Specific Aims – The Role of Rabbit POC1B in Sperm Centrioles

Sperm centrioles are vital for sperm differentiation and embryo development and are implicated in infertility and miscarriages. However, the precise way sperm centrioles function remains unresolved (Avidor-Reiss et al., 2020). A key reason for this lack of understanding is that many proteins are only transiently present in the sperm centriole because of a process known as Centrosome Reduction (Schatten, 1994) and sperm centrioles exhibit dramatic species-specific adaptions (Avidor-Reiss et al., 2019). It was once widely accepted that structural degeneration and functional inactivation occur at one or two of the centrioles in mammals, resulting in a vestigial remnant. This idea led to the belief that human spermatozoa have only one functional centriole (the Proximal Centriole). While Centrosome Reduction happens in mice, our studies suggest that two centrioles are present, and both are essential in non-murine mammals. While the proximal centriole is modestly modified, the Distal Centriole has a highly atypical structure and composition (Fishman et al., 2018; Khanal et al., 2021) (**Fig 1**). These findings led us to a new model: *Centriole Remodeling*, which states that preexisting spermatid centrioles, along with their pericentriolar material, go through a structural and compositional transformation during differentiation resulting in remodeled centrioles and pericentriolar material that functions as a dynamic basal complex in the spermatozoon (**Fig 1**) and as a precursor for the embryonic centrioles. A vital mystery remaining is: what are the centrioles' functions? To answer this question, we propose studying the sperm centrioles in a mutant with partially disabled sperm centrioles. We will use rabbits—which, unlike mice, have paternal centriole inheritance—that are mutant in a structural sperm centriole component, POC1B (Centriolar Protein 1B).

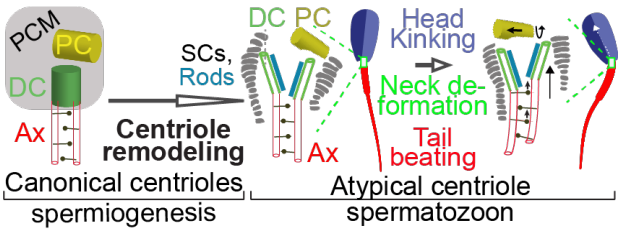

Fig 1: The centriole remodeling model. Early spermatids have two canonical centrioles, the proximal centriole (PC) and distal centriole (DC), enclosed by canonical pericentriolar material (PCM). The distal centriole forms the axoneme (Ax). Then, remodeling creates a dynamic basal complex (green box) with the atypical distal centriole at its core, containing rods and splayed microtubules, a modified proximal centriole, and segmented columns (SCs). In bovines, the sperm tail beats to the left, and the dynamic basal complex's right side slides rostrally relative to its left side. Its proximal centriole shifts to the left, and the head kinks to the left. The sperm tail beats to the right in rabbits, and the dynamic basal complex's right side slides caudally relative to its left side.

Our ***long-term goal*** is to reveal the properties of sperm centrioles, including their formation, maintenance, role, evolution, and clinical implications. We know that sperm have unique, atypical centrioles with significant species-specific differences (Fishman et al., 2018). We also know that the atypical and typical centrioles are part of a dynamic basal complex that moves in coordination with the sperm's tail and head (Khanal et al., 2021). However, we do not know the physiological significance of the atypical structures. Therefore, this project aims to provide the first insight into the atypical centriole's essential function in the sperm and, thereby, obtain veritable evidence for the Centriole Remodeling paradigm. The atypical centriole contains rods that move during the tail beating, a unique feature (Khanal et al., 2021). The rods include the conserved centriole structural protein POC1B. Therefore, our **central hypothesis** is that *POC1B is essential for distal centriole rod formation and normal function*. Indeed, our preliminary data suggest that distal centriole rods are moving in control rabbits but not moving in *Poc1B* mutant rabbits. We will test this hypothesis by pursuing three specific aims:

Aim 1: To determine rod movement during control and *POC1B mutant* rabbit sperm swimming. We hypothesize that POC1B is essential for normal rod movement. We will obtain substantive evidence by correlating the rod structure with sperm tail beating, gaining insight into distal centriole's POC1B and rod function.

Aim 2: To determine the Centriole Remodeling mechanism in control and *POC1B mutant* rabbit testes. We hypothesize that POC1B is essential for normal centriole rod formation. We will obtain substantive evidence by correlating the spermatid centriole structure and composition during spermiogenesis gaining insight into atypical distal centriole formation.

Aim 3: To map POC1B interactions with other rod components in vitro and in vivo: We hypothesize that POC1B interacts with POC5 and FAM161A in the sperm. We will obtain substantive evidence by mapping the interaction sites of these proteins and examining the interaction in testes and sperm.

We will accomplish these goals by identifying the precise locations of POC1B and other centriole proteins during sperm swimming and differentiation using superresolution microscopy and correlating them statistically with tail and head positions in rabbit sperm. We will gain further genetic insight by using inheritable rabbit *Poc1B* loss-of-function mutations. Undergraduate students will be engaged in experimentation from design to execution, analysis, writing papers, and presentations at conferences. Discoveries from these studies will advance our understanding of centrioles, leading to new studies focused on the contribution of sperm centrioles to infertility and developmental abnormalities.

Research Strategy

Significance

Scientific Significance: Early spermatids have two canonical centrioles surrounded by canonical pericentriolar material (**Fig 1**). During spermiogenesis, the centrioles and pericentriolar material are modified (Manandhar et al., 2000). The extent to which the centrioles' structure changes and their impact on centriole function vary in different animals (**Fig 2**) (Avidor-Reiss et al., 2015). In most basal animals (e.g., worms and fish), the pericentriolar material proteins are removed, with little or no noticeable change to the centrioles' structure (**Fig 2**, **black**). In these animals, the distal centriole is the spermatozoa axoneme base, and the two spermatozoa centrioles are vital for embryonic development (O'Connell et al., 2001; Yabe et al., 2007). In contrast, both spermatozoa centrioles in mice and other murines are unrecognizable using standard electron microscopy and thought to disappear following centrosome reduction (**Fig 2**, **green**) (Woolley and Fawcett, 1973). In non-murine mammals (e.g., humans and rabbits), there is only a single recognizable sperm centriole (the proximal centriole) in the spermatozoon (**Fig 2**, **blue**). In these mammals, we, and others, recently showed that the spermatozoon has an additional centriole, the distal centriole, attached to the axoneme base but has a dramatically atypical structure and composition with a species-specific size (Fishman et al., 2018; Leung et al., 2021). Both the proximal centriole and distal centriole appear to be essential for the early embryo's microtubule cytoskeleton organization in non-murine mammals (Cavazza et al., 2021; Schneider et al., 2020). Yet, little is known about the mechanisms that change sperm centrioles or the physiological reason for the change.

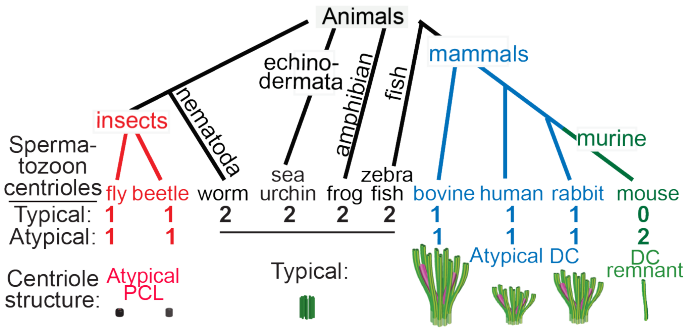

Fig 2: The number and size of spermatozoon centrioles vary in evolution. Four animal groups (color-coded) are shown based on their number and types of spermatozoon centrioles. Animals with just one canonical centriole also have an atypical centriole: the proximal centriole-like (PCL) in insects and atypical distal centriole (DC) in mammals.

Significance of centrioles in sperm anomalies: Sperm centrioles are essential for human fertility due to their vital role in sperm tail growth and the organization of the zygote's microtubule cytoskeleton (Avidor-Reiss et al., 2020). Centrioles contribute to several human infertility types, including acephalic spermatozoa, teratospermia, and unexplainable infertility (Avidor-Reiss et al., 2020; Garanina et al., 2019). Reduced centriole quality may underlie up to 15% of human infertility cases with no defects in sperm morphology, up to 75% of patients with teratospermia, and a significant portion of unexplained couples infertility (Jaiswal et al., 2022; Turner et al., 2021). Mathematical models predict that microtubules sliding at the base of the sperm tail – the centrioles' location – shapes the tail's waveform (Riedel-Kruse et al., 2007). We have visualized such sliding for the first time, suggesting that this is likely the atypical centriole's role in shaping sperm behavior (Khanal et al., 2021). *Revealing sperm centrioles' functions and the remodeling will help* ***increase scientific understanding of the possible causes of clinical human male infertility, miscarriages, and developmental diseases, and open novel avenues to develop next-generation infertility diagnoses and treatments.***

Significance of centrioles in cellular abnormalities: Besides their roles in reproduction, centrioles are critical cellular structures, but their function is incompletely understood (Nigg and Raff, 2009). Centrioles are usually made up of nine sets of short microtubule triplets arranged in a cylinder, playing a role in cell division by organizing the mitotic spindle. A centrosome comprising two centrioles organizes the cell microtubules and provides structure to the cell. Centrioles play a greater role in cell movement and signaling with the formation of cilia and flagella. Dysfunctional centrioles lead to many diseases, such as cancer and ciliopathies (Godinho and Pellman, 2014; Reiter and Leroux, 2017). Ciliopathies are systemic disorders that affect many organs. For example, retinal photoreceptors degeneration is a common feature of ciliopathic disorders and manifests as a progressive loss of peripheral vision. Like centriole remodeling in sperm, centrioles also vary during the differentiation of other cell types, e.g., cardiomyocytes (Ng et al., 2020; Zebrowski et al., 2015). For over a hundred years, it has been known that centrosomes' size and number are amplified during carcinogenesis. ***Yet, little progress has been made in elucidating their contribution to cancer.*** *Thus, understanding centrioles in sperm will provide* ***insights into other centriole-dependent cellular programs, contributing to greater understanding across cell types, tissues, and pathologies.***

Undergraduate research: This project involves undergraduate students investigating a potentially paradigm-shifting hypothesis. Undergraduate participation has been built into every part of the experimental design. Each undergraduate will play a significant, hands-on role in all activities, design, data collection, analysis, writing, and

presentations, gaining comprehensive research experience, exposing them to scientific discovery, and training them as scientists.

Innovation

Changes in sperm centrioles have been observed in many animal groups. Yet, their mechanistic contribution to sperm function is unknown (Fawcett and Phillips, 1969; Gordon, 1972; Manandhar and Schatten, 2000; Manandhar et al., 1998). We propose studying the changes in centrioles at the molecular and functional levels. This project is innovative because it overcomes a prominent technical barrier in sperm analysis: the difficulty of precisely determining both head and tail movement simultaneously in sperm. This difficulty results from the sperm's high beating frequency, the sperm's rotational motion, and the immense size diameter difference between the head and tail. We found a way to overcome this barrier by snap freezing moving sperm and analyzing them with superresolution microscopy that captures sperm head and tail position at the micron scale as well as the details of the centriole substructure at the nano-scale (Khanal et al., 2021). The project is also innovative because we put forward *a novel hypothesis that centriole remodeling creates a* ***dynamic basal complex*** *that defines tail beating and transmits tail movement to the neck and head*. An evolutionarily conserved program executes the remodeling with species-specific modifications that produce asymmetric centrioles of different sizes in different species (**Fig 1-2**). To test our hypothesis, we will use an interdisciplinary and comparative biological approach and innovative state-of-the-art methods, including superresolution microscopy and a genetically modified, non-standard but apt model (rabbits). *Our proposed research shifts the current paradigm and challenges the widespread idea that centrosome reduction degrades the human, rabbit, and other mammals' distal centriole; it seeks to open new directions centered around centriole remodeling*.

Approach

Overall Justification, Feasibility, & Preliminary Studies

A comparative biology approach benefits the sperm centrioles study: In all mammals studied to date, the distal centriole undergoes dramatic changes, suggesting a common mechanism (Avidor-Reiss, 2018; Fawcett and Phillips, 1969; Gordon, 1972; Manandhar et al., 1998). The distal centriole has significant size differences, pointing to species-specific adaptations (**Fig 2, 3a-b**) (Khanal et al., 2021). Correlations of distal centriole and other sperm neck properties with sperm behavior will likely shed light on the centriole's role. Hence, we will study rabbits in this project and compare them to published information on bovines and humans. Rabbits help study sperm centriole function because, unlike mice, their centriole inheritance resembles humans (Sutovsky et al., 1996; Terada, 2007). Rabbits are the smallest non-murine mammal suitable for genetic manipulation (Liu et al., 2004). Thus, we have generated mutations in one of the main distal centriole rod proteins, POC1B, in rabbits to facilitate our studies.

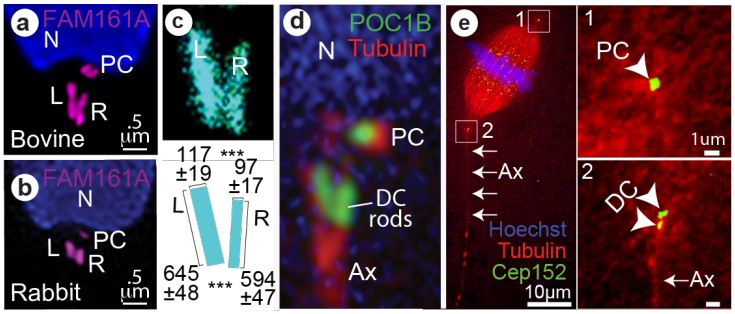

Fig 3: The distal centriole is atypical. **a-b)** Confocal image of FAM161A immuno-staining shows bovine distal centriole is twice the size of rabbit distal centriole. **c)** A side view of a FAM161A-labeled bovine distal centriole from a STORM image and a cartoon depiction of the distal centriole's left (L) and right (R) rod sizes; note that the left rod is longer and thicker than the right rod. **d)** SIM-3D microscopy shows that POC1B rods flank the splayed microtubules of the human distal centriole. **e)** Mitotic spindle (red) with an attached sperm axoneme (red, Ax, arrows), a pair of CEP152-labeled centrioles derived from the sperm distal centriole (arrowheads, inset 2) at one pole, and CEP152-labeled proximal centriole at the other pole (arrowhead, inset 1). ***, p<0.001; N, nucleus. ***Methods: a-b:*** Confocal HyVolution of sperm stained with FAM161A antibody (Sigma, HPA032119). ***c:*** STORM microscopy of methanol-fixed sperm. NIS-Elements imaging software was used to determine the distance along a line with over 50% intensity points relative to both sides' backgrounds. ***e:*** *see* (Fishman et al., 2018).

The spermatozoon distal centriole is an atypical functional centriole attached to the axoneme: Our published data show that sperm distal centriole has an atypical structure, protein content, and protein distribution relative to centrioles of other cell types (Fishman et al., 2018). The distal centriole of humans, bovines, and rabbits contain five centriole lumen scaffold members — POC5, POC1B, CENT1, WDR90, and FAM161A (**Fig 3a-d**) (Fishman et al., 2018; Khanal et al., 2021). All five proteins appear as two rods enveloped by splayed microtubules in the distal centriole when using superresolution microscopy (**Fig 3a-d**). This rod-like distribution differs from the radial distribution in canonical centriole proteins (Galletta et al., 2016; Le Guennec et al., 2020; Mennella et al., 2014). In bovine zygotes, the sperm distal centriole forms a centrosome and localizes to the spindle poles periphery (**Fig 3e).** These findings are corroborated by independent studies that suggest that the

atypical distal centriole is functional despite its attachment to the axoneme (Amargant et al., 2021; Cavazza et al., 2021; Schneider et al., 2021).

Centriole rod orientation-based sperm analysis (COSA): The sperm neck links the tail to the head and comprises the proximal centriole, distal centriole, and striated columns. These structures are aligned with the tail-beating plane but have lateral asymmetry (Ishijima, 2019; Ounjai et al., 2012). The neck is attached to the head via the implantation fossa situated asymmetrically at the nucleus base (Nicander and Bane, 1966). We found that the rods are also laterally asymmetric (**Fig 3c**). Stochastic Optical Reconstruction Microscopy (STORM) imaging of stained bovine FAM161A, POC1B, and POC5 shows that the left rods are consistently longer and thicker than the right rod.

The sperm tail can beat asymmetrically, and in murine mammals, the head's asymmetry identifies pro-hook and anti-hook tail bends (Chang and Suarez, 2011; Ishijima et al., 2002). However, whether the tail of non-murine mammal sperm beats in any particular direction was previously unknown because it was not possible to study the head or neck asymmetry and the tail curvature simultaneously (Woolley et al., 2009). **We have overcome this challenge by snap-freezing sperm while moving by adapting an existing standard method** (Lin et al., 2012; Woolley et al., 2006). Then, we assessed the rod asymmetry and the initial tail curvature using STORM with a 1,000X magnification. We oriented the sperm images with the large rod on the left side and the small rod on the right side of the neck's midline (**Fig 4a**). The rods' consistent reference allows a unique description of the flagellar and head bending relative to the sperm neck, despite the cell's complex rolling motions and the internal neck sliding movements (**Fig 4b**). We refer to this evaluation as centriole rod orientation-based sperm analysis (COSA) (Khanal et al., 2021). Using centriole rod orientation-based sperm analysis, we found a left beating bias (**Fig 4b**). These observations demonstrate that centriole rod orientation-based sperm analysis *is a novel way to study sperm behavior*.

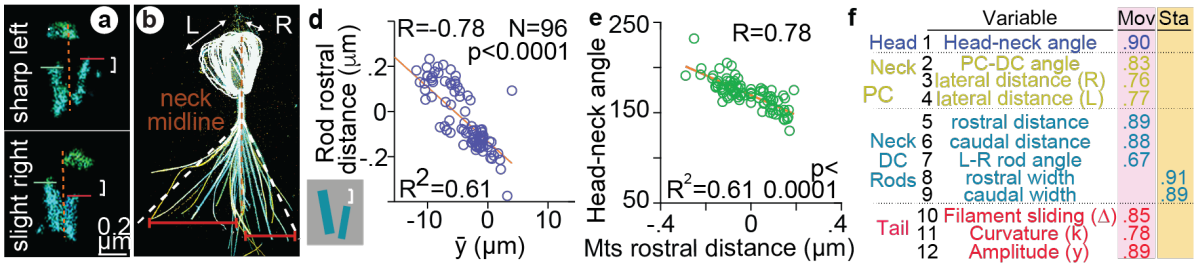

Fig 4: The bovine sperm tail beats to the left in coordination with neck sliding and head kinking. (a) FAM161A-labeled rods in sperm with sharp left and slight right bending. The left and right rods' rostral ends are marked with green and red lines, indicating rod sliding and generating a rod rostral distance (white bracket). **(b)** Superimposed sperm with their necks aligned to show a tail and head position bias to the left. To visualize the shape of the sperm, we enhanced the intensity of the low, non-specific signal in the sperm head and tail. **(e)** The correlation analysis between head-neck angle and distal centriole microtubules (Mts) rostral distance shows neck-head coordination. **(f)** exploratory factor analysis explored a data set with 12 variables. Two latent factors were identified: the tail-neck-head coordinated movement (Mov) factor and the no-movement of distal centriole center (Sta) factor. Factor loadings are provided for each variable in the pink and yellow columns. All factor loadings are above 0.5, indicating significant contributors with at least 25% of the variance. ***Method***: see Activity 1.1 and Fig 3c. **c-d:** a semi-automated bespoke image processing algorithm calculated mean flagellum beating amplitude by extracting coordinate values of points along the flagellum relative to the sperm head $\boldsymbol{r}(s) = (\mathrm{x}(s), \mathrm{y}(s))$, parametrized by arc length s (Gadêlha, 2018). Flagellar shapes are rotated and translated to align the head's length with the x-axis and center the neck as the origin. DATA is from 3 independent staining explements.

The distal centriole structure is consistent with the basal sliding model for sperm motility: The classic model for cilia beating, the microtubule sliding model, depicts the active sliding movement of the axoneme microtubules which are restricted at their base by the centriole (King and Sale, 2018). This model is consistent with the canonical centriole's solid structure at the base of most cilium types. Another model has also been proposed for mammalian sperm beating, the basal sliding model. In this model, the flagellum base microtubule slides to modulate the tail-beating pattern (Riedel-Kruse et al., 2007; Vernon and Woolley, 2004). To test the distal centriole structure's applicability to the basal sliding model, we compared the position of the left and right rods' rostral ends using STORM and FAM161A labeling in bovine sperm snap-frozen while moving. We found that the left rod is positioned caudally relative to the right rod when the tail bends to the left; the left rod moves rostrally when the tail bends to the right. Linear regression shows a strong correlation between the vertical distance of the rod ends and average flagellum beating amplitude (**Fig 4d).** We found similar results with distal centriole's left and right microtubules (Khanal et al., 2021). The rod and microtubules' total relative movement is ~300nm. *These findings demonstrate for the first time that the distal centriole's lateral sides slide relative to each other during sperm tail beating, as expected from the basal sliding model.*

A dynamic basal complex modulates bovine sperm behavior: The current prevailing view holds that the neck is cemented to the head. Some neck changes were observed, but they lacked correlation with tail beating; therefore, the sperm head is thought to passively follow the tail swimming movement (Ounjai et al., 2012; Riedel-Kruse et al., 2007; Woolley et al., 2008). **In contrast, we observed coordinated head motion and tail beating**. We named this 2D head movement relative to the neck's long axis "kinking" (**Fig 1**). The head kinking correlates with distal centriole microtubule sliding (**Fig 4e**) and other neck parameters (Khanal et al., 2021), suggesting the tail beating and neck movement mediate the head kinking**.** Furthermore, we found additional internal neck movements. For example, the proximal centriole changes its lateral position relative to the distal centriole midline, moving its right edge ~200 nm to the left (Khanal et al., 2021). To better determine the coordinated sperm movement, we performed exploratory factor analysis, measuring a total of 21 variables during sperm beating (Khanal et al., 2021). This analysis found that two main factors explained the underlying sperm behavior: (i) a significant tail-to-head coordinated movement synchronized with the distal centriole periphery and (ii) no movement of the distal centriole's center (**Fig 4f**). This data supports a model where axoneme sliding generates tail beating and neck sliding that subsequently kinks the head. *However, whether neck movement or sperm protein is essential for kinking is unknown. Here, we aim to characterize the role of the rods by mutating POC1B.*

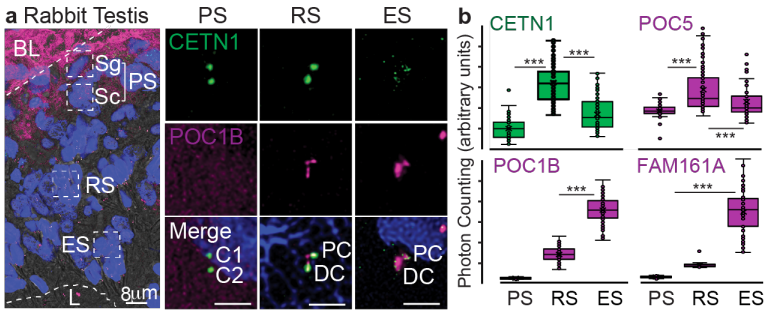

Fig 6: Rabbit centriole remodeling is a two-step process. **(a**) Different cell types were identified based on location relative to the basal lamina (BL) and seminiferous tubal lumen (L), size, shape, and peanut agglutinin staining. CETN1 labels the two centrioles in all cell types in all three species. POC1B labeling marks two centrioles in round spermatids (RS) and elongated spermatids (ES) but not the two centrioles (C1 and C2) of Pre-spermatid (PS). (**b**) Protein quantitation in different sperm cell stages in the rabbit. CETN1, POC5, and POC1B are enriched in the round spermatid stage. POC1B enrichment continues in the elongated spermatids when FAM161 is recruited to the centrioles. *** P<0.0001. ***Method***: see activity 2.4. DATA is from 3 independent staining explements.

The rod proteins are recruited and redistributed stepwise during remodeling: To get an overview of the remodeling process, we analyzed the localization of various proteins using HyVolution immunofluorescence microscopy in bovine, rabbit, and human testes. **We saw similar results in all three species** using immunofluorescent and confocal microscopy of CETN1, POC5, POC1B, and FAM161A (data not shown); due to space limitation, we show only some of the rabbit data with CETN1 and POC1B in **Fig 6a**. Three stages of spermatid cells were stained: pre-spermatids (PS), round spermatids (RS), and elongated spermatids (ES). CETN1 and POC5 are transiently enriched in the round spermatid's distal centrioles during remodeling (**Fig 6b**). POC1B is gradually enriched in the centriole, initially appearing in the distal centrioles of round and elongated spermatids. FAM161A is the last to be incorporated into the distal centrioles and is present in the elongated spermatids. *These findings suggest that the rod proteins are assembled in rabbits similarly to bovines and humans.*

Rabbit and bovine sperm tail beating are distinct, but the rod's sliding direction is conserved: We performed preliminary STORM imaging using FAM161A immunostaining, centriole rod orientation-based sperm analysis, and control rabbits under the same conditions used above for bovine. Unlike the left tail beating of bovine sperm, the rabbit sperm tail beats mainly to the right. As expected from a right bent tail, the right rod slides more caudally (**Fig 8b**), suggesting that rod movement directionality relative to the tail bending is the same in bovines and rabbits. Moreover, as expected from the right tail beating, the rabbit head kinks to the right, and the proximal centriole angle is much smaller. These data indicate that rabbit and bovine rods and dynamic basal complex have the same directionality as their tails. Yet, the tail, dynamic basal complex, and head move together, but to the opposite sides in bovines and rabbits. The control of the bias in the beating side is unknown, but it may involve Ca^{+2} signaling, as mice sperm tail beating can have a bias to either right or left, depending on Ca^{+2} signaling. (Chang and Suarez, 2011). We also found that, like in bovines, the left and right rods are asymmetrical, with the left rod being wider than the right rod. However, unlike in bovines, the two rods have a similar length in rabbits. As expected, the rods in rabbits are much shorter than in bovines (compare **Fig 3c** to **Fig 8a**). We also found that, like in straight bovine sperm, the right rod is more caudal than the left rod, and the rods are organized like a V shape with an acute angle (**Fig 8a**). *Here, we propose to perform a comprehensive sperm tail, neck, and head coordination analysis in rabbits to gain insight into the role of the dynamic basal complex.*

Generation of Poc1b-mutant rabbits: We started studying POC1B in rabbits several years ago for numerous reasons. First, POC1B was the first protein we identified as an atypical centriole-specific protein in flies, humans,

and bovines (Fishman et al., 2018; Khire et al., 2016). Second, in mammals, POC1B is a component of the distal centriole rods, novel structures that we recently hypothesized allow sliding of the sperm tail base (Khanal et al., 2021). Third, at that time, the composition of the centriole lumen scaffold was unknown, but it was known that POC1 family proteins were essential for centriole stability (Pearson et al., 2009). Finally, POC1B is enriched in the distal centriole relative to the proximal centriole, suggesting it has a different function there. Because mice have no spermatozoon centrioles, we used an alternative mammalian model for genetic studies. Therefore, we collaborated with Dr. Genevieve Jolivet (INRA, France) using CRISPR-Cas9 technology as part of an R21 (R21HD092700). We kept two *Poc1b* alleles, deleting 78 or 5 amino acids from the protein's C-terminal end. This project mainly uses the most severe allele, which lacks the last 78 amino acids, including the conserved coiled-coil domain (Δ78) (**Fig 8c-e)**. The Δ5 will be used as a control if needed. We produced 8 Δ78 homozygote males by crossing Δ78 heterozygote males and females that generated 34 offspring, suggesting a Mundelein inheritance. At puberty (~6 months), the Δ78 sperm have normal general morphology and is motile; this is expected since POC1B is dispensable for flagellum formation (Pearson et al., 2009; Roosing et al., 2014). *The generation of Poc1b-mutant rabbits with normal morphology and motility lays the groundwork to study POC1B and the rods' specific roles in sperm head movement and sperm sublet behaviors.*

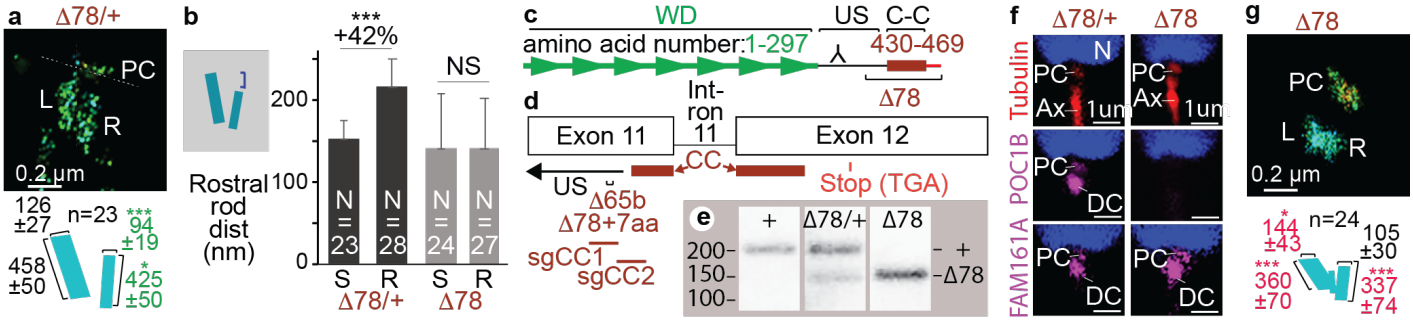

Fig 8: *The Poc1b* mutant-rabbit model. (**a**) STORM images of control (Δ78/+) sperm labeled with FAM161A (Sigma, HPA032119). A green font marks significantly different values in the right rods from the left rods. Orange and white dashed lines mark the distal centriole and proximal centriole midlines. A cartoon depicts the distal centriole rods. (**b**) The size of rostral rod distance is presented as Av ± SD in straight (S) and right (R) bent control (Δ78/+) and homozygotes (Δ78) sperm. (**c**) POC1B domain organization. WD, US, and C-C are the tryptophan-aspartic acid, unstructured, and coiled-coil domains, respectively. "Y" mark the antibody PA5-24495 (Thermo Fisher) target site (amino acid 320-349). (**d**) The strategy employed to obtain *Poc1b* mutants. The figure depicts exons 11 and 12 and intron 11 of the rabbit *Poc1b* gene and the protein domains they encode (the end of the unstructured domain (US), the coiled-coil domain (brown boxes), and the stop codon (red)). Two sgRNAs (sgCC1 and sgCC2) were designed to target the 11th exon and generate the Δ78 alleles by deleting 65 bases, which is predicted to result in a loss of 78 amino acids (b). We injected the Cas9 mRNA and one of the sgRNAs into a zygote. (**e**) Control (+), heterozygote (Δ78/+), and homozygotes (Δ78) genotyping. (**f**) Confocal images of heterozygote (Δ78/+) and homozygous mutant (Δ78) sperm labeled by tubulin (sheep Ab; ATN02, Cytoskeleton), POC1B (PA5-24495, Thermo Fisher), FAM161A (HPA032119, Sigma). (**g**) STORM images of Δ78 sperm labeled by FAM161A. Red fonts mark significantly different values in Δ78 sperm compared to control. **, P≤0.01; ***, P≤0.001. DATA is from two experiments.

Poc1b-mutant rabbits have abnormal sperm centrioles: By now, we have analyzed four mutant homozygote rabbits with similar results. Confocal immunofluorescence shows POC1B is undetectable in Δ78 spermatozoa proximal centriole and distal centriole (**Fig 8f**, middle right panel), indicating that the Δ78 protein is dysfunctional. In contrast, tubulin and FAM161A immunolabel the sperm centrioles, indicating that the centrioles are formed. Careful quantification of sperm from an aged-matched cohort of four mutant and three control rabbits with parallel immunostaining and imaging found increased FAM161A immunoreactivity in the sperm centriole and axoneme (PC: 1.41-fold, P<0.001, Cohen's d=0.32; DC: 1.44-fold, P<0.001, Cohen's d=0.38, Ax 10.63-fold, P<0.001, Cohen's d = 0.62). The cohort was analyzed three times independently, examining a total of 436 control sperm and 336 Δ78 sperm). This increased staining suggests that POC1B is dispensable for FAM161A recruitment and POC1B absence induces FAM161A increase in the centrioles with an extra that leaks to the axoneme.

Poc1b-mutant rabbits have abnormal and paralyzed rods: Preliminary STORM immunofluorescence with FAM161A found major structural abnormalities of the distal centriole rods in the Δ78 mutant. The Δ78 rods are shorter (~20% of original size) and barely distinct, suggesting they have not separated into two rods (**Fig 8g**). The right sides of the rod's caudal movement are diminished and not observed when the tail beats to the right (**Fig 8b**). These *observations are valuable to our studies because they indicate that the* Δ78 homozygote *has a specific centriolar defect, namely preventing the bifurcation of the rods, yet sperm motility is preserved, facilitating testing of our scientific premise on the rods' crucial role in tail-neck-head coordination.*

Male poc1b-mutant rabbits can fertilize female rabbits: Mating experiments of Δ78 homozygote male with a control female can produce live offspring. This data suggests that POC1B and rod movement are dispensable for rabbit sperm general movement and embryo development, possibly due to redundancy or compensation by the striated columns. Here, we will test if POC1B and the rods are essential for **efficient** sperm movement. We expect to find slight sperm movement abnormalities, providing insight into the rods' role.

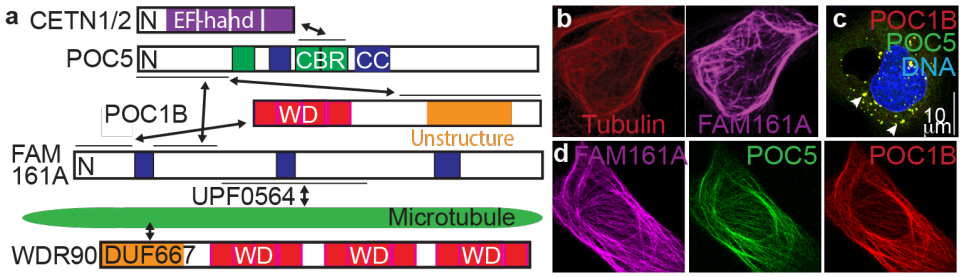

Fig 9: Rod proteins interact with each other. (**a**) Interactions of the rod proteins (arrows) as observed from yeast two-hybrid assays and co-expression in U2OS cells. (**b**) FLAG-FAM161A localizes to the microtubules. (**c**) Co-expressed POC1B-mCherry and POC5-GFP form cytoplasmic aggregates (arrowheads). (**d**) When POC1B-mCherry and POC5-GFP are co-expressed with FLAG-FAM161A, they colocalize to the cytoplasmic microtubules. DATA was repeated in at least three independent staining experiments.

The C-terminus of POC1B interacts with the N-terminus of POC5. The rod proteins POC1B, FAM161A, POC5, WDR90, and CETN1, interact with each other or the microtubules; FAM161A (via domain UPF0564) and WDR90 (via domain DUF667) interact with microtubules (Le Guennec et al., 2020; Rolland et al., 2014; Roosing et al., 2014; Zach et al., 2012). We mapped the rod protein domains that mediate the interactions using readily available human proteins (**Fig 9a**). Since FAM161A is a microtubule interacting protein, it localizes to the cytoplasmic microtubules when expressed in U2OS cells (**Fig 9b).** Since FAM161A also binds to other proteins, it efficiently recruits them to the microtubules when co-expressed (**Fig 9d**). Using this recruitment assay and expressing rod proteins with domain deletions in U2OS cells, we found that POC1B interacts with FAM161A's N-terminus (aa 1-110) (**Fig 9a**). Also, we discovered that POC5's N-terminus (aa 1-122) interacts with FAM161A's N-terminus (aa 111-230) (summarized in **Fig 9a**). Additionally, we found that POC1B's C-terminus (aa 365-478) interacts with POC5's N-terminus (aa 1-122) using the yeast 2-hybrid system (not shown). We observed that overexpressed POC1B localized specifically to form cytoplasmic aggregates in U2OS cells (**Fig 9c**). Detailed analyses found that the aggregates are specific structures with a composition similar to rods but are not centrioles or non-specific aggregates of unfolded proteins. These aggregates recruit endogenous POC1B, POC5, and CETN1/2 but do not recruit CEP152 or γ-tubulin. The overexpressed rod proteins are recruited only if they have the domain required for the specific interaction (not shown). *We will use these systems to map rabbit POC1B interactions and assess the impact of rabbit Poc1b mutations on rod protein interactions.*

Experimental Design

Aim 1: To determine rod movement during swimming of control and *Poc1B* mutant rabbit sperm

Introduction: The mature spermatozoon has centrioles with atypical structures and composition (Fishman et al., 2018), but their functions remain unknown. Our studies in bovine sperm suggest that the sperm centrioles form a dynamic basal complex that participates in a novel sperm tail-neck-head coordination. Based on our robust preliminary data, we hypothesize that rabbit sperm also have a dynamic basal complex and a coordinated movement and that POC1B is essential for the dynamic basal complex normal function. The objective of **Aim 1** is to identify the relationship between the centriole's properties, tail beating, neck sliding, and head kinking in rabbits in control and *Poc1B* mutant. The rationale is that decoding the sperm tail-neck-head coordination and regulation in rabbits, whose centrioles can be manipulated genetically, will help understand the hitherto elusive function of the sperm centrioles. We expect the centrioles' properties to correlate with tail beating, neck sliding, and head kinking patterns in rabbit sperm. This study will provide mechanistic information on how these structures function. It takes advantage of the high-resolution microscopy by directly visualizing the remodeling process and performing an exhaustive analysis of tail-neck-head coordination. We will carry out these investigations in conjunction with cutting-edge genetic approaches and multiparametric statistical analyses.

Activity 1.1. Determine the sperm's tail-neck-head coordination: We have determined bovine sperm's tail-neck-head coordination, but we do not know if the same occurs in other mammals. Here, we will test rabbit sperm, which have smaller distal centrioles than bovine (**Fig 2, 3a-b**). We will analyze the rabbit sperm and compare the results to our past studies of bovine sperm's tail-neck-head coordination. The **study design** will include three experiments. Each experiment will be independent, using sperm ejaculate from a distinct male. Each experiment will image and analyze 200 sperm for a total of 600 sperm. This number is sufficient to obtain statistically significant values based on our data with bovine sperm (Khanal et al., 2021).

Rabbit Breeding - We will breed and grow rabbits in a DLAR facility following United State Department of Agriculture guidelines (Institutional Animal Care and Use Committee protocol #108767). Individual rabbits are housed in separate cages in a six-cage rack. Females will be placed in the male's cage for mating until the male successfully mounts and ejaculates. Mounts will be done once an hour and repeated three times. Expectant pregnant females are kept in cages in a room separate from all other rabbits. A nesting material and box will be provided to the female 28 days after copulation. Kits will be kept with the mother until six weeks old, at which point they will be weaned. *Generating age-matched control and mutant cohort* - We will produce adult *Poc1b* mutant rabbits by crossing Δ78 heterozygote males with Δ78 heterozygote females to generate five Δ78 homozygotes and five control males. A single female can have 1–14 kits per litter with an average of 8. We expect 25% of kits to be homozygous mutants and 25% control rabbits on average. Of these, ½ will be males, giving us one Δ78 homozygote male and one control male per breeding cycle on average. ***This breeding design provides age-matched control and a mutant sperm sample.*** Breeding is recommended every two months, and we expect to generate the full experimental cohort within a year. *Sperm genotyping* - We will genotype the rabbits by extracting genomic DNA from the ear clips of newborns. The primers we generated to amplify the deleted region produce a 210 bp amplicon (WT) and a 145 bp amplicon (Δ78), which will help distinguish them (**Fig 8e**). *Sperm handling* - We will obtain freshly ejaculated sperm from our rabbits using an artificial vagina. Semen properties will be determined using standard semen analysis. After collecting the ejaculated semen, motile sperm will be separated from seminal fluids using a swim-up technique (PureSperm Wash kit, from Nidacon). This procedure results in a suspension enriched for motile sperm in the M199 buffer. *Immunofluorescence* - Sperm motility on the coverslip will be confirmed under a microscope, and slides with 90% free-swimming sperm will be used. The swimming sperm will be snap-frozen while motile in liquid nitrogen, fixed with cold menthol, and immunostained using standard conditions (Khanal et al., 2021). We will use a rabbit anti-FAM161A primary antibody (Sigma, HPA032119) with an anti-rabbit secondary antibody labeled by a far-red fluorophore (Alexa 647) to label the distal centriole rods, proximal centriole, and sperm. The anti-FAM161A primary antibody is validated in rabbits (Khanal et al., 2021). *STORM Imaging* - labeled sperm will be imaged using a 3D-STORM fluorescent microscope in our core instrumentation facility. Pictures will be taken at 1,000X magnification to resolve the proximal centriole and distal centriole rods (e.g., **Fig 4a**) and then at 600x to see the overall sperm head and tail shape. *STORM quantification* - We will quantitate a total of 12 variables for each sperm, including distal centriole rod caudal distance, distal centriole rod rostral distance, left proximal centriole lateral distance, proximal centriole-distal centriole angle, and head-neck angle. We will measure using Nikon's NIS-Elements imaging software. To measure rod length and width, a line, starting at 50% of the first intensity peak through 50% of the last intensity peak, will be drawn and measured along the rods' length and width. Caudal and rostral distance will be measured for rod sliding analysis. Sperm cells will be rotated to make the neck straight, the proximal centriole tip pointing to the right side and the bigger rod on the left side of the neck midline. This consistent reference will define the left and right rods and microtubules. Measurements will be taken at the caudal and rostral ends of the right rod and microtubules relative to the left rod and microtubules at the respective side. Measurement will be assigned a "-" value when the right rod or microtubule is below the left rod or microtubules and a "+" value when the right side is above the left side. *Tail variables* - Interfilament sliding, tail curvature, and tail amplitude will be determined with a semi-automated customized image processing algorithm (Khanal et al., 2021). Dr. Hermes Gadelha (University of Bristol, UK), an expert in analyzing and modeling sperm movement and a collaborator on our recently published paper, will help analyze these data (see letter of support).

This activity will study the relationship between the 12 variables in control rabbits. An **undergraduate student** will participate in all the activity stages, including design, data collection, analysis, writing, and presentations. The undergraduate will do STORM experiments related to sperm neck and head variables in control sperm from STORM images, plotting the relationship of the parameter's magnitude as a function of the three tail variables, performing linear regression to test if the neck/head variables correlate with the tail variables, and perform Fisher's r to z transformation to compare the rabbit sperm correlations to our past published bovine sperm correlations. We expect to see the various structures of the rabbit sperm neck and head move in coordination with the tail beating cycle, like in bovines. However, based on the preliminary data, we expect that the head and tail beating would be biased to the right in rabbits compared to the left bias observed in bovines.

Dr. Barbara Saltzman, an epidemiologist at The University of School of Population Health, our long-term collaborator and collaborator on this application, will guide the statistical analysis (Jaiswal et al., 2022; Khanal et al., 2021; Turner et al., 2021). With her help, we will perform an exploratory factor analysis like that described in **Fig 4f**. With this analysis, we will **ask:** what are the latent factors (the inferred variables that are indirectly observed) that control sperm tail and head movement in control rabbits, and which variables contribute to the moving factor? We expect to find one latent factor that underlies the coordinated tail-neck-head movement

revealing the contribution of the dynamic basal complex to the tail, neck, and head variables. We also expect the latent factor's polarity to be the same in rabbits as observed in bovines regardless of the bias in tail beating direction. For example, the bovine sperm beat is biased to the left, while rabbit sperm is biased to the right. These biases appear to correlate with the right rod's caudal sliding in rabbits and rostral sliding in bovines. Thus, in both species, the right rods move relative to the tail beating with the same polarity. Together, these findings are expected to establish that sperm's tail-neck-head coordination occurs in rabbits.

Activity 1.2: Determine the Poc1b mutant rabbit sperm's tail-neck-head coordination: Because the sperm distal centriole was thought to be absent, it was concluded that the dynein motors' physical forces are transmitted to the flagellum base only through the striated columns found around the centrioles (Lindemann and Lesich, 2016). However, our data indicate that the distal centriole is present. Its sides slide coordinately with the tail, neck, and head, suggesting that its participation is needed to transmit the dynein forces adequately. POC1B plays a central role in centriole assembly, centriole stability, and ciliogenesis (Pearson et al., 2009); it is also a component of the rabbit rods (Khanal et al., 2021). Here, we will analyze the sperm's tail-neck-head coordination using the ∆78 *Poc1b* rabbit, where the conserved coiled-coil domain is completely removed (**Fig-8**). The ∆78 protein is missing in the centrioles, suggesting that the distal centriole is abnormal (**Fig 8f**). We will use a similar approach to that described in Activity 1.1 and compare the phenotypes of five homozygote ∆78 males to five control males. Power analysis with a Cohen's d of 2 and an alpha of 0.05 has a power of at least 80%.

An **undergraduate student** will participate in breeding the aged-matched rabbit cohort, including designing the mating, performing the mating, genotyping, and determining statistically if the ∆78 allele behaves according to Mendelian inheritance. Also, we will test the presence of *∆78* proteins in the ejaculated spermatozoa (see the method in Activity 1.1) using western blot analysis. Proteins will be extracted using RIPA buffer, sonicated (4 times of 25% for 5 seconds with 15 seconds intervals), and boiled with Laemmli buffer for 10 minutes. The gel will be loaded with equal amounts of protein extract, determined using Pierce™ BCA Protein Assay Kit (Thermo-Fisher 23225). Protein will be separated on an SDS/PAGE and transferred to a membrane. The membrane will be probed with a mouse anti-POC1B antibody (Thermo-Fisher H00282809-B01P) and a secondary goat anti-mouse antibody conjugated to peroxidase (Jackson ImmunoResearch 115-035-003). Protein bands will be imaged and quantified using Western Blot Imaging Azure C500. Tubulin loading control (Dma1 antibody, Thermo-Fisher, 62204) will be analyzed to help correct small deviations in protein amounts. T-tests will be performed on band intensity results obtained from 5 independent experiments to determine if the *∆78* proteins are stable. These experiments will confirm the successful translation of the *Poc1b*-mutant allele and show the effect of ∆78 mutation on protein stability and length (*∆78* predicted to be 46 kDa instead of 54 kDa in control).

We will analyze the *Poc1B* mutant and control semen to determine sperm motility, count, morphology, and viability from 3 ejaculates per male (Williams et al., 1990). Dr. Tariq Shah (Director of the University of Toledo Andrology Laboratory), a semen analysis expert, will assist us with the analysis (see letter of support). Based on observation from the single male generated, we expect a minimal effect on average sperm count, morphology, and viability. In contrast, we expect an increase in linearity (LIN) and straightness (STR) but a reduction in wobble (WOB) in the *Poc1b* mutant (Mortimer, 1997). To quantitate the effect of the mutation, washed sperm (PureSperm Wash kit, from Nidacon) will be loaded into 20 μm depth Leja slides for motility, and images will be taken using a Nikon microscope equipped with a high-speed camera. Images will be analyzed by tracing sperm head movement and determining movement variables using the SpermQ computer program (Hansen et al., 2018). The findings will also immensely support the new role of POC1B in sperm movement. An **undergraduate** will participate in all these activity stages, including designing, and troubleshooting the semen collecting, sample preparation, Western blot execution, performing the statistical analysis, and communicating it.

Next, we will study the tail-neck-head coordination in *∆78* homozygotes. We will perform five independent experiments for each genotype; in each experiment, we will analyze 200 sperm for a total of 1,000 sperm per genotype. We will study the relationship between the 12 variables in the tail, neck, and head, as described in Activity 1.1 and **Fig 4f**. Unlike in control sperm**,** we expect that the rod variables will not contribute to the coordinated latent factor in *∆78* homozygotes, indicating a dysfunction in tail-neck-head coordination. We may find that the coordination of other variable factors is affected due to the *Poc1b* mutation. We also expect the correlation of distal centriole, rod, proximal centriole, and head movements with tail parameters to be abnormal in the mutants compared to control. These findings will support our hypothesis that POC1B is essential for rod movement and regular tail-neck-head coordination and provide insight into the specific function of POC1B. **An undergraduate** will participate in all these activity stages, including, designing, executing, and troubleshooting the sperm motility experiments, focusing on quantifying two sperm neck variables in mutant and Control sperm. **Finally, the undergraduate** will calculate the average and standard deviation, plot the relationship of the parameter magnitude as a function of the three tail variables, and perform Fisher's r to z transformation to

compare the mutant rabbit sperm correlations to the control sperm correlations to test if the *Poc1b* mutation significantly changes this parameter.

Aim 2: To determine the Centriole Remodeling mechanism in control and POC1B mutant rabbit testes

Introduction: Sperm centrioles are remodeled during spermiogenesis and form an atypical distal centriole (Fishman et al., 2018). However, the mechanism of the centriole remodeling and centriole's protein reorganization remains unknown. Revealing an active program modifying the canonical centriole into an atypical centriole with unique rod protein organization will prove the remodeling model over the degeneration model. We propose studying the molecular organization at the protein level in rabbit centriole remodeling. Our **working hypothesis** is that POC1B is essential for rod formation. The **objective** is to find the responsible changes, which will provide an insight into the mechanism generating the rods. For example, POC1B's recruitment to the centrioles during spermatogenesis may help form two distinct rods and determine their length. We expect to identify the specific role of POC1B in the sperm and the formation of the rods and dynamic basal complex.

Activity 2.1. Track the locations of the rod proteins in centriole remodeling in rabbits during spermiogenesis (spermatids) and in mature sperm (spermatozoa): One of the mechanisms maintaining the canonical centriole rigidity is a cylindrical luminal protein scaffold that interconnects the microtubules triplets (Le Guennec et al., 2020). This scaffold includes the same proteins found in the rods (POC1B, CETN1, POC5, FAM161A, and WDR90); mutating these proteins destabilizes canonical centriolar structure, demonstrating they are essential for the long-term rigidity of the canonical centriole (Pearson et al., 2009; Steib et al., 2020). In contrast, in the spermatozoon, microtubule doublets are splayed apart along the two rods, providing a scaffold to the distal centriole's moving sides. Our preliminary analysis with confocal microscopy and some rod proteins finds that the remodeling program is conserved in rabbits (**Fig 6)**. In the mature spermatozoon, the rods are nested like a Russian doll in their distribution in the bovine distal centriole (Khanal et al., 2021). Here, we propose determining the remodeling program by mapping the recruitment stages of five-rod proteins and their distribution in the centrioles before, during, and after the remodeling in control and mutant rabbits.

We will obtain testes from discarded New Zealand rabbits, embed them in the Optimal Cutting Temperature (OCT) medium, and section them in the University of Toledo histology facility (see letter of support from Dr. Andrea Kalinoski). STORM Immunofluorescence will be used as described in Activity 1.1 using the CETN1 antibody (Santa Cruz, Clone 2A6), FAM161A antibody (Sigma Aldrich, HPA032119), POC1B antibody (Thermo Fisher, PA5-24495 and H00282809-B01P), POC5 antibody (Thermo Fisher, PA5-84503), or WDR90 (Sigma Aldrich, HPA061785). The primary antibodies to each rod protein are validated (**Figs 3b, 8**, **11a**). We will compare the proteins before remodeling in spermatocytes (pre-spermatids), two stages during spermiogenesis (round and elongated spermatids), and spermatozoa. If significant differences between elongated spermatids and spermatozoa are observed, we will also investigate spermatozoa from the epididymis's caput and cauda to find the sperm stage where these differences originate. Different cell types in the testes will be identified based on their location relative to the basal lamina and lumen, size, shape of the DAPI labeled nucleus, and staining with the lectin Peanut Agglutinin (PNA). We will analyze the five-rod proteins in 4-6 sperm stages with at least 40 cells from each sperm stage as follows below.

First, labeled sperm will be imaged using a confocal fluorescent microscope equipped with HyVolution superresolution. The picture's stack will be taken at 630x with 4096x4096 pixel per picture to 60 nm pixel size to resolve the rods (**Fig 3b**). Projection images will be made from a z-stack covering 20 μm with a 0.3 μm separation between each picture. Staining intensity will be obtained by photon-counting imaging with standardized laser powers, correlating fluorophore intensity to protein amount. We will quantify the proximal and distal centrioles signal in distinct 0.75 by 0.75 μm areas using Leica Application Suite X imaging software (LASX) and calculate the changes relative to spermatocyte centrioles. We expect rod proteins' location and amount to change during remodeling in a protein-specific way, indicating they play distinct roles in the remodeling. For example, the CETN1 level is reduced in the late-spermatid stage while POC1B increases (**Fig 6a**), suggesting that rod proteins have distinct roles in the remodeling process. Together, the data will generate a detailed spatiotemporal map of centriole remodeling. **An undergraduate student** will participate in all these activity stages, including designing, troubleshooting, and quantifying the images, and studying one of the rod proteins in conjunction with CETN1, which acts as a control/centriolar marker.

Next, we will further define the distal centriole's remodeling by determining the proteins' relative position by performing double-labeling and STORM imaging in spermatozoa. For example, we will use anti-POC1B antibodies coupled with ATTO 488 with either FAM161A, POC5, or tubulin antibodies, each conjugated with Alexa 647 (Dempsey et al., 2011). We will then perform 3D sub-volumes in real space by translating, tilting, and in-plane rotating the volumes by cross-correlation of pixel intensities to obtain an average structure while also

measuring the variance of the individual protein structures. To determine if the rabbit rods differ structurally from bovine rods, we will calculate the length and width of all five-rod proteins in rabbits with 30 sperm as we did in bovines (Khanal et al., 2021). We expect to generate a model for rod protein organization in rabbits throughout remodeling. Differences will be traced to distinct functions and protein interactions (see **Activity 2.2 and Aim 3**) and sperm movement (see **Aim 1**). These findings will uncover the precise molecular basis of distal centriole organization and help understand the changes leading to atypical centrioles' formation. **An honors-level undergraduate student** will be involved in all aspects of this experiment, from design to execution, analysis, writing, and presentations, working with a Ph.D. student.

Activity 2.2. Determine how rabbit Poc1b mutation affects centriole remodeling and rod structure: Here, we will determine POC1B's essential role in centriole remodeling by analyzing the Δ78 rabbits. Our preliminary data found that Δ78 protein is missing in the ejaculated spermatozoa centrioles and that FAM161A labeled rods are abnormal, suggesting that POC1B is essential for distal centriole structure (**Fig 8f**). This abnormality may arise during remodeling due to a developmental defect or during sperm swimming due to mechanical failure during the tail beating. We hypothesize that the remodeling is defective because the rods appear shorter, likely due to the POC1 family protein's role in determining centriole size (Keller et al., 2009)(**Fig 8g**). We will test this hypothesis by analyzing the Δ78 centrioles in testis spermatids using confocal and STORM imaging as described in **Activities 2.1**. We will perform the ex vivo experiments with five Δ78 homozygotes and five control rabbits. We will harvest the testes from rabbits at one year old. We expect the seminiferous tubules and sperm to develop normally, indicating that POC1B is dispensable for testes development. In spermatids and spermatozoa, we expect POC1BΔ78 to be absent from the centrioles, and CETN1, POC5, FAM161A, and WDR90 to have abnormal levels, position, or recruitment time to the distal centriole and proximal centriole compared to control, indicating that POC1B is essential for rod formation. If the rods have a role in shaping the distal centriole, we may find that the distal centriole microtubules' organization is affected. Unlike control POC1B, Δ78 is expected not to interact with POC5 in immunoprecipitation experiments confirming our *in vitro* observation that POC1B C-terminus is essential for this interaction. Overall, we expect these results to support that POC1B is vital for adequately forming the rods during remodeling and providing insight into its specific function. **An honors-level undergraduate student** will participate in all these activity stages, including designing, troubleshooting, quantifying, analyzing, writing, and presentations.

Aim 3: To map POC1B interactions with other rod components in vitro and in vivo

Our preliminary mapping of the human rod protein interactions (**Fig 9)** indicates that protein interactions are crucial in assembling the centriole and its organization. We found that POC1B's C-terminus interacts with POC5's N-terminus, which also interacts with FAM161A's N-terminus. Here, we will study the interactions of rabbit orthologs to determine if the rabbit's differences can be attributed to their amino acid sequence variations. When aligned with BLAST, human POC1B's, POC5's, and FAM161A's amino acid sequences show 92%, 76%, and 74% identity to their respective rabbit orthologs. This observation provides clues that species specificity mainly arises from the sequences of POC5 and FAM161A. Narrowing further, most of the amino acid changes in POC5 are in its putative interaction site with POC1B and FAM161A (POC5 N-terminus). Therefore, we will test the interaction between POC1B's C-terminus and POC5's N-terminus using rabbit proteins so that we will be able to correlate the *in vitro* findings with *in vivo* phenotypes.

Activity 3.1. Determine the POC1B protein interactions in vitro: We will test these interactions by co-expressing the proteins in U2OS cells, a cell line commonly used in centriole biology, as demonstrated in **Fig 9**. To facilitate the study, we will tag the centriolar proteins POC1B, POC5, and FAM161A with mCherry, GFP, and FLAG. We have rabbit POC1B cDNA. The other two rabbit cDNA clones are not readily available, so we will generate them by reverse transcribing mRNA from rabbit testes using the ProtoScript First Strand cDNA Synthesis Kit (Biolabs). We will then amplify the open reading frame using PCR and gene-specific primers flanking the start and stop codons. We will subclone the cDNAs into a pIC194 plasmid and fuse them with mCherry, GFP, and FLAG tags, respectively. An **undergraduate student** will participate in all these activity stages, including designing, troubleshooting, analyzing, writing, presenting, and cloning the rabbit POC5.

We will co-express these proteins in U2OS cells and determine their interactions using immunofluorescent colocalization and co-immunoprecipitation studies. We will study the colocalization of the proteins using confocal fluorescent microscopy to observe the signals of mCherry, GFP, and FLAG-tag staining with a specific antibody, as seen in **Fig 9b-d**. We expect rabbit POC1B-GFP and POC5-mCherry to colocalize in the centrioles (identified by anti-γ-tubulin staining) and not be labeled in cytosolic aggregates labeled by anti-γ-tubulin (GTU-88, Sigma) staining. We expect these aggregates to recruit endogenous CETN2 (labeled by the 20H5 antibody, Sigma), demonstrating that the expressed proteins are folded correctly and are functional. Finally, we expect FAM161A-

FLAG to label the cellular microtubule network (E7, Sigma); when FAM161A-FLAG is expressed with POC1B-GFP and POC5-mCherry, it will recruit them to the microtubule network. An **undergraduate student** will participate in all these activity stages, including designing, troubleshooting, analysis, writing, and presentations; the student research will focus on the interaction of rabbit POC1B with rabbit POC5.

Activity 3.2. Determine POC1B protein interactions in vivo: We will extend our studies to co-immunoprecipitation and pull-down assays with tag-specific antibodies and observe the other proteins' presence with western blot analysis. We expect rabbit POC1B-GFP and POC5-mCherry to co-immunoprecipitate and co-express with FAM161A-FLAG; in fact, all three proteins may co-precipitate. In that situation, we will use Chromo's Tek GFP-Trap and RFP-Trap for immunoprecipitation beads. We will test if the human homologous domains (as in **Fig 9a**) of rabbit POC1B are essential by deleting them (using standard site-directed mutagenesis) and testing their ability to retain the interactions. All experiments will be confirmed with reverse pull-down assays. We expect that, like in humans, the C-terminus of rabbit POC1B will interact with the N-terminus of POC5. However, this interaction may have a distinct effect on POC5's interaction with FAM161A.

Next, we will test the interactions *in vivo* using co-immunoprecipitation studies in control rabbit testis extracts. We will covalently link the anti-POC1B antibody to beads, mix it with testis extract, and identify the co-precipitated proteins by western blot using commercial antibodies (e.g., rabbit anti-FAM161A, Novus NBP1-91508, and rabbit anti-POC5, Thermo Fisher PA5-57981). We will verify the results with reverse pull-down assays by covalently linking anti-FAM161A and anti-POC5 antibodies to beads and performing the same experiments. We expect to detect a complex between POC1B and POC5, and possibly FAM161A. Later, to comprehensively describe rod formation, we will expand this interaction study to include additional rod proteins (CETN1 and WDR90) and tubulin. Based on the sequential interaction findings, we will generate an interaction map in rabbits.

Aim 1-3 expected outcomes, potential problems, alternative strategies, & future directions

Our team and collaborators have extensive experience in each proposed study area and do not expect insurmountable technical hurdles. We expect to determine the centriole remodeling that forms the atypical distal centriole as part of the dynamic basal complex in rabbits. These outcomes will substantiate and confirm the centriole remodeling hypothesis. We expect to generate spatial and temporal interaction maps of protein localization in distal centriole during spermiogenesis in rabbits. We expect these findings will ultimately demonstrate that, for the first time, a spermatids' centriole remodeling results in a spermatozoon neck's dynamic basal complex.

We expect that POC1B is essential for efficient tail-neck-head coordination (**Aim 1**). However, the striated columns may be compensating and reducing the phenotype. In this case, we will challenge the sperm to reveal the phenotype using capacitating conditions and a denser medium. A possible complication is that the Δ78 homozygotes will not develop a detectable sperm motility phenotype using semen analysis because this method identifies major motility defects based on head movement. In this case, we will analyze the sperm tail beating using high-speed movies. Imaging will be performed using a phase-contrast lens at ~500 frames per second, with a pixel size of 8µm, using a microscope we routinely use in our core instrumentation center. Dr. Hermes Gadelha (University of Bristol, UK), an expert in analyzing and modeling sperm movement, will assist in analyzing these data sets (see letter of support). Finally, to associate the above phenotypes with the *Poc1b* mutation and not a background mutation, we will repeat these experiments with the other Poc1B mutants' alleles we generated (not shown), expecting similar results. Our **future direction** will determine the mutation's effects on the striated columns and embryo development. We will examine the sperm neck ultrastructure by collaborating with Dr. Tzviya Ben Mordehai to perform cryo-electron microscopy of the rabbit sperm's neck (see letter of support). We expect that the striated columns will be affected by Poc1B mutation and compensate for the distal centriole defect. We will study the fertility, fecundity, and embryonic phenotype produced by the homozygote mutant male and control mothers. We expect fertility and fecundity to be reduced under conditions that challenge sperm function (e g., sperm competition in a double-mating experiment (Dziuk, 1965)). Overall, we have assembled a strong team with the needed expertise and the means and resources to meet challenges if any arise.

Another potential pitfall is that STORM dual-labeling can be challenging depending on the primary antibody used (Aim 2). If necessary, we will use an alternative approach called the activator-reporter system, which takes more time, but has a higher success rate (Xu et al., 2017). Another potential pitfall is that the Δ78 rods are disorganized in ejaculated spermatozoa but may not be disorganized in epididymis spermatozoa, suggesting that mechanical stress and not remodeling defect generates the abnormal rods (Aim 2). In this case, we will study the sperm movement inducing this abnormality. If no interaction is observed in our immunoprecipitation studies (Aim 3), it will raise the possibility that post-translational modification or other proteins might be involved, and we will identify them in the future with mass spectroscopy (paid service by Dr. Ken Greis's lab, University of Cincinnati). This

interaction may be stage-specific, and we will test for this possibility with epididymis or ejaculated spermatozoa. Our **future studies** will focus on the coordination between centriole remodeling with pericentriolar material remodeling (striated column formation). We are interested in performing clinical investigations and studying the impact of sperm centriole remodeling defects on human reproductive diseases.

Rigor, Reproducibility, Statistics, and Observer bias: The described studies will be carried out independently at least three times. Significant differences (p-value <0.05) will be determined using ANOVA (e.g., two-tailed T-test). Western blots will be developed using a digital imager, in triplicate, and normalized against tubulin. Experiments with visual phenotype assessment such as fluorescent microscopy will be blinded; experimental conditions will be concealed, and samples, slides, or plates will be coded before quantification. Data would be excluded from statistical analysis only if technical issues indicated by positive or negative controls were failing. Studies will be designed using The Experimental Design Assistant tool and the Animal Research: Reporting of In Vivo Experiments guidelines 2.0.

Bio-Hazardous Materials and Safety: All personnel handling hazardous, chemical, and biological materials undergo training entailing proper use of personal protective equipment, disposal of hazards, along all procedures to maintain a safe workplace. The ex vivo analysis of sperm and testis involves biohazards. Biohazards are stored in the BSL-2-marked area and stored in labeled freezers and liquid Nitrogen tanks. Biohazardous waste is discarded in designated biohazard bins as per the institutional protocol and as described in the approval with Institutional Biosafety Committee and Institutional Animal Care and Use Committee. Lab members will be trained annually in a biohazard class provided by the University of Toledo (UToledo) Department of Environmental Health and Radiation Safety. Animals are grown based on United State Department of Agriculture guidelines and under the UToledo Department of Laboratory Animal Resources supervision. Biohazardous and chemical waste disposal will follow the Occupational Safety and Health Administration regulations.

Undergraduate students' role in this project: I have over 20 years of experience working with undergraduate students, starting in my graduate studies. My approach to undergraduate research has been successful at the UToledo, and this project will follow that model. My undergraduates participate in all aspects of a project, enabling them to gain a holistic research experience from beginning to end. The research team will be composed of two PhD students and 2-4 undergraduates at any point in time. Undergraduates apply directly to work with the lab and are selected after an interview to determine that their interests align with the lab's research. Third-year or later students are encouraged first to take my course-based undergraduate research experience (BIOL3910), where students learn the background information related to the sperm centriole and do immunofluorescent and confocal microscopy. Remarkably, the 2022 BIOL3910 class wrote a paper (titled *Tubulin posttranslational modifications modify the atypical spermatozoon centriole*) that we submitted to publication in the microPublication Biology journal. First- and second-year students will start by shadowing the lab research, participating in lab meetings, and performing the safety class and other regulatory preparations during the first semester. After that, all students join our research lab as part of the independent study course BIOL4700-022, honors studies BIOL4700-202, or paid research assistants from this grant funding. Each student participates in two weekly meetings: a general lab meeting with all lab members and a focus group meeting related to a project led by a PhD student. Typically, undergraduates start the hands-on research the following semester and then spend 3-8 semesters working on 1-2 subprojects. Honors undergraduates can spend up to four years in the lab doing research. Specific activities that undergraduates will carry out are identified in the Research Strategy section. Additionally, students deliver a lab seminar each semester and are encouraged to present their research in multiple local and national avenues. Undergraduates contributing meaningfully to the project appear as co-authors of published works. My students frequently become published authors because of their lab research (see **Biographical Sketch** for details).

Other official and unofficial events in the lab facilitate student interactions with professional scientists, such as visiting faculty from other institutions and weekly lab meetings with other students. Students enhance their writing and public speaking skills via formal research reports and presentations at the lab's poster or seminar sessions and other events. Overall, the students learn how to perform scientific research, how to write scientifically, and how to present their findings. The impact of this experience is assessed through one-on-one, weekly meetings between the me and the students, and by helping in post-graduation placement. Many of my undergraduate students have gone on to graduate programs in biology, medicine, and public health; student placement data is provided in the Biographical Sketch.