption of adipocyte insulin signaling to whole body insulin resistance remains unclear.

Immune cells of the adipose tissue

Cells of both the innate and adaptive immune system have roles in the development of adipose inflammation. Macrophages are the primary form of immune cells within adipose and obesity is associated with macrophage accumulation through both infiltration and proliferation (Weisberg et al., 2003; Xu et al., 2003; Zheng et al., 2016). While it was known that inflammation occurred during obesity, a major finding in the field was that HFD induces a macrophage polarization switch from M2 (anti-inflammatory) to M1 (pro-inflammatory) (Lumeng et al., 2007a; Lumeng et al., 2007b). This switch is associated with the development of chronic low-grade inflammation throughout the body.

The polarization switch of adipose macrophages is preceded by a cascade of immune cell activation and signaling. An accumulation of CD4+, CD8+, and Th1 T cells in adipose tissue is observed preceding macrophage accumulation (Kintscher et al., 2008; Nishimura et al., 2009; Winer et al., 2009). In both mice and humans, adipose T cell accumulation was associated with obesity and the development of insulin resistance (Kintscher et al., 2008; Pacifico et al., 2006; Winer et al., 2009). Early in DIO, antigen-presenting B cells activate both CD4+ and CD8+ T cells, leading to the production of IFNγ (DeFuria et al., 2013; Winer et al., 2011). IFNγ production leads to M1 macrophage recruitment in adipose tissue (Nishimura et al., 2009; Rocha et al., 2008). Supporting this idea, combined CD4+ and CD8+ T cell deficiency prevents macrophage recruitment and adipose inflammation (Khan et al., 2014). STAT3 expression initiates a switch from Treg cells to inflammatory Th1 cells via IL6 signaling, also promoting DIO and insulin resistance (Priceman et al., 2013). Natural killer (NK) cells, classified as innate lymphoid cells, are another cell type of the innate immune system activated in obesity. NK cells, like T cells,

promote M1 macrophage polarization through IFNγ and TNFα production in both mice and humans (Lee et al., 2016a; O'Rourke et al., 2009; O'Rourke et al., 2014; Wensveen et al., 2015). Deletion of NK cells from adipose tissue leads to decreased macrophage accumulation and an attenuation of diet-induced insulin resistance (Lee et al., 2016a; O'Rourke et al., 2014; Wensveen et al., 2015). NK cells function primarily in visceral adipose (Lee et al., 2016a), while in subcutaneous adipose, other group 1 innate lymphoid cells (ILC1s) are activated during obesity to produce IFNγ and recruit macrophages (O'Sullivan et al., 2016). Overall, adipose immune cells have a prominent part in inducing adipose inflammation and contributing to obesity and insulin resistance.

Inflammation in beige/brown adipose

While there have been numerous investigations of obesity-induced inflammation and immune cell infiltration in white adipose, these pathways in brown adipose are largely unexplored. Interestingly, BAT demonstrates a resistance to inflammation compared to WAT (Sierra Rojas et al., 2016). An initial report showed that after 13 weeks of HFD, BAT had almost no immune cell infiltration (Fitzgibbons et al., 2011). TLRs are expressed on brown adipocytes and are upregulated in BAT of obese mice, indicating inflammatory signaling is intact in this tissue (Bae et al., 2014). Activation of TLRs on brown adipocytes decreases basal and stimulated brown fat-selective genes (Bae et al., 2014). Interestingly, activation of TLR4 in mice causes a slight decrease in cold-activation of BAT, but a significant block in subcutaneous browning (Okla et al., 2015). These data again indicate there must be a protective mechanism unique to brown adipose. TNFα also decreases cold-responsiveness in BAT and SAT (Sakamoto et al., 2016). Together this suggests reducing inflammatory responses protects thermogenic function. Further studies are needed to determine the mechanism of resistance and better characterize inflammatory signals downstream of TLRs that negatively regulate *Ucp1* and brown fat function.

VI. Type I Interferon Pathway

Type IFNs

The type I IFN pathway has been extensively studied since it was first discovered 60 years ago (Isaacs & Lindenmann, 1957). In humans the type I IFN pathway is comprised of five family members IFN- α, β, ε, κ and ω (Pestka et al., 2004). In contrast, the type II IFN family is comprised of only IFNγ. The most well characterized type I IFNs are IFNα and IFNβ. Multiple species of IFNα are expressed while there is only one form of IFNβ. Type I IFNs are induced by viral infection, primarily downstream of PRRs (Noppert et al., 2007; Ozato et al., 2002). While almost any cell can secrete type I IFNs, IFNβ is most often produced by non-immune cells such as fibroblasts and IFNα is produced by innate immune cells such as macrophage and dendritic cells (Ivashkiv & Donlin, 2014). The primary role of type I IFNs is to activate immune cells. For example, IFNα strongly activates the differentiation of monocytes into mature dendritic cells (Santini et al., 2000; Santodonato et al., 2003). Type I IFN also primes mature dendritic cells to activate cells of the adaptive immune system such as CD8+ T cells, Th1 cells, and NK cells (Dean & Virelizier, 1983; Lapenta et al., 2006; Parlato et al., 2001; Santini et al., 2011), demonstrating type I IFNs as important modulators of both innate and adaptive immune responses.

IFNAR and downstream signaling

Type I IFNs signal through the IFN alpha receptor (IFNAR), a heterodimer of two subunits, IFNAR1 and IFNAR2 (Ivashkiv & Donlin, 2014). The binding of IFNs to IFNAR initiates a signaling cascade, starting with the activation of receptor-associated proteins Janus kinase 1 (JAK1) and Tyrosine kinase 2 (TYK2). These kinases go on to phosphorylate signal transducer and activator of transcription 1 (STAT1) and STAT2 (Stark & Darnell, 2012). STAT1 and STAT2 then dimerize and form a complex with IFN regulatory factor 9 (IRF9) called IFN-stimulated gene factor 3 (ISGF3). This complex is then transduced into the nucleus, where it binds sequences identified as IFN-sensitive response elements (ISRE) [GAAANNGAAAG/CT/C] present at the

promoters of many interferon-stimulated genes (ISGs) (Fig 1.5). Other cytokines, such as IFNγ, primarily activate STAT1 homodimers which bind to gamma-activated sequences (GAS). This specificity of motif binding presents a way in which type I IFNs activate a unique subset of genes for antiviral response (Rusinova et al., 2013; Samarajiwa et al., 2009; Schoggins et al., 2011). While ISGF3 complex formation is the canonical signaling cascade activated by type I IFN, STAT-3, 4, and 5 can also be utilized to promote gene expression (Su & David, 2000; Yang et al., 1996; Ziegler-Heitbrock et al., 2003), demonstrating a diversity in the type I IFN antiviral signal depending on pathogen and cell type.

Interferon regulatory factors

Interferon regulatory factors are critical for activating the type I IFN pathway. There are nine IRF family members (IRF1-9), which are transcription factors that contain a 120 amino acid N-terminal DNA-binding domain that is well conserved in the family of proteins (Taniguchi et al., 2001). This DNA-binding element recognizes sequences called IRF binding elements (IRF-E) [G(A)AAAG/CT/CGAAAG/CT/C], which are remarkably similar to ISREs. IRF1 was the first to be discovered as a factor that binds the promoter of IFNβ (Fujita et al., 1988; Miyamoto et al., 1988). IRF2 shares the most homology to IRF1 and binds the same DNA sequences, but represses many functions of IRF1 by competing for binding sites (Harada et al., 1989; Harada et al., 1990; Tanaka et al., 1993; Yamamoto et al., 1994). IRF3 and IRF7 are very similar proteins, which were both discovered for their homology to IRF1 (Au et al., 1995; Zhang & Pagano, 1997). Both IRF3 and IRF7 activate type I IFN expression; however, IRF3 and IRF7 can homo-dimerize or hetero-dimerize, with each dimer activating a different *Ifna* gene or *Ifnb* (Marie et al., 1998; Sato et al., 1998a; Sato et al., 2000; Sato et al., 1998b; Yoneyama et al., 1998) (Fig 1.5). IRF9 is another important factor for type I IFN signaling and was initially discovered as a component of the ISGF3 complex (Fu et al., 1990; Kessler et al., 1988) and later identified as a member of the IRF family of proteins (Veals et al., 1992). IRF4, 5, 6, and 8 all have varying functions, including lymphoid

cell development (Eisenbeis et al., 1995; Matsuyama et al., 1995; Nelson et al., 1996), but their roles in type I IFN signaling are less well known.

IRFs can not only activate type I IFN production to initiate downstream signaling, but can also bind to promoter elements of ISGs to amplify responses (Ivashkiv & Donlin; Stark & Darnell). It has been shown that IRF1 and IRF3 can directly activate certain ISGs by binding upstream promoters (Grandvaux et al., 2002; Xu et al., 2016). Many ISG promoters contain not only ISREs, but also IRF-E and GAS elements, suggesting cooperative binding of these promoters by multiple activators (Harada et al., 1996; Kimura et al., 1996). Additionally, ISGF3 binds the promoters regions of *Stats* and *Irf1*, *Irf7*, and *Irf9* to induce expression and form a feed-forward loop to maximally activate of other ISGs (Cheon et al., 2013; Harada et al., 1989; Marie et al., 1998; Sato et al., 1998a) (Fig 1.5). Together this demonstrates the importance of IRFs in regulating type I IFN signaling by both stimulating *Ifn* expression and coordinating promoter activation at ISGs to achieve maximal responsiveness.

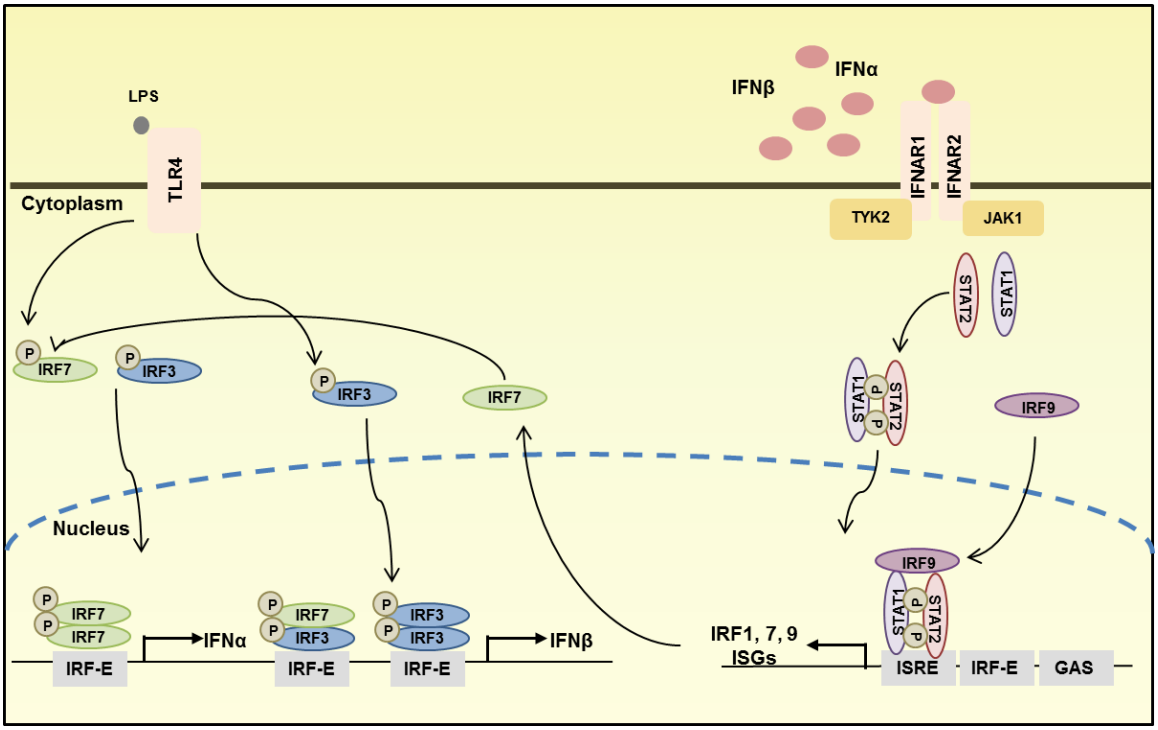

Figure 1.5 Type I Interferon Signaling

Toll-like receptors (TLR) on cell surfaces detect viruses or pathogens. Lipopolysaccharide (LPS), a component of many bacteria, binds TLR4 and stimulates a signaling cascade. This leads to the phosphorylation of interferon regulator factors, IRF3 and IRF4. These factors dimerize and translocate into the nucleus where they bind IRF elements (IRF-E) to activate Interferons, IFNα and IFNβ. Type I IFNs are released by the cell and bind to IFNα receptor (IFNAR). Receptor-associated proteins, TYK2 and JAK1, phosphorylate STAT1 and STAT2 that go on to form a complex with IRF9. The complex enters the nucleus and binds IFN-sensitive response elements (ISRE) to promote transcriptional activation of IFN-stimulated genes (ISGs). Adapted from Decker et al, 2005 and Ivashkiv and Donlin, 2014.

Type I IFN and IRFs in adipose tissue and insulin resistance

While the type II, IFNγ, has been shown to be an initiator of inflammation in adipose [discussed above], very little work on type I IFNs in adipose tissue has been done. TLRs, the initiators of innate immune signaling, are expressed on preadipocytes and adipocytes (Lin et al., 2000) and activation of adipocyte TLR3 in culture induces type I IFNs and downstream antiviral protein expression (Yu et al., 2014). This demonstrates that adipose cells are equipped with an innate antiviral system, although the function of adipose in viral infection is unclear. How this signaling affects adipocyte function is another remaining question. Certain studies have suggested that ectopic IFN signaling blocks adipogenesis and promotes apoptosis of mature

adipocytes (Birk & Rubinstein, 2006; Lee et al., 2016b), however, this work was only done in one adipogenic cell line. Other studies have shown that both type II and type I IFNs elicit insulin resistance in adipocytes (Wada et al., 2011). This is consistent with patient studies that demonstrate IFNα induces insulin resistance by decreasing glucose uptake in tissues (Imano et al., 1998; Koivisto et al., 1989). Further studies are needed to elucidate the role of type I IFN signaling in adipose *in vivo.*

All IRFs are expressed in preadipocytes and adipocytes, although they display different expression patterns through differentiation and many repress adipogenesis (Eguchi et al., 2008). IRF3 and IRF4 have increased expression in mature adipocytes and both of these factors have been found to regulate adipocyte function. IRF4 is required for lipolysis in both brown and white adipose and loss of IRF4 in adipocytes leads to increased weight gain and adiposity on HFD (Eguchi et al., 2011). IRF4 also promotes brown fat function through cooperation with PGC1α to bind and activate brown fat-selective and mitochondrial gene expression (Kong et al., 2014). IRF4 ablation in brown adipose leads to increased weight gain on HFD due to reduced energy expenditure (Kong et al., 2014). IRF4 is not only a critical regulator of adipocyte function, but also of macrophage polarization within adipose tissue. IRF4 promotes M2 macrophage polarization and an anti-inflammatory state in adipose which reduces diet-induced weight gain and insulin resistance (Eguchi et al., 2013). Alternatively, IRF3 promotes diet-induced adipose inflammation leading to weight gain and insulin resistance. Ablation of IRF3 also increases browning of subcutaneous adipose and increases energy expenditure (Kumari et al., 2016). Similarly, IRF7 deficiency ameliorated diet-induced weight gain and systemic inflammation (Wang et al., 2013b). Thus, IRF family members appear to have diverse roles in both white and brown adipose. *In vivo* investigations of other IRF family members in adipose tissue are still needed. While the critical nature of IRFs in adipose is clear, none of the studies directly address how the type I IFN signaling may contribute to adipose dysfunction and obesity.

CHAPTER 2 : PRDM16 represses the type I Interferon response in adipocytes to promote mitochondrial and thermogenic programing

This chapter is adapted from

Megan Kissig[1,2,], Jeff Ishibashi[1,2], Matthew J. Harms[1,2], Hee-Woong Lim[1,3], Rachel R. Stine[1,2], Kyoung-Jae Won[1,3], Patrick Seale[1,2,] (2017) PRDM16 represses the type I Interferon response in adipocytes to promote mitochondrial and thermogenic programing. *EMBO Journal* (Accepted)

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I. Abstract

Brown adipose has the potential to counteract obesity and thus identifying signaling pathways that regulate the activity of this tissue is of great clinical interest. PRDM16 is a transcription factor that activates brown fat-specific genes while repressing white fat and muscle-specific genes in adipocytes. Whether PRDM16 also controls other gene programs to regulate adipocyte function was unclear. Here, we identify a novel role for PRDM16 in suppressing type I Interferon (IFN)-stimulated genes (ISGs), including *Stat1*, in adipocytes *in vitro* and *in vivo*. Ectopic activation of type I IFN signaling in brown adipocytes induces mitochondrial dysfunction and reduces Uncoupling protein 1 (UCP1) expression. *Prdm16*-deficient adipose displays an exaggerated response to type I IFN, including higher STAT1 levels and reduced mitochondrial gene expression. Mechanistically, PRDM16 represses ISGs through binding to promoter regions of these genes and blocking the activating function of IFN regulatory factor 1 (IRF1). Together, these data indicate that PRDM16 diminishes responsiveness to type I IFN in adipose cells to promote thermogenic and mitochondrial function.

II. Introduction

There are three general classes of adipocytes: white, brown and beige. White adipocytes store and release energy according to systemic demand, whereas brown and beige adipocytes burn energy to produce heat. Brown and beige adipocytes are characterized by a high density of mitochondria that contain Uncoupling Protein-1 (UCP1) in their inner membrane. UCP1, when activated by fatty acids, permits proton leak across the inner mitochondrial membrane (Klingenberg et al., 1999). Dissipation of the mitochondrial proton gradient by UCP1 drives the oxidation of available substrates and results in heat production. The thermogenic function of brown and beige fat defends mammals against hypothermia upon cold exposure. Additionally, brown and beige fat activity counteracts many of the negative harmful metabolic effects of a high fat diet in mice, including obesity and insulin resistance (Auffret et al., 2012; Cederberg et al., 2001; Feldmann et al., 2009; Guerra et al., 1998; Seale et al., 2011). In humans, brown adipose tissue (BAT) activity levels correlate with leanness (Saito et al., 2009; van Marken Lichtenbelt et al., 2009).

PRD1-BF1-RIZ1 homologous-domain-containing protein 16 (PRDM16) is a critical regulator of the brown fat-selective gene program in brown and beige adipocytes (Harms et al., 2015; Kajimura et al., 2008; Ohno et al., 2012; Seale et al., 2008; Seale et al., 2011; Seale et al., 2007). PRDM16 increases the transcription of brown fat-specific genes such as *Ucp1* by co-activating various transcription factors, including peroxisome proliferator-activated receptor gamma (PPARγ), PPARγ coactivator 1-alpha (PGC1α), and CCAAT/enhancer-binding protein beta (CEBP-β) (Kajimura et al., 2009; Seale et al., 2008; Seale et al., 2007). The co-activator function of PRDM16 is mediated, at least in part, through recruitment of the Mediator complex to brown fat-specific gene enhancers (Harms et al., 2015; Iida et al., 2015). PRDM16 also represses the transcription of certain white adipocyte-specific and muscle-specific genes in adipose cells by interacting with C-terminal-binding proteins (CtBPs) and euchromatic histone-lysine N-methyltransferase 1 (EHMT1) (Harms et al., 2014; Kajimura et al., 2008; Ohno et al., 2013). Of

note, the N-terminal PR domain of PRDM16 contains methyltransferase activity and is able to methylate histone H3K9 (Li et al.; Pinheiro et al.; Zhou et al., 2016)

Genetic loss-of-function studies in mice show that PRDM16 is required for the maintenance of BAT activity and for beige adipocyte biogenesis in white adipose tissue (WAT) (Cohen et al., 2014; Harms et al., 2014; Seale et al., 2011). PRDM16 also plays an important role in the development and function of other cell types, including hematopoietic stem cells (HSCs) and neural stem cells (NSCs) (Aguilo et al., 2011; Chuikov et al., 2010). Deletion of PRDM16 in HSCs and NSCs increases the levels of reactive oxygen species (ROS) and promotes cell death (Chuikov et al., 2010). Similarly, loss of PRDM16 in astrocytoma cells leads to mitochondrial dysfunction and apoptosis (Lei et al., 2016). In HSCs, PRDM16 induces the expression of Mitofusin 2 to promote mitochondrial function and reduce endoplasmic reticulum stress (Luchsinger et al., 2016).

In this study, we identify a previously unrecognized role for PRDM16 as a repressor of type I Interferon (IFN) responses. The type I IFN pathway is best known for its critical role in anti-viral defense. However, type I IFN also regulates the activity of certain stem cell populations (Essers et al., 2009; Sato et al., 2009; Yu et al., 2015). We found that PRDM16 blocked both the basal and IFNα-induced expression of type I IFN-stimulated genes (ISGs) in adipogenic cells. Conversely, deletion of *Prdm16* from brown adipose cells and from BAT *in vivo* increased ISG expression. *Prdm16*-deficient BAT was also hyper-responsive to induced-IFN signaling *in vivo*. Ectopic activation of type I IFN signaling in brown adipocytes caused profound mitochondrial dysfunction and reduced thermogenic capacity. Mechanistically, PRDM16 bound to ISG promoters and blocked the binding and transcriptional activating function of IFN regulatory factor 1 (IRF1). We conclude that PRDM16-mediated ISG-repression plays an important role in maintaining mitochondrial and thermogenic function in adipocytes.

III. Results

PRDM16 is required to repress type I IFN-stimulated genes (ISGs) in adipocytes

PRDM16 binds and activates the transcription of many brown fat-specific genes in adipocytes (Harms et al., 2015). To identify additional PRDM16-regulated genes in adipocytes, we performed an unbiased analysis of gene expression in *Prdm16*-deleted versus control adipocytes using cDNA microarrays. Adipogenic precursor cells were isolated from the inguinal (subcutaneous) WAT (ingWAT) of *Rosa26*^{CreER};*Prdm16*^{flox} (*R26*$^{Cre+}$) mice and treated with 4-hydroxytamoxifen (4OHT) to induce *Prdm16* deletion or with vehicle (ethanol) as control. *Prdm16*-knockout (KO) and control cells were then induced to undergo adipocyte differentiation in the presence or absence of the PPARγ ligand rosiglitazone (rosi), which activates mitochondrial and brown fat-selective genes (Digby et al., 1998; Ohno et al., 2012; Petrovic et al., 2008; Tai et al., 1996). *Prdm16* KO and control cells underwent efficient conversion into mature lipid-droplet containing adipocytes that expressed equivalent levels of general adipocyte-specific genes such as *Adiponectin* (*AdipoQ*) and *Pparg2* (Fig 2.1A). *Prdm16*-deleted adipocytes expressed drastically reduced levels of *Ucp1* and other brown fat-selective genes in response to rosi (Fig 2.1A), in agreement with published results (Ohno et al., 2012).

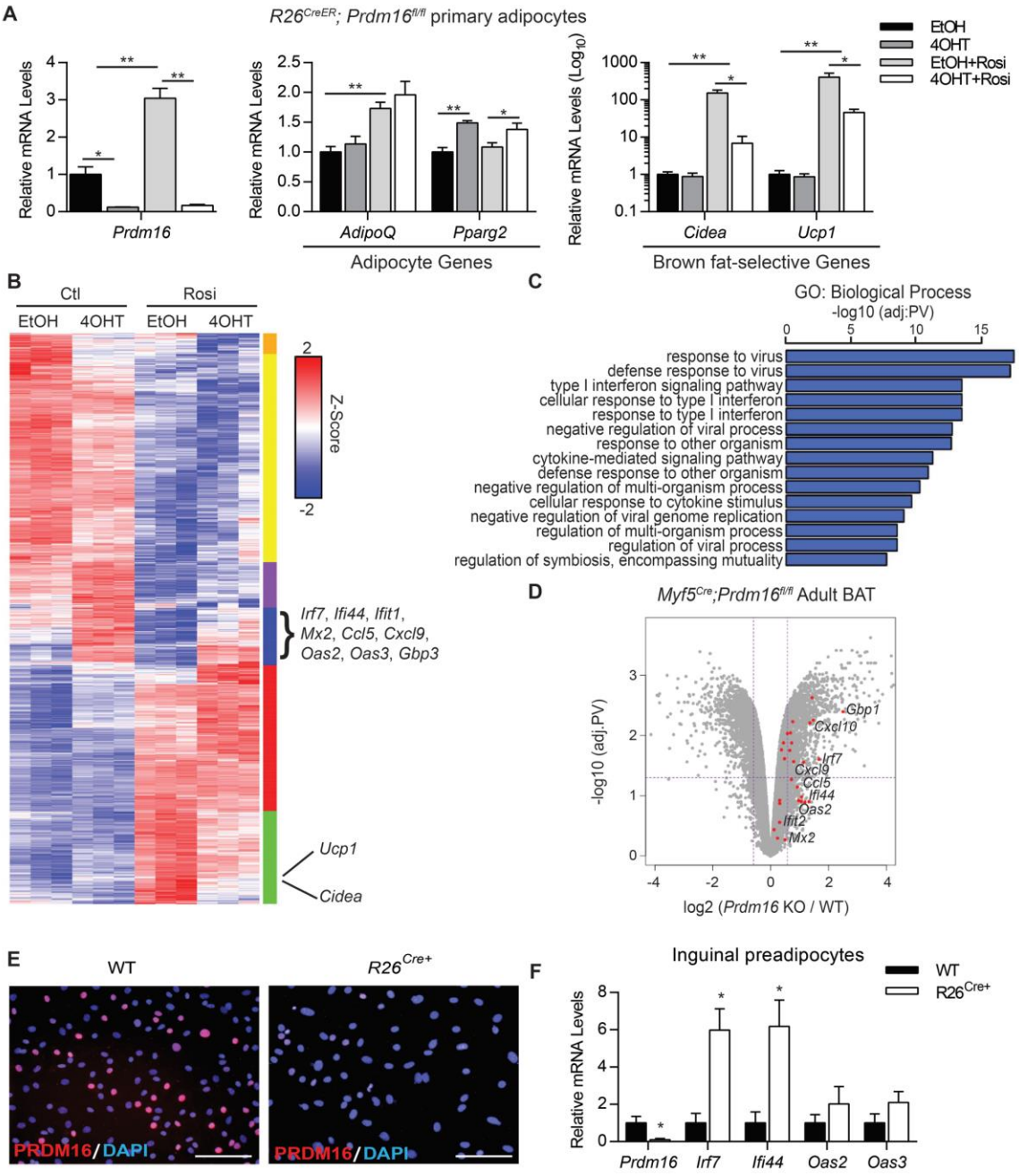

Figure 2.1 PRDM16 is required to repress type I IFN-stimulated genes (ISGs) in adipocytes

A) Relative mRNA levels of Prdm16, pan-adipogenic genes (AdipoQ, Pparg2), and brown fat-selective genes (Cidea, Ucp1) in R26CreER; Prdm16fl/fl inguinal adipocytes treated with ethanol (EtOH) or 1μM 4-hydroxytamoxifen (4OHT) to induce knockdown of *Prdm16*, then differentiated +/- 1 μM rosiglitazone (rosi).

B) Heat map depicting global gene expression levels in control (EtOH) and *Prdm16* KO (4OHT) cells under control (Ctl) or rosi treatment.

C) Gene ontology (GO) analysis of upregulated genes (blue cluster, Fig 2.1B).

D) Volcano plot depicting log-fold change of gene expression in *Prdm16*$^{fl/fl}$ (WT) and *Myf5*^{Cre}; *Prdm16*$^{fl/fl}$ (KO) adult mice. Red dots identify type I IFN-stimulated genes (ISGs) found in the blue cluster of the Fig 2.1B heat map.

E) Immunofluorescence analysis of PRDM16 expression (red) and nuclei (DAPI, blue) in WT and *R26*^{CreER}; *Prdm16*$^{fl/fl}$ (R26^{Cre+}) primary inguinal preadipocytes treated with 4OHT. Scale bar = 100 μm.

F) Relative mRNA levels of *Prdm16* and ISGs in WT and R26^{Cre+} primary inguinal preadipocytes. Data information: In (A, F), data are presented as mean ± standard deviation. *P≤0.05, **P≤ 0.01 (Student's t-test).

Global analyses revealed that many brown fat-selective and mitochondrial genes were induced by rosi in a PRDM16-dependent manner (Fig 2.1B, S2.1A; green cluster). Gene ontology (GO) analysis of the most up-regulated genes in *Prdm16* KO versus control adipocytes, both with and without rosi-treatment, identified the type I IFN and viral defense pathways as prominent PRDM16-repressed pathways (Fig 2.1B, C; blue cluster). The majority of the genes in this group were ISGs, including *Irf7*, *Ifi44*, *Mx2*, *Cxcl9*, and *Oas2*. These ISGs were also greatly increased in 4OHT-treated adipocytes from *R26*$^{Cre+}$ but not from *Prdm16*$^{fl/fl}$ wildtype mice, confirming that ISG activation was not caused by 4OHT (Fig S2.1B). Importantly, ISG levels were increased in *Prdm16* KO BAT from adult *Myf5*^{Cre};*Prdm16*$^{fl/fl}$ mice compared to control WT BAT (Fig 2.1D). The induction of ISGs was not apparent in the BAT of young *Prdm16* KO mice (Fig S2.1C), which have intact thermogenic function (Harms et al., 2014). Additionally, the cold-induced beiging of subcutaneous inguinal (ing) WAT, which occurs in a PRDM16-dependent manner (Cohen et al., 2014), was accompanied by decreased expression levels of many ISGs, including *Ifi27l2*and *Ccl5* (Fig S2.1D)

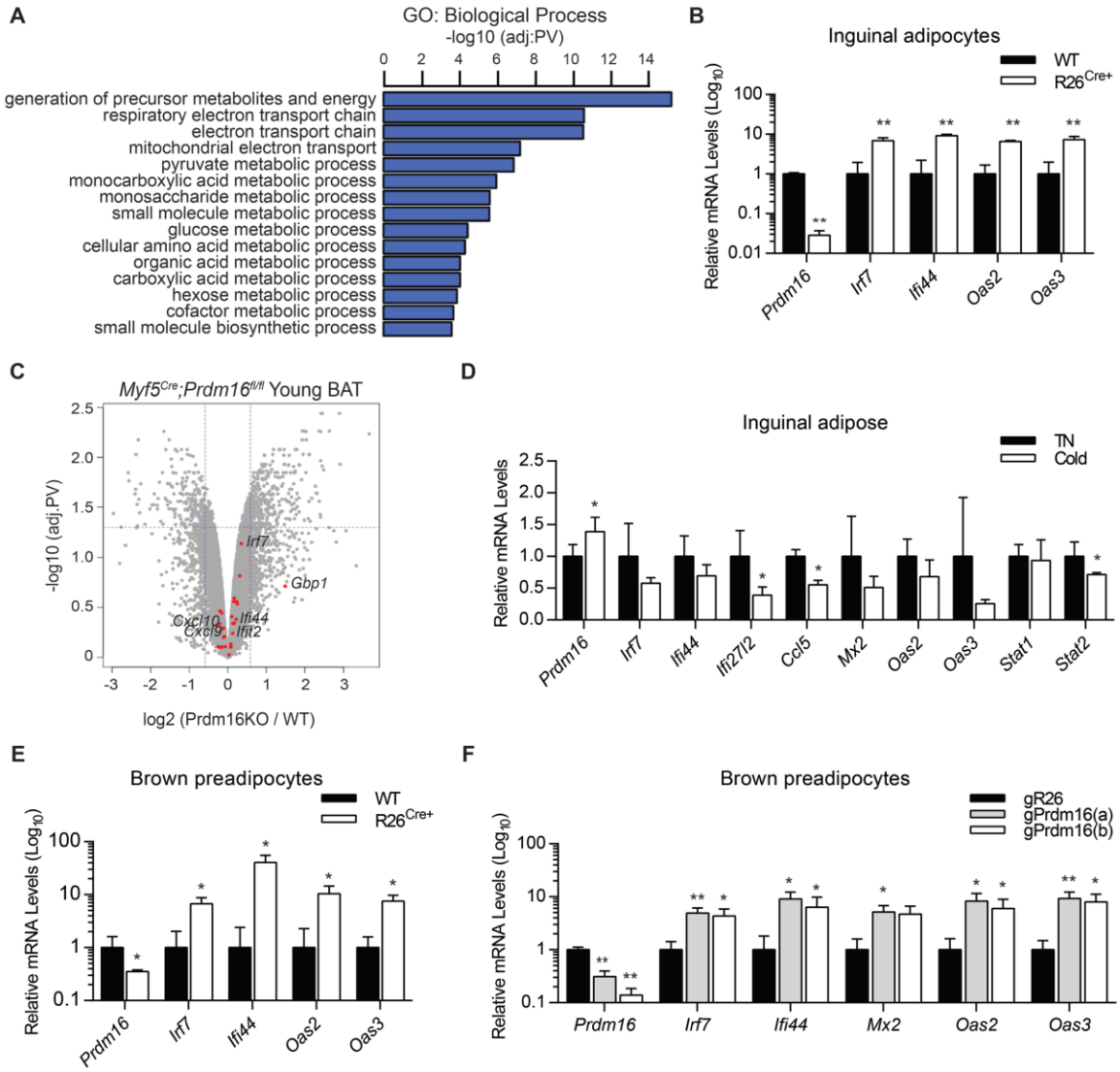

Figure S2.1 PRDM16 is required to repress type I IFN-stimulated genes (ISGs) in adipocytes

A) Gene ontology (GO) of downregulated genes in Prdm16 KO cells (green cluster Figure 1B).

B) Relative mRNA levels of *Prdm16* and ISGs in *Prdm16*$^{fl/fl}$ (WT) and *R26*^{CreER}; *Prdm16*$^{fl/fl}$ (*R26*$^{Cre+}$) inguinal adipocytes treated 1μM 4-hydroxytamoxifen (4OHT).

C) Volcano plot comparing gene expression between young *Prdm16*$^{fl/fl}$ (WT) and *Prdm16* KO BAT. Red dots indicate type I ISGs found in the blue cluster of Fig 2.1B heat map.

D) Relative mRNA levels of *Prdm16* and ISGs in inguinal adipose from wildtype mice incubated in TN (n=5) or cold (n=5).

E) Relative mRNA levels of *Prdm16* and ISGs in WT and *R26*$^{Cre+}$ brown preadipose cells treated with 4OHT.

F) Relative mRNA of *Prdm16* and ISGs in brown adipocyte precursor cells transduced with CRISPR lentiviral vectors expressing Cas9 and guide RNA sequences for *Rosa26* (gR26) or *Prdm16* (gPrdm16a, gPrdm16b).

Data information: Data are presented as mean ± standard deviation (B, E, F) and mean ± SEM (D). *P≤0.05, **P≤ 0.01(Student's t-test).

PRDM16 expression increases during the course of adipocyte differentiation (Seale et al., 2011; Seale et al., 2007) and thus the role of PRDM16 in regulating genes at the preadipocyte stage is largely unknown. We reliably detected nuclear PRDM16 protein in precursor cells isolated from the ingWAT of WT mice, while the addition of 4OHT eliminated PRDM16 protein signal only from $R26^{Cre+}$-derived cells (Fig 2.1E). As observed in mature adipocytes, *Prdm16*-deletion in ingWAT- (Fig 2.1F) and BAT- (Fig S2.1E) derived precursor cells led to increased expression of many ISGs. Similarly, CRISPR/Cas9-mediated reduction of PRDM16 (PRDM16-CRISPR) expression in brown adipocyte precursors increased ISG expression (Fig S2.1F). Together, these results establish a requirement for PRDM16 in repressing a broad set of type I ISGs in adipocytes and adipocyte precursor cells both *in vitro* and *in vivo*.

PRDM16 blocks type I IFN responses downstream of IFNAR receptor.

To determine if ectopic PRDM16 expression is sufficient to repress ISGs, we transduced *Prdm16* KO brown adipocyte precursors with either control or PRDM16-expressing retroviral vectors. PRDM16 decreased both the mRNA and protein levels of Signal Transducer and Activator of Transcription 1 and 2 (STAT1 and STAT2) (Fig 2.2A, B), which are transcription factors that mediate many effects of type I IFN (Bromberg et al., 1996; Horvath et al., 1996; Leung et al., 1995; Meraz et al., 1996; Park et al., 2000). The reduced protein levels of STAT1 and STAT2 corresponded with reduced levels of the phosphorylated (active) forms of these factors (Fig 2.2B). PRDM16 also strongly blocked the expression of many other ISGs, including a 10-20 fold reduction in the mRNA levels of *Irf7*, *Ifi44*, *Oas2* and *Oas3* (Fig 2.2A). PRDM16 did not reduce the levels of STAT3, another transcription factor involved in the IFN cascade (Fig 2.2B).

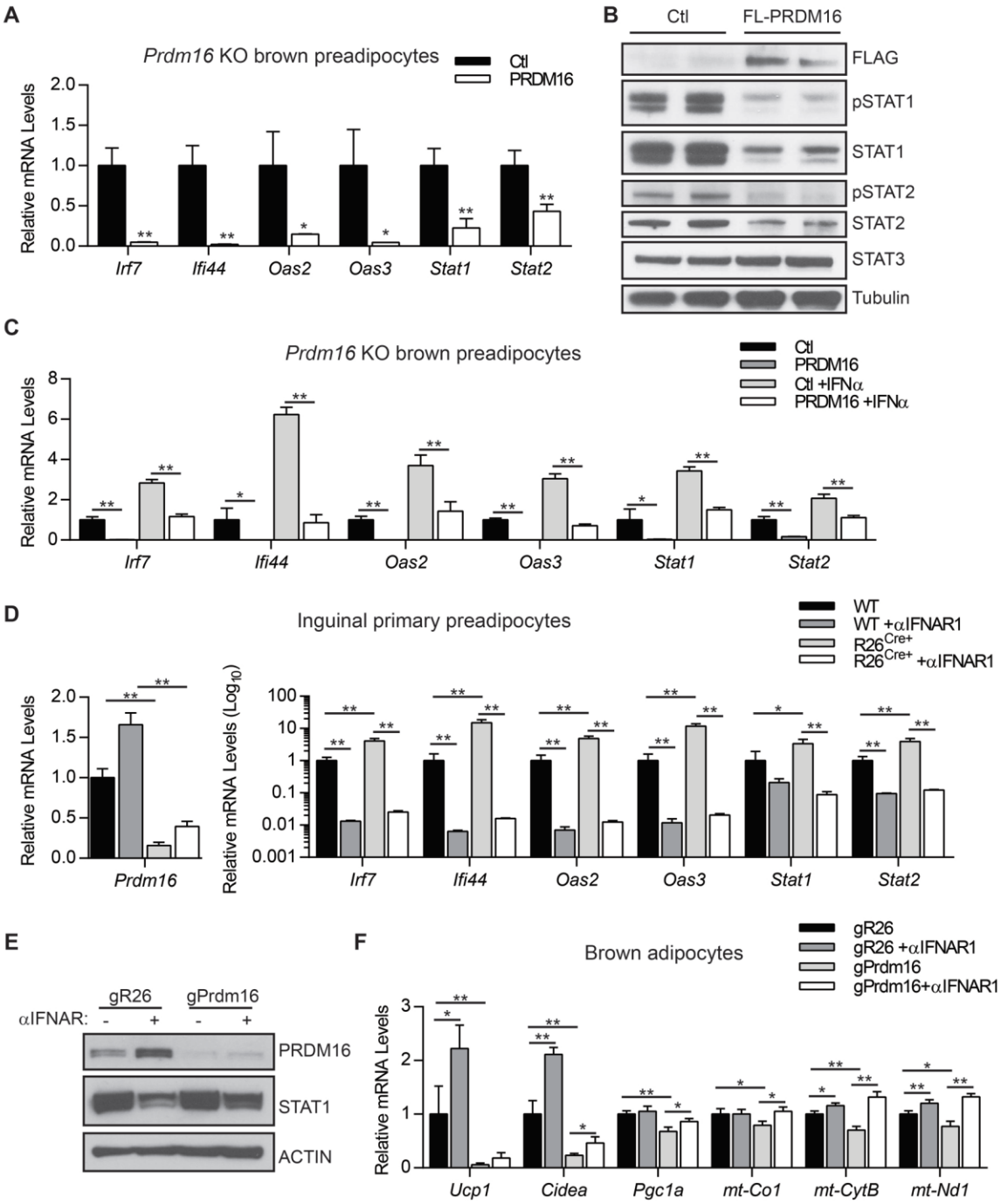

Figure 2.2 PRDM16 blocks type I IFN signaling downstream of IFNAR receptor

A) Relative mRNA levels of IFN-stimulated genes (ISGs) in *Prdm16* KO brown adipocyte precursors infected with control (Ctl) or PRDM16 retrovirus. Data are presented as mean ± standard deviation. *P≤0.05, **P≤ 0.01(Student's t-test).

B) Western blot analysis of FLAG, phosphorylated STAT1 (pSTAT1), STAT1, phosphorylated STAT2 (pSTAT2), STAT3, and Tubulin (loading control) protein in *Prdm16* KO precursors infected with control (Ctl)

or FLAG-PRDM16 retrovirus.

C) Relative mRNA levels of ISGs in control (Ctl) and PRDM16-expressing preadipocytes +/- recombinant mouse IFNα. Data are presented as mean ± standard deviation. *P≤0.05, **P≤ 0.01(Student's t-test).

D) Relative mRNA levels of ISGs in WT and $R26^{Cre+}$ inguinal preadipocytes treated with 4OHT and vehicle or anti-IFNAR1 neutralizing antibody (αIFNAR1) for 4 days. Data are presented as mean ± standard deviation. *P≤0.05, **P≤ 0.01 (Paired two-way ANOVA).

E) Western blot analysis of PRDM16, STAT1, and Actin (loading control) protein in brown adipocytes expressing gR26 (control) and gPrdm16 CRISPR/Cas9 constructs and treated +/- αIFNAR1 throughout differentiation.

F) Relative mRNA levels of brown-selective (*Ucp1*, *Cidea*, *Pgc1a*) and mitochondrial (*mt-Co1*, *mt-CytB*, *mt-Nd1*) genes in brown adipocytes expressing gR26 and gPrdm16 CRISPR/Cas9 constructs +/- αIFNAR1 throughout differentiation. Data are presented as mean ± standard deviation. *P≤0.05, **P≤ 0.01 (Two-way ANOVA).

We next examined if PRDM16 regulates transcriptional responses to exogenous type I IFN. Ectopic PRDM16 expression reduced basal ISG levels and blunted IFNα-induced ISG expression, including *Stat1* and *Stat2* (Fig 2.2C). Conversely, ISGs were induced to higher levels in *Prdm16* KO cells than in control cells in response to varying doses of IFNα (Fig S2.2A), indicating that *Prdm16*-deficiency sensitizes cells to IFNα-treatment.

Type I IFNs bind to the Interferon alpha and beta receptor (IFNAR) on the cell surface which activates a downstream signaling cascade. This response can be efficiently and specifically blocked in brown preadipocytes through treatment with an IFNAR-neutralizing antibody (αIFNAR) (Fig S2.2B). Notably, αIFNAR1 treatment eliminated ISG expression in both WT and *Prdm16* KO cells, indicating that receptor signaling is active under basal conditions and that ISG-induction due to loss of PRDM16 requires IFNAR function (Fig 2.2D).

To determine whether basal IFN signaling influences brown adipogenesis, we differentiated control and PRDM16-depleted brown preadipose cells with or without αIFNAR1. PRDM16-expression was efficiently reduced using CRISPR/Cas9 technology (Fig 2.2E), resulting in a corresponding decrease in the levels of brown fat-specific (*Ucp1*, *Cidea, Pgc1a*) and mitochondrial (*mt-Co1*, *mt-CytB, mt-Nd1*) genes in differentiated brown adipocytes (Fig 2.2F). Anti-IFNAR treatment rescued the expression of mitochondrial genes but not *Ucp1* in *Prdm16*-

deleted adipocytes (Fig 2.2F). Altogether, these results demonstrate that PRDM16 acts downstream of the type I IFN-receptor to repress transcriptional responses to type I IFN and safeguard mitochondrial gene expression in adipocytes.

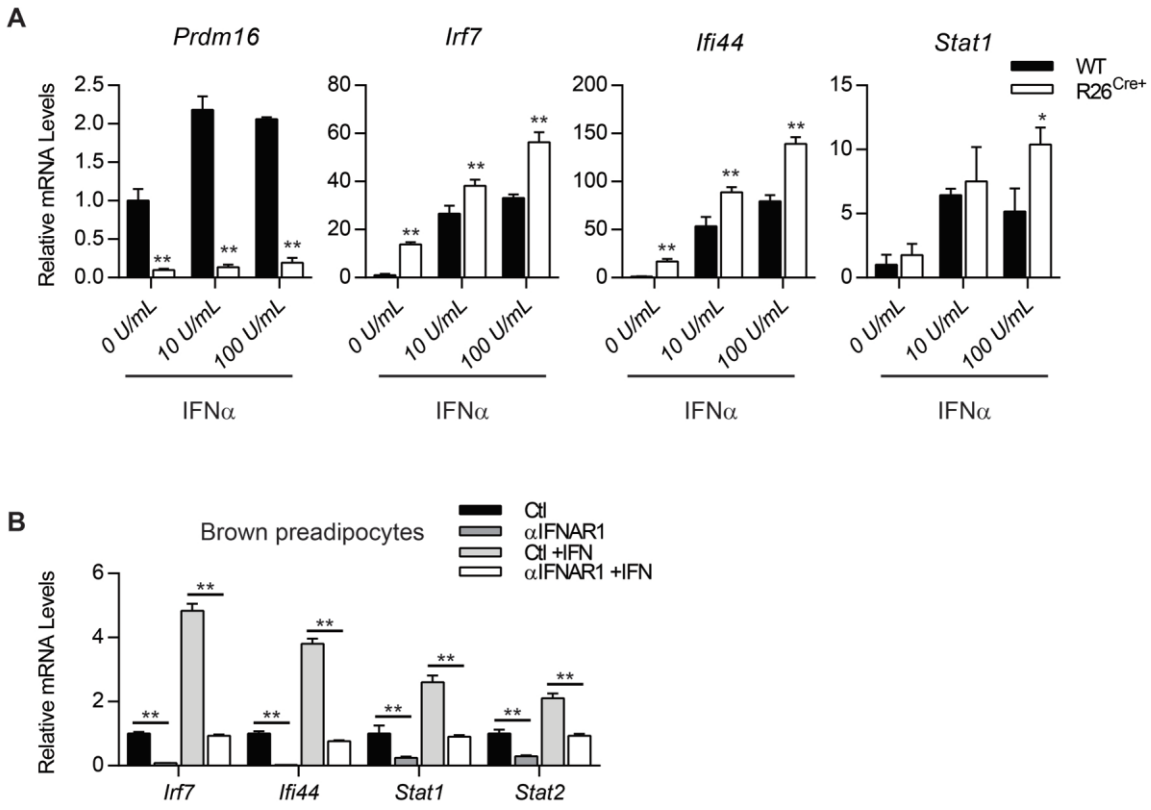

Figure S2.2 PRDM16 blocks type I IFN signaling downstream of IFNAR receptor

(A) Relative mRNA levels of *Prdm16*, *Irf7*, *Ifi44*, and *Stat1* in WT and *R26*$^{Cre+}$ inguinal precursors treated with increasing doses of recombinant mouse IFNα.

B) Relative mRNA levels of ISGs in brown preadipocytes treated with vehicle, anti-IFNAR (αIFNAR) neutralizing antibody, mouse IFNα, or a combination of αIFNAR and IFNα. Data information:

Activation of type I IFN signaling disrupts mitochondrial structure and function in adipocytes

The above studies suggested that IFN-activation may have an inhibitory effect on brown fat cell differentiation and/or function. To evaluate this, we treated brown adipocyte precursor cells with recombinant IFNα or vehicle control and induced adipocyte differentiation. We used a dose of IFNα that increased STAT1 mRNA and protein levels and elevated ISG levels to a similar extent as that observed in *Prdm16* KO cells (Fig 2.3A, C). A previous study reported that IFNα inhibits adipogenesis of 3T3-L1 cells (Lee et al., 2016b). By contrast, we found that IFNα-treated and control-treated cells differentiated into oil-red-o stained mature adipocytes with equivalent efficiency and expressed similar levels of the general adipocyte marker genes *Fabp4* and *Pparg2* (Fig 2.3B). Strikingly however, IFNα-treated adipocytes expressed drastically lower levels of UCP1 at the mRNA and protein level (Fig 2.3C, D) with no change in PRDM16 protein levels (Fig S2.3A). IFNα-treatment also decreased the expression of the brown fat marker gene *Cidea* and several mitochondrial genes, including *Cox7a1* and mitochondrial-encoded genes *mt-Cytb* and *mt-Co1* (Fig 2.3D). Pre-treatment of cells with αIFNAR prevented the decrease of *Ucp1* and *mt-CytB* expression in mature adipocytes (Fig S2.3B), confirming that the inhibitory effect of IFNα on brown fat and mitochondrial programming was due to elevated canonical IFN-signaling. IFNα-treatment similarly inhibited the beige fat program in inguinal adipocytes, including reducing the basal levels of mitochondrial genes and repressing (by ~50-fold) the rosi-stimulated expression of *Ucp1* (Fig S2.3C).

We found that IFN-treatment early in brown fat differentiation (day 0 to 4) led to a permanent reduction in the expression of brown fat- and mitochondrial- genes in mature adipocytes (5 days later); this included a ~60% reduction of *Ucp1* levels (Fig S2.3D). By contrast, IFNα-treatment during later stagers (day 5 to 9) had less of an impact on the brown fat gene program, including a ~35% reduction in *Ucp1* and no significant change in *Cidea* expression. Overall, these results show that activation of the type I IFN-system in brown preadipocytes and

during early stages of differentiation impairs activation of brown fat-specific genes with no effect on the general adipocyte program.

IFNα reduced the expression of specific mitochondrial proteins in brown fat cells, including MT-CO1, a subunit of complex IV, without affecting the levels of other mitochondrial components (Fig 2.3E). Control and IFNα-treated cells had comparable amounts of mitochondria DNA (Fig 2.3F), suggesting that IFNα-treatment does not reduce mitochondrial biogenesis *per se*. However, transmission electron microscopic analyses revealed that IFNα-treatment had profound effects on mitochondrial morphology. The mitochondria in control brown adipocytes contained dense and well-organized cristae whereas the mitochondria in IFNα-treated adipocytes had severely disorganized cristae with a highly reticular morphology (Fig 2.3G). Consistent with these morphological effects on mitochondria, IFNα-treated adipocytes displayed a 40% reduction in oxygen consumption as compared to control adipocyte cultures (Fig 2.3H).

To determine if increased PRDM16 expression can protect brown fat cells against the inhibitory effects of exogenous IFNα, we transduced brown preadipocytes with control or PRDM16-expressing retroviral vectors and induced the cells to differentiate in the presence of IFNα or vehicle control. Remarkably, PRDM16-expression completely rescued *Ucp1* expression in IFNα-treated adipocytes. PRDM16 also mitigated the inhibitory effects of IFNα on the expression of *Cidea* and mitochondrial genes (*Cox7a1*, *mt-Cytb*) (Fig 2.3I). Taken together, these results demonstrate that type I IFN signaling suppresses mitochondrial function and decreases the thermogenic capacity of brown and beige adipocytes and this effect can be blocked by elevating PRDM16 levels.

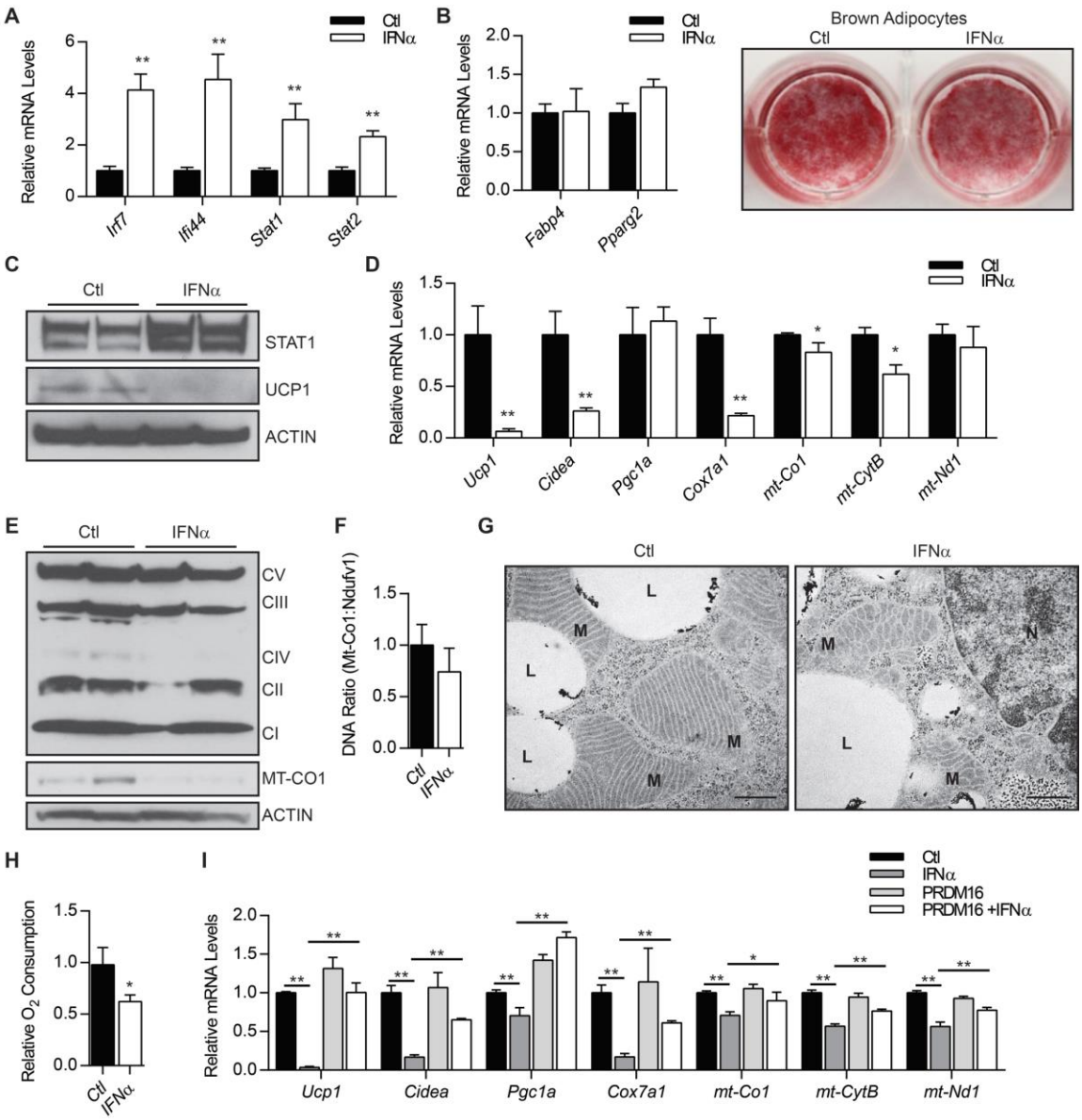

Figure 2.3 Type I IFN disrupts mitochondrial structure and function in adipocytes

A-I) Brown adipocytes were treated with 1000 U/mL mouse IFNα or vehicle (Ctl) throughout differentiation.

A) Relative expression levels of ISGs. Data are presented as mean ± standard deviation. *P≤0.05, **P≤ 0.01(Student's t-test).

B) Oil red O staining of lipid droplets and relative mRNA levels of pan adipogenic genes (*Fabp4*, *Pparg2*). Data are presented as mean ± standard deviation. *P≤0.05, **P≤ 0.01(Student's t-test).

C) Western blot analysis of STAT1, UCP1, and Actin (loading control) protein levels.

D) Relative mRNA levels of brown fat-selective (*Ucp1*, *Cidea*, *Pgc1a*) and mitochondrial (*Cox7a1*, *mt-Co1*, *mt-Cytb*, *mt-Nd1*) genes. Data are presented as mean ± standard deviation. *P≤0.05, **P≤ 0.01(Student's t-test).

E) Western blot analysis of mitochondrial complex proteins and Actin (loading control).

F) Relative ratio of mitochondrial DNA (mt-Co1) to nuclear DNA (Ndufv1) (n=6 replicates for each group). Data are presented as mean ± standard deviation. *P≤0.05, **P≤ 0.01(Student's t-test).

G) Transmission electron micrograph of representative brown adipocytes showing mitochondria (M), lipid droplets (L), and nuclei (N). Scale bar = 500 nm.

H) Relative oxygen consumption rates of adipocytes (n=6 replicates for each group). Data are presented as mean ± standard deviation. *P≤0.05, **P≤ 0.01(Student's t-test).

I) Relative mRNA levels of brown selective genes (*Ucp1*, *Cidea*, *Pgc1a*) and mitochondrial genes (*mt-Co1*, *mt-CytB*, *mt-Nd1*) in brown adipocytes infected with control (Ctl) or PRDM16 retrovirus +/- mouse IFNα. Data are presented as mean ± standard deviation. *P≤0.05, **P≤ 0.01(Paired two-way ANOVA).

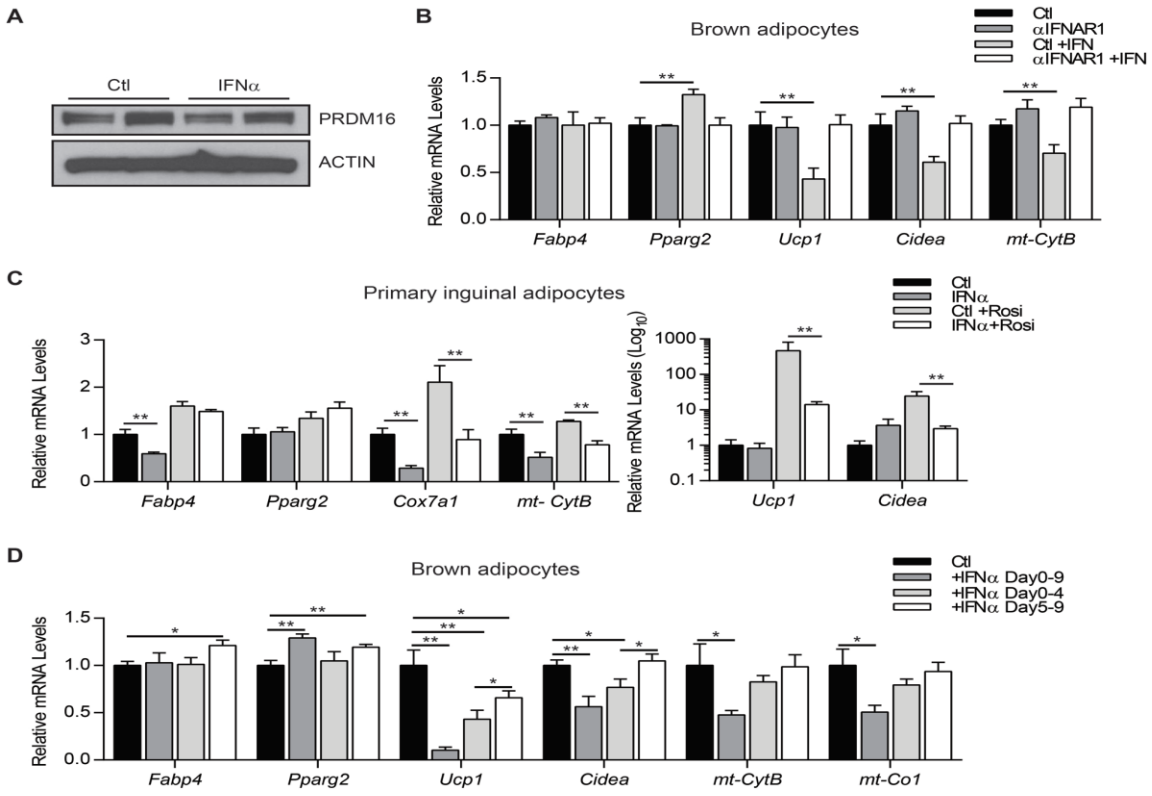

Figure S2.3 Type I IFN disrupts mitochondrial structure and function in adipocytes

(A-B) Western blot analysis of PRDM16 and Actin protein (A) and relative mRNA levels of pan-adipogenic genes (*Fabp4*, *Pparg2*) and brown selective genes (*Ucp1*, *Cidea*) (B) in brown adipocytes treated with vehicle, anti-IFNAR (αIFNAR) neutralizing antibody, mouse IFNα, or αIFNAR + IFNα.

C) Relative mRNA levels of general adipocyte markers (*Fabp4*, *Pparg2*), mitochondrial genes (*Cox7a1*, *mt-Cytb*), and brown fat-selective genes (*Ucp1*, *Cidea*) in primary inguinal adipocytes treated with IFNα or vehicle (Ctl) +/- 1 μM rosiglitazone (Rosi).

D) Relative mRNA levels of general adipocyte markers (*Fabp4*, *Pparg2*), brown fat-selective genes (*Ucp1*, *Cidea*), and mitochondrial genes (*mt-Cytb*, *mt-Co1*) in brown adipocytes treated with vehicle (Ctl) or mouse

IFNα for varying periods during differentiation.

Data information: Data are presented as mean ± standard deviation. *P≤0.05, **P≤ 0.01(Student's t-test).

PRDM16 opposes type I IFN signaling *in vivo*.

An important question is whether PRDM16 is required to reduce the response to type I IFN in BAT *in vivo.* To address this question, we treated 6 to 7 week old BAT-selective *Prdm16* KO (KO) (*Myf5*^{Cre};*Prdm16*^{flox}) and littermate control mice with either vehicle or recombinant IFNα over a two week period. At this young age, ISGs are expressed at similar levels in control and KO BAT (Fig S2.1C). However, the IFNα-treatment of mice induced STAT1 protein to much higher levels in KO BAT than in WT BAT (Fig 2.4A). Furthermore, the mRNA levels of *Stat1* and several other ISGs were increased by IFNα-treatment only in KO BAT (Fig 2.4B, S2.4A). In the ingWAT, there was no difference in ISG levels between WT and KO tissues with IFN treatment (Fig S2.4C).

IFNα-treatment had little impact on the morphology of BAT from control mice. Under basal conditions, the KO mice had paler BAT with larger lipid droplets and reduced *Ucp1* gene levels (Fig 2.4C, D). The loss of brown fat character in KO BAT was exacerbated by IFNα-treatment. This included diminished expression of UCP1 and brown fat-selective genes in BAT from IFNα-treated relative to control-treated KO animals (Fig 2.4C, D, S2.4B). Hematoxylin and eosin (H&E) staining of BAT sections revealed that there was greater lipid accumulation in the BAT of IFNα-treated KO mice relative to that in saline-treated KO mice (Fig 2.4C). In the ingWAT, where basal PRDM16 expression is low, the IFN treatment caused equivalent reduction in mitochondrial encoded genes (*mt-Co1*, *mt-Cytb*) in both WT and KO mice.

We studied the effect of IFNα treatment on the thermogenic capacity of WT and BAT-selective *Prdm16* KO mice by measuring oxygen consumption (respiration) using metabolic cages. To specifically evaluate BAT-activity, we monitored respiration in anesthetized mice before and after stimulation with norepinephrine (NE), the physiological inducer of brown fat thermogenesis. Interestingly, there was no significant difference in NE-stimulated respiration

between WT (control) mice treated with vehicle or IFNα. However, KO mice treated with IFNα displayed a significant reduction in NE-induced respiration compared to vehicle-treated KO mice ($p= 0.002$) (Fig 2.4E). These results suggest that the PRDM16-mediated suppression of type I IFN-responses is required for preserving BAT function.

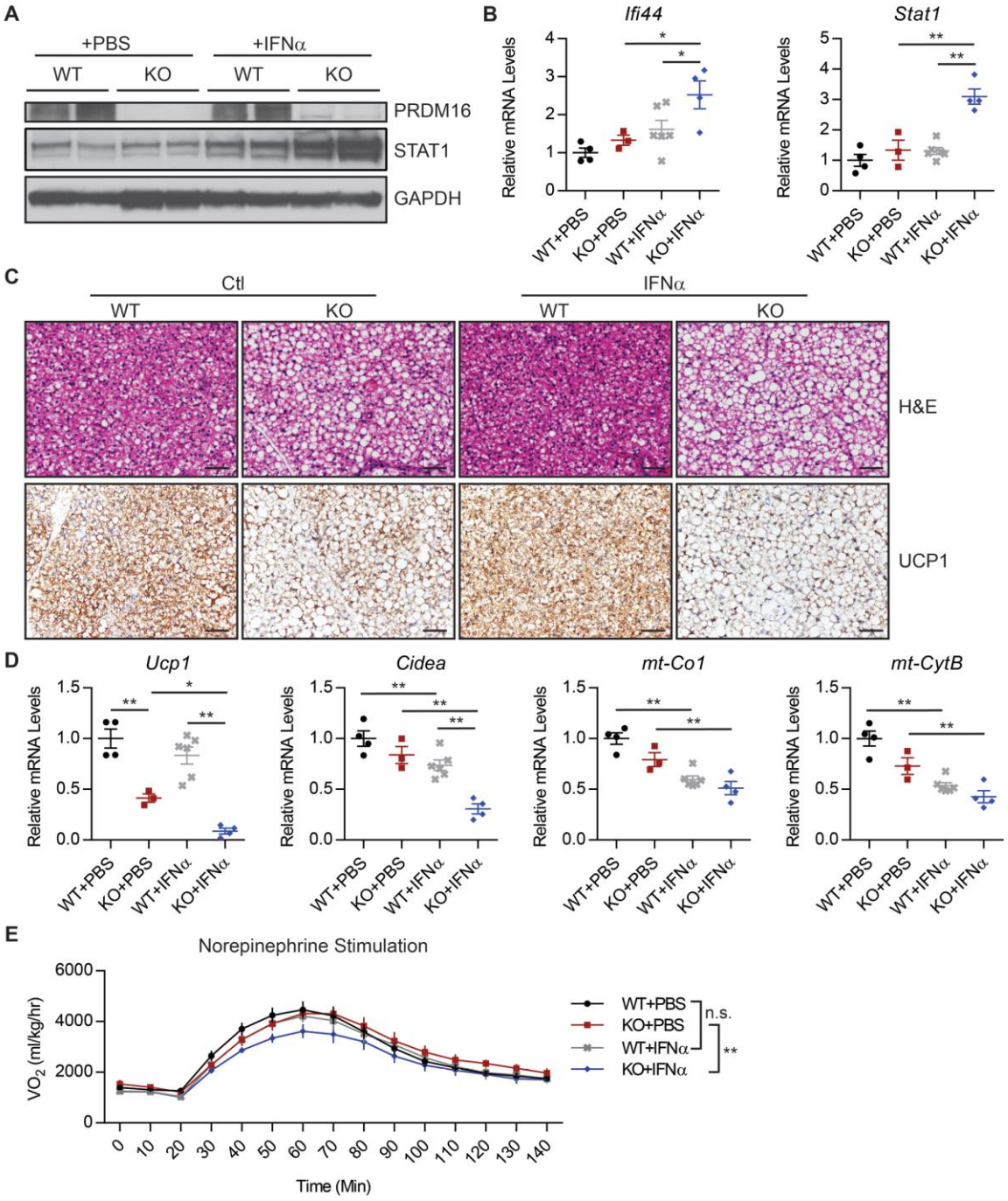

Figure 2.4 PRDM16 opposes type I IFN signaling *in vivo*

A-D) *Prdm16*$^{fl/fl}$ (WT) and *Myf5*^{Cre}; *Prdm16*$^{fl/fl}$ (KO) mice treated with IFNα or phosphate buffered saline (PBS) for two weeks prior to analysis of brown adipose tissue (BAT). Experimental groups: WT+PBS (n=4), KO+PBS (n=3), WT+IFN (n=6), KO+IFN (n=4).

A) Western blot analysis of PRDM16, STAT1, and GAPDH (loading control) protein levels.

B) qPCR analysis of *Ifi44* and *Stat1* mRNA levels. Data are presented as mean ± SEM. *P≤0.05, **P≤ 0.01(Paired two-way ANOVA).

C) Hematoxylin and eosin (H&E) and anti-UCP1 immunohistochemical staining. Scale bar = 50 μm.

D) Relative mRNA levels of brown-fat specific genes (*Ucp1*, *Cidea*) and mitochondrial genes (*mt-Co1*, *mt-CytB*). Data are presented as mean ± SEM. *P≤0.05, **P≤ 0.01(Paired two-way ANOVA).

E) Volume of O_2 (VO_2) consumed before and after norepinephrine injection. Experimental groups: WT+PBS (n=9), KO+PBS (n=6), WT+IFN (n=6), KO+IFN (n=7). Data are presented as mean ± SEM. **P≤ 0.01 (Paired two-way ANOVA).

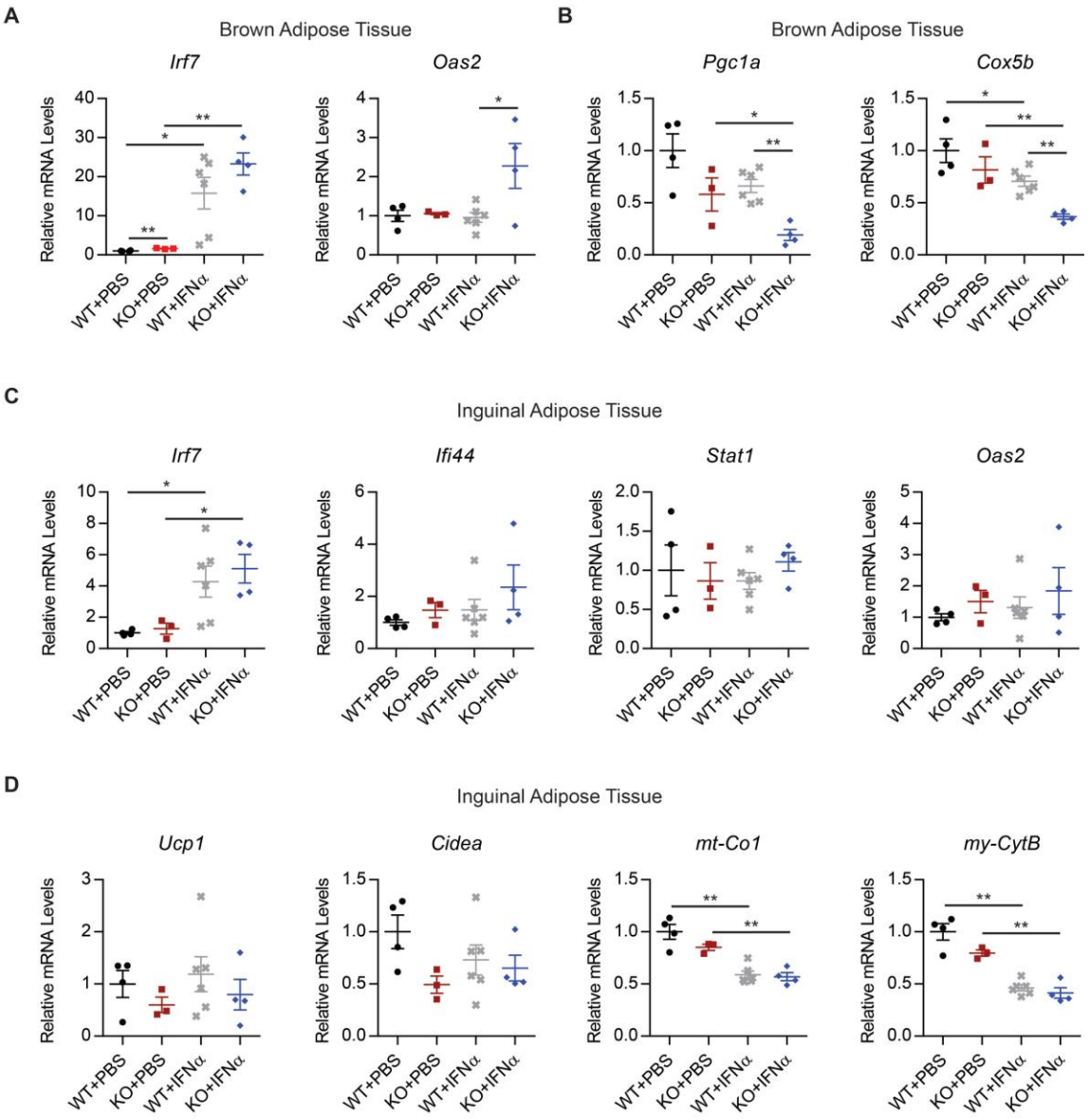

Figure S2.4 PRDM16 opposes type I IFN signaling *in vivo*

A-B) Relative mRNA levels of ISGs (A) and mitochondrial genes (B) in brown adipose of *Prdm16*$^{fl/fl}$ (WT) and *Myf5*^{Cre}*; Prdm16*$^{fl/fl}$ (KO) mice treated with IFNα or phosphate buffered saline (PBS) for two weeks.

C-D) Relative mRNA levels of ISGs (C), as well as brown fat-selective genes (*Ucp1*, *Cidea*) and mitochondrial genes (*mt-Co1, mt-CytB*) (D) in inguinal tissue from the same experimental mice in (A-B).

Data information: Experimental groups: WT+PBS (n=4), KO+PBS (n=3), WT+IFN (n=6), KO+IFN (n=4). Data are presented as mean ± SEM. *P≤0.05, **P≤ 0.01(Paired two-way ANOVA

PRDM16 represses ISG transcription through direct binding at promoter/enhancers

PRDM16 is a transcriptional factor that binds at brown fat-selective gene enhancers to activate gene transcription. Chromatin immunoprecipitation combined with high-throughput sequencing (ChIP-seq) in brown preadipocytes showed that PRDM16 also binds at or near the promoter regions of many ISGs, including *Ifi44* and *Oas3* (Fig 2.5A). These PRDM16-bound regions had lower levels of the activating histone mark H3K27-acetylation in PRDM16-expressing cells, suggesting that they are functional sites (Fig 2.5A). ChIP-qPCR experiments confirmed that PRDM16 binds proximal to many of the ISGs that are reduced in expression by PRDM16 (Fig 2.5B).

PRDM16 has several domains, including an N-terminal PR domain with methyltransferase function, two zinc finger clusters (ZF1 and ZF2) that can bind to DNA, and a transcriptional repressor domain that interacts with C-terminal binding proteins (CtBPs) (Ishibashi & Seale, 2015; Nishikata et al., 2003). To investigate which, if any, of these PRDM16-domains/activities are critical for ISG repression, we expressed mutant forms of PRDM16 in *Prdm16* KO brown preadipose cells. PRDM16 mutants that lack CtBP-binding or methyltransferase activity (e.g. PR-domain mutant) repressed the expression of STAT1 and other ISGs to a similar degree as wildtype PRDM16 (Fig 2.5C, D). However, a DNA-binding mutant form of PRDM16 harboring a point mutation in the second zinc-finger cluster (R998Q) had almost no capacity to repress ISGs (Fig 2.5C, D), though it activates brown fat genes and represses white fat genes normally (Fig S2.5A, S2.5B, & Seale et al, 2007). These results suggest that DNA-binding is critical for PRDM16-mediated suppression of ISGs, but not activation of brown-fat selective genes.

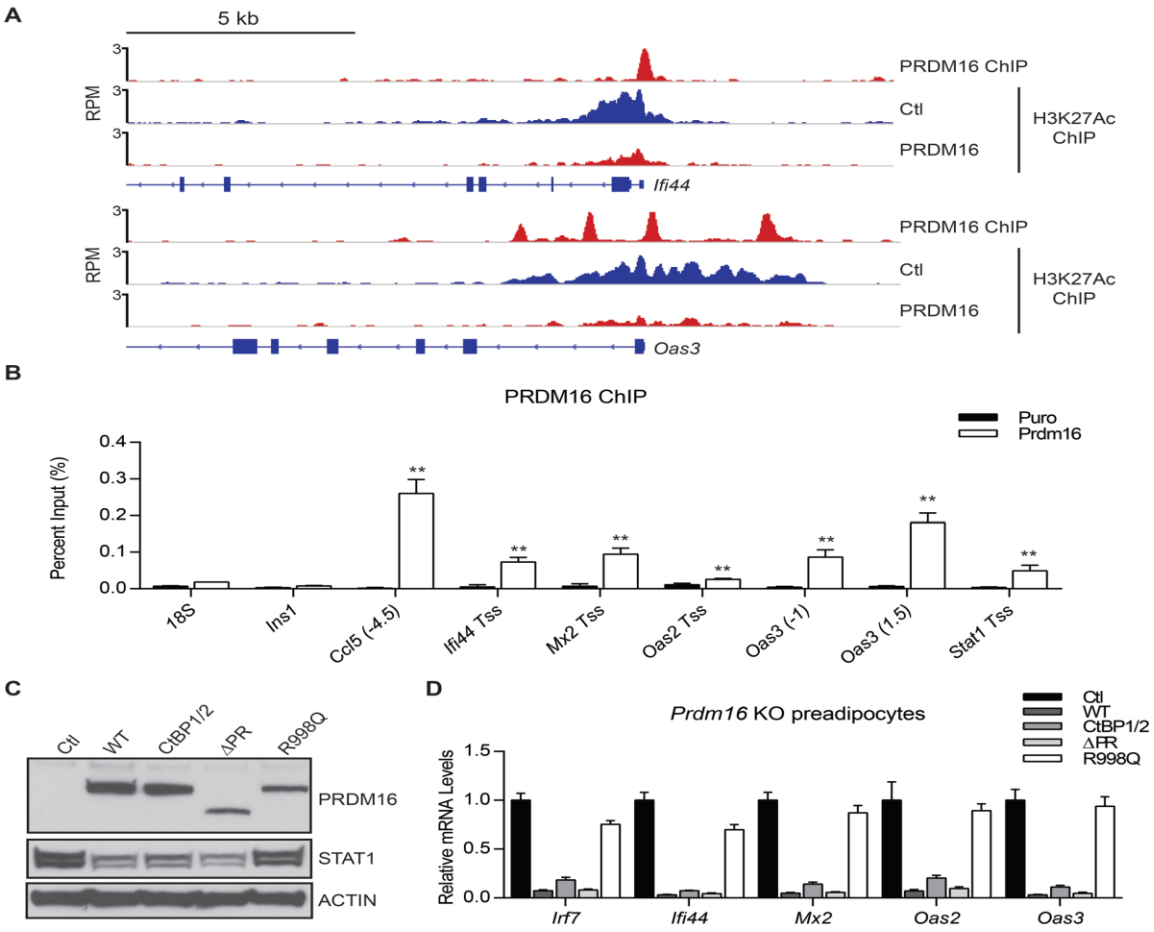

Figure 2.5 PRDM16 represses ISGs through direct binding at gene promoters

A) ChIP-seq stack-height profiles in reads per million (RPM) for PRDM16 and H3K27-Acetylation (Ac) at *Ifi44* and *Oas3* in *Prdm16* KO brown adipocyte precursors that express PRDM16 or a control (Ctl) retrovirus.

B) ChIP-qPCR analysis of PRDM16 binding at ISG promoters/enhancers in control (Ctl) or PRDM16-expressing brown preadipose cells (n= 3 replicates per group). *Ins1* and *18S* were used as non-specific binding site controls. Data are presented as mean ± SEM. **P≤ 0.01(Student's t-test).

C) Western blot analysis of STAT1 and PRDM16 protein levels in *Prdm16* KO brown preadipose cells transduced with retroviral vectors that express different forms of PRDM16: wildtype (WT), CtBP-binding mutant (CtBP1/2), PR-domain deletion mutant (ΔPR), DNA-binding mutant (R998Q) or empty vector (Ctl). Loading control, Actin.

D) Relative mRNA levels of ISGs in cells from (C). Data are presented as mean ± standard deviation. *P≤0.05, **P≤ 0.01(Student's t-test).

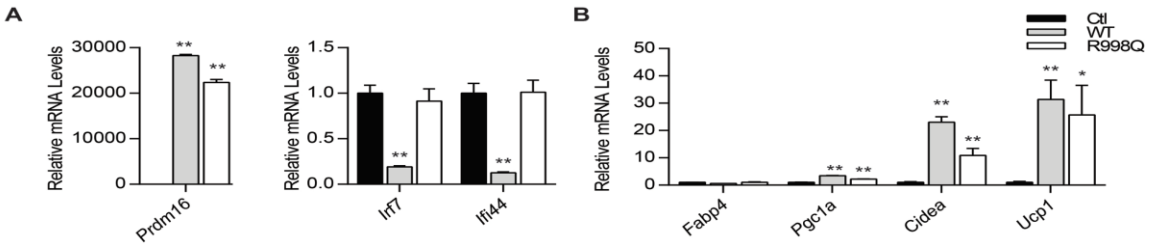

Figure S2.5 PRDM16 represses ISGs through direct binding at gene promoters

(A-B) Relative mRNA levels of *Prdm16* and ISGs (A) and relative mRNA levels of adipogenic (Fabp4) and brown fat-selective genes (*Pgc1a*, *Cidea*, *Ucp1*) in *Prdm16* KO brown adipocytes cells transduced with retroviral vectors expressing wildtype (WT) or DNA-binding mutant (R998Q) PRDM16, or empty vector (Ctl).

Data information: Data are presented as mean ± standard deviation. *P≤0.05, **P≤ 0.01(Student's t-test).

PRDM16 blocks the activation of ISGs by IRF1

We identified an overlapping IFN-stimulated response element (ISRE) and IRF binding element (IRF-E) at many of PRDM16 binding sites at ISG promoters, including at the *Ifi44* promoter (Fig S2.6A). IFN regulatory factors (IRFs) are critical transcriptional effectors of the IFN signaling circuitry (Fujita et al., 1989; Harada et al., 1990; Kimura et al., 1994; Sato et al., 2000; Schafer et al., 1998). Moreover, various IRFs have been shown to regulate adipocyte differentiation and function (Eguchi et al., 2011; Eguchi et al., 2008; Kong et al., 2014; Kumari et al., 2016). Among the IRF family members, we found that *Irf1* and *Irf7* were particularly highly expressed in brown preadipocytes (Fig S2.6B). IRF1 was a prime candidate for further study because it is known to activate a similar ISG signature as IFNα, including STAT1 (Xu et al., 2016). IRF1 expression levels are relatively constant throughout the process of adipocyte differentiation (Fig S2.6C). To test if IRF1 was required for ISG-induction in *Prdm16*-deficient brown adipocytes, we used lentiviral delivery of short hairpin RNAs (shRNAs) to knockdown *Irf1* expression. Two shRNA sequences were effective in reducing IRF1 protein levels and resulted in 50-70% reductions in the expression of many ISGs, including *Irf7*, *Ifi44* and *Stat1* (Fig 2.6A, 2.6B). The shRNA-mediated reduction in ISG expression was reversed by co-expression of the shRNA-resistant human form of IRF1 (Fig S2.6D). CRISPR/Cas9-mediated reduction of IRF1 in

Prdm16-deficient brown adipocytes also decreased ISG expression (Fig S2.6F). In mature brown adipocytes with endogenous levels of *Prdm16* expression, knockdown of *Irf1* did not affect ISG or brown fat gene expression (Fig S2.6E), whereas ectopic IRF1 expression in fibroblasts increased ISG levels (Fig 2.6C, 2.6D).

We then explored whether PRDM16 functionally interacts with IRF1 to regulate ISG expression. We used the proximal *Ifi44* promoter, containing an identified PRDM16-binding site (Fig S2.6A), to drive expression of a *Luciferase* reporter. IRF1 robustly activated the *Ifi44* promoter and this induction was very effectively blocked by co-expression of WT but not the R998Q mutant form of PRDM16 (Fig 2.6E). We were unable to detect any evidence of a physical interaction between IRF1 and PRDM16 using a variety of approaches and PRDM16 expression did not change IRF1 levels (Fig S2.6G, S2.6H). Furthermore, PRDM16 did not repress the activating function of a GAL4 DBD (DNA-binding domain)-IRF1 fusion protein on a *Gal4 DBD*-driven reporter (Fig S2.6I). These results suggest that PRDM16 does not repress IRF1 function through physical binding and that the repressive effect of PRDM16 requires the IRF-binding site and/or other nearby promoter elements.

PRDM16 suppressed IRF1-mediated gene activation in a dose-dependent manner (Fig 2.6F), suggesting that PRDM16 may compete with IRF1 for binding to the *Ifi44* promoter. Consistent with this, the isolated IRF-E/ISRE site alone was sufficient to confer responsiveness to both IRF1 and PRDM16 in transcription assays (Fig 2.6G). Additionally, we found that PRDM16 expression decreased IRF1 binding at several native ISG promoters using ChIP-qPCR (Fig 2.6H). Finally, in WT brown preadipocytes with endogenous PRDM16 levels, recombinant IFNα increased IRF1 binding, while *Prdm16* KO cells had basally higher IRF1 binding (Fig S2.6J). Together, these results suggest that PRDM16 represses ISG target genes by binding to IRF-elements and blocking access to the transcriptional activator, IRF1(Fig 2.6I).

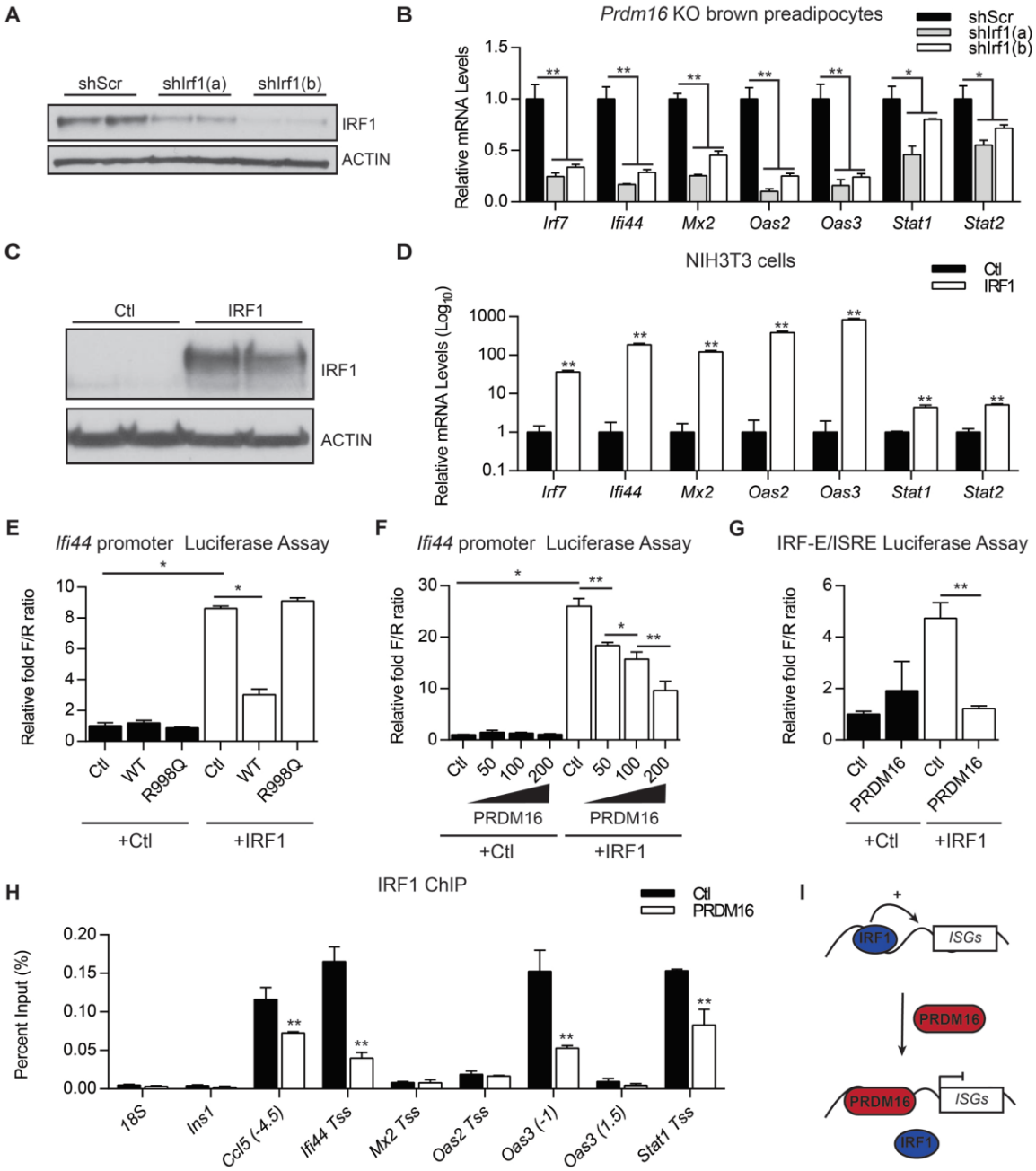

Figure 2.6 PRDM16 blocks the activation of ISGs by IRF1

A-B) Western blot analysis of IRF1 and Actin (loading control) protein levels (A) and relative mRNA levels of ISGs (B) in *Prdm16* KO brown preadipose cells transduced with lentiviral short-hairpin RNA directed against *Irf1* (shIrf1a, shIrf1b) or a scrambled control (shScr).

C-D) Western blot analysis of IRF1 and Actin (loading control) protein levels (C) and relative mRNA levels of ISGs (D) in cells from in NIH3T3 cells transfected with CMV6 (Ctl) or CMV6-IRF1.

E) Transcriptional activity of the *Ifi44* promoter in NIH3T3 cells upon expression of IRF1 and wildtype (WT)

or DNA-binding mutant (R998Q) forms of PRDM16.

F) Transcriptional activity of the *Ifi44* promoter in response to IRF1- and increasing amounts of PRDM16-expression.

G) Transcriptional activity of the IFN regulatory factor binding element (IRF-E)/IFN-stimulated response element (ISRE) in *Ifi44* in response to IRF1- and/or PRDM16.

H) ChIP-qPCR analysis of IRF1 binding at ISGs in brown preadipose cells transduced with PRDM16 or control (Ctl) retrovirus. *Ins1* and *18S* were used as non-specific binding site controls.

I) Proposed model for PRDM16-action at ISG promoter regions.

Data information: Data are presented as mean ± standard deviation (B,D) and mean ± SEM (E-H) . *P≤0.05, **P≤ 0.01(Student's t-test)

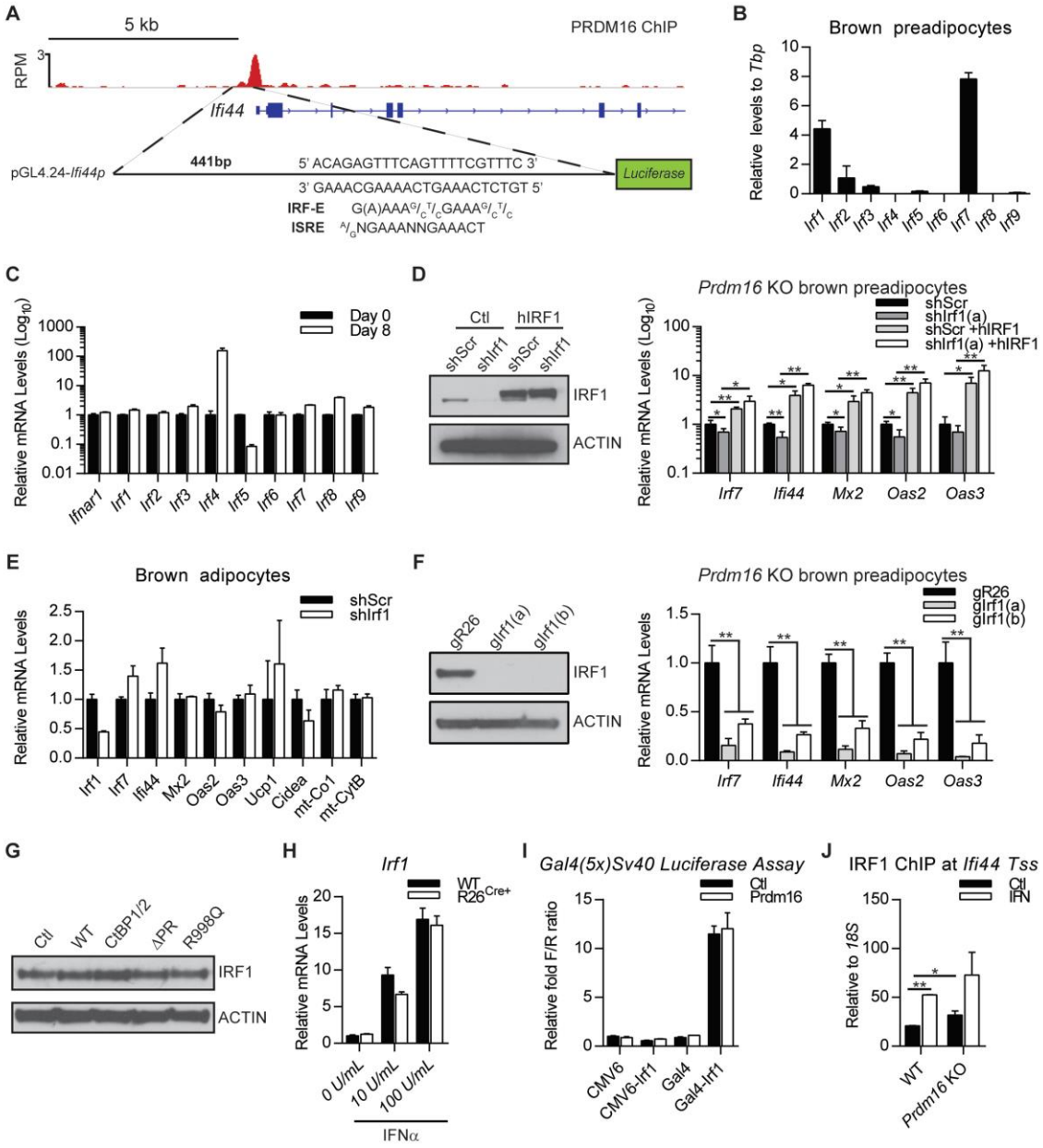

Figure S2.6 PRDM16 blocks the activation of ISGs by IRF1

(A) Schematic showing the ChIP-seq track of PRDM16 binding at *Ifi44* promoter and the identified IFN-stimulated response element (ISRE)/ IRF binding element (IRF-E) element that was inserted into the luciferase reporter plasmid (pGL4.24-*Ifi44*p).

B) Relative mRNA levels of IRF genes in brown preadipose cells.

C) Relative mRNA levels of *Ifnar1* and *Irfs* in brown preadipocytes (D0) and mature brown adipocytes (D8).

D) Western blot analysis of IRF1 and Actin protein levels and relative mRNA levels of ISGs in *Prdm16* KO brown adipocytes cells transduced with lentiviral short-hairpin RNA directed against *Irf1* (shIrf1) or a

scrambled control (shScr) and either retroviral expression vectors expressing human IRF1 (hIRF1) or puromycin control (Ctl).

E) Relative mRNA levels of *Irf1* and ISGs and of brown fat-selective genes (*Ucp1*, *Cidea*) and mitochondrial genes (*mt-Co1*, *mt-CytB*) in brown adipocytes transduced with lentiviral short-hairpin RNA directed against *Irf1* (shIrf1) or a scrambled control (shScr).

F) Western blot analysis of IRF1 and Actin protein levels and relative mRNA levels of ISGs in *Prdm16* KO brown adipocytes cells transduced with lentiviral transduced with CRISPR lentiviral vectors expressing Cas9 and guide RNA sequences for *Rosa26* (gR26) or *Irf1* (gIrf1a, gIrf1b).

G) Western blot analysis of IRF1 and Actin (loading control) protein levels in cells from Figure 5C.

H) Relative mRNA levels of *Irf1* in *Prdm16*$^{fl/fl}$ (WT) and *R26*^{CreER}; *Prdm16*$^{fl/fl}$ (*R26*$^{Cre+}$) inguinal adipocytes treated 1μM 4OHT and increasing doses of IFNα.

I) Transcriptional activity of a Gal4 UAS-driven *luciferase* gene in response to expression ofGAL4 DNA-binding domain alone (Gal4), IRF1, or a GAL4-IRF1 +/- PRDM16. J) ChIP-qPCR showing IRF1 binding at *Ifi44 Tss* in WT and *Prdm16* KO cells +/- IFNα.

Data information: Data are presented as mean ± standard deviation (B-F, H, J) and mean ± SEM (I). *P≤0.05, **P≤ 0.01(Student's t-test).

IV. Discussion

PRDM16 is a critical transcriptional regulator of brown and beige adipocyte identity. PRDM16 co-regulates other DNA-binding factors to promote the transcription of brown fat genes in adipocytes (Kajimura et al., 2009; Seale et al., 2008; Seale et al., 2007). We show here that, in addition to its direct transcriptional activating effect on brown fat-specific genes, PRDM16 reinforces and maintains brown fat identity by suppressing the type I IFN response. PRDM16 blocks the activation of IFN-induced genes by competing with IRF1 for binding to IRF-E binding motifs. While the interaction between PRDM16 and IRF1 plays an important role in regulating the IFN-response in adipogenic cells, whether PRDM16 also functionally interacts with other IRFs remains to be determined. Notably, PRDM1, a related family member, also binds to the IRF1 binding element to repress activation of the IFN pathway in other cell types (Doody et al., 2007; Kuo & Calame, 2004; Mould et al., 2015), suggesting that these two factors may have overlapping roles in regulating the type I IFN pathway.

Type I IFNs are best known for their role in mounting powerful antiviral responses. Virus-infected cells secrete and respond to type I IFNs, including IFNα and IFNβ. These cytokines establish an anti-viral state through multiple mechanisms, including the production of anti-microbial proteins that act directly on viruses and modulation of the adaptive immune response (Honda et al., 2005). The importance of this pathway is underscored by the finding that mice lacking the type I IFN receptor (IFNAR) rapidly succumb to viral infections (Hwang et al., 1995). Similarly, humans with mutations in STAT1, a key effector of the IFN-response, die from viral infection (Chapgier et al., 2006; Dupuis et al., 2003). The suppressive effect of PRDM16 on IFN-responses may be important for preserving the thermogenic function of BAT in virus-infected animals. This may be especially important in small animals such as newborns to survive cold-exposure while dealing with viral infection. PRDM16 may also be needed to protect BAT activity during viral infection in order to support hyperthermia (fever).

Low levels of type I IFN, particularly IFNβ are also present in many cells/tissues in the absence of infection (Abt et al., 2012; Hamilton et al., 1996; Hata et al., 2001; Hida et al., 2000;

Tovey et al., 1987). Constitutive type I IFN expression is believed to be a priming mechanism for rapid induction of the pathway upon viral infection (Abt et al., 2012; Ganal et al., 2012; Hata et al., 2001; Kawashima et al., 2013; Vogel & Fertsch, 1984). Similarly, increased type I IFN signaling can increase cellular responsiveness to other cytokines, such as IFNγ by increasing the levels of common signaling intermediates like STAT1 (Gough et al., 2010; Hamilton et al., 1996). Interestingly, the type I IFN pathway is required to achieve the proper balance of proliferation and maturation of hematopoietic stem cells (HSCs) (Essers et al., 2009; Kim et al., 2016). In this context, elevated IFN signaling leads to stem cell exhaustion (Essers et al., 2009; Sato et al., 2009), highlighting the importance of a tightly regulated IFN system in HSCs. Notably, PRDM16 is also a critical regulator of HSCs (Chuikov et al., 2010), suggesting that regulation of IFN-signaling may be a key function of PRDM16 in this compartment. Along these lines, it will now be important to determine if the PRDM16 and IFN regulate the proliferation and/or maintenance of brown adipose precursors.

A prominent effect of IFN-activation in brown adipocytes is reduced mitochondrial function and abnormal mitochondrial morphology (Fig 2.3). This result agrees with previous studies showing that IFNα inhibits the expression of mitochondrial-encoded genes in lymphoid cells (Lewis et al., 1996; Shan et al., 1990). We found that IFNα-activation leads to a loss of cristae structure and a striking reduction of specific mitochondrial proteins like MT-CO1 in brown adipocytes. The mechanism(s) by which IFN-activation reduces mitochondrial function is unclear. One possibility is that IFN-activated STAT1/2 directly represses the transcription of *Ucp1* and mitochondrial encoded genes. However, type I IFN induces a large number of downstream ISGs, any of which could have as yet undetermined roles in regulating mitochondrial function and cellular metabolism. Importantly, blocking JAK-STAT signaling in human adipocytes decreases IFN signaling and induces brown fat-like characteristics (Moisan et al., 2015), suggesting a potentially important role for this pathway in human metabolism.

In summary, PRDM16 regulates the brown fat gene program through multiple mechanisms, including via direct actions at brown fat gene enhancers and indirectly by

suppressing the type I IFN-driven gene program. Our study also suggests potentially important links between innate immune and metabolic pathways in adipocytes that warrant further investigation and predicts a potential role for IFN-signaling in metabolic regulation. In support of this, genetic manipulations that influence the type I IFN pathway in mice have revealed significant metabolic phenotypes. For example, IRF3 knockout mice have increased energy expenditure due to the browning of the inguinal adipose (Kumari et al., 2016). Moreover, both IRF7 and IRF3, knockout mice are protected from diet-induced obesity and have improved insulin sensitivity (Kumari et al., 2016; Wang et al., 2013b). Additional focus on the role of type I IFN in adipocytes may reveal new approaches to preserve and/or increase brown fat activity for the treatment of obesity and metabolic disease.

CHAPTER 3 : High fat diet-induced type I Interferon signaling leads to increased obesity and decreased glucose tolerance

I. Abstract

Diet-induced inflammation leads to obesity-related co-morbidity, thus determining the contributing immune pathways is important for potential therapeutic treatments. Both adaptive and innate immune signaling in adipose tissue has been implicated in the progression of obesity. Type I Interferon (IFN) signaling is an innate pathway which is activated in response to viruses and other pathogens. We reveal here a novel role for type I IFN signaling in the inflammatory milieu during diet-induced obesity (DIO). Short-term high-fat diet (HFD) feeding activates interferon-stimulated genes in all adipose tissues as well as other tissues, suggesting activation of systemic signaling. Eliminating IFN responses by ablating the IFN receptor alpha 1 (IFNAR1 KO) in mice resulted in improved weight gain and glucose sensitivity while on HFD. The subcutaneous adipose tissue of IFNAR1 KO mice had higher brown fat-selective and mitochondrial gene expression than control mice. After short-term HFD, IFNAR1 KO mice displayed increased energy expenditure compared to controls. Overall, our data suggests HFD-induced type I IFN signaling contributes to the development of metabolic syndrome in obesity, including weight gain and dysfunctional glucose homeostasis, by reducing whole body energy expenditure.

II. Introduction

Obesity is a disease with many associated co-morbidities such as type 2 diabetes, non-alcoholic fatty liver disease, and cardiovascular disease (Bornfeldt & Tabas; James & Day, 1998). Inflammation has been implicated as a leading cause for the development of other diseases during obesity (Johnson et al., 2012; Odegaard & Chawla, 2013). As adipose expands, macrophages, B cells, Th1 cells, and natural killer (NK) cells infiltrate and create a pro-inflammatory state within the tissue (Nishimura et al., 2009; Strissel et al., 2010; van der Heijden et al., 2015; Weisberg et al., 2003; Wensveen et al., 2015; Winer et al., 2011). Specifically, macrophages switch from an anti-inflammatory M2 polarization to a pro-inflammatory M1 polarization (Lumeng et al., 2007b). In this state, adipose begins to secrete cytokines such as interleukin-6 (IL6) and tumor necrosis factor alpha (TNFα) (Fried et al., 1998; Hotamisligil et al.; Mohamed-Ali et al., 1997), leading to systemic inflammation (du Plessis et al., 2015; Park et al., 2010; Varma et al., 2009).

Toll-like receptors (TLRs) are upregulated and activated in adipose tissue during diet-induced obesity and insulin resistance (Kim et al.; Reyna et al., 2008; Shi et al., 2006; Song et al., 2006). Classically, TLRs are pathogen-recognition receptors, which activate multiple downstream transcriptional programs to initiate immune cell responses (Akira et al., 2001). One such pathway is the type I Interferon (IFN) signaling pathway (Noppert et al., 2007). While the role of type I IFNs, IFNα and IFNβ, have been well defined in response to viral infection, whether type I IFN signaling is involved in diet-induced obesity (DIO) has not been investigated. Certain interferon regulatory factors (Irfs), which regulate the expression of IFN as well as IFN-stimulated genes (ISGs) (Au et al.; Sato et al.), are highly expressed in adipocytes (Eguchi et al.). Interestingly, IRF3 and IRF7 are upregulated within the adipose during diet-induced obesity. Knocking out these factors in mice ameliorates diet-induced weight gain and insulin resistance by reducing adipose inflammation (Kumari et al., 2016; Wang et al.).

There are three defined adipose tissues: white, brown, and beige adipose. Activated brown and beige adipose produce heat through uncoupling of the electron transport chain by Uncoupling

Protein 1 (UCP1) (Klingenberg et al., 1999). Interestingly, IRF3 deficiency also causes an increase in browning of the subcutaneous adipose tissue (Kumari et al., 2016). We have previously shown that IFNα treatment causes mitochondrial and thermogenic defects in brown and beige adipocytes, while the transcription factor PRDM16 opposes these actions by blocking IFN responses (Chapter 2). In animal models and humans, the attenuation of brown and beige adipose function is associated with obesity, while activating thermogenic adipose leads to increased energy expenditure and improvement of diet-induced metabolic syndrome (Auffret et al., 2012; Cederberg et al., 2001; Feldmann et al., 2009; Guerra et al., 1998; Seale et al., 2011).

In the current study, we found mice fed HFD for two weeks had increased ISG expression in multiple metabolic tissues compared to mice fed normal chow diet, suggesting activation of systemic IFN signaling. IFN alpha receptor 1 knockout (IFNAR1 KO) mice, which are unable to respond to type I IFN (Muller et al., 1994), were protected from diet-induced weight gain and disrupted glucose tolerance compared to WT mice. Additionally, aging-induced weight gain and glucose homeostasis were improved in the IFNAR1 KO mice. After two weeks of HFD, the IFNAR1 KO mice had significantly higher energy expenditure. While the brown adipose thermogenic capacity was equivalent in WT and IFNAR1 KO mice, the inguinal adipose had increased brown fat-specific and mitochondrial gene expression in KO tissue. We conclude that HFD-induced type I IFN signaling causes mitochondrial defects in the inguinal adipose tissue that leads to a reduction in energy expenditure contributing to weight gain and dysfunctional glucose homeostasis.

III. Results

Type I Interferon signaling is induced early in high-fat diet feeding

To determine whether the type I IFN pathway was activated in adipose during obesity, we examined multiple adipose depots from diet-induced obese (DIO) and chow-fed (control) mice. ISGs, such as *Irf7*, *Ifi44*, *Mx2*, *Oas2*, and *Oas3,* were increased in both the epididymal (eWAT) and inguinal (iWAT) adipose depots of DIO mice compared to that of controls (Fig 3.1A, B). Notably, *Ifi44* was expressed 200-fold higher in the iWAT of DIO mice. In the brown adipose, ISGs were expressed at similar levels in the control and DIO mice (Fig 3.1C). This was consistent with a previous study showing BAT is more resistant to inflammation compared to WAT (Sierra Rojas et al., 2016).

Inflammatory factors, such as type II IFN (IFNγ), are activated in WAT only after long term of HFD treatment (<4 weeks) (Strissel et al., 2010). To determine whether type I IFN signaling was induced earlier in the course of HFD, we fed C57Bl6 mice HFD for two weeks at thermoneutrality (TN). After two weeks, mice fed HFD gained more weight than mice fed a chow diet (Fig 3.1D). At this time-point, we observed increased *F480* expression in the iWAT and BAT tissues of the HFD-fed mice, suggesting increased macrophage infiltration or proliferation (Fig S3.1A, B). There was predominantly an increase in M2 polarized macrophage associated genes (*Clec7a*, *Retnla*, *Arg1*) (Fig S3.1A, B). Similar to the WAT of the DIO mice, we observed a two- to six-fold induction of ISG expression in both eWAT and iWAT of HFD-fed compared to chow-fed mice (Fig 3.1E, F). Interestingly, we found that two weeks of HFD caused a similar induction of ISGs in BAT, unlike what we observed in the DIO mice (Fig. 3.1G). Inflammation in other tissues, such as liver and muscle, is also a hallmark of obesity (Khan et al., 2015; Weisberg et al., 2003). To determine whether the increase in type I IFN signaling was adipose-specific, we also examined the liver and muscle. ISGs were increased more than 100% in both liver and muscle of HFD- compared to chow-fed mice (Fig 3.1H, I). In muscle, we observed a corresponding increase in macrophage-associated gene signature without an increase in other inflammatory signals (*Il6*,

Tnfα), but no changes in macrophage gene expression in the liver (Fig S3.1C, D). Together, these results suggest type I IFN signaling is systemically activated early in the course of HFD feeding.

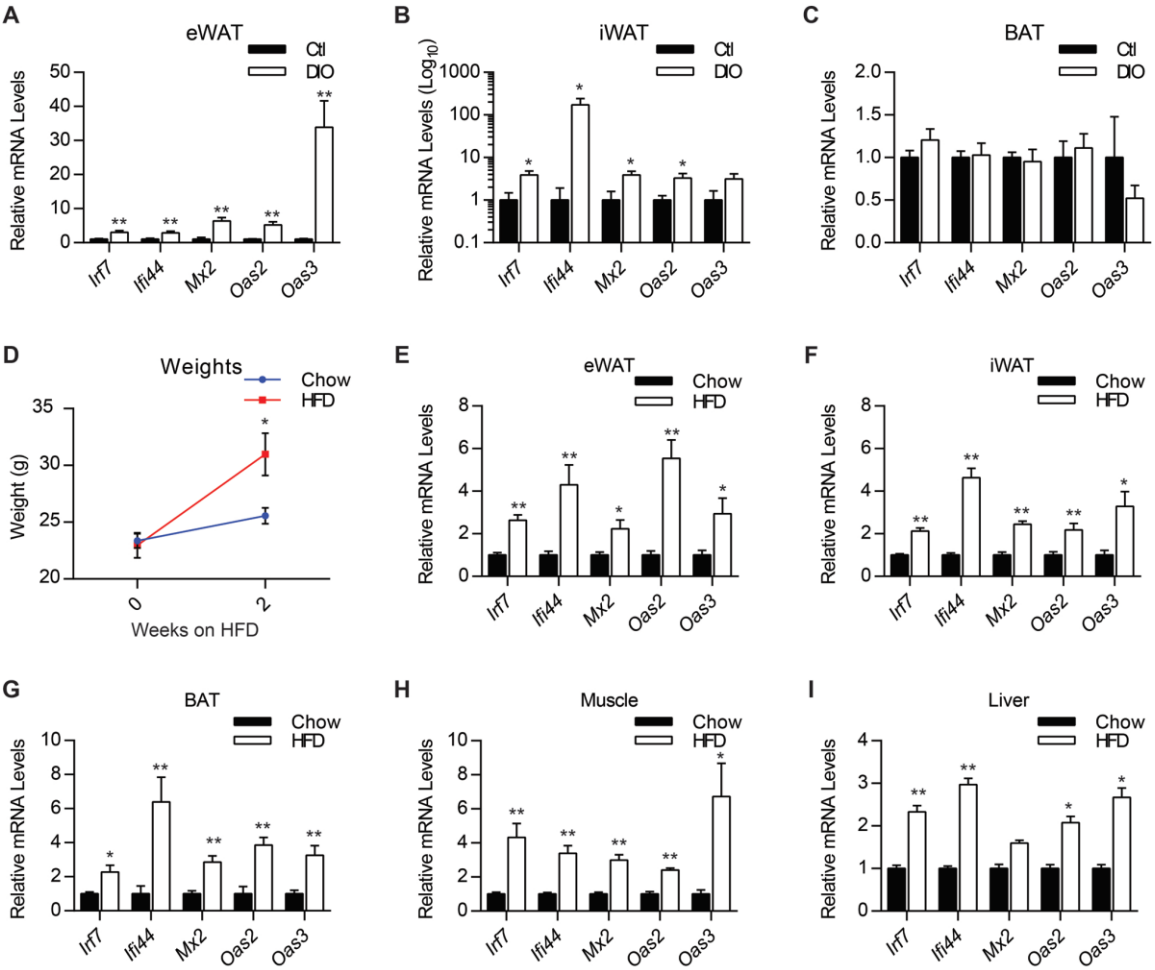

Figure 3.1 Type I Interferon signaling is induced with high-fat diet

A-C) Relative mRNA expression of ISGs (*Irf7*, *Ifi44*, *Mx2*, *Oas2*, *Oas3*) in epididymal (eWAT) (A), inguinal (iWAT) (B), and brown (BAT) (C) adipose tissue of control and diet-induced obese (DIO) mice (n=5 for both groups).

D) Average weights of C57Black6 mice fed chow or high-fat diets for two weeks (n=5 for both groups).

E) Relative mRNA expression of ISGs in eWAT (E), iWAT (F), BAT (G), muscle (H), and liver (I) of C57Black6 mice fed chow or high-fat diet for two weeks (n=5 for both groups).

Data information: Data are presented as mean ± SEM. *P≤0.05, **P≤ 0.01(Student's t-test)

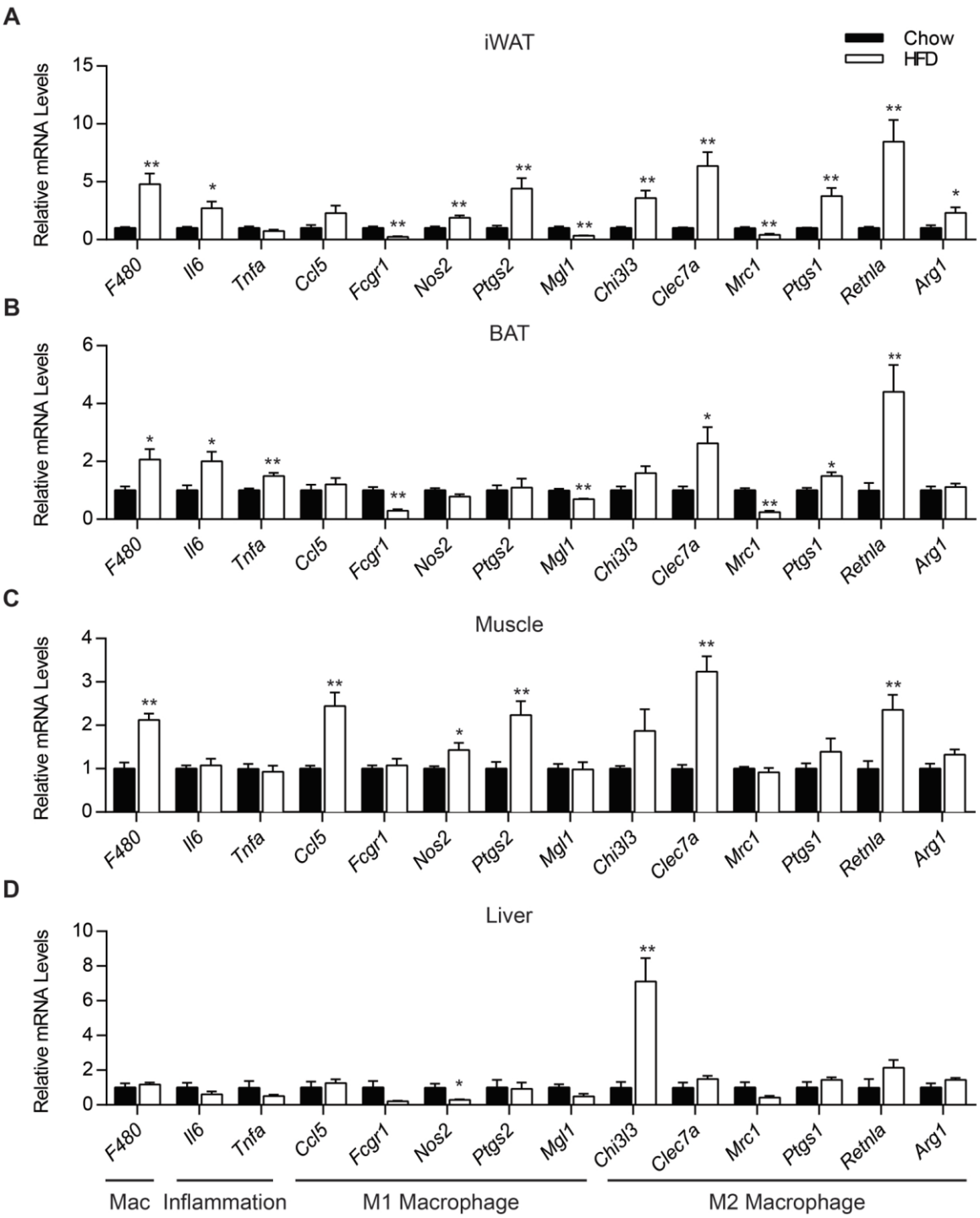

Figure S3.1 Macrophage infiltration/proliferation increased after two weeks HFD feeding

A-D) Relative mRNA expression of total macrophage marker (*F480*), inflammatory genes (*Il6*, *Tnfa*), M1 macrophage genes (*Ccl5*, *Fcgr1*, *Nos2*, *Ptgs2*, *Mgl1*), and M2 macrophage genes (*Chi3l3*, *Clec7a*, *Mrc1*, *Ptgs1*, *Retnla*, *Arg1*) in iWAT (A), BAT (B), muscle (C), and liver (D) of C57Black6 mice fed chow or high-fat diet (HFD) for two weeks (n= 5 for both groups).

Data information: Data are presented as mean ± SEM. *P≤0.05, **P≤ 0.01(Student's t-test)

Type I IFN signaling contributes to diet-induced weight gain and glucose tolerance

Type I IFNs, including IFNα and IFNβ, initiate downstream signaling by binding to the IFNAR receptor, leading to the activation of ISGs. To investigate the role of type I IFN in the development of diet-induced obesity, we utilized IFNAR1 KO mice which have no ability to respond to type I IFN signaling. These animals are healthy and comparable to WT mice until challenged with viral infection (Muller et al., 1994). We fed WT and IFNAR1 KO mice HFD for 18 weeks at TN. The weights of the mice diverged after 14 weeks with IFNAR1 KO mice weighing less than WT mice (Fig 3.2A). The IFNAR1 KO mice gained less weight than the WT mice from the second to the sixth week of HFD feeding (Fig 3.2B), indicating a role for type I IFN in the early stages of diet-induced obesity. Interestingly, there was no weight gain difference between WT and IFNAR1 KO mice fed HFD at room temperature (RT) (Fig S3.2A).

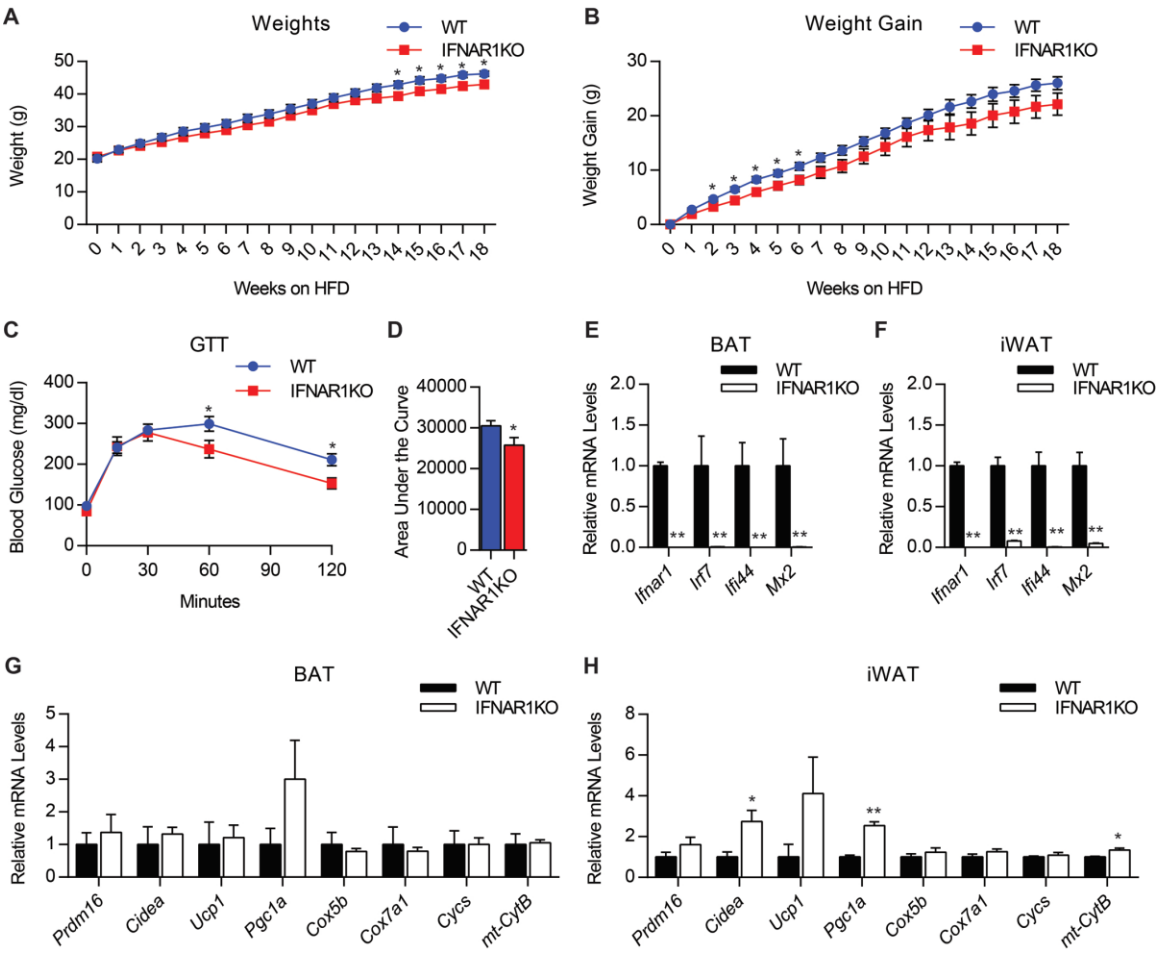

Figure 3.2 IFNAR1 ablation ameliorates diet-induced weight gain and glucose tolerance

A-H) Wildtype (WT) (n=14) and IFNAR1 knockout (KO) (n=11) mice fed high-fat diet (HFD) for 18 weeks at thermoneutrality.

A) Weekly weights in grams (g) of mice while on HFD.

B) Weekly weight gain in grams (g) of mice while on HFD.

C) Blood glucose response (mg/dl) over 120 minutes during a glucose tolerance test (GTT).

D) Area under the curve calculations for GTT blood glucose curves shown in (C).

E-F) Relative mRNA expression of *Ifnar1* and ISGs (*Irf7*, *Ifi44*, *Mx2*) in BAT (E) and iWAT (F) of mice after 18 weeks HFD.

G-H) Relative mRNA expression of brown fat-selective (*Prdm16*, *Cidea*, *Ucp1*, *Pgc1a*) and mitochondrial genes (*Cox5b*, *Cox7a1*, *Cycs*, *mt-CytB*) of BAT (G) and iWAT (H) of mice after 18 weeks HFD.

Data information: Data are presented as mean ± SEM. *P≤0.05, **P≤ 0.01(Student's t-test)

A

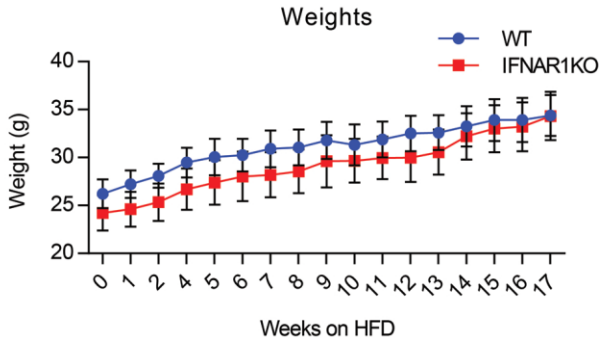

Figure S3.2 IFNAR1 ablation has no effect on weight gain at room temperature

A) Average weekly weights of wildtype (WT) (n=4) and IFNAR1 knockout (KO) (n=3) mice fed high-fat diet for 17 weeks at room temperature. Data are presented as mean ± SEM.

Ectopic type I IFN leads to a disruption in insulin signaling and glucose uptake in both mice and humans (Imano et al., 1998; Koivisto et al., 1989). To test whether the IFNAR1 KO mice had improved glucose tolerance, we conducted a glucose tolerance test (GTT) after 18 weeks on HFD. The IFNAR1 KO mice had a significantly better response compared to WT mice with lower maximal blood glucose and overall 15% lower area under the curve calculated for the GTT response curve (Fig. 3.2C, D). These results suggest HFD-induced type I IFN signaling contributes to decreased glucose tolerance in obesity.

We next assessed whether there were gene expression differences in the adipose tissue of WT and IFNAR1 KO mice. We observed that *Ifnar1*expression was completely eliminated in both BAT and iWAT and this corresponded with a 20-100 fold reduction in ISG expression (*Irf7*, *Ifi44*, *Mx2*) (Fig 3.2 E, F). These results confirmed that ISG stimulation in the adipose is type I IFN signaling-dependent. Increased browning of both BAT and WAT leads to reduced weight gain in many models (Auffret et al., 2012; Cederberg et al., 2001; Feldmann et al., 2009; Guerra et al., 1998; Seale et al., 2011). We next wanted to determine whether brown fat-selective or mitochondrial gene expression was altered in the adipose of IFNAR1 KO mice. In brown fat there was no difference in gene expression between WT and IFNAR1 KO mice (Fig 3.2G). Notably, in

the iWAT brown fat-selective genes (*Cidea*, *Pgc1a*) and the mitochondrial gene *mt-CytB* were more highly expressed in IFNAR1 KO mice compared to WT mice. Taken together, these results suggest that blocking type I IFN leads to less weight gain and improved glucose sensitivity, potentially due to increased browning of WAT.

Type I IFN contributes to aging-induced weight gain and glucose tolerance

Aging is associated with increased adiposity and the development of insulin resistance as well as inflammation (Horber et al., 1997; Lumeng et al., 2011; Pascot et al., 1999). Of note, increased type I IFN signaling with aging has been shown to lead to inflammation in the brain (Baruch et al., 2014). To determine whether type I IFN signaling contributed to aging-induced weight gain, we kept WT and IFNAR1 KO mice on chow diet for 6 months at room temperature (RT) and then moved them to TN for another 6 months. At 6 months of age, there was not a significant weight difference between WT and IFNAR1 KO mice (Fig 3.3A). At 12 months (including 6 months at TN), the WT mice weighed significantly more than IFNAR1 KO mice (Fig 3.3B), suggesting IFN signaling leads to increased weight gain during aging. We next want to assess whether IFNAR1 KO mice had improved glucose sensitivity. We conducted a GTT on the 12 month old WT and IFNAR1 KO mice. The aged IFNAR1 KO mice had an improved glucose response with lower blood glucose after 60 min and an overall reduced area under the curve for the glucose response curve (Fig 3.3C, D). We then used MRI to determine whether the weight gain differences observed were due to reduced fat or lean mass. While the percentage fat mass by body weight and total fat mass trended lower in the IFNAR1 KO mice, the differences were not significant (Fig 3.3E, F). The lean mass of the WT and IFNAR1 KO mice was the same (Fig 3.3G), suggesting overall differences in total weight were due to lower fat mass. Together this data suggests that type I IFN signaling during aging can lead to increased weight gain and decreased glucose tolerance.

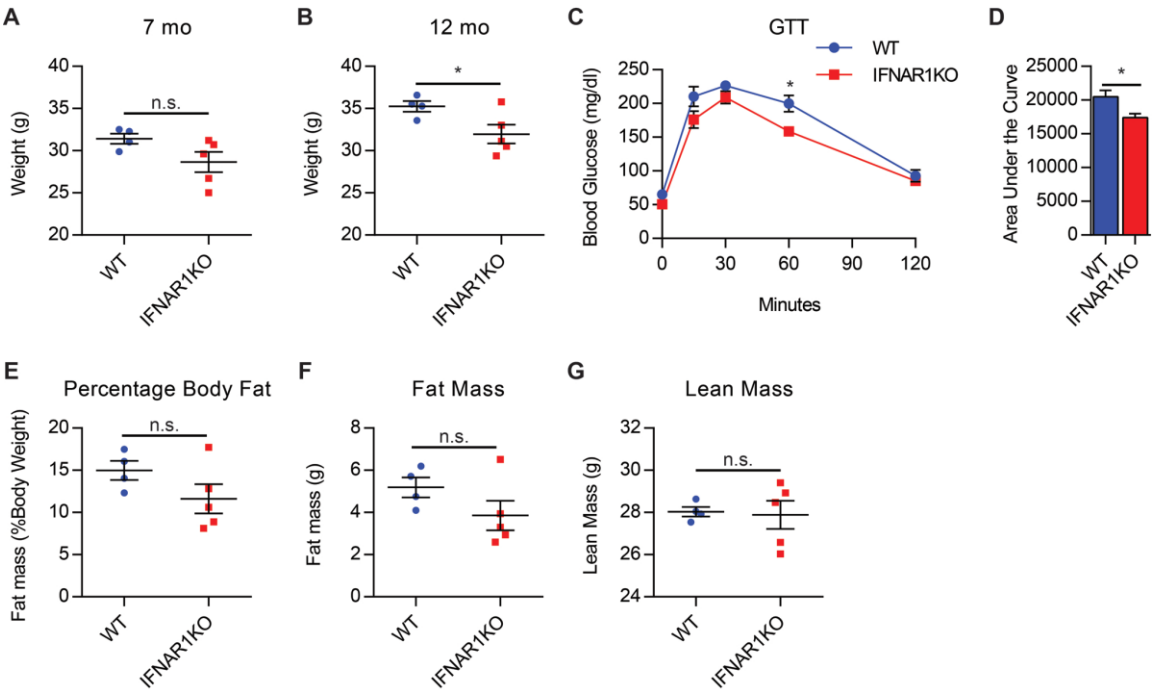

Figure 3.3 IFNAR1 ablation ameliorates weight gain and glucose tolerance associated with aging

A) Weights in grams (g) of wildtype (WT) (n=4) and IFNAR1 knockout (KO) (n=5) mice aged 7 months at room temperature.

B) Weights in grams (g) of WT and IFNAR1 KO mice aged 12 months (5 months at thermoneutrality).

C) Blood glucose response (mg/dl) during glucose tolerance test (GTT) of WT (n=4) and IFNAR1KO (n=4) mice at 12 months of age.

D) Area under the curve calculations for GTT blood glucose curves shown in (C).

E-G) Body composition measurements of WT and IFNAR1 KO mice at 12 months of age showing fat mass represented as percentage of total body weight (E), total fat mass in grams (g) (F), and total lean mass in grams (G).

Data information: Data are presented as mean ± SEM. *P≤0.05, **P≤ 0.01(Student's t-test)

High-fat diet-induced type I IFN signaling leads to reduced energy expenditure

The observed weight gain differences could be caused by reduced food consumption and/or increases in energy expenditure. To determine the cause of the weight differences between WT and IFNAR1 KO mice, we put chow-fed WT and IFNAR1 KO mice in the CLAMS unit to monitor their metabolic rates. The mice had comparable weights (Fig 3.4A), but the IFNAR1 KO mice displayed a higher respiration rate than the WT during light cycles (Fig 3.4B). There was no difference in activity or food consumption between the WT and IFNAR1 KO mice (Fig 3.4C, D). We next assessed whether the type I IFN signaling induced after two weeks HFD could cause changes in energy expenditure. The WT and IFNAR1 KO mice maintained comparable weights at this time point (Fig 3.4E) and macrophage-associated gene expression (*F480*) was comparable in iWAT and BAT (Fig S3.3A). We observed that IFNAR1 KO mice on HFD had significantly higher respiration rate compared to WT mice through both light and dark cycles (Fig 3.4F). This energy expenditure difference was not due to more activity in the IFNAR1 KO mice (Fig 3.4G) and food consumption was similar between WT and IFNAR1 KO mice (Fig 3.4H). Together this data indicates that type I IFN signaling reduces energy expenditure without modifying activity, suggesting a possible reduction in brown and/or beige fat thermogenesis.

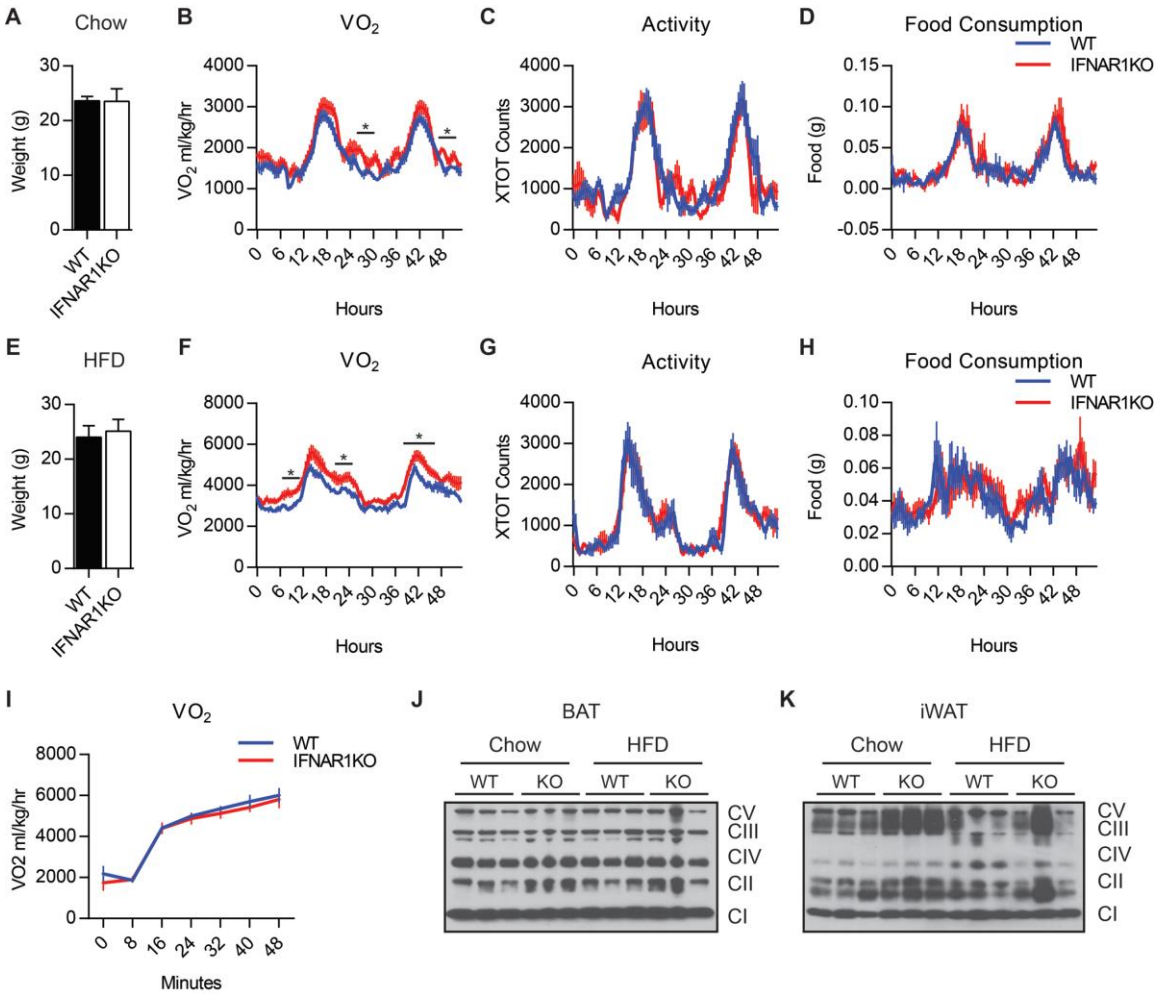

Figure 3.4 High-fat diet-induced type I IFN signaling leads to reduced energy expenditure

A-D) Weights in grams (g) (A), respiration rate represented as volume O_2 (VO_2), activity measurements (C), and food consumption in grams (g) (D) from wildtype (WT) (n=7) and IFNAR1 knockout (KO) (n=5) mice fed chow diet at thermoneutrality (TN).

E-H) Weights in grams (g) (A), respiration rate represented as volume O_2 (VO_2), activity measurements (C), and food consumption in grams (g) (D) from wildtype WT (n=5) and IFNAR1 KO (n=7) mice fed high-fat diet at TN for two weeks.

I) Volume of O_2 (VO_2) consumed after CL- injection by WT (n=4) and IFNAR1 KO (n=4) mice fed HFD for two weeks.

J-K) Western blot analysis of mitochondrial complex proteins in BAT (J) and iWAT (K) of WT and IFNAR1 KO mice fed chow or HFD for two weeks at TN.

Data information: Data are presented as mean ± SEM. *P≤0.05 (Student's t-test)

We next investigated whether the brown adipose of IFNAR1 KO mice had increased thermogenic potential compared to WT mice. We injected two week HFD-fed WT and IFNAR1 KO mice with pentobarbital to lower their respiration to basal levels. We then injected the mice with a β_3-agonist above the brown fat pad and measured respiration. The WT and IFNAR1 KO mice both had a maximal stimulated respiration of approximately 6000 ml/kg/hr (Fig 3.4I), indicating there was no difference in brown fat thermogenic capacity. While maximal capacity was similar, basal differences in mitochondrial function could account for changes in energy expenditure. We've previously shown type I IFN signaling can cause a disruption of mitochondrial function in brown adipocytes (Chapter 2). We next assessed whether there were differences in mitochondrial protein expression in the adipose of both the chow and HFD-fed WT and IFNAR1 KO mice. We found that mitochondrial complexes IV and II were slightly elevated in BAT of chow-fed IFNAR1 KO compared to WT, but this was not worsened by HFD treatment (Fig 3.4J). In iWAT, we observed a more dramatic increase in complex IV and II in the chow-fed IFNAR1 KO mice, but not in the HFD-fed mice (Fig 3.4I). Overall, this data indicates that blocking type I IFN signaling can increase energy expenditure, although this is not due to increased BAT thermogenic capacity, increased mitochondrial function particularly in the iWAT may be a contributing factor.

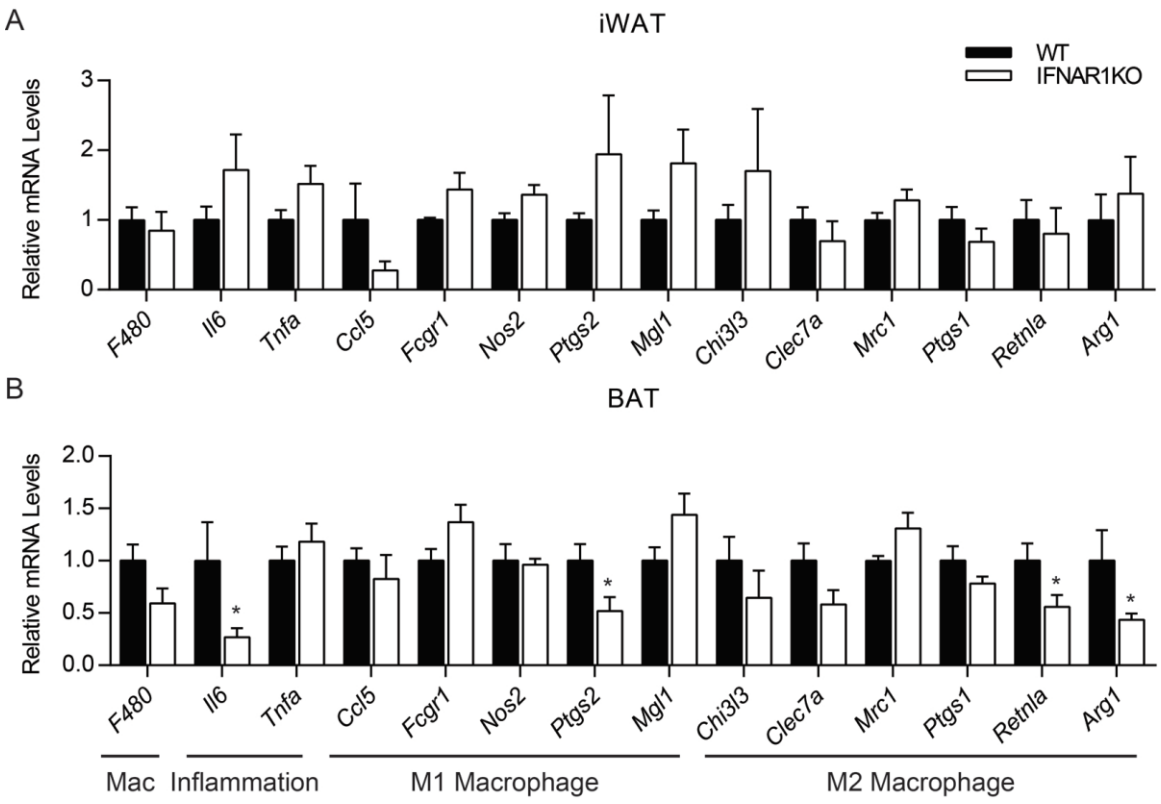

Figure S3.3 High-fat diet-induced macrophage infiltration/proliferation is unchanged by IFNAR1 ablation

A-B) Relative mRNA expression of total macrophage marker (*F480*), inflammatory genes (*Il6*, *Tnfa*), M1 macrophage genes (*Ccl5*, *Fcgr1*, *Nos2*, *Ptgs2*, *Mgl1*), and M2 macrophage genes (*Chi3l3*, *Clec7a*, *Mrc1*, *Ptgs1*, *Retnla*, *Arg1*) in iWAT (A) and BAT (B) of wildtype (WT) (n=5) and IFNAR1 knockout (KO) (n=7) mice fed high-fat diet for two weeks at thermoneutrality.

Data information: Data are presented as mean ± SEM. *P≤0.05 (Student's t-test)

IV. Discussion

Type I IFN is rapidly activated upon viral infections and is critical for initiating innate immune responses as well as priming the adaptive immune system (Pestka et al., 2004). Numerous cell types secrete type I IFNs including lymphocytes, macrophages, dendritic cells (DCs), plasmacytoid DCs, as well as non-immune cells such as fibroblasts (Ivashkiv & Donlin, 2014; Siegal et al., 1999). The importance of this pathway is underscored by the fact the IFN alpha receptor (IFNAR) is expressed ubiquitously through the body (Constantinescu et al., 1995), suggesting any tissue can respond to type I IFN. We show here that HFD feeding activates ISGs in multiple tissues, but it is not clear whether the signaling is local or systemic. This signaling is activated after only two weeks of HFD, earlier than many other inflammatory pathways (Strissel et al., 2010). As the first sensor of nutritional state, the gut has been implicated as an inflammatory activator in obesity (Winer et al., 2017). Determining where the signal is initiating will be an important next step in elucidating this pathway.

The switch from M2 to M1-polarized macrophages is a later event in DIO (Lumeng et al., 2007a) following the induction of IFNγ (Wensveen et al., 2015). We found that two weeks of HFD-feeding was associated primarily with an increased M2 macrophage gene signature in adipose and muscle, which is characterized as an anti-inflammatory state (Gordon, 2003). While ISG levels were increased in all observed tissues at this time point, *Il6* and *Tnfα* were not yet induced in most tissues. Specifically, the muscle appeared to have higher macrophage proliferation and/or infiltration with no change in *Il6* or *Tnfα*. This suggests that the type I IFN signaling event may be prior to other inflammatory factors. Interestingly, there was no difference in either M1 or M2 macrophage gene signatures in the adipose of WT and IFNAR1 KO mice after two weeks on HFD. However, it has been shown that IFNAR1 KO mice are not able to activate certain immune cells during viral infection (Muller et al., 1994). Characterizing differences in immune populations of WT and IFNAR1 KO adipose after two weeks HFD may further elucidate early events of diet-induced inflammation that are dependent on type I IFN.

Brown adipose is more resistant to inflammation compared to white fat (Sierra Rojas et al., 2016). In our current work, we observed higher ISG expression in the BAT of HFD- compared to chow-fed mice after two weeks, but not after 11 weeks. Conversely, WAT had increasingly higher fold changes of ISG expression with longer HFD. We have previously shown that the transcription factor PRDM16 blocks the type I IFN response in brown adipose to maintain brown fat character and mitochondrial function (Chapter 2).The higher expression of PRDM16 in BAT compared to WAT may explain normalization of the ISG signature after long-term HFD. We also found no brown fat-selective or mitochondrial gene expression differences in BAT of WT and IFNAR1 KO mice after long-term HFD. However, the iWAT in IFNAR1 KO mice had higher levels of *Cidea*, *Pgc1a*, and *mt-CytB*. This data suggests HFD-induced type I IFN signaling leads to mitochondrial dysfunction in iWAT, while PRDM16 blocks this effect in BAT.

Ectopic type I IFN signaling causes adipocytes and hepatocytes to become insulin resistant (Wada et al., 2011). We observed improved glucose tolerance in IFNAR1 KO compared to WT mice after long-term HFD feedings and aging, but which tissues had improved insulin sensitivity is not clear. Inflammation and disruption in insulin signaling in adipose, liver, and muscle have all been implicated in the development of insulin resistance (Johnson et al., 2012; Odegaard & Chawla, 2013). Hyperinsulinemic-euglycemic clamp studies would be required to discover any differences in insulin sensitivity of tissues in WT and IFNAR1 KO mice. Additionally, future studies involving tissue-specific knockout of the IFNAR1 are necessary to further elucidate the initiation of type I IFN metabolic defects.

Chapter 4 : Methods

I. In vivo studies

All animal experiments were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee.

Animals

Chapter 2:

Rosa26^{CreER}, *Prdm16*^{flox} mice were maintained on a mixed 129Sv/C57Black6 genetic background (Harms et al., 2014). *Myf5*^{Cre};*Prdm16*^{flox} mice were backcrossed into the C57Black6 background for 10 generations (Harms et al., 2014). Male mice were used for all experiments. Mice (6-7 weeks old) were injected with 25,000 U of recombinant mouse Interferon alpha A (PBL Assay Science) or an equal volume of phosphate buffered saline (PBS) as control six times over two weeks.

Chapter 3:

Control and diet-induced obese (DIO) mice were ordered from The Jackson Laboratory (# 380050). DIO mice were fed HFD (60 kcal% fat) for 11 weeks at room temperature (RT). C57Black6 (Taconic, B6M) mice (6-8 weeks old) were acclimated to 30°C, which is mouse thermoneutrality (TN), for two weeks while fed normal chow diet, then fed 45 kcal% fat HFD (Research Diets Inc., D12451) for two weeks. IFNAR1 KO mice were backcrossed toC57Black6 background for 7 generations (32045-JAX). For all experiments littermate controls were used. WT and IFNAR1 KO (B6.129S2-Ifnar1tm1Agt/Mmjax) mice were housed at RT or acclimated to TN for two weeks. Mice were then fed 45% HFD for up to 18 weeks while weekly weight gain was monitored. For aging experiments, WT and IFNAR1 KO mice were housed at RT on normal chow diet for 7 months, and then mice were kept at TN while maintained on chow diet for an additional 5 months.

Respiration Monitoring

Chapter 2:

For NE injections, mice were first placed in CLAMS metabolic chambers at 33°C, then sedated with 75 mg/kg Nembutal, followed 20 min later by injection with 1 mg/kg NE (Sigma A9512-1G). Data were collected until mice recovered from barbiturate sedation.

Chapter 3:

For energy expenditure experiments WT and IFNAR1 KO mice were acclimated to TN for two weeks, then fed chow or HFD for 10 days after which they were placed in CLAMS metabolic chambers at TN. Mice were allowed to acclimate to cages for 24 hours and then basal respiration was measured for 48 hours. For β_3-agonist injections, mice were first placed in CLAMS metabolic chambers at 33°C, and then sedated with 75 mg/kg Nembutal, followed 20 min later by injection with 1 mg/kg CL 316,243 (Sigma-C5976) diluted in PBS. Data were collected until mice recovered from barbiturate sedation.

Histology

Chapter 2:

For immunohistochemistry, BAT was fixed in 4% PFA overnight, dehydrated, and embedded in paraffin for sectioning. Sections were stained with hematoxylin and eosin or probed with antibodies for UCP1 (R&D Systems). For transmission electron microscopy, adipocytes were fixed with 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C, and then postfixed with 2.0% osmium tetroxide for 1 hr at room temperature. Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1010 electron microscope.

Glucose Tolerance Test

Chapter 3:

Mice were fasted overnight (~16 hours). Basal blood glucose was measured from small cut in the tail using a glucose meter (Bayer Contour). Mice were then injected intraperitoneally with 1.5mg/kg glucose (Sigma G8644). Blood glucose was measured 15, 30, 60, and 120 minutes after injection. Area under the curve was calculated in PRISM for glucose response curves.

II. Cell Culture

Chapter 2:

Primary inguinal preadipocytes were isolated from *Prdm16*^{flox} and *Rosa26*^{CreER}, *Prdm16*^{flox} mice as previously described (Rajakumari et al., 2013). Recombination in *Rosa26*^{CreER}, *Prdm16*^{flox} adipocytes was induced by treating cells with 1 μM of 4-hydroxy-tamoxifen (Sigma) for 3 days in growth phase. Cells were differentiated with medium containing 10% FBS, 0.5 μM isobutylmethylxanthine, 125 nM indomethacin, 1 μM dexamethosone, 20 nM insulin, and 1 nM T3 without or with 1 μM rosiglitazone. To block type I IFN signaling, cells were treated with 1 μ g/mL anti-IFNAR1 antibody MAR1-5A3 (Leinco Technologies, Inc) during growth for 4 days. Immortalized brown and primary ingWAT adipocytes were treated with vehicle or 1000 U/mL recombinant mouse Interferon alpha A (PBL Assay Science) throughout differentiation to determine effects of IFN on differentiation. For CRISPR/Cas9 mediated gene editing, guide RNA sequences against mouse Prdm16 were cloned into LentiCRISPR (Shalem et al., 2014), a gift from Feng Zhang (Addgene, 49535). A guide targeted at the mouse Rosa26 locus was used as a negative control. gRNA-Prdm16(A): 5' CGGCGTGCATCCGCTTGTGC 3'; gRNA-Prdm16(B): 5' CCAACCTGTGCCGGCACAAG 3'; gRNA-R26: 5' AAGATGGGCGGGAGTCTTCT 3'; gRNA-Irf1(A): 5' AGCACGCTGCTAAGCACGGC 3'; gRNA-Irf1(B): 5' GCACGCTGCTAAGCACGGCT 3'. Short-hairpin RNA (sh-RNA) constructs were generated by the High-Throughput Screening Core (University of Pennsylvania). shIrf1 (a): 5' AGATGGACATTATACCAGATA 3'; shIrf1(b): 5' CTCTTCTGTCTATGGAGACTT 3'. Oil red O staining and retrovirus production were performed as described previously (Seale et al., 2007).

Real-Time qPCR

Total RNA was extracted by TRIzol (Invitrogen) followed by purification using PureLink RNA columns (Invitrogen). Isolated mRNA was reverse transcribed using the High-Capacity cDNA Synthesis kit (Applied Biosystems) and used in real-time qPCR reactions with SYBR Green master mix (Applied Biosystems) on a 7900 HT (Applied Biosystems). Tata-binding protein (*Tbp*) was used as an internal normalization control.

Microarray data

Microarray services were provided by the UPENN Molecular Profiling Facility, including quality control tests of the total RNA samples by Agilent Bioanalyzer and Nanodrop spectrophotometry. All protocols were conducted as described in the Ambion Expression Manual and the Affymetrix GeneChip Expression Analysis Technical Manual. In microarray data of control (EtOH) and Prdm16 KO (4OHT) cells under control (Ctl) or rosiglitazone (rosi) (GSE86018), differentially expressed genes were selected for clustering analysis by fold-change > 1.5 and adjusted p.value < 0.05. Hierarchical clustering was performed using (1−Spearman correlation coefficient) as a distance measure for genes and samples. Gene ontology analysis was conducted using Enrichr (Chen et al., 2013; Kuleshov et al., 2016) and top enriched biological process terms were presented. For Prdm16-KO-BAT gene expression data, we used previously published microarray data (Harms et al., 2014; Harms et al., 2015).

Cell Immunostaining

Briefly, cells were fixed with 4% (wt/vol) paraformaldehyde (PFA) for 10 min, permeabilized with 0.5% Triton X-100 for 15 min, and then blocked in 4% goat serum for 30 min. Cells were then incubated with primary antibody anti-Prdm16 1:200 (Seale et al., 2007), followed by secondary antibody Alexa Fluor 647 donkey anti-rabbit IgG 1:500 (Invitrogen), and DAPI (Invitrogen) for nuclear staining.

Western Blot

Protein extracts were prepared as previously described (Rajakumari et al., 2013). Proteins were separated in 4%–12% Bis-Tris NuPAGE gels (Invitrogen) and transferred to PVDF membranes. For Western blot, antibodies used: anti-PRDM16 (Seale et al., 2007), anti-FLAG (Sigma, F1804), anti-pSTAT1 (Santa Cruz, sc7988), anti-STAT1 (Santa Cruz, sc-346), anti-pSTAT2 (Millipore, 07-224), anti-STAT2 (Cell Signaling Technology, 4597S), anti-STAT3 (Cell Signaling Technology, 9139S), anti-Tubulin (Sigma, T6199), anti-UCP1 (R&D Systems, MAB6158), anti-Actin (Millipore), total OXPHOS antibody cocktail (Abcam, ab110413), anti-MT-CO1 (Abcam, ab14705), anti-IRF1 (Cell Signaling Technology, 8478S).

Chromatin Immunoprecipitation

Immortalized brown preadipocytes infected with MSCV-Puromycin or MSCV–Prdm16 were grown to confluency and fixed in 1% formaldehyde for 15 min, then quenched with 125 mM glycine for 5 min. ChIP was performed as described previously (Harms et al., 2015). Chromatin was probed with 1 µg of the following antibodies: anti-PRDM16 (Harms et al., 2014) or anti-histone H3K27Ac (Abcam, ab4729). Bound fragments were eluted at 65°C overnight in 20 mM Tris pH 8, 1mM EDTA and 1% SDS and subsequently treated with RNaseA and proteinase K before undergoing column purification (Qiagen, 28104). Target enrichment was calculated as percent input. ChIP-seq reads for Prdm16 and H3K27-Ac (GSE86017) were aligned to mouse genome, mm9, and further processed for peak-calling and genome browser track creation as previously described (Harms et al., 2015).

O_2 Consumption

Differentiated brown adipocytes were trypsinized, pelleted, and resuspended in a buffer comprised of 2% BSA, 1.1 mM sodium pyruvate, and 25 mM glucose in PBS. Samples were placed in an MT200A Respirometer Cell (Strathkelvin), and oxygen consumption was measured for approximately 5 min. Oxygen consumption was normalized to total cell number.

Transcription Assays

The *Ifi44* promoter/luciferase reporter plasmid (pGL4-Ifi44p) was constructed by PCR cloning of genomic sequence from C57Bl/6 DNA corresponding to 441 bp of Ifi44 proximal promoter and 76 bp 5' UTR into the XhoI and NcoI sites of pGL4.24, replacing the existing minimal promoter (Promega). pGL4-Ifi44p-ISRE (IRF-E/ISRE) was built by inserting a 55 bp linker centered at the Ifi44 transcriptional start site (and ISRE) into the KpnI and XhoI sites of pGL4.24, retaining the minimal promoter. CMX-Gal4(DBD)-hIRF1 was cloned by PCR amplifying human IRF1 from CMV6-hIRF1, with BamHI and NotI sites appended for insertion into CMX-Gal4(DBD). pRL-CMV was used for internal normalization of the dual luciferase assays. The CMX-Gal4(DBD) (containing 447 bp of the Gal4 DNA binding domain), Gal4(5x)SV40-Luc, and pRL-CMV plasmids were provided by Mitch Lazar (University of Pennsylvania). CMX-hIRF1 was provided by Kathleen Sullivan (Children's Hospital of Pennsylvania). Reporter and expression plasmids were co-transfected into NIH3T3 cells (ATCC) using Lipofectamine 2000 (Invitrogen; 11668019). At 48 hours post-transfection, cells were harvested into passive lysis buffer for dual luciferase assays (Promega; E1960) using a Synergy HT plate reader (BioTek).

III. Statistical Analysis

Chapter 2:

Energy expenditure data were analyzed in R using a paired three-way ANOVA over all time points after NE injection with significance level, $\alpha=0.05$. Subsequent paired two-way ANOVAs for treatment effects over all time points were performed in individual genotype arms if interaction terms were significant at $\alpha=0.05$. For ANOVA calculations, D'Agostino-Pearson test was performed for normality with deviations significant at p-value less than 0.05. For Student' T-test, data were visualized and appeared approximately normal; no formal testing was performed. For data shown as log scale, statistical tests were performed that did not assume equality of underlying variances. For non-log scale, equal variance was assumed. Statistics for microarray data is discussed in Methods under Microarray data.

Chapter 3:

All qPCR and weight gain data were analyzed in PRISM using unpaired Student's t-test. Significance was considered p-value less than 0.05. For Student's T-test, data were visualized and appeared approximately normal; no formal testing was performed.

Chapter 5 : Conclusion and Future Directions

I. Summary

We investigated which pathways regulated by PRDM16 affect brown/ beige adipose function (Chapter 2). Through unbiased methods, we discovered that PRDM16 represses type I IFN responses in both brown and stimulated-beige adipocytes. We went on to show that PRDM16-dependent type I IFN repression is IFNAR-dependent. Ectopic IFNα signaling in brown adipocytes causes a reduction in thermogenic and mitochondrial function that can be rescued by Increasing PRDM16 expression. Similarly, PRDM16 expression blocks IFNα-induced ISG activation and BAT dysfunction *in vivo*. We went on to investigate the mechanism by which PRDM16 represses ISG activation using ChIP-sequencing. We found that PRDM16 binds proximal to promoter regions of regulated ISGs and DNA-binding is required for repression of this gene set. IFN regulatory factor 1 (IRF1) is highly expressed in brown preadipocytes and loss of IRF1 expression reduces ISGs in *Prdm16* KO cells. Using transcriptional assays, we next showed that PRDM16 can block binding and activation by IRF1.

We also discovered that type I IFN signaling is induced by high-fat diet (HFD) feeding in mice after only two weeks (Chapter 3). Blocking type I IFN response using an IFNAR1 KO mouse, we observed an improvement in weight gain and glucose homeostasis after long-term HFD feeding. We observed a similar improvement in aging mice lacking IFNAR1. To determine the metabolic differences between WT and IFNAR1 KO mice, we utilized metabolic chambers to measure energy expenditure and food consumption. We found that IFNAR1 KO mice have higher respiration rates compared to WT mice while on HFD, although brown fat capacity was the same between the groups. We also discovered increased brown fat-selective and mitochondrial gene expression in the iWAT of IFNAR1 KO mice compared to controls after long-term HFD. These data suggest that HFD-induced type I IFN may cause mitochondrial defects in iWAT which decrease energy expenditure and contribute to diet-induced weight gain.

In the following sections, I will further discuss the implications of the results found in Chapter 2and Chapter 3 and propose potential future investigations.

II. PRDM16 investigations

PRDM16 is a transcription factor with diverse regulatory roles, functioning as both a repressor and an activator. PRDM16 is highly expressed in brown adipose tissue (Seale et al., 2007), where it simultaneously transcriptionally activates the brown fat-selective genes and represses both muscle and white adipose gene programs (Kajimura et al., 2008; Ohno et al., 2013). In certain stem cell populations, such as HSCs, PRDM16 expression blocks the harmful effects of cellular stresses (Chuikov et al.). Before our work, it was not known whether PRDM16 also performed similar actions in brown adipocytes. In Chapter 2, we found that PRDM16 protects brown and beige adipocyte function by repressing type I IFN responses. While PRDM16 is expressed at the highest levels in mature brown adipocytes, we discovered that PRDM16 is also required in adipocyte precursors to block ISGs. Further studies are required to determine the roles of PRDM16 and the type I IFN pathway in brown/ beige precursor maintenance and proliferation. Type I IFN signaling, while critical for its antiviral actions, is cytotoxic at high levels. An example of this is in HSCs, where type I IFN has an important role in maturation (Essers et al., 2009; Kim et al., 2016), but increased signaling can lead to stem cell exhaustion (Essers et al., 2009; Sato et al., 2009). Determining whether PRDM16 opposes type I IFN responses in HSCs will be an important path for future investigations.

Previously, a genetic loss of function model revealed that PRDM16 is dispensable for brown fat development, but is required for maintaining brown fat character and mitochondrial function (Harms et al., 2014). We showed here that in addition to BAT dysfunction there is a coordinate increase in ISG expression in *Prdm16 KO* mice as they mature. Our lab showed that PRDM3/EVI, a PRDM16 homolog, is able to compensate for PRDM16 function early in BAT development (Harms et al., 2014). The progressive loss of ISG regulation would suggest that this

action of PRDM16 is also being compensated through BAT development. Another PRDM family member PRDM1/BLIMP1 has previously been shown to block IRF1 activation of target genes in the intestine (Mould et al., 2015), suggesting that this regulation may be a common action of the PRDM family. It remains unclear whether PRDM3/EVI1 or another PRDM family member is repressing type I IFN response in the absence of PRDM16. The necessity of compensatory factors indicates that repression of type I IFN responses is critical for developing BAT tissue.

To determine the requirement of PRDM16 in blocking ectopic IFN signaling *in vivo*, we injected young WT and *Prdm16* KO mice with recombinant IFNα while BAT function was still intact. We found that ISG expression was increased only in *Prdm16* KO mice injected with IFNα, suggesting that PRDM16 is able to block this response in WT mice. The ISG response in iWAT was the same in WT and *Prdm16* KO mice, confirming that this effect was specific to the *Prdm16* deficiency in BAT. Interestingly, ectopic IFN signaling led to decreased brown fat-selective and mitochondrial gene expression only in the *Prdm16* KO mice. We showed that these molecular changes resulted in a decrease in BAT respiratory capacity in the *Prdm16* KO mice injected with IFNα. Together these data demonstrated that PRDM16 protects brown fat from the effects of type I IFN signaling *in vivo*.

Previous studies have made clear the direct role of PRDM16 in activating brown fat gene expression, in particular *Ucp1* (Harms et al., 2015; Kajimura et al., 2009; Seale et al., 2007). Therefore, the contribution of increased endogenous ISG expression to the development of dysfunctional BAT in the *Prdm16* KO mice remains unclear. In adipocytes we showed that blocking ectopic IFN responses using an IFNAR-neutralizing antibody could rescue the defect in brown fat-selective and mitochondrial gene expression. Similarly, the neutralizing antibody fully restored mitochondrial gene expression in *Prdm16* deficient brown adipocytes, but could not rescue defects in *Ucp1* expression. This experiment clearly demonstrated the requirement for PRDM16 for *Ucp1* expression, but suggests the mitochondrial dysfunction may be indirect. The adult *Prdm16* KO mouse displays a loss of mitochondrial content and severe dysfunction (Harms

et al.). To determine whether this is due to type I IFN signaling, we could cross the *Prdm16* KO mouse to the IFNAR1 KO mouse and monitor the development of BAT dysfunction. These studies would more fully elucidate the role of the PRDM16-type I IFN axis in BAT maintenance and function.

To begin to uncover the mechanism by which PRDM16 regulates ISGs, we first used the IFNAR-neutralizing antibody to show that PRDM16 was blocking responses in a receptor-dependent manner. Next, we utilized ChIP combined with deep sequencing to show that PRDM16 binds proximal to the promoters of many negatively regulated ISGs. Interestingly, we found that a point mutation in the DNA-binding domain of PRDM16 (R998Q) abolished its ability to repress ISG expression. This was a surprising discovery since DNA-binding has been found to be dispensable for many PRDM16 actions in brown adipose thus far (Seale et al., 2007). In fact, no endogenous PRDM16-binding motif has been identified. One potential explanation for this is that PRDM16 directly binds DNA at a very small percentage of regulated sites, making identification of a binding motif very difficult.

When we analyzed the sequence under the PRDM16 ChIP binding peak at the *Ifi44* promoter, we found a putative ISRE overlapping an IRF-E. At many ISGs, IRFs will coordinately bind along with ISGF3 for maximal gene activation (Harada et al., 1996; Kimura et al., 1996). Both *Irf1* and *Irf7* are highly expressed in brown preadipocytes. Irf7 is strongly repressed by PRDM16, while IRF1 expression is not. Interestingly, IRF1 has recently been shown to activate a similar gene program as type I IFN (Xu et al., 2016). When we knocked down IRF1 in *Prdm16* deficient preadipocytes, ISG expression was decreased, indicating IRF1 was contributing to their activation in the absence of PRDM16. All of our transcriptional assays demonstrated that PRDM16 could block activation by IRF1, while R998Q could not. This again indicated direct DNA-binding was essential for the PRDM16 regulation of ISGs. Eliminating the entire promoter region except for the ISRE/IRF-e site, PRDM16 still blocked IRF1 activation, suggesting PRDM16 can bind directly to the IRF-E motif. While our study focused on one promoter region, further work

conducting unbiased analysis of PRDM16, R998Q, and IRF1 binding may identify a specific PRDM16 binding motif.

While PRDM16 expression decreased IRF1 binding at multiple ISG promoters, IRF1 was not bound to the promoter region of every PRDM16-regulated ISG. All IRFs bind the IRF-E sequence (Taniguchi et al., 2001), suggesting that PRDM16 may block the binding of other IRFs at certain ISG promoters. This will be important to explore further. In particular, IRF3 has been shown to block browning of subcutaneous adipose (Kumari et al., 2016), but it is unclear whether PRDM16 can antagonize IRF3 actions. Additionally, the role of IRF1 in brown/ beige adipose remains unclear. We found the overexpression of IRF1 did not affect brown adipogenesis; however, genetic loss of function *in vivo* may reveal more.

Taken together, this work has revealed a new role for PRDM16 in maintaining brown adipose function. Notably, we have also demonstrated a novel mechanism for PRDM16 transcriptional regulation. Future work on the PRDM16-IRF1 competitive binding model may uncover an endogenous PRDM16 binding motif. This regulatory pathway may also be critical in maintaining function of other cell types. Overall, this study has uncovered multiple critical lines of investigation.

III. Type I IFN in brown fat investigations

Type I IFN signaling is a vital pathway in the innate immune response to viruses and pathogens (Honda et al., 2005). In both mice and humans, loss of type I IFN responsiveness leads to susceptibility to infection (Chapgier et al., 2006; Dupuis et al., 2003; Hwang et al., 1995). Our major finding in Chapter 2 was that PRDM16 represses type I IFN responses in adipocytes. Unbiased comparison of WT and *Prdm16* KO cells uncovered a distinct group of negatively regulated genes, which gene ontology identified specifically as viral response and type I IFN response genes. While type I IFNs and IFNγ can activate overlapping gene sets (Ivashkiv &

Donlin, 2014), PRDM16 appeared to regulate type I IFN responses specifically. Interestingly, previous work suggested that type I IFN blocked adipogenesis (Birk & Rubinstein, 2006; Lee et al., 2016b). However, when we treated brown preadipocytes with IFNα throughout differentiation, we observed normal differentiation into lipid-containing adipocytes. One explanation for this discrepancy may be that the previous studies were done in 3T3-L1 adipocytes which are classic white adipocytes that have no detectable PRDM16 expression, whereas we used a brown adipocyte cell line. This suggests that PRDM16 blocks the type I IFN pathway in preadipocytes to protect general adipogenesis. We could test this hypothesis by treating *Prdm16* KO preadipocytes with IFNα and then observing the differentiation progression. Notably, it has been suggested that type I IFN causes cell cycle arrest which blocks adipogenesis (Lee et al., 2016b). We have found that cells overexpressing PRDM16 proliferate at a faster rate than control cells (data not shown). Whether PRDM16 functions to block the effects of type I IFN on the cell cycle is still unclear.

While we observed normal adipogenesis of brown adipocytes treated with IFNα, brown fat-selective and mitochondrial gene expression was significantly decreased. In addition, IFNα-treated adipocytes had severe mitochondrial dysfunction, leading to decreased cellular respiration. Previous studies have shown that IFNα/β inhibits mitochondrial function in lymphoid cells (Lewis et al., 1996; Lou et al., 1994; Shan et al., 1990). In agreement with these studies, we found that IFNα specifically blocks the transcription of mitochondrial-encoded genes (Lou et al., 1994; Shan et al., 1990). The ISG responsible for the repression of these genes is unidentified. In addition to mitochondrial genes, *Ucp1* expression is also strongly repressed by IFNα treatment. Whether this is a result of the mitochondrial dysfunction or an independent action of type I IFN signaling is unclear. One way to address these questions is to conduct a shRNA screen of our known PRDM16-regulated ISGs in brown preadipocytes. We would then treat the cells with IFNα through differentiation and determine if there was an improvement in mitochondrial and/or *Ucp1* expression.

We also found that PRDM16 protected BAT function in IFNα-injected WT mice. However, WT cells treated with IFNα had decreased respiratory function. IFNα did not reduce PRDM16 levels, but it may disrupt PRDM16 function contributing to the loss of brown adipocyte function. Interestingly, overexpressing PRDM16 in these cells rescued brown fat-selective and mitochondrial gene expression, indicating PRDM16 levels are critical. In agreement with this conclusion, we observed decreased mitochondrial gene expression in the iWAT of both WT and *Prdm16* KO mice. IWAT has lower PRDM16 levels (Seale et al., 2007) and thus is not protected from IFN signaling effects like BAT. In cells, we found that IFNα treatment through differentiation and early in differentiation caused a more profound reduction of brown-fat selective gene expression than acute late treatment. These data suggest ectopic IFNα signaling could be particularly detrimental during cold exposure when differentiation of precursors in both BAT and iWAT is critical. To test this hypothesis, we could pretreat WT mice with IFNα and continue treatment through a three week cold exposure. Under these conditions, we may observe dysfunction in BAT even with normal endogenous levels of PRDM16, consistent with our *in vitro* data.

Interestingly, we found that cold exposed mice had lower endogenous ISG expression in iWAT than mice at thermoneutrality (TN). Multiple papers have now documented an increased level of immune activity at TN, leading to more inflammation (Giles et al., 2016; Stemmer et al., 2015; Tian et al., 2016). We hypothesize that decreased *Prdm16* expression at TN in adipose leads to increased type I IFN responsiveness. To test this hypothesis, we would utilize WT and *Fabp4-Prdm16*, which overexpress PRDM16 in adipose tissue (Seale et al., 2011). After housing the mice at TN for two weeks, we would expect the *Fabp4-Prdm16* mice to have significantly lower ISG expression in adipose compared to WT mice.

In our current study, we have used recombinant IFNα to demonstrate the effects of this signaling pathway on brown adipose tissue. One important consideration is that type I IFN is endogenously activated by viral infection (Ivashkiv & Donlin, 2014). In viral infection both IFNα

and IFNβ are produced and can activate different gene programs (Schoggins et al., 2011). To better mimic the effect of infection on brown and beige adipose, we would inject WT and *Prdm16* KO mice with vehicle or poly(I:C), a synthetic double-stranded RNA (Rice et al., 1970), and observe them at TN and after cold exposure. These experiments would better replicate the antagonism between thermoregulation and viral defense uncovered by our experiments shown in Chapter 2 of this thesis.

Finally, the question of whether the PRDM16-type I IFN axis is relevant in human adipose tissue must still be addressed. Preliminary experiments in human adipocyte stem cells have shown that human recombinant IFNα blocks brown fat-selective gene induction by rosiglitazone (data not shown). Additional experiments are required to determine whether overexpressing PRDM16 can rescue this effect. In biopsies of adult human brown fat, PRDM16 is expressed at a normal distribution (Sharp et al., 2012), thus we could profile ISG expression in human brown fat and determine whether an inverse correlation exists with *PRDM16*. In order to determine whether viral infection affects brown fat function in humans, we could utilize FDG-PET scans to monitor cold-exposed individuals with lupus compared with healthy individuals. Lupus is an autoimmune disease characterized by sustained type I IFN signaling in blood and tissues (Crow, 2014). Alternatively, IFNα is used as a therapy in individuals with chronic viral infections such as hepatitis C virus. We could assess brown fat activity via FDG-PET scans in patients receiving IFNα treatment compared to patients with the same infection receiving other treatments. These studies have the potential to reveal previously unidentified side effects of increased type I IFN signaling in patients.

IV. Type I IFN in diet-induced obesity investigations

After observing reduced brown adipocyte function *in vitro* and *in vivo*, as described in Chapter 2, we next asked whether type I IFN is involved in the progression of diet-induced obesity (DIO). In Chapter 3, we first observed that HFD-feeding of mice induced ISG expression

in white adipose after 11 weeks and in many other tissues including brown adipose, muscle, and liver after just two weeks of HFD feeding. A time-course study starting with 12 hours of HFD feeding and progressing to two weeks will be an important follow-up to determine how early ISGs are induced. The previous experiments were conducted at TN in order to promote maximal weight gain, thus conducting the time-course study at TN and RT would be critical to determine whether mice at RT block this response.

The increased ISG expression in multiple tissues is indicative of systemic type I IFN signaling; however, it is possible local type I IFN is being induced in each tissue. To determine whether type I IFN levels are increased in the blood of HFD-fed mice, we will isolate serum from both experimental groups (chow and HFD-fed mice) and then conduct a viral protection assay in lymphoid cells treated with dilutions of the serum compared to a standard curve of recombinant type I IFN (Orange & Biron, 1996). This method has been shown to be more sensitive than currently available ELISA assays (Katakura et al., 2005). If we observed increased type I IFN in the serum of HFD-fed mice, it would suggest systemic activation of the pathway.

TLR4 is also increased in diet-induced obesity (Kim et al., 2012) and it has been proposed that free fatty acids (FFAs) activate downstream TLR signaling (Shi et al., 2006; Song et al., 2006). While TLR4 activation can initiate type I IFN production (Takeda & Akira, 2001), it is unclear whether the induction of ISGs in HFD feeding is TLR4-dependent. To address this question we could assess ISG levels after two weeks in WT and TLR4 KO mice (Shi et al., 2006). If ISGs are not in induced in the TLR4 KO mice, it would indicate type I IFN is stimulated by activated TLR signaling. We could further elucidate this pathway using a TLR4 tissue-specific knockout in adipose to determine whether the IFN is being produced by the adipose as has been described for TNFα (Hotamisligil et al., 1993). Determining the tissue of origin for the type I IFN signaling will be a primary focus of further experiments.

Since we observed ISG activation in multiple tissues, we used a whole-body IFNAR1 KO mouse model to determine the effects of increased type I IFN signaling during the progression of

DIO. We found that IFNAR KO mice gained less weight and had improved glucose tolerance after 18 weeks on HFD compared to WT mice. Interestingly, this difference was observed only when mice were housed at TN. This once again suggests cold exposure, even at RT, is enough to block activation of type I IFN signaling. As discussed previously, further studies are needed to directly compare ISG expression in mice at TN and RT during HFD-feeding.

Immune cell proliferation, infiltration, and activation in adipose tissue have all been shown to contribute to the development of DIO (Kintscher et al., 2008; Nishimura et al., 2009; Weisberg et al., 2003; Winer et al., 2009). The switch from M2 polarized macrophages to M1 macrophages is one the most well defined changes in the pro-inflammatory milieu during obesity (Lumeng et al., 2007a; Lumeng et al., 2007b). We observed an increase primarily in molecular markers for M2 macrophages in brown and white adipose after two weeks of HFD, indicating more proliferation and/or infiltration of anti-inflammatory macrophages at this time point. Unexpectedly, HFD-induced macrophage proliferation/ infiltration were not dependent on type I IFN signaling. DIO is also characterized as having an increased number of T cells and B cells in adipose (Kintscher et al., 2008; Pacifico et al., 2006; Winer et al., 2009; DeFuria et al., 2013; Winer et al., 2011). To determine whether HFD-induced type I IFN induces infiltration of other immune populations, we would use flow cytometry to quantify immune cells in HFD-fed WT and IFNAR KO mice.

While we observed increased ISG expression in brown fat after two weeks of HFD, after 11 weeks ISGs were expressed at similar levels. In Chapter 2 we showed that the BAT of WT mice is resistant to persistent ISG induction. We hypothesize from these data that high PRDM16 levels in BAT protect it from long-term HFD-stimulated ISG activation. Consistent with BAT being protected from increased type I IFN signaling, we found no difference in brown fat-selective genes between HFD-fed WT and IFNAR1 KO brown fat. The BAT capacity for maximal respiration was also the same in both groups. Interestingly, we did observe increased thermogenic and mitochondrial gene expression in the iWAT of IFNAR1 KO mice on long-term

HFD. We also found that certain mitochondrial complexes appear to be expressed higher in the iWAT of IFNAR1 KO mice. In future experiments, we will determine whether there is improved thermogenic and/ or mitochondrial function in the iWAT of IFNAR1 KO mice compared to WT mice by measuring respiration of isolated iWAT with a respirometer under basal and stimulated conditions.

IFNAR1 KO mice also had a higher rate of whole-body respiration than WT mice, which appeared to be amplified when mice were fed HFD. This indicates increased type I IFN signaling may reduce energy expenditure. To determine whether this is due specifically to defects in adipose tissue, we would develop an adipose-specific IFNAR1 KO mouse using the *Adiponectin*-Cre (AdipoQ-IFNAR1 KO). After two weeks of HFD-feeding, we would measure whole-body respiration in both control and AdipoQ-IFNAR1 KO mice. Additionally, we will assess mitochondrial and brown fat-selective expression in these mice. If these mice have a similar decrease in respiration as the whole-body IFNAR1 KO mice, we can conclude that HFD-induced defects in adipose are leading to decreases in energy expenditure and increased weight gain.

V. Final Thoughts

The interplay between inflammatory signaling and metabolism has become a major area of focus in the study of obesity and obesity-related diseases. While the field of brown/ beige adipose research continues to grow, very little work has been done investigating immune signaling in these thermogenic tissues. During this thesis work, we uncovered a novel mechanism by which PRDM16 promotes brown fat function by repressing type I IFN signaling. Additionally, we have shown that type I IFN signaling is induced in adipose as well as other tissues early in the development of diet-induced obesity leading to increased weight gain and disrupted glucose tolerance. Together these data indicate that immune therapies may be important for promoting brown/ beige adipose function and preventing DIO in humans. However,

there are many remaining questions to pursue to increase our understanding of the role of the type I IFN pathway in adipose tissue biology.

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