DEVELOPMENTAL SIGNALING PATHWAYS IN ADULT ENERGY HOMEOSTASIS

by

Patrick Antonellis

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STATEMENT OF COMMITTEE APPROVAL

Dr. Nicolas Berbari, Chair

Department of Biology

Dr. A.J. Baucum

Department of Biology

Dr. Benjamin Perrin

Department of Biology

Dr. Andrew Adams

Eli Lilly and Company

Approved by:

Dr. Theodore R. Cummins

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ABSTRACT

Many signaling pathways which are classically understood for their roles in early development are also known to be involved in tissue maintenance and adult energy homeostasis. Furthermore, dysfunction of these signaling pathways results in human diseases such as cancer. An in depth understanding of how developmentally important signaling pathways function in the adult will provide mechanistic insights into disease and potential new therapeutic targets. Here in Chapter 1, the Wnt, fibroblast growth factor (FGF), and Hedgehog (Hh) signaling pathways are discussed and examples of their relevance in development, adult homeostasis, and disease are provided. Wnt signaling provides an example of this concept as it has well described roles during both development and adult metabolism.

Work included in Chapter 2, investigates the regulation of adult energy homeostasis by a member of the endocrine FGF family, FGF19. The three endocrine FGFs, FGF19 (FGF15 in mice), FGF21, and FGF23 have well described roles in the regulation of metabolic processes in adults. While FGF23 is primarily involved in the regulation of phosphate and vitamin D homeostasis, FGF19 and FGF21 have shown similar pharmacological effects on whole body metabolism. Here, the importance of adaptive thermogenesis for the pharmacological action of FGF19 is explored. Using UCP1KO animals we show that whole-body thermogenesis is dispensable for body weight loss following FGF19 treatment.

Finally, the potential involvement of Hh signaling in mediating the hyperphagia driven obesity observed in certain ciliopathies is explored in Chapter 3. Emerging evidence suggests cilia play an important role in the regulation of feeding behavior. In mammals, the hedgehog pathway is dependent on the primary cilium as an organizing center and defects in hedgehog signaling share some clinical symptoms of ciliopathies. Here, we characterized the expression of core pathway components in the adult hypothalamus. We show that neurons within specific nuclei important for regulation of feeding behavior express Hh ligand and members of its signaling pathway. We also demonstrate that the Hh pathway is transcriptionally upregulated in response to an overnight fast. This work provides an important foundation for understanding the functional role of Hh signaling in regulation of energy homeostasis. In its entirety, this work highlights the emerging clinical relevance of developmentally critical pathways in diseases associated with dysfunction of adult tissue homeostasis, such as obesity.

CHAPTER 1. INTRODUCTION

The development of a complex, multicellular organism from a single cell requires precise organization. Embryonic development is a genetically controlled process which coordinates cellular events such as differentiation, proliferation, and migration. Among the most important signaling pathways for these processes are the FGF, Hedgehog, Wnt, TGFß, and Notch signaling pathways. These pathways are repeatedly utilized in both regional and temporal manors to produce the adult body plan. In addition to their critical role during development, these signaling pathways are also important for proper functioning and maintenance of adult tissues and emerging evidence now suggests that they have fundamental roles in adult energy homeostasis.

While not intended to be a comprehensive review, the following section provides specific examples of signaling pathways which are important in both embryonic development and adult homeostasis. First, the FGF signaling pathway is introduced to provide context for the included work on the role of FGF19 in the regulation of whole-body metabolism (Chapter 2). Next, Wnt signaling is discussed as a pathway with many well-known roles in both development and the maintenance and functioning of adult tissues. Furthermore, there are many similarities between the Wnt and Hedgehog signaling pathways, which is a major focus of this dissertation (Chapter 3). Finally, the role of the primary cilia in the regulation of energy homeostasis is explored by highlighting ciliopathy associated obesity. Examples of cilia-mediated hedgehog signaling in the functioning of metabolic tissues are also provided and further discussed.

1.1 The FGF pathway

1.1.1 FGF Signaling in development

Fibroblast Growth Factor (FGF) signaling is critical for the earliest stages of embryonic development and remains active in the adult where it functions as homeostatic factors important for tissue maintenance and metabolism. The FGF family consists of 18 secreted proteins which bind to and activate 4 tyrosine kinase FGF receptors (FGFRs). Binding of an FGF ligand to an FGFR induces dimerization and autophosphorylation of the receptor. Activated FGFR mediates intracellular signaling through the MAPK, AKT, PLCγ, and signal transducer and activator of

transcription (STAT) signaling pathways (1). The canonical FGFs act in an autocrine or paracrine fashion to regulate cell proliferation, differentiation, and survival. Additionally, there are three endocrine FGFs which regulate phosphate, bile acid, carbohydrate, and lipid metabolism (2, 3).

During early embryogenesis *Fgf4* is expressed in the epiblast of the inner cell mass where it is required for proliferation and formation of the primitive ectoderm (4, 5). Indeed, mice lacking *Fgf4* die early in embryogenesis due to impaired proliferation of the inner cell mass (5). FGF signaling is required throughout organogenesis where it regulates interactions between epithelial cells and mesenchyme. The apical ectodermal ridge (AER) is a specialized thickening of epithelium at the tip of the limb bud that is required for limb proximal-distal differentiation and growth (6). It has been shown that *Fgf10* is expressed in the lateral plate mesoderm where it signals the ectoderm to initiate formation of the AER (7-9). FGF10 is also required for lung development. While global loss of *Fgf10* leads to early post-natal death, conditional loss of *Fgf10* or *Fgfr2* in the mouse results in defective lobe formation and epithelial branching of the lung (10-12). In the developing nervous system, *Fgf8* is expressed in the neuroepithelium where it regulates anterior–posterior patterning of the neocortex, midbrain and hindbrain and mice lacking *Fgf8* die by embryonic day 9.5 (13-17). Induction and refinement of the otic placode, which gives rise to the entire inner ear, is regulated by FGF10 and FGF3 (18, 19). Later, *Fgf20* expressed in the prosensory epithelium signals to FGFR1 to regulate differentiation of the cochlear sensory epithelium (20, 21). A subfamily known as the intracellular FGFs (iFGFs) are not secreted and have no known interaction with FGFRs (22). Members of this subfamily, which includes FGF11, FGF12, FGF13, and FGF14, interact with voltage gated sodium channels to regulate their localization and gating properties (23-25). Animals lacking *Fgf13* or *Fgf14* exhibit neuronal defects which results in learning and memory deficits (26-28). Given the diversification and utilization of FGF ligands and receptors for process like cell proliferation, organogenesis, differentiation, and survival it comes as no surprise that this complex signaling machinery would also be utilized for adult tissue homeostasis and even organ-organ endocrine communication and metabolism.

1.1.2 The endocrine FGFs: Regulation of adult metabolism

The endocrine FGF subfamily consists of FGF19 (FGF15 is the mouse ortholog of human FGF19), FGF21, and FGF23. In contrast to the canonical FGFs, the endocrine FGFs have a low heparin binding affinity which allows them to escape the extracellular matrix and enter circulation. Instead of using heparin as a cofactor for FGFR binding and activation the endocrine FGFs rely on the Klotho family of transmembrane proteins. FGF15/19 and FGF21 signaling requires β-Klotho while FGF23 signaling requires α-Klotho (29).

In the adult, FGF23 is primarily involved in the regulation of phosphate and vitamin D homeostasis. FGF23, which is mainly expressed in osteocytes, mediates these processes through binding and activation of FGFR1, FGFR3, and FGFR4 in addition to the cofactor α-Klotho (30-32). α-Klotho restricts the activity of FGF23 and is highly expressed in the kidney and choroid plexus in the brain (33). FGF23 regulates serum phosphate levels by reducing expression of sodium-phosphate transporters within renal proximal tubular membranes, resulting in increased phosphate excretion (34). Concurrently, FGF23 inhibits renal *Cyp27b1* expression and promotes *Cyp24a1* expression, resulting in reduced levels of $1,25(OH)_2D$ and subsequent phosphate absorption by the gut and bone (35). Interestingly, animals lacking either *Fgf23* or α-Klotho exhibit very similar phenotypes in which there is no embryonic lethality but develop early postnatal hyperphosphatemia and hypercalcemia leading to premature death (33, 35). The importance of FGF23 for the maintenance of proper phosphate homeostasis is exemplified by human diseases associated with *FGF23* mutations. Gain of function mutations of *FGF23* in humans underlie autosomal dominant hypophosphatemic rickets (AHDR) which is associated with excessive urinary phosphate wasting (36). Similarly, a human patient with hypophosphatemic linear nevus sebaceous syndrome was identified as having elevated circulating levels of FGF23 (37). An inactivating mutation of *FGF23* was found in familial tumoral calcinosis, which is an autosomal recessive disorder characterized by ectopic calcifications and elevated serum phosphate levels (38).

FGF21 has been shown to be an important regulator of carbohydrate and lipid metabolism as well as whole body energy homeostasis. FGF21 is expressed in metabolic tissues such as liver, white adipose tissue, brown adipose tissue, muscle, and pancreas (39-43). Supportive of its role in regulation of metabolic processes, expression of its co-factor β-Klotho is restricted to

metabolically active tissues (44). Mice lacking *Fgf21* appear normal but gain significantly more weight than wild type mice when fed either a high fat or ketogenic diet (45, 46). Congruent with these findings *Fgf21* overexpressing animals exhibit a lean phenotype with accompanying improvements in glycemic control and insulin sensitivity (47). Furthermore, mice treated by pharmacological administration of recombinant FGF21 demonstrate profound metabolic effects such as reductions in serum glucose, insulin and triglycerides as well as a dose dependent decrease in body mass (48, 49). Interestingly, animals treated with FGF21 exhibit elevated expression of thermogenic genes, most notably uncoupling protein 1 (UCP1), in white and brown adipose tissue (50). This elevated thermogenic capacity of adipose tissue is accompanied by increased whole body energy expenditure following treatment with FGF21 (49, 51). Somewhat surprisingly, animals lacking UCP1 lost a similar amount of body weight as wild type animals when treated with FGF21 despite ameliorated UCP1-dependent thermogenesis (52). The body weight lost by UCP1KO animals treated with FGF21 was in part driven by an underlying reduction in food intake. Consistent with a potential roll in regulation of feeding behavior, it has been demonstrated that central infusion or overexpression of FGF21 modulates food intake and energy expenditure and these effects are lost in animals lacking β-Klotho in the brain (53-55). In support of these findings, genome analysis in humans have revealed mutations in the *FGF21* gene and locus that correlate with increased carbohydrate intake (56, 57). Furthermore, a polymorphism in the 3' untranslated region of *FGF21* in humans was found to be associated with obesity (58).

While deletion of *Fgf15* in mice is embryonically lethal, few do survive into adulthood (59). FGF19, the human ortholog of mouse Fgf15, has similar effects as FGF21 on whole body metabolism. Both animals treated with or overexpressing FGF19 exhibit increased metabolic rates, improved glucose tolerance and insulin sensitivity, and lose more body weight than control animals (60-62). However, in adult mice, the primary biological role of Fgf15 is regulation of bile acids. Post-prandial release of bile acids stimulates Fgf15 production in the ileum where it subsequently signals to the liver to inhibit bile acid synthesis (63). Blockade of bile acid flow into the small intestine through bile duct ligation results in elevated liver expression of *Cyp7a1*, which catalyzes the rate-limiting step in bile acid synthesis. Furthermore, *Fgf15* knockout mice have increased CYP7A1 mRNA, protein, and enzyme activity levels and a corresponding increase in fecal bile acid excretion (64). Fgf15 has been shown to bind to and activate FgfR4 and this

interaction is required for its action in the liver (65, 66). In addition to regulation of bile acid homeostasis, Fgf15 has also been shown to be important for liver regeneration and hepatocyte proliferation. Increased hepatocyte proliferation was observed in animals treated with FGF19 and this proliferative effect has been shown to be dependent on FgfR4 activation in the liver (66, 67). Following partial hepatectomy *Fgf15* knockout mice displayed extensive liver necrosis and mortality compared to wild type animals (68). Commensurate with its role in mediating hepatocyte proliferation, FGFR4 has been implicated in the progression of hepatocellular carcinoma (HCC) in humans. A study which analyzed FGFR4 expression in HCC tissue samples found eight genetic alterations including two highly frequent polymorphisms (69). Furthermore, it has been demonstrated that *FGF19* transgenic mice develop HCC (70). However, treatment of transgenic animals with an FGFR4 neutralizing antibody or crossing them with FGFR4 knockout mice, results in reduced liver tumor formation (71). The beneficial pharmacological effects of FGF19 and FGF21 treatment has generated interest in their use as therapeutic agents for metabolic diseases such as type 2 diabetes and non-alcoholic steatohepatitis (NASH). Therefore, it will be critical to gain a greater understanding of the mechanisms which underly their beneficial metabolic effects such as increased whole-body energy expenditure and weight loss.

1.2 The WNT signaling pathway

1.2.1 WNT Signaling in development

In Drosophila, Wingless (*wg)* was shown to regulate segment polarity during larval development while the mammalian *Wnt1* gene was originally identified as an oncogene in a mouse model of breast cancer (72, 73). In canonical Wnt signaling, in the absence of ligand cytoplasmic β-catenin is constantly degraded by the axin complex which includes Axin, Adenomatous Polyposis Coli (APC), casein kinase 1 (CK1), glycogen synthase kinase 3 (GSK3) (74, 75). CK1 and GSK3, phosphorylate β-catenin, which results in its ubiquitination and proteasomal degradation (76). Binding of a Wnt ligand to the receptor Frizzled (FZD), a seven-pass transmembrane G-protein-coupled receptor (GPCR), leads to recruitment of the Axin complex and inhibition by Dishevelled (77-79). Subsequently, stabilized β-catenin accumulates and travels to the nucleus where it complexes with TCF/LEF and activates target gene expression (80, 81). To date, 19 members of the Wnt family have been identified in mammals, along with 10 members of

the FZD family of receptors. Wnt proteins are hydrophobic, indeed it has been shown that when expressed *in vitro* they are primarily found associated with cell membranes or the extracellular matrix (82). It was further demonstrated that lipid modification by attachment of a palmitate moiety on a conserved cysteine residue is important for ligand secretion and signaling activity (83).

In addition to canonical signaling, the Wnt pathway also transduces non-canonical signaling which is often referred to as the β-catenin independent pathway. Non-canonical Wnt signaling can be further divided into the Planar Cell Polarity (PCP) and the Wnt/Ca2+ pathway. The PCP pathway primarily functions in regulating the actin cytoskeleton for the organization of cellular structures and cell migration (84). PCP signaling is initiated upon FZD activation and is transduced by asymmetric accumulation of a complex of proteins, including Dishevelled, at the plasma membrane (85). Downstream of Dishevelled, activation of the small GTPases RHO and RAC is critical for establishment of cell polarity (86, 87). The Wnt/Ca2+ pathway, which interacts with both canonical and PCP signaling, is defined by Wnt or FZD induced intracellular calcium release from the ER (88). The release of intracellular calcium has been shown to be dependent on the activity of heterotrimeric G-proteins (89). The increase of intracellular calcium results in activation of calcium sensitive signaling proteins such as protein kinase C (PKC) and calcium/calmodulin-dependent kinase II (CamKII) (90).

Studies in vertebrates and flies demonstrated that Wnt signaling plays a highly conserved role in embryonic development. In the fly, mutations of *wg*, dishevelled, armadillo (the fly homolog of β-catenin), or shaggy/zeste-white 3 (the fly homolog of GSK3), cause developmental abnormalities in patterning of the cuticle which normally exhibits alternating denticle and naked belts (73, 91-93). In studies using Xenopus embryos, injection of mouse *Wnt1* mRNA resulted in a duplication of the body axis (94). During mammalian development Wnt ligands are expressed in the primitive streak and early gastrula organizer and Wnt signaling is required for establishing the anterior-posterior axis, mesoderm development, and specification of neural crest stem cells as sensory neural cells (95-99)

1.2.2 WNT signaling in adult tissues and disease

Outside of its role in development, Wnt signaling remains important in adult tissue homeostasis through regulation of self-renewal. In the epithelium of the small intestine, proliferative progenitor cells located in the crypt region produce new epithelial cells at a high rate. Disruption of the Wnt pathway in mice results in loss of the crypt progenitor region (100). It has also been demonstrated that Wnt signaling is important for activation of multipotent epidermal stem cells found in the bulge region of the hair follicle. When β-catenin is conditionally ablated in mice, bulge stem cells do not produce hair lineage precursors resulting in hair loss (101, 102). Of particular relevance, it has been established that neurogenesis persists into adulthood in various regions of the brain. The subgranular zone (SGZ) of the dentate gyrus in the hippocampus contains neural stem cells that give rise to neuronally committed cells that mature into granule cells (103, 104). In mammals there is evidence that adult neurogenesis impacts not just structural plasticity but functions of the hippocampus such as spatial learning and memory (105-107). Not only are Wnts expressed in neural progenitor cells of the adult hippocampus, but neurogenesis is induced or inhibited *in vivo* through activation or inhibition of Wnt signaling respectively (108, 109).

The developmental and homeostatic importance of Wnt signaling is underscored by the myriad of human genetic disorders and cancers which result from its disfunction. Patients with a hereditary form of cancer called Familiar Adenomatous Polyposis inherit a single defective allele of *APC* and develop large numbers of colon polyps in early adulthood (110). Additionally, a majority of sporadic colorectal cancers result from loss of both *APC* alleles (111). Loss of APC function in intestinal epithelial cells causes constitutive β-catenin activity and subsequent inappropriate progenitor cell proliferation (112, 113). Defects in Wnt signaling have also been implicated in the progression of Alzheimer's disease (AD). Rodent models of AD exhibit reduced rates of neurogenesis (114, 115). Congruent with these findings, patients with AD pathology exhibit reduced adult hippocampal neurogenesis compared to neurologically healthy human subjects (116, 117). Interestingly, defects in Wnt signaling have been observed in animal models of AD (118-120). In human AD patients, altered expression of components of the Wnt signaling pathway has also been demonstrated (121-124). Furthermore, a mutation of the Wnt co-receptor, LRP6, exhibits reduced signaling activity and is associated with late-onset AD in humans (125, 126). The contribution of Wnt signaling dysfunction to these human diseases suggests that the Wnt

pathway is important for the proper functioning of adult tissues important for the regulation of energy homeostasis like the colon and brain.

1.3 WNT and Hh similarities

1.3.1 Signal transduction

Like Wnt signaling, the Hedgehog (Hh) pathway also has well characterized functions in embryonic development and emerging roles in adult energy homeostasis. There are notable similarities in the functioning of the Wnt and Hh signaling pathways. First, the Hh pathway member Smoothened (SMO) shares structural homology with the 10 FZD receptors and together make up a distinct class of GPCRs known as the Frizzled family (127). Additionally, secreted ligands of both pathways are modified by the posttranslational addition of palmitate (83, 128). Hh and Wnt proteins are palmitoylated by members of the membrane-bound O-acyltransferase (MBOAT) family (129, 130). Furthermore, this palmitoylation is critical for both their secretion and proper signaling (83, 131, 132). In addition to palmitoylation, Hh ligands are also modified by the addition of cholesterol which makes them more hydrophobic and enhances distribution (133, 134). Similar to WNT signaling, the Hh pathway has both canonical and non-canonical signaling activity. The Hh signaling pathway is discussed in more detail in subsequent sections. Briefly, canonical activation of Hh signaling occurs when Hh ligands bind the twelve-pass transmembrane receptor Patched (Ptch), which inhibits Smo (135, 136). Active Smo initiates an intracellular cascade that leads to the activation of Glioma (Gli) transcription factors (137-139). In general, non-canonical Hh signaling refers to Hedgehog-dependent signals that are independent of Gli activation. Type I non-canonical Hedgehog signaling works through Ptch and is independent of Smo, while type II functions through Smo (140-142). Additionally, Gli activation independent of Smo has been observed and referred to as a form non-canonical Hedgehog signaling (143, 144).

1.4 Progenitor cells and cancer

It has become increasingly clear that these pathways critical for early development remain active into adulthood where they mediate tissue homeostasis. Additionally, dysregulation of these pathways causes a myriad of diseases such as cancer. It has been proposed that there are a small

number of cancer stem cells (CSCs) within a tumor which are long-lived, proliferative, and multipotent. The existence of CSCs would help explain certain characteristics of tumors such as their recurrence, dormancy, and metastasis. Transplantation studies in many types of cancer, such as leukemia and colon cancer, have identified CSCs that possess the capability to initiate and sustain tumor growth through differentiation and proliferation (145-148).

A reoccurring theme for both Wnt and Hh signaling is their functioning in the maintenance of progenitor cell niches which are required for tissue turnover and repair. Furthermore, mutations in these pathways are known to result in enhanced cell proliferation and cancer. In an animal model of intestinal cancer in which the Wnt pathway is activated in progenitor cells through conditional deletion of *APC*, lineage tracing identified the presence of CSCs in tumors (149). Furthermore, *APC* loss of function mutations underlie both familial and sporadic forms of colorectal cancers (110, 111). For these reasons, the Wnt pathway has become a therapeutic target for the treatment of colorectal cancers (150).

Similar to Wnt signaling, the Hh pathway has been shown to be important for proper development of hair follicles (101, 102, 151). Consistent with this function, mutations which activate the Hh pathway have been identified in patients with basal cell carcinoma (BCC), which has been proposed to originate from the bulge region of the hair follicle (152-155). Both lineage tracing performed in *Ptch1* deficient animals as well as conditional knockout of *Ptch1* have identified CSCs within the hair follicle region as a source of BCC tumors (156, 157). Congruently, it has been demonstrated in mice that inappropriate activation of the Hh pathway in skin leads to BCC (158, 159). Furthermore, defects in the Wnt and Hh pathways represent 2 of the 4 distinct classes of medulloblastoma (MB), which is the most common malignant pediatric brain tumor (160-162). MB is thought to originate from granule-cell precursors (GCPs) within the external granule cell layer (163). It has been shown that not only does Hh signaling promote proliferation of GCPs, but genetic activation of the Hh pathway in GCPs results in MB (164-167). In depth understanding of the molecular mechanisms responsible for proper tissue maintenance and how their disfunction causes certain types of cancers will certainly lead to new investigational avenues for the discovery of therapeutics.

1.4.1 Regulation of metabolic tissues in the adult by Wnt and Hh

The Wnt and Hh signaling pathways have been implicated in the proper development and function of important metabolic tissues such as the pancreas and adipose tissue. In the developing pancreas, not only are multiple Wnt ligands and FZD receptors expressed but altered expression of ligand or antagonism of Wnt signaling results in inappropriate patterning of the pancreas (168-170). During organogenesis, Hh signaling has been shown to inhibit formation of the pancreas. Studies in mice have shown that upregulation of Hh signaling results in loss of pancreatic tissue while inhibition of signaling leads to expansion of the developing pancreas (171-175).

Studies in human cohorts identified mutations in transcription factor 7-like 2 (TCF7L2), a transcription factor in the Wnt pathway, that are associated with increased risk of type 2 diabetes (176-178). TCF7L2 has been shown to be important for the function of the pancreas, when it is knocked down in human and mouse islets it causes reduced glucose stimulated insulin secretion (179, 180). Furthermore, in mice conductional deletion of *TCF7L2* in the pancreas or in β-cells results in glucose intolerance and impaired glucose stimulated insulin secretion (181, 182). Congruent with these findings, mice expressing a dominant negative form of *TCF7L2* in β-cells display alterations in glucose homeostasis and insulin secretion (168, 183). Hh signaling has also been implicated in the functioning of the pancreas as ligand and receptor expression has been observed in islets (184). Additionally, animals heterozygous for global knockout of *Ptch1* or pancreatic knockout of *Smo* exhibit glucose intolerance (173, 185).

Related to their function in the maintenance of progenitor cells, both the Wnt and Hh pathways have been shown to inhibit adipogenesis by restricting differentiation of preadipocytes. It has been demonstrated *in vitro* that induction of Wnt signaling by overexpression of *Wnt10b* or other forms of pathway agonism inhibits differentiation of adipocytes while Wnt pathway inhibition causes adipocyte differentiation (186, 187). Indeed, overexpression of *Wnt10b* in the adipose of mice results in a lean phenotype due to an overall reduction in the amount of white and brown fat (188). Interestingly, a mutation in *WNT10B* has been described to be associated with obesity in humans (189). A summary of the role of Hh signaling in the differentiation of preadipocytes is covered in more detail in the following section **1.1.5.**

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1.5 Cilia signaling and obesity

Obesity is a major growing public health concern (190). The secondary health consequences including heart disease, high blood pressure, diabetes mellitus and osteoarthritis can reduce quality of life and lead to early mortality (191). Historically, obesity has been viewed as increased body fat due to overconsumption of food combined with a sedentary lifestyle. This viewpoint largely focuses on environmental and social factors; it fails to take into account evidence that indicates a profound role for genetic contributions (for a review on obesity-associated genetic syndromes see (192)). While most monogenetic human conditions involving morbid obesity are rare, it is clear that the study of their molecular and cellular etiology will offer insights into the mechanisms that regulate appetite and satiety. The objectives of this review are to discuss syndromic forms of obesity that impact genes required for the formation or function of small hair-like cellular appendages called cilia. We also discuss the potential that cilia genes may play a role in common forms of obesity. How cilia dysfunction in both the brain and peripheral tissues may contribute to obesity is discussed by reviewing mouse and cell model data. Interesting data from genetic mouse models of ciliopathies has revealed the importance of cilia in both development and homeostasis. **Of particular interest, the hedgehog signaling pathway depends on primary cilia for proper signal transduction in mammals. We highlight evidence connecting dysfunctions in cilia-mediated hedgehog signaling to the obesity phenotype observed in ciliopathies**. Ultimately, an understanding of how these organelles function to regulate energy homeostasis may reveal opportunities to address a major public health concern.

1.5.1 Ciliopathy syndromes and obesity

Ciliopathies are genetic disorders associated with deficits in cilia formation, maintenance and function. As cilia are nearly ubiquitous throughout the body, these disorders present with a wide range of features impacting all organ systems (193). Certain ciliopathies present with pediatric obesity including Bardet-Biedl syndrome (BBS, OMIM #209900), Alström syndrome

(ALMS, OMIM #203800), Mental retardation, truncal obesity, retinal dystrophy and micropenis syndrome (MORM, OMIM #610156) and Carpenter syndrome (CRPT1, OMIM #201000).

BBS affects 1 in 100,000 newborns, with higher prevalence in consanguineous and geographically isolated populations, where the disease can affect as many as 1 in 17,000 individuals (194). In addition to obesity, these children present with developmental delay, renal failure, retinitis pigmentosa, hypogonadism and polydactyly (195, 196). They also show a higher rate of metabolic syndrome including an increased risk for type 2 diabetes (196). To date, mutations in 22 genes have been identified to cause the disease (196-201). Several BBS genes encode for proteins that are associated with two larger complexes, one called the BBSome which is important for transport of signaling proteins and receptors to and from cilia (202-206) and the other is a chaperone complex associated with BBSome assembly (207).

Unlike the heterogenetic nature of BBS, Alström syndrome (ALMS) is monogenic, with loss-of-function mutations found in the ALMS1 gene (208, 209). ALMS is rare with an apparent worldwide prevalence of less than 1 in a million individuals (210). While ALMS patients present with childhood truncal obesity and hypogonadism, they also have an earlier onset of type 2 diabetes, visual loss, progressive hearing loss, short stature and cardiomyopathy (209, 211). ALMS1 protein localizes to centrioles and the base of cilia where it is thought to be important for proper cilia function, maintenance and allowing signaling proteins in and out of the organelle (212-216).

Clinical efforts are being made to treat the obesity in BBS and ALMS patients. Currently, phase 3 clinical trials of Setmelanotide, a Melanocortin-4 receptor (MC4R) agonist, are underway with the aim to reduce appetite and increase energy expenditure (ClinicalTrials.gov Identifier: NCT03746522) (217, 218). Interestingly, it has also recently been reported that Methionine aminopeptidase 2 inhibitors (MetAP2i) are able to reduce hyperphagia in obese ciliopathy mouse models (219). Previous clinical studies of MetAP2i in Prader-Willi syndrome (PWS, OMIM #176270) patients showed weight loss through reduced food intake (220, 221). Though clinical trials of MetAP2i have been suspended, development of next generation MetAP2i compounds could be beneficial for ciliopathy patients.

In addition to BBS and ALMS, other rare syndromes such as MORM and Carpenter syndromes, as well as monogenic alleles of adenylate cyclase 3 (*ACIII)*, *MC4R*, and the inorganic pyrophosphate transporter *ANKH*, have been associated with cilia dysfunction and obesity. Both MORM and Carpenter syndromes are extremely rare autosomal recessive ciliopathies (195, 222). MORM syndrome is clinically defined by its acronym, mental retardation, early onset truncal obesity, non-progressive retinal dystrophy and micropenis in males. Carpenter syndrome includes similar features with skeletal and craniofacial malformations, polydactyly, heart and eye defects, childhood obesity as well as hydrocephaly and intellectual disability (223-225). Both arise from mutations in genes that encode for cilia-specific enzymes. In MORM, the lipid phosphatase, inositol polyphosphate-5-phosphatase E (*INPP5E)* is mutated (195, 222, 226). INPP5E is found predominantly in the cilia transition zone where it is thought to help establish cilia sub-compartmentalization important for proper signaling (227-229). The Rab-GTPase, *RAB23* is mutated in Carpenter syndrome (223-225). Rab23 is important for proper trafficking of ciliary proteins and receptors as well as negative regulation of hedgehog signaling. (230-233).

The primary cilium is required for proper hedgehog signaling in mammals, for an in-depth review of hedgehog signaling and primary cilia see Kopinke, Norris and Mukhopadhyay in this edition (234). Interestingly, disruption in hedgehog signaling produces severe clinical features such as abnormal development of the nervous system, facial structure, and limbs. Mutations in pathway ligand sonic hedgehog as well as downstream genes result in a disorder known as Holoprosencephaly (HPE), in which patients develop an abnormal brain and facial structure, with frequent midfacial clefts (235-237). Additionally, patients with mutations in the ligands Indian hedgehog and desert hedgehog exhibit defects in skeletal and sexual development (238-242). Many ciliopathy phenotypes such as polydactyly, external genitalia anomalies, and craniofacial defects are reminiscent of Shh pathway deficiencies (243, 244). While there are currently no functional data linking hedgehog defects to human obesity, it is possible that hedgehog deficiency contributes to ciliopathy-associated obesity given that the hedgehog pathway relies on the primary cilium in mammals and that human genetic defects in this pathway cause phenotypes observed in ciliopathies. In this review, we further discuss animal and *in vitro* studies of the hedgehog pathway and potential implications in obesity.

Other ciliary genes implicated in monogenic forms of obesity are ANKH and centrosomal protein 19 (CEP19) (245, 246). The *ANKH* gene encodes an inorganic pyrophosphate transporter that is involved in bone calcium homeostasis and localizes to cilia and basal bodies (247, 248). While ANKH is known for its role in mineralization, mutations of the gene identified in a subset of Russian population are associated with increased BMI, waist-to-hip ratio and levels of plasma leptin (245). How ANKH or its ciliary localization play a role in energy homeostasis is not known. However, a recent study shows mice lacking osteoblast-specific genes are fat and glucose intolerant indicating a role of the skeleton in endocrine regulation (249). This combined with the correlation of ANKH with leptin suggests an indirect role for the protein in peripheral tissues such as adipocytes. On the other hand, autosomal recessive mutations in *CEP19* result in obesity in a large Israeli family (246). CEP19 exclusively localizes to basal bodies of cilia to facilitate ciliogenesis and trafficking of ciliary GPCRs. (246, 250, 251). Furthermore, CEP19 knockout mice recapitulated the human phenotype and displayed hyperphagia along with an increase in adipogenesis (246).

1.5.2 Cilia in the central nervous system and feeding behavior

Neurons and most cell types throughout the central nervous system possess primary cilia (252-254). Yet, understanding the function of neuronal cilia has remained a challenge. As new approaches and tools have begun to be established to allow their visualization (255) it has become clear that neuronal cilia likely play diverse signaling roles throughout the brain and impact several behaviors including feeding.

Initial conditional mouse approaches to disrupt cilia formation through intraflagellar transport (IFT) alleles, (for a review of IFT see (256)), were useful for obtaining a broad understanding of phenotypes that emerge upon cilia dysfunction, including altered energy homeostasis. Near ubiquitous cilia loss in adult mice results in hyperphagia and subsequent obesity (257). Controlling for food intake in pair-feeding experiments of conditional IFT mutants prevented obesity, indicating that cilia restrict weight gain by inhibiting the consumption of food rather than by affecting metabolism or locomotor activity. Using this same approach to remove cilia from neurons recapitulated the obesity phenotype and demonstrated a role for neuronal cilia in feeding behavior (257).

1.5.3 Neuronal cilia GPCRs and obesity

GPCR misregulation has emerged as a focus of many studies on how cilia centrally regulate appetite and satiety. Several GPCRs appear preferentially enriched at the membrane of primary cilia, some of which have well recognized roles in feeding behavior or energy homeostasis, including melanin-concentrating hormone receptor 1 (MCHR1), MC4R, neuropeptide Y receptors 2 and 5 (NPY2R and NPY5R), somatostatin receptor 3 (SSTR3), kisspeptin 1 receptor (KISS1R), serotonin receptor 6 (5HT6), dopamine receptor 1 (DRD1) (203, 253, 254, 258-261).

Interestingly in mouse models of BBS, some GPCRs that are normally found in cilia no longer appear to localize to the compartment such as MCHR1 and SSTR3 (202). Additionally, NPY2R fails to localize to primary cilia in mice lacking the BBSome subunit BBIP10 (*Bbip10)* (259). NPY2R ciliary localization was also found to be decreased when *Bbs1* is lost specifically from POMC or AGRP neurons (262). Furthermore, recent studies have revealed a role for the BBSome in dynamically transporting GPCRs across the transition zone of cilia (263-265). These data indicate that the BBSome is important for proper localization of GPCRs and suggests that impaired signaling due to their mislocalization could contribute to obesity in BBS.

In ALMS mouse models, ciliary GPCRs appear to localize properly. However, the cilia signaling protein, ACIII, shows decreased localization (213). Without ACIII, which is activated via $G\alpha_s$-coupled GPCRs, the normal downstream intracellular signaling events required to maintain a normal body weight may be impaired. In fact in an ACIII knock out mouse model, obesity occurs through decreased activity and increased food intake (266). Furthermore, inhibiting ACIII specifically in a population of neurons in the PVN also leads to weight gain (261).

The most recent GPCR found to localize to primary cilia is MC4R (261). Using *in situ* hybridization, MC4R was found to be expressed throughout the brain of rodents including the hypothalamus (267). In mice, loss of *Mc4r* results in hyperphagia and obesity (268). Using a GFP tag, MC4R can be seen in cilia of neurons within the paraventricular nucleus of the hypothalamus (PVN). Intriguingly, some of the mutations in *MC4R* that result in obesity in humans described above result in decreased localization of MC4R in cilia (261). It remains to be seen if mutations associated with ciliopathies impact MC4R function.

Several proteins are needed to properly transport GPCRs into and out of the cilium. It is likely that the mechanisms are different for different ciliary GPCRs. Proteins like the atypical small GTPase RAB-like 2 (RABL2), CEP19, and TUB all have roles in transporting GPCRs to the cilia and also play a role in maintaining normal adult mouse body weight. *Rabl2* knockout mice become obese with age and recently it was found that without RABL2, ciliary GPCRs like GPR161 and 5HT6 are absent from primary cilia (251, 269). Similarly, *Cep19* knockout mice are obese and fail to localize GPR161 to cilia (246, 251). Obese tubby knockout mice also fail to localize SSTR3, MCHR1, and NPY2R to cilia (259, 270, 271).

The impact of the subcellular localization of these receptors in regulating feeding is not well understood. Perhaps they localize to the ciliary compartment in order to interact with specific G-proteins to produce appropriate second messengers or even to interact with other GPCRs that are also found within the cilium. For example, it has been shown that SSTR3 and MCHR1 found in the same cilium physically interact by forming heteromers (272). Other studies have shown that ciliary GPCR signaling interacts with the ciliary hedgehog pathway. For example, GPR161, is known to influence Hedgehog pathway activity in development but little is known about functions in adult neurons (273, 274). In the olfactory system, when smoothened, a GPCR in the hedgehog pathway, is knocked out from olfactory sensory neurons, odorant GPCRs cilia localization is attenuated (275). Other data supporting interactions between the hedgehog pathway and GPCR signaling comes from primary hypothalamic cultures. Pharmacological activation of smoothened inhibits response to the MCHR1 agonist melanin-concentrating hormone (276). Further studies will reveal how these pathways interact and how their localization to cilia influences their signaling and ability to modulate feeding behavior.

1.5.4 Cilia in peripheral tissues and metabolic phenotypes

In addition to obesity, ciliopathies like BBS and ALMS are also associated with clinical features such as type 2 diabetes and non-alcoholic fatty liver disease (277). How cilia in peripheral tissues impact metabolism and energy homeostasis is unclear. A growing body of literature suggests that functional cilia on preadipocytes are critical for coordinating adipogenesis. Additionally, cilia have been suggested to be important for normal pancreatic morphology and

function. In this section, we outline a few examples of how cilia are involved in the proper functioning of adipose tissue and the pancreas.

1.5.5 Cilia in adipose tissue

White adipose tissue not only serves as a site of calorie storage after feeding and the source of circulating free fatty acids during fasting but also plays an important endocrine role (278, 279). Metabolic diseases such as obesity, type 2 diabetes, and lipodystrophies can be characterized by dysfunction of adipose tissue (280). While the predominant cell type in adipose is mature adipocytes, the tissue is comprised of a variety of other cell types such as endothelial cells, blood cells, fibroblasts, pericytes, and preadipocytes (281). Preadipocytes arise from mesenchymal stem cells, which can produce mature adipocytes through the process of adipogenesis (282). However, the regulation and cellular signaling associated with this differentiation process is not fully understood (283). It has been demonstrated that preadipocytes possess a primary cilium during differentiation and that it plays a critical role in their ability to become adipocytes (284-286). Specifically, knockdown of BBS proteins (BBS10 and BBS12) which localize to the primary cilia induces adipogenesis. Furthermore, BBS patient derived preadipocytes accumulate more fat than controls upon differentiation *in vitro* (285). Commensurate with these findings knockdown of *BBS12* in human primary mesenchymal stem cells also facilitated adipogenesis (287). Additionally, this study showed that *Bbs12* knockout mice, despite being obese, were glucose and insulin tolerant and that this phenotype recapitulates observations in patients (287). These results suggest that BBS patients are predisposed to adiposity through increases in adipogenesis.

Two recent studies provide further evidence for the roles of cilia in adipocyte differentiation. Kopinke *et al.* demonstrate that cilia-mediated hedgehog signaling is important for restricting Fibro/adipogenic progenitors in muscle from becoming adipocytes (284), suggesting an active role for hedgehog in adipogenesis. In another recent study by Hilgendorf *et al.*, conditional knockout of cilia in mouse preadipocytes resulted in reduced body weight due to a reduction in total fat mass. Furthermore, the GPCR, omega-3 fatty acid receptor FFAR4/GPR120, was found to localize to the primary cilia in preadipocytes. Interestingly, FFAR4 agonists and omega-3 fatty acids triggered mitosis and adipogenesis (288). These findings suggest that cilia are important for adipogenesis mediated by preadipocytes in response to external cues. Future studies assessing the

roles of cilia in different adipose tissue cell types and diseases such as obesity and lipid dystrophies may reveal interesting themes regarding cilia signaling.

Data from multiple systems suggest hedgehog signaling is important in adult energy homeostasis. A genome-wide RNAi screen in adult *Drosophila* identified the Hedgehog pathway as being fat specific and obesity related (289). It has also been demonstrated in the fat body of *Drosophila* that lipid accumulation is repressed or enhanced by Hedgehog activation and inhibition, respectively (290). Furthermore, the *Drosophila* lipoprotein-associated form of hedgehog is secreted from the gut and signals to the fat body to broadly regulate lipid mobilization in order to couple growth to development (291, 292).

As described, hedgehog signaling is critical for proper adipocyte differentiation, and this process is perturbed in ciliopathy model systems. Further evidence that hedgehog pathway deficiency underlies ciliopathy phenotypes after development is suggested by data showing that the BBSome plays roles in proper ciliary trafficking of both patched and smoothened and mislocalize in *Bbs7* knockout cells (293). In mice, hedgehog inhibits adipogenesis and pathway activity is reduced in the adipose of obese animals (290). Furthermore, it was demonstrated that adipose specific conditional activation of the hedgehog pathway results in white adipose reduction, but not brown (289). However, in a separate study it was shown that *in vivo* activation of hedgehog signaling in adipose through deletion of Ptch1 or overexpression of SmoM2 both inhibited the formation of brown adipose tissue (294). Through the use of inducible promoters it was further shown that hedgehog pathway activation resulted in a lean phenotype with a reduction in white adipose and improvements in whole-body glucose tolerance and insulin sensitivity (295). It is interesting that both hedgehog pathway activation and cilia loss inhibits adipogenesis. Similarly, as we have previously discussed, BBS deficient preadipocytes are prone to adipogenesis. It should be noted that BBS defects do not often lead to complete cilia loss (296-299). Taken as a whole, these findings suggest that properly functioning cilia on preadipocytes are required to coordinate multiple signaling pathways for appropriate adipogenesis to occur.

1.5.6 Cilia in the pancreas

The pancreas is primarily responsible for glucose homeostasis and the release of digestive enzymes. It is composed of multiple cell types including endocrine cells, acinar cells, and ductal cells (300). Initial links between cilia and pancreatic function come from polycystic kidney disease patients and their associated pancreatic pathologies (301, 302). Indeed, cilia are present in both islet and ductal cells and the classic cilia hypomorphic Oak Ridge Polycystic Kidney (*Orpk)* mouse model of polycystic kidney disease exhibits changes in pancreas morphology and function (303, 304). In *Bbs4* knockout mice, defects in glucose homeostasis are observed prior to the onset of obesity and insulin secretion is impaired following knockdown of *Bbs4 ex vivo* (305). Data also suggest that the insulin receptor is directly trafficked by BBS proteins to the cell surface (306). More recently, a critical role of cilia in β-cells was highlighted when conditional loss of β-cell cilia in adult mice led to reductions in glucose homeostasis and insulin secretion (307). It is clear that cilia are present on diverse pancreatic cell types and future work will reveal new roles for cilia in pancreatic physiology.

The data discussed here implicate primary cilia as being critical for the proper functioning of adipose tissue and the pancreas, two peripheral organs critical for energy homeostasis. The role of primary cilia in adipogenesis and pancreatic function underlie not just the clinical phenotypes of certain ciliopathies but points to a potential involvement in general obesity and type 2 diabetes.

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CHAPTER 2. THE ANTI-OBESITY EFFECT OF FGF19 DOES NOT REQUIRE UCP1-DEPENDENT THERMOGENESIS

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Patrick Antonellis was involved in the design of all experiments, data interpretation, and wrote the manuscript. He performed all experiments and analysis except hepatic bile acid analysis, metabolic cage studies, lipid tolerance tests, and 2-DG and C-14 tissue uptake experiments.

2.1 Abstract

2.1.1 Objective

Fibroblast growth factor 19 (FGF19) is a postprandial hormone which plays diverse roles in the regulation of bile acid, glucose, and lipid metabolism. Administration of FGF19 to obese/diabetic mice lowers body weight, improves insulin sensitivity, and enhances glycemic control. The primary target organ of FGF19 is the liver, where it regulates bile acid homeostasis in response to nutrient absorption. In contrast, the broader pharmacologic actions of FGF19 are proposed to be driven, in part, by the recruitment of the thermogenic protein uncoupling protein 1 (UCP1) in white and brown adipose tissue. However, the precise contribution of UCP1-dependent thermogenesis to the therapeutic actions of FGF19 has not been critically evaluated.

2.1.2 Methods

Using WT and germline UCP1 knockout mice, the primary objective of the current investigation was to determine the in vivo pharmacology of FGF19, focusing on its thermogenic and anti-obesity activity.

2.1.3 Results

We report that FGF19 induced mRNA expression of UCP1 in adipose tissue and show that this effect is required for FGF19 to increase caloric expenditure. However, we demonstrate that neither UCP1 induction nor an elevation in caloric expenditure are necessary for FGF19 to induce weight loss in obese mice. In contrast, the anti-obesity action of FGF19 appeared to be associated

with its known physiological role. In mice treated with FGF19, there was a significant reduction in the mRNA expression of genes associated with hepatic bile acid synthesis enzymes, lowered levels of hepatic bile acid species, and a significant increase in fecal energy content, all indicative of reduced lipid absorption in animals treated with FGF19.

2.1.4 Conclusion

Taken together, we report that the anti-obesity effect of FGF19 occurs in the absence of UCP1. Our data suggest that the primary way in which exogenous FGF19 lowers body weight in mice may be through the inhibition of bile acid synthesis and subsequently a reduction of dietary lipid absorption.

2.2 Introduction

There has been a significant increase in the prevalence of individuals clinically diagnosed as overweight or obese in the United States (1). The relevance of increased adiposity is exemplified by the relationship between obesity and multiple chronic metabolic diseases including type 2 diabetes, non-alcoholic steatohepatitis, and cardiovascular disease (2-5). As such, there remains a need to identify therapeutics with the capability of treating this key underlying driver of metabolic disease (6). In line with this, the endocrine hormone fibroblast growth factor 19 (FGF19) has emerged as a potential therapy for the treatment of obesity and its associated comorbidities(7-10).

FGF15, the mouse ortholog of FGF19, is an ileum derived metabolic factor that functions in the postprandial period to regulate bile acid homeostasis (11). Promoting lipid absorption, bile acids are made in the liver, stored in the gall bladder, and subsequently released into the small intestine (12). Deletion or overexpression of FGF15 causes defects in bile acid production (11, 13). Furthermore, regulation of bile acids by FGF19/15 is dependent upon its action in the liver, where it binds to FGF receptor 4 (FGFR4) and inhibits the expression of cholesterol 7 alpha-hydroxylase (CYP7A1), the rate limiting enzyme in bile acid production (14, 15).

Both FGF19 and its endocrine FGF family member FGF21, appear to have broadly similar effects on whole-body metabolism. Animals overexpressing FGF19 or FGF21 as well as those treated with recombinant protein, exhibit elevated metabolic rates and decreased fat mass in

addition to improvements in glucose utilization, insulin sensitivity, and lipid profiles(16-20). We and others have previously shown that induction of UCP1 by FGF21 is required for its thermogenic effects. However, the majority of the metabolic benefits characteristic of FGF21 action remain in the absence of UCP1 (21). Similar to FGF21, the therapeutic benefits associated with FGF19 administration have also been proposed to be linked to the recruitment of UCP1 and other key thermogenic pathways in white and brown adipose tissue (14, 16, 17). However, the role of UCP1 in mediating the metabolic effects observed with FGF19 has not been fully investigated. To determine the specific contribution of UCP1 to the known pharmacology of FGF19, we treated wild-type (WT) and UCP1 null (UCP1KO) mice chronically with FGF19. We show that chronic FGF19 administration reduced body weight but did not increase energy expenditure in UCP1KO mice. UCP1 null animals treated with FGF19 exhibited a down regulation of hepatic genes linked to bile acid production, lowered levels of certain hepatic bile acid species, and a significant increase in fecal energy content. Taken together, these data suggest that the anti-obesity action of FGF19 may be driven by its classical role in the regulation of hepatic bile acid synthesis rather than the upregulation of thermogenic pathways in adipose tissue.

2.3 Materials and Methods

2.3.1 Animals

All animal studies were approved by the Eli Lilly and Company Institutional Animal Care and Use Committee. Obese male wild-type C57Bl/6NTac mice were obtained from Taconic Farms, male UCP1KO mice (B6.129-ucp1tmkz/J) and their WT siblings were obtained from the Jackson Laboratory. All animals were individually housed in a temperature-controlled (24°C–27 °C) environment with 12 h/12 h light/dark cycle. Wild-type and UCP1KO mice were fed a calorie-rich diet consisting of 40% fat, 39% carbohydrate, and 21% protein caloric content (TD95217; Envigo) for a minimum of 16 weeks prior to the start of treatment and had free access to food and water. During the study period, mice received a daily intraperitoneal injection with either vehicle (PBS) or recombinant human FGF19 (0.2 or 2 mg/kg). Recombinant human FGF19 (FGF19) was generated in house at Eli Lilly and Company. Food intake and body weights were recorded daily. Body composition was determined by quantitative nuclear magnetic resonance (QNMR) using an

Echo System instrument (ECHO MRI, 3-1 Composition Analyzer; Echo Medical Systems, Houston, TX).

2.3.2 Indirect calorimetry

Changes in metabolic rate associated with chronic dosing of FGF19 were assessed over an 8 day period using an open respirometer system (LabMaster System; TSE Systems, Bad Homburg, Germany). Briefly, oxygen consumption (VO2, mL/kg/h) and carbon dioxide production (VCO2, mL/kg/h) were measured throughout the 8 day dosing period. VO2 (mL/kg/h) and VCO2 (mL/kg/h) were used to calculate energy expenditure and respiratory exchange ratio (RER = VCO2/VO2). Animals had ad libitum access to water and high-fat diet when in metabolic cages. All measurements were made at approximately 24 °C. The effects of FGF19 on metabolic rate in UCP1KO mice and their WT siblings was determined during the final 24 h of dosing using the CLAMS™ animal monitoring system (Columbus instruments, Columbus, OH USA). Animals had ad libitum access to water and high-fat diet when in the CLAMS. All measurements were made at approximately 24 °C.

2.3.3 Tissue collection

Animals were euthanized by CO2 asphyxiation followed by exsanguination via cardiac puncture. Blood was collected in EDTA coated tubes; plasma was separated by centrifugation, aliquoted, and frozen for future analysis. Adipose tissues (epididymal white (eWAT), inguinal white (iWAT) and interscapular brown (iBAT)) and liver were removed and flash frozen in liquid nitrogen.

2.3.4 Determination of insulin sensitivity

Following 7 days of FGF19 treatment, insulin sensitivity was determined in wild-type and UCP1KO mice. Briefly, on the morning of the procedure, animals were fasted for four hours. The animals were anesthetized with isoflurane throughout the entire procedure. A blood sample was collected by tail clip method. Each animal received 10 μCi of [3H] 2-Deoxyglucose (Perkin–Elmer) and 0.5 U/kg of insulin (Humilin R, Eli Lilly and Company, Indianapolis, IN) by retro-orbital injection. Additional blood samples were taken at 2, 5, 10, 15, 20, and 30 min after injection.

The blood samples were treated with Barium Hydroxide and then precipitated with Zinc Sulfate. The samples were centrifuged, the supernatant was collected, and the radioactivity was measured by liquid scintillation. After the final blood collection, the animals were euthanized, and tissues were collected. The tissue samples were clamp frozen in liquid nitrogen. For in vivo glucose uptake, tissue samples were weighed and homogenized in 0.1% perchloric acid. The homogenates were combined with either water to determine total 2-deoxyglucose or barium hydroxide/zinc sulfate to determine free 2-deoxyglucose. Radioactivity was measured by liquid scintillation. Data are presented as μmol/100 g/min.

2.3.5 Lipid tolerance test

Following 7 days of FGF19 treatment, wild-type and UCP1KO mice were fasted overnight (14–16 h) in standard cages with ad libitum access to water. Mice were gavaged with 0.5 mL of olive oil and blood was collected for measurement of triglyceride content. Blood samples were collected via tail bleed using a Microvette® CB 300 K2E (Sarstedt) at 0, 1, 2, 3, and 5 h of the lipid challenge. Serum levels of triglycerides were quantified using a triglyceride assay kit (Liquicolor (Mono®)).

2.3.6 Hepatic bile acids and fecal energy content

To determine fecal energy content, feces were collected from WT and UCP1KO animals dosed with vehicle or FGF19 over the last 3 days of treatment. Fecal energy content was measured via bomb calorimetry (Covance Laboratories, Madison). Hepatic bile acids were analyzed as previously described (22).

2.3.7 Analysis of metabolites and circulating factors

Plasma triglycerides, cholesterol and free fatty acids were measured using a Hitachi 912 Clinical Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN). Insulin (Crystal Chem Inc., Downers Grove, IL, USA), total Adiponectin (BioVendor Inc., Asheville, NC, USA) and FGF21 (R&D Systems, Minneapolis, MN, USA) were measured by ELISA.

2.3.8 2.8. RNA isolation, RT and real-time quantitative PCR

Total RNA was isolated from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) and RNeasy lipid mini kit (Qiagen, Venlo, Netherlands). RNA concentration was determined with a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific). One μg of RNA was used to synthesize cDNA using a High-Capacity cDNA RT Kit (PE Applied Biosystems). Expression of mRNA was determined on a QuantStudio 7 system using Universal PCR Master Mix and TaqMan primers (Applied Biosystems). Data were normalized to β-actin and fold-change calculated using $2^{-\Delta\Delta}$CT.

2.3.9 Statistical analysis

All data were graphed and analyzed using GraphPad Prism, version 7.03. Data are presented as mean ± SEM. Statistical analyses performed included student unpaired t-test, one-way ANOVA or two-way ANOVA, followed by Dunnett's multiple comparisons test where appropriate. Differences were considered significant when $p < 0.05$*.

2.4 Results

2.4.1 FGF19 lowers body weight in obese mice

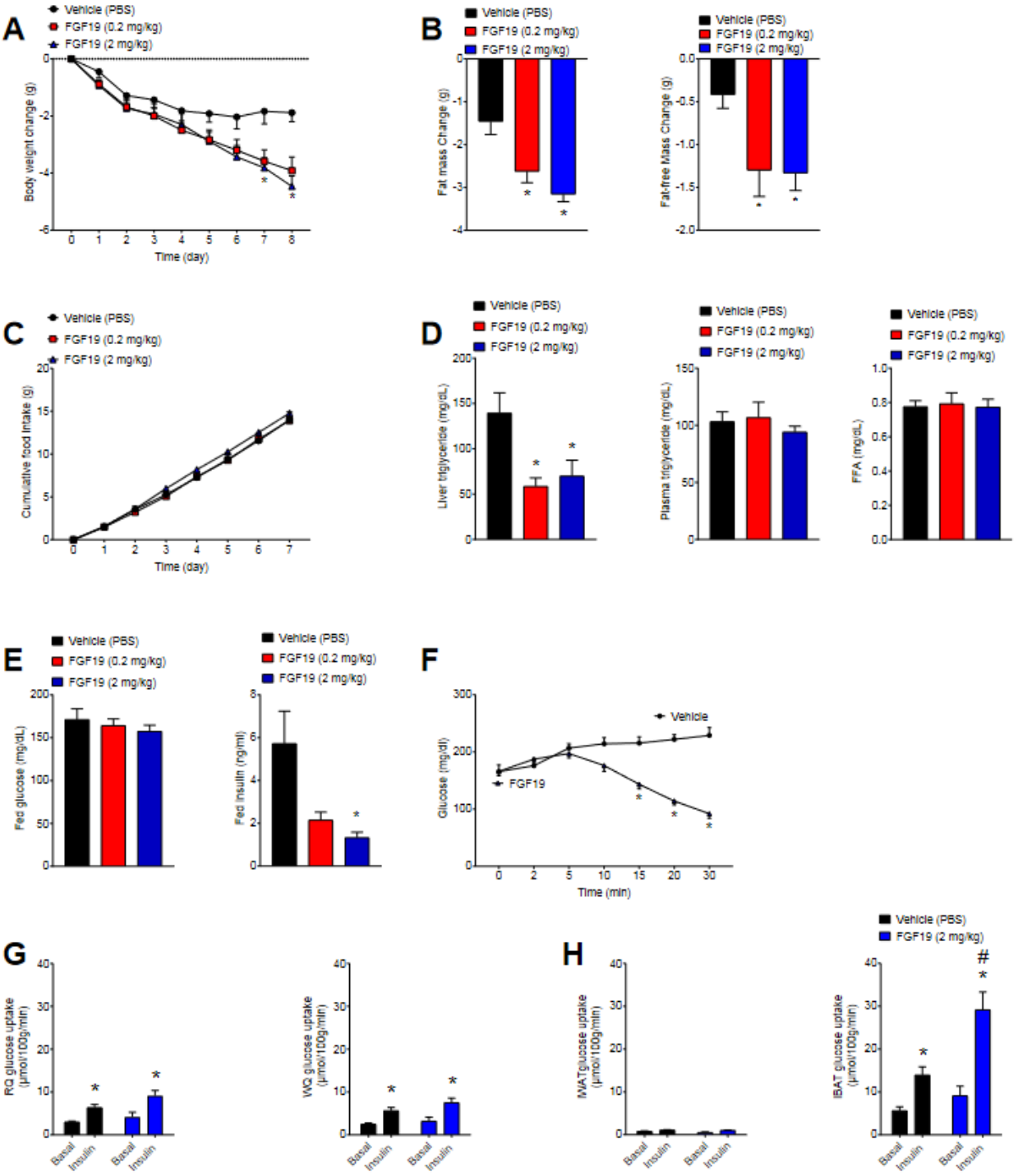
The anti-obesity action of FGF19 is suggested to be linked, in part, with its ability to induce a negative energy balance via an increase in whole-body caloric expenditure (16, 17). However, the kinetics of the onset of weight loss and the increase in caloric expenditure following FGF19 administration has not been shown. To address this, we evaluated the effect of daily subcutaneous administration of FGF19 (0.2 or 2 mg/kg) in obese mice over an 8 day period. As previously reported (16, 18), obese animals treated with FGF19 lost a significant amount of body weight, an effect which appeared to be saturated at a dose of 0.2 mg/kg of FGF19 per day (**Figure 2.1A**). This loss of body mass was a result of reductions in both fat mass and fat-free mass (**Figure 2.1B**) and was independent of changes in daily food intake (**Figure 2.1C**). Additionally, while there was no effect on circulating levels of triglycerides or free fatty acids (FFA), FGF19 treatment led to reductions in liver triglyceride content after 8 days of treatment (**Figure 2.1D**).

2.4.2 FGF19 enhances insulin stimulated glucose disposal

Next, we characterized the effect of FGF19 on glucose metabolism. While there was no effect of FGF19 on fed blood glucose, 8 days of FGF19 treatment did cause a significant reduction in fed plasma insulin levels (**Figure 2.1E, F**). Tissue insulin sensitivity was assessed in vivo by quantifying radiolabeled glucose uptake in response to a submaximal insulin dose (0.5 U/kg). Glucose clearance in response to insulin was significantly increased in FGF19 treated animals (**Figure 2.1F**). Tissue glucose uptake was determined in muscle (red and white quad) and adipose tissue (subcutaneous inguinal white (iWAT) and interscapular brown (iBAT)), to determine which tissues contributed to the enhanced glucose disposal. Basal glucose uptake was not different between vehicle and FGF19 treated mice however, insulin stimulated glucose disposal was significantly enhanced in iBAT of FGF19 treated animals (**Figure 2.1G, H**).

Figure 2.1. FGF19 lowers body weight in obese mice.

Obese mice (C57Bl/6NTac) were treated once a day with either vehicle (saline, n = 6) or FGF19 (n = 6 per dose group). Eight days of FGF19 treatment significantly lowered body weight when compared to control mice (A). $F(16, 20) = 6.416$, $p<0.001$ by two-way ANOVA. Weight loss was associated with a reduction in fat and fat free mass (B). $F = 11.57$, $p = 0.009$ and $F = 5.045$, $p = 0.0211$ by one-way ANOVA respectively. There was no effect of FGF19 treatment on daily food intake (C). $F(14, 105) = 0.9382$, $p = 0.5212$ by two-way ANOVA. FGF19 significantly reduced liver triglyceride content but had no impact on circulating triglycerides or free fatty acids (FFAs) at the end of the 8 day dosing period (D). $F = 5.958$ $p = 0.134$, $F = 0.4621$ $p = 0.664$, and $F = 0.04753$ $p = 0.9537$ by one-way ANOVA respectively. While there was no effect of FGF19 on fed glucose levels, there was a significant reduction in fed plasma insulin in animals treated with FGF19 (E and F). $F = 0.5354$ $p = 0.5962$ and $F = 6.638$ $p = 0.0086$ by one-way ANOVA respectively. In support of improved glycemic control, obese animals treated with FGF19 (n = 6) exhibited lower glucose levels in response to an insulin challenge (n = 6, F). $F(6, 60) = 57.75$, $p < 0.0001$ by two-way ANOVA. FGF19 treatment significantly enhanced insulin stimulated glucose disposal in interscapular brown adipose tissue (iBAT), but not muscle (red quadriceps [RQ], white quadriceps [WQ]) or white adipose tissue (iWAT) (G and H). $F(1,20) = 5.206$ $p = 0.0336$, $F(1,19) = 0.7176$ $p = 0.4075$, $F(1, 20) = 0.5382$ $p = 0.4717$, and $F(1, 18) = 1.223$ $p = 0.2833$ by two-way ANOVA respectively. Data are presented as mean ± SEM. $P < 0.05$* compared to vehicle and $p < 0.05$# compared to treatment.


A
Vehicle (PBS)
FGF19 (0.2 mg/kg)
FGF19 (2 mg/kg)
Body weight change (g)
Time (day)
B
Fat mass Change (g)
Fat-free Mass Change (g)
C
Cumulative food intake (g)
Time (day)
D
Liver triglyceride (mg/dL)
Plasma triglyceride (mg/dL)
FFA (mg/dL)
E
Fed glucose (mg/dL)
Fed insulin (ng/ml)
F
Vehicle
FGF19
Glucose (mg/dl)
Time (min)
G
RQ glucose uptake (μmol/100g/min)
WQ glucose uptake (μmol/100g/min)
Basal
Insulin
H
MAT glucose uptake (μmol/100g/min)
IBAT glucose uptake (μmol/100g/min)

2.4.3 FGF19 rapidly increase caloric expenditure in obese mice

To determine the impact of FGF19 on whole body metabolism, animals were housed in a TSE indirect calorimetry system for 7 days. FGF19 treated animals exhibited a time-dependent and dose-dependent increase in energy expenditure, which was statistically significant 48 h after study onset, and remained elevated throughout the treatment period (**Figure 2.2A**). There was no effect of FGF19 on carbohydrate and lipid oxidation rates (respiratory exchange ratio (RER)) when compared to vehicle treated animals (**Figure 2.2B**).

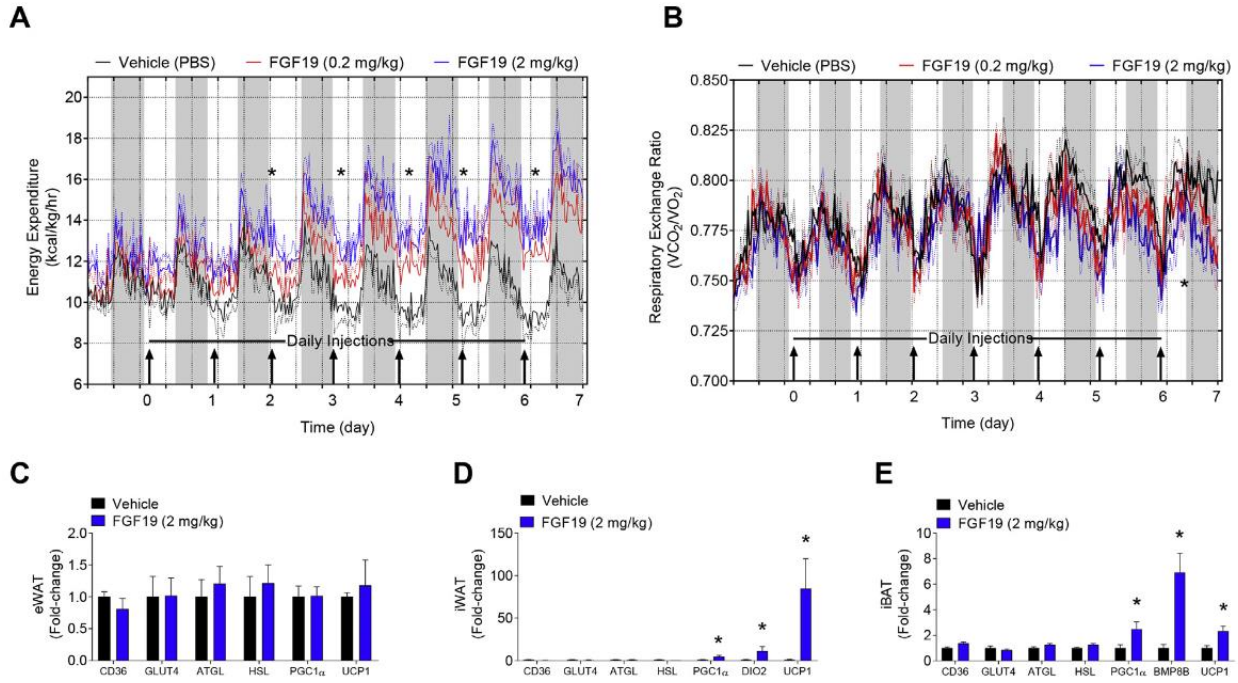


Figure 2.2. FGF19 increases caloric expenditure and thermogenic gene expression.

Energy expenditure was measured for 7 days in obese wild-type (C57Bl/6NTac) animals treated with FGF19 daily. FGF19 treated (n = 6) mice exhibited a time and dose dependent increase in caloric expenditure (kcal/kg/hr) when compared to vehicle (n = 6, A). $F(1005, 6700) = 2.595$, $p < 0.0001$ by two-way ANOVA. There was no effect of FGF19 on carbohydrate or lipid oxidation rates (respiratory exchange ratio (RER), VCO2/VO2, B). Data are presented mean ± SEM (dotted line in graphs). The expression of genes associated with thermogenic capacity was assessed in epididymal white (eWAT, C), inguinal white (iWAT, D) and interscapular brown (iBAT, E) adipose tissue. Thermogenic gene expression was elevated in both iWAT and iBAT, but not eWAT of animals treated with FGF19. $F(5, 60) = 1.319$ $p = 0.2682$, $F(6, 70) = 19.26$ $p < 0.0001$, and $F(5, 60)$ 0.1976 $p = 0.9622$ by two-way ANOVA respectively. Data are presented as mean ± SEM. $P < 0.05$* compared to vehicle

2.4.4 FGF19 induces thermogenic gene expression in adipose tissue

In support of FGF19 recruiting adipose tissue thermogenic pathways, gene expression analyses indicated a clear impact of FGF19 on gene transcripts associated with enhanced thermogenic activity at the high dose of FGF19 (2 mg/kg/day). In both white and brown adipose tissue, there was a significant increase in the mRNA expression of key regulators of mitochondrial capacity as well as thermogenic activity. Specifically, in subcutaneous white adipose tissue (iWAT) there was a significant increase in mRNA expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) as well as type II iodothyronine deiodinase (DIO2) (**Figure 2.2D**). Furthermore, in interscapular brown adipose tissue (iBAT) there was a significance increase in PGC1α and bone morphogenic protein 8b (BMP8b) (**Figure 2.2E**). Importantly, mRNA expression of UCP1 was significantly elevated in both iWAT and iBAT following 7 days of FGF19 treatment (**Figure 2.2D, E**). There was no effect of FGF19 on these transcripts, in visceral, epididymal (eWAT) white adipose tissue (**Figure 2.2C**).

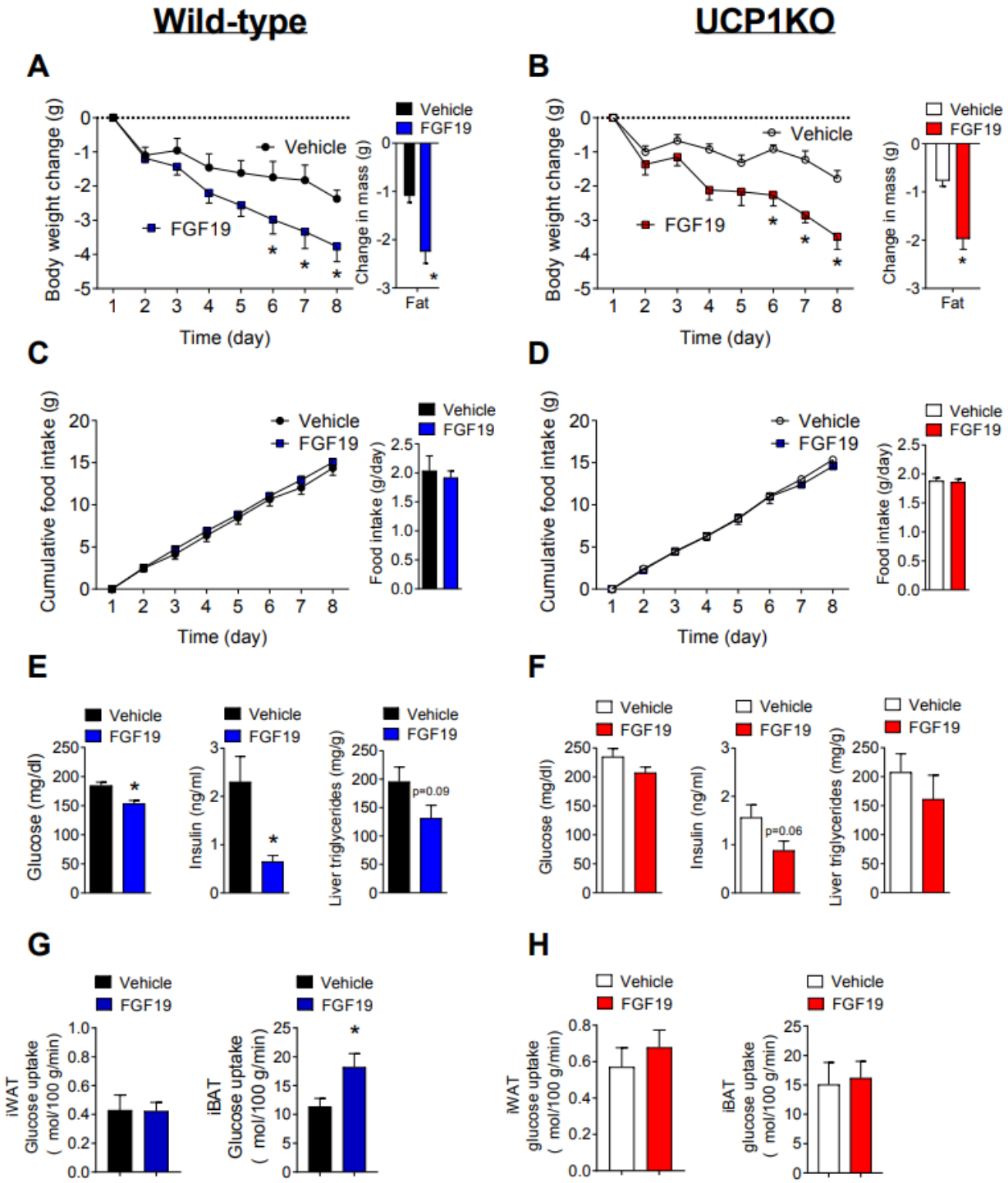
2.4.5 FGF19 does not require UCP1 to lower body weight in mice

To determine which facets of FGF19's effects require the activity of UCP1, we compared the pharmacological effects of FGF19 in mice with whole body deletion of UCP1 to their wild-type littermate controls (23). Surprisingly, chronic administration of FGF19 (2 mg/kg/d for 7 days) was equally efficacious at reducing body weight in obese WT (**Figure 2.3A**) and UCP1 null mice (**Figure 2.3B**). Furthermore, in an important contrast to our earlier work on FGF21, there was no effect of FGF19 on daily caloric intake in WT (**Figure 2.3C**) or UCP1KO (**Figure 2.3D**) mice. There was a significant reduction in fed glucose concentration, as well as a significant decrease in plasma insulin levels in WT (**Figure 2.3E**) treated with FGF19. However, in UCP1KO animals this effect of FGF19 on fed glucose was absent and there was only a trend ($p = 0.06$) towards significantly lowered insulin concentrations (**Figure 2.3F**). Of note, FGF19's therapeutic impact on insulin stimulated glucose disposal in iBAT of WT animals was not observed in UCP1 null animals (**Figure 2.3G, H**). To determine if the thermogenic effects of FGF19 depend on the presence of UCP1, whole body energy expenditure was evaluated at the end of the 7 day treatment period in both WT and UCP1KO animals treated with FGF19. Once again, we found that FGF19 treatment led to elevated energy expenditure in both the light and dark phase without affecting

substrate utilization rates in WT mice (**Figure 2.4A**). Conversely, the effect of FGF19 treatment on energy expenditure was entirely absent in UCP1KO animals (**Figure 2.4B**).

Figure 2.3. UCP1 is not required for the antiobesity effects of FGF19.

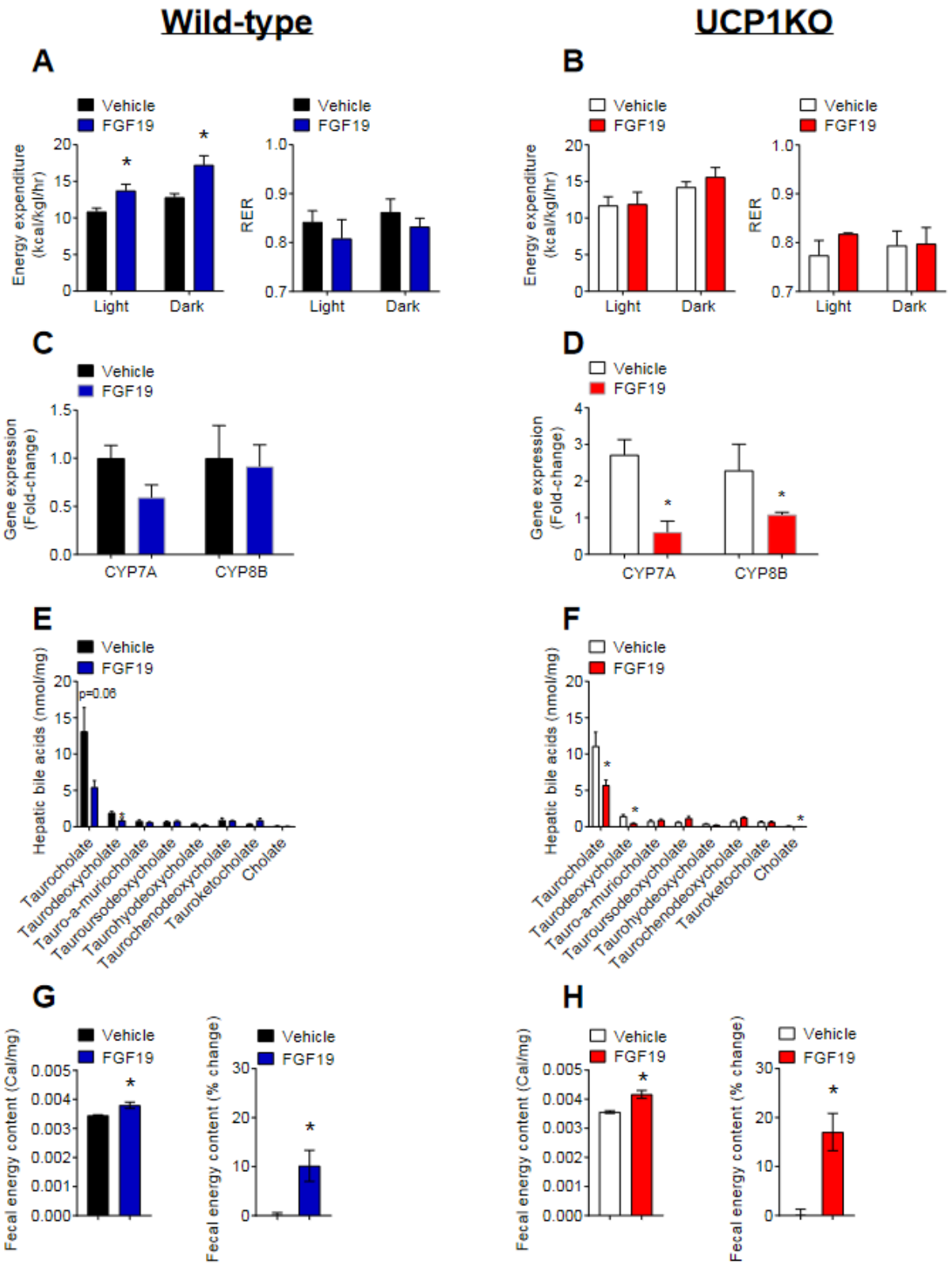
Both wild-type sibling controls (WT, B6.129-ucp1tmkz/J) and uncoupling protein 1 knockout (UCP1KO, B6.129-ucp1tmkz/J) mice were treated once daily with either vehicle (n = 6) or FGF19 (n = 6, 2 mg/kg). Chronic administration of FGF19 was equally efficacious at reducing body weight in obese WT and UCP1 null mice (A and B) without effecting food intake (C and D). $F(21, 154) = 3.91$ $p < 0.0001$ and $F(21, 140) = 0.6443$ $p = 0.8791$ by two-way ANOVA respectively. While there was not a significant reduction in fed glucose, FGF19 treatment led to reduced insulin levels in WT and UCP1KO animals (E and F). $F(3, 22) = 12.48$ $p < 0.0001$ and $F(3, 22) = 4.92$ $p = 0.0092$ by one-way ANOVA respectively. While insulin stimulated glucose uptake was increased in interscapular brown adipose tissue (iBAT) of WT animals, this effect was absent in UCP1KO treated animals (G and H). $F(3, 20) = 1.111$ $p = 0.3682$ by one-way ANOVA. Data are presented as mean ± SEM. $p < 0.05$* compared to vehicle.


Wild-type
UCP1KO
A
B
Body weight change (g)
Vehicle
FGF19
Time (day)
Change in mass (g)
Fat
C
D
Cumulative food intake (g)
Food intake (g/day)
E
F
Glucose (mg/dl)
Insulin (ng/ml)
Liver triglycerides (mg/g)
p=0.09
p=0.06
G
H
iWAT
iBAT
Glucose uptake (mol/100 g/min)
glucose uptake (mol/100 g/min)

The most well described biological function of FGF19 is its role in regulation of bile acid homeostasis (11). We found that FGF19 significantly reduced the mRNA expression of CYP7A and CYP8B in the liver of UCP1KO mice and although it did not reach significance, CYP7A was also decreased (approx. 50%) in WT animals when compared to vehicle (**Figure 2.4C, D**). To further characterize the effect of FGF19 on bile acid homeostasis, we measured hepatic bile acid content. FGF19 significantly lowered the amount of certain bile acid species in the liver of both WT and UCP1KO animals (**Figure 2.4E, F**). To determine if the reduction in body weight was associated with a reduction in energy absorption, we measured fecal energy content via bomb calorimetry in feces collected during the last 3 days of treatment in both WT and UCP1KO mice. There was a significant increase in fecal energy content in both WT (10% increase, **Figure 2.4G**) and UCP1KO (17% increase, **Figure 2.4H**) animals treated with FGF19 when compared to vehicle. The effect of FGF19 treatment on fecal energy content was significantly greater in UCP1KO animals compared to WT animals ($p = 0.035$).

Figure 2.4. UCP1 is required for the thermogenic effect of FGF19.

Whole body energy expenditure was measured in wild-type sibling controls (WT, B6.129-ucp1tmkz/J) and uncoupling protein 1 knockout (UCP1KO, B6.129-ucp1tmkz/J) animals dosed once daily with vehicle (saline) or FGF19 (2 mg/kg). FGF19 significantly increased caloric expenditure in WT mice during the light and dark phase (A) of their daily cycle, this effect was absent in UCP1 null animals (B). $F(3, 30) = 0.3191$ $p = 0.8115$ by two-way ANOVA. There was no effect of FGF19 treatment on substrate utilization rates in both genotypes (A and B). $F(3, 30) = 0.2452$ $p = 0.8641$ by two-way ANOVA. Hepatic expression of CYP7A and CYP8B mRNA was measured in both WT and UCP1KO animals (C and D). $F(3, 46) = 0.236$ $p = 0.8708$ by two-way ANOVA. Bile acid species were measured from livers of animals treated with FGF19 or vehicle (E and F). $F(27, 210) = 3.622$ $p < 0.0001$ by two-way ANOVA. While both WT and UCP1KO animals showed a significant increase in fecal energy content (G and H) this effect was significantly greater in UCP1KO animals compared to WT animals ($p = 0.035$). $F = 12.36$ $p < 0.0001$ by one-way ANOVA. Data are presented as mean ± SEM. $p < 0.05$* compared to vehicle.


Wild-type
UCP1KO
A
B
Vehicle
FGF19
Energy expenditure (kcal/kgl/hr)
RER
Light
Dark
C
D
Gene expression (Fold-change)
CYP7A
CYP8B
E
F
Hepatic bile acids (nmol/mg)
p=0.06
Taurocholate
Taurodeoxycholate
Tauro-a-muriocholate
Tauroursodeoxycholate
Taurohyodeoxycholate
Taurochenodeoxycholate
Tauroketocholate
Cholate
G
H
Fecal energy content (Cal/mg)
Fecal energy content (% change)

2.5 Discussion

Administration or overexpression of FGF19 in preclinical models of obesity and T2DM provides protection from weight gain and enhances glycemic control. The metabolic efficacy of FGF19 appears to be linked to its ability to increase whole-body caloric expenditure, an effect in rodents underpinned by the action of the thermogenic protein UCP1, in white and brown adipose tissue (16, 17). The primary objective of the current study was to determine the contribution of UCP1-dependent thermogenesis to the pharmacology of FGF19.

Firstly, we attempted to delineate in wild type mice, the impact of FGF19 treatment on caloric expenditure and weight loss. To do this, we conducted a time-course study monitoring both body weight and metabolic rate throughout FGF19 treatment. We found that the effect of FGF19 on body weight was significant by day 7 with no significant difference between the high and low treatment groups. FGF19 treated animals exhibited reductions in both fat mass and fat-free mass, furthermore, these effects were independent of changes in food intake. Since the liver is a major site of FGF19 action, we sought to determine the effect of FGF19 treatment on hepatic lipid content. While we found hepatic triglyceride content was lower in animals treated with FGF19, circulating triglycerides and FFAs were unchanged.

Next, we assessed the effects of FGF19 on glucose metabolism. While there was no effect on fed glucose levels, FGF19 treatment led to a reductions in fed insulin as well as improved insulin sensitivity. Importantly, we found that FGF19 treatment led to improved insulin stimulated glucose disposal in brown adipose tissue. Taken together, these data may indicate that FGF19 is an insulin sensitizer and that adipose tissue is an important mediator of its anti-diabetic action.

In agreement with previously published data (16, 17, 24), whole-body calorimetry showed that FGF19 treatment in WT animals does indeed cause an elevation in whole-body caloric expenditure, an effect that reached significance 48 h following the onset of treatment. Concordant with this finding, we also show that FGF19 treatment leads to increased expression of genes involved in thermogenesis, most notably UCP1, in both white and brown adipose tissue. These data show that an elevation of energy expenditure precedes weight loss, supporting the contention that the anti-obesity activity of FGF19 may be linked to its action on UCP1.

To directly test whether the pharmacologic effects we saw in our time-course study are dependent on UCP1-mediated thermogenesis, we treated both WT and UCP1KO animals chronically with FGF19. The primary novel finding of the current investigation is that FGF19 is able to induce weight loss in the absence of UCP1 and an elevation of whole-body caloric expenditure. In fact, in contrast to prior hypotheses, we show here that the activity of UCP1 in adipose tissue is dispensable for FGF19's anti-obesity action, illustrated by the fact that UCP1KO animals lost equivalent weight when compared to their WT counterparts. These results are in agreement with our previously published findings that activation of the predominant FGF receptor in adipose, FGFR1, is not required for FGF19-induced weight loss (24). However, in contrast to its anti-obesity activity, our data suggest that the action of UCP1 is required for FGF19 to enhance tissue specific insulin sensitivity. Specifically, in UCP1KO animals, FGF19 is no longer able to induce whole-body thermogenesis or insulin stimulated glucose disposal in brown adipose tissue. These findings support the contention that the pharmacologic effects of FGF19 treatment on caloric expenditure and glucose uptake in brown adipose tissue require the action of UCP1, potentially through FGF receptor 1 (FGFR1). In support of this, it has been demonstrated that an FGF19 variant unable to bind and activate FGFR1 was unable to improve blood glucose levels in *ob/ob* mice (25). Conversely, FGF19 retained its ability to improve glucose tolerance in FGFR4 null animals (14).

We have previously shown that weight loss in UCP1KO animals in response to FGF21 is associated with a reduction in caloric intake when energy expenditure is not increased (21). Interestingly, FGF19-induced body weight lowering in UCP1KO mice was not associated with a change in food intake. While neither FGF19 nor FGF21 are expressed in the brain, both their receptors and their requisite co-receptor (β-Klotho) are expressed centrally (26, 27). Indeed, central infusion of FGF19 or FGF21 is able to modulate feeding behavior and glucose homeostasis in rodents (16, 28-31). The importance of central signaling is highlighted by studies showing that many of the beneficial metabolic effects of both FGF19 and FGF21 are abrogated in animals deficient in central β-Klotho (32, 33). However, it is important to note that FGF21 crosses the blood-brain barrier more readily than FGF15/19 (34, 35); therefore, it may be the pharmacodynamic properties of FGF19 and FGF21, rather than differential FGFR activation, that delineates the differences observed between the current study and our previous work.

Next, we investigated whether the ability of FGF19 to induce weight loss independently of UCP1-mediated thermogenesis is tied to its known role in bile acid and lipid homeostasis. We found that FGF19 robustly inhibited hepatic transcription of CYP7A1 and CYP8B, proteins required for bile acid synthesis, in UCP1 null mice. While in this experiment there was only a trend towards FGF19 lowering CYP7A a key enzyme associated with bile acid production in WT animals, this action of FGF19 has been shown previously (14). However, it should be noted that this is effect is potentially due to the elevated hepatic expression of both CYP7A1 and CYP8b in UCP1KO animals compared to WT animals. When individual hepatic bile acid species were measured, we found that FGF19 treatment did indeed lead to significant reduction of certain bile acids in both WT and UCP1KO animals. As bile acids are crucial for absorption of lipophilic nutrients, we assessed fecal energy content in WT and UCP1KO animals treated with FGF19. Importantly, we found that FGF19 increased fecal energy content in WT mice and that this effect was greater in UCP1 null animals ($p = 0.035$). These data suggest that the primary way in which pharmacologic administration of FGF19 reduces body weight may be through the inhibition of bile acid synthesis and subsequent impairment of lipid uptake in the gut.

2.6 Conclusions

Taken together, we report here for the first time that the anti-obesity effect of FGF19 occurs in the absence of the key thermogenic protein UCP1. Our data suggest that the primary way in which pharmacologic administration of FGF19 reduces body weight may be through the inhibition of bile acid synthesis and a subsequent reduction of dietary lipid absorption.

2.7 Acknowledgements

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CHAPTER 3. THE HEDGEHOG SIGNALING PATHWAY IS EXPRESSED IN THE ADULT MOUSE HYPOTHALAMUS AND MODULATED BY FASTING

This work has been submitted for publication at *eNeuro*

Patrick Antonellis was the primary contributor to the design and execution of all experiments as well as all data interpretation and manuscript preparation.

3.1 Abstract

Primary cilia are found on almost all mammalian cell types and their dysfunction underlies a class of human genetic disorders known as ciliopathies (1). Obesity is a core clinical phenotype of certain ciliopathies such as Bardet-Biedl (BBS, OMIM #209900) and Alström (ALMS, OMIM #203800) syndromes (2, 3). Ciliopathy patients present a wide range of clinical symptoms, some of which are also associated with genetic defects in hedgehog signaling such as bone, limb patterning and genitalia malformations (4-8). Furthermore, the ciliopathy Carpenter syndrome (OMIM #20100) results from mutations in Rab23, a negative regulator of hedgehog signaling. Clinical features of patients with Carpenter syndrome include both skeletal malformations, and obesity (9, 10). Thus, these genetic disorders implicate a potential role for hedgehog signaling in regulation of energy homeostasis in humans.

Ciliopathy mouse models, as well as conditional animal models of cilia loss, have implicated hypothalamic neuronal cilia-mediated feeding behaviors in obesity (11-15). Neuronal primary cilia preferentially localize G protein-coupled receptors (GPCRs) known to be important for regulation of energy homeostasis and feeding behavior, such as neuropeptide Y receptors 2 and 5, melanin-concentrating hormone receptor 1 (MCHR1) and melanocortin-4 receptor (MC4R) (12, 16, 17). However, the impact of ciliary localization on their signaling capabilities is not well understood. We have previously demonstrated in primary mouse hypothalamic neurons *in vitro*, interactions between a ciliary GPCR and hedgehog signaling, suggesting that the hedgehog pathway may modulate GPCR activity at the cilium in differentiated neurons (18).

In mammals, the hedgehog pathway is dependent on the primary cilium as an organizing center (19-23). Components of the hedgehog pathway such as patched (PTCH1), smoothened

(SMO) and the Glioma (Gli) transcription factors GLI2 and GLI3 dynamically localize to the primary cilia (24-26). Upon binding to the ligand sonic hedgehog (SHH), PTCH1 leaves the cilia allowing for SMO to enter primary cilia. GLI2 is activated into its transcriptional activator (GLI2A) form while GLI3R formation is inhibited, leading to expression of Gli target genes, which includes *Ptch1* and *Gli1*, (27-30). Numerous other genes are known to regulate hedgehog signaling in embryonic development, such as *Gpr161*, an orphan GPCR shown to localize to cilia and negatively regulate pathway activity (31).

Beyond embryonic development, cilia and hedgehog signaling continue to play an important role in the postnatal brain for growth and the maintenance of neural progenitors (32-36). However, the role of hedgehog signaling in the adult hypothalamus is less clear. *In situ* hybridization studies have shown that *Shh*, *Ptch1*, and *Smo* mRNA are expressed in several regions of adult rat brain, including the hypothalamus (37-40). Here we characterize in greater detail the expression and transcriptional activity of the hedgehog pathway in the feeding centers of the adult hypothalamus *in vivo*. We also demonstrate that hedgehog pathway activity changes based on feeding status and this response is absent following the onset of obesity, suggesting a role for hedgehog signaling in the modulation of adult behaviors.

3.2 Materials and Methods

3.2.1 Animals

All procedures were approved by the Institutional Animal Care and Use Committee at Indiana University-Purdue University Indianapolis. Male C57BL/6J (stock 000664) mice were ordered from The Jackson Laboratory and housed on a standard 12-hour light dark cycle and given food and water *ad libitum* except for experiments as described. Chow fed mice were maintained on a standard chow diet consisting of 13% fat, 67% carbohydrate, and 20% protein caloric content (2014; Envigo). High fat diet (HFD) fed animals were maintained on a diet consisting of 40% fat, 39% carbohydrate, and 21% protein caloric content starting at 6 weeks of age (TD95217; Envigo).

3.2.2 In situ hybridization

Brains from 8 to 10 week old male C57BL/6J mice were harvested and fixed as previously described (41). Sections were cut at a thickness of 15 μm and mounted directly on slides then post-fixed with 4% paraformaldehyde for 16 hr at 4ºC. Detection of transcripts in brain sections was performed using the RNAscope 2.5 HD Duplex Assay (ACD, Newark, CA). Tissue pretreatment was performed according to technical note 320534 Rev A. Probe hybridization, counterstaining, and mounting of slides was performed according to user manual no. 322500-USM Rev A. Slides were assayed using probes to SHH (Cat No. 314361), SMO (Cat No. 318411), GLI1 (Cat No. 311001), PTCH1 (Cat No. 402811), GPR161 (Cat No. 318111), AGRP (Cat No. 400711-C2), POMC (Cat No. 314081-C2), MC4R (Cat No. 319181-C2), MCHR1 (Cat No. 317491-C2), or GFAP (Cat No. 313211-C2) transcripts (ACD). Sections were counterstained with hematoxylin, dehydrated, and mounted using VectaMount (Vectorlabs, Burlingame, CA). Slides with positive control probe (PPIB-C1/POLR2A-C2; Cat No. 321651) and negative control probe (DapB; Cat no. 320751) were ran with each experiment. At least 3 animals were analyzed for each group.

3.2.3 Quantitative real-time PCR

RNA was isolated, cDNA prepared, and quantitative real-time PCR performed as previously described (18). Assays-on-Demand Gene expression probes (Applied Biosystems) were as follows: Shh Mm00436528_m1; Ptch1 Mm00436026_m1; Smo Mm01162710_m1; Gli1 Mm00494654_m1; Gpr161: Mm01291057_m1. CT values were normalized to β-actin, and relative expression was calculated by the ΔΔCT method and fold change was calculated by normalizing relative expression to the proper control.

3.2.4 Experimental design and statistical analyses

Whole hypothalamus was collected from 35 to 36 week old, C57Bl/6, lean and obese animals that were either allowed *ad libitum* access to food or fasted overnight. Region specific micropunches were collected from 7 to 8 week old, lean, C57Bl/6 mice using 1.0 mm Militex Biopsy Punch (Electron Microscopy Sciences). There was a minimum of 6 animals per treatment group. Statistical analysis was performed using two-way ANOVA and corrected for multiple

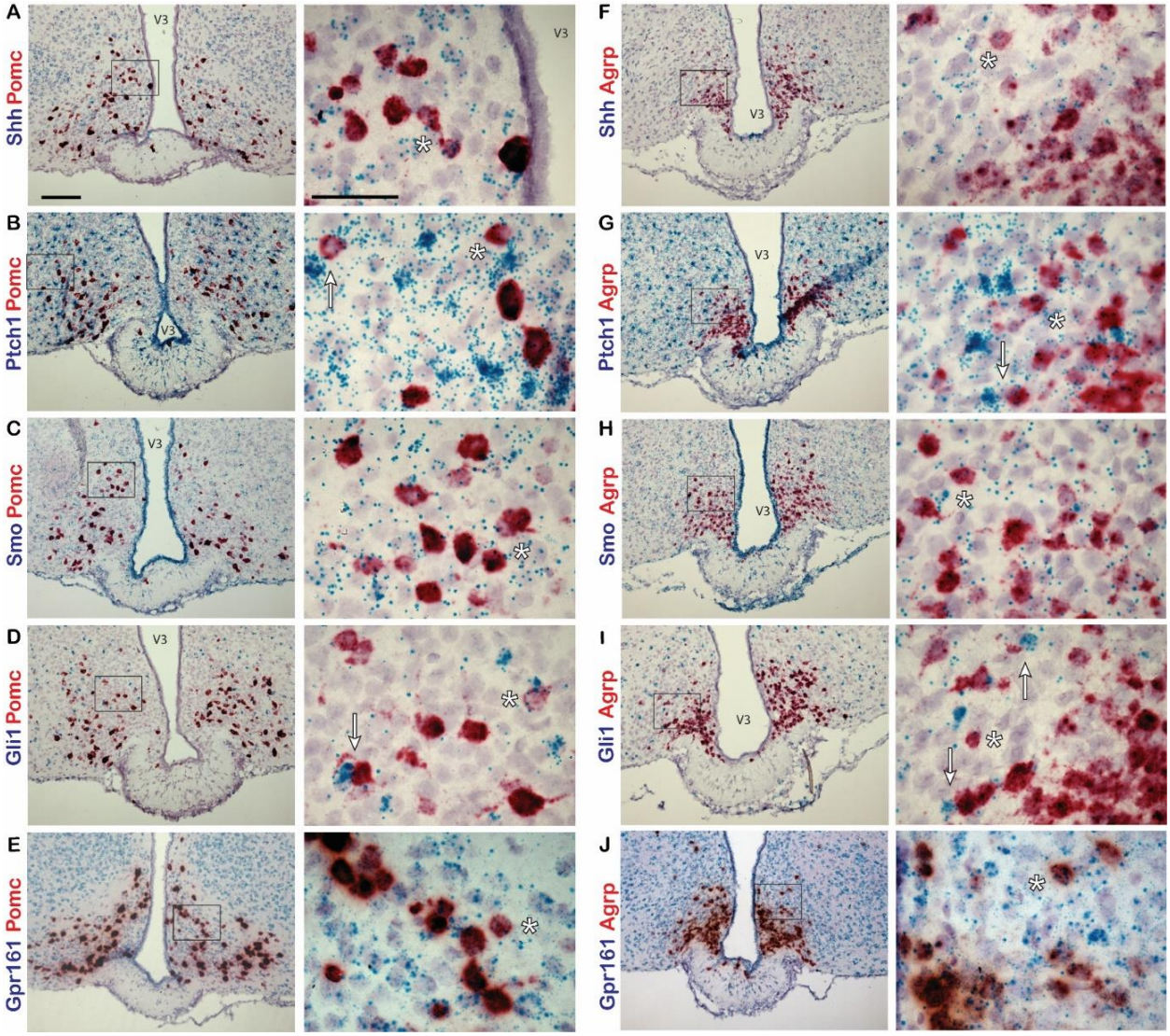
comparisons. Differences were considered significant when $P < 0.05$. Data are presented as mean ± SEM.

3.3 Results

To determine whether *Shh* and components of the signaling pathway are expressed in the adult mouse hypothalamus we performed *in situ* hybridization studies. Using a dual labeling approach, we first assessed whether neurons of one of the feeding centers, the arcuate nucleus of the hypothalamus (ARC), express hedgehog pathway genes. Two major neuronal subtypes within the ARC, the anorexigenic proopiomelanocortin (POMC) expressing neurons and orexigenic agouti-related protein (AgRP)/neuropeptide Y (NPY) coexpressing neurons, are crucial for normal energy homeostasis (42). Hypothalamic sections from adult, C57/Bl6 mice were labeled with probes to *Shh*, *Ptch1*, *Smo*, *Gli1*, or *Gpr161* and colabeled with probes to either *Pomc* or *Agrp*. In the ARC, we found that all hedgehog pathway genes assayed were coexpressed in neurons expressing *Pomc* (**Figure 3.1A-D**) and *Agrp* (**Figure 3.1F-I**). We also found that *Gpr161* is expressed at a relatively high level throughout the ARC (**Figure 3.1E** and **3.1J**). For each experiment, sections were labeled with positive and negative control probes (**Figure 3.2**).

Figure 3.1. Hedgehog pathway expression in the adult mouse ARC.

Dual probe in situ hybridization labeling of the ARC with probes to hedgehog pathway and neuronal gene transcripts. Pomc (A-E) and Agrp (F-J) probes are labeled in red while Shh (A and F), Ptch1 (B and G), Smo (C and H), Gli1 (D and I), and Gpr161 (E and J) probes are labeled in blue. Examples of cells colabeled by both probes are denoted by an asterisk (*). Pomc or Agrp expressing cells adjacent to highly expressing Ptch1 (B and G) or Gli1 (D and I) cells are denoted by an arrow. Right hand panels are magnified images of region in black box on left hand side. Scale bar 100 μm on left panels and 50 μm right panels. V3 indicates third ventricle.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V3
V3
B
Ptch1 Pomc
V3
C
Smo Pomc
V3
D
Gli1 Pomc
V3
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F
Shh Agrp
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V3
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Gpr161 Agrp

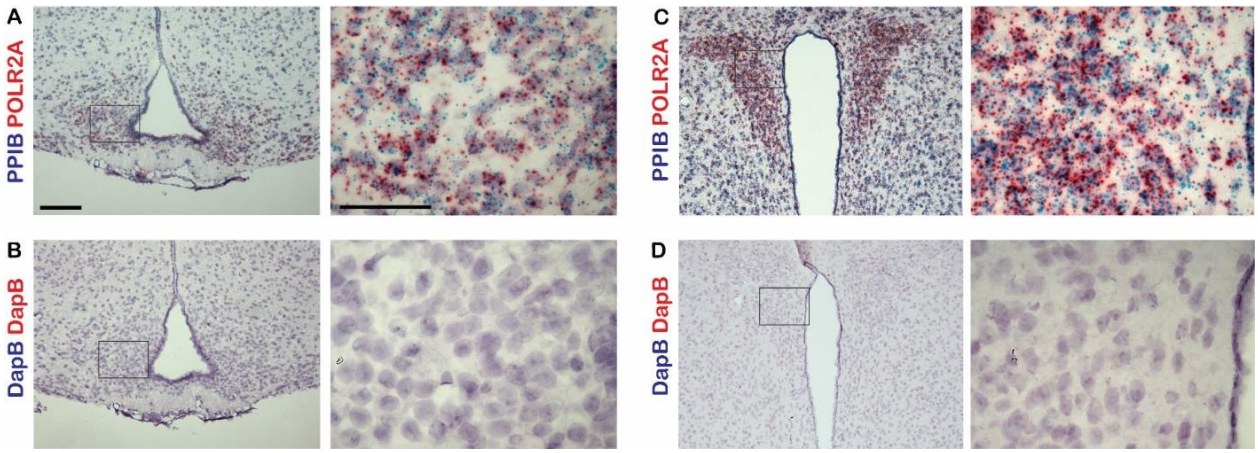


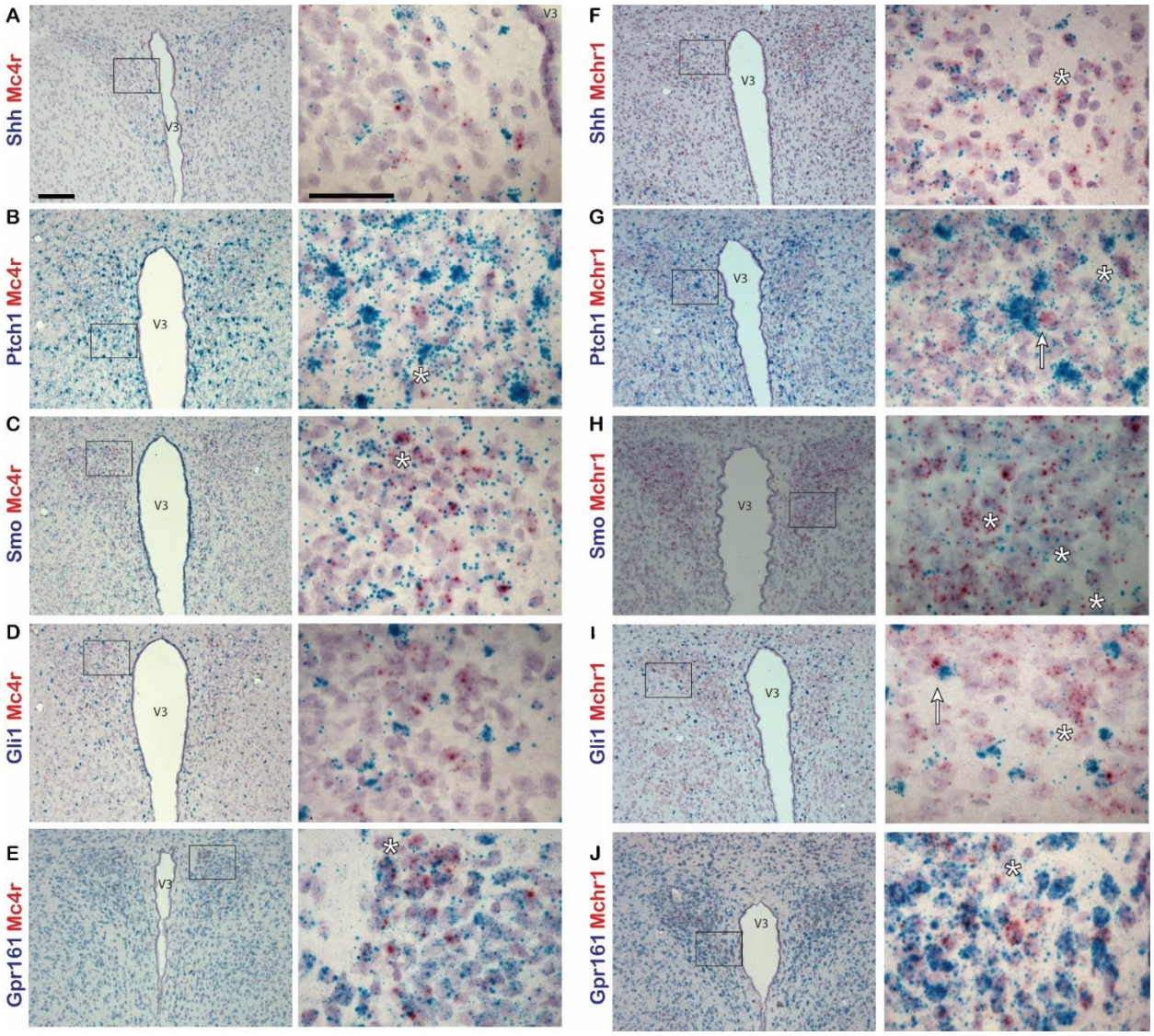
Figure 3.2. Controls for dual probe in situ hybridization studies.

Sections of adult mouse ARC and PVN were labeled with either positive control probes targeting the mouse genes Ppib and Polr2a (A and C) or negative control probes to the bacterial gene dapB (B and D). Right hand panels are magnified images of region in black box on left hand side. Scale bar 100 μm on left panels and 50 μm right panels

We next assessed whether *Shh* and its pathway components are also expressed in another feeding center, the paraventricular nucleus of the hypothalamus (PVN). We labeled sections of hypothalamus with probes to *Shh*, *Ptch1*, *Smo*, *Gli1*, or *Gpr161* and colabeled with probes to either *Mchr1* or *Mc4r*. Both MCHR1 and MC4R are known to be important for regulation of energy homeostasis and feeding behavior and localize to primary cilia in neurons (Berbari et al., 2008;Siljee et al., 2018). In the PVN, we observed relatively few *Mc4r* positive neurons with sparse incidence of colabeling with probes to hedgehog pathway genes (**Figure 3.3A-D**). However, *Mchr1* positive neurons were much more abundant and were frequently colabeled with probes to all hedgehog pathway genes used (**Figure 3.3F-I**). As in the ARC, *Gpr161* is expressed abundantly throughout the PVN (**Figure 3.3E** and **J**).

Figure 3.3. Hedgehog pathway expression in adult mouse PVN.

Dual probe in situ hybridization of probes for hedgehog pathway gene transcripts colabeled with probes to neuronal gene transcripts in the PVN of adult mice. Mc4r (A-E) and Mchr1 (F-J) probes are labeled in red while Shh (A and F), Ptch1 (B and G), Smo (C and H), Gli1 (D and I), and Gpr161 (E and J) probes are labeled in blue. Example of cells colabeled by both probes are denoted by an asterisk (*). Mchr1 expressing cells adjacent to highly expressing Gli1 cells are denoted by an arrow (I). Right hand panels are magnified images of region in black box on left hand side. Scale bar 100 μm left panels and 50 μm right panels. V3 indicates third ventricle.


A
Shh Mc4r
V3
V3
B
Ptch1 Mc4r
V3
C
Smo Mc4r
V3
D
Gli1 Mc4r
V3
E
Gpr161 Mc4r
V3
F
Shh Mchr1
V3
G
Ptch1 Mchr1
V3
H
Smo Mchr1
V3
I
Gli1 Mchr1
V3
J
Gpr161 Mchr1
V3

Interestingly, we observed cells highly positive for either *Gli1* or *Ptch1* expression throughout the ARC and PVN. Some of these cells appeared adjacent to neurons expressing *Pomc*, *Agrp*, or *Mchr1* (**Figure 3.1B, D, G, I,** and **3.3I**, **G**, arrows). Previously it has been reported that astrocytes of the adult mouse brain are responsive to hedgehog signaling and increase *Gli1* expression upon pathway activation (43). Therefore, we sought to determine if these hedgehog responsive cells were astrocytes by colabeling with probes to either *Gli1* or *Ptch1* and glial fibrillary acidic protein (*Gfap*), an astrocyte marker. In the ARC (**Figure 3.4A** and **C)** and PVN (**Figure 3.4B** and **D**), cells highly expressing *Gfap* were observed colabeled with *Gli1* or *Ptch1*, suggesting that some of the hedgehog responsive cells adjacent to neurons may be astrocytes in the hypothalamus.

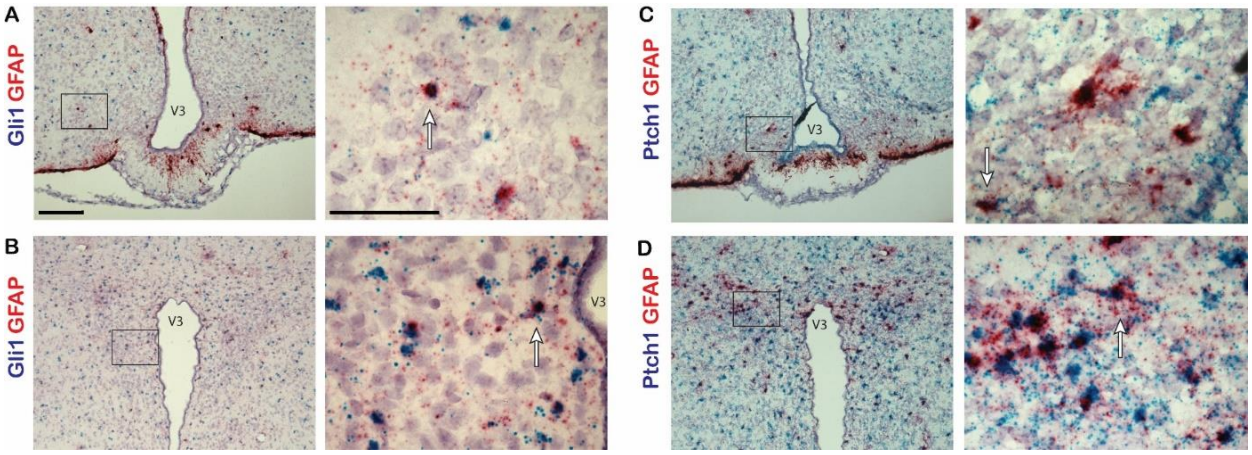


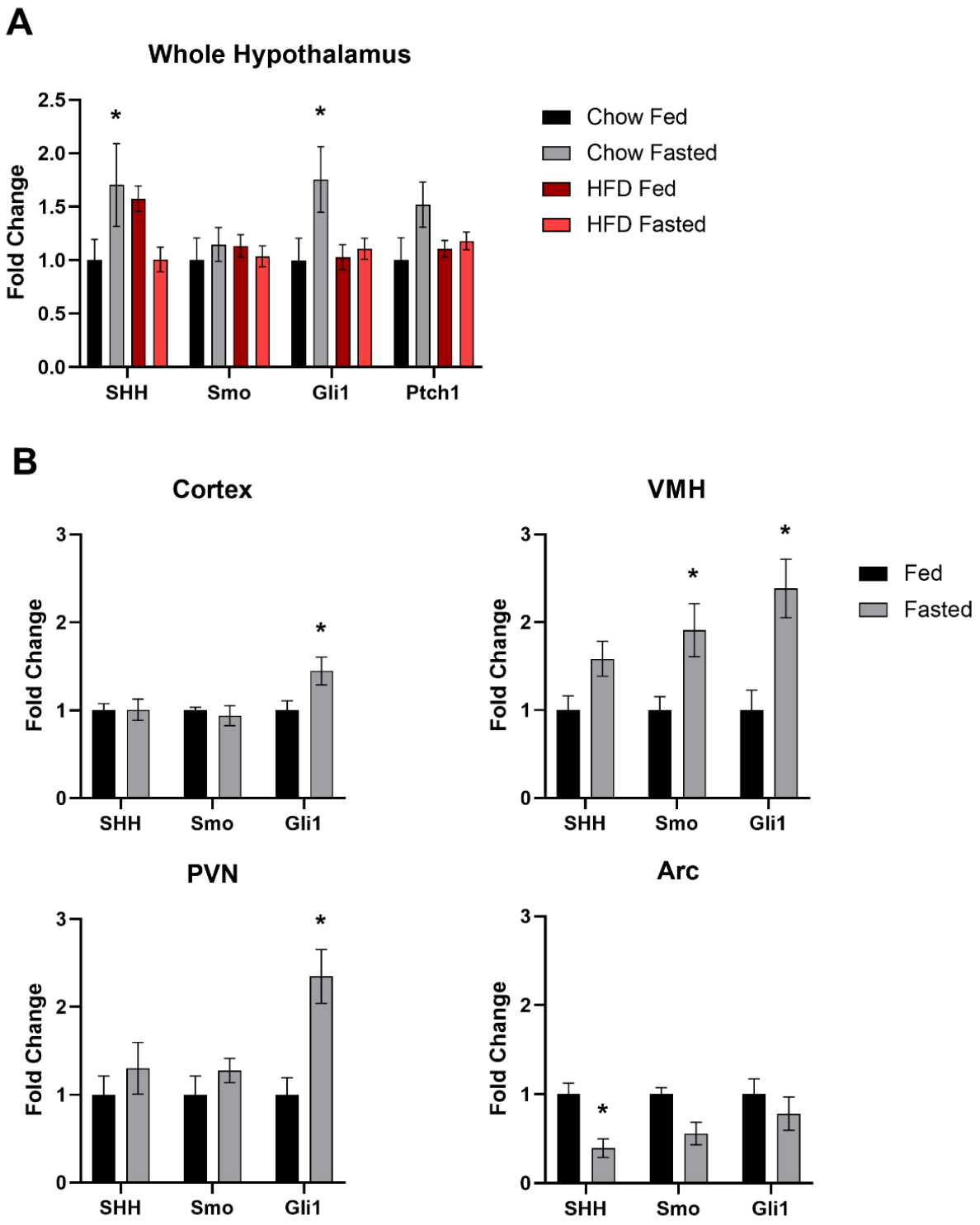
Figure 3.4. Gli1 and Ptch1 expression in astrocytes in adult mouse hypothalamus.

Dual probe in situ hybridization was performed with probes for Gli1 or Ptch1 and astrocyte marker Gfap gene transcripts. Sections of the ARC (A and C) and PVN (B and D) from adult mice were colabeled with probes to Gli1 or Ptch1 labeled in blue and probes to Gfap labeled in red. Example cells which are highly positive for both probes are denoted by an arrow. Right hand panels are magnified images of region in black box on left hand side. 100 μm scale bar for left side panels, 50 μm scale bar on right side. V3 indicates third ventricle.

Since we determined that genes of the hedgehog pathway are expressed in neurons of the adult hypothalamus, we next wanted to determine whether these genes are transcriptionally regulated by physiological changes associated with the normal function of this brain region, such as nutritional status. This was accomplished by gene expression analysis of the whole hypothalamus from chow fed lean and high fat diet (HFD) fed obese animals in the fed and fasted state. HFD fed animals weighed significantly more than their chow fed counterparts at 35 weeks of age (57.1g +/- 0.97 versus 34.8g +/- 0.75; mean +/- SEM; $p < 0.05$, Student's t-test). Lean and obese animals were allowed either *ad libitum* access to food or fasted overnight, then whole hypothalamic RNA was collected for qPCR analysis. In lean animals there was a significant increase in both *Shh* and *Gli1* expression in the hypothalamus after an overnight fast (**Figure 3.5A**). The expression of *Ptch1* also was elevated in fasted compared to fed animals, but this effect was not significant (**Figure 3.5A**). Strikingly, there were no significant changes in gene expression in the hypothalamus of obese fasted vs fed animals (**Figure 3.5A**). Since transcriptional regulation was observed in response to fasting in control chow fed animals at the level of the whole hypothalamus, we wanted to assess which specific nuclei were contributing to this effect. Once again control, normal weight C57Bl/6J animals were either allowed *ad libitum* access to food or fasted overnight. Micropunches were then collected from the cortex, ventromedial hypothalamus (VMH), PVN, and ARC for qPCR analysis. We found the fasting induced increase in *Gli1* expression in the whole hypothalamus is driven by increases in *Gli1* expression specifically in the VMH and PVN but not the ARC (**Figure 3.5B**). We also found that *Gli1* was upregulated, to a lesser extent, in the cortex (**Figure 3.5B**). Additionally, expression of *Smo* was increased in the VMH, while *Shh* gene expression was reduced in the ARC following an overnight fast (**Figure 3.5B**). Finally, we performed qPCR on whole hypothalamus and cortex, a brain region known to exhibit hedgehog pathway activity (43-45), collected from adult animals. We found that all hedgehog pathway genes measured were more highly expressed in the hypothalamus than the cortex (**Figure 3.6).** Overall, these data demonstrate that not only does expression of the hedgehog pathway continue into adulthood in the hypothalamus, a region critical for energy homeostasis, but that specific nuclei respond with transcriptional changes based upon feeding status.

Figure 3.5. Feeding status mediated changes in hypothalamic hedgehog pathway gene expression.

(A) Hedgehog pathway gene expression in the whole hypothalamus of adult mice. Lean animals fed a standard chow or obese animals fed high-fat diet were allowed ad libitum access to food or fasted overnight. A minimum of 6 animals were used per treatment group. Whole hypothalamic RNA was used for qPCR. $F(9, 84) = 1.027$ $p = 0.4256$ by two-way ANOVA. (B) Hedgehog pathway gene expression in brain micropunches of adult mice. A total of 16 animals, 8 per treatment group, fed a chow diet were allowed ad libitum access to food or fasted overnight. Micropunches were taken from the cortex, VMH, PVN, and for qPCR analysis. $F(2, 36) = 2.708$ $p = 0.0802$, $F(2, 42) = 1.416$ $p = 0.205$, $F(2, 39) = 3.283$ $p = 0.0481$, $F(2, 33) = 0.9542$ $p = 0.3955$ by two-way ANOVA respectively. Asterisks denote a p-value of < 0.05.


A
Whole Hypothalamus
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
*
*
SHH
Smo
Gli1
Ptch1
Chow Fed
Chow Fasted
HFD Fed
HFD Fasted
B
Cortex
VMH
PVN
Arc
Fold Change
3
2
1
0
*
SHH
Smo
Gli1
Fed
Fasted

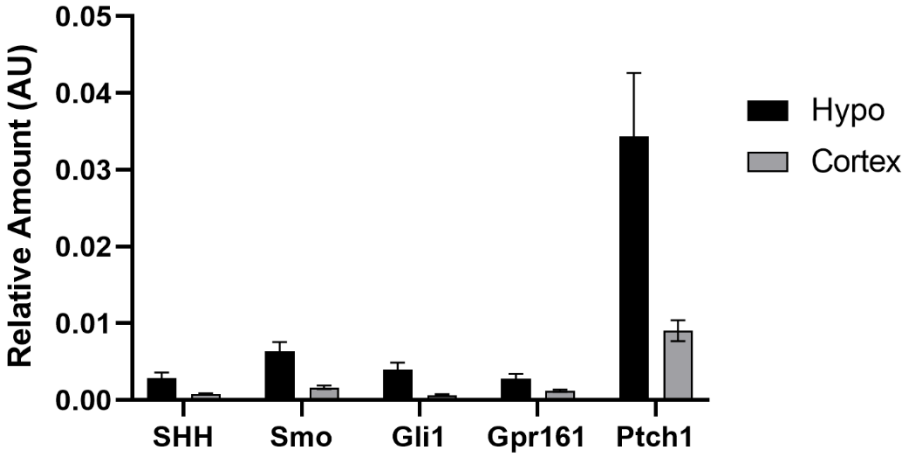


Figure 3.6. Expression of selected hedgehog pathway transcripts in the adult mouse brain. Whole hypothalamus and cortex were collected for qPCR analysis from 6 adult animals allowed ad libitum access to food. $F(4, 50) = 6.853$ $p = 0.0002$ by two-way ANOVA.

3.4 Discussion

Primary cilia are crucial for mediating hedgehog signaling in mammals and furthermore, certain ciliopathies are associated with pediatric obesity (46). Therefore, we focused our efforts on evaluating expression of the hedgehog pathway in hypothalamic feeding centers of adult mice. Because reliable commercial antibodies for many pathway proteins are currently unavailable, making immunolabelling studies difficult, we utilized a dual-labeling *in situ* hybridization approach which allowed for sensitive detection of hedgehog pathway gene transcripts in specific adult neurons. Our *in situ* data revealed the broad expression of *Shh* and core pathway components throughout the hypothalamus of adult mice. We found that mRNA for *Shh*, *Ptch1*, *Smo*, *Gli1*, and *Gpr161* could be detected in both the ARC and PVN. Within the ARC, each one of these transcripts are detected in neurons co-expressing *Pomc* or *Agrp*. While a similar labeling pattern was observed in the PVN, transcripts for hedgehog pathway genes were more readily found colabeled with probes to *Mchr1* than *Mc4r*. However, this could potentially be due to the relatively low abundance of MC4R expressing neurons or low mRNA expression for this particular GPCR. Our results expand upon prior studies which found hedgehog pathway expression in the adult rat brain (37-39) by demonstrating that neurons in two nuclei of the hypothalamus important for regulation of

feeding behavior express *Shh* and members of its signaling pathway. Somewhat surprisingly, we also found that *Gpr161* is expressed abundantly throughout both the ARC and PVN. This is contrary to previously published findings which used digoxigenin probes *for in situ* analysis of the adult mouse brain and showed a more restricted expression pattern of *Gpr161* in the nucleus accumbens and amygdala with low hypothalamic expression (47). Analysis of the amygdala and accumbens was outside the scope of the present study, and relative expression of *Gpr161* between these brain regions was not determined. However, a thorough understanding of this GPCR negative regulator of hedgehog signaling in the adult brain may reveal themes for its roles in cilia mediated behaviors.

Interestingly, in both the ARC and PVN we observed cells with high expression of either *Ptch1* or *Gli1* immediately adjacent to neurons expressing *Pomc*, *Agrp*, or *Mchr1*. By colabeling sections of hypothalamus with probes to *Gfap* and *Gli1* or *Ptch1 we* were able to identify some of these hedgehog responsive cells as *Gfap* positive astrocytes. While neurons of the adult mouse hypothalamus express *Shh* and components required for its signal transduction, the cells which are most responsive to hedgehog signaling, as indicated by high levels of *Gli1* and *Ptch1* expression, may in fact be astrocytes. This supports previous findings which showed that not only do neurons produce *Shh*, but subpopulations of mature astrocytes in the forebrain are responsive to hedgehog signaling (43). It remains unclear why these *Gfap* positive cells outside of known neurogenic niches exhibit high levels of *Gli1* and *Ptch1* relative to neighboring neurons.

There is growing evidence suggesting that the hedgehog pathway is involved in regulation of whole-body energy homeostasis. It has been demonstrated in both the fat-body of drosophila and adipose tissue of mice that hedgehog signaling regulates adipocyte differentiation (48, 49). Circulating forms of Hedgehog have been detected and during drosophila larval development shown to be secreted from the gut and act on multiple tissues to coordinate development with nutrient availability (50, 51). Given these findings, we also analyzed the expression of *Shh*, *Smo*, and *Gli1* in the adult mouse hypothalamus in response to changes in metabolic state and nutritional status. We compared transcriptional regulation of the hedgehog pathway in both lean, control diet fed animals and obese, HFD fed animals. Animals fed a HFD are a well-established model of non-insulin dependent type II diabetes which exhibit many hallmarks of metabolic dysfunction such as reduced glucose tolerance (52, 53). In the whole hypothalamus, expression of *Gli1* was

upregulated in lean mice following an overnight fast. Congruent with this finding, expression of *Ptch1* exhibited on non-significant upregulation in lean, fasted animals. In contrast, we observed no significant transcriptional regulation in the hypothalamus of fasted obese animals compared to fed controls. These data suggest that hedgehog signaling is involved in the physiological response to fasting and may be dysregulated in obese animals. To determine if this fasting-induced upregulation of *Gli1* is a response generated by the whole hypothalamus or specific nuclei, micropunches were collected from hypothalamic nuclei as well as the cortex. We found that in fasted animals *Gli1* expression was elevated specifically in the VMH, PVN, and to a lesser extent the cortex, but not the ARC. These data suggest that increased hedgehog pathway activity upon fasting in the hypothalamus is primarily driven by the VMH and PVN. Future studies will determine the cell types responsible within these nuclei for this increase in *Gli1* expression. Additional work is also required to determine the source of pathway activation observed in these studies. It is possible ligand is produced outside of the hypothalamus, therefore, further analysis could potentially reveal the primary source of *Shh* following an overnight fast.

Taken as a whole, this data shows that neurons of the hypothalamus express both *Shh* and members of its signaling pathway required for signal transduction and that activity of this pathway is upregulated in response to fasting in discrete hypothalamic nuclei. Given our *in situ* data identifying astrocytes as being highly positive for *Gli1* and *Ptch1* in the hypothalamus, it is possible that astrocytes or other support cells are primarily responsible for this fasting induced upregulation of hedgehog signaling. We have previously shown in primary hypothalamic cultures consisting of both neurons and glia, that modulation of the hedgehog pathway alters the electrophysiological response to melanin-concentrating hormone (18). Interestingly, it has also been demonstrated in primary cortical cultures that the presence of astrocytes alters the response of neurons to agonism of the hedgehog pathway (54). Furthermore, astrocyte specific inhibition of hedgehog signaling *in vivo* was shown to disrupt early postnatal organization and remodeling of cortical synapses resulting in increased neuronal excitability (44). Together, these findings suggest novel potential roles for hedgehog signaling outside of its roles as a classical developmental morphogen or in stem cell niche regulation.

The data presented here on the expression and transcriptional regulation of the hedgehog pathway in the adult mouse hypothalamus lays the foundation for future mechanistic studies to

determine its role in the proper functioning of the hypothalamus. Given that mammalian hedgehog signaling is coordinated by primary cilia, our future studies will focus on how hypothalamic hedgehog expression may contribute to the obesity phenotype seen in ciliopathies such as Bardet-Biedl syndrome and Alström syndrome (2, 3). Interestingly, certain ciliopathy clinical features such as skeletal and external genitalia abnormalities are also observed in patients with genetic defects in the hedgehog pathway (4-8). Therefore, it would be of interest to determine whether hedgehog signaling is dysregulated in the hypothalamus of animal ciliopathy models. Further mechanistic studies are needed to determine whether hedgehog signaling modulates neuronal activity critical for the physiological response to fasting and if genetic modulation of the hedgehog pathway in the hypothalamus alters feeding behavior. In conclusion, elucidating the involvement of this developmentally important signaling pathway in feeding behavior and body composition is an exciting new avenue of the hedgehog pathway to explore. Greater understanding of the hedgehog pathway in adult energy homeostasis may also reveal common themes for this pathway in regulation of other behaviors.

3.5 Acknowledgements

The following people are co-authors of this manuscript: Staci E. Engle, Kathryn M. Brewer, Nicolas F. Berbari

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CHAPTER 4. DISCUSSION

4.1 Endocrine FGFs in adult energy homeostasis

The endocrine FGFs have well described roles in the regulation of metabolic processes in adults. Two family members, FGF19 and FGF21, have shown similar effects on whole body metabolism. Both FGF19 and FGF21, when either overexpressed or administered pharmacologically in mice results in increased metabolic rates, improved glucose tolerance and insulin sensitivity, and reduced body weight (1-4). Due to these beneficial metabolic effects, FGF19 and FGF21 have been pursued as therapeutics for the treatment of metabolic disease such as obesity and its comorbidities. Non-alcoholic steatohepatitis (NASH) is a subset of non-alcoholic fatty liver disease (NAFLD). NASH is characterized by steatosis, lobular inflammation, cellular ballooning and fibrosis and can lead to cirrhosis and hepatocellular carcinoma (HCC) (5). Indeed, in animal models of NASH, treatment with FGF19 and FGF21 analogs have demonstrated improvements in pathological features such as steatosis (6, 7).

While FGF19 and FGF21 exhibit similar pharmacological effect there are distinct differences in the biological function of these two related proteins. FGF15/19 is produced in the ileum where it signals to the liver to inhibit bile acid synthesis in the post-prandial state (8). FGF21 is expressed in the liver where it is upregulated in response to metabolic stressors such as fasting, glucose challenge and protein restriction (9-11). FGF21 signals to adipose where it enhances glucose and lipid uptake as well as stimulating adipogenesis and reducing lipolysis (2, 12-14). It has also been shown that central action of FGF21 is able to mediate its metabolic effects such as modulation of food intake and energy expenditure (15, 16). The disparate biological effects of FGF15/19 and FGF21 are likely due to differing receptor complexes which mediate their signaling. While both FGF15/19 and FGF21 require the presence of the co-factor β-Klotho (KLB) to signal, they exhibit differing functional interactions with FGFR/β-Klotho complexes (17, 18). FGF21 has high affinity for FGFR1 and to a lesser extent FGFR3, whereas FGF19 has high affinity for FGFR4 but is also able to activate FGFR1 (19, 20). The FGFR1/KLB complex is expressed in regions of the brain and in adipocytes while the FGFR4/KLB complex is expressed in hepatocytes (20, 21). Biologically, the primary sites of action of FGF21 are adipose tissue and the CNS, while FGF19 acts in a paracrine fashion on hepatocytes.

The data presented here on the role of UCP1 in the pharmacological effect of FGF19 supports the contention that activation of adipose tissue mediates specific aspects of FGF19 pharmacology. We demonstrate that FGF19 treatment does not induce whole-body thermogenesis or insulin stimulated glucose disposal in brown adipose tissue in UCP1KO animals. This suggests that these pharmacologic effects of FGF19 treatment require the action of UCP1, potentially through FGFR1 activation. Somewhat surprisingly, we found that UCP1KO animals lose the same amount of body weight compared to wild type animals following FGF19 treatment. Importantly, we found that in response to FGF19 treatment UCP1KO animals exhibited an enhanced suppression of bile acids and increased fecal energy content compared to wild type animals. This suggests that impaired lipid uptake in the gut due to inhibition of bile acid synthesis contributed to the effect of FGF19 on body weight.

FGF15/19 signaling also has a described role in hepatocyte proliferation and has been linked to the development of HCC. It has been demonstrated that animals treated with FGF19 exhibit hepatocyte proliferation and that this effect is mediated by FGFR4 activation (22, 23). Furthermore, animals overexpressing FGF19 develop HCC (24). Therefore, a comprehensive understanding of the biology and signaling requirements of FGF19 will greatly aid in avoiding potential mitogenic risks of FGF19 based therapeutics for NASH. Indeed, an engineered variant of FGF19 has been generated which does not induce cell proliferation through the STAT3 pathway while maintaining FGFR4 activity (25). In an animal model of NASH, treatment with this FGF19 variant leads to improvements in steatohepatitis and fibrosis while avoiding tumor formation observed with wild type FGF19 (26).

4.2 Hedgehog signaling in the adult hypothalamus

Our work described here provides detailed characterization of the expression of core components of the hedgehog signaling pathway in the adult hypothalamus. Previous studies found that certain hedgehog pathway components are expressed in the adult rat hypothalamus (27, 28). However, through the use of dual labelling *in situ* hybridization, we are able to expand upon these findings by showing that neurons within specific nuclei of the hypothalamus important for regulation of feeding behavior express *Shh* and members of its signaling pathway. We also assessed the expression of a recently described negative regulator of hedgehog signaling, *Gpr161*,

which has been previously reported to have a somewhat restricted expression pattern in the nucleus accumbens and amygdala with low hypothalamic expression (29). In contrast to these findings, we found that *Gpr161* is expressed abundantly throughout both the ARC and PVN. We also identify cells responsive to hedgehog signaling, as indicated by high levels of *Gli1* and *Ptch1*. By colabeling with probes to *Gfap*, a marker for astrocytes, we were able to identify some of these hedgehog responsive cells as *Gfap* positive astrocytes. Overall these findings support previous studies in the forebrain which showed that neurons produce *Shh* while subpopulations of mature astrocytes are responsive to hedgehog signaling (30).

To determine whether the hedgehog pathway is involved in the functioning of the adult hypothalamus *in vivo*, we measured transcriptional activity via qPCR following an overnight fast. Indeed, we found that expression of *Gli1* was significantly upregulated in the hypothalamus of fasted animals compared to fed animals. Interestingly, the same upregulation of *Gli1* following an overnight fast was not observed in the hypothalamus of obese animals fed a high fat diet. Furthermore, through analysis of micropunches from specific hypothalamic nuclei we found that the upregulation of *Gli1* expression in response to an overnight fast is driven primarily by the VMH and PVN. These findings suggest that hypothalamic hedgehog signaling is upregulated in response to fasting in a nuclei-specific manor and may be dysregulated in obese animals.

Overall, these findings provide strong evidence that the hedgehog pathway is both expressed and active in the adult mouse hypothalamus. These observations suggest that hedgehog signaling is required in the biological functioning of the adult hypothalamus, however its mechanistic role is yet to be determined. Additionally, this work does not address the potential source of pathway activation. While we observed that neurons do indeed express *Shh*, we did not detect an upregulation of *Shh* expression in the fasted state. It is possible that ligand originates from brain regions outside the hypothalamus or even peripheral tissues. Therefore, a much more comprehensive characterization of *Shh* both centrally and peripherally would need to be performed. It should be noted that while ligand-independent activation of Gli1 has been observed, this has been primarily seen in cancer tissues (31).

This work identifies astrocytes within the hypothalamus as being responsive to hedgehog signaling, as well as the VMH and PVN as specific nuclei within the hypothalamus exhibiting

pathway activation in response to fasting. Future work will be directed at understanding which cell types within the PVN and VMH are mediating this transcriptional response. Specifically, it would be of interest to determine whether astrocytes within the VMH and PVN are responsible for hedgehog pathway transcriptional activity following a fast. This could potentially be assessed using commercially available fluorescent in situ assays. The *in situs* performed in these studies were chromogenic, which is semi-quantitative, and limited to labelling of two probes at a time. Fluorescent multiplex in situs can co-label up to 4 probes, which would allow for detection of *Gli1* transcripts within the PVN and VMH in both neurons and astrocytes. Furthermore, artificial intelligence-based analysis currently in use in the lab would greatly aid in cell type specific quantification. Another potential avenue to address this question would be to perform flow cytometry to separate astrocytes and neurons followed by qPCR. Such studies would need to be performed using hypothalamic micropunches, so material availability would need to be considered.

Another interesting finding was that obese animals fed a high fat diet do not exhibit the hypothalamic transcriptional upregulation of the Hh pathway observed in lean animals. This suggests that Hh pathway dysregulation contributes to obesity. However, these studies were unable to determine whether alteration in Hh signaling in the hypothalamus is a secondary consequence to the obese phenotype. In order to understand whether changes in central Hh signaling alter feeding behavior and energy homeostasis, experiments utilizing mouse ciliopathy models or genetic modulation of pathway components can be pursued. As previously discussed, hedgehog signaling is dependent on the primary cilia in mammals, therefore it would also be of interest to perform characterization studies in ciliopathy and cilia loss animal models. Multiple animal models of Bardet-Biedl syndrome such as *Bbs2*, *Bbs4*, and *Bbs6* KO mice recapitulate characteristics of the human syndrome including hyperphagia and obesity (32-34). Additionally, our lab and others have used a ubiquitously-expressed, tamoxifen-inducible Cre recombinase (CAGCreER) crossed to a conditional null allele of IFT88 to systemically disrupt cilia. When whole body cilia loss is induced in adult mice they become hyperphagic and obese (35-37). Characterization of hypothalamic hedgehog signaling in these models before and after the onset of obesity would help to determine whether dysregulation of hedgehog signaling due to cilia defects contributes to the obesity phenotype of these animals.

In addition to having a more detailed understanding of regional and cell type hedgehog pathway activity, it will be important to determine the functional consequence of pathway activation. Specifically, whether hedgehog signaling in the hypothalamus regulates neuronal activity. Using primary hypothalamic cultures, which consist of both neurons and glia, we have previously shown that modulation of the hedgehog pathway alters the electrophysiological response to melanin-concentrating hormone (MCH) (38). This is congruent with earlier studies using primary cortical cultures which showed that the presence of astrocytes alters the response of neurons to agonism of the hedgehog pathway (39). Electrophysiological recordings from brain slices could be performed to further elucidate whether the hedgehog pathway impacts neuronal activity. Neuronal activity can be measured following either treatment with hedgehog pathway activators and inhibitors alone, or pretreatment with hedgehog pathway modulators followed by application of signaling peptides such as MCH. Such studies would provide insight into the role of hedgehog signaling in the regulation of electrophysiological activity in the hypothalamus.

Perhaps one of the most important questions to be addressed is whether alterations in hypothalamic hedgehog signaling effects energy homeostasis and feeding behavior *in vivo*. Due to the developmental and post-natal role of Shh in the nervous system it would be beneficial to use genetic, inducible animal models to modulate hedgehog signaling. Because our findings show that transcriptional activity of the hedgehog pathway is highest in the VMH and PVN following a fast, future studies will focus on these brain regions. As discussed in previous sections MCHR1 is a ciliary GPCR that is expressed in the hypothalamus and is known to regulate feeding behavior and energy homeostasis (40). Our lab has generated and previously described a transgenic mouse where the *Mchr1* promotor drives expression of tamoxifen inducible CreER recombinase (41). To assess the effect of cilia loss on *Mchr1* expressing neurons, this transgenic animal can be crossed with the IFT88 floxed allele. Phenotypic characterization could then be performed to assess impacts on feeding behavior and energy homeostasis. Additionally, characterization of transcriptional regulation of the hedgehog pathway and electrophysiological activity of the hypothalamus via the methods previously discussed could also be performed. The Mchr1-CreER model can also be used to genetically modulate the hedgehog pathway in *Mchr1* positive neurons. For example, activation of the hedgehog pathway can be achieved by crossing the Mchr1-CreER line to a line carrying an inducible, constitutively active form of Smo (SmoM2) (42). In the

resulting animals Smo activity can be conditionally induced in *Mchr1* positive neurons. Once again phenotypic profiling as well as interrogation on the effects on hypothalamic hedgehog signaling will be performed. In addition to the hypothalamus, *Mchr1* is expressed in many brain regions including the striatum, hippocampus, and amygdala and has been shown to be involved in the regulation of anxiety, learning and memory (43-47). Therefore, interpretation of these studies will have to consider potential contributions of *Mchr1* activity outside of the hypothalamus. Furthermore, additional behavioral phenotyping of animals generated using the Mchr1-CreER line would likely need to be performed.

Another potential avenue of research would be to determine whether hedgehog signaling alters the ciliary localization of other GPCRs. As mentioned previously, when smoothened is knocked out from olfactory sensory neurons, cilia localization of odorant GPCRs is altered (48). Therefore, it is possible that changes in hedgehog signaling alter the cilia localization of GPCRs known to be involved in feeding behavior and energy homeostasis, such as Mchr1. One way to investigate this would be to measure ciliary localization of Mchr1 in brain sections from SmoM2 Mchr1-CreER animals by performing immunofluorescent labelling studies. Similar studies could be performed to assess localization of other ciliary GCPCRs following genetic or pharmacologic manipulation of the hedgehog pathway.

The role of hedgehog signaling in metabolic tissues such as the pancreas and adipose is related to its function in the maintenance of progenitor cell niches. It is possible that hedgehog signaling modulates the functioning of the hypothalamus through regulation of neurogenesis. In the adult brain, neurogenesis has been shown to occur in the hippocampus where granule-cell precursors (GCPs) in the subgranular zone (SGZ) of the dentate gyrus (DG) generate new granule cells, the excitatory neurons of the DG (49). Neurogenesis also occurs in the subventricular zone (SVZ), the area immediately adjacent to the lateral ventricles, where progenitor cells differentiate into new interneurons that reach the olfactory bulb (50). In both the SGZ and SVZ, GFAP positive progenitors have been identified as major contributors to neurogenesis (51-53). Furthermore, it has been demonstrated that hedgehog signaling regulates neural progenitors in the SGZ. Post-natal neurogenesis is enhanced by increases in hedgehog signaling while it is inhibited by reductions in hedgehog signaling (54, 55).

More recently, it has been shown that neurogenesis occurs in the adult hypothalamus and that it can be stimulated by neurogenic factors (56-58). The neural precursor cells of the hypothalamus have been identified by the expression of neural stem cell markers as tanycytes, some of which express GFAP (59-61). Tanycytes are radial-glial like cells located in the adult hypothalamic ventricular zone (60). Genetic, *in vivo* cell fate mapping studies have shown that tanycytes are capable of self-renewal and differentiation into other cell types, including mature neurons (61-63). Of particular significance, it has been demonstrated that changes in metabolic state such as feeding animals a high fat diet and physical activity alters the rate of neurogenesis in the hypothalamus (64-67). Additionally, intermittent fasting has been shown to induce neurogenesis in the hippocampus and is associated with increased memory in mice (68-70). It would be of interest to characterize neurogenesis in the hypothalamus of intermittently fasted mice and whether there are associated changes in the hedgehog pathway. Furthermore, because we observed GFAP positive, hedgehog responsive cells we could also investigate whether these cells contribute to any observed neurogenesis. This could be accomplished by performing BRDU labelling studies using GFAPCreER animals crossed to a reporter allele to label GFAP positive cells (71). This GFAPCreER line could also be used to explore the importance of hedgehog signaling in GFAP positive cells in the hypothalamic control of fasting.

These future mechanistic studies will help to elucidate potential roles of cilia mediated Hh signaling in the hypothalamic regulation of energy homeostasis. Importantly, these studies will determine whether Hh pathway dysfunction in feeding centers of the brain contributes to obesity. Insights such as these could potentially lead to novel therapeutic targets for the treatment of syndromic and non-syndromic forms of obesity.

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