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# SPAG17 is Important for Protein Trafficking in Mammalian Spermiogenesis

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# *SPAG17* is Important for Protein Trafficking in Mammalian Spermiogenesis

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University

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Abbreviations

ARL13B = ADP Ribosylation Factor 13B
AZ11 = Centrosomal Protein 131
BBS4 = Bardet-Biedl Syndrome Type 4
BSA = Bovine Serum Albumin
CEP164 = Centrosomal Protein 164
CPC = Central Pair Complex
DDB1 = DNA Damage Binding Protein 1
DEFB1 = β-Defensin 1
Dhh signaling = Desert hedgehog signaling
DMEM = Dulbecco's Modified Eagle Medium
DMP1 = Dentin Matrix Protein 1
ER = Endoplasmic Reticulum
FRT = Flippase Recognition Target
GATA4 = GATA-binding Transcription Factor 4
GLI1 = Glioma Transcription Factor 1
GOPC = Golgi-associated PDZ-domain and coiled-coil Motif-containing Protein
Hh signaling = Hedgehog signaling
HTCA = Head-tail coupling apparatus
IF = Immunofluorescence
IFT = Intraflagellar Transport
IFT121 = Intraflagellar Protein 121
IFT20 = Intraflagellar Protein 20
IFT74 = Intraflagellar Protein 74
IFT88 = Intraflagellar Protein 88
IMT = Intramanchette Transport
INCENP = Inner Centromere Protein
IP = Immunoprecipitation
IPA = Ingenuity Pathway Analysis
KO = Knockout
LacZ = β -galactosidase encoding Lac Operon
LoxP = Lox-flanked cassette
LRGUK = Leucine-rich Repeat (LRR) and Guanylate Kinase-like Domain-containing Isoform 1
MEIG1 = Mouse Meiosis-expressed Protein 1
MORN3 = Membrane Occupation and Recognition Nexus Repeat-containing Protein 3
NEK4 = Nima-related Protein 4
ODF1 = Outer Dense Fiber Protein 1
PACRG = Parkin Co-regulated Gene
PBS = Phosphate-buffered Saline
PCDP1 = Primary Ciliary Dyskinesia Protein 1

PCR = Polymerase Chain Reaction
PFA = Paraformaldehyde
PRKCSH = Protein Kinase C Substrate 80K-H
RAB10 = Ras-related Protein 10
RAB5B = Ras-related Protein 5B
RAB6A = Ras-related Protein 6A
RAB8 = Ras-related Protein 8
RCC1 = Regulator of Chromosome Condensation Protein 1
SEPT2 = Septin 2
SMO = Smoothened
SPAG17 = Sperm-associated antigen-17
SPAG6 = Sperm-associated antigen-6
SPEF2 = Sperm Flagellar Protein 2
SSC = Spermatogonial Stem Cell
SUN3 = Sad/Unc84 domain-containing Protein 3
Trypsin-EDTA = Trypsin-ethylenediaminetetraacetic acid
WT = Wild-type
ZNF217 = Zinc Finger Protein 217

## ABSTRACT

### *SPAG17* is Important for Protein Trafficking in Mammalian Spermiogenesis

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University

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Spermiogenesis is the process through which undifferentiated germ cells develop into mature spermatozoa. While spermiogenesis is a very well-regulated process, the protein-protein interactions regulating it remain poorly understood. A knockout (KO) mouse for the ciliary protein SPAG17 was generated by our lab. Loss of SPAG17 has been shown to disrupt the transport of proteins important for acrosome biogenesis and manchette functions. With this information, we hypothesized that SPAG17 plays an essential role in protein trafficking during mammalian spermiogenesis. To further investigate this, immunofluorescence (IF) studies were performed in germ cells collected from both WT and SPAG17 KO mice to visualize proteins of interest. Results showed GOPC, AZI1, KIF3A, INCENP, RAB6A and DDB1 to be missing from the manchette in the SPAG17 KO, suggesting they are part of the SPAG17 interactome of proteins. We then used IPA to map a possible interactome of proteins that may be regulated by SPAG17. Altogether, these findings reveal that SPAG17 is involved in the intracellular

trafficking of proteins and it influences manchette formation, and thus acrosome and tail biogenesis in elongating spermatids by disturbing the recruitment of essential proteins to the manchette.

## 1. Introduction

### *1.1 Spermatogenesis*

Spermatogenesis is a well-regulated process that occurs throughout the reproductive lifetime of an organism in which undifferentiated germ cells develop into mature spermatozoa (Chemes, 2017; Griswold, 2016). Spermatogenesis begins within the seminiferous tubule of the testes and is aided by Sertoli cells which provide support to germ cells and aid in their migration to the tubule lumen (Chemes, 2017; Neto et al., 2016). This process can be divided into three phases: mitosis, meiosis and spermiogenesis (Fig. 1).

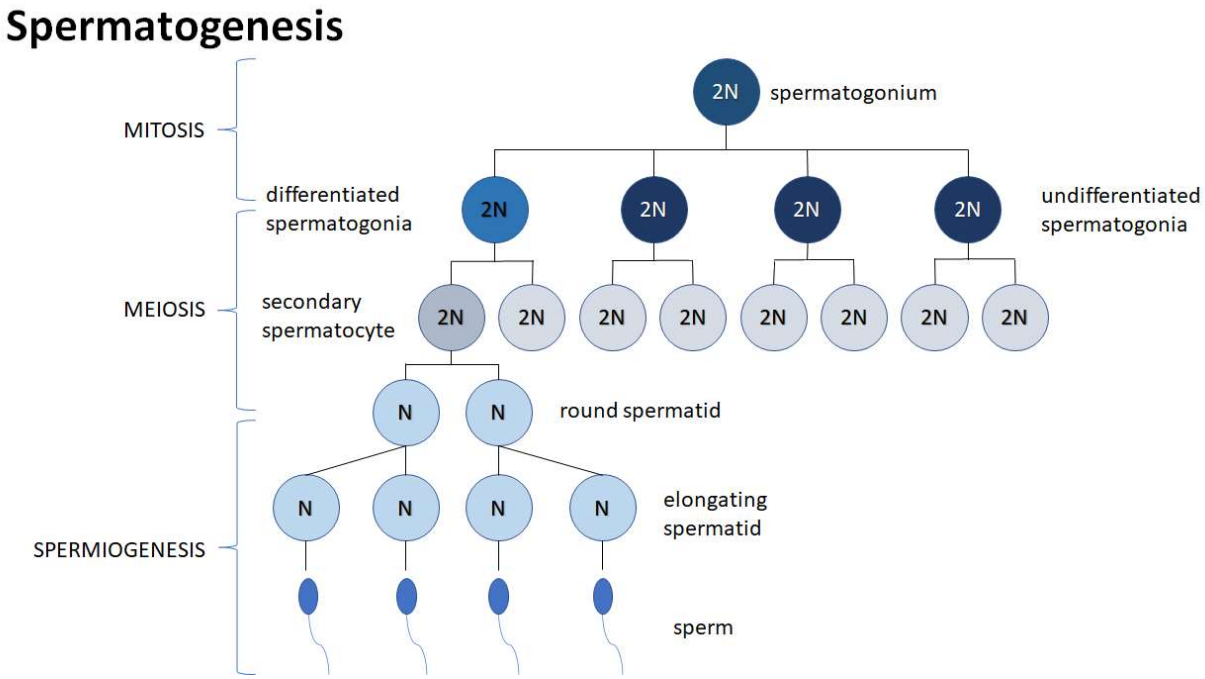



**Figure 1: Schematic representation of the different phases of spermatogenesis. Mitosis, involving division of spermatogonia.** Meiosis I is a special type of cell division in sexually-reproducing organisms used to produce the gametes, such as sperm. It creates two diploid secondary spermatocytes. Meiosis II is a cell division that results in four haploid spermatids. Spermiogenesis is the last step where the cells differentiate to sperm.

The **mitotic phase** consists of diploid stem cells, called spermatogonia, multiplying. During this proliferative phase, two types of spermatogonia are produced (undifferentiated and

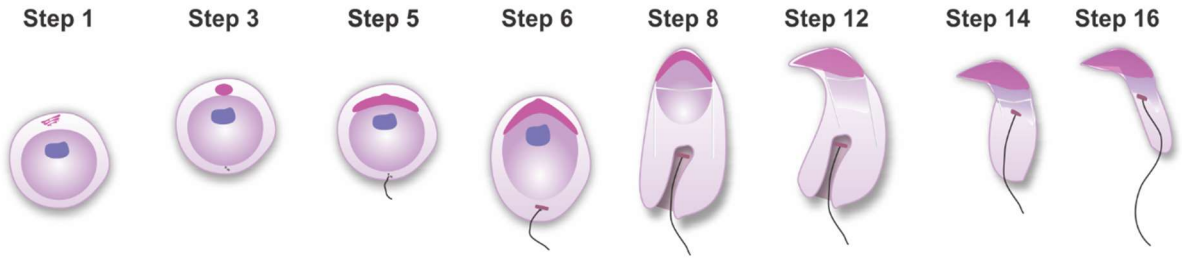
differentiated spermatogonia). Undifferentiated spermatogonia are normally present in all stages of the seminiferous epithelium. They are responsible for the renewal of their own stock of cells and to produce differentiated spermatogonia. Differentiated spermatogonia are cells committed to sperm production. This process halts at birth and begins again with the **meiotic phase** at puberty. Two meiotic divisions take place and for the first division, some spermatogonia stop proliferating and differentiate into diploid primary spermatocytes (Alberts et al., 2002). These primary spermatocytes exhibit changes in morphological features in terms of nuclear and chromatin morphology and can be divided in five types of spermatocytes of varying sizes with unique morphological features (preleptotene, leptotene, zygotene, pachytene, and diplotene) (Auger, 2018). After meiosis I is completed, diploid secondary spermatocytes undergo meiosis II to produce four round haploid spermatids (Fig. 1) (Alberts et al., 2002).

**Spermiogenesis** is the final differentiation phase in which the haploid round spermatids produced at the end of meiosis II undergo significant morphological changes to become mature spermatozoa (Fig. 1) (Auger, 2018). These changes include chromatin condensation, nuclear formation, flagella and acrosome development, and elimination of excess cytoplasm (Lehti & Sironen, 2017; Russell et al., 1993). This final step is essential for the successful completion of spermatogenesis and requires the intracellular trafficking of proteins to shape and create normal spermatozoa (Teves et al., 2020).

In the mouse, spermiogenesis can be divided into 16 steps, each of which is characterized by the morphological appearance of the developing acrosome and nuclear shape (Fig. 2). Steps 1-8 begin with a round, centralized nucleus that becomes compacted and polarized to one side of the cell at the end of step 8. The acrosome flattens over the surface of the nucleus and polarizes to one side of the nucleus (Lehti & Sironen, 2017; O'Donnell, 2014). At step 3, the axoneme, a

9+2 microtubule core structure of the sperm flagella, begins to elongate from the distal centriole (Lehti & Sironen, 2017). The manchette, a transient microtubular structure, begins to form at step 8 (O'Donnell, 2014). Steps 9 to 14 are defined by the presence of the manchette surrounding the caudal part of the sperm head. It is believed that this transitory structure modulates the acrosome and nuclear shape. The manchette also acts as a trafficking mechanism for delivering proteins to the developing tail (Teves et al., 2020). During steps 15-16, most cytoplasmic organelles such as the endoplasmic reticulum, Golgi apparatus, and lysosomes are eliminated as well as the cytoplasm (Chubb, 1992).

**Figure 2: Spermiogenesis steps.** Spermiogenesis is the final phase of spermatogenesis during which haploid round spermatids become mature sperm. This phase can be divided into 16 steps, each of which is defined by morphological changes. Changes during this phase include




chromatin condensation, nuclear formation, acrosome and flagella development and elimination of excess cytoplasm.

Spermiogenesis ends with spermiation in which elongated spermatids leave the epithelium of the seminiferous tubules and become free spermatozoa in the lumen (Chemes, 2017; O'Donnell, 2014). Sperm then pass into the epididymis where they fully mature and are stored (Alberts et al., 2002). Recently the importance of correct protein transport during spermiogenesis has been recognized and defects during this process have been known to result in male infertility (Pleuger et al., 2020; Teves et al., 2020).

*1.2 Relevance of the Manchette*

The manchette is a transitory skirt-like structure that aids in nuclear shaping and elongation, chromatin condensation, flagellar formation, and protein trafficking (Lehti & Sironen, 2017; Pleuger et al., 2020). The manchette is present only during spermatid differentiation (Lehti & Sironen, 2016). The manchette is made of microtubules attached to a perinuclear ring that surrounds the lower part of the nucleus with actin filaments and associated motor proteins interspersed between the microtubules (Fig. 3) (Kierszenbaum et al., 2011; Lehti & Sironen, 2016; Wei & Yang, 2018). Formation of the microtubules requires a nucleation site, the identity of which is still under debate. Multiple hypotheses exist with many claiming the perinuclear ring, the centrosome, or even other microtubules as potential nucleators (Lehti & Sironen, 2016). While there is some evidence for each hypothesis, none have proven to be true thus far.

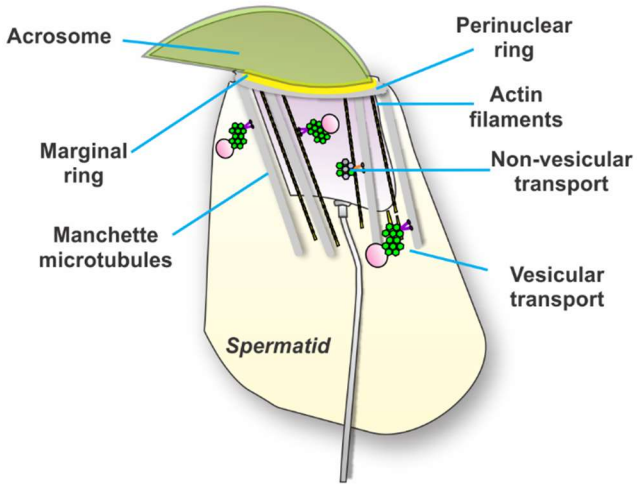



**Figure 3: Model illustrating intramanchette transport mechanism.** The manchette is a transitory organelle surrounding the elongating spermatid nucleus. It consists of bundles of microtubules connected to a perinuclear ring and filaments of actin intercalated between the microtubules. Proteins are transported on these tracks to specific intracellular sites during the process of sperm differentiation. Some proteins form large complexes that can transport vesicular as well as non-vesicular cargos. (Teves et al., 2020)

Microtubules and actin both provide a scaffolding for the intracellular trafficking of proteins and vesicles between the nucleus and cytoplasm as well as to the base of the developing sperm tail and to the centrosome (Fig. 3) (Kierszenbaum & Tres, 2004; Lehti & Sironen, 2016; Sun et al., 2011). Proteins are transported using motor proteins kinesin and dynein along microtubules and using myosin along actin filaments during intramanchette transport (IMT) (Lehti & Sironen, 2016; Sun et al., 2011). Intraflagellar transport (IFT) also occurs using the microtubule scaffolding and motor proteins to deliver proteins to the developing sperm tail (Lehti & Sironen, 2016).

As spermiogenesis continues, manchette microtubules and actin filaments begin to move down toward the axoneme in a zipper-like fashion and reduces its diameter to aid in shaping of the spermatid head and nucleus (Lehti & Sironen, 2016; Sun et al., 2011). This movement of the manchette is believed to assist with chromatin condensation which in turn influences nuclear shaping (Lehti & Sironen, 2016).

*1.3 Mechanisms of Protein Trafficking During Spermiogenesis*

There are three main paths for protein trafficking during spermiogenesis: Golgi transport, IMT and IFT. Trafficking of proteins from the Golgi apparatus is essential throughout sperm differentiation. During acrosome biogenesis Golgi-derived vesicles are transported from the trans-Golgi and fused to generate the acrosome. Then they interact with the inner acrosomal membrane, with the acroplaxome, and with the outer and inner nuclear membrane. Microtubules and F-actin tracks present in this region are responsible for this process (Kierszenbaum, Rivkin, & Tres, 2011). Additionally, numerous proteins have been shown to use these tracks for delivery purposes and are important for acrosome biogenesis (Teves et al., 2020). Perturbation of the developing acrosome results in male infertility and round-headed spermatozoa

(globozoospermia) (Coutton et al., 2015; Kang-Decker et al., 2001; Kierszenbaum & Tres, 2004; Xiao et al., 2009; Yao et al., 2002).

Intramanchette transport assists with protein trafficking using the manchette. Proteins and vesicles are transported between the nucleus and cytoplasm, to the centrosome, and to the developing sperm tail (Pleuger et al., 2020; Teves et al., 2020). The manchette is located at the nucleo-cytoplasmic interchange to allow for protein transport through nuclear pores (Kierszenbaum, Rivkin, & Tres, 2011). Ran, a GTPase of the Ras superfamily, has been suggested to help control the trafficking of nuclear proteins, especially during condensation of the nucleus (Kierszenbaum, 2002). Ran exists in GTP- and GDP-bound conformations with the active GTP-bound Ran binding proteins for transport (Clarke & Zhang, 2001). The Ran GTPase system processes the transition between histones to transition proteins to protamines during chromatin condensation (Kierszenbaum, 2002).

IMT is assisted by motor proteins kinesin and dynein which move cargo in the anterograde and retrograde direction, respectively (Lehti & Sironen, 2016; Lehti et al., 2013; Rosenbaum et al., 1999). Kinesin traffics structural sperm tail proteins to the base of the tail while dynein transports turnover proteins back to the cytoplasm (Lehti & Sironen, 2017; Rosenbaum et al., 1999). Motor proteins are able to carry passenger proteins using protein rafts made of a complex of IFT particles. There are about seventeen IFT particles organized into two complexes, IFT-A and IFT-B. These cargo, raft, and motor proteins move bi-directionally along microtubules during both IMT and IFT (Cole et al., 1998).

The mechanism of IMT is not well known but it is believed to resemble IFT (Lehti & Sironen, 2016). Several gene mutations associated with IMT lead to head and tail deformities

that cause male infertility. These mutations can disturb protein trafficking to the correct site during spermiogenesis and create deformities in the sperm (Gunes et al., 2018).

IFT functions to transport proteins from the base of the developing sperm tail to the tip using a system of microtubules and motor and raft proteins similar to IMT (Lehti & Sironen, 2016; Rosenbaum et al., 1999). Mutations associated with IFT microtubules can cause head and tail abnormalities similar to IMT as well (Gunes et al., 2018).

*1.4 Sperm-associated antigen-17 (SPAG17)*

The *SPAG17* gene is known to encode a protein localized to the central pair complex (CPC) of the axonemal sperm flagella and believed to play a role in sperm flagella motility (Rupp et al., 2001; Zhibing Zhang et al., 2005). SPAG17 is the mammalian orthologue of PF6, a protein located at the C1a projection of the CPC in *Chlamydomonas reinhardtii* (Zhang et al., 2005). PF6 is an essential component for the C1a assembly at the CPC, confirmed by a *PF6* mutant mouse displaying a twitchy and non-functional flagellum (Rupp et al., 2001; Zhibing Zhang et al., 2005). The C-terminal of PF6 has been shown to be necessary for flagellar motility and C1a assembly while the N-terminal is responsible for stabilization of the C1a complex (Goduti & Smith, 2012). Though murine *Spag17* also encodes a 250 kDa protein in the CPC, the mammalian gene is known to show greater complexity in expression patterns and function (Teves et al., 2016).

*SPAG17* co-localizes to Golgi vesicles, the acrosome, manchette microtubules and the sperm tail (Fig. 4a, 4b) (Kazarian et al., 2018). *SPAG17* knockout mice have been found to develop a primary ciliary dyskinesia phenotype identified by disrupted cilia motility in addition to the areas listed above. The delivery of some axonemal proteins and intraflagellar transport during spermiogenesis is also disrupted. In addition, proteins important for sperm tail

development fail to localize in the manchette in the absence of SPAG17. This suggests that SPAG17 is involved in the intracellular trafficking of proteins.

*1.5 Hypothesis*

In this study, we hypothesize that *SPAG17* is essential for protein trafficking during mammalian spermiogenesis, and the loss of *SPAG17* results in disrupted transport of proteins important for acrosome biogenesis and manchette functions.

*1.6 Specific Aims*

The first aim with this study is to characterize the transport of proteins during spermiogenesis. The second aim is to determine the proteins associated with the *SPAG17* complex. The third aim is validation of protein interactions.

## 2. Materials and Methods

*2.1 Animals*

All experimental protocols involving animal use were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and animal protocol AM10297 approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. All efforts were made to minimize the potential for animal pain and stress.

Heterozygous B6N(Cg)-*Spag17*$^{tm1b(KOMP)Wtsi}$/J (Stock No. 026485) mice from Jackson Laboratories were used to generate homozygous mice with disrupted expression of the *Spag17* gene. The *Spag17* knockout mutant vector was generated by the Knockout Mouse Phenotyping Program (KOMP2) at The Jackson Laboratory. The L1L2_Bact_P cassette, which is composed of an FRT site followed by lacZ sequence and a loxP site, was inserted on Chromosome 3. The

first loxP site is followed by neomycin under the control of the human β-actin promoter, SV40 polyA, a second FRT site, and second loxP site. A third loxP site is inserted downstream of the targeted exon 5. The critical exon is thus flanked by loxP sites. The mouse strain was generated by the KOMP2 at The Jackson Laboratory using embryonic stem cells provided by the International Knockout Mouse Consortium. The construct was introduced into C57BL/6N-derived JM8.N4 embryonic stem cells, and correctly targeted embryonic stem cells were injected into B6(Cg)-Tyrc-2J/J (Stock No. 58) blastocysts. The resulting chimeric males were bred to C57BL/6NJ (Stock No. 005304) females and then to B6N.Cg-Tg(Sox2-cre)1Amc/J mice (Stock No. 014094) to remove the floxed neomycin cassette and critical exon sequences. The resulting offspring were bred to C57BL/6NJ mice to remove the Cre-expressing gene.

*2.2 Mouse genotyping*

The mice were genotyped by PCR using the following primers: forward 5'-CTGTCTTGATGAGAATGTAATG-3' (this sequence is present in the wild-type genomic DNA but absent in the mutant mouse), reverse 5'-GAGTGAGCAACTTTCCTCAGGAG-3' (this sequence is present in the wild-type genomic and mutant mouse DNA), and forward 5'-CCCTGAACCTGAAACATAAA-3' (this sequence is present upstream of the first LoxP site in the vector sequence). The expected PCR for wild-type animals is 96bp. The mutant band is larger at 300bp due to an extra sequence present in the vector.

*2.3 Germ cell preparation*

The testis from adult wild-type and *Spag17* knockout mice were decapsulated and placed in an incubation buffer of 5mL DMEM (Gibco by Life Technologies, Grand Island, NY, USA), 1.0 µg/mL DNase I (Sigma-Aldrich, St. Louis, MO, USA), and 0.5mg/ml collagenase IV (Sigma-Aldrich, St. Louis, MO, USA), then incubated for 30 min at 32°C to allow for cell dissociation.

Cells were then washed three times with PBS and fixed in 5mL of 4% PFA (Sigma-Aldrich, St. Louis, MO, USA) and 0.1M sucrose shaking at room temperature for 15 min. Cells were washed again in PBS and resuspended in 7mL PBS.

*2.4 Immunofluorescence*

Cells were plated on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA), allowed to partially dry, and permeabilized in 0.1% Triton X-100 (Fisher Scientific, Fair Lawn, NJ, USA) for 5 min at 37°C. Then, cells were washed three times with PBS and blocked in 10% Goat serum (Vector Laboratories, Inc., Burlingame, CA, USA) or 0.5% BSA (Sigma-Aldrich, St. Louis, MO, USA) in a wet box at room temperature for 60 min. Cells were then incubated overnight with several primary antibodies for the respective protein of interest (Table 1) and mouse anti-acetylated tubulin or rabbit anti-SPAG6 as manchette markers. After washing, the cells were incubated with anti-rabbit Alexa Fluor 488-labeled, anti-mouse Alexa Fluor 488-labeled, anti-rabbit Cy3-labeled, anti-mouse Alexa Fluor 594-labeled, and/or anti-goat Alexa Fluor 594 labeled secondary antibodies (Jackson ImmunoResearch Laboratory Inc., Grove, PA, USA) for 60 min. Cells were then washed with PBS, mounted with VectaMount with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) to stain for the nucleus and sealed using nail polish. Images were captured using a Zeiss LSM 700 confocal laser-scanning microscope. Microscopy was performed at the VCU Microscopy Facility, supported, in part, by funding from the NIH-NCI Cancer Center Support Grant P30 CA016059. Images were processed using ZEISS Zen 3.2 (blue edition) imaging software from Carl Zeiss Microscopy GmbH. Experiments were performed in three or more independent samples per genotype. Localization of the proteins to the manchette was determined by a randomized and blind analysis in and average of 20 cells/protein per genotype.

**Table 1: Antibodies used for immunofluorescence studies.** These antibodies were chosen based on previous research implicating them in spermiogenesis.

| Primary Antibodies Used for Immunofluorescence Studies |
|---|
| GOPC (Proteintech Group, Rosemond, IL, USA) |
| AZI1 (Proteintech Group, Rosemond, IL, USA) |
| ZNF217 (Sigma-Aldrich, St. Louis, MO, USA) |
| HOOK1 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) |
| IFT74 (Antibody Verify, Las Vegas, NV, USA) |
| IFT88 (Proteintech Group, Rosemond, IL, USA) |
| IFT121 (Antibody Verify, Las Vegas, NV, USA) |
| LRGUK (Novus Biologicals USA, Centennial, CO, USA) |
| KIF3A (BD Biosciences, San Jose, CA, USA) |
| MEIG1 (Antibody Verify, Las Vegas, NV, USA) |
| MORN3 (developed by our lab) |
| ODF1 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) |
| PACRG (developed by our lab) |
| RCC1 (developed by our lab) |
| SEPT2 (Proteintech Group, Rosemond, IL, USA) |
| SPAG6 (Thermo Fisher Scientific, Rockford, IL, USA) |
| SPEF2 (Sigma-Aldrich, St. Louis, MO, USA) |
| SUN3 (Invitrogen, Carlsbad, CA, USA) |
| ARL13B (Proteintech Group, Rosemond, IL, USA) |
| CEP164 (Proteintech Group, Rosemond, IL, USA) |
| INCENP (Thermo Fisher Scientific, Rockford, IL, USA) |
| RAB5B (LifeSpan BioSciences, Inc., Seattle, WA, USA) |
| RAB6A (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) |
| RAB8 (MyBioSource, Inc., San Diego, CA, USA) |
| RAB10 (Cell Signaling Technology, Inc., Danvers, MA, USA) |
| BBS4 (Proteintech Group, Rosemond, IL, USA) |
| GLI1 (Sigma-Aldrich, St. Louis, MO, USA) |
| SMO (Biomatik USA, LLC, Wilmington, DE, USA) |
| DDB1 (Thermo Fisher Scientific, Rockford, IL, USA) |
| DEFB1 (Boster Biological Technology Co., Ltd, Pleasonton, CA, USA) |
| DMP1 (Abcam, Cambridge, MA, USA) |
| GATA4 (OriGene Technologies, Inc., Rockville, MD, USA) |
| anti-acetylated tubulin (Sigma-Aldrich, St. Louis, MO, USA) |

*2.5 SPAG17 interactome analysis using IPA*

We developed an interactome using Ingenuity Pathway Analysis (IPA), version 49932394 (https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis), software from QIAGEN Inc., for protein interaction analysis. We examined the proteins

presented in this study and focused on partners for which protein-protein binding was validated by published data using IP, yeast-two hybrid screen, tubulin binding assay, affinity purification columns and/or co-transfection. Additionally, the tool "Path Explorer" in the IPA software was used to generate an interactome. This tool elucidates the shortest path between molecules based on specific criteria. Several filters were employed. The software was only allowed to consider "direct" interactions. "Relationship types" were limited to only "protein-protein interactions. There are several types of molecules, or "node types," that IPA can consider when making its connections. However, these molecules were filtered to include only the following, in order to capture only proteins or protein complexes: complex, cytokine, enzyme, G-protein coupled receptor, group, growth factor, ion channel, kinase, ligand-dependent nuclear receptor, peptidase, phosphatase, transmembrane receptor, and transporter. "Species" were limited to human, mouse, rat, and uncategorized data. All other criteria were left as the default settings.

## 3. Results

Immunofluorescence studies were performed in WT and *Spag17* KO mice to determine the localization of several proteins previously implicated in IMT. Mixed germ cells from both WT and *Spag17* KO mice were isolated and co-immunostained with primary antibodies and anti-acetylated tubulin as a microtubule marker. Co-localization of the primary antibody with microtubules structures was indicated by the presence of yellow due to overlap of red and green staining.

### *3.1 Golgi Proteins*

Golgi proteins are essential for trafficking pro-acrosomal vesicles originating from the Golgi apparatus during early spermiogenesis (Khawar et al., 2019). We focused on Golgi-

associated PDZ-domain and coiled-coil motif containing protein (GOPC) for our studies as it has been previously implicated in vesicle transport during spermiogenesis (Suzuki-Toyota et al., 2007).

GOPC is a coiled-coil protein that plays a significant role in vesicle transport from the Golgi apparatus and in membrane fusion (Suzuki-Toyota et al., 2007). GOPC is localized to the trans-Golgi and perinuclear ring in round spermatids and later to the cytoplasm in elongating spermatids which is consistent with Golgi location at that stage. Pro-acrosomal vesicles in *Gopc* KO male mice fail to fuse to the nuclear envelope resulting in a fragmented acrosome leading to malformations of the head, manchette, post-acrosomal sheath, and posterior ring (Suzuki-Toyota et al., 2007; Yao et al., 2002). Tail elements seem to form normally until spermiation when the tail coils around the nucleus (Suzuki-Toyota et al., 2007).

We found GOPC to localize to the Golgi, acrosome granules, the manchette, centrosomes, and to the mature sperm tail in WT germ cells. In *Spag17* KO germ cells, GOPC was no longer visible in the acrosome and had a much weaker signal in the manchette area. The protein was still present in the Golgi in round spermatids suggesting GOPC may move to the mid-point at maturation. While GOPC is present in the cytoplasm of elongating spermatids, it is no longer present in the manchette suggesting that it is part of the SPAG17 interactome of proteins (Fig. 4).

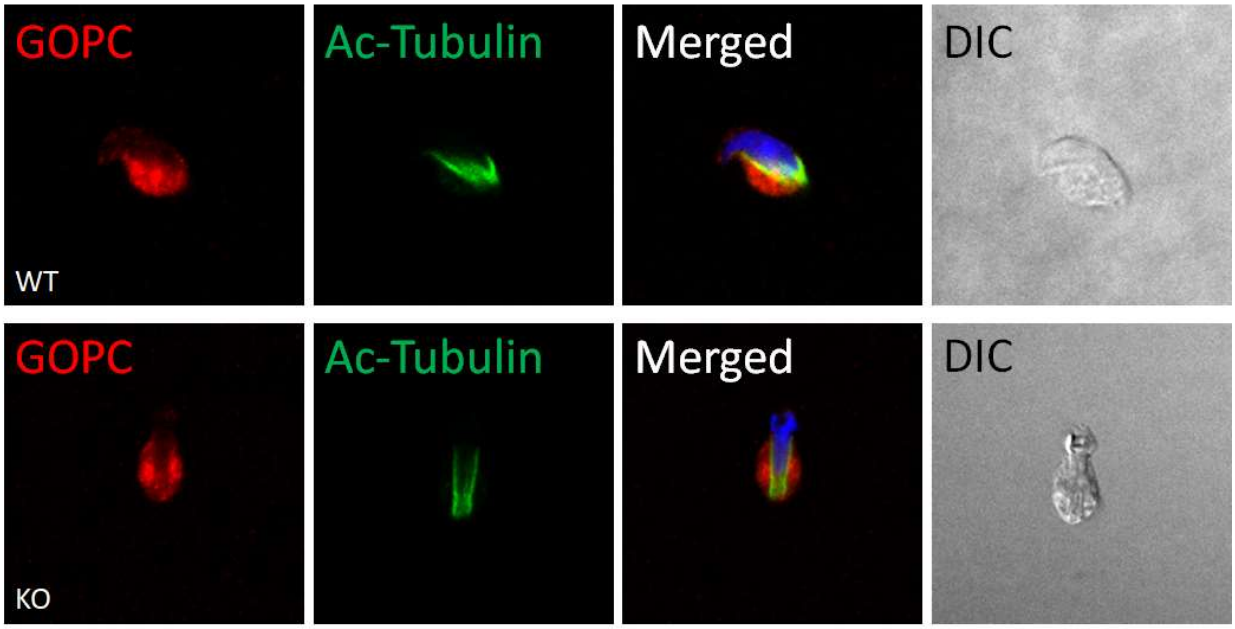



**Figure 4. GOPC localization to the manchette is dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult and stained for GOPC and manchette structures. GOPC failed to localize to the manchette in elongating spermatids.

*3.2 Acrosomal Proteins*

Acrosome biogenesis requires a number of proteins to ensure pro-acrosomal vesicles fuse properly to the nuclear envelope to form the acrosomal cap. Additional proteins are needed to tether the acrosomal cap to the acroplaxome to ensure the acrosome is anchored to the sperm head so proper sperm head shaping can occur (Khawar et al., 2019). There are also several proteins that reside in the mature acrosome that participate in the capacitation and fertilization process.

Zinc fingers proteins are transcriptions factors that are often found in the nuclei of sperm and bind to DNA, RNA, and histones to regulate chromatin organization, gene expression, histone modification and meiosis during spermatogenesis (Castillo et al., 2013; Noce et al., 1992). Zinc finger proteins bind to DNA, RNA, or histones and regulate their functions by transcriptional activation or repression (Castillo et al., 2013). Several zinc fingers proteins such

as ZFP318, basonuclin, CTfin33, CTfin51 and CTfin92 have been localized to the nucleus of germs cells in all stages of spermatogenesis (Ishizuka et al., 2016; Mahoney et al., 1998; Noce et al., 1992). However, zinc finger proteins were also associated with lysosome vesicles like the acrosome (Li et al., 2009). Mutants of zinc finger proteins are infertile. Though ZNF217 has not yet been implicated in spermiogenesis, we chose to focus on it to see if it plays a role in spermiogenesis at all. We found this protein to localize to the acrosome (a lysosome like organelle) in both WT and *Spag17* KO germ cells suggesting that the transport of this protein does not depend on SPAG17 (Fig. 5).

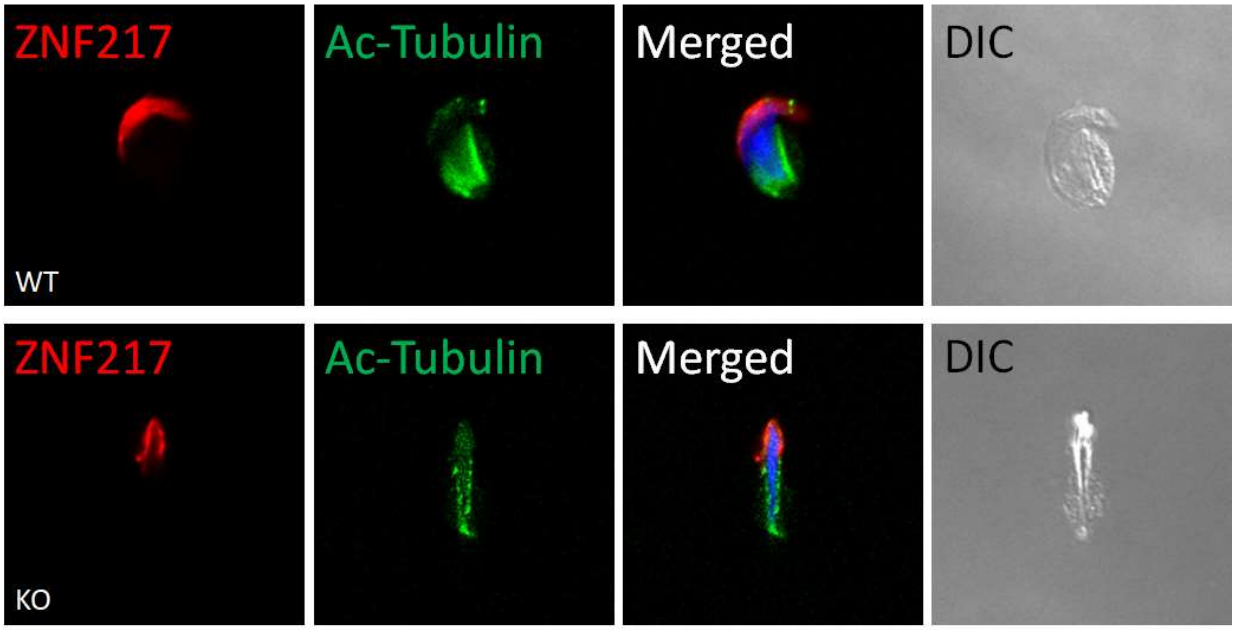



**Figure 5. ZNF217 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for ZNF217 and manchette structures. ZNF217 localizes to the acrosome in both WT and KO elongating spermatids.

*3.3 Microtubule Associated Proteins*

As mentioned above, the manchette is a transient skirt-like microtubular structure that provides a scaffolding for protein and vesicular trafficking (Pleuger et al., 2020; Teves et al., 2020). Several proteins have been found to localize to the manchette during spermiogenesis and

they have a number of functions, including stabilizing microtubules at the perinuclear ring, manchette assembly and disassembly and the trafficking of proteins in a retro- and anterograde fashion.

HOOK1 is a coiled-coil protein shown to participate in IMT due to its microtubule binding capabilities (Mendoza-Lujambio et al., 2002). HOOK1 ensures correct positioning and elongation of the manchette microtubules so IMT is properly carried out for flagellar formation (Kierszenbaum, Rivkin, & Tres, 2011; Mendoza-Lujambio et al., 2002). Localization of HOOK1 shifts throughout spermatogenesis with initial detection in the trans-Golgi along with pro-acrosomal vesicles and eventually in the acrosome-acroplaxome after membrane fusion (Kierszenbaum, Rivkin, & Tres, 2011). During spermatid elongation, HOOK1 relocates from the acrosome-acroplaxome to the manchette from where it is finally seen in the head-tail coupling apparatus (HTCA) (Kierszenbaum, Rivkin, & Tres, 2011; Mendoza-Lujambio et al., 2002). No HOOK1 staining is seen in mature sperm cells so it is not likely to be a structural component of sperm. Loss of HOOK1 results in abnormal head morphology and severe tail defects resulting in male infertility due to microtubular defects (Mendoza-Lujambio et al., 2002).

Our experiments showed HOOK1 to localize to the manchette, around the nucleus in the form of vesicles, and to the cytoplasm in WT germ cells. HOOK1 also localized to the tail in mature sperm. HOOK1 expression remained in the manchette and cytoplasm in *Spag17* KO mice suggesting its localization is independent of SPAG17 (Fig. 6).

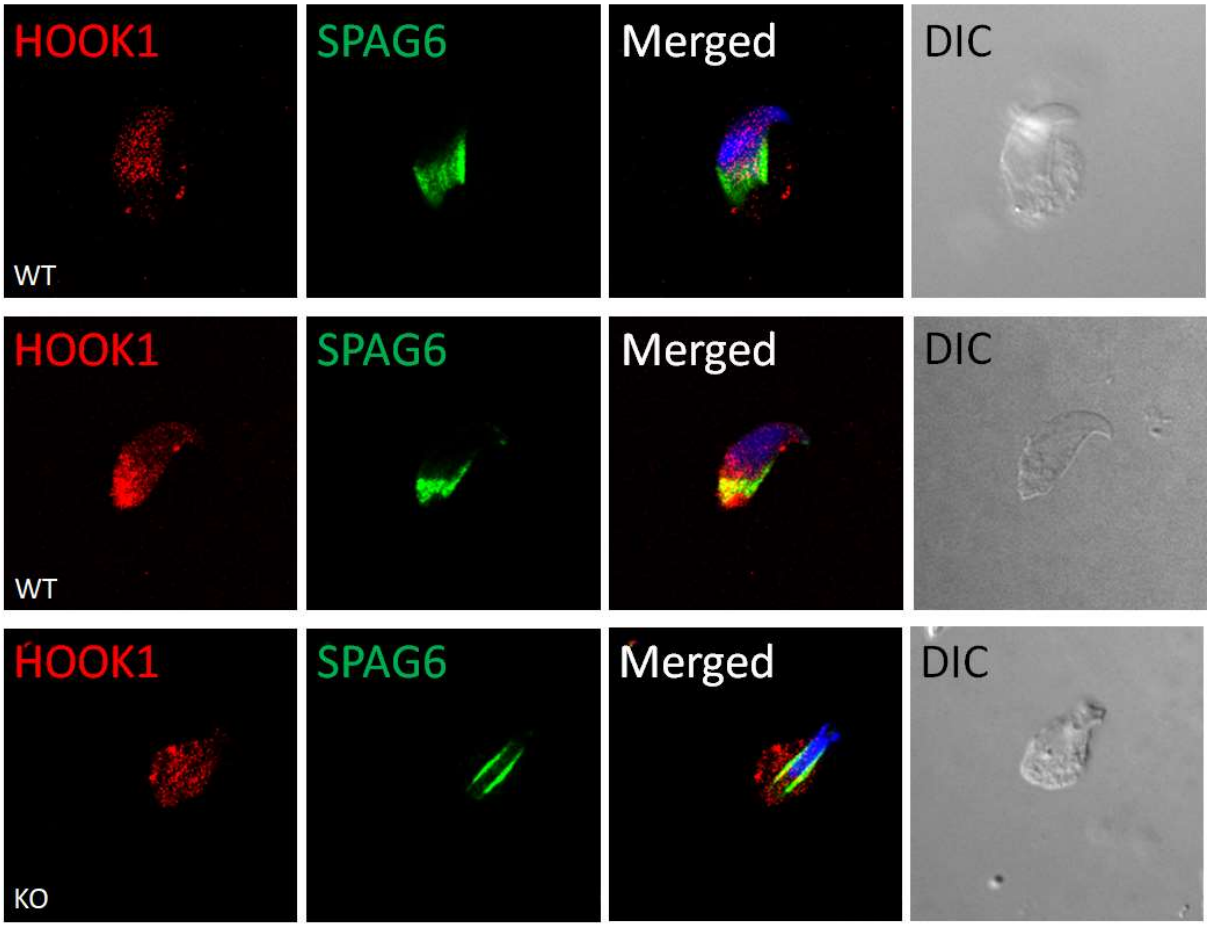



**Figure 6. HOOK1 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for HOOK1 and manchette structures. HOOK1 localized to the manchette, around the nucleus, and to the cytoplasm of elongating spermatids in both WT and KO germ cells.

Intraflagellar protein 74 (IFT74) is a 74 kDa protein of the IFT-B complex and is essential for mature sperm formation. IFT74 stabilizes the IFT-B complex, transports tubulin, and controls cilium formation and length. It is found in the vesicles of spermatocytes and round spermatids, in the acrosome and centrosome of elongating spermatids and in developing sperm tails. *Ift74* KO mice are infertile, and their sperm are immotile with misshaped heads and short tails (Shi et al., 2019).

Our studies found IFT74 to localize to the manchette and acrosome of both WT and *Spag17* KO germ cells suggesting that the transport of this protein does not depend on SPAG17 (Fig. 7).

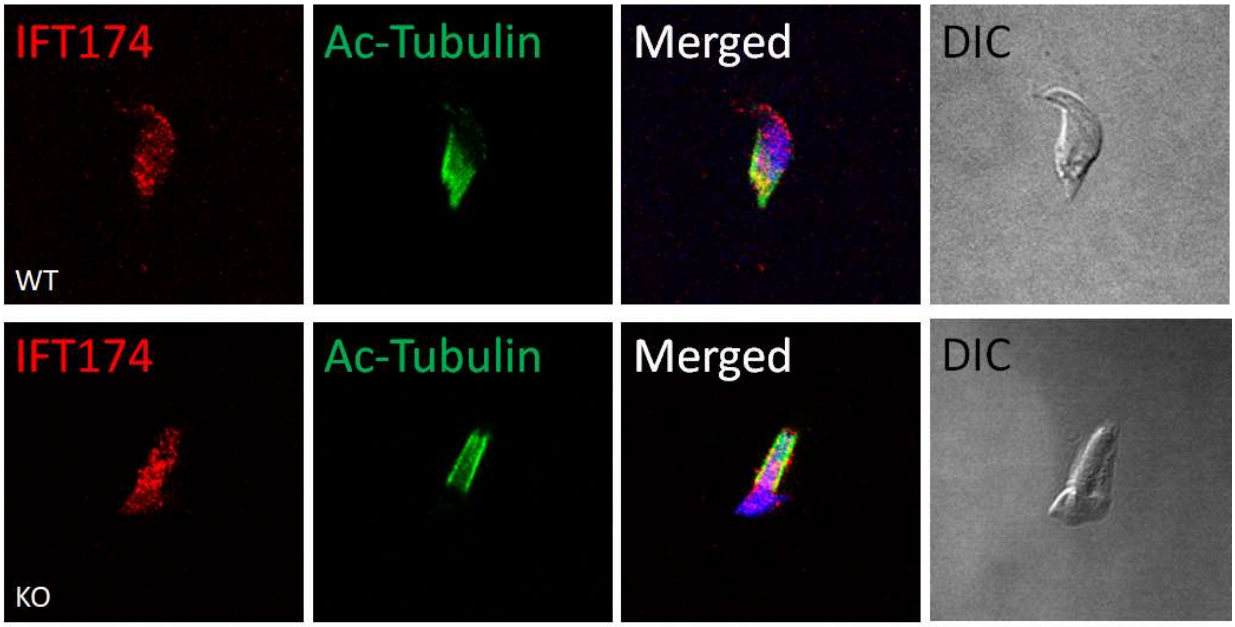



**Figure 7. IFT74 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for IFT74 and manchette structures. IFT74 localized to the manchette and acrosome of both WT and KO elongating spermatids.

Intraflagellar protein 88 (IFT88) is another protein of the IFT-B complex that is involved in sperm tail formation along with acrosome-acroplaxome and HTCA development. IFT88 is found in the trans-Golgi network, pro-acrosomal vesicles, along the acrosomal membrane, in the manchette, in the HTCA, and in the developing sperm tail. *Ift88* KO mice present with abnormal head shaping and lack of flagella (Kierszenbaum, et al., 2011).

Our studies found IFT88 to localize to the manchette, cytoplasm, and nuclear area of WT and *Spag17* KO elongating spermatids (Fig. 8).

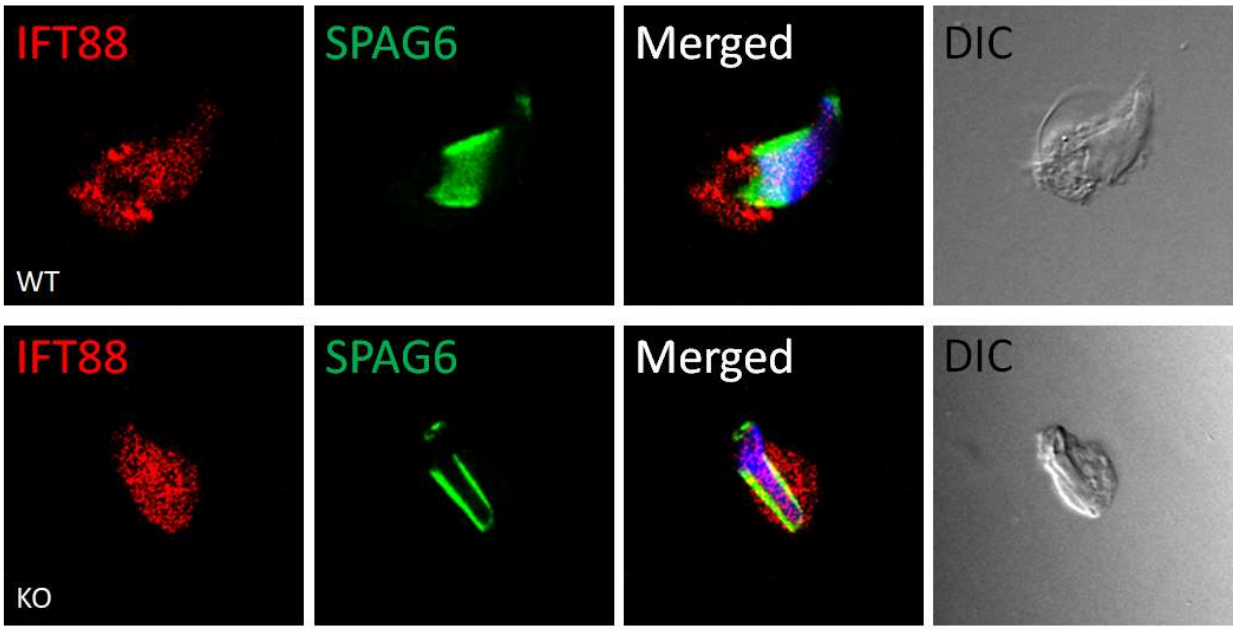



**Figure 8. IFT88 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for IFT88 and manchette structures. IFT88 localized to the manchette, cytoplasm, and nuclear area of both WT and KO elongating spermatids.

IFT121, or WD Repeat Domain 35 (WDR35), is a protein of the IFT-A complex that regulates retrograde protein trafficking during cilium assembly. IFT121 has also been found to aid in the fusion of RAB8 vesicles at the base of the cilium (Fu et al., 2016). Mutations in IFT121 have been implicated in ciliopathies and disrupt retrograde IFT and the localization of IFT121 to the axoneme and basal body (Antony et al., 2017). Cilia appear short and bulbous with abnormal axoneme and ciliary membrane morphology (Fu et al., 2016).

Our studies found IFT121 to localize to the manchette of both WT and *Spag17* KO germ cells suggesting that the transport of this protein does not depend on SPAG17 (Fig. 9).

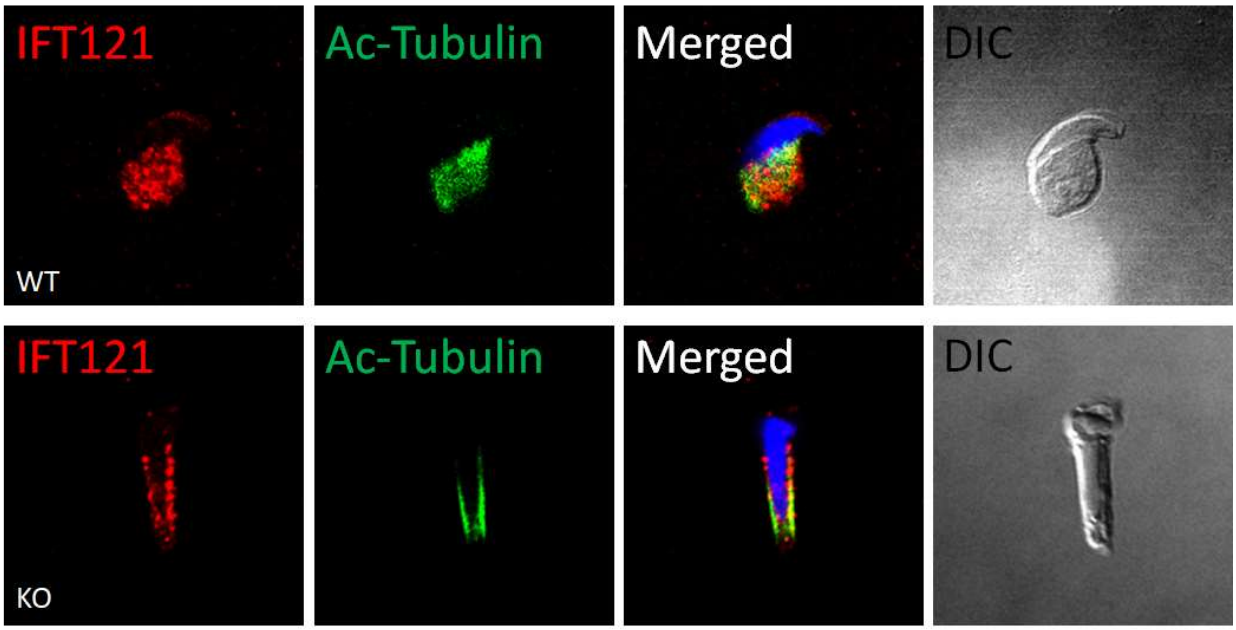



**Figure 9. IFT121 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for IFT121 and manchette structures. IFT121 localized to the manchette, cytoplasm, and nuclear area of both WT and KO elongating spermatids.

Leucine-rich repeat (LRR) and guanylate kinase-like domain containing, isoform-1 (LRGUK) plays several roles during spermatogenesis including acrosome attachment, sperm head shaping, IMT, and flagella formation (Liu et al., 2015; Okuda et al., 2017). LRGUK is first found in the acrosome and acrosome-acroplaxome area where it regulates acrosome attachment to the nuclear envelope (Liu et al., 2015). It is also seen in the manchette where it plays a role in nuclear head shaping and manchette microtubule function and organization (Okuda et al., 2017). Finally, LRGUK localizes to the basal body where it is essential for basal body attachment to the plasma membrane to ensure axoneme formation. *Lrguk* KO mice exhibit acrosome detachment, abnormal head shaping, and absence of the axoneme suggesting it is required for spermatogenesis and male fertility. While the manchette does form at the correct time in elongating spermatids, it is highly disorganized and disrupts proper IMT resulting in the phenotypes described above (Liu et al., 2015; Okuda et al., 2017).

Our experiments show LRGUK to localize to acrosome vesicles and the manchette in both WT and *Spag17* KO mice (Fig. 10), suggesting that the localization of this protein is independent of SPAG17.

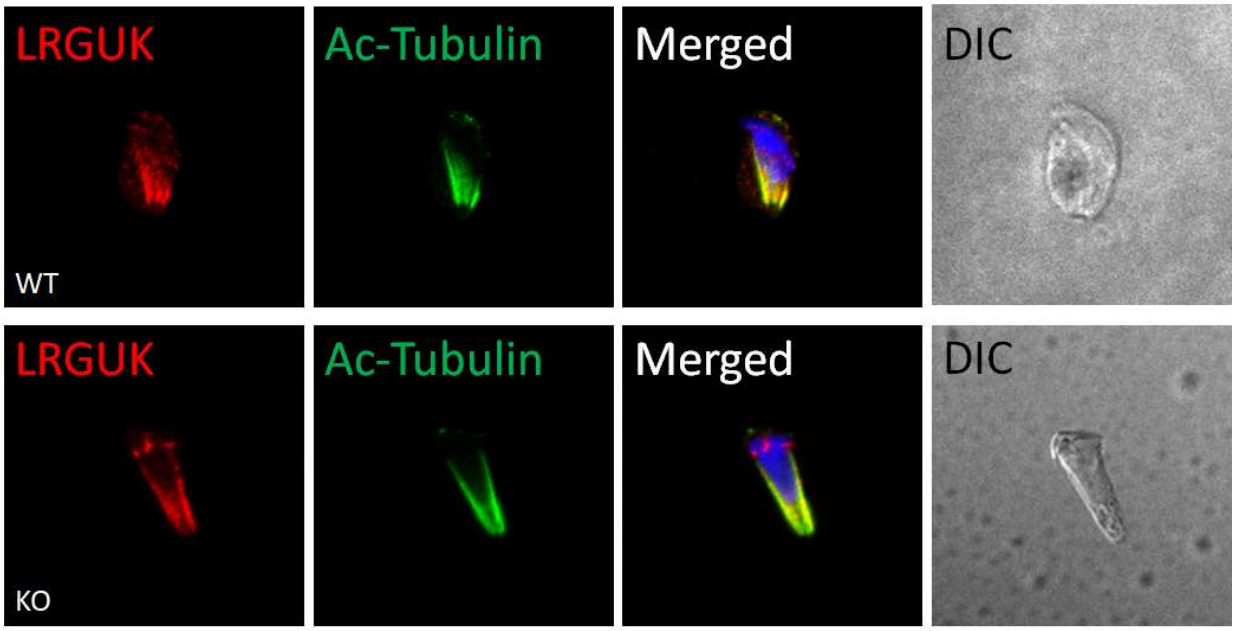



**Figure 10. LRGUK localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for LRGUK and manchette structures. LRGUK localized to acrosome vesicles and the manchette in both WT and KO elongating spermatids.

KIF3A is a kinesin motor protein responsible for protein trafficking and microtubule organization during axoneme formation. KIF3A has been localized to the manchette and principal piece of the sperm tail, further providing evidence of its role in manchette microtubule organization. KIF3A mutant mice show abnormal head morphology and lack an axoneme due to disrupted manchette organization. While microtubules, outer dense fibers, and the fibrous sheath are present, all are mislocalized in the KIF3A mutant and show abnormally long manchettes. No phenotypically normal sperm are seen in adult mice and they are usually infertile (Lehti et al., 2013).

Our experiments found KIF3A to localize around the nucleus in both WT and *Spag17* KO germ cells. However, it seems to fail to localize to the manchette in the *Spag17* KO (Fig. 11).

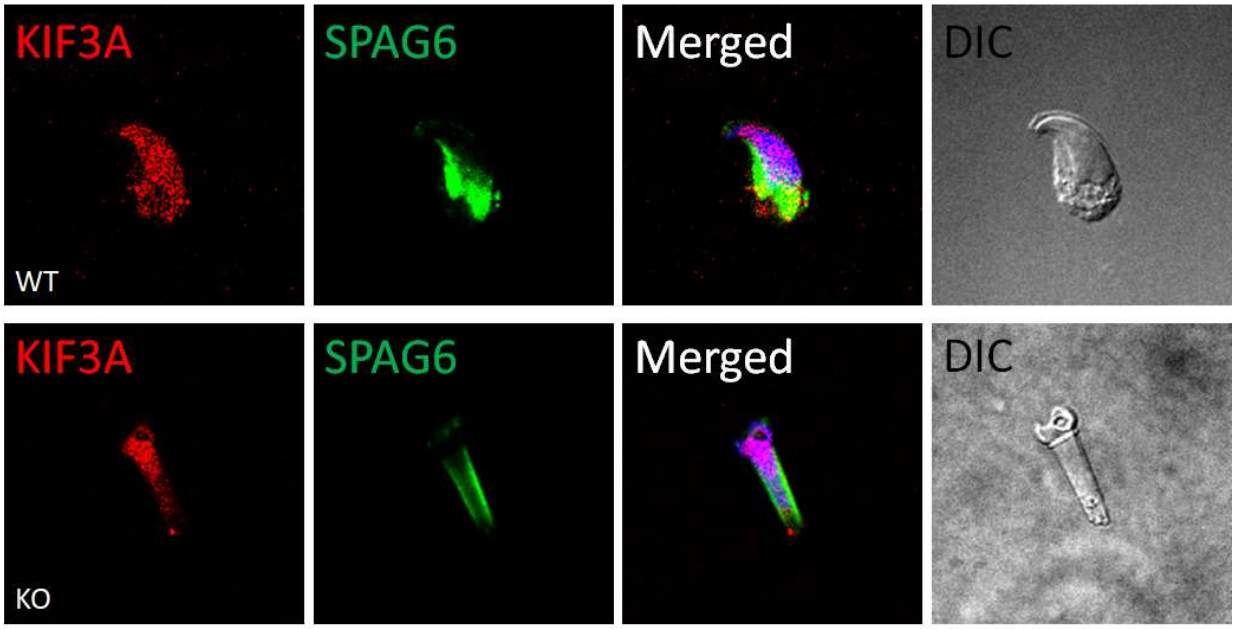



**Figure 11. KIF3A localization to the manchette is dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for KIF3A and manchette structures. KIF3A failed to localize to the manchette in *Spag17* KO elongating spermatids.

Mouse meiosis-expressed protein 1 (MEIG1) is a 31kDa protein expressed primarily in ciliated cells and thought to regulate spermiogenesis (Salzberg et al., 2010; Zhang et al., 2015). Deletion of the *Meig1* gene in the mouse has shown MEIG1 to play an essential role in maintaining manchette stability and function during spermiogenesis (Zhang et al., 2009). MEIG1 is first seen in spermatocytes from where it migrates to the manchette of elongating spermatids. MEIG1 is responsible for protein transport in the manchette for normal flagella formation (Li et al., 2015). *Meig1* KO mice phenotypes include abnormal head shaping, disrupted manchette organization, and lack of flagella (Salzberg et al., 2010; Zhang et al., 2009). Manchette microtubules are present but not organized in the correct arrangement (Zhang et al., 2009).

We found MEIG1 to localize to the acrosome, manchette and tail of WT germ cells. MEIG1 was still present in the manchette but not in the acrosome in the *Spag17* KO (Fig. 12).

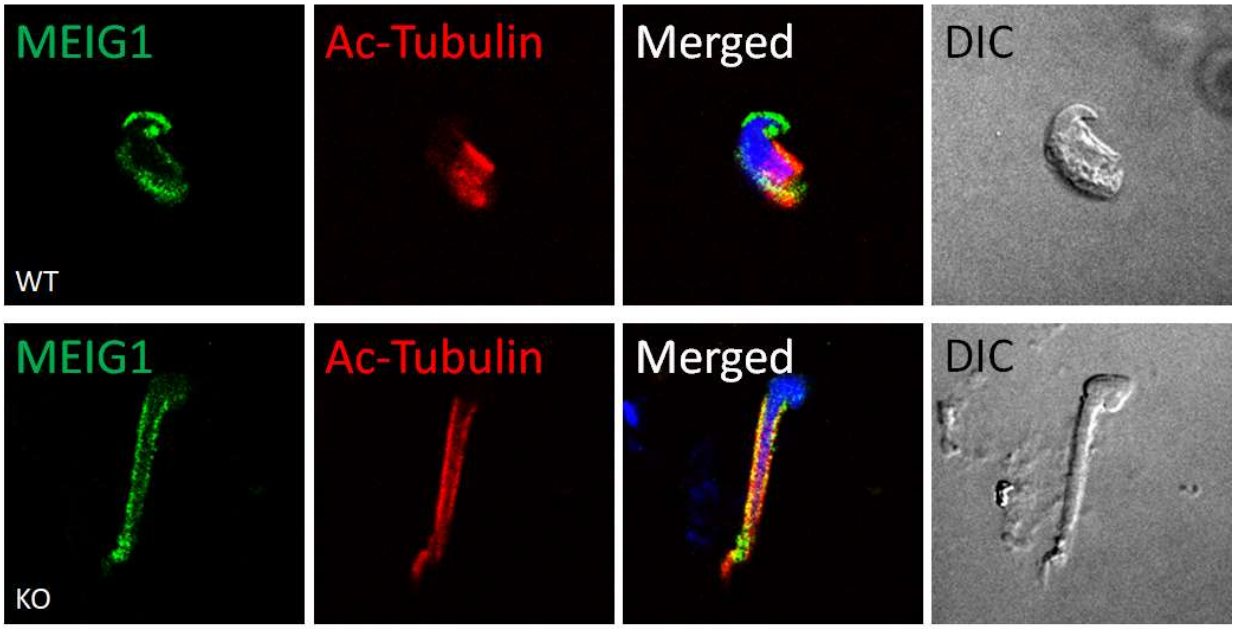



**Figure 12. MEIG1 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for MEIG1 and manchette structures. MEIG1 localized to the acrosome, manchette, and tail of WT elongating spermatids. While MEIG1 was still present in the *Spag17* KO manchette, it was missing from the acrosome.

Membrane occupation and recognition nexus repeat-containing protein 3 (MORN3) is a 25 kDa protein expressed during spermiogenesis. MORN3 has been previously implicated in flagellar formation and regulation of spermatogenesis. *Morn3* mRNA is abundantly present in mouse testes with the protein first being seen in the acrosome of round spermatocytes. Acrosome staining persists throughout spermiogenesis and manchette staining is seen in elongating spermatids (Zhang et al., 2015).

In our experiments, MORN3 was also found to localize to the acrosome and manchette of both WT and *Spag17* KO germ cells. (Fig. 13).

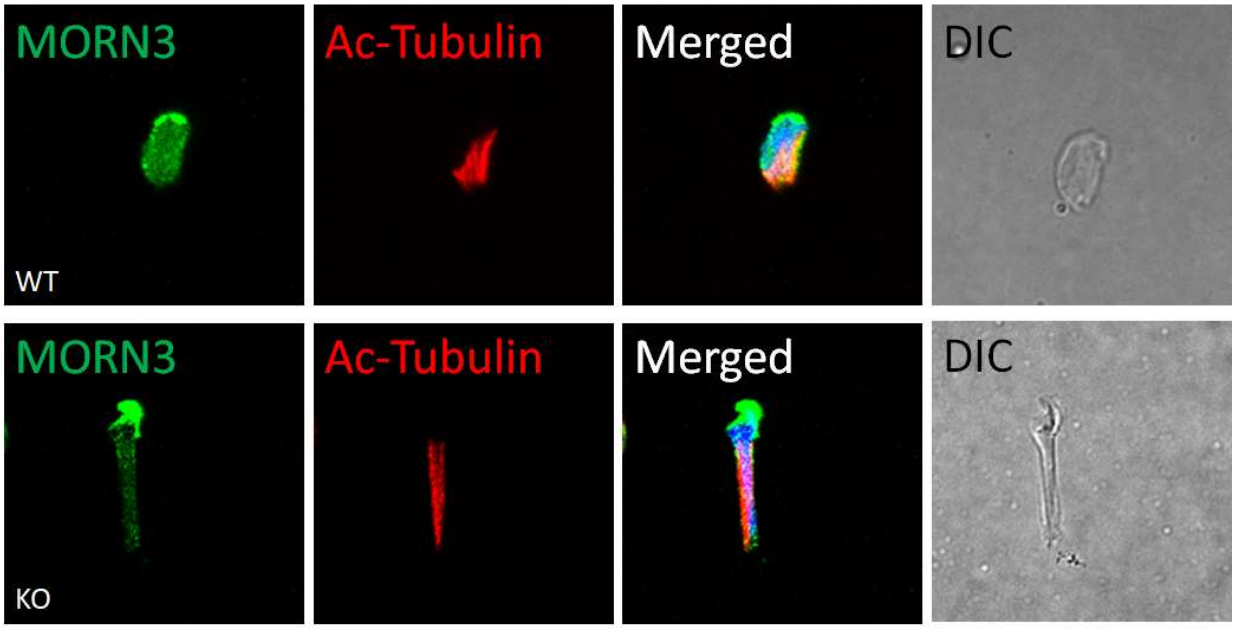



**Figure 13. MORN3 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for MORN3 and manchette structures. MORN3 localized to the acrosome and manchette of both WT and KO elongating spermatids.

Outer dense fiber protein 1 (ODF1) is a 27kDa protein localized to the outer dense fibers (ODFs) of the mature sperm tail (Shao et al., 1999). ODF1 has been localized to the manchette of elongating spermatids (Contreras & Hoyer-Fender, 2019; Kierszenbaum & Tres, 2002; Schalles et al., 1998). *Odf1* deficiency results in infertile male mice due to detachment of the sperm head though headless tails are still motile. Mitochondrial sheath and ODF organization are disrupted as well with no clear alignment to the nine ODFs in the sperm tail. Spermatogenesis is normal up to the spermatid stage where head-tail linkage is weakened and tails coil and detach from the head. Functional mature sperm are rarely found in the *Odf1* KO mice. Sperm also failed to perform acrosome reaction (Yang et al., 2012).

Our studies found ODF1 to localize around the nucleus in the form of vesicles, to the manchette and in the centrosome area of both WT and *Spag17* KO elongating spermatids (Fig. 14).

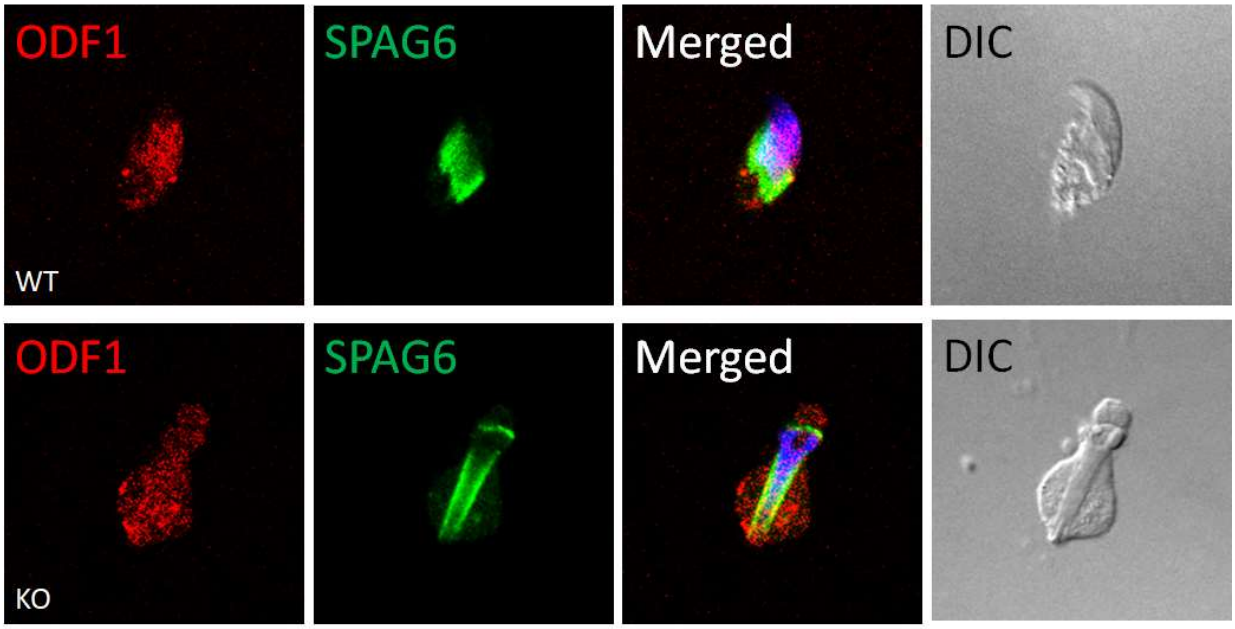



**Figure 14. ODF1 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for ODF1 and manchette structures. ODF1 localized to around the nucleus in the form of vesicles, to the manchette, and in the centrosome area of both WT and KO elongating spermatids.

Parkin co-regulated gene (PACRG) is a 28 kDa protein found to play a role in manchette structure and function (Li et al., 2015; Lorenzetti et al., 2004). PACRG is known to bind tubulins suggesting it may play a role in manchette attachment to the nuclear ring (Lorenzetti et al., 2004). Expression of *Pacrg* is limited to spermatogenic cells with localization to the post-acrosomal region of the sperm head, the manchette, and midpiece of the tail (Li et al., 2015; Lorenzetti et al., 2004). *Pacrg* KO mice phenotypes include abnormal head shaping, abnormal elongated manchette and failure of microtubules to maintain 9+2 arrangement resulting in immotile or absent flagella and male infertility.

We found PACRG to localize to the acrosome and manchette. PACRG was still present in the acrosome and manchette in the *Spag17* KO (Fig. 15).

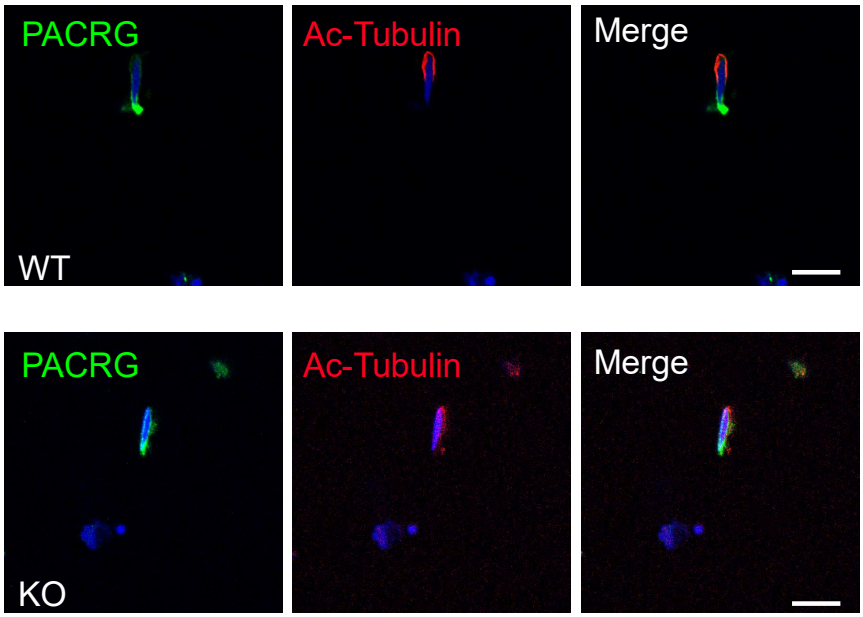



**Figure 15. PACRG localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for PACRG and manchette structures. PACRG localized to the acrosome and manchette of both WT and KO elongating spermatids.

Regulator of chromosome condensation protein 1 (RCC1) is a 45 kDa Ran guanine nucleotide exchange factor (RanGEF) that plays a role in nucleocytoplasmic transport during spermatogenesis (Ohtsubo et al., 1989; Zou et al., 2002). RCC1's role as a GEF allows it to shuttle between the nucleus and cytoplasm depending on GTP or GDP activation (Zou et al., 2002). As its name suggests, RCC1 regulates chromosome condensation by replacement of somatic histones by transition proteins which are in turn replaced by protamine proteins at the end of spermiogenesis (Wang et al., 2012). RCC1 localization in the acrosome and manchette further supports this as the manchette functions in nuclear shaping and condensation during spermiogenesis as well (Pittoggi et al., 1999; Zou et al., 2002). Proteins in the RCC1 superfamily have been found to localize to the acrosome as well, implicating them in the protein trafficking to the acrosome during acrosome biogenesis (Wang et al., 2012). It is also thought to aid in the

assembly and function of manchette microtubules, similar to its role in spindle microtubule nucleation of somatic cells (Carazo-Salas et al., 1999; Zou et al., 2002). *Rcc1* KO studies in somatic cells show microtubular disarray.

We found RCC1 to localize to the acrosome and manchette. RCC1 was still present in the acrosome and manchette in the *Spag17* KO (Fig. 16).

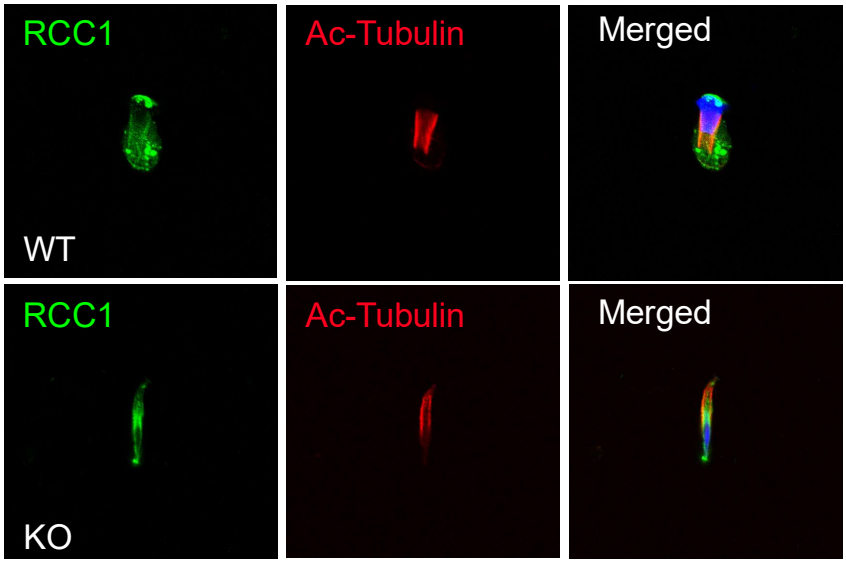



**Figure 16. RCC1 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for RCC1 and manchette structures. RCC1 localized to the acrosome and manchette of both WT and KO elongating spermatids.

*SEPTIN* genes generally encode polymerizing GTP-binding cytoskeletal proteins that function in mitosis, cytoskeletal remodeling, cell polarity, and vesicle trafficking (C. Y. Huang et al., 2018). Septin 2 (SEPT2) localizes to the head-tail connecting apparatus and annulus in sperm where it forms a complex with other septin proteins to maintain the connections. Defects in the formation of these complexes results in a disorganized HTCA and annulus and bent sperm tails (Kuo et al., 2015; Shen et al., 2020).

Our studies show SEPT2 to localize to the pro-acrosomal vesicles of the Golgi, the manchette, and the centrosome area. SEPT2 expression was not affected in the *Spag17* KO (Fig. 17).

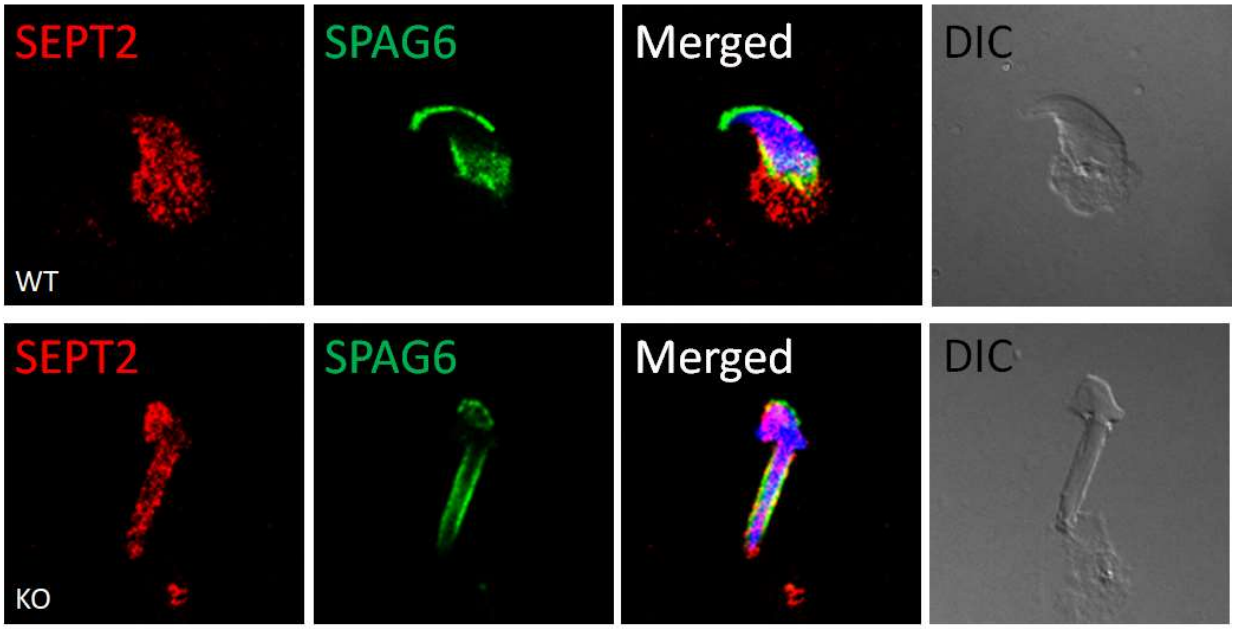



**Figure 17. SEPT2 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for SEPT2 and manchette structures. SEPT2 localized to the pro-acrosomal vesicles of the Golgi, the manchette and the centrosome area of both WT and KO elongating spermatids.

Sperm-associated antigen 6 (SPAG6) is a 56kDa protein responsible for sperm flagellar motility and organization (Liu et al., 2019; Sapiro et al., 2002). About half of *Spag6* KO animals die from hydrocephalus before adulthood and those that do survive are infertile due to sperm motility defects (Sapiro et al., 2002). Mutant sperm phenotypes include abnormal head formation or loss of head, disorganized axoneme microtubules, and missing flagella. Other areas of localization include vesicles in spermatocytes, the acrosome and manchette in elongating spermatids, and the tail in mature sperm (Liu et al., 2019; Sapiro et al., 2000). This suggests a role for SPAG6 in vesicle trafficking, acrosome biogenesis, and IMT (Liu et al., 2019).

Our studies show SPAG6 to localize to the acrosome and manchette of elongating spermatids and to the tail of mature spermatozoa. These localizations are preserved in the *Spag17* KO (Fig. 18).

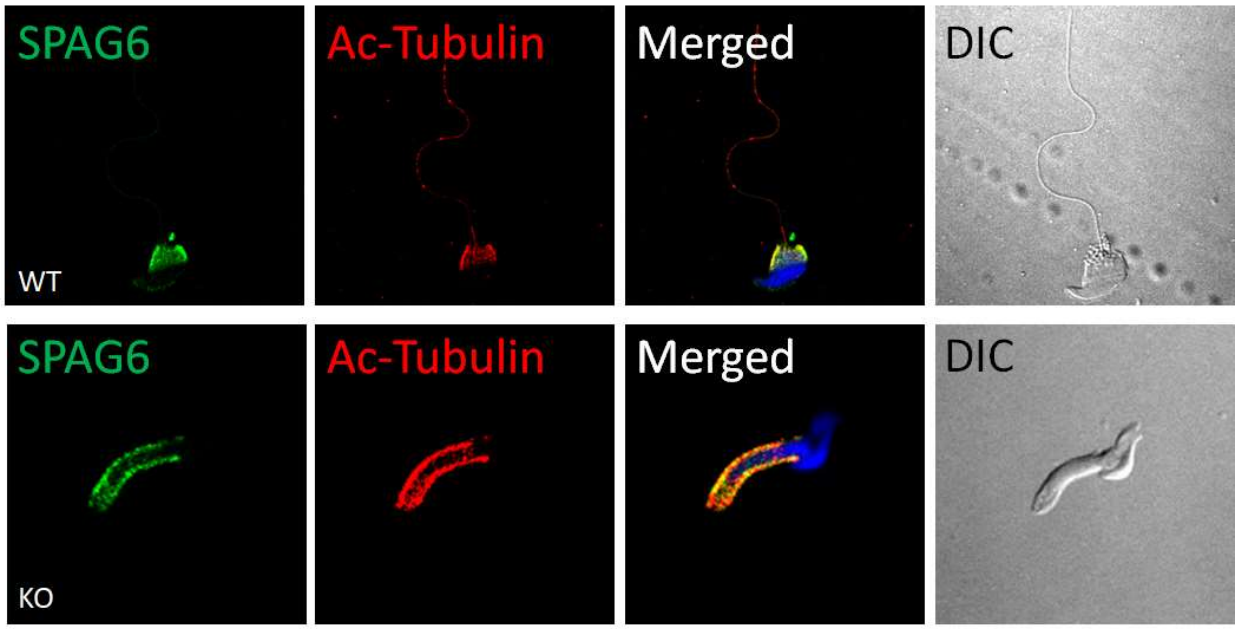



**Figure 18. SPAG6 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for SPAG6 and manchette structures. SPAG6 localized to the manchette of both WT and KO elongating spermatids.

Sperm flagellar protein 2 (SPEF2) is a 200kDa protein necessary for correct cilia and sperm tail development. In germ cells, SPEF2 localizes to the Golgi complex where it functions in Golgi vesicle transport. It is also found in the manchette where it is involved in sperm head shaping, IMT, and dismantling of the manchette at the end of spermiogenesis. Finally, SPEF2 localizes to the basal body, neck region, and the mid-piece of the sperm tail. Male infertility develops in the absence of *Spef2* due to abnormal head shape, short/thick tails, basal body defects resulting in two or no tails, and failure of manchette migration (Lehti et al., 2017; Sironen et al., 2010).

Our studies show SPEF2 to be present in the manchette of both WT and *Spag17* KO elongating spermatids (Fig. 19).

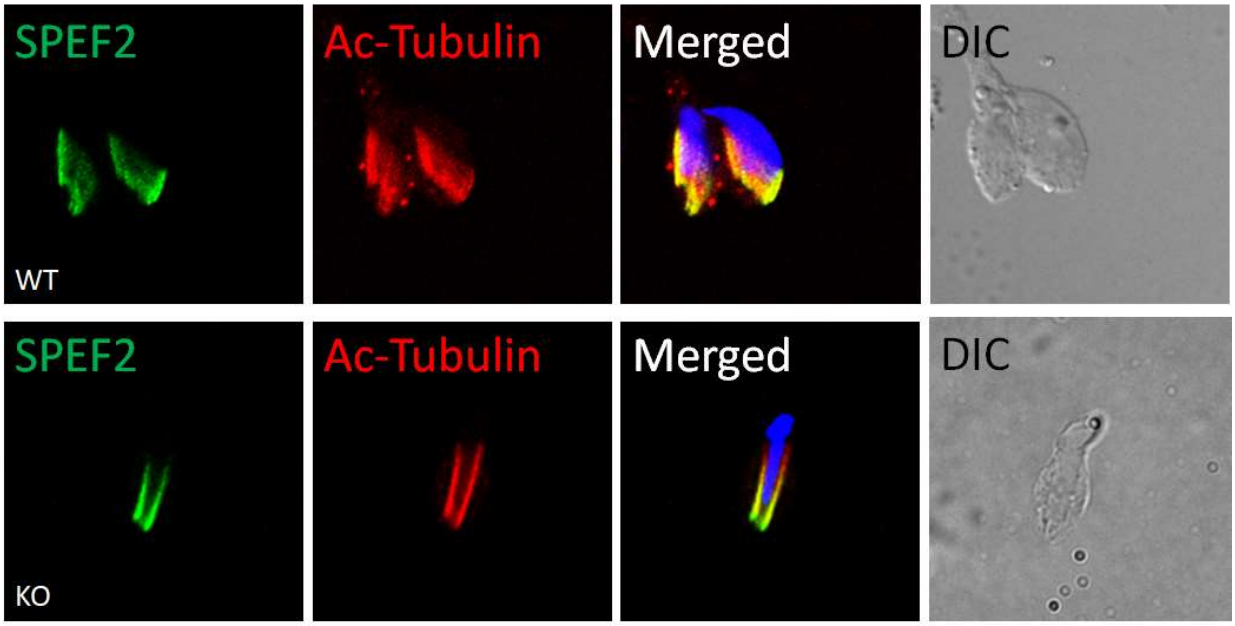



**Figure 19. SPEF2 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for SPEF2 and manchette structures. SPEF2 localized to the manchette of both WT and KO elongating spermatids.

Sad/Unc84 domain-containing protein 3 (SUN3) is part of SUN3/NESPRIN1 LINC complex that connects the nucleus with the peripheral cytoskeleton (Göb et al., 2010). LINC, or linker of nucleoskeleton and cytoskeleton, complexes have been thought to play a role in nuclear shaping by connecting manchette microtubules to the nuclear envelope for transmission of force to properly shape sperm nuclei (Gao et al., 2020; Göb et al., 2010). The role of SUN3 in LINC complexes was further confirmed by studies reporting SUN3 localization exclusively in regions where microtubule bundles attach to the nuclear envelop (Göb et al., 2010; Pasch et al., 2015). SUN3 KO mice are infertile with reduced sperm counts and abnormal sperm morphology (Gao et al., 2020). Phenotypes typical of SUN3 KO animals include missing/fragmented acrosome,

abnormal/round head shape, missing manchette and coiled/bent tails. These results show the importance of SUN3 in manchette formation and organization throughout spermatogenesis.

We found SUN3 to be localized to the manchette of both WT and *Spag17* KO elongating spermatids suggesting it does not depend on SPAG17 for manchette localization (Fig. 20).

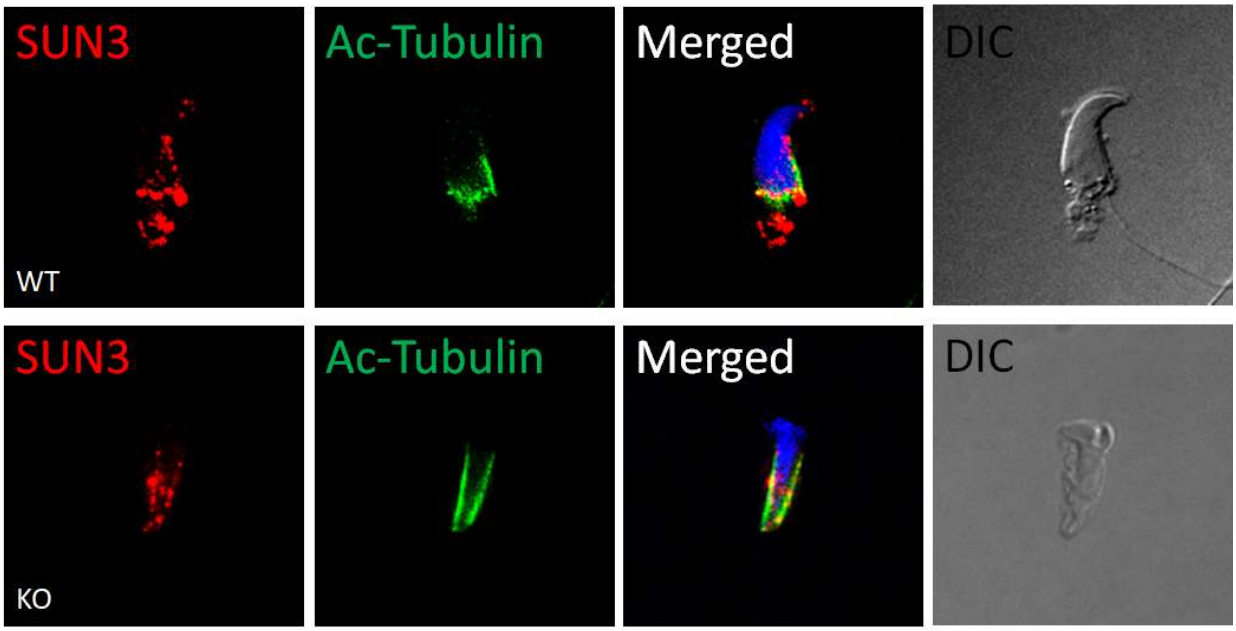



**Figure 20. SUN3 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for SUN3 and manchette structures. SUN3 localized to the manchette of both WT and KO elongating spermatids.

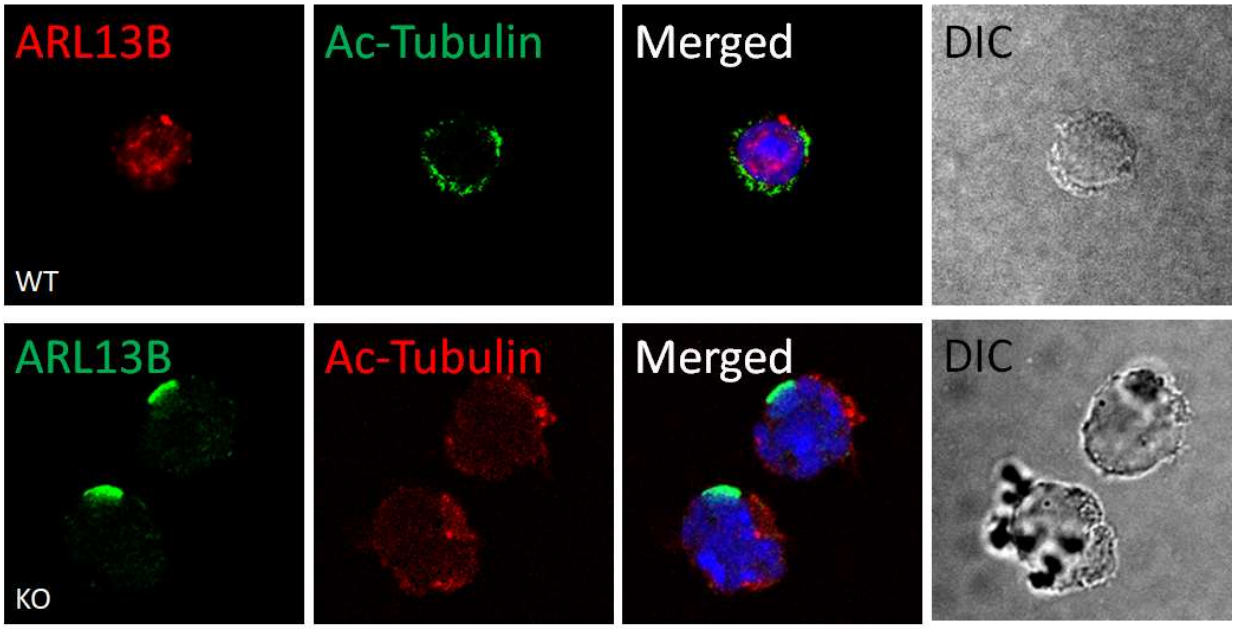
*3.4 Centriolar Proteins*

ADP ribosylation factor 13B (ARL13B) is a 48kDa protein that is a member of the small GTPase superfamily, Arf/Arl (Caspary et al., 2007). Arl13b has been previously implicated in primary cilia formation and Sonic Hedgehog (Shh) signaling (Caspary et al., 2007; Li et al., 2010). Arl13b localizes to the ciliary membrane and along the length of the axoneme (Caspary et al., 2007). Its role at the ciliary membrane includes ciliary transmembrane protein localizations and anterograde IFT stability. Other speculated functions include vesicle trafficking, cellular differentiation, cell movement, and cytoskeletal processes (Cevik et al., 2010). *Arl13b* gene

disruption results in the Hnn mutation causing Joubert syndrome, an autosomal recessive disorder displaying abnormalities in the central nervous system and various ciliopathies (Caspary et al., 2007; Li et al., 2010). Cilia in mutant mice are often short and present with a structural defect in the ciliary axoneme (Caspary et al., 2007). There is also a disrupted association between IFT subcomplexes A and B and reduced IFT speeds (Cevik et al., 2010; Li et al., 2010). No studies using ARL13B to study spermatogenesis have been completed as of yet.

In our experiments, ARL13B was present in the acrosome and manchette of both WT and *Spag*17 KO round and elongating spermatids (Fig. 21).

A



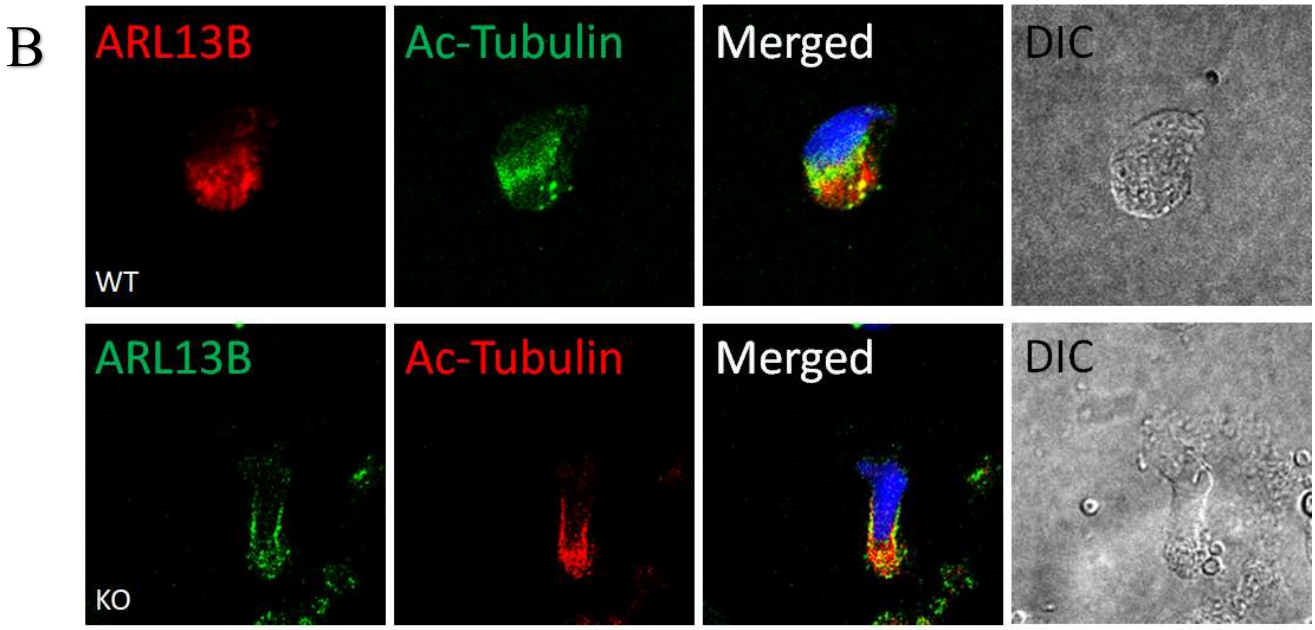



**Figure 21. ARL13B localization to the manchette is not dependent on SPAG17 in round spermatids and elongating spermatids.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for ARL13B and manchette structures. A) ARL13B localized to the acrosome of both WT and KO round spermatids. B) ARL13B localized to the manchette of both WT and KO elongating spermatids.

AZI1, or centrosomal protein 131 (CEP131), is a 131kDa centrosomal protein that is highly expressed during flagellogenesis and expected to play a role in protein trafficking at that stage (Aoto et al., 1995; Hall et al., 2013). This is further supported by AZI1 localization shifting from the acrosome in early spermatids to the centrosome of the head-tail coupling apparatus (HTCA) in later stage spermatids. AZI1 has been visualized trafficking along microtubules in a retro- and anterograde fashion confirming its use of IMT during spermatogenesis. Loss of *Azi1* disturbs both IMT and IFT and results in club-shaped heads and lack of flagella ultimately leading to male infertility (Hall et al., 2013).

Our studies show AZI1 to localize to the acrosome, the manchette, and the centrosome. Though AZI1 is still present in the acrosome and centrosome in *Spag17* KO spermatids, it is missing from the manchette (Fig. 22).

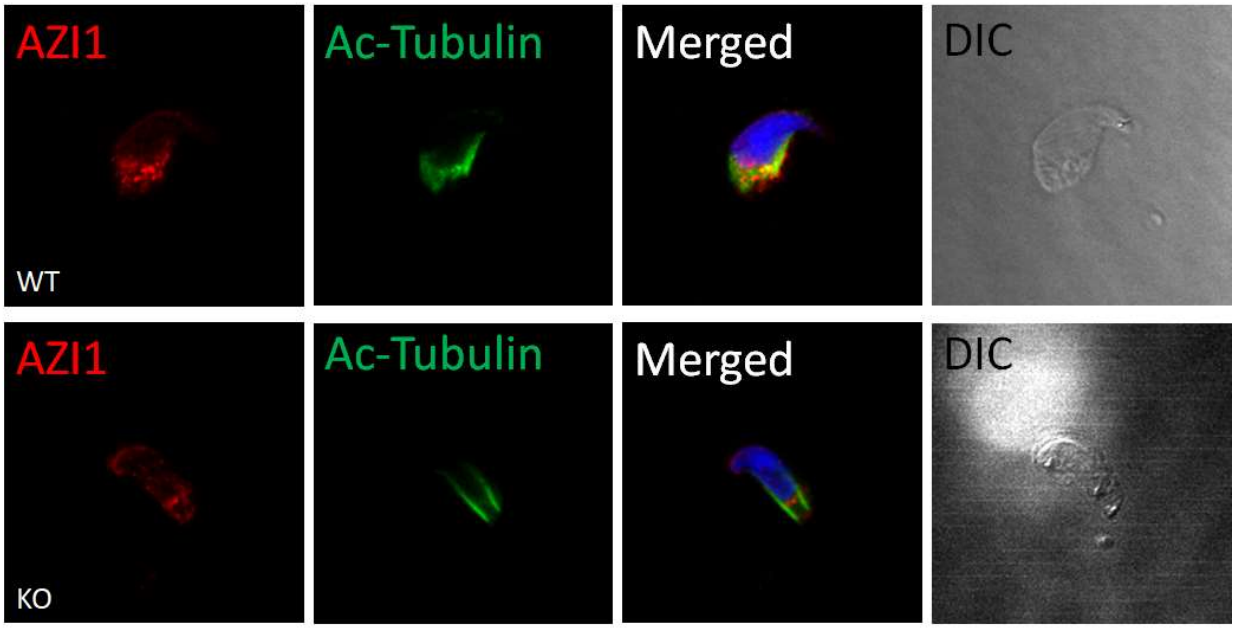



**Figure 22. AZI1 localization to the manchette is dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for AZI1 and manchette structures. AZI1 failed to localize to the manchette in *Spag17* KO elongating spermatids. However, protein is still present in the acrosome area and the centrosome.

Centrosomal protein 164 (CEP164) is a 164kDa protein that is required for primary cilia formation (Devlin et al., 2020; Graser et al., 2007). *Cep164* weakly expresses in developing spermatogonia and spermatocytes and strongly expresses in spermatid tails (Devlin et al., 2020). It localizes to the distal appendages of the mature centriole from which the primary cilia is formed (Graser et al., 2007). The distal ends of mature centrioles have two sets of appendages that anchor cytoplasmic microtubules to allow docking of the centriole to the cell membrane during primary cilium formation (Goetz & Anderson, 2010). This in turn allows vesicular docking that initiates cilia membrane biogenesis (Schmidt et al., 2012). *Cep164* KO male mice are completely infertile with no mature sperm detectable in the epididymis. Both sperm flagella and primary cilia are lacking in the mutant mice (Siller et al., 2017). *Cep164* KO mice did not seem to have any defects in microtubule-dependent transport (Schmidt et al., 2012).

Our studies found CEP164 to localize to the acrosome and manchette of WT and *Spag*17 KO mice (Fig. 23).

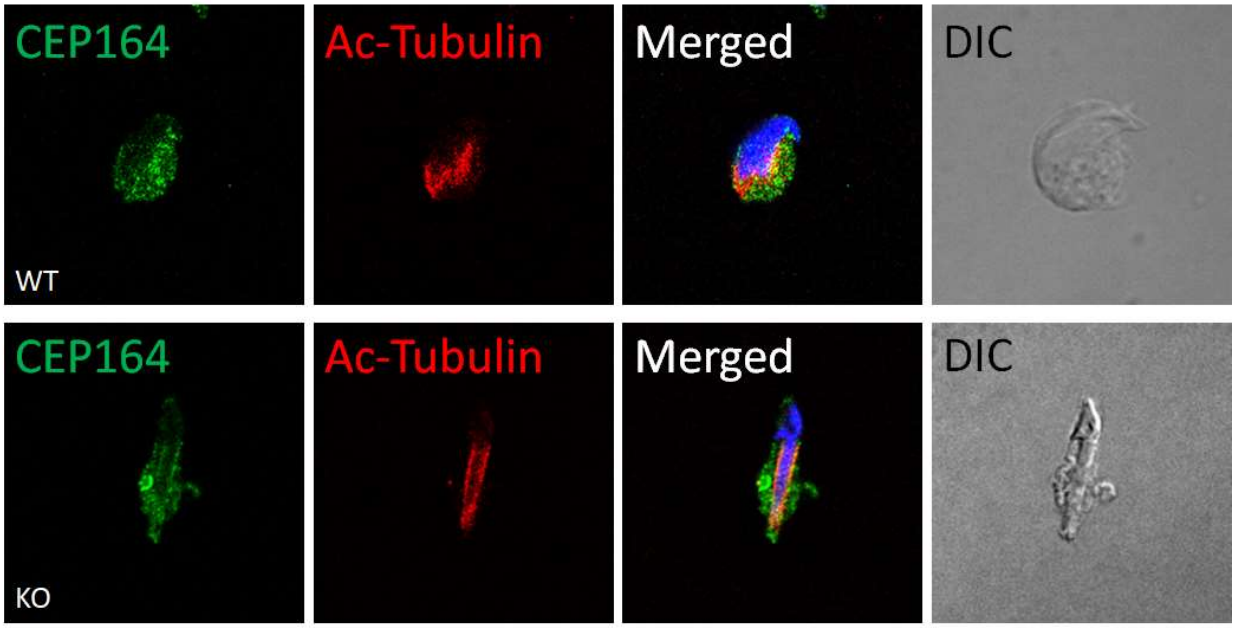



**Figure 23. CEP164 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for CEP164 and manchette structures. CEP164 localized to the acrosome and manchette of both WT and KO elongating spermatids.

Inner centromere protein (INCENP) has been implicated in reproductive disorders due to its role in mitosis and meiosis to ensure proper sister chromatid separation (Liu et al., 2016; Parra et al., 2003). INCENP specifically interacts at the centromere to ensure cohesion of sister chromatids until segregation. Studies have found INCENP to play a role in sperm functions, but no intensive studies have yet been done (Hering et al., 2014). In mouse spermatocytes, INCENP localizes to the inner domain of metaphase I centromeres and at a connecting strand joining sister kinetochores in metaphase II centromeres (Parra et al., 2003). INCENP has also been known to attach to microtubules to get to its proper location, further implicating it in spermatogenesis. *Incenp* KO mice display disruptions in mitosis and cytokinesis and abnormal microtubule aggregates (Wheatley et al., 2001).

Our results showed this protein localizes to the manchette microtubules in the WT spermatids but is mis-located in the *Spag17* KO spermatids (Fig. 24).

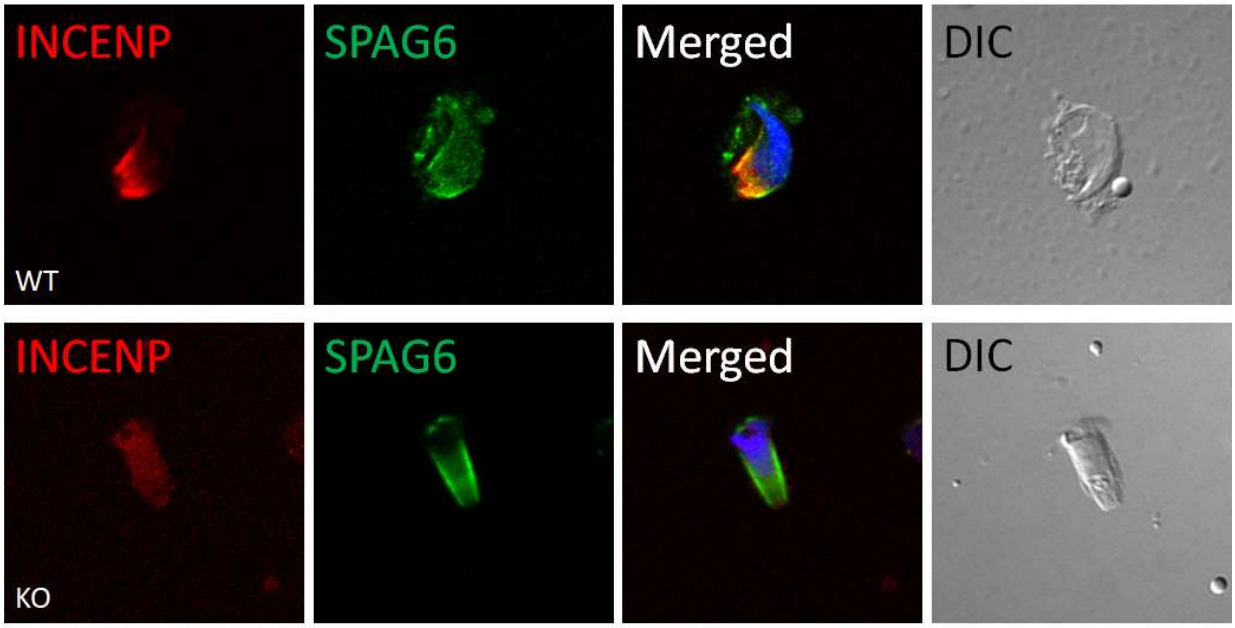



**Figure 24. INCENP localization to the manchette is dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for INCENP and manchette structures. INCENP failed to localize to the manchette in *Spag17* KO elongating spermatids.

3.5 *Ras-related proteins*

Ras-related, or RAB, proteins are a family of small GTPases that regulate vesicular trafficking in both the anterograde and retrograde directions. They are also known to interact with motor proteins along microtubules and actin filaments to ensure cargo delivery to the proper location. Several members of the RAB protein family have been found to facilitate membrane fusion and/or participate in the acrosome reaction (Del Nery et al., 2006; Teves et al., 2020). We focused on Ras-related proteins 5B, 6A, 8 and 10 as they have been previously implicated in spermiogenesis.

RAB5B is a 25 kDa protein that is localized to the plasma membrane where it contributes to intracellular trafficking and endocytosis (Khawar et al., 2019; Lin et al., 2017; D. B. Wilson & Wilson, 1992).

Our studies found RAB5B to localize in the manchette of both WT and *Spag17* KO germ cells suggesting it does not depend on SPAG17 for its function (Fig. 25).

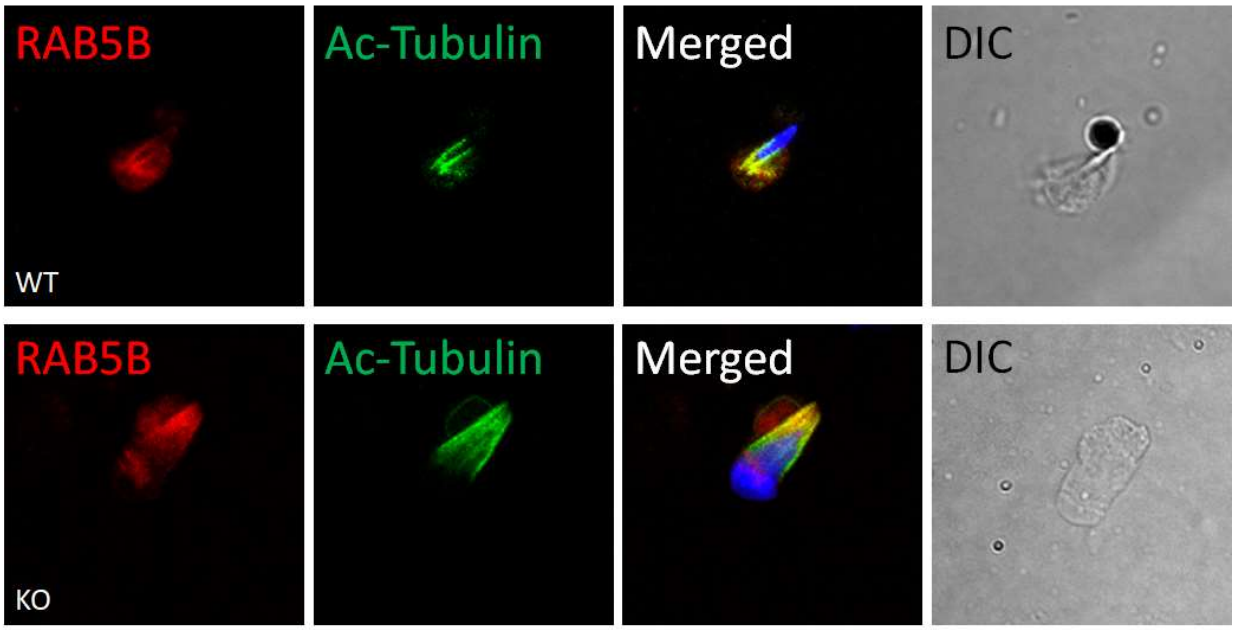



**Figure 25. RAB5B localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for RAB5B and manchette structures. RAB5B localized to the manchette of both WT and KO elongating spermatids.

RAB6A has been shown to regulate vesicular trafficking within the Golgi and post-Golgi compartments (Del Nery et al., 2006). Studies have also found RAB6 to localize to the acrosome and manchette of elongating spermatids (Lin et al., 2017). Knockdown of RAB6A disrupts intracellular transport and disrupts Golgi-associated protein recycling along with causing swelling in the Golgi. Golgi transport is not affected (Del Nery et al., 2006).

We found RAB6A to localize to the manchette of elongating spermatids in WT germ cells. RAB6A is missing from *Spag17* KO elongating spermatids (Fig. 26).

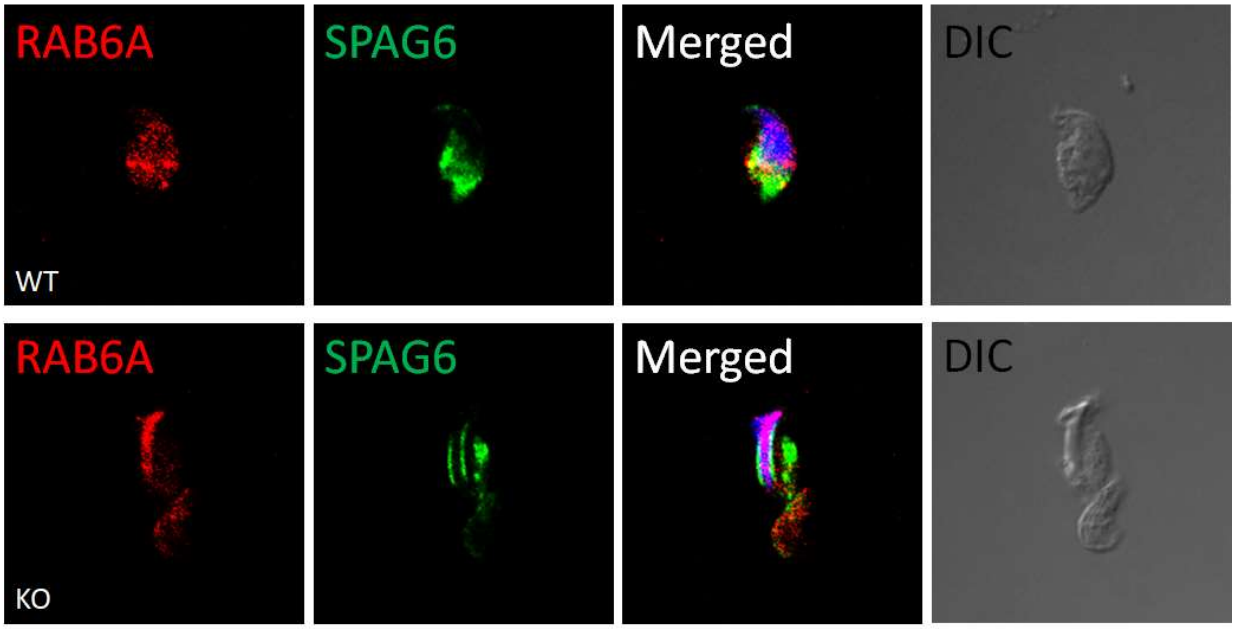



**Figure 26. RAB6A localization to the manchette is dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for RAB6A and manchette structures. RAB6A failed to localize to the manchette in *Spag17* KO elongating spermatids.

RAB8 is a 24kDa small GTPase that aids in vesicular trafficking and facilitates the assembly of ciliary vesicles and membranes at the distal appendage at the base of the cilia once it is recruited by CEP164 (Lau & Mruk, 2003; Siller et al., 2017). Rabin8, a RAB8 GEF, loads GTP onto RAB8 which can then enter the cilium to promote the docking and fusion of post-Golgi vesicles to the base of the ciliary membrane. RAB8 also works with a multi-protein BBS protein complex to recruit cargo to the basal body for transport to the ciliary membrane and regulate membrane trafficking for primary ciliogenesis (Nachury et al., 2007). RAB8 has also been found to promote membrane transport through the reorganization of actin and microtubules in fibroblasts (Peränen et al., 1996). RAB8 requires CEP164 for centrosome binding and localizes to the primary cilium (Nachury et al., 2007). It has also been found to localize to the basal compartment of the testis, which contains spermatocytes and spermatids, in all stages of spermatogenesis (Lau & Mruk, 2003). Though no *Rab8* KO mice studies have been completed, it

has been found that expression of GDP-RAB8, the inactive form, results in the accumulation of vesicles at the base of cilium in photoreceptors (Nishimura et al., 2004).

Our experiments found RAB8 to localize to the acrosome and manchette in elongating spermatids and to the mitochondria and mid-piece in mature sperm. In *Spag17* KO mice, RAB8 still co-localized with anti-acetylated tubulin in the manchette. (Fig. 27).

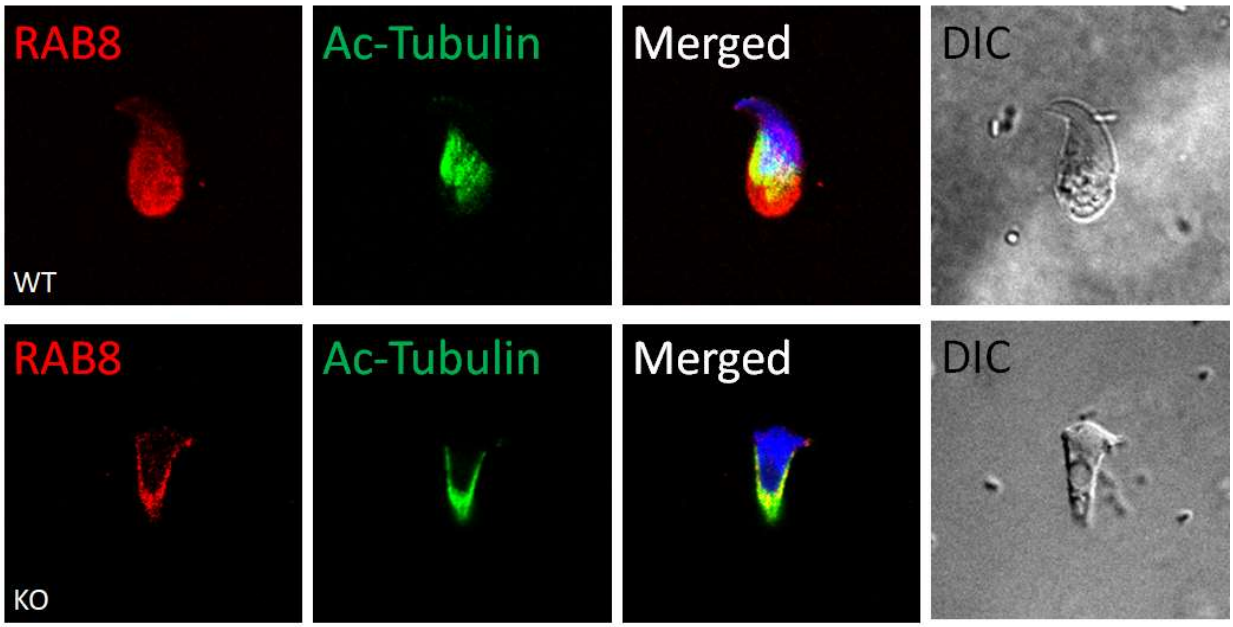



**Figure 27. RAB8 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for RAB8 and manchette structures. RAB8 localized to the acrosome, manchette, mitochondria and mid-piece of both WT and KO elongating spermatids.

RAB10 is 20-40kDa small GTP-binding protein that regulates membrane trafficking and fusion events. RAB10 has been previously implicated in IMT and sperm head and tail formation. Its function in spermatogenesis is further emphasized by its localizing to the perinuclear ring, the manchette, and the midpiece of the sperm tail. Loss of *Rab10* disrupts the development of early mammalian embryos (Lin et al., 2017). A double mutant even leads to embryonic lethality at E7.5 (Lv et al., 2015).

Our studies found RAB10 to localize around the nucleus in the form of vesicles and in the centrosome area of both WT and *Spag*17 KO mice. The protein was not found in the manchette. (Fig. 28).

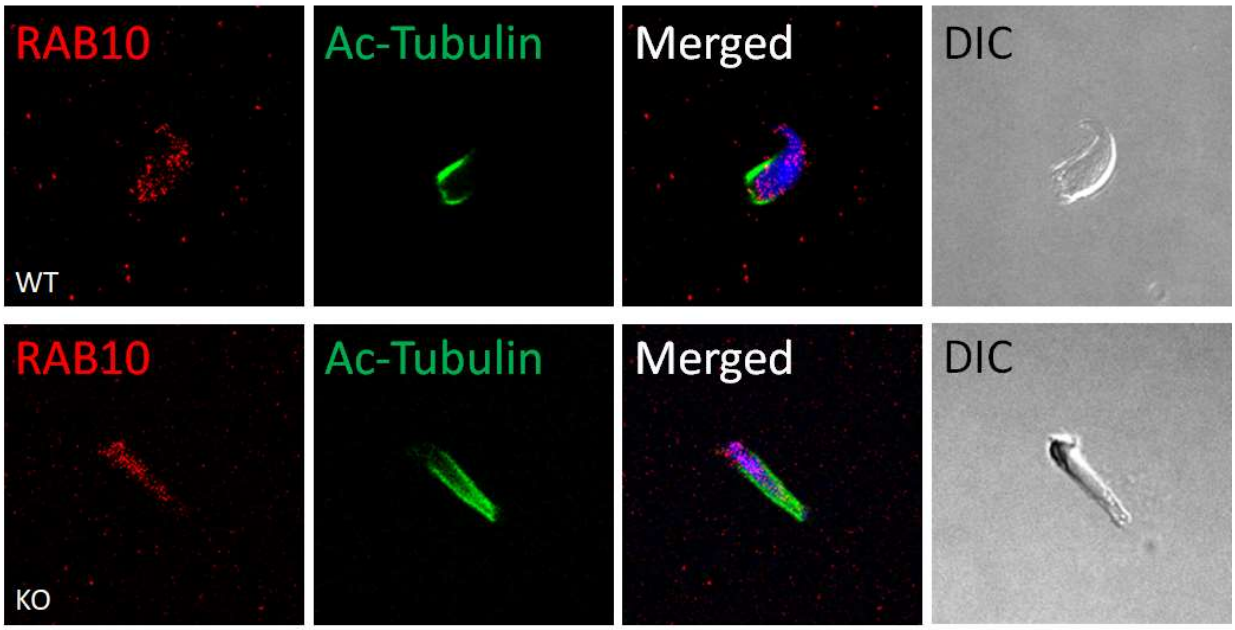



**Figure 28. RAB10 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for RAB10 and manchette structures. RAB10 localized to around the nucleus and in the centrosome area both WT and KO elongating spermatids.

*3.6 Hedgehog (Hh) signaling proteins*

A number of proteins known to play a role during spermatogenesis are also part of the Hh signaling cascade. These proteins include BBS4, GLI1, and SMO, as well as ARL13B which was described above. The Hh signaling pathway plays an important role during embryonic development to drive cell proliferation, promote cell survival, and direct differentiation (Szczepny et al., 2006). The Hh signal binds to and inactivates Patched (Ptc), a transmembrane domain protein on the cell surface, so it no longer inhibits Smoothened (Smo), another transmembrane domain protein (Hooper & Scott, 2005). Smo then initiates a signaling cascade that dissociates Ptc-Smo-Gli complex allowing Gli1 to reach the nucleus where it can activate

target genes (Morales et al., 2009). BBS4, GLI1, SMO and ARL13B were visualized in germ cells from both WT and *Spag17* KO mice using the appropriate antibody and anti-acetylated tubulin as a microtubule marker.

Bardet-Biedl syndrome type 4 (BBS4) is known to cause Bardet-Biedl syndrome, a genetically heterogeneous autosomal recessive disorder characterized by obesity, retinal degeneration, polydactyly, hypogenitalism and renal defects (Chamling et al., 2013). These phenotypes are said to be the result of a lack of cilia form or function (Mykytyn et al., 2004). BBS4 has also been found to be implicated in the trafficking of various ciliary cargo proteins. In sperm, BBS4 is responsible for trafficking essential proteins to the sperm tail and localizes to the annulus for this purpose (Chamling et al., 2013). In somatic cells, BBS4 localizes to the centrosome and basal body of primary cilium (Chamling et al., 2013; Mykytyn et al., 2004). BBS4 may play a role in IFT as rates of movement for BBS4 have been found to approach known IFT rates (Follit et al., 2006; Nachury et al., 2007). *Bbs4* KO sperm lack a sperm flagellum resulting in male infertility (Chamling et al., 2013; Mykytyn et al., 2004).

Our studies show BBS4 to localize to the manchette and to the nucleus and nuclear membrane in WT mice. BBS4 is still present in the manchette and nucleus in *Spag17* KO mice (Fig.29).

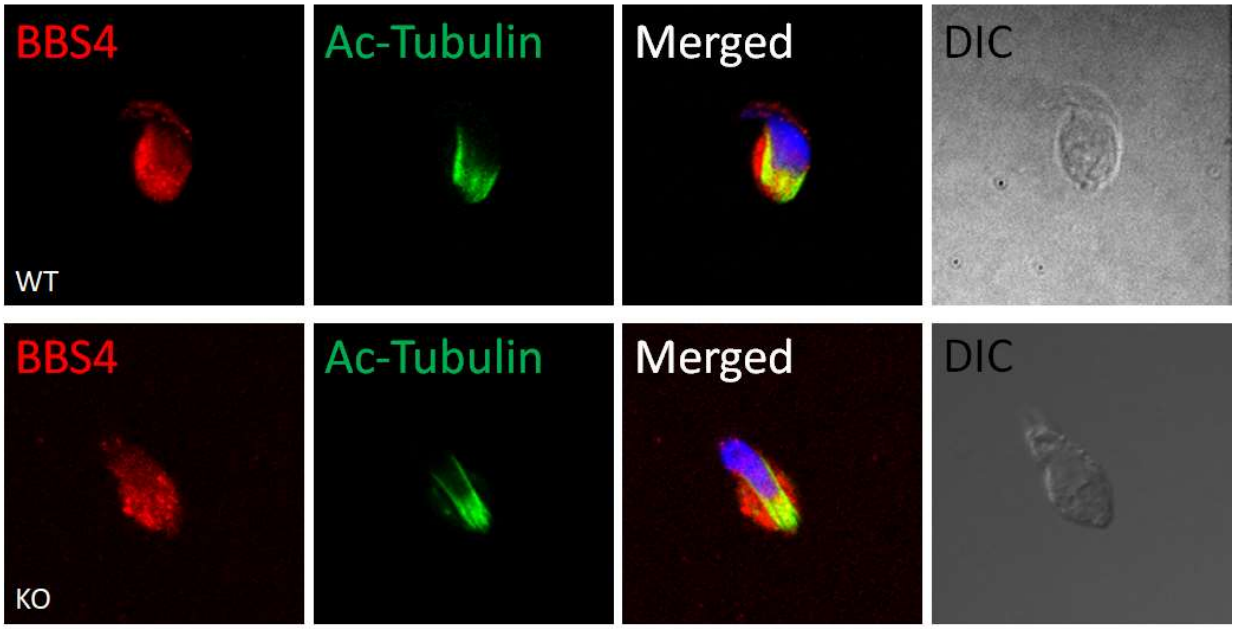



**Figure 29. BBS4 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for BBS4 and manchette structures. BBS4 localized to the manchette and to the nucleus and nuclear membrane of both WT and KO elongating spermatids.

Glioma transcription factor 1 (GLI1) is a transcription factor involved in Shh signaling (Sahin et al., 2014). *Gli1* mRNA has been found in spermatogonia, spermatocytes, and elongating spermatids with GLI1 localization shifting between the cytoplasm and nucleus (Kroft et al., 2001; Szczepny et al., 2006). *Gli1* is initially expressed in the cytoplasm and shifts to the nucleus in pachytene cells and round spermatids (Kroft et al., 2001). Expression shifts once again from the nucleus to the cytoplasm in elongating spermatids indicating *Gli1*-mediatd transcription is switched off at this stage (Kroft et al., 2001; Szczepny et al., 2006). The presence of *Gli1* mRNA during spermatogenesis suggests Hh functions within germ cells, specifically during Desert hedgehog (Dhh) signaling, an Hh homologue expressed in the testis, to regulate spermatogenesis (Kroft et al., 2001; Sahin et al., 2014). This was further confirmed by Dhh KO studies in which male mice were sterile (Kroft et al., 2001). In addition, overexpression of *Gli1* disrupted spermatogenesis and blocks germ cells at the pachytene stage providing further

evidence that the Hh pathway is essential for spermatogenesis and that Hh signaling may be needed to sustain spermatogenic stem cells (Sahin et al., 2014; Szczepny et al., 2006). *Gli1* KO animals develop normally and are fertile in adult hood. Spermatogenesis is not affected in KO animals possibly due to the redundant function of GLI1 and GLI2 (Barsoum & Yao, 2011).

Our studies found GLI1 to localize to the manchette of both WT and *Spag17* KO spermatids (Fig. 30).

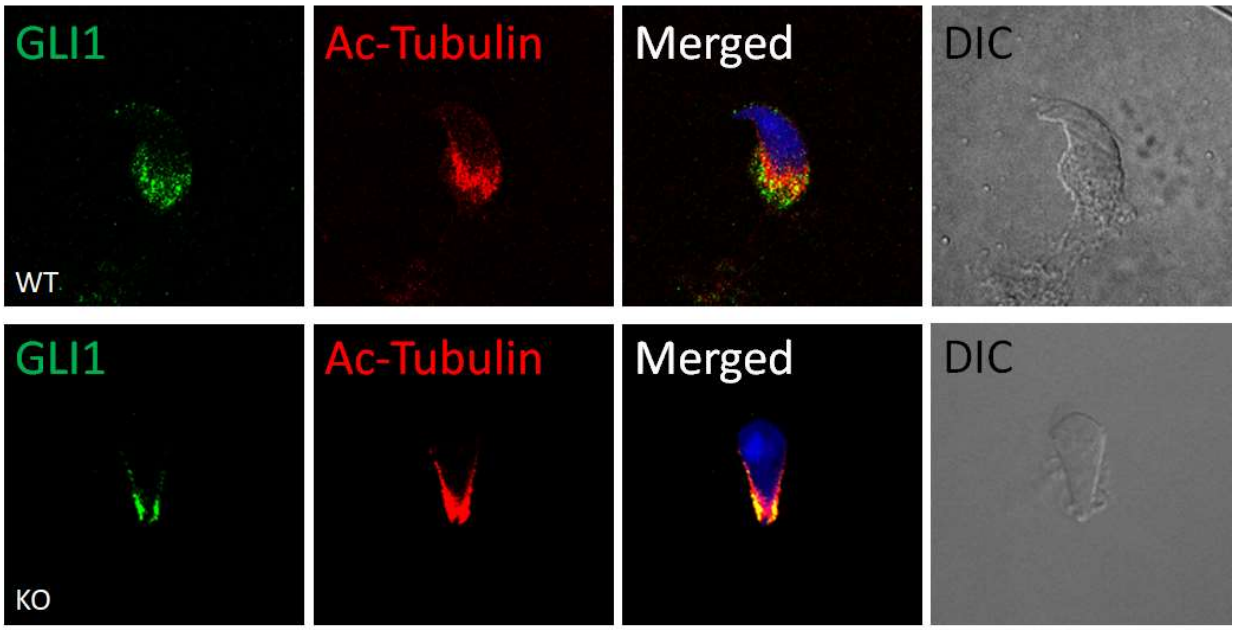



**Figure 30. GLI1 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for GLI1 and manchette structures. GLI1 localized to the manchette of both WT and KO elongating spermatids.

Smoothened (SMO) is a ciliary transmembrane protein also involved in the Hh signaling cascade (Caspary et al., 2007). Activation of SMO leads to translocation of GLI1 to the nucleus and initiates transcription of targeted genes. Smo has been found to be localized in the cilia of Hh responding cells in the mouse embryo, suggesting a requirement for cilia in Hh signal transduction (Caspary et al., 2007; Corbit et al., 2005). During spermatogenesis, *Smo* is expressed in primary spermatocytes and in round and condensing spermatids. SMO localizes to

the cytoplasm of late pachytene and secondary spermatocytes and elongating spermatids. There is also a high level of SMO immunoreactivity in the head of condensing spermatids (Morales et al., 2009). Mutation of *Smo* in somatic cells prevents ciliary localization and eliminates its activity (Corbit et al., 2005).

Our studies show SMO to localize to the acrosome, manchette and cytoplasm of elongating spermatids and to the tail in mature sperm. At later developmental stages, acrosome staining is less visible. However, SMO is still present in the manchette of *Spag17* KO spermatids (Fig. 31).

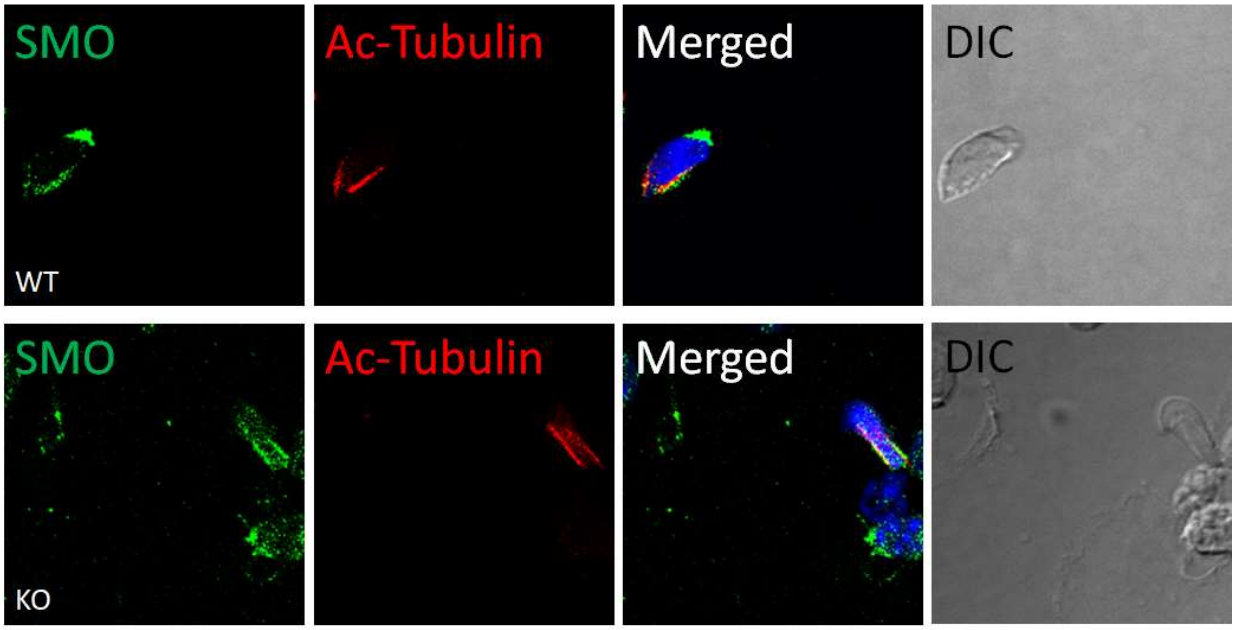



**Figure 31. SMO localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for SMO and manchette structures. SMO localized to the acrosome, manchette and cytoplasm of both WT and KO elongating spermatids and to the tail of both WT and KO mature spermatozoa.

*3.7 Other proteins*

DNA damage binding protein 1 (DDB1) is involved in Sertoli cell proliferation, remodeling of the testis cord via the TGFβ pathway, and spermatogonial stem cell maintenance in mice (Yu et al., 2016; Zheng et al., 2019). Previous studies have found DDB1 to localize to the nuclei of perinatal and juvenile testis (Zheng et al., 2019). Depletion of DDB1 in Sertoli cells results in disruption of the functions listed above as well as spermatogonial stem cell deficiency. *Ddb1* KO mice are infertile and lack germ cells (Yu et al., 2016; Zheng et al., 2019).

We found DDB1 to localize to the manchette in WT elongating spermatids. No signal was seen in WT mature sperm. In *Spag17* KO spermatids, DDB1 failed to localize to the manchette (Fig. 32).

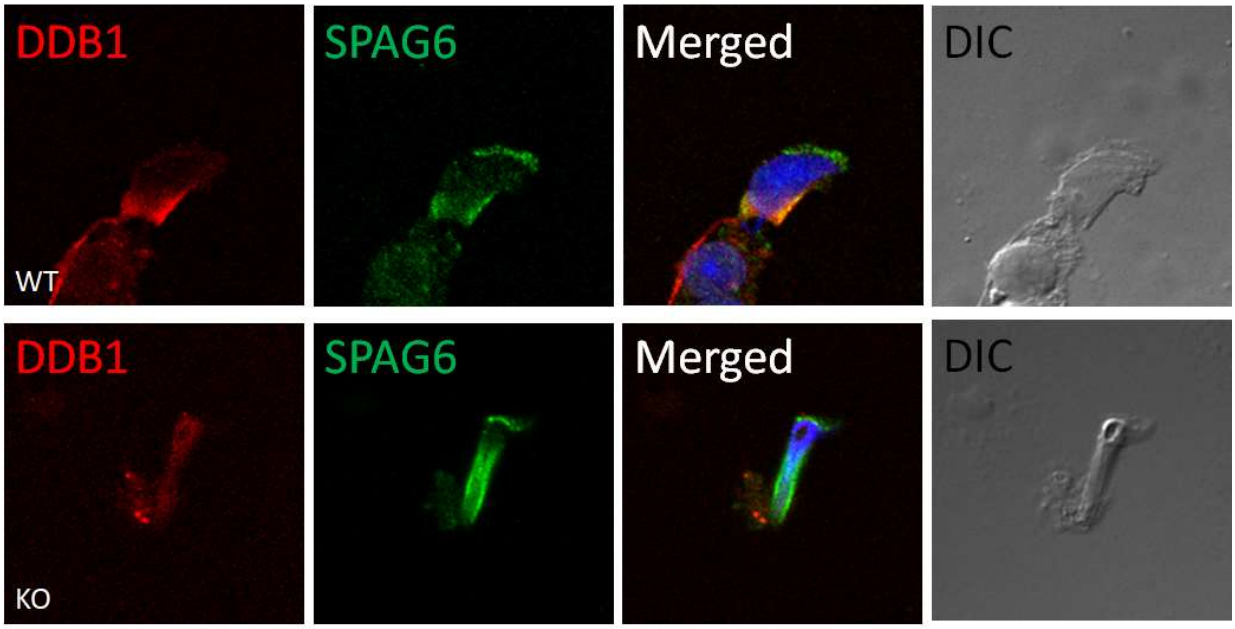



**Figure 32. DDB1 localization to the manchette is dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for DDB1 and manchette structures. DDB1 localized to the manchette of WT elongating spermatids but failed to localize to the manchette in *Spag17* KO elongating spermatids.

β-defensin 1 (DEFB1) is part of the β-defensin family of antimicrobial peptides that plays a role in host defense and regulation of sperm function such as sperm motility (Diao et al., 2014). DEFB1 is typically seen in the mucosa of the male genito-urinary tract where it prevents infection and protects sperm motility through the female reproductive tract by forming an antimicrobial shield around sperm (Zupin et al., 2019). DEFB1's role in host defense was further confirmed by studies showing low levels of *Defb1* in sperm from infertile men exhibiting either leukocytospermia, a presence of leukocytes in the seminal tract, or asthenozoospermia, reduced sperm motility. When comparing sperm from normal and asthenozoospermia patients, *Defb1* expression was positively correlated for sperm motility meaning increased *Defb1* expression resulted in increased sperm motility. Comparing sperm from normal and leukocytospermia patients found *Defb1* expression to inversely correlate to the number of semen leukocytes present, meaning decreased *Defb1* resulted in a higher number of leukocytes. DEFB1 has been found to localize to the lower portion of the sperm head and in the mid-piece of the sperm. Depletion of *Defb1* results in reduced sperm motility and antimicrobial activity along with reduced staining for DEFB1 in patients with asthenozoospermia or leukocytospermia (Diao et al., 2014). *Defb1* KO mice are infertile due to fragile sperm and spontaneous acrosome reactions prior to fertilization. There are also disordered microtubule structures, including lack of arrangement in the 9+2 structure (Dorin, 2015).

DEFB1 was found to localize to the manchette of both WT and *Spag17* KO elongating spermatids. DEFB1 was seen in the Golgi and surrounding cells in the *Spag17* KO (Fig. 33).

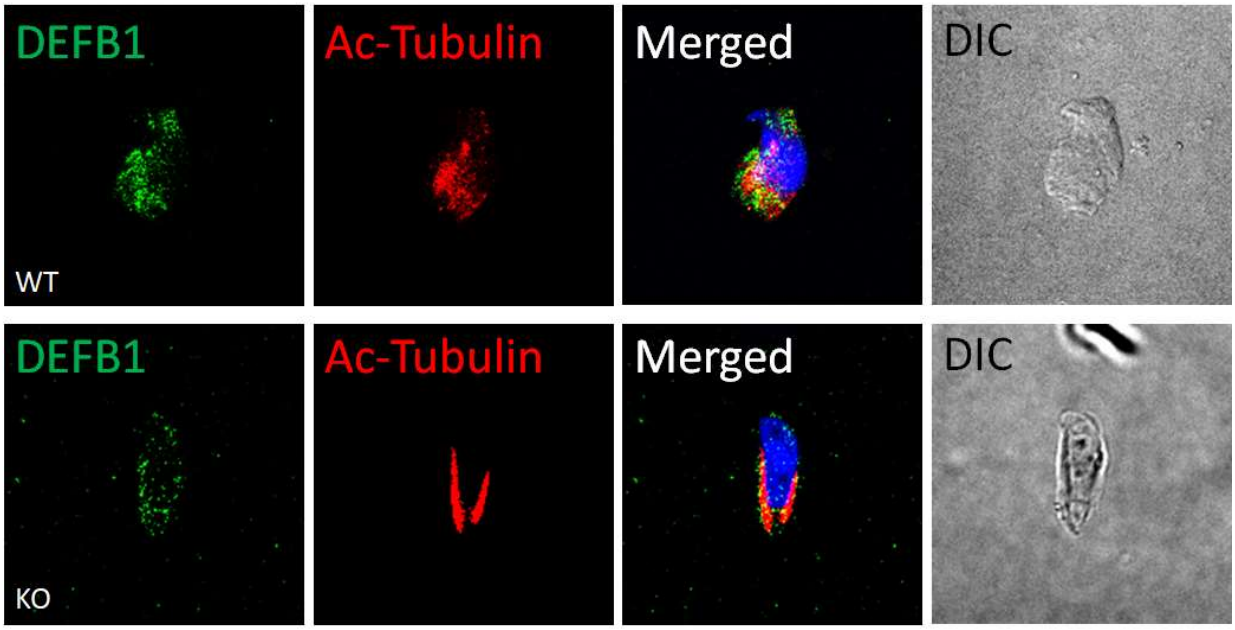



**Figure 33. DEFB1 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for DEFB1 and manchette structures. DEFB1 localized to the manchette of both WT and KO elongating spermatids.

Dentin matrix proteins (DMP) are a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family and have been found to bind and activate matrix metalloproteases. DMP expression has been shown in testis cord but its role in spermiogenesis has not been proven (Wilson et al., 2005)

Our studies found DMP1 to localize to the manchette of WT and *Spag17* KO elongating spermatids. (Fig. 34).

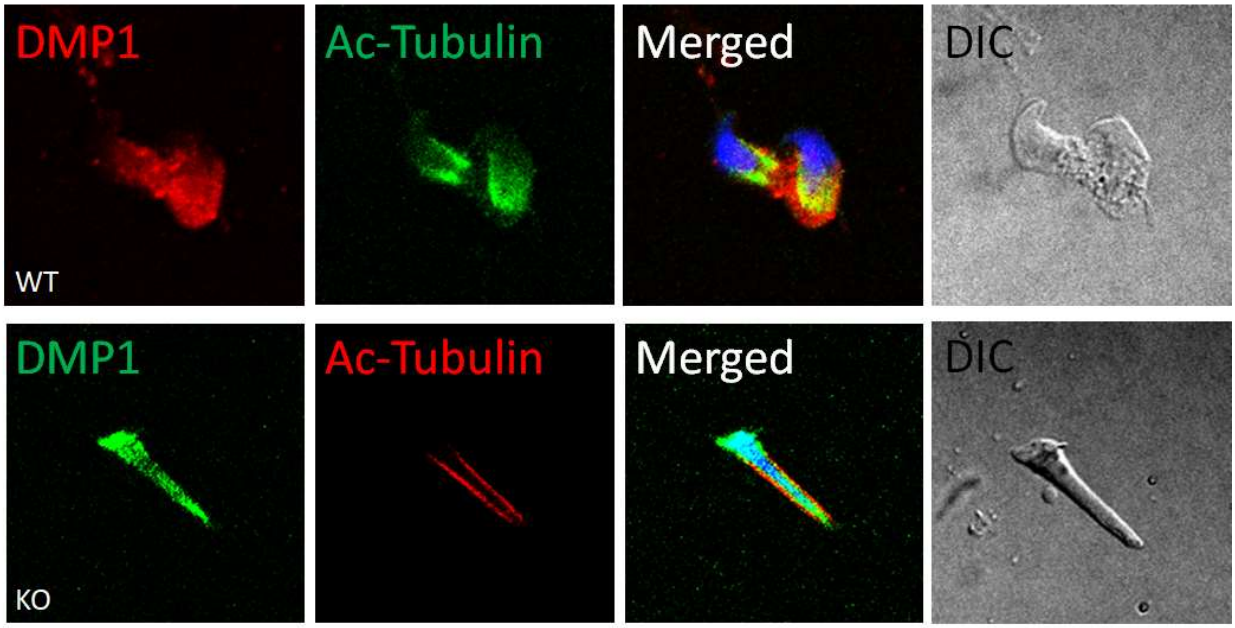



**Figure 34. DMP1 localization to the manchette is dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for DMP1 and manchette structures. DMP1 localized to the manchette of WT elongating spermatids but seemed to be missing from the manchette in *Spag17* KO elongating spermatids.

GATA-binding transcription factor 4 (GATA4) is zinc finger transcription factor that has been previously implicated in the development and function of the mammalian testes (Chen et al., 2015; Kyrönlahti et al., 2011). GATA4 is mostly expressed in somatic cells within the testes, including Sertoli cells, and maintains the spermatogonial stem cell (SSC) pool (Chen et al., 2015). *Gata4* KO mice develop age-dependent testicular atrophy accompanied by loss of fertility, decreased sperm quantity and decreased motility (Kyrönlahti et al., 2011). Mice also display a loss of Sertoli cells and thus a loss in the SSC pool (Chen et al., 2015).

Our studies found GATA4 to localize around the nucleus in the form of vesicles in both WT and *Spag17* KO germ cells (Fig. 35).

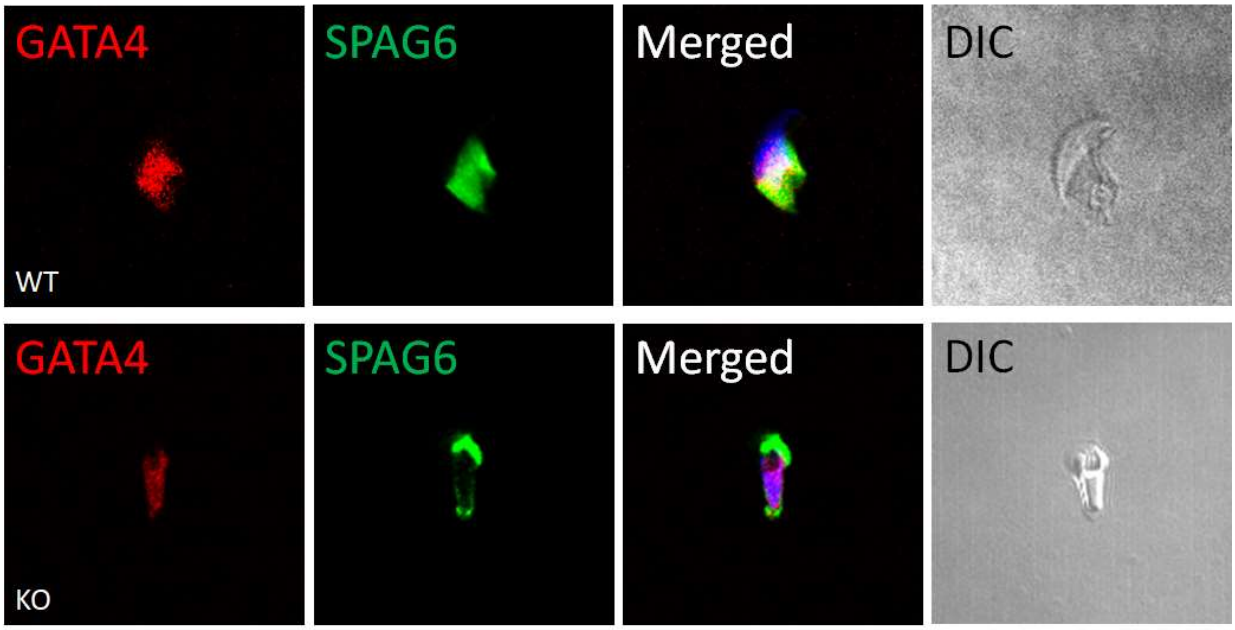



**Figure 35. GATA4 localization to the manchette is not dependent on SPAG17.** Germ cells were collected from WT and *Spag17* KO adult mice and stained for GATA4 and manchette structures. GATA4 localized to around the nucleus of both WT and KO elongating spermatids.

*3.8 SPAG17 Interactome*

The interactome created was very complex due to the number of proteins analyzed. Figure 36 shows a simplified interactome with intermediate proteins listed in Table 2 and Table 3. All of the proteins were found to interact with SPAG17 via either Nima-related protein 4 (NEK4) or protein kinase C substrate 80K-H (PRKCSH), which the software found to be direct binding partners of SPAG17. SPAG6 was also found to be a direct binding partner of SPAG17. NEK4 is from a group of serine-threonine kinases and is essential for cell division, primary cilia formation, DNA damage response, and microtubule stabilization (Basei et al., 2015). PRKCSH is a protein kinase found in the endoplasmic reticulum (ER) that is involved in carbohydrate processing, folding and translocation of newly synthesized glycoproteins (Gao et al., 2010).

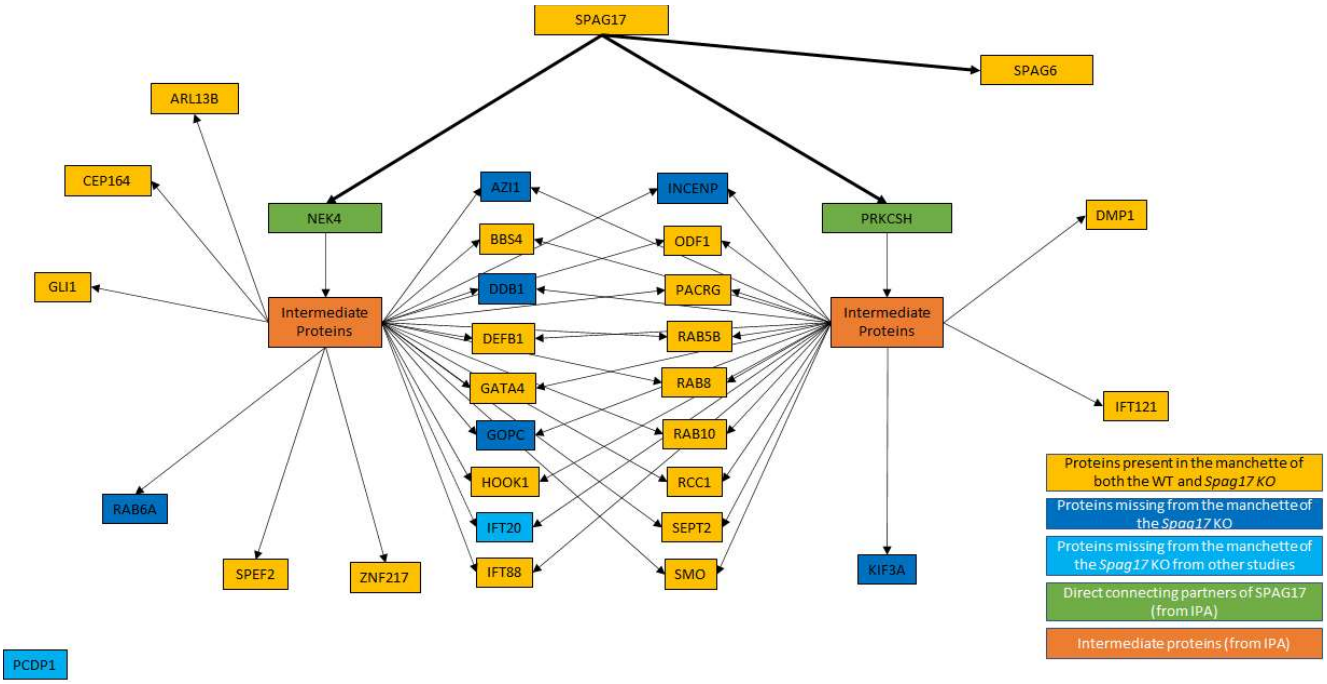



**Figure 36. SPAG17 interactome of proteins.** The Ingenuity Pathway Analysis (IPA) software, from QIAGEN Inc., was used for protein interaction analysis. Only proteins used in our study were analyzed. Yellow boxes indicate proteins used in our study present in the manchette of both WT and *Spag17* KO spermatids. Dark blue boxes indicate proteins from our study missing from the manchette of *Spag17* KO spermatids. Light blue boxes indicate proteins other studies have found to be missing from the manchette of *Spag17* KO spermatids. Green boxes indicate the two major binding partners of SPAG17, according to IPA, along with SPAG6. Blue boxes indicate intermediate proteins the IPA software found (Table 2).

| NEK4-Connecting Intermediate Proteins | | | | | | |
|---|---|---|---|---|---|---|
| ABCF1 | DDX17 | HSP90AB1 | NUP98 | PRKAB2 | SKAP1 | TRAK2 |
| ACTC1 | DDX50 | HSPA6 | OTUD4 | PRKDC | SLC25A11 | TRIO |
| AIFM1 | DDX56 | ITGB4 | P4HB | PRPF19 | SLC25A13 | TUBAL3 |
| ALDOC | DGKD | KDM2B | PARP1 | PRPF4B | SLC25A3 | UBE2E1 |
| ALLC | DHX15 | KHSRP | PCNA | PSMA1 | SLC25A4 | UEVLD |
| APOB | DHX9 | KPNB1 | PDIA3 | PSMA2 | SLC25A5 | UQCRC2 |
| ATP2A3 | DNAJA2 | LGALS3BP | PFKL | PSMA4 | SLC25A6 | VPS18 |
| ATP5F1A | DUSP26 | LPL | PGAM5 | PSMA7 | SLC39A10 | XPO1 |
| ATP5F1B | EFTUD2 | MAP3K7 | PKM | QPCT | SMG6 | XRCC1 |
| ATP5F1C | EIF2AK3 | MAPK3 | PKN2 | QSOX1 | SNRNP200 | XRCC6 |
| ATP5PF | ENO1 | MAPK8 | PLAUR | RABGGTA | SRPK1 | XRN1 |
| BLM | HACE1 | MARK1 | PLOD3 | RACK1 | SSB | YWHAZ |
| CAD | HAT1 | MCM3 | PPP1CC | RBBP4 | STK24 | ZDHHC17 |
| CAPN15 | HECTD1 | MCM4 | PPP2CB | RGPD4 | STUB1 | |
| CBR3 | HERC2 | MCM5 | PPP2CP | RIOK1 | TENM1 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| CCAR2 | HLA-B | MINDY2 | PRDX1 | RPN1 | TIMM50 | |
| CERT1 | HNRNPA1 | MYH9 | PRKAA1 | RPS3 | TNFRSF19 | |

**Table 2. List of NEK4-connecting intermediate proteins.** The Ingenuity Pathway Analysis (IPA) software, from QIAGEN Inc., was used for protein interaction analysis. This list contains all proteins that connect to NEK4 as a part of the SPAG17 interactome.

| PRKCSH-Connecting Intermediate Proteins | | |
|---|---|---|
| COPG1 | HSPA5 | SLC27A2 |
| CUL7 | IKBKE | SOD1 |
| EDEM3 | NTRK1 | TRAF6 |
| ESR1 | PHOSPHO1 | TRIM9 |
| FBX032 | PKD2 | UBE2M |
| GANAB | PLPPR4 | |

**Table 3. List of PRKCSH-connecting intermediate proteins.** The Ingenuity Pathway Analysis (IPA) software, from QIAGEN Inc., was used for protein interaction analysis. This list contains all proteins that connect to PRKCSH as a part of the SPAG17 interactome.

## 4. Discussion

Previous *Spag17* KO studies have shown disruption of SPAG17 to affect the recruitment of proteins to the manchette, including primary cilia dyskinesia protein 1 (PCDP1) and intraflagellar protein 20 (IFT20) (Kazarian et al., 2018). Using IF, we similarly found GOPC, AZI, KIF3A, INCENP, RAB6A and DDB1 localization to the manchette to be disrupted in the *Spag17* KO. Our results suggest these proteins are part of the SPAG17 interactome and depend on SPAG17 for their function and localization.

### *4.1 Disruption of Spag17 affects recruitment of several proteins to the manchette*

PCDP1 is a central pair protein that localizes to the cytoplasm, manchette, and sperm flagella (Kazarian et al., 2018; Lee et al., 2008). *Pcdp1* KO mice develop primary cilia dyskinesia and typically lack mature sperm with flagella leading to male infertility. Sperm heads

are often seen with no attached tails (Lee et al., 2008). In *Spag17* KO germ cells, PCPD1 localization to the manchette is disrupted (Fig. 37) (Kazarian et al., 2018).

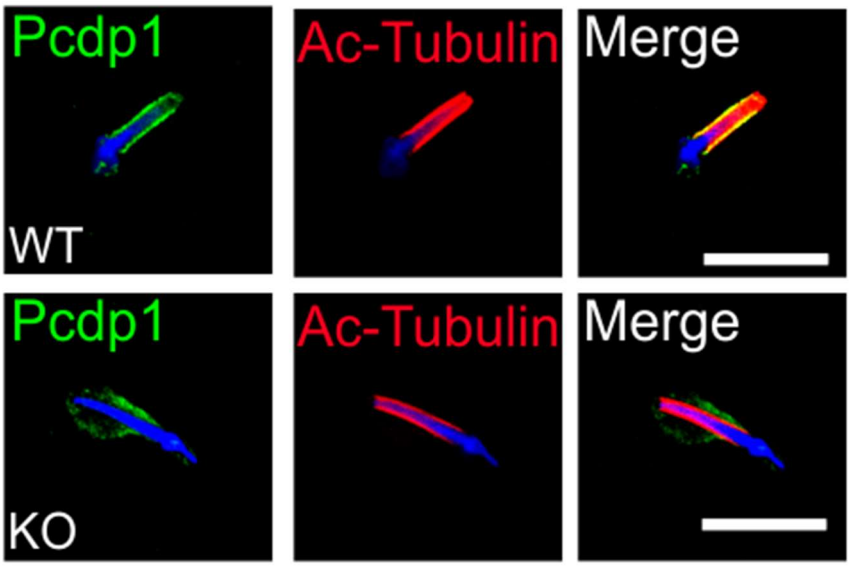



**Figure 37. PCDP1 localization in the manchette is dependent on SPAG17.** Germ cells from wild-type and knockout mice were collected from adult mice and stained for proteins associated to manchette structures. PCDP1 failed to localize to the manchette in the elongating spermatids from *Spag17* knockout mice and its localization appears diffused in the cytoplasm. (Kazarian et al., 2018)

IFT20 is the smallest of the IFT proteins at 15kDa (Sironen et al., 2010; Zhang et al., 2016). IFT20 participates in the transport of cargo proteins for sperm flagella formation from the Golgi complex and along manchette microtubules (Follit et al., 2006, 2008; Huang et al., 2020; Zhang et al., 2016). Once pro-acrosomal vesicles have fused, IFT20 can be seen in the acrosome of round spermatids (Huang et al., 2020; Zhang et al., 2016). In elongating spermatids, IFT20 is found in the manchette and basal body (Sironen et al., 2010). In mature sperm, IFT20 is seen only in the sperm tail. *Ift20* KO mice are infertile with reduced sperm counts and motility (Zhang et al., 2016). Previous *Spag17* KO studies have found IFT20 transport along manchette microtubules to be disrupted in elongating spermatids (Fig. 38) (Kazarian et al., 2018).

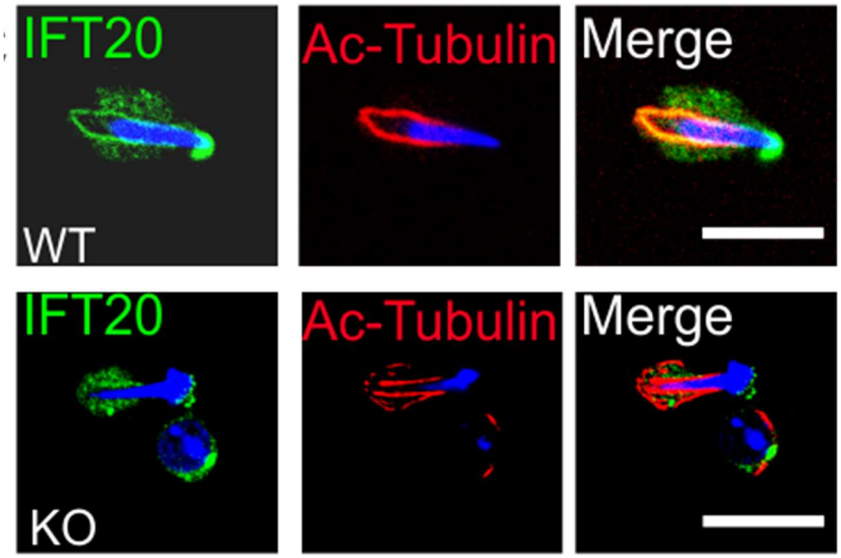



**Figure 38. IFT20 localization in the manchette is dependent on SPAG17.** Germ cells from wild-type and knockout mice were collected from adult mice and stained for proteins associated to manchette structures. IFT20 failed to localize to the manchette in the elongating spermatids from *Spag17* knockout mice and its localization appears diffused in the cytoplasm. (Kazarian et al., 2018)

With this information in mind, we hypothesized that *SPAG17* is essential for protein trafficking during mammalian spermiogenesis, and the loss of *SPAG17* will result in disrupted transport of proteins important for acrosome biogenesis and manchette functions. Consistent with our proposal, the localization of several proteins, including GOPC, AZI1, KIF3A, INCENP, RAB6A and DDB1 was disrupted in the *Spag17* KO (Fig. 4, 5, 24, 26, 32). IF studies staining for the protein of interest and anti-acetylated tubulin or SPAG6 as manchette markers showed the stated proteins to be missing in the manchette of KO elongating spermatids. Their absence in the *Spag17* KO suggests these proteins rely on SPAG17 for proper localization and function and are part of the SPAG17 interactome.

GOPC, AZI1, KIF3A, INCENP, RAB6A and DDB1 have a number of functions similar to SPAG17 and localize to many of the same areas, including the Golgi apparatus, acrosome,

manchette, and sperm tail. Many of these functions are similar to that of SPAG17 and depletion of each protein in their respective KO models results in phenotypes similar to those seen in the *Spag17* KO mouse. For example, GOPC has been implicated in vesicle transport from the Golgi apparatus and in membrane fusion of pro-acrosomal vesicles and its KO model displays a fragmented acrosome and malformations of the head, manchette and tail (Suzuki-Toyota et al., 2007). SPAG17 localizes to Golgi vesicles and its KO model similarly displays malformations of the sperm (Kazarian et al., 2018). This suggests disruption of SPAG17 may in turn disrupt GOPC which causes the phenotypes typical to the *Spag17* KO to appear.

Similar relationships can be made with AZI1, KIF3A, INCENP, RAB6A and DDB1 as well. AZI1 is involved in protein trafficking and localizes to the acrosome, microtubules and HTCA (Aoto et al., 1995; Hall et al., 2013). Loss of *Azi1* disturbs both IMT and IFT and results in malformations of sperm which is similar to malformations seen in the *Spag17* KO (Hall et al., 2013; Kazarian et al., 2018). KIF3A is another protein involved in protein trafficking that localizes to the manchette. Its KO model show abnormal morphology as well as manchette disorganization (Lehti et al., 2013). INCENP ensures proper sister chromatid separation during mitosis and meiosis and has been implicated in reproductive disorders (Liu et al., 2016; Parra et al., 2003). RAB6A regulates vesicle trafficking within the Golgi and has been shown to localize to the acrosome and manchette of elongating spermatids. Loss of RAB6A disrupts intracellular transport (Del Nery et al., 2006; Lin et al., 2017). DDB1 maintains the spermatogonial stem cell population and its depletion results in SSC deficiency infertility (Yu et al., 2016; Zheng et al., 2019). Many of the functions of each of the proteins that fail to localize to the manchette in the *Spag17* KO are similar to SPAG17 itself. Their failure to localize to the manchette may reveal an

upstream defect in SPAG17 (Fig. 39) that in turn affects these proteins to cause the phenotypes that are so often seen in the *Spag17* KO.

The remaining proteins maintained their localization to the manchette in both WT and *Spag17* KO germ cells suggesting they do not rely on SPAG17 for their localization and function. They may be upstream of SPAG17 and/or not a part of the SPAG17 interactome (Fig. 39).

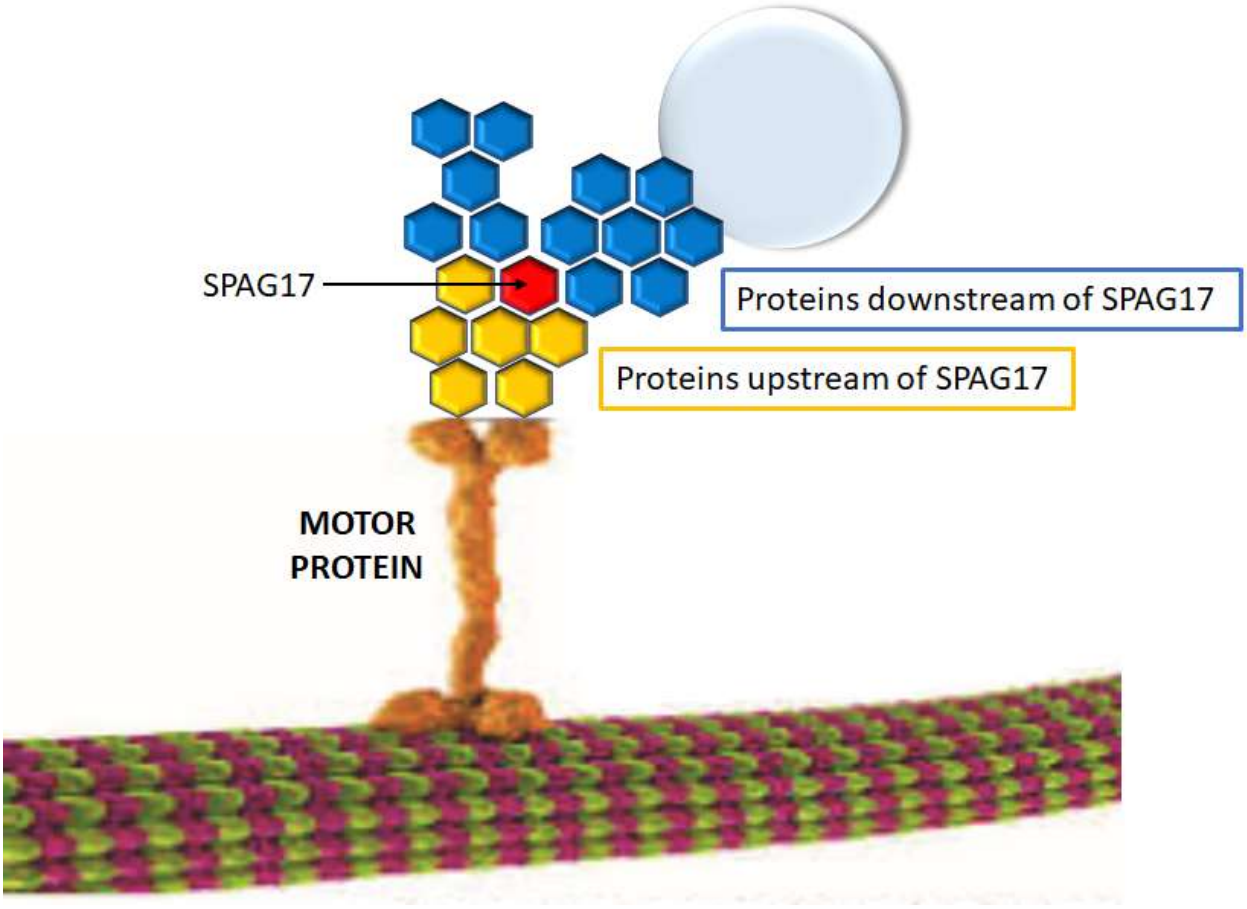



**Figure 39. Schematic of the possible SPAG17 interactome.** We found DDB1, RAB6A, GOPC, AZI1, KIF3A, and INCENP (blue) to be missing from the manchette of *Spag17* KO mice, suggesting they downstream of SPAG17 and rely on it for their function. The remaining proteins may either be upstream of SPAG17 (yellow) or not a part of the interactome.

*4.2 Conclusion and Future Directions*

Our current findings show a possible interactome of SPAG17 binding partners, but the identity of these direct interacting partners is not yet known. Using the interactome we developed, future studies will include IF experiments using several of the proteins suggested by the interactome as well as immunoprecipitation studies to confirm binding partners. These studies have the potential to reveal direct binding partners of SPAG17 as well as the specific function of SPAG17 and other proteins in male fertility. In conclusion, we present possible binding partners of SPAG17 and show that several proteins are not recruited in the *Spag17* KO suggesting they are in the interactome.

Understanding protein trafficking during spermatogenesis and the SPAG17 interactome has clinical implications as well. Defects in spermiogenesis lead to male infertility and so it is crucial to focus on establishing the interactome of proteins and discovering the specific mechanism of protein transport during spermiogenesis.

## Vita

Virali M. Bhagat was born on April 15, 1994 in South Plainfield, New Jersey. She later moved to Virginia where she resided in Chesapeake, Virginia. Virali went on to attend Virginia Commonwealth University in Richmond, Virginia and received her Bachelor of Science in Biology and minors in Chemistry, Mathematics, and French in May 2016. Virali worked in France as a primary school English teacher after graduating. She returned to VCU for the Premedical Graduate Health Sciences Certificate Program and continued on to earn a Master's in Physiology and Biophysics under Dr. Maria Teves' mentorship.

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