CHARACTERIZATION OF THE
CHIBBY FAMILY OF CENTROSMAL PROTEINS

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Abstract

The Chibby protein antagonizes Wnt/Beta-Catenin signaling, a prominent regulator pathway of development, via direct interaction with the transcriptional activator domain of Beta-Catenin. Chibby mutant mice have a defect in the formation of motile cilia in the airway epithelium. The centrosome is the major microtubule organizing center of animal cells and includes two centrioles, termed mother and the daughter based on their age and replicative history. The mother harbors subdistal and distal appendages, which, respectively, anchor the interphase microtubule array and are required for ciliation. Several lines of published evidence suggest a link between the primary cilium, a single, non-motile structure found on many cells, and Wnt/Beta-Catenin signaling. Based on this evidence I investigated Chibby, and related members of the Chibby protein family, to determine its role in centrosome and cilium structure and function.

I report here that the Chibby family of proteins is associated physically and functionally with the centrioles of the centrosome in mammalian cells. The Chibby family consists of Chibby (Cby1), Nurit/Cby2, and the previously uncharacterized, Chibby3 (Cby3). Fluorescence microscopy revealed that Cby1 localized to the mother centriole of the centrosome, and that this localization was partially shared with Nurit/Cby2 and Cby3. Cby1 colocalizes at the mother centriole with phospho-Beta-Catenin, a modified form of Beta-Catenin that, in some contexts it is targeted for proteasome-mediated degradation. I also found that Cby1 partially colocalized with phospho-Beta-Catenin at a ring-like structure at the midbody during cytokinesis, where they might regulate abscission.
The localization of Cby1 to the mother centriole was similar to that described for proteins for the distal appendages of the mother centriole. I found that Cby1 colocalized with, and exhibited cell cycle dynamics similar to, Cep164, a known distal appendage protein that is required for primary cilium formation.

Consistent with a role for Cby1 in these centriolar structures, Cby1^{-/-} mouse embryonic fibroblasts exhibited a strong reduction in primary cilium formation that could be complemented by expression of the wild-type Cby1 protein. Remarkably, overexpression of either GFP-tagged Beta-Catenin or GFP-Cby1 in hTERT-RPE1 cells resulted in fewer, but longer, cilia than controls suggesting that both of these proteins are important for structure and function of the primary cilium. In summary, I propose that Cby1 is associated with the distal appendages of the mother centriole, that defects in those structures are the likely cause of the failure of cilium formation in cells lacking Cby1, and that both primary and motile cilia display this requirement for Cby1.
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Chapter 1:

Introduction
Increasingly, sub-fields in cell biology that were previously thought to be discrete are revealed to have considerable overlap. A resulting question is which cellular processes and structures that currently seem distinct actually function in symphony? A first step toward making this determination is to catalog molecules that are important for multiple structures and processes. This question is of particular importance when such processes and structures are essential for development or malfunction in diseases. Ultimately we might find that interconnectedness is the rule rather than the exception.

There is a poorly understood interplay between cilia and Wnt/Beta-Catenin signaling that is interconnecting these fields (Gerdes and Katsanis, 2008; He, 2008). Here, we have made several new discoveries concerning a protein called ‘Chibby’, which is known to potently antagonize the key intracellular Wnt/Beta-Catenin signaling component, Beta-Catenin through direct physical interaction (Takemaru et al., 2003; Li et al., 2007). Intriguingly, Chibby is also important for the formation of motile cilia in the airway epithelium, which are cell projections that beat in a coordinated fashion to clear the airway of debris (Voronina et al., 2009).

In this study we have performed protein characterizations that reveal Chibby as a member of a family of mother centriole proteins. We have also established further molecular interconnectedness of cilia and Wnt/Beta-Catenin signaling pathway components and found a new molecular connection to the midbody ring-like structure, not to be confused with the actin-myosin contractile ring, which regulates cellular abscission, the final step of cytokinesis. On a function level, we have found evidence that both Chibby and Beta-Catenin are important for primary
cilium formation. Below, I will review the pertinent cellular processes and structures.

**Wnt/Beta-Catenin signaling**

In multi-cellular organisms, Wnt/Beta-Catenin signaling is profoundly important for cell proliferation and cell fate and thereby regulates development and mature tissue homeostasis. Wnts are secreted glycoproteins that direct recipient cell expression levels of proliferation and migration regulating genes (Nelson and Nusse, 2004; Moon, 2005; MacDonald *et al.*, 2009). Defective Wnt/Beta-Catenin signaling correlates with a variety of diseases, especially cancers (Klaus and Birchmeier, 2008; Takemaru *et al.*, 2008).

The oncoprotein Beta-Catenin was originally identified as a product of the segment polarity gene *Armadillo* (G. Jürgens, 1984) and later found to be the key intracellular Wnt signaling component (Riggleman *et al.*, 1990; Peifer *et al.*, 1991). Beta-Catenin resides in multiple cellular pools which are described below.

The cytoplasmic pool of Beta-Catenin is thought to be the relevant pool in Wnt/Beta-Catenin signaling. In absence of Wnt stimulation, cytoplasmic Beta-Catenin levels are kept low in the cytoplasm by the following mechanism. The amino terminus of Beta-Catenin is sequentially phosphorylated by two serine/threonine kinases, casein kinase 1 (CK1) at Serine 45 (Amit *et al.*, 2002; Liu *et al.*, 2002) and glycogen synthase kinase 3 (GSK3) at Serine 33, Serine 37 and Threonine 41 (Yost *et al.*, 1996). CK1 and GSK3 complex with the Beta-Catenin binding scaffold proteins Axin and Adenomatous polyposis coli (APC) which forms
the so-called, ‘destruction complex’. This phosphorylated form of Beta-Catenin is called ‘phospho-Beta-Catenin’ and it is recognized by Beta-Trcp which is an E3 ubiquitin ligase subunit that targets it for ubiquitin-proteasome-mediated degradation (MacDonald et al., 2009).

Upon Wnt ligand binding to a plasma membrane residing receptor complex that consists of Frizzled (Fz) and the low-density lipoprotein receptor-related proteins, LRP5 and LRP6, a scaffolding protein called Dishevelled (Dsh) stimulates Axin recruitment to the plasma membrane, triggering Beta-Catenin dissociation from the destruction complex and subsequent stabilization and cytoplasmic accumulation. Beta-Catenin then translocates to the nucleus where it directly interacts via the carboxy terminal region with the transcriptional coactivator T cell factor/lymphoid enhancer factor (TCF/Lef) family of proteins to promote transcription of cardinal cell cycle regulators including MYC and CYCD1 (Clevers, 2006).

Beta-Catenin also functions as a component of the cadherin complex in cell-cell adhesion. Beta-Catenin acts as a scaffold and a linker by binding the cytoplasmic domain of type 1 cadherins and also to Alpha-Catenin, which organizes the actin cytoskeleton (Gumbiner, 2000; Jamora and Fuchs, 2002). It is unclear if both the Wnt/Beta-Catenin pathway and cell-adhesion are indeed branches of a super pathway or if molecular parts that interact with each other have been used as components of separate processes (Nelson and Nusse, 2004).
The basic structure and functions of the Centrosome

Centrosomes are non-membrane bound organelles of about 1µm$^3$ that are in close proximity to the nucleus. Centrosomes organize primary cilia, the interphase microtubule (MT) array and reside at the poles of the bipolar spindle. Centrosomes are composed of centrioles and the pericentriolar material (PCM). The PCM is a fibrous matrix of proteins that nucleates and organizes MTs during interphase and mitosis (Bettencourt-Dias and Glover, 2007). Key PCM components include the coiled-coil proteins pericentrin and AKAP450 (Bornens, 2002). The gamma tubulin ring complex is the key MT nucleator of the PCM (Jeng and Stearns, 1999; Moritz and Agard, 2001).

Centrioles

The centrioles reside within the PCM and morphologically are nine triplets of microtubules in a radial spoke configuration and are typically about 0.5 µM long and 0.2 µM in diameter (Bornens, 2002). Centriole MTs are stabilized via post-translational tubulin modifications including acetylation and polyglutamylation (Piperno et al., 1987; Bobinnec et al., 1998). The centrioles differ in age, form and function. The mother centriole templates the daughter centriole from the side of the proximal end and harbors two sets of appendages at the distal end (Nigg and Raff, 2009). The subdistal appendages anchor the interphase MT array and the distal appendages are required for cilium formation (Graser et al., 2007; Nigg and Raff, 2009). The centriole distal end protein Odf2 regulates the development and/or maintenance of both appendage sets. Following deletion of Odf2 in mouse cells,
both sets of appendages disappear by both immunofluorescence and ultrathin-section electron microscopy (Ishikawa et al., 2005). The microtubule anchoring subdistal appendage components include epsilon-tubulin, EB1, Centriolin and dynactin (Bornens, 2002; Badano et al., 2005). Both sets of appendages are thought to be recruited in G2 phase. The distal appendages, as evidenced by the exclusively distal appendage protein Cep164, persist throughout mitosis (Graser et al., 2007). The subdistal appendages are lost during mitosis (Guarguaglini et al., 2005; Graser et al., 2007). In absence of either Odf2 or Cep164, cells fail to ciliate, indicating that the distal appendages are required for this process (Ishikawa et al., 2005; Graser et al., 2007).

The centrosome cycle

To ensure that each daughter cell receives an equal complement of chromatids during cell division, it is key that both the centrosome and DNA duplicate exactly once per cell cycle (Tsou and Stearns, 2006; Nigg, 2007). A failure of centrosome duplication or overduplication of the centrosome can result in chromosome instability, a common feature of cancers (Ganem et al., 2009).

At either pole of the metaphase spindle resides a centrosome. At the end of mitosis the centrioles are released from their tight orthogonal arrangement in a process termed ‘disengagement’ which is mediated by the activities of Polo kinase and separase (Tsou and Stearns, 2006; Tsou et al., 2009). Disengagement licenses the centrioles for a new round of replication (Tsou and Stearns, 2006).
Shortly after disengagement, a dynamic fibrous protein linker develops that connects the centrioles via their proximal ends. This linker is composed of Centrosomal Nek2-associated protein 1 (C-Nap1), Rootletin (Bahe et al., 2005; Yang et al., 2006) and Beta-Catenin (Bahmanyar et al., 2008).

In S phase a procentriole begins to develop in a tight orthogonal arrangement at the proximal side each of both the mother and daughter centriole. The two centrioles are now called ‘mother centrioles’ and when the procentrioles develop into centrioles they are called ‘daughter centrioles.’ This process is regulated by the kinase activities of Cdk2-cyclin E and the centriole localizing Plk4, although the nature of their targets is poorly understood. Depletion of either Cdk2-cyclin E or Plk4 prevents centrosome duplication. Overexpression of Plk4 results in centriole amplification implicating Plk4 as a cardinal regulator of centriole-genesis (Meraldi et al., 1999; Bettencourt-Dias et al., 2005; Habedanck et al., 2005). Following the priming of unknown factors at the centrosome, there is a recruitment of procentriole assembly factors including CPAP, Cep135 and gamma-tubulin which is followed by MT assembly and the MT capping protein CP110 which seems to regulate centriolar length (Kleylein-Sohn et al., 2007).

Procentriole elongation continues until the final length is reached in late G2 and at about the same time the younger of the two mother centrioles reaches what is called ‘maturity’ by attaining the subdistal and distal appendages. Thus, the formation of the procentriole to the maturation of a mother centriole occurs over two cell cycles (Habedanck et al., 2005).
For the development of the bipolar spindle the pairs of centrioles and their surrounding PCM must separate which is called ‘centrosome separation.’ This process is regulated at the onset of mitosis when the NIMA-related centrosomal kinase Nek2 phosphorylates C-Nap1 and Rootletin resulting in the dissociation and an ultimate un-tethering of centrosomes (Hayward and Fry, 2006). To prepare for the formation of the microtubule rich mitotic spindle, the centrosomes must also acquire more PCM and gamma-tubulin ring complexes, a process called ‘centrosome maturation’ (Bornens, 2002).

**Cytokinesis**

Cytokinesis is the final stage of cell division. Cytokinesis temporally follows anaphase and it is mechanistically the terminal separation of the cytoplasm and membrane of the two daughter cells. The midbody is a narrow hourglass-like pinch between the two daughter cells and is formed by cleavage furrow ingression of the actin-myosin ring. This hourglass-like region contains overlapping antiparallel track-like acetylation stabilized microtubules that originate from the central spindle (Steigemann and Gerlich, 2009). Remarkably, the mother and daughter centrioles separate during cytokinesis and one or both mother centrioles migrate to the midbody whereby the midbody microtubules disappear by an unknown mechanism. Subsequently, the mother and daughter centrioles reunite and abscission is initiated (Murray, 2001).

Abscission is the concluding step of cytokinesis whereby the daughter cells are bisected. Not to be confused with the actin-myosin II contractile ring, the
protein Centriolin localizes to a small ring-like structure encircling the midbody microtubules (Gromley et al., 2005). Centriolin, which ironically is also a mother centriole protein, recruits Golgi-derived secretory vesicles and recycling endosomes to a point near the midbody via SNAREs and the exocyst complex. Cytokinesis is complete when the midbody microtubules are severed and the recruited vesicles fuse with the plasma membrane to separate the daughter cells (Steigemann and Gerlich, 2009).

**Beta-Catenin at the centrosome and midbody**

Beta-Catenin was noticed at the midbody MTs and at the poles of the mitotic spindle and upon depletion of Beta-Catenin by siRNA it was discovered to be required for centrosome duplication (Kaplan et al., 2004). A phospho-Beta-Catenin specific antibody revealed this form of Beta-Catenin at the centrosome and the midbody microtubules (Huang et al., 2007) and more specifically at the mother centriole (Gerdes et al., 2007) where its functions are unknown.

Beta-Catenin (non-phosphorylated) was later fine mapped to the polylinker region of interphase cells where it physically interacts with Rootletin and is itself a Nek2 kinase substrate. In mitosis when Nek2 activity increases, Beta-Catenin re-localizes to Rootletin independent sites on centrosomes. Strikingly, centrosome splitting is induced by the overexpression of Beta-Catenin with stabilization mimicking mutations found in cancer which possibly links its oncogenic activity to a process other than Wnt/Beta-Catenin signaling (Bahmanyar et al., 2008).
Cilia

Cilia are whip-like microtubule based organelles that project from nearly all vertebrate cell types and are generally made in G0/G1 phase of the cell cycle. There are basically two types of cilia, primary cilia and motile cilia (Marshall, 2008).

Primary cilia were long thought to be a vestigial organelle and have recently become widely recognized as a key regulator of development and mature tissue integrity. It is our understanding that primary cilia typically function as sensory organelles of stimuli of mechanical and/or chemical nature from outside the cell; however, there are exceptions such as the motile primary cilia of the mouse node (discussed below). In contrast, some tissues such as the multiciliated cells of airway epithelium and the oviduct utilize motile cilia generate mechanical motion to clear the airway of debris or move the oocyte respectively (Marshall, 2008).

Ciliogenesis

The genesis of both motile cilia and primary cilia are understood to follow largely parallel pathways although there are differences in the mode of generation of the templating centrioles. Once a centriole has templated a cilium it is called a ‘basal body.” In cells that only make one cilium, the primary cilium, it is always the mother centriole that serves as the template and by electron microscopy, mother centrioles and the centrioles that template motile cilia in multiciliated cells are indistinguishable. A pressing question in cell biology is which molecular
differences allow mother centrioles but not daughter centrioles to template cilia (Marshall, 2008).

Ciliogenesis occurs in G1/G0 phase and is thought to be initiated by the fusing of the distal end of the mother centriole with a vesicle of unknown origin (Fliegauf et al., 2007). Elongation of the centriolar MTs then proceeds which establishes the beginnings of the ciliary axoneme, which occurs the cytoplasm (Snell et al., 1974; Fliegauf et al., 2007). The ciliary axoneme and the basal body MTs are continuous and so the nine-fold symmetry of the basal body is conveyed to the axoneme (Marshall, 2008).

Structurally, we know that motile cilia and primary cilia differ in the presence of two factors, both of which function in motility: 1) a pair of microtubules at the center of the axoneme and 2) along the sides of the axonemes are arm like structures of axonemal dynein which is not to be confused with cytoplasmic dynein. The membrane surrounding the axoneme grows with the developing axoneme until fusion of this membrane with the plasma membrane occurs, a process that requires a hole in the actin cortex (Pan et al., 2007). This event establishes a gate called the transition zone, which separates the cytoplasm and the cilioplasm as well as the plasma membrane and the ciliary membrane (Sorokin, 1962; Deane et al., 2001; Alieva and Vorobjev, 2004). Like for centrioles, the axonemal MTs are stabilized via post-translational tubulin modifications including acetylation and polyglutamylation (Piperno et al., 1987; Bobinnec et al., 1998).
The transition zone is thought to be synonymous with the distal appendages (Hoyer-Fender, 2009). The axoneme grows to maturity via a process called intraflagellar transport (IFT) whereby ciliary cargo docks at the transition zone and then moves anterogradely along the axoneme via the motor kinesin-2 and retrograde via cytoplasmic dynein. IFT persists after the final ciliary length is reached suggesting that ciliary length is maintained by equilibrium of constituent addition and removal (Marshall and Rosenbaum, 2001; Marshall et al., 2005; Engel et al., 2009) and/or a balance of the loading and unloading of IFT particles at the transition zone (Dentler, 2005).

**The primary cilium and signaling**

Primary cilia are important for several vertebrate signaling pathways. The determination of left-right asymmetry during development occurs at the node. Node cells have motile primary cilia, which is an exception to the rule. Node cilia have ciliary dynein but not the central pair of microtubules and they move differently than the motile cilia of multiciliated cells. Node cilia direct a leftward flow over the node and disruption of this process can result in *situs inversus* (Nonaka et al., 2002).

In kidney tubules, primary cilia are mechanosensors of changes in fluid flow to modulate the levels of intracellular calcium as a messenger (Praetorius and Spring, 2001, 2003). Cilia may also regulate kidney tubule structure formation or maintenance as defects in IFT proteins in this tissue cause renal cyst development (Fliegauf et al., 2007).
The primary cilia of fibroblasts have platelet-derived growth factor alpha (PDGFRalpha) receptors. Upon ligand-dependent stimulation the Akt and the Mek1/2-Erk1/2 cell survival and proliferation inducing pathways are potentiated (Schneider et al., 2005).

The Hedgehog (Hh) pathway is of particular interest due to its recognition as a major regulatory factor in many cancers and because cilia seem to be essential for this pathway. Extracellular Hh protein binds membrane bound Patched (Ptc) which liberates membrane bound Smoothened (Smo) to activate Gli transcription factors (Lum and Beachy, 2004; Ingham and Placzek, 2006). In a series of elegant experiments, Hh signaling was found to require IFT proteins (Huangfu et al., 2003). It was later found that Smo functions at the primary cilium (Corbit et al., 2005) and that Ptc regulates Hh signaling specifically at the primary cilium (Rohatgi et al., 2007).

Signaling by the primary cilium seems to be essential for development. The IFT component IFT88/Polaris is thought to be essential for the formation of all cilia (Murcia et al., 2000; Pazour et al., 2000). A non-conditional mouse model deleted of the key IFT component IFT88/Polaris is embryonic lethal. Homozygous IFT88/Polaris mice die mid gestation and exhibit random left-right body axis development and neural tube defects presumably as a result of the loss of node cilia (Murcia et al., 2000; Pazour et al., 2000) and IFT88/Polaris is required for the formation of renal cilia (Yoder et al., 2002a; Yoder et al., 2002b). Similar defects were observed upon the analysis of the cilium specific Kinesis II subunits Kif3A
and Kif3B. Homozygous deletions of either protein led to embryonic lethality and left-right patterning randomization (Nonaka et al., 1998; Marszalek et al., 1999).

Evidence for a role of the primary cilium in Wnt/Beta-Catenin signaling is emerging. Inversin (Inv) is a gene that when mutated causes situs inversus and renal cysts and coincidently inhibits Wnt/Beta-Catenin signaling (Simons et al., 2005). Two independent studies subsequently found increased Wnt/Beta-Catenin activity in absence of primary cilia. Downstream Wnt/Beta-Catenin target gene amplification was measured upon stimulation with Wnt ligand following depletion of Kinesin-2 (Gerdes et al., 2007; Corbit et al., 2008).

Confoundingly, Wnt/Beta-Catenin signaling appears normal in early gestation Kif3a−/−, IFT88/Polaris−/− embryos which were assayed prior to lethality (Ocbina et al., 2009). Surprisingly, IFT88/Polaris−/− zebrafish are viable and seem to have normal Wnt/Beta-Catenin signaling. As expected, Hh signaling is attenuated. This result differed from that in mouse where cilia seem essential for Hh signaling (Huangfu et al., 2003; Corbit et al., 2005; Rohatgi et al., 2007). Furthermore, zebrafish disrupted of Hh signaling via a Smo mutant are not viable (Aanstad et al., 2009). These results raise the question of whether it is cilia or actually basal bodies that regulate Wnt/Beta-Catenin signaling (Huang and Schier, 2009; Ocbina et al., 2009).

The Chibby protein family

Chibby (Cby1) is a small (14.5 kDa) highly conserved protein. RNAi mediated depletion of Cby1 led to ectopic activation of the Wnt/Beta-Catenin
pathway and mechanistically, Cby1 competes with TCF/Lef for physical interaction with the C-terminal activation domain of Beta-Catenin (Takemaru et al., 2003; Li et al., 2007). Subsequently, a Cby1<sup>−/−</sup> mouse was generated. About seventy five percent of Cby1<sup>−/−</sup> mice succumbed to complications of unknown origin by P2. Survivors exhibited defects of both diminished fat storage and the generation of motile cilia in the airway epithelium (Voronina et al., 2009).

Intriguingly, Chibby was identified in a yeast two hybrid screen for proteins that interact with polycystin-2, a ciliary protein that when mutated correlates with cystic kidneys (Hidaka et al., 2004; Nigg and Raff, 2009). Chibby was also shown to interact with both polycystin-2 (PC2) and the Golgi component protein, GM130 in tissue culture cells. Called ‘PIGEA-14’ (polycystin-2 interactor, Golgi- and endoplasmic reticulum-associated protein with a molecular mass of 14 kDa) in this study, Chibby might play a role in the transport of PC2 through the endoplasmic reticulum and Golgi to the cilium (Hidaka et al., 2004).

In a brief report Chibby was found to interact with the proteins NBPF11 and Clusterin which seem to be important for neuroblastoma (Vandepoele et al.; Vandepoele et al., 2008). Clusterin is implicated in a broad range of cellular processes but of interest, polycystic kidney disease (Rosenberg and Silkensen, 1995a, b). Little more is known of NBPF11. Neither NBPF11 nor Clusterin interaction with Chibby affected Wnt/Beta-Catenin signaling (Vandepoele et al., 2010).

The Chibby interactions with PC2, GM130, NBPF11 and Clusterin were characterized using the exogenous expression of tagged constructs (Vandepoele et
and it will be important for future studies to confirm these interactions with antibodies directed against these proteins.

A phylogenetic analysis of Chibby as part of a protein family is provided by TreeFam: a curated database of phylogenetic trees of animal gene families (Li et al., 2006; Ruan et al., 2008). However, no alignment of the Chibby family or rationale for family designation has been found. The Chibby protein family consists of Cby1, Nurit/Cby2 and Chibby (Cby3) in mammals and just Cby1 in lower organisms (Li et al., 2006).

Nurit localizes to the flower like structure in mouse, which is a transient structure during spermatogenesis that is thought to extrude cytoplasmic components that are unnecessary for the mature sperm (Feige et al., 2002). Cby3 was uncharacterized prior to this study.
Chapter 2:

The Chibby Family of Centriolar Proteins
Abstract

Chibby (Cby1) antagonizes Wnt/Beta-Catenin signaling and functions in the generation of the motile cilia of the airway epithelium. The centrosome is the major MT organizing center in animal cells and encompasses the two centrioles, the mother and the daughter. The mother centriole has two sets of appendages, the subdistal and the distal end appendages. Although the proteins of both appendage sets are thought to be recruited in the G2 cell cycle stage, the subdistal appendages disappear from the mother centriole during mitosis while the one protein that is thought to exclusively reside at the distal appendages, Cep164, persists. The subdistal appendages anchor the interphase MT array and the distal appendages are important for ciliation.

To learn more about Chibby we looked for paralogs and found Nurit/Cby2 and the previously uncharacterized Chibby 3 (Cby3). Nurit localizes to the flower like structure, which is a transient structure during the process of spermatogenesis that is thought to be involved in the removal of cytoplasmic components that are not needed by the mature sperm.

All three Chibby proteins are present in mammals but fly and lower organisms have only Cby1. We found conserved amino acid sequence that defines the Chibby family as having these three proteins and only these three proteins. We found all three proteins at the mother centriole. The Chibby family members share an amino acid sequence motif with other known mother centriole proteins and proteins that are implicated in ciliogenesis. Moreover, Cby1 gene presence correlates with ciliation in unicellular organisms. Unexpectedly, Cby2 and Cby3
appear to be recruited to the mother centriole prior to G2. The temporal recruitment of Cby1 to the mother centriole is consistent with G2 recruitment. Interestingly, All three Chibby proteins reside at the mother centriole during mitosis like Cep164.
Introduction

Chibby is a potent antagonist of Wnt/Beta-Catenin signaling in fly and human cells and regulates ciliation of the airway epithelium (Takemaru et al., 2003; Li et al., 2007; Voronina et al., 2009). Wnt/Beta-Catenin signaling is a key regulator of development and mature tissue regulation even in the simple multicellular organism, sea anemone (Nelson and Nusse, 2004; Kusserow et al., 2005).

Centrioles and ciliary axonemes are composed of MTs modified with acetylation and glutamylation (Piperno et al., 1987; Bobinnec et al., 1998) and antibodies against these modifications allow cilia and centrioles to be clearly discerned from other organelles. Although the mother centriole MTs are continuous with the axonemal MTs, antibodies against acetylated and polyglutamylated tubulin often do not stain the region where the mother centriole MTs end and the axoneme MTs begin (Graser et al., 2007). We call this location ‘the ciliary gap.’ The ciliary gap allows for a clear distinction between the mother and the daughter centriole and more specifically, the proximal from the distal end of the mother centriole (Graser et al., 2007). The basal body and axonemal MT structure is highly conserved in multicellular organisms, an exception being the cilia of C. elegans (Inglis et al., 2007).

Centrosome duplication initiates in S phase and the resulting two centrosomes move to opposite sides of the nucleus by the onset of mitosis for the establishment of the mitotic spindle (Tsou and Stearns, 2006). The distal appendages are thought to be required for ciliogenesis (Ishikawa et al., 2005;
Graser et al., 2007) and the subdistal appendages anchor the interphase MT array (Nigg and Raff, 2009). Although both appendage set proteins are recruited in G2 the exclusively distal appendage protein, Cep164, persists at the mother centriole throughout mitosis whereas the subdistal appendage proteins disappear (Guarguaglini et al., 2005; Graser et al., 2007).
Materials and Methods

Protein Sequence Alignment

Human Chibby family UniProtKB/Swiss-Prot amino acid sequences were gathered at the ExPASy Proteomics Server (www.expasy.ch/sprot/) (Gasteiger et al., 2003). For the Chibby protein family ortholog alignments sequences were gathered from NCBI Protein, the ExPASy Proteomic server (Gasteiger et al., 2003), identified with reciprocal BLASTP analysis (Altschul et al., 1990) and gathered at TreeFam (www.treefam.org) (Li et al., 2006; Ruan et al., 2008). The alignment algorithms used were CLUSTAL W (Thompson et al., 1994), MUSCLE (Edgar, 2004) and T-Coffee (Notredame et al., 2000). Alignments were manually curated with Jalview (www.jalview.org) (Waterhouse et al., 2009). Alignments were prepared for presentation with BOXSHADE for which there is no publication. Sequences were selected to show probable sequence differences while keeping the number of sequences low.

Phylogeny

For phylogenetic analysis a compound tree consisting of Chibby protein family member paralogs manually aligned with the human Chibby protein family orthologs with Jalview (Waterhouse et al., 2009). A maximum parsimony based tree was generated with the compound alignment. 1000 standard bootstrap cycles were performed (Goloboff P., 2000; Dereeper et al., 2008). The tree was graphically rendered with TreeDyn (Chevenet et al., 2006). Cby1 was identified in lower organisms with reciprocal BLASTP analysis (Altschul et al., 1990).
dendrobatidis Cby1 was identified with the BLAST Similarity Search engine at the Broad Institute (www.broadinstitute.org).

Cell lines and culture

hTERT-RPE1 cells (Clontech) and WT and Cby1\(^{+/-}\) MEFs, which have been described previously (Li et al., 2007). All cells were grown by ATCC guidelines in 5% CO\(_2\) at 37°C. All cells were cultured in DMEM + 10% fetal bovine serum except when serum starved when 0.5% was added + 1% Antibiotic-Antimycotic (Invitrogen). To induce ciliation, 80% confluent cells were arrested in G\(_0\) with DMEM + 0.5% FBS for 20-48 hours.

Plasmid

Full-length human Cep164 cDNA was amplified from clone KIAA1052 (Kazusa DNA Research Institute) by PCR and sub-cloned into pER5/FRT/LAP-DEST Gateway (Invitrogen) resulting the GFP containing Lap tag at Cep164 N-terminus. All constructs have been verified by DNA sequencing.

Transfection

Cells were transfected at ~80% confluency in six well plates (Falcon) for three hours with 2µg plasmid DNA using 10µL Lipofectamine 2000 (Invitrogen). The Lipofectamine 2000 and the DNA were incubated in 500µL Opti-MEM reduced serum medium (Invitrogen) for 30 minutes prior to transfection.
**Antibodies**

All antibodies were diluted in PBSBT (PBS + 3% BSA + 0.1% Triton X-100 + 0.02% Sodium Azide). Primary antibodies used were mouse anti-acetylated alpha-tubulin clone 6-11B-1 (1:5000, Sigma), mouse anti-Chibby (8-2) (1:200, Santa Cruz Biotechnology, Inc.), mouse anti-Centrin (clone 20H5; 1:2000; gift from J. Salisbury, Mayo Clinic, Rochester), mouse anti-polyglutamylated tubulin (GT335; 1:2000; gift from C. Janke, Centre de Recherches de Biochimie Macromoléculaire, Montpellier, France), rat anti-GFP (GF090R) (1:2000, Nacalai Tesque, Inc.), rabbit anti-Nurit (Cby2) (1:400, gift from Benny Motro, Bar-Ilan University, Israel), rabbit anti-Cby3 seras 1 and 3 (1:400, gift from Ken-Ichi Takemaru, Stony Brook University). Secondary antibodies used were Alexa 488 or 594-conjugated goat anti-mouse, anti-rabbit and anti-rat (1:200, Invitrogen).

**Immunofluorescence**

Cells were grown on acid washed poly-L-lysine coated #1.5 coverslips (Thermo Scientific) and fixed in -20°C methanol for 2 minutes. Following fixation cells were washed twice with PBS and blocked for 30 minutes at room temperature with PBST. Cells were incubated with the following primary antibodies at room temperature for two hours: anti-Cby1. For one hour: anti-Cby3 seras 1 and 2. For 30 minutes: anti-acetylated alpha-tubulin, anti-polyglutamylated tubulin, and anti-GFP and anti-Centrin. Cells were incubated with secondary antibodies for 30
minutes and DAPI for ten seconds. Coverslips were mounted on Superfrost Plus slides (Fisher Scientific) using Antifade (Invitrogen) and sealed with clear nail polish (Sally Hansen). Images were processed using Adobe Photoshop. DNA was stained with DAPI (1µg/mL).

**Microscopy**

Immunofluorescence images were captured using an Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc.), with a Plan-NEOFLUAR 100X 1.3 NA objective (Zeiss), a Plan-APOCHROMAT 63X 1.4 NA objective (Zeiss), an Orca ER CCD camera (Hamamatsu) and Openlab 4.0.4 (Improvision). Images were processed using Photoshop 7.0 (Adobe).

**Acknowledgments**

Results

Chibby is a member of a family of three protein in mammals

To gain further insights into Chibby function we asked if Chibby is a member of a protein family. The Phylogenetic tree database of animal genes (TreeFam) (Li et al., 2006; Ruan et al., 2008) was searched. Although fly was listed to have just Chibby, TreeFam indicated that mammals have Chibby and two Chibby orthologs, Nurit/Cby2 and the uncharacterized Cby3. At TreeFam and elsewhere, no alignment or other rationale for protein family designation was found (Li et al., 2006; Ruan et al., 2008). To investigate why the Chibby family might consist of these three proteins and just these three proteins, UniProtKB/Swiss-Prot amino acid sequences of the human Chibby protein family members were aligned. Our alignment indicated two domains (Figure 1). For each domain we noted motifs of amino acids shared by all three proteins (Figure 2, 3) and searched the entire Swiss protein database with the ScanProsite tool at the ExPASy Proteomics Server for proteins with those motifs. Retrievals for both Domain 1 and Domain 2 only included Cby1, Cby2 and Cby3 from various mammals.

We reasoned that a compound alignment of Chibby paralogs and orthologs from a variety of organisms (Figure 5-7) should support a phylogenetic tree that ensured that the domains indicated in the alignment were well conserved. We assembled such an alignment and generated a parsimony-based tree that met our criteria and perfectly matched the branching pattern of the tree provided by TreeFam (Figure 8) (Chevenet et al., 2006; Dereeper et al., 2008). Based on these
results, we conclude that Chibby is indeed part of a protein family that contains these three proteins and just these three proteins.

**Chibby protein family sequence suggests mother centriole localization and ciliary function**

Seeking insight into possible functions of the Chibby protein family, we searched ScanProsite with sub motifs from the Chibby domains reasoning that searches of increasingly smaller sub motifs might retrieve related proteins. A Domain 2 sub domain search retrieved just 14 different mammalian proteins (Figure 3). Three of the fourteen, Ninein (Delgehyr et al., 2005), Ninein like protein (Nlp) (Rapley et al., 2005) and Centlein (Makino et al., 2008), are mother centriole proteins. Well characterized Ninein and Nlp are subdistal appendage proteins (Delgehyr et al., 2005; Rapley et al., 2005). Next, we searched The Mouse Tracheal Epithelial Cell (MTEC) data set which is a transcriptional analysis of ciliating epithelia cells (Ramona Hoh, personal communication) as well as published ciliation data sets at the so called, ‘The Ciliome’ (Inglis et al., 2006) (http://www.sfu.ca/~leroux/ciliome_database.htm). Seven of the fourteen retrievals are published at the mother centriole and/or found to be implicated in ciliation in the MTEC data set and/or The Ciliome (Figure 3).

To further investigate the potential of a role for Chibby proteins in ciliation we asked if there are correlations between harboring a Cby1 gene, making a cilium and possessing Wnt/Beta-Catenin signaling. We performed reciprocal BLASTP analysis of multicellular and unicellular eukaryotes. For visualization purposes, we
generated a simple taxonomic tree containing ciliated representatives of the Animalia, Fungi and Plantae kingdoms (adapted from (Hedges, 2002)) (Figure 4). We found that in multicellular organisms, with the exception of *C. elegans*, that cells that make cilia have the Chibby family of proteins (mammals (Figure. 7)) or Cby1. However, Cby1 gene presence also strongly correlated with the property of ciliation in unicellular organisms, which do not, by nature, possess Wnt/Beta-Catenin signaling. However, the highly divergent Plantae representative, *Chlamydomonas* apparently lacks a Chibby gene. We conclude that our phylogenetic analysis suggests a role for Cby1 in ciliation that is independent of Wnt/Beta-Catenin signaling.
Figure 1. Alignment of the Chibby family human Swiss-Prot sequences (sp). Amino acids that are identical (black) or similar (grey) in at least two of the proteins. Red boxes contain Domain1 and Domain2. Note that Domain 1 is split between two lines.
Figure 2. Chibby Domain 1 motif. Top: Arrows indicate amino acids that are conserved in all three human Chibby family members and were included in the motif definition. X denotes a conserved amino acid that was not included because it is far from the others. Bottom: Domain 1 motif, which was found to be exclusive to the Chibby family. Letters indicate identical amino acids, x indicates interspersed non-identical amino acids and numbers indicate the number of xs between identical amino acids. Similar amino acids were not considered.
Figure 3. Chibby Domain 2 motif, shortened Domain 2 motif and other proteins published at the mother centriole and/or implicated in ciliogenesis. Top: Chibby Domain 2 motif which was found to be exclusive to the Chibby family when we searched ScanProsite at the ExPASy. Middle: We searched with increasingly shorter fragments of the Domain 2 motif until we retrieved just 14 different mammalian proteins, three of which are published at the mother centriole and the rest of which are implicated in ciliation. See Figure 2 for motif definition.
Figure 4. Ciliated unicellular Animalia and Fungi have Chibby but not Wnt/Beta-Catenin signaling. BLASTP searches were performed to determine the correlations between having a cilium, Chibby genes and Wnt/Beta-Catenin signaling. 1) *C. elegans* lack Chibby and have a basal body and cilia of unusual MT arrangement. 2 & 3) Unicellular organisms with both Chibby genes and typical cilia but not Wnt/Beta-Catenin signaling. 4) Chlamydomonas has cilia with typical MT structure but apparently lacks a Chibby gene.
of the organisms.

Amino acids that are identical (gray) or similar (black) in at least two
sequences. Arrows indicate amino acids that are the same in all three human ChlPAMP proteins and arrows indicate amino acids that are the same in two of the three human ChlPAMP proteins. A range of organisms was selected to highlight sequence identities, similarities, and differences.

Figure 5. CYP1 is highly conserved. A range of organisms was selected to highlight sequence identities, similarities, and differences.

Domain 2

Domain 1

Domain 2

Domain 1
same in two of the three human Chidsky proteins. Among residues that are identical (black) or similar (gray) in at least two of the organisms, arrows indicate amino acids that are the same in all three human Chidsky proteins and arrow heads indicate amino acids that are the

Figure 6. CPyR is highly conserved. A range of organisms was selected to highlight sequence identity, similarities, and differences.
same in two of the three human ChpY proteins. Amino acids that are identical (black) or similar (gray) in at least two of the organisms.

Figures 7 C/fy3 is highly conserved. A range of organisms was selected to highlight sequence identities, similarities, and differences.
Figure 8. Phylogenetic analysis of the Chibby protein family. Top: TreeFam generated tree. Nurit/Cby2 is Sper, which is short for spermatid associated. Bottom: Bootstrapped maximum parsimony tree generated from a compound alignment of Chibby protein orthologs and paralogs, which were loaded onto the human Chibby protein family alignment. Bar indicates distance.
**Cby1 localizes to the distal end of the mother centriole**

Although the mother centriole MTs are continuous with the axonemal MTs, antibodies against acetylated and polyglutamylated tubulin don’t stain the region where the mother centriole MTs terminate and the axoneme MTs begin (Fig 9).

To determine if Cby1 is a centrosomal protein we stained serum starved hTERT-RPE1 cells with anti-Cby1 and anti-polyglutamylated tubulin. Cby1 localized to the ciliary gap and was not observed at the daughter centriole or at the central or proximal regions of the mother centriole (Figure 9).

The distal appendage protein Cep164 localizes to the ciliary gap. Seeking confirmation that Cby1 localizes to the distal end of the mother centriole, a GFP-Cep164 fusion construct was transiently transfected into hTERT-RPE1 cells proceeding serum starvation and staining with anti-GFP and anti-acetylated tubulin. GFP-Cep164 localized to the ciliary gap (Figure 10). Cby1 and GFP-Cep164 colocalized in serum starved hTERT-RPE1 cells (Figure 11) and we conclude that Cby1 localizes to the distal end of the mother centriole.

**Cby1 appears at the mother centriole by prophase and persists there throughout mitosis**

Having established Cby1 localization at the distal end of the mother centriole, we sought insight into the centriolar function of Cby1 reasoning that if Cby1 localization is consistent with G2 recruitment, Cby1 likely functions as an appendage protein and that Cby1 presence or absence at the mother centriole during mitosis would be suggestive of Cby1 mother centriole function.
Asynchronous hTERT-RPE1 cells were stained with DAPI to visualize DNA, anti-Cby1 and the commonly used marker of both centrioles, anti-Centrin (Figure 10). Based on nuclear morphology, the presence of duplicated centrosomes and if they had separated, we categorized mitotic cells as Prophase, Metaphase, Anaphase or Telophase. Interphase cells were conservatively categorized as G1/S or S/G2 depending on the number of centrioles and/or procentrioles and the distance between centrosomes if they had separated. Cby1 persisted at the mother centriole throughout mitosis like Cep164 does and stains one centriole in G1/S and S/G2 cells but is seen at one of each centriole pair, or centrosome, at either side of prophase cell nuclei.
Figure 9. Primary cilia visualization and Cby1 localization in serum starved hTERT-RPE1 cells. hTERT-RPE1 cells stained with anti Cby1 and anti polyglutamylated tubulin. DNA was labeled with DAPI. The mother and daughter centriole and the axoneme can be distinguished from each other when staining with anti acetylated or anti polyglutamylated tubulin (shown here) in part because of a staining gap between the mother centriole and the axoneme. Cby1 localizes at the distal end of the mother centriole as indicated by staining of anti Cby1 in the staining gap. G0 was induced by serum starvation.
Figure 10. Cby1 and Cep164 colocalize. hTERT-RPE1 cells were transiently transfected with GFP-Cep164, serum starved and stained with anti acetylated tubulin and anti Cby1. DNA was labeled with DAPI. Top: GFP-Cep164 localizes at the ciliary gap. Bottom: Cby1 and GFP-Cep164 colocalize. G0 was induced by serum starvation.
number of centriole pairs and distance between centriole pairs.
and with centriole DNA was labeled with DAPI.
Figure 11. Cpy1 is present at the mother centriole throughout mitosis and the recruitment of Cpy1 to the new mother centriole is consistent with G2 phase recruitment. Asynchronous PtK2 cells were stained with anti-Cpy1.

Figure 11. Cpy1 is present at the mother centriole throughout mitosis and the recruitment of Cpy1 to the new mother
centriole is consistent with G2 phase recruitment. Asynchronous PtK2 cells were stained with anti-Cpy1.
Cby2 and Cby3 are centriolar proteins

To see if Cby2 is a centrosomal protein, hTERT-RPE1 cells were stained with anti-Cby2 and anti-acetylated-tubulin. Mother centriole appendage proteins are typically recruited in G2 so when viewing appendage proteins in G1/S cells, one usually sees staining at one centriole. We observed an unusual G1/S staining pattern. Some centrosomes harbored Cby2 at one centriole while others clearly showed Cby2 staining at both centrioles. Moreover, in many instances, Cby2 localized in either a letter U or donut shaped morphology at all cell cycle stages including mitosis (Figure 12).

Two anti-sera were raised against Cby3. Staining of hRTET-RPE1 cells didn’t reveal centrosome staining or discernable staining of any other cellular compartments. We turned to WT mouse embryonic fibroblasts (MEFs) because we were studying Cby1 with both WT and Cby1−/− MEFs (discussed in Chapter 3). We observed a staining distribution that was similar to that of Cby2 in two ways and quite dissimilar in one way. Some centrosomes harbored Cby3 at one centriole while others clearly showed Cby3 staining at both, like for Cby2. Cby3 was seen at one centriole of each spindle pole during mitosis; however, we observed punctuate and not letter U or donut shaped localization, unlike for Cby2 (Figure 12,13). We did not observe a difference in this unusual staining distribution between the two sera. We conclude that Cby2 and Cby3 are both mother centriolar proteins.
Figure 12. Cby2 is a centriolar protein that persists at the centrosome during mitosis. Asynchronous hTERT-RPE1 cells were stained with anti Cby2 and anti acetylated tubulin to visualize centrioles. DAPI labeled DNA. Top two rows: Cells with two apparent centrioles were conservatively considered to be G1/S. Of 100 G1/S cells scored, Cby2 localized at one, one plus (not shown) or two centrioles (30|35|35). Bottom: A mitotic cell exhibiting centriolar Cby2.
Figure 13. Cby3 is a centriolar protein that persists at the centrosome during mitosis. Asynchronous WT MEF cells were stained with anti Cby2, anti acetylated tubulin to visualize centrioles. DAPI labeled DNA. Top: Cells with two apparent centrioles were conservatively considered to be G1/S. Of 100 G1/S cells scored, Cby3 localized at one, one plus (not shown) or two centrioles (42|23|35). Bottom: A mitotic cell exhibiting centriolar Cby3.
Cby2 and Cby3 both partially colocalize with Cby1 and neither requires Cby1 for mother centriolar localization

Given the apparent differences in the time of recruitment and staining morphology of the Chibby family members, we hypothesized that Cby1 would not perfectly co-localize with Cby2 or Cby3. However, a partial colocalization was observed in interphase and mitosis of Cby1 and Cby2 in hTERT-RPE1 cells (Figure 15) and Cby1 and Cby3 in WT MEF cells (Figure 16). We were curious to see if either Cby2 or Cby3 requires Cby1 for centriolar recruitment. Cby1+/− MEFS and WT MEFs as a control were stained with anti-Cby1 and either anti-Cby2 or anti-Cby3. Both the centriolar levels of Cby2 and Cby3 appeared equivalent in the presence or absence of Cby1 (data not shown). We conclude that Cby2 and Cby3 partially colocalize with Cby1 and that both reside at mother centrioles independently of Cby1.
Figure 14. Cby2 and Cby1 partially colocalize. Asynchronous hTERT-RPE1 cells were stained with anti Cby2, anti Cby1 and DAPI to label DNA. Both nuclear morphology and the number of and distance between centriole pairs determined cell cycle stage.
Figure 15. Cby3 and Cby1 partially colocalize. Asynchronous hTERT-RPE1 cells were stained with anti Cby3, anti Cby1 and DAPI to stain DNA. Both nuclear morphology and the number of and distance between centriole pairs determined cell cycle stage.
Chapter 3:

Chibby and Beta-Catenin in Ciliogenesis and at the midbody
Abstract

Cby1 is known to antagonize Wnt/Beta-Catenin signaling and function in the establishment of the motile cilia of the airway epithelium. Here, we found that Cby1^{-/-} MEFs ciliated at a drastically reduced frequency than WT controls. This defect was rescued by the exogenous expression of a Cby1-GFP fusion construct but not by the expression of unfused GFP.

In absence of Wnt stimulation cytoplasmic Beta-Catenin levels are kept low by the kinase activity of a protein complex called the ‘destruction complex’ which phosphorylates Beta-Catenin resulting in the so called ‘phospho-Beta-Catenin.’ Recently, phospho-Beta-Catenin has been found at both the mother centriole and at the midbody MTs, a structure that forms from the midzone MTs during cytokinesis.

We noticed that hTERT-RPE1 cells expressing GFP-Cby1 seemed to make longer cilia than their non-transfected neighbors on the same coverslips. We confirmed this by comparing the length of cilia of cells expressing GFP-Cby1 with their non-transfected neighbors on the same coverslips as well as with the cilia of cells expressing unfused GFP. Additionally, we performed the same protocol of comparisons with the exogenous expression of a GFP-Beta-Catenin fusion lacking the phosphorylation for destruction sites at the N-terminus that target it for degradation (GFP-Beta-Catenin*). We found that both GFP-Cby1 and GFP-Beta-Catenin* expressing cells make cilia about four times less often than cells expressing GFP only. Of those cells that ciliated, the cilia were significantly longer than those of controls.
We found that Cby1 partially colocalized with phospho-Beta-Catenin at the mother centriole. To our surprise, Cby1 localized to a recently discovered and poorly understood ring-like structure at the midbody, reminiscent of another mother centriole protein, Centriolin. Additionally, we found phospho-Beta-Catenin at the midbody MTs as expected but surprisingly, also colocalizing with Cby1 at a ring-like structure where they both might regulate cellular abscission which is the terminal cytokinetic step and a process where Centriolin plays a role.
Introduction

Signaling by the primary cilium is thought to be essential for development. Mice deleted IFT88/Polaris or the of the essential ciliary Kinesin II subunits Kif3A or Kif3B die early to mid gestation and these embryos exhibit left-right patterning defects that likely result from loss of node cilia (Nonaka et al., 1998; Marszalek et al., 1999; Murcia et al., 2000; Pazour et al., 2000).

Cilia, whether motile or non-motile are different in function yet very similar in structure. Ciliogenesis in both instances are thought to follow largely parallel pathways although the mode of the generation of centrioles is known to differ (Vladar and Stearns, 2007; Marshall, 2008).

Cby1 is a potent antagonist Wnt/Beta-Catenin signaling (Li et al., 2007; Voronina et al., 2009). A Cby1<sup>-/-</sup> mouse was generated. About seventy five percent of mice with homozygous deletion of Cby1 succumbed to complications of unknown origin by the second day after birth. Survivors exhibited defects of diminished fat storage and motile airway cilia generation (Voronina et al., 2009).

In absence Wnt ligand stimulation, cytoplasmic Beta-Catenin is phosphorylated by the ‘destruction complex’ at the N-terminus targeting it for degradation. However, phospho Beta-Catenin has recently been found to localize to midbody microtubules where its function is unknown and at the centrosome where it seemed to affect MT growth and/or anchoring (Huang et al., 2007) and more specifically at the mother centriole (Corbit et al., 2008).
The primary cilium is always templated by the mother centriole and genesis of the primary cilium occurs in G1/G0 phase (Fliegauf et al., 2007) and cilium formation can be induced by inducing G0 phase by serum starvation (Satir and Christensen, 2007).
Materials and Methods

Cell lines and culture

hTERT-RPE1 cells (Clontech) and WT and Cby1+− MEFs, which have been described previously (Li et al., 2007). All cells were grown by ATCC guidelines in 5% CO₂ at 37°C. All cells were cultured in DMEM + 10% fetal bovine serum except when serum starved when 0.5% was added + 1% Antibiotic-Antimycotic (Invitrogen). To induce ciliation, 80% confluent cells were arrested in G₀ with DMEM + 0.5% FBS for 20-48 hours.

Plasmids

The GFP tagged Cby1 constructs (GFP-Cby1 and Cby1-GFP) have been described previously (Li et al.) but briefly, they are the only human Cby1 isoform fused N- or C-terminally to GFP in the pC2+ vector. GFP-Beta-Catenin N-terminal delete (GFP-Beta-Catenin*) (gift from Angela Barth and James Nelson, Stanford University) has been described previously (Barth et al., 1999; Bahmanyar et al., 2008) but briefly, the N-terminal region of Beta-Catenin that is phosphorylated for degradation targeting is excised and the backbone is pEGFP-C1 (Clontech).

Transfection

Cells were transfected at ~80% confluency in six well plates (Falcon) for three hours with 2µg plasmid DNA using 10µL Lipofectamine 2000 (Invitrogen). The Lipofectamine 2000 and the DNA were incubated in 500µL Opti-MEM reduced serum medium (Invitrogen) for 30 minutes prior to transfection.
Antibodies

All antibodies were diluted in PBSBT (PBS + 3% BSA + 0.1% Triton X-100 + 0.02% Sodium Azide). Primary antibodies used were mouse anti-acetylated alpha-tubulin clone 6-11B-1 (1:5000, Sigma), mouse anti-Chibby (8-2) (1:200, Santa Cruz Biotechnology, Inc.), mouse anti-Centrin (clone 20H5; 1:2000; gift from J. Salisbury, Mayo Clinic, Rochester), rat anti-GFP (GF090R) (1:2000, Nacalai Tesque, Inc.), rabbit anti-phospho-Beta-Catenin (1:200, Cell Signaling Technology). Secondary antibodies used were Alexa 488 or 594-conjugated goat anti-mouse, anti-rabbit and anti-rat (1:200, Invitrogen).

Immunofluorescence

Cells were grown on acid washed poly-L-lysine coated #1.5 coverslips (Thermo Scientific) and fixed in -20°C methanol for 2 minutes. Following fixation cells were washed twice with PBS and blocked for 30 minutes at room temperature with PBST. Cells were incubated with the following primary antibodies at room temperature for two hours: anti-Cby1, anti-phospho-Beta-Catenin. For 30 minutes: anti-acetylated alpha-tubulin, and anti-GFP and anti-Centrin. Cells were incubated with secondary antibodies for 30 minutes and DAPI for ten seconds. Coverslips were mounted on Superfrost Plus slides (Fisher Scientific) using Antifade (Invitrogen) and sealed with clear nail polish (Sally Hansen). Images were processed using Adobe Photoshop. DNA was stained with DAPI (1µg/mL).
**Microscopy**

Immunofluorescence images were captured using an Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc.), with a Plan-NEOFLUAR 100X 1.3 NA objective (Zeiss), a Plan-APOCHROMAT 63X 1.4 NA objective (Zeiss), an Orca ER CCD camera (Hamamatsu) and Openlab 4.0.4 (Improvision). Images were processed using Photoshop 7.0 (Adobe).

**Quantification of primary cilium length**

Cilia were measured using ImageJ 1.42q segmented line tool. Student’s t-test and SEM analysis was done using Excel (Microsoft).

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Results

Cby1 deleted mouse embryonic fibroblasts exhibit a severe primary cilium formation defect

Organismal level defects in primary cilium formation are thought to cause a much broader range of disease conditions than are exhibited by the Cby1−/− mouse so we hypothesized that mouse embryonic fibroblast (MEF) cells liberated from Cby1−/− embryos would ciliate at levels comparable to WT MEFs.

WT and Cby1−/− MEFs were serum starved and stained with anti-Cby1 and anti-acetylated tubulin. Cby1 staining at centrioles was present in WT cells and absent from Cby1−/− cells (Figure 16). 36 % of WT MEFs ciliated whereas, surprisingly, 0.07% of Cby1−/− MEFs ciliated (1000 cells scored) (Figure 17). We reasoned that the Cby1−/− cells could fail to ciliate for two reasons: 1) Cby1 regulates embryonic fibroblast ciliation or 2) the Cby1−/− MEFs exhibit a secondary defect that affects ciliation. Cby1−/− MEFs transiently transfected with Cby1-GFP or GFP only as a control, were serum starved and stained with anti-GFP and anti-acetylated tubulin.

We noticed ciliated cells with GFP-Cby1 localizing to the ciliary gap (Figure 18). We asked if GFP-Cby1 was merely localizing to ciliated cells or facilitating ciliation. Although 3.2 % of Cby1−/− MEFs that were transfected with the GFP control construct visibly expressed GFP, only 0.02% of that total population ciliated. Similarly, 3.8% of the Cby1−/− MEFs that were transfected with the Cby1-GFP construct visibly expressed GFP. However, 3.7% of the total population ciliated and 2.4% exhibited Cby1-GFP localization at the mother
centriole yielding a conservative rescue frequency of 65% as evidenced by both
ciliation and Cby1-GFP localization at the mother centriole (Table 1). We conclude
that expression of Cby1-GFP but not un-fused GFP rescues ciliation in Cby1−/−
MEFs.
Figure 16. Anti Cby1 does not stain centrioles in Cby1<sup>+/−</sup> MEFs. Asynchronous MEFs were stained with anti Cby1, anti Centrin and DAPI to label DNA. A cell with extra centrioles was chosen for demonstration. Anti Cby1 stains mother centrioles of WT MEFs (not shown).
Figure 17. Cby1<sup>-/-</sup> MEFs exhibit a ciliation defect. WT and Cby1<sup>-/-</sup> MEFs were serum starved to induce ciliation and stained with anti acetylated tubulin to stain cilia. 1000 cells from each population were scored for the presence of cilia.
Figure 18. GFP-Cby1 localizes to the ciliary gap of Cby1$^{+/+}$ MEFs. Cby1$^{+/+}$ MEFs were transiently transfected with GFP-Cby1 and stained with anti GFP, anti acetylated tubulin and DAPI to label DNA.
Table 1: Exogenous Cby1 expression rescues the Cby1⁻/⁻ MEF ciliation defect.
Cby1⁻/⁻ MEFs were transiently transfected with Cby1-GFP or GFP. For both conditions, the percentage of transfected cells and cells that ciliated was calculated. The percentage of cells exhibiting Cby1-GFP at the basal body was also calculated. Unfused GFP was not discernable at basal bodies. 1000 cells were counted for each category.
Exogenous expression of Cby1 or Beta-Catenin alters primary cilium length and ciliation frequency in hTERT-RPE1 cells

hTERT-RPE1 cells exhibiting transfected GFP-Cby1 at the mother centriole, seemed to make longer cilia than their non-transfected neighbors (Figure 19). To confirm this, hTERT-RPE1 cells were transiently transfected with GFP-Cby1 or GFP only as a control and stained with anti-GFP and anti-acetylated tubulin. We developed a non-biased protocol to correlate ciliary length with expression. While viewing GFP or GFP-Cby1, we searched the coverslips from side to side in a manner that insured that the same cell would not be scored twice. When a candidate cell was identified, we switched filters to view acetylated tubulin. An image was taken if a cilium was present. To score non-transfected neighbor cells for cillum length comparison, we moved the objective randomly in the acetylated tubulin-viewing channel and then imaged the primary cilium closest to the center of view only if that cell was not transfected. Cells expressing GFP-Cby1 made significantly longer cilia than their non-transfected neighbors on the same coverslips (Figure 20); although, they made cilia approximately four times less frequently than cells expressing GFP only.

A phosphorylation sequence deleted Beta-Catenin construct was fused to GFP at the remaining N-terminus. GFP-Beta-Catenin* transfected cells made significantly longer cilia than their non-transfected neighbors. Intriguingly, GFP-Beta-Catenin* also made cilia approximately four times less frequently than GFP only expressing cells (Figure 20). We conclude that both Beta-Catenin and Cby1 regulate the formation and/or maintenance of primary cilia.
Figure 19. GFP-Cby1 expressing cells appeared to have longer cilia than their non-transfected neighbors. hTERT-RPE1 cells were transiently transfected with GFP-Cby1 and stained with anti GFP, anti acetylated tubulin and DAPI to stain DNA. Arrows point to the nuclei and the cilia of a transfected cell (top) and a non-transfected cell (bottom). The cilia length discrepancy shown here is an extreme example.
Figure 20. The primary cilia of hTERT-RPE1 cells expressing either GFP-Cby1 or GFP-Beta-Catenin* are longer than those of neighboring cells on the same coverslips and of cells expressing un-fused GFP. Cells expressing GFP-Cby1 or GFP-Beta-Catenin* ciliated about four times less frequently than cells expressing un-fused GFP. hTERT-RPE1 cells were transiently transfected with either GFP-Cby1 or GFP-Beta-Catenin-Delta-N (GFP-Beta-Catenin*), which lacks the N-terminal region that is phosphorylated for degradation. Cells were serum starved to induce ciliation and stained with anti GFP, anti acetylated tubulin and DAPI to label DNA. About 100 cilia over two experiments were measured for each condition with ImageJ. The ratios of ciliary lengths did not vary significantly between individual experiments. Statistical significance via the Student’s t-test. Error bars are Standard Error of the mean.
Cby1 and phospho-Beta-Catenin partially colocalize at the mother centriole

Cby1 and phospho-Beta-Catenin partially colocalized at the mother centriole in hTERT-RPE1 (Figure 21). In the majority of cells viewed, phospho-Beta-Catenin and Cby1 signals overlapped to some extent but not entirely. Cby1−/− MEFs and WT control MEFs were stained for Cby1 and phospho-Beta-Catenin. Phospho-Beta-Catenin levels at the mother centriole seemed equivalent in the presence or absence of Cby1 (data not shown). We were unable to achieve complete depletion with siRNA of Beta-Catenin at the centrosome as viewed by indirect IF and Cby1 levels there appeared normal (data not shown). We conclude that the localizations Cby1 and Beta-Catenin differ somewhat at the mother centriole and that Cby1 is not required for phospho-Beta-Catenin localization.

Cby1 and phospho-Beta-Catenin partially colocalize at the midbody

Midbody MTs are modified with acetylation (Steigemann and Gerlich, 2009). Cby1−/− MEFs and WT controls were stained with anti-Cby1 and anti-acetylated tubulin. Cby1 localized to a ring-like structure at the midbody of WT MEFs but not Cby1−/− MEFs (Figure 22), demonstrating that Cby1 is a midbody protein and suggesting that it localizes to the midbody ring, a poorly understood structure that recruits vesicles necessary for the final stages of cytokinesis, abscission (Steigemann and Gerlich, 2009).

To determine if Cby1 and Beta-Catenin colocalize at the midbody, we returned to hTERT-RPE1 cells as the midbodies of these cells easily visualized. As phospho-Beta-Catenin localizes to midbody MTs, we hypothesized that Cby1 and
phospho-Beta-Catenin would not colocalize. Surprisingly, we observed a dynamic localization of phospho-Beta-Catenin. In some fixed cells, phospho-Beta-Catenin localized exclusively to midbody MTs while in others we observed both midbody MT staining and ring-like staining that colocalized with Cby1 (Figure 23). We conclude that Cby1 is a midbody protein that colocalizes with phospho-Beta-Catenin in some late telophase cells.
Figure 21. Cby1 and phospho-Beta-Catenin partially colocalize at the mother centriole. hTERT-RPE1 cells were stained with anti Cby1, anti phospho-Beta-Catenin and DAPI to label DNA.
Figure 22. Cby1 localizes to a midbody ring-like structure. Both Cby1$^{-/-}$ MEFs and WT MEFs were stained with anti Cby1, anti acetylated tubulin to visualize midbody MTs and DAPI to label DNA. Top row: WT MEF. Bottom row: Cby1$^{-/-}$ MEF.
Figure 23. Cby1 and phospho-Beta-Catenin partially colocalize at a ring-like structure in cytokinetic cells. hTERT-RPE1 cells were stained with anti Cby1, anti phospho-Beta-Catenin and DAPI to label DNA. Cytokinetic cells were identified by chromatin morphology and the presence and location of phospho-Beta-Catenin decorated MTs. The vertical sequence of rows is not meant to indicate a hypothesis of the temporal progression of these proteins during cytokinesis.
Chapter 4:

Discussion
Chibby is a highly conserved protein that directly interacts with Beta-Catenin to antagonize Wnt/Beta-Catenin signaling (Takemaru et al., 2003; Li et al., 2007) and regulates motile cilium formation in the airway epithelium (Takemaru et al., 2003; Li et al., 2007; Voronina et al., 2009).

To gain further insights into Chibby functions, we looked for Chibby paralogs and found that Chibby is a member of a family of three proteins in mammals including Chibby (Cby1), Nurit/Cby2 and Cby3 that share unique sequence that suggested mother centriole localization. Indeed, all three proteins localized to the mother centriole although there were differences in staining morphology and apparent time of recruitment.

Cby1 partially co-localized with Cby2 and Cby3 at the mother centriole and was more precisely resolved at the distal end where it colocalized well with and shared similar cell cycle dynamics with the distal appendage protein, Cep164 (Graser et al., 2007).

Unexpectedly, the distribution of Cby2 and Cby3 suggested G1 recruitment. Appendage proteins are generally recruited to the new mother centriole in G2 (Nigg and Raff, 2009). It would be interesting to determine if the time of recruitment of Cby2 and Cby3 are important for centriolar function.

Cby2 exhibited centriolar letter U and donut morphology like staining which is reminiscent of the MT anchoring subdistal appendage protein Ninein (Ou et al., 2002). Ninein also happens to share the prospective mother centriole localization motif. It will be important to mutate the this motif in the Chibby family members to see if this sequence is indeed mother centriole targeting.
Odf2 localizes to both the subdistal and the distal appendages and loss of Odf2 results in the loss of these structures (Ishikawa et al., 2005). If Cby1 localizes to the distal appendages and Cby2 localizes to the subdistal appendages, these results would suggest that the appendages are of shared lineage.

Signaling by the primary cilium seems to be essential for mouse development. Mice deleted of IFT88/polaris or the of the ciliary Kinesin II subunits Kif3A or Kif3B die early to mid gestation (Nonaka et al., 1998; Marszalek et al., 1999; Murcia et al., 2000; Pazour et al., 2000; Ocbina et al., 2009). Clearly, the Kinesin II subunit Kif3A and the IFT component IFT88/polaris are required for the generation of the primary cilium. However, the functions of these proteins are not exclusively ciliary in nature. For example, myeloid and lymphoid cells of the immune system lack cilia yet have IFT88/polaris and other IFT proteins that are essential for proper functions (Finetti et al., 2009). Disruption of Kinesin II function results in aberrant mitosis (Haraguchi et al., 2006). It is conceivable that the loss of either of these proteins disrupts the same non-ciliary process resulting in phenotypes attributed to the loss of the primary cilium.

Of the Cby1−/− mice, ~25% survive past P2, those that survive do not exhibit the spectrum of developmental defects that would be expected from a loss of ciliation fidelity in multiple tissue types (Voronina et al., 2009). However, Cby1−/− MEFs ciliated at a drastically reduced frequency when compared with WT MEFs, suggesting that Cby1 functions in the generation of both motile and primary cilia, at least those of fibroblasts.
Cby1 was identified in fly, which has one Chibby family member (Cby1) (Takemaru et al., 2003) and flies without cilia are viable (Basto et al., 2006) suggesting that Cby1 primarily functions in Wnt/Beta-Catenin signaling in fly. However, Cby1 is highly conserved down to ciliated single cell organisms that lack Wnt/Beta-Catenin signaling components.

Surprisingly, Nurit/Cby2−/− mice seem normal and healthy (Benny Motro, personal communication), yet this protein is very highly conserved in mammals as are both Cby1 and Cby3. It is possible that the individual Chibby protein family members have redundant functions and can compensate for each other in Wnt/Beta-Catenin signaling and/or the formation of cilia in most tissue types and that the airway epithelium and fibroblasts are exceptions. This would explain the less severe than expected for a loss of all cilia spectrum of phenotypes observed in the Cby1−/− mouse. To explore this possibility it will be important to determine if Cby1 is required for ciliation fidelity in other tissue types. If so, future studies might discover that primary cilia are less important than the current belief.

Ciliation is initiated in the cytoplasm upon the docking of the distal end of the templating centriole with vesicles of unknown origin (Fliegauf et al., 2007). This is likely to occur via the distal appendages, which are thought to become the ‘transition zone’ that separates the compositionally distinct cilioplasm from the cytoplasm and the ciliary membrane from the plasma membrane (Hoyer-Fender, 2009). In addition, this region is the presumptive docking site of the intraflagellar transport (IFT) particles. Ciliary length control is poorly understood but there is evidence suggesting control by a balance of intraflagellar transport of cargo up and
down the ciliary axoneme (Marshall and Rosenbaum, 2001; Marshall et al., 2005; Engel et al., 2009) and/or a balance of the loading and unloading of IFT particles at the transition zone (Dentler, 2005).

We exogenously expressed either GFP-Cby1 or GFP-Beta-Catenin that has had the N-terminal sequence that is phosphorylated targeting it for destruction deleted (GFP-Beta-Catenin*), in hTERT-RPE1 cells. Quizzically, exogenous expression of either protein resulted in a reduced ciliation frequency when compared to an unfused GFP control (~ 4 X) and those cells that did ciliate, made significantly longer cilia than their non transfected neighbors on the same coverslips and cells expressing unfused GFP.

We propose that both Cby1 and Beta-Catenin participate in both the docking of the distal appendages with vesicles destined to be ciliary membrane and cargo at the transition zone.

We hypothesize that in cells over expressing either protein, vesicle docking at the distal appendages is disrupted by the presence of excess Cby1 or Beta-Catenin in the cytoplasm that competes for binding with said vesicles.

However, if docking and transition zone establishment is successful, extra copies of these proteins at the transition fibers significantly facilitates the binding of extra IFT complexes at the transition zone leading to extra ciliary constituent shipment up the axoneme; however, for this model to be correct, extra copies of said proteins in the cytoplasm don’t compete with the transition fibers for IFT complex binding. Such a role for Beta-Catenin is plausible as it has recently been found at the basal body and within the cilium (Corbit et al., 2008) where it might
function in IFT. Additionally, by TEM, the airway epithelia ciliation defect in Cby1−/− mouse appeared to result from a failure of templating centriole docking (Voronina et al., 2009).

Cellular abscission, the last stage of cytokinesis is essential for proper cell division yet poorly understood due its difficulty of study (Steigemann and Gerlich, 2009). We found that Cby1 localizes to a ring-like structure that surrounds the breakpoint of midbody MTs. Intriguingly, these MTs are apparently decorated with phospho-Beta-Catenin, which has only recently been considered as a functional molecule rather than exclusively, cytoplasmic Beta-Catenin that is targeted for destruction in absence of Wnt stimulation. Alternatively, phospho-Beta-Catenin at the mother centriole and at the midbody might differ chemically from that targeted for destruction, an intriguing question warranting further study. New is an apparent localization of both phospho-Beta-Catenin and Cby1 at a ring-like structure at the midbody. Ironically, another mother centriole protein, Centriolin is the canonical midbody ring-like protein and it recruits vesicles necessary for abscission via exocyst and SNARE complexes (Gromley et al., 2005; von Dassow and Bement, 2005). The idea that Cby1 recruits vesicles to the distal end of the mother centriole meshes well with a role for doing the same thing at the midbody and a similar role might be played by Beta-Catenin, a new and potentially revealing role for this heavily studied oncoprotein.
Appendix A:

Sperm Centrosome Proteomics
Acknowledgments

I was very fortunate to have worked with Sarah Elliot and benefited greatly from her biochemical approach.

Tim Stearns, Sarah Elliot and I designed the Bull sperm fractionation scheme. I performed the bull sperm fractionation scheme, the tables and analysis within and the anti C9orf9 staining of bull sperm. Sarah Elliot did all aspects of BC1 antibody preparation and characterization to the best of my knowledge.

We thank Jeffery Salisbury for the Centrin antibody.
Materials and Methods

Cell lines and culture

hTERT-RPE1 cells (Clontech) were grown by ATCC guidelines in 5% CO₂ at 37°C. All cells were cultured in DMEM + 10% fetal bovine serum except when serum starved when 0.5% was added + 1% Antibiotic-Antimycotic (Invitrogen). To induce ciliation, 80% confluent cells were arrested in G₀ with DMEM + 0.5% FBS for 24 hours. Bull semen (JLG Enterprises) was thawed and washed 3X with PBS.

Antibodies

All antibodies were diluted in PBSBT (PBS + 3% BSA + 0.1% Triton X-100 + 0.02% Sodium Azide). Primary antibodies used were mouse anti-acetylated alpha-tubulin clone 6-11B-1 (1:5000, Sigma), mouse anti-Centrin (clone 20H5; 1:2000; gift from J. Salisbury, Mayo Clinic, Rochester) and rabbit anti-BC1 was raised against the N-terminal half of human BC1 expressed and purified as a soluble GST fusion protein from E. coli using glutathione sepharose (GE Healthcare), according to the manufacturer's instructions. The protein was then used to immunize rabbits (Josman, LLC). Anti-BC1 antibodies were affinity purified from serum using MBP-N-BC1 bound Affi-Gel 15 resin (Bio-Rad) according to the manufacturer’s protocol. This rabbit anti-BC1 affinity purified antibody (BC1-Aa) was used at 1:100 for immunostaining.
Sperm fractionation

4 mL semen was thawed and rinsed with 3X PBS to remove the cryopreservation solution. Sperm were then resuspended in original semen volume of PBS and sonicated to break sperm apart into heads midpieces and tails. Sperm heads separated from midpieces and tails using a 30% glycerol cushion. After centrifugation at 1500 g for 2 min the heads pelleted and the tails and midpieces remained at the cushion interphase. Tails were then sequentially extracted with extraction buffer 1 (50 mM Tris pH 8, 2 mM EGTA, 2 mM EDTA, 500 mM NaCl, 1% NP-40, 0.1% β-mercaptoethanol, 1 mM DTT), extraction buffer 2 (50 mM Tris pH 8, 600 mM KSCN, 2 mM DTT) and extraction buffer 3 (50 mM Tris pH 8, 4 M urea). Each extraction was occurred at 4°C for 1 hour. Protease inhibitor cocktail tablets (Roche) were added to all solutions. The soluble components from each extraction were precipitated with TCA and fractions were resuspended in sample buffer and analysed by SDS-PAGE. Protein bands were identified following trypsinization using MS/MS Ion Search using ESI-TRAP. Mowse Score determined statistical significance. Trypsinization and mass spectrometry were performed by the Vincent Coates Foundation Mass Spectrometry Laboratory, Stanford University Mass Spectrometry (http://mass-spec.stanford.edu).

Immunofluorescence

Cells were grown on acid washed poly-L-lysine coated #1.5 coverslips (Thermo Scientific) and fixed in -20°C methanol for 2 minutes. Following fixation cells were washed twice with PBS and blocked for 30 minutes at room temperature with
PBST. Cells were incubated with the following primary antibodies at room temperature 30 minutes. Cells were incubated with secondary antibodies for 30 minutes and DAPI for ten seconds. Coverslips were mounted on Superfrost Plus slides (Fisher Scientific) using Antifade (Invitrogen) and sealed with clear nail polish (Sally Hansen). Images were processed using Adobe Photoshop. DNA was stained with DAPI (1µg/mL).

**Microscopy**

Immunofluorescence images were captured using an Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc.), with a Plan-NEOFLUAR 100X 1.3 NA objective (Zeiss), a Plan-APOCHROMAT 63X 1.4 NA objective (Zeiss), an Orca ER CCD camera (Hamamatsu) and Openlab 4.0.4 (Improvision). Images were processed using Photoshop 7.0 (Adobe).
Report

Centrosomes organize cilia the interphase MT array and reside at the poles of the bipolar spindle (Bettencourt-Dias and Glover, 2007). We sought to identify new centrosome components and turned to bull semen as a readily available and abundant source of centrosomes.

Mature mammalian sperm can be described in three sections: head, midpiece and the tail (Figure 24) (Inaba, 2003). Sperm contain very little cytoplasm and the nucleus fills most of the head. The sperm and ciliary axonemes are essentially the same MT based structure (Ibanez-Tallon et al., 2003). The sperm axoneme like that of the cilium is anchored by the templating centriole as part of the centrosome, which is adjacent to the head. The midpiece is the portion of the axoneme that is closest to the head and is surrounded by mitochondria. The tail is essentially motile in function and is basically axoneme surrounded by plasma membrane (Inaba, 2003).

We developed a bull sperm fractionation scheme (Figure 25, 26) based on the methods of prior studies where various sperm components were isolated (Vijayaraghavan et al., 1999; Ricci and Breed, 2001; Egydio de Carvalho et al., 2002).

We identified 16 genes with statistical significance (p<0.05) (Table 2) and asked which of these genes were implicated in centrosome, centriole and/or ciliary processes. The Mouse Tracheal Epithelial Cell (MTEC) data set is a transcriptional analysis of ciliating epithelia cells (Ramona Hoh, personal communication). The Ciliome (Inglis et al., 2006) (http://www.sfu.ca/~leroux/ciliome_database.htm) is a
compilation of published ciliation datasets. All, with the exception of Espin have been published at sperm, cilia or centrosomes or were identified in the MTEC or The Ciliome data sets. However, Espin is interesting because it interacts huntingtin interacting protein 1 (HIP1) (Rao et al., 2001) which was identified but below the statistical significance threshold (Table 3 and below).

There were many genes identified below the statistical significance threshold and we selected 16 to report here (Table 2) because they have been published at sperm, cilia or centrosomes or were identified in the MTEC or The Ciliome data sets. Ccdc150 is an exception that was selected because it is a coiled-coil protein which are highly enriched at centrosomes (Raff, 2002).

We chose two proteins, Ccdc146 (named BC1 here) and C9orf9 for further investigation. Ccdc146 was chosen because it has orthologs in Chlamydomonas (MBO2) and T.brucel (TbMBO2) with relevant functions. MBO2 localizes at centrioles and flagella and is required for flagellar function (Tam and Lefebvre, 2002). TbMBO2 is also required for flagellar function and is essential for cytokinesis as well (Broadhead et al., 2006).

C9orf9 was chosen because it localizes to fallopian tube motile cilia (www.proteinatlas.org) (Ponten et al., 2009) and because it was identified in the MTEC dataset. Additionally, we were interested in C9orf9 because it is adjacent to another protein identified in the MTEC dataset, C9orf98 and they are divergently transcribed in both mouse and human, a relationship deserving of further study.

To obtain more evidence that C9orf9 and BC1 are sperm centrosome proteins, we stained bull sperm with anti Centrin and with either anti BC1 or anti
C9orf9. Both proteins colocalize with Centrin indicating sperm centrosomal localization (Figure 27).

Seeking confirmation that BC1 is a centrosomal component we stained serum starved hTERT-RPE1 cells with anti acetylated tubulin and anti BC1 (Figure 28). BC1 colocalized with centrioles as indicated by anti acetylated tubulin staining so we conclude that BC1 is a centrosomal protein. It will be important to further characterize both BC1 and C9orf9.
Figure 24: Bull sperm components. Left: sperm can be divided into three sections: the head, which houses the DNA, the midpiece which contains part of the axoneme, the mitochondria and the centrosome. The tail is the remainder of the axoneme. Right: Bull sperm stained with anti Centrin to mark centrioles and DAPI to label DNA. Additionally, sperm were visualized with differential interference microscopy (DIC).
Figure 25: Bull sperm fractionation scheme. Left: sperm were broken into heads, midpieces and tails using sonication. Heads were then removed from midpieces and tails by glycerol cushion sedimentation. Mitochondria were then solubilized with detergent and reducing agents. The insoluble material was treated with KSCN and then with 4M urea. Right: legend and example of SDS-PAGE visualization of sperm fractionation. Red-boxed numbers on the left correspond to red-boxed numbers on the right.
Figure 26: Determination of centrosome constituent containing fraction and mass spectrometric analysis of that fraction. Right: tracking of a mitochondrial marker, MtHsp70 and the centrosome marker Centrin by Western blot analysis. The three bands correspond to different concentrations of DTT (1 mM, 10 mM and 100 mM), which did not affect the separation. Note: the centrosome marker Centrin was essentially all in fraction 2. Bottom: an SDS-PAGE separation of the fractions. Bands present in fraction 2 but not the other fractions were excised and analyzed by mass spectrometry.
Table 2. Statistically significant genes identified by mass spectrometry and other places where these genes have been identified. We named Ccdc146 Bull Centriole 1 (BC1) and confirmed both BC1 and C9orf9 at the sperm centrosome (below).

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We named Ccdc146 Bull Centriole 1 (BC1) and confirmed both BC1 and C9orf9 at the sperm centrosome (below).
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Table 3. Non-statistically significant genes identified by mass spectrometry and other places where these genes have been identified.
Figure 27. BC1 and C9orf9 localize to sperm centrosomes. Bull sperm were stained with anti Centrin and either anti BC1 (top row) or anti C9orf9 (bottom row). Chromatin was stained with DAPI.
Figure 28. BC1 localizes to the centrosome in hTERT-RPE1 cells. hTERT-RPE1 cells were stained with anti BC1 and anti acetylated tubulin.
References


Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting,


