Analysis of Two Centrin-Binding Proteins, Poc5 and Sfr1, in *Tetrahymena thermophila* Basal Bodies

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The final copy of this thesis has been examined by the signatories, and we
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ABSTRACT

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Analysis of Two Centrin-Binding Proteins, Poc5 and Sfr1, in *Tetrahymena thermophila*

Basal Bodies

Dissertation directed by Professor Mark Winey

Basal bodies are microtubule-based structures which template, anchor, and orient cilia at the cell surface. Although basal bodies contribute to vital cell functions, the molecular contributors of their assembly and maintenance are poorly understood. Previous studies in *Tetrahymena thermophila* revealed important roles for centrins in basal body assembly, separation of new basal bodies, and stability. Here, I characterized the basal body function of two centrin-binding proteins, Sfr1 and Poc5, in Tetrahymena. Sfr1 is the only centrin-binding protein in Tetrahymena that localizes to all cortical row and oral apparatus basal bodies. Poc5, on the other hand, transiently localizes to basal bodies with enrichment in assembling basal bodies and removal prior to the onset of cilia formation. On an ultrastructural level, Sfr1 resides at the microtubule scaffold from the proximal cartwheel to the distal transition zone. Tetrahymena Poc5 ultrastructural localization is not fully characterized, but localization was observed in the proximal and distal ends of the basal body microtubule scaffold. Complete genomic knockouts of *SFR1 (sfr1Δ)* and *POC5 (poc5Δ)* caused a significant increase in cortical row basal body density, contributing to an overall overproduction of basal bodies. Reintroduction of *SFR1* in *sfr1Δ* cells led to a marked reduction in basal body density and total cortical row basal bodies. Therefore, Sfr1 directly modulates...
cortical row basal body production. This study revealed an inhibitory role for Sfr1 and Poc5 (and potentially centrins) in Tetrahymena basal body production. Given the modulatory roles of Sfr1 and Poc5 in basal body production, a double knockout was generated that resulted in an exacerbated basal body overproduction phenotype in poc5Δ; sfr1Δ cells. This Tetrahymena work highlights a redundant role for Sfr1 and Poc5 in modulating the production of basal bodies along cortical rows. Through a collaborative effort, Xenopus Poc5 appears to have an important role in ciliogenesis since depletion of Poc5 resulted in ciliopathy-like defects in wholemount embryos and a reduction in the number of ciliated cells in the ciliated epithelium of Xenopus. Collectively, this work shed light on the function of Sfr1 and Poc5 in Tetrahymena basal bodies and Xenopus cilia formation, of which nothing was previously known.
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Chapter One: Introduction

I. Basal Bodies and Centrioles are Microtubule-Organizing Centers

Microtubule-organizing centers (MTOCs), such as centriole-comprised centrosomes and basal bodies, are widely conserved structures in eukaryotic cells that nucleate microtubules for cellular control of vital processes. Centriole-comprised centrosomes utilize microtubules to assemble and organize the mitotic spindle during cell division, while basal bodies act to anchor and template cilia formation at the cell surface. Centrosomes typically contain two orthogonally oriented centrioles of roughly 450 nm in length and 240 nm in diameter (Gönczy, 2015). Centrioles exhibit a nine-fold radially symmetric array of microtubules, ranging from singlets as in *C. elegans* to triplets, which is found in humans and many other eukaryotes (Li et al., 2004). The pair of centrioles are surrounded by a proteinaceous matrix known as the pericentriolar material (PCM) that acts primarily to concentrate tubulin and nucleate microtubules in order to build the bipolar mitotic spindle (Gönczy, 2015; Rieder et al., 2001).

Multiple evolutionary studies have traced the origins of basal bodies and centrioles, finding that centrioles are preserved in organisms that also utilize basal bodies and cilia (Carvalho-Santos et al., 2010; Hodges et al., 2010). Higher plants and budding yeast, both of which lack cilia, also lack centrioles/basal bodies, therefore the evolutionary selective pressure seems to be on maintaining basal bodies and cilia. Supporting these evolutionary studies, basal bodies have been found to be absolutely required for the formation of cilia, while Drosophila and Xenopus studies have revealed that mitosis can be successfully performed in the absence of centrosomes albeit spindle
assembly is slowed and the length of mitosis is extended (Basto et al., 2006; Gogendeau and Basto, 2010; Heald et al., 1996).

II. Centrosome Duplication

Cells entering the cell cycle have one centrosome consisting of two centrioles, with centrosome duplication occurring only once per cell cycle beginning at the G1/S transition (Fig. 1-1) (Hinchcliffe and Sluder, 2001; Nigg and Raff, 2009; Nigg and Stearns, 2011). In S phase, daughter centrioles or procentrioles are assembled orthogonally and at the proximal end of existing, mother centrioles. These procentrioles then elongate during S and G2 phases to reach the approximate length of the mother centrioles, before finally segregating as mother-daughter pairs during mitosis to form the spindle poles. Precise duplication of centrosomes is critical, as emphasized by centriolar aberrations and overduplicated centrosomes commonly found in tumor cells (Godinho et al., 2014; Lingle et al., 2005; Nigg, 2002). These centrosomal abnormalities have been shown to lead to genomic instability as well as tumor aggressiveness, both of which are hallmarks of cancer.

III. Centriole to Basal Body Interconversion

Centrioles are interconverted to basal bodies in multiple cell types during quiescence (Carvalho-Santos et al., 2011; Hodges et al., 2010; Kobayashi and Dynlacht, 2011). This transition entails the centriole pair migrating to the cell surface where the mother centriole acts as a basal body, nucleating microtubules now for the purpose of templating and building a cilium (Fig. 1-1). Interestingly, this process is reversible: when cells reenter the cell cycle the cilium is disassembled and basal bodies migrate away from the cell surface to act as centrioles (Kobayashi and Dynlacht, 2011).
Although basal bodies and centrioles serve distinct functions, they are structurally similar (enabling their interconversion) and proteomic studies have revealed that they share many components (Andersen et al., 2003; Graser et al., 2007; Hodges et al., 2010).

Figure 1-1. The Centrosome Duplication Cycle. This schematic representation shows procentriole (in grey) formation beginning at S phase and orthogonally from existing mother centrioles (in dark green). Procentriole elongation and maturation occurs during G2 phase before mother-daughter pairs segregate at the onset of mitosis for bipolar spindle assembly. If cells enter a quiescent state (G0), the mother centriole now acts as a basal body docking to the cell surface and giving rise to a cilium. (Nigg and Raff, 2009).
Despite the importance of basal bodies and centrioles, the fundamental mechanisms for assembly and maintenance of these structures, especially on a molecular level, remain poorly understood. Nonetheless, the morphological structure of basal bodies is well studied and has been recently characterized across many eukaryotes using primarily transmission electron microscopy and electron tomography (Allen, 1969; Bayless et al., 2015; Jana et al., 2016; Marshall and Osborn, 2016; Meehl et al., 2016; Pearson and Winey, 2009; Zhang and Mitchell, 2015).

IV. Basal Body Structure and Function

Basal bodies, like centrioles, are cylindrical, microtubule-based structures that typically display a nine-fold radially symmetric array of triplet microtubules (see Fig. 1-2) (Allen, 1969; Pearson and Winey, 2009). Through electron microscopy and proteomic analysis, basal bodies have been further organized structurally into three distinct domains (Kilburn et al., 2007). The proximal region contains the cartwheel structure, encompassing nine radial spokes emanating from a central hub to the microtubule triplets. The cartwheel is postulated to provide the necessary template for assembling a basal body, as well as initiating the nine-fold symmetry (Strnad and Gönczy, 2008). Microtubules extend from the proximal to the distal end of the basal body, forming the microtubule scaffold. At the distal end of the basal body is the transition zone or the region of transition from the microtubule triplets of the basal body to the microtubule doublets of the ciliary axoneme (Kilburn et al., 2007; Pearson and Winey, 2009). Additionally, the central core region, or midpoint, lies between the proximal and distal regions of the basal body and contains the lumen. The luminal density within this region is distinct from the cytoplasm and its function is unknown.
New basal body assembly is tightly regulated for specific cellular requirements, with assembly either limited to one site on an existing structure or occurring through an amplification process of mass assembly (Gönczy, 2012). Assembly generally occurs from an extant structure in a 1:1 ratio (as shown with centrioles in Fig. 1-1) with the aid of molecular assembly factors (Azimzadeh and Marshall, 2010; Culver et al., 2009; Pearson and Winey, 2009; Ross et al., 2013). Additionally, basal bodies can be assembled de novo, as well as from a deuterosome, serving to mediate the amplification of these structures in specific cell types (Al Jord et al., 2014; Hagiwara et al., 2004; Klos Dehring et al., 2013). Upon assembly, basal bodies are stabilized by post-translational tubulin modifications as well as molecular stability factors (Bayless et al., 2012; Bobinnec et al., 1998; Gudi et al., 2011; Pearson et al., 2009a; Shang et al., 2002a; Vonderfecht et al., 2011). The specialized microtubules that comprise basal bodies lend a unique, hyper-stabilized property allowing for this structure to remain functional through multiple cell divisions, compared to the dynamic microtubules that perform other essential cellular functions (Bobinnec et al., 1998; Pearson et al., 2009). This stabilized property of basal bodies is important in ciliated cell types and organisms that rely on stable, functional basal bodies to assemble and maintain cilia.

V. Cilia Structure and Function

Cilia are highly conserved, microtubule-based structures that are built at the cell surface from microtubules extending from the basal body (Fig. 1-2). The cilium, at its
Figure 1-2. Ciliary Ultrastructure and Structural Comparison between Cilia Types.
Cilia are found on almost all cells in the human body. The top 3 panels (A-C) show scanning electron micrograph images of mouse nodal cilia, motile cilia within the mammalian trachea, and primary cilia in the renal tubule epithelia, respectively. Panel D shows schematic representations of cross sections of the two major types of cilia, motile and non-motile (primary), as well as nodal cilia. Nodal and non-motile/primary cilia have a 9+0 axonemal pattern. Motile cilia have in addition a central pair of microtubules (9+2 pattern). Schematic also shows the radial spokes, inner and outer dynein arms, as well as nexin that connects microtubule doublets. This representative cilium is nucleated from a basal body at the cell surface. (Praveen et al., 2015).
core, is microtubule-based and composed of nine outer doublet microtubules (forming the axoneme) surrounded by a ciliary membrane that is continuous with the plasma membrane. The nine outer doublet microtubules are an extension of the doublets found within the transition zone of the basal body. Most human cells, across almost all organ systems in the body, are ciliated (Praveen et al., 2015). These hair-like projections are utilized for a broad spectrum of biological functions, including generation of directional fluid flow, locomotion, sensory reception, and signal transduction (Marshall and Nonaka, 2006). Historically, cilia have been classified into two categories, motile and primary cilia, based on the observation that some cilia noticeably move (motile) while others are immotile (Ishikawa and Marshall, 2011).

Motile cilia have defined roles in clearing mucus from sinuses and the airway, generating cerebrospinal-fluid flow, sperm movement, organismal motility, and other important functions. Primary cilia, on the other hand, were long thought to be vestigial organelles given their lack of obvious movement until intraflagellar transport (IFT) was discovered in the 1990s in *Chlamydomonas reinhardtii* and a mouse mutant for a critical IFT component (IFT88) was linked to polycystic kidney disease (PKD) (Kozminski et al., 1993; Pazour et al., 2000). Primary cilia do not contain ribosomes therefore proteins required for cilium assembly and function are transported bi-directionally through IFT, along ciliary axonemal microtubules by anterograde kinesin motors and retrograde dynein motors (Hao and Scholey, 2009; Sánchez and Dynlacht, 2016). Since this initial discovery, primary cilia are now well-established organelles for facilitating signaling pathways, such as Wnt and Hedgehog signaling, as well as acting like a cellular antenna for receiving sensory information (chemo-, mechano-, and photosensory) from
the environment (Ishikawa and Marshall, 2011; Marshall and Nonaka, 2006). Adding to the complexity, recent studies have questioned the clear distinction between primary and motile cilia (aside from ultrastructural differences) now that sensory reception has been discovered in both primary and motile cilia (Bloodgood, 2010; Shah et al., 2009). Aside from primary and motile cilia, an additional category of cilia exists known as nodal cilia (Babu and Roy, 2013). Nodal cilia are a type of motile cilia that contribute to establishing left-right asymmetry in vertebrate embryos during gastrulation. Collectively, cilia are now recognized as important organelles during development and in adult physiology.

In addition to the distinct functions of primary and motile cilia, the two major classes of cilia are structurally unique (Fig. 1-2). The ciliary axoneme is composed of nine doublet microtubules in both primary and motile cilia, but the two classes differ in the presence of a central pair of singlet microtubules in motile cilia. This distinction gives rise to the typical “9+2” axonemal pattern seen on an ultrastructural level for motile cilia and the “9+0” axonemal pattern for primary cilia (Marshall and Nonaka, 2006; Satir and Christensen, 2007). In order to beat in a rhythmic fashion, motile cilia also have radial spokes emanating from the central pair to the outer doublets, along with inner and outer dynein arms (Fig. 1-2). The dynein arms generate a minus-end directed sliding motion by binding adjacent doublets and hydrolyzing ATP, ultimately leading to ciliary beating (Ishikawa, 2016; Satir, 1980). Cilia also coordinate properly oriented fluid flow through utilizing planar cell polarity and basal body accessory structures such as ciliary rootlets and kinetodesmal fibers (Marshall and Kintner, 2008).

VI. Ciliopathies
Given that cilia are present on almost every cell in the human body and contribute to a wide spectrum of biological functions, it should not be surprising that improper formation or function of cilia can lead to diseases with equally broad symptoms. Cilia have been implicated in human disease since 1976 when male subjects were found to have immotile sperm, improper mucus and sinus clearance, and *situs inversus* (organs were reversed or mirrored relative to their normal position), all of which were a result of ciliary dysfunction from missing dynein arms (as seen by electron microscopy) (Afzelius, 1976). This condition is known as Kartagener syndrome and is now a part of a recognized class of human diseases, called ciliopathies. Ciliopathies are complex, pleiotropic diseases with underlying genetic mutations (an excess of 50 causal loci are now known) in both basal body and ciliary components, giving rise to a diverse set of disease manifestations (Fig. 1-3) (Badano et al., 2006; Lee and Gleeson, 2011; Waters and Beales, 2011; Wheway et al., 2015). Ciliopathies are individually rare diseases (average incidence of 1:100,000) but collectively approach the incidence frequency of more common diseases such as Down syndrome (Davis and Katsanis, 2012; Hook, 1983). Although ciliopathies are phenotypically heterogeneous, common overlapping manifestations include cognitive impairment, skeletal abnormalities, organ malfunction, retinal degeneration, polydactyly, obesity, among other maladies (Fig. 1-3) (Waters and Beales, 2011).

Through proteomic and comparative genomic work, a better understanding of the protein composition of a cilium is now attained, revealing upwards of 1000 proteins predicted to be important for the formation and maintenance of cilia (www.ciliaproteome.org) (Gherman et al., 2006). Although this serves as an important
beginning, characterizing the function(s) of these identified components and gaining an understanding of the composition of the cilium in a more cell type-specific manner will be critical. In addition, whole genome sequencing of patients will be necessary given the complexity of these diseases, where mutations in the same gene can contribute to different diseases and mutations in different genes are associated with the same disease (Davis and Katsanis, 2012).

Figure 1-3. Ciliopathies are Complex, Pleiotropic Diseases. This collection of diseases affect almost every organ in the human body. Common clinical manifestations encompass cognitive impairment, retinal degeneration, polydactyly, skeletal abnormalities, etc. For each manifestation, the disease acronyms are listed (figure and full description of each disease in Lee and Gleeson, 2011).
Lastly, ciliary proteins often have distinct non-ciliary functions in specific tissues/cell types and it is currently not well understood how ciliary-independent functions influence disease pathology (Davis and Katsanis, 2012; Vertii et al., 2015).

VII. Diversity of Centrin Function in Basal Bodies and Centrioles

Centrins are small (~20 kDa) calcium-binding proteins that are widely conserved in eukaryotes and ubiquitously associate with MTOCs, including centrioles, basal bodies, and the yeast centrosome (spindle pole body; SPB) (Andersen et al., 2003; Hodges et al., 2010; Keller et al., 2005; Kilburn et al., 2007; Liu et al., 2007). Centrin, also known as caltractin, was first identified as a component of the calcium-sensitive striated flagellar roots of the green algae *Tetraselmis striata* and has since been characterized in many eukaryotes (Dantas et al., 2011; Keller et al., 2005; Salisbury et al., 1984; Stemm-Wolf et al., 2005; Vonderfecht et al., 2011, 2012; Yang et al., 2010). Humans have three centrins (Cetn1-3), two of which localize ubiquitously to centrioles (Cetn2 and Cetn3) while Cetn1 is expressed only in male germ cells (Errabolu et al., 1994; Middendorp et al., 2000). *Saccharomyces cerevisiae* (budding yeast) have one centrin, Cdc31, that localizes to the site of new SPB assembly, known as the half-bridge, where it was found to be required for SPB duplication (Baum et al., 1986; Spang et al., 1993). The yeast SPB is structurally divergent from basal bodies and centrioles but acts as a functional equivalent of a centrosome (Jaspersen and Winey, 2004; Kilmartin, 2014).

Previous Winey laboratory work in *Tetrahymena thermophila* identified four Tetrahymena centrins, two of which (Cen1 and Cen2) share homology with human centrins 2 and 3 (Stemm-Wolf et al., 2005). As shown with tagged alleles, only the
centrins sharing homology with human centrins, Cen1 and Cen2, localized to basal bodies (Stemm-Wolf et al., 2005). On an ultrastructural level, Cen1 localizes to the site of new basal body assembly, the midzone, and the transition zone, while Cen2 localizes to the site of new basal body assembly and the transition zone but is largely absent from the midzone (Kilburn et al., 2007; Stemm-Wolf et al., 2005; Vonderfecht et al., 2012). Complete genomic knockouts of CEN1 and CEN2 revealed a requirement for centrins in proper basal body assembly, particularly orienting newly assembled basal bodies, as well as maintenance (Stemm-Wolf et al., 2005; Vonderfecht et al., 2011, 2012). Interestingly, Tetrahymena Cen2 and human Cetn3 are functionally conserved with induction of human Cetn3 capable of rescuing basal body defects in Tetrahymena CEN2 null cells (Vonderfecht et al., 2012).

Centrin function in basal bodies seems to be conserved in vertebrates, with germline depletion of mouse CETN1 leading to defective basal body maturation (Avasthi et al., 2013). Additionally, centrins are required for the formation and maintenance of both primary and motile cilia in vertebrates, as exemplified in three previous studies. Loss of mouse CETN2 resulted in (motile) olfactory cilia loss and impaired ciliary trafficking (Ying et al., 2014). Secondly, zebrafish studies where centrin2 was depleted led to ciliopathy-like embryonic phenotypes and cyst formation (Delaval et al., 2011). Thirdly, chicken DT40 lymphocytes depleted of CETN2 were unable to make primary cilia (Prosser and Morrison, 2015). Interestingly, despite their inability to make cilia, their centrioles were intact. This result supports other studies of centrin function in centrioles and suggests that centrins are not required for centriole assembly (Kleylein-Sohn et al., 2007; Strnad et al., 2007). In fact, a deletion of all three
centrins in chicken DT40 cells did not affect cellular division or normal centrosome structure (Dantas et al., 2011). Although centrins are not required for centriole assembly, they are capable of modulating centriole production and influence the timing of centriolar component incorporation (Sawant et al., 2015; Yang et al., 2010). HeLa cell studies identified an overproduction of centrioles when Cetn2 was overexpressed (promotes centrosome duplication), whereas Cetn3 acts oppositely by inhibiting centrosome duplication (Sawant et al., 2015). Collectively, this work highlights the requirement for centrins in basal bodies across eukaryotes but sheds light on the uncertainty surrounding centrin function in centrioles.

As a brief aside, centrins have been implicated in other biological processes including DNA damage repair, nuclear export, and regulating fibroblast growth factor (FGF)-mediated signaling (Dantas et al., 2011; Nishi et al., 2005; Resendes et al., 2008; Shi et al., 2015). In fact, the aforementioned chicken DT40 study where cells depleted of all centrins still exhibited normal centrosomal structure, also found that these cells were highly sensitive to UV irradiation due to the lack of centrin functioning in nucleotide excision repair upon DNA damage (Dantas et al., 2011).

VIII. Centrin-Binding Proteins Contain Sfi1-Repeats

Although centrins have been extensively studied with respect to basal bodies and centrioles, the function of centrin-binding proteins is poorly understood. Given the multifaceted functions of centrin, it is thought that temporal and spatial constraints on centrin are provided by centrin-binding partners. Previous studies revealed hydrophobic interactions between *Saccharomyces cerevisiae* centrin (Cdc31) and an alpha-helical protein, Sfi1, at the yeast SPB, where both proteins are essential for SPB duplication
A hydrophobic pocket in centrin’s c-terminal domain binds an internal sequence repeat within Sfi1 with a 33 amino acid periodicity (yeast Sfi1 contains 21 repeats), now known as a Sfi1-repeat or centrin-binding repeat (PFAM entry: http://pfam.xfam.org/family/PF08457). A crystal structure has been solved for Sfi1 bound to Cdc31 through its Sfi1-repeats (Fig. 1-4) (Li et al., 2006). Each repeat has the capacity to bind one centrin molecule, and contains an embedded, conserved sequence motif, Ax7LLx3F/Lx2WK/R, that is required for this interaction (Kilmartin, 2003; Li et al., 2006).

Utilizing this sequence motif information obtained from yeast Sfi1, centrin-binding proteins have been uncovered in ciliates and vertebrates, highlighting a similarly high degree of conservation in eukaryotes between centrin and its binding partners (Azimzadeh et al., 2009; Gogendeau et al., 2007, 2008; Kilmartin, 2003; Stemm-Wolf et al., 2013). Notably, human Sfi1 and another centrin-binding protein, human Poc5 (hPoc5), both localize to centrioles (Azimzadeh et al., 2009; Kilmartin, 2003). In Paramecium tetraurelia, centrin-binding proteins have an important role in maintaining the structural integrity of the centrin-rich cytoskeleton, called the infraciliary lattice (Gogendeau et al., 2007, 2008). With centrin-binding proteins displaying a conserved localization to MTOCs across eukaryotes, it is important to gain a better understanding of how these proteins are functioning in regards to proper assembly and maintenance of these structures.
Figure 1-4. Yeast Sfi1 Binds Centrin through its Sfi1-Repeats. A crystal structure has been solved showing the Sfi1-centrin complex containing two Sfi1-repeats and a molecule of centrin bound at each repeat. The residues important for this interaction are indicated. The sequence logo below shows the amino acid variation at each given amino acid position across all 21 Sfi1-repeats within budding yeast Sfi1. Within this repeat is the conserved sequence motif, Ax7LLx3F/Lx2WK/R. (Li et al., 2006).
IX. Sfi1-Repeat Protein Family in *Tetrahymena Thermophila*

A large family of 13 Sfi1-repeat (SFR) proteins, named Sfr1-13, was discovered by mining the Tetrahymena genome for proteins containing the Sfi1-repeat conserved sequence motif (Fig. 1-5) (Stemm-Wolf et al., 2013). Interestingly, localization studies (by using tagged alleles) revealed that the majority of these proteins localize to basal bodies. Furthermore, image averaging of basal bodies (including the basal body periphery) from cells co-expressing tagged alleles of a particular SFR protein and a known basal body marker (Poc1), revealed that members of the SFR family reside in distinct domains that heavily overlap with centrin localization (Fig. 1-5) (Stemm-Wolf et al., 2005, 2013, Vonderfecht et al., 2011, 2012). Functional studies have previously only been conducted on a single member of this family, Sfr13, which was shown to bind centrin and have an important role in separating and stabilizing Tetrahymena basal bodies (Stemm-Wolf et al., 2013). Upon loss of Sfr13, basal bodies became unstable, resulting in a significant reduction in the total number of cortical row basal bodies. This dissertation describes the characterization of Sfr1 in Tetrahymena basal bodies, serving as only the second body of work on this family of proteins.

X. Poc5 Vertebrate Functions

Poc5, also known as C5orf37/FLJ35779, was initially identified as a centrin-interacting protein by using a two-hybrid approach (Azimzadeh et al., 2009). Given its homology with a Chlamydomonas protein found in centriole fractions called POC5 (proteome of centriole 5), the human protein was called hPoc5 and the Tetrahymena
Figure 1-5. Sfi1-Repeat (SFR) Family of Proteins in Tetrahymena. The SFR family contains 13 Sfi1-repeat proteins of varying length and number of Sfi1-repeats (represented by blue boxes). Members range from containing 3 Sfi1-repeats (Sfr1, the focus of this dissertation) to 44 Sfi1-repeats in Sfr13. Each Sfi1-repeat is 33 amino acids in length (stemming from initial yeast Sfi1 studies). The panel on the right is a schematic showing the relative basal body positions of Sfi1-repeat proteins (the listed number is reflective of the family member, e.g. Sfr1 is listed as “1”). This data was obtained from image averaging relative to the basal body marker, Poc1, which resides at the basal body scaffold. Only Sfr1 resides within the basal body scaffold, with the remaining members mostly overlapping in the vicinity of the Tetrahymena accessory structures, including the kinetodesmal fiber (KF), transverse microtubules (T), and post ciliary microtubules (PC). (Stemm-Wolf et al., 2013).
orthologue identified in this dissertation is called ttPoc5 (Keller et al., 2005). The Poc5 family is conserved across eukaryotes that have centrioles and/or basal bodies but is not found in organisms (including budding yeast, ciliates) that lack these structures (Fig. 1-6) (Azimzadeh et al., 2009). Poc5 family members have two or three centrin-binding repeats, all containing the Sfi1-repeat conserved sequence motif, Ax7LLx3F/Lx2WK/R (Kilmartin, 2003; Li et al., 2006). The centrin-binding repeats are primarily organized as an isolated repeat towards the N-terminus (CBR1) and a tandem repeat (CBR2, CBR3). Interestingly, CBR1 is not present in a subset of Poc5 family members (Trypanosoma, Giardia, Paramecium, etc.) and centrin-binding studies revealed that CBR1 does not bind centrin in vitro (Azimzadeh et al., 2009). On the other hand, hPoc5 binds both hCetn2 and hCetn3 directly through the tandem CBR2+3 repeat that is found in all Poc5 family members. The overall sequence conservation between Poc5 family members is moderate (mean 16% identity, 29% similarity). Despite this overall moderate sequence conservation, all Poc5 orthologues have a highly conserved (mean 57% identity, 81% similarity) 21 amino acid signature sequence motif (or Poc5 box) (Fig. 1-6). Due to the high sequence similarity between Poc5 orthologues within the Poc5 box, this motif (along with the presence of centrin-binding repeats) was utilized for mining annotated genomes to find Poc5 family members (the Poc5 box is not found outside of the Poc5 family), including ttPoc5 in this dissertation (Azimzadeh et al., 2009).

Previous work on Poc5 is limited and has been mostly focused on elucidating the functions of Poc5 in centrioles of cultured cells (Azimzadeh et al., 2009; Dantas et al., 2013). By using immunofluorescence microscopy, localization of hPoc5 (63 kDa; 575 amino acids) is restricted to centrioles (Azimzadeh et al., 2009). hPoc5 localizes to both
Figure 1-6. Poc5 is a Conserved Centrin-Binding Protein in Eukaryotes. The top left panel shows three Poc5 orthologues with characteristic centrin-binding repeats (pink), coiled-coil regions (green), and the highly conserved Poc5 box (indicated in orange). Below is a multiple sequence alignment across Poc5 orthologues with similarly colored sequence features from the top panel. Residues that are similar across orthologues at a given amino acid position are shaded in grey, while identical residues are shaded in black. The top right panel shows the wide conservation of Poc5 across eukaryotes. (Azimzadeh et al., 2009).
the mother and daughter centrioles throughout the cell cycle and is recruited to procentrioles during G2 phase. This recruitment is later in the cell cycle than centrin recruitment, which occurs during early S phase (Middendorp et al., 1997; Piel et al., 2000). hPoc5 localization to centrioles was confirmed using immunoelectron microscopy (immunoEM) and was further revealed to be enriched at the distal part of centrioles (Azimzadeh et al., 2009). HeLa cells depleted of hPoc5 by siRNA treatment showed an extended S phase followed by ensuing cell death. In addition, hPoc5-depleted cells have procentrioles that are roughly 40% as long as full-length centrioles, a defect that is distinct from cells arrested in S phase by drug-induction (Azimzadeh et al., 2009). Intriguingly, Poc5 is not found in *Drosophila melanogaster* or *Caenorhabditis elegans*, both of which assemble short centrioles (Callaini et al., 1997; Pelletier et al., 2006). These results show that centriole initiation can occur normally in the absence of hPoc5 but subsequent centriole elongation and maturation of the distal end of centrioles requires hPoc5.

Aside from the aforementioned primary study of Poc5 function in centrioles, the chicken Poc5 orthologue was found to also localize to centrioles in chicken DT40 cells (Dantas et al., 2013). In addition to centriolar localization, Poc5-containing “fibers” were visible in cells where Poc5 was overexpressed. These fibers recruit centrin, as centrin co-localizes throughout these linear assemblies. Also, DT40 cells devoid of all centrins never form these fibers, indicating a requirement for centrin. Lastly, these fibers when analyzed using transmission electron microscopy are distinct from cilia and overelongated centrioles, instead resembling linear protein aggregates (Dantas et al., 2013).
Although Poc5 has an important function in centrioles, there are no published studies highlighting the role of Poc5 in basal bodies and cilia formation. There are, however, intriguing studies that may implicate Poc5 in these structures (Patten et al., 2015; Young et al., 2015). Mutations in hPoc5 were identified in multiple patients affected with idiopathic scoliosis and amazingly, expression of these variants of hPoc5 in zebrafish resulted in spinal deformities reflective of scoliosis (Patten et al., 2015). Although the scoliosis-inducing mutations in Poc5 have not been studied in the context of ciliogenesis, an independent study linked idiopathic scoliosis (and spine development) with cilia motility genes important for generating motile cilia-driven cerebrospinal fluid flow (Grimes et al., 2016). Genome-wide association studies (GWAS) have also implicated Poc5 in obesity, which is a common clinical manifestation found in ciliopathies (Young et al., 2015). Lastly, the overall frequency and composition of mutated POC5 in the human population was estimated from a large human database, the Exome Aggregation Consortium (exac.broadinstitute.org), revealing 403 variants including 163 missense and 7 loss-of-function alleles. No individuals were found to be homozygous for predicted loss-of-function mutations but homozygous missense mutations were identified (http://exac.broadinstitute.org/gene/ENSG00000152359). The lack of homozygous loss-of-function mutations suggests that these alleles are either rare in the population (and thus were not picked up even in this larger scale study) or they are embryonic lethal.

**XI. Tetrahymena as a Model System for Studying Basal Bodies**

The free-swimming ciliate, *Tetrahymena thermophila*, is a powerful system for studying basal body dynamics because it has a multitude of basal bodies, organized in
cortical rows running along the anterior-posterior axis of the cell, as well as a basal body-comprised feeding structure known as the oral apparatus (Fig. 1-7) (Allen, 1969). Each cell typically contains 18-21 cortical rows of basal bodies and the oral apparatus comprises roughly 150 basal bodies segregated into four membranelles (hence Tetrahymena for Tetra- (four) and -hymena (membranes)). Importantly, Tetrahymena basal bodies share structural similarities and conserved molecular components with humans, including centrins as well as centrin-binding proteins like Poc5 (Allen, 1969; Bayless et al., 2015; Kilburn et al., 2007; Li et al., 2004).

Figure 1-7. *Tetrahymena* Possess a Highly Organized Cytoskeleton. *Tetrahymena* cells have a highly structured network of basal bodies with cortical rows of basal bodies running along the anterior-posterior axis of the cell (anterior is towards the oral apparatus) and a basal body-comprised feeding structure, known as the oral apparatus (as seen in the live image on the left). The image on the left also shows the oral primordium or developing oral apparatus, indicating that the cell is preparing to divide. The panel on the right shows an immunostained cell with basal bodies in red (centrin), cilia in green (tubulin), and DNA in blue. Most basal bodies give rise to a cilium that allows *Tetrahymena* to swim. (Live cell image on left taken by Westley Heydeck, image of immunostained cell from University of Georgia Biomedical Microscopy Core (Kandasamy)).
There is a rich body of historical literature that characterizes the remarkable ability of Tetrahymena cells to maintain basal body homeostasis, or a regulated near constant number of cortical row basal bodies (Frankel, 1980; Nanney, 1966, 1968). This process entails adjusting either basal body density or the number of cortical rows in order to keep a near constant total number of basal bodies. The mechanism underlying this process is unknown but a molecular contributor has been found and characterized in this dissertation.

During logarithmic growth, basal body assembly is not synchronous with low levels of assembly at any given time. This changes dramatically when cells prepare for cell division, where basal bodies duplicate to ensure that daughter cells receive a normal complement of basal bodies and cilia. Basal bodies along cortical rows do not all nucleate cilia (known historically as “naked” basal bodies), leaving approximately 30 regularly spaced cilia along a given row that are used for cellular locomotion. Given the extensive nature of the cytoskeleton, Tetrahymena has been a valuable system for studying basal bodies and has brought upon assays not well established in other systems that can importantly dissect molecular contributions to assembly or maintenance/stability (Culver et al., 2009; Ross et al., 2013; Stemm-Wolf et al., 2005). These assays were essential for the completion of my thesis research and contribute to a competitive advantage for using Tetrahymena to study basal body dynamics.

Tetrahymena have many experimental advantages over other regularly used model organisms. Tetrahymena cells are relatively large at a common width of 20 µm and length of 35 µm, making this system ideal for cytological analysis. Tetrahymena are bi-nucleated, with a germline micronucleus and a transcriptionally active somatic
macronucleus. The ability to utilize homologous recombination in this system has critically allowed for replacement of genes with selective markers, ultimately enabling the generation of complete genomic knockouts (Turkewitz et al., 2002). In addition to genetic tractability, the Tetrahymena genome is annotated, tagged alleles can be made, and an inducible promoter system has been extensively used (Eisen et al., 2006). Collectively, there are clear experimental advantages of Tetrahymena for studying basal bodies, but Tetrahymena has also been used for other important discoveries, including but not limited to the motor protein dynein, telomerase, and catalytic RNA (Asai and Wilkes, 2004).

XII. Scope of Thesis Research

My thesis research entailed characterizing the basal body function of two centrin-binding proteins, Sfr1 and Poc5, in *Tetrahymena thermophila*. My analysis of Sfr1 is only the second study of the large SFR family of centrin-binding proteins in Tetrahymena. I have found that Sfr1 localizes to all Tetrahymena basal bodies, including all of the basal bodies organized in cortical rows and the oral apparatus. Ultrastructurally, Sfr1 localizes broadly throughout the full extent of the basal body scaffold with significant enrichment at the basal body midpoint. Upon loss of Sfr1, Tetrahymena cells surprisingly overproduce basal bodies, as evident by a significant increase in both basal body density and the number of cortical rows (combining for an increase in total cortical row basal bodies). When Sfr1 is reincorporated in cells lacking Sfr1, this observed increase in basal body density is rescued to wild-type levels. This portion of my thesis work identified a novel role for centrin-binding proteins and Sfr1
stands as the first known molecular contributor to the well-established process of maintaining basal body constancy in Tetrahymena.

The second significant portion of my thesis research focused on identifying a role for Poc5 in basal bodies, of which nothing has been previously published. Tetrahymena Poc5 localizes in a unique pattern with enrichment at assembling basal bodies and removal in mature basal bodies prior to cilia formation. Co-localization of Poc5 with a known basal body marker (Poc1) places Poc5 at the basal body scaffold. Preliminary ultrastructural localization using immunoEM supports this basal body localization. Upon loss of Poc5, Tetrahymena cells overproduced basal bodies similarly to the increase observed in cells lacking Sfr1. Given the sequence homology between Sfr1 and Poc5 along with the similar modulatory role on basal body production, a double knockout was generated that elicits basal body defects ultimately leading to cell death. Collectively, my thesis research contributes to the paltry body of work on centrin-binding proteins and sheds light on novel basal body functions. This work can be extended to exploring the vertebrate function of Poc5. Using *Xenopus laevis* in collaboration with the Klymkowsky Lab (MCDB), we have begun to gather evidence suggesting that Poc5 could have an important role in ciliogenesis, serving as an exciting new direction for this work.
I. Introduction

Previous studies in the ciliate *Tetrahymena thermophila* revealed important roles for two centrin family members in basal body assembly, separation of new basal bodies, and stability (Stemm-Wolf et al., 2005; Vonderfecht et al., 2011, 2012). Despite the extensive study of centrins in Tetrahymena, little is known about how centrin-binding proteins contribute to the multiple roles of centrins in basal bodies. Through my thesis studies, I characterized the basal body function of a centrin-binding protein, Sfr1, in *Tetrahymena*.

A previous study identified a large family of 13 centrin-binding proteins (Sfr1-Sfr13) in the Tetrahymena genome, all of which contain a motif (Sfi1-repeat) originally identified in *Saccharomyces cerevisiae* Sfi1 that binds centrin (Kilmartin, 2003; Stemm-Wolf et al., 2013). This study revealed a role for Sfr13 in stabilizing and separating basal bodies, both of which are also important functions of centrins. In addition, the basal body localization pattern across the SFR family was determined by mCherry-tagged or GFP-tagged fusion proteins of each member, revealing basal body localization for 9 of the 13 family members. Through image-averaging of cortical row basal bodies, it was shown that these 9 SFR proteins localize asymmetrically around the basal body periphery except for Sfr1, which exhibits no asymmetry relative to the basal body and resides at the basal body proper (Fig. 1-5) (Stemm-Wolf et al., 2013).
The unique basal body location of Sfr1 relative to other SFR family members suggests non-redundant basal body function(s) and prompted further investigation of the role of Sfr1 in basal bodies.

Sfr1 contains three Sfi1-repeats organized as a tandem repeat and an isolated C-terminal repeat (Fig. 2-1A). Each Sfi1-repeat is 33 amino acids in length (based off of yeast Sfi1) and were identified by the presence of an embedded Sfi1-repeat consensus sequence motif (Ax7LLx3F/Lx2WK/R) (Kilmartin, 2003; Li et al., 2006). In this study, Sfr1 was found to localize to all Tetrahymena basal bodies and more specifically, primarily along the microtubule scaffold. Upon generation of a complete genomic SFR1 knockout, Tetrahymena cells overproduced basal bodies resulting in increased basal body density along cortical rows and an overall increase in total cortical row basal bodies.

II. Results

Sequence confirmation of *T. thermophila* Sfr1 and homology with *P. tetraurelia*

Sfr1

Microarray expression data available on the Tetrahymena Genome Expression Database (ciliate.org) indicates that Sfr1 is the most highly expressed SFR protein during logarithmic growth (Fig. 2-1B) (Miao et al., 2009). To confirm the cDNA sequence of Sfr1, RNA was isolated from wild-type (WT) Tetrahymena cells and RT-PCR revealed a cDNA sequence containing a previously unannotated 88bp intron in the
Figure 2-1. Sfr1 is a Highly Expressed Centrin-Binding Protein. (A) Sfr1 contains three Sfi1-repeats organized as a tandem repeat and an isolated C-terminal repeat. (B) Expression profile of Sfr1 from microarray data (Tetrahymena Genome Expression Database). The red and blue lines are two separate experiments. Cells were cultured in three different conditions (L = logarithmic growth, S = starvation, C = conjugation). Sfr1 is highly expressed in logarithmic growth (Miao et al., 2009).
N-terminus (N-terminal to the Sfi1-repeats). This intronic sequence was not annotated in the Tetrahymena Genome Database and as a result the confirmed cDNA sequence of Sfr1 was not used in the previous study of Tetrahymena SFR proteins (Stemm-Wolf et al., 2013). Importantly, the previous annotation of Sfr1 was still in-frame but exhibited a different 5' end. Sfr1 is 212 amino acids in length (27.8 kDa) and contains three Sfi1-repeats, organized as a tandem-repeat (similar to human Poc5; (Azimzadeh et al., 2009)) and as an isolated C-terminal repeat (Fig. 2-2A). Interestingly, the Basic Local Alignment Search Tool (BLAST) revealed that Sfr1 is restricted to only a subset of ciliates, Tetrahymena thermophila and Paramecium tetraurelia. The Paramecium Sfr1 homologue, derived from a reciprocal best BLAST search, shares Sfi1-repeat organization and amino acid sequence homology both within and outside of the Sfi1-repeats (overall identities 55/194 (28%), positives 108/194 (55%)) (Arnaiz and Sperling, 2011).

**Sfr1 localizes to all Tetrahymena basal bodies**

In order to observe the localization of Sfr1 using live cell analysis, the confirmed Sfr1 sequence was N-terminally tagged with GFP, cloned into the exogenous RPL29 locus through homologous recombination, and placed under the control of the cadmium-inducible *MTT1* promoter (Matsuda et al., 2010; Winey et al., 2012). *MTT1*pr-GFP-Sfr1 was then transformed into cells endogenously expressing a known basal body marker, Poc1-mCherry (Pearson et al., 2009a). Overexpressed Sfr1 co-localizes with Poc1 in all basal bodies along the highly organized cortical rows and in both the immature and mature basal body-comprised feeding structures of the cell, the oral apparatus (Fig. 2-2B). Of note, endogenous Poc1 exhibits an uneven expression pattern along cortical
Figure 2-2. Sfr1 localizes to all basal bodies in *T. thermophila*. (A) Sequence alignment of Sfr1 homologues in two ciliates, *Tetrahymena thermophila* (THERM_00463380) and *Paramecium tetraurelia* (GSPATP00030126001). Both homologues contain 3 Sfi1-repeats or centrin-binding repeats (CBRs) highlighted in colored, dashed boxes. CBR1 and CBR2 are oriented in tandem while CBR3 is isolated in the C-terminus. Identical residues are shaded in black and similar residues are shaded in gray. Residues marked with asterisks denote the conserved sequence motif found within CBRs, Ax7LLx3F/Lx2WK/R. Sequence comparison between both Sfr1 homologues: overall identities 55/194 (28%), positives 108/194 (55%). (B) Live cell imaging of N-terminally tagged MTT1pr-GFP-Sfr1 co-localized with C-terminally tagged Poc1-mCherry (44). MTT1pr-GFP-Sfr1 localizes to all cortical row basal bodies, the mature oral apparatus (arrow), and the developing oral apparatus (arrowhead). Scale bar: 10µm.
rows, reflecting a previously reported slow incorporation into newly assembled basal bodies and gradual accumulation in maturing basal bodies (Pearson et al., 2009b). Similar to the previous analysis of SFR proteins in Tetrahymena, Sfr1 was unable to be endogenously tagged, therefore all analysis was conducted using $MTT1_{pr}$-GFP-Sfr1 (Stemm-Wolf et al., 2013).

**Localization of Sfr1 truncations**

In order to identify what domain of Sfr1 drives basal body localization, truncated versions of Sfr1 were placed in the same $MTT1_{pr}$-GFP plasmid as the full-length protein (Fig. 2-3A). This experimental strategy utilized an N-terminal GFP tag for visualization in live cells and overexpression of truncated Sfr1 was under the control of the cadmium-inducible $MTT1$ promoter. For domain analysis, Sfr1 was broken into three distinct domains: the N-terminus that does not contain a Sfi1-repeat, the central region that only contains the tandem Sfi1-repeats, and the C-terminus that contains both the isolated Sfi1-repeat and non-repeat sequence (Fig. 2-3A). For completeness, the N-terminus and central 2-repeat domains (N+2-repeat) were combined, as well as the central 2-repeat and C-terminus regions (2-repeat+C).

The results of this domain analysis were not conclusive due to a complete lack of signal in cells transformed with the N+2-repeat and C-terminus fragments. These cells were successfully transformed (due to survival after drug selection) but may not be expressing these truncated versions of Sfr1, which will require immunoblot analysis to verify expression levels (should be applied for all truncations made). Despite these inconclusive results, the N-terminal Sfr1 region exhibited strong basal body localization, with clear localization to cortical row basal bodies (Fig. 2-3B). Interestingly, this
Figure 2-3. Localization of Sfr1 Truncations. (A) Schematic representation of domain analysis. Sfr1 was broken into five truncations: N-term, tandem Sfi1-repeats (2-repeat), C-term (including Sfi1-repeat), N-term+2-repeat, and 2-repeat+C-term. All 5 truncations were expressed using the MTT1 promoter and visualized by GFP. (B) N-term Sfr1 localizes to cortical row basal bodies. (C) The central region containing the tandem Sfi1-repeats does not localize to basal bodies. (D) The combined regions of the tandem repeats and the C-terminus localizes to basal bodies but expression is uneven. Scale bar = 10µm.
truncation does not contain centrin-binding repeats, suggesting that Sfr1 may be capable of localizing to basal bodies in a centrin-independent manner. The central 2-repeat region does not seem to localize to basal bodies, instead it is enriched in membranes in a similar manner to GFP alone (Fig. 2-3C). Surprisingly, the 2-repeat+C fragment localized unevenly to cortical row basal bodies and to both the mature and immature oral apparatus (Fig. 2-3D). This observed cortical row localization pattern differs from full-length Sfr1 and needs to be further explored. In conclusion, two truncations of Sfr1 (the N-terminus and 2-repeat+C) were capable of localizing to basal bodies but do not overlap in sequence. This suggests potential bimodal recruitment of Sfr1 to basal bodies (begging the question of the requirement for centrin in this process) but ultimately could not be confirmed, remaining inconclusive.

**Sfr1 localizes broadly throughout the basal body microtubule scaffold**

Precise basal body localization was determined by immunoelectron microscopy (immunoEM) using *MTT1pr*-GFP-Sfr1 cells and a gold-conjugated antibody against GFP. As seen in serial transverse sections from a cortical row basal body, Sfr1 localizes primarily to the microtubules extending from the proximal cartwheel structure to the distal transition zone of the basal body (red arrowheads; Fig. 2-4A).

Through comprehensive analysis of seven complete serials from three different *MTT1pr*-GFP-Sfr1 cells, the ultrastructural basal body localization was quantified within known basal body domains (Fig. 2-4B). Sfr1 is concentrated at the midpoint of the basal body (52.2%) and at the proximal cartwheel (33.3%), with a smaller population found at the distal transition zone (5%). Only 9.5% of Sfr1 was detected in the basal body periphery, often adjacent to basal body accessory structures that aid in properly
Figure 2-4. Sfr1 localizes broadly throughout the basal body microtubule scaffold. Immunoelectron microscopy of N-terminally tagged MTT1pr-GFP-Sfr1 cells. (A) Serial sections reveal a majority of Sfr1 (gold particles) within the midpoint region of the basal body. Sfr1 localizes primarily along the microtubule scaffold (arrowheads). Scale bar: 100nm. The schematized basal body indicates the proximal, midpoint, and distal regions used for quantification in (B). (B) Quantification of the ultrastructural basal body localization of Sfr1 is based on data from 7 complete serial sections from 3 different cells (201 total gold particles). Sfr1 localizes primarily to the proximal and midpoint regions (85.5% total), with a small population at the distal end (5%) and peripheral to the basal body along the accessory structures (9.5%).
orienting basal bodies along cortical rows (Pearson and Winey, 2009). The ultrastructural basal body localization of Sfr1 partially overlaps with that of centrin, which was previously found to be enriched in the microtubules at the basal body midpoint and at the distal transition zone, but also resides at the site of new basal body assembly where Sfr1 is notably absent (Kilburn et al., 2007; Stemm-Wolf et al., 2005). Enriched localization of Sfr1 primarily along the microtubule scaffold suggested a role for Sfr1 in basal body assembly and/or maintenance rather than modulating basal body orientation along cortical rows. It is thought that other SFR proteins that localize to accessory structures, particularly the kinetodesmal fiber, function to properly orient basal bodies (Bayless et al., 2015).

**Overexpressed Sfr1 leads to basal body defects**

Full-length Sfr1 under control of the *MTT1* promoter localizes to all Tetrahymena basal bodies, including cortical row basal bodies and the basal bodies that comprise the oral apparatus (Fig. 2-2B). Given that Sfr1 was being controlled by a cadmium-inducible promoter in localization experiments, it was possible to drive overexpression of *MTT1pr-GFP-Sfr1* to higher levels by increasing the cadmium concentration to 1 µg/ml CdCl$_2$ (versus 0.5 µg/ml CdCl$_2$ used in localization experiments). Importantly, doubling the cadmium concentration may not have resulted in a corresponding increase in the level of Sfr1 expression, therefore immunoblot analysis should be performed. By increasing the cadmium concentration, Sfr1 overexpression led to multiple observed basal body defects indicative of a breakdown in regulating basal bodies, including mis-oriented basal bodies along cortical rows (curvy cortical rows) and large gaps in cortical rows (Fig. 2-5). Large gaps in cortical rows are often indicative of either improper
Figure 2-5. Overexpression of Sfr1 leads to mis-regulated basal bodies. Top live-cell image shows localization of full-length GFP-Sfr1 using 0.05µg/ml CdCl₂ (used for all localization experiments). Bottom live-cell images show full-length GFP-Sfr1 using 1µg/ml CdCl₂. Overexpression of Sfr1 with increased cadmium led to mis-oriented basal bodies along cortical rows (left panel) and gaps in cortical rows (combined with clustered basal bodies; right panel). Scale bar = 10µm.
assembly of basal bodies or destabilized basal bodies, both of which can disrupt the spacing of basal bodies along cortical rows (Pearson et al., 2009b). Interestingly, when the spacing between basal bodies gets too large it can lead to orientation defects (due to missing interconnections between basal bodies), potentially linking the two observed overexpression defects (Vonderfecht et al., 2011). Although overexpressed Sfr1 led to basal body defects, this experiment was not pursued further since overexpression of a protein could lead to confounding results. In order to determine the basal body function of Sfr1, emphasis was placed instead on generating a complete genomic knockout.

**Generation of complete genomic knockout of SFR1**

To determine the function(s) of Sfr1, a complete genomic knockout strain was generated by replacing the SFR1 open reading frame (ORF) with a neo2-cassette conferring paromomycin resistance for selection. Knockout cells were confirmed by PCR using two oligonucleotide pairs: one pair (wild-type) was designed to amplify a sequence specific to the wild-type SFR1 ORF, whereas the second pair (neo cassette) was designed to amplify sequence specific to the neo2-cassette in order to detect the presence of the knockout construct (Fig. 2-6A). Isolated genomic DNA from sfr1Δ cells showed a robust PCR product from the neo cassette amplification but no evidence of wild-type SFR1. RNA was also isolated from WT and sfr1Δ cells in order to perform RT-PCR analysis to confirm that sfr1Δ cells completely lacked SFR1 (Fig. 2-6B). RT-PCR validated the complete genomic knockout strain, revealing expression of SFR1 only in WT cells and expression of NEO only in sfr1Δ cells.

Cells lacking SFR1 (sfr1Δ) are viable and persist indefinitely, therefore SFR1 is not an essential gene for viability in Tetrahymena. Time course analysis of growth rates
Figure 2-6. *sfr1Δ* cells are viable and do not exhibit growth defects. **Complete micronuclear knockout of SFR1.** (A) PCR confirming complete micronuclear knockout of SFR1. The schematic depicts the homologous recombination strategy employed for generation of *sfr1Δ* cells. The arrows indicate the primers used to amplify both wild-type (WT) and deletion-specific PCR products. Only WT cells contain the SFR1 ORF and only *sfr1Δ* cells contain the Neo2 cassette. (B) RT-PCR analysis confirmed knockout of SFR1. Expression of SFR1 was only seen in WT cells while only *sfr1Δ* cells expressed NEO. (C) Logarithmic growth curves for WT and *sfr1Δ* cells grown in SPP at either 30°C or 37°C for a duration of 8 hours. Cell density (cells/mL) measurements were taken at the initiation of the growth experiment, as well as 4 and 8 hours after initiation. *n* = 2 samples per analyzed strain and growth temperature. Error bars indicate standard deviation.
for \textit{sfr1}\textDelta{} and WT cells was conducted at both the optimal growth temperature for
Tetrahymena (30°C) and at a temperature typically used to identify temperature-
sensitive mutants (37°C) (Fig. 2-6C). WT and \textit{sfr1}\textDelta{} had similar growth rates at both
temperatures.

\textbf{Loss of Sfr1 leads to increased basal body production along cortical rows}

To investigate potential basal body functions of Sfr1, phenotypic analysis was
conducted on growing and starved cells (starved for 24 or 48 hours) at both 30°C and
37°C. WT and \textit{sfr1}\textDelta{} cells were examined for three major cellular parameters: cell
length (µm), basal body density along cortical rows (basal bodies/10µm), and the total
number of cortical rows (Fig. 2-7A). From these measurements, an estimate of the total
number of cortical row basal bodies was generated. These parameters are influenced
by the ability of Tetrahymena cells to properly assemble and maintain basal bodies,
both of which are critical processes for sustaining spatial organization of cortical rows.
Cells analyzed under growth conditions were assayed for the capacity to assemble
basal bodies, while starvation conditions prevent new basal body assembly and
exacerbate any existing underlying deficiencies in basal body maintenance/stability
(Pearson et al., 2009a).

In all experimental conditions, WT and \textit{sfr1}\textDelta{} cells properly orientated basal
bodies along cortical rows and maintained the correct gross morphology of their oral
apparatus (Fig. 2-7B). Given the fact that Sfr1 resides primarily along the basal body
scaffold (Fig. 2-4A), it was not unexpected that \textit{sfr1}\textDelta{} cells maintain appropriate basal
body orientation. As seen in Fig. 2-7B and quantified in Fig. 2-7C, differences in cell
length were only observed during growth at 37°C where \textit{sfr1}\textDelta{} cells exhibited a
Figure 2-7. Loss of Sfr1 leads to an overproduction of basal bodies along cortical rows. *sfr1Δ* cells maintain a highly organized cytoskeleton with proper orientation of cortical rows but overproduce basal bodies. (A) Representative *Tetrahymena* cell fixed and stained with Cen1 antibody to label basal bodies. Three cellular parameters were measured for phenotypic analysis: cell length (in µm), basal body density (number of basal bodies per 10µm), and the number of cortical rows. The total number of cortical row basal bodies is derived from: cell length X basal body density X number of cortical rows. (B) Centrin staining of WT and *sfr1Δ* cells in growth and starvation conditions. Phenotypic analysis was conducted at both 30°C and 37°C. Scale bar: 10µm. (C, D, E, F) Quantification from phenotypic analysis of effect on cellular parameters from loss of Sfr1. For quantification, grey bars show the results from WT cells and white bars were used for *sfr1Δ* cells. n = 15 per strain per condition. Error bars indicate standard error of mean. P-values were derived from a Student’s t-test calculation. * = p<0.05, ** = p<0.01, *** = p<0.001.
significant reduction in length compared to WT cells. This indicates that deleting SFR1 may lead to altered cell length at a higher growth temperature, but not as a result of aberrant basal body orientation (Fig. 2-7B).

Although loss of Sfr1 does not seem to have a large effect on cell length, sfr1Δ cells grown at 30°C (but not at 37°C) had a significantly greater basal body density along cortical rows (7.91 basal bodies/10µm) compared with WT cells (6.98 basal bodies/10µm) (Fig. 2-7B, 2-7D). Even though sfr1Δ cells assemble more basal bodies during growth, excess basal bodies are not maintained, as sfr1Δ cells have a basal body density and a total number of basal bodies similar to WT cells after starvation (Fig. 2-7F). In addition to increased basal body density, loss of Sfr1 leads to an increase in the number of cortical rows during both growth and starvation (Fig. 2-7E). This increase in the number of cortical rows likely accommodates the excess basal bodies produced during growth. When basal body numbers return to normal in starvation, it may take a longer time for the cell to reorganize its cortex such that the number of cortical rows reverts to WT levels (Nanney, 1971). Overproduction of basal bodies upon depletion of SFR1 is a surprising result because it suggests an antagonistic role for Sfr1 in the production of basal bodies.

**Increased basal body production is the direct consequence of loss of Sfr1**

In order to determine that the observed increase in cortical row basal body density was a direct result of the loss of Sfr1, a strain was generated that reintroduced SFR1 in sfr1Δ cells (sfr1Δ rescue). The sfr1Δ rescue strain was made by transforming a cadmium chloride (CdCl₂)-inducible rescuing construct, MTT1pr-GFP-Sfr1, into the SFR1 null strain. This approach resulted in proper localization of GFP-Sfr1 (compared
with Fig. 2-2B) to all cortical row and oral apparatus basal bodies without the presence of endogenous Sfr1 (Fig. 2-8A). To assess rescue, cell length, basal body density, and the number of cortical rows were analyzed at 30°C with CdCl$_2$ (+CdCl$_2$) and without CdCl$_2$ (-CdCl$_2$). An immunoblot verified that GFP-Sfr1 was expressed in the presence of CdCl$_2$ and at a higher level than without CdCl$_2$ (Fig. 2-8C). Importantly, GFP-Sfr1 expression was not observed in WT cells and was only seen in sfr1Δ rescue cells. Due to the leakiness of the MTT1 promoter, GFP-Sfr1 was expressed at a detectable level without adding CdCl$_2$ but at roughly half of the level (based on normalized mean fluorescence) than that observed with CdCl$_2$ (Fig. 2-8D). Similar to this analysis, leaky expression of the MTT1 promoter after depletion of CdCl$_2$ has been observed previously (Shang et al., 2002b).

As seen in Fig. 2-8B, sfr1Δ rescue cells maintained cortical basal body organization and proper orientation along basal body rows upon treatment with CdCl$_2$. When WT and sfr1Δ rescue cells were incubated with CdCl$_2$, cell length was also unaffected (Fig. 2-8E). This was not a surprising result since cell length did not differ between WT and sfr1Δ cells grown at 30°C (Fig. 2-7C). Unlike cell length, cortical row basal body density was significantly higher in sfr1Δ rescue cells incubated without CdCl$_2$, and this density decreased to WT levels when GFP-Sfr1 expression was induced with CdCl$_2$ (Fig. 2-8F). This increase in basal body density, despite leaky expression of GFP-Sfr1, suggests that the level of expression without CdCl$_2$ was below a potential threshold needed for driving changes in basal body production. Of note, the increased basal body density seen in sfr1Δ rescue cells without CdCl$_2$ was similar to that observed in sfr1Δ cells (Fig.
Figure 2-8. Overproduction of basal bodies is the direct consequence of loss of Sfr1. Rescue of increased basal body density and the total number of cortical row basal bodies observed in sfr1Δ cells through the reintroduction of MTT1pr-GFP-Sfr1 (sfr1Δ rescue strain). (A) Live cell analysis of sfr1Δ rescue cells shows proper localization of MTT1pr-GFP-Sfr1 in the absence of SFR1 (compared with localization in WT cells containing SFR1 in Fig. 1B). (B) Centrin staining of WT and sfr1Δ rescue cells after overnight induction of GFP-Sfr1 (+CdCl2; 0.05 µg/mL CdCl2) or in the absence of CdCl2. Scale bar: 10µm. (C) Western blot analysis of both WT and sfr1Δ rescue cells in -CdCl2 and +CdCl2. Antibodies against GFP (for GFP-Sfr1) and Alpha-tubulin (as loading control) were used. The left panel shows levels of GFP-Sfr1 expression, the middle panel shows alpha-tubulin expression, and the right panel is a merged image of both channels. (D) Normalized mean fluorescence was calculated for both WT and sfr1Δ rescue strains in the presence and absence of CdCl2. (E,F,G,H) Quantification of the rescuing effect of induced GFP-Sfr1 expression in cells lacking Sfr1. For quantification, gray bars show the results from WT cells and white bars were used for sfr1Δ rescue cells. n = 15 per strain per condition. Error bars indicate standard error of mean. P-values were derived from a Student’s t-test calculation: * = p<0.05, ** = p<0.01, *** = p<0.001.
Since the presence of CdCl$_2$ did not have any effect on basal body density in WT cells, Sfr1 modulates basal body production along cortical rows.

Interestingly, although the $sfr1\Delta$ rescue strain when uninduced had increased basal body density like the $sfr1\Delta$ strain, it did not have additional cortical rows (Fig. 2-8G). This could be due to leaky expression from the rescuing construct, thus limiting the amount of overduplication normally observed in the null strain, and circumventing the cell’s need to add cortical rows to accommodate extra basal bodies. Lastly, $sfr1\Delta$ rescue cells grown without CdCl$_2$ had a significantly higher total number of cortical row basal bodies, as was seen in $sfr1\Delta$ cells (Fig. 2-7F, 2-8H). When CdCl$_2$ was added, the total number of cortical row basal bodies decreased to WT levels, therefore Sfr1 also regulates the total number of cortical row basal bodies (Fig. 2-8H). This increase in the total number of cortical row basal bodies was a direct result of increased basal body density since the number of cortical rows did not differ between WT and $sfr1\Delta$ rescue (Fig. 2-8G). Collectively, the results of this rescue experiment demonstrate that Sfr1 is directly responsible for limiting the number of basal bodies in Tetrahymena.

III. Discussion

Tetrahymena have a highly organized cytoskeleton that requires proper assembly, orientation, and maintenance of basal bodies along cortical rows, all of which are critical processes that rely on centrin (Kilburn et al., 2007; Stemm-Wolf et al., 2005; Vonderfecht et al., 2012). It is largely unclear how centrin performs multiple, essential basal body functions that require precise timing and distinct centrin populations at basal body subdomains, but it is thought to be through interacting partners (Kilburn et al., 2007; Stemm-Wolf et al., 2005; Vonderfecht et al., 2012). Intriguingly, Tetrahymena
have a large family of centrin-binding proteins (Sfr1-13) that may individually be responsible for subsets of overall centrin function and collectively provide both the necessary spatial and temporal regulation of centrin at basal bodies (Stemm-Wolf et al., 2013). This potential spatial regulation of centrin is reflective of the previous finding that SFR family members are each restricted to distinct regions of the basal body periphery (except for Sfr1 at the basal body proper), coinciding with regions known to also contain centrin (Stemm-Wolf et al., 2013).

Sfr1 is the only SFR family member that localizes to the basal body microtubule scaffold, indicating a potentially non-redundant basal body function for Sfr1. Basal body localization of Sfr1 was further dissected at the ultrastructural level by utilizing immunoelectron microscopy, which revealed a high percentage of Sfr1 broadly residing along the basal body microtubule scaffold from the proximal cartwheel to the distal transition zone. The ultrastructural localization of Sfr1 overlaps with Tetrahymena Cen1 and Cen2 at the transition zone and both Sfr1 and Cen1 are heavily enriched at the basal body midpoint (Kilburn et al., 2007; Stemm-Wolf et al., 2005; Vonderfecht et al., 2012). Aside from localization along the microtubule scaffold, Cen1 and Cen2 predominantly localize to the site of new basal body assembly, where Sfr1 is largely absent. Interestingly, previous ultrastructural analysis of Sfr13 differs from Sfr1 in that it is highly enriched at the site of new basal body assembly, supporting the hypothesis that centrin-binding proteins may be guiding the spatial positioning of centrin (Stemm-Wolf et al., 2013).

Tetrahymena cells lacking SFR1 (sfr1Δ) are viable and display a marked overproduction of basal bodies along cortical rows. The observed overproduction of
basal bodies upon the loss of Sfr1 was rescued by reintroducing Sfr1 under the control of an inducible promoter, therefore revealing a direct role for Sfr1 in modulating basal body production along cortical rows. Tetrahymena cells are strikingly capable of maintaining a near constant number of cortical row basal bodies by adjusting basal body density relative to the number of cortical rows (Frankel, 1980, 2008, Nanney, 1966, 1968, 1971; Nelsen and Frankel, 1979). Basal body number constancy has been shown to be regulated based on cellular corticotype (the number of cortical rows). The total basal body number in elevated corticotypes is balanced by a decreased basal body density and conversely, lower corticotypes exhibit increased basal body density. Furthermore, maintenance of corticotype was initially documented to be around a “stability center” at 19 cortical rows but was later refined to be around a “stability range” of 18-20 rows (Frankel, 1980; Nanney, 1966). It is unknown what mechanisms or signaling cues underlie the ability of Tetrahymena to regulate overall basal body number and how they direct the distribution of basal bodies to compensate for aberrant basal body density or an abnormal number of cortical rows. In the absence of Sfr1, sfr1Δ cells unexpectedly lost this compensatory mechanism for maintaining constancy in cortical row basal body number during logarithmic growth and instead exhibit both increased basal body density and an increased number of cortical rows relative to WT cells (and therefore a significant increase in total basal bodies). Although sfr1Δ cells overproduce basal bodies during growth, the condition of having excess basal bodies does not appear to be stable as starved sfr1Δ cells have a similar total number of cortical row basal bodies as WT cells. It is not yet understood if this function for Sfr1 is unique or if other SFR family members have an inhibitory effect on basal body number.
Similar to the basal body number constancy observed in Tetrahymena, centriole number was also found to be intrinsically constant in some cancer cell lines (Wong et al., 2015). Cancer cell lines with supernumerary centrioles treated with a specific inhibitor of the centriole assembly regulator, Polo-like kinase 4 (Plk4), showed a reduction in centriole number due to cell division in the absence of centriole assembly. Upon removal of the inhibitor, centriole number reverted back to the previous supernumerary state. This suggests the existence of a mechanism in cells that maintains a set number of basal bodies and centrioles - a mechanism that may be conserved across eukaryotes. More research is needed to address the molecular underpinnings of this process.

Centrins and their interacting proteins are good candidates for participants in such a process. The role for Sfr1 in modulating basal body production does not correspond with reported functions of centrins in Tetrahymena. This could be because Sfr1 acts independently of centrin or because this role for centrin may have been undetectable in previous studies of centrin-null cells that display the full effects of centrin loss (Stemm-Wolf et al., 2005; Vonderfecht et al., 2011, 2012). Intriguingly, it has recently been shown that human Cetn3 can antagonize human Cetn2 function in centriole assembly, highlighting a role for Cetn3 in inhibiting centrosome duplication (Sawant et al., 2015). Depletion of Cetn3 in HeLa cells resulted in the overproduction of centrioles, providing an exciting potential parallel to our observed overproduction of basal bodies upon loss of the centrin-binding protein Sfr1. Further analysis of centrin-binding proteins is needed to understand their contribution to known centrin functions and how binding specificity to the two functionally distinct centrin families is attained.
Given evidence that centrins can act antagonistically in regards to centriole assembly, centrin-binding proteins may act to bi-directionally modulate basal body production and therefore basal body number through interaction with a specific centrin.
Chapter Three: Characterizing the Role of Poc5 in Tetrahymena Basal Bodies

I. Introduction

Previous studies in the ciliate *Tetrahymena thermophila* revealed important roles for two centrin family members (ttCen1 and ttCen2) in basal body assembly, separation of new basal bodies, and stability (Stemm-Wolf et al., 2005; Vonderfecht et al., 2011, 2012). Despite the extensive analyses of centrin basal body functions in Tetrahymena, little is known about how centrin-binding proteins contribute to the multiple roles of centrins in basal bodies. In the previous chapter I highlighted the function of the centrin-binding protein Sfr1 in modulating basal body production.

Sfr1 is a member of the SFR family of centrin-binding proteins (Sfr1-13) that was previously identified in the Tetrahymena genome (see Fig. 1-5; (Stemm-Wolf et al., 2013)). Two members of the SFR family have been studied, with Sfr1 functioning to modulate basal body production (characterized in this thesis) and Sfr13 functioning to stabilize and separate basal bodies (Stemm-Wolf et al., 2013). Sfr1 localizes to all Tetrahymena basal bodies (primarily along the microtubule scaffold) and has a role in inhibiting basal body production. It is not yet understood if the function of inhibiting basal body number for Sfr1 is unique within the SFR family. Intriguingly, although this role for Sfr1 does not correspond with reported functions of centrins in Tetrahymena, it does provide an exciting potential parallel to a known inhibitory function of human Centrin3 (Cetn3) in centriole production (Sawant et al., 2015). Collectively, the work done on SFR family members is sparse but prompted the desire to further explore the functions of other centrin-binding proteins in Tetrahymena.
All SFR family members were identified by searching the Tetrahymena genome for a motif (Ax7LLx3F/Lx2WK/R) originally found in *Saccharomyces cerevisiae* Sfi1 to bind centrin (Kilmartin, 2003; Stemm-Wolf et al., 2013). This consensus motif is embedded in Sfi1-repeats/centrin-binding repeats, which are 33 amino acids in length (based off of yeast Sfi1) and have been shown to bind centrin in a 1:1 manner (one repeat binding one molecule of centrin) (Kilmartin, 2003; Li et al., 2006). Although all SFR family members have Sfi1-repeats, the number and spatial organization of repeats varies significantly (Stemm-Wolf et al., 2013). Sfr1 contains 3 Sfi1-repeats organized as a tandem repeat and an isolated C-terminal repeat, which is in stark contrast to the 44 Sfi1-repeats extending along Sfr13.

Outside of the SFR family in Tetrahymena, centrin-binding proteins have been uncovered in ciliates and vertebrates by utilizing the consensus sequence motif (Ax7LLx3F/Lx2WK/R) for mining genomes. Through a previous analysis, humans were found to have five centrin-binding proteins, of which two (Poc5 and Sfi1) have been studied in some capacity (Azimzadeh et al., 2009; Kilmartin, 2003). Of note, these two human centrin-binding proteins both localize to centrioles, highlighting a conserved localization of centrin and its binding partners to microtubule-organizing centers (Azimzadeh et al., 2009; Kilmartin, 2003; Stemm-Wolf et al., 2013). Furthermore, human Poc5 (hPoc5) is required for centriole elongation and maturation, making hPoc5 the only human centrin-binding protein with a published function (Azimzadeh et al., 2009). In the previous functional analysis of hPoc5, Poc5 orthologues were also identified across eukaryotes that have centrioles and/or basal bodies but were notably absent from budding yeast that lack these structures (yeast have structurally distinct
centrosomes known as spindle pole bodies). This suggests that the driving force behind Poc5 conservation in eukaryotes is potentially due to an important role(s) in centrioles, basal bodies, and/or cilia.

Although Poc5 has an important known function in centrioles, there are no published studies highlighting the role of Poc5 in basal bodies or cilia formation. A significant portion of my thesis studies was dedicated to characterizing the basal body function of Tetrahymena Poc5. Tetrahymena Poc5 localizes in a unique pattern (compared with other known Tetrahymena basal body components) with enrichment at assembling basal bodies and removal in mature basal bodies prior to cilia formation. Co-localization of Poc5 with a known basal body marker (Poc1) places Poc5 at the basal body scaffold. Preliminary ultrastructural localization using immunoelectron microscopy (immunoEM) supports localization to the basal body microtubule scaffold. Upon loss of Poc5, Tetrahymena cells were viable and overproduced basal bodies, unexpectedly revealing similar roles for Poc5 and Sfr1 in modulating basal body production. Given the sequence homology between Sfr1 and Poc5 along with a similar modulatory role on basal body production, a double knockout was generated that elicited basal body defects and resulted in cell death.

II. Results

Identification of the Poc5 orthologue in Tetrahymena thermophila

Similar to Sfr1, Poc5 family members predominantly have three centrin-binding repeats, organized as an isolated repeat and a tandem repeat (see Fig. 1-6;
Unlike Sfr1 (see Fig. 2-1A), the isolated repeat is N-terminal to the tandem repeat instead of C-terminal (Azimzadeh et al., 2009). The functional consequences of this organizational difference is unknown. Based on the previous analysis of hPoc5, the overall sequence conservation between Poc5 family members is moderate (mean 16% identity, 29% similarity) (Azimzadeh et al., 2009). Despite this overall moderate sequence conservation, Poc5 orthologues have a highly conserved (mean 57% identity, 81% similarity) 21 amino acid signature sequence motif known as the Poc5 box. The Poc5 family, across eukaryotes, is unique compared to other known centrin-binding proteins in that orthologues have this second signature motif (the Poc5 box) that is not found outside of the Poc5 family, in addition to the presence of centrin-binding repeats. Due to the high sequence similarity between Poc5 orthologues within the Poc5 box, this motif (along with the presence of centrin-binding repeats) was utilized to identify the Tetrahymena Poc5 orthologue.

Using the human Poc5 protein sequence as the query sequence, a BLAST search of the *Tetrahymena thermophila* genome (ciliate.org) identified the Tetrahymena Poc5 orthologue (TTHERM_00079160) with the following sequence similarity to hPoc5: overall identities 68/291 (23%), positives 135/291 (46%), gaps 20/291 (6%). *TtPOC5* encodes a 78.5 kDa (684 amino acids in length) protein containing both signature sequence motifs characteristic of the Poc5 family, three centrin-binding repeats and the Poc5 box. Although Tetrahymena and humans are evolutionarily distant in terms of eukaryotes, the Poc5 box sequence is highly similar (Fig. 3-1). Of note, the previous study that identified the SFR family used a different approach for finding centrin-binding proteins in the Tetrahymena genome, which ultimately may have accounted for *ttPoc5*.
Figure 3-1. Poc5 has Two Signature Sequence Motifs. Poc5 contains three Sfi1-repeats organized as a tandem repeat and an isolated C-terminal repeat. Poc5 uniquely also contains a second motif, the Poc5 box. A multiple sequence alignment across multiple eukaryotes shows high sequence conservation within this domain. Expression profile of Poc5 from microarray data (Tetrahymena Genome Expression Database). The red and blue lines are two separate experiments. Cells were cultured in three different conditions (L = logarithmic growth, S = starvation, C = conjugation). Poc5 is moderately expressed in logarithmic growth with increased expression in starvation and conjugation (mating) (Miao et al., 2009).
not appearing in the previous search (Stemm-Wolf et al., 2013). SFR family members were uncovered by using the conserved sequence motif (Ax7LLx3F/Lx2WK/R) found within centrin-binding repeats, while the search for ttPoc5 used the entire hPoc5 sequence containing centrin-binding repeats and the highly conserved Poc5 box.

Microarray expression data available on the Tetrahymena Genome Expression Database (ciliate.org) indicates that ttPoc5 is moderately expressed during logarithmic growth with a gradual increase in expression during starvation and further increased expression during early and late conjugation (Fig. 3-1) (Miao et al., 2009). Importantly, the different culturing conditions used to gather the available microarray data did not provide direct information about potential roles for ttPoc5 or potential localization to basal bodies. The levels of expression for ttPoc5, however, were an optimistic indicator that localization in live cells may be detectable even when endogenously tagged with a fluorescent marker.

**Poc5 transiently localizes to assembling Tetrahymena basal bodies**

Although hPoc5 was found to localize to centrioles, there is no published evidence that Poc5 family members also localize to basal bodies (Azimzadeh et al., 2009). In order to ascertain whether ttPoc5 localizes to Tetrahymena basal bodies, Poc5 was endogenously tagged at the C-terminus with either mCherry or GFP (Stemm-Wolf et al., 2013). Using live cell analysis, cells transformed with Poc5-mCherry exhibited a unique pattern of localization (compared with other previously analyzed Tetrahymena basal body components), with fluorescent foci marking what appeared to be basal bodies but not all basal bodies (Fig. 3-2). Interestingly, in addition to Poc5-mCherry only marking a subset of apparent cortical row basal bodies it was also highly
Figure 3-2. Endogenously Expressed Poc5-mCherry Localizes to a Subset of Apparent Basal Bodies. Poc5 was endogenously tagged at the C-terminus with mCherry. Live-cell analysis revealed Poc5-mCherry signal along cortical rows but not at all basal bodies. Localization was observed at the developing oral apparatus (arrowhead) but not at the mature oral apparatus (arrow).
enriched in the oral primordium (developing oral apparatus, arrowhead) but undetectable in the mature oral apparatus (indicated with an arrow).

In order to confirm that Poc5 was indeed localizing to basal bodies, Poc5-GFP was colocalized with an endogenously expressed known basal body marker, Poc1-mCherry (Pearson et al., 2009a; Stemm-Wolf et al., 2013). As seen in Fig-3-3, Poc5-GFP localized to basal bodies along cortical rows with no apparent localization outside of these structures. Of note, when imaging live Tetrahymena cells their large circular cellular vacuoles are autofluorescent and were not an indicator of Poc5-GFP signal. Interestingly, endogenous Poc5-GFP only localized to a subset of basal bodies compared with endogenous Poc1-mCherry. To dissect this localization further, two biological clues were taken into account. First, endogenous Poc1 was previously reported to slowly incorporate into newly assembled basal bodies and then gradually accumulate in maturing basal bodies (Pearson et al., 2009a). Thus, Poc1 signal could be used as a tool for assessing the maturity of cortical row basal bodies. Second, Tetrahymena cells assemble new basal bodies anterior to existing mature basal bodies, where the mature oral apparatus defines the anterior end of a Tetrahymena cell (Bayless et al., 2015). This means that basal bodies along cortical rows are typically organized as pairs with the anterior basal body arising from the posterior basal body, serving as an additional method for distinguishing between newly assembled versus mature basal bodies.

Given this information, cortical row basal body pairs (containing a mature and assembling basal body) were analyzed in cells co-expressing endogenous Poc5-GFP and endogenous Poc1-mCherry. In respect to a given basal body pair, collected
Figure 3-3. Endogenously expressed Poc5-GFP Localizes to a Subset of Basal Bodies. Poc5 was endogenously tagged at the C-terminus with GFP and co-expressed with a known basal body marker, Poc1-mCherry. Live-cell analysis revealed an interesting pattern of Poc5-GFP localization at basal bodies along cortical rows. Poc5-GFP does not localize to all cortical row basal bodies.
images showed clear Poc5-GFP signal at the anterior basal body and no detectable signal at the posterior (mature) cortical row basal body (Fig. 3-4 top panels). Furthermore, the Poc5-GFP+ basal body in a given pair was marked with very little Poc1-mCherry, supporting that the anterior Poc5-GFP+ basal body was in the assembly process. Image averaging was conducted in order to verify this localization pattern, as well as assess any potential asymmetrical pattern of Poc5-GFP signal relative to the basal body (marked by Poc1-mCherry). Images were collected of 58 pairs of cortical row basal bodies from cells co-expressing endogenous Poc5-GFP and endogenous Poc1-mCherry (Fig. 3-4 bottom panels). Image averaging confirmed that Poc5-GFP is highly enriched at the anterior, assembling basal body in a given pair. In addition, the Poc5-GFP and Poc1-mCherry signals at the anterior basal body colocalized to the center of the basal body (the basal body proper including the microtubule scaffold). The center of the basal body was determined by the center of the Poc1 signal since Poc1 is known to accumulate in the basal body proper rather than the basal body periphery. The region of the basal body scaffold was then calculated by drawing a 200nm diameter circle from this center point, representing the average diameter of a Tetrahymena basal body (Bayless et al., 2015; Meehl et al., 2016; Pearson and Winey, 2009). This observed localization of Poc5 suggested distinct localization to a domain(s) within the basal body proper rather than the basal body periphery (in the proximity of accessory structures). Poc5 localizing predominantly to assembling basal bodies also shed light on the initial localization pattern seen in cells transformed with only Poc5-mCherry, where Poc5 signal was apparent in the developing oral apparatus but not the mature oral apparatus (see Fig. 3-2). Collectively, this data indicates that Poc5 has dynamic
behavior with enrichment at assembling basal bodies but removal at some point in the maturation process.

Figure 3-4. Endogenously expressed Poc5-GFP Localizes to Assembling Basal Bodies. Poc5 was endogenously tagged at the C-terminus with GFP and co-expressed with a known basal body marker, Poc1-mCherry. Image averaging was performed on images of basal body pairs (marked with a dashed box on a representative cell). Arrow to left of representative cell marked anterior (A) – posterior (P) axis of cell. Each basal body pair contained an assembling (anterior) and mature (posterior) basal body. Image averaging revealed Poc5-GFP localization exclusively to the anterior, assembling basal body, as compared with Poc1-mCherry. The distance between the Poc5-GFP signal at the assembling basal body and the Poc1-mCherry signal at the mature basal body is 450nm.
Although this behavior suggested that Poc5 was acting as an assembly factor, the dynamics were still unclear so I sought to gain a better understanding of the timing of basal body incorporation and removal.

**Dynamic incorporation of Poc5 in Tetrahymena basal bodies**

In order to better understand the dynamic behavior of Poc5, Tetrahymena cells were transformed with only endogenously tagged Poc5-GFP and cultured in three conditions: growth, media starvation, as well as a starve and release method. These three conditions elicited different cellular responses to basal body assembly and maintenance. Basal body assembly during logarithmic growth is not synchronous in Tetrahymena cells, meaning the majority of cortical row basal bodies are mature with a minimal number of newly assembled basal bodies. In fact, Tetrahymena basal bodies remain docked at the cell surface where they are stabilized for the entirety of the cell cycle (Bayless et al., 2015). Under media starvation, Tetrahymena cells are arrested in G1 which prevents new basal body assembly so only mature basal bodies persist. Lastly, when cells are grown, shifted into media starvation for 24 hours to inhibit basal body assembly, and then released back in growth media (starve and release) this causes a synchronous wave of new basal body assembly. If Poc5 is localizing only to basal bodies at some point in the assembly process prior to full maturation, then one would expect to see some Poc5 signal during logarithmic growth, no signal during media starvation, and then elevated levels of signal during starve and release. As shown in Fig. 3-5, Poc5-GFP was detectable in a small proportion of basal bodies during logarithmic growth at 30°C (optimal growth temperature), as expected since only
Figure 3-5. Poc5-GFP is Highly Enriched in Assembling Basal Bodies. Poc5 was endogenously tagged at the C-terminus with GFP. During logarithmic growth, there was minimal basal body assembly and correspondingly low levels of Poc5 signal (marked with arrowheads). In starvation, cells were arrested in G1 preventing any new basal body assembly, which resulted in no Poc5 signal. When Poc5-GFP cells were starved for 24 hours and then released into growth media it stimulated new basal body assembly and enriched Poc5-GFP signal.
minimal basal body assembly occurs during growth. When cells were shifted into media starvation conditions for 24 hours this signal disappeared, once again supporting the notion that Poc5-GFP does not localize to mature basal bodies. This depleted signal was in stark contrast to the observed enrichment of Poc5-GFP signal after cells were released into growth media for five hours (starve and release). This enrichment of Poc5-GFP corresponded well with a higher proportion of newly assembled basal bodies after starve and release. This experiment elucidated the dynamic nature of Poc5 basal body localization and served as a secondary method for confirming that Poc5 localizes to assembling basal bodies (secondary to the image averaging results). Additionally, the observed dynamic behavior of Poc5 prompted interest in uncovering the timing of this process.

To better understand the timing of Poc5 incorporation in assembling basal bodies, endogenous Poc5-GFP was colocalized with endogenous Poc1-mCherry or Sas6a-mCherry. As mentioned previously, Poc1 is slow to incorporate into basal bodies but gradually accumulates during maturation, making this marker ideal for a live-cell readout of basal body maturation (Pearson et al., 2009a). Sas6, on the other hand, localizes to all Tetrahymena basal bodies equally and is an early marker of basal body assembly because it is a critical, conserved component of the cartwheel structure (templates the nine-fold symmetry of a basal body) (Culver et al., 2009). For all analyses of Poc5 incorporation, cells were starved and released to increase the number of basal bodies with Poc5 signal. Since Sas6 acts as an early marker of basal body assembly, cells co-expressing Poc5-GFP and Sas6a-mCherry were used to observe how early Poc5 is incorporated into basal bodies. For this, cells were starved for 24
hours and then released into growth media for five hours at 30°C. Interestingly, Poc5-GFP and Sas6a-mCherry signals did not overlap in the same imaging plane when capturing multiple images in a collection (Z-stack), therefore single images from a Z-stack were analyzed for this experiment (instead of an image projection combining multiple images). Previous reports have placed Sas6 at the proximal cartwheel of Tetrahymena basal bodies and have placed human Poc5 at the distal end of centrioles, therefore if ttPoc5 localizes to the distal end of basal bodies then this may have accounted for the observed spatial separation (see immunoEM section of this chapter; (Azimzadeh et al., 2009; Culver et al., 2009)). Also, using single images from this analysis limited the ability to do image averaging of Poc5-GFP⁺; Sas6a⁺ basal bodies. This did not, however, preclude analyzing the timing of incorporation between these two basal body components. As expected, Sas6a-mCherry marked all cortical row basal bodies (since in Tetrahymena basal bodies the cartwheel is a permanent structure unlike other organisms) while Poc5-GFP was restricted to a subset of basal bodies (Fig. 3-6 top panels). By focusing on a representative region of a cortical row (drawn with a dashed box), it was apparent that Poc5-GFP resided at the anterior basal body in a pair and Poc5-GFP⁺ basal bodies were always also marked with Sas6a-mCherry (Sas6 marks both the anterior and posterior basal bodies) (Fig. 3-6 bottom panels). This means that Poc5 incorporation does not precede Sas6 incorporation, as Poc5-GFP signal is only at Sas6a-mCherry⁺ basal bodies. This was not unexpected since Sas6 is a very early component of the cartwheel structure templating the assembling basal body.
To gain information about incorporation later in the assembly process, another basal body marker was used (Poc1-mCherry). As mentioned previously, Poc1 is not an early marker of basal bodies due primarily to slow incorporation into basal bodies but once incorporated, Poc1 signal increases as basal bodies mature (Pearson et al., 2009).

Figure 3-6. Poc5-GFP Basal Body Localization does not Precede Sas6a-mCherry. Endogenously expressed Poc5-GFP was co-expressed with a basal body cartwheel protein, Sas6a-mCherry in starved and released cells. Poc5-GFP localized to a subset of basal bodies and Sas6a-mCherry localized to all basal bodies. A representative section of a cortical row (marked with a dashed box) was highlighted in the bottom panels. Poc5-GFP signal at the anterior, assembling basal bodies always coincided with the presence of Sas6a-mCherry. Poc5-GFP was absent at the posterior (mature) basal bodies.
In a similar manner to the Sas6a analysis, cells co-expressing Poc5-GFP and Poc1-mCherry were starved for 24 hours and then released into growth media for five hours at 30°C to allow for increased new basal body assembly and enriched Poc5-GFP signal (Fig. 3-7 top panels). Focusing specifically on the timing of incorporation between these two basal body components, a representative region of a cortical row was enhanced to show Poc5-GFP signal relative to Poc1-mCherry signal (Fig. 3-7 bottom panels). Intriguingly, Poc5-GFP marked anterior, assembling basal bodies that were devoid of Poc1-mCherry signal. This region of a cortical row displayed three pairs of basal bodies with Poc1-mCherry at the mature basal body of all three pairs. In contrast, Poc5-GFP localized to the assembling basal body of all three pairs, of which only two had apparent Poc1-mCherry signal. Although this indicates that there is overlap in when Poc1 and Poc5 reside at assembling basal bodies, it suggests different temporal requirements for each component. Of great interest for future analysis, the assembling basal body that is Poc5-GFP⁺; Poc1-mCherry⁺ (marked by an arrow) is at the greatest distance from the mature basal body (compared with the other two pairs), suggesting that it is at a later stage in basal body assembly/maturation. Assembling basal bodies also appear to have different levels of Poc5-GFP fluorescence intensity, potentially in direct correlation with the distance from the mature (mother) basal body (the assembling basal body separates from the mother as it assembles and matures). Collectively, Poc5 is incorporated after the cartwheel structure (marked by Sas6a-
Figure 3-7. Poc5-GFP Basal Body Localization Precedes Poc1-mCherry localization. Endogenously expressed Poc5-GFP was co-expressed with a known basal body protein, Poc1-mCherry in starved and released cells. Poc1-mCherry is slow to incorporate in basal bodies and accumulates during basal body maturation (Pearson et al., 2009). A representative section of a cortical row (marked with a dashed box) was highlighted in the bottom panels. Poc1-mCherry marked all posterior (mature) basal bodies. Poc5-GFP localized to assembling basal bodies before Poc1-mCherry incorporation. Poc5-GFP signal was strongest in the assembling basal body nearest to the mature basal body (marked with an arrowhead) and Poc5-GFP signal was weakest in the assembling basal body furthest from the mature basal body (marked with an arrow).
mCherry) is formed but before basal body assembly/maturation is completed (using a later stage basal body marker, Poc1-mCherry).

**Dynamic removal of Poc5 in Tetrahymena basal bodies**

In addition to ascertaining the timing of Poc5 basal body incorporation, it was important to gather information about when removal of Poc5 from basal bodies occurs. Since Poc5 only localizes to a subset of basal bodies, removal from basal bodies could be prompted at some stage in basal body assembly/maturation or at the onset of cilia formation. It is also possible that Poc5 removal requires centrin or an unknown interactor. In fact, human Centrin2 (Cetn2) regulates the removal of a distal end basal body protein known as CP110, which is a critical step in initiating cilia formation (Prosser and Morrison, 2015). Drawing a potential parallel, human Poc5 was found to be a distal end component of centrioles so it was plausible that Poc5 could also reside at the distal end of basal bodies and potentially behave in a similar manner as CP110 (Azimzadeh et al., 2009). To delve into the timing of Poc5 removal, Poc5-GFP was transformed into cells expressing the radial spoke head 9 protein (RSPH9-mCherry, see Fig. 1-2 for underlying ciliary structure), which marks Tetrahymena cilia for live-cell analysis (a generous gift from Chad Pearson, University of Colorado Anschutz Medical Campus). For this analysis, cells co-expressing Poc5-GFP and RSPH9-mCherry were starved for 24 hours and then released into growth media for three hours at 30°C before imaging. As seen in Fig. 3-8 top panels, RSPH9-mCherry evenly labeled ciliary axonemes and there was no signal overlap with Poc5-GFP. By visualizing the Poc5-GFP and RSPH9-mCherry signals separately in an inset of a cortical row, it was more apparent that Poc5-GFP+ basal bodies were not ciliated (Fig. 3-8 bottom panels). This
Figure 3-8. Poc5-GFP+ Basal Bodies are not Ciliated. Endogenously expressed Poc5-GFP was co-expressed with a ciliary marker, RSPH9-mCherry in starved and released cells. RSPH9-mCherry evenly labelled ciliary axonemes. A representative section of a cortical row (marked with a dashed box) was highlighted in the bottom panels. Poc5-GFP signal did not overlap with that of RSPH9-mCherry. Based on this analysis, Poc5-GFP+ basal bodies were not ciliated, suggesting that removal of Poc5-GFP was prior to cilia formation.
result suggested that Poc5 is removed before cilia formation or at the onset of cilia formation, both of which would lead to basal bodies devoid of Poc5 at the base of cilia.

To gain a better perspective of the timing of removal, a basal body marker was added so cells were co-expressing Poc5-GFP, Poc1-mCherry (basal body marker), and RSPH9-mCherry (ciliary marker). Both Poc1-mCherry and RSPH9-mCherry could be used because they localize exclusively to the basal body and ciliary axoneme, respectively. These cells were starved for 24 hours and then released into growth media for 5 hours at 30°C before imaging. Co-expression of all three markers allowed for mature basal bodies to be visualized at the base of cilia, in addition to ciliary axonemes and Poc5-GFP signal (Fig. 3-9 top panels). By visualizing the individual channels in an inset of a cortical row, Poc1-mCherry resided in basal bodies at the base of cilia, whereas Poc5-GFP+ basal bodies were not ciliated (Fig. 3-9 bottom panels). Interestingly, this analysis also revealed Poc1-mCherry+ mature basal bodies (enriched Poc1-mCherry signal) that were not ciliated nor was there any detectable Poc5-GFP signal (indicated with an arrow in Fig. 3-9 bottom panels). Tetrahymena cells provided a good system for studying removal of Poc5 relative to cilia formation, because not all mature basal bodies are ciliated (known as naked basal bodies) in a given cell (Nanney, 1975). Given the presence of two types of mature basal bodies (naked and ciliated), it was evident that some Poc1-mCherry+ mature naked basal bodies were devoid of Poc5-GFP signal. This provided evidence that Poc5 removal occurs prior to cilia formation, potentially at a defined stage in basal body maturation.

**Preliminary ultrastructural basal body localization of Poc5**
Figure 3-9. Poc5-GFP Removal was prior to Cilia Formation. Endogenously expressed Poc5-GFP was co-expressed with a ciliary marker (RSPH9-mCherry) and a basal body marker (Poc1-mCherry) in starved and released cells. RSPH9-mCherry evenly labelled ciliary axonemes while Poc1-mCherry marked mostly mature basal bodies (slow incorporation in assembling basal bodies). A representative section of a cortical row (marked with a dashed box) was highlighted in the bottom panels. Based on this analysis, Poc5-GFP+ basal bodies were not ciliated, suggesting that removal of Poc5-GFP was prior to cilia formation. In addition, removal of Poc5-GFP appeared to be before the onset of cilia formation since mature basal bodies marked with Poc1-mCherry (labeled with an arrow) were not ciliated nor did they have Poc5-GFP signal.
To determine a more precise localization pattern of Poc5 within the basal body ultrastructure, I utilized the starve and release method in cells expressing Poc5-GFP that was capable of enriching the Poc5-GFP signal in live-cell localization studies. By increasing the number of Poc5-GFP+ basal bodies, it was possible to use immunoelectron microscopy (immunoEM) to conduct preliminary analysis of the ultrastructural basal body localization of Poc5. All immunoEM was performed by Janet Meehl (Winey Lab – CU Boulder). For this analysis, Poc5-GFP signal was enriched (due to increased basal body assembly) by starving cells for 24 hours and then releasing them into growth media at 30°C for five hours. Since Poc5 was tagged with GFP, it was possible to use a gold-conjugated antibody against GFP in order to visualize Poc5 with the presence of gold particles. As seen in a longitudinal section of a cortical row basal body, Poc5 appeared to be heavily enriched at the distal end of the basal body (Fig. 3-10 left panel). Poc5 was also notably restricted to the distal end of the basal body with no apparent Poc5 in any other domain. This observed ultrastructural localization of ttPoc5 was similar to the previously reported localization of human Poc5 to the distal end of centrioles (Azimzadeh et al., 2009). Interestingly, this particular basal body was oriented towards the cell surface, a sign that it was not newly assembled since assembling basal bodies are perpendicular to the mother basal body and then tilt upwards towards the cell surface before docking (Pearson and Winey, 2009). Although localization studies conducted in live cells with Poc5-GFP and Poc1-mCherry were not able to determine this precise of a localization pattern, they did reveal Poc5-GFP+ basal bodies that were not in close proximity to the mature basal body and
Figure 3-10. Preliminary Ultrastructural Basal Body Localization of Poc5. Cells endogenously expressing Poc5-GFP were starved and released for immunoelectron microscopy (immunoEM). ImmunoEM was performed by Janet Meehl (Winey Lab-CU Boulder). A gold-conjugated antibody against GFP was used to visualize Poc5 with the presence of gold particles. The left panel shows a longitudinal view of a cortical row basal body with Poc5-GFP enrichment restricted to the distal end of the basal body. The right panel shows a transverse view of a cortical row basal body with Poc5-GFP enrichment near the proximal end of the basal body (proximal based off of the presence of the cartwheel structure).
contained Poc1-mCherry signal (an indicator of basal body maturity), drawing a parallel between the two localization studies.

Adding to the complexity of Poc5 basal body localization, a transverse section of a cortical row basal body showed enrichment of Poc5 towards the proximal end (given the presence of the cartwheel structure) of the basal body (Fig. 3-10 right panel). It was not apparent how mature this particular basal body was (due to a transverse view), but this indicates that Poc5 is potentially dynamic on an ultrastructural level, residing in multiple domains within the ultrastructure of the basal body. It will be important to first gain a more precise understanding of how Poc5 localizes within cortical row basal bodies by increasing the size of the immunoEM data set. Then it will be intriguing to determine if Poc5 exhibits temporal changes in ultrastructural localization during the basal body assembly and maturation process, which would potentially bridge the two pieces of evidence that Poc5 localizes to the proximal and distal ends of the basal body.

**Overexpressed Poc5 leads to basal body defects**

Endogenously expressed Poc5 transiently localizes to assembling basal bodies along cortical rows and is removed before cilia formation. The transient nature of this localization suggested specific temporal requirements for Poc5 at basal bodies. In order to potentially disrupt this timing and assess the cellular consequences, a construct was built that placed Poc5-mCherry under the control of the cadmium-inducible *MTT1* promoter. Cells that were transformed with *MTT1pr*-Poc5-mCherry and incubated without cadmium, exhibited Poc5 localization at only a subset of basal bodies, which was similar to endogenously expressed Poc5 (Fig. 3-11A). On the other hand, when cells expressing *MTT1pr*-Poc5-mCherry were incubated with 0.5 µg/ml CdCl₂ overnight
Figure 3-11. Overexpressed Poc5-mCherry leads to Basal Body Defects and Poc5+ fibers. Poc5-mCherry was exogenously expressed by the MTT1 cadmium-inducible promoter. (A) When cells transformed with MTT1pr-Poc5-mCherry were incubated without cadmium, Poc5-mCherry signal was similar to endogenously expressed Poc5 (only a subset of basal bodies are Poc5+). (B) When incubated in 0.5µg/mL CdCl₂, Poc5-mCherry localized to all cortical row basal bodies and resulted in the formation of line Poc5 fibers (marked with an arrowhead). (C) Cells that were incubated in 0.5µg/mL CdCl₂ were fixed and stained with an anti-centrin antibody to determine if centrin localized to Poc5 fibers. Centrin was found at a fiber, revealing that Poc5+ fibers are also centrin+. 
this resulted in a different localization pattern than what was observed at endogenous levels, as well as the presence of Poc5+ fibers (Fig. 3-11B). Overexpressed Poc5-mCherry appeared to have the capacity to localize to all cortical row basal bodies, instead of solely assembling basal bodies. The Poc5-mCherry signal at basal bodies was also aberrantly elongated instead of the punctate foci representative of basal bodies. Of note, it will likely require the resolution of EM to determine if this is the result of over-elongated basal bodies, improperly docked basal bodies (potentially lying parallel to the cell surface), or another basal body defect. Interestingly, overexpression of Poc5-mCherry altered the localization pattern of Poc5 along cortical rows but Poc5 remained restricted to the developing oral apparatus and not in the mature oral apparatus. This suggested potentially different requirements for Poc5 in cortical row and oral apparatus basal bodies.

Potentially independent of basal bodies, a signature feature unique to Poc5-mCherry overexpression was the presence of Poc5 fibers (Fig. 3-11B). These structures varied in length and were found in multiple locations of the cell, mostly arising in the proximity of a cortical row or an oral apparatus. The presence of Poc5+ fibers was not unique to this study, in fact a previous analysis of Poc5 in chicken DT40 cells found linear Poc5+ assemblies upon Poc5 overexpression (Dantas et al., 2013). Centrin was also found to reside in these linear structures and depletion of centrin abolished their formation (centrin-dependency). In this previous study, they conducted EM analysis and found that these linear aggregates did not appear centriole- or cilia-like, instead they resembled protein aggregates. It is unclear why overexpression of Poc5 resulted in assembly of these fibers since overexpression of Sfr1 (and other basal body
components the Winey Lab has studied) did not lead to fiber formation. Nonetheless, cells overexpressing *MTT1pr-Poc5-mCherry* (incubated overnight in 0.5 µg/ml CdCl₂) were fixed and stained with an anti-centrin antibody to observe centrin basal body localization and to determine if centrin localized to Poc5⁺ fibers (Fig. 3-11C). Centrin staining revealed aberrant basal bodies once again along cortical rows (basal bodies appeared elongated or pointed compared to punctate foci) and fibers were apparent, indicating that these fibers also contained centrin. In conclusion, *MTT1pr-Poc5-mCherry* overexpression led to Poc5-mCherry marking all cortical row basal bodies and the developing oral apparatus, but not the mature oral apparatus. Also, overexpression of Poc5-mCherry resulted in the formation of linear fibers apparent with light microscopy, containing both Poc5 and centrin. Given the interesting dynamic localization pattern of Poc5 and the unique Poc5 overexpression phenotype, emphasis was placed on generating a complete genomic knockout in order to determine the basal body function of Poc5.

**Generation of complete genomic knockout of *POC5***

To determine the function(s) of Poc5, a complete genomic knockout strain was generated using established methods (Hai et al., 2000). This entailed replacing the *POC5* open reading frame (ORF) with a codon-optimized neo2-cassette conferring paromomycin resistance for selection. Knockout cells were confirmed by PCR using an oligonucleotide pair that was designed to amplify a sequence specific to the wild-type *POC5* ORF (Fig. 3-12A). Isolated genomic DNA from poc5Δ cells showed no evidence of wild-type *POC5*, whereas genomic DNA isolated from WT cells exhibited clear amplification of wild-type *POC5*. Cells confirmed to completely lack *POC5* (*poc5Δ* cells)
Figure 3-12. poc5Δ Cells are Viable and Maintain Organized Cortical Rows. Complete micronuclear knockout of POC5. (A) PCR confirming complete micronuclear knockout of POC5. An oligonucleotide pair was used to amplify the wild-type POC5 ORF. Only WT cells contain the POC5 ORF. (B) Representative Tetrahymena cell with three cellular parameters measured for phenotypic analysis of poc5Δ cells: cell length (µm), basal body density (basal bodies/10µm), and the number of cortical rows. (C) WT and poc5Δ cells were grown at 30°C and 37°C for immunofluorescence microscopy. Cells were fixed and stained with an anti-centrin antibody. Scale bar: 10µm.
are viable and persist indefinitely, therefore POC5 is not an essential gene for viability in Tetrahymena. From a gross morphological standpoint, poc5Δ cells appeared healthy (are not rounded or lysing) and did not exhibit abnormal swimming behavior. In order to study the effects of loss of Poc5 specifically on basal bodies, immunofluorescence microscopy was performed on WT and poc5Δ cells.

**Loss of Poc5 leads to increased basal body production along cortical rows**

To investigate potential basal body functions of Poc5, phenotypic analysis was conducted on growing cells at both 30°C and 37°C. WT and poc5Δ cells were examined for three major cellular parameters: cell length (μm), basal body density along cortical rows (basal bodies/10μm), and the total number of cortical rows (Fig. 3-12B). From these measurements, an estimate of the total number of cortical row basal bodies was generated. These parameters are influenced by the ability of Tetrahymena cells to properly assemble and maintain basal bodies, both of which are critical processes for sustaining spatial organization of cortical rows. Cells analyzed under growth conditions assayed primarily for the capacity of poc5Δ cells to assemble basal bodies (Pearson et al., 2009). The use of two growth temperatures, 30°C and 37°C, tested for deficits in assembling basal bodies at an optimal growth temperature and under additional temperature-induced stress, respectively.

At both growth temperatures, WT and poc5Δ cells properly orientated basal bodies along cortical rows and maintained the correct gross morphology of their oral apparatus (Fig. 3-12C). Given the fact that Poc5 appears to reside along the basal body microtubule scaffold (based on image averaging and preliminary immunoEM), it was not unexpected that poc5Δ cells maintained the appropriate basal body orientation.
As seen in Fig. 3-12C and quantified in Fig. 3-13A, differences in cell length were only observed during growth at 37°C where poc5Δ cells exhibited a significant increase in cell length compared to WT cells. This was a different cellular response to the loss of Poc5 compared with the loss of Sfr1, which resulted in decreased cell length at 37°C. Cell length measurements in poc5Δ cells indicated that deleting POC5 may lead to altered cell length at a higher growth temperature, but not as a result of aberrant basal body orientation (Fig. 3-12C).

Although loss of Poc5 does not seem to have a large effect on cell length, poc5Δ cells grown at 30°C and at 37°C had a significantly increased basal body density along cortical rows (7.49 basal bodies/10µm at 30°C and 7.60 basal bodies/10µm at 37°C) compared with WT cells (6.60 basal bodies/10µm at 30°C and 6.13 basal bodies/10µm at 37°C) (Fig. 3-13B). Surprisingly, loss of Poc5 led to increased basal body density but the number of cortical rows in poc5Δ cells remained similar to WT cells during growth at both 30°C and 37°C (Fig. 3-13C). This result was surprising because loss of Sfr1 led to a similar increase in basal body density and also an increase in the total number of cortical rows, which was thought to accommodate the excess basal bodies produced during growth. Despite the lack of difference between poc5Δ and WT cells with respect to the number of cortical rows, poc5Δ cells had a significantly higher total number of cortical row basal bodies at both growth temperatures compared with WT cells (Fig. 3-13D). In conclusion, loss of Poc5 led to an overproduction of basal bodies along cortical rows, primarily as a result of increased basal body density. The manner in which poc5Δ cells overproduced cortical row basal bodies differed slightly from the overproduction seen in sfr1Δ cells, but the parallels in knockout phenotypes could not
Figure 3-13. Loss of Poc5 leads to an overproduction of basal bodies along cortical rows. poc5Δ cells exhibited increased basal body density and a higher total number of cortical row basal bodies during logarithmic growth at 30°C and 37°C. Three cellular parameters were measured for phenotypic analysis: cell length (in µm), basal body density (number of basal bodies per 10µm), and the number of cortical rows. The total number of cortical row basal bodies is derived from: cell length X basal body density X number of cortical rows. (A,B,C,D) Quantification from phenotypic analysis of effect on cellular parameters from loss of Poc5. For all measured parameters, grey bars were used to show the results from WT cells and white bars were used for poc5Δ cells. n = 15 per strain per condition. Error bars indicate standard error of mean. P-values were derived from a Student’s t-test calculation. * = p<0.05, ** = p<0.01, *** = p<0.001.
be ignored. Collectively, the work carried out in this thesis identified two centrin-binding proteins that seem to have an antagonistic role in basal body production. Given the similarities between the basal body phenotypes observed in *poc5Δ* and *sfr1Δ* cells, a double knockout was generated to explore a potential functional overlap between these two basal body components.

**Double knockout of *POC5* and *SFR1* results in high levels of basal body production and cell death**

Due to the shared similarities between Poc5 and Sfr1, it was enticing to see if a combined loss of both basal body components would lead to an exacerbated basal body phenotype. To delve deeper into these similarities, Poc5 and Sfr1 are both centrin-binding components with a similar organizational layout of three centrin-binding/Sfi1-repeats, organized as a tandem repeat and an isolated repeat. In addition to the organization of the three centrin-binding repeats, the amino acid sequence within the centrin binding repeats of Poc5 and Sfr1 share similarity. This became evident when hPoc5 was used as the query sequence to search the *Tetrahymena thermophila* genome (ciliate.org) for ttPoc5. In this search, ttPoc5 was the top hit (e value: 3e-13, overall identities 68/291 (23%), positives 135/291 (46%), gaps 20/291 (6%)) and Sfr1 was the second hit (e value: 0.001) with the following sequence similarity to hPoc5: overall identities 40/172 (23%), positives 74/172 (43%), gaps 18/172 (10%). Sfr1 does not contain the characteristic Poc5 box but does share sequence similarity within the centrin-binding repeats, potentially providing centrin binding specificity that is not currently understood. Aside from sequence similarity, ttPoc5 and Sfr1 are the only two characterized centrin-binding proteins that localize to the basal body microtubule
scaffold rather than the basal body periphery (in proximity to accessory structures). Lastly, loss of either ttPoc5 or Sfr1 resulted in an overproduction of basal bodies along cortical rows, which is to our knowledge a novel knockout basal body phenotype in Tetrahymena and is suggestive of a potential functional overlap in regards to modulating cortical row basal bodies.

Despite these similarities, it is worth noting that there are differences between these two centrin-binding proteins. Of these two components, only ttPoc5 contains the Poc5 box that was found to be highly conserved in previously identified Poc5 orthologues across many eukaryotes (Azimzadeh et al., 2009). It is completely unknown why the Poc5 box has been so highly conserved in the Poc5 family but it could potentially serve as an important domain for binding a non-centrin interactor. As previously mentioned, Sfr1 and ttPoc5 both localize to the microtubule scaffold of basal bodies but they exhibit notably different localization patterns. The fact that Sfr1 localizes to all Tetrahymena basal bodies and ttPoc5 only localizes to assembling basal bodies suggests different spatial and/or temporal basal body requirements for each component. However, this difference in localization pattern has a caveat since Sfr1 was only able to be exogenously expressed by the MTT1 promoter, which ultimately could have obscured the underlying dynamics of Sfr1 basal body localization. Lastly, loss of either component led to an overproduction of basal bodies but loss of Poc5 did not affect the number of cortical rows.

The process of generating the double knockout of POC5 and SFR1 utilized the same established method that successfully generated both single knockouts (Hai et al., 2000). This entailed replacing the POC5 ORF with a codon-optimized neo2-cassette
and replacing the \textit{SFR1} ORF with a neo cassette, both of which conferred paromomycin resistance for selection. Unlike \textit{poc5}\textsuperscript{$\Delta$} and \textit{sfr1}\textsuperscript{$\Delta$} single mutants, \textit{poc5}\textsuperscript{$\Delta$}; \textit{sfr1}\textsuperscript{$\Delta$} double mutants were not viable, suggesting that Poc5 and Sfr1 have functional redundancy. Fortunately, Tetrahymena is an advantageous system for studying the function of essential genes (or gene combinations) because complete genomic knockouts are only generated when two different mating types of heterokaryons are mated together (Hai et al., 2000). Heterokaryons take advantage of Tetrahymena genetics and rely on the binucleated nature of Tetrahymena cells. In the case of the generation of the \textit{poc5}\textsuperscript{$\Delta$}; \textit{sfr1}\textsuperscript{$\Delta$} double knockout, each heterokaryon had a transcriptionally active wild-type nucleus (contained both \textit{POC5} and \textit{SFR1}) and a transcriptionally silent germline nucleus that lacked both components. Isolated genomic DNA from potential heterokaryons were confirmed by PCR to ensure that \textit{POC5} and \textit{SFR1} were replaced by a codon-optimized neo2 cassette and a neo cassette, respectively (Fig. 3-14). The PCR used two oligonucleotide pairs: one pair was designed to amplify a sequence specific to the codon-optimized neo2-cassette in order to detect the presence of the knockout construct in the \textit{POC5} locus (was also used to confirm \textit{poc5}\textsuperscript{$\Delta$} cells), whereas the second pair (neo cassette) was designed to amplify sequence specific to the neo-cassette in order to detect the presence of the knockout construct in the \textit{SFR1} locus. Since selection of both neo cassettes was through the same drug (paromomycin), it was essential to test for successful generation of heterokaryons by PCR on all drug-resistant clones. This resulted in identifying paromomycin-resistant cells that were only missing \textit{POC5}, only missing \textit{SFR1}, or were heterokaryons (marked with arrows) (Fig. 3-14). When these confirmed heterokaryons were mated together to generate the double
knockout, the transcriptionally active nucleus was entirely derived from the germline nuclei of each parental strain, which both lacked *POC5* and *SFR1*.

**Detection of codon-optimized Neo2-cassette in *POC5* locus**

**Detection of Neo-cassette in SFR1 locus**

**Figure 3-14. PCR Confirmation of Heterokaryons to generate poc5Δ; sfr1Δ Cells.** Drug-resistant cells were tested for the presence of the codon-optimized Neo2-cassette that replaced the *POC5* ORF, as well as the presence of the neo-cassette that replaced the *SFR1* ORF. Heterokaryons were identified (marked by arrows) that exhibited successful replacement of *POC5* (left panel) and *SFR1* (right panel).
This resulted in \( poc5\Delta; sfr1\Delta \) cells that were not viable, therefore genomic DNA could not be isolated like for \( poc5\Delta \) and \( sfr1\Delta \) cells.

The experimental strategy employed for studying \( poc5\Delta; sfr1\Delta \) cells was to carry out mass matings in order to have enough progeny (prior to cell death) for phenotypic analysis with immunofluorescence microscopy. Once mating was finished, growth media was added without drug since Tetrahymena mating only occurs in starvation. From a gross morphological standpoint, mass mated cultures of \( poc5\Delta; sfr1\Delta \) cells at this point contained mostly rounded cells (thought to be double knockout progeny) and healthier cells (thought to be non-maters). After four hours in growth media, paromomycin was added to select against non-mated cells (since mass matings were never 100% efficient), serving as time point 0. By 24 hours after drug addition, the culture consisted of mostly smaller cells (potentially an early indicator of dying cells) and at 48 hours after drug was added there were no living \( poc5\Delta; sfr1\Delta \) cells. At 48 hours, the presence of only dead cells appeared to be a combinatorial effect of double knockout progeny dying from loss of both Poc5 and Sfr1, as well as non-maters dying from drug-selection.

In order to specifically focus on the consequences of loss of both Poc5 and Sfr1, the double knockout was mated in parallel with control crosses: a cross between two wild-type strains, heterokaryons crossed with wild-type, and a non-mated wild-type control. Since basal body rearrangements (and potential changes in basal body number) do occur in Tetrahymena cells during mating, the cross between two wild-type strains served as a basal body number control for phenotypic analysis. Even though these cells went through mating, the progeny do not have a neo cassette therefore they
were not drug-resistant (serving as a control for potential basal body organization defects after mating or through drug selection). The cross between heterokaryons and wild-type served as a control for drug-resistant progeny (resistance obtained from the heterokaryon) that were not devoid of either POC5 or SFR1 (heterozygotes since mated with wild-type). Lastly, the non-mated wild-type culture controlled for the effects of drug selection on non-maters that were inevitably in the double knockout culture (and were not drug-resistant).

All of the above mentioned cultures were used for immunofluorescence analysis, with fixation occurring at 24 and 48 hours after drug was added and staining was with anti-centrin to mark basal bodies. Importantly, paromomycin was not an efficient drug (in terms of killing cells) so at 24 hours after drug addition all cultures still had living cells even though some cultures contained only cells that were not drug-resistant. Also, the double knockout cross ultimately led to cell death but at 24 hours there were still intact, living cells that could be analyzed. At 24 hours, double knockout and control cells maintained basal body organization along cortical rows, despite the presence of smaller cells (Fig. 3-15 top panels). By 48 hours after drug addition, the wild-type X wild-type culture and the non-mated wild-type control contained only dead cells because of drug-selection, as expected. As previously mentioned, the double knockout progeny (and non-maters that were selected against) were also dead by 48 hours. On the other hand, at 48 hours the heterokaryon X wild-type culture was dense with growing drug-resistant cells (heterozygotes). This experiment confirmed that poc5Δ; sfr1Δ cells were not viable because of loss of Poc5 and Sfr1.
Figure 3-15. Double Knockout Cells exhibited an Exacerbated Basal Body Overproduction Phenotype.  *poc5Δ; sfr1Δ* cells were generated by mating two heterokaryons together (per established methods, Hai et al., 2000).  *poc5Δ; sfr1Δ* cells were inviable and died within 48 hours after drug addition, alongside control cultures (non-mated wild-type and wild-type crossed with wild-type).  Heterokaryons mated with wild-type (making heterozygotes) were drug-resistant and contained both *POC5* and *SFR1*, therefore they did not die.  Scale bar: 10µm.  At 24 hours, the basal body density was measured in fixed and stained (centrin) cells.  *poc5Δ; sfr1Δ* cells had a significantly higher basal body density than control cultures.  *n* = 15.  Error bars indicate standard error of mean.  *P* - values were derived from a Student’s t-test calculation.  *** = *p*<0.001.
The presence of living cells in all cultures at 24 hours after drug was added allowed for an estimate of basal body density to be quantified in double knockout cells prior to death, in comparison to the control cells (Fig. 3-15 bottom panel). Excitingly, $poc5\Delta; sfr1\Delta$ cells exhibited high levels of basal body production with significantly increased basal body density along cortical rows (8.84 basal bodies/10µm), exceeding what was observed in $poc5\Delta$ cells (7.49 basal bodies/10µm) or $sfr1\Delta$ cells (7.91 basal bodies/10µm). Interestingly, at this point in drug selection non-maters were still present so this basal body density measurement may be an underestimate. Of note, non-significant differences in basal body density were measured between cells from control cultures. The double knockout progeny had significantly higher basal body density compared with all three control cultures. This observed overproduction of basal bodies suggests that Poc5 and Sfr1 have redundant roles in modulating basal body production along cortical rows. When both components were genetically deleted, it resulted in basal body production potentially exceeding what the cell could tolerate, eventually leading to cell death. For further studies, it would be of great value to construct a full length Poc5 shut-off construct under control of the $MTT1$ promoter. This could provide the ability to maintain $poc5\Delta; sfr1\Delta$ cells with the presence of cadmium (allowing for selection against non-maters), before removing cadmium and potentially revealing the primary cause of cell death. Uncovering the primary cause of lethality in $poc5\Delta; sfr1\Delta$ cells is critical and will require further analysis and experimental tools. Nonetheless, this exciting preliminary study revealed an exacerbated basal body overproduction phenotype in $poc5\Delta; sfr1\Delta$ cells.

III. Discussion
Tetrahymena have a highly organized cytoskeleton that requires proper assembly, orientation, and maintenance of basal bodies along cortical rows, all of which are critical processes that rely on centrin (Stemm-Wolf et al., 2005; Kilburn et al., 2007; Vonderfecht et al., 2012). It is largely unclear how centrin performs multiple, essential basal body functions that require precise timing and distinct centrin populations at basal body subdomains, but it is thought to be through interacting partners (Stemm-Wolf et al., 2005; Kilburn et al., 2007; Vonderfecht et al., 2012). Intriguingly, Tetrahymena have multiple centrin-binding proteins, including ttPoc5 and a large family of centrin-binding proteins (Sfr1-13) that may individually be responsible for subsets of overall centrin function and collectively provide both the necessary spatial and temporal regulation of centrin at basal bodies (Stemm-Wolf et al., 2013). This potential spatial regulation of centrin is reflective of the previous finding that centrin-binding proteins in Tetrahymena are each restricted to distinct regions of the basal body periphery (except for Poc5 and Sfr1 at the basal body proper), coinciding with regions known to also contain centrin (Stemm-Wolf et al., 2013).

Outside of Tetrahymena, a centrin-binding protein known as hPoc5 was studied in human cultured cells and was found to be required for building the distal end of centrioles for successful completion of centriole elongation and maturation (Azimzadeh et al., 2009). There is currently no published information about the function of Poc5 in basal bodies. Through my thesis studies, I aimed to characterize the basal body function of the Poc5 orthologue in the ciliate *Tetrahymena thermophila*, ttPoc5. The Tetrahymena Poc5 orthologue has two signature domains found in all Poc5 family
members: three centrin-binding repeats (containing the embedded Ax7LLx3F/Lx2WK/R motif) and the highly conserved Poc5 box of unknown function (Azimzadeh et al., 2009). Interestingly, endogenously expressed ttPoc5 localized to basal bodies, but to only a subset of cortical row basal bodies and to the developing oral apparatus (not the mature oral apparatus). By colocalizing ttPoc5 with known basal body markers, it became apparent that ttPoc5 was enriched in assembling basal bodies and this enrichment could be increased by stimulating new basal body assembly. Colocalization studies with additional basal body markers may be required in order to better define the stage in basal body assembly that ttPoc5 becomes incorporated. Since ttPoc5 did not localize to all cortical row basal bodies, I sought to gain a better understanding of when ttPoc5 was being removed from assembling/maturing basal bodies. By using a basal body and ciliary marker, ttPoc5 removal was revealed to be prior to the onset of cilia formation and at some stage in late basal body assembly/maturation. Similar to the dynamic basal body incorporation exhibited by ttPoc5, additional late stage (relative to basal body assembly/maturation) markers will be required to dissect this localization pattern further. Through image averaging and immunoEM, ttPoc5 was found to localize to the basal body microtubule scaffold, with enrichment preliminarily observed at both the proximal and distal ends of the basal body. Going forward, a larger immunoEM dataset needs to be collected from Poc5-GFP cells that have been starved and released (to enrich Poc5-GFP signal and increase basal body assembly). This would provide ultrastructural basal body localization information for ttPoc5 and it would allow for ttPoc5 to be visualized within basal bodies with the resolution needed to also correlate localization (and domains of localization) with stages of basal body
assembly/maturation. This will be especially important if ttPoc5 exhibits temporal
changes in basal body localization on the ultrastructural level, potentially explaining the
observed enrichment of ttPoc5 to the distal and proximal ends of the basal body.
The dynamic nature of Poc5 basal body incorporation and removal is unique among
known centrin-binding proteins (and characterized basal body components in
Tetrahymena), suggesting spatial and/or temporal requirements for ttPoc5 basal body
function. When ttPoc5 was overexpressed, potentially disrupting the required timing of
Poc5 function, it resulted in localization to all cortical row basal bodies and the
developing oral apparatus, but not the mature oral apparatus. Interestingly, driving
ttPoc5 localization to all cortical row basal bodies also resulted in an aberrantly
elongated basal body signal instead of characteristic punctate foci. Electron microscopy
should be employed in further studies to delve deeper into potential basal body
structural defects upon ttPoc5 overexpression. In addition to this aberrant basal body
signal, ttPoc5 overexpression led to the formation of Poc5+, centrin+ linear fibers, which
were not observed in cells overexpressing Sfr1. These linear aggregates have been
previously reported when Poc5 was overexpressed in chicken DT40 cells (Dantas et al.,
2013). It is currently unclear why overexpression of Poc5 and not Sfr1 (or other centrin-
binding proteins) leads the formation of these fibers.

Tetrahymena cells lacking POC5 (poc5Δ) are viable and displayed a marked
overproduction of basal bodies along cortical rows. Loss of Poc5 led to an
overproduction of basal bodies along cortical rows, primarily as a result of increased
basal body density (and not an increase in the number of cortical rows). Despite the
lack of difference between poc5Δ and WT cells with respect to the number of cortical
rows, poc5Δ cells had a significantly higher total number of cortical row basal bodies at both tested growth temperatures compared with WT cells. Phenotypic analysis should be expanded to starvation conditions, similar to the phenotypic analysis conducted on sfr1Δ cells, to assess basal body stability in poc5Δ cells. The modulatory role of Poc5 in the production of basal bodies was similar to the apparent antagonistic function of Sfr1 described in Chapter 2 of this thesis (and Heydeck et al., 2016 mSphere in press). Of note, the observed overproduction of basal bodies upon the loss of Poc5 needs to be rescued by reintroducing Poc5 under the control of the MTT1 inducible promoter, in order to confirm a potentially direct role for Poc5 in modulating basal body production along cortical rows. This approach rescued the effect of loss of Sfr1 on basal body density and the total number of basal bodies in sfr1Δ cells.

Analyses of ttPoc5 and Sfr1 revealed notable similarities between these two centrin-binding proteins. Both components localized to the microtubule scaffold of basal bodies, a location that is not occupied by any other known Tetrahymena centrin-binding proteins (Stemm-wolf et al., 2013). In addition, ttPoc5 and Sfr1 have three centrin-binding repeats with sequence similarity within the repeats, suggesting potential centrin-binding specificity that is currently not understood. Surprisingly, loss of ttPoc5 or Sfr1 resulted in viable cells but led to an overproduction of total cortical row basal bodies, primarily as a result of increased basal body density. Given these similarities, it was suspected that ttPoc5 and Sfr1 could have functional overlap with respect to modulating basal body production. To pursue this further, a double knockout was generated (poc5Δ; sfr1Δ cells) that resulted in cell lethality. Since poc5Δ and sfr1Δ single mutants were viable but loss of both resulted in cell death, this suggested that these two centrin-
binding proteins have functional overlap (drawing similarities to synthetic lethal relationships; (Kaelin, 2005)). Before cell death, poc5Δ; sfr1Δ cells were analyzed in parallel with wild-type controls to assess the effects of loss of both components on basal body density. Interestingly, poc5Δ; sfr1Δ cells were organized along cortical rows but exhibited an increase in basal body density that surpassed what was observed in poc5Δ and sfr1Δ cells. This exacerbated basal body overproduction phenotype suggests that ttPoc5 and Sfr1 are paralogues with functional redundancy in terms of modulating basal body production. This would not be unique in Tetrahymena with previous reports of the cartwheel protein Sas6 separated into Sas6a and Sas6b in Tetrahymena, as well as the presence of multiple paralogues that may regulate the size of Tetrahymena cilia (Culver et al., 2009; Wloga et al., 2006). With respect to Poc5 in Tetrahymena, when only POC5 was deleted (poc5Δ cells), Sfr1 may have been compensating to maintain viability and diminish the full effects of loss of Poc5 on basal body production (and vice versa in sfr1Δ cells). It is still unclear how Poc5 or Sfr1 are modulating basal body production, prompting the need for further phenotypic analysis of poc5Δ; sfr1Δ cells. Also, reincorporation of either POC5 or SFR1 in poc5Δ; sfr1Δ cells should rescue cell lethality if these components have functional redundancy and would be a critical experiment to conduct.

To our knowledge, there are no published studies that uncovered a basal body overproduction phenotype leading to cell death. Without parallel examples, it complicates the ability to predict what is driving cell death in poc5Δ; sfr1Δ cells. However, abolished centrin-binding or aberrant timing of the Poc5-centrin/Sfr1-centrin interaction in poc5Δ; sfr1Δ cells may be the underlying cause of basal body
overproduction and/or cell death. Combining the localization information for ttPoc5 and the previously published detailed basal body localization mapping for the SFR family, only Sfr1 and ttPoc5 localize to the basal body microtubule scaffold (Stemm-Wolf et al., 2013). This is important because Tetrahymena Cen1 was found to localize to the midzone of the basal body and both Cen1 and Cen2 localize to the transition zone of the basal body microtubule scaffold (Stemm-Wolf et al., 2005; Kilburn et al., 2007; Vonderfecht et al., 2012). It is thought that centrin-binding proteins are functioning to properly localize centrins to specific domains, therefore Sfr1 and ttPoc5 may be the only centrin-binding proteins bringing centrin to the basal body proper. Given that a complete knockout in Tetrahymena of CEN1 is lethal, proper localization and basal body function of Cen1 is critical for Tetrahymena cell viability (Stemm-Wolf et al., 2005). Also, human Centrin 3 (hCetn3) was found to inhibit centrosome duplication in HeLa cells, while hCetn2 promotes centrosome duplication (Sawant et al., 2015). This published study found a modulatory role for centrins in centrosome duplication, therefore it is possible that centrins also regulate (both positively and negatively) basal body assembly. Furthermore, the proposed negative regulation of basal body assembly by centrins could be influenced by centrin-binding proteins, namely Sfr1 and Poc5. In the initial analysis of poc5Δ; sfr1Δ cells, I used an antibody that was specific to Cen1. Through previous analysis of Tetrahymena centrins, an antibody against Cen2 was also generated (Vonderfecht et al., 2011; Vonderfecht et al., 2012). For further analysis, it is possible to look for potential displacement of Cen1 or Cen2 from the basal body proper in poc5Δ; sfr1Δ cells by image averaging the basal body signal of each centrin (using specific centrin antibodies) with signal from an antibody that marks the basal body
proper, a commercially available antibody against glutamylated tubulin (GT335). The pattern of centrin localization relative to the basal body proper could then be compared with image averaging results from poc5Δ and sfr1Δ cells (that similarly compare centrin and GT335 signals). Since poc5Δ and sfr1Δ cells are viable, this comparative analysis with poc5Δ; sfr1Δ cells could shed light on compensation occurring in the single knockout cells that keeps centrin at the basal body proper. This could be further elucidated by mutating the centrin-binding repeats of either ttPoc5 or Sfr1 (to abolish binding of centrin) and observing whether this results in an overproduction of cortical row basal bodies as was seen in poc5Δ, sfr1Δ, and poc5Δ; sfr1Δ cells. Intriguingly, only ttPoc5 contains the highly conserved Poc5 box, suggesting that the function of this domain is probably not the main contributor to the observed basal body phenotype in poc5Δ cells. Divergence of Poc5 in Tetrahymena with only ttPoc5 containing the highly conserved Poc5 box could provide an additional unique opportunity to explore the specific function of the Poc5 box.

In conclusion, Tetrahymena may be a unique model system for studying the function of Poc5 because of the potential divergence of Poc5 into two centrin-binding proteins, ttPoc5 and Sfr1. This relationship is intriguing to study and could shed light on centrin-dependent and centrin-independent roles of Poc5 in basal bodies, of which nothing has been published. Furthermore, it is not fully understood how centrin-binding proteins contribute to known centrin functions and how binding specificity to the two functionally distinct centrin families is attained. Given evidence that centrins can act antagonistically in regards to centriole assembly, centrin-binding proteins may act to bi-directionally modulate basal body production and therefore basal body number through
interaction with a specific centrin. The work highlighted in this thesis provides an exciting claim for pursuing future studies of Tetrahymena Poc5, Poc5 function in vertebrates, and centrin-binding proteins in all systems.
Chapter Four: Preliminary Analysis of Vertebrate Poc5 Function in *Xenopus laevis* (in collaboration with Dr. Jianli Shi and Professor Michael Klymkowsky)

I. Introduction

In the previous chapter I described studies of Poc5 in Tetrahymena, where ttPoc5 localizes to assembling basal bodies and is removed before cilia formation. While studies on Tetrahymena can provide important information on basal body assembly and function, such unicellular organisms cannot provide information about roles of Poc5 in a multi-cellular, developing organism. There are multiple vertebrate systems that have been used to study basal bodies, cilia, and on a limited basis, the underlying molecular mechanisms of ciliopathies. Mammalian cells (RPE1 and HeLa cells) were used previously to localize hPoc5 to centrioles and uncover a role for Poc5 in building the distal end of these structures (Azimzadeh et al., 2009). Although mammalian cell culture was valuable for elucidating a centriolar function for Poc5, the system can only provide limited information about the role of Poc5 in cilia formation/maintenance (especially in multiciliated tissues since RPE1 cells only have primary cilia), as well as in the context of development. The Winey Lab has previously collaborated with the Klymkowsky Lab because of the strength of *Xenopus laevis* as a system for studying basal body and ciliary functions in the context of development (Shi et al., 2014, 2015; Zhao et al., 2016).

We chose *Xenopus laevis* because with exception to unicellular ciliated organisms like Tetrahymena, ciliated tissues are often internal (e.g. motile cilia in the trachea) limiting the analysis that can be conducted in other mammalian models. Unlike other organisms, frogs respire through their skin and possess an external, accessible
ciliated epithelium containing both ciliated cells and non-ciliated mucus-secreting cells. The ciliated epithelium of Xenopus has been studied to address biological questions concerning ciliary function, including providing the first evidence of planar cell polarity signaling and directional fluid flow affecting the polarity of cilia/basal bodies (Brooks and Wallingford, 2015; Park et al., 2008). Furthermore, Xenopus embryos have all three types of cilia: motile, primary, and nodal. The motile cilia of the epithelium generate directional fluid flow, replenishing oxygen over the skin. Primary cilia are found in the frog neural tube and dysfunction of these cilia result in aberrant embryonic development due primarily to impaired signaling. Thirdly, nodal cilia are present in the gastrocoel roof plate (analogous to the embryonic node in mammals and the kupffer’s vesicle in zebrafish) where they contribute to establishing left-right asymmetry and patterning the embryo.

_Xenopus laevis_ (and more recently _Xenopus tropicalis_) has also served as an important model system for studying vertebrate embryology and development. Xenopus produce an abundance of large embryos that are tolerant to manipulation and develop externally (therefore accessible throughout development). Furthermore, egg production in Xenopus can be controlled for research purposes by stimulation in response to mammalian hormones (chorionic gonadotropin). Additionally, Xenopus embryos undergo fast development from the initial 90-minute cell cycle to the next eleven divisions (at 20-30 minute intervals) resulting in upwards of 4,000 cells (Heasman, 2006). At this stage, the mid-blastula embryo has an animal cap, an equatorial or marginal zone, and the vegetal mass, which give rise to the ectoderm, the mesoderm, and the endoderm, respectively. Dissected animal caps of blastula-stage
embryos, also known as ectodermal explants, have been used in previous studies because this multiciliated tissue provides exceptional accessibility and experimental tractability for analyses focusing on ciliogenesis (Dubaisi and Papalopulu, 2011; Hayes et al., 2007). Furthermore, ectodermal explants have been shown to differentiate at a similar rate to intact embryos to form epidermal tissue, containing ciliated cells and non-ciliated mucus secreting cells, in the absence of the mesoderm and endoderm (as well as their signaling systems) (Zhao et al., 2016). Due to these experimental advantages, ectodermal explants have been used extensively in previous collaborations between the Winey Lab and Klymkowsky Lab, as well as in my thesis work (Shi et al., 2014; Shi et al., 2015; Zhao et al., 2016).

As embryonic development progresses, zygotic transcription (as opposed to maternal transcription) begins at the end of the 12th cell cycle during a point in development known as the mid-blastula transition (Heasman, 2006). The mid-blastula transition initiates transcription of zygotic genes that in turn, provide important developmental cues for the cellular movement required for establishing the germ layers (endoderm, mesoderm, and ectoderm) during gastrulation. The endoderm forms the inner lining of internal organs and the gut. The ectoderm gives rise to the skin, brain, nervous system, and other external tissues. Lastly, the mesoderm forms muscle, the skeletal system, and the circulatory system. Since Poc5 has been implicated in scoliosis, it is important to note that the notochord is derived from mesoderm and during neurulation the notochord induces neural plate formation, eventually giving rise to the neural tube and then the spinal cord.
There are currently two Xenopus species used in biological research, *Xenopus laevis* and *Xenopus tropicalis*. *Xenopus tropicalis* has a small, diploid genome making it a useful model for generating transgenic reporter lines. In comparison, *Xenopus laevis* is an allotetraploid species with genetic redundancy that led to limited genetic tractability before the genome was sequenced and annotated. The sequenced genomes of both Xenopus species are available on the Xenopus Genome Database (www.xenbase.org), revealing orthologues of many human genes and enhancing the utility of Xenopus in the study of human disease. Despite the allotetraploid state of the *Xenopus laevis* genome, the introduction of translation-blocking and RNA splice-inhibiting antisense oligonucleotides known as morpholinos propelled the ability to knockdown expression of specific genes and were employed in this study to target POC5 (Heasman et al., 2000).

In this chapter, I describe preliminary analysis of Xenopus POC5 function (carried out in collaboration with Jianli Shi and Mike Klymkowsky), and focused on revealing a potential role for POC5 in the motile cilia (and the underlying basal bodies) of the epithelium.

**II. Results**

A BLAST search using human POC5 as the query sequence identified a POC5 ortholog in *Xenopus laevis*. Using the latest version of the *Xenopus laevis* genome (9.1 on Xenbase) we identified two POC5-related chromosomal scaffolds (due to *X. laevis* being tetraploid): the first (listed as poc5.L) is fully annotated and was used in the design of our studies, while the second (listed as poc5.S) is not annotated and therefore was not used. As visualized through a multiple sequence alignment, the *X. laevis* Poc5-L protein (572 amino acids in length) shares a high degree of sequence similarity
(overall identities 260/595 (44%), positives 359/595 (60%), gaps 43/595 (7%)) with the human Poc5 protein (575 amino acids in length), including the three characteristic centrin-binding repeats as well as the Poc5 box (Fig. 4-1 top panel). The Poc5 box is present in all Poc5 orthologues but is not found outside of the Poc5 family, providing confidence that we were studying the Xenopus Poc5 orthologue (Azimzadeh et al., 2009).

In addition to available sequence information, Xenbase provides a temporal expression profile for POC5-L as determined by microarray analysis (Fig. 4-1 bottom panel) (Yanai et al., 2011). This microarray data was generated from whole embryos at multiple stages of development using both *Xenopus laevis* and *Xenopus tropicalis* embryos. *Xenopus laevis* and *tropicalis* POC5 RNA appears to be supplied maternally at high levels and expression remains fairly constant throughout early development, including at the onset of cilia formation (Zhang and Mitchell, 2016).

**Localization of POC5 on the Ciliated Epithelium**

Human Poc5 has been previously shown to localize to centrioles in human cultured cells (Azimzadeh et al., 2009). *Tetrahymena* Poc5 localizes to cortical row basal bodies and basal bodies within the oral primordium (developing oral apparatus) but is absent from ciliary axonemes. Additionally, wholemount *in situ* hybridization data from the Zebrafish Poc5 scoliosis study revealed ubiquitous expression of *poc5* early in development followed by expression restricted to the head and bud region (and eventually the brain), suggesting a potential role in patterning the anterior-posterior axis (Patten et al., 2015).
In order to characterize POC5 localization in *X. laevis*, a chimeric polypeptide was constructed that contains the full-length POC5 sequence in-frame with a C-terminal GFP tag for visualization. This plasmid was sequenced prior to use to confirm the absence of mutations from cloning and POC5-GFP RNA was made for injection into Xenopus embryos.

![Figure 4-1. The Xenopus POC5 Orthologue is Highly Expressed.](image)

The top panel is a multiple sequence alignment showing a high degree of similarity between human and *X. laevis* POC5, with identical residues shaded black and similar residues shaded grey. Centrin-binding repeats are marked with dashed red boxes and the highly conserved Poc5 box is indicated by a dashed blue box. The bottom panel shows log expression of POC5 from microarray analysis in *X. laevis* and *X. tropicalis* whole embryos through early development (Yanai et al., 2011).
Localization of POC5-GFP within the ciliated epithelium was determined in ectodermal explants dissected from mid-blastula stage embryos (see Chapter 6: Materials and Methods). Fertilized eggs were injected with POC5-GFP RNA and ectodermal explants from these embryos were analyzed when intact, control embryos reached developmental stage 24, a point in development after ciliogenesis has occurred (Zhang and Mitchell, 2016). Explants expressing POC5-GFP were fixed and analyzed by confocal microscopy. A top-down view of an ectodermal explant shows POC5-GFP enrichment in multiciliated cells (detected by anti-acetylated α-tubulin) compared with the non-ciliated cells of the epithelium (Fig. 4-2A). As a control, fertilized eggs were injected with RNA encoding a membrane bound form of GFP (Shi et al., 2014). The POC5-GFP localization pattern differed from the membrane-bound GFP control that labels cell boundaries and suggests that POC5 is driving the observed localization pattern in multiciliated cells (Fig. 4-2B). Of note, it remains essential that the chimeric POC5-GFP polypeptide is deemed functional by rescuing morphant phenotypes (next section). At higher magnification, the observed localization is punctate and appears to be in the vicinity of the ciliary axonemes, but also to a high degree in the cytoplasm (Fig. 4-2C). High resolution structured-illumination microscopy (SIM) confirmed the presence of punctate foci that partially overlap with ciliary signal but did not reveal a discernible localization pattern within the ciliary axoneme (Fig. 4-2D). Unfortunately, localization of POC5-GFP was not consistent between repeated experiments (or across explants that were processed together) and was often found to be diffuse in the cytoplasm rather than accumulating in multiciliated cells. It was unclear whether this lack of consistency was
due to technical issues or real biological consequences of overexpressing POC5 in this tissue.

Figure 4-2. Localization of POC5-GFP. (A) Top-down view of an ectodermal explant injected with POC5-GFP, fixed, and stained with anti-acetylated α-tubulin (cilia) and GFP. The tissue contains ciliated cells and non-ciliated mucus secreting cells. Imaged at 20X. (B) Left panel shows membrane-bound GFP marking cell boundaries. Right panel shows POC5-GFP. Imaged at 40X with 4X zoom. (C) Side-on view of multiciliated cells imaged at 40X. (D) SIM image of a single multiciliated cell at 100X.
Also, if POC5-GFP localizes to the ciliary axoneme, then this result would be unexpected given the lack of Poc5 ciliary signal in RPE1 cells or Tetrahymena cells (Azimzadeh et al., 2009). Nonetheless, this localization should be further pursued since RPE1 cells only possess primary cilia, therefore differences in localization may be due to the type (or function) of cilia as well as differences between unicellular eukaryotes (Tetrahymena) and vertebrates.

Due to the complex results from cellular localization of the POC5-GFP fusion protein, a secondary method of localization was employed that utilized a rabbit anti-human POC5 polyclonal antibody that was previously untested in Xenopus (Bioss USA, catalog number bs-7822R). Ectodermal explants were prepared from uninjected embryos and fixed explants were stained with both mouse anti-acetylated α-tubulin to mark cilia and the rabbit anti-POC5 antibody. Similar to localization of POC5-GFP, the anti-POC5 antibody revealed an accumulation of POC5 at ciliated cells and at higher magnification, this localization seemed to be at ciliary axonemes and primarily basal bodies (Fig. 4-3). Once again, the result of using this antibody was not consistent across explants that were processed together or in additional repeated experiments. The POC5 signal was often diffuse throughout the cytoplasm and repeated experiments did not recapitulate the observed enrichment to multiciliated cells. In conclusion, localization of POC5 to multiciliated cells is not unambiguous. By using POC5-GFP and an anti-POC5 antibody, there is promising potential for POC5 localizing to a structure found within ciliated cells (ciliary axonemes and/or basal bodies). Despite the inconsistencies, both methods of localizing POC5 resulted in accumulation in the multiciliated cells of the epithelium and potentially to the ciliary axoneme.
The Effect of Loss of POC5 on the Ciliated Epithelium

To investigate the functional role of POC5 in cilia formation, we had GeneTools, LLC. design and synthesize a translation-blocking antisense oligonucleotide (morpholino, MO).

**Figure 4-3. Localization of POC5 with Anti-POC5 Antibody.** Top-down view of an ectodermal explant from an uninjected embryo, fixed, and stained with anti-acetylated α-tubulin (cilia) and a rabbit anti-human POC5 polyclonal antibody (Bioss USA). Top panels were imaged at 40X and bottom panels show higher magnification image of a single multiciliated cell at 63X.
When the POC5 MO sequence was used in a BLAST search against the 9.1 version of the *X. laevis* genome, the only match was a site within POC5.L therefore the MO should specifically target POC5. The designed POC5 MO is 25 nucleotides in length and spans the translation start site (blocking translation) with binding occurring 2 nucleotides upstream (Fig. 4-4 top panel). The effect of down-regulating POC5 was characterized by injecting fertilized eggs with either a control MO or the POC5 MO. Initially, the effect of depleted POC5 was assessed on whole Xenopus embryos to provide a clue as to how POC5 may be contributing to proper embryonic development. Control and POC5 morphant embryos were allowed to develop to stage 33 and then live embryos were analyzed on a light microscope. POC5 morphant embryos were distinct from control MO-injected embryos in that POC5 morphants displayed two gross anatomical defects commonly seen in ciliopathy mutants: a marked body axis curvature and edema (Fig. 4-4 bottom panel). Intriguingly, POC5 morphant embryos that were allowed to develop to a later stage alongside uninjected embryos displayed a localized kinking of the body (Fig. 4-5 top panel) and the notochord (Fig. 4-5 bottom panel) reflective of the deformation observed in the POC5 idiopathic scoliosis zebrafish study (albeit prior to spine formation) (Patten et al., 2015).

Since ciliopathy-like defects can arise from either underlying mechanical or inductive signaling issues, both avenues were initially explored. Previous collaborative work between the Winey and Klymkowsky Labs found that the Centrin 2 (CETN2) MO affected the levels of BMP4 and FGF8, two signaling molecules important for embryonic development (Shi et al., 2015). In addition, EFHC1 was found to localize to the ciliary
axoneme and down-regulation of EFHC1 using a MO increased the levels of the signaling molecule Wnt8a (Zhao et al., 2016).

Figure 4-4. POC5 Morphants Display Ciliopathy-Like Defects. The top panel is a schematic of the POC5 MO binding to the translation start site to inhibit translation of POC5. The bottom panels highlight wholemount embryonic defects (twisted body axis and edema) in embryos injected with the POC5 MO that are not present in control MO-injected embryos. Arrow points to edema. Image taken by Jianli Shi.
Figure 4-5. Late Stage POC5 Morphants Display Scoliosis-Like Defects. The top panels show idiopathic scoliosis in mutant POC5 zebrafish from Patten et al., 2015. Below the zebrafish data, late stage POC5 morphant Xenopus embryos exhibit scoliosis-like kinking of the body axis when compared with uninjected embryos. Xenopus POC5 morphant embryos stained to show notochord development reveal additional late stage scoliosis-like defects. Arrow indicates region of abnormal notochord development (bottom panel).
Taking these effects on signaling into account, POC5 and control morphant embryos at stage 11 (prior to ciliogenesis) were tested for changes in gene expression using standard RT-PCR. Unlike these previous studies, down-regulation of POC5 did not have a detectable effect on the levels of BMP4, Wnt8, FGFR1a, or FGF8 (Fig. 4-6). It would be important to repeat this RT-PCR procedure by testing for gene expression changes in POC5-MO injected embryos after the onset of ciliogenesis to determine if signaling is affected later in development. Also, the effectiveness of the POC5 MO has not been assessed so the lack of effect on translation could be due to low efficacy of the morpholino. Nonetheless, due to the lack of apparent effect on signaling seen from RT-PCR the focus was shifted to determining if the ciliopathy-like defects observed in wholemount POC5 morphant embryos was a structural consequence of improper cilia formation.

To determine the effect of loss of POC5 on cilia formation, fertilized eggs were co-injected with either a control MO or the POC5 MO and a RNA encoding a membrane-bound GFP as a tracer for injections. The experimental approach for generating ectodermal explants was identical to the above mentioned localization strategy, where explants were isolated from mid-blastula embryos and fixed at stage 24. These explants were then stained with an anti-acetylated α-tubulin antibody to mark ciliary microtubules and an anti-GFP antibody to determine the region of injection (morpholino). As shown in Fig. 4-7, ectodermal explants from control morphant embryos exhibited an even distribution of ciliated cells whether overlapping with the site of morpholino injection (high GFP) or not. In contrast, ectodermal explants from POC5 morphants displayed fewer ciliated cells, especially within the region of high GFP (MO).
It is also worth noting that the region marked with GFP was smaller than the region of cilia depletion, suggesting that the MO was capable of diffusing through the embryo a greater distance than the GFP RNA (as expected).

Figure 4-6. Signaling Gene Expression is Not Altered in POC5 Morphants. The left panel shows an increase in BMP4 levels and a marked decrease in FGF8 levels (while Wnt8 remains unchanged) when Centrin2 (CETN2) is down-regulated (Shi et al., 2015). The right panel shows the results from standard RT-PCR of stage 11 embryos injected with a control (Con) MO or the POC5 MO. Gene expression of four major signaling molecules (BMP4, Wnt8, FGFR1a, and FGF8) was not effected by down-regulation of POC5. ODC is an unchanged control.
Figure 4-7. Explants from POC5 Morphant Embryos Have Fewer Ciliated Cells. The top series of panels show an ectodermal explant injected with a control MO, fixed, and stained with anti-acetylated α-tubulin and GFP. Explants from control MO-injected embryos have an even distribution of ciliated cells even in regions of high GFP (MO). The bottom panels show an explant injected with the POC5 MO. Explants from POC5 MO-injected embryos have a reduced number of ciliated cells in the region of high GFP (MO). All images taken at 20X.
Further quantification of this phenotype is needed in order to determine the extent of ciliated cell reduction on the epithelium, as well as whether down-regulation of POC5 also leads to a reduced number of cilia per multiciliated cell.

III. Discussion

Even though POC5 is conserved across eukaryotes, there is currently no published information about a potential role for POC5 in the assembly or maintenance of basal bodies, as well as cilia. Determining what tissues and regions of the Xenopus embryo that POC5 localizes to during development is a critical first step. Two methods were employed and described in this thesis that ultimately did not generate conclusive cellular localization information. It is possible that given the biological function of POC5 a large tag is not tolerated on its C-terminus. The functionality of a chimeric POC5 polypeptide should be assessed by co-injecting this polypeptide with the POC5 MO (or in CRISPR-mediated mutagenesis experiments) to determine if it can rescue morphant/mutant phenotypes. For future work, localization of an N-terminally tagged GFP-POC5 should be attempted. In addition, a cloning strategy has been initiated to determine localization of POC5 with a smaller HA tag on either the N- or C-terminus. Aside from utilizing fluorescent tags, generating a probe from a subcloned fragment of the POC5 coding region for in situ hybridization studies could provide important information about the spatiotemporal expression of POC5 during embryonic development (ubiquitous expression versus restricted expression to specific tissues). The pattern of expression observed from in situ hybridization may guide further analysis to the ciliated epithelium or a tissue not hypothesized to require POC5 function. Thus far, POC5 seems to have a conserved localization to microtubule-organizing centers,
including the basal bodies of Tetrahymena cells and the centrioles of human cultured cells (Azimzadeh et al., 2009). Once localization in Xenopus is established, it will be worthwhile gaining a higher resolution perspective to determine precisely what structure POC5 localizes to within that given tissue. In the ciliated epithelium, utilizing antibodies to mark cilia and basal bodies (centrin) should be sufficient. Lastly, this pilot study focused on localization of POC5 to the motile cilia of the epithelium but follow-up analysis could also reveal both potential localization and functionality in primary cilia, as well as nodal cilia in the gastrocoel roof plate. Extending localization and functional analysis to other cilia types would potentially provide novel information to the field and could highlight different functions for a basal body/ciliary gene depending on the type of cilia.

Despite the preliminary nature of the Xenopus POC5 work highlighted in this thesis, the wholemount embryonic defects seen in POC5 morphants are exciting because they mirror defects commonly observed when basal body and ciliary genes are down-regulated. It is not clear what mechanistically is driving these embryonic defects, but ectodermal explants from POC5 morphants displayed a marked reduction in the number of ciliated cells. This morphant phenotype may provide a structural clue for what is contributing to the wholemount defects. In order to understand the function of POC5 in basal bodies and/or cilia, further work that builds upon this preliminary data is necessary. First, the effectiveness of down-regulating POC5 using the POC5 MO should be determined. Immunoblot analysis could examine this down-regulation by comparing embryos injected with RNAs encoding GFP-tagged POC5 (assuming a construct is built that can localize POC5 consistently) and either the control MO or the
POC5 MO. The protein levels of GFP-POC5 (visualized with an anti-GFP antibody) should only be reduced in embryos injected with the POC5 MO not in the control MO-injected embryos. This version of tagged POC5 could also be used to potentially rescue the observed reduced number of ciliary cells by co-injecting the POC5 MO and tagged POC5. Furthermore, conditions for morphant analysis in ectodermal explants need to be determined that can consistently recapitulate the effect on the number of ciliated cells, in order to quantify this effect through a semi-automated ImageJ script (Shi et al., 2014). Furthermore, this can be extended to analyzing the effect of the POC5 MO on the number of cilia per multiciliated cell. Aside from cilia, POC5 has been shown to preferentially localize to basal bodies in Tetrahymena and may be restricted to basal bodies in Xenopus. Aberrant functions of a basal body component can lead to improper cilia formation and/or maintenance. Adding to the complexity, a reduction in the number of cilia does not always stem from a reduction in the number of basal bodies (Zhao et al., 2016). Electron microscopy may have to be used to determine if there are underlying basal body defects, such as issues with full elongation and maturation of basal bodies (drawing a parallel to the observed phenotype in hPoc5-depleted HeLa cells), docking of basal bodies, or cilia formation specifically (Azimzadeh et al., 2009).

As a future plan for the POC5 work, guide RNAs for CRISPR-mediated mutagenesis have been designed and constructed to compare mutant versus morphant phenotypes (in collaboration with Tyler Square in the Medeiros Lab (EBIO)). In zebrafish, mutant phenotypes across multiple genes using zinc finger nucleases/TALENS/CRISPR were found to poorly correlate with morphant phenotypes, suggesting that different methods for mutational analysis could result in varying
outcomes (Kok et al., 2015). Once further studies have been conducted to ascertain
the function of POC5 through phenotypic analysis of morphant embryos, similar
analysis could be conducted using CRISPR-mediated mutagenesis. This sort of
comparative analysis has not been previously conducted in Xenopus. The preliminary
work in this thesis and the above mentioned follow-up work would collectively explore
how POC5 may be functioning in basal bodies and cilia, both of which are vital
structures for the developing Xenopus embryo. Additionally, it could shed light on the
validity of conducting mutant analysis with solely morpholinos or CRISPR.
Chapter Five: Materials and Methods

*T. thermophila* strains and culture media

The wild-type strain used as a control comparison for assaying growth rates and for phenotypic analysis of the *sfr1Δ*, *poc5Δ*, *sfr1Δ rescue*, and *poc5Δ; sfr1Δ* strains was derived from the progeny of a cross between B2086 and CU428 (each parental strain from the Tetrahymena Stock Center, Cornell University, Ithaca, NY). Cells were grown in 2% super-peptose (SPP) media (Orias et al., 2000) at 30°C for Sfr1 and Poc5 localization experiments. To assess dynamic Poc5 localization, cells were either grown in SPP media, starved overnight in 10 mM Tris-HCl (pH 7.4), or starved overnight in 10 mM Tris-HCl (pH 7.4) and then released back into SPP media for 3-5 hours before imaging. For phenotypic analysis of the *sfr1Δ* strain, cells were grown in EPP media (Orias and Rasmussen, 1976) at either 30°C or 37°C, as described. For phenotypic analysis of the *sfr1Δ* strain during starvation, cells were grown overnight in EPP to mid-log density (cell density measured by Z2 Coulter Counter by Beckman Coulter) at 30°C or 37°C, washed with 10 mM Tris-HCl (pH 7.4), and resuspended in 10 mM Tris-HCl (pH 7.4) for either 24 or 48 hours at 30°C or 37°C. Analysis of the *sfr1Δ rescue* strain was performed by growing cells overnight in EPP and starving cells in 10 mM Tris-HCl (pH 7.4) for 5 hours (to eliminate dividing cells) before fixation. EPP media was used for phenotypic analysis of all *sfr1Δ* strains for consistency between analyses and because EPP lacks a metal chelator, therefore less CdCl$_2$ needed to be used for induction. For phenotypic analysis of the *poc5Δ* and *poc5Δ; sfr1Δ* strains, cells were grown in SPP media (61) at 30°C or 37°C, as described.

*cdNA synthesis and sequence comparison of Sfr1 homologues*
Tetrahymena *SFR1* (TTHERM_00463380) cDNA was generated with the Superscript® II One-Step RT-PCR system (Invitrogen) and then cloned into the pBluescript KS(-) construct for sequencing. The confirmed cDNA sequence of *SFR1* was logged at the Tetrahymena Genome Database Wiki with the gene information now searchable by name (“Sfr1”) in addition to its TTHERM identifier. The Tetrahymena Sfr1 protein sequence was used as the query sequence for a BLAST search of the *Paramecium tetraurelia* genome (Paramecium Genome Database) to uncover the Paramecium Sfr1 homologue (GSPATP00030126001), which serves as the reciprocal best BLAST hit. Multiple sequence alignment of the two Sfr1 homologues and boxshading of residues was performed using ClustalW and Boxshade, respectively (ExPASy).

**Plasmids and strain construction**

The exogenous N-terminal GFP fusion used to localize Sfr1 was generated by cloning *SFR1* first into the pENTR4 Dual Selection Vector (Invitrogen) and then subcloning into pBS-*MTT1*-GFP-gtw (Winey et al., 2012) utilizing the Gateway cloning system (Invitrogen). This resulted in N-terminally tagged GFP-Sfr1 under control of the *MTT1*-inducible promoter and at the rpl29 locus (conferring cycloheximide resistance). Exogenous Sfr1 was co-expressed with endogenous Poc1 (Pearson et al., 2009a) tagged with mCherry at its C-terminus for co-localization studies. The endogenous C-terminal mCherry or GFP fusions used to localize Poc5 were built by using the pmCherry-LAP-Neo2 or pGFP-LAP-Neo2 vectors (conferring paromomycin resistance) and by cloning 1kb sequences flanking *POC5* upstream and downstream (Stemm-Wolf et al., 2009). This was designed for homologous recombination into the *POC5* locus.
with a C-terminal mCherry or GFP tag under control of the endogenous promoter. The endogenous C-terminal Poc5-GFP fusion was used for starve and release experiments and immunoEM. Endogenous Poc5-GFP was co-expressed with endogenous Poc1-mCherry (similar to Sfr1 colocalization; Pearson et al., 2009), Sas6a-mCherry (Culver et al., 2009), or RSPH9-mCherry (courtesy of Chad Pearson, University of Colorado Anschutz Medical Campus) for co-localization studies. Overexpression of Poc5 used an N-terminal Poc5-mCherry fusion in the endogenous POC5 locus under control of the cadmium-inducible MTT1 promoter. Macronuclear transformation of DNA constructs into T. thermophila was performed using a PDS-1000 particle bombarder (Biorad) in accordance with previous studies (Bruns and Cassidy-Hanley, 2000). For all experiments using the inducible MTT1 promoter, constructs were induced by incubating cells overnight in CdCl₂ (0.5 µg/ml for SPP and 0.05 µg/ml for EPP).

**Generation of sfr1Δ, sfr1Δ rescue, poc5Δ, and poc5Δ; sfr1Δ strains**

The strains used for micronuclear transformation (as described in (Bruns and Cassidy-Hanley, 2000)) were B2086/crNeo and CU428/crNeo, both of which contain two frame-shift mutations in the NEO gene in the macronucleus to prevent DNA elimination of the selectable marker following micronuclear transformation (Mochizuki et al., 2002; Yao et al., 2003). The construct used to delete SFR1 contains the NEO2 cassette along with 1kb of flanking sequence upstream of the start codon and 1kb of flanking sequence downstream of the stop codon (Gaertig et al., 1994). The construct used to delete POC5 contains a codon-optimized NEO2 cassette along with 1kb of flanking sequence upstream of the start codon and 1kb of flanking sequence downstream of the stop codon. DNA was coated on gold particles and biolistic
bombardment into conjugating cells (using the Biorad PDS-1000) was conducted. Cells were allowed to finish conjugation after bombardment by maintaining them in 10 mM Tris-HCl (pH 7.4) overnight before transfer into SPP media for ensuing drug selection. Integration into the SFR1 and POC5 loci was confirmed by PCR. Micronuclear knockout heterokaryons of two different mating types were generated using star strains (Hai et al., 2000). Heterokaryons were mated to eliminate SFR1 and/or POC5 from both nuclei (somatic and germline), creating sfr1Δ, poc5Δ, and poc5Δ; sfr1Δ cells. Deletion of SFR1 and replacement with the Neo2 cassette was validated by PCR and RT-PCR. Wild-type SFR1 forward primer:

TAAGTTTAGTTTAGAGAAACAATATAGGCTTATGAGAAGG. Wild-type SFR1 reverse primer: TCAATCAAATTTCTTTTTTTATTTATGAAAGG. Neo-cassette forward primer: AATCTACTAATTTCATTTTTTCATAAGC. Neo-cassette reverse primer: TCCATACTTTGAAGATATCAAGC. Wild-type POC5 forward primer: ATGAATTCAAATAAGAATCAACCAAAGAAGAAA. Wild-type POC5 reverse primer: TTTTTGTTAGTTGTATGTGTTTATTGC. For RT-PCR, RNA was isolated from WT and sfr1Δ cells using the RNeasy Mini and QIAshredder kits supplied by Qiagen. cDNA was generated with the Superscript® IV One-Step RT-PCR system (Invitrogen). Ensuing standard PCR used a pair of primers to detect SFR1 and a pair to detect NEO. SFR1 forward primer: TAAGTTTAGTTTAGAGAAACAATAT- AGGCTTATGAGAAGG.

SFR1 reverse primer: TCAATCAAATTTCTTTTTTTATTT- AATTTATGAAAGG.

NEO forward primer: ATGGCAAGCTTGGATGGATTGCACGC. NEO reverse primer: TCAGAAGAACTCGTCAAGAAGGCG. The sfr1Δ rescue strain was made by re-introducing SFR1 in sfr1Δ cells by biolistics in order to incorporate pBS-MTT1-GFP-Sfr1
into the somatic macronucleus. Transformed cells were selected by adding cycloheximide (pBS-MTT1-GFP-Sfr1 confers cycloheximide resistance) and SFR1 reintroduction in the sfr1Δ rescue strain was through CdCl₂ induction.

**Fluorescence imaging**

Image collection was all conducted at room-temperature using an Eclipse Ti inverted microscope (Nikon, Japan) with a CFI Plan Apo VC 60x H numerical aperture 1.4 objective (Nikon, Japan) and a charge-coupled device camera (CoolSNAP HQ2, Photometrics, Tuscon, AZ). Image acquisition was through the Metamorph Imaging Software (Molecular Devices, Sunnyvale, CA).

For live-cell imaging of GFP- and mCherry-tagged proteins, cells were washed in 10 mM Tris-HCl (pH 7.4), pelleted and placed on microscope slides (VWR, Radnor, PA). Image averaging was conducted by drawing a 1μm X 1μm box around a given basal body pair (for 58 total basal body pairs). These images were then averaged by making a Z-stack projection with summed fluorescence. For immunofluorescence, cells were fixed with 3% formaldehyde followed by 15% ethanol (Stuart and Cole, 2000). Fixed cells were placed on poly-L-lysine coated multi-well slides (Polysciences Inc., Warrington, PA) and blocked for 1 hour with PBS + 1% BSA before primary antibody incubation. The Tetrahymena Cen1 antibody was used at 1:2,000 (Stemm-Wolf et al., 2005) and antibody incubation was carried out overnight at 4°C. The secondary antibody used was the anti-rabbit Alexa Fluor 488 (Invitrogen) at 1:1,000 and for a two-hour room-temperature incubation. Primary and secondary antibodies were in PBS + 1% BSA and washes in between antibody incubations were performed using PBS + 0.1% BSA. Cells were mounted in Citifluor (Ted Pella Inc., Redding, CA).
Immunoelectron microscopy

Tetrahymena cells were prepared for ultrastructural localization by high-pressure freezing with a Wohlwend Compact 02 high-pressure freezer (Technotrade International, Manchester, NH) followed by freeze substitution (Giddings et al., 2010; Meehl et al., 2009). Cells were freeze-substituted in 0.25% glutaraldehyde and 0.1% uranyl acetate in acetone and embedded in Lowicryl HM20. Immunoelectron microscopy was conducted by making 70-nm-thick sections that were incubated with a rabbit polyclonal GFP antibody (generously gifted by Chad Pearson) and then a 15 nm gold-conjugated anti-rabbit secondary antibody (Ted Pella, Redding, CA). Samples were imaged using a Philips CM 100 transmission electron microscope. The location of gold particles was analyzed relative to known structural domains within basal bodies as previously described (Kilburn et al., 2007).

Western blot analysis

Cells were grown overnight at 30°C in EPP to mid-log density and then starved for 5 hours (incubated with or without 0.05 µg/ml CdCl₂ to induce the GFP fusion). Whole-cell extracts were made by lysing ~30,000 cells (based on cell density measurements from Z2 Coulter Counter by Beckman Coulter) in lysis buffer made of 50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, plus protease inhibitors (0.2 mg/ml soybean trypsin inhibitor, 10 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin A, 10 µg/ml TAME, and 10 µg/ml benzamidine). 6,000 cells were loaded per lane onto a 10% SDS-PAGE gel and were transferred to an Immobilon-P PVDF membrane (EMD Millipore, Billerica, MA) using a transfer apparatus (Bio-Rad Labs, Hercules, CA). The membrane was incubated in
TBS + 0.05% Tween 20 with 1% BSA. Primary antibodies were a polyclonal A.v. rabbit anti-GFP primary antibody (Takara Bio USA, Mountain View, CA) at 1:1,000 and a monoclonal mouse anti-Atu1 (12G10; a generous gift of Joseph Frankel, University of Iowa, Iowa City, USA) at 1:2,500 (both in TBST + 1% BSA). Secondary antibodies were anti-rabbit IR 800 and anti-mouse IR 680 both at 1:10,000 (in TBST + 1% BSA) and signal was detected using a LI-COR Odyssey infrared imager (LI-COR, Lincoln, NE).

The normalized mean fluorescence from split-channel acquired images was calculated by drawing a box within the GFP signal and obtaining the mean fluorescence value using ImageJ. This value was then background subtracted by using the same size box to measure the background mean fluorescence. Finally, the ratio of GFP to alpha-tubulin (loading control) fluorescence was taken to get the final, normalized mean fluorescence values.

**Analysis of sfr1Δ, poc5Δ, and poc5Δ; sfr1Δ strains**

**Calculation of growth rates**

A Z2 Coulter Counter (Beckman Coulter) was used to measure the cell density of log-phase cultures to determine growth rates. The time-course of growth was initiated with all strains set to a beginning density of 0.5x10^5 cells/mL. Cells were allowed to grow for 8 hours in SPP at 30°C or 37°C with measurements taken at 0, 4, and 8 hours. The wild-type strain used is progeny from B2086 x CU428 (as described in *T. thermophila* strains and culture media).

**Quantification of cell length and cortical row basal bodies**

Measuring the cell length (in μm), basal body density (basal bodies/10 μm), and the number of cortical rows was through ImageJ analysis on fixed cells (wild-type, sfr1Δ,
poc5Δ, poc5Δ; sfr1Δ, or sfr1Δ rescue) with basal bodies labelled with the Cen1 antibody. Cell length was calculated using the line tool function on scaled images. A Z-stack of the entire cell was captured using Metamorph Imaging Software (Molecular Devices, Sunnyvale, CA) in order to count the number of cortical rows in the entirety of the cell. Basal body density was determined by measuring a 10 µm region of three separate cortical rows using ImageJ, with two rows on the side containing the oral apparatus and the third row on the opposite side of the cell. The total number of cortical row basal bodies was determined by multiplying all three cellular parameters as previously described (Stemm-Wolf et al., 2013).

**Xenopus laevis embryo manipulation and analysis**

Xenopus embryos were staged and ectodermal explants were generated by following standard procedures (Nieuwkoop and Faber, 1967; Shi et al., 2011; Zhao et al., 2016). mMessage mMachine kits (supplied by Ambion) were used to make transcribed capped mRNAs from linearized plasmid. For ectodermal explant studies, two-cell stage embryos were injected in the animal hemisphere region (performed by Dr. Jianli Shi). To trace injections, RNAs encoding GFP or GFP-CAAX (membrane-associated GFP) were co-injected with POC5 GFP fusions or the POC5 MO (Zhao et al., 2016). Ectodermal explants/animal caps were isolated from stage 8 mid-blastula embryos in 0.5x Marc’s Modified Ringers (MMR) solution and allowed to develop past ciliogenesis (~stage 18) (Shi et al., 2011; Zhao et al., 2016). RNA isolation, cDNA synthesis, and RT-PCR were conducted as previously described (Shi et al., 2015; Zhao et al., 2016). cDNA was synthesized from 1 µg of purified RNA using a Verso cDNA kit (Thermo-Scientific). The oligos used for RT-PCR analysis were BMP4 [U 5′-TGG TGG
ATT AGT CTC GTG TCC -3’ D 5’-TCA ACC TCA GCA GCA TTC C -3’]; Wnt8a [U 5’-TGA TGC CTT CAC TTC TGT GG-3’ D 5’-TCC TGC AGC TTC TCC TCT CC -3’]; FGFR1a [U 5’-GCG CAT TGG TGG ATA TAA GG -3’ D 5’- AGA CCG GCT TGT AGG ATT GG-3’]; FGF8 [U 5’-TGG TGA CCG ACC AAC TAA GC D 5’-CGA TTA ACT TGG CGT GTG G 3’]; Ornithine decarboxylase (ODC) [U 5’-CAG CTA GCT GTG GTG TGG -3’ D 5’-CAA CAT GGA AAC TCA CAC-3’].

**Morpholinos and plasmids**

Gene Tools, Inc. designed the POC5 morpholino that spans the translation start site (AAATGTCTTCTGACGAGGAGTATCG). The control morpholino used as a comparison in phenotypic analyses has been described previously (Shi et al., 2015; Zhao et al., 2016). The POC5 coding sequence was amplified by cDNA from wholemount embryos and then was subcloned into the pCS2mt-GFP plasmid to produce a C-terminally tagged GFP fusion.

**Immunofluorescence microscopy and imaging**

Embryos were fixed and stained as previously described (Dent et al., 1989). A mouse monoclonal anti-acetylated α-tubulin antibody was used to mark ciliated cells, while a chicken anti-GFP antibody (Immunology Consultants Lab, Inc.) was used to visualize GFP (POC5-GFP or co-injected GFP in morpholino studies) in ectodermal explants (excised ciliated epithelium). For the Poc5 antibody test, a rabbit anti-human POC5 polyclonal antibody was used, as supplied by Bioss USA. Imaging was performed using a Zeiss 510 Confocal Laser Scanning Microscope. To visualize notochord in late stage wholemount embryos, embryos were bleached and then stained
with the mouse monoclonal anti-keratin sulfate antibody MZ15 (Developmental Studies Hybridoma Bank).
Chapter Six: Conclusions

Basal bodies and centrioles are microtubule-organizing centers that utilize microtubules to template cilia at the cell surface and organize the mitotic spindle, respectively. These microtubule-based structures are conserved across many eukaryotes and dysfunctional assembly and/or maintenance of basal bodies and centrioles are associated with disease. Although basal bodies and centrioles have distinct functions, their structures are similar. In fact, centrioles are interconverted to basal bodies in multiple cell types during quiescence (Hodges et al., 2010; Carvalho-Santos et al., 2011; Kobayashi and Dynlacht, 2011). Basal bodies, like centrioles, are cylindrical, microtubule-based structures that typically display a nine-fold radially symmetric array of triplet microtubules (Allen, 1969; Pearson and Winey, 2009). Through electron microscopy and proteomic analysis, basal bodies have been further organized structurally into three distinct domains: the proximal end containing the cartwheel structure, the midzone, and the distal end containing the transition zone (Kilburn et al., 2007). In addition to characterizing the basal body ultrastructure, a molecular inventory of basal body components has been assembled, revealing conserved basal body components across eukaryotes (Hodges et al., 2010; Carvalho-Santos et al., 2011). Although an inventory exists, how these components function in assembling and maintaining the structure of basal bodies is still mostly unclear. Elucidating the functions of these components could provide important insight into the underlying mechanisms of diseases associated with these structures, especially ciliopathies.
Centrins are small (~20 kDa) calcium-binding proteins that are widely conserved in eukaryotes and ubiquitously associate with MTOCs, including centrioles, basal bodies, and the yeast centrosome (spindle pole body; SPB) (Andersen et al., 2003; Keller et al., 2005; Kilburn et al., 2007; Liu et al., 2007; Hodges et al., 2010). Humans have three centrins (Cetn1-3), two of which localize ubiquitously to centrioles (Cetn2 and Cetn3) (Errabolu et al., 1994; Middendorp et al., 2000). Previous Winey laboratory work in the ciliate, *Tetrahymena thermophila*, identified four Tetrahymena centrins, two of which (Cen1 and Cen2) share homology with human centrins 2 and 3 and localize to basal bodies (Stemm-Wolf et al., 2005). Complete genomic knockouts of *CEN1* and *CEN2* revealed a requirement for centrins in proper basal body assembly, particularly orienting newly assembled basal bodies, as well as maintenance (Stemm-Wolf et al., 2005; Vonderfecht et al., 2011; Vonderfecht et al., 2012). This previous work identified multiple important roles for centrins in basal bodies but it is not understood how centrins carry out these functions that have specific temporal and spatial requirements.

Although centrins have been extensively studied with respect to basal bodies and centrioles, the function of centrin-binding proteins is poorly understood. Given the multifaceted functions of centrin, it is thought that temporal and spatial constraints on centrin are provided by centrin-binding partners. Centrin-binding proteins have been identified across eukaryotes by searching for proteins that contain centrin-binding repeats/Sfi1-repeats with an embedded, conserved sequence motif, Ax7LLx3F/Lx2WK/R (Kilmartin, 2003; Li et al., 2006). Using this conserved sequence motif, a large family of 13 centrin-binding proteins (SFR family; Sfr1-13) was uncovered in Tetrahymena (Stemm-wolf et al., 2013). Prior to my thesis studies, only Sfr13 had
been characterized, revealing a role for Sfr13 in separating and stabilizing basal bodies. Outside of the SFR family in Tetrahymena, five centrin-binding proteins have been identified in humans, of which only Sfi1 and Poc5 have been analyzed to any extent (Kilmartin, 2003; Azimzadeh et al., 2009). The only published functional information about a human centrin-binding protein is for human Poc5 (hPoc5), which was found to localize to centrioles and have an important function in building the distal end of these structures by aiding in full elongation and maturation (Azimzadeh et al., 2009). There is currently no published study that characterizes the function of Poc5 in basal bodies.

My thesis work entailed the characterization of two centrin-binding proteins, Sfr1 and Poc5, in *Tetrahymena thermophila*. Tetrahymena cells have a highly organized cytoskeleton with cortical rows of basal bodies that run along the anterior-posterior axis of the cell and a basal body-comprised feeding structure, known as the oral apparatus (Bayless et al., 2016). Tetrahymena has served as a valuable system for studying basal bodies because of the high level of organization of basal bodies as well as the ability to dissect basal body functions specific to assembling or maintaining these structures.

Sfr1 is a member of the SFR family of centrin-binding proteins in Tetrahymena, while ttPoc5 lies outside of this family and contains a distinguishable second signature motif (first being centrin-binding repeats) called the Poc5 box that is highly conserved across Poc5 orthologues (Azimzadeh et al., 2009). Sfr1 and ttPoc5 both localized to basal bodies but behaved in notably different ways. Exogenously expressed Sfr1 under control of the cadmium-inducible *MTT1* promoter localized to all Tetrahymena basal bodies, including cortical row basal bodies and the mature oral apparatus. It is unclear
what domain of Sfr1 is driving this basal body localization, but preliminary domain analysis showed that expression of the N-terminus of Sfr1 (which is N-terminal to the centrin-binding repeats) was capable of localizing to basal bodies. In contrast to Sfr1, endogenously expressed ttPoc5 localized to only a subset of cortical row basal bodies and the developing oral apparatus but not to the mature oral apparatus. This localization pattern was further elucidated, with enriched ttPoc5 observed specifically in assembling basal bodies. To support localization of ttPoc5 to assembling basal bodies, Poc5-GFP signal was moderate during logarithmic growth (only minimal basal body assembly occurs during growth), disappeared in media starvation conditions (no new basal body assembly in starved cells), and was highly enriched in starved and released cells (corresponding with a synchronous wave of new basal body assembly).

Localization of ttPoc5 to basal bodies was revealed to be dynamic, with ttPoc5 basal body incorporation occurring after the cartwheel structure was built (using Sas6a-mCherry as a marker) but before the slow incorporating basal body component, Poc1-mCherry, resides in basal bodies. Removal of ttPoc5 from basal bodies was shown to be prior to the onset of cilia formation at some stage in basal body maturation. Further analysis of this dynamic incorporation and removal would be valuable to correlate this dynamic behavior with stages in basal body assembly/maturation (from a structural perspective) using electron microscopy.

The localization of Sfr1 and ttPoc5 within basal bodies was uncovered by performing immunoelectron microscopy (immunoEM). For both analyses, a gold-conjugated antibody against GFP was utilized to visualize Sfr1 and ttPoc5 with the presence of gold particles. Serial sections of Tetrahymena basal bodies revealed Sfr1
localization throughout the basal body microtubule scaffold (proximal, midzone, and distal regions) with enrichment primarily in the basal body midzone and proximal domains. The ultrastructural basal body localization of Sfr1 partially overlapped with that of centrin, which was previously found to be enriched in the microtubules at the basal body midpoint and at the distal transition zone, but also resided at the site of new basal body assembly where Sfr1 was notably absent (Stemm-Wolf et al., 2005; Kilburn et al., 2007). The ultrastructural localization of ttPoc5 was also elucidated using immunoEM but with starved and released cells (to enrich Poc5 signal) that expressed Poc5-GFP at endogenous levels. Although this analysis was preliminary, Poc5 was found to localize to both the proximal and distal ends of the basal body, suggesting a potentially dynamic population of Poc5 within the basal body ultrastructure. An expansion of the immunoEM dataset from starved and released Poc5-GFP cells will be required to dissect this ultrastructural basal body localization further and potentially uncover temporal changes in localization.

Overexpression of Sfr1 and ttPoc5 led to basal body defects, indicating that the level of expression of both centrin-binding proteins is important. Sfr1 overexpression resulted in multiple basal body defects indicative of a breakdown in regulating basal bodies, such as mis-oriented basal bodies along cortical rows and aberrant spacing of basal bodies. With regards to Poc5, the transient nature of endogenously expressed ttPoc5 incorporation and removal from basal bodies, suggested specific temporal functional requirements for Poc5 in basal bodies. To explore this further, Poc5 was exogenously expressed by placing Poc5 under the control of the cadmium-inducible MTT1 promoter. Overexpression of Poc5 resulted in localization of Poc5 to all cortical
row basal bodies rather than localization limited to assembling basal bodies as was seen with endogenously expressed Poc5. Interestingly, the cortical row basal body signal was altered with overexpressed Poc5 but overexpression did not drive localization to the mature oral apparatus, indicating a potentially different requirement for Poc5 in cortical row and oral apparatus basal bodies. Unique to Poc5 overexpression, these cells also exhibited linear Poc5+ fibers that were previously seen in chicken DT40 cells (Dantas et al., 2013). Similar to that previous analysis, centrin was found to also localize to these fibers. It is unclear why overexpression of ttPoc5 and not Sfr1 (or other basal body components in Tetrahymena) led to formation of these fibers (of unknown function).

To determine the function of Sfr1 and ttPoc5 in Tetrahymena basal bodies, complete genomic knockouts were generated and confirmed by PCR and RT-PCR (for sfr1Δ cells) (Hai et al., 2000). Cells lacking SFR1 (sfr1Δ cells) or POC5 (poc5Δ cells) were viable and could be passaged indefinitely. From a gross morphological standpoint, both sfr1Δ and poc5Δ cells appeared healthy (not rounded or dying) and did not display abnormal swimming behavior. Time-course analysis of growth rates using WT and sfr1Δ cells revealed a similar growth rate at both 30°C (optimal growth temperature) and 37°C (temperature used to identify temperature-sensitivity). Both sfr1Δ and poc5Δ cells were fixed and stained with centrin to visualize basal bodies for phenotypic analysis. Basal body organization along cortical rows as well as proper orientation was maintained in sfr1Δ and poc5Δ cells. Interestingly, loss of Sfr1 did not affect cell length but resulted in a significant increase in basal body density (along cortical rows), an increase in the number of cortical rows, and the total number of
cortical row basal bodies. Even though sfr1Δ cells assembled more basal bodies during growth, excess basal bodies were not maintained, as sfr1Δ cells had a basal body density and a total number of basal bodies similar to WT cells after starvation. Importantly, when SFR1 was reincorporated in sfr1Δ cells (using the cadmium-inducible MTT1 promoter) the basal body overproduction phenotype was rescued, suggesting a potentially direct role for Sfr1 in modulating basal body production. Similar to sfr1Δ cells, poc5Δ cells during logarithmic growth did not have altered cell length and exhibited a significant increase in basal body density as well as an increase in the total number of cortical row basal bodies. This overproduction of cortical row basal bodies was observed at both 30°C (optimal growth temperature) and 37°C. Phenotypic analysis of poc5Δ cells is not complete and needs to be expanded to starvation (similar to phenotypic analysis in sfr1Δ cells). Also, reincorporation of Sfr1 was capable of rescuing the basal body overproduction phenotype in sfr1Δ cells, therefore this experimental approach should be employed for attempting to rescue the basal body overproduction phenotype in poc5Δ cells. Interestingly, loss of Sfr1 or ttPoc5 resulted in an overproduction of basal bodies along cortical rows which is to our knowledge a novel knockout basal body phenotype in Tetrahymena. Collectively, the work carried out in this thesis identified two centrin-binding proteins, Sfr1 and Poc5, which seem to have a similar antagonistic role in basal body production.

To explore a potential functional overlap between Sfr1 and Poc5 in regards to modulating the production of cortical basal bodies, a double knockout of SFR1 and POC5 was generated. Unlike sfr1Δ and poc5Δ single mutants, poc5Δ; sfr1Δ double knockout cells were not viable. Of note, the combined loss of both Sfr1 and Poc5 led to
cell death, whereas loss of either component individually resulted in viable cells. This is suggestive of functional redundancy between Sfr1 and Poc5 and an ability for either component to compensate. In order to specifically focus on the consequences of the combined loss of both Poc5 and Sfr1 on modulating basal body production, poc5Δ; sfr1Δ cells were fixed and stained with centrin alongside wild-type control cells. Excitingly, poc5Δ; sfr1Δ cells exhibited high levels of basal body production with significantly increased basal body density along cortical rows (8.84 basal bodies/10µm), exceeding what was observed in poc5Δ cells (7.49 basal bodies/10µm) or sfr1Δ cells (7.91 basal bodies/10µm). This observed overproduction of basal bodies in poc5Δ; sfr1Δ cells that surpassed what was seen in poc5Δ or sfr1Δ cells suggests that Poc5 and Sfr1 have redundant roles in modulating basal body production along cortical rows. When both components were genetically deleted, it resulted in basal body production potentially exceeding what the cell could tolerate, eventually leading to cell death. Uncovering the primary cause of lethality in poc5Δ; sfr1Δ cells is critical and will require further phenotypic analysis and experimental tools.

Intriguingly, it has recently been shown that human Cetn3 can antagonize human Cetn2 function in centriole assembly, highlighting a role for Cetn3 in inhibiting centrosome duplication (Sawant et al., 2015). Depletion of Cetn3 in HeLa cells resulted in the overproduction of centrioles, providing an exciting potential parallel to our observed overproduction of basal bodies upon loss of the centrin-binding proteins Sfr1 and Poc5. Given evidence that centrins can act antagonistically in regards to centriole assembly, centrin-binding proteins may act to bi-directionally modulate basal body production and therefore basal body number through interaction with a specific centrin.
Also, Tetrahymena cells are strikingly capable of maintaining a near constant number of cortical row basal bodies by adjusting basal body density relative to the number of cortical rows (Nanney, 1971; Frankel, 2008). Although it is unknown what mechanisms or signaling cues underlie the ability of Tetrahymena to regulate overall basal body number, ttPoc5 and Sfr1 are good candidates for molecular contributors of this process.

Collectively, my thesis research contributes to the paltry body of work on centrin-binding proteins by shedding light on novel basal body functions for two centrin-binding proteins, Sfr1 and Poc5. This work in Tetrahymena provides support for the notion that centrin-binding proteins are performing multiple basal body functions and prompts the need for future studies of Tetrahymena Poc5, Poc5 function in vertebrates, and centrin-binding proteins in all systems. In addition to characterizing the function of Poc5 in Tetrahymena, exploration of vertebrate Poc5 functions has been initiated in my thesis research. Using *Xenopus laevis* in collaboration with the Klymkowsky Lab (CU Boulder-MCDB), we have begun to gather evidence suggesting that vertebrate Poc5 could have an important role in ciliogenesis, serving as an exciting new direction for this work.

Upon depletion of Poc5 (using a morpholino, MO), wholemount Xenopus embryos displayed a curved body axis and body edema, both of which are hallmarks of ciliopathy mutants. Later in development, POC5 MO-injected embryos had scoliosis-like defects, similar to what was observed in a previous zebrafish study focusing on the role of Poc5 in scoliosis (Patten et al., 2015). To delve deeper into a potential function of Poc5 in cilia formation, the ciliated epithelia of POC5 MO-injected Xenopus embryos were dissected away from the rest of the embryos and immunofluorescence microscopy was conducted. Interestingly, the ciliated epithelia from Poc5 morphant embryos have fewer
ciliated cells compared with the ciliated epithelia from control MO-injected embryos. Collectively, the work performed in Xenopus uncovered a potential role for Poc5 in cilia formation, of which nothing has been published.

The Winey Lab is interested in conducting further work on Poc5 in both Tetrahymena and vertebrates. In Tetrahymena, the mechanism underlying the role of Sfr1 and Poc5 in modulating the production of cortical row basal bodies is unknown. To our knowledge, there are no parallel examples of basal body overproduction leading to cell death, which intensifies our interests in understanding this observed phenotype but also complicates the ability to predict potential mechanisms without further information. It is thought that centrin-binding may be an important aspect of this function, which can be pursued further with a domain analysis and mutagenesis approach. Since Sfr1 and ttPoc5 only have three centrin-binding repeats, it is possible to mutate the conserved consensus sequence motif (Ax7LLx3F/Lx2WK/R) or specific residues thought to be important for binding centrins within these repeats. This approach has not been undertaken with centrin-binding repeats and could elucidate the importance of centrin-binding in the uncovered functions of Sfr1 and Poc5, as well as potentially reveal important sequence specificity driving this interaction. If abolished centrin binding is driving overproduction of basal bodies, then mutating the centrin-binding repeats of Sfr1 or Poc5 could mimic the phenotype seen in poc5Δ and sfr1Δ cells. Outside of centrin-binding repeats, ttPoc5 has a second signature motif, the Poc5 box, which does not have any known function. A similar mutational approach could be employed to assess the function of the Poc5 box and furthermore, identify a potential interactor that binds to this motif. There is no published information on non-centrin interactors of centrin-
binding proteins. Lastly, the Xenopus portion of my thesis research uncovered a potential role for Poc5 in cilia formation. The Winey Lab is interested in pursuing this further using CRISPR-mediated mutagenesis in human RPE-1 cells, which have a primary cilium and could serve as a model for studying the role of Poc5 in vertebrate basal bodies as well as for cilia formation/maintenance. Using RPE-1 cells to study vertebrate Poc5 functions could also provide an opportunity to identify non-centrin interactors in humans and potentially a conserved binding partner (based on Tetrahymena). Collectively, further work on Poc5 will focus on elucidating the underlying mechanism driving basal body overproduction and eventual cell death upon loss of Sfr1 and/or Poc5. Also, we aim to build on the Xenopus Poc5 work and use our Tetrahymena Poc5 analysis to guide a human cultured cell study that explores the function of Poc5 in vertebrate basal bodies and for proper assembly/maintenance of cilia.
References:


