PKM2: A Novel Therapeutic Target for the Prevention and Treatment of Obesity

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DEDICATION

I dedicate my thesis work to my parents, Steven and Connie Dowker, for giving me this extraordinary chance at life.

-Your China Doll

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I stand amazed by the opportunities and blessings that have filled the chapters of my life. To God be given all the glory in everything I do, for it is His mercy and grace that has allowed me to come thus far and provide me with everything I have, will have, and will become.

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ABSTRACT

The obesity epidemic continues to be a growing concern as it closely parallels with the exponential rise of associated co-morbidities including cardiovascular disease, type 2 diabetes, and other metabolic diseases. Furthermore, this epidemic has harnessed a wide interest to investigate dynamics of adipose tissue and its function. Although pharmaceuticals targeting weight loss and obesity are currently available, safety concern and off-target side effects propel the need for novel prevention and treatment options. In the last decade, development and regulation of brown adipose tissue (BAT) has become an attractive therapeutic strategy to promote weight loss and improve metabolic homeostasis. Additionally, recent studies have identified the glycolytic enzyme pyruvate kinase M2 (PKM2), as a novel regulator of brown adipogenesis and the browning of white adipose tissue (WAT). However, the molecular mechanisms remain to be elucidated. The current study investigates the role of β-catenin and its downstream target high mobility group AT-hook 2 (HMGA2) in mediating PKM2's role in the browning of WAT. We demonstrate that PKM2 deficiency promotes the browning of white primary pre-adipocytes via modulation of the β-catenin/HMGA2 axis. Disruption of the β-catenin/HMGA2 signaling pathway abolished the effects of PKM2 deficiency on the browning of white adipocyte. While these findings are still preliminary, our study suggests that targeting PKM2 could constitute a strategy to prevent excess fat accumulation, enhance whole body energy expenditure, and improve glycemic control.

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CHAPTER I
INTRODUCTION

Wnt Pathways Overview

The Wnt signaling pathway participates in a wide variety of different biological processes and serves as a fundamental mechanism for cell fate, proliferation, migration, polarity, and stem cell self-renewal. Therefore, aberrant or mutated Wnt signaling has been associated with various disorders that affect embryonic developmental or linked to human disease, most notoriously to cancer [1-3]. However, before examining the diversities of dysfunctional Wnt signaling, this section will give a general overview of the historical and mechanistic properties of the Wnt signaling cascades. The early 1980's launched the introduction of the *Wnt*-1 gene, formerly referred to as *Int-1* [4]. Later into this decade, experiments identified the fly *Wingless* (*wg*) as a gene homolog to Wnt-1 of which, participates to establish larval segment polarity [5, 6] and importantly, reinforces the gene's developmental pertinency. These two genes pioneer the Wnt family and encode for secreted, cysteine-rich glycoproteins that are relatively 40 kDa in size and can be modified by lipids to enhance their productivity [7]. Wnt signaling enhancement via lipid alteration is supportively demonstrated by Porcupine's (Porc), a multi-pass transmembrane O-acyltransferase- 'like', indispensability. Loss of the highly conserved Wnt component instigates defective Ser209-dependent acylation and ultimately the retainment of Wnt3A within the endoplasmic reticulum (ER), thus limiting the protein's secretion and transport [8-10]. Likewise, mutations to *Wntless* (*Wl*), another transmembrane protein, exhibited Wg accumulation within the Golgi [11]. Previously, Wnt proteins were categorized based on their axis-inducing ability and grouped as either canonical (transforming; Wnt1, Wnt3A) or noncanonical (non-transforming; Wnt5A, Wnt11). However, upon further investigation, this categorization was found to be oversimplified as the Wnt protein binding capacity with their receptors is promiscuous and can 'inductively' activate multiple intracellular signaling pathways with the same Wnt. Wnts also encompass a morphogenic signaling modality that spans across developing tissue, mainly within short-range distances [12, 13]. However, while more rarely, long-range signaling has also been proven. For example, lipid-modified Wg was shown to interact with specific binding associates to execute distant

signaling [14]. The Wnt signaling complex is comprised of three main branches: canonical, noncanonical (also referred to as planar cell polarity, PCP), and Wnt/Ca^{2+} pathways [15]. The highly conserved canonical pathway was initially established through studies utilizing Drosophila and Xenopus models [16, 17]. However, it wasn't until the work of the late-90s to early-2000s that further elucidated the pathway's signaling transduction. Accordingly, the identification of nuclear targets and receptor/co-receptor components such as T-cell factor/lymphoid enhancer factor (TCF/LEF) and Frizzled (Fzs)/ LRPs (a.k.a. as Arrow in *Drosophila*) helped seal a variety of major breaches within the canonical pathway [18-21]. In essence, the canonical Wnt pathway promotes the stabilization of β-catenin, a transcriptional activator. The conservation of β-catenin further facilities the activation of Wnt target genes through association with the DNA-binding factor TCF/LEF. The presence or absence of translated *Wnt* products (e.g., Wnt ligands) determine the pathway's 'on' v. 'off' state. When Wnt ligands are absent, target gene transcription remains inactive, thus the canonical pathway is considered to be switched 'off'. This dormancy is emanated by the actions of the destruction complex which is made up of a variety of scaffolding proteins (axis inhibition protein-1, axin-1; adenomatous polyposis coli, APC), Ser/Thr kinases (casein kinase 1α, CK1α; glycogen synthase kinase 3β, GSK3β), and cytoplasmic effector proteins (dishevelled, Dvl). This complex works together in congruence to mediate phosphorylation of the transcriptional activator, consequently marking β-catenin for protein degradation by β-transductin-repeat-containing protein (β-Trcp), an E3 ubiquitin ligase subunit [22-24]. The phosphorylation of β-catenin is site-specific and sequential. CK1α first phosphorylates the transcription factor at Ser45 to prime the Thr41 site for cytoplasmic GSK3β-mediated phosphorylation, which also subsequently phosphorylates β-catenin at sites Ser33 and Ser37. The successive modifications of β-catenin allow for β-Trcp's docking and destabilization and further degradation of β-catenin [25-27]. The lower kept cytoplasmic levels of this protein promote the nuclear binding interaction between TCF/LEF and transcriptional repressors such as Groucho (Gro) and histone deacetylases which prevents target gene transcription activity [28, 29]. The canonical Wnt signaling is also

negatively regulated by Ring finger protein 43 (RNF43) through a direct ubiquitin effect on Fz [30]. Similarly, Dickkopf1 (DKK1) maintains its prototypical inhibitory status, at the receptor level within Wnt signaling, through which the protein modifies LRP6 functioning. Two key models have been proposed to help elucidate the actions of DKK1. One, an antagonistic effect that prevents Fz-LRP6 complexation and two, the internalization and degradation of the coreceptor thus lowering its cell surface concentration and thereby, limiting Wnt binding availability [31-33]. Alternatively, upon the presence and binding of Wnt ligands to Fz's cysteine rich domain (CRD) and coreceptor, LRP5/6, the destruction complex is rendered inactive through dissociative measures. This hindrance subsequently stabilizes β-catenin levels while also promoting the transcription factor's translocation to the nucleus to act as a coactivator of Wnt target genes.

The scaffolding protein, Axin, contains binding sites that host both kinase and phosphatase activity, as well as for the principle canonical factor β-catenin. Notably, this protein has been reported to possess a unique attribute known as the "scaffolding effect" which expedites the concentration effectiveness by anchoring the enzyme and substrate into close proximity of each other. Furthermore, kinase activity has been shown to be countered by the efforts of the Ser/Thr protein phosphatases-1 (PP1) and phosphatases-2A (PP2A). These phosphatases function to dephosphorylate β-catenin and Axin, of which, accordingly, shifts these components toward nuclear translocation and the plasma membrane [34, 35]. Likewise, Dishevelled (Dsh/Dvl) also is recruited to the plasma membrane where it binds to Fz and provides a scaffolding framework for the Axin-GSK-3β arrangement. Along with CK1γ, the Axin-GSK-3β complex activates LRP5/6, while both CK1ε and CK2 have been reported to modulate the activity of Dvl, thus ultimately driving the stabilization of cytosolic β-catenin. Wnt signaling is enhanced through the binding of R-spondin to leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), thus countering the inhibitory effects of RNF43 [35-37]. Post-translocation of β-catenin prompts several actions within the constructs of the nucleus. β-catenin works as a transcriptional co-activator that complexes with TCF/LEF family transcription factors and promotes their activation state to further induce target gene transcription.

Other relevant β-catenin binding partners include Legless/BCL9, Pygopus [38, 39] and Hyrax (Hyx)/Parafibromin [40], all which have reported different influences on β-catenin's nuclear retention, transactivation capacity of target gene expression, and overall nuclear Wnt signaling transduction [41].

Non-canonical Wnt signaling includes two sub-categories: planar cell polarity signaling and the Wnt/Ca^{2+} pathway. Both sub-pathways mediate signaling that controls aspects of cell behavior such as migration, adhesion, and polarity, and furthermore, is β-catenin-independent. Cellular polarization is a common yet, complex interplay that guides developing tissues toward a particular shape and functionality [42]. The *Drosophila* model is one of the most acclaimed study representations that has made extensive advances in our understanding of principle factors within the PCP pathway, detailing the various regional polarities of the fly most notably, the wing and eye [43, 44]. Additionally, convergence and extension (C&E) gastrulation movements in vertebrates were also shown to exploit the mechanisms of PCP signaling [45]. Although Wnt ligand requirement for PCP signaling remains controversial for the Drosophila model [46, 47], vertebrate studies have established both Wnt5a and Wnt11 as PCP pathway initiators [48, 49]. Upon binding to Fz, the PCP pathway, likewise to canonical signaling, initiates specific component recruitment to the plasma membrane, such as Dishevelled. Importantly, Dsh establishes a critical branch point between canonical and non-canonical signaling, as it positionally succeeds common receptors in both pathways, yet accordingly, conveys differential signaling cascades [50]. Downstream of Dsh's membrane enlistment, incites the subsequent employment and poorly detailed actions of the core PCP-specific proteins, namely Prickle (Pk), Van Gogh (Vangl)/Strabismus (Stbm), Flamingo (Fmi)/Starry night (Stan), and Diego (Dgo)/Diversin. Presumed functioning of these different components include the mediation of protein-protein interaction, intercellular adhesion, component recruitment, and protein localization [51]. Upon recruitment to the apical membrane, PCP core components are complexed and then diligently placed either proximally (Stbm-Pk) or distally (Fz–Dsh–Dgo) of the cell to promote planar polarization [52]. Moreover, Dvl claims interaction with downstream effectors (e.g., small GTPases or RING finger proteins)

[53] that to activate different pathways leading to cytoskeleton remolding (Dvl-WGEF-RhoA-ROCK-MRLC) [54], cellular growth, movement, polarity (Dvl-Rac1-JNK-cJUN) [55, 56] and motility (Dsh-XRNF185-paxillin) [57].

As implied its name, the Wnt/Ca^{2+} signaling cascade is reported to play a role in regulating the levels of intracellular Ca^{2+} [58]. The implication of calcium modulation to the Wnt field was addressed by a zebrafish model that found an enhancement of embryonic Ca^{2+} signaling via exogenous application of the non-transforming Wnt ligand, Wnt5A [59]. The Wnt/Fz interaction activates the membrane-bound enzyme, phospholipase C (PLC), which cleaves and converts phosphatidylinositol-4,5-bisphosphate (PIP_2) into secondary messengers. Diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) promotes the direct intracellular release of Ca^{2+} as well as activating the Ca^{2+}-dependent modulator, protein kinase C (PKC). The increase of intracellular Ca^{2+} also positively regulates the preceding modulator, in addition to calcium calmodulin mediated kinase II (CAMKII) and Calcineurin (CNA), a ubiquitous Ser/Thr phosphatase. Briefly, subsequent activities of these Ca^{2+}-dependent modulators include gene transcription activation (nuclear factor of activated T-cells, NF-AT), 'crosstalk' inhibition of the canonical nuclear β-catenin via Nemo-like kinase (NLK), and cytoskeletal formation downstream of the *plausible* Cdc42 activation [15, 58, 60-62].

Wnt and White Adipogenesis

As previously described, Wnt signaling is initiated upon binding of secreted Wnt-encoded glycoproteins to Fz receptors where this operation of reception is internalized and can activate the canonical and non-canonical pathways. Unfortunately, the signal specificity of these cascades remains undetermined due to the ambidextrous temperament of certain ligands and receptors. As such, Wnt11 has been demonstrated to maintain a dual aptitude to activate both β-catenin-dependent and independent signaling, whereas Wnt5a and Wnt8 more selectively activate either the non-canonical or canonical pathway, respectively [63, 64]. Similarly, Frizzled receptors also bear the capacity to operate and transduce activation

within both pathways despite their role as the primary upstream reception centric for Wnt ligands in general [41]. Although the clarity of how these components decide on which pathway to deploy remains unclear, the more fundamental Wnt 'on v. off' switch has been shown to modulate a variety of developmental and homeostatic maintenance processes, including adipogenesis. Wnt family members such as Wnt-1 and Wnt10b are recognized as potent inhibitors of adipogenesis. This is exhibited by their ability to block differentiation within 3T3-L1 pre-adipocytes through the stabilization β-catenin. As presented by the MacDougald laboratory, cell system manipulations either promoting the synthetic downstream activation of the canonical pathway or Wnt-1 overexpression, lead to the failure of differentiation as evidenced by lack of lipid droplet accumulation and absence of 422/ap2 expression, a notable adipocyte marker [65]. It is furthermore suggested that Wnt signaling represses the progression of adipogenesis through the inhibition of the key regulators PPARγ and C/EBPα [65-67]. The introduction of either principle factors into Wnt-1 expressing cells resulted in a vivid-to-moderate Oil Red O (ORO) staining, while the cells without application remained fibroblastic [65]. Supportively, another study demonstrated the inhibition of the two master adipogenic transcription factors through Wnt signaling activation with the use of CHIR 9902, a specific inhibitor of glycogen synthase kinase 3 (GSK3) and synthetic Wnt signaling activator in 3T3-L1 pre-adipocytes. Moreover, this study discovered that levels of Wnt10b mRNA expression share an inverse relationship with the differentiation status of pre-adipocytes, with an undetectable amount of expression at 36 hours post-induction of differentiation [68]. Notably, β-catenin has been shown to be regulated by PPARγ through proteasomal degradation while also through the functional interaction between the TCF/LEF binding domain of β-catenin and catenin binding domain of PPARγ [69, 70]. Another study suggested that PPARγ and β-catenin share a reciprocating relationship as the induction of PPARγ was shown to be obstructed on the account of Wnt-1 expression, while suppression of β-catenin liberates the differentiation blockade within Wnt-1 expressing pre-adipocytes [71]. Cell cycle dysregulation has also been underlined as a corresponding factor that modulates adipogenesis as mitotic clonal expansion was

found to be blunted at the enforcement of Wnt signaling [72]. Other components of the Wnt system, such as the dispensability of certain of Fz receptors and LRP co-receptors, in addition to the Wnt signaling inhibitors, namely secreted Frizzled-related protein-1 or -2 (sFRP-1 or sFRP-2) or a dominant-negative TCF4 (dnTCF4), have also helped characterize the regulation of adipogenesis [65, 68, 73]. For instance, while the activation of Wnt-Fz1 and Wnt-Fz2 were both found to block the adipogenesis of 3T3-L1 pre-adipocytes, only Fz-1 was shown to follow expectations of classical stabilization of β-catenin and subsequent dependent signaling. Conversely, Fz2 was proposed to utilize an alternative mechanism that is independent of β-catenin [74]. Wnt ligands such as Wnt5b have been shown promote adipogenesis through an inhibitory effect on the canonical pathway [75]. Interestingly, cells that overexpress Wnt5b were demonstrated to have a downregulatory effect on known upregulated genes during canonical activation including insulin like-growth factor-1 (IGF-1), vascular endothelial growth factor-C (VEGF-C), and WNT1 inducible signaling pathway protein 1(WISP-1). Furthermore, this overexpression was shown to possess the capacity to partly counter Wnt-3a-induced canonical activation through the reduction of nuclear translocation of β-catenin [76]. The upregulation of several genes related to adipocytokine expression such as *apM1* and *Lep* in 3T3-L1 cells that overexpress Wnt5b has been demonstrated and further suggested that Wnt5b may be moderately associated with diabetic susceptibility [77]. Additionally, the Wnt co-receptor LRP5 was shown to play a crucial role in the maintenance of glucose and cholesterol metabolism through the examination of $LRP5^{-/-}$ mice, of which experienced glucose intolerance, increased plasma cholesterol, and decreased insulin secretion [78]. Taken together, these of studies advance the Wnt pathway into the more clinical aspect of adipogenesis thus broadening its scope of implication.

While many studies examining adipogenesis from the Wnt perspective operate upstream of nuclear translocation using stabilization of the target protein as a focal point, Kennell and colleagues investigated the signaling cascade's nuclear regulation [79]. Of note, it has been suggested that β-catenin may serve an alternative role in adipogenesis other than suppression, as evidenced by its ability to co-activate non-

TCF/LEF transcription factors and relatively higher nuclear resilience in comparison to subsiding expression levels of Wnt10b throughout differentiation [80-82]. TCF7L2N, an alternatively spliced isoform of mouse TCF-4, was demonstrated to co-activate the adipogenic master regulator, C/EBPα, through synergetic complexation with β-catenin and p300. Furthermore, the splicing isoform was shown to partially rescue adipocyte differentiation upon ectopic expression of β-catenin as cells with co-expression with TCF7L2N, rather than β-catenin alone, experienced a greater magnitude of ORO stain while also rescuing C/EBPα expression [79]. More recently, a study perturbing the expression of TCF/LEF through a quadruple KO method discovered the transcriptional activation of a certain set of genes, of which the authors refer to as the GHOST (genes hidden outside the standard targets) response, that are capable of by-passing the traditional TCF/LEF complex with support from an indispensable nuclear β-catenin. Interestingly, while previous reports already support the ability of β-catenin to co-activate transcription factors outside of the constraints of TCF/LEF [83, 84], the proposed GHOST genes are uniquely expressed only upon the loss or strict inhibition of TCF/LEF expression [85]. It has been suggested that the inhibitory effect on adipogenesis through Wnt signaling may be confined within the first three days of pre-adipocyte differentiation, as 3T3-L1 cell sensitivity to CHIR 9902 was only partially sufficient on day four of treatment [68]. Likewise, the elevated nuclear expression of β-catenin from the latter experiment was considerably maintained until day 4 of differentiation while C/EBPα expression peaked at day 3 and TCF-4N expression was found to be substantial throughout the process [79]. Moreover, Chibby (Cby), a nuclear β-catenin antagonist, was shown to positively regulate the adipogenic program as demonstrated by the RNAi silencing of Cby, of which, profoundly reduced the induction of the master adipogenic transcription factors, PPARγ and C/EBPα [86, 87]. Interestingly, Wang and Di demonstrated the influential effects of the AMP-activated protein kinase (AMPK) cascade and activators, such as 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), on Wnt/β-Catenin and adipogenesis. In this study, AICAR-activated AMPK increased β-catenin stabilization and nuclear activity with TCF leading to GATA3 expression and subsequent anti-adipogenic

effects including downregulation of PPARγ and C/EBPα expression [88]. The Rho/Rho associated kinase (ROCK) pathway was also found to cross-talk with Wnt signaling to modulate adipogenesis. Li and colleagues demonstrated that ROCK inhibition negatively regulates β-catenin abundance while mRNA expression of PPARγ was shown to be markedly increased. Co-treatment with lysophosphatidic acid (LPA) rescued the inhibitory effects of ROCK and furthermore produced a delayed, yet significant upregulation of several canonical Wnt constituents including Wnt10b and β-catenin [89].

Notably, microRNAs (miRNAs) have also been demonstrated to play a role in adipogenesis through canonical Wnt signaling. For instance, miR-8 family members were demonstrated antagonize Wnt signaling from various angles that span across properties from ligand secretion to target gene transcription [90]. Similarly, 3T3-L1 cells transfected with miR-210 resulted in a distinctive lipid accumulation, while application of an miR-210 inhibitor decreased the differentiation markers PPARγ and ap2. Meanwhile, Tcf7l2, a proposed miR-210 target and Wnt target gene modulator, was found to be transcriptionally hampered in addition to decreased levels luciferase reporter activity upon miR-210 co-transfection [91]. Another study found that miRNA-135a-5p negatively regulates the differentiation process of 3T3-L1 pre-adipocytes, as overexpression of miRNA-135a-5p blunts triglyceride content, lipid accumulation, and hinders the expression of adipogenic genes. Moreover, it was suggested that miRNA-135a-5p activates canonical Wnt signaling through the targeting of a scaffolding component, adenomatous polyposis coli (Apc) [92]. Together, these studies implicate this class of small non-coding RNAs into the larger scale intricacy of Wnt signaling and adipogenesis. Wnt ligands have also been acknowledged to take a part in overall mesenchymal stem cell (MSC) fate. Cen and colleagues highlighted TNF receptor-associated factor 4 (TRAF4) as a negative adipogenic regulator of MSCs. These authors suggest that TRAF4 enhances the kinase activity of pyruvate kinase isozyme type M2 (PKM2) along with the subsequent activation of the canonical pathway upon TRA4 overexpression, thus attenuating the adipogenic differentiation potential of MSCs. It was further proposed that during adipogenesis, the downregulation of TRAF4 may be due to the

demethylation modifications that are promoted by ALKBH5-mediated N6-methyladenosine [93]. In addition to Wnt10b, other Wnt members including Wnt10a and Wnt6 also have been demonstrated to regulate MSC fate specifically promoting osteoblastogenesis while also suppressing adipogenesis. Conversely, Wnt knockdown cells resulted in the impairment of osteoblastogenesis coincided with progression of adipogenesis following the decline of the canonical target protein, β-catenin [94]. Additionally, it has been reported that Wnt/β-catenin signaling inhibition is able to influence a cell-fate shift from osteoblasts to adipocytes within cells that were already pre-committed to the osteoblastic lineage [95]. Intrinsic transcription factors from both cell lineages have been shown to regulate one another's differentiation processes. Furthermore, it has been suggested that the inhibitory effects of adipocytes on osteoblast differentiation and vice-versa, may be due to the over precedence of each other's set of transcription factors [95, 96]. Moreover, ablation of tuberous sclerosis complex 1 (TSC1), a tumor suppressor, was found to favor bone marrow adiposity cooperatively with bone mass reduction and associative decreased Wnt signaling thereby supporting the differentiation balance between adipocytes and osteoblasts. Importantly, it was proposed that the downregulation of Wnt signaling may be attributable to the suppression of autophagy that promotes protein levels of Notch1 within TSC1-deficient bone marrow stromal cells (BMSCs) [97]. Another group has suggested that irisin induced-autophagy conversely promotes factors of osteogenesis, as well as Wnt signaling components, in BMSCs while osteogenic differentiation is negatively regulated by autophagic inhibition via bafilomycin A1 [98]. Interestingly, Wnt signaling has been demonstrated to, as opposed to its more well-established differential role in the early stage adipogenesis, also front significance in terminally differentiated adipocytes specifically, lipid metabolism [99]. It was further reported that the transcriptional activity of and therefore, metabolic regulation by, TR4 is repressed by a Wnt/β-catenin-Slug signaling complex and in turn inhibits 3T3-L1 pre-adipocyte lipid accumulation and adipogenic potential [100].

Wnt and Brown Adipogenesis

In addition to inhibitory its effect on white adipogenesis, canonical Wnt signaling has also been demonstrated to regulate brown adipogenesis and metabolism. Comparatively, signaling activation via Wnt10b overexpression in two in vitro cell models, $Rb^{-/-}$ MEFs and HIB-1B cells, successfully blocked brown adipogenesis. This hindrance was evidenced by blunted lipid accumulation, impaired induction of the adipogenic master transcription factors, and repression of downstream adipogenic gene expression including FABP4 and UCP1. Moreover, the rescue of UCP1, a hallmark of the brown thermogenic program, required co-expression of both the master regulator PPARγ and brown-fat specific co-activator PPARγ co-activator 1-α (PGC1α) [101]. Similarly, Cantwell and colleagues suggest that brown fat differentiation and importantly, the thermogenic capacity, is also moderated by components of the JAK-STAT pathway. $STAT3^{-/-}$ (KO) primary brown pre-adipocytes experienced a robust downregulation of general adipocyte markers namely, PPARγ and C/EBPα, in addition to brown fat-specific markers including PRDM16, CIDEA, and the functional protein marker, UCP1. Furthermore, it was revealed that inhibition targeting of the canonical Wnt pathway or genetic knockdown (KD) of the canonical target protein, β-catenin, in opposition to the non-canonical calcium-calmodulin/calcineurin pathway, were shown to successfully restore UCP1 levels and rescue differentiation in $STAT3^{-/-}$ brown preadipocytes, respectively. Moreover, these authors suggest that the dispensability of STAT3 increases following differentiation phases that are post-induction [102]. In another study, Kim et al. demonstrated that myostatin regulates brown adipogenesis in early stages through the stabilization of β-catenin that is Smad3-mediated. Brown preadipocytes treated with myostatin were subjected to a decrease in brown adipogenic markers including PGC-1a, PRDM16, and UCP-1. Congruently, myostatin-induced phosphorylation of Smad3 subsequently activates the canonical pathway during brown adipocyte differentiation, thus also decreasing expression levels of these brown adipogenic transcription factors [103]. Microarray analysis of *Irs1* KO cells revealed that these cells exhibit an increased expression level of several Wnt members and components including *Wnt4, Wnt5a,*

Wnt6, *Wnt10a* and *Tcf7*, of which, the latter two were significant up-progressors and more negatively associated with differentiation status of cells [104]. Interestingly, Wnt10a was further reported to have influence over brown adipogenesis. The expression levels of Wnt10a were shown to share an inverse relationship with the expression of levels of insulin receptor substrate-1 (IRS-1) and further, the differentiation functionality of brown adipocyte precursor cells [105]. Another study found that the downregulation of necdin, an upstream differential inhibitor, within IRS-1 KO cells, prompts the suppression of Wnt10a and Pref-1 and restored the differentiation capacity of these cells. Furthermore, it was suggested that modulation of cyclic AMP response element binding protein (CREB) activity may regulate some of the repressive downstream attributes of the insulin/IRS pathway including *Wnt10a* and *necdin.* A constitutively active CREB was shown to be able to reverse high expression levels of both in *Irs1* knockout preadipocytes and partially rescue adipocyte differentiation [104]. More recently, Wnt3a has been demonstrated to regulate mitochondrial biogenesis through non-canonical Wnt signaling and partly, along the p38/CREB axis. While treatment of Wnt3a upregulated ATP synthase FO subunit 6 (Atp6), cytochrome c oxidase subunit I (Cox I), NADH dehydrogenase subunit 1 (Nadh1), Nadh5, selective p38 inhibition via SB23906 was shown to impair the ability of Wnt3a to induce these mitochondrial genes [106]. Epigenetic compounds such as lysine-specific demethylase 1 (LSD1) inhibitors have also been demonstrated to negatively regulate brown adipogenesis. Normally, LSD1 functions to repress Wnt signaling through component demethylation at H3K4 promoter regions; however, inhibition of LSD1 was shown to reduce canonical Wnt signaling repression and progression of brown fat differentiation [107]. Another study suggested that the H3K2 methyltransferase activity of Ezh2 is necessary for repression of several Wnt inhibitors and subsequent progression of adipocyte differentiation. Severe defects to brown adipogenesis and associated brown-fat specific markers were partially rescued upon ectopic expression of Ezh2 in $Ezh2^{-/-}$ brown preadipocytes. Moreover, immortalized and primary $Ezh2^{-/-}$ preadipocytes exhibited an

increased expression level of Wnt, elevated cytosolic accumulation of β-catenin, and markedly increased *Axin2* expression, of which is indicative of canonical Wnt signaling [108].

Wnt and "Browning"

While Wnt signaling has been abidingly associated to MSCs biology and adipogenesis in general, its specific implications to the "browning" program remains elusive. Inhibition of Wnt signaling using both pharmacological and genetic approaches was shown to significantly enhance the browning of primary white adipocytes from the inguinal depot of mice, as demonstrated by the increase in thermogenic markers including *Ucp1* and *Cidea*. Furthermore, albeit more modest, the regulation of Wnt inhibition on intrascapular brown primary adipocytes was also found to be positively significant [109]. Similarly, increased beige adipogenesis was observed in peripheral WAT in response to sclerostin, an osteocytic secreted Wnt inhibitory factor. Specially engineered osteocytes that exhibit a high secretion rate of sclerostin were demonstrated to have an increased expression level of UCP1, whilst partially reduced expression level of the functional thermogenic marker was indicated upon sclerostin depletion [110]. In a more recent report, varying concentrations (0–100 μM) of Ginsenoside Rb1 (G-Rb1) were found to blunt the inhibitory effects of Wnt signaling and promote browning within 3T3-L1 pre-adipocytes as inversely illustrated by the gradual reduction of oil Red O stain to increasing G-Rb1 concentrations concomitant with decreased β-catenin levels. Moreover, C-3f and SKL2001, Wnt signaling activators, were used to confirm the role of Wnt/β-catenin signaling 3T3-L-1 browning. Both activators were shown to be partly efficient in eliminating the browning effect of G-Rb1 via canonical Wnt signaling activation [111]. A previous study suggested that G-Rb1's browning capacity was induced through the regulation of PPARγ. Mu and colleagues revealed that the activity of PPARγ was appreciably increased in response to G-Rb1 treatment in Cos-1 cells. Additionally, browning of mature 3T3-L1 adipocytes was evidenced by the upregulation of brown-specific genes including, PGC-1α, PRDM16, and brown functional protein UCP-1. Notably, G-Rb1 was correspondingly shown to improve glucose tolerance and increase energy expenditure of these cells

[112]. In a following study, Park et al. also confirmed the modulation of 3T3-L1 PPARγ activity in response to G-Rb-1. These authors also investigated the effects of black ginseng (BG) and found that both G-Rb1 and BG were able to induce browning properties within 3T3-L1 and primary white adipocytes derived from C57BL/6 mice, however, mediated through the AMPK signaling pathway [113]. Furthermore, Sun et al. demonstrated that the browning program of white adipocytes could be blunted by fibroblast growth factor 9 (FGF9) [114]. In this study, FGF9 treatment and genetic overexpression was shown to inhibit beige adipogenic potential of SVF precursor cells whereas shRNA knockdown led to the significant increase in mRNA levels of Ucp1, PRDM16, Pgc1α, and Cidea. Interestingly, several WNT-related genes were observed to be transiently repressed in response to FGF9 at Day 0 and thus, indicating a plausible 'early-onset' regulatory role for the Wnt signaling pathway in the browning process [114].

Therapeutic Potential of Targeting Wnt/ β-catenin

Although it's more modernly recognized that white adipose tissue (WAT) serves an important role in normal physiological maintenance, a simple energy imbalance between input and expenditure has the inherent ability to promote the excess accumulation of WAT leading to obesity and associated complications including type 2 diabetes (T2DM), cardiovascular disease (CVD), chronic kidney disease (CKD), and non-alcoholic fatty liver disease (NAFLD) [115]. Since it has also been established that Wnt signaling plays a crucial role in several developmental and homeostatic processes including stem cell pluripotency or mature tissue maintenance, it doesn't come as a surprise that pathway aberration has been correlated to overall health status [41]. Therefore, targeting the Wnt pathway may hold relevance as a therapeutic potential to mitigate obesity and its associative metabolic disorders.

As previously described, in vitro work has exceedingly implicated Wnt signaling in the process of adipogenesis [65, 101]. Notably Wnt10b, when constitutively expressed, inhibits adipogenesis through the stabilization of β-catenin and repression of key adipogenic transcription factors [65]. In vivo studies investigating Wnt10b overexpression found that transgenic FABP4-Wnt10b mice experience a decrease in

whole body adiposity by 50% with a 60% depot reduction of the epididymal and perirenal fat pads [116]. In addition, these mice were also resistant to adipocyte expansion under subjection to genetic or diet-induced obesity. Importantly they also exhibited an improved metabolic profile including an increased insulin sensitivity and improved glucose tolerance that is potentially acquired through leptin maintenance [116, 117]. Furthermore, it was concluded that Wnt10b-induced inhibition of adipogenesis yields lean mice that exhibit the lack lipodystrophic diabetes [116]. Additionally, studies have proposed that variable effects of Wnt10b expression on brown adipogenesis are contingent upon the use of different promoters [101, 116]. While the intrascapular tissue of FABP4-Wnt10b and UCP1-Wnt10b mice both host a white phenotype and lack certain brown-specific markers, only Wn10b expression under control of the UCP1 promoter was shown to endorse the expression of white adipogenic markers, thus suggesting a unique trans-differential property of the latter promoter [101, 116]. Interestingly, using a mutational screening design, Christodoulides and colleagues identified WNT10B C256Y as a naturally occurring Wnt10b variant that may contribute to early onset obesity in humans. It was proposed that C256Y renders the capability of Wnt10b to activate the canonical complex and inhibit adipogenesis [118]. Likewise, genome-wide association studies have suggested a link between the related risk and susceptibility of type 2 diabetes development to variants of other Wnt components such as transcription factor 7-like 2 (TCF7L2) and Wnt5b [77, 119]. While some researchers suggest TCF7L2 gene variation condones a positive correlation such as improving glycemic control or body fat mass reduction [120], others opposingly disagree. In contrast, Haupt and colleagues argues that TCF7L2 gene variation produces less weight loss in patients following a lifestyle intervention [121]. Follow-up Wnt5b in vitro work found that overexpression of Wnt5b in 3T3-L1 pre-adipocytes not only promotes their differentiation but was also related to adipocytokine gene expression enrichment [77]. Gene polymorphisms of the Frizzled co-receptor, low-density lipoprotein receptor-related protein 5 (LRP5), similarly found obese phenotypic associations upon intronic variants of this gene [122]. Additionally, mice lacking LRP5 were found to experience high plasma cholesterol levels and glucose

intolerance when fed either a high-fat or normal diet, respectively. Moreover, LRP5-deficient islets lacked the ability to secrete insulin in response to Wnt3a stimulation [78]. A missense mutation in epidermal growth factor–like domain of the LRP6 gene was shown to obstruct the Wnt signaling cascade and further suggested to harbor a casual association with early coronary artery disease (CAD) and its metabolic risk factors [123]. $Lrp6^{mut/mut}$ mice were associated with hyperlipidemia and development of fatter liver disease (FLD). However, Wnt3a administration was shown to be sufficient enough to normalize heighten triglycerides (TGs) and plasma LDL, in addition to, reducing hepatic lipid content [124]. Patients carrying a LGR4 low-frequency functional missense variant (A750T) were linked to a higher risk for obesity. However, Lgr4 homozygous mutant ($Lgr4^{m/m}$) mice or Lgr4 ablation from the SVF of epididymal WAT were able to demonstrate improved glucose metabolism and potentiate brown adipogenesis, respectively [125]. In another study, Wnt5a was demonstrated to contribute to metabolic dysregulation mediated through non-canonical Wnt signaling across various study models. Fuster et al. revealed that their genetically engineered Wnt5a- knockout (KO) mice were able to attenuate high-fat/high-sucrose (HFHS) diet-induced metabolic dysfunction, including attributes such as IR and glucose intolerance, in contrast to their wild type (WT) littermates. Comparatively, Wnt5a transcriptional and protein expression levels were found to be more prominent in the visceral than subcutaneous AT of obese human subjects and the mRNA levels of Wnt5a were directly proportional to the clinical parameter, waist-to-hip ratio [126]. Wnt5a has also been suggested to play a role in WAT inflammation and atherosclerosis progression [126, 127]. Moreover, immunohistochemistry performed on human AT biopsies from both obese diabetic and lean subjects revealed that the macrophages infiltrating this tissue were positive for Wnt-5a expression. A functional follow-up report further revealed that cells incubated in a specially formulated macrophage-conditioned medium arrested their adipogenic potential and therefore, suggesting macrophages may contribute to adipocyte development via Wnt molecule secretion and signaling [128]. Another report suggests that loss of macrophage-derived Wnts may improve cardiac repair and function following a myocardial infarction

(MI) incident, as a shift towards M2-like macrophages was presented by Wntless-deficient macrophages [129].

Inhibition of Wnt signaling has also been seen consequent of the actions prompted by Dickkopf (DKK) family members. DKKs function to interfere with complexation of the two Wnt surface receptors Frizzled and LRP. IIIC3a, a DKK inhibitor, was found to improve glucose tolerance and insulin sensitivity of *db/db* mice via the inhibition of DKK2 [2, 130]. Ablation of DKK3 in ApoE-deficient mice were shown to exhibit a decrease in atherosclerotic lesions accumulation in addition to, a marked reduction in multiple proinflammatory cytokines while increasing plaque stability [131]. Similarly, another ApoE-deficient mouse model demonstrated that Wnt5a silencing also inhibited atherosclerotic development, downregulated of MAPK/NF-κB pathway mediators, and increased plaque stability without affecting blood lipid levels [132]. Interestingly, administration of DKK1 gene therapy was found to downregulate fibrotic expression markers associated with pathogenesis of an obstructive nephropathy model [133]. The regulation of Wnt inhibitors, such as secreted frizzled-related proteins (SFRPs), have additionally been cited as important Wnt signaling modulators that influence metabolic homeostasis. For instance, SFRP5 has been shown to be positively correlated with adipose tissue expansion [134], while also function as an anti-inflammatory adipokine that can modulate obesity-related metabolic dysfunction [135, 136]. Mice deficient of SRFP5 fed high fat-high sucrose (HF-HS) diet established a severe glucose intolerance and activated macrophages residing in their adipose tissue were further associated with elevated c-Jun N-terminal kinase (JNK), c-Jun, and IRS-1 phosphorylation, contributing to outcomes such as insulin resistance [135]. In contrast, SRFP1-deficient mice exhibited a decrease in percent body adiposity by 22%, however, enhanced orbital adipogenesis seen in patients with Graves' ophthalmopathy (GO) may be stimulated by heightened levels of SRFP1 [137, 138]. Furthermore, the human gene expression of Srf2 was found more prominently in visceral adipose tissue (VAT) upon comparison to the subcutaneous (SQ) depot [139].

Wnt Modulators

As previously mentioned, the Wnt signaling cascade has been implicated in numerous accounts of developmental and maintenance processes and moreover, perturbation to the pathway's signaling sets the stage for a catalog of different diseases [140]. Therefore, researchers have been diligently at work to identify various modulators aimed to manipulate the Wnt system in the efforts to help alleviate the associated costs.

From a natural perspective, most modulators, either Wnt activators or inhibitors, have been sourced from a plant basis. However, other source organisms have included fungi [141, 142], bacteria [143, 144], algae [145], lichens [146], and marine life [147, 148]. Endogenous activation of canonical signaling upon the binding of Wnt ligands propagates downstream inhibitory actions against the β-catenin destruction complex, of which includes, glycogen synthase kinase 3 (GSK3) and casein kinase I-alpha (CKIα) [149]. Mammals host two GSK3 enzymatic isoforms, GSK3-α and GSK3- β, and within the scope of the Wnt signaling cascade, the two isoforms have been proposed to function redundantly. Agitation to this Wnt component has also been associated with the pathologies of multiple conditions including obesity, inflammation, and diabetes mellitus thus, making it an appreciable therapeutic target [150, 151]. Moreover, many natural products used to modulate Wnt signaling have been shown to target GSK3 to promote canonical Wnt activation. Indirubins, a natural product from sources including indigo-producing plants [152], bacteria [153], and mollusks [154], were originally discovered as cyclin-dependent kinase (CDK) inhibitors [155]. However, indirubins have also been highlighted to blunt the inhibitory modes of GSK3 [154, 156] and with slight modifications at position 6, results in an enhanced synthetic derivative, 6-bromoindirubin-3-oxime (BIO), that favors GSK3 inhibition over CDK. BIO was shown to disrupt and inhibit the phosphorylation of Tyr276/216 on GSK3-α/ -β, respectively, thus blunting its inhibitory activity. Furthermore, BIO was demonstrated to comparatively mimic embryonic *Xenopus* Wnt signaling [154]. More recently, the formerly screened and identified the Wnt activator, indirubin-3′-oxime (I3O), was used to examine its effect on adipogenesis and obesity. 3T3-L1 pre-adipocyte treated with I3O found cell

differentiation apprehended, as the expression of major adipogenic markers such as C/EBPα and PPARγ were downregulated. In vivo effects demonstrated that I3O sufficiently perturbed induced weight gain and attenuated the accumulation of visceral adiposity of mice under subjection to a high-fat diet [157]. Furthermore, the mechanisms of indirubins and their associative derivatives, such as the potent analog, indirubin-5-nitro-3'-oxime (INO) [158], have been denoted as the graded benchmark of Wnt signaling activation, in comparison to the already acknowledged use of inorganic lithium salts [159]. GSK3 works closely and sequentially with CKIα to promote the ubiquitous 'tagging' and subsequent degradation of β-catenin [160]. Therefore, studies have similarly targeted CKIα inhibition as a potential mechanism for Wnt activation. For example, an in vitro study utilizing a pyridone alkaloid from *Ricinus communis*, ricinine, found that cells treated with this compound exhibited lower levels of phosphorylated β-catenin at Ser site 45, Ser33, Ser37, and Thr41, along with higher overarching levels of β-catenin. Furthermore, a combinatory treatment of ricinine and pyrvinium, a CKIα activator, found that these cells exhibited an increased level of β-catenin in comparison to lower levels in cells treated with only pyrvinium [161]. An isoflavone extract obtained from *Butea monosperma*, formononetin (FNT), was found to play a role in the sensitive balancing act between adipocyte and osteoblast differentiation. Gautam and colleges revealed that FNT downregulates adipogenic differentiation, while increasing osteoblastic potential through the stabilization of β-catenin. Moreover, these authors found that in vivo FNT attenuated obesity and rescued the decreased effect on trabecular bone volume from a high fat-induced diet [67]. Similarly, it was demonstrated that a polyphenol, curcumin, and a triterpenoid, toosendanin, possessed anti-adipogenic properties via Wnt signaling activation [162, 163]. Another natural plant extract, embelin, was shown capable of attenuating obesity against a high-fat induced diet (HFD) in mice through Wnt activation. Importantly, HFD-fed mice were shown to exhibit an improved status of insulin sensitivity and glucose tolerance, in concurrence with an expression inhibition of Dkk1 under influence of embelin [164]. Other proposed mechanisms of natural product-induced Wnt activation includes stabilizing cytoplasmic levels of β-catenin [165, 166], increasing

nuclear β-catenin accumulation or β-catenin transcription [167, 168], and alterations to Wnt ligands, receptors, or receptor modulators [169-171]. On the other hand, natural products that have been used to inactivate Wnt signaling exploit contrasting mechanisms to destabilize cytoplasmic β-catenin [172], decrease accumulation of nuclear β-catenin or perturb transcriptional interactions [173, 174] or modulate the activity of key inhibitory enzymes, Wnt receptors, and ligands [175-177]. For example, separate reports identified two marine sponge-derived metabolites, aeroplysinin-1 and smenospongidine, as Wnt signaling inhibitors via the mechanisms of increased β-catenin proteasomal degradation [144, 178]. Additionally, an isodonrubescens var. lushanensis-derived diterpenoid, Henryin was shown to work as a Wnt signaling antagonist that impairs the nuclear interactions of the β-catenin/TCF4 transcriptional complex [179].

In addition to the use of natural compounds, great efforts have been made to develop synthetic small-molecule drugs that also modulate the activities of Wnt signaling. The majority of these small-molecule inhibitors have been demonstrated to specify their activity towards one target element of the pathway. For example, inhibitory molecules such as NSC668036 [180] and 3289-8625 [181] were revealed to disrupt an essential interaction between the PDZ domain of disheveled and an internal sequence of Frizzled thus impeding the signaling transduction of Wnt activation [182]. Other inhibitors denote their actions by stabilizing different components of the destruction complex. As such, pyrvinium has been identified to bind and activate a supporting component of the destruction complex, casein kinase 1α (CK1α), and therefore, subsequently promoting degradation of β-catenin [183]. Additionally, select inhibitors has been reported to affect the interactions of nuclear β-catenin such as the interference between β-catenin and cyclic AMP response-element binding protein (CBP) that is prompted by ICG-001. This infringement further propagates a disturbance to the overall maintenance of certain Wnt target genes [184]. Moreover, IWP-L6 and XAV939 have been demonstrated to modulate their inhibitory actions more indirectly by either blunting the palmitoylation process of Wnt proteins or averting the antagonistic activity of TNKS, respectively [185, 186]. Activation of Wnt signaling cascade has been heavily reliant on inhibiting the

proceeding actions of GSK-3β. Most notably, LiCl [187] and CHIR99021 [188] have been used to mediate terms of activation. The dysregulation of several lncRNAs have been identified and associated with obesity [189-191]. For instance, SRA $^{(-/-)}$ mice were resistant to a high-fat feeding model, as evidenced by a leaner profile and enhanced insulin sensitivity [192]. In another study, adipose tissue collected from obese mice found an increased expression of the oncogenic lncRNA, PVT1, in comparison to their non-obese counterparts [193]. Additionally, lncRNAs have more recently been highlighted as novel regulators in the adipogenic program and moreover, selectively in Wnt signaling [194]. For example, lncRNA AC092834.1 has been suggested to be a positive adipogenic regulator through directly enhancing the induction of *DKK1* expression and subsequently antagonizing canonical signaling. Consequently, this suppression lead to the promotion of various adipogenic transcription factors such as C/EBPα, PPARγ, and FABP4 and differential advancement of human adipose-derived mesenchymal stem cells (hADSCs) [195].

CHAPTER II
RESEARCH PROJECT

Beta-Catenin Mediates the Suppressive Role of Pyruvate Kinase M2 on the Browning of White Adipocytes

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ABSTRACT

The obesity epidemic continues to be a growing concern as it closely parallels with the exponential rise of associated co-morbidities including cardiovascular disease, type 2 diabetes, and other metabolic diseases. Furthermore, this epidemic has harnessed a wide interest to investigate dynamics of adipose tissue and its function. Although pharmaceuticals targeting weight loss and obesity are currently available, safety concern and off-target side effects propel the need for novel prevention and treatment options. In the last decade, development and regulation of brown adipose tissue (BAT) has become an attractive therapeutic strategy to promote weight loss and improve metabolic homeostasis. Additionally, recent studies have identified the glycolytic enzyme pyruvate kinase M2 (PKM2), as a novel regulator of brown adipogenesis and the browning of white adipose tissue (WAT). However, the molecular mechanisms remain to be elucidated. The current study investigates the role of β-catenin and its downstream target high mobility group AT-hook 2 (HMGA2) in mediating PKM2's role in the browning of WAT. We demonstrate that PKM2 deficiency promotes the browning of white primary pre-adipocytes via modulation of the β-catenin/HMGA2 axis. Disruption of the β-catenin/HMGA2 signaling pathway abolished the effects of PKM2 deficiency on the browning of white adipocyte. While these findings are still preliminary, our study suggests that targeting PKM2 could constitute a strategy to prevent excess fat accumulation, enhance whole body energy expenditure, and improve glycemic control.

Introduction

Obesity remains one of the largest health concerns worldwide. The exponential growth of this epidemic is reasonably alarming as over one billion individuals are considered obese as according to the World Health Organization [196]. In the United States (US), statistical data collected from 2020 reported that roughly one third of the nation's states had an adult obesity prevalence that was either at or *above* 35%. Obesity is characterized by an excess accumulation of white adipose tissue and its ability to pose a threat to human health [197]. Importantly to note, as obesity trends upwards, known associative co-morbidities, such as diabetes mellitus (DM), cardiovascular disease (CVD), and metabolic syndrome closely parallel [198]. Although standard therapeutics to combat this disease do exist, their limitations and/or undesirable side effects make them counterproductive. For example, lifestyle interventions (e.g., diet, exercise, cognitive behavior) are limited by low adherence, lack of sustainability, and even patient weight regain [199-201]. Therefore, pharmacology is usually prescribed alongside lifestyle modifications. However, due to modest efficacy and subsequently higher dosing to reach a meaningful potential, pharmaceuticals on the market have reported numerous adverse and often intolerable off-target effects [202, 203]. While bariatric surgeries are the more effective option in comparison to non-surgical regimens [204], these approaches are typically reserved for a subset of candidates that meet a more specified criteria, in addition to being linked to several significant post-operative complications [205-208]. Consequently, there is a need for more novel treatments to manage obesity and alleviate its associated comorbidities. Additionally, for effective therapy, a better understanding of adipose biology and adipogenesis is crucial.

Adipose tissue is an important metabolic organ that assimilates a wide array of homeostatic processes and is an important contributor to whole-body glucose homeostasis and energy metabolism [209]. White adipose and brown adipose are the two major types of adipose tissues in mammals. White adipose tissue (WAT) is the primary site for triglyceride (TG) storage and fatty acid release, whereas brown adipose tissue (BAT) generates heat via the uncoupling of electron transfer from ATP synthesis [210]. In the last decade, development and regulation of BAT has gained traction due to its potential role in the protection against obesity and its associated disorders [211, 212]. This widespread interest in using BAT as a novel therapeutic strategy for the treatment of obesity and its complications was further emphasized by several studies demonstrating that adult humans have distinct brown fat deposits and BAT activity inversely correlates with overall adiposity [213-216]. Indeed, positron emission tomography (PET) and computed tomography (CT) imaging studies have identified functional BAT in in adult humans [214] despite prior assumptions of BAT's insignificance at this stage of life [217, 218]. However, BAT's therapeutic candidacy remains relatively elusive due to the surrounding confines of PET/CT methodology and further, the sheer displacement of volume between white to brown [219-221]. Consequently, the trans-differential properties of WAT have emerged as an additional therapeutic strategy to mitigate the metabolic dysfunction and stress of human obesity [222]. The "browning" process of WAT leads to the accumulation of brown fat-like adipocytes usually referred to as brite or beige. This sub-group of adipose encompass the morphological and functional dynamics of classical BAT yet is interspersed within WAT [223]. Notably, the white-to-brown transdifferentiating mechanism has been demonstrated to protect knockout mice against obesity induced by high fat diets (HFDs) [224, 225]. However, this process of interconversion remains incompletely understood and

moreover, additional studies are warranted to fully comprehend the underlying mechanisms of beige/brite development including key molecular players that regulate browning and brown adipogenesis.

Several transcription factors and transcriptional co-activators have been identified as critical regulators of browning and classical thermogenesis [226-228]. This includes the pyruvate kinase M2 (PKM2), a rate-limiting glycolytic enzyme that catalyzes the final step of glycolysis by converting the high energy molecule phosphoenolpyruvate (PEP) to pyruvate. Mammals express four different isoforms of which, PKM2 and PKM1 are derived from the PKM gene while the L and R isoforms are established by the PKLR gene. Although PKM1 and -2 are splicing isoforms, PKM2 is more characteristically expressed during cell proliferation and embryonic development as opposed to PKM1's predominance in more differentiation tissue [229]. PKM2 has been thoroughly investigated within the paradigms of cancer such as facilitation of the advantageous Warburg effect [229, 230] however, its metabolic role and functioning in adipose tissue, adipogenesis, and its contribution to obesity remain largely uncharted.

Recent studies have demonstrated that inhibition of PKM2 using shikonin, a natural herbal compound extracted from Lithospermun erythrorhizon (LE) Sieb. Et Zucc, negatively regulates adipocyte differentiation [231, 232]. Conversely, Kimura and colleagues opposingly report PKM2 overexpression suppresses the differential capacity of pre-adipocytes while inhibition enhances adipocyte differentiation markers including C/EBPB, FABP4, and PPARy, concomitant with increased lipid accumulation [233]. Moreover, it has been further proposed that PKM2 represses thermogenic potential due the promotion of thermogenic gene expression, including fibroblast growth factor 21 (Fgf21) and uncoupling protein 1 (Ucp1), demonstrated by PKM2 depletion in

brown adipocytes [234]. Therefore, due to controversy among the collective evidence, additional research is necessary to elucidate the exact role of PKM2 on white and brown adipogenesis and further, the potential therapeutic potential of targeting PKM2 for the treatment and prevention of obesity. Moreover, data on the nutritional regulation of PKM2 is lacking. Thus, in the present study, we investigated the effects of diet-induced obesity on PKM2 expression and the effects of modulating PKM2's activity on adipocyte differentiation. Our study revealed that PKM2 phosphorylation at Try105 ($PKM2^{Tyr105}$) and therefore, its enzymatic activity, is nutritionally regulated. We also found that high fat feeding decreased $PKM2^{Tyr105}$ phosphorylation and negatively correlated with metabolic status across various study models including rodents, primates, and humans [235]. In the present study, we also uncover a novel role for PKM2 in adipose function and demonstrate that PKM2 deficiency activates a brown fat-like program in white adipocytes.

Material and Methods

Reagents. Reagents purchased from Invitrogen (Carlsbad, CA) include trypsin, penicillin/streptomycin, puromycin, fetal bovine serum (FBS), G418, and Dulbecco's Modified Eagle Medium (DMEM). B-Actin antibodies were purchased from Upstate Biotechnology (Lake Placi, NY). Antibodies for PKM1, and PKM2 were purchased from Cell Signaling Technology (Beverly, MA). Additionally, antibodies for Ucp1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from BioResources International (Carlsbad, CA). Furthermore, unless otherwise specified, additional chemicals were purchased from Millipore-Sigma (St. Louis, MO).

Cell Culture and Immunofluorescence. Isolation of white and brown adipocytes as well as their precursor cells were sourced from the corresponding white inguinal and brown fat pads of newborn wild type (WT) mice via collagenase digestion, as previously described [235]. Human telomerase reverse transcriptase (TERT) was used to immortalize cells (GeneCopoeia, Inc.; Rockville, MD). Cells were maintained in a 50 μg/ml streptomycin, 10% FBS, 25 mM glucose, and 50 U/ml penicillin DMEM cocktail. Three different hairpins from (GeneCopoeia), were used to achieve PKM2 silencing in pre-adipocytes. Lentivirus packaging system (GeneCopoeia) was used to generate Lentiviruses carrying shRNA in HEK293FT cells (GeneCopoeia), following the manufacturer's instructions. The generated Lentivirus particles were then used to transfect pre-adipocytes and cells were selected with 200 μg/ml of hygromycin. To eliminate any off-target results, PKM2 knockdown cells (M2KD) were reconstituted with lentivirus carrying the human wild-type PKM2 and PKM2 rescued cells (M2R) were selected using 400 μg/ml of Geneticin (G418) for 14 days. To differentiate cells into fully mature adipocytes, pre-adipocytes were cultured and grown to confluency in complete growth medium containing 10% FBS. then switched to differentiation media (1 nM triiodothyronine [T3], 10% FBS, and 20 nM insulin) in order to induce cell differentiation. After 48 hrs, differentiation media was replaced with freshly prepared induction media (0.125 mM indomethacin, 5 μM dexamethasone, and 0.5 mM isobutyl methylxanthine). 48 hrs post-induction, cells were switched back to differentiation media which was replaced with a freshly prepared once every 48 hrs until cells (≥85%) exhibited a fully differentiated phenotype characterized by the formation of a large number of lipid droplets.

Oil Red O Staining. Cells were fixed at room temperature (RT). for 1 hr in a 10% buffered formalin solution. Next, plates were rinsed twice with PBS, followed by a third wash with 60%

isopropanol, and air dried for at least 1 hr at room temperature. Cells were then stained with a filtered oil red O solution (5 g/L in isopropyl alcohol) for approximately 1 hr, rinsed with distilled water, and imaged using Leica DMI8 inverted microscope (Leica Microsystems Inc. Buffalo Grove, IL). Oil red O staining was extracted using 100% isopropanol and absorbance (indicative of lipid accumulation) was determined using the Synergy™ HTX Multi-Mode microplate reader (BioTek Instruments, Inc. Winooski, VT) at a wavelength of 500 nm.

Fluorescence Microscopy. Coverslip-grown cells were fixed using 100% ice-cold methanol (10 mins) then blocked for 1 hr in a 3% BSA-PBS solution. Next primary antibodies were applied, and cells were incubated for 12 hrs at 4°C, followed by a second incubation with a fluorescent-conjugated secondary antibody for 1 hr at RT. Cells were then mounted using Mowiol (Sigma) and assessed using Olympus FluoView Laser Scanning Confocal Microscopy. Likewise, tissue from subcutaneous white and brown adipose fat pads were fixed using 10% formalin, embedded in paraffin then co-stained with DAPI (nuclear) and PKM2 (green fluorescence). Slide images were taken using the Lecia DMI3000B inverted microscope.

Biochemical Analyses. As previously described [236], radio-immunoprecipitation assay buffer (RIPA: 1 mM sodium orthovanadate and protease inhibitors, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM NaF, and 0.1% sodium dodecyl sulfate [SDS]) was used to homogenize cells/tissues. Lysate purification was achieved via centrifugation at 13,000 rpm (10 mins.) and proteins were quantified using the bicinchoninic acid assay kit (Pierce Chemical). 5-10 μg of proteins were resolved in electrophoresis then eluted onto membranes (polyvinylidene fluoride; PVDF). Transferred membranes were blocked for 45-60 mins with TBST (TBST: 0.1% tween and 5% BSA, pH: 7.4,

at RT) prior to the application of specified primary antibodies (at RT) (Table 1). After 1 hr of primary antibody incubation, membranes were washed in TBST (3X/5 mins) and corresponding secondary antibodies were applied (for 1 hr at RT) then washed with TBST (4X/15 mins) before signal development. Protein signals were visualized using Luminata™ Western Chemiluminescent HRP Substrate (Millipore Corp., Billerica, MA, USA). Fluorchem Q Imaging software (Alpha Innotech Corp., San Jose, CA, USA) was used to quantify total expression and posttranslational modifications of proteins.

Subcellular fractionation. Subcellular fractionation was performed as previously described [237], with modification. Briefly, 10X 10^6 cells were washed with 500 ul buffer 1 (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4) then resuspend in 500 ul buffer 2 (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, 5% Percoll, 0.01% digitonin, 2 mM sodium orthovanadate, 10 mM aprotinin, 10 mM pepstatin A, 10 mM leupeptin, 25 mM calpain inhibitor I and 1 mM PMSF, pH 7.4). lysates were further passed a 30-gauge syringe (10X) then incubated for 15 mins in ice and centrifuged for 10 mins at 500 rpm to pellet intact cells. Then nuclei were pelleted by centrifuging the supernatant for 10 minutes at 3,500 rpm at 4°C. In order to pellet mitochondria, the resultant supernatant was further centrifuged for 15 minutes at 15000 rpm and washed twice with buffer 2 then centrifuged an additional time for 1 h at 100,000 rpm. The resulting supernatant was designated as the cytosolic fraction while the pellet was designated as the microsomal fraction and was resuspended in 100 µl of buffer 3 (10 mM Tris-HCl, pH 7.5, 2.5 mM KCl, 2.5 mM MgCl2, 2 mM sodium orthovanadate, 10 mM aprotinin, 10 mM pepstatin A, 10 mM leupeptin, 25 mM calpain inhibitor I and 1 mM PMSF). The purity of subcellular fractions was verified using

GAPDH, Histone B, cytochrome C, and PTP1B for the cytosolic, nuclear, mitochondrial, and microsomal fractions, respectively.

Animal Studies. Age matched C57Bl/6J male mice (Jackson Laboratories, CA) were maintained on a 12-hour light-dark cycle and fed ad libitum. Mice were placed on standard lab chow (Purina lab chow, # 5001) then switched to HFD (60% kcal from fat, # D12492, Research Diets). At the indicated age, mice were sacrificed and different adipose depots were collected and flash frozen in liquid nitrogen then stored at -80ºC. For cold exposure experiments, a group of C57Bl/6J mice was acclimated to cold temperature 4°C for 6 hours/day for 5 consecutive days prior to the terminal exposure (4°C for 24hrs). Core body temperature was measured 2 hours prior to euthanasia. All rodent studies were approved by the Institutional Animal Care and Use Committee at University of Tennessee, Knoxville.

Results

Nutritional regulation of adipose PKM2: To gain insight into the molecular mechanisms underlying PKM2 action in the adipose tissue, we first verified its expression in both WAT and BAT depots of mice that were provided a regular chow diet. PKM2 expression, while not found in muscle nor liver, was exhibited in various WAT depots including retroperitoneal (Ret), subcutaneous inguinal (SQ), visceral omental (Vis), and epididymal (Epi) as well as in intrascapular BAT (**Fig. 1A**). Conversely, expression of PKM1, PKM2's splicing isoform, was also found in WAT and BAT depot in addition to muscle. Notably, the expression of PKM2 was comparable to whole lysate and purified fat derived from SQ adipose digested by collagenase thereby suggesting that adipocytes accommodate significant levels of expression (**Fig. 1B**). It is well established that phosphorylation of PKM2 on tyrosine 105 inhibits PKM2 activity [238].

However, whether PKM2 activity is nutritionally regulated remains elusive. Thus, in the current study, we investigated whether PKM2 Tyr105 phosphorylation directly correlates adiposity and its associated metabolic alterations. Specifically, we examined whether high fat diet (HFD)-induced obesity alters PKM2 expression and phosphorylation on tyrosine 105 in white and brown subcutaneous fat of mice at various stages of high fat feeding. Western blotting analysis revealed a decrease in PKM2 Tyr105 phosphorylation in both white and brown adipose tissue of mice fed an HFD for 6 and 22 weeks (**Fig. 1C**). Interestingly, while the expression levels of total PKM2 and PKM1 remained unchanged in both Bat and WAT, changes in PKM2 Tyr105 phosphorylation were concomitant with altered insulin sensitivity of obese mice as judged by the decrease in the phosphorylation, and subsequent activation of the insulin receptor (pIR Tyr1162/Tyr1163) and its downstream target; AKT (AKT Ser473) compared with controls (**Fig. 1C**). Similar observation was obtained in tissue lysates from the subcutaneous brown adipose tissue of obese mice (**Fig. 1D**). Next, to determine the clinical relevance of our findings in mice and whether our results translate to humans, we examined changes in PKM2 Tyr105 phosphorylation in the perirenal fat from female (denoted in red) and male (denoted in black) subjects with various BMI. Notably, while PKM2 and PKM1 total protein expression levels remained unchanged, PKM2 Tyr105 phosphorylation was significantly less in subjects with higher BMI (≥30) compared to those with lower BMI (<30) (**Fig. 1E**). Collectively, our findings demonstrate that adipose PKM2 Tyr105 phosphorylation directly correlates with the metabolic state in rodent and humans. However, it remains unclear whether alterations in Tyr105 phosphorylation are causative or consequences of the metabolic state and further studies are warranted to investigate the clinical significance of

preventing changes in PKM2 Tyr105 phosphorylation for the treatment of obesity and its associated metabolic disorders.

Differential regulation of adipose PK isoforms in white and brown adipocytes: To begin to address the role of PKM2 in adipogenesis, we first examined changes in PKM1 and PKM2 expression during the differentiation of white and brown primary pre-adipocytes. In **Fig. 2**, we demonstrate that pre-adipocytes and differentiating brown and white adipocytes express PKM2 (**Fig. 2A-D**). Immunofluorescence also demonstrated the expression of PKM2 in pre-adipocytes, BAT, and WAT thus supporting previous observations (**Fig. 2A**). Interestingly, while PKM2 in WAT seem to exhibit both, a cytosolic and a nuclear localization, most PKM2 in BAT was restricted to the cytosol (**Fig. 2A**). Although, these findings suggest that PKM2 in BAT exhibit a glycolytic function consistent with the higher glycolytic rate of BAT compared to WAT, additional studies are warranted to determine the significance of this differential subcellular localization of PKM2 in BAT Vs. WAT. On the other hand, the nuclear presence of PKM2 in WAT suggest a potential role in regulating gene expression consistent with findings from a large number of cancer studies [229, 239]. To further confirm these findings, we performed fractionation studies in white and brown adipocytes at early and late stage of differentiation. As shown in **Fig. 2B**, PKM2 nuclear expression was significantly higher in white versus brown pre-adipocytes. On the other hand, PKM1 localization was restricted to the cytosolic fraction only, confirming that adipose PKM2 exhibits other functions in addition to its glycolytic role. Furthermore, adipocytes, PKM2 levels, but not PKM1 were significantly increased in both white and brown primary adipocytes (**Fig. 2C-D**). Taken together, these findings advocate for differential role of PK isoforms in white and brown adipocyte homeostasis and function.

PKM2 knockdown in white adipocytes leads to brown-adipocyte like phenotype: Next, to examine the effects of PKM2 on adipocyte function, a stable knockdown of PKM2 (M2KD) within 3T3 established pre-adipocyte and primary white pre-adipocytes was generated using lentiviral shRNA. On the other hand, a scramble shRNA (SCR) was used to generate control cells. The reconstitution of knockdown cells with a shRNA-resistant wild type human PKM2 (M2R) was necessary to help rule out any potential off-target effect. To ensure that our findings are not specific to a particular cell system, primary white pre-adipocytes were isolated from the inguinal fat pads of C57Bl/6J male mice and cultured as detailed in the Material and Methods section. Upon immortalization using human telomerase reverse transcriptase (hTERT), cells were then subjected to the same genetic manipulations as the established 3T3 white pre-adipocytes. As shown in **Fig. 3A**, PKM2 expression, as assessed by immunoblot analysis, was demonstrated to be significantly reduced in the 3T3-L1 white knockdown cells, while expression of PKM2 was found to be similar to endogenous levels in reconstituted cells (M2R; **Fig. 3A**). Likewise, we were successful in generating primary white pre-adipocytes with a significant reduction in PKM2 expression. Importantly, knockdown of PKM2 expression did not alter the expression of the splicing isoform (PKM1), as evidenced by an immunoblot analysis using a specific anti-PKM1 antibody. Moreover, immunoblot analysis utilizing an antibody that proportionately recognizes both splicing isoforms (M1 and M2) revealed a significant reduction in total PKM (**Fig. 3A**), thus suggesting that PKM2 is more predominate isoform in these cells.

Adipocyte differentiation is an intricate mechanism that involves a wide variety of hormones and nutrient stimuli [240] In order to evaluate the role of PKM2 in adipocytes, oil red O was used to stain and monitor lipid accumulation among differentiated control, knockdown and

reconstituted adipocytes (**Fig. 3B**). Control cells (SCR) exhibited a fully differentiated phenotype with more than 90% of cells containing distinguished lipid droplets. Interestingly, PKM2 knockdown 3T3-L1 and primary pre-adipocytes were found to accumulate lipid droplets comparable to those observed in classical brown adipocytes (**Fig. 3B-D**) while reconstituted PKM2 (M2R) white pre-adipocytes exhibited lipid accumulation similar to controls (SCR). Moreover, oil red O-staining in white adipocytes revealed that cells with PKM2 knockdown exhibited higher lipid accumulation compared with controls (SCR; **Fig. 3B-D**), while PKM2 rescued cells exhibited a significant reduction in oil red O staining and lipid accumulation (**Fig. 3B-D**).

Next, in order to decipher whether or not PKM2 deficiency contributes to the "browning" process of WAT, we assessed expression of the classical BAT marker, UCP1 [241]. As a positive control, we used brown adipose tissue from mice exposed to cold temperature (4°C for 24 hrs), a condition previously shown to increase UCP1 levels and brown fat activity [242, 243]. Not surprisingly, the expression of UCP1 was found in BAT of wild type mice and was significantly increased in response to cold exposure (**Fig. 3E**). Differentiated brown adipocytes and notably, differentiated PKM2 knockdown 3T3 and primary adipocytes were also found to express UCP1 (**Fig. 3E**). Importantly, the reconstitution of PKM2 in 3T3-L1 knockdown cells abrogated the induction of the thermogenic marker (UCP1) prompted by PKM2 depletion (**Fig. 3E**).

β-catenin mediates PKM2's suppressive role on the browning of white adipose tissue. A growing body of evidence suggest that β-catenin mediates many PKM2 functions under a plethora of physiological and non- physiological conditions [244, 245]. β-catenin, a core component of the Wnt canonical signaling pathway, has been also identified as a vital player in adipogenesis for both white and brown tissue. Of note, β-catenin is a direct downstream target of

PKM2 and also acts as a direct interacting partner. For instance, epidermal growth factor (EGF)-induced β-catenin transactivation in human glioblastoma cells requires interactions between K433 of PKM2 and phosphorylated Y333 (pY333) of β-catenin and involves PKM2 protein kinase activity at the promoter of target genes [246, 247]. Additionally, PKM2 can also promote the expression and stabilization of the β-catenin oncogene target gene c-Myc [248], which was identified as a critical regulator of early stages of white and brown adipogenesis [249, 250]. Hence, we examined whether PKM2 deficiency alters β-catenin expression and function during white adipocytes differentiation. Consistent with previous studies [87, 100], the expression of β-catenin was significantly reduced during the differentiation process of white adipocytes. Brown adipocytes exhibited lower levels of β-catenin compared with white adipocytes both, basally and upon differentiation. Of note, these effects were concomitant with a reduction in β-catenin tyrosine 333 phosphorylation (PKM2 interacting site). On the other hand, PKM2 deficiency in white and brown adipocytes resulted in significant reduction of β-catenin, both basally prior to and also during the process of adipogenic differentiation (**Fig. 4A**). Consistent with these findings, the expression levels of two β-catenin direct downstream target; c-Myc and the architectural transcription factor high mobility group AT-hook protein 2 (HMGA2), mirrored the expression pattern of β-catenin in both white and brown adipocytes. Together, these findings suggest a correlation between PKM2 and β-catenin activity during the process of adipogenesis. To confirm this observation, we examined the expression levels of β-catenin and its phosphorylation at tyrosine 333 and Serine 33 (a site that promotes β-catenin ubiquitination and proteasomal degradation) in the perirenal fat of male and female subjects with various BMI. As shown in **Fig. 4B**, we observed a significant stabilization of β-catenin protein levels in subjects with higher BMI (≥30) compared to those with

lower BMI (<30) as judged by the reduction in β-catenin Ser 33 phosphorylation and the increase in Tyr 333 phosphorylation. In line with the increased β-catenin levels, both c-Myc and HMGA2 protein levels were significantly higher is subject with obesity compared to lean subjects (**Fig. 4B**). Collectively, these findings support the correlation between PKM2 and β-catenin activity during the process of adipogenesis. To further validate this hypothesis, we expressed a constitutively active mutant of β-catenin (S33A, S37A, T41A), which resists GSK3β phosphorylation and subsequent degradation [251], in PKM2 knockdown or reconstituted white and brown pre-adipocytes and examined changes in adipocyte differentiation as well as the browning of white adipocytes. As demonstrated in **Fig. 4C**, the expression of the constitutively active form of β-catenin (CA) resulted in higher levels of c-Myc and HMGA2 compared to cells transfected with the empty vector (V). This effect was concomitant with the abolishment of adipocytes differentiation of both white and brown precursor cells. Furthermore, CA- β-catenin abolished the effects of PKM2 deficiency on the browning of white adipocytes as judged by the reduced UCP1 expression (**Fig. 4C**). In sum, our findings demonstrate that PKM2 deficiency promotes adipogenic and thermogenic gene expression by reducing the expression and transcriptional activity of β-catenin, and that β-catenin mediates PKM2's repressive role in adipogenesis and thermogenesis.

PKM2/β-catenin axis suppresses adipogenesis and the browning of white adipocytes through HMGA2. Although the role of β-catenin as a suppressor of adipogenesis is well established, the molecular mechanisms are yet to be determined. Emerging evidence suggest that β-catenin's downstream target HMGA2 is a key player in the regulation of early stages of adipocytes differentiation [252-255]. Together with CCAAT-enhancer binding protein β (C/EBP β) family proteins, HMGA2 orchestrates the clonal expansion step through modulating the

expression of transcriptional factor PPARγ [254], the master regulator of adipogenic differentiation [256]. Therefore, in order to determine whether HMGA2 mediates the effects of PKM2/β-catenin action on adipogenesis and the browning of white adipocytes, we used the shRNA-mediated lentivirus to knockdown HMGA2 in both M2R and M2KD pre-adipocytes. We also, overexpressed the human wild type HMGA2 in both M2R and M2KD and examined the subsequent effects of HMGA2 knockdown (KD) and over expression (OV) on the differentiation and the browning of white adipocytes. As shown in **Fig. 5**, HMGA2 was effectively overexpressed and silenced as intended (**Fig. 5A**). Neither HMGA2 knockdown nor its overexpression abolished the effects of PKM2 deficiency on β-catenin levels, confirming that HMGA2 acts downstream of both, PKM2 and β-catenin. Notably, HMGA2 KD cells exhibited a brown-like phenotype similar to M2KD cells which was further enhanced in cells that are deficient in both HMGA2 and PKM2 (**Fig. 5B**) as judged by the size of lipid droplets and UCP1 expression (**Fig. 5B-C**). On the other hand, HMGA2 over expression not only abolished the differentiation of white pre-adipocytes, but also reversed the browning effects of PKM2 deficiency (**Fig. 5B-C**), suggesting that HMGA2 mediates the suppressive role of the PKM2/β-catenin axis on the browning of white adipocytes. In conclusion, while additional research is needed, our findings suggests that targeting the PKM2/β-catenin/HMGA2 axis could be a promising strategy to promote the browning of white adipose tissue, enhance energy expenditure while preventing obesity and its associated metabolic alterations.

Discussion

White adipose tissue (WAT) is a major organ that critically functions beyond its role as an energy reservoir [257]. However, WAT, in excess, has the potential to threaten human health

through obesity and its associated comorbidities [258]. Although, multiple approaches have been designed to prevent and/or alleviate the concomitant pathophysiology of obesity, most of these treatments are also challenged by various discrepancies including low adherence, inefficacy and safety concern [199-203] thus, warranting for novel therapeutic alternatives. In addition to WAT, brown adipose tissue (BAT) can also be found in mammals and emerging evidence support its therapeutic potential to counter the obesity crisis [213, 214, 221]. Although BAT is a tempting remedial angle, WAT undoubtedly remains more dominate in quantity [259]. Furthermore, a third sub-category of adipose tissue, namely beige/brite, has been demonstrated to arise from the residency of WAT under the subjection of various stimuli [260]. These conditional brown-like adipocytes, although residing in WAT depots, histologically mirror classical BAT (cBAT) as well as express functional UCP1 thus making them an attractive therapeutic target [261]. Although the implications of beige/brite adipocyte synthesis positively correlate with metabolic status including averting the consequences of high fat diet (HFD)-induced obesity in experimental models of human obesity [224, 225, 262], a substantial amount of research is still necessary to delineate the exact molecular mechanisms behind the "browning" process. Moreover, although targeting activation of cBAT or browning of WAT via pharmacology exist, both lack of efficacy [263] and safety concerns [264] constrain human translatability and manipulation.

PPARy, PGC1a, and Prdm16 have all been identified as critical modulators for the development and maintenance of classical brown adipogenesis and WAT browning [226-228]. Notably, recent studies have identified pyruvate kinase M2 (PKM2) as a novel modulator of adipogenesis and brown fat thermogenic potential in particular. While, the role of this catabolic

enzyme has been comprehensively investigated within the margins of cancer [229, 230], it's contribution to obesity and metabolic dysfunction, has yet to be fully elucidated.

As previously mentioned, WAT is a versatile and *necessary* organ to systemic homeostasis; however, in excess, WAT can contribute as major source of obesity-related inflammation and consequently, metabolic dysfunction [265]. Our current and previous findings identified PKM2 as a potential IR contributor in adipose tissue as increased phosphorylation of PKM2 at tyrosine site 105 ($PKM2^{Y105}$) was associated with a favorable metabolic profile across three independent study models [235]. PKM2 is also evolving as an attractive therapeutic target for obesity. Accordingly, a more recent report marked PKM2 as a potential modulator of brown fat adipogenesis. This study demonstrated PKM2 was more significantly expressed in brown than white adipose and further, repressed the thermogenic program of brown adipocytes. Genetic depletion of PKM2 resulted in the upregulation of both UCP1 and fibroblast growth factor 21 (Fgf21), of which, are known thermogenic makers and gene regulators, respectively [234]. In line with these findings, our study demonstrates that PKM2 exhibit differential regulation and localization patterns in white versus brown adipocytes. Notably, PKM2 deficiency in white adipocytes leads to a brown adipose-like phenotype. These effects were concomitant with decreased total pyruvate kinase activity and increased expression of brown fat markers in WAT. These findings demonstrate that PKM2 not only regulate brown fat adipogenesis and function [234], but also the browning of WAT.

At the molecular levels, we demonstrate that the suppressive role of PKM2 on browning of WAT are mediated, at least in part through β-catenin and its downstream target HMGA2. β-catenin is a core component of Wnt canonical signaling pathway and regulates the transcription of several genes involved in adipogenesis [23]. Activation of β-catenin by most wnt isoforms was

shown to stabilize β-catenin and inhibit adipogenesis [266, 267]. Unfortunately, PKM2's effect on WAT browning is not clearly understood nor sufficiently characterized; however, the Wnt signaling cascade is a key and evolutionarily conserved signaling pathway that governs many essential developmental and homeostatic processes [41]. Moreover, various Wnt proteins and aberrant Wnt/β-catenin signaling has been associated with chronic metabolic diseases [15] and thereby targeting the Wnt/β-catenin system may hold therapeutic potential against obesity and its associated comorbidities. WNT10b has been identified as a potent inhibitor of white and brown adipogenesis that is highly expressed in pre-adipocytes and promptly repressed upon differentiation [68, 116]. FABP4-WNT10b transgenic mice were shown to have an approximate 50% reduction of total body fat and additionally, protected against high fat diet-induced adipose accumulation [116]. Furthermore, another study utilizing FABP4-WNT10b transgenic mice on either an ob/ob or lethal yellow agouti (A(y)) background, revealed that Wnt10b mediated activation of β-catenin positively protected against weight gain, reduced subcutaneous and visceral adipose by 70%, and averted metabolic abnormalities including glucose intolerance and insulin insensitivity [117]. Among an early-onset obese population, Christodoulides and colleagues detected two non-synonymous Wnt10b variants, C256Y and P301S. The authors further investigated the mutants' effect on Wnt/β-catenin activation and subsequently, adipocyte differentiation. WNT10b P301S was found to increase the stabilization of β-catenin and TCF promoter activity while WNT10b C256Y opposingly had contrasting effects that rendered WNT10b signaling and promoted pre-adipocyte differentiation and lipid accumulation [118]. Opposingly, a later study demonstrated that WNT10b variations, in fact, do not contribute to monogenic obesity in humans. The non-synonymous variant identified in obese patients, R228Q,

failed to co-segregate in obesity among the three investigated biological siblings [268]. Although conflicting, these studies suggest that additional research is necessary to uncover the molecular and genetic ties between Wnt/β-catenin signaling and obesity.

Recent investigations have implicated the role of β-catenin in adipogenesis; however, the molecular mechanisms as well as upstream modulators have yet to be determined. The Wnt signaling complex is traditionally categorized into either the canonical (β-catenin dependent) and non-canonical (β-catenin independent) cascades, of which, the latter half is further sub-categorized into the planar cell polarity (PCP) and Wnt/Ca^{2+} pathways [41]. Activation of canonical Wnt signaling is dependent upon the binding of translated Wnt ligands. Binding of these secreted glycoproteins to the Frizzled (Fz) receptor's cysteine-rich domain causes the downstream stabilization and nuclear translocation of β-catenin where it can further mediate the regulation of Wnt target genes [269, 270]. Wnt-1 and Wnt10b are recognized as potent inhibitors of adipogenesis. Studies by Ross and colleagues demonstrated that overexpression with Wnt-1 or synthetic downstream canonical activation, prompted the failure of 3T3-L1 pre-adipocyte differentiation, as evidenced by the lack of adipocyte marker 422/aP2 and lipid accumulation [65]. Wnt-1 was also found to block adipogenesis in vivo. In contrast to the pLXSN-infected control cells, cells infected with Wnt-1 were demonstrated to form an undifferentiated and more fibroblastic-like fat pad [65]. It has been suggested that Wnt-1 and Wnt-10b block adipogenesis via accumulation and stabilization of β-catenin and further, the repression of key adipogenic regulators including PPARγ and CEBPα [65-67]. Notably, it was also insinuated that PPARγ activity and β-catenin expression may function in a reciprocal dynamic. Pre-adipocytes with ectopic expression of Wnt-1 impaired the induction of PPARγ gene expression while ectopic

PPARγ expression suppressed levels of β-catenin and rescued the adipogenic potential of Wnt-1-expressing cells [71]. Furthermore, Liu et al. proposed that glycogen synthase kinase (GSK-3β) may serve as key regulator that balances β-catenin and PPARγ activity. The constitutively active β-catenin (S37A), a stable mutant that averts GSK-3β phosphorylation, was demonstrated to resist PPARγ-programmed degradation and block the expression of traditionally induced adipogenic genes (perilipin, aP2, adiponectin) concomitant with decreased levels of C/EBPα [69]. Additionally, Wnt10b mRNA levels have been demonstrated to be inversely related to the induction of 3T3-L1 adipocyte differentiation with complete depletion 36-hours after induction [68]. More recently, Cen et al. demonstrated that TNF receptor-associated factor 4 (TRAF4) negatively regulates the adipogenic potential of mesenchymal stem cells (MSCs). Here it was demonstrated that TRAF4 mechanistically activates the kinase activity of PKM2 via direct binding and subsequent β-catenin signaling renders MSC adipogenesis. However, another report opposingly found that bone marrow mesenchymal stem cells (BMSCs) treated with a PKM2 activator (DASA-58) resulted in reduced levels of active β-catenin concomitant with increased expression of adipogenic gene markers (PPARγ, FABP4, Adipsin) while PKM2 inhibition via C3k treatment produced opposite effects [271]. In addition to white adipogenesis, canonical Wnt signaling has also been shown to negatively regulate brown fat differentiation [266]. Indeed, Wnt10b overexpression was demonstrated to similarly inhibit brown adipogenesis in two independent in vitro cell models including Rb−/− MEFs and HIB-1B. Comparatively, the over expression of Wn10b or a dominant-stable form of β-catenin (β-cat^{S33Y}) blunted the induction of adipogenic master regulators and reduced lipid droplet accumulation [101]. Cantwell and colleagues suggest that brown fat differentiation, in addition to the thermogenic program, is

mediated by several components along the JAK-STAT axis. As such, conventional markers of adipogenesis including PPARγ and CEBPα, together with brown fat-specific markers such as PRDM16, CIDEA, UCP1, were found to be downregulated in STAT3$^{-/-}$ (KO) primary brown pre-adipocytes. However, restoration of the functional thermogenic protein, UCP1 and differential rescue of STAT3$^{-/-}$ brown preadipocytes were respective responses of canonical Wnt signaling inhibition and β-catenin knockdown [102]. In another study, myostatin was shown to negatively regulate brown adipocyte differentiation through a Smad3-mediated stabilization of β-catenin of which, subsequently decreased brown fat-specific markers including PGC-1α, PRDM16, and UCP1 [103]. Regulation of brown adipogenesis by epigenetic modulation was highlighted by a more recent study that investigated the histone demethylase, Lysine-Specific Demethylase 1 (LSD1). LSD1 usually serves to downregulate the transcription of several Wnt pathway genes via H3K4 demethylation thereby repressing Wnt signaling and promoting brown adipogenesis. Chen et al. demonstrated that LSD1 depletion or inhibition obstructs brown adipocyte differentiation while LSD1 knockout mice exhibited brown fat defects. Importantly, LGK974, an inhibitor of canonical signaling, was demonstrated to rescue defective brown adipocyte differentiation that is associated with LSD1 deficiency, thus indicating that LSD1 functioning in brown adipose is accomplished, to a large degree, via canonical Wnt repression [107].

While Wnt signaling is widely accepted as a general regulator of adipogenesis, it's specific role in the “browning” of white adipose (WAT) has yet to fully elucidated. However, Lo and colleagues demonstrated that inhibitory targeting of Wnt signaling resulted in an upregulation of thermogenic genes within primary inguinal mouse adipocytes. Furthermore, primary white adipocytes collected from the epididymal fat pad were found, in contrast to the inguinal depot,

resistant to Wnt inhibition browning. These observations suggest that Wnt inhibition browning of WAT is more relevant to precursor cells (e.g., inguinal SVF) rather than previously established or committed white adipocytes [109]. In a more recent report, varying concentrations (0–100 μM) of Ginsenoside Rb1 (G-Rb1) were found to blunt the inhibitory effects of Wnt signaling and promote browning within 3T3-L1 pre-adipocytes as inversely illustrated by the gradual reduction of oil Red O stain to increasing G-Rb1 concentrations concomitant with decreased β-catenin levels. Moreover, C-3f and SKL2001, Wnt signaling activators, were used to confirm the role of Wnt/β-catenin signaling 3T3-L-1 browning. Both activators were shown to be partly efficient in eliminating the browning effect of G-Rb1 via canonical Wnt signaling activation [111]. A previous study suggested that G-Rb1's browning capacity was induced through the regulation of PPARγ. Mu and colleagues revealed that the activity of PPARγ was appreciably increased in response to G-Rb1 treatment in Cos-1 cells. Additionally, browning of mature 3T3-L1 adipocytes was evidenced by the upregulation of brown-specific genes including, PGC-1α, PRDM16, and brown functional protein UCP-1. Notably, G-Rb1 was correspondingly shown to improve glucose tolerance and increase energy expenditure of these cells [112]. In a following study, Park et al. also confirmed the modulation of 3T3-L1 PPARγ activity in response to G-Rb-1. These authors also investigated the effects of black ginseng (BG) and found that both G-Rb1 and BG were able to induce browning properties within 3T3-L1 and primary white adipocytes derived from C57BL/6 mice, however, mediated through the AMPK signaling pathway [113]. Furthermore, Sun et al. demonstrated that the browning program of white adipocytes could be blunted by fibroblast growth factor 9 (FGF9). In this study, FGF9 treatment and genetic overexpression was shown to inhibit beige adipogenic potential of SVF precursor cells whereas shRNA knockdown led to the

significant increase in mRNA levels of Ucp1, PRDM16, Pgc1α, and Cidea. Interestingly, several WNT-related genes were observed to be transiently repressed in response to FGF9 at Day 0 and thus, indicating a plausible 'early-onset' regulatory role for the Wnt signaling pathway in the browning process [114].

Importantly, it was established that the propensity of this browning phenomena is more significantly conspicuous upon differentiation perturbance at an earlier, rather than terminal, phase. In line with this observation, our study demonstrates that the suppression of browning through activation of the PKM2/β-catenin axis is mediated, at least in part, through HMGA2, a key player in the regulation of early stages of adipocytes differentiation [252-255]. Together with CCAAT-enhancer binding protein β (C/EBP β) family proteins, HMGA2 orchestrates the clonal expansion step through modulating the expression of transcriptional factor PPARγ [254]. Of note, recent studies have implicated HMGA2 in the adipogenesis process, as *Hmga2* null mice exhibited a significant reduction in body weight accompanied by reduced fat mass compared to their control wild type littermates. *Hmga2* null mice were also resistant to high fat-induced obesity both under normal or high fat feeding conditions [252, 253, 255]. While whether these affects are directly caused by browning of WAT remain to be determined. Nevertheless, previous studies have shown that HMGA2 is also a downstream target of the microRNAs miR-365 [272] and miR-328 [273], both of which promote brown adipogenesis [274].

In summary, our findings suggest that PKM2 deficiency in white adipocytes could constitute a strategy to enhance whole body energy expenditure and prevent excess fat accumulation. Additional studies are necessary to determine the precise mechanisms underlying the role of PKM2 in white adipocyte browning and how regulation of adipose function by PKM2

and its downstream effectors might be effectively utilized to fight obesity and its associated disorders.

List of Abbreviations:

AICAR: 5-aminoimidazole-4-carboxamide ribonucleoside

AMPK: AMP-activated protein kinase

APC: Adenomatous Polyposis Coli

Atp6: ATP synthase FO subunit 6

Axin-1: Axis inhibition protein-1

BAT: brown adipose tissue

BG: black ginseng

BIO: 6-bromoindirubin-3-oxime

BMSC: bone marrow stromal cells

C&E: convergence and extension

C/EBPα: CCAAT/enhancer-binding protein alpha

CAD: coronary artery disease

CAMKII: calcium calmodulin mediated kinase II

CBP: cyclic AMP response-element binding protein

Cby: Chibby

CDK: cyclin-dependent kinase

CK1α: casein kinase 1α

CKD: chronic kidney disease

CKIα: casein kinase I-alpha

CNA: Calcineurin

Cox I: cytochrome c oxidase subunit I

CRD: cysteine rich domain

CREB: cyclic AMP response element binding protein

CVD: cardiovascular disease

DAG: diacylglycerol

Dgo: Diego

DKK: Dickkopf

DKK1: Dickkopf1

dnTCF4: dominant-negative TCF4

Dvl/Dsh: Dishevelled

ER: endoplasmic reticulum

FABP4: fatty acid binding protein 4

FASN: fatty acid synthase

FGF9: fibroblast growth factor 9

FLD: fatty liver disease

Fmi: Flamingo

FNT: formononetin

Fz: Frizzled

GATA3: GATA-binding factor 3

GHOST: genes hidden outside the standard targets

GO: Graves' ophthalmopathy

G-Rb1: Ginsenoside Rb1

Gro: Groucho

GSK3: glycogen synthase kinase 3

GSK3: glycogen synthase kinase 3

GSK3β: glycogen synthase kinase 3β

hADSCs: human adipose-derived mesenchymal stem cells

HFD: high fat diet

HF-HS: high-fat/high-sucrose

Hyx: Hyrax

I3O: indirubin-3′-oxime

IFG-1: insulin like-growth factor-1

INO: indirubin-5-nitro-3'-oxime

IP3: inositol-1,4,5-triphosphate

IR: insulin resistance

IRS-1: insulin receptor substrate-1

JNK: Jun N-terminal kinase

KD: knockdown

KO: knockout

Lgr4m/m: Lgr4 homozygous mutant

LGR5: leucine-rich repeat-containing G-protein coupled receptor 5

lncRNA: Long noncoding RNA

LPA: lysophosphatidic acid

LRP: lipoprotein receptor-related protein

LSD1: Lysine-Specific Demethylase 1

MI: myocardial infarction

miRNA: microRNA

MSC: mesenchymal stem cell

Nadh; NADH dehydrogenase subunit

NAFLD: non-alcoholic fatty liver disease

NF-AT: nuclear factor of activated T-cells

NLK: Nemo-like kinase

ORO: oil red O

PCP: planar cell polarity

PDZ: Postsynaptic density 95, PSD-85; Discs large, Dlg; Zonula occludens-1domains

PGC1α: PPARγ co-activator 1-α

PIP2: phosphatidylinositol-4,5-bisphosphate

Pk: Prickle

PKC: protein kinase C

PKM2: Pyruvate kinase isozyme type M2

PLC: phospholipase C

Porc: Porcupine

PP1: protein phosphatase 1

PP2: protein phosphatase 2

PPARγ: Peroxisome proliferator- activated receptor gamma

RNF43: Ring finger protein 43

ROCK: Rho/Rho associated kinase

sFRP-1: secreted Frizzled-related protein-1

sFRP-2: secreted Frizzled-related protein-2

sFRPs: secreted frizzled-related proteins

SQ: subcutaneous depot

Stan: Starry night

Stbm: Strabismus

SVF: stromal vascular fraction

T2DM: type 2 diabetes

TCF/LEF: T-cell factor/lymphoid enhancer factor

TCF7L2: transcription factor 7-like 2

TG: triglyceride

TRAF4: TNF receptor-associated factor 4

TSC1: tuberous sclerosis complex 1

UCP1: uncoupling protein 1

Vangl: Van Gogh

VAT: visceral adipose tissue

VEGF-C: vascular endothelial growth factor-C

WAT: white adipose tissue

Wg: Wingless

WISP-1: WNT1 inducible signaling pathway protein 1

Wl: Wntless

WT: wild type

β-Trcp: β- transductin-repeat-containing protein

Declarations:

Ethics approval: All mice studies were conducted in accordance with federal regulations upon approval from the Institutional Animal Care and Use Committee at the University of Tennessee, Knoxville.

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APPENDIX

TABLES

Table 1: List of Antibodies Used in the Reported Experiments

Antibodies	Source	Catalog Number	MW (kDa)	Host	Dilution
AKT	Cell Signaling Technology	4685	60	Rabbit	1:1,000
c-Myc	Santa Cruz Biotechnology	sc 40	67	Mouse	1:1,000
Cyt C	Santa Cruz Biotechnology	sc 13156	14	mouse	1:1,000
FAS	Santa Cruz Biotechnology	sc 48357	270	Mouse	1:1,000
GAPDH	Santa Cruz Biotechnology	sc 25778	36	Rabbit	1:1,000
Histone 3	Santa Cruz Biotechnology	sc 10809	17	Rabbit	1:1,000
HMGA2	Santa Cruz Biotechnology	sc 13024	23	Mouse	1:1,000
IR	Santa Cruz Biotechnology	sc 711	93	Rabbit	1:1,000
Lamin A	Santa Cruz Biotechnology	sc 376248	68	Mouse	1:1,000
Perilpin	Santa Cruz Biotechnology	Sc 390169	60	Mouse	1:1,000
Phospho-AKT ^{S473}	Cell Signaling Technology	4058	60	Rabbit	1:1,000
Phospho-IR $^{Y1162/Y1163}$	Santa Cruz Biotechnology	sc-25103-R	93	Rabbit	1:1,000
Phospho-PKM2^{Y105}	Cell Signaling Technology	3827	61	Rabbit	1:1,000
Phospho-β-catenin ^{S33}	Santa Cruz Biotechnology	sc 57535	90	Mouse	1:1,000
Phospho-β-catenin ^{Y333}	AbCam	Ab119363	90	Rabbit	1:1,000

Table 1. Continued

Antibodies	Source	Catalog Number	MW (kDa)	Host	Dilution
PKM1	Millipore-Sigma	SAB4200094	61	Rabbit	1:1,000
PKM1/2	AbCam	Ab181693	61	Goat	1:1,000
PKM2	Cell Signaling Technology	4053	61	Rabbit	1:5,000
PTP1B	Santa Cruz Biotechnology	sc 1718-R	50	Rabbit	1:1,000
UCP1	Santa Cruz Biotechnology	sc 6529	33	Goat	1:1,000
β-actin	Santa Cruz Biotechnology	sc-47778	44	Mouse	1:20,000
β-catenin	Santa Cruz Biotechnology	sc 7963	90	Mouse	1:1,000

FIGURES

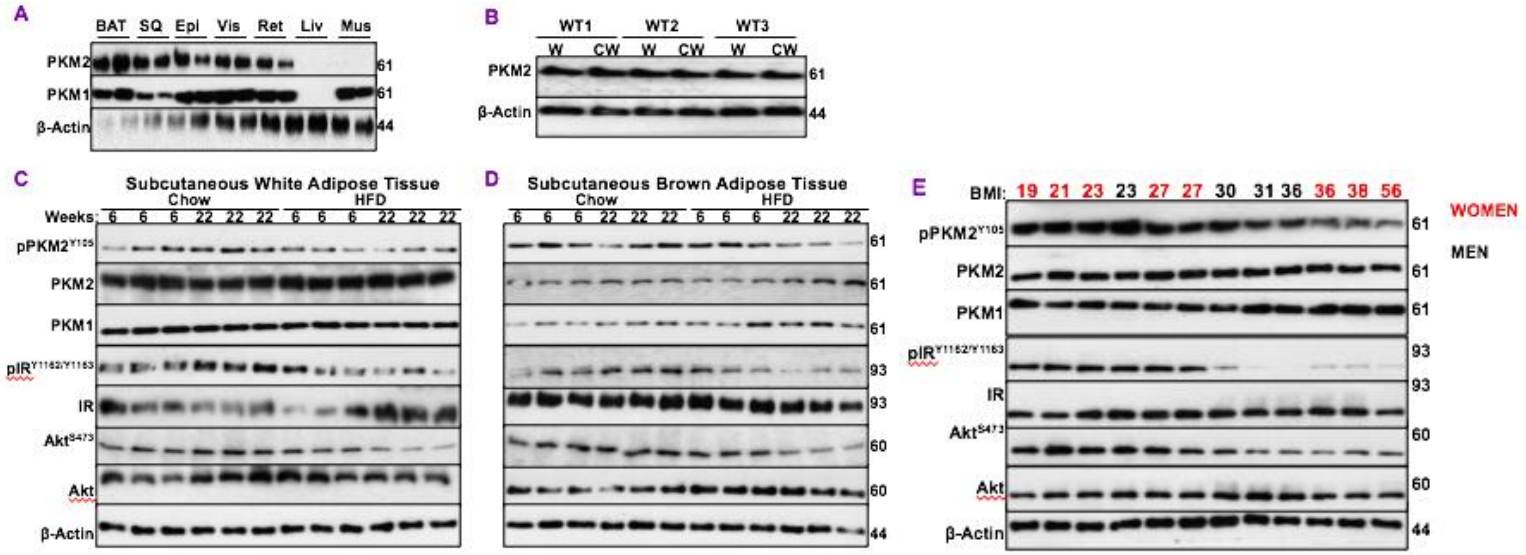


Figure 1: Nutritional Regulation of PKM2. A) Representative immunoblot of PKM1, PKM2, and β-Actin, serving as a loading control, in various tissues harvested from 6-week-old C57bl6/J mice housed at room temperature and fed ad libitum a regular chow diet. **B)** Immunoblots of PKM2 and β-Actin in lysates of subcutaneous adipose tissue (W) and collagenase-treated subcutaneous adipose tissue (CW) from three 6-week-old C57bl6/J mice housed at room temperature and fed ad libitum a regular chow diet. **C-D)** Representative immunoblot of PKM1, PKM2, $PKM2^{Y105}$, IR, $IR^{Y1162/Y1163}$, AKT, AKT^{S473}, and β-Actin in lysates of subcutaneous white and brown adipose tissue depots of mice fed chow or HFD for the specified times. Each lane represents a sample from a separate animal. **E)** Representative immunoblot of PKM1, PKM2, $PKM2^{Y105}$, IR, $IR^{Y1162/Y1163}$, AKT, AKT^{S473}, and β-Actin in lysates of retroperitoneal adipose tissue biopsies from female (denoted in red) and male (denoted in black) human subjects across different BMIs.

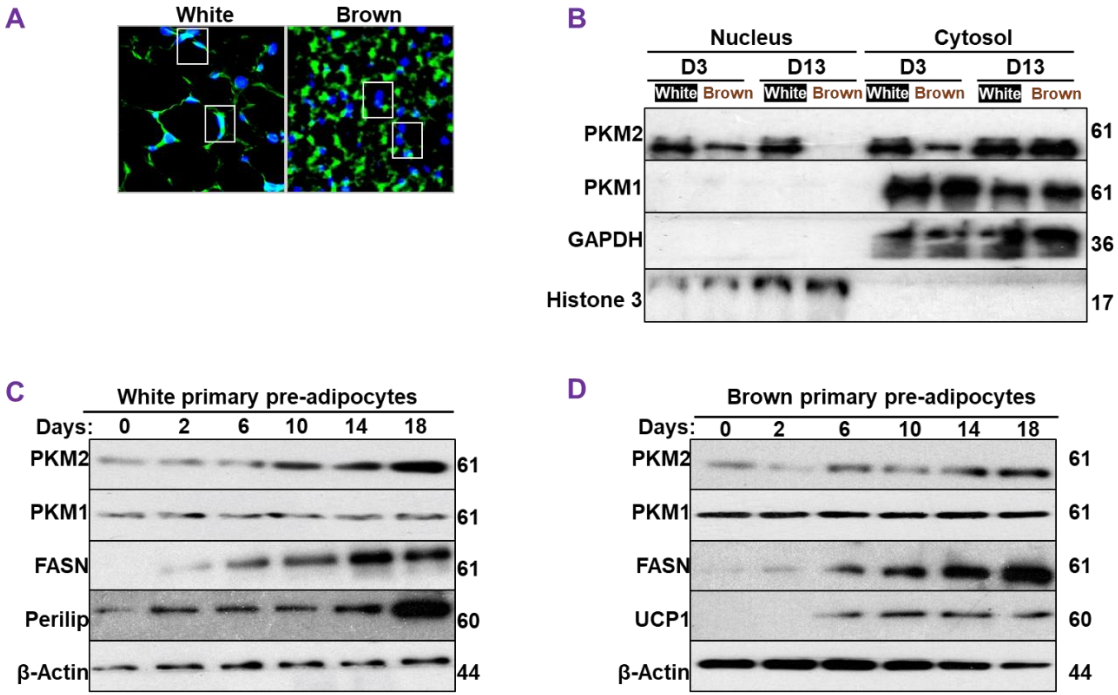


Figure 2: Differential Regulation of Adipose PK Isoforms in White and Brown Adipocytes. A) Immunostaining for PKM2 (Green), and nuclear DNA (Blue) in white subcutaneous and brown adipose tissue of 24 weeks old mice fed a chow diet. Scale bar: 100 µm. **B)** Immunoblots of PKM2 and PKM1 in cytosolic and nuclear fractions of brown (Br) and 3T3-L1 white (white) adipocytes at day 3 and 13 of differentiation. The purity of subcellular fractions was verified using GAPDH and Histone B for the cytosolic and nuclear fractions, respectively. **C**-**D**) PKM2 expression in white (**C**) and brown (**D**) adipocytes at variable stages of differentiation. Additionally, as a loading control, lysates were blotted for β-Actin.

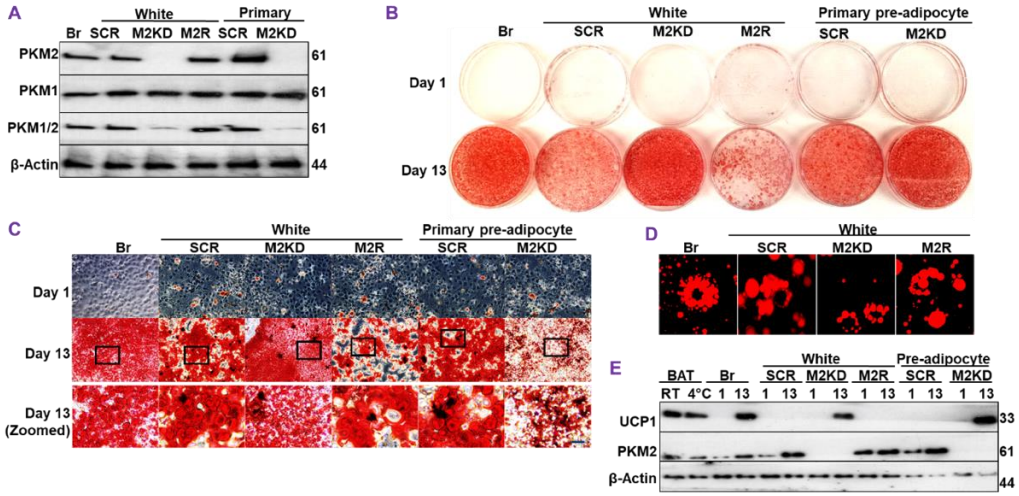


Figure 3: PKM2 Knockdown in White Adipocytes Leads to Brown-Adipocyte Like Phenotype. A) Immunoblots of PKM2 in lysates of brown (Br), scramble shRNA (SCR), PKM2 knockdown (M2KD) and reconstituted (M2R) 3T3 cells and primary inguinal pre-adipocytes infected with SCR and PKM2 lentiviral shRNA. Lysates were also blotted for PKM1, PKM1/2 and β-Actin to control for loading. **B)** Oil red O-stained image of differentiated brown, SCR, M2KD and M2R in 3T3 white (white) adipocytes and SCR and M2KD primary adipocytes difference between M2KD and SCR. **C**) Phase contrast images of oil red O-stained undifferentiated and differentiated brown, SCR, M2KD and M2R in 3T3 white adipocytes and SCR and M2KD primary adipocytes. Images in bottom panel (Day 13 Zoomed) are zoomed captures of the boxed areas on Day 13 of differentiation. Scale bar: 100 μm. **D**) Immunofluorescence staining of lipid droplets in brown primary preadipocytes (Br) and SCR, M2KD, M2R 3T3 white pre-adipocytes using LipidSpot™ red dye. **E**) Immunoblots of Ucp1 and PKM2 in BAT of wild type mice housed at room temperature (RT) or cold-exposed, and in pre-adipocytes (day 1) and differentiated (day 13) brown, SCR, M2KD, M2R 3T3 cells, SCR and M2KD primary adipocytes. β-Actin was also blotted to serve as a control for loading.

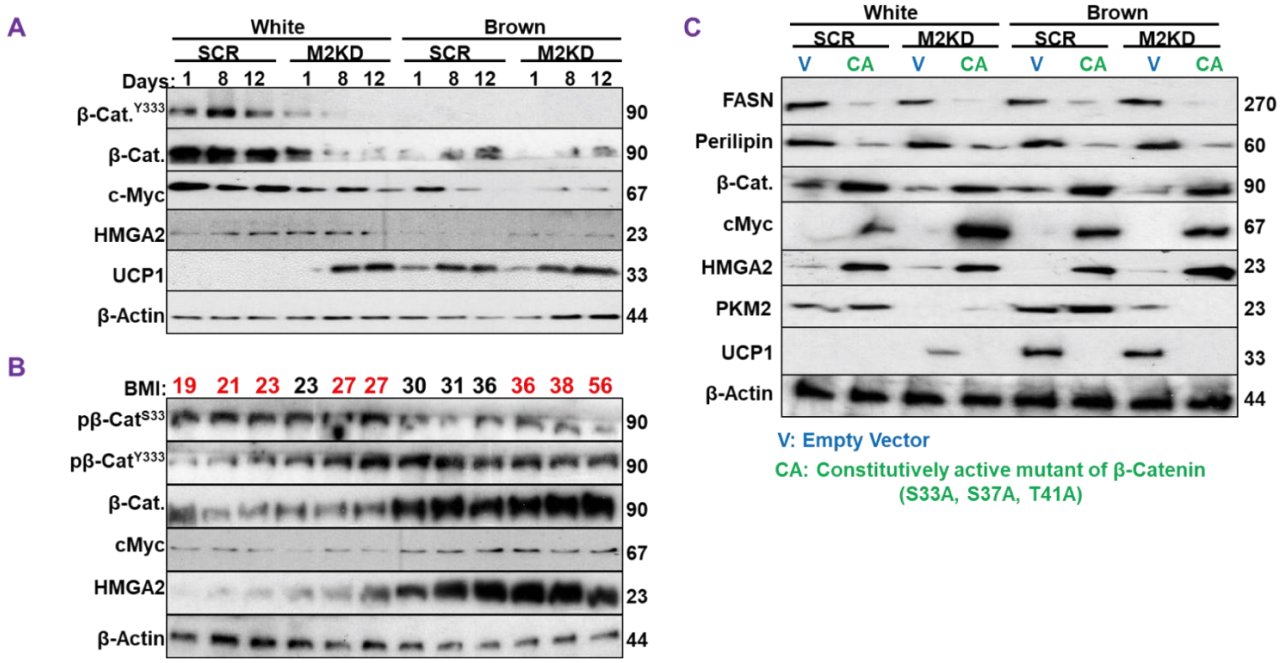
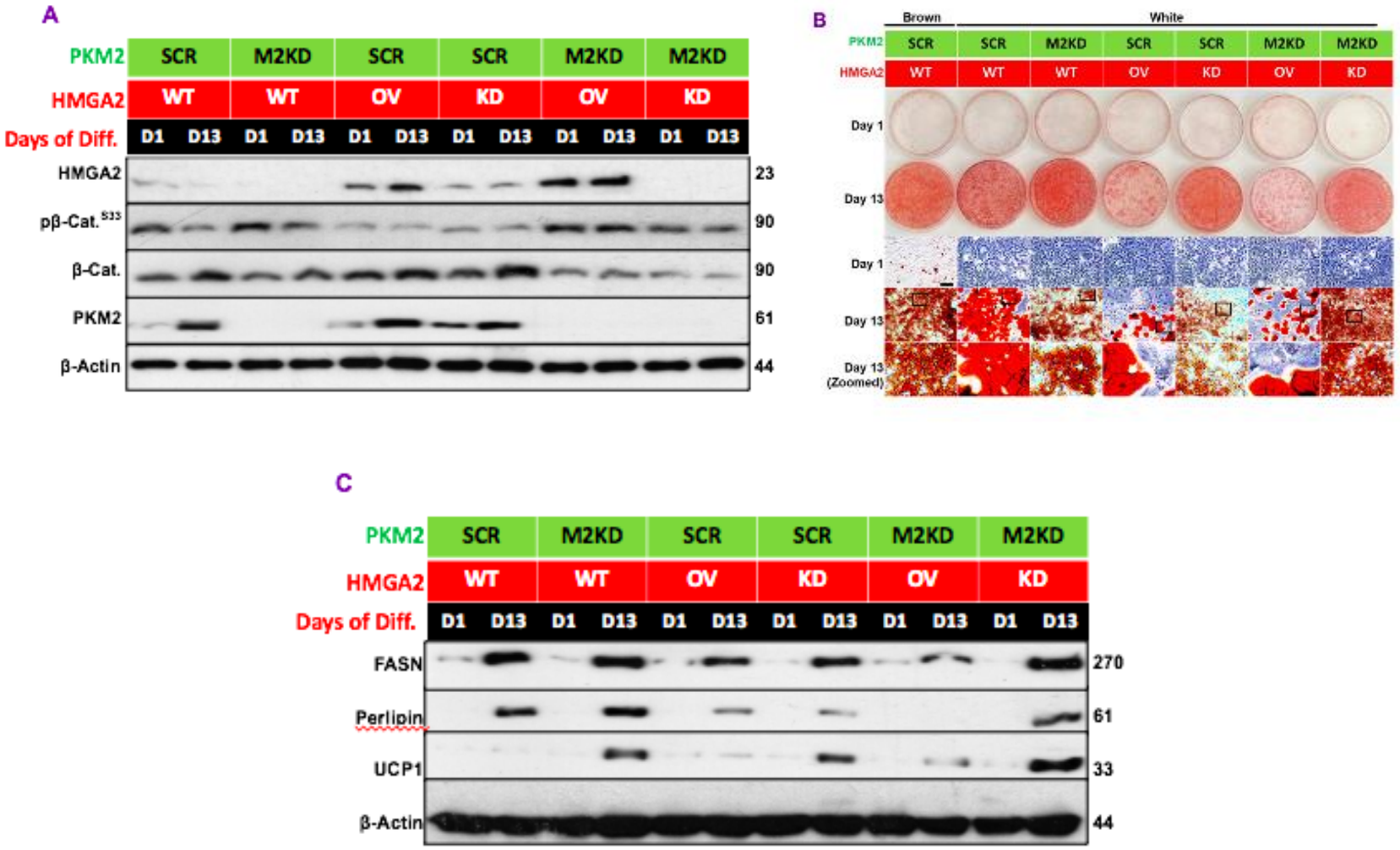


Figure 4: β-catenin Mediates PKM2's Suppressive Role on Thermogenesis. A) Representative immunoblot of β-CateninY333, β-Catenin, c-Myc, HMGA2, UCP1, and β-Actin in lysates of white and brown SCR and M2KD cells on days 1, 8 and 12 of differentiation. **B)** Representative immunoblot of β-CateninS33, β-CateninY333, β-Catenin, c-Myc, HMGA2, UCP1, and β-Actin in lysates of retroperitoneal adipose tissue biopsies from female (denoted in red) and male (denoted in black) human subjects across different BMIs. **C)** Representative immunoblot of markers of differentiation (FASN and Perilipin), browning (UCP1), β-Catenin, c-Myc, HMGA2, PKM2, and β-Actin as a loading control in fully differentiated (day 13) white and brown SCR and M2KD cells transfected with the empty pCS2+ vector (**V**) or pCS2 plasmid expressing a constitutively active β-catenin mutant (**CA**) plasmids prior to the initiation of the differentiation process.

Figure 5: PKM2/β-catenin Axis Suppresses Adipogenesis and the Browning of White Adipocytes Through HMGA2. A) Representative immunoblot of HMGA2, β-CateninS^{33}, β-Catenin, PKM2, and β-Actin as a loading control in undifferentiated (D1) and fully differentiated (day 13) white SCR and M2KD cells infected with lentivirus particles carrying shRNA (KD) or the opening reading frame of human HMGA2 (OV). **B)** Upper panel: Oil red O staining of undifferentiated (D1) and fully differentiated (day 13) white SCR and M2KD cells infected with lentivirus particles carrying shRNA (KD) or the opening reading frame of human HMGA2 (OV). Brown primary pre-adipocytes were used as a positive control. Lower panel: Phase contrast of oil red O-stained images of differentiated brown, SCR, M2KD and M2R cells infected with lentivirus particles carrying shRNA (KD) or the opening reading frame of human HMGA2 (OV). Images in bottom panel are zoomed captures of the boxed areas on Day 13 of differentiation. Scale bar: 100 μm. Brown primary pre-adipocytes were used as a positive control. **C)** Representative immunoblot of FASN, Perilipin, UCP1, and β-Actin as a loading control in undifferentiated (D1) and fully differentiated (day 13) white SCR and M2KD cells infected with lentivirus particles carrying shRNA (KD) or the opening reading frame of human HMGA2 (OV).


A
PKM2
HMGA2
Days of Diff.
SCR
M2KD
WT
OV
KD
D1
D13
HMGA2
pβ-Cat.S33
β-Cat.
PKM2
β-Actin
23
90
90
61
44
B
Brown
White
Day 1
Day 13
Day 1
Day 13
Day 13 (Zoomed)
C
FASN
Perlipin
UCP1
β-Actin
270
61
33
44

CONCLUSION, LIMITATIONS, AND FUTURE DIRECTIONS

Despite the innumerable lines of therapeutics that have been designed and employed throughout the decades, the obesity epidemic continues to progress at a distressing rate. Obesity thoroughly extends far beyond its formal definition as the accumulation of excess of white adipose tissue and further encompasses the competence to induce metabolic disturbances [277]. Additionally, this epidemic is costing the United States (US) roughly $173 billion dollars a year which relatively constitutes ten percent of the nation's medical budget [278]. Fundamental strategies including diet, exercise, and bariatric surgeries encounter many tribulations pertaining to disability, sustainability, and accessibility. Moreover, pharmaceutical or adjunctive phytotherapy, in addition, experience concerting difficulties such as efficacy, human translational fidelity, as well as, the lack of understanding of their molecular mechanisms.

Emerging evidence suggest that targeting brown adipose tissue and function along with modulation of the fate of white adipogenesis in a way that promotes the browning of WAT holds the key for novel anti-obesity preventative and therapeutic strategies. However, the mechanisms that regulate the browning of WAT remain incompletely understood. In this regard, a growing body of literature suggests that activation of the intracellular energy sensor adenosine monophosphate-activated protein kinase (AMPK) promotes fatty acid oxidation and browning of white adipose tissue [279, 280]. Additionally, activation of AMPK in white adipocytes (using Metformin, 5-aminoimidazole-4-carboxyamide-1-beta-D-ribofuranoside (AICAR), shikonin, resveratrol and other phytochemicals) results in the downregulation of sterol regulatory element binding protein (SREBP)-1c and inhibition of lipogenesis. AMPK activation also leads to increased glucose oxidation and enhanced fatty acid oxidation as well as the activation of mTOR

signaling pathway, which is known to inhibit white fat adipogenesis in-vitro yet potentiates brown fat cells activity and the differentiation of brown fat progenitor cells in-vivo [88, 281-286]. Contrary to these initial reports, recent studies demonstrated that activation of AMPK by AICAR in human white adipocytes induces a brown-like phenotype with increased markers of beige/brown adipocytes including TBX1, UCP1, CIDEA, PRDM16 and TMEM26) [287]. Likewise, prolonged activation of AMPK by AICAR led to the browning of rat white adipocytes [288]. Notably, several phytochemicals and natural compounds such quercetin [289], Zeaxanthin [290], trans-cinnamic acid [291], cordycepin [292], xanthohumol [293], and many more were shown to induce the browning of white adipocytes, both in-vitro and in-vivo, through the activation of AMPK. It is unclear why studies investigating the effects of AMPK activation on browning have not produced consistent results. Importantly, several pharmacological AMPK activators (including metformin) have several concerns regarding their safety and efficacy [294-299]. ranging from lactic acidosis to micronutrient deficiencies and impairments in cognitive functions. Thus, additional targets are needed.

The present study identified PKM2 as a novel therapeutic target for the treatment and prevention of obesity. Here, we demonstrate that PKM2 exhibit differential regulation and localization patterns in white versus brown adipocytes. Notably, PKM2 deficiency in white adipocytes leads to a brown adipose-like phenotype. These effects were concomitant with decreased total pyruvate kinase activity and increased expression of brown fat markers in WAT. Together, our study establishes that PKM2 not only regulate brown fat adipogenesis and function [234], but also the browning of WAT. At the molecular levels, we demonstrate that the suppressive

role of PKM2 on browning of WAT are mediated, at least in part through β-catenin and its downstream target HMGA2.

Despite its novel findings, there are several limitations to the current study. First, although the scope of the study is to establish the role of PKM2 in the browning of WAT, investigating PKM2's function in brown adipogenesis and identification of the potential mediating mechanisms would strengthen the evidence related to the therapeutic potential of targeting PKM2 for the treatment of obesity and its associated metabolic disorders. Secondly, we did not consider the existent Wnt isoforms that are involved in the regulation of the PKM2/β-catenin/HMGA2 axis in the process of adipogenesis. However, given that Wnt ligands differentially regulate the canonical Wnt signaling system, investigating each of these isoforms is beyond the scope of this study due to the precedence that is needed to first identify the role of Wnt signaling in the regulation of PKM2 activity in adipogenesis. Follow-up studies should be focused on examining *which* wnt isoform(s) is/are specifically involved in regulating the PKM2/β-catenin/HMGA2 dynamic in adipogenesis in order to gain insights into the role of the Wnt signaling pathway in association to metabolic disease. Future studies should also give priority toward investigating the role and function of PKM2/β-catenin/HMGA2 axis in brown adipogenesis. In order to ensure translatability of these results and confirm the therapeutic potential of targeting this axis, future studies should also be focused on the role of the PKM2/β-catenin/HMGA2 axis explicitly in an experimental model of human obesity. Moreover, further studies on the Wnt/ β-catenin signaling pathway and its particular role in 1) browning of WAT, 2) brown adipogenesis, 3) obesity and metabolic homeostasis are warranted. Finally, while our study directly focused on the effects of genetic ablation of PKM2 on WAT browning, additional research examining the effects of

pharmacological activators and inhibitors of PKM2 on adipogenesis and the browning of WAT both, in vitro and in vivo, are necessary.

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"Spirit lead me where my trust is without borders; Let me walk upon the waters
Wherever You may call me; Take me deeper than my feet could ever wander;
And my faith will be made stronger; In the presence of my Savior"

-Hillsong United