

KINESIN-BASED TRANSPORT OF ECTOPLASMIC SPECIALIZATIONS
AND LOCALIZATION OF DYNAMIN 3 IN THE TESTIS

by

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Abstract

The Sertoli cell cytoskeleton plays a number of roles including intracellular transport, maintenance of cell shape, and formation of adhesion junctions. During spermatogenesis, the cytoskeleton plays a number of roles to ensure the proper formation of spermatids in the seminiferous epithelium. The epithelium is composed of the spermatogenic cells as well as Sertoli cells. Sertoli cells act as nurse cells and provide support and nourishment to the developing spermatids. The spermatids are attached to Sertoli cells through actin-rich adhesion complexes known as ectoplasmic specializations. During spermatogenesis, these junctions and the associated spermatids are hypothesized to be transported along polarized microtubule tracts in the Sertoli cell to the base of the seminiferous epithelium before retreating back to the apex of the epithelial tissue. Upon reaching the apex of the tissue, the ectoplasmic specializations are disassembled and spermatids are released into the lumen of the seminiferous epithelium. The study of this junction transport and disassembly will help to better understand mechanisms of spermatogenesis. Recent work has provided evidence that a dynein motor protein is the likely mechanism which transports the spermatid/ectoplasmic specialization junction to the apex of the epithelium. In addition, past work using mRNA GeneChip data arrays has identified the Rab6KIFL kinesin to be highly expressed in Sertoli cells. In chapter 2 of this thesis, I present evidence that antibodies raised against the Rab6KIFL protein are associated with ectoplasmic specializations and that this motor may be involved in spermatid entrenchment. Results indicate localization of antibodies at ectoplasmic specializations at times of spermatogenesis when entrenchment occurs and localization of antibodies on the cytoplasmic face of ectoplasmic specializations. In chapter 3 of this

thesis I demonstrate that dynamin 3 is present at tubulobulbar complexes and when transfected into eGFP-nectin-2 γ MDCK cells tube like structures form. Dynamin 3 is highly expressed in testis however its function has been unexplored. Here I demonstrate that dynamin 3 may facilitate formation of these structures. The findings summarized in this thesis are integrated around the predominant subject of adhesion junction transport and disassembly. This work provides insight into mechanisms involved in spermatid translocation and release during spermatogenesis.

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List of Abbreviations

°C	Degrees Celsius
mg	Micrograms
mm	Micrometers
cDNA	Complementary DNA
COS-7	Transformed African Green Monkey Kidney Fibroblast cells
DAPI	4',6-diamidino-2-phenylindole
ddH ₂ O	Double distilled H ₂ O
DMEM	Dulbecco's Modified Eagle's Media
DTT	Dithiothreitol
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced Green Fluorescent Protein
ES	Ectoplasmic specialization
ESs	Ectoplasmic specializations
FBS	Fetal Bovine Serum
FHA	Fork-Head Associated
IgG	Immunoglobulin G
IgY	Immunoglobulin Y
KLH	Keyhole limpet hemocyanin
M	moles per liter
MDCK	Madin-Darby canine kidney
MES	Ethyl methyl sulfide

mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
NaCl	Sodium chloride
NCIgY	Normal Chicken Immunoglobulin Y
NRIG	Normal Rabbit Immunoglobulin G
NGS	Normal goat serum
OCT	Optimal Cutting Temperature
PBS	Phosphate buffered solution
pCMV-HA	Plasmid containing mammalian vector - hemagglutinin
PMSF	Phenylmethylsulphonylfluoride
RIPA	Radio-Immunoprecipitation Assay
RPM	Revolutions per minute
SDS	Sodium dodecyl sulfate
TBC	Tubulobulbar complex
TBS	Tris buffered solution
TBST	Tris buffered solution with Tween
TPBS-BSA	Tween phosphate buffered solution – bovine serum albumin

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Chapter 1

General Introduction

General Statement

Sertoli cells in the mammalian testis comprise a major component of this organ and play a key role during spermatogenesis. Sertoli cells position spermatogenic cells in the seminiferous epithelium, play a role in spermatid release and are involved in establishing, maintaining and turning over junction complexes at the base of the epithelium. Adhesion junction turnover occurs on a grand scale, particularly these junctions between adjacent Sertoli cells and between Sertoli cells and spermatids. These junctions, known as ectoplasmic specializations, assemble and are broken down on a continuous basis. This provides an excellent model for studying junction turnover as this occurs in an in vivo system. This also provides an ideal situation to explore how intercellular adhesion junctions are coupled to microtubule transport machinery in a cell to move adjacent cells. In this thesis, I present evidence that is consistent with the hypothesis that a kinesin motor is associated with adhesion junction components and is therefore likely to be responsible for spermatid translocation to the base of the Sertoli cell. Once spermatids complete their translocation to the base of Sertoli cells and then move back to the apex, the adhesion junctions are then broken down and spermatids are released. In the second portion of my thesis I provide evidence that dynamin 3 is associated with tubulobulbar complexes (TBCs) that internalize junctions and that the protein may be involved in the morphogenesis of these structures.

Cytoskeleton

A cell has a network of filamentous structures that is similar to a skeleton of a multi-cellular organism. These structures, collectively known as the cytoskeleton are composed of intermediate filaments, actin filaments and microtubules, as well as multiple associating proteins. Each of the three major cytoskeletal elements plays a significant role in cellular function, including transport, cellular integrity and cell shape.

Intermediate Filaments

Intermediate filaments are classified into groups based on their substructure and sequence homology (Coulombe and Wong 2004). They are 8-12 nm in diameter filaments and form a mesh-like network around the nucleus of a cell and extend peripherally to attach to cell/cell adhesion junctions (Fuchs and Cleveland 1998). These filamentous structures lack polarity and therefore no motor proteins are dependant on these filaments (Coulombe and Wong 2004). There also is recent evidence indicating that these filaments are flexible and extensible and allow dramatic stretching (Fudge, Gardner et al. 2003; Kreplak, Bar et al. 2005). It is thought that the primary function of these filaments is mechanical support and to accommodate cellular deformation (Fuchs and Cleveland 1998).

Actin

Actin is the most abundant protein in the cell and is formed of globular subunits. Actin filaments provide mechanical support, are a determinant of cell shape, and enable cell movement (DeMali, Wennerberg et al. 2003; Winder and Ayscough 2005; Kovar 2006). Actin filaments also play a major role in muscle contraction. The filaments are 6 nm in diameter and are controlled by capping, severing and sequestering proteins that

associate with actin and regulate this filament (May, Hall et al. 1999). Actin also plays a fundamental role in all cell types and is responsible for cytokinesis and involved in junction formation.

Microtubules

Microtubules are long slender filamentous structures which are 25 nm in diameter. They also are generally longer than actin filaments and are formed by globular α and β tubulin subunits. Microtubules are polarized structures with the plus end, the fast growing end, and the minus end known to be the slow growing end. Microtubule growth begins at nucleation sites termed microtubule organizing centres. From these sites, the microtubules grow outwards to the periphery of the cell membrane with their plus ends generally directed outwards to the cell membrane. Microtubules play a major role in many cell types in addition to Sertoli cells including general epithelial cells, and neural cells. Functions include parallel formation of the mitotic spindle, cell shape and secretion (Allen, Metuzals et al. 1982; Dustin 1984; Hayden and Allen 1984; Brady, Lasek et al. 1985). In various cells, microtubules play an intracellular vesicle transport role. In these cells, various types of cargo including organelles, proteins and RNA are transported throughout the cell along arrays of microtubules (Flickinger and Fawcett 1967; Olmsted and Borisy 1973; Brady, Lasek et al. 1982; Vogl, Linck et al. 1983; Koonce and Schliwa 1985; Schnapp, Vale et al. 1985; Vale, Reese et al. 1985; Vale, Schnapp et al. 1985; Vale, Schnapp et al. 1985; Duden, Ho et al. 1990; Kelly 1990; Kreis 1990). The direction of movement of cell cargo is partly based on the polarity of microtubules and also on the type of motor protein involved.

Motor proteins

Microtubules act as highways for the transport of cellular machinery and materials from one region of the cell to another. This movement occurs as a result of microtubule-based motor proteins that walk along microtubule tracts. There are two major types of microtubule-based motors, the dyneins and the kinesins. Dyneins are relatively large proteins that form large protein complexes with associated proteins (dynactin complex) and travel almost exclusively towards the negative ends of microtubules, however there is evidence they can move bidirectionally (Ross, Wallace et al. 2006). The kinesins can also form protein complexes however these proteins move toward the positive ends of microtubules (Vale 1987).

Kinesins are a superfamily of microtubule-associated proteins that is comprised of 318 members and 45 genes in humans alone (Miki, Setou et al. 2001). Both kinesins and dyneins have a conserved motor domain which allows them to bind to and travel along microtubules. This motor domain also has a conserved ATP hydrolysis-binding region which allows for the addition of ATP and conversion of this into mechanical energy (Farrell, Mackey et al. 2002). Kinesins are composed of a head motor which binds to microtubules and is generally highly conserved among families. As all kinesins travel along polarized microtubules, it is not unusual to expect the head regions of kinesins to be highly conserved. They also contain a neck region and a tail which bind cargo and is usually specific to each type of kinesin. The tail regions are divergent as these portions of the kinesin bind specific cargos to transport various proteins and organelles around the cell (Allan, Thompson et al. 2002; Seog, Lee et al. 2004). Other roles of kinesins include

forming and walking along spindles during mitosis (Thaler and Haimo 1996; Robertson and Allan 1997).

There are different types of kinesins, some with one or two heads and it is unclear how all these proteins bind cargo and walk along microtubules. Generally, kinesins have two heavy and two light chains. It is believed that the heavy chains contain components for movement and walking along microtubules while the light chains bind the cargo. Affinity for cargo and binding of microtubules has been shown to be regulated by phosphorylation of light chains and regulation of protein levels (Reilein, Rogers et al. 2001), however not all species contain light chain components (Steinberg and Schliwa 1995; Kirchner, Woehlke et al. 1999) suggesting they may not be required for cargo binding.

The kinesin family is divided into numerous groups. The major groups are based on where the conserved motor region is located (N-terminus, C-terminus, or internally) and to which cargo they are able to bind. These groups are then broken down based on homology in the motor domain (Lawrence, Dawe et al. 2004). Adjacent to this motor region is the coiled-coil stalk that dimerizes two heavy chains. Conventional kinesin is a heterotetramer composed of two heavy and two light chains in the C-terminus which form a tail for organelle (cargo) binding (Bowman, Kamal et al. 2000; Byrd, Kawasaki et al. 2001; Reilein, Rogers et al. 2001; Verhey, Meyer et al. 2001). Interestingly some kinesins are able to travel to the negative end of microtubules. When the head region of a kinesin is moved to the C-terminus of the protein, a change in the direction of movement occurs (Henningsen and Schliwa 1997; Wade and Kozielski 2000; Menetrey, Bahloul et

al. 2005). In addition, direction of movement is changed when amino acids in the neck of the kinesin are changed (Salanova, Stefanini et al. 1995; Endow and Higuchi 2000).

Intercellular adhesion Junctions

Multicellular organisms are composed of cells which are attached to each other by cell-cell junctions. There are many types of junctions which include desmosomes, tight and gap junctions and adherens junctions.

Adherens junctions are composed of transmembrane junctions that anchor to the actin cytoskeleton. Their adhesion molecules include cadherins, integrins and nectins which bind to the extracellular matrix and allow for folding and bending of sheets of cells.

Desmosomes are junctions that result in strong points of adhesion between adjacent cells (Green and Gaudry 2000). These junctions form plaques which act as anchors for intermediate filaments to attach through linking proteins and desmosomal cadherins.

Tight junctions are junctions that form barriers between two different regions of epithelial tissue. The junctions include the claudins, occludin, which interacts with the ZO family of proteins and JAMs which attach to the actin cytoskeleton of the cell. These junctions formed by these cells form a barrier that separates apical from basolateral compartments of the epithelium.

Gap Junctions are intercellular junctions that act as channels for hydrophilic molecules to pass through. These junctions selectively allow certain molecules to cross the membrane while blocking others (de Wit, Hoepfl et al. 2006). These channels are composed by connexin proteins which combine six together to form a connexon hemi-

channel within a cell membrane that then links another hemi-channel in the adjacent membrane to form a gap junction. The number of channels found in these junctions can vary greatly from a few to several thousand. The opening and closing of these junctions is also regulated and this can control the communication between cells (de Wit, Hoepfl et al. 2006).

Junction disassembly

The breakdown of junctions has been studied extensively, however there is a lot of detail about the precise mechanisms still missing. Endocytosis is believed to play a major role in the disassembly of junctions and is thought to work in recycling and degrading adhesion junction components (Le, Yap et al. 1999; Le, Joseph et al. 2002). Generally, it is believed that junction molecules in adjacent cells separate from each other and are endocytosed by each cell (Gaietta, Deerinck et al. 2002). The situation is different with gap junctions in that they are internalized as intact junctions by one or the other cell (Laird 2006). Endocytosed vesicles containing gap junctions have been shown to stain with late endosomal markers and may be directed for lysosomal degradation (Laird 2006). Also adhesion molecules such as $\beta 1$ integrins have been shown to be internalized in an endocytic manner (Ng, Shima et al. 1999). Based on junction dynamics, it appears that endocytosis may be the major factor which is responsible for junction turnover.

The Testis

The testis is the organ where spermatozoa and testosterone are produced. Generally, this organ can be divided into two major components, the interstitial and germinal components. The interstitial component is the area outside of the seminiferous tubules and contains the Leydig cells. These cells are responsible for production and secretion of

testosterone. The germinal component is composed of the seminiferous tubules and is the site of spermatogenesis. The seminiferous tubules are lined by a seminiferous epithelium which sits on a basement membrane that separates the epithelium from the tubule wall. The epithelium consists of two types of cells: the Sertoli cells which form the architectural component of the epithelium and spermatogenic cells which lie between the Sertoli cells and differentiate into spermatids. Spermatogonia are the most undifferentiated of the spermatogenic cells, and sit on the basal lamina and undergo mitosis. Some of these cells then differentiate into spermatocytes while others remain to maintain the spermatogonial population. The spermatocytes on the other hand undergo meiosis, pass through the basal junction complexes between adjacent Sertoli cells, and then give rise to spermatids.

Spermatids begin as round cells, and during the process known as spermiogenesis, elongate and differentiate into mature spermatozoa. During this process, the spermatid nucleus condenses, excess cytoplasm is removed from the spermatids and a flagellum and acrosome (a secretory vesicle) are produced. Once this process is complete the spermatozoa are released into the lumen of the seminiferous epithelium and are known as spermatozoa. However this maturing process is dependant on the other cell in the seminiferous epithelium - the Sertoli cell.

Sertoli Cells

Sertoli cells are the component of the seminiferous epithelium that maintain epithelial structure and play a vital role during the development of spermatogenic cells. They are columnar cells that line the seminiferous epithelium and are responsible for positioning, structural support, developing, nourishing and structurally supporting

spermatogenic cells. These cells contain a large abundance of cytoskeletal components including intermediate filaments, microtubules and actin. Intermediate filaments are concentrated at the base of the cell around the nucleus and project towards desmosome junctions as well as around the invaginations containing the attached spermatids. Microtubules are also very abundant however their orientation is starkly different from other cell types.

Microtubules play a factor in the development not only of spermatogenesis but also in the formation of Sertoli cells. Sertoli cells are columnar cells and this is due to microtubules playing a role in the shaping of these cells (Russell, Malone et al. 1981; Vogl, Linck et al. 1983; Amlani and Vogl 1988). In the rat, during the first ten days of age, microtubules stain densely at the base of the Sertoli cell. By day 15 microtubules extend toward the centre of the seminiferous epithelium and begin a parallel pattern longitudinally in the cell. By 20 days, the microtubules reach the centre of the tubule and formation of the Sertoli cell begins to appear more columnar. By 25 days, the microtubules reach the area of the tubule lumen and appear as a spoke like pattern (Redenbach, Hall et al. 1995).

The microtubules in Sertoli cells are oriented with their slow growing minus ends pointing towards the periphery of the cell (Redenbach and Vogl 1991). They are oriented parallel to the long axis of the Sertoli cell and appear nucleated at periphery sites (Redenbach and Vogl 1991; Vogl, Weis et al. 1995; Guttman, Janmey et al. 2002) however it is not completely clear how microtubules form in Sertoli cells. Microtubules generally are described as growing peripherally from central organizing centres, usually a centrosome around the nucleus (Mitchison and Kirschner 1984; Kimble and Kuriyama

1992; Rose, Biggins et al. 1993). This is not the case in Sertoli cells, nor in other columnar epithelial cells, where the positive ends of microtubules are located basally (Vogl, Weis et al. 1995). There is evidence to support the conclusion that nucleation occurs at the cell apex (Vogl, Weis et al. 1995; Johnson, Hall et al. 1996) and projects basally into Sertoli cells possibly linking into the intermediate filament network around the nucleus (Mogensen, Tucker et al. 1989) and not projecting from a specific centrosome microtubule organizing centre (Vogl, Weis et al. 1995).

Regardless of their orientation, Sertoli cell microtubules are unstable reflecting the high degree of remodeling that occurs during spermatogenesis (Hermo, Oko et al. 1991; Wenz and Hess 1998). Microtubules also are susceptible to various toxins at certain stages of spermatogenesis (Nakai, Miller et al. 2002). Sertoli cells treated with carbendazim show sloughing of elongating spermatids in stages VI and VII (mid to late staged) indicating that only microtubules at these stages have susceptibility to this treatment (Nakai, Miller et al. 2002).

Actin filaments are the third component of the cytoskeleton and this filament is mainly seen to associate at unique adhesion junctions in the testis known as ectoplasmic specializations.

Junctions in the seminiferous epithelium

The seminiferous epithelium contains a large number of junctions between adjacent Sertoli cells including tight junctions, gap junctions, desmosomes and unique adhesion junctions known as ectoplasmic specializations. Many of these junctions appear to overlap with one another. The junction complex formed between adjacent Sertoli cells divides the seminiferous epithelium into an apical and basal region. Tight junctions in

this complex form the blood-testis barrier. Spermatocytes cross this site before completing meiosis and the junction reforms behind the passing spermatocytes. Tight junctions also act as an immunological barrier to give spermatogenic cells apical to the barrier a homeostatic environment to develop (Boekelheide, Fleming et al. 2003). Unique intercellular adhesion junctions known as ectoplasmic specializations (ESs) form a significant component of the basal junction complexes between adjacent Sertoli cells and also are the major attachment junction between Sertoli cells and elongate spermatids. These adhesion junctions in the testis have become the focus of intense interest recently due to their possible role as molecular targets for possible male contraceptives (Cheng and Mruk 2002).

Ectoplasmic Specializations

Ectoplasmic specializations are adhesion junctions exclusively seen in Sertoli cells between adjacent Sertoli cells and between Sertoli cells and spermatids. They are composed of the Sertoli cell plasma membrane, a layer of hexagonally packed actin filaments and a cistern of ectoplasmic reticulum facing the cytoplasm of the Sertoli cell (Fawcett, Leak et al. 1970; Russell 1977; Vogl and Soucy 1985; Vogl, Soucy et al. 1985; Vogl, Pfeiffer et al. 1991; Vogl 1996; Chen, Li et al. 1999) (Figure 1.1). They are bilateral structures between adjacent Sertoli cells and unilateral structures at apical sites between Sertoli cells and spermatids (Dym and Fawcett 1970; Grove and Vogl 1989). It is believed that ectoplasmic specializations form as an adhesive component between two cells. When the seminiferous epithelial tissue is fragmented, apical ectoplasmic specializations remain attached to the elongating spermatid heads (Romrell and Ross 1979; Vogl 1996). In addition, adhesion molecules, particularly $\alpha 6 \beta 1$ integrin and

nectin-2 have been seen to concentrate at these junctions (Pfeiffer and Vogl 1991; Palombi, Salanova et al. 1992; Ozaki-Kuroda, Nakanishi et al. 2002). Actin, α -actinin, vinculin, espin, fibrin, myosin VIIa, rac1, and afadin have all been shown to localize at these junction complexes (Toyama 1976; Jockusch and Isenberg 1981; Vogl, Linck et al. 1983; Suarez-Quian and Dym 1984; Vogl and Soucy 1985; Vogl, Soucy et al. 1985; Camatini, Anelli et al. 1986; Vogl, Grove et al. 1986; Camatini and Casale 1987; Masri, Russell et al. 1987; Suarez-Quian and Dym 1988; Fouquet, Kann et al. 1989; Grove and Vogl 1989; Pfeiffer and Vogl 1991; Palombi, Salanova et al. 1992; Hasson and Mooseker 1995; Guttman, Janmey et al. 2002; Ozaki-Kuroda, Nakanishi et al. 2002; Velichkova, Guttman et al. 2002). Cadherin proteins also have been reported at this site (Cheng and Mruk 2002) however this is still controversial. Ectoplasmic specializations at apical sites also are transported to the base of the seminiferous epithelium with the attached spermatids during spermatogenesis.

Junction transport

During spermatogenesis, spermatids are attached to the Sertoli cell through an apical ectoplasmic specialization. At this time, spermatids begin as round spermatids and then elongate. Spermatids attach to Sertoli cells in apical invaginations termed apical crypts in the Sertoli cell. These crypts deepen during the elongation process and the spermatid translocates to the base of the Sertoli cell. The retreat of the spermatid back to the apex is the result of the apical invagination becoming shallow again. The biological significance of this translocation event is not known, however it is believed to be required for the development of functional spermatids. Throughout this process, the ectoplasmic specialization remains attached to the spermatid. It is hypothesized that this transport of

the spermatid is due to a microtubule based transport system. It is hypothesized that the spermatid is transported to the base of the Sertoli cell and then back to the apex by motor proteins. Motor proteins attach to the endoplasmic reticulum of the ectoplasmic specialization and walk along the Sertoli cell's microtubule tracts (Figure 1.2). Based on the polarity of the microtubules in Sertoli cells, a kinesin motor protein attached to the endoplasmic reticulum of the ectoplasmic specialization would be responsible for the movement to the base of the Sertoli cell and a dynein would be responsible for movement to the apex.

There are a number of pieces of evidence that support the microtubule-based spermatid translocation hypothesis. First, ectoplasmic specializations attached to spermatids bind exogenous microtubules (Vogl 1996). Second, in vitro motility assays have shown microtubules translocating over ectoplasmic specialization plaques (Beach and Vogl 1999). Also this motility was also shown to occur in both the plus and minus end directions (Guttman, Kimel et al. 2000). A final piece of evidence to support this translocation hypothesis is a cytoplasmic dynein chain (IC-74) has been shown to localize to the ectoplasmic specialization at stages when the spermatids are moved back to the apex of the seminiferous epithelium (Miller, Mulholland et al. 1999). A number of kinesins have been identified in the testis, however none of these motor proteins has been shown to associate with the ectoplasmic specialization (Johnson, Hall et al. 1996; Miller, Mulholland et al. 1999; Lai, Fernald et al. 2000; Navolanic and Sperry 2000; Junco, Bhullar et al. 2001; Macho, Brancorsini et al. 2002; Zou, Millette et al. 2002). Chapter 2 of this thesis explores the hypothesis that a specific kinesin is associated with ectoplasmic specializations.

Junction turnover

Little is known about the turnover of adhesion junctions in the testis. Turnover of junctions occurs during two specific events – when spermatocytes pass through the basal junction complexes between adjacent Sertoli cells, and during spermatid release by the Sertoli cell into the lumen of the seminiferous epithelium. At basal sites, the adhesion junctions and their associated proteins must disengage to allow the spermatocyte to cross this junction and then form behind these cells.

Apical ectoplasmic specializations form adjacent to early elongate spermatids adjacent to the developing acrosome region. This adhesion junction remains attached to the spermatid until just prior to spermatid release. At this time, finger-like projections, termed tubulobulbar complexes begin emanating from the spermatid as the junction plaque disassembles. Little is known about this turnover, however tubulobulbar complexes have been implicated in the process (Russell, Goh et al. 1988; Guttman, Takai et al. 2004). Projections from the spermatid protrude into the Sertoli cells cytoplasm forming double-membrane invaginations (Russell 1979). They are composed of a long proximal tube, a bulbous regions and a short distal tube. Some of the components found in ESs are also found in tubulobulbar complexes (Figure 1.3). The long proximal tube is believed to increase the surface area of the plasma membrane being internalized, however this matter is open to debate. In any case, these complexes are the last structures seen prior to spermatid release. The role of tubulobulbar complexes in the internalization of ectoplasmic specializations is discussed in Chapter 3.

Dynamin 3

Dynamin 3 is an endocytic GTPase that has been shown to be involved in membrane invagination and tubular excision (Hinshaw and Schmid 1995). The Dynamin family of proteins has three isoforms and multiple spliced variants that are present throughout the organism (Cao, Garcia et al. 1998). Dynamins have been shown to act as pinchases of membranes for the formation of vesicles during endocytosis although the precise function of dynamin is still under debate (McNiven 1998; Hill, van Der Kaay et al. 2001). It is also unclear as to whether dynamin is required for the initiation of the formation of endocytic vesicles or plays a role during the formation of tubular invaginations of the membrane (Hill, van Der Kaay et al. 2001; Roux, Uyhazi et al. 2006). Here I look at the role of dynamin 3 in the testis. In addition to dynamins, tubular invaginations of the membrane appear to be cuffed by a network of actin filaments (Tehrani, Faccio et al. 2006).

Arp2/3

A major complex that is involved in the assembly of actin networks is the Arp2/3 protein complex. Arp2/3 is a seven-subunit protein that is believed to play a role in actin assembly. This complex is the nucleation site of actin filaments and initiates the growth of new actin filaments from preexisting actin filaments at a characteristic seventy degree angle (Pollard and Beltzner 2002). It has been shown that this complex plays a role in actin assembly at sites associated with membrane invaginations which include podosomes (Ochoa, Slepnev et al. 2000) and comet tails (Orth, Krueger et al. 2002).

Thesis Problem

In chapter 2, I test the hypothesis that a Rab6KIFL kinesin motor protein is associated with ectoplasmic specializations and therefore may be a candidate for spermatid translocation to the base of the Sertoli cell. I approached this by generating antibodies specific to Rab6KIFL to determine the localization of this protein at the light microscopic and electron microscopic levels in the testis. In chapter 3, the second portion of my thesis, I demonstrate the novel finding that dynamin 3 is a component of tubulobulbar complexes and may be part of the mechanism by which these structures are formed.

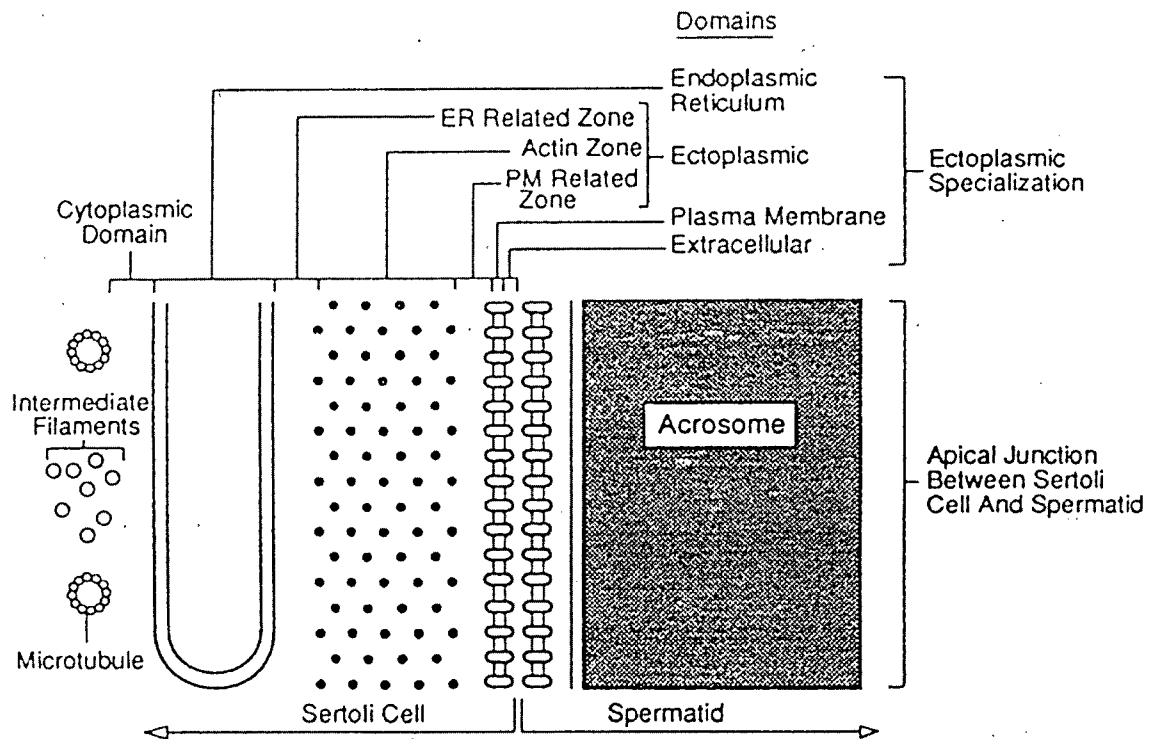


Figure 1.1: Diagram of an apical ectoplasmic specialization (cross-section). These junctions occur between Sertoli cells and spermatids on the Sertoli cell side and between adjacent Sertoli cells where they occur as bipartite structures and are known as the blood-testis barrier and provide a homeostatic environment. The ectoplasmic specialization is defined by the Sertoli cell plasma membrane, the actin zone consisting of a hexagonally packed layer and a cistern endoplasmic reticulum which faces into the cytoplasm of the Sertoli cell.

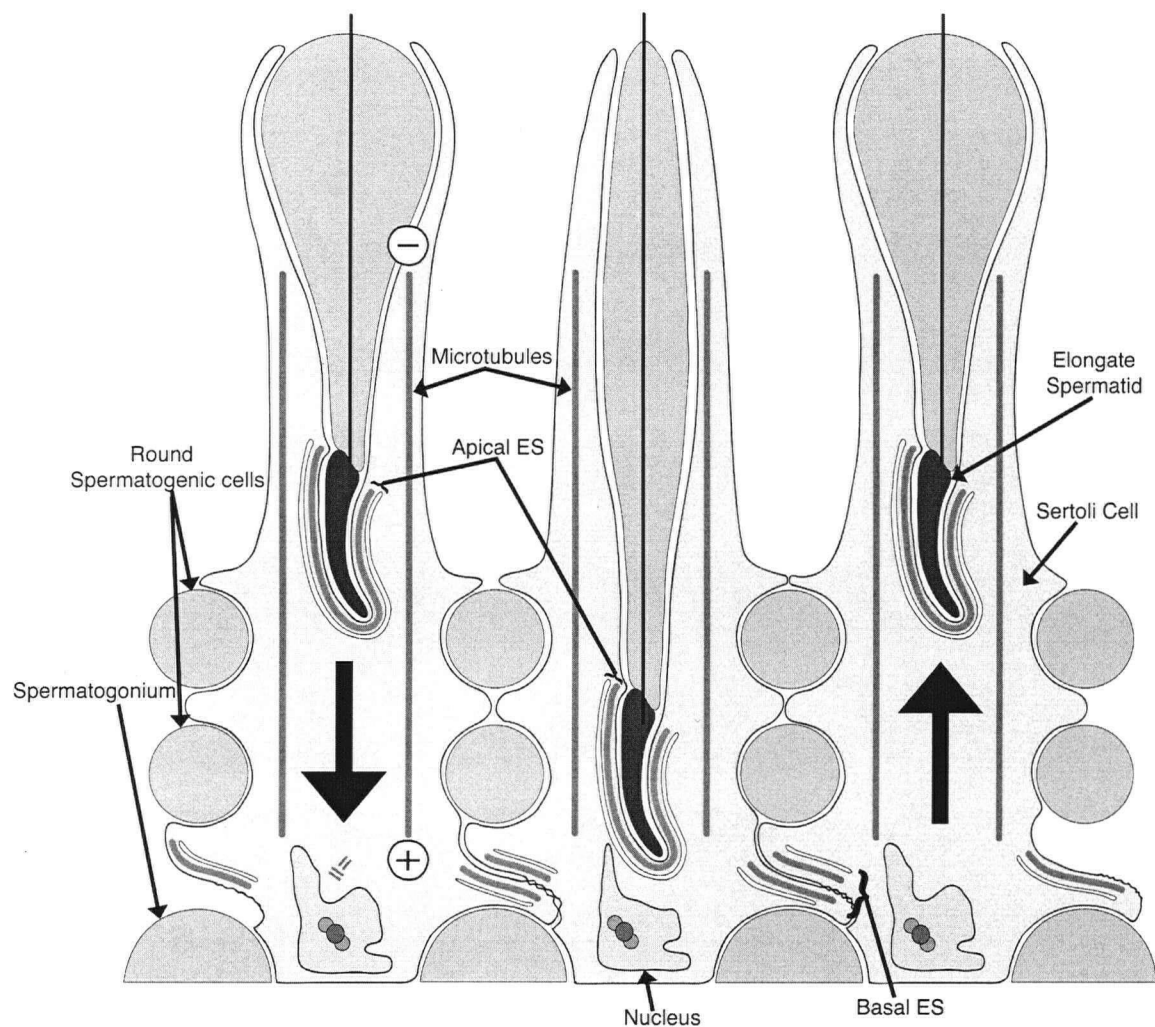


Figure 1.2: Diagram of the Spermatid Translocation hypothesis. Round spermatogonium cross basal ectoplasmic specialization junction sites also known as the blood-testis-barrier and these round spermatogenic cells attach to the Sertoli cell through apical ectoplasmic specializations. The attached spermatid translocates to the base of the Sertoli through an apical crypt in the Sertoli cell before retreating back to the apex of the cell and being released into the lumen of the seminiferous epithelium. Running adjacent to the ectoplasmic specializations in the Sertoli cell cytoplasm are polarized microtubules with their positive ends at the base of the cell and their negative ends at the apex. It is hypothesized that this translocation event occurs as a result of the apical ectoplasmic specialization and spermatid complex being pulled to the base by a kinesin based motor protein running along the microtubule tracts. A dynein (IC-74) has previously been shown to associate with the ectoplasmic specialization and is likely responsible for the retreat of the spermatid to the apex of the Sertoli cell.

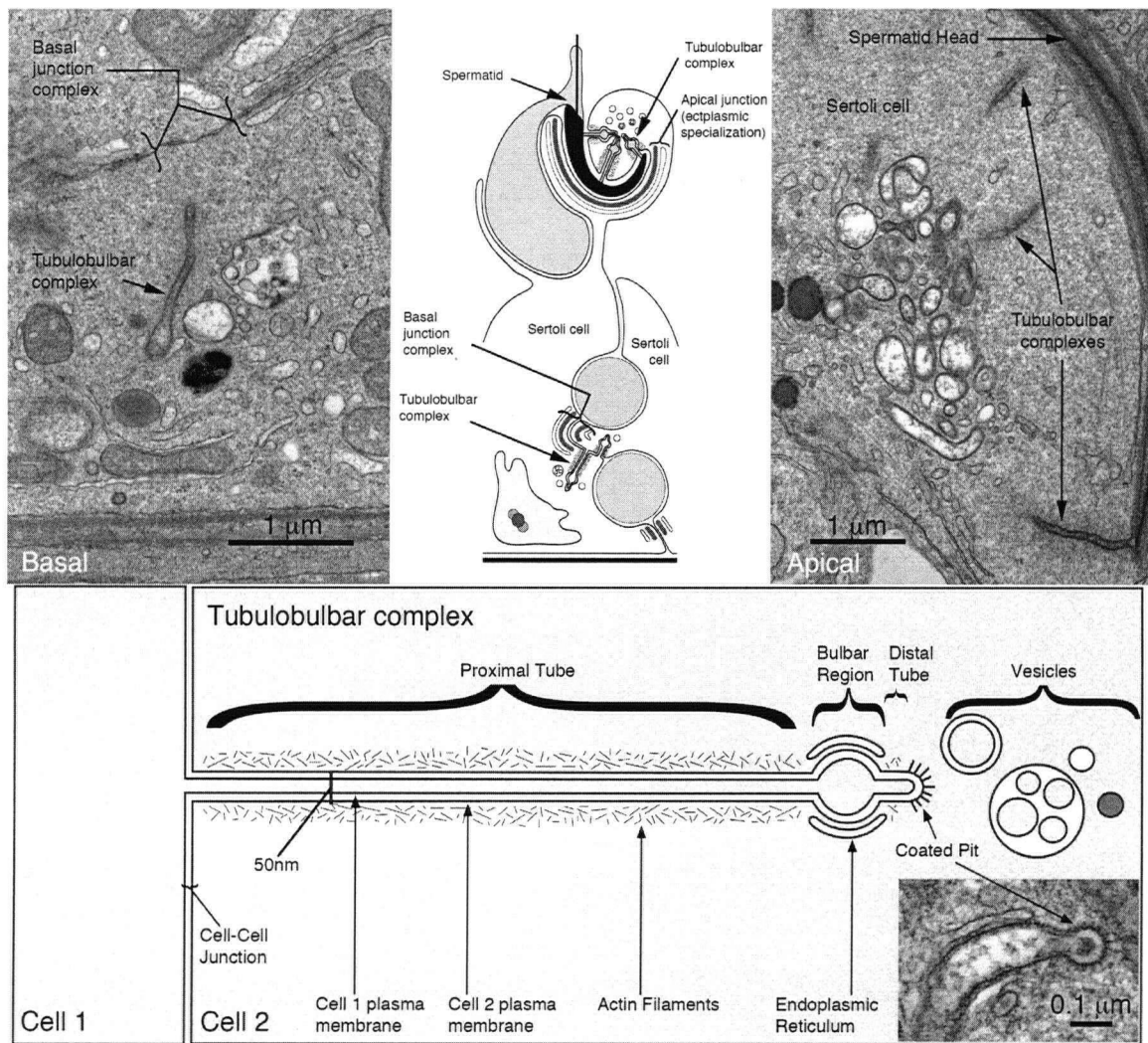


Figure 1.3: Diagrams and electron micrographs illustrating the position and structure of tubulobulbar complexes in the seminiferous epithelium. Basal tubulobulbar complexes occur in association with junctions between two neighboring Sertoli cells. Apical complexes occur in regions where Sertoli cells are adherent to spermatids. Each tubulobulbar complex consists of an elongate invagination into a Sertoli cell of the plasma membranes of two cells at the site of intercellular junction. The complex consists of tubular and bulbar regions. A coated pit occurs at the end of the complex. Note that the proximal tube has a diameter of 50 nm, is a double membrane and vesicles budding from the distal tube also consist of a double membrane.

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Chapter 2

Introduction¹

In the mammalian seminiferous epithelium, spermatid development and maturation occur in conjunction with the Sertoli cell. Adjacent Sertoli cells form basal junction complexes. Tight junctions in these complexes make up the blood testis barrier. In the epithelium, Sertoli cells also form junctions with spermatids. These cells develop from round into elongate spermatids before release. It is believed that the cytoskeleton of the Sertoli cell plays an important role in this development.

Sertoli cells contain polarized microtubules with their negative end at the apex or peripheral end and the positive end located around the nucleus of the Sertoli cell (Redenbach and Vogl 1991; Redenbach and Boekelheide 1994). This arrangement is similar to other columnar epithelial cells, but different that other cells such as axons which have their fast growing positive end located at the cell periphery (Bergen, Kuriyama et al. 1980; Euteneuer and McIntosh 1980; Burton and Paige 1981; Vale 1987). Microtubules project to the periphery of the cell and appear as bundles extending along the crypts of the Sertoli cell in addition to acting as structural components of cells.

When microtubules are disrupted by nocodazole or colchicine, Sertoli cells lose their columnar shape and become cuboidal (Vogl, Linck et al. 1983; Nakai and Hess 1994; Nakai, Hess et al. 1995). This change in shape also has a dramatic effect on

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spermatogenesis and is detrimental to the formation of germ cells. Microtubules in Sertoli cells have roles including structural organization, physical support (Russell, Malone et al. 1981; Vogl, Linck et al. 1983; Allard, Johnson et al. 1993), paracrine support (Achler, Filmer et al. 1989; De Almeida and Stow 1991), and secretion for germ cell development (Rindler, Ivanov et al. 1987; Achler, Filmer et al. 1989; Eilers, Klumperman et al. 1989; Johnson, Patel et al. 2000) as these functions have microtubule dependent components. When Sertoli cell microtubules are disrupted with over expression of the microtubule nucleating protein γ -tubulin, the Sertoli cell is unable to provide these benefits to germ cells and results in an impairment of spermatogenesis (Fleming, Shank et al. 2003).

Although microtubules display a general pattern of orientation positioned parallel to the long axis of the cell (Redenbach and Vogl 1991; Vogl, Weis et al. 1995), they change in their distribution during the stages of spermatogenesis (Wolosewick and Bryan 1977). Microtubules do not congregate around early spermatogenic cells to the same degree as elongating spermatids and are only in Sertoli cell cytoplasm flanking elongating spermatids in apical crypts (Vogl 1988). Microtubules are seen in Sertoli cell cytoplasm surrounding the acrosome of developing spermatids and form elaborate networks in the regions associated with maturing spermatids. These observations suggest microtubules may play a role in the shaping of the acrosome and spermatid nucleus as structural components (Kondo, Hasegawa et al. 1988).

During spermatogenesis, microtubules play a vital role in the formation of functional spermatids. During spermatogenesis, the microtubule bundles are aligned parallel to the apical crypts containing the spermatid heads. Spermatid heads are attached to Sertoli cells by intercellular adhesion complexes known as ectoplasmic specializations

(ESs) (Russell 1977). These complexes are also located at basal sites between adjacent Sertoli cells as bipartite structures. This junction is composed of the Sertoli cell plasma membrane, a hexagonally packed layer of actin four to six filaments deep and a layer of endoplasmic reticulum (ER) in the Sertoli cell cytoplasm (Vogl, Pfeiffer et al. 1991). The plasma membranes are connected through nectin-based and integrin-based adhesion molecules and the ER is contained on the cytoplasmic side of the Sertoli cell and is associated with microtubules (Sato-Horikawa, Nakanishi et al. 2000; Ozaki-Kuroda, Nakanishi et al. 2002). Nectin-2 molecules on the Sertoli cell plasma membrane are believed to bind to nectin-3 molecules on the spermatid membrane while the ligand for the $\alpha 6 \beta 1$ integrin on the adjacent membrane is not clear. $\gamma 3$ laminin is a possible candidate (Yan and Cheng 2006). These adhesion molecules are thought to bind to their ligands across the intercellular space and the absence of these molecules results in abnormal spermatid formation and male specific infertility (Salanova, Ricci et al. 1998; Mueller, Rosenquist et al. 2003; Takai and Nakanishi 2003). N-cadherin also has been reported to be present specifically in ESs (Chen, Lee et al. 2003), but still needs to be confirmed.

Links have been shown between this junction and microtubules on the cytoplasmic side of the Sertoli cell (Russell 1977; Redenbach, Boekelheide et al. 1992). During spermatogenesis, one of the most dramatic events occur when the entire ES along with the attached spermatid in the apical crypt, translocates to the base of the Sertoli cell before retreating back to the apex of the cell and being released into the lumen of the seminiferous epithelium (Vogl, Pfeiffer et al. 1991). It is hypothesized that this translocation is due to motor proteins walking along the microtubule bundle tracts

indicating that microtubules may be responsible for facilitating the position of elongating spermatids (Russell 1977; Vogl 1988; Redenbach and Vogl 1991; Vogl, Pfeiffer et al. 1991; Redenbach, Boekelheide et al. 1992).

It has been proposed ESs are moved by microtubule motor proteins (kinesins and dyneins) that are attached to the cytoplasmic side of the endoplasmic reticulum in the Sertoli cell (Russell 1977; Vogl, Pfeiffer et al. 1991). The specific attachment between the motor proteins and the endoplasmic reticulum is not known. The translocation process is accomplished by these microtubule-based motor proteins transporting entire ESs and attached spermatids down into the deep recess of Sertoli cells in the seminiferous epithelium along adjacent Sertoli cell microtubule tracts which run parallel to the direction of movement of the ES/spermatid complex. This spermatid translocation hypothesis is supported by several key pieces of evidence. First, microtubules are arranged along the long axis of the Sertoli cell which would allow motor proteins to travel along them (Redenbach and Vogl 1991). Second, binding assays have been performed which show that isolated ES junctions bind to microtubules in a nucleotide ATP dependent fashion. Third, motility assays were also conducted and it was shown that ESs could transport microtubules (Beach and Vogl 1999; Guttman, Kimel et al. 2000). Finally, a dynein motor (IC-74) has previously been localized to ESs using immunofluorescence and may be responsible for the upward movement of the ES/spermatid complex towards the apex (Guttman, Kimel et al. 2000).

Pan specific kinesin antibodies were produced against conserved sequences of kinesins using three conserved peptide sequences. All partially react with ESs on fixed frozen sections or fixed epithelial fragments, however the staining pattern of one

antibody raised to the LAGSE (LNLVDLAGSE) conserved sequence of kinesins associated more prominently with the junction sites in addition to other sites known to contain kinesins (Guttman 2003). This data combined with mRNA transcript screens using mouse GeneChip arrays and Sertoli cell cDNA (performed by Jim Shima from Dr. Michael Griswold's Laboratory; Washington State University) indicated that a possible kinesin for spermatid translocation was Rab6KIFL. A second group of candidate kinesins identified using mass spectroscopy was the UNC-104/KIF1 group that was identified on the basis of two peptide stretches.

Rab6KIFL is a predicted 100 kDa protein which belongs to the kinesin 6 family or the KIF20A family of kinesins and is distantly related to MKLP-1 (Miki, Setou et al. 2001). Its main role is in spindle formation during mitosis localizing mainly during late anaphase as well as during cytokinesis. Studies of this protein have also revealed it is able to interact with the GTPase Rab6 and may be involved in transport of vesicles within the Golgi and between the Golgi and ER. Three isoforms have been isolated of the Rab6 GTPase, one of which is involved with binding to Rab6KIFL and functions in the movement of vesicles between Golgi and endoplasmic reticulum although the specific function of Rab6 GTPase binding remains unclear (Echard, Jollivet et al. 1998). Another previously described function of RabKIFL is it may play a role in cell division (Hill, Clarke et al. 2000). Rab6-KIFL has a conserved motor region with two microtubule-binding sites for ATP hydrolysis, one at each the N-terminal and C-terminal and the Rab-6 binding domain separating the two. mRNA levels of this protein also are expressed highly in tissues with high proliferation rates including testis. Sequence conservation in this protein family is mainly limited to the 350 amino acid motor domain which is the

region involved in microtubule binding and ATP hydrolysis suggesting that this protein is able to bind multiple cargo proteins.

The UNC-104/KIF1 family is a group of monomeric motors whose primary function is organelle transport. Most kinesins move along microtubules by using two motor heads that alternatively attach to the microtubule, however it is not known how a monomeric kinesin travels along a microtubule. There are two hypotheses. First monomeric kinesins have been shown to move by having a second binding site that allows processive movement along the microtubule. Secondly, this motor protein family also has been shown to have the ability to dimerize via coiled-coil regions adjacent to the motor protein resulting in a two headed motor which can then move end over end along the microtubule (Tomishige, Klopfenstein et al. 2002). This family also has two features that are unique to this group. First, they have a conserved insertion in loop 3 near the nucleotide binding pocket and second they have a presence of a fork head homology (FHA) domain C-terminal on the motor domain (Vale 2003). The two sequences used to identify UNC-104/KIF1 from the mass spectroscopy of testis samples are identical sequences in all the KIF1-like kinesins.

The goal of this study was to generate antibodies to Rab6KIFL and KIF1-like kinesin sequences identified by mass spectroscopy, and use them as probes to determine if these proteins are associated with adhesion junctions in the testes. This is part of a larger study to test the hypothesis that spermatid translocation in the seminiferous epithelium involves the transport of adhesion junctions and attached spermatids along adjacent cellular microtubules.

Methods and Materials

Antibody Production

Using gene chip data arrays and mass spectroscopy, Rab6KIFL and a KIF1-like kinesin were identified. Polyclonal antibodies to each of these proteins were produced by selecting hydrophilic regions. Hydrophilic regions are generally exposed regions of the protein and allow for access for antibody binding. Sequences were selected not only based on hydrophilicity, but also on specificity. Specificity was determined by performing blast searches on the chosen hydrophilic region using the NCBI protein database and regions which were unique to Rab6KIFL were selected. Problematic amino acids such as phenylalanine and cysteine were avoided where possible due to problems with incorporating these amino acids into a peptide as well as the formation of disulphide bonds that may change the structure of a peptide sequence. On the other hand proline, tryptophan and methionine were included where possible due to their uniqueness as these are relatively rare amino acids.

Three sequences were chosen from the Rab6KIFL protein and 2 sequences from the kif1-like family of kinesins. The 3 Rab6KIFL sequences include KEDKADSDLEDSPED, GQASAKKRLGANQENQQ, and DLRSVVRKDLLSDCS while the 2 kif1-like sequences included TVAATNMNETSSP and ANSTGATGARLK. These 5 sequences were synthesized by Invitrogen Canada. All peptides were conjugated to KLH and antibody production was performed by Invitrogen using a standard 10 week injection protocol. Briefly, 2 New Zealand white rabbits 3-9 months in age were each injected with each of the 5 peptide sequences for a total of 10 rabbits. Before initial injection, a 2-5 ml pre-immune test bleed was taken from each animal. Initial

immunization was performed with 0.2 mgs of antigen (complete Freund's adjuvant). At week 2 this was followed by 0.10 mgs of antigen (incomplete Freund's adjuvant). At 4 weeks a 20 ml bleed was performed and Elisa readings were taken to determine if an immune response had been initiated. This was followed by another immunization at week 7 with 0.10 mg antigen (incomplete Freund's adjuvant). At week 8 another 20 ml bleed was taken with an Elisa test and another immunization boost of 0.10 mg antigen (incomplete Freund's adjuvant). This was followed by a final bleed of 40 ml at week 10.

Following the 10-week protocol, ELISA titer readings were taken in each rabbit to determine immune response. Due to low readings, additional aggressive and double antigen boosts were performed on rabbits. These included boosts with complete Freund's adjuvant followed by a boost with incomplete Freund's adjuvant and double antigen boosts using twice the standard antigen amount followed by a bleed. These boosts were performed for an additional 3 months followed by terminal bleeds of animals. Collected rabbit serum was collected and affinity purified using the peptide to which each antibody was raised. Affinity purification was performed by Invitrogen Corporation using standard protocols. In total 6 antibodies were purified for the Rab6KIFL protein and 2 antibodies for the kif1-like protein as 2 peptide sequences failed to illicit an immune response.

Animals

All animals used in these studies were reproductively active male Sprague Dawley rats and CD1 male mice. These animals were obtained from the University of British Columbia animal care colony and were maintained according to the guidelines established by the Canadian Council on Animal Care (Appendices 1 and 2). All experiments were repeated at a minimum in duplicate using different animals.

Chemicals and Reagents

All chemicals and reagents were obtained from Sigma-Aldrich (Alberta, Canada) unless otherwise indicated. Paraformaldehyde and NaCl were obtained from Fisher Scientific (Nepean, Ontario) and all immunoglobulins and secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson ImmunoResearch (Baltimore, Maryland). All secondary antibodies conjugated to Alexa fluorochromes for immunofluorescence were obtained from Molecular probes (Burlington, Ontario) while Polybed embedding resin was from EM Sciences (Hornby, Ontario).

Sectioned Tissue

Testicular tissue for examination by immunofluorescence was collected from reproductively active male Sprague-Dawley rats between 250 and 500 grams. Animals were anesthetized using Halothane inhalation and testis were removed from the animals. Each testis was perfused with warm (33°C) PBS (150 mM NaCl, 5 mM KCl, 0.8 mM KH₂PO₄, 3.2 mM Na₂HPO₄, pH 7.3) for 2 min to clear organ of blood through the spermatic artery using a 26-gauge needle attached to a gravity fed perfusion apparatus. This was followed by fixation with warm 3% paraformaldehyde in PBS for 30 min using gravity fed perfusion. Following fixation, the testis was perfused for an additional 30 min with PBS. This was followed by mounting the fixed tissue on an aluminum stub by OCT compound (Sakura Finetek USA) and at the same time freezing in liquid nitrogen. Tissue was then cut using a cryo-microtome at 5-10 micron thickness and placed on poly-l-lysine coated slides. Slides were then immediately dunked into -20° C acetone for 5 min before being allowed to air dry.

Fragmented Tissue

Fixation for fragmented tissue was identical to sectioned tissue. However, upon completion of perfusion, the testis was removed from the canula and the seminiferous epithelium was mechanically fragmented into small pieces in PBS using scalpels. Tissue was passed through an 18 gauge needle twice followed by two passes through a 21 gauge needle to further fragment tissue. This tissue was then placed into a 15 ml falcon tube and allowed to sediment by gravity for 5 min at room temperature. The upper layer was transferred to another tube and pelleted by centrifugation. This pellet, re-suspended in a small amount of PBS was added to poly-l-lysine coated slides and placed in humidity chambers for 10 min. Following the incubation, all excess PBS was removed and the slides were dunked into -20°C acetone for 5 min before allowing material to air dry.

Immunofluorescence

Sectioned and fragmented tissue was re-hydrated and blocked with 5% Normal Goat Serum in TPBS-BSA (PBS containing 0.05% Tween-20 and 0.1% bovine serum albumin) and placed in a humidity chamber for 20 min. This was followed by addition of 50 μl of primary antibodies made up in TPBS-BSA with 1% NGS (Sigma-Aldrich, Canada) overnight at 4°C in a humidity chamber. All primary antibodies were added at a final concentration of 0.01 mg/ml. Following the overnight incubation, 3 times 10 min washes were performed with TPBS/BSA buffer followed by addition of 50 μl of secondary antibody (goat anti rabbit ALEXA 488) for 1 hour at 37°C . This was followed by an additional 3 times 10 min washes with TPBS/BSA buffer followed by mounting with Vectashield (pH 8.2) containing DAPI. Tissue was then viewed using a Zeiss Axiophot microscope fitted with fluorescent filters. Controls included replacing primary

antibody with IgG, replacing primary antibody with buffer alone and replacing both primary and secondary antibodies with buffer alone.

Filamentous actin was labeled using Alexa 568 phalloidin (Molecular probes). The stain was made up in TPBS-BSA and tissue was incubated in stain at room temperature for 20 min before being washed in TPBS-BSA.

Peptide Blocking

Peptide blocking of antibodies was performed using peptides to which antibodies were raised. Protocol was identical to procedures for immunofluorescence or western blotting. Except that primary antibodies were incubated with either 5 ul of peptide or ddH₂O (final concentration of peptide twice that of the antibody) or 40 ul of peptide or ddH₂O (final concentration of peptide at ten times that of antibody) at identical concentrations overnight on a LabQuake rotator at 4°C. After incubation, these dilutions were added to the tissue or blots as per normal protocols.

Western Blotting

Western Blots were performed using standard protocols. Briefly, rat tissue was homogenized in RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 5 mM EDTA, 1% Nonidet P-40, 1% deoxycholic acid [sodium salt], 10% SDS, protease inhibitor capsule [Roche, Mississauga, Ontario]) using electric homogenizer with 10 gentle passes at setting 3. Tissue was boiled for 10 min and then loaded at equal concentrations (1 mg/ml) on a 1 mm thick 5% SDS page gel. The gel was run at 70 volts and transferred overnight at 4°C onto a nitrocellulose membrane. Membranes were then washed in TBST (500 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 5 min followed by blocking in 4% non-fat milk (Santa Cruz Biotechnology, Santa Cruz, California) for eight hours. This was

followed by 3 times 10 min washes in TBST followed by the addition of primary antibodies in 1% BSA in TBST overnight at 4°C. All antibodies were used at a final concentration of 0.001 mg/ml. The following day membranes were washed 3 times 5 min washes followed by 3 times 10 min washes in TBST and then secondary antibodies conjugated to horse radish peroxidase were added in 1% BSA in TBST for 1 hour at room temperature. This was followed by 3 times 5 min washes and 3 times 10 minute washes in TBST followed by 3 times 5 min washes and 3 times 10 min washes in TBS. Upon completion of washes the membranes were reacted with ECL reagents (Amersham, Baie d'Urfe, Quebec) and exposed to Kodak Biomax film for various durations. Controls consisted of replacing the primary antibodies with IgG at identical primary antibody concentrations.

Line Blots

A nitrocellulose membrane, identical to membranes used for Western Blotting, was incubated with 10 ml of peptide solution in TBST at 10 ug/ml for 1 hour. 3 times 5 min washes were then performed on membrane before blocking using 4% non-fat milk overnight. The following day, the membrane was placed into a Bio-Rad multi-protean II multi screen apparatus which was then placed into a humidity chamber. Each lane of the Bio-Rad apparatus was then incubated with a specific antibody for 4 hours at room temperature followed by successive washes and secondary antibody for 1 hour. Blots were then washed and treated identical to Western Blots.

Gelsolin Treated ESs

Testes were removed from Sprague-Dawley rats under deep anesthetic (Halothane) and placed in a Petridish containing cold PEM-250 (80 mM Pipes, 1 mM

EGTA, 1 mM MgCl₂, 250 mM sucrose, 10 ug/ml Soybean trypsin inhibitor, 0.5 ug/ml Leupeptin, 0.5 ul/ml Pepstatin, 0.1 mM PMSF) and decapsulated. The seminiferous tubule mass was then transferred to fresh PEM-250 and cut with scalpels into small pieces. Under a Zeiss dissecting microscope fitted with a dark field condenser and using microprobes, the epithelium was squeezed out of individual tubules and collected using a 37 mm capillary 0.3-200 ul microflex pipette tip (National Scientific Supply Company, Claremont, California) and placed into a conical glass tube on ice. During this fragmentation procedure, spermatids remain attached to ectoplasmic specializations. Collection of tissue was performed for up to 30 min. Upon completion of collection, tissue was sedimented using a clinical centrifuge. Upon centrifugation, supernatant was removed and discarded and pellet was resuspended in 150 ul cold PEM-250 buffer and aspirated to gently fragment the epithelium.

During this time, three 5% step sucrose gradients were prepared from 60% to 30% in 0.8 ml ultraclear centrifuge tubes (Beckman). Each gradient step was prepared using stock of PEM-60% sucrose (80 mM Pipes, 1 mM EGTA, 1 mM MgCl₂, 30 grams sucrose, 10 ug/ml Soybean trypsin inhibitor, 0.5 ug/ml Leupeptin, 0.5 ul/ml Pepstatin, 0.1 mM PMSF, pH 6.8) and PEM (80 mM Pipes, 1 mM EGTA, 1 mM MgCl₂, 10 ug/ml Soybean trypsin inhibitor, 0.5 ug/ml Leupeptin, 0.5 ul/ml Pepstatin, 0.1 mM PMSF) and adding 93 ul of each step to centrifuge tubes and cooling gradients on ice. Seminiferous epithelium sample size of 50 ul was then added to the top of each gradient tube and tubes were placed in rotor buckets at 4°C and centrifuged in SW55Ti rotor for 6 min at 5000 RPM. Upon completion of centrifugation elongate and late spermatid fractions with attached ectoplasmic specializations material forms a band between the 40% and 45%

interface of the sucrose gradients. This material was then collected using a 23 gauge needle on a 1 ml syringe to collect the fraction through the tube wall. The collected sample was then collected into a centrifuge tube and diluted in 1 ml of PEM-250 before being pelleted by centrifugation for 2 min at setting 6 on desktop Eppendorf centrifuge and then rotating the tube 180 degrees and repeating centrifugation for an additional 2 min. This procedure was repeated for 3 animals to obtain 3 pellets to ensure enough tissue was collected.

The three centrifuged pellets were then resuspended in 100 ul each cold MES buffer (50 mM MES, 5 mM DTT, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 6.3) and pooled into one tube. an additional 700 ul of MES buffer was then added and resuspended pooled fraction was placed on ice for 10 min. A sample of this fraction was then taken to treat for actin as the pre-Gelsolin treatment to ensure the ectoplasmic specializations were intact and attached to spermatids after processing. The fraction was pelleted by centrifuging 2 times 2 min at setting 6 in Eppendorf desktop centrifuge and rotating the tube 180 degrees after the first spin. The supernatant was discarded and the pellet was suspended in 300 ul MES buffer containing 0.4 mg/ml gelsolin. This suspension was then incubated for two hours at room temperature on a LabQuake rotor. This was followed by aspiration of the material through a thin tipped pipette to rip apart any remaining endoplasmic specializations. Following aspirations a 5 ul sample was collected to stain for actin as a post-Gelsolin control to ensure ectoplasmic specializations were no longer attached to the elongate spermatids. The remaining fraction was then centrifuged 2 times 2 min at setting 10 on a desktop Eppendorf centrifuge and rotating the tube 180 degrees after the first spin. Following centrifugation, the supernatant was collected and a sample

visually inspected under phase microscopy to ensure spermatids were present. The supernatant and pellet were then frozen separately as blocks in nitrogen and stored at -70 degrees Celsius.

Immunoprecipitation

Immunoprecipitations were performed using previously described standard protocols. Briefly, 500 ul of homogenized isolated seminiferous epithelium or other tissue was diluted in a tube to 1 mg/ml in TBS with protease inhibitors. 5 ug of Normal Rabbit IgG and 25 ul of protein A beads (Sigma) were added to samples and incubated at 4°C for 1 hour. This was followed by centrifugation at 4°C at setting 10 in Eppendorf desktop centrifuge for three minutes. Following centrifugation, the supernatant was transferred to a new tube and 5 ug of primary antibody added to sample at 4°C for 4 hours followed by addition of 25 ul of protein A beads overnight. The following day the beads were washed extensively 5 times in PBS buffer (pH 7.3) and the supernatant discarded. 45 ul of sample buffer was then added to the beads and boiled for 10 min. The remainder of the protocol was identical to Western Blot analysis.

Immunoelectron Microscopy

Rat testes were removed and perfused first with warm (33°C) PBS pH 7.3 for two minutes to clear the organ of blood. This was followed by perfusion with 3% paraformaldehyde fixative for 30 min followed by perfusion with PBS for an additional 30 min. Each testis was then removed from the canula, minced into small pieces with scalpels and aspirated through an 18 gauge and then a 21 gauge needle. The material was then allowed to settle in a falcon tube followed by the collection of the upper layer which was concentrated by centrifugation at 7300 x g for 4 min. The material was then divided

into four equal volumes, reconcentrated by centrifugation and resuspended in antibody or control solutions. Control 1 consisted of primary antibody being replaced with NRIgG or NCIgY at the same concentration, for control 2 the primary antibody was replaced with buffer and for control 3 the primary and secondary antibodies were replaced with buffer. These were left to incubate at 4°C overnight. The following day the samples were washed in PBS containing 1% BSA on a rotator for 15 min using 1 ml of buffer each. Upon completion of washing, samples were resuspended in secondary antibody conjugated to 5 nm colloidal gold (Sigma-Aldrich) and incubated for two hours. The tissue was washed in PBS, fixed for an additional hour with 1% paraformaldehyde in PBS with 0.1% gluteraldehyde. Another wash with 0.1 M sodium cacodylate pH 7.3 for ten minutes was performed before post fixation on ice for 1 hour with 1% Osmium Tetraoxide (2% OsO₄ and 0.2 M NaCac). Upon fixation, the tissue was washed 3 times 10 min each with ddH₂O and stained at room temperature for 1 hour with 1% aqueous uranyl acetate. An additional 3 times 10 min washes were performed with ddH₂O then dehydrated in an ascending alcohol series (50%, 70%, 95%, 100%), for 10 min at each concentration. Three washes were performed with 100% ethanol. This was followed by two incubations of 30 min each in propylene oxide. Infiltration was then performed in a 1:1 solution of propylene oxide:Polybed overnight (EM Sciences, Hatfield, PA) followed by 3 times 100% Polybed for 1 hour each. The material was then embedded in 100% Polybed in embedding capsules and incubated at 60°C for 24 h. Sections were viewed and photographed on a Philips 300 electron microscope operated at 60 kV.

Transfection

Full length Rab6KIFL (Kif20a) cDNA was obtained from Open Biosystems (Image ID #5720408) and cloned into pCMV-HA (Clontech Laboratories Inc. Mountain View, California) using PCR based cloning to generate the correct reading frame. The plasmid was sequenced to ensure accuracy, and transfected into COS-7 cells using Eugene 6 reagent (Roche, Mississauga, Ontario). 24 hours after transfection, cells were lysed in RIPA buffer and Western Blot analysis was performed.

Results

Antibody Production

As Rab6KIFL was previously identified to be present in Sertoli cells using GeneChip data arrays (Michael Griswold's Laboratory), this protein was selected to produce antibodies to determine its localization pattern. Selected peptide stretches were selected and are highlighted in Figure 2.1a. Antibodies were raised against these peptide regions as they were identified to be specific to Rab6KIFL according to the NCBI protein database and due to their avoidance of problematic amino acids that may have caused difficulty synthesizing the peptide or misfolding of the peptide. These include amino acids phenylalanine for synthesis problems and multiple cysteines which would form disulphide bonds thereby possibly changing the structure of the peptide.

KIF1 family kinesins were previously identified using mass spectroscopy. Identification of this motor family was the result of two peptide sequences - TVAATNMNETSSP and ANSTGATGARLK (Figure 2.1b). These sequences are conserved in KIF1-like kinesin proteins. These sequences were used to produce antibodies that were predicted to be pan-specific antibodies to the KIF1-like family of kinesins.

The peptide regions that were selected to produce antibodies were first identified based on hydrophilicity. A hydrophilicity plot was performed on the Rab6KIFL protein to identify hydrophilic regions of the protein due to these regions generally being exposed surfaces on the protein (Figure 2.2a). Hydrophilicity is based on the Hopp-Woods scale where each amino acid is assigned a value based on its hydrophilic properties. A value greater than zero indicates a hydrophilic amino acid while values

below zero indicate hydrophobic regions of the protein. Using this process, hydrophilic regions were selected and another hydrophilic plot was performed on these peptide stretches to ensure hydrophilicity (Figure 2.2b). The three peptide regions against Rab6KIFL were deemed to be hydrophilic and specific to this protein and were therefore selected to raise antibodies. The two peptide regions against kif1-like kinesins were also determined for hydrophilicity to determine their suitability to produce antibodies. While these peptide stretches were not as hydrophilic as the Rab6KIFL peptide stretches, they were both partially hydrophilic and were the only peptide sequences that were identified using mass spectroscopy.

Of the ten antibodies produced (2 against each peptide), the two antibodies raised against one of the KIF1-like peptide stretches failed to produce an immune response. Therefore purified antibodies included 6 against Rab6KIFL and 2 against KIF1-like kinesins. Antibodies C1397 and C1398 were raised against the Rab1 peptide stretch, antibodies C1454 and C1455 against the Rab2 stretch and antibodies C1457 and C1459 against the Rab3 protein stretch. Antibodies C1463 and C1464 were produced against the Kin2 peptide stretch. The antibodies raised against the Kin1 peptide stretch failed to produce an immune response in the rabbits injected and therefore were not analyzed further. The naming of Kin1 and Kin2 were simply used to identify the peptide stretches. The remaining 8 antibodies were then probed to determine specificity to the peptide to which they were raised (Figure 2.3). Of the eight antibodies, only three, C1455, C1457 and C1459 reacted against the peptide to which they were raised indicating these antibodies were worth pursuing.

Immunofluorescence

Prior to the production of these antibodies, pan-specific kinesin antibodies were produced as an initial probe to determine if a kinesin was indeed present in Sertoli cells and if any of these antibodies reacted with ectoplasmic specializations. Three chicken antibodies were produced, anti-LAGSE (LNLVDLAGSE), anti-HIPYR (CHIPYRESTKLT) and anti-FAYGQ (GYNTIFAYGQTG). These antibodies were raised against conserved regions of kinesins that when taken together are present among a large majority of kinesin proteins. I re-assessed these antibodies by reacting them with sectioned fixed rat tissue (Figure 2.4a). Of these antibodies, the LAGSE and FAYGQ antibodies displayed some positive staining on sectioned tissue while the LAGSE antibody displayed the best defined staining in the ectoplasmic specialization around the spermatid head on fragmented fixed rat tissue (Figure 2.4b) as previously described (Guttman 2003). Some staining was also seen using the FAYGQ antibody however it was not as prominent as the LAGSE staining. Due to the staining pattern of LAGSE producing the best results in sectioned as well as fragmented tissue, this antibody was pursued further at the electron microscopy level, which had not previously been performed.

Immunofluorescence was also performed using the eight produced antibodies, six against the Rab6KIFL protein and two against the KIF1-like kinesins on fixed sectioned (Figure 2.5a) and fixed fragmented (Figure 2.5b) tissue. Staining was seen with the C1455, C1457 and C1459 (anti-Rab1 and anti-Rab3) antibodies while the remaining antibodies were all negative. Staining appears to be localized around spermatid heads and based on sectioned tissue the antibodies localize to this area. This result is consistent with

the results of the line blots in figure 3 as these were the only antibodies that reacted with their peptide. Also as can be seen from Figure 5, C1455 displays very bright staining in the ectoplasmic specialization as compared to the C1457 and C1459 antibodies. The C1457 and C1459 antibodies do however also display staining in the ectoplasmic specializations and are likely reacting to a protein or proteins in this region. The C1398 antibody is also important to note. There is some immunofluorescence staining at the base of the ectoplasmic specialization which corresponds to the staining of the C1457 antibody. This staining is confirmed by western blot analysis.

Controls for these above mentioned antibodies was also performed by replacing the antibodies with Normal Rabbit IgG for the C1397, C1398, C1454, C1455, C1457, C1459, C1463, and C1464 antibodies and Normal Chicken IgY for the LAGSE, HIPYR and FAYGQ antibodies. Figure 2.6a displays controls staining fixed sectioned rat tissue. As expected the staining is mainly negative with some non-specific diffuse staining seen throughout the section. This staining pattern is in stark contrast to the staining of the experimental antibodies where staining is brighter and specific to regions of the Sertoli cell. On fragmented tissue no staining is seen in the spermatid head itself or in any region of the ectoplasmic specialization (Figure 2.6b). The IgG and IgY controls as well as the secondary antibody controls are all negative further indicating that previous staining displayed by the C1455, C1457, C1459 and the chicken antibodies is specific. As another approach to determine specificity of all the antibodies produced, western blot analysis was performed.

Western Blots

Western blot analysis was performed using all the antibodies produced. These blots were initially performed on isolated seminiferous epithelium and whole testis lysates (Figure 2.7a). Results were generally in concordance with immunofluorescent data previously described. Due to results from fluorescence, antibodies C1455, C1457 and C1459 were of greatest interest. As previously mentioned, these were the only antibodies to result in positive staining in Sertoli cells. C1455 stains for a specific band at approximately 128 kDa in seminiferous epithelium and a fainter band in whole testis lysates. The C1457 and C1459 antibodies, however stain a specific band at approximately 140 kDa. The C1457 antibody also stains a band at approximately 100 kDa however this band is likely non-specific as the C1459 antibody does not react at this molecular weight but reacts to the same peptide as the C1457 antibody as previously described in Figure 2.3. The C1398 antibody also reacts at a band at approximately 140 kDa indicating that this band may be the coincide with the staining of the C1457 antibody at this location.

The LAGSE antibody, as expected, reacted with multiple bands, however it should be noted that it too reacts with a band at approximately 128 kDa and there is high probability that this band coincides with the same band as with the C1455 antibody based on fluorescent staining being of similar characteristics. Reactivity specificity of the eight produced antibodies was also further determined by performing western blots on multiple tissues.

In an effort to determine if the antibodies reacted with similar bands in other tissues, western blot analysis was performed using the antibodies on brain, lung, liver, kidney, spleen and heart rat tissue lysates and compared to whole testis and seminiferous epithelium (Figure 2.7b). As expected, antibodies C1397 and C1454 failed to produce

any specific bands in any of the tissues. Antibodies C1398, C1457 and C1459 produce a specific band at approximately 140 kDa in seminiferous epithelium and whole testis as previously described, however in other tissues they produced multiple bands which cannot all be accounted for when staining with the normal rabbit IgG control antibody. This indicated some non-specificity of these antibodies. The C1455 antibody however, produced a specific band at 128 kDa which is specific to seminiferous epithelium and whole testis. The bands in other tissues using this antibody are accounted for when compared to the rabbit IgG control. This is unusual as this indicated that the reactive antigen is specific to testis. Finally the C1463 and C1464 antibodies raised against kif1-like kinesins failed to produce any specific bands in any tissue. Based on this western blot data as well as previously discussed immunofluorescence, the C1455 antibody was pursued to determine if staining of this antibody was specific to stages when the spermatid translocates to the base of the seminiferous epithelium.

Stage Specificity of the C1455 Antibody

Based on fluorescent staining, the C1455 antibody displayed the brightest staining pattern as compared to the other antibodies as well as being localized to the ectoplasmic specialization (region around the spermatid head). Therefore an indepth look of the C1455 antibody's staining was performed. Specifically, staining of the C1455 antibody was compared during the spermatid elongation period (Figure 2.8). Staining at the ectoplasmic specialization (staining around the spermatid head) by the C1455 antibody is seen to occur at early stages of spermatogenesis and begins to fade at later stages. At early stages, when the spermatid begins its descent to the base of the Sertoli cell, staining is seen at the ES. Furthermore, this staining is seen to begin to fade and disappear as the

spermatid translocates back to the apex of the Sertoli cell and is released into the lumen. This staining pattern is consistent with what would be expected for a kinesin based transport system. The staining of this antibody was also seen to be specific on tissue when blocked with its peptide.

Peptide Blocking

Peptide blocking experiments were also done to verify specificity. The C1455 antibody was incubated with its peptide prior to reacting on tissue. This competitive block was performed on sectioned and fragmented tissue (Figures 2.9a and 2.9b respectively). In cases where the C1455 antibody was reacted to its peptide before incubation with tissue, immunofluorescence was dramatically reduced and was mainly non-specific staining throughout the Sertoli cell. No staining was localized to the ectoplasmic specialization and the staining was weaker and similar to the rabbit IgG controls. A dramatic decrease in staining was seen in comparison to normal staining with the C1455 antibody. Western blot analysis was also performed using antibodies blocked with peptide (Figure 2.9c). In this case no bands are visible when the antibody was blocked with peptide before being added to the blots. This indicated the antibody was specific to its peptide and the band visible when the no peptide was used containing the same antigen as the peptide. Based on this and previous data, the C1455 antibody specificity across species was determined.

Antibody specificity in mouse tissue

To determine the specificity across species, staining was performed in mouse tissue. The Rab6KIFL sequence from which the peptide stretches for the antibodies was taken was also taken from the mouse sequence. Mouse tissue was also chosen due to the

size of the protein band determined by western blot analysis was larger than the 100kDa molecular weight as described in other work. The first question required to be addressed was to determine if localization of the antibody was at ectoplasmic specializations. Sectioned mouse tissue (Figure 2.10a) and fragmented mouse tissue (Figure 2.10b) were stained with the C1455 antibody. In both cases, staining is seen in regions around the spermatid head. Figure 2.10b describes these results in a more conclusive manner. However in both the sectioned and fragmented tissue, staining is seen to be specific and surrounding the spermatid heads which are highlighted with DAPI. These results are consistent with staining seen in rat tissue and they provide support for cross species conservation. In addition, western blot analysis was performed on mouse testis lysate and compared to rat seminiferous epithelium as well as rat whole testis lysate (Figure 2.10c). Interestingly, a band at approximately 128 kDa is observed which is consistent with rat tissue. This band at approximately 128 kDa has been localized to the seminiferous epithelial tissue of the testis, however it was not known if this band was isolated to the cytoplasmic face of the ectoplasmic specialization. For this reason, electron microscopy was performed to determine the specific localization pattern of the C1455 antibody at the ultrastructural level.

Ultrastructural Localization

Specific localization of the LAGSE and C1455 antibodies were determined using electron microscopy (Figures 2.11a and 2.11b respectively). If these antibodies do in fact react against a kinesin that is responsible for spermatid translocation, the antibodies should localize to regions of the ES where they are able to interact with microtubules. When secondary antibodies conjugated to colloidal gold were used, the gold particles

generally associated with the cytoplasmic face of the endoplasmic reticulum of the ES. The gold particles conjugated to secondary antibody specifically were found concentrated in this area when conjugated the C1455 antibody as well as the LAGSE antibody were used as compared to rabbit and chicken IgG controls respectively. This localization was further confirmed using western blotting of ER enriched fractions from gelsolin treated spermatid/junction fractions.

Localization to Cytoplasmic face of the Ectoplasmic Specialization

To further verify that the 128 kDa band localized to the ER, the ectoplasmic specialization was separated at the actin layer using gelsolin. Prior to gelsolin being added to the fragmented spermatid and attached ES, actin is seen to localize around the spermatid head. Post treatment of gelsolin displays no actin present around the spermatid. Figure 2.12a describes the location of actin before and after the addition of gelsolin. Once gelsolin treated, the ectoplasmic reticulum component of the ES is separated from the spermatid at the actin layer. Upon centrifugation of the sample, the spermatids form a pellet and the endoplasmic reticulum portion of the ectoplasmic specialization is left in the supernatant. The supernatant containing the endoplasmic reticulum and the pellet, resuspended in an equal volume as the supernatant containing the spermatids, were then added to the gels in equivalent volumes and then run out on a blot and compared to isolated seminiferous epithelium. Staining with the C1455, C1457 and C1459 antibodies resulted in bands at a similar molecular weight as the seminiferous epithelium in the endoplasmic reticulum layer (Figure 2.12b). The C1455 antibody resulted in a band at 128 kDa while staining with the C1457 and C1459 antibodies resulted in bands at approximately 140 kDa. However a faint band also is visible in the pellet layer of the

ectoplasmic specialization containing the spermatid layer. This band was likely the result of slight contamination as separation of the two layers was only performed using centrifugation and manual removal of the supernatant. Of particular interest is the C1457 and C1459 antibodies reacting to a protein with a slightly higher molecular weight than the C1455 antibody but also localizing to the cytoplasmic face of the ectoplasmic specialization. Due to this results of bands at multiple molecular weights, an attempt was made to identify the proteins which resulted from these blots.

Transfection of the Rab6KIFL cDNA

In an attempt to identify these bands to determine if in fact they were Rab6KIFL mass spectroscopy of the bands was initially performed. These results however were inconsistent and failed to identify a specific protein. Therefore, a Rab6KIFL mouse cDNA was obtained and transfected into kidney cos-7 cells transfected in a pCMV-HA vector. In this case, the C1455, C1457 and C1459 antibodies all reacted with a band at a molecular weight of approximately 110kDa in transfected cells as compared with non-transfected cells (Figure 2.13). The anti-HA antibody was used as a control to identify the HA tag in the vector used to identify the Rab6KIFL protein and it also identified a band at approximately 110kDa. These results indicate that all of these three antibodies do recognize the full length Rab6KIFL protein.

Discussion

The spermatid translocation hypothesis predicts that a kinesin associated with ectoplasmic specializations is responsible for the translocation of the spermatid to the base of the Sertoli cell. If true, this hypothesis would explain how spermatids are transported in an apical crypt of the Sertoli cell to the base of the epithelium and back to the apex of the epithelium. The hypothesis is supported by a number of pieces of evidence. First, microtubules arrange along the long axis of the Sertoli cell which would allow motor proteins to travel along these structures (Amlani and Vogl 1988; Vogl 1988). Second, binding assays show that isolated ES junctions bind to microtubules in a nucleotide dependent fashion indicating motor proteins are present (Redenbach, Boekelheide et al. 1992). Third, motility assays show that ESs can transport microtubules and that movement occurs in both directions (Guttman, Kimel et al. 2000). Finally, dynein was localized to ESs using immunofluorescence (Guttman, Kimel et al. 2000). In this study I have localized a protein we suspect to be a kinesin to the ES.

The mammalian testis is known to contain a large number of motor proteins including multiple kinesins (Hall, Eveleth et al. 1992; Sperry and Zhao 1996). Kinesins from many families have been identified in the testis by using a conserved pan-specific antibody against kinesins (Sperry and Zhao 1996), however the localization pattern of many of these proteins is unclear. Rab6KIFL is an example of one of these kinesin proteins (Guttman 2003). This protein has been shown to associate with spindles during mitosis. However, Sertoli cells, which express high levels of mRNA for this protein, are non-dividing cells. The presence of this mRNA detected in GeneChip arrays is unusual

and may signify a unique function for this protein. For this reason, we decided to determine the localization pattern of this protein.

Another candidate kinesin is a kif1-like kinesin, based on mass spectroscopy of isolated seminiferous epithelium. Using mass spectroscopy, two peptide sequences were identified that are conserved for the kif1-like family of kinesins. Therefore an attempt was made to produce antibodies against these two conserved sequences that were identified.

Antibodies were produced against the Rab6KIFL protein as well as against kif1-like kinesins. Due to poor immune responses in the rabbits, only three antibodies, C1455, C1457 and C1459 resulted in antibodies that reacted well against their peptides and provided a positive result. These antibodies were all against the Rab6KIFL protein and the C1455 antibody obtained the best results by reacting at ectoplasmic specializations. However, all three antibodies reacted with a protein at the cytoplasmic face of the ectoplasmic specialization, as well as with the full length Rab6KIFL protein produced from transfected cells. The difference with these antibodies however was the C1455 antibody reacted with a band at 128kDa while the C1457 and C1459 antibodies reacted with a band at approximately 140kDa while no other specific bands correlate to the same molecular weight between the three antibodies. This leaves the possibility that the Rab6KIFL protein may have multiple isoforms in Sertoli cells that react at different molecular weights or possibly sliced variants or post-transcriptionally modified.

While the C1457 and C1459 antibodies reacted with a band at approximately 140kDa in seminiferous epithelium and whole testis, both of these antibodies also reacted with multiple bands that did not correspond to any bands in the rabbit IgG control.

Therefore it is possible that these antibodies react against multiple proteins in the testis and are not specific to Rab6KIFL. Therefore these antibodies were not pursued in depth. On the other hand, the C1455 antibody reacted well with a band at 128kDa while no other specific bands were seen as all remaining bands could be accounted for when compared to the rabbit IgG control. This antibody was also specific when immunoprecipitations were performed (data not shown). For this reason, the C1455 antibody was further characterized and attempts were made to confirm the identity of this protein.

Verification of the localization pattern was obtained by performing a number of experiments. Firstly, peptide blocking indicated that this antibody reacted with its peptide and specific staining was blocked on sectioned and fragmented tissue. Specific localization of the C1455 antibody was determined using electron microscopy, and cross-species specificity was determined when staining was performed on mouse tissue. Site specific staining was further reinforced by western blot analysis on regular seminiferous epithelium and testis lysates in addition to western blot analysis using gelsolin treated ectoplasmic specializations. This data conclusively determined that the C1455 antibody did in deed react with a specific protein and that this protein was localized to the cytoplasmic face of the ectoplasmic specialization.

The staining obtained from this antibody not only localized to the cytoplasmic face of ectoplasmic specializations, it also was specific to stages of spermatogenesis when the spermatid translocated to the base of the Sertoli cell. Staining is also present at ESs with both the C1455 antibody as well as with the LAGSE antibody. This data along

with both antibodies reacting with a specific band at 128 kDa suggest that the two antibodies may be reacting with the same protein.

Corroboration of the identity of the protein that the C1455 antibody reacted against came from transfecting Rab6KIFL cDNA into cos-7 cells. The C1455 antibody was identified to react with the full length Rab6KIFL protein at approximately 100kDa and at the same molecular weight as an anti-HA antibody. This verification provided support that the C1455 antibody was in fact reacting to Rab6KIFL.

A dilemma faced in this study was the fact that in all blots a band at approximately 128 kDa routinely appeared to react with the C1455 antibody. Rab6KIFL has a predicted molecular weight of 100 kDa and a heavier band was troublesome. As Rab6KIFL was only shown to be highly expressed enough in mitotic cells to perform visible blots, no positive controls existed where easy comparison could be performed. In addition, the full-length Rab6KIFL transfected cDNA reacted with a smaller band. This leaves the possibility that this protein may have multiple isoforms that may be present in the testis. This assumption would not be implausible as testis is known to contain multiple proteins of varying molecular weight as compared to other organs in an organism. A prime example is espin which has been identified in the testis to be of a higher molecular weight as compared to other tissues as this protein is modified (Bartles, Wierda et al. 1996). This is also an area that would require further evidence before any conclusions can be drawn.

For a kinesin to play a role in the spermatid translocation, the kinesin must be isolated in an area of the ES that allows it to react to microtubules in the Sertoli cell. This is important not only for spermatid translocation of the spermatid, but also for the

translocation of the entire junction which is seen to move with the spermatid to the base and then to the apex of the Sertoli cell. Gelsolin treated ESs were therefore run on western blot and a band was seen on the ES which faced the cytoplasmic side of the ectoplasmic specialization. A faint band was also seen in the pellet. The cytoplasmic face of the Sertoli cell and the spermatids were only able to be separated by centrifugation and it is highly likely that the pellet was contaminated with the isolated ES layer. It is important to note that the band that appeared in the pellet sample was dramatically fainter than the band in the isolated ES. Further analysis using electron microscopy specifically shows staining to be isolated to the cytoplasmic face of the ES and noticeably absent from the spermatid side of the ES. However, there is some non-specific staining seen in the actin layer which could account for staining in the spermatid pellet layer. In any case, staining was generally present in the ectoplasmic reticulum layer of the ES as predicted by the kinesin based transport hypothesis.

The translocation of spermatids along microtubules is potentially a significant component of spermatogenesis. Human biopsies from some infertile men have phenotypes consistent with defects in the translocation machinery such as failure of the entrenchment and malpositioning of spermatids in the epithelium (Vogl, unpublished data).

The presence of microtubules and the localization of antigens reactive with antibodies raised against kinesins to the cytoplasmic region of the ES are consistent with a kinesin playing a role in spermatid translocation specifically in the translocation of spermatids to the base of the Sertoli cell. Our data is consistent with, but does not prove that the identified protein is Rab6KIFL as this protein contains the peptide sequences

from both the C1455 and LAGSE antibodies. This still requires confirmation. The pathway that activates this kinesin, the possible translocation event itself and other possible mechanisms also remain to be explored. This study has provided evidence that is consistent with the spermatid translocation hypothesis and further work is required to determine if this kinesin is required for the translocation event. In any case, the results are still consistent with the possibility that a kinesin is associated with ectoplasmic specializations.

A

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1      mshrilsppa gllsdedvvd spilestaad lrsvvrkdll sdcsvisasl edkqalledt
61     sekvkylri rpfltseldr qedqgcvcie ntetlvlqap kdsfalksne rgvgqathkf
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241    kkrvhtesri gasnsfdsgv aglsstsqft sssqldetsq lwaqpdtpvp svpadirfsv
301    wisffeiyne llydllepps hqhkrqtlrl cedqngnpyv kdlnwihvrd veeawkllkv
361    grknqsfast hmqgqssrsh sifsirilhi qgegdivpki selslcldag serckhqksg
421    erlkeagnin tslhtlgrci aalrqnqqnr skqnlipfrd skltrvfqgf ftgrgrscmi
481    vvnnpcasty detlhaakfs alasqlvhap pvhlgipslh sfikkhspqv gpglekedka
541    dsdledsped eadvsvygke ellqvveamk alllkergek lqleiglree icnemveqmq
601    qregwcserl dnqkelmeel yeeklkilke slttfyqeqi qerdekieel etllqeakqq
661    paaqsgsgls llrrsqrlaa sastqqfgev kaeleqckte lssttaelhk yqqvlpkpppp
721    akpftidvdk kleegqknir llrtelqklg qslqsaerac chstgagklr qaltncddil
781    ikqnqtlael qnmvvlvkld lqkkaaciae qyhtvklqg qasakkrlga nqenqgpnhq
841    ppgkpkflrn llprtptcqs stdsspyari lrsrhspllk spfgkky

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B

TVAATNMNETSSR

ANSTGATGARLK

Figure 2.1: Protein Sequences. (A) The sequence of the Rab6KIFL protein in single letter amino acid code. This protein is an 887 amino acid kinesin protein and was identified to be present in Sertoli cells using GeneChip data arrays. Highlighted in red is the LAGSE sequence illustrating that this protein contains this pan specific sequence. Green highlights indicate the RAB1 sequence to which antibodies C1397 and C1398 were raised. Blue highlights indicate the RAB2 sequence to which antibodies C1454 and C1455 were raised while yellow highlights the RAB 3 sequence to which antibodies C1457 and C1459 were raised. (B) The sequences of the conserved KIF1-like family peptides. These peptides were identified by mass spectroscopy. The first sequence indicates the KIN1 sequence to which antibodies C1460 and C1461 were raised and the second sequence indicates the KIN2 sequence to which antibodies C1463 and C1464 were raised. C1460 and C1461 failed to produce an immune response in the rabbits injected therefore no antibodies were produced.

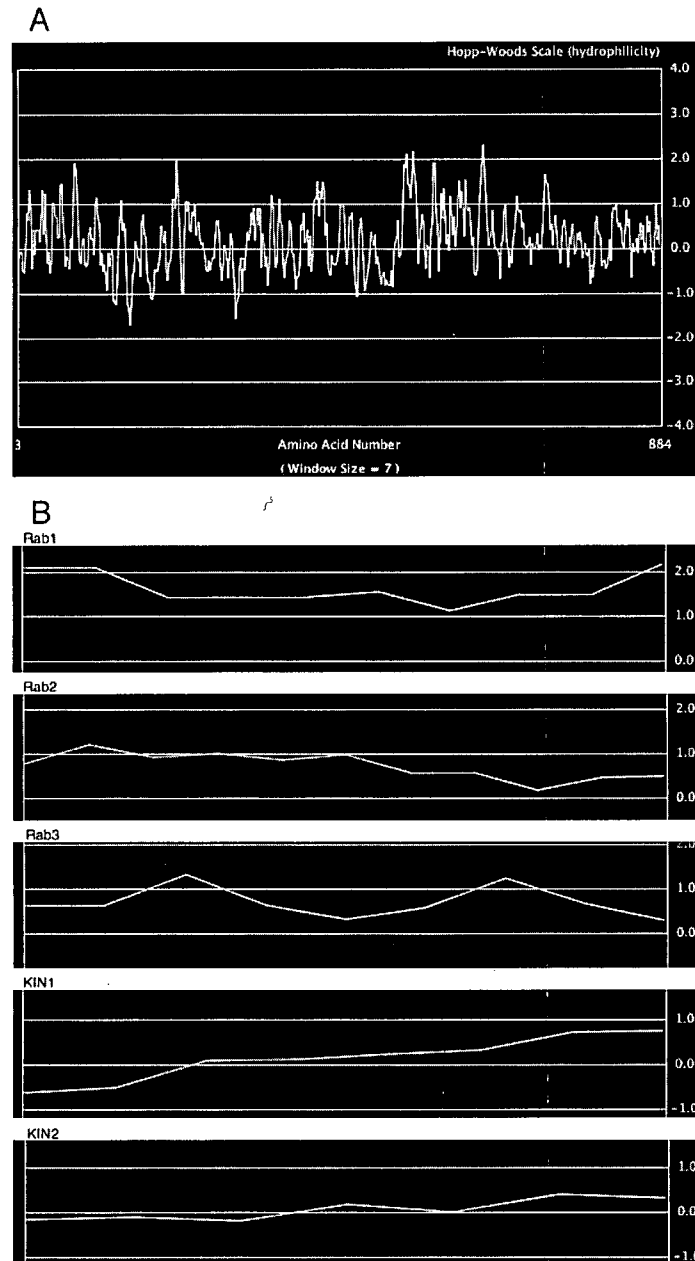


Figure 2.2: Hydrophilicity plots of RabKIFL and sequences to which antibodies were raised. (A) Hydrophilicity plot of the full-length Rab6KIFL protein sequence. A hydrophilicity plot was performed to identify hydrophilic regions of the protein which would be suitable candidates for antibody production. Regions of the plot above 0 indicate hydrophilic regions of the protein sequence while regions of the plot below 0 indicate hydrophobic regions. Hydrophilic regions are desirable as these sections are likely exposed regions of the protein and would allow for antibodies to react to the full length protein. After suitable peptide regions were identified and verified to ensure no problematic amino acids were included in the sequence, another hydrophilicity plot was performed on these sequences. (B) Hydrophilicity plots of the peptide sequences to which antibodies were raised. In total 5 peptides were synthesized and 10 antibodies were to be produced. The RAB1, RAB2 and RAB3 sequences were all chosen due to their high hydrophilicity while the KIN1 and KIN2 regions were selected due to their identification in KIF1-like kinesin proteins. THE KIN1 and KIN 2 peptide regions are not highly hydrophilic and the probability of producing an immune response in rabbits to produce antibodies was low. As was the case, KIN1 did not produce an immune response in the animals in which it was injected.

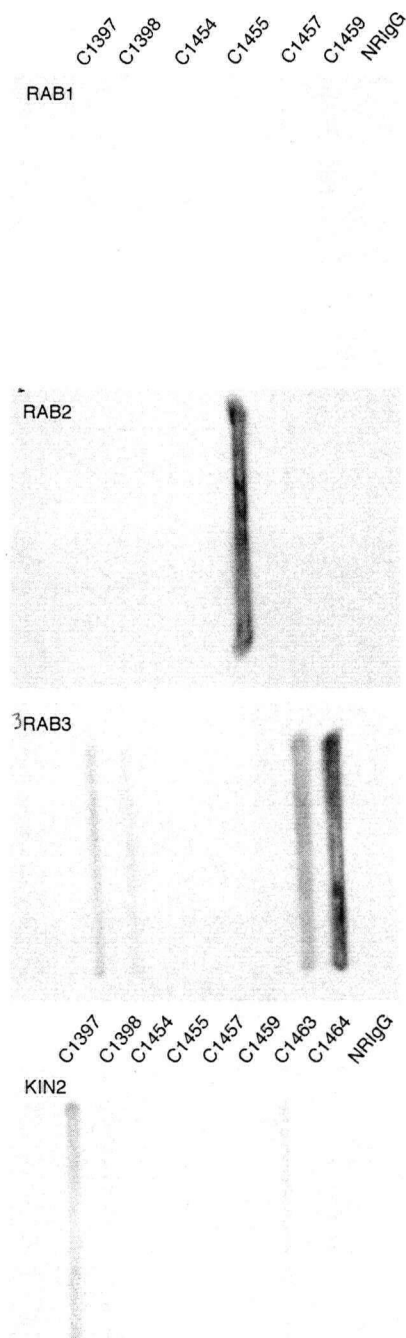


Figure 2.3: Line blots of all produced antibodies on nitrocellulose membranes coated with the various peptides to which they were raised. Four blots were coated with the four peptides that were used to produce the antibodies and stained in sequence with the antibodies. The KIN1 peptide (antibodies C1460 and C1461) failed to induce an immune response and was not included in experiments. From the figure it can be seen that the C1397 and C1398 (anti-Rab1) antibodies do not react to the peptide to which they were raised. From C1454 and C1455 (anti-Rab2), only C1455 reacts with its peptide while C1457 slightly and C1459 (anti-Rab3) react to the peptide to which they were raised. Also C1463 slightly reacts with its peptide while C1464 (anti-KIN2) does not. NRIgG was also reacted against the various peptides and did not react with any peptide. Line blots show specificity of which antibodies are specific to the peptides to which they were raised and helped to deduce which antibodies would merit further study. Based on these results the best antibodies appear to be C1455, C1457 and C1459.

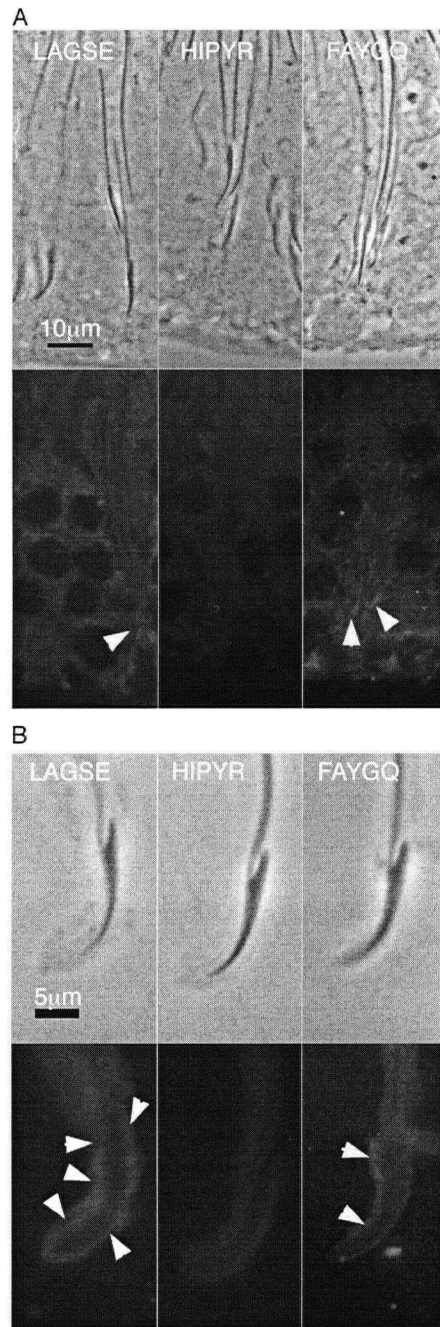


Figure 2.4: Immunofluorescence of chicken antibodies raised against pan-specific conserved sequences of kinesins on rat tissue. (A) Immunofluorescence of chicken antibodies raised against pan-specific conserved sequences of kinesins on sectioned rat tissue. LAGSE, HIPYR and FAYGQ are sequences that are each conserved among a large number of kinesins. As can be seen from the immunofluorescence, LAGSE is seen to associate around spermatids in regions of ectoplasmic specializations while the HIPYR and FAYGQ fail to localize to this region. (B) Immunofluorescence of chicken antibodies raised against pan-specific conserved sequence of kinesins on fragmented rat tissue. As can be seen by immunofluorescence, LAGSE staining can be seen at ectoplasmic specializations in the region around the spermatid head with some similar staining with the FAYGQ antibody. However, of the 3 antibodies raised against conserved sequences, LAGSE displays the best staining at ectoplasmic specializations and is the only sequence which is contained in the Rab6KIFL protein sequence. Peptide blocking was also seen to inhibit these antibodies (Guttman 2003). Arrowheads indicate ectoplasmic specialization junction staining.

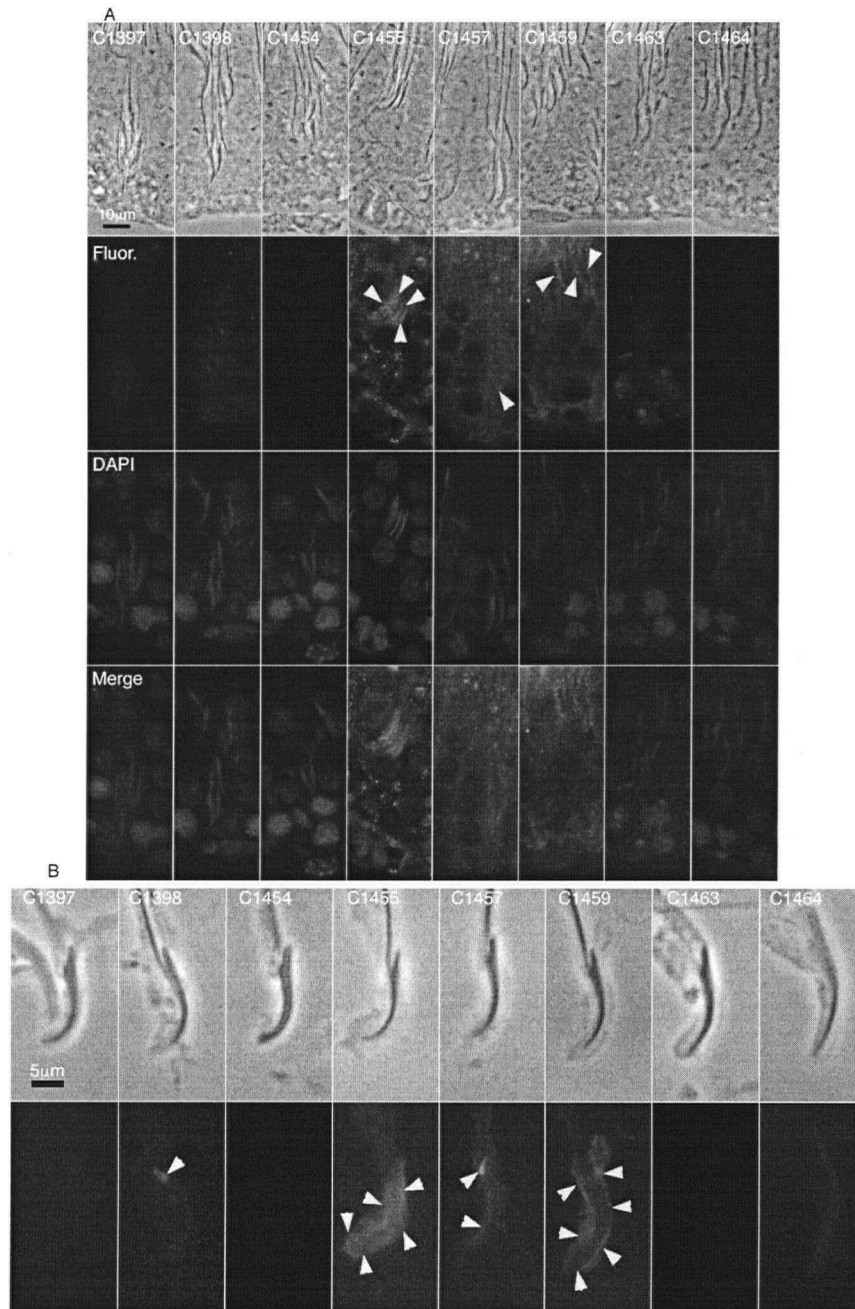


Figure 2.5: Immunofluorescence of rat tissue of all 8 produced antibodies. (A) Immunofluorescence of sectioned rat tissue of all 8 produced antibodies. Phase, fluorescence, DAPI and merged sections are seen for each antibody. Based on the staining patterns, only 3 antibodies, C1455 (anti-Rab2), C1457 (anti-Rab3) and C1459 (anti-Rab3) appear to have a specific staining pattern as would be expected due to only these 3 antibodies reacting strongly with their respective peptides. From the merged figures, it can be seen that C1455 reacts in regions of the ectoplasmic specializations around early staged spermatids as would be expected for a kinesin which is associated with spermatid translocation. Antibodies C1463 and C1464 which were raised against kif-1-like kinesins appear to be negative. Arrowheads indicate ectoplasmic specialization junctions. (B) Immunofluorescence of fragmented rat tissue with all 8 produced antibodies. As can be seen from fluorescence, C1455, C1457 and C1459 appear to have significant staining. In addition C1455 appears to have prominent staining in the ectoplasmic specialization region around the spermatid with some staining also present with C1457 and C1459. Antibodies C1463 and C1464 raised against kif1-like kinesins appear to be negative. Arrowheads indicate ectoplasmic specialization junctions.

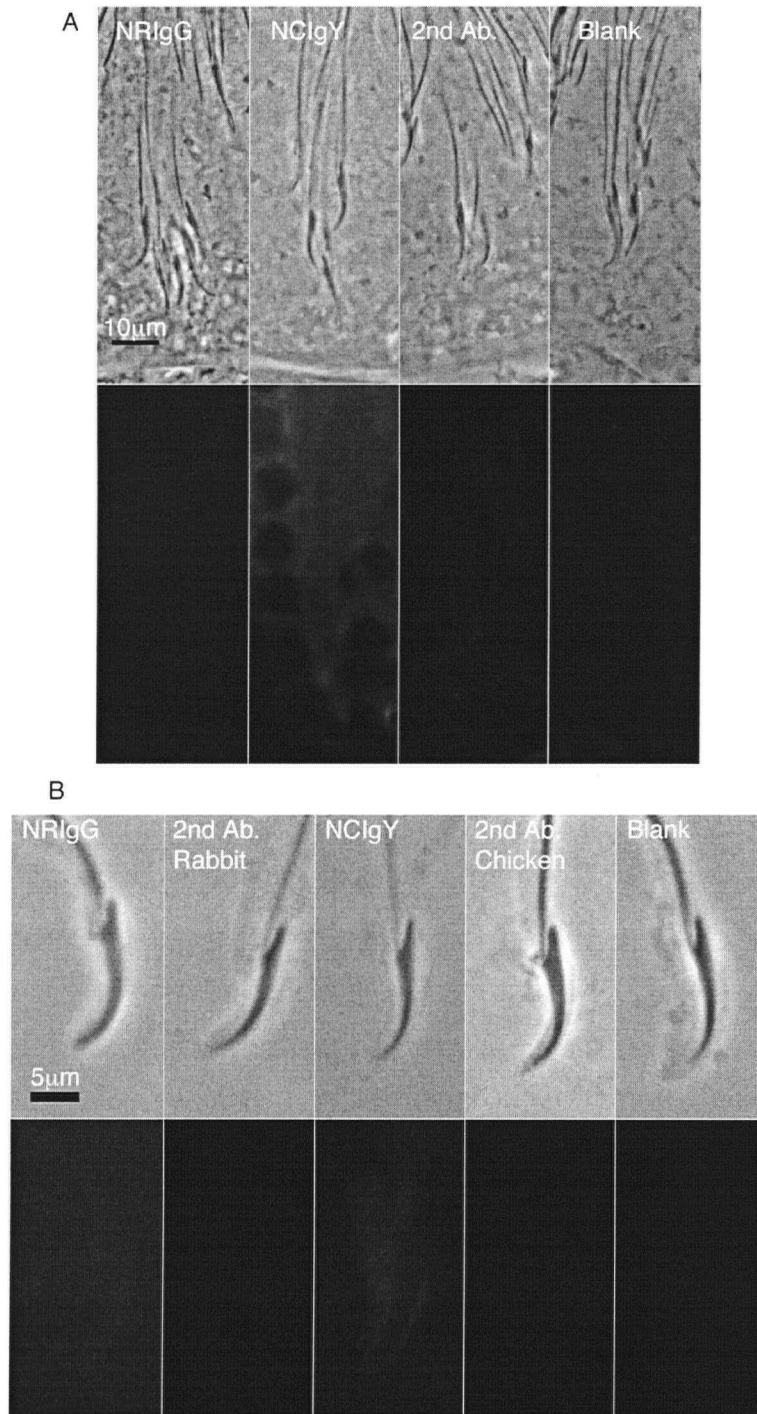


Figure 2.6: Immunofluorescence of rat tissue with control rabbit IgG and chicken IgY antibodies. (A) Immunofluorescence of sectioned rat tissue with control rabbit IgG and chicken IgY antibodies. No significant specific staining is seen with any control antibodies and compared to previous figures of C1455, C1457 and C1459 antibodies. No specific staining is seen to associate anywhere in the Sertoli cell or in the areas around the spermatid. Secondary antibody and blank controls are also negative. (B) Immunofluorescence of fragmented rat tissue with control IgG and IgY antibodies. Mid-staged spermatid fragments were taken and no specific staining is seen with the rabbit IgG and chicken IgY antibodies at ectoplasmic specializations as compared to previous figures displaying C1455, C1457 and C1459 staining. Secondary antibody and blank controls are also negative.

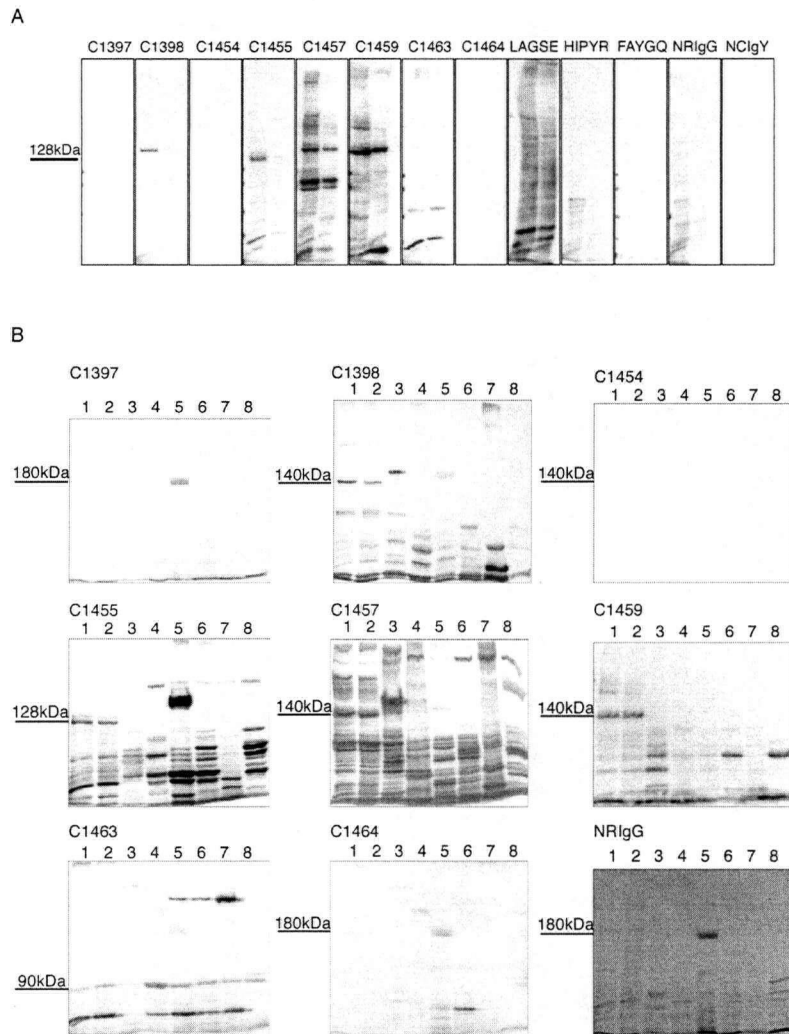


Figure 2.7: Western Blots of all antibodies produced against Rab6KIFL and the kif1-like proteins as well as chicken antibodies raised against conserved sequences of kinesins and control blots. (A) First lane is seminiferous epithelium and second lane is whole testis lysate. As is clear in the blots, C1397, C1454, C1463 and C1464 do not produce and any specific bands as was expected based on previous immunofluorescence data. C1398 displays a band at approximately 140 kDa as does C1457 and C1459. C1457 also produces a specific band at approximately 100 kDa while both C1457 and C1459 produce many smaller bands that appear specific as compared to the rabbit IgG control. The C1455 antibody produces a specific band at approximately 128 kDa. Due to insignificant immunofluorescence of the C1398 antibody, it was not pursued. The C1457 and C1459 antibodies reacted to many bands in both seminiferous epithelium and whole testis indicating that these antibodies were not specific. A faint band at approximately 128 kDa is also seen in the blot stained for LAGSE indicating that this band may be localizing to the same band as the C1455 antibody. Smaller bands in the C1455 blot appear to be non-specific based on higher exposure times of the IgG controls (data not shown). Multiple bands are seen in the LAGSE blot as expected. (B) Western blots of all antibodies produced and rabbit IgG control on multiple tissues. Tissues are as follows: lane 1 – seminiferous epithelium, lane 2 – whole testis, lane 3 – brain, lane 4 – lung, lane 5 – liver, lane 6 – kidney, lane 7 – spleen, lane 8 – heart. Again, C1397, C1454 do not produce any specific bands. C1463 and C1464 produce some bands, however they appear to be non-specific when compared to higher exposure times of the rabbit IgG controls (data not shown). C1398 appears to display a specific band at 140 kDa in seminiferous epithelium and whole testis. C1457 and C1459 produce multiple bands in multiple tissues indicating non-specificity. C1455 displays a band at 128kDa that is specific to seminiferous epithelial tissue and whole testis lysate and non-specific bands in other tissues when compared to the NRIG control.

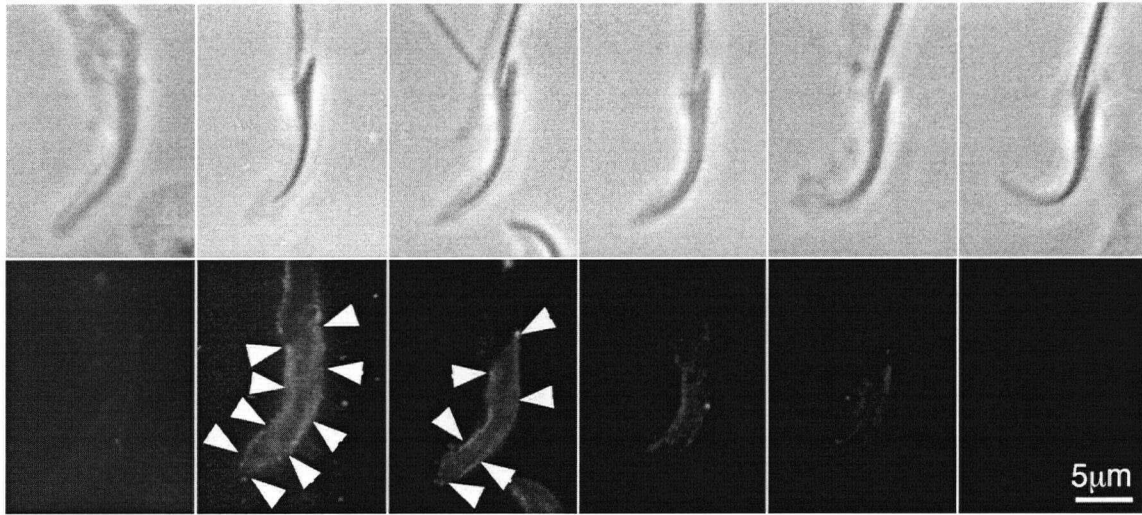


Figure 2.8: Immunofluorescence of C1455 antibody on fragmented rat tissue of stage sequences. Staining is of sequential stages of spermatogenesis from early spermatids to late spermatids. As can be seen from the staining, no staining is seen at early stage spermatids prior to the formation of the ectoplasmic specialization. As spermatids begin to elongate and translocate to the base of the Sertoli cell, C1455 is seen to localize to the ectoplasmic specialization. Staining is seen through the middle stages of spermatid development and then begins to fade and disappear at later stages when the spermatid moves to the apex of the Sertoli cell. Stages of spermatids are determined by curvature of the end of the spermatid heads and early spermatids can be identified by the absence of a sperm tail. Arrowheads indicate ectoplasmic specialization junctions.

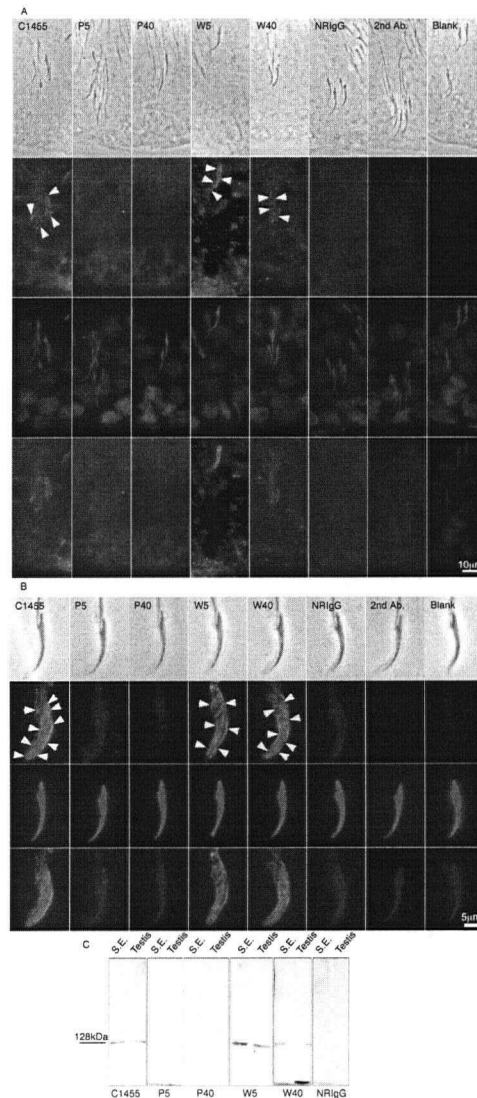


Figure 2.9: Immunofluorescence of C1455 antibody and peptide blocking. (A) Immunofluorescence of C1455 antibody and peptide blocking on sectioned rat tissue. Normal C1455 staining is seen and compared to peptide blocking. A dramatic decrease is seen in staining when the antibody is blocked with twice the concentration of peptide (P5) and also with ten times the concentration of peptide (P40). W5 and W40 controls were performed where the volume of peptide added was replaced with ddH₂O to correct for volume changes. Minimal non-specific staining is seen when the C1455 antibody is blocked with peptide. DAPI staining (blue) and merged image display staining of C1455 antibody is localized to ectoplasmic specializations. Rabbit IgG and secondary antibody controls are also negative. Arrowheads indicate ectoplasmic specialization junctions. (B) Immunofluorescence of C1455 antibody and peptide blocking on fragmented rat tissue. C1455 staining, DAPI and merged figures are seen. Similar to sectioned tissue, C1455 staining is seen in regular staining and when the peptide is replaced with ddH₂O. When peptide is added at double the concentration (P5) or ten times the concentration (P40) of the antibody, no staining is seen as similar to rabbit IgG and secondary antibody controls. W5 and W40 controls were performed where the volume of peptide added was replaced with ddH₂O to correct for volume changes. Merged images indicate staining of C1455 is localized to the ectoplasmic specialization. Arrowheads indicate ectoplasmic specialization junctions. (C) Western Blot analysis of C1455 antibody and peptide blocking on isolated seminiferous epithelium and whole testis lysates. A band at 128kDa is seen when stained with the C1455 antibody. When the peptide is added at double the concentration (P5) or ten times the concentration (P40) of the antibody, no band is visible. However, when the peptide is replaced with ddH₂O, a band is again visible at 128kDa. Absence of a band at P5 and P40 indicate the antibody is specific to its peptide.

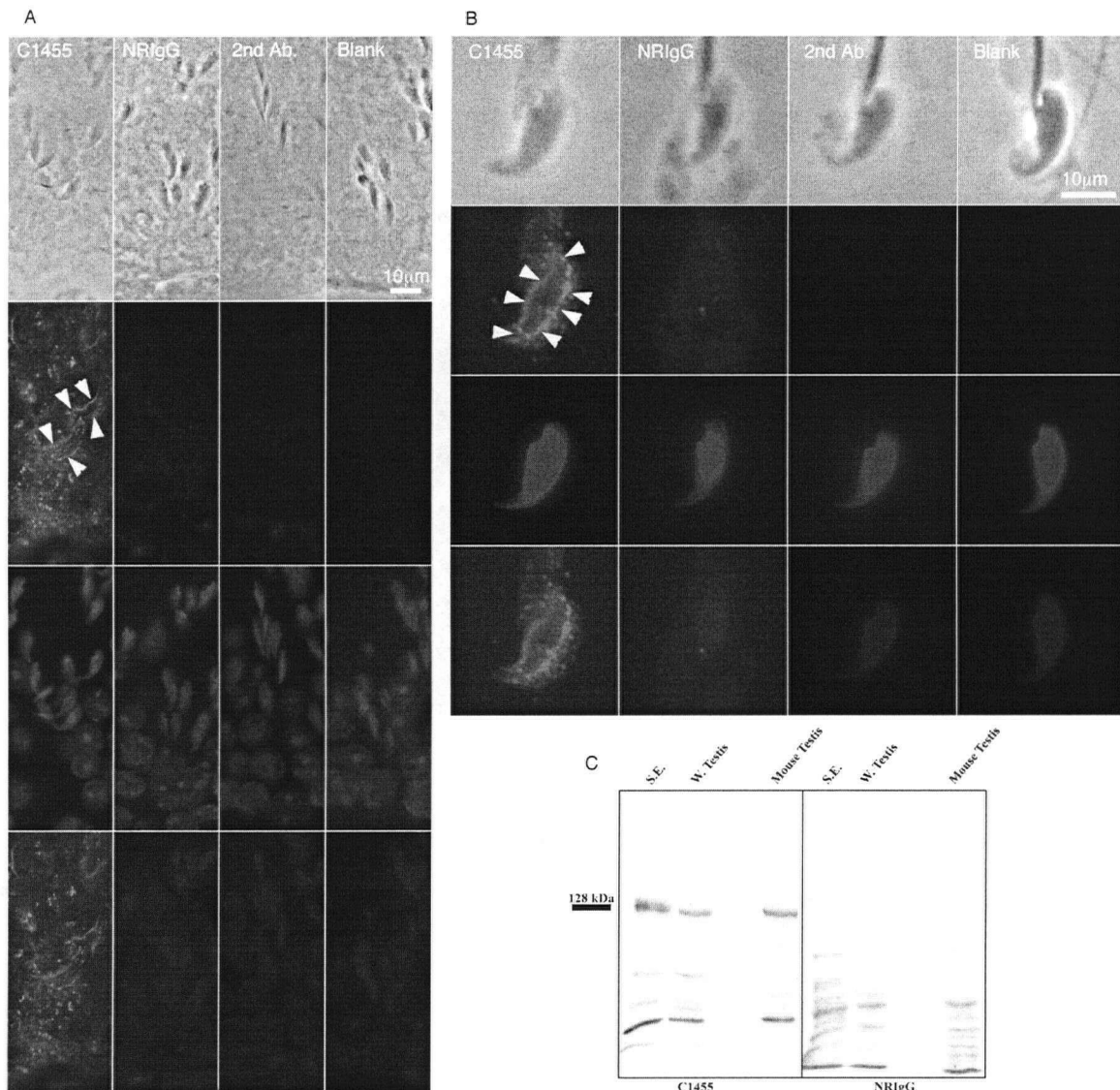


Figure 2.10: Immunofluorescence of the C1455 antibody on mouse tissue. (A) Immunofluorescence of the C1455 antibody on mouse sectioned tissue. C1455 fluorescence (Green and Gaudry), DAPI (blue) and merged figures can be seen for C1455 and associated controls. C1455 staining is seen to localize in regions around the spermatid as can be seen in the merged image. Staining with rabbit IgG and secondary antibody controls is negative. Arrowheads indicate ectoplasmic specialization junctions. (B) Immunofluorescence of the C1455 antibody on mouse fragmented tissue. C1455 fluorescence (Green and Gaudry), DAPI (blue) and merged figures indicate staining of the C1455 antibody to be isolated to the ectoplasmic specialization around the spermatid head. Staining with rabbit IgG and secondary antibody controls is negative for specific staining. Peptide sequences from the Rab6KIFL protein were obtained from the mouse protein sequence. Arrowheads indicate ectoplasmic specialization junctions. (C) Western Blot analysis of whole mouse testis as compared to rat seminiferous epithelium and whole testis stained with C1455. In all cases a specific band at 128kDa is observed as compared to the IgG control blot. Results indicate a specific protein across species.

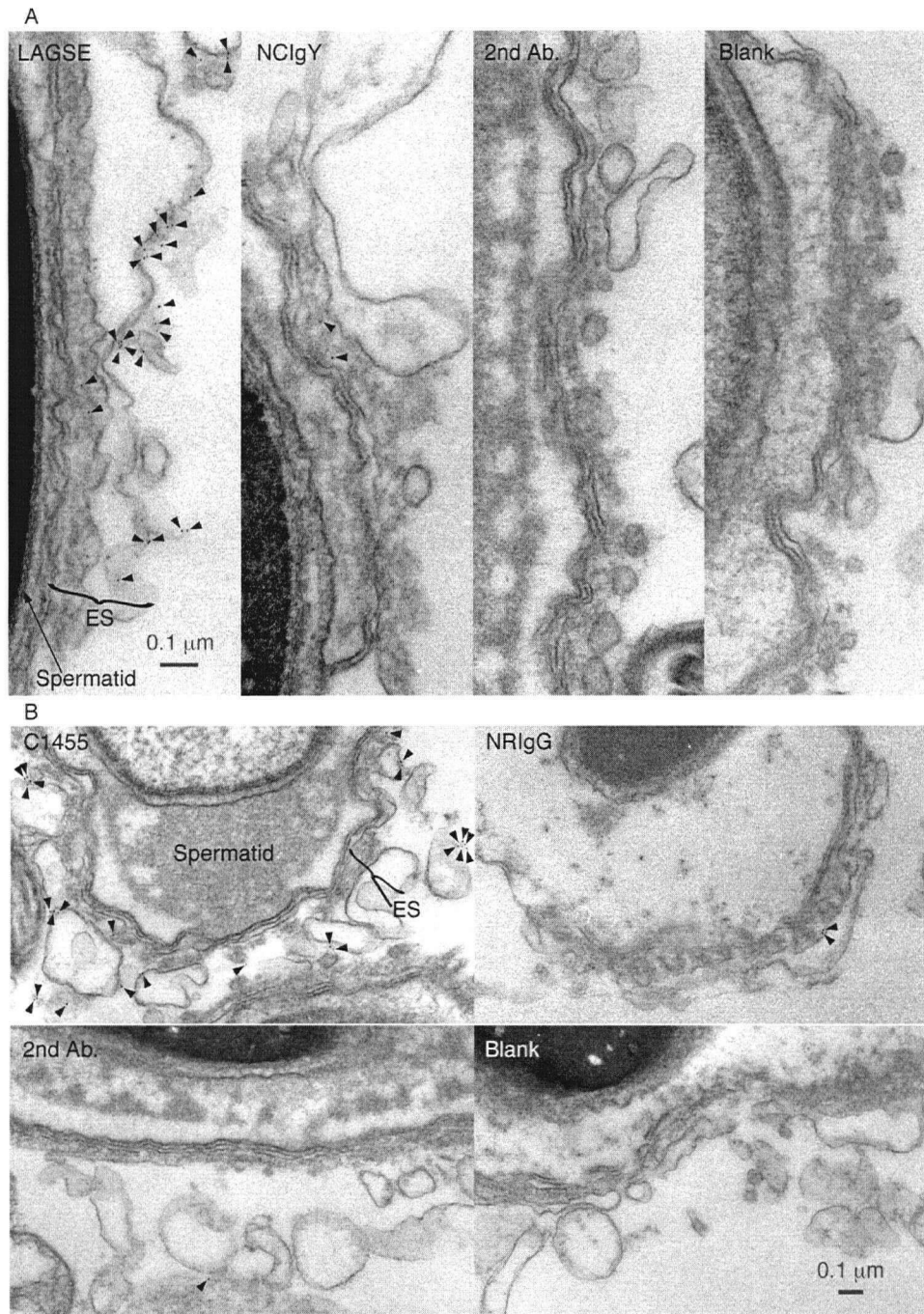


Figure 2.11: Electron microscopy of custom antibodies. (A) Electron microscopy of the LAGSE antibody and associated controls on sectioned rat tissue. Immuno staining using 5nm gold particles indicates staining to be localized on the cytoplasmic face of the Ectoplasmic Specialization along the endoplasmic reticulum (arrowheads). This is the site where staining would be predicted if this antibody was staining for a kinesin that was associated with spermatid translocation. Chicken IgY control and secondary antibody control are both negative for colloidal gold staining. (B) Electron microscopy of the C1455 antibody and associated controls on sectioned rat tissue. Immuno staining using 5 nm gold particles indicates staining to be localized on the cytoplasmic face of the Ectoplasmic Specialization along the endoplasmic reticulum (arrowheads). Some non-specific staining is also seen in the actin layer. This is the site where staining would be predicted if this antibody was staining for a kinesin that was associated with spermatid translocation. Rabbit IgG and secondary antibody controls are both negative for colloidal gold staining.

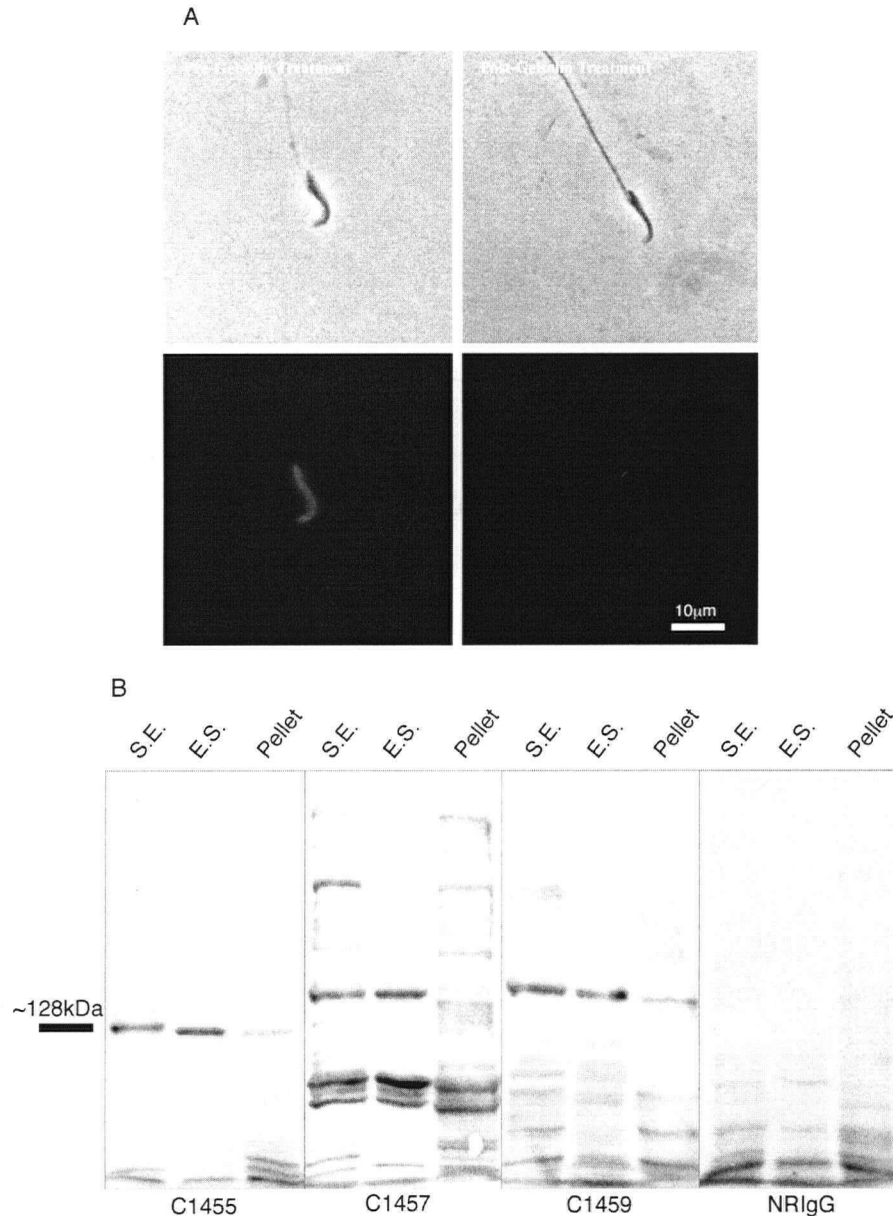


Figure 2.12: Gelsolin Treated Ectoplasmic Specializations. (A) Immunofluorescence of pre and post Gelsolin treated ectoplasmic specializations stained with a phalloidin staining actin. As displayed, actin is present in pre-Gelsolin treatments of isolated seminiferous epithelial tissue but not present in epithelial tissue post treatment of Gelsolin. Treatment of Gelsolin removes the ectoplasmic specialization from the spermatid by disassembling the actin layer. The ectoplasmic specialization layer containing the endoplasmic reticulum is separated from the spermatids by centrifuging the spermatids into a pellet. The endoplasmic specialization layer is mainly free from contamination however the pellet layer may be slightly contaminated from the supernatant. (B) Western Blot analysis of seminiferous epithelium compared to post-Gelsolin treated isolated ectoplasmic specialization and pellet containing the spermatids. A band is seen at 128 kDa for isolated seminiferous epithelium, as well as in the isolated ectoplasmic specialization when stained with the C1455 antibody. A faint band is also observed in the pellet tissue containing the spermatids as would be expected due to slight contamination from the isolation procedure as centrifugation is not able to remove all proteins completely. The C1457 and C1459 antibodies produce a band at approximately 140 kDa in all tissues as well as many lower bands some of which can be accounted for when compared to the rabbit IgG control antibody however some bands remain present further indicating cross reactivity to other proteins.

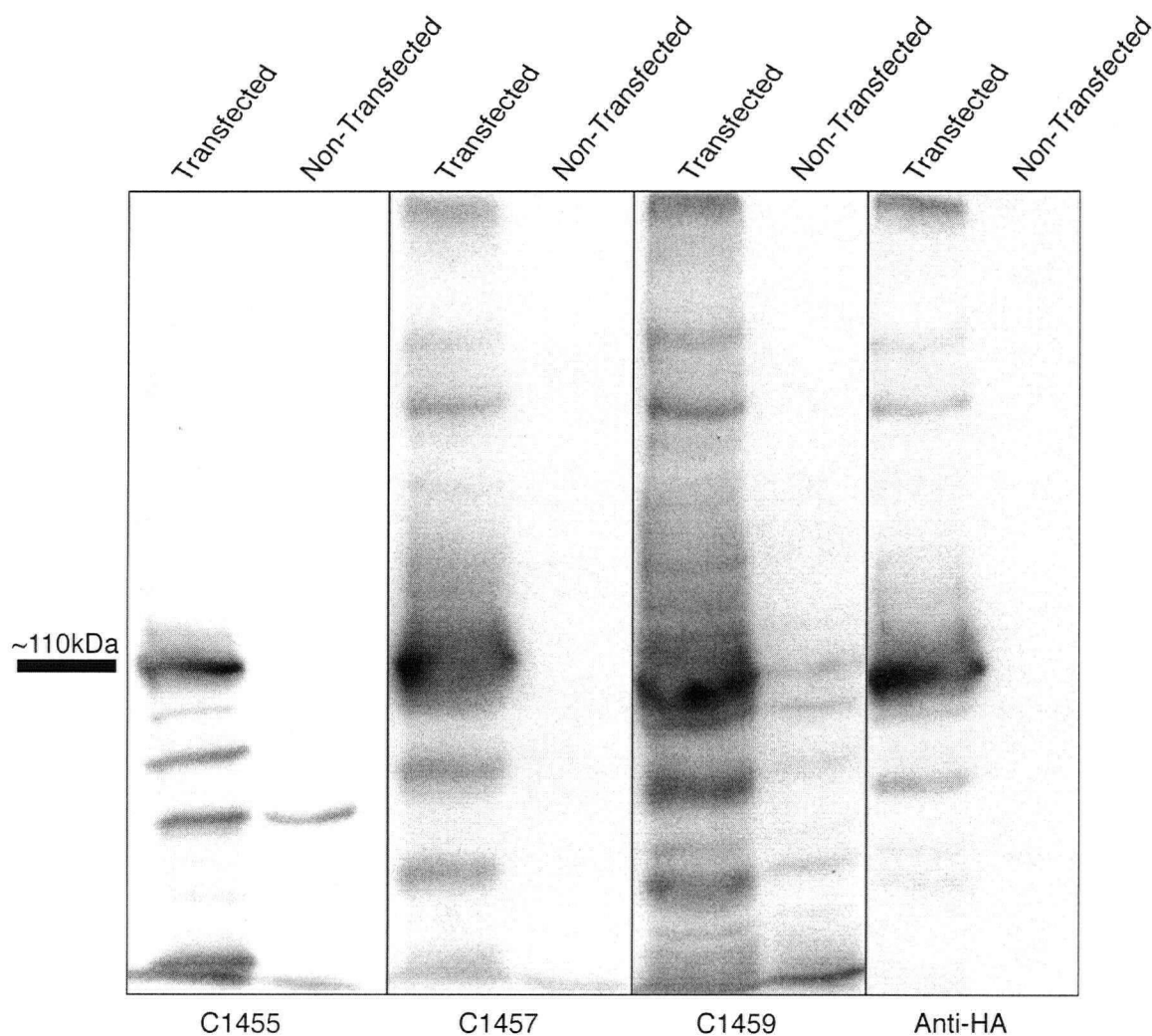


Figure 2.13: Westerns Blot analysis of monkey kidney cos-7 cells transfected with Rab6KIFL cDNA in a pCMV-HA vector. A band with a molecular weight of approximately 110 kDa appears when using C1455, C1457, C1459 and an anti-HA antibody. The predicted molecular weight of Rab6KIFL based on current literature is 100kDa and the addition of the HA tag in the vector are likely causes for the slight increase in molecular weight. The band at 100kDa appears only in transfected cells as compared to non-transfected cells.

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Chapter 3

Introduction²

A massive turnover of intercellular junctions occurs during sperm release and the movement of spermatocytes through the blood-testis barrier. At apical sites of attachment between Sertoli cells and spermatids, adhesion junctions disassemble as spermatozoa are released into the duct system. New adhesion junctions form in association with the next generation of spermatids as these cells begin to elongate. At basal sites of attachment between Sertoli cells, junction complexes containing adhesion, tight and gap junctions disassemble above and assemble below spermatocytes as these cells translocate between basal and adluminal compartments of the epithelium. Structures termed tubulobulbar complexes develop in association both with apical and basal junctions (Russell and Clermont 1976), and are thought to be the mechanism by which large surface areas of intercellular contact are internalized during junction turnover (Russell 1979; Pelletier 1988; Guttman, Takai et al. 2004).

Tubulobulbar complexes consist of elongate tubular evaginations of one cell together with the corresponding invaginations and related cuffs of actin filaments of the adjacent cell. Bulbar swellings, devoid of associated actin filaments, but closely related to cisternae of endoplasmic reticulum, occur near the ends of the structures and each of the

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complexes is capped by what has been described as a “bristle-coated” pit (Russell and Clermont 1976; Russell 1979; Russell 1979; Russell and Malone 1980; Guttman, Takai et al. 2004). The large double membrane bulbar regions of the complexes appear to “bud” from the complexes and fuse with lysosomes (Russell 1979; Russell 1979; Guttman, Takai et al. 2004). Junction molecules (Guttman, Takai et al. 2004) and morphologically identifiable junctions (Russell 1979; Pelletier 1988) are present in tubulobulbar complexes and in the double membrane vesicles associated with them. Our current working hypothesis is that tubulobulbar complexes are elongate actin-related tubular structures that function to internalize intercellular junctions during junction disassembly.

Because the dynamins in other systems are known to associate with endocytic mechanisms (McNiven 1998), to generate tubular structures in certain situations (Takei, McPherson et al. 1995; Sweitzer and Hinshaw 1998; Ochoa, Slepnev et al. 2000), and to influence the maintenance and growth of actin-based structures (Ochoa, Slepnev et al. 2000; Orth, Krueger et al. 2002; Schafer 2002; Orth and McNiven 2003; Gray, Kruchten et al. 2005), we predicted that one of the components of tubulobulbar complexes in Sertoli cells may be a dynamin. The dynamins are large (100 kDa) GTPases classically described as being involved with the formation of vesicles both from the plasma membrane (McNiven 1998) and from the trans-Golgi network (Jones, Howell et al. 1998). These proteins are thought to function as mechanoenzymes that constrict the necks of forming vesicles (McNiven 1998; Sweitzer and Hinshaw 1998). When the system is under cytoskeletal tension, possibly provided by the actin cytoskeleton, this constriction facilitates separating the vesicles from their parent membranes (Roux, Uyhazi et al. 2006). The dynamins also are implicated as playing roles in determining the structure and

maintenance of dendritic spines (Gray, Kruchten et al. 2005), podosomes (Ochoa, Slepnev et al. 2000) and “comet tails” (Orth, Krueger et al. 2002), most likely through an influence on the dynamics of the actin cytoskeleton (Schafer 2002; Orth and McNiven 2003).

There are three dynamin isoforms each encoded by a separate gene with a total of over 25 alternatively spliced variants (Cao, Garcia et al. 1998). Dynamin 1 is exclusively expressed in neuronal cells (Nakata, Iwamoto et al. 1991; Cao, Garcia et al. 1998). Dynamin 2 is ubiquitously expressed in all tissues including testis (Cao, Garcia et al. 1998) while Dynamin 3 is expressed in a variety of tissues including testis, brain, heart, and lung and has a total of at least 13 variants (Cao, Garcia et al. 1998). Localization patterns of the various variants show each spliced form has a unique expression pattern and therefore may interact with different organelles and have different functions (Cao, Garcia et al. 1998). Dynamins 2 and 3 are both highly expressed in the testis (Kamitani, Yamada et al. 2002) and the proteins have been localized to Sertoli and spermatogenic cells (Nakata, Takemura et al. 1993; Iguchi, Watanabe et al. 2002; Kamitani, Yamada et al. 2002); however, the subcellular distribution of the proteins has not previously been defined.

We show here that dynamin 3, but not dynamin 2, is localized in Sertoli cells along the elongate tubular components of tubulobulbar complexes. Moreover, when dynamin 3 is expressed in MDCK cells that have been stably transfected with nectin-2, junction regions become irregular and have tubular structures projecting from them into the cytoplasm. Nectins are adhesion molecules connecting adjacent plasma membranes of cells. Our results are consistent with the hypothesis that dynamin 3 may be involved with

determining the elongate structure of tubulobulbar complexes in Sertoli cells. Regulated constriction by dynamin 3 also could be one of the steps involved in the budding of large double membrane vesicles from the complexes and internalization of junction proteins, although this remains to be verified. The role of dynamin 3 in spermatogenic cells, where the protein is localized in the connecting piece, may be to reinforce the base of the sperm tail during flagellar beats or to serve as a protein reservoir for future use.

Methods and Materials

Animals

Animals used in this study were reproductively active male Sprague Dawley rats. They were obtained from the University of British Columbia animal care colony and were maintained in accordance with the guidelines established by the Canadian Council on Animal Care.

Chemicals and Reagents

Unless otherwise indicated, all chemicals and reagents were obtained from Sigma Aldrich Canada. The paraformaldehyde and NaCl were obtained from Fisher Scientific. All control immunoglobulins (IgGs) as well as all secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories, Inc. Secondary antibodies conjugated to ALEXA fluorochromes were purchased from Molecular Probes. Polybed embedding resin was obtained from EM Sciences.

Immunofluorescence

Tissue Preparation

Testes were removed from rats under deep anesthesia induced with halothane. Warm (33°C) PBS (150 mM NaCl, 5 mM KCl, 0.8 mM KH_2PO_4 , 3.2 mM Na_2HPO_4 , pH 7.3) was perfused through the spermatic artery using a 26-gauge needle attached to a gravity-fed perfusion apparatus for 2 min to clear the organ of blood. Following this, warm 3% paraformaldehyde in PBS was perfused through the testis for 30 min, then reperfed with PBS to wash out any remaining fixative.

Frozen Sections

Fixed testes were frozen (using liquid nitrogen) and attached to an aluminum stub using OCT compound (Sakura Finetek USA, Torrance, CA). 5 μ m frozen testis sections were cut, attached to poly-l-lysine-coated glass slides, immediately plunged into -20°C acetone for 5 min, air dried, and then processed for immunostaining.

Fragmented Material

Perfusion fixed testes were decapsulated in PBS and the seminiferous tubule mass was minced into approximately 1-2 mm cubes. The pieces were transferred into a 50 ml plastic Falcon tube in approximately 10 ml of PBS and gently passed through an 18-gauge, then 21-gauge needle for 2–5 passes each. The fragmented material was left to sediment by gravity at room temperature for 10 min. The upper layer, containing spermatogenic cells with attached junctions and tubulobulbar complexes, was transferred to another tube and the cells pelleted by centrifugation (5 min at 4000g), then resuspended in 2 ml of PBS. The cells were added to poly-L-lysine-coated slides and allowed to incubate in a humidity chamber for 10 min. All excess PBS was then removed and the slides were immediately treated with -20°C acetone for 5 min and allowed to air dry.

Epididymal Spermatozoa

The epididymides of anesthetized rats were excised and placed in fixative (3% paraformaldehyde in PBS). The organ was cut open and spermatozoa squeezed into solution. The cells were collected, placed in a conical tube and fixed for 30 min. The spermatozoa were collected by centrifugation then washed three times with PBS, placed on poly-L-lysine-coated slides, treated with -20°C acetone for 5 min and then air dried.

Immunostaining

Slides with attached tissue fragments or cryosections were rehydrated and blocked with 5% normal goat serum (NGS) in TPBS-BSA (PBS containing 0.05% Tween-20 and 0.1% bovine serum albumin) for 20 min at room temperature. Primary antibodies were diluted in TPBS-BSA containing 1% NGS and consisted of a rabbit anti-dynamin 3 antibody used at 5 μ g/ml (Cao, Garcia et al. 1998), mouse anti-dynamin 2 antibody used at 5 μ g/ml (BD Transduction Labs) and mouse anti-Arp3 antibody used at 10 μ g/ml (Sigma-Aldrich). Antibodies were added to the tissue and incubated overnight at 4°C in a humidity chamber. The following day the material was washed extensively with TPBS-BSA, then incubated for 60 min at 37°C with goat anti-rabbit or goat anti-mouse antibodies conjugated to ALEXA 488. The slides were again washed and coverslips mounted using Vectashield (Vector Labs, Burlington, ON). The tissue was visualized using a Zeiss Axiophot microscope fitted with appropriate filter sets for detecting fluorescence and with the appropriate optics for phase microscopy.

Filamentous actin was labeled using ALEXA 568 phalloidin (Molecular Probes). The stain was made up in TPBS-BSA and incubated at room temperature for 20 min before washing extensively in TPBS-BSA.

Controls for immunofluorescent localization consisted of replacing the primary antibodies with normal immunoglobulin (IgG) from the host animal species at identical concentrations to the primary antibody, replacing the primary antibodies with buffer alone and replacing both the primary and secondary antibodies with buffer alone.

Western Blotting

Western blots were performed on both whole testis and seminiferous epithelium lysates. Testes were removed from rats under deep anesthesia and seminiferous epithelium was isolated as described elsewhere (Vogl 1996). Briefly, testes were decapsulated and the seminiferous tubule masses were cut into small pieces in ice cold PEM/250 buffer (Vogl 1996). Isolated seminiferous tubules were collected, placed in fresh PEM/250 buffer and the epithelium separated from tubules walls using microprobes. The isolated sheets of epithelium were collected and then pelleted by centrifugation. Pellets were resuspended and extensively homogenized in RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 1% Nonidet P-40, 1% deoxycholic acid [sodium salt], 10% SDS) before being loaded into wells of 1-mm-thick 5% SDS-PAGE gels at equal concentrations as determined by protein assay and run according to standard protocols (Laemmli 1970). Proteins were transferred onto Immobilon-P transfer membrane (Millipore, Billerica, MA) and then washed for 5 min at room temperature with TBST (500 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20). The blots were blocked for 8 h at 4°C using 4% nonfat milk (Blotto, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed three times, 10 min each wash, and then incubated overnight at 4°C with a rabbit anti-dynamin 3 antibody (Cao, Garcia et al. 1998) or mouse anti-Arp3 antibody used at 0.5 µg/ml overnight at 4°C. The following day, blots were washed extensively with TBST followed by a 1 hour, room temperature goat anti-rabbit or anti-mouse horseradish peroxidase conjugated antibody incubation. After washing with TBST followed by TBS, blots were reacted with ECL (Amersham) and bands visualized with X-OMAT film (Eastman Kodak, Rochester, NY).

Controls consisted of replacing the primary antibodies with normal rabbit IgG or normal mouse IgG at the identical primary antibody concentration.

Electron Microscopy

Rat testes were removed from animals under deep halothane anesthesia and perfused first for 2 min with PBS then for 30 min with 0.1 M sodium cacodylate, 1.5% paraformaldehyde, 1.5% glutaraldehyde pH 7.4. Each testis was cut into small pieces and immersion fixed for an additional 90 min in same fixative. Samples were washed three times, 10 min each wash, with 0.1 M sodium cacodylate and then fixed on ice for 60 min in 1% osmium fixative in 0.1 M sodium cacodylate (pH 7.3). Samples were washed three times with ddH₂O, 10 min each wash, then stained for 1 h with 0.1% uranyl acetate. The material again was washed three times in ddH₂O, and then dehydrated through a graded concentration series of ethyl alcohols (50%, 70%, 95%, 100%). This was followed by two 15 min incubations with propylene oxide. The samples then were left in a 1:1 solution of propylene oxide:EMbed-812 overnight. The material was embedded in 100% Embed-812 (Electron Microscopy Sciences, Hatfield, PA) and then incubated at 60°C for 24 h. The tissues were sectioned and viewed using a Philips 300 electron microscope operated at 60 kV.

Immunoelectron microscopic localization of dynamin 3

Testes were fixed using a modified version of the protocol described by (Tokuyasu and Singer 1976). Testes were removed from animals anesthetized with halothane and perfused for 30 min with 3% paraformaldehyde and 20mM ethylacetimidate in PBS (pH 7.3). The testes were cut into small pieces and fixed again by immersion for 1 hour in 3% paraformaldehyde and 0.1% glutaraldehyde in PBS (pH

7.3). Following fixation, samples were treated with PBS containing 50 mM ammonium chloride, and then washed three times with PBS, dehydrated through a graded series of cold (0 to -20°C) ethyl alcohols, and infiltrated with Unicryl (Electron Microscopy Sciences, Hatfield, PA) at -20°C. Samples were left overnight in Unicryl, and then transferred to fresh Unicryl and polymerized at -20°C under ultraviolet light. Thin sections were placed on carbon/formvar coated grids and blocked with 5% normal goat serum (NGS) in PBS/0.1% BSA/0.05% Tween 20 (TPBS). The sections then were incubated overnight at 4°C with primary antibody (at a 1:10 or 1:20 dilution of 1 mg/ml anti-dynamin 3 stock) in 1% NGS in TPBS. Sections were washed with TPBS then incubated for 1 hour with a goat anti-rabbit antibody conjugated to 5nm colloidal gold (Sigma-Aldrich Canada) diluted 1:25 in TPBS containing 5% fetal bovine serum. The grids were washed with PBS, fixed with 0.1% glutaraldehyde in PBS, washed with ddH₂O, stained with 1% aqueous uranyl acetate, washed again with ddH₂O, and then air dried. Sections were evaluated and photographed using a Philips 300 electron microscope operated at 60 kV.

Controls for staining paralleled those for immunofluorescent localization and included replacing the primary antibody with the same concentration of normal rabbit, replacing the primary antibody with buffer alone, and replacing both the primary and secondary antibodies with buffer alone.

Transfections

Nectin-2δ cDNA was subcloned from a pCAGIPuro-nectin-2δ construct (Takahashi, Nakanishi et al. 1999) into the pEGFP-N1 plasmid (BD Biosciences, Ontario Canada) using *Hind*III and *Bam*HI restriction enzymes (New England Biolabs, Ontario,

Canada). The generated eGFP-tagged nectin-2 δ was transfected using Lipofectamine 2000 into MDCK cells. Stable clones were selected using 500 μ g/ml of G418 in DMEM + 10% FBS + 1% Penn/Strep for 2 weeks then maintained after selection in DMEM + 10% FBS containing 300 μ g/ml G418.

Dynamin 3 cDNA was subcloned from the pEGFP-C1-Dynamin-3 plasmid (Kamioka, Fukuhara et al. 2004) into a dsRed-C1 plasmid (BD Biosciences, Ontario, Canada) using *Eco*RI and *Bam*HI restriction enzymes (New England Biolabs). Stably transfected eGFP-tagged nectin-2 δ MDCK cells were transiently transfected with the dsRED-dynamin-3 construct using FuGene-6 as described by the manufacturer. Transfection controls consisted of transfecting the dsDed plasmid alone, using Fugene-6 only, and using nectin-2 MDCK cells that were not exposed to the transfectant or the plasmid at all.

Results

Dynamin 3 localizes to tubulobulbar complexes and the base of spermatid tails in the testis

Dynamin 3 previously has been demonstrated to be abundant in the testis (Diatloff-Zito, Gordon et al. 1995). To determine the accurate localization and possible function of this protein, we immunostained dynamin-3 in testis sections. Using previously characterized antibodies (Cao, Garcia et al. 1998), we localized dynamin-3 to apical (Figure 3.1A,B) and basal (Figure 3.1C) tubulobulbar complexes and to the base of elongate spermatid tails (Figure 3.1B). Tubulobulbar complexes are finger-like projections that form at intercellular junctions prior to sperm release and translocation of spermatocytes from basal to adluminal compartments of the epithelium. Staining patterns were confirmed on fragmented testis material and protein abundance was assessed by Western blotting showing increased levels of dynamin-3 in seminiferous epithelium versus whole testis lysates when run at equivalent protein concentrations (Figure 3.1B). Labeling at ectoplasmic specializations and antibody controls all were negative (Figure 3.1A,B).

Ultrastructural localization of dynamin 3 at tubulobulbar complexes and the base of spermatid tails

In order to determine the ultrastructural localization of dynamin-3 at tubulobulbar complexes, we performed immunoelectron microscopy. Dynamin-3 specifically labeled the membranous portion of the proximal tubular region of the tubulobulbar complex (Figure 3.2). Ultrastructural localization of dynamin-3 at the base of spermatid tails encircled the amorphous, flocculent material adjacent to the capitulum and striated

column between the spermatid head and the mitochondrial sheath (Figure 3.3). This staining was again absent from the ectoplasmic specializations.

Dynamin 3 localization in spermatozoa

The localization of dynamin-3 at the base of spermatid tails suggests a role during morphogenesis of the sperm tail or possibly some function in mature sperm or during fertilization. To provide insight into a possible role for dynamin 3 in the sperm tail, we investigated whether dynamin-3 also stained the connecting piece in spermatozoa stored in the epididymis, a result that would be consistent with a role in mature spermatozoa or during fertilization. Using spermatozoa from the cauda epididymis, we immunolocalized dynamin-3 and found it to be retained at the base of spermatozoa (Figure 3.4).

Dynamin 3 tubularizes nectin-2 *in vitro*

Dynamin proteins can function to tubularize membranes. To determine if dynamin-3 has this potential, we transiently transfected dsRed-tagged dynamin-3 into stably transfected eGFP-Nectin-2d MDCK cells. Nectin-2 is one of the key adhesion proteins at ectoplasmic specializations and the eGFP-Nectin-2d protein provides us a visual marker for the effects of dynamin-3 on junctions in MDCK cells. Using this *in vitro* approach, we demonstrated tubularization of eGFP-Nectin-2d at the cell periphery when transfected with the dsRed-dynamin-3 as compared to controls (Figure 3.5). In addition, there was an abundance of eGFP-Nectin-2d vesicles in the cytoplasm of cells transfected with dsRed-dynamin-3 relative to controls.

Dynamin 3 does not co-localize with dynamin 2

Dynamin 2 is concentrated in spermatids in regions where the acrosome is related to the nucleus and does not colocalize with dynamin 3 that concentrates at the base of

spermatid tails and at tubulobulbar complexes (Figure 3.6a,c). As dynamin 2 disappears from early and mid-staged spermatids, dynamin 3 transiently appears in regions along the dorsal curvature of spermatids (Figure 3.6c).

Actin and Arp2/3 are present at tubulobulbar complexes

Dynamin 2 can regulate the assembly of actin by the Arp2/3 complex (Schafer, Weed et al. 2002), and Arp2/3 is postulated to generate the actin networks associated with the tubular membrane invaginations of podosomes (Linder, Nelson et al. 1999), that also contain dynamin 2 (Ochoa, Slepnev et al. 2000). Actin networks are also associated with TBCs. In order to determine if Arp 2/3 may play a role in tubulobulbar formation, we determined the localization of the Arp 3 component of the Arp2/3 complex at tubulobulbar complexes. We found that probes for Arp 3 stained tubulobulbar complexes in a similar pattern to those for actin (Figure 3.7).

Discussion

Here we report that dynamin 3 is a component of tubulobulbar complexes and may be involved in their morphogenesis. We also have found that dynamin 3 is deposited in dense material surrounding major components of the connecting piece at the base of spermatid tails, and that it is retained in this position in epididymal spermatozoa.

Tubulobulbar complexes develop at sites of intercellular attachment between Sertoli cells and spermatids at the apex of the seminiferous epithelium, and between neighboring Sertoli cells at the base of the epithelium (Russell and Clermont 1976). The structures consist of elongate invaginations consisting of the plasma membranes of the two attached cells that protrude from sites of intercellular junctions into one or the other of the cells. A network of actin filaments “cuffs” each of the complexes and is classically described as part of the structures (Vogl 1989). A swollen bulbar region occurs near the end of each complex. This region lacks an associated actin network, but is closely related to cisternae of endoplasmic reticulum. A short tubular region ending in a “bristle-coated” pit occurs at the tip of each complex (Russell 1979; Russell 1979). The bulbar regions “bud” from the complex and are thought to fuse with lysosomes as part of the mechanism of junction disassembly. Double membrane vesicles occur next to the complexes (Pelletier 1988; Guttman, Takai et al. 2004) and adhesion molecules (nectins) have been immunolocalized to the vesicles (Guttman, Takai et al. 2004). Also, gap and tight junctions occur in tubular and bulbar regions of the complexes that form at basal junctions between neighboring Sertoli cells (Russell 1979), and in related vesicles (Pelletier 1988). In addition, some tubulobulbar complex associated vesicles react positively for acid phosphatase (Russell 1979) and lysosomal markers (Guttman, Takai et

al. 2004). All of these observations are consistent with the conclusion that tubulobulbar complexes are “junction internalization machines” used by Sertoli cells to eliminate large surface areas of intercellular contact during two processes fundamental to spermatogenesis and male fertility: sperm release, and translocation of spermatocytes from basal to adluminal compartments of the seminiferous epithelium.

Little is known about the morphogenesis of tubulobulbar complexes, however the tubular morphology of the system suggested to us that one of the components involved might be a dynamin GTPase. One of the classical described functions of the dynamins is to facilitate vesicle separation from parent membranes during endocytosis (McNiven 1998). The proteins associate with the necks of vesicles and function as a mechanoenzyme to “pinch off” vesicles from parent membranes when the systems are under tension, likely provided by the actin cytoskeleton (Roux, Uyhazi et al. 2006). In the absence of GTP or in the presence of non-hydrolyzable analogues of GTP, the dynamins can generate tubules with diameters of 25 nm from artificial membranes (Takei, McPherson et al. 1995; Sweitzer and Hinshaw 1998). When GTP_i is added to the system, the tubules vesiculate. In addition to a role in vesiculation, the dynamins can influence actin-membrane dynamics (Gray, Kruchten et al. 2005) and Arp2/3 mediated actin network formation (Schafer 2002). Tubulobulbar complexes are tubular in structure, include Arp2/3 enriched actin networks as part of their structure (this paper), and are associated with vesicle formation and internalization, albeit the vesicles are much larger than those involved in conventional endocytosis.

Dynamins are not the only proteins that initiate membrane elongation and tubulation in the cell. Other proteins associated with this function are sar1p, ephrins, and

amphiphysin. Tubular structures are also seen in the endoplasmic reticulum. Sar1p is essential for vesicle formation from the endoplasmic reticulum (Barlowe, d'Enfert et al. 1993). Ephrins play a role in cell-cell interaction and have been shown to play roles in embryonic patterning (Adams, Diella et al. 2001), neuronal targeting (Wilkinson 2001) and vascular assembly (Poliakov, Cotrina et al. 2004; Davy and Soriano 2005). This protein is believed to use the clathrin-mediated endocytic pathway to form invaginations and vesicles between adjacent cells (Parker, Roberts et al. 2004). Amphiphysin on the other hand has been implicated in the recruitment of dynamin resulting in tubular invagination of membranes as well as vesicle formation (Grabs, Slepnev et al. 1997). Tubular membrane structures similar to membrane invaginations associated with dynamins have also been shown to occur in the endoplasmic reticulum (Bard and Malhotra 2006). These associating proteins may also be associated with TBCs, however localization of these proteins has not been determined. In addition, tubular formation at endoplasmic reticulum around the bulbous regions of the TBC may play a role in the recycling of the plasma membrane.

Immunological probes for dynamin 3 label the length of the tubular parts of tubulobulbar complexes, both at apical and at basal sites of intercellular contact. At the ultrastructural level, the probes label regions directly adjacent to the Sertoli cell plasma membrane between this membrane and the actin "cuff". Probes for dynamin 2 were not reactive above background with tubulobulbar complexes. We conclude that dynamin 3, not dynamin 2, is a major component of tubulobulbar complexes and is located along the plasma membrane of the tubular invaginations of the Sertoli cell. Dynamin 2 is localized to the acrosome of spermatids and is generally consistent with previous accounts of

where staining was observed in early maturation spermatids and spermatocytes (Iguchi, Watanabe et al. 2002).

Although tubulobulbar complexes of the seminiferous epithelium are unique to this tissue, they bear a striking resemblance to podosomes that occur at sites of cell/substrate adhesion in osteoclasts and in cells transformed by the Rous sarcoma virus (Ochoa, Slepnev et al. 2000). Podosomes in these systems and tubulobulbar complexes both consist of cores of tubulbar membrane surrounded by networks of actin filaments; however, tubulobulbar complexes differ from these podosomes in a number of important ways. Tubulobulbar complexes are formed by two attached plasma membranes at sites of intercellular contact whereas podosomes are formed by the invagination of the plasma membrane of a single cell usually at sites of cell/substratum attachment. As a consequence, the membrane cores of tubulobulbar complexes are roughly twice the diameter of podosome cores. Another difference is that tubulobulbar complexes are associated with membrane internalization (Russell 1979; Russell 1979), whereas podosomes likely are not (Ochoa, Slepnev et al. 2000). Also, tubulobulbar complexes have at their tips what appear to be "bristle-coated" pits (Russell 1979; Russell 1979), while podosomes are not obviously associated with clathrin coated pits or vesicles (Ochoa, Slepnev et al. 2000). Finally, tubulobulbar complexes are associated with dynamin 3, as we show here, whereas podosomes contain dynamin 2 (Ochoa, Slepnev et al. 2000). This latter difference may be related to differences in the diameters of the two structures.

Our observation that the transfection of dynamin 3 into MDCK cells stably transfected with nectin 2 generates irregular staining patterns at intercellular adhesion

junctions and results in the formation of nectin 2 containing tubular structures at intercellular attachment sites is consistent with the conclusion that dynamin 3 may somehow influence junction membrane patterning, possibly through an influence on junction related actin networks.

Based on previous morphological descriptions of tubulobulbar complexes (Russell and Clermont 1976; Russell 1979; Russell 1979) and on our findings, we propose the following model for the development of tubulobulbar complexes (Fig. 3.8). A "coated" pit forms at the plasma membrane of one of the cells and initiates the invagination of the junction into the Sertoli cell. The recruitment of dynamin 3 at the neck of the forming vesicle together with the initiation of Arp2/3 mediated growth of an actin cuff begins the process of elongation of the neck into a double membrane tube. The continued recruitment of dynamin 3 and growth of the actin network extend the tube. It is possible that dynamin 3 influences actin network formation through actin binding proteins such as cortactin. Cortactin is present in actin containing structures associated with adhesion junctions where tubulobulbar complexes form (Kai, Irie et al. 2004), has been found to mediate the effect of dynamin 2 on actin kinetics in other systems (McNiven, Kim et al. 2000; Schafer, Weed et al. 2002), and has been implicated with dynamin 3 in the formation of dendritic spines (Gray, Kruchten et al. 2005). Formation of the bulbar region of the complex may be associated with loss of dynamin 3 and the related actin cuff. Separation of the bulbar region from the proximal tubular part of the complex could result from activation of the "pinchase" function of dynamin 3. Alternatively, or in conjunction with dynamin 3, changes in the status of the actin cuff may lead to "budding" from or vesiculation of the complex. Supporting the involvement

of actin in the process of membrane internalization is the observation that tubulobulbar complexes either do not form properly or vesiculate following intratesticular injection of cytochalasin D (Russell, Saxena et al. 1989).

The finding of large deposits of dynamin 3 in regions of the sperm tail that connect the head to the tail is unexpected, novel and also perplexing. The deposits mainly surround the capitulum and striated columns of the connecting piece and are not obviously associated with actin networks, membranes, or endocytic structures. Although we can not rule out the possibility that dynamin 3 may play a role in the morphogenesis of the connecting piece, the presence of these deposits both in mature spermatids of the seminiferous epithelium and in spermatozoa stored in the cauda epididymis suggest additional functions as well. The protein may play a structural role in reinforcing the connecting piece during flagellar beat. Another, and potentially more exciting possibility is that the deposits are a reservoir of dynamin 3 for use during fertilization or for events that occur in the ovum subsequent to fertilization. The presence of these deposits in regions of the tail nearest the equatorial segment of the head where fusion with the egg occurs is consistent with this possibility.

Although dynamin 3 previously has been shown to be expressed in Sertoli and spermatogenic cells, we have now localized the protein to specific structures. In Sertoli cells, dynamin 3 is concentrated in tubulobulbar complexes and may function in morphogenesis and vesiculation of the structures. In spermatogenic cells, the protein is present in large deposits in the connecting piece. The function of dynamin 3 in this location has yet to be determined.

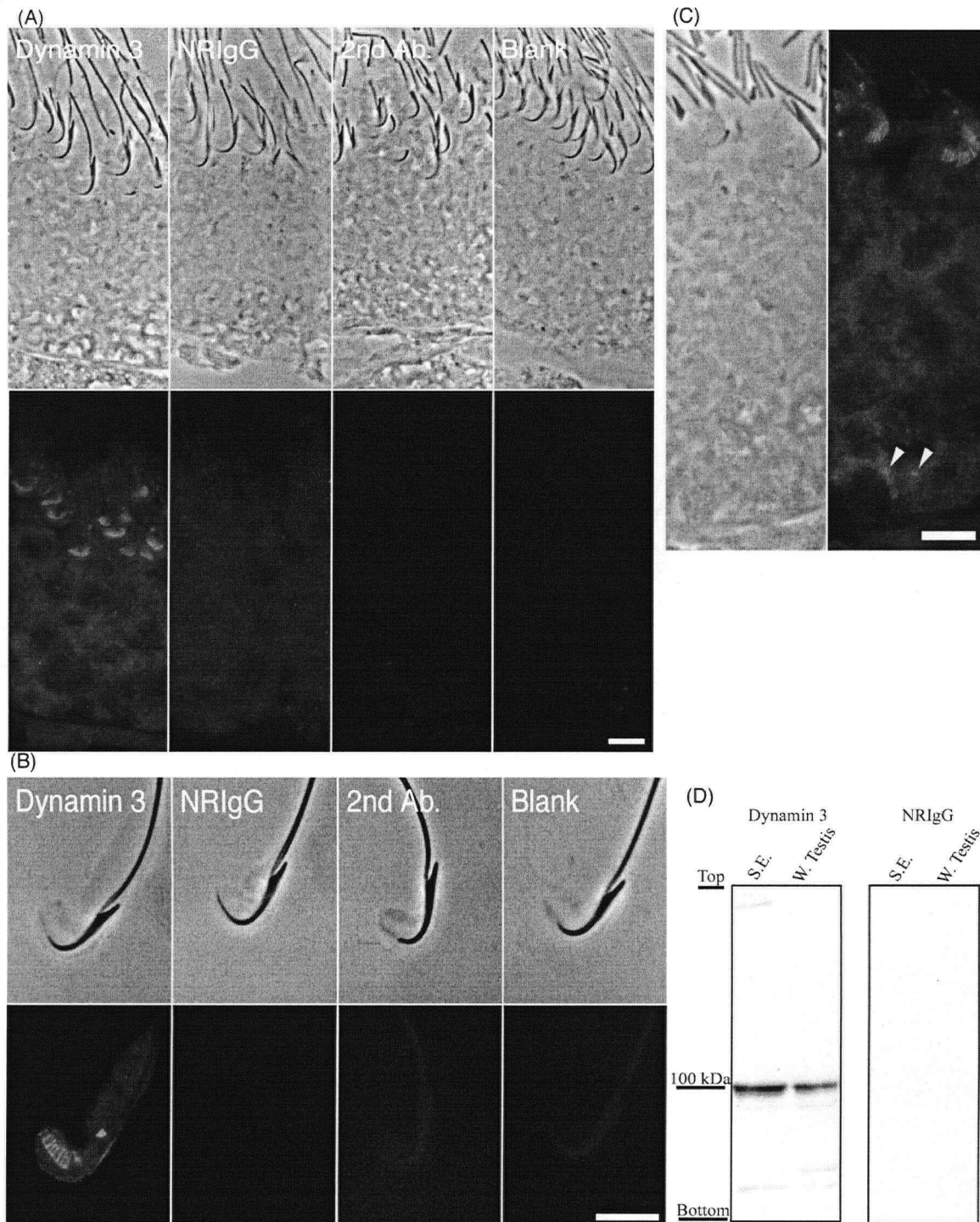


Figure 3.1: Localization of dynamin 3 on fixed sectioned (A) and fragmented (B) tissue at apical sites. Staining is prominent at TBCs (arrows) as compared to IgG and secondary antibody controls. In fragmented tissue, staining also is present at the base of the spermatid tail where the head and tail meet (arrowhead). (C) Dynamin 3 staining of TBCs at basal sites. At these sites, pairs of bright dots (arrows) are seen. This staining pattern is consistent with basal tubulobulbar complexes which are much smaller than the complexes at apical sites. (D) Western blot analysis of dynamin 3 on isolated seminiferous epithelial tissue as well as whole testis. A specific band at 100 kDa is seen in both lanes compared to the IgG control. Also, the intensity of staining is higher in isolated seminiferous epithelial tissue as compared to whole testis when loaded at equal concentrations, a result consistent with dynamin 3 localization to the epithelium.

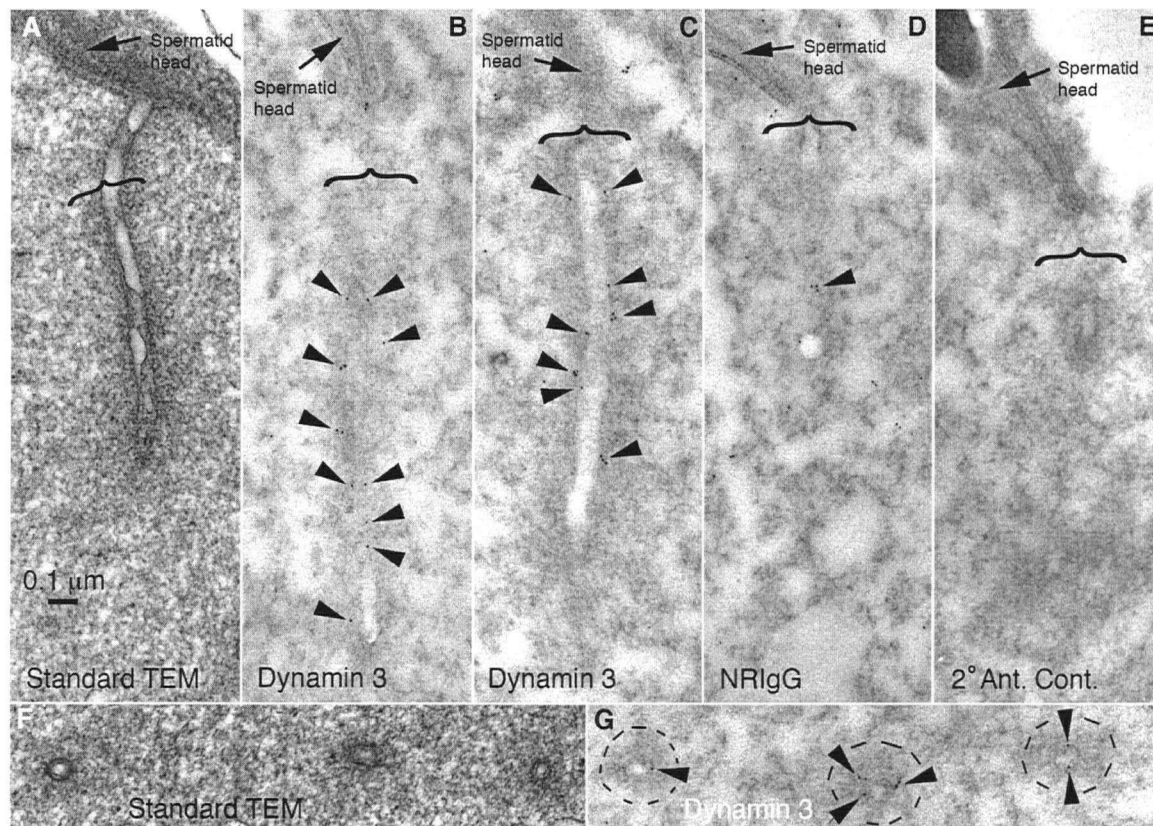


Figure 3.2: Immunoelectron microscopy of longitudinal and cross sections of TBCs labeled for dynamin 3. (A) Standard TEM of an apical tubulobulbar complex. The TBC forms as an invagination into the Sertoli cell. (B) and (C) Longitudinal sections of apical tubulobulbar complexes labeled first with an antibody to dynamin 3 then with a second antibody conjugated to 5 nm colloidal gold particles. Gold particles are localized along the proximal tubule of the tubulobulbar complex adjacent to the Sertoli cell plasma membrane. (D) NR1gG control. While some colloidal gold is still present, staining does not specifically localize to the tubulobulbar complex membrane and the pattern of staining is random. (E) Secondary antibody only. No staining is present. ({}): indicates the location of the tubulobulbar complex and arrowheads highlight colloidal gold particles. (F) Standard TEM of cross-sectioned tissue containing tubulobulbar complexes. (G) Cross-sectioned tissue containing tubulobulbar complexes labeled for dynamin 3. Gold particles are associated with the Sertoli cell plasma membrane. TBCs are circled with a dashed line.

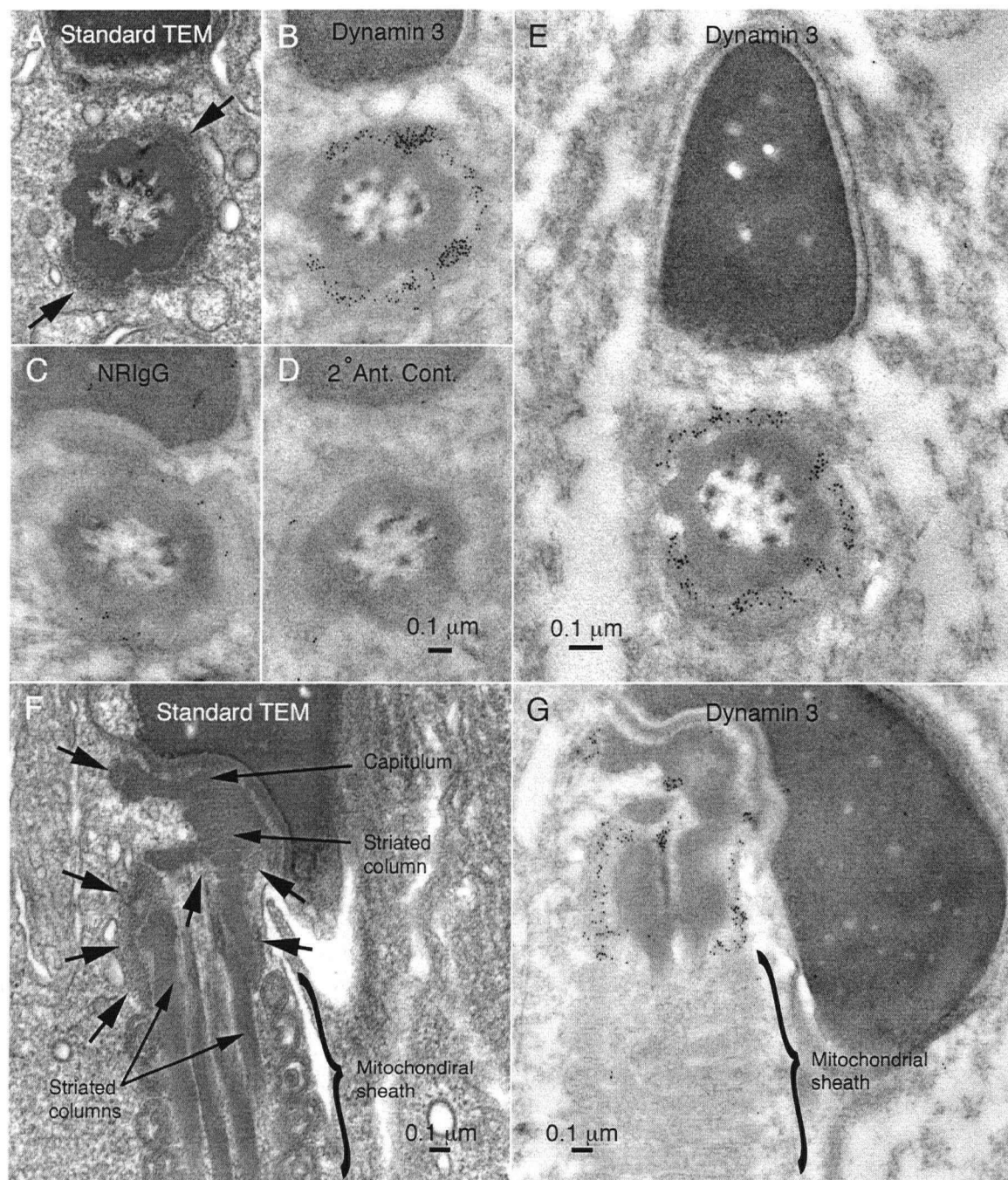


Figure 3.3: Immunoelectron microscopy of longitudinal and cross sections of spermatid tails labeled for dynamin 3. (A) Standard TEM of a cross-sectioned spermatid at the base of the spermatid tail. Arrows indicate the location of dense material that is shown to be positive for dynamin 3 in (B) and (E). (C) Normal rabbit IgG control and (D) secondary antibody control. While some colloidal gold particles are seen associating around the base of the spermatid tail, there is a dramatic reduction as compared to sections treated with primary antibody. (F) Standard TEM of a longitudinal section of a spermatid. The capitulum, striated columns and the mitochondrial sheath of the spermatid are indicated. Arrows indicate the dense flocculent material shown to label with the dynamin 3 antibody in (G).

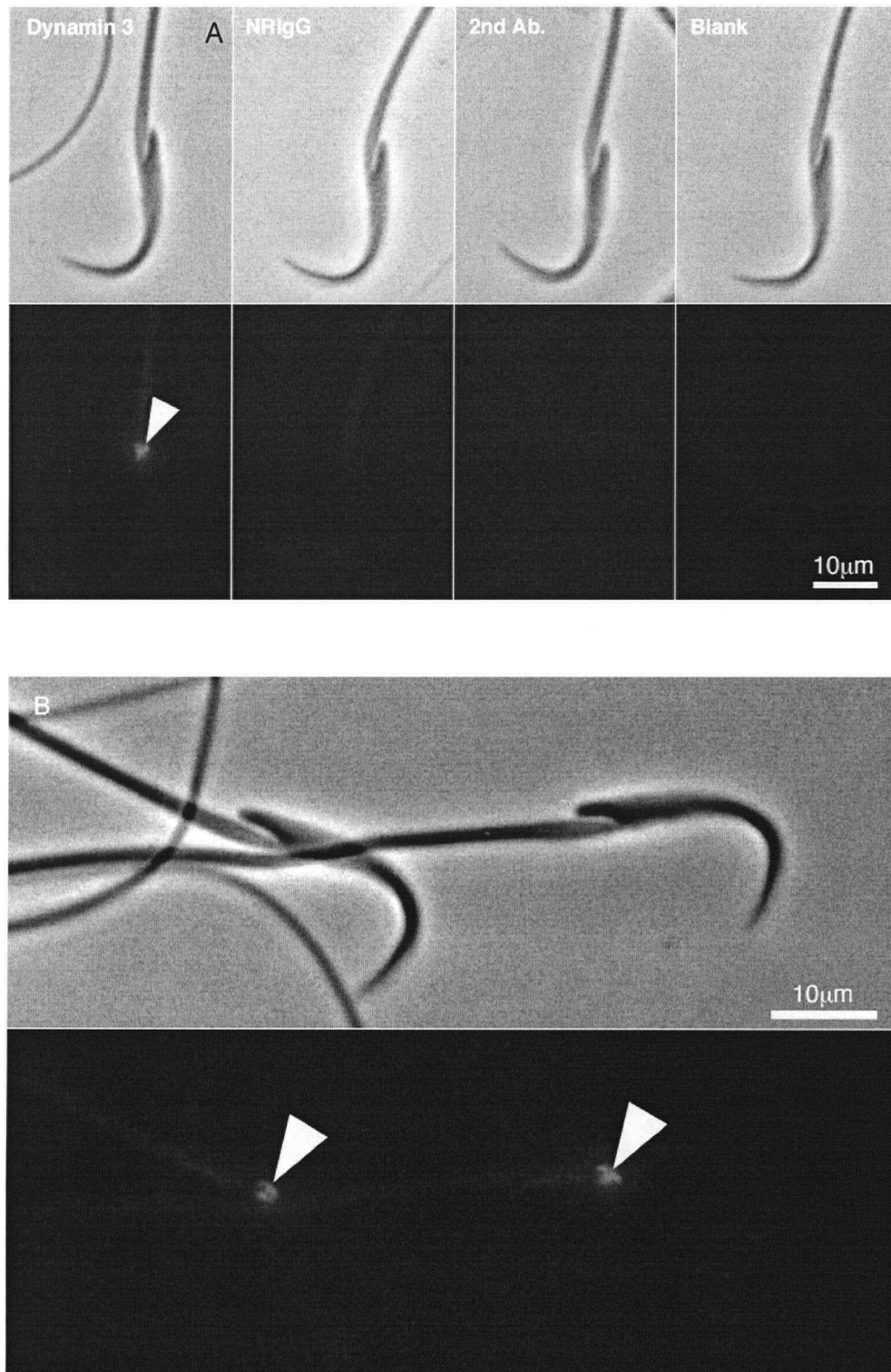


Figure 3.4: Immunofluorescence of dynamin 3 in the tails of epididymal spermatozoa. (A) Epididymal spermatozoa labeled for dynamin 3 and associated IgG, secondary antibody and autofluorescence (blank) controls. Dynamin 3 staining is present at the base of the sperm tail in the same location as was observed in testicular spermatids. (B) Magnified view of dynamin 3 staining at the base of the spermatid tail. Arrowheads indicate localization of dynamin 3. In addition to intense staining in the connecting piece, notice that weak staining extends distally approximately 10 mm into the midpiece of the tail.

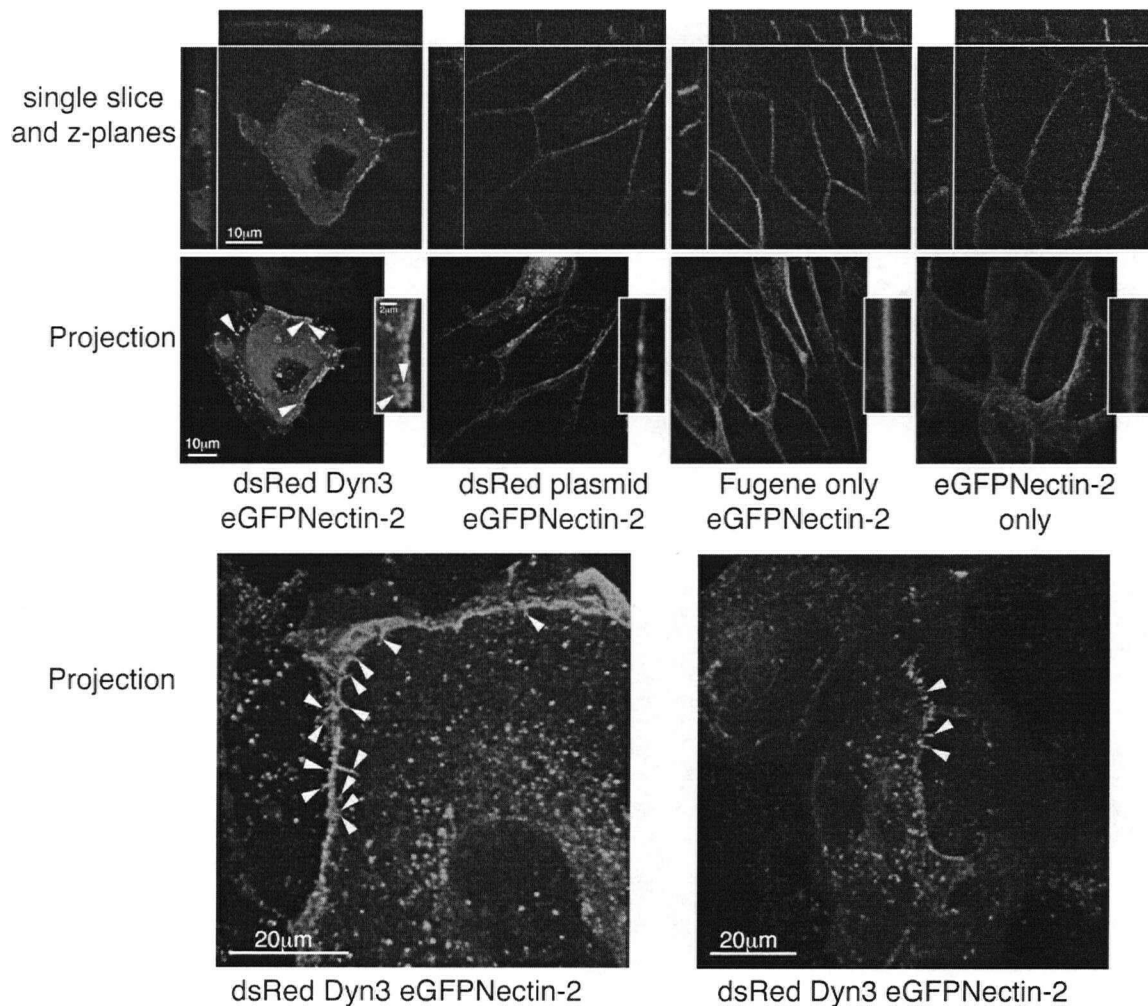


Figure 3.5: Stably transfected eGFP nectin-2 MDCK cells transfected with ds-red tagged dynamin 3. In cells transfected with dynamin 3, tubular structures are seen extending from the membrane (arrowheads). Tubular structures are absent in control cells transfected with the ds-tagged plasmid only, FuGene 6 only and nectin 2 transfected cells only. Projections, single slice and Z-planes have been taken to display tubule formation when dynamin 3 is present as compared to non-transfected cells. Picture insets provide a detailed view of irregular sites of intercellular contact and tubule formation compared to control projections. Large projections (bottom) display tubules and a general “ragged” appearance of nectin 2 at cell-cell adhesion contact points. Not all dynamin 3 transfected cells fluoresced red because the ds-red failed to tetramerize. However, differences in dynamin 3 transfected vs non-transfected cells are still evident.

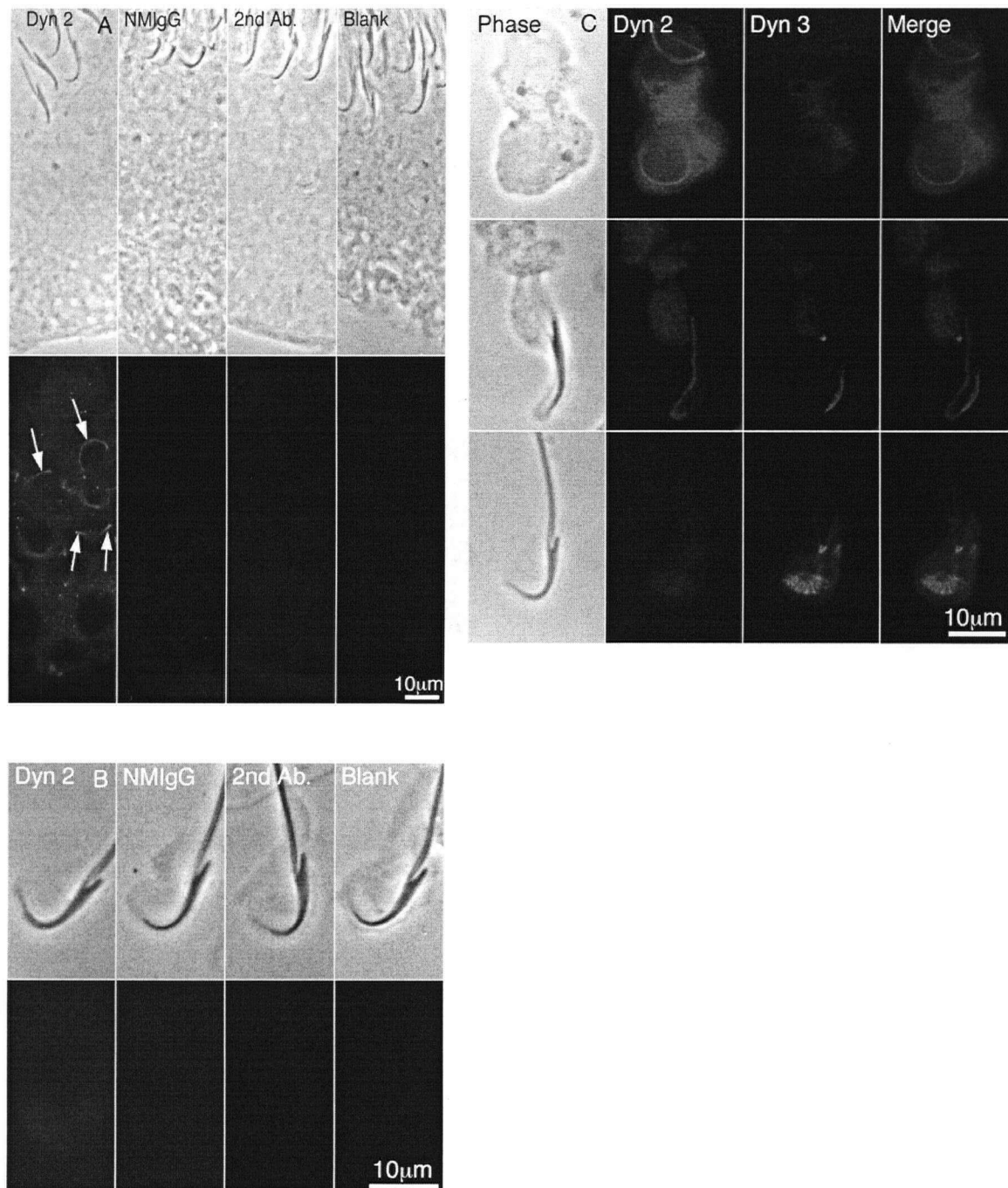


Figure 3.6: Localization of dynamin 2 in fixed sections and fragments of seminiferous epithelium. (A) Immunofluorescence of dynamin 2 on sectioned testicular tissue. Dynamin 2 is localized in early spermatids and spermatocytes. Particularly evident is localization to the acrosome in early spermatids (arrows). This staining is present in early stages and disappears during spermatid elongation. All antibody controls are negative for staining. (B) Immunolocalization of dynamin 2 associated with late spermatids. Staining is negative. (C) Co-localization of dynamin 2 and dynamin 3 at early, mid and late staged spermatids. Dynamin 2 localizes in early spermatids, is still present at mid staged spermatids along the concave surface of the cell and disappears at later stages. Dynamin 3 is not present at early stages, but does appear transiently along the convex curvature of the head in mid-staged spermatids. Dynamin 3 labels only tubulobulbar complexes associated with late spermatids. Merged figures indicate no co-localization of the dynamin 2 and dynamin 3 isoforms at any stages of spermatogenesis.

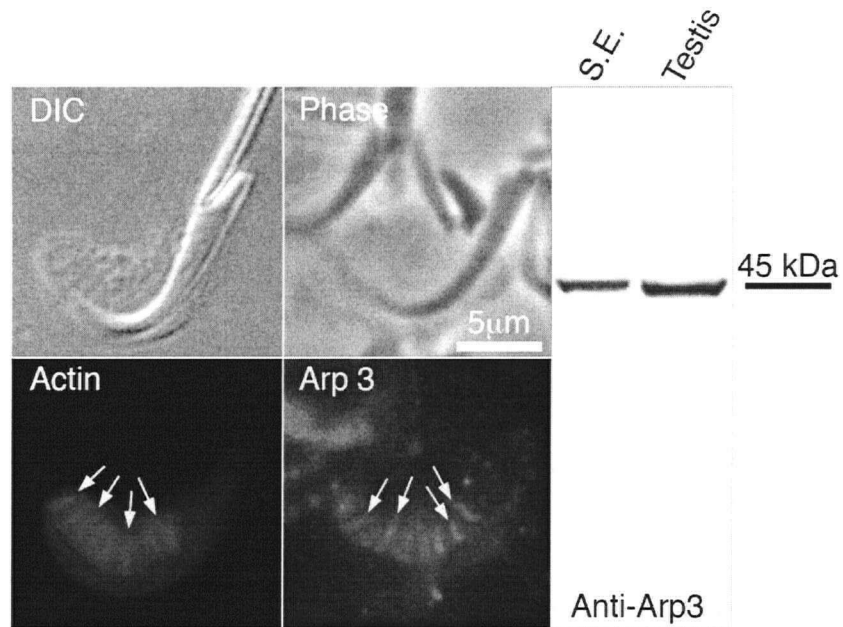


Figure 3.7: Actin and Arp 3 localization at tubulobulbar complexes. Actin and Arp 3 are localized along the proximal tube of tubulobulbar complexes (arrows). Anti-Arp3 antibody is specific and stains for a specific band at 45 kDa in isolated seminiferous epithelium and whole testis lysates.

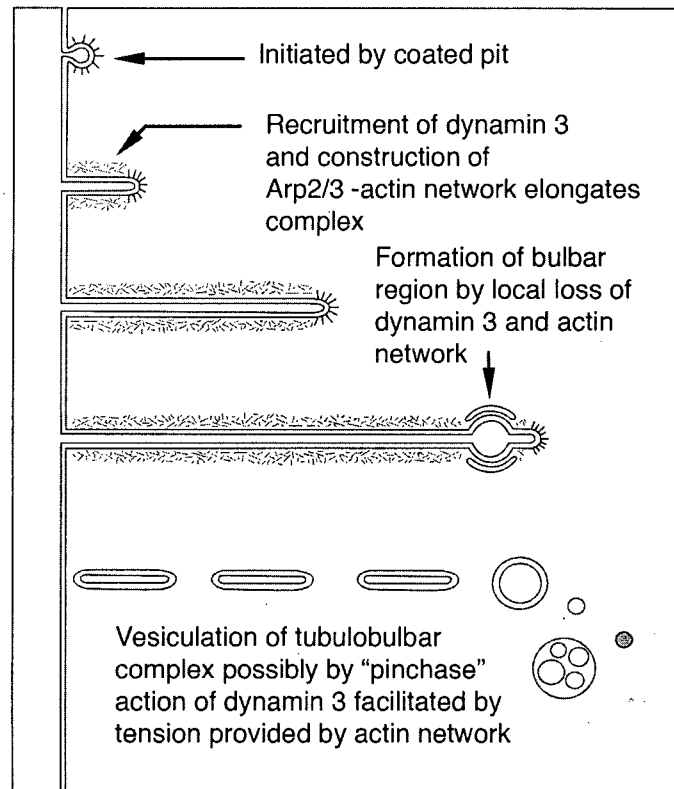


Figure 3.8: Proposed model for the formation and vesiculation of tubulobulbar complexes.

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Chapter 4

Final Discussion and Conclusions

The data presented in this thesis provides insight into spermatogenesis as well as to basic cell biology concepts. These include motor protein based transport systems as well as internalization of adhesion junction components. Work in this thesis supports the hypotheses that ectoplasmic specializations are transported by motor proteins along microtubule tracts and that dynamin 3 may be part of the mechanism by which these junctions are internalized by tubulobulbar complexes upon the completion of spermatogenesis.

Kinesin project

Microtubule based transport of adhesion junctions in the testis has been studied for some time (Redenbach, Boekelheide et al. 1992; Beach and Vogl 1999; Vogl, Pfeiffer et al. 2000; Guttman 2003). Most research has been focused on the orientation of the microtubule tracts, and on running microtubule binding and motility assays on isolated junctions. Although a kinesin has not been localized to the junctions, cytoplasmic dynein has (Guttman, Kimel et al. 2000).

Because dyneins have been shown to move towards the minus end of microtubules, and microtubules in Sertoli cells have their minus end at the apex of the cell, a likely candidate for movement to the positive end of microtubules or to the base of the Sertoli cell would be a kinesin. Previous work using mRNA screens of Sertoli cells indicated Rab6KIFL (a Rab kinesin) was expressed at a high levels and was the only kinesin highly expressed in Sertoli cells as compared to the whole testis. This gave a possible suggestion that this kinesin may be involved in spermatid translocation.

Rab6KIFL is a kinesin that was found to regulate membrane trafficking in Golgi as well as required for cytokinesis and localized to spindles during mitosis (Echard, Jollivet et al. 1998; Echard, Opdam et al. 2000; Hill, Clarke et al. 2000). However, as the functions of this protein differed in other cells, as well as Sertoli cells being non-dividing cells, the possibility existed that this kinesin functions in spermatid translocation.

A proteomics approach identified a Kif1-like kinesin as another candidate kinesin that was present in Sertoli cells. However, I was unable to produce any specific antibodies to this family.

The results of my research indicate that antibodies raised against Rab6KIFL localize to ectoplasmic specializations in Sertoli cells. The antibodies react with the cytoplasmic face of the adhesion junction as would be predicted for a kinesin that was involved in spermatid translocation. In addition the Rab6KIFL antibodies associate at ectoplasmic specializations at times when the spermatid is translocating to the base of the Sertoli cell.

One issue which remains to be addressed is the different molecular weights and staining patterns of the antibodies raised against Rab6KIFL. In total, three of the produced antibodies react with Rab6KIFL at two different molecular weights. Also the staining pattern of the two that react at a higher molecular weight are not specific to ectoplasmic specializations. Further characterization of these antibodies would be required to determine if they are reacting to a different isoform of Rab6KIFL or another non-specific protein. Multiple isoforms of this protein would not be atypical judging by the multiple functions of this protein.

Tubulobulbar Complexes Project

Tubulobulbar complexes are structures that have largely been overlooked in the past by researchers. These structures are small and are unique to Sertoli cells. The data presented in this thesis supports the hypothesis that dynamin 3 is a component of TBCs and may be involved in their morphogenesis.

Tubulobulbar complexes form at sites previously occupied by ectoplasmic specializations. These complexes are composed of a double membrane as they protrude from one cell into another and bud double membrane vesicles. Tubulobulbar complexes form just prior to spermatid release at apical sites and between adjacent Sertoli cells at basal junction sites. It is believed that these complexes internalize junction components to release spermatids and to allow spermatocytes to cross the blood-testis barrier at the base of the epithelium. Results from this thesis indicate that tubulobulbar complexes are associated with dynamin 3, a member of the dynamin proteins that are generally endocytic GTPase proteins that result in the pinching off of vesicles from parent membranes. Dynamin 3 is only found along the tubular stalk of tubulobulbar complexes and not at ectoplasmic specializations. The formation of tubular structures at intercellular adhesion sites in MDCK cells transfected with dynamin 3 indicates that dynamin 3 may be involved with the morphogenesis of TBCs in vivo.

Overall Significance

In terms of cell biology, the significance of the both projects is important. First, the kinesin project provides evidence that a kinesin motor protein couples an adhesion junction to a microtubule based transport system. Implicitly, this implies that a kinesin is capable of moving an entire cell which in this case is a developing spermatid. In addition, an antigen reactive to a Rab6KIFL antibody has been localized at ectoplasmic

specializations. If in fact this antigen is Rab6KIFL, spermatid translocation would be a new role for the Rab6KIFL kinesin. The possibility of multiple isoforms of this protein exists.

The Tubulobulbar complex project is significant in that very little work has been performed on these structures and very little is known about the mechanism of how these structures work. Here, we have demonstrated that dynamin 3 is a component of these structures and may play a role in their morphogenesis. If dynamin 3 also is involved in vesiculation of tubulobulbar complexes, the protein would be directly involved with junction internalization and junction turnover.

The identification of dynamin 3 at Tubulobulbar complexes offers insight into how ectoplasmic specialization disassembly occurs and possibly why these junctions contain endoplasmic reticulum (to recycle and package vesicles).

Both projects, in addition to their cell biology significance may in the future lead to a better understanding of spermatogenesis. This could lead to important advances in the study of male fertility as well as the identification of male contraceptive targets.

Future Directions

While the presented data is consistent with the hypothesis that a kinesin is associated with ectoplasmic specializations, it is still not certain that the kinesin is Rab6KIFL and that it is associated with spermatid translocation in vivo. To determine if in fact that this protein is involved in this translocation event, binding and motility assays would be required. These assays would determine if Rab6KIFL could bind to ectoplasmic specializations and microtubules and travel towards the positive polar end of the

microtubule. In addition, it may be interesting to determine the exact stage of Rab6KIFL localization occurs and the effects on spermatogenesis if this protein is blocked.

The Tubulobulbar project should be continued with a number of key questions remaining to be addressed. It is currently unknown what pathway recruits dynamin 3 to Tubulobulbar complexes. Tubulobulbar complexes are only present at specific stages of spermatogenesis when adhesion junctions are known to disassemble. Dynamin 3 is also only present at these stages. Further study is required to determine the mechanism that enlists dynamin 3 to the adhesion junction site and if Tubulobulbar complexes play other roles in spermatogenesis. Other specific experiments that need to be performed are electron microscopy of transfected MDCK cells and colocalization of dynamin 3 with Arp3. It may also be interesting to determine if junctions between MDCK cells are broken down or if tubular invaginations into adjacent cells remain. Electrical conductance experiments measurements could answer this question.

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Animal Care Certificate

Application Number: A05-0094

Investigator or Course Director: Wayne A.W. Vogl

Department: Anatomy, Cell Biology & Physiology

Animals Approved:

Mice
10
Rats
57

Start Date: 2003-3-1

Approval Date: 2005-4-24

Funding Sources:

Funding Agency:	Canadian Institutes of Health Research
Funding Title:	Kinesin associated with an adhesion junction: spermatid translocation.

Unfunded title: n/a

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility

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ANIMAL CARE CERTIFICATE

Application Number: A05-0094

Investigator or Course Director: Wayne A.W. Vogl

Department: Cellular & Physiological Sc.

Animals:

Mice
CD
10
Rats
SD
57

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