USING MOUSE MODELS TO INVESTIGATE CILIOPATHIES

by

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A DISSERTATION

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Cilia and flagella are microtubule based organelles found on nearly every cell type in the mammalian body. Flagella have an essential role in sperm locomotion and reproduction, while motile cilia have a known role in fluid movement in the brain, lungs, and portions of the female reproductive tract. While the role of most primary cilia remained unknown for decades, they are now recognized as being essential to mammalian development, renal homeostasis, and satiety, just to name a few.

Because of their widespread presence and importance, dysfunction of cilia and flagella can lead to a variety of human diseases, collectively referred to as ciliopathies. These diseases present with a variety of symptoms including developmental defects, infertility, and obesity, reflecting the various functions of cilia and flagella. Studying the pathogenesis and molecular mechanisms of these diseases in humans is often made difficult due to both practical and ethical issues, and as such animal models are often used to determine the function of genes and proteins that are linked to human ciliopathies, in addition to elucidating what signaling pathways are being affected.

In particular, the mouse has proven to be an excellent model organism for studying ciliopathies. Being mammals, their physiology often closely matches that of humans, and the creation of cilia mutant mice has provided valuable information on how cilia are built, maintained, and what role they play in body. In the following thesis, I will report my findings documenting a previously uncharacterized mammalian cilia gene, Cluap1. I
will show that *Cluap1* mutant mice have phenotypes closely resembling that of mice with mutations in known Meckel-Gruber syndrome genes, a perinatally lethal ciliopathy. Similarly, my research into *Ccde42* has uncovered that loss of this gene causes azoospermia and male infertility in mice due to dysfunctional flagella assembly. This gene is highly conserved and is thus a good candidate loci that may be involved in infertility in human males. Finally, I will also discuss my work into the link between cilia and obesity. Previous studies have indicated the hyperphagia/obesity phenotype associated with cilia dysfunction is due to the development of leptin resistance. However, I show that a defect in leptin signaling is not the primary cause of the obesity phenotype, but instead involves a currently unknown mechanism.
DEDICATION

For my loving wife, Johanna.
ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Bradley Yoder, for his instruction and guidance during my graduate school education. He has taught me many valuable laboratory techniques, ranging from tissue microdissection to immunofluorescence microscopy to keg stands, and I will carry these lessons with me throughout my scientific career. I would also like to thank my committee members – Dr. Robert Kesterson, Dr. Elizabeth Sztul, Dr. Michael Miller, and Dr. David Schneider – who have guided me from my earliest days as a student at UAB. My gratitude also goes to Dr. Nicholas Berbari, who together we worked through the many ups and downs of the obesity project, in addition to instructing me on how to be a better graduate student.

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<td>FAA</td>
<td>food anticipatory activity</td>
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<td>FS</td>
<td>fibrous sheath</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GPCR</td>
<td>G protein coupled receptor</td>
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<td>Het</td>
<td>Heterozygous</td>
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<td>HTCA</td>
<td>head-tail coupling apparatus</td>
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<td>IFT</td>
<td>intraflagellar transport</td>
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<tr>
<td>IMCD</td>
<td>inner medullary collecting duct</td>
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<td>MCH</td>
<td>melanin concentrating hormone</td>
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<td>MRapX</td>
<td>marginal ring of the acroplaxome</td>
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<td>MSH</td>
<td>melanocyte stimulating hormone</td>
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<td>NL</td>
<td>nexin link</td>
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<td>NPY</td>
<td>neuropeptide Y</td>
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<td>ODF</td>
<td>outer dense fiber</td>
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<td>PCD</td>
<td>primary ciliary dyskinesia</td>
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<td>POMC</td>
<td>pro-opiomelanocortin</td>
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<td>Ptch1</td>
<td>Patched-1</td>
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<td>RMR</td>
<td>resting metabolic rate</td>
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<td>radial spoke</td>
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<td>round spermatid</td>
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<td>seminal vesicle</td>
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<td>Spermatogonia</td>
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<tr>
<td>SuFu</td>
<td>Suppressor of Fused</td>
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<td>WT</td>
<td>Wildtype</td>
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INTRODUCTION

Introduction to Cilia and Flagella

Primary cilia, motile cilia, and flagella are evolutionarily conserved organelles widespread through eukaryotic life [1-5]. Among vertebrates, motile cilia have a well-established role in fluid movement, as seen in the ventricles of the brain where the synchronized beating of motile cilia keep cerebral spinal fluid in constant circulation [6]. Motile cilia are also abundant in the trachea and larger airways of the lungs where they carry debris and other potentially harmful pathogens out of the respiratory tract [7]. Conversely, the flagellum is not involved in the day to day health of mammals, but they do impart locomotor abilities on the male sperm, thus proving essential for fertilization to occur and subsequent propagation of the species [8]. In contrast to the well-studied functions of motile cilia and flagella, the seemingly inert primary cilia seemed to play no observable role in most tissues. While it was widely recognized that highly modified primary cilia had a known role in vision and olfaction, this organelle in most other mammalian tissues was believed to be vestigial [9, 10]. Recent studies however, indicate the primary cilium has a wide variety of roles in both developmental biology and in the health of an adult organism. The widespread importance of cilia and flagella in human health is further reflected in the ciliopathies, a variety of diseases that are caused due to dysfunctional cilia and flagella [11].
As will be discussed in the following sections, all cilia and flagella share common structural elements and are assembled through the same fundamental process. It is because of these two facts that the words cilia and flagella are often used interchangeably.

Structure of Mammalian Cilia and Flagella

Nearly all eukaryotic cilia and flagella share a highly similar structure (Figure 1). The core of the organelle is a microtubule based axoneme that extends towards the tip of the cilium or flagellum. Transverse cross sections of motile cilia and flagella reveal 9 microtubule doublets that form a circle on the inside of the plasma membrane, and also an additional pair of microtubules at the center of the axoneme itself. This arrangement is referred to as a 9 +2 structure [12, 13]. Immotile primary cilia also have 9 microtubule doublets that form a circle on the inside of the plasma membrane, but lack the central microtubule pair, and are therefore referred to having a 9 + 0 structure [14, 15]. Motile cilia and flagella also possess nexin links and dynein arms that attach to the microtubule doublets, as well as radial spokes that project into the center of the axoneme [16, 17]. These structures are necessary for proper movement of the organelle. The axoneme is anchored to the cell through a basal body, a centrosome derived structure consisting of nine microtubule triplets arranged in a helical pattern [18]. Intraflagellar transport (IFT) complexes move up and down the length of the axoneme and are necessary for the assembly of cilia and flagella themselves, and they will be discussed in the following section.

The basal body serves as a nucleation site for the microtubule assembly that will make up the axoneme. In addition to this structure, transition-fiber and transition zone
Figure 1. Structure of eukaryotic cilia and flagella. Primary cilia possess an axoneme that contains nine microtubule doublets, and are referred to as being “9+0”. Motile cilia possess an additional pair of microtubules in the middle of their axoneme and thus have a “9+2” structure. Additionally, motile cilia have nexin links (NL) connecting the doublets, as well as dynein arms (DA) and radial spokes (RS) that help facilitate movement. Motile cilia and flagella are typically anchored to the cell itself through a centrosome derived basal body, while transition fibers regulate the entry and exit of proteins. IFT complexes move up and down the microtubule axoneme via molecular motors and are necessary for cilia and flagella assembly.
proteins are present in a specialized region between the basal body and the beginning of 
the axoneme [19]. This region is thought to regulate protein entry and exit from the cilia 
compartment, though this process remains poorly understood [20, 21].

The flagellum is anchored to the nucleus through a centrosome derived structure 
called the head-tail coupling apparatus (HTCA), which contributes the basal body and 
serves as a nucleation of the flagellum microtubule axoneme [22]. Unlike the basal body 
of mammalian cilia however, the basal body of the flagellum axoneme degrades once the 
sperm is mature [23].

While primary and motile cilia share many of these common elements, the exact 
protein composition and structure can vary depending on the cell type. This permits the 
cell to elicit unique responses to external cues. For example, while cilia and flagella both 
have microtubule based axonemes, those found inside flagella are typically much longer 
(in mice, approximately 115 μm for sperm flagella and 13 μm, for motile cilia) [24, 25]. 
The processes that control cilia length are poorly understood, but available levels of solu-
tible tubulin and cAMP signaling have been shown to play a role [26, 27].

Sperm flagella themselves can be divided into three main components, a mid-
piece, a principal piece, and an end piece (Figure 2). In the midpiece, mitochondria are 
wrapped into a highly compact sheet below the plasma membrane [28]. This mitochon-
drial sheet itself surrounds nine cytoskeletal based structures called outer dense fibers, 
which, unlike the mitochondrial sheath, also extend into the principal piece [29]. The 
outer dense fibers are highly modified cytoskeletal elements that provide elastic strength 
to the beating flagella [30]. The end piece is devoid of both the mitochondrial sheath and 
outer dense fibers, and instead simply contains the microtubule based axoneme and
Figure 2. Mature sperm cell. The sperm cell is made up of two main regions, the head and the tail. The anterior portion of the head is covered by the acrosomal cap and the head is joined to the tail by the connecting piece/head-tail coupling apparatus (HTCA). The tail is divided into three regions: the midpiece; principal piece; and the end-piece. The electron micrographs showing cross-sections (not to scale) of each region highlights the main components of the tail structure: the axoneme; outer dense fibers (ODF); and the mitochondrial sheath (midpiece) and fibrous sheath (FS) (principal piece). The end piece consists solely of the axoneme and plasma membrane.

plasma membrane [31]. Additional unique structures are also found in sperm cells. For example, the acrosome is a Golgi derived organelle that releases proteolytic enzymes during ova fertilization [32]. This structure is in turn anchored to the nucleus through the acroplaxome, an F-actin containing cytoskeletal structure than is necessary for shaping of the nucleus during spermatid development [33]. All of these structures must be built and maintained properly in order for the sperm to be fully motile and have reproductive function.

Intraflagellar Transport and Intramanchette Transport

The conserved structure of cilia and flagella across vastly different eukaryotic cells is a reflection of the fact that they are all built and maintained through the same fundamental process, intraflagellar transport (IFT). Although originally described in the green algae, *Chlamydomonas reinhardtii*, IFT is now known to be an evolutionarily conserved process and has since been found to be necessary for cilia and flagella assembly in *Caenorhabditis elegans*, *Tetrahymena*, *Danio rerio*, mammals, and others [34-37]. Cilia assembly, or ciliogenesis, thus appears to have arisen very early in the evolution of eukaryotes.

In minimalistic terms, IFT is the bidirectional movement of multi-protein complexes along the cilia axoneme [38]. These multi-protein complexes, or IFT particles, move in between the microtubule axonemes and ciliary membrane [34]. Work in *Chlamydomonas* has demonstrated the existence of two different classes of IFT complexes in the IFT particle, a larger IFT B complex, composed of 14 currently identified scaffolding proteins, and a smaller IFT A complex, which consists of 6 currently identified scaffold-
ing proteins [35, 39]. Current data suggests the two complexes play complementary roles in context of the cilia. Anterograde movement, or movement towards the tip of the cilia, is accomplished through the association of the IFT B complex with a heterotrimeric kinesin-II motor [40, 41]. This complex collectively allows the transport of cargo towards the tip of the cilia. Conversely, movement back towards the base of the cilia, or retrograde transport, is accomplished via the IFT A complex, which associates with an isoform of cytoplasmic dynein to ferry its cargo [42, 43]. Functional redundancy does not appear to be a property of the IFT proteins, as loss of function mutations in even a single IFT gene can prevent assembly of the entire complex [44].

The differing functions of the IFT B and IFT A complexes can be seen in the phenotypes that are present in mammalian cells with loss of function mutations in genes that belong to either group. For example, IFT88 is a protein which was biochemically demonstrated to be a member of the IFT B complex in *Chlamydomonas*. Mice with loss of function mutations in the mammalian homolog, *Ift88*, display a total loss of cilia on their cells, a reflection of the fact that the cells in these animals are no longer able to perform anterograde IFT [45]. Another example exists with a member of the *Chlamydomonas* IFT A complex, IFT144. Unlike *Ift88* mutants, which are lacking in cilia altogether, mouse *Ift144* mutants possess cilia. However, the cilia are stumpy and have bulbous tips indicative of an accumulation of proteins due to a non-functional IFT A complex to bring them back out [46]. Thus, classifying previously unrecognized mammalian IFT genes is possible based on the differing properties of the IFT B and IFT A complexes.

Although less understood than IFT, a highly similar process called intramanchette transport (IMT) also occurs in developing sperm cells [47]. During sperm maturation, a
transient microtubule based structure called the manchette surrounds the nucleus. The manchette is well noted for being vital in shaping and condensing the nucleus, proper placement of the HTCA, and development of the flagella [48]. Along the manchette, proteins are transported towards the HTCA and developing flagella [49]. IMT shares many similar molecular components to IFT. At its core, IMT consists of a raft protein complex transporting cargo proteins along microtubules by molecular motors. IFT proteins such as Ift88 and Ift20 have been implicated in this process, demonstrating the strong functional similarities between IFT and IMT [50]. The two processes appear to cooperate in assembling the sperm flagella; IMT places the HTCA and brings proteins to the flagella base, while IFT builds the flagella itself.

Human Ciliopathies and their Mouse Models

Diseases caused by dysfunctional cilia and flagella, collectively referred to as ciliopathies, comprise a heterogeneous group of disorders associated with mutations in genes resulting in abnormal cilia function (Table 1). As cilia are present on almost all mammalian tissue, cilia dysfunction can adversely affect a wide variety of biological systems and organs. Although there are too many ciliopathies to go into detail in this thesis, some of the most notable ciliopathies, and what mouse models have taught us about their pathogenesis, deserve special mention.

Meckel-Gruber syndrome

Some ciliopathies have such severe physiological consequences that they are not compatible with human life. Such is the case with Meckel-Gruber syndrome (MKS), an
**Table 1. Examples of ciliopathies and their symptoms.** An incomplete table of some of the most notable human ciliopathies in addition to the genes that have been linked to the diseases. Note that the same gene can be linked to different diseases, and symptoms can be common to different ciliopathies.

<table>
<thead>
<tr>
<th>Ciliopathy</th>
<th>Associated Genes</th>
<th>Affected Tissues</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alström Syndrome</td>
<td>ALMS1</td>
<td>Eyes, inner ear, adipose tissue</td>
<td>Blindness, hearing loss, obesity</td>
</tr>
<tr>
<td>Bardet-Biedl Syndrome</td>
<td>BBS1-BBS16</td>
<td>Eyes, olfactory nerves, kidneys, bone, central nervous system, adipose tissue</td>
<td>Blindness, anosmia, renal dysfunction, polydactyly, mental retardation, obesity</td>
</tr>
<tr>
<td>Joubert Syndrome</td>
<td>AHI1, NPHP1, RPRG1P1L, CEP290, ARL13B, MKS3</td>
<td>Eyes, kidneys, bone, central nervous system</td>
<td>Blindness, renal dysfunction, polydactyly, ataxia</td>
</tr>
<tr>
<td>Meckel-Gruber Syndrome</td>
<td>MKS1, MKS3, NPHP6, RPRG1P1L, B9D1, B9D2, MKS6, IFT88</td>
<td>Kidneys, bone, central nervous system, heart</td>
<td>Perinatal death, renal dysfunction, polydactyly, neural tube defects, congenital heart malformations</td>
</tr>
<tr>
<td>Nephronophthisis</td>
<td>NPHP1-NPHP6, AHI1</td>
<td>Eyes, kidneys</td>
<td>Blindness, renal dysfunction</td>
</tr>
<tr>
<td>Orofaciodigital Syndrome</td>
<td>OFD1</td>
<td>Kidneys, bone, central nervous system</td>
<td>Renal dysfunction, craniofacial abnormalities, polydactyly, cerebellar defects</td>
</tr>
<tr>
<td>Primary Ciliary Dyskinesia</td>
<td>DNAI1, DNAH5, DNAH11, DNAH2, CCDC39, CCDC40, RSPH4A, RSPH9</td>
<td>Lungs, internal organs, reproductive system</td>
<td>Chronic respiratory infections, situs inversus, infertility</td>
</tr>
<tr>
<td>Polycystic Kidney Disease</td>
<td>PKD1, PKD2, PKHD1</td>
<td>Kidneys, vasculature, liver</td>
<td>Renal dysfunction, aneurysms, hepatic and renal fibrosis</td>
</tr>
<tr>
<td>Senior-Loken Syndrome</td>
<td>NPHP1, NPHP3, NPHP4, NPHP6, IQCB1</td>
<td>Eye, kidneys</td>
<td>Blindness, renal dysfunction</td>
</tr>
</tbody>
</table>
autosomal recessive disorder resulting in perinatal lethality of the affected fetus [51]. The disease is characterized by severe defects in the development of the nervous system, failure of neural tube closure, skeletal malformations, polydactyly, and both renal and hepatic cysts [52]. MKS can be caused by mutations in several known genes, including MKS1, MKS3, NPHP6, RPGRIP1L, B9D1, B9D2, MKS6, and IFT88 [53-59]. Consistent with its role as cilia protein, studies in mice reveal that Mks proteins are widely expressed in various ciliated tissues, including kidney liver, brain and the developing limb buds and localization studies demonstrate that MKS proteins localize to the base or axoneme of the cilia in mice [53]. Mouse and other animal models of Mks genes have shed light on the importance of these genes in ciliogenesis. Loss of Mks1 causes phenotypes mimicking MKS symptoms, including renal cysts and patterning defects. Examination of the Mks1 mutant mice revealed a defect in cilia formation and a lack of floor plate specification, which is necessary for proper development [60]. Similar findings have also been reported for mutants of other MKS linked genes including, Ifi88 and B9d1 [36, 61]. Mouse models of NPHP6, RPGRIP1L, and B9D2 also have defective cilia assembly leading to disruptions in development and retinal degeneration [62-64]. In contrast, an MKS3 mouse model display elongated cilia but still have symptoms indicating defects in cilia function such as cystic kidneys [65]. These models have contributed to the growing body of evidence that cilia are necessary for developmental pathways and renal homeostasis, and have thus proven critical in implicating defective cilia as the pathological cause of MKS [66]. It is now believed that loss of function mutations in MKS genes lead to loss of cilia mediated sensory and signaling activities.
Previous to being linked to MKS in humans, *Ift88* and its homologs had a well-established role in the IFT B particle and ciliogenesis in various eukaryotic organisms [44, 45, 67]. The finding that mutations in this gene could cause human disease opens up the possibility that other mammalian IFT genes could likewise be linked to known ciliopathies.

**Primary Ciliary Dyskinesia and Infertility**

One of the first diseases to be attributed to dysfunctional cilia was primary ciliary dyskinesia (PCD) [68]. Patients with PCD display chronic upper and lower respiratory tract infections, as well as sinusitis [69]. Examination of the lungs of these patients revealed defects in the beating of the motile cilia lining the airways [70]. Electron microscopy further indicates that the motile cilia often have structural defects in the microtubule axoneme, dynein arms, or radial spokes that are needed for cilia to beat properly [71-73]. These defects interrupt the mucociliary escalator that normally clears the airways of pathogens and other noxious stimuli, thus disruption in their function leads to the persistent infections in PCD patients [74]. Additionally, approximately 50% of all PCD patients present with *situs inversus*, which is a reversal of internal body asymmetry [75, 76]. When *situs inversus* is present with chronic respiratory disease and sinusitis, the condition is referred to as Kartagener syndrome, and is recognized to be a subset of PCD [77]. The genetic component behind PCD is less well understood than other ciliopathies, however several genes encoding dynein motor components (*DNAI1, DNAH5, DNAH11, DNAI2, CCDC39, CCDC40*) or proteins associated with the radial spokes of motile cilia and flagella (*RSPH4A, RSPH9*) have been linked to the disease [17, 78-81].
Strikingly, many patients with PCD are not diagnosed until defects in fertility are also observed [82]. Spermatozoa from affected males often have defects in flagella movement, and motile cilia in female patients also display defects in the motile cilia in their oviducts [83, 84]. Given the importance that motile cilia in the oviduct play in the transport of the oocyte to the uterus, and the necessity of the flagella in successful fertilization of the egg, these observations readily account for the infertility often experienced by PCD and Kartagener syndrome patients.

A variety of cilia mutant mouse models replicate the dysfunctional motile cilia phenotype that causes disease in human PCD patients. For example, inv mice (short for inversion of embryonic turning) show complete reversal of the internal body asymmetry [85]. This phenotype is accompanied by defects in fluid flow in the embryonic node during development caused by dysfunctional motile cilia. Likewise, mice mutant for Kif3b, an essential component of the IFT B motor complex, lack motile cilia and thus fluid flow in the embryonic node [86]. While these embryos die during midgestation, they also can display reversal of body asymmetry. Work with these models have demonstrated that the presence of motile cilia in the embryonic node, an early gastrulation state organizing center important for defining body axis during development, is critical for proper left-right body axis patterning. These nodal cilia will beat in a vortical fashion that causes fluid to flow towards the left side of the embryo. Through a mechanism that remain somewhat controversial, this action determines internal body asymmetry. Several hypotheses posit that the motile cilia cause the delivery of Sonic hedgehog ligand and retinoic acid filled vesicles to be preferentially delivered to the left side of the embryo, initiating asymmetrical development [87]. It has also been proposed that non-motile cilia also exists in the
embryonic node, and these cilia bend in response to fluid flow created by motile cilia, resulting in a calcium spike on the left side of the embryo that leads to asymmetric organ placement [88].

Regardless of the exact mechanism, these mouse models implicate defective nodal cilia during human development as being responsible for the situs inversus phenotype found in Kartagener syndrome patients. However, both the *inv* and *Kif3b* mice are embryonically lethal, and so far these genes have not been linked to PCD or Kartagener syndrome. In contrast, animal models have proven useful in the discovery of other novel PCD genes. A family of Old English Sheepdogs with chronic respiratory infections was found to possess dysfunctional motile lining their airways [89]. Radiography was performed on the animals and one was also found to have *situs inversus*, much like Kartagener syndrome patients. Genetic analysis revealed the dogs shared homozygous mutations in *Ccdc39*. Furthermore, knockdowns of this gene in zebrafish was also associated with laterality defects, further implicating *Ccdc39* in motile cilia function and proper *situs*. Concurrent to this study, findings on a gene with related structural motifs, *Ccdc40* was also published [90]. Mice homozygous for null alleles of *Ccdc40* died before weaning age, but like *Ccdc39* mutant dogs, displayed randomization of internal organ asymmetry. Much like the *inv* and *Kif3b* mice, *Ccdc40* mutants also had defects in nodal cilia, accounting for the *situs inversus* like phenotype in these mice. Importantly, structural analysis indicated that both *Ccdc39* and *Ccdc40* mutant animals displayed defects in the inner dynein arms necessary for proper cilia motility. These same structural defects have been observed in the motile cilia of human PCD patients. Subsequent genetic analysis found that among unrelated human PCD patients who had inner dynein arm defects in their mo-
tile cilia, a striking 88.2% were biallelic for mutations in either CCDC39 or CCDC40, indicating a clear link with these genes to human disease [81]. Furthermore, abnormal flagella assembly was also common among these same male patients, leading to infertility.

CCDC39 and CCDC40 are part of a broader group of proteins that are named after their coiled-coil domains. Currently, it remains unclear of how ubiquitous the necessity of “CCDC” proteins in motile cilia and flagella function are, as studies detailing the functions of other CCDC proteins are currently lacking. Mouse models have proven excellent resources in the study of both the CCDC proteins and human male infertility, as most genes and physiological properties necessary for sperm production are conserved between the two species [91]. Unfortunately however, Ccdc39 mutant mice have not yet been reported, and Ccdc40 mutant mice die before sexual maturity, making the study of the infertility in CCDC39 and CCDC40 human patients more difficult.

Equally important, most patients that experience reduced fertility or infertility do not suffer from PCD or Kartagener syndrome. This becomes particularly evident in light of the fact that as many as 1 in 25 males experience infertility [92, 93]. Some of these patients have mutations in genes previously linked to flagella assembly or sperm maturation, but the vast majority of cases of infertility are caused by unknown physiological reasons.

In both mice and men, sperm maturation is a complex process that begins in the seminiferous tubules of the testis [94]. In brief, developing germ cells undergo meiosis to produce haploid spermatids. As these spermatids develop, they move away from the basement membrane and towards the center of the seminiferous tubule lumen. The sper-
matids then undergo further morphological changes, including nuclear condensation and flagellum assembly, to become spermatozoa. Sertoli cells, which lay on the basement membrane on the inside of the seminiferous tubules, nourish the developing sperm cells and consume residual products of sperm development. As sperm cell mature, they leave the testes and are stored in the latter portions of the epididymis until copulation occurs. Importantly, sperm cells become fully motile only and have the ability to fertilize an ovum only once inside the female reproductive tract, and these conditions can be mimicked \textit{in vitro} [95]. An interruption in any of the noted processes can lead to infertility in males. However, since sperm cells move both away from the seminiferous tubule basement membrane as they mature and move into the epididymis, the expression of genes and the localization of proteins necessary for these different events are thus temporally and spatially restricted. That is to say, a gene necessary for the later parts of flagella assembly is typically not expressed in cells that have yet to undergo meiosis. Thus, when studying a mouse model of male infertility, determining the temporal expression of the affected gene and the localization of its protein product can provide clues as to what process is being disrupted in human patients.

More studies on other “Coiled-coil domain containing proteins” and other proteins necessary for sperm development will be useful in determining how defects in flagella function contribute to human diseases like PCD and infertility.

\textit{Bardet-Biedl Syndrome}

Despite the severity of MKS, not all ciliopathies that adversely affect development are perinatally lethal. Bardet-Biedl syndrome (BBS) is a rare genetically heteroge-
neous disease where in patients can generally live to adulthood. Affected individuals present with symptoms involving multiple organ systems including renal dysfunction, retinal degeneration, polydactyly, cognitive deficiencies, and hypogonadism [96]. Strikingly, one of the defining features of BBS is obesity, which together with polydactyly and retinal degeneration, constitutes the core diagnostic criteria of the disease [97]. To date, 16 genes, named *BBS1*-*BBS16*, have been identified as causing BBS [98-112]. Mutations in *BBS1*-16 are responsible for approximately 70% of known BBS cases, indicating that other BBS genes have yet to be found [107, 108, 110, 113]. BBS proteins typically localize to or near the cilia, yet global cilia assembly is generally not impaired in mouse models of BBS, suggesting a role in cilia signaling or cilia protein tracking instead of cilia assembly [114]. Seminal work in the study of BBS by Nachury et al. supports this idea, and has shown that BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, and BBS9 form a complex called the BBSome [115]. The BBSome is thought to function in the transport of proteins along microtubules to or into the cilium. Other BBS proteins, including BBS6, BBS10, and BBS12, have protein chaperone like homology and are believed to facilitate assembly of the BBSome itself [116]. The remaining BBS proteins, including BBS3, BBS11, BBS13, BBS14, BBS15, and BBS16 have a less well defined role in cilia biology.

To date, mutant alleles in mice exist for *BBS1*, *BBS2*, *BBS3*, *BBS4*, *BBS6*, *BBS8*, *BBS11*, *BBS13*, and *BBS14*. Remarkably, phenotypes observed in the mouse models of the various BBS genes replicate the human symptoms very closely. In particular, a knock-in mouse with the M390R mutation in *Bbsl*, the most commonly reported mutation in human BBS patients, experience retinopathy, ventriculomegaly, male infertility, and hyperphagia induced obesity [117]. Similar phenotypes are also reported seen in
Bbs2, Bbs4, and Bbs6 mutant mice [118-120]. In contrast, obesity has not been reported in Bbs3 mutants, however an increase in fat pad size is observed [121]. Conversely, Bbs11 mutant mice are significantly larger than controls, but this is due to an increase in lean mass instead of adiposity [122].

Anosmia, a reported symptom of BBS, has also been reported in the Bbs2 and Bbs8 mutant mice [123]. Strikingly, when Bbs8 mutant mice were crossed to an olfactory receptor reporter line (M72TL), defects in axonal targeting became apparent, where the axonal fibers of olfactory neurons wandered during development instead of targeting to a single distinct glomerulus like they did in the control animals [124]. This developmental defect indicates that several of the symptoms of human BBS patients may be caused by similar defects in neuronal patterning.

Alström Syndrome

Like BBS, Alström syndrome (ALMS) is a human ciliopathy causing retinal degeneration and obesity [125]. It is unique however, in that ALMS patients lack the cognitive defects, renal dysfunction, and skeletal abnormalities that often affect other ciliopathies. Two mouse models of ALMS currently exist, with one containing a spontaneous 11bp deletion in exon 8 of the ALMS1 gene (foz), and the other with a genetrap allele in exon 11 (Alms1Δ11) [126, 127]. Both mouse models have identical phenotypes that closely mimic the symptoms of ALMS. Much like their human counterparts, foz and Alms1Δ11 mice are born at a normal body weight, but hyperphagic behavior leads to an obesity phenotype that is accompanied by hyperinsulinemia and type 2 diabetes. Both ALMS mouse models also display male infertility and cochlear defects indicative of hearing loss,
indicating that Alms1 has functions in a multiple tissues. Despite this, the exact function of ALMS1 in either humans or mice is unknown. Localization studies demonstrate that the protein product of the gene localizes to the basal body and cilia in both human and mouse cells [128, 129]. However, neither ALMS patients nor mouse models of the disease show signs of global defects in cilia assembly. Interestingly however, a reduction of hypothalamic neuronal cilia has been observed in the ALMS1 mutant foz mouse [130]. Given the importance of the hypothalamus in feeding behavior, this further implicates hypothalamic neuronal cilia in feeding behavior. Similarly, in vitro knockdown of ALMS1 has also causes a reduction in ciliogenesis in kidney cells lines [131]. It remains possible then that ALMS1 has a role in cilia mediated signaling or trafficking, much like the BBS proteins.

**Ciliopathies and Conditional Ift88 Mice**

As mentioned previously, loss of function in genes necessary for cilia assembly or proper cilia function can result in perinatal lethality. Thus, the study of cilia function in adult organisms is made more difficult by the necessity of these genes for proper development. To circumvent this problem, conditional alleles of cilia genes can be utilized to induce cilia loss or dysfunction in a temporal or tissue specific manner. One such allele, ift88<sup>em1Bly</sup>, has been used extensively. In this allele, exons four, five, and six are flanked by loxP sites that undergo recombination when exposed to a cre-recombinase, thus creating a null Ift88 allele and ablating cilia on the cells expressing the cre [132, 133].

To analyze ciliary function without interfering with any aspects of development, it is necessary to utilize a line where cre-recombinase can be regulated. This can be ac-
complished using the creER<sup>TM</sup> line where cre-recombinase has been fused to a modified estrogen receptor. This modification prevents the fusion cre from binding estrogen, but allows it to instead bind to the estrogen analog tamoxifen. In the absence of tamoxifen, the fusion cre is excluded from the nucleus; however, once exposed to tamoxifen the cre translocates to the nucleus to mediate excision of any DNA flanked by loxP sites [134]. In the case of the CAGG-creER<sup>TM</sup> line, the fusion cre is under the control of an actin promoter and expressed ubiquitously. Thus, when the CAGG-creER<sup>TM</sup> allele is present in mice homozygous for the Ifi88 conditional allele, injection of tamoxifen causes systemic loss of cilia [135].

The effects of systemic cilia loss using the conditional Ifi88 allele have provided valuable insights into cilia function. One of the most surprising findings was that the severity of one of the phenotypes was dependent on when cilia loss was induced. For example, if cilia loss was induced at 17.5 days post fertilization (E17.5), renal cysts formation was rapid, and occurred within two weeks of tamoxifen injection. However, if cilia loss was induced at 8 – 12 weeks of age, cysts formation occurred at an extremely slow rate, taking 6 months before the phenotype was evident [135]. The cystic kidney phenotype seen in the conditional Ifi88 mutants is reminiscent to the renal dysfunction seen in human ciliopathies of Meckel-Gruber syndrome and polycystic kidney disease. Strikingly however, the conditional Ifi88 mutants also displayed another phenotype found in both Bardet-Biedl syndrome and Alström syndrome, obesity. Loss of cilia at 8 weeks caused an increase in food intake beginning within one week of the initial tamoxifen injection, and a significant increase in body weight was observable by four weeks post tamoxifen. The hyperphagic behavior observed after tamoxifen injection in adult mice
raised the possibility that loss of ciliary function on neurons was driving the obesity phenotype. To test this hypothesis, conditional Ifi88 mice were crossed with synapsin1-cre mice. The synapsin1 promoter is primarily active in neurons, and thus conditional Ifi88 mice with the synapsin1-cre allele lose cilia on their neurons, but retain them on most all other tissues. Importantly, the synapsin1-cre is a congenital allele expressed during development, meaning tamoxifen is not required to induce loss of Ifi88, and cilia are theoretically absent from neurons during development as well as in adulthood [136]. Much like inducible Ifi88 mutants using the CAGG-creERTM allele, ablation of cilia in the conditional Ifi88 mice using the synapsin1-cre also induced hyperphagia and obesity, indicating that loss of neuronal cilia was enough to induce the phenotype.

Many neurons within the brain have been implicated in regulating feeding behavior. In particular, the arcuate nucleus of the hypothalamus contains ciliated POMC expressing cells, which, as will be discussed shortly, have a critical role in regulating satiety [137]. To further define on which neurons cilia function is needed to prevent the hyperphagic phenotype, conditional Ifi88 mutant mice were crossed to a mouse line expressing cre recombinase in only these POMC expressing neurons. Loss of Ifi88 on just POMC expressing neurons in the brain was enough to induce the obesity phenotype. This finding is consistent with cilia on hypothalamic POMC neurons having a role in satiety signaling, and that this process may be disrupted in obese ciliopathy patients [135].

In addition to the cystic kidney and obesity phenotype mentioned previously, the conditional Ifi88 mutant mice have also shown signs of other phenotypes. Although not thoroughly studied or well reported, retinal degeneration, infertility, and airway inflammation, have all been casually observed in conditional Ifi88 mutants. Thus these mice
may prove as valuable models for studies of these conditions which afflict human ciliopathy patients.

**Cilia Mediated Signaling**

Cilia have been implicated in a wide variety of signaling pathways and are essential in regulating and integrating pathways and environmental responses. While the role of cilia in some of these signaling pathways remains well established, sometimes the necessity of cilia to other signaling events remains more ambiguous. In the following section, I will cover some of these biological pathways and how cilia are believed to be involved.

**Cilia as Environmental Sensors**

Cilia play an important role in allowing vertebrates to sense the outside environment. In the eyes of both mammals and fish, the retina contains neurons called photoreceptors. These photoreceptors extend toward the retinal surface and terminate with a structure called the outer segment [138]. The outer segment itself contains flattened discs that contain rhodopsin and initiate the first events in the perception of light. Furthermore, the outer segment is connected to the rest of the photoreceptor through a highly modified primary cilium called the connecting cilium. Not only is the connecting cilium built through IFT like more traditional cilia and flagella, but the transport of proteins such as rhodopsin to the outer segment is also dependent on IFT to occur [139, 140]. As the outer segment is highly dynamic structure that continuously undergoes protein turnover, loss of IFT leads to degeneration of the photoreceptor outer segments and vision loss [141].
Unsurprisingly, retinal degeneration and blindness is a common phenotype of both cilia mutant mice and is often a defining symptom of many ciliopathies, including Alström syndrome and Bardet-Biedl syndrome [11].

Highly modified primary cilia are also present in the olfactory system of mammals. Here, sensory neurons extend axons through the cribiform plate and into the olfactory nerve, while dendrites are projected into the mucus covering the surface of the olfactory epithelium in the nose [142, 143]. Each dendrite terminates with a cluster of approximately 15 modified sensory primary cilia. Odorant G-protein coupled receptors (GPCRs) localize to these primary cilia and serve as olfactory receptors [144]. In brief, once odorant ligands come into contact with their respective GPCR, the receptor induces adenylyl cyclase III through a stimulatory G(olf) protein. This leads to an increase in cAMP and the opening of cyclic nucleotide gated ion channels. This ultimately leads to a depolarization of the neuron and leading to the sensation of smell. Defects in olfaction, or anosmia, have been reported in both cilia mutant mice and human ciliopathy patients, confirming the importance of the cilia in smell perception [123].

Cilia and Sonic Hedgehog Signaling

Sonic hedgehog (Shh) is a secreted protein necessary for a variety of developmental processes in vertebrates. Failure or interruption of this pathway results in a variety of phenotypes affecting development of the neural tube, brain, and limbs [145]. Strikingly, both human ciliopathy patients and mouse models of ciliopathies commonly present with characteristics of defective Shh signaling. The first connection between the cilium and Shh signaling was made when a mutagenesis screen searching for patterning defects in
mice found two mutants that had neural tube closure failure and polydactyly, two phenotypes characteristic of Sonic hedgehog (Shh) signaling defects [146]. Positional cloning and complementation analysis of the affected mutants found both mutations disrupted IFT genes, *Ift172* and *Ift88*. These findings were consistent with previous reports of both the hypomorphic and null *Ift88* alleles, which also showed polydactyly and neural tube closure defects, respectively [45, 147]. Examination of the neural tubes of the new *Ift172* and *Ift88* mutants found a lack of primary cilia in the neural tubes of mutant animals, as well as severe disruptions in ventral patterning of the neural tube with a loss of ventral neurons and expansion of the dorsal fates.

This finding started a cascade of research into the link between the cilium and Sonic hedgehog signaling. Subsequent studies clarified the role of cilia in hedgehog signaling as the Shh receptor, Patched-1 (Ptch1), is now known to localize to the primary cilia in the developing vertebrate embryo [148]. In the absence of Shh, Ptch1 represses the activity of the transmembrane protein Smoothened (Smo), and also prevents its entry into the cilium. In the presence of Shh, ligand binds to Ptch1, and the ligand-receptor complex internalizes, causing the derepression of Smo and its subsequent entry into the cilium [148, 149]. Smo then activates the Gli transcription factors to propagate Shh signaling. While Gli1 is primarily a pathway activator, Gli2 and Gli3 have dual roles as both an activator and repressor [150, 151]. In contrast, Gli3 predominates in a truncated repressor form to keep target genes off in the absence of ligand. Importantly, the processing of Gli2 and Gli3 occurs at the cilium itself [152]. The Gli transcription factors then control the expression of genes important for dorsal-ventral neural tube and digit patterning. Mammals with loss of function in IFT complex genes often display a signifi-
cant loss in Shh signaling, as cilia are no longer present for the pathway receptor to localize to or for the Gli transcription factors to be processed [146, 153]. It is for this reason that neural tube closure defects and polydactyly are common phenotypes/symptoms in cilia mutant mice and ciliopathy patients.

**Cilia and Leptin Signaling**

The regulation of feeding behavior remains a very active field of research and is clinically very important. Countless studies have demonstrated that the pathways involved are often very complex and are influenced by a wide variety of factors secreted by different organs of the body. Different pathways and neurons can act synergistically or antagonistically to fine tune appetite and regulate feeding behavior in extremely complex webs of cell signaling. A large portion of my dissertation research will focus on the possible involvement of cilia in the leptin signaling axis and how disruption of the cilium can lead to obesity. As such, in the following sections, I will discuss in more detail the current knowledge about the leptin signaling axis in the hypothalamus, a critical pathway in appetite regulation.

*Discovery of leptin and leptin resistance.* In 1949, a spontaneous mutation arose in the mouse colony at Jackson Laboratory resulting in morbid obesity. The mutant allele was appropriately named *obese*, and mice homozygous for the allele are referred to as *ob/ob* [154]. The gene responsible for the obese phenotype in these animals remained unknown for over 40 years, until Zhang et al. successfully identified and cloned the affected gene [155]. The product of that gene, named leptin, is a 16 kDa protein hormone
that is secreted into the blood by white adipose tissue to reduce food intake. Thus, as the amount of fat increases, the level of circulating leptin increases to reduce feeding activities [156]. Although the details of which won’t be covered in this thesis, leptin is also critical in regulating other physiological aspects including female fertility and thermoregulation [157, 158].

Since ob/ob mice have a loss of function mutation in the leptin gene, these mice are lacking in this critical satiation pathway. Indeed, exogenous injections of leptin into lean mammals and ob/ob mice causes a decrease in feeding behavior [159]. The implications of this finding created much excitement in the field of obesity research, as it was hypothesized that leptin could be used as a form of weight loss therapy [160]. Unfortunately, the excitement surrounding this discovery was greatly attenuated when it was determined that a barrier to the anorectic effects of leptin must exist in an obese individual as nearly all obese rodents and human patients are unresponsive to exogenous doses of leptin [161, 162]. The molecular mechanism responsible for this phenomenon, called leptin resistance, remains an active area of research. Furthermore, leptin resistance also complicates research into obesity as it can become difficult to discern if leptin resistance was the initiating factor leading to obesity, or merely a secondary consequence of obesity itself with other factors leading to the obese state.

*Cell signaling through the leptin receptor.* The leptin receptor (LepR) is found in various parts of the brain including the hypothalamus, hippocampus, hindbrain, pyriform cortex, and olfactory centers to name a few [163, 164]. Although six variants of LepR transcript can be produced, only the long form (LepRb) has been shown to be necessary
for the anorectic effects of leptin signaling [165]. The binding of leptin ligand to LepRb causes an activation of the Janus kinase – signal transducer and activator of transcription (JAK-STAT) pathway [166]. In brief, binding of leptin leads to the phosphorylation of tyrosine residues on LepRb which not only propagate the leptin signaling to reduce feeding behavior, but also to regulate the pathway with feedback inhibition [167, 168]. Feedback inhibition is accomplished through initiating the transcription of suppressor of cytokine signaling 3 (SOCS3), which acts by binding to one of the tyrosine residues of LepRb to block its phosphorylation and subsequently prevent further leptin signaling [169].

**Leptin signaling in the hypothalamus.** LepRb is found in multiple areas of the brain, but it exerts its primary influence on feeding behavior in the arcuate nucleus (ARC) of the hypothalamus (Figure 3) [170, 171]. We now know that two antagonistic populations of neurons exist in the ARC that control feeding behavior and food intake. The first of which are pro-opiomelanocortin (POMC) neurons. POMC neurons are stimulated by leptin to secrete alpha-melanocyte stimulating hormone (α-MSH), an anorectic peptide that activates the G-protein coupled melanocortin 3 receptor (MC3R) and the melanocortin 4 receptor (MC4R) in other regions of the hypothalamus, including the paraventricular nucleus (PVN) [171-173]. The ultimate result is the reduction of food intake and an increase in energy expenditure. The ARC is also home to a second class of appetite regulating cells, the agouti-related peptide/neuropeptide Y (AgRP/NPY) secreting cells. AgRP/NPY neurons secrete their namesake peptides to cause an increase in feeding behavior [174, 175]. Like α-MSH, AgRP activates MC3R and MC4R [176]. However, unlike POMC, which acts as an agonist on MC3R and MC4R to increase their
Figure 3. Hypothalamus and Feeding. The arcuate nucleus of the hypothalamus is located near the 3rd ventricle on the ventral side of the brain. Panel A shows a Nissl stained coronal section of the mouse brain. The inset shows the arcuate nucleus (ARC) of the hypothalamus, the third ventricle (3V), and the paraventricular nucleus (PVN) of the hypothalamus. Images modified from the Allen Brain Atlas http://www.brain-map.org/. Panel B shows two populations of neurons (POM in red and AgRP/NPY in green) involved in feeding behavior and are located in the ARC. The peptides released by these neurons act on receptors located on ‘higher’ second order neurons of the PVN. This signaling ultimately results in changes in neuronal activity that led to either feeding behavior.
or satiation. The inset shows cultured hypothalamic neurons labeled for NPY in green and the cilia receptor MCHR1 in red. Cilia are indicated by arrows. BBB corresponds to the blood brain barrier.

activity, AgRP acts as an inverse agonist to cause a decrease in their activity and subsequently increase feeding behavior [177]. Concurrent to the effects of AgRP, NPY binds to the GPCRs neuropeptide Y1 and neuropeptide Y5, which causes an increase in feeding behavior and a decrease in energy expenditure [178, 179]. Unlike POMC neurons, AgRP/NPY neurons are inhibited by leptin. Thus, not only does leptin stimulate POMC neurons to decrease food intake, but it also inhibits AgRP/NPY neurons that otherwise signal to increase food consumption.

**Bardet-Biedl syndrome genes and leptin signaling.** Hyperphagia and obesity is a common phenotype among various cilia mutant mouse models. Likewise, it is also a defining symptom of both Alström syndrome (ALMS) and Bardet-Biedl syndrome (BBS) in human patients. These findings indicate that cilia themselves have a role in regulating feeding behavior, and speculation soon began as to what signaling pathway was being disrupted to cause the obesity phenotype. Intriguingly, abnormally high leptin levels have been reported in both cilia mutant mice and obese ALMS and BBS patients [120, 180, 181]. Furthermore, loss of cilia on just leptin responsive POMC neurons was sufficient to induce the obesity phenotype [135]. These findings led to a model where defective leptin signaling might be driving the obesity phenotype observed in the ciliopathies [182].

An initial investigation into Bbs mutant mice and leptin resistance was first reported by Rahmouni et al. Their findings showed that three different mouse models of BBS (Bbs2−/−, Bbs4−/−, and Bbs6−/−) were not only obese due to hyperphagia, but were also resistant to the anorectic effects of exogenous leptin injections [120]. This leptin re-
sistance phenotype was also accompanied by a significant decrease in the expression of 
POMC mRNA in the brains of the Bbs mutants when compared to their controls. Thus, they proposed that defects in the leptin responsive POMC neurons are contributing to the obesity phenotype.

These findings however are complicated by the fact that leptin resistance is well reported secondary consequence of obesity [183]. That is to say, it is unclear if an innate leptin resistance is what causes the obesity phenotype or if it is secondary to it. To resolve this dilemma, a follow-up study was reported in which the three different Bbs mutant mice lines (Bbs2−/−, Bbs4−/−, and Bbs6−/−) were kept under a calorically restricted diet to prevent the obesity phenotype. Thus, feeding behavior in response to an exogenous dose of leptin would not be complicated by the fact that circulating leptin levels are markedly elevated and the animals leptin resistant due to a secondary consequence of obesity. In this paradigm, the researchers kept Bbs mutant animals lean by giving them 70-80% of food consumed by controls every day until the day before leptin sensitivity was assayed. As expected, control animals responded to leptin with a decrease in food consumption when compared to those that received a control injection. In contrast, lean Bbs mutants displayed no significant difference in food intake in response to leptin [184]. This finding indicated that leptin resistance in the Bbs mutants is unlike to be a secondary consequence of obesity. In vitro studies in the same report also provided evidence that the leptin receptor (LepRb) physically interacted with the protein Bbs1, and that depletion of either Bbs1 or Bbs2 with shRNA caused mislocalization of LepRb. Given the role that the Bbs proteins are proposed to play in trafficking proteins to the cilia, the authors concluded that Bbs mutant mice were unable to properly traffic the leptin receptor to the cilia.
or other areas of the plasma membrane, thus causing obesity in both the Bbs mouse models and human Bardet-Biedl syndrome patients.

The findings of leptin resistance in mouse models of BBS led to speculation that defects in leptin signaling would account for the obesity phenotype in other cilia mutant mouse models as well, and may even account for the obesity in human ciliopathy patients with BBS or ALMS. Thus, the cilium as a whole has been proposed to be a mediator of leptin signaling [182]. Caveats to this interpretation need to be considered however. The first of which is that leptin sensitivity assays have not been reported for other cilia mutant mouse models. Thus it is ambiguous as if the leptin resistance phenotype is specific to the Bbs mutants, that maintain a cilium that is dysfunctional, or if it is a characteristic of other models with defective cilia assembly. Additionally, a peculiar phenomenon called food anticipatory activity (FAA) is known to occur in rodents that are given limited access to food over extended periods of time [185]. FAA is characterized by changes in the physical activity and feeding behavior of the animal in response to being calorically restricted. Strikingly, these changes in activity and feeding behavior do not immediately cease when mice are returned to an ad libitum diet [186]. When leptin sensitivity was tested in Bbs mutant mice, they were kept on a calorically restricted diet until one day before being tested for leptin sensitivity [184]. Thus, it remains possible that a FAA response complicated the interpretation of the leptin sensitivity assay done on the Bbs mutant mice one day after their caloric restriction ceased.

This would not be the first time that a role for cilia in a signaling pathway has been disputed. The Wnt signaling pathways is an evolutionarily conserved process that utilizes β-catenin and intracellular calcium levels to pattern the developing embryo [187,
One group reported that \textit{in vitro} knockdown of cilia proteins causes alterations in Wnt signaling, and thus cilia themselves are important players in the Wnt pathway [189]. However, IFT mutants do not show obvious phenotypes characteristic of Wnt pathway mutants [190]. Furthermore, \textit{in vivo} experiments utilizing Wnt reporters failed to show any change in Wnt activity in cilia mutant mice [191]. As such, these conflicting pieces of data have called into question the true role of cilia in Wnt signaling.

\textbf{Continued Studies in Cilia Assembly and Function}

The objective of my studies described in the following chapters is to further elucidate the assembly of cilia and flagella, and to determine the consequences of cilia loss on the development, reproductive capabilities, and behavior in mammalian system. The first chapter documents my studies of \textit{Cluap1}, a gene which has cilia related functions in other organisms. For example, the \textit{Cluap1} homolog in \textit{C. elegans} is necessary for proper cilia assembly in that model organism, and zebrafish with mutations in their \textit{Cluap1} homolog developed cystic kidneys, a classic symptom of cilia dysfunction [192, 193]. To date however, the role of \textit{Cluap1} in a mammalian system had yet to be determined. By creating a \textit{Cluap1} knockout mouse, I was able to assess the consequences of \textit{Cluap1} loss on both the assembly of the cilia and the developmental consequences on the organism as a whole.

In contrast to \textit{Cluap1}, less is known about the possible function of \textit{Ccde42}. \textit{Ccde42} has homologs in various eukaryotic organisms with motile cilia and flagella, but is oddly absent from those with just primary cilia or without cilia altogether. However, a clue to its possible function lies in the fact that \textit{Ccde42} shares homology and structural
motifs to a family of other proteins necessary for motile cilia function [89, 90]. I thus hypothesized that \textit{Ccdc42} likewise had a role in motile cilia biology. To investigate this hypothesis, we generated \textit{Ccdc42} knockout mice to determine the function of this gene in motile cilia or flagella function. We also created transgenic \textit{Tetrahymena thermophila} to further resolve its subcellular localization and to use as a future tool in studies of the molecular function of \textit{Ccdc42}.

The role of cilia and flagella in fluid movement and cell motility has been well established. Likewise, our understanding of primary cilia in proper development has expanded rapidly over the past decade. Yet one of the most elusive roles cilia have been implicated in is that of appetite regulation. Bardet-Biedl syndrome and Alström syndrome are two human diseases caused by dysfunctional cilia, and in each one, obesity is a prominent symptom [105, 128]. Likewise, perturbation of cilia function or structure in mouse models can also result in hyperphagic induced obesity, implicating the cilium as a key regulator of appetite and satiety [182]. Previous reports propose that mouse models of Bardet-Biedl syndrome are obese due to dysfunctional leptin signaling, and may be the cause of obesity in other cilia mutant mice, as well as in human ciliopathy patients [120, 184]. Using systemically inducible \textit{Ift88} mutants, we have ablated cilia in adult mice and assessed for the effects of exogenous leptin on feeding behavior during different stages of adiposity. Together, these findings will provide a better understanding of the assembly and function of cilia and flagella.
MAMMALIAN CLUSTERIN-ASSOCIATED PROTEIN 1 IS AN EVOLUTIONARILY CONSERVED PROTEIN REQUIRED FOR CILIOGENESIS

by

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Abstract

Background: Clusterin-associated protein 1 (CLUAP1) was initially characterized as a protein that interacts with clusterin, a gene whose mRNA expression is elevated in cystic kidney diseases, a marker for immature collecting ducts in nephrons, and as a gene frequently up-regulated in colon cancer. Although the consequences of these observations remain unclear, research of CLUAP1 homologs in C. elegans and zebrafish indicate that it is needed for cilia assembly and maintenance in these models. To begin evaluating whether Cluap1 has an evolutionary conserved role in cilia in mammalian systems, and to explore the association of Cluap1 with disease pathogenesis and developmental abnormalities, we generated mice lacking Cluap1.

Methods: Cluap1 mutant embryos were generated and examined for gross morphological and anatomical defects using light microscopy. Reverse transcription PCR, β-galactosidase staining assays, and immunofluorescence analysis were used to determine the expression of the gene and localization of the protein in vivo and in cultured cell lines. We also used immunofluorescence analysis and qRT-PCR to examine defects in the Sonic hedgehog signaling pathway in mutant embryos.

Results: Cluap1 mutant embryos die in mid-gestation indicating that it is necessary for proper development. Mutant phenotypes include a failure of embryonic turning, an enlarged pericardial sac, and defects in neural tube development. Consistent with the diverse phenotypes, Cluap1 is widely expressed. Furthermore, the Cluap1 protein localizes to primary cilia and mutant embryos were found to lack cilia at embryonic day 9.5. The phenotypes observed in Cluap1 mutant mice are indicative of defects in Sonic hedgehog
signaling. This was confirmed by analyzing hedgehog signaling activity in Cluap1 mutants which revealed that the pathway is repressed.

Conclusions: These data indicate that the function of Cluap1 is evolutionarily conserved with regards to ciliogenesis. Further, the results implicate mammalian Cluap1 as a key regulator of hedgehog signaling and as an intraflagellar transport B complex protein. Future studies on mammalian Cluap1 utilizing this mouse model may provide insights into the role for Cluap1 in intraflagellar transport, and the association with colon cancer and cystic kidney disorders.

Keywords: Intraflagellar transport; Sonic hedgehog; Clusterin-associated protein 1; IFT Complex B
Background

Cilia are complex organelles requiring hundreds of different genes for their assembly and function [1]. The assembly of the cilium is dependent on intraflagellar transport (IFT), a molecular motor driven process that mediates the bidirectional movement of proteins between the base and tip of the cilium [2, 3]. IFT was initially described in the green algae, *Chlamydomonas reinhardtii*, and subsequently in multiple other ciliated eukaryotes thereby suggesting a highly conserved function.

Biochemical analysis has revealed the presence of two large distinct complexes of IFT proteins termed IFT complex A and B. Complex B is thought to mediate movement in an anterograde direction towards the tip of the cilium, while IFT complex A appears to facilitate retrograde movement to bring proteins back to the cilium base [4, 5]. Each complex is necessary for proper cilia maintenance and is important for cilia mediated signaling activities. For example, the Sonic hedgehog (Shh) pathway requires the cilium, with mutations in complex B proteins resulting in a repressed pathway while complex A mutants having elevated signaling. [6-9]. In humans, loss of ciliary function is responsible for a variety of diseases collectively referred to as ciliopathies [10]. The ciliopathies are characterized by a broad range of clinical features including neural tube defects, skeletal abnormalities, cystic kidneys, retinal degeneration, and obesity just to name a few [11]. How loss of ciliary function contributes to this wide range of phenotypes is unknown. Therefore, the identification of novel mammalian IFT associated genes and the generation of corresponding mutant models will provide insights into the ciliary connection to human disease and development defects.

In this regard, invertebrate model organisms have proven invaluable. One example can be seen in the case of *dyf-3*, a gene recently demonstrated to be necessary for
proper ciliogenesis in the nematode worm *C. elegans* [12, 13]. Subsequent studies demonstrated that a homolog of *dyf-3*, named *qilin*, is also present in zebrafish [14]. Interestingly, not only was *qilin* found to be necessary for cilia assembly and maintenance in zebrafish, but loss of function mutations in *qilin* cause a polycystic kidney disease like phenotype similar to that observed for mutations in known IFT genes [15, 16]. Although a *Chlamydomonas* homolog of DYF-3/qilin was not biochemically purified as a key component of the IFT complex, fluorescently tagged DYF-3 has been observed undergoing IFT in the cilia of *C. elegans* [17]. Further, mutations in *dyf-3* result in ciliary defects indicating that the protein may be a previously unrecognized component of either the IFT B or IFT A complex [4, 5, 17].

There is also a human homolog of DYF-3/qilin, originally referred to as ‘hypothetical protein KIAA0643’, but later renamed Clusterin-associated protein 1 (CLUAP1). Cluap1 was described as a coiled-coil protein that localized to the nucleus whose expression changed with the cell cycle. Further, *Cluap1* was commonly up-regulated in numerous colorectal carcinomas and suppression of Cluap1 expression reduced the growth of colon cancer [18]. In addition, Cluap1 interacts with clusterin, a protein induced by cell injury and elevated in cyst fluid in multiple cystic kidney disorders [18, 19]. The cellular properties and physiological importance of CLUAP1 is unknown despite its association with cell cycle, and demonstrated alterations of CLUAP1 expression in various human disorders and diseases, as well as *in vitro* interaction with the protein clusterin, [18, 20].

Based on the findings in *C. elegans* and zebrafish, it was hypothesized that the mammalian homolog would have roles in IFT and cilia mediated signaling. To test this
hypothesis, a Cluap1 knockout mouse model was generated to assess the role of Cluap1 in an *in vivo* mammalian system.

**Methods**

*Generation of Cluap1 Knockout Allele Mice*

The Cluap1 knockout allele (*Cluap1*<sup>tm1a(KOMP)Wtsi</sup>, Knockout Mouse Project Repository, Davis, CA; hereinafter referred to as *Cluap1*<sup>KO</sup>) was generated using embryonic stem cells in which a β-galactosidase-neomycin resistance fusion cassette was inserted into intron 2 of Cluap1. The insertion site was confirmed by genomic PCR and sequence analysis. PCR primers for genotyping were designed based on the insertion site (sequences available upon request). The embryonic stem cells containing the targeted allele were on the C57BL/6N background and were injected into albino C57BL/6 blastocysts (C57BL/6J-*Tyrc-2J*; JAX Laboratories) by the UAB Transgenic Mouse Facility using standard procedures. Chimeras were then crossed with albino C57BL/6 females and germline transmission was confirmed by coat color of the offspring and subsequent PCR genotyping. After obtaining no homozygous mutant offspring from heterozygous matings, timed pregnancies were established to isolate embryos at the indicated gestational timepoint with the morning of vaginal plug being considered embryonic day 0.5 (E0.5). Embryos were genotyped from DNA isolated from yolk sac by PCR. All mice were provided standard laboratory chow and water ad libitum, and maintained in accordance with IACUC regulations at the University of Alabama at Birmingham.
Reverse Transcription PCR Analysis

RNA was isolated from Cluap1WT, Cluap1Het, and Cluap1KO E9.5 embryos with Trizol reagent according to the manufacturer’s protocol (15596-026, Life Technologies, Carlsbad, CA). Once extracted, RNA was used to synthesize cDNA using Verso cDNA kit according to the manufacturer’s protocol (AB-1453, Thermo Scientific, Pittsburgh, PA). PCR analysis was then performed using the following primers (written 5’ to 3’) which flank the sequence between the first and last exons of the Cluap1WT allele: GGACTCGAGACCATGTCT and GGACCCGGGAAGAAGTCA. The following primers were also used as a positive control to confirm the presence of actin in all samples: ATGGGTCAGAAGGACTCCTA and GGTGTAAAACGCAGCTCA. All results were confirmed by repeating the experiment in at least two additional animals.

Cluap1 Antibody Generation

Antisera against Cluap1 was generated in rabbits by using a 19-residue peptide (KPSRRIRKPEPLDESNDNDF) starting at position 395 of the mouse protein according to the standard protocol established by Open Biosystems (Huntsville, AL, USA). Specificity of the antisera against Cluap1 was confirmed by Western blot analysis of protein extracts isolated from Cluap1WT, Cluap1Het, and Cluap1KO embryos.

Cell Culture

IMCD3 cells (ATCC, Manassas, VA) were maintained in DMEM: F12 medium supplemented with 10% FBS, 1.2 g/l of sodium bicarbonate, 0.5 mM sodium pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin. NIH3T3 cells were cultured in
DMEM with 10% FBS containing 100 U/ml penicillin, and 100 mg/ml streptomycin. Creation of 176-6C renal epithelial cells was derived by microdissection of cortical collecting duct segments of the kidney as previously described by Croyle et al. [21]. To induce cilia formation, cells were serum starved for 24 – 48 hours prior to analysis. All cells were grown at 5% CO₂/95% air at 37°C.

**Immunoblotting**

Embryonic day 9.5 embryos were isolated into ice-cold lysis buffer (137 mM NaCl, 20 mM Tris pH 8.0, 1% Triton X-100, 10% glycerol, and complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)). Embryos were disrupted by passage several times through a syringe attached to a 30.5 gauge needle. The lysates were incubated on ice for 30 minutes and vortexed every 5 minutes. Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Protein samples were resolved on a denaturing 10% Tris-HCL gel (Bio-Rad Laboratories, Hercules, CA) and transferred to Immobilon-Psq transfer membrane (Millipore, Billerica, MA). Membranes were blocked in TBS-T (10mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) with 5% milk for 1 hour and incubated with primary antibody diluted in TBS-T with 2% BSA for 16-24 hours at 4°C. Membranes were probed with horseradish peroxidase (HRP) conjugated secondary antibodies diluted in TBS-T with 1% milk for 1 hour at room temperature. Secondary antibodies were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Waltham, MA) and bands were visualized using Blue Ultra Autorad Film (Bioexpress ISC). The following primary antibodies and dilutions were used: anti-actin (Sigma; rabbit polyclonal; 1:1000), and anti-Cluap1
(1:1000). The secondary antibody was HRP conjugated anti-rabbit (#31460) and was used at 1:5000 (Pierce/Thermo Scientific, Waltham, MA).

β-Galactosidase Assays

Whole kidney and heart were extracted from Cluap1WT and Cluap1Het mice at 8 weeks of age. Tissues were fixed overnight at 4°C in 4% PFA in PBS and subsequently washed in PBS. Tissues were then cryoprotected with 30% sucrose in PBS for 24 h, and snap frozen in OCT freezing compound. Ten micron sections were cut with a Leica CM1900 cryostat and sections were attached to Superfrost Plus microscope slides (12-550-15, Fisher Scientific, Pittsburgh, PA). Sections were postfixed in 4% PFA in PBS for 10 minutes, washed three times with lacZ wash buffer (2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40, in 100 mM sodium phosphate buffer, pH 7.3), and then incubated in X-gal staining solution (2 mM MgCl₂, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mg ml⁻¹ X-Gal, in PBS) at 37°C overnight. Sections were then counterstained in Fast Red for 5 minutes. Similarly, for whole-mount analyses E9.5 embryos and lung tissue from 8 week old mice were fixed in 4% PFA in PBS, washed three times with lacZ wash buffer, and then incubated in X-gal staining solution at 37°C overnight.

Immunofluorescence

Embryos and cells grown on coverslips were fixed in 4% PFA and permeabilized with 0.3% Triton X-100 in PBS with 2% donkey serum, 0.02% sodium azide and 10 mg/ml bovine serum albumin (BSA). Embryos were then cut to make 10 micron sec-
tions. Cell and embryos were labeled with the following antibodies: anti-acetylated α-tubulin, 1:1000 (T-6793; Sigma-Aldrich, St. Louis, MO), anti-Arl13b, 1:1000 (a gift from Dr. Tamara Caspary, Emory University), anti-Cluap1, 1:1000 (generated as described above) and anti-ShhN (5E1, dilution, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). All incubations and washes were carried out in PBS with 2% normal donkey serum, 0.02% sodium azide and 10 mg/ml BSA. Primary antibody incubations were performed for 16-24 hours at 4°C and secondary antibody incubations were performed for 1 hour at room temperature. Secondary antibodies included Alexa Fluor-594 and 488 conjugated donkey anti-mouse and anti-rabbit (A-21203 and A-11001, Invitrogen, Carlsbad, CA). Nuclei were visualized by Hoechst nuclear stain (Invitrogen, Carlsbad, CA). Sections were mounted onto glass slides and mounted using DABCO mounting media (10 mg of DABCO (D2522; Sigma-Aldrich, St. Louis, MO) in 1 mL of PBS and 9 mL of glycerol). Slides were sealed using nail polish.

Confocal Microscopy

All fluorescence images were captured on Perkin Elmer ERS 6FE spinning disk confocal microscope and images were processed and analyzed in Volocity version 6.1.1 software (Perkin Elmer, Shelton, CT).

Quantitative Real Time PCR Analysis

Quantitative real-time (qRT) PCR analysis of RNA isolated from embryonic day 9.5 embryos was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) with the CFX96 real-time PCR detection system (Bio-Rad) as previously reported
Primer pairs (from 5’ to 3’) used for qRT-PCR analysis were as follows, Patched-1: GCCAAGCCCTAAAAAAAT and ACCACAATCAATCTCCTG (previously reported by Croyle et. al. [23], Gli1: TCGACCTGCAAACCGTAATCC and TCCTAAA-GAAGGGCTCATGGTA. The following primers for peptidylprolyl isomerase A (Ppia) were used as an internal control: CAGACGCCACTGTCGCTTT and TGTCTTT-GGAACCTTTGTC (both Gli and Ppia primers previously reported by Hellstrom et. al. [24]). Samples were run in triplicate using RNA from at least three different embryos per genotype.

**Statistical Analysis**

The difference in gene expression between Cluap1<sup>WT</sup> and Cluap1<sup>KO</sup> embryos was assessed using a Student’s *t*-test on log transformed values of the relative normalized quantity of template. Significance was established at *P* < 0.01. All calculations were performed using Microsoft Excel.

**Results**

*Loss of Cluap1 is Embryonically Lethal*

Analysis of the homolog of *Cluap1* in *C. elegans* and zebrafish suggest that it is a component of the Intraflagellar transport (IFT) machinery necessary for cilia assembly [17]. To assess if this role for *Cluap1* is evolutionarily conserved in mammals, a mouse embryonic stem cell line harboring a β-galactosidase cassette in intron 2 of *Cluap1* was obtained and used to generate a knockout mouse line (hereinafter referred to as *Cluap1<sup>KO</sup>*)(Figure 1A, B). We crossed Cluap1 heterozygotes (*Cluap1<sup>Het</sup>*) to produce
Figure 1: Clusterin-associated protein 1 (Cluap1) knockout mice are embryonic lethal. (A) Schematic of the wild-type Cluap1 allele (Cluap1WT) and the Cluap1 knockout allele (Cluap1KO). The relative position of the β-galactosidase cassette is indicated by the blue box. (B) PCR genotyping of Cluap1WT, Cluap1Het, and Cluap1KO embryos. (C) At E9.5, Cluap1KO embryos are runted, have enlarged pericardial sacs (arrow), and fail to turn properly (asterisk). (D) RT-PCR gel showing the expression of Cluap1 transcript in both Cluap1WT and Cluap1Het embryos and the absence in Cluap1KO embryos. Actin served as a positive template control in all samples. Reactions treated with reverse transcriptase (“+”) are alongside negative RT control samples (“-“). (E) Loss of the wild-type Cluap1 protein in Cluap1KO embryos was determined by Western blot. Actin was used as a control.
homozygous Cluap1 knockouts ($Cluap1^{KO}$). More than 15 different $Cluap1^{Het}$ intercrosses producing over 150 offspring failed to yield any $Cluap1^{KO}$ pups, indicating that loss of $Cluap1$ is embryonically lethal. To determine the timing of $Cluap1$ mutant lethality, we set up timed pregnancies (embryonic day 0.5 (E0.5) was the morning of copulatory plug visualization). This revealed no surviving $Cluap1^{KO}$ embryos between E10.5 and E18.5. However, surviving $Cluap1^{KO}$ embryos (determined by the presence of a beating heart), were detected at E9.5. Analysis of $Cluap1^{KO}$ embryos revealed that they were runted and exhibited enlarged pericardial sacs (Figure 1C, arrow). Most strikingly however, was the failure of proper embryonic turning marked with kinks in the neural tube (Figure 1C, asterisk) when compared to wildtype siblings ($Cluap1^{WT}$). These phenotypes are similar to those of known IFT mutants [25, 26]. To determine if our $Cluap1^{KO}$ allele was a null, we looked at both transcript and protein levels in $Cluap1^{KO}$ embryos. Both analyses demonstrated a total loss of Cluap1 transcript and protein in the $Cluap1^{KO}$ embryos (Figure 1D, E, Supplemental Figure 1).

**Cluap1 is Widely Expressed in the Adult and Embryonic Mouse**

Previous studies of IFT genes have indicated they are widely expressed [27, 28]. Similarly, RT-PCR analysis revealed Cluap1 expression in all tissues tested (Figure 2A). We analyzed spatial expression of Cluap1 using the β-galactosidase (β-gal) reporter present in the $Cluap1^{KO}$ allele (Figure1A). Heart, kidney, and lung tissue taken from $Cluap1^{Het}$ mice showed β-galactosidase positive staining (Figure 2B). The expression of Cluap1 is markedly elevated in multi-ciliated cells such as the bronchioles of the lung (Figure 2B) and ependymal cells of the brain (data not show), but was absent in the
**Figure 2.** *Cluap1* is expressed in ciliated cells with a wide tissue distribution. (A) RT-PCR gel showing expression of *Cluap1* in the indicated tissues, Sk. Muscle, skeletal muscle. Actin is used as a positive control. Reactions treated with reverse transcriptase (“+”) are alongside negative RT control samples (“-”). (B) β-galactosidase staining assay showing *Cluap1* expression in *Cluap1*Het tissue in the ventricles of the heart, cortex of the kidney, lung tissue, and whole E9.5 embryo. Asterisks mark non-ciliated cells in lung parenchyma. *Cluap1*WT control tissue samples. Heart and kidney sections were counterstained in nuclear fast red. Scale bars are 10 µm in heart sections, 30 µm in kidney sections, 1000 µm for whole lung tissues and embryos.
alveolar parenchyma (Figure 2B, asterisks). Cluap1 β-gal expression was also detected in cells with a single primary cilium (Figure 2B, heart and kidney).

We also stained Cluap1\textsuperscript{WT} and Cluap1\textsuperscript{Het} embryos at embryonic day 9.5, the last timepoint in which Cluap1\textsuperscript{KO} embryos are viable. In Cluap1\textsuperscript{Het} embryos, β-galactosidase positive staining was present along the entire anterior-posterior axis (Figure 2B). These results show that Cluap1 is widely expressed in ciliated tissues.

**Cluap1 Localizes to the Primary Cilia in vitro**

To assess Cluap1 subcellular localization, we co-immunolabeled NIH3T3 cells with our Cluap1 antibody and the cilia marker acetylated α-tubulin (Figure 3A,C). Cluap1 localizes to the primary cilia, and was visualized throughout the length of axoneme (Figure 3B,C). We confirmed the cilia localization in two additional independent cell lines derived from renal collecting ducts of adult mice (176-6C cells, Figure 3D-F and IMCD3 cells, Figure 3 G-I).

**Cluap1\textsuperscript{KO} Embryos Lack Primary Cilia**

The improper embryonic turning and enlarged pericardial sac phenotypes seen in Cluap1\textsuperscript{KO} animals are similar to phenotypes observed in IFT mutants [25, 26]. This finding combined with the cilia localization of Cluap1 raised the possibility that mammalian Cluap1 is required for ciliogenesis. To test this hypothesis, E9.5 Cluap1\textsuperscript{KO} embryos were immunostained for the presence of cilia. Antibodies to acetylated α-tubulin showed a complete absence of cilia in sections of the lateral plate mesenchyme of Cluap1\textsuperscript{KO} embryos (Figure 4B,D,F), while in control Cluap1\textsuperscript{WT} embryos, a single primary cilium was
**Figure 3.** *Cluap1 localizes to primary cilia in vitro*. Antibody against acetylated α-tubulin (red) and Cluap1 (green) label primary cilia (arrows) in (A-C) NIH3T3 cells (scale bars are 14 µm), (D-F) 176-6C collecting duct epithelium, (scale bars are 21 µm) and (G-I) IMCD3 cells (scale bars are 20 µm). Arrows indicate primary cilium. Nuclei are stained blue with Hoechst.
Figure 4: **Cluap1<sup>KO</sup> embryos fail to form primary cilia.** (A,C,E) Cluap1<sup>WT</sup> E9.5 embryos were immunolabeled for the cilia marker acetylated α-tubulin (red) and Cluap1 (green) in the lateral plate mesenchyme of Cluap1<sup>WT</sup> embryos. (B,D,F) Cluap1<sup>KO</sup> embryos show a total loss of cilia in the same region. Hoechst nuclear stain in blue. Scale bar is 31.5 µm.
detected on nearly every cell (Figures 4A,C,E). Thus, *Cluap1* is necessary for cilia formation in mice. Also in *Cluap1* mutant cells, the immunofluorescence showed an increase in acetylated α-tubulin staining similar to another Ift mutant [26].

**Loss of Cluap1 Disrupts Sonic Hedgehog Signaling**

Cilia are necessary for normal activation as well as repression of the Sonic hedgehog signaling (Shh) pathway and the phenotypes in *Cluap1* mutants is consistent with defects Hh activity [29]. To evaluate this possibility, we performed immunofluorescence analysis on the neural tubes of E9.5 *Cluap1*KO embryos. As expected, *Cluap1*WT embryos possessed a properly defined Shh immunopositive floorplate (Figure 5A,E arrowhead). In contrast, *Cluap1*KO embryos stained positive for Shh ligand, but lacked a defined Shh positive floorplate (Figure 5B,F). Furthermore, staining for Arl13b, a small GTPase that localizes to primary cilia and is necessary for Shh signaling, confirmed an absence of cilia in the neural tubes of *Cluap1*KO embryos (Figure 5D) [30, 31]. To further confirm defects in Hh signaling, whole embryos were analyzed for overall Shh pathway activity by qRT-PCR analysis of *Patched-1* and *Gli1*, two downstream target genes induced by Hh. *Cluap1*KO samples showed a significant reduction in both *Patched-1* and *Gli1* (53.3% and 20.8% of wildtype transcript levels, respectively. p < 0.001, Figure 6). Aside from indicating a defect in the Shh pathway, the down regulation of *Patched-1* and *Gli1* is also informative about the role of Cluap1 within the cilium itself. As previously reported, loss of function mutations in IFT complex B genes cause a down regulation of *Patched-1* and the *Gli1* transcription factors. Conversely, mutations in genes encoding IFT A complex proteins cause an increase in the *Gli1* and *Patched-1* expression [32-34]. Thus, these data
indicate that Cluap1KO embryos are defective in Sonic hedgehog signaling most likely due to the loss of IFT B complex function.

**Figure 5:** Cluap1KO embryos have defects in floor plate induction. (A,C,E) Cluap1WT E9.5 embryo stained for Arl13b (green) show cilia in the neural tube and surrounding tissue. Staining for Sonic hedgehog ligand (red) shows a Shh immunopositive floorplate. (B,D,F) Cluap1KO embryos show an absence of cilia as indicated by the lack of Arl13b staining. Note the lack of a clearly defined Shh immunopositive floorplate. Hoechst nuclear stain in blue. Scale bars are 21 µm.
Figure 6: *Cluap1*KO embryos have downregulated expression of *Patched-1* and *Gli1*. Real-time PCR results for the expression of *Patched-1* and *Gli1* in E9.5 *Cluap1*WT and *Cluap1*KO embryos demonstrate a significant decrease in expression of both *Patched-1* and *Gli1*. Expression levels are relative to control peptidylprolyl isomeras A (PPIA). Bars represent mean fold expression and error bars are ± SEM. Asterisks represent significant difference from control (**P < 0.01, Student’s t-test).

Discussion

Previous data implicate homologs of *Cluap1* in cilia assembly. For example, in *C. elegans*, the *Cluap1* homolog *dyf-3* is necessary for normal cilia structure, with mutant worms failing to assemble the cilia distal segment [13]. *Dyf-3* mutant worms also display defects in cilia regulated behaviors [12]. Similarly, in zebrafish *qilin/Cluap1* mutant cilia degenerate in the pronephric duct leading to subsequent cystogenesis [14, 16]. Here we provide the first evidence that mammalian *Cluap1* is also a cilia protein required for cilia formation, and show that mutants have characteristics consistent with Cluap1 being an IFT B complex protein.

Loss of *Cluap1* is embryonically lethal. In addition to being runted, *Cluap1*KO mutants also failed to be properly turned by E9.5 and have an enlarged pericardial sac,
indicating that cardiac insufficiency could be contributing to the midgestational lethality. Defects in embryonic turning with altered left-right axis specification along with an enlarged pericardial sac have been observed in several IFT mutant mouse models [25, 26, 35]. Aside from having a known role in left-right asymmetry of the heart, cilia have also been implicated in being necessary for early cardiac development through the Sonic hedgehog (Shh) signaling pathway [36, 37]. Thus, it remains possible that a defect in Shh signaling during heart development could be driving the pericardial defects we observe in Cluap1KO embryos.

In mice, deletion of Cluap1 causes a total loss of cilia within the developing embryo, but this phenotype diverges slightly from studies of Cluap1 homologs in other model organisms. An initial publication in zebrafish stated that mutants of the Cluap1 homolog, qilin, were still capable of cilia assembly, leading to speculation that the protein has an accessory role in cilia maintenance or signaling [14, 19]. This belief was further supported by the fact that the Chlamydomonas homolog of Cluap1 was not found in biochemical analysis of IFT particles isolated from this organism’s flagella [4, 5]. A follow up report on the function of qilin in zebrafish did demonstrate that cilia in qilin mutants degenerate over time [16]. However, an independent study utilizing a Morpholino approach to knockdown qilin revealed a more severe developmental phenotype with pronounced cilia loss [15]. This suggests maternal contribution of qilin mRNA in the genetic mutant is masking a role for qilin in early ciliogenesis. Our Cluap1KO mutant mouse provides further support that this protein has an important role in ciliogenesis conserved across a diverse range of eukaryotic species.
Analysis of the Cluap1KO mutant mice revealed that the Shh signaling pathway is severely disrupted. Cluap1KO embryos lack a Shh positive floor plate by E9.5 and have markedly reduced levels of Patched-1 and Gli1 mRNA. Significantly, mutations affecting complex A or complex B IFT proteins have different effects on the activity of the Shh pathway. IFT B gene mutations show a decrease in Shh signaling activity, while loss of IFT A genes have increased levels of Shh signaling [32-34]. Thus, the complete loss of cilia seen in Cluap1KO mutants combined with the reduction in Patched-1 and Gli1 expression implies that Cluap1 is a component to the IFT B complex involved in anterograde cilia transport. However, we cannot unequivocally exclude a role for Cluap1 in ciliogenesis outside of IFT complex B.

Conclusions

This study demonstrates a highly conserved role for mammalian Cluap1 in cilia biology. Cluap1 is necessary for proper mouse development, is expressed with a wide tissue distribution, and the protein localizes predominantly to the cilium axoneme. Cluap1KO mutant embryos display an enlarged pericardial sac and have defects in neural tube development, possibly related to impaired Shh signaling activity. Importantly, these findings on the role of Cluap1 in ciliogenesis and cilia mediated signaling support the possibility of Cluap1 being a candidate loci affected in human ciliopathy patients.

Abbreviations
IFT: Intraflagellar transport; Cluap1: Clusterin-associated protein 1; Shh: Sonic hedgehog; WT: Wildtype; Het: Heterozygous; KO: Knockout
Competing Interests

The authors have no conflicts or competing interests to disclose

Author Contributions

RCP and NFB designed and performed experiments and wrote the manuscript. WRL performed experiments. RAK created the mouse model. BKY designed experiments and wrote the manuscript.

Acknowledgements

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References


Supplemental Figure 1. *Loss of Cluap1 protein in Cluap1*KO* embryos.* Western blot analysis showing loss of Cluap1 protein expression in Cluap1 null embryos. A higher molecular weight nonspecific band is also detected but is not altered in Cluap1 mutant embryos.
COILED-COIL DOMAIN CONTAINING PROTEIN 42 IS NECESSARY FOR SPERM FLAGELLA FORMATION IN THE MALE MOUSE

by

RAYMOND C. PASEK, LAURA TRES, NEERAJ SHARMA, ROBERT A. KESTERSON, BRADLEY K. YODER, AND ABRAHAM KIERSZENBAUM
Abstract

Flagella and motile cilia are highly conserved eukaryotic organelles necessary for cell movement. Amongst many vertebrates, flagella confer locomotor abilities on individual sperm cells and thus allow fertilization occurs. If the maturation of the sperm cell itself is interrupted, or flagella are unable to be assembled, the organism is left with a severely reduced chance of siring offspring. This is particularly relevant to human beings as defects in flagella assembly is a common cause of infertility among males.

This conserved nature of the flagella is reflected in the fact that, regardless of the organism, flagella are both built and maintained through the process of intraflagellar transport (IFT). However, the assembly of this organelle in sperm also relies on other complex processes to occur, including intramanchette transport (IMT). IMT occurs along the microtubule based manchette that surround the elongating nuclei in the developing sperm cells. Much like IFT, IMT consists of protein complexes that assemble together and ferry cargo. It is through this process that the centrosome derived head-tail coupling apparatus (HTCA) is anchored to the nucleus and serves as a nucleation site for the assembling flagella, and it is also how proteins are delivered to the flagella base. If this process is interrupted, the sperm cell is unable to properly build a flagella tail.

Here, we report our findings on Ccdc42, a gene necessary for assembly of the sperm flagella in the mouse. Our findings show that in contrast to many other flagella and motile cilia genes, Ccdc42 is expressed specifically in the testes in a spatially and temporally dependent manner. Furthermore, Ccdc42 mutant male mice are sterile due to a loss in flagella assembly and defects in the HTCA.
Introduction

Defects in fertility are a common symptom among human ciliopathies, including primary ciliary dyskinesia, Bardet-Biedl syndrome, Alström syndrome, and even polycystic kidney disease [1]. Many more human patients also have infertility or subfertility due to unknown physiological or genetic reasons. In this regard, mouse models have proven valuable in studying both the infertility phenotype of human ciliopathy patients and infertility in general [2].

Like motile cilia, sperm flagella are a microtubule based structure possessing a 9 + 2 organization of microtubule doublets that are assembled through intraflagellar transport (IFT) [3]. The flagellum is anchored to the head of the sperm through a structure called the head-tail coupling apparatus (HTCA), a centrosome based structure that serves to attach the sperm tail to the nucleus. This same structure also contributes a basal body to initiate assembly of the microtubule axoneme of the flagella [4, 5]. In contrast to ciliated cells however, the flagellated sperm also contains various unique structures. Such an example is the acrosome-acroplaxome complex. While the acrosome is a Golgi derived structure that releases digestive enzymes necessary for successful fertilization of the oocyte, the acroplaxome anchors the acrosome to the nucleus, and it has also been proposed to have a role in nuclear shaping and condensation [6, 7].

During sperm development, a microtubule based structure, called the manchette, surrounds the elongating nucleus and it is along these microtubule tracts that proteins are ferried to the HTCA and developing flagellum through a process referred to as intra-manchette transport (IMT) [8]. Proper IMT is necessary for transport of many sperm cell proteins as well as development of the flagellum. IMT shares many common compo-
nents as intraflagellar transport (IFT), as they both involve molecular motors mobilizing with protein rafts to carry cargo [9]. This is further evidenced by the fact that Ift88 is found in both the manchette and the flagellum and mutations in Ift88 affect both processes [10]. Importantly, in order for the sperm to become fully motile and have the ability to successfully fertilize an oocyte, the developing sperm cell must correctly undergo all of these radical morphological changes and processes including proper assembly of the acrosome-acroplaxome complex, intramanchette transport, and growth of a flagella tail.

Disruptions in these events can lead to infertility or total sterility, which remains a problem affecting both ciliopathy patients and the male population as a whole [11, 12]. As such, the use of animal models has provided an excellent tool to find potential genes linked to flagella dysfunction.

Such an example can be seen in the genes Ccdc39 and Ccdc40, both of which are necessary for the proper assembly and function of flagella and motile cilia in a variety of vertebrates. For example, a cohort of Old English Sheepdogs with homozygous mutations in Ccdc39 suffered from chronic respiratory infections and can display situs inversus, two phenotypes caused by defective motile cilia in these animals [13]. Likewise, mice with mutations in Ccdc40 display not only situs inversus, but also hydrocephalus, again reflecting dysfunctional motile cilia [14]. The clinical relevance to these genes was demonstrated when numerous primary ciliary dyskinesia (PCD) patients were found to be homozygous for mutant alleles of CCDC39 or CCDC40 and suffered from infertility and other motile cilia related symptoms [15].

Other proteins with similar structural motifs to Ccdc39 and Ccdc40 also exist, including Ccdc42. Additionally, the widespread presence of Ccdc42 homologs exclusively
to organisms with flagella or motile cilia indicate it may have a role in the function of these organelles. Despite these facts, the function of Ccdc42 in flagella and cilia biology remains unexplored. Based on the findings of Ccdc39 and Ccdc40 mutant animals, we hypothesized that Ccdc42 also has a role in motile cilia or flagella assembly and would thus be a candidate ciliopathy or infertility gene.

**Methods**

**Generation of Ccdc42 Knockout Mice**

The Ccdc42 knockout allele (Ccdc42^tm1(KOMP)Vlcg, Knockout Mouse Project Repository, Davis, CA; hereinafter referred to as Ccdc42^KO) was generated using embryonic stem cells in which all seven exons of the wild-type Ccdc42 allele were removed and replaced with a β-galactosidase cassette. The correct targeting of the construct into Ccdc42 was confirmed by genomic PCR and sequence analysis. PCR primers for genotyping were designed based on the insertion. The embryonic stem cells containing the targeted allele were on the C57BL/6N background and were injected into albino C57BL/6 blastocysts (C57BL/6J-Tyrc-2J; JAX Laboratories) by the UAB Transgenic Mouse Facility using standard procedures. Chimeras were then crossed with albino C57BL/6 females and germline transmission was confirmed by coat color of the offspring and subsequent PCR genotyping. All mice were provided standard laboratory chow and water ad libitum, and maintained in accordance with IACUC regulations at the University of Alabama at Birmingham.
Reverse Transcription PCR Analysis

To assess Ccde42 expression among various organs, RNA was isolated from kidney, heart, liver, brain, lung, oviduct, ovary, seminal vesicle, and testes. Likewise, RNA was also extracted from testes at the various indicated timepoints. In both cases, Trizol reagent was used to extract RNA and was used according to the manufacturer’s protocol (15596-026, Life Technologies, Carlsbad, CA). Once extracted, RNA was used to synthesize cDNA using the Verso cDNA kit according to the manufacturer’s protocol (AB-1453, Thermo Scientific, Pittsburgh, PA). PCR analysis was then performed using the following primers (written 5’ to 3’) which flank the sequence between the first and last exons of the Ccde42WT allele: GGACTCGAGACCAGTTTGGGATGGGAA and GTACCCGGGACATCCGGACTTGTTG. The following primers were also used as a positive control to confirm the presence of actin in all samples: ATGGGTCAGAAGGACTCCTA and GGTGTAAAACGCAGCTCA. All results were confirmed by repeating the experiment in at least two additional animals.

β-Galactosidase Assays

Whole testes were extracted from Ccde42WT and Ccde42Het mice at 8 weeks of age. Tissues were fixed overnight at 4°C in 4% PFA in PBS and subsequently washed in PBS. Tissues were then cryoprotected with 30% sucrose in PBS for 24 h, and snap frozen in OCT (4583, Tissue-Tek, Torrance, CA) freezing compound. Ten μm sections were cut with a Leica CM1900 cryostat and sections were attached to Superfrost Plus microscope slides (12-550-15, Fisher Scientific, Pittsburgh, PA). Sections were postfixed in 4% PFA in PBS for 10 minutes, washed three times with lacZ wash buffer (2 mM
MgCl$_2$, 0.01% sodium deoxycholate, 0.02% NP-40, in 100 mM sodium phosphate buffer, pH 7.3), and then incubated in X-gal staining solution (2 mM MgCl$_2$, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mg ml$^{-1}$ X-Gal, in PBS) at 37°C overnight. Sections were then counterstained in Fast Red for 5 minutes.

**Testing Fertility of Ccdc42$^{KO}$ Mice**

To determine if Ccdc42$^{KO}$ mice were capable of producing offspring, one Ccdc42$^{KO}$ mutant would be set up with one wildtype mouse of the opposite sex. Copulation was determined by the presence of a vaginal plug the morning after the mating pair was set up. The female would then be separated into her own cage. The number of pups born, if any, was then counted 21 days after observation of the plug.

**Histology**

Testes were extracted from Ccdc42$^{WT}$ and Ccdc42$^{KO}$ mice, cut into halves, and fixed with Bouin’s fixative solution (1120-16, Ricca Chemical Company, Arlington, TX) for approximately 24 hours. Tissue was then rinsed thoroughly in tap water, dehydrated, embedded in paraffin, and cut into 10 μm sections following standard procedures. Sections were stained with Periodic acid-Schiff stain and counterstained with hematoxylin.

**Statistical Analysis**

The difference in fertility between Ccdc42$^{WT}$ and Ccdc42$^{KO}$ mice was assessed using a Wilcoxon rank sum test with continuity correction. Significance was established at $P < 0.01$. All calculations were performed using Microsoft Excel.
**Ccdc42 Antibody Generation**

Antisera against Ccdc42 was generated in rabbits by using a 19-residue peptide (DLSDIWTEVKKEQQQVRM) starting at position 294 of the mouse protein according to the standard protocol established by Open Biosystems (Huntsville, AL, USA).

**Immunofluorescence and transmission electron microscopy of mouse testes**

Samples were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 6.9) and postfixed in 2% osmium tetroxide in the same buffer, and embedded in a plastic resin. For electron microscopy, sections were stained with 5% uranyl acetate in methanol followed by lead citrate and examined using a JEM-100CX transmission electron microscopy operated at an accelerating voltage of 60 kV. For immunofluorescence, cells were incubated with an anti-Ccdc42 primary antibody (1:500) generated in rabbits. Fluorescence signal was detected with an anti-rabbit IgG conjugated with fluorescein isothiocyanate secondary antibody (1:200). Specimens were mounted with Vectashield (Vector Laboratories, Burlingame, CA) containing propidium iodide (to detect nuclei by a red emission color).

**Creation of GFP-Ccdc42 Tetrahymena thermophila**

To express *Ccdc42* in *Tetrahymena thermophila* cells, the coding region of Ccdc42 cDNA was cloned into pMTT1-GFP to create pMTT1-GFP-Ccdc42. The cDNA was amplified with primers carrying MluI (5’-TATATACGCGTCATGATTAATAAAATAAATCTAAT-3’) and BamHI (5’-TAATTGGATCCCTATCAATAGTTGTATTTAGTTTATTT-3’) sites. To introduce
transgene, starved CU522 *Tetrahymena thermophila* cells were bombarded with a SacII- and XhoI-digested pMTT1-GFP-Ccdc42 plasmid, and transformants were selected on SPP medium (1% proteose peptone, 0.2% glucose, 0.1% yeast extract, and 0.003% EDTA ferric sodium salt) with 20 μM paclitaxel. Using this approach, the transgene integrates by homologous recombination into the nonessential *BTU1* gene that carries a mutation conferring sensitivity to paclitaxel (for a thorough description of this process, see Shang et al. [16]). The copy number of the transgene was increased by allowing cells to assort the mutant *BTU1* allele during vegetative propagation in the presence of paclitaxel. To induce expression of GFP-Ccdc42, cells were incubated in 2.5 μg/ml CdCl₂ (202908, Sigma-Aldrich, St. Louis, MO).

*Immunofluorescence of Tetrahymena thermophila*

Approximately 100 induced or uninduced GFP-Ccdc42 *Tetrahymena* cells were isolated on coverslips, and were simultaneously fixed and permeabilized with 2% paraformaldehyde and 0.5% Triton X-100 in PBS buffer. Cells were then air dried at 30°C and blocked with 1% BSA for 1 hour. Immunofluorescence with 12G10 anti-α-tubulin monoclonal antibody (1:10) and anti-polyglycylation polyclonal (1:300) was performed by incubating cells in primary antibody diluted in 1% BSA overnight at 4°C. Cells were then washed with PBS and incubated in secondary antibodies for one hour at room temperature. Secondary antibodies included Alexa Fluor-647 and 594 conjugated donkey anti-rabbit and anti-mouse (A-21244 and A11012, Invitrogen, Carlsbad, CA). Nuclei were visualized by Hoechst nuclear stain (Invitrogen, Carlsbad, CA). Stained cells were
mounted using DABCO mounting media (10 mg of DABCO (D2522; Sigma-Aldrich, St. Louis, MO) in 1 mL of PBS and 9 mL of glycerol). Slides were sealed using nail polish.

**Results**

*Ccdc42*<sup>KO</sup> Mice are Viable

To determine the cellular function of *Ccdc42* in a mammalian system, a mouse embryonic cell line harboring a null allele of *Ccdc42* was obtained and used to generate a knockout mouse line (hereinafter referred to as *Ccdc42*<sup>KO</sup>). The null allele was created by using homologous recombination to target all seven exons of the wildtype *Ccdc42* allele and replace it with a β-galactosidase cassette (Figure 1A). We then crossed Ccdc42 heterozygotes to produce wild-type (*Ccdc42*<sup>WT</sup>), Ccdc42 heterozygous (*Ccdc42*<sup>Het</sup>), and homozygous Ccdc42 knockout mice (*Ccdc42*<sup>KO</sup>) (Figure 1B). Surprisingly, in contrast to *Ccdc39* and *Ccdc40* mutant animals, *Ccdc42*<sup>KO</sup> mice were born at a rate that did not significantly deviate from the Mendelian ratio, and showed no obvious signs of improper development or ill-health (Figure 1C).

*Ccdc42 is expressed in a tissue and age dependent manner*

The apparent lack of a developmental phenotype led us to analyze where and when the gene was being expressed in order to better understand what biological processes were being disrupted by the loss of *Ccdc42*. RT-PCR analysis revealed *Ccdc42* is expressed specifically in the testes. This expression did not extend to all areas of the male reproductive system as the seminal vesicles, organs that secrete fluid to make semen, tested negative (Figure 2A) [17]. Furthermore, this expression was time dependent, as
Figure 1. Creation of Coiled-coil domain containing 42 (Ccdc42) knockout mice. (A) The Ccdc42 knockout allele was created by deleting the endogenous seven exons of the gene and replacing it with a β-gal reporter cassette through homologous recombination. (B) PCR genotyping of Ccdc42<sup>WT</sup>, Ccdc42<sup>Het</sup>, and Ccdc42<sup>KO</sup> mice. (C) An anesthetized Ccdc42<sup>WT</sup> mouse next to a Ccdc42<sup>KO</sup> sibling.
Figure 2. *Ccdc42* is expressed in the testes in a time and spatially dependent manner. (A) RT-PCR gel showing *Ccdc42* is expressed specifically in the testes of all organs tested. (S. vesicle = seminal vesicle). All samples come from 8 week old male mice except for the oviduct and ovaries, which came from 8 week old female mice. β-Actin is used as a positive control for template. (B) RT-PCR gel showing *Ccdc42* expression in the testes only begins at approximately 10 days of age. β-Actin is used as a positive control for template. (C) β-galactosidase staining assay showing *Ccdc42* expression in the maturing sperm cells of the seminiferous tubules of Ccdc42^Het^ testes. Sections from Ccdc42^WT^ testes were used as a negative control. Sections were counterstained with Fast Red. Scale bar represents 60 µm.
testicular expression of Ccdc42 was not detected until 10-15 days of age and then continued to be expressed at all future timepoints tested (Figure 2B). The spatial pattern of Ccdc42 expression was assessed using the β-galactosidase (β-gal) reporter gene found in the mutant allele to determine where in the testes Ccdc42 is expressed. In testes from adult Ccdc42Het, mice revealed that the strongest expression of Ccdc42 was found in flagellated spermatids closest to the lumen of the seminiferous tubules. Conversely, cells closest to the basement membrane of the seminiferous tubules and cells that are found in-between tubules displayed very little, if any, expression of Ccdc42 (Figure 2C).

_Cccdc42 localizes to the acrosome-acroplaxome complex and manchette in developing spermatids_

To determine the localization of Ccdc42 protein in developing spermatids, sections of testes from adult mice where immunolabeled with a Ccdc42 antibody. In agreement with the β-gal expression data, immature spermatozoa closest to the basement membrane of the seminiferous tubules did not express the Ccdc42 protein. In contrast, Ccdc42 was detected at the acrosome-acroplaxome complex in round spermatids, a structure which anchors the acrosome to the spermatid nucleus (Figure 3A). Elongating spermatids likewise stained positive for Ccdc42, but in addition to the localization at the acroplaxome, Ccdc42 was also present at the leading edge of the acroplaxome, a structure called the marginal ring of the acroplaxome (MRapx) and is believed to be necessary for nuclear shaping [18]. Interestingly, Ccdc42 was also observed in puncta along the manchette, the microtubule structure which surrounds the nucleus (Figure 3B). These findings strongly implicate a role for Ccdc42 in sperm maturation and may imply a defect in this process in the Ccdc42KO mice.
Figure 3. Ccdc42 localizes to the acrosome-acroplaxome and manchette in developing spermatids. (A) At the round spermatid stage (rspd), Ccdc42 localizes to acrosome-acroplaxome complex, while at the late spermatid stage (lspd) Ccdc42 is found along the elongated and condensed nucleus. No localization is seen in spermatogonia (spg) or spermatocytes. Arrowhead indicates seminiferous tubular wall. (B) During the elongating spermatid stage (espd), Ccdc42 localizes to the acrosome-acroplaxome complex and the marginal ring of the acroplaxome (MRapx), as well as discrete puncta along the manchette. Arrows indicate intramanchette transport along microtubules of the manchette. Nuclei are stained with propidium iodide. Scale bars are 5 μm.
Male Ccdc42\textsuperscript{KO} mice are sterile

The specific expression of Ccdc42 in the testes, the induction during adolescence, in addition to localization of the protein to developing spermatozoa, led us to hypothesize that the gene had a role in fertility. To test this, we set up timed pregnancies of Ccdc42\textsuperscript{KO} mice crossed with wildtype mice of the opposite sex. Ccdc42\textsuperscript{KO} males mated with wildtype females as evidenced by a vaginal plug the morning after a mating pair was set up; however, no pups were ever produced as a result of these matings. Conversely, Ccdc42\textsuperscript{KO} females mated with wildtype males and produced litters of a size that did not significantly deviate from wildtype by wildtype matings. Thus, male Ccdc42 mutants are sterile while females have normal fertility (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>Ccdc42\textsuperscript{WT} (♂) x Ccdc42\textsuperscript{WT} (♀)</th>
<th>Ccdc42\textsuperscript{WT} (♂) x Ccdc42\textsuperscript{KO} (♀)</th>
<th>Ccdc42\textsuperscript{KO} (♂) x Ccdc42\textsuperscript{WT} (♀)</th>
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<tr>
<td>Fertility (Litters/plug)</td>
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<td>0/12</td>
</tr>
<tr>
<td>Mean Litter Size (pups)</td>
<td>7.8 ± 0.4</td>
<td>6.3 ± 0.6</td>
<td>0*</td>
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Table 1. Ccdc42\textsuperscript{KO} males are infertile. Males and females (8 – 12 weeks of age) from both Ccdc42\textsuperscript{WT} and Ccdc42\textsuperscript{KO} mice were tested for fertility (number of litters per copulatory plug) and for average litter size. No significant difference was found in the mean litter size between Ccdc42\textsuperscript{WT} and Ccdc42\textsuperscript{KO} females according to a Wilcoxon Rank Sum test (*P < 0.01). In contrast, Ccdc42\textsuperscript{KO} males were completely sterile. Copulation was determined by the presence of a vaginal plug the morning after a mating was established.

Defective spermatozoa development in Ccdc42\textsuperscript{KO}

We next performed histological analysis on testes from Ccdc42\textsuperscript{KO} mice to determine if any morphological defects could account for the male infertility phenotype. Ccdc42\textsuperscript{WT} mice displayed normal morphology as evidence by the production of flagellat-
ed spermatozoa projecting into the lumen of the seminiferous tubules (Figure 4A). In contrast, sections of testes from Ccdc42\(^{KO}\) mice revealed seminiferous tubules with an absence of a lumen containing flagellated spermatozoa (Figure 4B). This finding indicated that Ccdc42 was necessary for proper spermatozoa development, thus explaining the male infertility of Ccdc42\(^{KO}\) mice.

Figures 4. Ccdc42\(^{KO}\) mice show abnormally testicular morphology. (A) A histological cross section of testes taken from a Ccdc42\(^{WT}\) mouse. Developing sperm project their thread-like flagella into the lumen of the seminiferous tubules. (B) Cross section of testes taken from Ccdc42\(^{KO}\) mice displays an absence of a vesicular lumen in addition to the absence of sperm flagella. Sections are stained with Periodic Acid-Schiff and counterstained with hematoxylin. Scale bar represents 60 µm.

To better characterize the spermatozoa phenotype in Ccdc42\(^{KO}\) males, we utilized electron microscopy to assess possible ultrastructural defects in individual spermatozoa. As indicated by the histology, a near complete absence of flagella was observed among Ccdc42\(^{KO}\) spermatozoa in our electron microscopy analysis (Figures 5,6). In place of flagella, an accumulation of transport vesicles was sometimes observed. Strikingly howev-
er, defects in the number and positioning of the head-tail coupling apparatus (HTCA), the centrosome based structure homologous to the ciliary basal body, were found. While wildtype spermatozoa possess one HTCA that anchors the developing flagellum to the rest of the cell, $Ccdc42^{KO}$ spermatozoa contained anywhere from zero to three HTCA structures that were either aberrantly assembled and/or abnormally placed within the cell (Figure 5A,B). Even in cases where the HTCA appeared to be placed properly, morphological defects such as a lack of a flagella and abortive acrosome formation were still observed (Figure 6A). Other morphological phenotypes, such as abnormal nuclear folding and condensation, were also noticed (Figure 6B).

*Tetrahymena thermophila* have a potential homolog of *Ccdc42* that localizes to the base of the cilia

Assessing the function of a gene in sperm cells can be difficult. By their nature, sperm are short lived and non-dividing cells, making them difficult to maintain in culture. Furthermore, the highly condensed nature of the sperm nucleus renders the cell essentially transcriptionally silent making the expression of fluorescently tagged proteins nearly impossible. Therefore, we sought other organisms with potential homologs of *Ccdc42* where gene function could be more easily manipulated. Interestingly, organisms with only a primary cilium (e.g. *C. elegans*) did not possess possible *Ccdc42* homologs. In contrast, most organisms with motile cilia do have a putative homolog. In *Tetrahymena thermophile* there are two potential protein homologs, one of which was TTHERM_00730320. Unlike sperm cells, *Tetrahymena* are easy to culture, and genetically manipulate. To determine if TTHERM_00730320 had characteristics consistent
Figure 5. Electron microscopy reveals abnormalities in the head-tail coupling apparatus (HTCA) in Ccdc42KO spermatids. (A) Ccdc42KO spermatids lacking a microtubule based flagellum. Instead, transport vesicles have accumulated where the flagella should be. Additionally, three head-tail coupling apparatuses (HTCA) are present instead of the usual one. The acrosome and microtubule based manchette appear normal. (B) Another example of a Ccdc42KO spermatid lacking a flagellaum tail, with two HTCA's instead of the usual one. Additionally, mitochondria have failed to migrate properly and the acrosome has started to degrade, as evidence by its dissociation from the nucleus.
Figure 6. Electron microscopy reveals abnormalities in acrosome formation and nuclear folding in Ccdc42\textsuperscript{K0} spermatids. (A) Ccdc42\textsuperscript{K0} spermatid with an abortive acrosome and lack of flagellum. Placement of the head-tail coupling apparatus (HTCA) appears normal. (B) A Ccdc42\textsuperscript{K0} spermatid with severe disruptions in nuclear condensation and nuclear folding.
with being a homolog of *Ccdc42*, we created transgenic *Tetrahymena* wherein THER_00730320 was tagged with GFP and placed under the control of a promoter inducible with cadmium chloride (CdCl₂). In the uninduced state, no GFP signal is detected in the *Tetrahymena* cells positive for the transgene. However, once cadmium chloride is added, robust expression of the GFP-THERM_00730320 fusion protein is observed. Co-staining the *Tetrahymena* cells with an antibody against α-tubulin (12G10) to label cilia tips and an antibody against polyglycylation (2302) which labels cilia in *Tetrahymena* cells, revealed that GFP localizes specifically to the base of each motile cilia (Figure 7). This observation is consistent with the *Ccdc42*KO mice where defects are seen in the HTCA, a structure which localizes to the base of the flagella. We therefore propose that THERM_00730320 is a homolog of mammalian *Ccdc42*, and for the sake of brevity refer to the transgene as GFP-Ccdc42.

**Discussion**

Our studies of *Ccdc42* reveal a highly specific defect in sperm maturation leading to male infertility. This gene is thus distinct from the recently characterized *Ccde39* and *Ccde40* genes that encode two proteins with structural motifs similar to Ccdc42 and were found to be necessary for both flagella and motile cilia function [13, 14]. In agreement with the testes specific expression of *Ccde42*, *Ccde42KO* mice displayed no obvious signs of improper development or otherwise ill-health and were visually indistinguishable from wildtype siblings. Unlike many other knockout mouse models, where a portion of the endogenous gene remains in the mutant allele, the entire coding sequence of *Ccde42* is missing from our *Ccde42KO* allele, making the possibility that alternate transcripts are
Figure 7. GFP-Ccdc42 localizes to the base of the cilia in transgenic Tetrahymena thermophila. (A-D) In the uninduced state, no GFP signal can be seen in transgenic GFP-Ccdc42. Cilia axonemes are labeled with a polyglycylation (2302) antibody. Cilia tips are labeled with an α-tubulin antibody (12G10). (B-L) Two hours after the addition of cadmium chloride (CdCl₂), GFP-Ccdc42 localizes to the base of the cilia. Cilia axonemes are labeled with a polyglycylation (2302) antibody. Cilia tips are labeled with an α-tubulin antibody (12G10). All nuclei are labeled with Hoechst. Scale bar is 30 μm.
masking other phenotypes highly unlikely. However, it remains possible that another
gene is compensating for the loss of Ccdc42 in other cell types.

The observation that Ccdc42 expression was upregulated in the testes between 10 – 15 days of age is informative, as previous reports found that at approximately 12 days of age the expression of many genes necessary for sperm development and flagella assembly is significantly increased [19]. Male mice are not capable of producing mature sperm cells until approximately 35 days of age and it is believed that the increased expression of genes allows the first wave of morphological changes (including nuclear condensation and flagellum assembly) necessary to create mature sperm [20]. That is to say, before 12 days of age, cells within the testes are generally undergoing mitotic cell divisions, and are not yet building a flagellum or undergoing nuclear condensation. The fact that Ccdc42 is not expressed before 10 – 15 days of age indicate that the gene is dispensable for these early cell divisions, and instead may be have a role with the morphogenesis of the cells into mature sperm.

In addition to developing sperm and germ stem cells, Sertoli cells are also found in the seminiferous tubules [21]. These cells are attached to the inside basement membrane on the seminiferous tubules, and are responsible for nourishing the developing spermatozoa. In the interstitial space between the seminiferous tubules reside Leydig cells, which are responsible for the production of testosterone and other androgens [22]. The β-galactosidase staining assays for Ccdc42 expression suggests that cells closest to the basement membrane and cells in the interstitium do not express detectable levels of Ccdc42 arguing in favor of a spermatozoa autonomous function of Ccdc42. Also of importance is the fact that Ccdc42KO males still produced copulatory plugs and thus were
actually mating with females, confirming that the sterility phenotype was not due to reproductive behavior abnormalities.

While \(\text{Cc}dc42^{\text{KO}}\) males were sterile, \(\text{Cc}dc42^{\text{KO}}\) females have normal fertility, consistent with the fact that expression was found in the testes, but not in the ovaries or oviducts. As motile cilia are needed to move oocytes to the uterus, the normal fertility of \(\text{Cc}dc42^{\text{KO}}\) females indirectly supports the notion that \(\text{Cc}dc42\) is not necessarily for motile cilia assembly or function [23].

Localization studies using a Ccdc42 antibody revealed that in developing spermatozoa the protein localizes to the acrosome-acroplaxome complex, a characteristic that is also reported for Ift88, a protein needed for assembly of cilia and flagella [10]. Previous publications further demonstrated that Ift88 mutants display abnormal sperm head shaping in addition to a lack of flagellum [10]. While we also observed these phenotypes in \(\text{Cc}dc42^{\text{KO}}\) spermatozoa, in \(\text{Cc}dc42^{\text{KO}}\) there are also defects in the placement and proper number of the head-tail coupling apparatus (HTCA). Furthermore, while expression of \(\text{Cc}dc42\) is restricted to the testes, Ift88 is ubiquitously expressed and is required for global cilia and flagella assembly. It thus remains likely that in spermatozoa, Ccdc42 has a molecular function at least partially distinct from IFT proteins.

Our recent work in \textit{Tetrahymena thermophila} corroborates our findings in mice. The basal body in \textit{Tetrahymena} is homologous to the HTCA of sperm cells. Therefore, the localization of GFP-Ccdc42 to the basal body in \textit{Tetrahymena} is consistent with the defects in HTCA placement and number in the \(\text{Cc}dc42^{\text{KO}}\) sperm. Unlike spermatozoa, \textit{Tetrahymena} are relatively easy to culture and manipulate, and we propose that this eukaryote will be a model organism for future studies of \(\text{Cc}dc42\). It remains possible that
evolutionary divergence has created distinct roles for the gene in these two different cell types. Conversely, functional redundancy could also exist for the gene, as another *Tetrahymena* gene, named TTHERM_00773210, was also found to be a potential homolog of mammalian *Ccdc42*. Further studies will help clarify this discrepancy.

The exact molecular function of *Ccdc42* is unclear, but several possibilities exist based on our observations. The Ccdc42 positive puncta that are seen along the manchette in elongating spermatids may hint to a role for Ccdc42 in intramanchette transport (IMT). IMT has been proposed to sort structural proteins to the HTCA and developing flagella, and it remains possible that Ccdc42 is an essential component of the IMT machinery [8]. In this scenario, loss of Ccdc42 would prevent the transport of IMT ferried proteins, leading to defects in the HTCA and inability to build a flagellum. However, the manchette or a manchette-like structure has not been described in *Tetrahymena thermophila* cells, and thus this hypothesis does not explain what purpose *Ccdc42* may have in that organism.

Conversely, in mice sperm cells, Ccdc42 might simply be transported by IMT and have some other role in the positioning of the HTCA. Alternatively, *Ccdc42* may be necessary for the transport or accumulation of proteins to the basal bodies of *Tetrahymena* cells in a manner similar to IMT, thus explaining the strong localization of GFP-Ccdc42 to the base of motile cilia.

The abnormal number of HTCA structures seen in *Ccdc42 KO* spermatozoa could also point to a role for the protein during meiosis. As mentioned previously, the HTCA is a centrosome based structure [4, 5]. During meiosis, the centrosomes duplicate and are sorted into what will become the two new daughter cells. Given the fact that some *Ccdc42 KO* sperm cells contain a loss of the HTCA while others contain an excess, it re-
mains possible that Ccdc42 is necessary for the sorting of the centrosomes in spermatozoa cell division. The abnormal number of centrosomes would then give rise to disruptions in flagella assembly as a secondary consequence. The abnormal number of HTCA structures might also mean that loss of Ccdc42 causes abnormal degradation and de novo assembly of new centrosomes, also leading to disruption of flagella assembly. Notably however, even Ccdc42KO spermatozoa that had the proper number of HTCA structures were still unable to assemble flagella, making these explanations only partially responsible for the phenotype at best.

The infertility phenotype in male Ccdc42KO mice in the absence of other maladies raises the possibility that homozygous loss of function mutations in CCDC42 are a cause of male infertility in human patients. As female Ccdc42KO mice show no defects in fertility, such a deleterious allele would have a higher chance of being passed onto future generations compared to those that affect both male and female fertility. Similar findings have been documented with other mutant mice. For example, azh mutant mice also have a male infertility phenotype due to abnormal sperm head shaping [24]. The azh mutant mice were later found to harbor mutations in the gene Hook1, whose protein product localizes to the acrosome-acroplaxome complex and manchette in a similar fashion to Ccdc42 [25]. However, unlike the Ccdc42KO we report here, azh mutants are still able to assemble flagella, indicating distinct differences in molecular function. Future studies screening male infertility patients for mutations in CCDC42 will help to address this question and provide more insights into its molecular function.
References


NEURONAL CILIA AND OBESITY

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*Both authors contributed equally to this work

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Format adapted for dissertation
The cilium is a small microtubule based cellular appendage found on most mammalian cells where it plays a crucial role as a complex sensory and signaling center (for a review on cilia signaling see (Berbari et al. 2009)). There is now an emerging class of genetic diseases coined ciliopathies that is associated with dysfunction of the cilium. Interestingly, the clinical features of ciliopathies range from renal cysts in humans with polycystic kidney disease (PKD1, OMIM #601313) to the cystic kidneys, skeletal anomalies, neurodevelopmental defects, heart malformations and perinatal lethality associated with Meckel-Gruber Syndrome (MKS1, OMIM #249000) (for an in-depth review on ciliopathies see (Sharma et al. 2008)). This broad scope of clinical features has been attributed to both the ubiquitous nature of the cilium and the types of mutations affecting its function. PKD, for example, is associated with mutations in signaling proteins that appear to have crucial roles in renal homeostasis, while the mutations in MKS are often associated with proteins found at the base of the cilium, where they are thought to play more general roles in cilia structural integrity and the regulation of cilia protein composition. Until relatively recently much of our knowledge of cilia has come from studies in model systems focusing on the process of building and maintaining the cilium, known as Intraflagellar Transport (IFT) (for a review on IFT machinery see (Pedersen and Rosenbaum 2008)). While the list of ciliopathies continues to grow at an incredible pace, our current understandig of how dysfunction of the cilium leads to some of the clinically observed phenotypes remains elusive. One of the clinical features where this is most evident is the hyperphagia-associated obesity that occurs in two ciliopathies, Alström Syndrome (ALMS, OMIM #203800) and Bardet-Biedl Syndrome (BBS, OMIM #209900).
The proteins affected in both of these disorders are associated with the cilium and are required for normal cilia function or formation.

Bardet-Biedl Syndrome (BBS) is a group of rare genetically heterogeneous disorders resulting in an array of seemingly unrelated symptoms and progressive degenerative defects. A triad of symptoms including obesity, retinitis pigmentosa/retinal degeneration, and polydactyly remains the hallmark for diagnosing the disease, as was the case when it was independently classified by Georges Bardet and Arthur Biedl in the early 20th century (Bardet 1920; Biedl 1922). Subsequent analysis has revealed that hypogonadism, renal dysfunction, and mental retardation are also highly prevalent among BBS patients. Nearly half of BBS patients are completely or partially anosmic and have deficits in thermosensation (Kulaga et al. 2004; Tan et al. 2007). Also of consequence are the secondary features of BBS, including diabetes mellitus, hypertension, and heart disease, which develop likely as a result of the obesity. Less commonly, BBS patients can have situs inversus, a defect often caused by dysfunctional cilia on the embryonic gastrulation stage structure called the node (Lorda-Sanchez et al. 2000; Deffert et al. 2007). Among North American and European populations, BBS is relatively uncommon with an estimated occurrence of 1 in 120,000 live births, while in Middle Eastern populations it has been reported to be as high as 1 in 13,500 (Klein and Ammann 1969; Croft et al. 1995; Beales et al. 1997). Although it is possible to detect the condition during gestation, patients are typically diagnosed when both obesity and retinal degeneration are apparent and polydactyly or mental deficits have been observed.

Confusion in diagnosing BBS exists, as a similar condition, Laurence-Moon Syndrome, is also a genetically-inherited disease in which patients present with retinitis pig-
mentosa and mental disability. This observation subsequently caused Solis-Cohens and Weiss to conclude that both diseases are synonymous and thus both disorders have been referred to as Laurence-Moon-Bardet-Biedl Syndrome. However, there is debate about the synonymous classification as the patients reported by Laurence and Moon displayed paraplegia of the lower extremities that is not typically associated with BBS. Furthermore, polydactyly and obesity are not generally traits of Laurence-Moon syndrome, while they are clinical hallmarks of BBS. Given that polydactyly is not fully penetrant among BBS patients, and that the obesity experienced by most BBS patients can be attenuated or eliminated with diet and exercise, the lack of these symptoms does not necessarily mean the two disorders are not allelic variations (Beales et al. 1999; Ghadami et al. 2000).

BBS has extensive genetic heterogeneity with 16 known loci (BBS1-BBS16, Table 1). Mutations in BBS1-10 are responsible for approximately 70% of known BBS cases, and even the identification of BBS11-16 only added fractional amounts to that number; thus it remains almost certain that other BBS genes have yet to be found (Chen et al. 2011; Stoetzel et al. 2006; Leitch et al. 2008; Chiang et al. 2006). Conversely, the genetic basis of Laurence-Moon is currently unknown, so it remains to be seen if the symptoms of Laurence-Moon are caused by mutations in BBS genes.

The mode of BBS inheritance is also complicated. Although originally believed to be a typical recessive Mendelian disorder, analysis by Katsanis et al. demonstrated this may not always be the case (Katsanis et al. 2001). In their study, the authors reported families where both affected and unaffected individuals could carry homozygous mutant alleles of BBS2. However, the affected individuals were also heterozygous for a mutation
Table 1. Bardet-Biedl syndrome genes. Known BBS genes are listed with their location in the human genome as well as their known protein motifs and domains. Members of the BBSome are indicated with an * and BBS chaperone proteins are indicated with a #.

<table>
<thead>
<tr>
<th>BBS</th>
<th>Gene</th>
<th>Human Locus</th>
<th>Motifs/Domains</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBS1</td>
<td>BBS1</td>
<td>11q13.2</td>
<td>Beta propeller*</td>
<td>(Mykytyn et al. 2002)</td>
</tr>
<tr>
<td>BBS2</td>
<td>BBS2</td>
<td>16q12.2</td>
<td>Beta propeller*</td>
<td>(Nishimura et al. 2001)</td>
</tr>
<tr>
<td>BBS3</td>
<td>ARL6</td>
<td>3q11.2</td>
<td>ADP ribosylation factor-like 6</td>
<td>(Chiang et al. 2004)</td>
</tr>
<tr>
<td>BBS4</td>
<td>BBS4</td>
<td>15q24.1</td>
<td>TPR repeats*</td>
<td>(Mykytyn et al. 2001)</td>
</tr>
<tr>
<td>BBS5</td>
<td>BBS5</td>
<td>2q31.1</td>
<td>Pleckstrin homology*</td>
<td>(Kulaga et al. 2004)</td>
</tr>
<tr>
<td>BBS6</td>
<td>MKKS</td>
<td>20p12.2</td>
<td>Chaperone-like&quot;</td>
<td>(Slavotinek et al. 2000)</td>
</tr>
<tr>
<td>BBS7</td>
<td>BBS7</td>
<td>4q27</td>
<td>Beta propeller*</td>
<td>(Badano et al. 2003)</td>
</tr>
<tr>
<td>BBS8</td>
<td>TTC8</td>
<td>14q31.3</td>
<td>TPR repeats*</td>
<td>(Ansley et al. 2003)</td>
</tr>
<tr>
<td>BBS9</td>
<td>PTHB1</td>
<td>7p14.3</td>
<td>Beta propeller*</td>
<td>(Nishimura et al. 2006)</td>
</tr>
<tr>
<td>BBS10</td>
<td>BBS10</td>
<td>12q21.2</td>
<td>Chaperone-like&quot;</td>
<td>(Stoetzel et al. 2007)</td>
</tr>
<tr>
<td>BBS11</td>
<td>TRIM32</td>
<td>9q33.1</td>
<td>E3 Ubiquitin Ligase</td>
<td>(Chiang et al. 2006)</td>
</tr>
<tr>
<td>BBS12</td>
<td>BBS12</td>
<td>4q27</td>
<td>Chaperone-like*</td>
<td>(Stoetzel et al. 2007)</td>
</tr>
<tr>
<td>BBS13</td>
<td>MKS1</td>
<td>17q22</td>
<td>B9/C2</td>
<td>(Leitch et al. 2008)</td>
</tr>
<tr>
<td>BBS14</td>
<td>CEP290</td>
<td>12q21.32</td>
<td>Coiled-coil</td>
<td>(Leitch et al. 2008)</td>
</tr>
<tr>
<td>BBS15</td>
<td>WDPCP</td>
<td>2p15</td>
<td>Coiled-coil, WD40</td>
<td>(Kim et al. 2010)</td>
</tr>
<tr>
<td>BBS16</td>
<td>SDCCAG8</td>
<td>1q43</td>
<td>Globular, coiled-coil</td>
<td>(Schaefer et al. 2011)</td>
</tr>
</tbody>
</table>
in a second BBS gene, BBS6. This phenomenon, which the authors named ‘triablelic inheritance’, demonstrates that manifestations of BBS phenotypes sometimes rely on mutations at a second locus and thus BBS may result from the overall genetic mutational load in a patient. However, analysis of the triallelic inheritance hypothesis in several other BBS cohorts did not reveal evidence of triallelism suggesting this may be an exception (Mykytyn et al. 2003; Abu-Safieh et al. 2012; Smaoui et al. 2006; Laurier et al. 2006). Information from these cohorts suggests that BBS is an autosomal recessive inherited disease. But these data cannot rule out possible contributions from unidentified BBS alleles and it remains possible that BBS can manifest through both an autosomal recessive fashion and triallelic inheritance.

The great degree of variability in both inheritance and symptoms presented by BBS patients leads to the question of whether there exists any correlation between the genes and mutations involved and the way the disease manifests itself. For example, while obesity and renal abnormalities are frequent, the degree of mental retardation or learning disabilities varies greatly, with patients from some families showing little or no mental deficits (Riise et al. 1997). Thus, the relation between BBS mutations and the expressivity of traits has been of great interest, but largely remains inconclusive with limited genotype-phenotype correlation.

Birth weight in BBS patients is usually normal, with obesity developing during childhood continuing into adulthood (Beales et al. 1999). This observation indicates that the obese phenotype may not be a direct consequence of defects in development, but rather due to errors in energy metabolism or appetite regulation. Evidence for this hypothesis is found in the fact that when compared to BMI matched controls, BBS patients did
not possess significant differences in body fat or resting metabolic rate (RMR) (Grace et al. 2003). Although this information indicates that body fat and RMR is the same, subsequent work demonstrated that circulating leptin and triglyceride levels were significantly higher in BBS patients compared to other BMI matched obese individuals, despite the fact that glucose tolerance and insulin resistance was comparable between the two groups (Feuillan et al. 2011). Leptin has a known role in suppressing appetite, and this finding supports the possibility that BBS patients have a higher degree of leptin resistance. Recent work in animal models have strongly implicated the primary cilia as being necessary for regulating appetite, and that cilia regulated signaling can be disrupted in mouse models of BBS (Davenport et al. 2007; Weatherbee et al. 2009). However, the molecular mechanism causing this disease in BBS patients remains uncertain.

The other human ciliopathy associated with obesity is Alström syndrome (ALMS), which was first classified in 1959 (for a review of ALMS see (Girard and Petrovsky 2011)). Human ALMS patients manifest with several symptoms including obesity, retinitis pigmentosa, and hearing loss with a tendency towards shorter stature, and a disruption in the growth hormone/Insulin-like growth factor 1 signaling axis. They also exhibit phenotypes likely related to their obesity that include diabetes mellitus and elevated leptin levels when compared to unaffected individuals (Maffei et al. 2007). ALMS is a rare autosomal recessive disorder with an occurrence at less than 1 in 100,000 and is caused by mutations in the gene ALMS1 (Collin et al. 2002; Hearn et al. 2002). To date, 81 different disease causing mutations in ALMS1 have been reported (Joy et al. 2007; Marshall et al. 2007). The exact function of ALMS1 remains unknown, but clues to its possible cellular function was uncovered when it was found that ALMS1 is widely
expressed and localizes to the centrosome and the basal body of the primary cilium in cultured human cells (Hearn et al. 2005; Knorz et al. 2010). Interestingly, dermal fibroblasts derived from an ALMS patient had normal basal body localization and primary cilia assembly suggesting that ALMS1 might be involved in ciliary related signaling pathways, but not in establishing cilia architecture itself (Hearn et al. 2005). In contrast, knockdown of Alms1 by siRNA in mouse inner medullary collecting duct (mIMCD3) cells caused a stunted cilia phenotype, and also impaired their mechanical stimuli sensing abilities of these cells. This discrepancy in phenotype could be due the nature of the mutation, which may not have a caused a complete lack of protein function, in the ALMS patients.

Although BBS and ALMS are relatively rare diseases, understanding how these proteins normally regulate satiation responses will provide important insights into molecular pathways that could be manipulated to control satiation and obesity.

**Ciliopathy Mouse Models of Obesity**

To better understand the causes of human obesity genetically manipulated mouse models are continuously being developed with the ultimate goal of elucidating the molecular mechanisms driving the phenotype. Surprisingly, over the past decade the primary cilium has emerged as key factor regulating satiation responses. The Bbs and Alms1 mouse models along with mutations affecting the Intraflagellar transport 88 (Ift88) gene have established a strong link between defects cilia mediated sensory or signaling activity and obesity (Table 2). In this section, we will review data derived from several of the
obesity mouse models that have been associated with ciliary dysfunction and highlight the proposed function of the affected proteins.

**Bbs Mutant Mouse Models**

Seminal work in the BBS field by Nachury *et. al.* has shown that BBS proteins 1, 2, 4, 5, 7, 8, and 9 form a ~450kDa complex called the BBSome (Nachury *et al.* 2007). The BBSome is thought to function in transport of membrane along with specific trans-membrane proteins to the cilium (Jin *et al.* 2010). Of these BBSome genes, mouse models of *Bbs1*, *Bbs2*, *Bbs4*, and *Bbs8* have been generated and characterized. The validity of utilizing mice to model human BBS was demonstrated when a knock-in allele of the *Bbs1* M390R mutation, one of the most common single human BBS disease alleles, was created and replicated many of the human symptoms of BBS, including retinal degeneration, male infertility, and obesity (Davis *et al.* 2007). Strikingly these hallmark features of BBS are shared phenotypes among other mouse models of BBS such as *Bbs2* and *Bbs4* mutants (Mykytyn *et al.* 2004; Nishimura *et al.* 2004). Neurological defects were also observed. For example, disruption of *Bbs1* or *Bbs4* caused cilia loss on the olfactory epithelium, and the same report demonstrated partial or total anosmia in a cohort of human BBS patients. These same studies found a common social dominance defect among *Bbs2* and *Bbs4* mutant mice, in which the mutants were more submissive to control mice. Although the olfactory and behavior phenotypes may not directly be related to the obesity seen in these mice, it does reflect the importance of the BBSome genes in the regulation of behavior.
As in BBS patients, obesity is not present in young Bbs mutant mice. In fact, most Bbs mutant mice are initially runted, and it has been proposed that this is possibly due to olfactory defects that make it difficult for pups to accomplish nipple searching and suckling (Eichers et al. 2006). However, mutants eventually developed hyperphagia and became obese (Figure 1A). The obesity phenotype also correlated with hyperleptinemia in Bbs1 M390R knock-in mice. More recently, Bbs8-null mice have been reported that also have defects in olfactory function, as has been shown in Bbs1 and 4 mutant mice.

When these Bbs8 mutant mice were crossed to an olfactory receptor reporter line (M72TL), severe defects in the targeting of olfactory sensory neurons became apparent, and individual axonal fibers seemed to wander, instead of terminating at single glomerulus as in the control mice (Tadenev et al. 2011). The axonal targeting defects reported in the Bbs8 mutant mice further confirm the importance of the BBSome in proper neuronal development and signaling.

Other genes involved in human BBS that do not encode direct BBSome components have been identified. These proteins share homology to chaperones, and there are indications that these too are necessary for normal activity of satiation pathways. These genes, include BBS6, 10, and 12, that encode proteins that may be necessary to stabilize the BBSome (Seo et al. 2010). Of these three, only a mouse mutant of Bbs6 (previously referred to as Mkks for its involvement in McKusick-Kaufman syndrome) has been reported. As seen in the other BBSome mutant mice, Bbs6 mutants display age dependent retinal degeneration and exhibit hyperphagic behavior leading to obesity with elevated leptin levels (Fath et al. 2005). Furthermore, male infertility was reported due to a failure
Table 2. *Cilia associated mouse models of obesity.* Mutant mice with defects in known or potential cilia genes are shown alphabetically, along with the allele listing according to Mouse Genome Informatics (MGI; http://www.informatics.jax.org/). Please note that the mouse phenotypes column includes some of the more prominent features, but for the sake of brevity, is not comprehensive.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele (MGI)</th>
<th>Type of Allele</th>
<th>Mouse Phenotypes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC3</td>
<td>Adcy3&lt;sup&gt;tm1Drs&lt;/sup&gt;</td>
<td>Knock-out</td>
<td>Obesity, anosmia</td>
<td>(Wang et al. 2009; Wong et al. 2000)</td>
</tr>
<tr>
<td>Alms1</td>
<td>Alms&lt;sup&gt;Cb(XH152)Byg&lt;/sup&gt;</td>
<td>Genetrap</td>
<td>Obesity, retinopathy, male infertility, late onset hearing loss</td>
<td>(Collin et al. 2005)</td>
</tr>
<tr>
<td>Alms1</td>
<td>Alms&lt;sup&gt;foz&lt;/sup&gt;</td>
<td>Spontaneous</td>
<td>Obesity, male infertility, late onset hearing loss</td>
<td>(Arsov et al. 2006)</td>
</tr>
<tr>
<td>Bbs1</td>
<td>Bbs&lt;sup&gt;tm1Vcs&lt;/sup&gt;</td>
<td>Knock-in</td>
<td>Obesity, retinopathy, male infertility, ventriculomegaly</td>
<td>(Davis et al. 2007)</td>
</tr>
<tr>
<td>Bbs2</td>
<td>Bbs&lt;sup&gt;tm1Vcs&lt;/sup&gt;</td>
<td>Knock-out</td>
<td>Obesity, retinopathy, renal cysts, male infertility, anosmia, social submissiveness, ventriculomegaly</td>
<td>(Nishimura et al. 2004)</td>
</tr>
<tr>
<td>†Bbs3/Aril6</td>
<td>Aril&lt;sup&gt;tm2Vcs&lt;/sup&gt;</td>
<td>Knock-out</td>
<td>Increased fat mass, retinopathy, male infertility, hydrocephalus, elevated blood pressure</td>
<td>(Zhang et al. 1994)</td>
</tr>
<tr>
<td>*Bbs4</td>
<td>Bbs&lt;sup&gt;tm1Vcs&lt;/sup&gt;</td>
<td>Knock-out</td>
<td>Obesity, retinopathy, male infertility, social submissiveness, ventriculomegaly</td>
<td>(Mykytyn et al. 2004)</td>
</tr>
<tr>
<td>*Bbs4</td>
<td>Bbs&lt;sup&gt;Ar1Nk&lt;/sup&gt;</td>
<td>Genetrap</td>
<td>Obesity (sex dependent penetrance and severity), retinopathy, social submissiveness, increased anxiety</td>
<td>(Kulaga et al. 2004; Eichers et al. 2006)</td>
</tr>
<tr>
<td>Bbs6/Mkks</td>
<td>Mkks&lt;sup&gt;tm1Vcs&lt;/sup&gt;</td>
<td>Knock-out</td>
<td>Obesity, retinopathy, male infertility, anosmia, elevated blood pressure, social submissiveness, ventriculomegaly</td>
<td>(Fath et al. 2005)</td>
</tr>
<tr>
<td>†Bbs11/Trim32</td>
<td>Trim32&lt;sup&gt;Q&lt;sub&gt;4DLDKAS55Myg&lt;/sub&gt;&lt;/sup&gt;</td>
<td>Genetrap</td>
<td>Increased body weight, muscular myopathy</td>
<td>(Kudryashova et al. 2011)</td>
</tr>
<tr>
<td>Ift88</td>
<td>Ift88&lt;sup&gt;tm1Bky&lt;/sup&gt;</td>
<td>Conditional Knock-out</td>
<td>Obesity, renal cysts, hepatic cysts</td>
<td>(Davenport et al. 2007)</td>
</tr>
<tr>
<td>Kif3a</td>
<td>Kif3a&lt;sup&gt;tm1Pan&lt;/sup&gt;</td>
<td>Conditional Knock-out</td>
<td>Obesity, renal cysts, hepatic cysts</td>
<td>(Davenport et al. 2007)</td>
</tr>
<tr>
<td>Tub</td>
<td>Tub&lt;sup&gt;tm1&lt;/sup&gt;</td>
<td>Spontaneous</td>
<td>Obesity, retinopathy, late onset hearing loss</td>
<td>(Coleman and Eicher 1990)</td>
</tr>
<tr>
<td>Tub</td>
<td>Tub&lt;sup&gt;tm1Mek&lt;/sup&gt;</td>
<td>Knock-out</td>
<td>Obesity, retinopathy, late onset hearing loss</td>
<td>(Stubdal et al. 2000)</td>
</tr>
</tbody>
</table>

†Bbs3 mutant mice do not become obese, but do display increased fat mass. Likewise, Bbs11 mutants have not been reported to be obese, but do display a significant and persistent increase in body weight starting at 2 months of age.

*Two different Bbs4<sup>-/-</sup> knockout mouse lines have been independently generated and different penetrance and severity of obesity have been reported for each.
Figure 1. Obese cilia mutant mouse models. Figure 1A shows a normal weight control mouse next to an obese Bbs4<sup>−/−</sup> mutant littermate. Figure 1B likewise shows a normal weight control mouse next to an obese conditional Ift88 mutant that expresses the pan-neuronal cre transgene, synapsin1-cre.
in the formation of spermatozoa flagella, similar to the findings of Bbs1, 2, and 4 mutant mice.

Not all known BBS genes fall into the category of being a BBSome complex member or having chaperone-like properties. This includes the BBS3 gene that encodes the small GTPase ARL6. Mouse Bbs3 mutants exhibit both retinal degeneration and male infertility due to loss of sperm flagella (Zhang et al. 1994). In addition, severe hydrocephalus accompanied by altered beating of ependymal cilia was found, but no loss or obvious defects in primary cilia morphology were evident. Most strikingly however, was the apparent lack of an overt obesity phenotype in the Bbs3 mutants. Likewise, leptin levels in the mutants were not statistically different than controls (Zhang et al. 1994). Although the Bbs3 mutants did not have a significant increase in body weight, they do have an increase in the amount of gonadal and retroperitoneal fat. The reason that Bbs3 mutant mice do not display an obesity phenotype is unknown. It was proposed this may be due to the early onset hydrocephalus; however, it should be noted that obesity along with hydrocephalus is seen in some of the other Bbs models. The lack of an obesity phenotype could also be related to different functions of the Bbs proteins and differential effects they have on protein trafficking. Melanin concentrating hormone receptor1 (Mchr1) is a ciliary localized G protein coupled receptor (GPCR) known to have orexigenic effects. Intriguingly, in obese models such as Bbs2 and Bbs4 mutants, Mchr1 is not present in the cilium while it does localize to in the cilia of cultured neurons from Bbs3 mutants (Zhang et al. 1994). It is also important to note that Bbs3 is neither a BBSome complex member nor a BBS chaperone protein, raising the possibility that Bbs3 has functions independent of the other BBS proteins.
In some cases, previously identified genes are now being recognized as belonging to the BBS family. For example, mutations in the E3 ubiquitin ligase TRIM32 was identified in BBS patient (hence called \textit{BBS11}) through the use of homozygosity mapping with SNP arrays (Chiang et al. 2006). \textit{Trim32}/\textit{Bbs11} mutant mice display muscular dystrophy and a decreased concentration of neurofilaments, as well as a reduction in myelinated motoraxon diameters (Kudryashova et al. 2009; Kudryashova et al. 2011). A small increase in body weight was found in the \textit{Bbs11} mice when compared to controls, but this was only a 10% difference at 8 weeks of age (Kudryashova et al. 2009). Much like Bbs3, Bbs11 is neither a BBSome complex member nor a BBS chaperon protein, and thus may also have independent or unique functions from the rest of the BBsome. However, it remains possible that the muscular dystrophy and reduction in motor axon myelination are precluding the emergence of an obesity phenotype. In addition, mutations in BBS11 can cause two distinct clinical disorders; BBS and limb-girdle muscular dystrophy type 2H (LGMD2H). BBS phenotypes were associated with N-terminal mutations (P130S) while LGMD2H appears to be caused by mutations in the C-terminal region (R394H, D487N, D588del, or T520TfsX13) that do not disrupt its ability to function in ubiquitination.

Studies of more recently identified BBS genes include \textit{MKS1}/\textit{BBS13} and \textit{CEP290}/\textit{BBS14}. However, current reports utilize \textit{Bbs13} and \textit{Bbs14} mutant mice that are either embryonically lethal and/or not true genetic nulls, thus making the potential role of \textit{Bbs13} and 14 in obesity and appetite regulation ambiguous (Tadenev et al. 2011; Weatherbee et al. 2009; Lancaster et al. 2011). Regardless, the fact that not all \textit{Bbs} mutant mice have the same phenotypes indicates complexity and diversity in the functions of
the different BBS genes along with differential effects of the specific mutations on gene function.

Mouse Models of Alström Syndrome

The other ciliopathy with obesity as a symptom is Alström syndrome. In contrast to BBS, Alström syndrome appears to be caused by mutations in a single gene ALMS1. The ALMS1 protein localizes to the basal body of ciliated cells, but the function of the protein is not certain (Hearn et al. 2005; Collin et al. 2005). Mouse models corresponding to Alström syndrome have also been reported and include a gene-trapped allele (Alms1<sup>−/−</sup>) and a spontaneous mutant (fat aussie, foz) (Arsov et al. 2006; Collin et al. 2005). Mice lacking functional Alms1 are born at a normal weight much like their human counterparts. However, hyperphagic behavior and obesity ensue that is accompanied by hyperinsulinemia and type II diabetes. The Alms1<sup>−/−</sup> mutant mice also have enlarged livers with the accumulation of lipid deposits, and the pancreas is hyperplastic. In addition to obesity, mice lacking Alms1 display male infertility, as well as retinal and cochlear defects, all of which are reminiscent of cilia associated defects in human patients.

Obesity in the Intraflagellar Transport Mutants

Cilia formation and maintenance, and possibly its signaling activity, is dependent on the intraflagellar transport (IFT) system to mediate bidirectional transport of proteins between the base and tip of the cilium. Null alleles of the Ift88 gene (originally referred to as Tg737 in mouse) caused early embryonically lethality and even hypomorphinc alleles
caused death prior to adulthood with systemic effects (Moyer et al. 1994; Murcia et al. 2000; Lehman et al. 2008). The necessity of cilia for normal mammalian development has made analyzing possible roles of the cilium in satiation and obesity difficult. This problem was circumvented with the creation of conditional alleles of *Ift88* and the IFT motor *Kif3a* (Marszalek et al. 1999; Haycraft et al. 2007). Using a tamoxifen-inducible cre recombinase expressed from the actin promoter (CAGG-creER<sup>TM</sup>) (Davenport et al. 2007), cilia loss could be induced systemically in adult mice. This was found to cause hyperphagia within three weeks of inducing cilia loss and subsequently caused obesity. Furthermore, the obesity phenotype was prevented by maintaining adult conditional cilia mutant mice on a restricted diet, wherein they were provided the same daily amount of food as normal controls consumed. This observation indicates that the obesity phenotype is caused by the lack of a satiation response that leads to the overconsumption and not a general alteration in metabolic or locomotor activity.

The change in feeding behavior observed in the *Ift88* and *Kif3a* conditional mutant mice led to the possibility that cilia on neurons may be responsible for the obesity phenotype. To test this hypothesis, conditional *Ift88* and *Kif3a* mutant mice were crossed to synapsin1-cre mice to cause loss of cilia exclusively in neurons (Zhu et al. 2001). As with the systemically induced cilia mutants, neuronal specific cilia mutant mice became morbidly obese and strongly implicated a previously unappreciated role for neuronal cilia in regulating appetite (Figure 1B).

The hypothalamus is a critically important signaling center of the brain known to regulate appetite. This action is done in large part by neurons that express either pro-opiomelanocortin (POMC) or agouti-related protein (AgRP) that release signaling factors
ultimately suppressing or enhancing appetite, respectively (for a review see (Mountjoy 2010)). Importantly, hypothalamic neurons each possess a single primary cilium, although the function of the cilium on these neurons is largely unexplored. To address the role of neuronal cilia and appetite, Ifi88 and Kif3a conditional mutants were crossed to POMC-cre or AgRP-cre expressing mice, to conditionally ablate cilia on POMC or AgRP expressing neurons, respectively (Xu et al. 2005b). By 6 weeks of age, both male and female POMC cilia mutant mice weighed significantly more than control mice, and continued to become morbidly obese into adulthood. This was not evident in the mice lacking cilia on AgRP neurons (Berbari and Yoder, unpublished data). Another observation that was reported in the POMC cilia mutant mice was an increase in the levels of leptin, fasting serum glucose, and insulin (Davenport et al. 2007). This was observed only in the obese state and not in mice kept lean by pair-feeding, indicating that these elevated levels were a secondary consequence of the obesity. This report was significant for providing some of the initial evidence indicating the importance of neuronal cilia in regulating obesity.

Other Nonhuman Obesity Mouse Models Associated with Ciliary Proteins

In addition to the Bbs, Alms1, and Ifi88 mouse models there are several other mutant mouse strains supporting a connection between cilia and obesity. One prime example is a mutation in the type III adenylyl cyclase (ACIII). ACIII localizes to the primary cilia throughout the adult rodent brain (Bishop et al. 2007) and loss of ACIII causes anosmia and obesity by 3 months of age. Interestingly, even when ACIII mutants do not weigh significantly more than wildtype siblings, they have an increased level of serum
leptin (Wang et al. 2009). It is interesting to note that a recent Genome Wide Association Study revealed there is a SNP near ACIII that is associated with obesity in humans (Hebebrand et al. 2010; Nordman et al. 2008).

Another example may be the tubby mouse that was first identified at the Jackson Laboratory as a spontaneous mutant causing a maturity-onset obesity phenotype (Coleman and Eicher 1990) and subsequently regenerated by gene targeting (Ashrafi et al. 2003). The tubby mutants have progressive loss of hearing and vision (Ohlemiller et al. 1995), similarities that are also shared with the Bbs and Alms1 mutant mice. The spontaneous tubby mouse possesses a single base pair mutation within a splice site of the gene Tub (named after the mutant mouse) resulting in the expression of an aberrant transcript. The functions of the Tub protein remain ambiguous, but it is found to be highly expressed in portions in the brain, including the arcuate nucleus of the hypothalamus (Kleyn et al. 1996). The Tub protein is dispensable in assembly of the cilia, and no defects in the cilia assembly process of intraflagellar transport (IFT) have been reported. Intriguingly, despite the fact that Tub has yet to be reported in mammalian primary cilia, a physical association between Tub and the IFT complex has been reported in an immortalized human cell line (Mukhopadhyay et al. 2010). Further evidence for a ciliary role of Tub can be found with the C. elegans homolog tub-1 that undergoes transport along the ciliary axoneme (Mukhopadhyay et al. 2005). Like the tubby mouse, C. elegans with a deletion of tub-1 show an increase in fat content, suggesting an evolutionarily conserved role of the gene in regulating fat storage. Other proteins in the tubby family of proteins have also been implicated as having ciliary roles. For example, tubby-like protein 1 (Tulp3) localizes to the primary cilia during mouse development, and is necessary
for proper Shh signaling but its association with obesity has not yet been determined (Norman et al. 2009).

Potential Molecular Mechanisms of Ciliopathy Associated Obesity

The hyperphagia induced obesity is one of the more intriguing phenotypes of ciliopathies that remains to be fully explained. There are indeed several possibilities described in the literature as to how loss of the cilia could alter states of satiety and appetite. Here we review a few of the candidate molecular pathways that may play roles in obesity associated with cilia dysfunction. These possibilities include primary deficits in leptin signaling, altered G-protein coupled receptor (GPCR) signaling, and abnormal regulation of mTor and hedgehog signaling pathways (Table 3).

Cilia and the Leptin Signaling Axis

While the conditional allele of Ift88 implicated a role for neuronal cilia in satiety and more specifically a role for POMC neuronal cilia, this study did not specify a molecular framework for the underlying hyperphagia phenotype (Davenport et al. 2007). More recent data has aimed to accomplish this by showing that Bbs1, a component of the BBSome, directly binds to the leptin receptor and that BBS proteins may have a role in leptin receptor trafficking (Figure 2A) (Seo et al. 2009). The initial identification of the leptin gene encoding a small protein hormone in the spontaneous obese mouse mutant ob/ob was the source of much excitement (Zhang et al. 1994). Importantly, leptin suppresses feeding activity and it is secreted into serum at levels proportionate to the amount
Table 3. Neuronal cilia signaling proteins that may contribute to obesity. Potential pathways behind the obesity phenotype are listed with the components that localize to the cilium or at the base of the cilium, as well as a current hypothesized mechanism. It is of note that a second MCH receptor, named Mchr2, also exists in humans, canines, and many predators but is absent in rodents.

<table>
<thead>
<tr>
<th>Candidate Molecule</th>
<th>Known Receptors</th>
<th>Localization</th>
<th>Potential Mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>Lep-R</td>
<td>Cell membrane, 'peri'-ciliary membrane</td>
<td>-changes in Leptin receptor localization</td>
<td>(Seo et al. 2009)</td>
</tr>
<tr>
<td>Melanin concentrating hormone</td>
<td>Mchr1, *Mchr2</td>
<td>Ciliary Membrane</td>
<td>-changes in Mchr1 localization</td>
<td>(Berbari et al. 2008)</td>
</tr>
<tr>
<td>Dopamine</td>
<td>D₁</td>
<td>Ciliary</td>
<td>-changes in Drd1 cilia localization</td>
<td>(Domire et al. 2011)</td>
</tr>
<tr>
<td>Serotonin</td>
<td>5-HT₆</td>
<td>Ciliary membrane in certain brain regions</td>
<td>-changes in ciliary receptor 5HT6 Signaling</td>
<td>(Brailov et al. 2000)</td>
</tr>
<tr>
<td>Adenylate Cyclase III</td>
<td>Olfactory GPCRs</td>
<td>Cilia throughout the brain</td>
<td>-altered coupling downstream of ciliary GPCRs</td>
<td>(Bishop et al. 2007)</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>Patched</td>
<td>Ciliary membrane in regions of adult neurogenesis</td>
<td>-Altered adult neurogenesis -Altered hypothalamic development -Non-canonical hedgehog signaling</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*Mchr2 is common to primates, canines, and other carnivores, but is absent among rodents.
of adipose tissue, the hormone’s primary source (Considine et al. 1996). Interestingly, these recent studies in *Bbs2*, *Bbs4*, and *Bbs6* mutant mice also revealed that they are hyper-leptinemic and importantly, they fail to reduce food intake in response to IP or ICV injection of leptin (Rahmouni et al. 2008). Thus, defects in leptin signaling were thought to contribute directly to the obesity phenotype in BBS.

The excitement surrounding leptin’s initial discovery was attenuated when it was determined that nearly all obese mice and humans have markedly elevated levels of circulating leptin, yet do not have normal leptin-mediated repression of appetite (Considine et al. 1996; Maffei et al. 1995). This barrier phenomenon is known as leptin resistance, the mechanism of which remains an active area of research. Thus, in obesity research, one challenge is determining whether leptin resistance is a primary cause or a consequence of the obesity. One approach used to overcome this situation is to decrease the amount of adipose tissue, and consequently the levels of circulating leptin, through caloric restriction. Interestingly, when this was performed on the BBS mutant mouse models to maintain body weight and leptin levels as seen in controls, they were still resistant to leptin suggesting leptin signaling defects are a primary cause of the phenotype (Seo et al. 2009). However, the study did not take into account a phenomenon called *food anticipatory behavior* wherein the mice alter their meal structure in response to the calorie restriction such that they consume nearly all of their calories within the first few hours of food access (for a review on anticipatory feeding behavior and methods see (Mistlberger 2009). Interestingly, during this entrained period the mice consume nearly the same amount of food as they were given during calorie restriction, even when they are given *ad libitum* amounts of food. This entrained behavior persists for over a week and during this
period the mice do not reduce food intake in response to leptin and thus appear leptin resistant (Berbari and Yoder unpublished). This feeding behavior resulting from the anticipation of food is in large part thought to be the result of a feeding clock, somewhat analogous to but distinct from the circadian clock (Mistlberger 2009). It remains to be seen whether either Bbs or Ift conditional mice would respond to leptin when both body weight and anticipatory feeding behavior are experimentally controlled. This would require testing animals for leptin sensitivity after deterioration of the food anticipatory behavior.

The BBS studies utilized congenital mutants and reported a loss of approximately 20 percent of the leptin responsive proopiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus (Seo et al. 2009). Thus, the authors propose that improper leptin receptor trafficking in POMC neurons of the arcuate nuclease leads to their inability to sense leptin and thus mediate its anorectic effects. While several possibilities exist for the loss of 20 percent of POMC neurons in Bbs mice, it is of note that alteration in the Foxo1/Insulin signaling pathway have resulted in similar changes in adult POMC neuronal populations (Plum et al. 2012). Loss of POMC neurons in Bbs models could be the result of neurodevelopmental changes or possibly maintenance of POMC neuron population through altered adult neurogenesis that lead to the hyperphagic phenotype. Similarly, the obesity phenotype observed in the hyperphagic Ift88 conditional POMC-cre model could be a hypothalamic developmental phenotype. However, the rapid onset of obesity upon ubiquitous loss of cilia induced by the actin promoter driving cre line (CaGG-CreER) in adult mice suggests that cilia play a direct role in appetite and satiety. What is needed is an investigation using inducible cilia mutants in POMC neurons or other
Figure 2. Potential ciliary signaling pathways necessary for appetite regulation. (A) Depicts the leptin receptor interacting with Bbs1 of the BBSome near the base of the cilia where it has been proposed to be available for leptin activation and subsequent phospho-Stat3 induction and translocation to the nucleus. (B) A depiction of cilia specific GPCRs such as Mchr1, Sstr3, Drd1, and 5HT6, and their potential effector ACIII and G proteins such as $G_{s,q,i,o}$. (C) The Hedgehog signaling pathway, with patched repressing smoothened translocation into the cilium until ligand stimulation, upon which Gli transcription factors are processed from the inhibitor to the activator forms followed by subsequent translocation to the nucleus.
neuronal populations implicated in feeding behavior in order to elucidate the role of primary cilia signaling in appetite and satiety.

_Cilia and Melanin-Concentrating Hormone Pathway_

While both genetic models and biochemical approaches have informed our current understanding of BBS the precise mechanism behind BBS-associated obesity is unknown. Many of the BBS proteins form a protein complex (BBSome) that is involved in proper cilia membrane formation (Nachury et al. 2007). Indeed there is evidence that the BBSome may be a membrane vesicle coat complex that is critical for establishing and maintaining the cilia membrane’s signaling capabilities by directing specific receptors to this compartment (Jin et al. 2010). Furthermore, _Bbs_ mutant mouse models appear to improperly localize several cilia-specific GPCRs, and most relevant with regard to obesity is the melanin-concentrating hormone receptor 1 (Mchr1) (Berbari et al. 2008). Mchr1 couples through G proteins to reduce cAMP and decreases the frequency of spontaneous action potentials in hypothalamus (Gao and van den Pol 2001, 2002). Mch injections induce feeding behavior while Mchr1 mutant mice are resistant to diet induced obesity (Gomori et al. 2003). Intriguingly, Mchr1 fails to localize normally in neuronal cilia of _Bbs2, Bbs3_, and _Bbs4_ mutant mice (Berbari et al. 2008; Zhang et al. 1994). Thus in both _Ift88_ and _Bbs_ obese mutants, Mchr1 fails to be properly localized creating a defect in Mchr1 signaling, possibly leading to the hyperphagic behavior in these models. Both pharmacological or genetic agonism of the Mchr1 pathway is associated with hyperphagia while antagonism is associated with anorectic behavior, as such antagonism of this receptor has been of interest to the pharmaceutical industry (Qu et al. 1996; Borowsky et
al. 2002; Ludwig et al. 2001; Shimada et al. 1998; Chen et al. 2002). However, assuming that the obese phenotype behind both Bbs and Ifi models is driven by a similar molecular pathway, then one would have to propose that in the absence of the cilium or the ability to reach the cilium that the Mchr1 pathway is ectopically activated or not efficiently desensitized after activation.

While there is circumstantial evidence for Mchr1 cilia mis-localization driving hyperphagia in cilia mutant mouse models, it is interesting to note that there is an emerging list of GPCRs that preferentially localize to neuronal cilia in different regions of the brain (Figure 2B). Some of these neuronal cilia specific GPCRs include somatostatin receptor 3 (Sstr3), serotonin receptor 6 (5HT6), and dopamine receptor 1(Drd1) (Handel et al. 1999; Schulz et al. 2000; Hamon et al. 1999; Brailov et al. 2000; Marley and von Zastrow 2010; Domire et al. 2011). While the significance of localizing these receptors within the ciliary compartment remain unknown, it is enticing to speculate that perhaps they may play a role in appetite and satiety, especially when one considers that the somatostatin, serotonin, and dopaminergic systems have all been implicated in either reward or feeding behaviors directly (Vijayan and McCann 1977; Aponte et al. 1984; Pollock and Rowland 1981; Salamone et al. 1990).

Cilia and the Mammalian Target of Rapamycin (mTOR) Pathway

Another pathway that may be involved in neuronal cilia regulation of satiation is the mammalian target of rapamycin (mTOR) pathway. mTOR signaling is complex and involves many factors (for an in depth review of mTOR and disease see (Dazert and Hall 2011)). mTOR is a serine/threonine protein kinase which as its name implies can be in-
hibited by the antifungal rapamycin. It has been established as a regulator/coordinator of cellular metabolic activity that responds to both the energy and stress levels experienced by the cell. It carries out these regulatory roles by participating in two protein complexes, the rapamycin-sensitive mTOR Complex 1 and the rapamycin-insensitive mTORC2. In general mTORC1 regulates translational control and mTORC2 is involved cytoskeleton organization. While the functions of mTOR and its interactors have been determined in considerable detail at the genetic and cellular levels, the effects of mTOR signaling on the organismal level continue to emerge. Interestingly, there are several reports associating the cilium or its signaling proteins with overactivation of mTOR activity or in changes in the cytoskeleton and cell size (Sharma et al. 2011; Bell et al. 2011; Boehlke et al. 2010). In addition, rapamycin is able to partially rescue renal cystic disease in mouse models of PKD, further supporting a connection between cilia and mTor (Shillingford et al. 2006; Shillingford et al. 2010). While the in vivo relevance of cilia and mTOR signaling with regards to the obesity phenotype in cilia mutants remains to be determined, it is of note that mTOR signaling within the hypothalamus has been associated with obesity in other animal models (Cota et al. 2006). It will be interesting to determine if mTOR signaling activity within adult neurons is regulated through neuronal cilia and influences feeding behavior.

Hedgehog Signaling and the Cilium

The final pathway that we will discuss with regard to neuronal cilia and obesity is the hedgehog (Hh) pathway. Hh and its role in cilia and neuronal development is reviewed in Chapter 2 of this work by Mariani and Caspary. Several groups have demon-
strated that canonical hedgehog signaling in mammalian cells utilizes the ciliary compartment. This is best demonstrated by the transient localization of several of the pathway components to the cilium and altered pathway activity when the cilium has been disrupted (reviewed in detail by (Goetz and Anderson 2010)). With regard to hedgehog signaling and neuronal cilia, there is a consensus emerging that primary cilia within the adult central nervous system sense hedgehog ligand and mediate the process of adult neurogenesis (Breunig et al. 2008; Han et al. 2008). Furthermore when neuronal cilia-mediated hedgehog signaling is altered in a gain of function fashion it can result in medulloblastoma and when it is disrupted in a loss of function fashion in the developing brain it is associated with a range of neurodevelopmental phenotypes (Chizhikov et al. 2007; Han et al. 2009).

If altered hedgehog signaling and cilia mutations have such profound effects on the adult and developing nervous system how may they account for the hyperphagia associated obesity in adults? In both Ift conditional and Bbs models the possibility that altered hedgehog signaling in the developing hypothalamus can lead to obesity in adulthood has yet to be thoroughly investigated. For example, POMC-cre conditional Ift88 mutant models appear normal other than the onset of hyperphagia and obesity, but the potential for a developmental phenotype remains. This becomes important when the expression pattern of POMC is taken into account. POMC is known to be expressed in places outside of the arcuate nucleas of the hypothalamus such as the nucleus tract solitarius of the hindbrain, and the anterior and intermediate lobe of the pituitary in neonatal animals (Xu et al. 2005a). This becomes particularly important when the crucial role of hedgehog not only in the developing neural tube but also in the developing hypothalamus
is taken into account (Szabo et al. 2009; Alvarez-Bolado et al. 2012). To address these potentials both *Ift* and *Bbs* conditional models need to be tested with inducible POMC-cre deletion. These experiments would also be useful in assessing whether different molecular mechanisms may be involved in driving obesity in Bbs and Ift mouse models. The possibility remains that hedgehog signaling required for adult neurogenesis is disrupted contributing to hyperphagia, and there are reports of adult neurogenesis within the hypothalamus (Kokoeva et al. 2005; Xu et al. 2005c; Pierce and Xu 2010; Lee et al. 2012). Perhaps this process is compromised in CAGG-creER;*Ift88* conditional mutants. However, hyperphagic behavior is observed within 3 weeks of cilia loss in these mice and thus it may not have sufficient time to be a result of altered adult neurogenesis.

Finally a third potential for a non-canonical form of hedgehog signaling exists in the adult hypothalamus that requires neuronal cilia. The relevance of a non-canonical Hh pathway emerges when the expression pattern of certain pathway components in the adult brain is analyzed. For example the Hh receptor, patched, is expressed in regions of the brain that do not co-express the Hh effector smoothened (Traiffort et al. 1999; Traiffort et al. 1998). This incongruence in pathway component expression pattern is especially true with regard to the hypothalamus (for a review of hedgehog in the adult brain see Traiffort et al. 2010). Furthermore it has been shown that hedgehog can directly alter neuronal activity (Bezard et al. 2003; Pascual et al. 2005). In developing spinal neurons, Hh stimulation causes a transient increase in Ca2+ activity that was dependent on Smo and Gai (Belgacem and Borodinsky 2011). Since hedgehog pathway components such as patched are expressed in the adult hypothalamus, it is feasible that cilia may alter satiation responses through regulation of neuronal firing activity (Traiffort et al. 1999;
Traiffort et al. 1998). Exploring whether loss of cilia alters this increase in Ca\(^{2+}\) in response to Hh in POMC neurons could prove to be a very fruitful avenue of investigation to connect cilia dysfunction to abnormal satiation.

**Non-Mammalian Ciliopathy Models of BBS**

Although more distantly related to human beings than mice, non-mammalian models have proven to be invaluable to the study of the role of human cilia and their relation to disease. This is particularly evident in the study of the assembly and maintenance of the cilium through IFT, and how disruption of this event can lead to certain phenotypes. This process, referred to as intraflagellar transport (IFT), was largely characterized biochemically using the small green algae, *Chlamydomonas*, and genetically using *C. elegans* (for an in depth review see (Pedersen and Rosebaum 2008)). In this section we focus solely on the genes and proteins known to be associated with the obesity phenotype observed in ciliopathies, as such it will largely focus on the functional roles of the BBS genes in both *Chlamydomonas reinhardtii* and *Caenorhabditis elegans*, two of the most well studied non-mammalian organisms in regards to cilia/flagella biology.

While *Chlamydomonas* has served as good model for biochemical purification of flagellar and IFT components it has also proven useful for comparative genomics studies in discovering new ciliopathy genes, such as *BBS5* (Kulaga et al. 2004). Through the use of this simple model, elegant studies have begun to shed new light on how the BBS proteins may function as modulators of ciliary signaling and even serve as structural components of the transition zone (Lechtreck et al. 2009; Craige et al. 2010).
Much of what we know about the molecular motors that mediate cilia formation and maintenance has come from studies visualizing IFT movement in the cilia of *C. elegans*. In *C. elegans* cilia of the sensory neurons it has been demonstrated that both BBS7 and BBS8 serve as adaptors to the IFT complexes and their cargoes (Blacque et al. 2004), however, whether they play similar roles in mammalian systems has not been determined. Interestingly, it has been shown that *C. elegans* ciliary morphology can change dependent on cilia-mediated signaling and that the phenotypes of *bbs* mutant worms can be ameliorated by altering the downstream second messengers (Tan et al. 2007; Mukhopadhyay et al. 2008; Mok et al. 2011). Recent work has also suggested that altered neuroendocrine signaling and exocytosis of factors such as insulin drives many of the phenotypes observed in *bbs* mutant worms (Lee et al. 2011). Interestingly, another study points to more general roles for bbs proteins in cilia membrane homeostasis (Kaplan et al. 2012). Although the invertebrates lack many of the organ systems present in mammals, it is clear that both *C. elegans* and *Chlamydomonas* models offer advantages in both cost, time and in some instances genetic tractability. These models will continue to provide insights into the fundamental processes that are mediated by the ciliopathy proteins and the cilium and thus further serve to inform our understanding of complex phenotypes such as feeding behavior and the regulation of appetite and satiety.

**Conclusion**

In summary, remarkable progress has been made in the past twenty years demonstrating the clinic importance of the cilium in may tissues and developmental processes. Despite this progress, there remain several key questions that must be addressed before
we can understand the molecular and cellular mechanisms responsible. Hopefully, as research on the rare ciliopathies advances we will gain an understanding of fundamental processes such as satiety and appetite that we can then apply to direct therapeutic strategies for an exceedingly common clinical feature such as obesity.
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LEPTIN RESISTANCE IS A SECONDARY CONSEQUENCE OF THE OBESITY IN CILIOPATHY MUTANT MICE

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Abstract

Although primary cilia are well established as important sensory and signaling structures, their function in most tissues remains unknown. Obesity is a feature associated with some syndromes of cilia dysfunction, such as Bardet-Biedl syndrome (BBS) and Alström syndrome (ALMS), as well as in several cilia mutant mouse models. Recent data indicate that obesity in Bbs mutant mice is due to defects in leptin receptor trafficking and leptin resistance. Furthermore, induction of cilia loss in leptin responsive POMC neurons results in obesity, implicating cilia on hypothalamic neurons in regulating feeding behavior. Here, we directly test the importance of the cilium as a mediator of the leptin response. In contrast to the current dogma, a longitudinal study of conditional Ifit88 cilia mutant mice under different states of adiposity indicates that leptin resistance is present only when mutants are obese. Our studies show that caloric restriction leads to an altered anticipatory feeding behavior that temporarily abrogates the anorectic actions of leptin despite normalized circulating leptin levels. Interestingly, pre-obese Bbs4 mutant mice responded to the anorectic effects of leptin and did not display other phenotypes associated with defective leptin signaling. Furthermore, thermoregulation and activity measurements in cilia mutant mice are inconsistent with phenotypes previously observed in leptin deficient ob/ob mice. Collectively, these data indicate that cilia are not directly involved in leptin responses and that a defect in the leptin signaling axis is not the initiating event leading to hyperphagia and obesity associated with cilia dysfunction.
Introduction

Obesity is a major health issue in developed countries and is associated with complications that cause significant morbidity and premature death. Thus, the initial identification of the gene encoding the small protein hormone leptin in the spontaneous obese mouse mutant \textit{ob/ob} was the source of much excitement (1). Leptin suppresses feeding activity and is secreted into serum at levels proportionate to the amount of adipose tissue, the hormone’s primary source (2). However, the excitement surrounding leptin’s discovery was attenuated when it was determined that a barrier to the action of leptin must exist in an obese individual as nearly all obese mice and human patients exhibit elevated levels of circulating leptin (2, 3). This barrier phenomenon is known as leptin resistance, the precise mechanisms of which remain an active area of research. In the field of obesity research, one challenge then becomes determining how individuals acquire resistance to the actions of leptin and whether this is a primary cause of the obesity phenotype or simply a consequence.

Recent findings revealed that obesity is associated with mutations in proteins disrupting the function of the primary cilium in mice and in human patients. Primary cilia are small, immotile, microtubule-based appendages that protrude from the surface of most mammalian cell types. Long thought to be vestigial, primary cilia are now known to serve as critical signaling hubs for diverse cellular pathways during embryonic development and in adult tissue homeostasis (for a review see (4)). The emergence of the primary cilium as a clinically important organelle was initiated by studies in model organisms such as \textit{Chlamydomonas reinhardtii}, \textit{Caenorhabditis elegans}, and mice. These studies led to the identification of proteins required for cilia formation and maintenance
through the bidirectional transport of cargo along the cilium in a process known as *Intraflagellar Transport* (IFT, for a review see (5)). Since IFT null mutations are embryonically lethal, research into the roles of cilia in adults was initially limited. However, the generation of conditional IFT alleles in mice and the realization that several human genetic syndromes, known as *ciliopathies*, result from cilia dysfunction has rapidly expanded our understanding of the organelle (for a review see (6)). Mutations affecting cilia function in humans lead to a wide spectrum of disease phenotypes. These range from prenatal lethality and neural tube defects in Meckel-Gruber syndrome (MKS, OMIM #249000) to obesity in human patients with Alström Syndrome (ALMS, OMIM #203800) or Bardet-Biedl Syndrome (BBS, OMIM #209900).

Bardet-Biedl syndrome is a pleiotropic, genetically heterogeneous syndrome associated with the following clinical features: retinopathy, cystic kidneys, cognitive deficits, polydactyly, anosmia, heart defects, and obesity (for a review of BBS see (7)). Several of the proteins involved in BBS form a complex called the BBSome which functions in membrane trafficking to and from the cilium and for normal cilia localization of G-protein coupled receptors (8-11). Numerous mouse models of BBS have been reported that recapitulate many of the phenotypes observed in patients (12, 13). A major distinction between mouse models of BBS and IFT mutants is that BBS models possess primary cilia. This is in contrast to mutations in IFT genes, such as *Ift88*, where null mutations lead to complete cilia ablation. Furthermore, data from multiple model systems suggest that BBS associated phenotypes result from defective cilia mediated signaling activity rather than loss of cilia structure or maintenance (8, 14, 15).
Recent data show that Bbs1, a component of the BBSome, directly binds to the leptin receptor and that BBS proteins may have a role in leptin receptor trafficking (16). Furthermore, recent studies in Bbs2, Bbs4, and Bbs6 mutant mice reveal that they are hyperleptinemic and importantly, they fail to reduce food intake in response to either intraperitoneal (IP) or intracerebroventricular (ICV) injection of leptin (17). This was observed in the mutant mice even after caloric restriction reduced fat mass and normalized circulating leptin levels to those of control mice (16). Thus, defects in leptin signaling are currently thought to contribute directly to the obesity phenotype in BBS.

We have previously shown that conditional disruption of Ifi88 throughout an adult mouse using the ubiquitously expressed CAGG-CreER (actin promoter) transgene leads to hyperphagia and obesity (18). Intriguingly, the obesity phenotype observed after systemic cilia loss can be recapitulated using POMC-Cre to disrupt Ifi88 in POMC neurons of the hypothalamus, a key satiety center that regulates leptin responses (18). Thus, our goal was to investigate whether cilia are directly involved in leptin signaling, whether cilia loss contributes to the development of leptin resistance, and whether the etiology of obesity in cilia mutant mice is similar to that observed in Bbs mutants where cilia are intact but are dysfunctional.

Materials and Methods

Mice

To generate cohorts, females homozygous for the Ifi88$flo$ conditional allele (Ifi88$^{tm1Bky}$) were crossed with CAGG-CreER transgenic; homozygous Ifi88$flo$ (B6.Cg-Tg(CAGG-cre/Esr1*)5Ame/J) mice to yield either experimental Ifi88$flo$; CAGG-
CreER mice or gender matched control Ifi88<sup>flax/flax</sup> (19, 20). Bbs4<sup>−/−</sup> congenital mutant mice were obtained from Jackson Labs (Bbs4<sup>tmVcs</sup>). All mice in this study were maintained on an inbred C57BL/6 genetic background in accordance with Institutional Animal Care and Use Committee (IACUC) regulations at the University of Alabama at Birmingham.

*Induction of Cilia Loss*

Tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) was administered to 8 week old mice by intraperitoneal injection for five consecutive days at a dose of 6.0mg/40g body weight as described previously (19). Initial induction of the Ifi88<sup>flax</sup> allele was confirmed through genotyping, followed by western blotting and immunofluorescence.

*Fixation and tissue processing*

Animals were anesthetized by a 0.1 ml per 10 g of body weight intraperitoneal injection of 2.5% tribromoethanol (Sigma-Aldrich), killed by cardiac puncture, and perfused with PBS followed by 4% paraformaldehyde (Amresco, Solon, OH, USA). The brains were then immersion fixed in 4% paraformaldehyde overnight at 4°C followed by cryoprotection in 30% sucrose in PBS overnight at 4°C. Cryoprotected brains were embedded in Optimal Cutting Temperature compound (VWR, Randor, PA, USA) and cryosectioned at 20 µm.

*Immunoblotting*

Whole hypothalami were dissected and isolated into ice-cold lysis buffer (137 mM NaCl, 20 mM Tris pH 8.0, 1% Triton X-100, 10% glycerol, and complete EDTA-
free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA)). After a 5 second sonication the tissue was incubated on ice for 30 minutes and then vortexed briefly before centrifugation at 10,000 × g at 4°C for 10 minutes. Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples were resolved on a denaturing 10% Tris-HCL gel (Bio-Rad Laboratories) and transferred to Immobilon-Psq transfer membrane (Millipore, Billerica, MA, USA). Membranes were blocked in TBS-T (10mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) with 5% milk for 1 hour and incubated with primary antibody diluted in TBS-T with 2% BSA for 16-24 hours at 4 °C. Membranes were probed with horseradish peroxidase (HRP) conjugated secondary antibodies diluted in TBS-T with 1% milk for 1 hour at room temperature. Secondary antibodies were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce/Thermo Scientific, Waltham, MA, USA) and bands were visualized using Blue Ultra Autorad Film (Bioexpress ISC). The following primary antibodies and dilutions were used: anti-actin (A2066; Sigma-Aldrich)1:1,000, anti-Adenylate cyclase III (sc-588; Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:500, and anti-Ift88 rabbit polyclonal 1:500 (Yoder Laboratory(49)), anti-Bbs4 rabbit polyclonal (gift of Kirk Mykytyn) 1:500. Secondary antibodies were HRP conjugated anti-rabbit (#31460) 1:5,000, HRP conjugated anti-mouse (#31430) 1:10,000 (Pierce/Thermo Scientific).

**Immunofluorescence**

Sections were permeabilized and blocked in PBS with 1% BSA, 0.3% Triton X-100, 2% donkey serum, and 0.02% sodium azide. Sections were incubated in primary
antibody overnight at 4°C, and secondary antibody incubations were carried out for 1 hour at room temperature. All incubations and washes were performed in PBS with 1% BSA, 0.3% Triton X-100, 2% donkey serum, 0.02% sodium azide. Primary antibodies included anti-Adenylate cyclase III (sc-588; Santa Cruz Biotechnology) 1:500 and anti-cFos (#2250; Cell Signaling, Beverly, MA, USA) 1:200. Secondary antibodies included: Alexa Fluor-546 conjugated donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) 1:1000. Nuclei were visualized by Hoechst nuclear stain (Invitrogen). Coverslips were mounted using DABCO.

Confocal Microscopy

All fluorescence images were captured on Perkin Elmer ERS 6FE spinning disk confocal microscope and images were processed and analyzed in Volocity version 6.0 software (Perkin Elmer, Shelton, CT, USA).

Serum Leptin Analysis

Daily handling of mice was performed to minimize effects of stress on serum leptin measurements. Individual mice were transported into a procedure room for blood collection from a tail clip. Samples were allowed to coagulate for 30 minutes at room temperature and then centrifuged at 2,500 g for 15 minutes at 4°C. Serum was collected and stored at -20°C for future analysis. To calculate leptin levels, a mouse leptin ELISA kit was used (Millipore, Billerica, MA, USA) according to the manufacturer’s protocol. Briefly, a reference curve was plotted using data from standards provided by the manufacturer, and the validity of the assay was confirmed using two separate quality control
samples per assay. The measurements of unknown samples were then calculated using a 4-parameter logistic function in Microsoft Excel.

**Feeding Studies**

All body weights were measured with a Mettler-Toledo digital scale (Columbus, OH, USA). All food intake measurements were conducted on individually housed mice with the BioDAQ episodic intake monitor (New Brunswick, NJ, USA). To induce weight loss in experimental animals for timepoint III analysis, food consumption was measured and averaged for each gender. Mice were then given a daily amount 10% less than what they would eat on an *ad libitum* diet for approximately one week. A further 10% reduction from *ad libitum* food consumption was applied the following week and so on until body weights did not differ from their control counterparts. All animals were monitored daily for signs of distress. Once normal bodyweight had been achieved through calorie restriction, mice were given the daily amount of food that controls consumed for 10 days. Finally, experimental mice were fed *ad libitum* until entrained behavior was lost. Timepoint III leptin sensitivity experiments were then performed as described below.

**Leptin Sensitivity Assays**

Intraperitoneal injections of 50 µg of recombinant mouse leptin (Phoenix Pharmaceuticals, Burlingame, CA, USA) in 20 mM Tris pH 8.0 were given just prior to initiation of the dark cycle. Food intake was assessed with a BioDAQ episodic intake monitor (New Brunswick, NJ, USA). Leptin sensitivity was assessed by comparing cumulative food intake for the dark cycle period post leptin injection with the cumulative food intake
from the previous night when a control injection of 20 mM Tris pH 8.0 was administered. The leptin sensitivity at each timepoint was carried out in 3 separate cohorts of mice.

Activity Measurements

For long-term locomotive activity, independent cages with a camera system (detecting infrared) recorded animal activity over a 24 hour period. The system consists of four home cages (30 x 30 cm) with a camera in the center of the top of each cage. The animal is put in the arena, is acclimated with the home cage for 24 hours, and is then observed for 24 hours with a camera-driven tracker system (Phenotyper, Noldus, Netherlands). The test measures the circadian activity pattern of the mice. The activity is measured on a 12:12 hour light/dark cycle, in which the lights turn on at 6:00 and turn off at 18:00. Animals are provided a small black box home in the corner so that they can sleep where movement is undetected by infrared light.

Quantitative Magnetic Resonance Body Composition Measurements

Body composition was measured using the EchoMRI™ 3-in-1 composition analyzer QMR instrument (Echo Medical Systems, Houston, TX, USA) as previously described (50).

Thermoregulation

Rectal temperatures were measured using a Thermalert TH-5 instrument (Physitemp, Clifton, NJ, USA). Mice were acclimated to the procedure by taking measurements once a day for three days prior to the cold temperature challenge. Baseline tem-
Temperatures were recorded the morning of the experiment. Thermoregulation of mice was then tested as previously described (51). Briefly, mice were housed without bedding or enrichment at 4°C for 4 hours during which measurements were taken at 30 minutes, 2 hours and 4 hours. Mice were then moved to 24°C and measurements were taken at 30 minutes, 2 hours and 4 hours. All mice had unlimited access to food and water during the course of the experiment. Cold challenge was terminated for an entire group when a single mouse body temperature fell below 32°C.

Statistical methods and analysis

The difference between mice strains and timepoints of QMR data and serum leptin levels, the difference in rectal temperature in cold exposure between IFT and ob/ob mice, and the activity chamber data for IFT and ob/ob mice was determined using one-way ANOVA, followed by Tukey's honestly significant difference (HSD) test. Student's t-test was used for assessing the effects of vehicle and leptin treatment on food intake and rectal temperature during cold exposure in Bbs mice. Statistics were analyzed using Microsoft excel and R 2.14.1 (the R Foundation for Statistical Computing).

Results

A longitudinal leptin sensitivity testing paradigm was designed to determine whether leptin resistance in cilia mutant mice is a direct result of cilia loss or a secondary consequence of obesity (Figure 1A). In order to initiate cilia ablation in adult mice, a previously described conditional allele of Ifit88 and ubiquitously expressed inducible CAGG-CreER line were utilized (18-20). Cohorts were generated such that transgene
Figure 1: Paradigm for analysis of leptin sensitivity in induced Ift88 conditional mutant mice and analysis of body composition and serum leptin profiles throughout paradigm. (A) Longitudinal paradigm showing the body weight data of induced cilia mutants (Ift88Δ/Δ) and control mice (Ift88floxflox) during cre induction (C), timepoint I (I) when mice are still lean but cilia are lost, timepoint II (II) when mice are obese, and timepoint III (III) when mice have been calorie restricted back to littermate control body weights. The number of mice analyzed in each group was: Ift88floxflox 9♀/9♂ and Ift88Δ/Δ 8♀/8♂. Points are mean weights ± SEMs. Asterisks indicate a significant difference (Students t-test; *P < 0.01). (B) Genotyping PCR on tamoxifen treated and untreated CAGG-CreER Ift88 conditional mice. The uppermost band indicates the presence or absence of the cre transgene (Cre), the lower bands indicate the conditional state of the Ift88 allele, floxed (Flox) or mutant (Δ). (C) Immunofluorescence for neuronal cilia marker ACIII (in red) in the arcuate nucleus of IFT88floxflox and IFT88Δ/Δ 10 days post cre induction. Note the absence of cilia in IFT88Δ/Δ. Scale bar is 21µm for upper row. Bottom row shows inserts with scale bar of 10.5 µm. Hoechst nuclear stain is blue. (D) Western blot on whole hypothalamic protein 10 days post cre induction in control (Ift88floxflox) and mutant (Ift88Δ/Δ) showing loss of Ift88 relative to the cilia marker Adenylyl Cyclase III (ACIII) and loading control (Actin). (E) QMR analysis of body weight, fat mass and lean mass between
IFT88^flox/flox^ and IFT88^Δ/Δ^ mice at timepoints I, II and III. The number of mice analyzed in each QMR group was: timepoint I (IFT88^flox/flox^ 7♀/4♂ and IFT88^Δ/Δ^ 9♀/7♂), timepoint II (IFT88^flox/flox^ 8♀/5♂ and IFT88^Δ/Δ^ 6♀/8♂), timepoint III (IFT88^flox/flox^: 8♀/5♂ and IFT88^Δ/Δ^: 6♀/8♂). The number of mice in each group for leptin serum analysis was: timepoint I (IFT88^flox/flox^ 13♀/7♂ and IFT88^Δ/Δ^ 15♀/8♂), timepoint II (IFT88^flox/flox^ 8♀/5♂ and IFT88^Δ/Δ^ 7♀/6♂), timepoint III (IFT88^flox/flox^ 8♀/5♂ and IFT88^Δ/Δ^ 7♀/6♂). Bars represent mean measurements ± SEMs. Asterisks represent a significant difference from other groups (one-way ANOVA followed by post hoc Tukey's HSD test; *P < 0.05).
CAGG-CreER positive animals were always compared to control CAGG-CreER negative littermates. *Ift88* deletion was induced at 8 weeks of age through tamoxifen injection (Figure 1A). Genotyping and western blotting confirmed CreER mediated deletion of *Ift88* within ten days after tamoxifen administration (Figure 1B, D). Subsequent immunostaining for the neuronal cilia marker adenylate cyclase III (ACIII) (21) confirmed the absence of hypothalamic neuronal cilia throughout the study (Figure 1C). These induced cilia mutant mice are hereafter referred to as *Ift88*Δ/Δ and control mice as *Ift88*flx/flx. Mice were challenged with IP injection of recombinant leptin and subsequent feeding behavior was assessed using a Biodaq electronic feeding monitoring system (22, 23). This system allows for monitoring food intake of individual mice in real-time. Mice were injected with leptin at three time points at which they had different body and serum leptin compositions, hereafter referred to as timepoints I, II and III (Figure 1E) corresponding to pre-obese, obese, and food restricted non-obese states, respectively.

At timepoint I, *Ift88*Δ/Δ mice are hyperphagic but have not yet diverged significantly in body weight or fat mass from *Ift88*flx/flx controls. Serum leptin measurements demonstrated no significant difference between the two groups at this point (*Ift88*flx/flx 1.07ng/mL and *Ift88*Δ/Δ 1.05ng/mL; *P* = 0.94 Student’s *t* test) (Figure 1A, E). Somewhat surprisingly, when feeding behavior was continuously monitored for 12 hours after IP leptin administration, a reduction in food intake for both *Ift88*Δ/Δ and *Ift88*flx/flx mice was observed when compared to vehicle injection (Figure 2A and D and insets). Furthermore, the difference in food intake between *Ift88*Δ/Δ mice treated with leptin and *Ift88*flx/flx mice treated with leptin was not significant (*Ift88*flx/flx 2.22g and *Ift88*Δ/Δ 2.26g; *P* = 0.93 Student’s *t* test) (Figure 2A and D insets). Additionally, both *Ift88*flx/flx
Figure 2: Feeding behavior and cFos induction in the arcuate nucleus in Ift88 conditional mutant mice after leptin injection at timepoints I, II, and III. (A-F) Twelve hour cumulative feeding data determined after leptin injection of Ift88flk/flox (A, B, C and insets) and mutant Ift88Δ/Δ (D, E, F and insets) mice at timepoints I, II, and III. The number of mice analyzed in each group was: timepoint I (Ift88flk/flox 7♀/8♂ and Ift88Δ/Δ 8♀/9♂), timepoint II (Ift88flk/flox 8♀/8♂ and Ift88Δ/Δ 8♀/8♂), timepoint III (Ift88flk/flox 6♀/6♂ and Ift88Δ/Δ 6♀/6♂). Insets show total cumulative food intake over the period. Points in graphs and bars in insets represent mean food intake ± SEMs. Asterisks represent a significant difference from vehicle (Student’s t-test; **P < 0.01) (G-I) Acute induction of nuclear cFos (red) in the arcuate nucleus of the hypothalamus 90 minutes after IP leptin or vehicle injection in Ift88Δ/Δ mutant and Ift88flk/flox control mice at timepoints I, II and III. Scale bar is 86µm. Hoechst nuclear stain is blue. Dotted lines indicate approximate border of arcuate nucleus based on morphology and nuclear density.
and *Ift88Δ/Δ* mice at timepoint I displayed increased cFos immunostaining within the arcuate nucleus of the hypothalamus 90 minutes after injection indicative of a response to leptin (Figure 2G) (24). These results indicate that at timepoint I, after both the Ift88 protein and cilia have been lost, the response to leptin remains intact as measured by both behavioral activity and neuronal markers of activation in cilia mutant *Ift88Δ/Δ* mice.

After 80 days of *ad libitum* feeding (timepoint II), *Ift88Δ/Δ* mutants are significantly heavier with greater fat mass, and greater serum leptin concentrations (body weight *Ift88flox/flox* 24.26g and *Ift88Δ/Δ* 40.87g; *P* < 0.01, fat mass *Ift88flox/flox* 3.36g and *Ift88Δ/Δ* 18.48g; *P* < 0.01, serum leptin *Ift88flox/flox* 4.46ng/mL and *Ift88Δ/Δ* 45.39ng/mL; *P* < 0.01 Student’s *t* test) (Figure 1A and E). Again, leptin was administered IP and feeding behavior was monitored for 12 hours. As observed in other models of increased adiposity and hyperleptinemia, the *Ift88Δ/Δ* mice did not diminish feeding in response to leptin, while control *Ift88flox/flox* responded with reduced food intake (Figure 2B, E and insets). Furthermore, cFos labeling indicated fewer positive cells in the arcuate nucleus of *Ift88Δ/Δ* mice compared to controls (Figure 2I). These results indicate that obese *Ift88Δ/Δ* mice have elevated leptin concentrations and are leptin resistant.

To test whether the increased fat mass and serum leptin observed at timepoint II drive the leptin resistant phenotype, mice were subjected to calorie restriction using a gradual stepwise process over a period of several weeks (Figure 1A and E). This restored adiposity and leptin levels back to those observed in controls. The mice were then allowed *ad libitum* access to food for several days prior to testing the leptin response (hereafter referred to as timepoint III). Leptin sensitivity was assessed prior to recommencing changes in bodyweight and serum leptin; a condition similar to timepoint I. Strikingly,
*Ifi88*Δ/Δ mice again demonstrated a strong anorectic behavior response to leptin administration (Figure 2C, F and insets) with activation of cFos in the arcuate nucleus (Figure 2I). This result suggests that the leptin resistance observed in a mouse model of cilia loss is a consequence of the onset of obesity and hyperleptinemia, and not a primary defect associated with cilia loss.

The finding that leptin signaling activity is maintained in adult cilia mutants (both prior to becoming obese and again after caloric restriction) was unexpected based on previous reports in the *Bbs* mutant mice (17); Seo, 2009 #15}. To further explore potential causes behind the discrepancy between *Bbs* and cilia null mice, we evaluated whether caloric restriction itself had effects on feeding behavior. This analysis revealed that animals that undergo calorie restriction greatly alter their feeding pattern structure (Figure 3A). These changes in feeding behavior are consistent with food anticipatory activity (FAA) observed previously in rodents (Figure 3A and B) (25-28). *Ifi88*Δ/Δ mutant mice undergoing calorie restriction eat a large majority of their daily food allowance within 3 hours of access to food (Figure 3B, days 1-3). This altered FAA meal structure persisted for 9 days after *ad libitum* food access was reinstated (Figure 3A and B, days 4-12).

Another observation was that for a couple of days after *ad libitum* food (Figure 3A days 5-7) the mutants exhibited a transient decrease in food consumption. We believe this may be due to the absence of a cued response previously associated with food addition during calorie restriction. Surprisingly, this FAA behavior was stronger than the action of leptin (Figure 3A day 7, 3C and inset). Even though there was no behavioral leptin response with regards to feeding activity, there was a change in cFos
**Figure 3:** Food anticipatory activity induced feeding behavior observed in ad libitum fed Ift88 conditional mutant mice that were calorie restricted. (A) Graph output of electronic real-time food intake monitoring cage system. Days 1-3 show Ift88\(^{\Delta\Delta}\) mutant anticipatory feeding behavior during calorie restriction, blue line (Calorie Restricted). Steep initial slope followed by a plateau indicates that Ift88\(^{\Delta\Delta}\) mutants consumed the majority of their food immediately upon its arrival. Ad libitum feeding began on day 4 (begin ad-lib). It took approximately 9 days for anticipatory feeding structure to deteriorate (Ad libitum Access but FAA Behavior) and normal feeding structure to resume (FAA Lost). Vehicle and leptin injection during the entrained timepoint are indicated (Vehicle TP E and Leptin TP E respectively) which showed no effects. Timepoint III vehicle and leptin injection nights are also indicated (Vehicle TP III and Leptin TP III respectively). Gray shading indicates dark cycle (night). (B) Graph showing the percentage of food consumed be-
tween 5-8 PM (corresponding to the period just after food addition during the caloric restricted paradigm) indicating the persistence of the FAA feeding structure over several days. During the first 3 hours of lights out the FAA Ift88$^{Δ/Δ}$ ad libitum mice ate the majority of their food when compared to Ift88$^{flx/flx}$ mice that consistently ate approximately a quarter of their nightly food during this same period. Asterisks represent a significant difference between Ift88$^{flx/flx}$ and mutant Ift88$^{Δ/Δ}$ (Student’s t-test; *P < 0.01). (C and inset) Twelve hour cumulative feeding data over time post leptin injection of Ift88$^{flx/flx}$ and mutant Ift88$^{Δ/Δ}$ in the FAA period. The number of mice analyzed in each group for A and B was: Ift88$^{flx/flx}$ 8♀/5♂ and Ift88$^{Δ/Δ}$ 6♀/8♂. Points in graphs and bars in insets represent mean food intake ± SEMs. No significant difference from vehicle was observed (n = 13, 14 Student’s t-test; P = 0.54). (D) Acute induction of nuclear cFos (red) in the arcuate nucleus of the hypothalamus 90 minutes after IP leptin or vehicle injection in Ift88$^{flx/flx}$ control and Ift88$^{Δ/Δ}$ mutant mice during the FAA period. Scale bar is 86µm. Hoechst nuclear stain is blue. Dotted lines indicate approximate border of arcuate nucleus based on morphology and nuclear density.
immunolabeling in the arcuate nucleus indicating that leptin signaling occurs during the FAA period (Figure 3D).

To further explore possible connections between leptin signaling defects in adult induced cilia mutants we performed both thermoregulation and activity experiments. Shortly after the discovery of the leptin deficient \textit{ob/ob} mice, it was reported that they displayed defects in thermogenesis, as evidenced by their lower basal body temperature and inability to maintain body temperature when challenged with a cold environment (29). To see if conditional cilia mutant mice develop a cold intolerance phenotype associated with defects in leptin signaling, we cold-challenged them both in the lean and obese states. In contrast to the \textit{ob/ob} mutant mice, \textit{Ift88}^Δ/Δ cilia mutant mice were able to regulate body temperature in response to cold challenge similarly to \textit{Ift88}^{flox/flox} and C57BL/6 controls (Figure 4A). Furthermore, activity analysis of \textit{Ift88}^Δ/Δ revealed a subtle hyperactivity prior to the onset of obesity and no changes in activity when obese (Figure 4B). In contrast, \textit{ob/ob} mice displayed diminished locomotor activity (Figure 4B; \textit{Ift88}^Δ/Δ 40064 beam breaks/24 hours and \textit{ob/ob} 20530 beam breaks/24 hours; \( P < 0.01 \) one way ANOVA with post hoc Tukey’s HSD) (30, 31). Collectively these results indicate that cilia loss in \textit{Ift88}^Δ/Δ mutant mice does not result in other phenotypes associated with leptin signaling deficits.

To determine if the phenotypes observed in cilia loss models (\textit{IFT88}^Δ/Δ) differ from those observed in BBS mouse models, leptin sensitivity and thermoregulation experiments were performed in \textit{Bbs4} mutants (congenital knockout of \textit{Bbs4}, hereafter \textit{Bbs4}^{−/−}). To avoid confounding effects of calorie restriction and altered body composition \textit{Bbs4}^{−/−} mutants were analyzed prior to the onset of obesity. \textit{Bbs4}^{−/−} mice genotyped null
Figure 4: Comparison of thermoregulation and locomotor activity between Ift88Δ/Δ and ob/ob mice and leptin sensitivity analysis in Bbs4−/− mice. (A) Body temperature of Ift88floxflox, Ift88Δ/Δ, and ob/ob mice measured in the morning for baseline, and then following times 30, 120 and 240 minutes, during exposure to 4°C. Body temperature was assessed at 270, 390 and 480 minutes during room temperature recovery. The number of mice analyzed in each group was: Ift88floxflox 5♂, Ift88Δ/Δ 4♂, ob/ob 4♂. Note that the ob/ob mice were pulled from the experiment during the 4°C challenge phase due to an inability to thermoregulate. Points represent mean temperatures ± SEMs. Asterisks represent a significant difference from other groups at each timepoint (one-way ANOVA followed by post hoc Tukey’s HSD test; *P < 0.05). (B) Locomotor activity at timepoint I comparing the number of beam breaks over a 24 hour period between Ift88floxflox, Ift88Δ/Δ and ob/ob mice. The number of mice analyzed in each group was: Ift88floxflox 12♂,
Ift88ΔΔ 8♂ and ob/ob 4♂. Bars represent mean breaks ± SEMs. Asterisks represent a significant difference from other groups at each timepoint (one-way ANOVA followed by post hoc Tukey's HSD test; *P < 0.05, **P<0.01). (C) Genotyping PCR on wildtype (Bbs4+/+) and Bbs4 mutant (Bbs4−/−) mice. (D) Western blot on whole hypothalamic protein of Bbs4+/+ and Bbs4−/− showing loss of Bbs4 compared to actin loading control. (E) Twelve hour cumulative feeding data collected after leptin and vehicle injection in Bbs4−/− mutant mice. The number of mice analyzed was: Bbs4−/− 5♀/6♂. (E inset) Total food intake at the end of the twelve hour period. Points in graph and bars in inset represent mean food intake ± SEMs. Asterisks represent a significant difference from vehicle (Student’s t-test; *P < 0.05). (F) Acute induction of nuclear cFos (red) in the arcuate nucleus of the hypothalamus 90 minutes after IP leptin or vehicle injection in Bbs4+/+ and Bbs4−/− pre-obese mice. Scale bar is 86µm. Hoechst nuclear stain is blue. Dotted lines indicate approximate border of arcuate nucleus based on morphology and nuclear density. (G) Body temperature of Bbs4+/+ and Bbs4−/− mice measured in the morning for baseline, and then following times: 30, 120 and 240 minutes, during exposure to 4°C. Body temperature was then assessed at 270, 390 and 480 during room temperature recovery. The number of mice analyzed in each group was: Bbs4+/+ 4♂ and Bbs4−/− 5♂. Points represent mean temperatures ± SEMs.
and showed loss of protein as previously described (Figure 4C, D) (12) as well as mis-localization of neuronal cilia G-protein coupled receptors (8). Interestingly, much like Ift88Δ/Δ mice, Bbs4−/− mice responded to leptin when compared to vehicle injection (Figure 4E). Furthermore, cFos immunolabeling within the arcuate nucleus of Bbs4−/− prior to obesity onset indicated a leptin response (Figure 4F). Also consistent with normal leptin signaling, non-obese Bbs4−/− mice did not display a defect in thermoregulation upon cold challenge (Figure 4G). These results indicate that in both a model of cilia loss (Ift88Δ/Δ) and a model of defective cilia signaling (Bbs4−/−) leptin signaling is not directly affected. Importantly, these results indicate that a defect in a yet to be determined satiation pathway is dependent on the cilium.

**Discussion**

We previously showed that loss of cilia on the POMC cells in conditional mouse mutants resulted in hyperphagia, and subsequent obesity (18). However, the molecular mechanisms behind this phenotype remain elusive. More recent studies in mouse models of Bardet-Biedl syndrome (BBS) suggest that the complex of BBS proteins known as the BBSome is critical to proper leptin receptor trafficking and pathway activity (16). Interestingly, BBS mutant mice retain their cilia, albeit with disrupted cilia receptor localization, while IFT mutations result in complete organelle loss (8, 18). While both the loss of BBSome function and IFT mutation lead to obesity, it is unclear if the same molecular mechanism drives these similar phenotypes. Here we directly test whether the obesity phenotype observed in mouse models of cilia ablation is primarily driven by defects in leptin signaling.
In order to achieve this goal, the CAGG-CreER inducible transgene was utilized. Although this does not lead to total loss of IFT88 it eliminates potentially confounding effects of cilia loss during development, an advantage that the previously utilized POMC-Cre transgene does not possess (19, 32). It was previously shown that obese, leptin resistant animals can regain leptin sensitivity upon regulation of body composition through controlled feeding (33, 34). Furthermore, the longitudinal study presented here allowed for the repetitive assessment of leptin sensitivity in the same cohort of mice at different adiposity and leptin levels. Somewhat surprisingly, Ift88Δ/Δ conditional mutant mice are only resistant to the actions of leptin after they are obese and have increases in serum leptin. Strikingly, Ift88Δ/Δ mice are sensitive to the actions of exogenous leptin both before weight gain and after weight loss.

Although the exact mechanism behind leptin action and resistance remains unclear, the downstream effects of leptin have been characterized. Leptin leads to the phosphorylation of STAT3 (pSTAT3) and the induction of Socs3, and subsequent neuronal activity results in increases in nuclear cFos (35). In the pre-obese and caloric restricted states (timepoints I and III), Ift88Δ/Δ mice responded to acute injections of exogenous leptin with an induction of cFos in the arcuate nucleus of the hypothalamus, in contrast to what was recently shown for lean Bbs mutant mice. It is interesting to note that in Bbs mutant mice leptin induced pSTAT3 is still observed, indicating that leptin signaling is not completely disrupted (16). These results suggest that the hyperphagia associated obesity in models of cilia dysfunction is not initiated by defects in leptin signaling.

To further investigate whether other phenotypes associated with leptin signaling defects were observed in Ift88Δ/Δ mice, both thermoregulation and locomotor activity ex-
periments were performed. Thermoregulation experiments in which Ifi88Δ/Δ mice were cold challenged demonstrated a normal phenotype. This stands in stark contrast to the leptin deficient ob/ob mouse, which is unable to maintain proper body temperature in a cold environment (31). To further assess leptin signaling in cilia mutants, locomotor activity was evaluated. Ifi88Δ/Δ mice were significantly more active in a 24-hour period relative to ob/ob mice. Taken together these data indicate that conditional Ifi88Δ/Δ cilia loss in adult mice does not lead to a primary defect in leptin signaling.

Previous reports demonstrated that restricted feeding in rodents can alter both behavior and certain aspects of physiology, independent of light-dark cycles (reviewed in (36)). For example, in response to restricted food access mice are known to display food anticipatory activity (FAA) and alter their feeding behavior and meal structure (25). Thus, FAA can confound results in experiments if not taken into account. Our analysis of Ifi88Δ/Δ mutant mice in the longitudinal paradigm clearly revealed the FAA phenomenon. The mutants consume the majority of their calories within the first 3 hours of the dark cycle during the paired feeding period (Figure 3A and B). In contrast, control mice on an ad libitum diet normally consume their calories gradually throughout the dark cycle. The FAA feeding behavior in the mutants persisted for nine days after ad libitum food access was initiated (see Figure 3B), an observation made possible by the use of the BioDaq real-time electronic monitoring system. In addition, after ad libitum access the mutants experienced a short period of depressed feeding activity (days 5-7). This diminished feeding may result from the loss of feeding cues established by the daily administration of food during caloric restriction. This cue would not occur during ad libitum access. If mutants are tested for leptin sensitivity during this FAA period they appear
leptin resistant with regards to their feeding activity. Despite the lack of a leptin mediated behavior response, staining for nuclear cFos in mutants after 90 minutes post IP leptin administration indicates the leptin signaling axis was intact and activated. Thus, FAA appears to be stronger than the appetite suppression effects of leptin. After mutants emerged from the FAA period and returned to a normal feeding pattern (timepoint III), they once again exhibited an anorectic response to leptin. The persistence of this FAA represents an important and underappreciated aspect of feeding behavior analysis in the obesity field. Interestingly, FAA is dependent on the suprachiasmatic nuclei (SCN) and not the arcuate nucleus where ciliary function is needed for satiation responses (25). Future studies will address whether the FAA observed in Ift88 conditional mutants differs from that observed in wildtype mice.

In contrast to our findings, current dogma indicates that cilia are needed for normal leptin sensitivity based on several previous studies in the Bbs ciliopathy mouse models. Data from Seo et al. utilized a caloric restriction paradigm up until leptin responsiveness was assessed to ensure that the Bbs mutant mice were kept lean (16). Although not directly addressed, this may have caused a FAA effect similar to what we observed in the Ift88Δ/Δ mice overriding the anorexogenic effects of leptin. To directly test whether Bbs mutant mice have a leptin signaling defect, we assessed both leptin sensitivity and thermoregulation in Bbs4−/− mice prior to the onset of obesity. Interestingly, Bbs4−/− mice responded to IP leptin injection and were able to maintain body heat when cold challenged, unlike ob/ob mice. Collectively, these data suggest that the leptin signaling defect reported previously in the Bbs mice is only secondary to either weight gain or the FAA
brought on by calorie restriction. This ultimately leaves the initiating molecular mechanism behind cilia dysfunction-associated obesity unknown.

Several potential molecular mechanisms for the obesity observed in ciliopathy mouse models exist. One must consider that the mechanism leading to obesity in Ift conditional models and Bbs models may indeed be different. It is now well appreciated that the cilium functions as a key regulatory organelle for multiple different pathways. Some of the potential pathways that could be involved in the obesity phenotype in these models include altered G-protein coupled receptor (GPCR) signaling or abnormal regulation of mTOR or hedgehog (Hh) signaling pathways. Importantly, the orexigenic GPCR melanin concentrating hormone receptor 1 (Mchr1) is present on neurons of the hypothalamus, but is mis-targeted in the Bbs mutant mice (8). Thus, the possibility exists that altered Mchr1 signaling in the absence of cilia in the Ift88 mutants, or due to its exclusion from the cilia in the Bbs mutants, could result in hyperphagia induced obesity. Cilia loss alters mTOR activity which has a well-documented role in energy homeostasis (37-41); however, altered mTor has not been evaluated in the Bbs mutant mice. Further, treatment of cilia mutant mice with rapamycin can partially correct some mutant phenotypes (42, 43). Arguably, defects in the Hh pathway are currently the most directly associated with abnormal cilia function (reviewed in (44)). Hh signaling is important for the development and patterning of numerous tissues, including the hypothalamus, and has critical roles during adult neurogenesis (45-48). Thus, obesity in congenital Bbs mutants could arise through mis-patterning of the hypothalamus. In fact, previous reports have indicated loss of POMC neurons in Bbs mutant mice (16). This seems less likely in the adult inducible Ift88 mutant as the hyperphagia phenotype is evident within two weeks of inducing cilia
loss. However, Hh pathway components are expressed in the hypothalamus of adult mice and thus, it will be particularly informative to evaluate whether feeding behavior and energy homeostasis can be altered by modulating Hh signaling activities specifically in the POMC neurons and whether this is influenced by the presence or absence of the cilium.
References


FUTURE STUDIES AND CONCLUDING REMARKS

The past few decades have shed much light on our understanding of cilia and flagella biology. Importantly, it is now recognized that cilia and flagella dysfunction are the cause of a wide variety of human diseases, the ciliopathies [11]. These diseases can manifest themselves through a wide variety of symptoms including developmental defects, retinal degeneration, anosmia, cystic kidneys, obesity, and infertility. Our understanding of the molecular mechanisms responsible for ciliopathies has come a long way, largely through the use of in vitro assays and animal models. Mouse models in particular have been a significant tool in the study of ciliopathies, as they have provided insights into the mechanisms of pathogenesis of several of the symptoms of ciliopathies including developmental defects, infertility, and obesity. For example, mouse models have shown us the importance of cilia in Sonic hedgehog signaling [146]. As such, mutations in cilia genes such as Mks1 or Ift88 leads to disease characterized by cystic kidneys, polydactyly, and neural tube defects in human ciliopathy patients [147, 194]. Mouse models of cilia genes have also provided insights into the infertility that is common amongst ciliopathy patients, as they revealed that function of motile cilia and flagella in the reproductive tract are often disrupted [195, 196]. We can therefore use infertile mice with mutations in novel genes to guide efforts in finding underlying genetic factors that can lead to infertility in human patients. Recent studies have also focused on the obesity phenotype that is found in ciliopathy patients with Alström syndrome (ALMS) and Bardet-Biedl syndrome (BBS) and common to several mice with mutations in cilia genes [126, 197]. Strikingly,
some of these studies in mice have shown that the obesity phenotype is due to loss of normal cilia function on neurons [135]. This demonstrated a previously unrecognized importance of this organelle and a novel requirement for cilia for neuronal activity. One of the reasons research into cilia and obesity remains so popular is that it could not only lead to understandings of obesity in ciliopathy patients, but it could also provide insight into appetite and obesity in the rest of the human population, possibly even leading to therapeutic interventions to control weight gain.

The studies that I have presented here have expanded our understanding of cilia and flagella biology in the areas of development, infertility, and obesity. In the following sections, I will discuss the implications of these findings and how our understanding of cilia and flagella and can be expanded by further by using the data presented in this thesis as a starting point.

**Cluap1 as a Novel Mammalian Cilia Gene needed for normal IFT**

Our findings for the first time implicate *Cluap1* as being necessary for cilia assembly and proper development in a mammal system. Prior reports have implicated homologs of *Cluap1* in cilia biology in other model organisms. Mutagenesis screens in the nematode worm *C. elegans* had uncovered a *Cluap1* homolog necessary for proper cilia assembly and cilia mediated behavior [198]. This gene, named *dyf-3*, is necessary for assembly of the distal portion of neuronal cilia [192]. Simultaneously to this finding, it was discovered that mutations in *qilin*, the *Cluap1* homolog in zebrafish, caused the same cystic kidney phenotype. This phenotype is shared with several other cilia mutant zebrafish [193]. More recent publications have also demonstrated that loss of *qilin* in
zebrafish causes defects in hedgehog signaling pathway as well as defect in cilia assembly [199].

As defects in cilia assembly and function are the known cause of ciliopathies, the potential for mutations in CLUAP1 to cause disease should not be overlooked. This is even more evident by the recent finding that mutations in IFT88 can cause Meckel-Gruber syndrome in humans [59]. Likewise, a very rare ciliopathy named Sensenbrenner syndrome has also been linked to IFT122, an IFT A complex gene [200]. This disease is characterized by heart defects, chronic renal failure and developmental abnormalities including microcephaly [201]. Ift122 mutant mouse models show similarities to the Cluap1 mutant mice reported here, including the absence of cilia and defects in Sonic hedgehog signaling. The similarities between Ift88 and Ift122 mutants to the Cluap1 mutant embryos are strikingly similar, as are their effects on cilia assembly. Importantly, the affected gene or genes in most cases of ciliopathies in human patients are unknown, and thus mutations in CLUAP1 could be an unrecognized component in human disease. Screening human ciliopathy patients for mutations in CLUAP1 will help clarify this possibility.

A more detailed understanding of the molecular function of Cluap1 will also be necessary to determine its exact role in cilia structure. Our data show that loss of Cluap1 leads to total loss of cilia in the developing mouse embryo and a disruption of Sonic hedgehog signaling, two phenotypes which are nearly identical to those seen if Ift88 and Traf3ip1 mutant mice [45, 202]. Both of these genes encode proteins that are known and critical members of the IFT B complex which are necessary to both cilia assembly and proper development, raising the possibility that Cluap1 is also an IFT B complex mem-
ber. The *Chlamydomonas* homolog of *Cluap1, FAP22*, is also upregulated in flagella regeneration, further arguing in favor of some ciliary role [203]. Future studies of *Cluap1* and the IFT complex can clarify the exact molecular function of the protein. Particularly, the *Cluap1* antibody we have developed to demonstrate localization of the protein to the cilia could be used in biochemical studies to determine if *Cluap1* interacts with known IFT proteins through co-immunoprecipitation. Alternate techniques, such as fluorescence resonance energy transfer (FRET) could also be used to identifying IFT proteins that are potentially interacting with *Cluap1* in cells.

Perhaps one of the biggest challenges in assessing the diverse roles for cilia genes is the severe developmental defects that are associated with loss of their function during embryogenesis. The work I have presented in this thesis demonstrates that, much like *Ift88* and *Traf3ip1, Cluap1* mutant mice die embryonically, thus making the study of this gene in adults problematic [45, 202]. Conditional alleles circumvent this problem, such as the case with the *Ift88* conditional allele we utilized in our study on cilia and leptin signaling. Fortunately, such a possibility exists with our *Cluap1KO* allele. The *Cluap1KO* allele contains a β-galactosidase trapping cassette which causes alternate splicing into the β-galactosidase gene followed by a transcription termination sequence. This allows the allele to act as a reporter in addition to being a constitutive null mutation in *Cluap1*. Importantly, the β-galactosidase trapping cassette is flanked by Flp recombinase target (FRT) sites. When exposed to a flippase enzyme (FLP), the FRT sites undergo recombination to remove the β-galactosidase trapping cassette. Once removed, the allele is able to produce wildtype *Cluap1* transcript. However, exon 3 is also flanked with loxP sites, which when exposed to a cre recombinase causes deletion of this exon and results in a
frameshift mutation causing a premature stop codon in the allele. This means we can use our Cluap1<sup>KO</sup> allele to create conditional Cluap1 mice where loss of the gene can be selectively controlled through the cre-loxP system.

Such an approach can be used to bypass the embryonic lethality of Cluap1<sup>KO</sup> mice. As our current studies indicate that Cluap1 is widely expressed in adult tissue, it remains possible that loss of the gene will adversely affect cilia formation in adults much like loss of Cluap1 inhibited ciliogenesis in the embryo. If this is the case, we can expect a wide variety of cilia related phenotypes in the adult mice, including retinal degeneration, renal cysts, infertility, and obesity. It would also be interesting to note if phenotypes not typically associated with loss of other cilia genes appear.

Cluap1 is named after an interaction with the protein clusterin (hence, CLusterin-Associated Protein 1) [204]. So far this finding has only been reported in overexpression experiments in non-ciliated cells, and its implications to ciliated cells or living organisms expressing the genes at normal levels remains unknown. Clusterin itself is an enigmatic protein that has been implicated in a variety of cellular events including cell clustering, complement inhibition, injury repair, and apoptosis [205]. Consistent with its proposed role in injury response, clusterin knockout mice experience more severe renal injury in response to renal ischemia-reperfusion [206]. Intriguingly, several studies have found that clusterin is also upregulated in mouse models of polycystic kidney disease [207]. This is particularly relevant given the fact that renal cysts are a common characteristic of ciliopathy patients and cilia mutant mouse models, and that cystic kidneys was a phenotype for the qilin mutant zebrafish. Despite the discovery of these implications, no clear
role of clusterin’s effects or molecular mechanism has been proposed, and it remains possible that some of its functions are mediated through an interaction with Cluap1.

Our findings are significant as they demonstrate the discovery of a previously unrecognized mammalian cilia gene. Previous reports in zebrafish demonstrated that loss of function mutations in qilin, a Cluap1 homolog, did not necessary inhibit cilia formation in those mutants, although it was undetermined if this was a true lack of phenotype or was caused by maternal loading of mRNA [193]. Thus, it remained ambiguous if Cluap1 and its homologs were truly necessary for cilia formation or not. Our studies using a null Cluap1 allele resolve this question in a mammalian system and conclusively show the necessity of this gene in both cilia assembly and proper Sonic hedgehog signaling. It is evident that future studies can further clarify the exact role of Cluap1 in cilia assembly and its potential involvement in human disease.

Ccdc42 and Infertility

Our finding that Ccdc42 mutant mice survive development with no obvious phenotypes reflecting dysfunction of primary cilia or motile cilia was a surprising one. Unlike genes linked to primary ciliary dyskinesia like DNAI1, DNAH5, Ccdc39 and Ccdc40, which have roles in both flagella and motile cilia function, Ccdc42 appears limited to strictly defects in flagella formation [78-81]. Furthermore, mutations in these genes usually only cause subtle defects in the axoneme structure that makes the beating of the cilia or flagella more difficult or uncoordinated. In contrast, our Ccdc42 mutant mice have a complete absence of flagella but do not appear to have any defects in other motile forms of cilia. This most likely indicates that Ccde42 may have a trafficking role in the assem-
bly of flagella akin to the Bbs proteins, and is not actually involved in the structural architecture of the flagellum itself. The localization of Ccdc42 in distinct puncta in developing wild-type spermatids indicates that the protein is a component of or is transported by intramanichette transport (IMT), the pathway to bring proteins to the head-tail coupling apparatus (HTCA) and developing flagella [47]. This could indicate that Ccdc42 has a specific role in IMT during sperm maturation. The fact that Ccdc42 is not expressed in immature spermatozoa lining the basement membrane of the seminiferous tubules and are not undergoing IMT, support this notion. The reason for Ccdc42 localization to the acro-some-acroplaxome complex remains more elusive. It is possible that the protein is part of the acroplaxome plate that helps anchor the acrosome to the nucleus, hence why some of the Ccdc42 mutant spermatids display abortive or detached acrosomes. The reason for the loss or duplication of HTCA in the Ccdc42 mutant spermatids also remains elusive, as it is unlikely that there are defects in mitosis based on the lack of Ccdc42 expression in immature sperm cells that have not yet finished dividing. It remains possible that loss of Ccdc42 results in severe dysfunction of pathways regulating centrosomes-HTCA in sperm cells, thus accounting for the abnormal number of HTCA structures found in Ccdc42 mutant sperm cells.

The short lived and dynamic nature of sperm cells complicates the study of the molecular mechanism of Ccdc42, as the cells are not easy to manipulate. Furthermore, very little cells are harvested from the epididymis of Ccdc42 mutant mice, indicating that the sperm cells have mostly degraded. Thus, to better elucidate the molecular function of this gene, future studies would benefit from the study of Ccdc42 homologs in other model organisms. We have started this process by finding a potential Ccdc42 homolog in
Tetrahymena thermophila. Creating transgenic GFP-Ccdc42 Tetrahymena reveals localization to the base of the motile cilia in these organisms, which complements the defects seen in the HTCA of our Ccdc42 mutant spermatozoa. Based off of the phenotypes seen in mouse spermatozoa, it is possible that Ccdc42 mutant Tetrahymena have defects in flagella assembly. Preliminary data indicates this is not the case however, and the Ccdc42 mutant Tetrahymena can still assemble motile cilia and swim at normal speeds. During our search for Ccde42 homologs in Tetrahymena, a second potential homolog was found. It remains possible that this other gene is compensating for the loss of Ccde42 in our Ccde42 mutant Tetrahymena. In mice, the Ccde42 protein was associated with the acrosome-acroplaxome complex and IMT particles, and theoretically it is at these locations where the protein functions in those cells. However, neither of these structures is present in Tetrahymena. Therefore, an alternate explanation is that the phenotype in Ccde42 mutant Tetrahymena might be more subtle, such as defects in trafficking of certain proteins to the motile cilia, reflecting an evolutionarily divergence of the gene. It also remains a possibility that functional redundancy is occurring with another gene in the mammalian genome.

Potential homologs of Ccde42 are found in many organisms, including Chlamydomonas reinhardtii, Plasmodium falciparum, and zebrafish, and studies in those model organisms will clarify the relationship between Ccde42 and motile cilia/flagella.

The sterility phenotype seen in Ccde42 mutant males immediately raises the possibility that loss of function mutations in the human homolog, CCDC42, will also cause male infertility. Mutations that effect flagella function or assembly in mammals often affect cilia in other parts of the body as well, hence the developmental patterning defects
and chronic respiratory infections in primary ciliary dyskinesia patients [208-210]. In contrast, the specific expression in the testes and lack of other observable phenotypes indicate that humans with loss of function mutations in *CCDC42* would have no other obvious symptoms besides male infertility. This would make identifying the cause of infertility as mutations in *Ccdc42* much more difficult. As most cases of infertility is due to unknown causes, the identification as CCDC42 would prove an advancement for reproductive science. Our lab is currently testing primers to start screening male human infertility patients to determine if in fact we can find a link between mutations in *CCDC42* and infertility.

**Cilia and Obesity**

The obesity epidemic in first world countries is rapidly becoming a major concern to the health of the population [211]. Obesity is associated with premature mortality caused by severe secondary consequences such as diabetes, heart disease, and asthma. In addition to causing major health concern, the continued rise of obesity in the United States has become an immense cost to the healthcare system that is predicted to only increase.

Obesity was once considered a strictly social issue brought on by overindulgence and lack of physical activity. However, the identification of mutations in mammalian genes resulting in hyperphagia and obesity caused a dramatic reassessment of the nature of the obesity epidemic [155]. We now know that the regulation of energy balance is a very complex process involving numerous diverging and converging pathways within the brain and peripheral tissues. The discovery that obesity was a symptom of both Bardet-
Biedl syndrome (BBS) and Alström syndrome (ALMS), as well as the finding that cilia mutant mice are often obese, implicated the primary cilium as an underappreciated player regulating satiety [182]. Despite previous publications implicating the leptin signaling pathway as causing obesity in cilia mutant mice, our data shows this is not the case and that leptin resistance in cilia mutant animals is a secondary consequence of obesity and abnormal feeding behavior caused by caloric restriction [121, 184].

Obesity induced leptin resistance is a well reported phenomenon, even if the reasons behind it are poorly understood. In contrast, changes in feeding behavior caused by caloric restriction, or food anticipatory activity (FAA), are less well recognized in the field of obesity research. FAA occurs when animals are given restricted food access, and as a result alterations in their behavior occur [186]. These behavioral changes are accompanied by physiologic changes including increases in plasma corticosterone levels, body temperature, ketone bodies, and free fatty acids [212]. It is believed that FAA serves an evolutionary role in helping an animal find food at times when it is known to be available as well as preparing the body for the sudden intake of calories and nutrients. This response is similar to and often compared to the circadian rhythms that control sleep cycles. However FAA has proven to have at least partially distinct mechanisms, as Clk mutant mice, which have severe disruptions in circadian rhythm, are still able to display FAA [186].

FAA activity and disruptions in a normal feeding pattern persists temporarily even when rodents are returned to an ad libitum diet [186]. Previous studies on leptin resistance in calorically restricted Bbs mutant mice did not take the FAA phenomenon into account, as evidence by the fact that leptin sensitivity was assayed one day after the
cessation of caloric restriction [184]. The fact that this concerning caveat in the methods was not detected by either the researchers or the peer review process indicates that FAA is not yet well recognized in the field of obesity research. Our findings will bring more attention to this issue, as we have conclusively demonstrated that cilia mutant mice will respond to the anorexic effects of leptin when not in the FAA period, but show feeding behavior similar to leptin resistance during the FAA period. This strongly implies that the “leptin resistance” that was previously reported to occur in lean Bbs mutant animals was in fact due to the effects of FAA, and not a mechanism rending the mice unable to respond to leptin itself.

Equally important, our findings will shift the focus of cilia and obesity research away from leptin signaling and towards other potential pathways that are driving the hyperphagia mediated obesity in both cilia mutant mice and human ciliopathy patients. Clues to what may actually being causing obesity in ciliopathy patients can be collected from other ciliary proteins and cilia mutant mouse models. Another ciliary protein implicated in appetite regulation is type III adenylyl cyclase (ACIII). Previous reports demonstrate that ACIII has a strong localization preference to the neurons of primary cilia [213]. Unlike the IFT and BBS genes however, there are no obvious signs of defective development in a mutant mouse model of ACIII (ACIII−/−). However, ACIII−/− mice do have hyperphagia induced obesity in a similar manner to the Ift88 and Bbs mutant mice [214]. Additionally, ACIII−/− are also anosmic, another phenotype shared with Bbs mutant mice and human BBS patients [215]. Mutations in ACIII are not currently reported to cause or contribute to any known ciliopathy. Strikingly however, ACIII gene polymorphisms have been associated with obesity in otherwise healthy human patients, demon-
strating the importance of this mouse model to human obesity research [216]. It remains possible that cilia mutant mice and human ciliopathy patients cannot properly localize ACIII, leading to interruptions in the proteins normal function, resulting in obesity.

Another neuroendocrine signaling protein known to regulate feeding behavior is G-protein coupled receptor melanin concentrating hormone receptor 1 (Mchr1), which like ACIII, has a strong localization preference to neuronal primary cilia in most regions of the brain [217]. Administration of melanin concentrating hormone (MCH), the ligand which binds to Mchr1, elicits an increase in feeding behavior that is believed to be mediated by a decrease in the frequency of spontaneous action potentials [218]. Pharmacological experiments collaborates the orexigenic role of Mchr1 stimulation as pharmacological antagonists of the receptor cause a decrease in feeding behavior [219]. Strikingly, mislocalization of Mchr1 has been observed in the neurons of obese Bbs mutant mice [220]. In a similar fashion, Mchr1 cannot properly localize in the conditional Ift88 mutants used in our studies either, as they are lacking in cilia altogether. Potentially, mislocalization of MCHR1 is also driving obesity in human ciliopathy patients.

The previous examples were only two of many possibilities potentially leading to hyperphagia and obesity in cilia mutant mice and obese ciliopathy patients, and ongoing work in our lab is currently pursuing these new avenues. Importantly, obesity research has also uncovered pharmacological agents that target ciliary proteins. For example, a pharmacological antagonist to Mchr1 has proven to be effective in reducing food intake and causing significant weight loss in mice [219]. The effects that this drug would have on the cilia mutant mice or humans have yet to be determined, but it demonstrates the
exciting potential of therapeutically targeting the cilia to fight the growing obesity epi-
demic.
GENERAL LIST OF REFERENCES


46. Ashe A, Butterfield NC, Town L, Courtney AD, Cooper AN, Ferguson C, Barry R, Olsson F, Liem KF, Jr., Parton RG, et al: Mutations in mouse Ift144 model the


commonly involved in Bardet-Biedl syndrome, a complex human obesity


203. Stolc V, Samanta MP, Tongprasit W, Marshall WF: Genome-wide transcriptional analysis of flagellar regeneration in Chlamydomonas reinhardtii identifies


APPENDIX A

IACUC APPROVAL
NOTICE OF RENEWAL

DATE: February 17, 2012

TO: BRADLEY K YODER, Ph.D.
NCLM-066 8009
FAX: (205) 934-0960

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Ciliary Dysfunction and Pathogenesis of Obesity
Sponsor NIH
Animal Project Number: 120206061

As of February 22, 2012, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

<table>
<thead>
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<th>Species</th>
<th>Use Category</th>
<th>Number In Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>A</td>
<td>2044</td>
</tr>
<tr>
<td>Mice</td>
<td>B</td>
<td>110</td>
</tr>
</tbody>
</table>

Animal use must be renewed by February 21, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 120206061 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7062.