DEPLETION OF DOCOSAHEXAENOIC ACID (DHA) AND ARACHIDONIC ACID CAUSES MALE INFERTILITY BY DISRUPTING BOTH SECRETORY GRANULE BIOGENESIS AND ADHERENS JUNCTION ASSEMBLY IN TESTIS

BY

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DISSERTATION

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Abstract

An insufficiency in omega-3 fatty acids has been linked to a wide variety of health concerns including Alzheimer’s disease, CHD, sub-optimal fetal development and male infertility. The long chain omega-3 docosahexaenoic acid (DHA) and omega-6 arachidonic acid (ARA) are highly unsaturated fatty acids (HUFAs) preferentially enriched in the sn2 position of membrane phospholipids and ARA is abundant in tissues throughout the body. DHA, however, is preferentially enriched in the brain, retina and testis, and although recent epidemiological and clinical evidence has established a relationship between DHA consumption and sperm health, the essential roles for DHA in the testis remain largely unexplored.

By disabling the rate-limiting step in the endogenous conversion of dietary essential 18-carbon polyunsaturated fatty acids to ARA and DHA, the fatty acid desaturase2 (Fads2) knockout mouse model allows for the creation of a HUFA-selective deficiency. Use of this Fads2 -/- model led to the discovery of HUFA essentiality in male fertility and supporting the proper maturation of germ cells within the testis. Here, we sought to define the molecular nature of this HUFA-deficient male infertility, with an emphasis on spermatid adhesion and acrosome formation.

Dietary ARA or DHA was found to be sufficient in restoring the distribution of vesicular trafficking proteins that promote the biogenesis of an organelle unique to sperm, the acrosome. In contrast, ARA was much less effective than DHA in restoring germ cell adhesion integrity. Assessments of cell-cell contacts which form transiently to adhere maturing germ cells to neighboring nurse cells (Sertoli cells), revealed a mislocalization of essential adhesion molecules nectin-2 and nectin-3 in animals deficient in both HUFAs. Further, the loss of nectin-2 localization in Sertoli cells was selective, with only Sertoli-spermatid contacts showing disruption, while Sertoli-Sertoli contacts of the Blood-testis barrier (BTB) displayed normal protein organization. Indeed, the BTB was intact by all accounts: by functional evaluations using biotin tracer injections, ultrastructural assessments using electron microscopy, and adhesion molecule localization using IF.
In conclusion, this study revealed essential roles for testis HUFAs as regulators of germ cell adhesion integrity, cell adhesion molecule localization and vesicular trafficking protein distribution. The selective impairment of Sertoli-spermatid adhesion, but not Sertoli-Sertoli adhesion (blood-testis barrier) by HUFA deficiency, taken together with the ineffective adhesion restoration seen with ARA feeding, suggests that dietary DHA intake, sufficient to maintain testis enrichment, is critical for the healthy production of spermatozoa.
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Chapter 1: Introduction

Major research question
What is the functional significance of reducing highly unsaturated fatty acids (HUFAs) from the lipid bilayers of cell types typically enriched in HUFAs?

It has long been understood that the fidelity of the primary structure of a protein is crucial to the function of that protein. Taking what should be a lysine residue, in the primary structure, and replacing it with a glycine residue is a structural change capable of inducing a profound effect on protein function. This research aims to ask, is there is a parallel fidelity required of the fatty acid composition of our cells? For example, if a cell type normally enriched in the omega-3 fatty acid docosahexaneoic acid (DHA), has a certain amount of this DHA replaced with some fatty acid that is not DHA (e.g. the omega-6 arachidonic acid), how does this change in structure affect function within the cell? This basic question serves as the foundation for the research presented in this document.

Are we underconsuming omega-3?
The idea that certain fatty acids might be indispensable components of the diet was introduced in earnest in 1929, when the dietary essentiality of an omega-6 fatty acid was demonstrated by Burr and Burr. Weanling rats were fed a diet completely devoid of fat and this treatment was shown to uniformly induce dermatitis and severe growth retardation (Burr and Burr, 1930). The authors went on to show that this dietary fat deficiency phenotype could be rescued by feeding the long-chain, polyunsaturated omega-6 fatty acid, linoleic acid (Burr and Burr, 1930). This early finding clearly established the existence of a minimum intake requirement for fat in the diet, however human evidence for linoleic acid deficiency could not be shown at this time (Holman, 1998) and the actual minimum dietary intake requirement for omega-6 fatty acids in humans remains poorly understood even today.

By 1960, although essential functions for omega-6 PUFAs had been established, other forms of dietary fat were increasingly being hypothesized as contributing factors to disease risk. In 1961, based largely on an increased prevalence of CVD in the United States, and an association between dietary fat intake and plasma cholesterol levels, the American Heart Association issued a report where they recommended limiting total fat intake to reduce the risk of heart attack and stroke (AHA, 1961). The data to support this recommendation was largely cross sectional, as the
diets of Americans consuming approximately 40-45% of their energy from fat were compared to populations in other parts of the world where daily fat intake added up to only roughly a third of what Americans were consuming, and where the risk of heart attack or stroke was much lower (AHA, 1961). This report went on to highlight saturated fat as a subgroup of fat to be reduced, recommending that polyunsaturated fats found in oils from fish or plants – both corn and soy are mentioned – should be used to replace some of the saturated fat in the diet coming from high fat dairy and meat. Whereas the findings of Burr and Burr in 1929 had established the existence of a minimum requirement for the consumption of certain PUFAs, this report hypothesized that an upper limit intake level of dietary fat was being exceeded in the U.S., although no specific threshold to stay below was provided at the time (AHA, 1961).

In the 1970’s, landmark work by Dyerberg and Bang began highlighting the differences among dietary sources of PUFAs and calling into question the relevance of reducing total fat intake as an effective strategy for lowering CVD risk. The researchers examined an indigenous Eskimo population from the western coast of Greenland, previously understood to have very low levels of heart disease (Bang et al., 1980), and compared them to a Danish reference group consuming a more Western diet. Analysis of plasma lipids revealed that despite a high intake of fat – between 35 and 60% of energy – Greenland Eskimos had significantly lower plasma cholesterol and triglyceride levels than Danish controls, and that this difference was even more pronounced among older adults (Bang et al., 1971). Later, more detailed analysis of fatty acid levels in the diet and plasma in this Eskimo population emphasized marked differences in omega-3 levels. Among polyunsaturated fatty acid intake, Eskimos consumed significantly less 18-carbon omega-6 linoleic acid than Danes (5% of energy vs. 10%) and substantially more long-chain omega-3 (13.1% vs. 0.8%) (Bang et al., 1980). Indeed, this dietary difference was reflected in fatty acid levels of phospholipids in the plasma, with long-chain omega-3 levels of DHA and EPA significantly elevated in Greenland Eskimos compared to Danes, and omega-6 linoleic and arachidonic acid levels significantly reduced (Dyerberg et al., 1975).

The dietary habits and fat intake of Greenland Eskimos were not entirely inconsistent with the recommendations put forward in the 1961 AHA report, but a very critical distinction needs to be drawn. Although the total amount of energy from fat in Greenland Eskimos was comparable to that of the average American diet (~40% of energy), the saturated fat was largely replaced with
the fat from fish, which is very highly enriched in the long-chain omega-3 fatty acids eicosapentaenoic acid (EPA) and DHA. The recommendations of the AHA in 1961 to increase PUFA from corn oil, soybean oil or fish, treated omega-3 and omega-6 fatty acids indiscriminately: corn oil contains very high levels of omega-6 and almost no omega-3, whereas the fatty acid profile of fish is completely the opposite. In terms of CVD risk reduction and the maintenance of healthy plasma lipid levels, the findings of Dyerberg and Bang suggested there may be critical distinctions to make between omega-3 and omega-6 PUFAs.

In 1982, the physiological essentiality of omega-3 fatty acids was at last definitively demonstrated (Holman et al., 1982). A young female patient receiving a safflower-based lipid emulsion (which contained abundant omega-6, but virtually no omega-3 fatty acids) on total parenteral nutrition (TPN), whose plasma phospholipid profile indicated dramatically reduced omega-3 levels, developed neuropathy. Following a lipid emulsion change from safflower oil to soybean oil (a source of omega-3), neuropathy symptoms resolved and the plasma phospholipid profile showed that omega-3 levels had been restored (i.e. increased) toward normal control levels, while the omega-6 levels had actually been reduced (Holman et al., 1982). This finding at last demonstrated that omega-6 fatty acids are not the only dietary essential fatty acids, but a distinct species of PUFA – omega-3 fatty acids – are essential components of the diet as well.

In 2000, informed by several large scale longitudinal studies assessing diet and disease incidence, the AHA issued another report relating fat intake to CVD risk (Krauss et al., 2000). Two specific recommendations added to the original recommendations made in 1961 were, 1) to consume at least 2 servings of fish per week, and 2) to reduce energy from saturated fat to less than 10% of total energy (Krauss et al., 2000). The recommendation to consume fish was based on a reduced risk of mortality from CVD among groups with the highest fish consumption, whereas the recommendation to reduce saturated fat to below 10% of total energy was based on its association with reduced plasma LDL cholesterol levels, and not to more concrete outcome measures like disease incidence or mortality (Krauss et al., 2000).

Assuming an individual at energy balance wanted to reduce their saturated fat intake to below this ceiling level (10% of energy), the question remained: what type of energy should be used to replace the energy from saturated fat? To answer this question, and evaluate the physiological relevance of achieving the AHA’s recommended saturated fat intake of no more than 10% of
energy, a 2009 meta-analysis evaluated the relative risk of coronary events or coronary death by replacing saturated fat in the diet with PUFA, MUFA or carbohydrates (Jacobsen et al., 2009). The analysis considered 11 cohort studies following roughly 345,000 people over 4-10 years and found that reducing saturated fat in the diet gave no reduction in risk if that saturated fat was replaced with energy from either carbohydrates or MUFAs. The only group that showed any benefit from a reduction in saturated fat intake was the group that replaced saturated fat with PUFA (Jacobsen et al., 2009). This finding, too, is essentially consistent with the initial recommendations of the AHA made in 1960 to replace saturated fat from meat and dairy with either vegetable oils or fat from fish (i.e. PUFAs). The work of Dyerberg and Bang, however, strongly suggested that making distinctions between omega-3 and omega-6 classes of PUFAs can be meaningful, physiologically.

A 2013 meta-analysis of 7 randomized control trials (RCTs) aimed to make precisely this distinction. The authors identified RCTs where dietary saturated fat was replaced with dietary PUFA sources of clearly known omega-3 and omega-6 levels. The trials were then separated based on which class of PUFA was used to replace saturated fat. The authors found that when saturated fat was replaced by dietary sources of PUFA containing only omega-6, there was no reduction in CVD risk, in fact there was a trend toward an increased risk (Ramsden et al., 2013). However, replacing saturated fat with a mixture of omega-3 and omega-6, or with oils containing mostly omega-3, significantly reduced the risk of death from CVD, as well as all-cause mortality (Ramsden et al., 2013). These findings indicate that, among the omega-6 and omega-3 classes of essential fatty acids, omega-6 requirements are routinely met by the average American, and an increased intake in this specific class of PUFA provides no clear benefit relevant to CVD risk. The fact that an increase in dietary omega-3 PUFA can significantly reduce the risk of CVD indicates that the current average intake level among Americans is insufficient to support the omega-3 requiring functions of the body, although they remain at this time largely undetermined. Determining what those biological and mechanistic functions of omega-3 are, and how their disruption might increase the risk of CVD, would be of tremendous value to larger efforts aimed at improving human health through diet.

To answer the question “Are we underconsuming omega-3?” under consumption must first be clearly defined. There is a pressing need to identify a physiological endpoint – a molecular
mechanism – demonstrated to be dependent on a sufficiency in the omega-3 fatty acid supply in the diet. It is the aim of this document to clearly demonstrate the potential to define this endpoint in the testis.
References


Chapter 2: Literature Review

Introduction
Male infertility is estimated to affect 7-8% of all men in the U.S., and currently known causal mediators can only explain approximately 50% of all cases (Krausz, 2011). That leaves, in the U.S. alone, roughly 10 million infertile men with no means to improve the health of their sperm and no explanation as to the origin of their dysfunction. Recent epidemiological and clinical evidence, however, has established a relationship between the dietary consumption of long chain omega-3 fatty acids (specifically docosahexaenoic acid (DHA)) and multiple parameters of sperm health, demonstrating the need to further explore the essential roles for DHA in the testis, and any sperm phenotype specific to insufficient omega-3 fatty acid consumption. This review aims to summarize recent research findings relating essential fatty acids to sperm health in both human and animal models, and to explore potential mechanisms of action for highly unsaturated fatty acids (HUFAs) in the testis.

Highly unsaturated fatty acids and male fertility

Human evidence for essential fatty acids in the maintenance of male fertility
Two essential fatty acid supplementation trials have successfully improved meaningful parameters of sperm health. In one clinical trial, oligoasthenoteratospermic idiopathic infertile males (n=211) supplemented with 1.85g EPA+DHA/day for 32 weeks showed significant improvements in sperm count, sperm motility and morphology, as compared to placebo controls, and these improvements were highly correlated with increases in sperm, red blood cell and seminal plasma DHA concentrations (Safarinejad, 2011). Another trial supplemented healthy young men with 75g of walnuts/day, which contain the 18-carbon n-3 alpha-linolenic acid as well as the 18-carbon n-6 linoleic acid, but not preformed longer chain n-3 or n-6 fatty acids EPA, DHA or ARA. After 12 weeks, increases in serum n-3 and n-6 fatty acids in the treatment group were correlated with improved sperm vitality (determined by a membrane exclusion eosin dye), as well as sperm motility and morphology (Robbins et al., 2012). A third supplementation trial with treatment arms receiving either 400mg DHA/day or 800mg DHA/day failed to show any significant improvements in parameters of sperm health for asthenozoospermic males
(Conquer et al., 2000); however this trial was brief in duration (12wks) and based on a small sample size (n=10). Additionally, epidemiological work has found higher n-3 fatty acid intake, as determined by food frequency questionnaires, to be associated with better sperm morphology (Attaman et al. 2012).

**Animal Model Evidence for Highly Unsaturated Fatty Acid Function in Male Fertility and Sperm Maturation**

Although the human data is strongly suggestive of a role for omega-3 fatty acids in the maintenance of sperm health and male fertility, it leaves many questions unanswered as to which specific fatty acid may be most critical. In an effort to clarify the physiological functions of the different classes of PUFAs enriched in the body and in the diet, the Delta-6 desaturase knockout (D6D -/-) mouse model was developed (Stroud et al., 2009). In this animal model, the endogenous conversion of the 18-carbon essential fatty acids –linoleic acid and alpha-linolenic acid – to the longer-chain products arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively, is disabled (Fig.1). This manipulation allows for D6D -/- mice to be fed 18-carbon PUFAs, but, because they have lost the capacity to utilize these fatty acids as substrates for desaturation and elongation into the highly unsaturated fatty acids (HUFAs) ARA, EPA and DHA, over time, tissue enrichment of these HUFAs is depleted. However, supplementing the diet with a preformed HUFA can serve to maintain tissue levels. In this way, the D6D -/- mouse can be used create a highly selective fatty acid depletion from tissues. For example, by feeding sufficient levels of the essential 18-carbon omega-3 and omega-6 PUFAs, as well as the 20-carbon omega-6 HUFA ARA, a DHA-selective deficiency can be induced in tissues throughout the body.

The tissues primarily responsible for carrying out the endogenous conversion of PUFA to HUFA, and for maintaining the enrichment levels of select tissues (i.e. brain, testis, retina), have not been clearly defined. It has been shown, at the level of mRNA expression in rats that D6D was abundantly expressed in the epididymis and testis, as well as the liver, and that testis expression was confined largely to Sertoli cells, with minimal expression in germ cells (Saether et al., 2003). These results indicate that Sertoli cells are capable of contributing to testis HUFA enrichment by endogenously converting available PUFA to HUFA and may transporting these HUFAs directly to germ cells. However, testis D6D expression in rats did not vary in response
to a fat-free diet, whereas the same dietary treatment brought about a dramatic increase in liver D6D (Saether et al., 2007), which may indicate that the liver is primarily responsible for endogenous HUFA synthesis. It is also important to note that when D6D knockout males were supplemented with dietary sources of preformed HUFA, tissue HUFA levels were maintained at or above wild type levels (Stroud et al. 2009), which demonstrates that without Sertoli cell (or liver) D6D activity testis HUFA enrichment can be maintained. This highlights the value of including preformed HUFA in the diet to support the enrichment of distant tissues. However, the complexity of fatty acid transport, and potential complications in the preferential distribution of select fatty acids should not be overlooked.

**HUFA-Deficient Male Infertility Phenotype**

The most striking and earliest onset phenotype shown to be induced after the selective depletion of tissue ARA and DHA, using the D6D -/- mouse, was male infertility (Stroud et al., 2009). Further investigation utilizing this HUFA-deficient male infertility model provided an initial characterization of several more detailed parameters of male fertility and sperm health (Roqueta-Rivera et al., 2010), and demonstrated the critical function of, specifically, the omega-3 HUFA DHA (Roqueta-Rivera et al., 2011).

Tissue HUFA depletion prevented successful breeding and dramatically reduced sperm counts. In all D6D -/- animals fed HUFA-free diets for at least 6 weeks, the selective depletion of ARA and DHA from male testis tissue was shown to completely prevent male mice from successfully siring offspring (Roqueta-Rivera et al., 2011). Sperm counts from these HUFA depleted males were also dramatically reduced compared to wild type (wt) controls (Roqueta-Rivera et al., 2011). Histological assessment of testis seminiferous tubule cross sections revealed a severely impaired morphological restructuring of spermatids, as the characteristic elongation of spermatid heads in the penultimate stages of spermatogenesis was shown to be disrupted (Roqueta-Rivera et al. 2010). A clear distinction in the effectiveness of omega-6 ARA feeding versus omega-3 DHA feeding was also demonstrated. Although restoring testis tissue enrichment of ARA with dietary supplementation of ARA in the knockout (ko) did show some improvement in the parameters of breeding success rate and spermatid morphology in the seminiferous epithelium, epididymal sperm counts remained low. Feeding DHA alone to ko males, however, completely
restored all parameters of sperm health and breeding success in the ko males to wt levels (Roqueta-Rivera et al. 2010). These findings collectively defined new parameters of HUFA-deficient male infertility, and clearly demonstrated the superiority of DHA over ARA in supporting the healthy production of spermatozoa.

The disruption of spermatid elongation in HUFA-deficient ko males was also shown to coincide with the complete loss of an organelle unique to spermatids, the acrosome (Roqueta-Rivera et al. 2011). These findings clearly demonstrated a critical function for HUFAs in male fertility (specifically DHA), and pointed to specific defects in acrosome biogenesis.

**Protein trafficking in spermatid maturation**

A potential connection between organelle biogenesis and HUFA action in testis tissue can be made through mechanisms of vesicular trafficking. Although essential roles for HUFAs as free fatty acids, or as components of bilayer phospholipids, are largely unknown, there is growing evidence to suggest they may serve as regulators of intracellular protein distribution by promoting specific types of vesicular fusion (Connel et al., 2007; Darios and Davletov, 2006; Mazelova et al., 2009). In this section, relevant findings on acrosome biogenesis and vesicular trafficking will be discussed.

**Acrosome biogenesis**

Following the completion of meiosis, male germ cells undergo a dramatic morphological maturation – a process known as spermiogenesis. Spermiogenesis can be broken down into four basic sequential phases, defined by morphological criteria related to the development of a perinuclear spermatid organelle, the acrosome. Periodic acid Schiff (PAS) reaction was found to stain a thin arc covering the heads of elongated spermatids in the seminiferous epithelium, and by tracing this region of PAS reactivity through progressively less advanced spermatids, the assembly of this head cap was first described (Leblond, 1950). The first phase of acrosome development, “Golgi phase,” consists of transformations that occur at or adjacent to the Golgi apparatus, including the appearance of lightly PAS staining vesicles which coalesce to form the more darkly stained proacrosomal granules. Eventually, all proacrosomal granules (typically 2-4) appear to fuse together to form one larger acrosomal granule (Leblond and Clermont, 1952).
which tethers tightly to the nuclear envelope by directly interacting with the testis specific cytoskeletal structure, the acroplaxome (Kierszenbaum et al. 2003). In the second phase, “Cap Phase,” the acrosomal granule transforms from a spherically shaped organelle to more of a flattened saccule that spreads over a majority of the anterior region of the nucleus (Hermo et al. 2010). During the third phase, the “Acrosomal phase”, the Golgi apparatus migrates away from the anterior pole of the spermatid, where the acrosome has been assembled, and the acrosome continues to stretch over nearly two-thirds of the spermatid nucleus as it begins to elongate. In the fourth phase, “Maturation phase”, the acrosome forms into its final shape, seen in morphologically mature spermatozoa (Leblond and Clermont, 1952).

The Golgi apparatus is widely recognized as a primary sorting station for organizing the intracellular distribution of newly synthesized proteins; however, many issues related to selective vesicle budding and fusion remain unresolved. The role of the Golgi apparatus in assembling the acrosome of developing spermatids has been described largely at the ultrastructural level.

Transmission electron microscopy assessments of D6D -/- spermatid ultrastructure demonstrated that in early Golgi phase acrosome biogenesis was disrupted. Dense core proacrosomal vesicles could be found in very early Golgi phase in both wt and ko animals, but the slightly larger proacrosomal granules were only found in the wt (Roqueta-Rivera et al. 2011), revealing a disruption in acrosome biogenesis that initiates at the transition from proacrosomal vesicle formation to proacrosomal granule formation. Further analysis of ko spermatids that had progressed beyond the Golgi phase revealed abnormal morphology of both the ER and Golgi apparatus as compared to wt, and abnormal accumulations of small translucent vesicles near the cis-Golgi were shown in ko spermatids (Roqueta-Rivera et al. 2011). This phenotype of proacrosomal vesicles failing to come together to form a proacrosomal granule, taken together with the abnormal accumulation of smaller translucent vesicles at the cis-Golgi, is indicative of a disruption in vesicle fusion. Next, recent evidence related to vesicle fusion during acrosome biogenesis will be presented.
Acrosome Formation and Endosomal Vesicle Trafficking
The acrosome has classically been described as an organelle derived from small vesicles of Golgi origin which are released from the TGN beginning at the onset of Golgi Phase in round spermatids (Leblond and Clermont 1952). The extensive involvement of the Golgi apparatus during acrosome formation has been confirmed primarily by ultrastructural analysis of transmission electron microscopy as well as periodic acid Schiff staining of glycoproteins of Golgi origin (Hermo et al. 2010). Recently, however, more indirect routes of vesicular trafficking involving movement through endosomal compartments have been proposed to play critical roles in acrosome formation.

Resident proteins of the endosome organize at the acrosome during spermatid maturation. Using confocal microscopy and immunofluorescence in mouse testis tissue, the endosomal marker early endosomal antigen 1 (EEA1) and endosomal sorting complex required for Transport-0 (ESCRT-0) were shown to relocalize at the onset of the Golgi Phase from a pattern of dispersed, cytosolic puncta to the acrosomal region, where these endosomal markers colocalize with markers of resident acrosomal proteins (Berruti et al. 2010). It was further shown that EEA1 and ESCRT-0 positive puncta appeared to move from the cytosol to the acrosome while displaying colocalization with the reverse transport protein VPS54 (Berruti et al. 2010), which suggests that some resident proteins of the acrosome are trafficked to the growing acrosome by first moving through endosomal compartments. Additionally, a novel acrosome related protein, acrosome formation associated factor (Afaf), which localizes at the acrosomal inner and outer leaflets during assembly but is absent from mature acrosomes, has been shown to colocalize with both early endosomes and the plasma membrane in transfected HeLa cells (Li et al. 2006). The transient nature of this protein’s localization at the developing acrosome, taken together with its distribution through endosomes in HeLa cells further suggest the involvement of endosomal trafficking during acrosome biogenesis. Taken together, it is likely that distribution of acrosomal resident proteins is critically reliant on both anterograde trafficking directly from the TGN, as has long been proposed, and on retrograde vesicular movement through various endosomal compartments.
Fusion proteins of acrosome formation
Vesicle-associated membrane protein 4 (VAMP4) is a membrane fusion protein of the SNARE family, a class of proteins that selectively interact to promote the coalescence of discreet, membrane enclosed vesicles (Wickner et al. 2010). SNARE protein interaction allows for dynamic subcellular compartmentation when SNARE transmembrane proteins on opposing membranes bind to one another in a characteristic Q-R pairing, based on center position glutamine (Q) or arginine (R) in heptad repeats. Typically, three Q-SNAREs interact in a cis conformation on one membrane and one R-SNARE, on the opposing membrane, can then bind this complex in trans (Wickner et al. 2010).

VAMP4 is an R-SNARE that has been localized primarily in the trans-Golgi network region in several cell types (Steegmaier et al. 1999, and Feldmann et al. 2009). IF was used to localize VAMP4 in the acrosomal region of elongating spermatids (Guo et al. 2010). Following an in vivo RNA-mediated knockdown of VAMP4, there was a failure of acrosome biogenesis, which, based on ultrastructural analysis, was arrested at the stage of proacrosomal vesicle fusion (Guo et al. 2010). This finding is consistent with the disruption of acrosome formation in HUFA-deficient D6D-/- spermatids, where acrosome formation is arrested just prior to the coalescence of proacrosomal vesicles into a proacrosomal granule (Roqueta-Rivera et al., 2011). A role for VAMP4 in dense-core granule formation has been demonstrated in other cell types. Secretory granules in acinar cells of the parotid gland contain high levels of VAMP4 in the early stages of granule formation, but following their maturation into high density granules, VAMP2 is much more abundant (Katsumata et al. 2007), which suggests VAMP4 is critical in the early stages of specialized granule assembly, but not for maintenance of more mature dense-core granules.

Intracellular compartments, apart from dense-core vesicles, rely on VAMP4 for assembly. In adipocytes, VAMP4 directs intracellular trafficking of GLUT4, localizing it to an insulin sensitive intracellular compartment (Williams and Pessin 2008). VAMP2 was critical for bringing about insulin-mediated GLUT4 translocation to the plasma membrane, but the effect of insulin was lost when siRNA treatment against VAMP4 prevented the assembly of this intracellular, insulin-sensitive GLUT4 compartment (Williams and Pessin 2008) indicating how the fusion protein interactions regulated by VAMP4 typically occur prior to protein distribution to the plasma membrane. And in cardiomyocytes, VAMP4 mediates the translocation of CD36
to the plasma membrane, whereas a distinct pairing of SNAREs is responsible for promoting GLUT4 translocation (Schwenk et al. 2010), demonstrating a potential role for proper VAMP4 movement on energy substrate preference.

**Additional vesicle trafficking regulators**

Many issues related to the selective vesicle budding and fusion of vesicles at the Golgi apparatus remain unresolved: the molecular determinants of cis to trans cisternae maturation; the relative importance of the ER-Golgi intermediate compartment; and the molecular stability of these membranous structures during periods of extensive budding and fusing of vesicles are current areas of research. Proper vesicle transport depends on more than just selective interactions among SNARE proteins. Appropriate trafficking is hypothesized to include at least these four steps: 1) vesicular budding/cargo selection, where cargo proteins are organized and released from a donor compartment; 2) vesicle movement, where motor protein mediate vesicle distribution along a cytoskeletal track made up of either microtubules or actin); 3) vesicle tethering, where the initial interaction between a vesicle and its target membrane is established; and 4) vesicle fusion, where the lipid bilayer of the delivery vesicle is fused together with the lipid bilayer of the target compartment, allowing vesicle contents to mix with the target compartment.

**Coats and adaptors**

Working in concert with SNAREs, coat proteins function to select cargo for recruitment to developing transport vesicles. Coat proteins (e.g. clathrin, COPI and COPII) interact with cargo by way of adaptor proteins (e.g. adaptor protein 2) and are also integral in bringing about the membrane deformation required to bud off a transport vesicle from the membrane of origin (Cai et al. 2007).

Adaptor protein complexes serve as hub proteins that link together the coats (clathrin lattice), transmembrane cargo and also interact with phosphatidylinositol at the plasma membrane. Various adaptor protein complexes can confer specificity in cargo selection and promoting
distinct routes of intracellular trafficking (McMahon and Boucrot 2011). HIV-Rev binding protein (Hrb) is a clathrin adaptor protein which has been shown to directly interact with the R-SNARE VAMP7 (Pryor et al. 2008). In spermatids, Hrb associates with proacrosomal vesicles during the Golgi phase of spermiogenesis, which coalesce into the larger acrosomal granule. When was made deficient in spermatids, these proacrosomal vesicles were seen in the early steps of the Golgi phase, but larger acrosomal granules were never formed (Kang-Decker et al. 2001.) Due to its clathrin adaptor and SNARE binding functions, Hrb may be necessary for the inclusion of the necessary SNAREs into proacrosomal vesicles.

**Golgins**

Golgins are resident proteins of the Golgi that may contribute to the molecular stability of the Golgi apparatus during periods of dynamic biogenesis. Golgins are long, coiled-coil molecules with curved membrane binding affinity at the N-terminus, by way of an amphipathic lipid packing sensor (ALPS) motif, and adaptor binding affinity at the C-terminus, by way of a GRIP-related Arf binding (GRAB) domain (Drin et al. 2008). By simultaneously binding to adaptor protein-containing regions of the Golgi apparatus and with lipid vesicles of a narrow range of sizes, golgins can serve to promote the tethering of vesicles destined for fusion with the Golgi. Golgin-97 tethers retrograde transport vesicles moving from endosomes to the TGN (Jing et al. 2010) and also to participate in the trafficking of the adhesion molecule E-cadherin in post-Golgi tubulovesicular carriers (Lock et al. 2005). TMF/ARA160 is another golgin which also contributes to retrograde trafficking from endosomes to the TGN (Yamane et al. 2007). Male mice deficient in TMF/ARA160 are infertile and spermatids fail to develop acrosomes during the Golgi phase of spermiogenesis (Lerer-Goldshtein et al. 2010), suggestive of an essential retrograde trafficking mechanism involved in acrosome biogenesis.
Cell-Cell Adhesion in the testis

Testis Adhesion under HUFA deficiency
The integrity of the cell-cell adhesions of spermatid development may be disrupted by a reduction in testis HUFA enrichment. Analysis of the HUFA-deficient male infertility phenotype using the D6D -/- mouse model revealed a conspicuous appearance of round germ cells in the epididymis of HUFA-deficient males (Roqueta-Rivera et al., 2010). In wt animals, the epididymis was filled exclusively with morphologically mature spermatozoa, but the epididymis of ko animals contained both abnormally formed spermatozoa and immature, round spermatids (Roqueta-Rivera et al., 2010). This appearance of round spermatids in the epididymis suggests that spermatids may not only be failing to properly mature into spermatozoa, they may also be failing to establish the critical cell-cell adhesions with Sertoli cells that support their retention in the seminiferous epithelium. As spermatozoa are released from seminiferous tubules into the epididymis (the tissue just distal to seminiferous tubules) following the active breakdown of spermatid adhesions, the appearance of round, immature spermatids in the epididymis could potentially be explained by a disruption in Sertoli-spermatid adhesion integrity. The next section examines relevant literature on the topic of Sertoli-spermatid adhesion and explores the function of potential molecular mediators in multiple tissues throughout the body.

Heterotypic Cell-Cell Adhesions
In building the adhesive structures necessary to support sustained interactions between neighboring cells, all multicellular organisms face the dual challenge of establishing a stabilizing architecture that also remains responsive enough to facilitate movement and cellular morphogenesis. Adherens junctions, a canonical form of cell-cell adhesion complex, sustain cell-cell contacts with direct interactions between extracellular regions of transmembrane adhesion proteins distributed to the plasma membrane of each cell involved in the interaction. Cytosolic regions of transmembrane adhesion proteins directly interact with cytosolic adapters, which, in turn, interact with cytoskeletal structures. The end result is tight associations between the cytoskeletal structures of adjacent cells (Harris and Tepass 2010).

Junctions that form between neighboring cells can be broadly classified into two distinct categories: 1) those that form symmetrical adhesions between like cells (homotypic adhesions)
and 2) those that form asymmetrical adhesions between non-like cells (heterotypic adhesions). Recently characterized knockout mouse models have revealed essential functions for a new class of transmembrane adhesion protein – nectins – in, specifically, heterotypic adhesion. Nectins are immunoglobulin-like, calcium independent cell adhesion molecules (CAMs) and consist of four family members (nectin-1 through nectin-4). An important characteristic that distinguishes nectins from more classic CAMs (e.g. cadherins), is their strong affinity for interacting heterophilically, in trans, with other family members (e.g. nectin-1 with nectin-3; nectin-2 with nectin-3). It is this property that allows non-like cells to establish points of contact based on their respective expression of the appropriate nectin protein (Takai et al. 2008).

Cell-cell adhesions of Sertoli cells
In the seminiferous epithelium, the Sertoli cells that form a monolayer along the basement membrane of these tubules participate in two distinct types of cell-cell adhesions. When adjacent Sertoli cells interact with one another to form a symmetrical class of adhesion between two like cell types, the result is the homotypic adhesions of the testis, which form the Blood-testis barrier (Cheng and Mruk, 2002). The Blood-testis barrier is a complex form of cell-cell adhesion that forms between adjacent Sertoli cells in the basolateral region of the seminiferous epithelium and serves as an immunological barrier separating the adluminal compartment, where spermiogenesis occurs, general circulation (Cheng and Mruk, 2010). Preleptotene spermatocytes must pass through the Blood-testis barrier into the adluminal compartment, which may occur as early as stage VI to VII of the cycle of the seminiferous epithelium (Yan et al., 2006), or more synchronized with spermiation at stage VIII (Cheng and Mruk, 2010): there is some disagreement as to whether or not spermiation is synchronized with the passage of preleptotene spermatocytes through the BTB (Pelletier, 2011).

Sertoli cells also interact with post-meiotic germ cells at their elongation phase to form an asymmetrical form of adhesion, which supports the contact between non-like cell types – these are the heterotypic adhesions of the testis. Nectin-2 is expressed in Sertoli cells and is a component of each of these adhesions. Characterization of the nectin-2 knockout mouse first demonstrated the essentiality of this CAM in male fertility (Bouchard et al., 2000), and later revealed its specialized role in supporting cell-cell adhesions between Sertoli cells and germ cells.
(Ozaki-Kuroda et al., 2002). The original nectin-2 knockout report on male infertility described defects in cytoskeletal morphology and mitochondrial localization in spermatozoa, with indentations in the nuclei and disorganized helical sheaths and outer dense fibers of the middle piece. There were no reported differences in sperm number from the epididymis, and sperm were described as motile, however, they appeared morphologically abnormal with misshapen heads and inconsistent acrosome structures. Also, later stage spermatids presented with an accumulation of large translucent vesicles in the heads (Bouchard et al., 2000). The succeeding report on testis in the nectin-2 knockout focused more on cytoskeletal organization. F-actin distribution was described as “scarce” covering elongated spermatids, apparently accumulating in regions not adjacent to spermatids. The nectin-actin adaptor protein afadin was also found to be disorganized in the knockout, with actin and afadin demonstrating nectin-2 dependent localization at Sertoli-spermatid contacts (Ozaki-Kuroda et al., 2002).

Nectin-3 is expressed in post-meiotic germ cells and appears exclusively at the heterotypic adhesions in the testis (Cheng and Mruk, 2010). Characterization of a nectin-3 knockout mouse model demonstrated a very similar phenotype to the nectin-2 knockout. The animals presented with male-specific infertility; abnormal spermatid morphology was described at the elongation phase of spermiogenesis; F-actin appeared disorganized in the tubules; and assessment of TEM ultrastructure was used to show the dissociation of Sertoli cells and spermatids. The authors went on to show how nectin-3 is critical for spermatids, and not for Sertoli cells, by transplanting wild type germ cells into nectin-3 null testis and finding normal morphological differentiation of these spermatids (Inagaki et al., 2006).

Junction adhesion molecule C (JAM-C) is another CAM expressed in spermatids and localized at the Sertoli-spermatid contacts. JAM-C deficient mice present with male infertility and show severe disruptions in the localization of polarity proteins. Par-3, par-6 and PATJ (discussed later), as well as aPKC and Cdc42, all failed to organize at the anterior poles of spermatids at the cap phase of spermiogenesis (Gliki et al., 2004), which would indicate a severe disruption in the polarization of spermatids in JAM-C -/- mice. Time course analysis of adhesion assembly in cultured MDCK cells suggested that distribution of nectin molecules acts upstream of either JAM or E-cadherin distribution to points of cell-cell contact (Fukuhara et al., 2002).
Cell-Cell Adhesions of the Brain

The omega-3 HUFA DHA is preferentially enriched, in addition to the testis, in the brain, another tissue with well-established dependence on heterotypic adhesion integrity and nectin protein distribution. Nectin-1 and nectin-3 are expressed in presynaptic and postsynaptic neurons, respectively, where they interact in trans and function as critical components of the puncta adherentia junctions, which provide the structural support for synapses (Honda et al. 2006). Intracellular translocation of nectin-1 to the synaptic region may be an important process of learning. Mice exposed to contextualized fear conditioning (CFC) showed an up regulation of nectin-1 at synaptic regions (isolated as a synaptoneurosome fraction) shortly after training, despite steady nectin-1 expression levels in the whole hippocampal fraction. When antibodies against a nectin-1 ectodomain were infused directly after CFC training, learning was impaired (Fantin et al. 2013), suggestive of a role for nectin-1 translocation to plasma membrane region of synaptic cell-cell contacts in learning.

Nectin-3 also appears to have functional significance in synaptic adhesions of the brain. Early life stress, spatial learning impairments, and dendritic spine loss in mice were associated with reduced protein and RNA levels of nectin-3 in the hippocampus. The dendritic spine loss and some learning behaviors were rescued by overexpression of nectin-3 (Wang et al. 2013). Furthermore, reduced DHA levels in the hippocampus have been shown in patients with Alzheimer’s disease (Lukiw et al. 2005), where hippocampal synapse loss is a histological hallmark of the disease. Therefore, it is possible that an interaction between DHA and nectin molecules may also be operating in the brain; although the function of DHA in the nervous system is poorly understood.

Nectin-interacting molecules

Specific molecular mediators of intracellular nectin molecule distribution have not yet been elucidated, however a series of proteins have been shown to directly interact with various nectin family members. A majority of these nectin binding proteins have prominent roles in promoting apico-basal cell polarization.

All nectin proteins associate with adjacent cytoskeletal structures through direct associations with the adaptor protein afadin (Tanaka-Okamoto et al., 2011). Nectin-afadin binding interactions have a feed forward effect on trans-heterotypic nectin binding and nectin-afadin
binding: nectin-3-afadin interactions promote the trans-heterotypic interaction of nectin-1 and nectin-3; nectin-1 trans-heterotypic interaction with nectin-3 promotes nectin-3 interaction with afadin (Kurita et al. 2011). Conditional hippocampal neuron selective afadin knockdown resulted in decreased synapse formation (measured by colocalization of pre and post synaptic proteins), reduced spine density. Reduced N-cadherin puncta at synapses was also shown, although N-cadherin expression was not changed (Beaudoin et al. 2012), suggestive of a critical role for afadin in promoting CAM distribution to the cell-cell adhesions of synapses.

Protein interacting with C Kinase 1 is a Bin/Amphiphsin/Rvs (BAR) domain containing protein highly expressed in the brain and the testis. In the brain, PICK1 plays a key role in the trafficking of AMPA receptors to synapses, which can regulate synaptic strength and may influence the acquisition of learning and memory (Rocca et al. 2008). In the testis, PICK1 deficiency was shown to result in round-headed sperm with severely impaired motility and fragmented acrosomes (Xiao et al., 2009). In cultured COS cells, IF staining showed colocalization of both nectins and JAMs at cell junctions, and a direct interaction for both nectin-2 and nectin-3 with PICK1 was demonstrated with co-IP and a yeast two hybrid model (Reymond et al., 2005).

In the neuroepithelial cells of mice, a direct interaction between PAR-3 and nectin-3 or nectin-1 was shown, but not nectin-2 (Takekuni et al., 2003). This direct interaction between par-3 and nectin-3 was confirmed in another study that went on to show how par-3, afadin and nectin were all required for the successful formation of adherens junctions, in an MDCK transfection model (Ooshio et al., 2007). Par-3 bundling was shown to support the positioning and preferential stabilization of microtubules in axons near the growth cone, where par-3 and microtubules directly interact (Chen et al., 2013).

Membrane palmitoylated protein 3 and 5 were shown to interact selectively with nectin molecules: MPP3 binds nectin-1 and nectin-3; MPP5 binds only nectin-3. This interaction was further shown to be critical for promotion of nectin distribution to cell contact sites at the plasma membrane in COS-7 cells (Dudak et al., 2011), suggestive of a necessary MPP-nectin direct interaction for proper intracellular distribution. The isoform MPP6 is highly expressed in the pachytene and spermatid cells of the testis, and their expression is up regulated at puberty (MRG
The PDZ domain containing polarity proteins Patj and MUPP1 have also been shown to directly bind nectins (Adachi et al., 2009).

**Cytoskeletal Function in Cell-Cell Adhesion**

Adherens junctions do not serve only to tether adjacent cells to one another; their organization into belts also serves to landmark the border between apical and basal regions of cells (Harris and Tepass, 2010). In order to establish and then maintain this polarized architecture, which must be both structurally sound and highly responsive, the molecular components of cell-cell adhesions show properties of dynamic redistribution that are as yet poorly described. However, indirect association with membrane adjacent cytoskeletal structures is clearly a major structural stabilizer for cell-cell adhesion. Here, recent findings focused on the cytoskeletal structures of adhesion complexes are briefly discussed.

Willin, an actin adaptor protein known to associate with par-3 and play a role in actomyosin contractility and epithelial morphogenesis, has been shown to directly interact with nectin at its juxtamembrane region, however, this willin distribution does not depend on nectin-1 expression or its proper intracellular distribution (Ishiuchi and Takeichi 2012).

There is further evidence of actin and nectin establishing localization within cells in an independent fashion. Chemical disruption of the actin cytoskeleton resulted in the dissociation of cell adhesion proteins JAM, E-cadherin and claudin, but not nectin (Yamada et al., 2004), which indicates that nectin distribution to points of cell-cell contact may be independent of actin cytoskeletal formation.

Cytoskeletal dynamics also play roles in modulating synapse integrity. Actin restructuring has been associated with the strengthening of synapses and lengthening of dendritic spine necks, as synaptic abundance was increased by stimulating actin reorganization. This synaptic increase occurred when either transcription or translation were inhibited (Shoji-Kasai et al. 2007), which may suggest a role for adhesion protein translocation, as opposed to up regulated expression, in supporting new synapse formation.
Cell Adhesion Molecule Trafficking

The adhesion complexes assembled at the interface between Sertoli cells and spermatids must be disassembled at step 16 of spermiogenesis in order to allow for the release of these morphologically mature spermatozoa into the lumen of the seminiferous epithelium. It has been proposed that the primary means of disassembling the adhesion complexes of Sertoli-spermatid contact is by Sertoli cell internalization. Ultrastructural analysis of the seminiferous epithelium in the late stages of spermiogenesis revealed the existence of internalized double membrane vesicles adjacent to the concave regions of step 15-16 spermatids. These vesicles appeared to be budding from larger tubular structures with bulbous ends extending from the plasma membrane of spermatids, invaginating into Sertoli cells. They were termed tubulobulbar complexes (Guttman et al. 2004). The molecular nature of these structures was revealed when colocalization of nectin-2, nectin-3, alpha-6 beta-1 integrin (another CAM of Sertoli-spermatid contacts) and early endosomal antigen 1 (EEA1) were shown at the bulbar ends of tubulobulbar complexes in elongated spermatids. All CAMs were also found along the tubular components, but EEA1 was absent (Young et al. 2009). Internalized CAMs colocalized with the early endosomal marker Rab 5 in the region directly adjacent to step 16 spermatids, but cytosolic puncta of CAMs (nectin-2) distributed closer to step 8 spermatid heads colocalized with the recycling endosomal marker Rab11 (Young et al. 2012), suggestive of an endosomal recycling of nectin proteins internalized through the tubulobulbar complex.

The fidelity of apical versus basolateral transmembrane protein sorting in polarized cells is critical for cell and tissue function. The molecular machinery and vesicular intermediates involved in protein transport to the basolateral plasma membrane domains have been well described (Mellman and Nelson, 2008). Select combinations of adaptor protein complex components, vesicle protein coats, as well as the tethers and SNAREs of vesicle fusion have been described for a variety of cargo proteins (Gravotta et al., 2012) and (Mellman and Nelson, 2008). However, regulation of protein delivery to the apical membrane is less well understood. Whereas basolateral sorting is thought to be driven primarily by cytosolic cargo motifs, apical sorting is likely driven by either extracellular or transmembrane domains, which may be peptide sequences intrinsic to the proteins or distinct glycosylations of the extracellular domain (Schuck and Simons, 2004). The preferential apical localization of many transmembrane proteins may also depend on a transcytosis mechanism (basolateral to apical trafficking through endosomal
compartments), which may be promoted by the posttranslational removal of basolateral sorting motifs (Wisco et al. 2003). In hepatocytes, nearly all apically localized transmembrane proteins are first delivered to basolateral plasma membrane domains and rely on transcytosis for their eventual apical localization (Tuma and Hubbard, 2003).

**Rab11 and the recycling endosome**

There are molecular and functional distinctions between early endosomes and recycling endosomes, as organellar protein sorting stations. Whereas the majority of protein recycling to the basolateral plasma membrane domain has been shown to occur directly through early endosome-derived vesicles, the majority of apically delivered, recycled proteins pass through a Rab11-positive, recycling endosomal compartment. Based on density gradient separation of MDCK endosomal compartments, early endosomes were shown to play a relatively minor role in recycling to the apical membrane, whereas Rab11-positive recycling endosomes were shown to play a major role (Sheff et al., 1999). Rab11a knockdown in MDCK cells prevented the apical delivery of Rhodopsin, but did not interfere with its basolateral distribution, and both Rab11a and rhodopsin were shown to pass through post-Golgi apical endosomal compartments prior to delivery to the apical plasma membrane (Thuenauer et al., 2014). The role of Rab11 in mediating protein distribution to the apical membrane domain has also been shown to be critical specifically for the localization of rhodopsin in developing *Drosophila* photoreceptors (Satoh et al., 2005).

Rab11 appears to have important roles in protein delivery in a variety of developmental processes. Localization of the beta catenin family protein p0071, which is thought to function as an adaptor protein linking cadherins to either actin or microtubules, was found to be regulated by Rab11 (Keil and Hatzfeld, 2013), providing further evidence that Rab11 serves as a critical component of adherens junction assembly and dynamic restructuring. Cerebral accumulations of beta-amyloid peptides, generated by catalytic action of the beta and gamma secretases on amyloid precursor proteins, are a prominent characteristic of Alzheimer’s disease. Rab11 has been shown to regulate the movement of beta secretase in cultured HeLa cell expressing the Swedish APP-mutation (Udayar et al., 2013), which suggests it may be involved in controlling
the abundance and accumulation of beta-amyloid peptides. Although Rab11 is typically considered to label recycling endosomes, imaging from cultured, nerve-growth factor stimulated PC12 cells revealed that conventional recycling endosomal markers Rab11, Arf6 and transferrin receptor displayed distinct localization patterns (Kobayashi and Fukuda, 2013). This finding indicates there may be cell specific compartmentalizations of the recycling endosome and there is much left to discover about its regulation and physiological influence.

**Summary and research objectives**

There exists an opportunity to clearly define HUFA function at the level of the cell by improving our understanding of sperm development. Both human and animal model evidence on fatty acid enrichment in the testis point toward critical functions for HUFAs, specifically the omega-3 fatty acid DHA, in supporting the integrity of spermatid maturation. Although human evidence demonstrating the importance of DHA enrichment for sperm health is limited to analysis of semen, animal models have provided more in depth clues as to the nature of testis dysfunction under DHA or HUFA deficiency.

This review has highlighted recent findings in the areas of acrosome biogenesis and Sertoli-spermatid adhesion formation, focusing on potential links between these fields of research and the phenotype of HUFA-deficient male infertility. To create a molecular context for the complete loss of acrosome formation and potential disruption in Sertoli-spermatid adhesion integrity found in the HUFA-deficient D6D -/- mouse, a non-exhaustive review of potential molecular mediators have been described here.

**Research aim I**

Strong connections made between the roles of vesicle trafficking proteins of the TGN or endosome to the success or failure of acrosome biogenesis suggest a role for HUFAs in promoting certain specialized modes of vesicular trafficking. The first aim of this research is to perform a molecular characterization of acrosome biogenesis and its failure under HUFA deficiency. We aim to determine how TGN and endosomal trafficking proteins might serve as
regulators of acrosomal cargo distribution, and how these regulators might be disrupted under HUFA deficiency.

**Research aim II**
The fact that critical functions for adhesion proteins of Sertoli-spermatid contacts have been described only in tissues where HUFAs (DHA specifically) are preferentially enriched (i.e. brain, eye, testis), taken together with the likely disruption in Sertoli-spermatid adhesion evidenced by the abnormal appearance of round spermatids in the epididymis of HUFA-deficient males, suggests that HUFAs may also serve to support the assembly of adhesions in the testis, and potentially other DHA-enriched cell types. The second aim of this research is to determine whether the presence of immature spermatids in the epididymis under HUFA deficiency is the result of disrupted cell-cell adhesion formation at the Sertoli-spermatid interface. We aim here to assess the integrity of both Sertoli-spermatid and Sertoli-Sertoli adhesions at the functional and molecular level.
Fig. 1. Pathway for the endogenous conversion of 18-carbon PUFA to long-chain highly unsaturated fatty acids arachidonic acid (ARA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA).
References


Chapter 3: Sertoli-spermatid adhesions are disrupted by docosahexaenoic and arachidonic acid deficiency

Abstract
Male fertility is dependent on the successful maturation of germ cells within the testis. This maturation relies on the assembly of intercellular adhesions that form between Sertoli cells (“Mother” cell of the seminiferous epithelium) and developing spermatids. Expression and localization of adherens junction molecules nectin-2 and nectin-3 is critical to the stability of this unique, heterotypic Sertoli-spermatid adhesion. Here, we report that highly unsaturated fatty acids (HUFAs) that are enriched in the testis, omega-3 docosahexaenoic acid (DHA) and omega-6 arachidonic acid (ARA), are essential physiologically as critical regulators of Sertoli-spermatid adhesion integrity and nectin protein localization. Using Fads2 -/- mice to create select HUFA deficiencies in the testis, we demonstrate that heterophilic binding partners nectin-2 and nectin-3 are expressed appropriately in their respective cell types, but mislocalized. Further, the loss of nectin-2 localization in Sertoli cells is selective, with only Sertoli-spermatid contacts showing disruption, while Sertoli-Sertoli contacts of the Blood-testis barrier (BTB) display normal protein organization. Indeed, the BTB is intact by all accounts: by functional evaluations using biotin tracer injections, ultrastructural assessments using electron microscopy, and adhesion molecule localization using immunofluorescence. Although nectin adhesion molecules fail to organize along Sertoli-spermatid adhesions at any phase of spermiogenesis, these points of cell-cell contact do initially recruit actin filaments in the Sertoli cell, and show several histological hallmarks of normal adhesion formation, before becoming disrupted in later development. These results identify the fatty acids DHA and ARA as critical mediators of nectin protein distribution to the heterotypic, Sertoli-spermatid adhesions of the testis.
Introduction
The omega-3 fatty acid docosahexaenoic acid (DHA) and the omega-6 fatty acid arachidonic acid (ARA) are the highly unsaturated fatty acids (HUFAs) most abundant in the body, serving critical physiological and cellular functions. These HUFAs are preferentially enriched in the sn2 position of membrane phospholipids and serve as precursors for the synthesis of signaling molecules, such as eicosanoids – ARA metabolites known to mediate inflammatory reactions throughout the body (Funk, 2001), and docosanoids – DHA metabolites thought to regulate neuroinflammation and promote cell survival (Bazan, 2013). Essential roles for these HUFAs as free fatty acids, or as components of bilayer phospholipids, are largely unknown, although growing evidence suggests they may serve to regulate intracellular protein distribution by promoting specific types of vesicular fusion (Connel et al., 2007; Darios and Davletov, 2006; Mazelova et al., 2009). Dietary essential 18-carbon polyunsaturated fatty acids (the omega-6 linoleic acid and the omega-3 alpha-linolenic acid) are endogenously converted to ARA and DHA by a series of desaturation and elongation reactions (Nakamura and Nara, 2004). In the fatty acid dasaturase2 (Fads2) knockout mouse, the rate-limiting step in this endogenous conversion pathway has been disabled. Use of the Fads2 -/- model allows for the creation of a HUFA-selective deficiency, while maintaining tissue levels of the essential 18-carbon fatty acids (Stroud et al., 2009), and led to the discovery that HUFAs are essential for male fertility and the spermatogenic action of the testis (Stroud et al, 2009).

The production of sperm in the testis culminates with an elaborate transformation of post-meiotic round spermatids into morphologically elongated, mature spermatozoa, a process known as spermiogenesis. This process, which takes place within the seminiferous tubule, consists of 16 developmental steps in mice, as spermatids change from round to elongated and highly polarized, before being released into the tubule lumen at the completion of spermiogenesis (Hess and Renato de Franca, 2008; Leblond and Clermont, 1952). This morphological maturation is dependent on a complex interaction between maturing spermatids and the unique epithelial cells that line seminiferous tubules – Sertoli cells. These large, polyhedral support cells directly interact with developing germ cells, anchoring them in the seminiferous tubules, and serve to regulate germ cell migration toward the tubule lumen (Pelletier, 2011). Sertoli cell regulation of spermatid retention in the seminiferous tubule is achieved, on a structural level, by the establishment of an adherens-like cell-cell adhesion unique to the testis, known as the apical
ectoplasmic specialization (ES), which tethers spermatids directly to Sertoli cells (Vogl et al., 2008; Cheng and Mruk 2010). Both the cell adhesion molecules (CAMs) and the actin-based cytoskeletal structure of the apical ES begin to organize in seminiferous tubules at the initiation of the elongation phase, steps 8-9 of spermiogenesis – or “Stage VIII-IX” in the cycle of the seminiferous epithelium (Hess, 1990) – when the nucleus migrates to the plasma membrane at its anterior pole (Hermo et al., 2010b; Ozaki-Kuroda et al., 2002) and the nuclear chromatin begins condensing. As the elongation phase progresses, the spermatid nucleus is elongated caudal to the anterior pole by a microtubule-based assembly known as the manchette (Hermo et al., 2010a). Following successful completion of the 16th step of spermiogenesis, morphologically mature spermatozoa are actively released from Sertoli cells, and from the seminiferous tubules, by the proteolytic action of matrix metalloproteinases (Siu and Chang, 2004), which act extracellularly to cleave CAMs of the apical ES, and by Sertoli cell invagination of Sertoli-spermatid adhesion complexes (Young et al., 2012), which are endocytosed as large, double-membrane vesicles.

In the seminiferous epithelium, Sertoli cells must establish two distinct types of cell-cell adhesions – one homotypic, and one heterotypic. Adjacent Sertoli cells adhere along their basolateral plasma membrane domains to form the Blood-testis barrier (BTB), a homotypic, symmetrical class of adhesion consisting of adherens junction, tight junction and gap junction CAMs. This Sertoli-Sertoli adhesion serves to form an immunological barrier in the testis that segregates the autoantigens of meiosis and post-meiotic germ cell maturation from general circulation; and the BTB is dynamic, being restructured with each new generation of spermatocytes that must migrate through the BTB without breaching its barrier function (Cheng and Mruk, 2010; Smith and Braun, 2012). The heterotypic adhesions are assembled in the crypts found along the apical plasma membrane domain of Sertoli cells, where contacts with elongating spermatids are established (Russell and Clemont, 1976). The integrity of this asymmetrical, Sertoli-spermatid adhesion is critically dependent on an interaction between the CAMs, and heterophilic binding partners, nectin-2 and nectin-3 (Ozaki-Kuroda et al., 2002; Inagaki et al., 2006; Takai et al., 2008). Prominently expressed in post-meiotic germ cells at the onset of spermatid elongation, nectin-3 localizes in the testis only at Sertoli-spermatid contacts (Inagaki et al., 2006); whereas nectin-2, expressed in Sertoli cells, is found at both the basolateral BTB adhesions and the apical Sertoli-spermatid adhesions (Takai et al., 2008; Bouchard et al., 2000).
Our recent characterization of HUFA deficient male infertility revealed disruptions in the elongation phase of spermiogenesis, with arrested spermatid morphogenesis and an abnormal release of immature spermatids from the seminiferous tubules into the epididymis (Roqueta-Rivera et al., 2010; Roqueta-Rivera et al., 2011), suggestive of a defect in Sertoli-spermatid adhesion. Little is known regarding the regulation of heterotypic adhesion assembly, although it should be noted that tissues where heterophilic interactions between nectin family members have been proven critical – synapses of the hippocampus (Honda et al., 2006), between pigment and non-pigment ciliary epithelial layers in the eye (Inagaki et al., 2005), and in the testis – are the very same tissues where HUFAs are preferentially enriched in the body. We aimed here to test the hypothesis that a selective reduction in the HUFAs ARA and DHA leads to the premature dissociation of spermatids from the seminiferous epithelium by disrupting the assembly of the nectin-based, heterotypic adhesions of the testis.
Results

HUFA deficiency disrupts Sertoli cell nectin-2 localization to apical adhesions, but not to basolateral adhesions.

Using immunofluorescent (IF) staining of intact testis tissue slices, we first investigated how testis HUFA deficiency affects Sertoli cell localization of nectin-2, through all steps of spermiogenesis. In the HUFA sufficient wild type (wt), we found nectin-2 had consistently organized into semicircles at the interface between spermatids and Sertoli cells (Fig. 2A; red arrows) by stage VI-VII of the cycle of the seminiferous epithelium, just prior to the initiation of spermatid elongation. This localization was maintained until just prior to the completion of spermiogenesis (Fig. 2B; steps 7, 9, 11 and 14), when spermatozoa are released from the seminiferous epithelia. By stage VI-VII in the HUFA deficient knock out (ko), however, regions of Sertoli cells adjacent to spermatids showed no clear pattern of nectin-2 organization (Fig. 2A; red arrows), and this loss of localization at Sertoli-spermatid contacts was consistent throughout all steps of spermiogenesis (Fig. 2B; all steps). Three dimensional images (composites of ~20 serial cross sections imaged every 300nm of depth in the z-axis) highlight the complete loss of nectin-2 distribution to spermatid-adjacent regions of Sertoli cell plasma membranes, at step 9 of spermiogenesis (Fig. 2C).

Sertoli cells organize nectin-2 not only at the heterotypic adhesions formed with maturing spermatids, but also at the homotypic adhesions formed with adjacent Sertoli cells. We found that nectin-2 distribution to these Sertoli-Sertoli adhesions, formed near the basement membrane of the seminiferous epithelium, was not altered by testis HUFA deficiency (Fig. 2A; white arrows), as both the wt and ko displayed a comparable pattern of organization.

These observations demonstrate that testis HUFA deficiency disrupts Sertoli cell localization of nectin-2 in a selective fashion. Sertoli cell organization of nectin-2 is lost only at the apical membrane subdomains where heterotypic adhesions with spermatids are assembled. Neither the expression of nectin-2 in Sertoli cells, nor its intracellular distribution to basally oriented Sertoli-Sertoli adhesions, is altered by testis HUFA deficiency.
Spermatid nectin-3 is expressed appropriately, but fails to organize at anterior poles, under HUFA deficiency

After learning that testis HUFA deficiency disrupts Sertoli cell distribution of nectin-2 to apical membrane domains, where Sertoli-spermatid contacts are assembled, we next asked if localization of nectin-3, the heterophilic binding partner to nectin-2, expressed in spermatids, was affected by testis HUFA deficiency. We used IF to visualize nectin-3 distribution in spermatids throughout all phases of their post-meiotic maturation.

When we probed for nectin-3 throughout all steps of spermiogenesis, we first found that a developmentally appropriate increase in spermatid nectin-3 expression at step 9 was apparent in both wt and HUFA deficient ko animals (Fig. 3A; step 9). Spermatids of the wt showed nectin-3 localization at both the anterior poles (Fig. 3A; red arrows), and the regions caudal to those poles (Fig. 3A; white arrows). However, in spermatids of the ko, nectin-3 remained localized in, exclusively, a region caudal to the anterior pole (Fig. 3A; white arrows), where the microtubule-based manchette forms. HUFA deficient spermatids displayed a conspicuous absence of nectin-3 localization at the rounded anterior poles (Fig. 3A; red arrows), where Sertoli-spermatid adhesions form. Three dimensional images of step 9 spermatids highlight the complete loss of nectin-3 at anterior poles of ko spermatids throughout the 5µm depth of these tissue slices (Fig. 3B). These results demonstrate that, under conditions of testis HUFA deficiency, both nectin-2 and nectin-3 are expressed and distributed normally to regions within their respective cell types that are distant from Sertoli-spermatid contacts; however at this contact, each of these heterophilic binding partners show a selective loss of localization.

Sites of Sertoli-spermatid contact are properly landmarked by actin cap assembly, but CAMs fail to colocalize under HUFA deficiency

After showing how both nectin-2 and nectin-3 fail to localize at sites of Sertoli-spermatid contact under conditions of HUFA deficiency, we next asked if the Sertoli-spermatid cell-cell interface was being properly landmarked by critical molecular components other than nectins. We used IF to visualize actin localization in Sertoli cells and to evaluate actin cap formation at sites of Sertoli-spermatid contact.
We found that in both wt and ko, actin caps were formed, and appeared as semicircles adjacent to spermatids beginning at steps 8-9 of spermiogenesis (Fig. 4A; red arrows). However, as development proceeded, actin in the ko began to delocalize, with little to no predictable pattern of localization found in Sertoli cells adjacent to step 14 spermatids (Fig. 4B; red arrows). The actin-based cytoskeletal structures organized at homotypic adhesions of adjacent Sertoli cells, formed near the basement membrane of the seminiferous epithelium, were comparable in wt and ko testis (Fig. 4A and B; white arrows).

After learning that actin caps were successfully assembled in Sertoli cells in the early phases of spermatid elongation in both wt and ko, we next sought to confirm that the loss of nectin localization in ko testis was true even in the narrow developmental window when actin initially assembles properly. We used sequential double staining IF to conduct colocalization experiments, simultaneously visualizing actin and CAM protein localization. In the wt, nectin-2 and actin displayed a high degree of colocalization at step 9 (Fig. 5A; top row). In the ko, however, nectin-2 showed no preferential colocalization with the adluminal actin caps (Fig. 5A; bottom row).

Integrin β1, an additional Sertoli cell adhesion molecule of the apical ES, organizes at sites of cell-cell adhesion in the punctate fashion of a focal adhesion complex. In the wt, we found punctate integrin β1 highly colocalized along actin caps formed adjacent to the slightly more mature step 11 spermatids (Fig. 5B; top row). Although, in the ko, regions of Sertoli cells interacting with spermatids at step 11 continued to show appropriately organized actin caps, no colocalization with integrin β1 was found (Fig. 5B; bottom row). Taken together, these findings demonstrate an inability of HUFA deficient Sertoli cells to organize adhesion proteins at the apical ES, despite proper actin organization.

We next simultaneously probed for actin and the spermatid-expressed CAM nectin-3. In the wt, nectin-3 was found distributed to both the anterior poles of spermatids, where it colocalizes with actin, and to caudal regions, where the manchette drives nuclear elongation (Fig. 5C; top row). In the ko, nectin-3 localization in spermatids is restricted to regions caudal to the anterior poles, and shows little to no colocalization with actin (Fig. 5C; bottom row). This mutually exclusive localization of Sertoli cell actin and spermatid nectin-3 demonstrates that HUFA deficiency, in addition to disrupting Sertoli cell protein localization, also disrupts spermatid organization of
adhesion proteins at the apical ES. This is true despite the proper alignment of spermatids and Sertoli cells, with the rounded anterior poles of spermatids oriented directly adjacent to Sertoli cell actin caps (Figs 3, 4).

**Spermatid and Sertoli cell plasma membranes dissociate, despite a normal apical ES ultrastructure at early developmental stages under HUFA deficiency**

To further assess the integrity of Sertoli-spermatid contacts, we used transmission electron microscopy (TEM) to evaluate several well-described ultrastructural characteristics of these unique, testis adhesions. The actin plaques and endoplasmic reticulum (ER) cisternae, characteristic of the apical ES, organize against spermatids at steps 8-9 of spermiogenesis in both wt and ko Sertoli cells (Fig. 6A). In the wt, the plasma membranes (PMs) of Sertoli cells and spermatids are closely apposed along the entire length of the apical ES, whereas, in the HUFA deficient ko, the spermatid PM appears ruffled and there is separation between Sertoli and spermatid PMs at multiple points along the length of the apical ES (Fig. 6A; asterisks).

By steps 10-11 of spermiogenesis, the Sertoli cell actin filaments arranged in between Sertoli PM and ER cisternae are more regularly organized into puncta-like bundles (Fig. 6B; white arrowheads) in the wt. In the ko, minimal actin bundling is evident and large stretches with no actin in between ER cisternae and the Sertoli PM can now be found (Fig. 6B; black arrowheads).

At step 12 in the wt, the puncta-like actin bundles are regularly ordered along the entire length of the apical ES (Fig. 6C; white arrowheads), and Sertoli-spermatid PMs are tightly apposed across the same distance. By step 12 in the ko, substantial Sertoli-spermatid PM separation (Fig. 6C; double-sided arrow) can be seen, in addition to multiple points of more moderate bilayer separation (Fig. 6C; asterisks). In the ko, no puncta-like actin bundles are found and several stretches of apical ES show no actin (black arrowheads); the nucleus also fails to elongate properly and an aberrant protrusion of the perinuclear ring can be seen.

These TEM observations, revealing an ultrastructurally normal actin and ER cisternae organization at early apical ES assembly in the HUFA deficient ko, are consistent with the IF protein data showing Sertoli cell formation of actin caps at Sertoli-spermatid contacts under HUFA deficiency. However, the dissociation of Sertoli cell and spermatid PMs, shown with TEM, is consistent with the IF protein data showing a loss of nectin-2 and nectin-3 localization.
at Sertoli-spermatid contacts; and provides further confirmation that these CAMs serve critical functions for heterotypic adhesions in the testis.

**Under HUFA deficiency, Sertoli-Sertoli adhesions are molecularly, morphologically and functionally intact.**

To confirm that HUFA deficiency disrupts CAM localization selectively – affecting only cell-cell adhesions between non-like cells of the testis, with no disruptive effect on adhesions between like cells – we next more closely examined the integrity of the basolateral, homotypic adhesions that form between adjacent Sertoli cells at the BTB. We focused our analysis on a developmental window of spermatogenesis that displays dramatic Sertoli-Sertoli cell adhesion restructuring, with predictable phases of CAM relocalization: the migration of pre-meiotic spermatocytes through the BTB.

We first used IF to visualize the localization of N-cadherin, a prominent adherens junction CAM of Sertoli-Sertoli contacts. When we examined Stage III-V tubules, which contain Intermediate and B spermatogonial germ cells that have not yet migrated through the BTB, we found that N-cadherin organized apically to these germ cells, in both wt and ko testis (Fig. 7A; left column). In Stage VI-VII, just prior to spermatocyte migration through the BTB, N-cadherin localization was shown to completely surround preleptotene spermatocytes in both the wt and ko (Fig. 7A; middle column). These results indicate that the assembly of new Sertoli-Sertoli adhesions, which occurs basal to preleptotene spermatocytes, is initiated prior to the disassembly of the apically oriented adhesions already present and that the timing of this development is consistent in wt and ko testis. As leptotene spermatocytes complete their migration through the BTB during stages IX-X, N-cadherin was removed from apical distribution and could only be found basal to leptotene spermatocytes, in both wt and ko testis (Fig. 7A; right column).

To confirm that the basally oriented Sertoli cell nectin-2 distribution shown in Figure 1 was, in fact, properly organized at the BTB, we used sequential double staining IF to visualize N-cadherin and nectin-2 together. We found robust colocalization of N-cadherin and nectin-2 in both wt and ko (Fig. 7B). This demonstrates that, despite HUFA deficiency, Sertoli cells retain the capability to distribute nectin-2 to regions of the basolateral membrane where Sertoli-Sertoli
adhesions form, and that these adhesions are successfully rebuilt with each new generation of spermatocyte that migrates through the BTB.

We next assessed the morphology of the Sertoli-Sertoli contacts, at the level of TEM. At this ultrastructural level, the basal ES in both the wt and ko appeared normal (Fig. 8). The Sertoli cell actin plaque arrangement (Fig. 8; white arrowheads), ER cisternae alignment, and tight apposition of adjacent Sertoli cell plasma membranes are all characteristic of a healthy basal ES.

Finally, we assessed the functional integrity of the BTB in HUFA deficient animals. Biotin was injected directly into the interstitium of the testis to evaluate the permeability of the BTB and fluorescence microscopy was used to determine how far into the epithelium of seminiferous tubules the tracer could penetrate. In both the wt and ko, the biotin tracer was held in regions of the seminiferous epithelium basal to Sertoli-Sertoli adhesions, surrounding, but not moving beyond, the basally oriented Intermediate and B spermatogonia (Fig. 9; Stage III-IV) and preleptotene spermatocytes (Fig. 9; Stage VII-VIII).

Taken together, these observations demonstrate that, under HUFA deficiency, Sertoli cells continue to form molecularly and functionally intact adhesions with neighboring Sertoli cells, but fail to form molecularly or functionally intact Sertoli-spermatid adhesions.
Discussion
We previously showed that a selective HUFA deficiency leads to male infertility, and that this HUFA-deficient male infertility phenotype was characterized by a disruption in the elongation phase of spermiogenesis, arrested spermatid morphogenesis, and an abnormal release of immature spermatids from seminiferous tubules into the epididymis (Roqueta-Rivera et al., 2010; Roqueta-Rivera et al., 2011). As this phenotype is suggestive of a loss in Sertoli-spermatid adhesion integrity, we aimed here to determine how nectin-2 and nectin-3 protein distribution and Sertoli-spermatid adhesion assembly in the testis might be affected by HUFA deficiency. We demonstrate for the first time that HUFAs have essential roles in supporting the unique adhesions of the testis, at the level of cell adhesion molecule localization. Based on a careful examination of precisely stage-matched seminiferous tubules, we show that under conditions of HUFA deficiency: 1) critical adhesion molecules nectin-2 and -3 are appropriately expressed in their respective cell types, but are mislocalized; 2) the blood-testis barrier is intact by all accounts – CAM and actin localization by IF, ultrastructure by TEM, permeability by biotin injection; 3) nectin-2 localization at Sertoli cell adhesions is selectively impaired.

The interpretation that a loss in the localization of both nectin-2 and nectin-3 at the apical ES in HUFA deficient males would be sufficient to bring about male infertility is consistent with the phenotypes of knockout mouse models for nectin-2 and nectin-3 – Pvrl2 -/- and Pvrl3 -/- (Bouchard et al., 2000; Ozaki-Kuroda et al., 2002; Inagaki et al., 2006). In Pvrl2 -/- mice, where no other overt developmental abnormalities beyond male infertility were described, spermatozoa were severely impaired morphologically, and the defects of spermatogenesis were confined to a disruption in germ cell maturation, first seen at the spermatid elongation phase of spermiogenesis (Bouchard et al., 2000) – the phase when nectin-2 organizes to form the apical ES in Pvrl2 +/+ mice (Ozaki-Kuroda et al., 2002). Consistent with these previous reports, we found nectin-2 localization in HUFA sufficient males to be established at Sertoli-spermatid contacts just prior to the initiation of spermatid elongation, at steps 7-8 (Fig.1b). The Pvrl2 +/- mouse also showed a selective disruption in Sertoli-spermatid adhesions. Despite a severely disrupted apical ES, the BTB retained normal localization of the nectin adaptor protein afadin, as well as F-actin and the tight junction protein ZO-1, demonstrating a critical role for nectin-2 in anchoring the adhesions between Sertoli cells and spermatids, but not those between adjacent Sertoli cells (Ozaki-Kuroda et al., 2002). This Pvrl2 +/- male infertility phenotype is comparable to our HUFA deficiency
model in that only the heterotypic adhesions of the apical ES in the testis are affected, while the BTB remains intact.

*Pvrl3*-/- mice produce morphologically abnormal spermatozoa, with the defects of spermatid maturation, also, first observed at the initiation of the elongation phase in spermiogenesis (Inagaki et al., 2006). Nectin-3 expression in germ cells was also shown to be crucial for nectin-2 localization at, specifically, the apical ES; whereas nectin-2 localization at the BTB was not affected in the *Pvrl3* -/- mice (Inagaki et al., 2006). This selective mislocalization of nectin-2 in the Sertoli cells of *Pvrl3* -/- mice is very similar to what we show under HUFA deficiency (Fig. 2A). However, transplantation of nectin-3 expressing spermatogonial stem cells into the testes of *Pvrl3* -/- mice resulted in the restoration of apical ES formation (Inagaki et al., 2006), demonstrating that nectin-3 expression in germ cells was sufficient to promote the proper localization of nectin-2 at the apical ES. Significantly, we show that under HUFA deficiency, although nectin-3 is abundantly expressed in spermatids, and expressed at the appropriate developmental stage (step 9 of spermiogenesis; Fig. 3A), selective mislocalization of Sertoli cell nectin-2 persists.

Seminiferous tubules in *Pvrl2* -/- mice also showed a disruption in F-actin localization at the apical ES as well as mislocalization of the nectin adaptor protein afadin (Ozaki-Kuroda et al., 2002). This differs slightly from our finding that actin was initially localized properly at the apical ES, and only later in spermiogenesis (steps 12-15; Fig. 4) was actin organization disrupted. It may be that our detailed, step-wise examination of seminiferous tubules allowed us to identify a narrow window of spermatid development when the cytoskeletal structures of the apical ES begin to form, which was previously overlooked. It is also possible that the phenotype of apical ES nectin mislocalization, despite initial actin cap formation, is specific to HUFA deficiency. It is nevertheless significant to note that despite a complete absence of nectin-2 localization under HUFA deficiency, the apical ES is initially landmarked properly by an appropriate actin organization (Fig. 5), which supports the theory that assembly of a polarized cytoskeletal structure is, either, hierarchically upstream of CAM localization during adherens junction formation, or independent of it (Pilot et al., 2006; Li and Gunderson, 2008; Harris and Tepass, 2010). This initial cytoskeletal polarization shown in spermatids of HUFA deficient males differs, also, from the spermiogenesis phenotype described in junctional adhesion.
molecule C (JAM-C) knockout mice – Jam3-/-, where, based on the mislocalization of polarity proteins like Par6 and Cdc42, spermiogenesis was shown to be disrupted by a loss of spermatid polarization (Gliki et al., 2004). These authors did not provide a step-wise assessment of actin localization, but did show that early in the elongation phase, actin organization at Sertoli-spermatid contacts was disorganized (Gliki et al., 2004). Indeed, our HUFA deficient model of male infertility differs from each of the previously mentioned knockout models (i.e. nectin-2, nectin-3 and JAM-C) in that actin cap formation occurs (Fig.3-5).

In conflict with a previous report suggesting that the integrity of the BTB was disrupted by HUFA depletion in the Fads2-/- mouse (Stoffel et al., 2009), we found that the BTB is molecularly (Fig. 7), ultrastructurally (Fig. 8), and functionally intact (Fig. 9). Stoffel et al. based their conclusions on tracer permeability challenges to the BTB, using the electron-opaque lanthanum nitrate as a tracer and the 10 kDa dextran, tetramethylrhodamine, as a tracer. Our contradiction in findings may be explained by methodological differences. The sole seminiferous tubule image provided by Stoffel et al. of Fads2-/- males challenged with the 10 kDa dextran tracer appears to show a tubule section that is oblique, and possibly transverse. Tubules cut in this fashion provide few histological cues as to where the basal to adluminal transition occurs, and therefore can be misleading if included in an analysis dependent on clearly identifying these compartments. For our analysis, we have included only tubules cut in cross sections, which provide obvious histological cues as to the basal or adluminal identity of any region within the tubule (Fig. 9). Also, unlike the Stoffel paper, we provide carefully stage-matched comparisons of tubule cross sections showing two distinct phases of spermiogenesis, both clearly indicating that the biotin tracer is held basal to the BTB in the HUFA deficient males (Fig. 9A, B). Further, the penetration Stoffel et al. show of a 10 kDa molecule into the basal compartment of Fads2+/+ seminiferous tubules is suspect, as a molecule of this size is typically retained in the microvessels of healthy testis and fails to diffuse into even the interstitial space (Kamimura et al., 2002). We chose to use a tracer known to diffuse, in healthy mice, from the microvessels of the testis into the interstitial space – but not beyond the tight junctions between adjacent Sertoli cells (Morrow et al., 2009; Bi et al., 2013), and show this pattern of diffusion in both HUFA sufficient and HUFA deficient mice. As for the electron-opaque lanthanum nitrate tracer method, a high likelihood of false positives and inconsistent staining of extracellular spaces has led to a caution against the use of this technique (Pelletier, 2011).
That the Sertoli cells of HUFA deficient mice successfully distribute nectin-2 to the homotypic adhesions at basolateral membrane domains, but not to the heterotypic adhesions at apical membranes, indicates that nectin-2 delivery to these distinct membrane destinations is mediated by distinct modes of protein delivery. Although the specific route of nectin-2 protein trafficking to the apically oriented Sertoli-spermatid contact is still unclear, Sertoli cell endocytosis of large, tubular structures (termed tubulobulbar complexes) is known to be the primary means of removing adhesion proteins from Sertoli-spermatid contact sites at the time of spermatozoa release (Guttmann et al., 2004). Vesicles budding from these tubular structures stained positive for the CAMs nectin-2, nectin-3, and integrin β1, and were colocalized with early endosomal antigen 1 (Young et al., 2009). The same CAMs, after internalization, colocalized with the early endosomal marker Rab 5 in the region directly adjacent to the releasing spermatozoa, but the CAM-positive cytosolic puncta that were distributed in the seminiferous tubules closer to step 8 spermatid heads, showed a colocalization with, not Rab5, but the recycling endosomal marker Rab11 (Young et al., 2012), suggestive of an endosomal recycling of nectin proteins to the newly forming apical ES following internalization through the tubulobulbar complex (Fig. 10A; purple arrows). According to this model, our data suggest that HUFA deficiency may selectively disrupt nectin-2 localization at the apical ES by selectively disrupting nectin-2 intracellular trafficking (Fig. 10B).

When regulators of endosomal recycling, with critical functions in the testis, have been impaired, the consequent defects in spermiogenesis are strikingly similar to the spermiogenesis phenotype of HUFA deficient male infertility. The C-terminal Eps-15 homology domain protein 1 (EHD1), which directly interacts with the Rab5 effector, rabenosyn 5, regulates protein trafficking from recycling endosomes to the plasma membrane by serving to vesiculate recycling endosome tubular elements (Naslavsky and Caplan, 2005). The male specific infertility described in the Ehd1 -/- mouse is characterized by a loss of morphologically mature spermatozoa, and an abnormal appearance of sloughed, immature spermatids in the epididymis (Rainey et al., 2010). Analysis of the seminiferous epithelium in Ehd1 -/- males revealed a severe disruption in spermatid maturation, as most spermatids failed to elongate properly; although pre-meiotic germ cell maturation, meiosis, and the early steps of post-meiotic maturation appeared normal (Rainey et al., 2010). The few spermatids that did successfully elongate, then failed to be released from the seminiferous epithelium at the appropriate developmental stage (Rainey et al., 2010). This
early release of immature, round spermatids, together with an inability to release mature spermatozoa, is suggestive of defects in Sertoli cell endocytosis and recycling, as well as Sertoli-spermatid adhesion integrity. In the HUFA deficient Fads2-/- we show these same two defects, with condensed nuclei of step 16 spermatids found retained in the seminiferous tubules after the developmentally appropriate stage of spermatozoa release (Fig. 4A; asterisk), further supporting the proposed defect of endosomal recycling in HUFA deficiency (Fig. 10B). The vacuolar protein sorting-associated protein 54 (Vps54) is a component of the Golgi-associated retrograde protein (GARP) complex and is known to regulate retrograde transport from early or late endosomes to the Trans-Golgi network (TGN) (Quenneville et al., 2006). Further suggestive of apical ES dependence on the integrity of endosomal redistribution, the male specific infertility of the Vps54 spontaneous recessive mutant is characterized by impaired spermatid maturation, with transmission electron microscopy of the seminiferous epithelia showing multiple gaps between Sertoli cells and immature germ cells (Paiardi et al., 2011). In order to clarify this proposed role for HUFAs in promoting endosomal recycling in the testis (Fig. 10), further investigation is required.

Among the two HUFAs studied here, ARA is rarely under consumed by humans eating a typical western diet, whereas insufficient DHA intake in the United States is widespread (Kris-Etherton et al., 2000). An estimated one half of all cases of male infertility are idiopathic (Krausz, 2011) and reduced levels of the omega-3 HUFA, DHA in this population have been found in both spermatozoa and blood plasma (Zalata et al., 1998; Safarinejad et al., 2010). Epidemiological data supports this association further, showing better sperm morphology among males with higher omega-3 fatty acid intake (Attaman et al., 2012); and a recent clinical trial successfully demonstrated that improvements in sperm health could be correlated with increases in sperm DHA concentration, brought about by simple fish oil consumption (Safarinejad, 2011). This suggests that dietary DHA intake, sufficient to maintain testis enrichment, is critical for the healthy production of spermatozoa and the maintenance of male fertility. We have shown here that the assembly of a unique cell-cell adhesion in the testis, critical to the healthy formation of sperm, is dependent on sufficient supply of the HUFAs ARA and DHA, providing a biochemical basis for these observations.
In conclusion, our study demonstrates a selective impairment of Sertoli-spermatid adhesion, but not Sertoli-Sertoli adhesion (blood-testis barrier) by HUFA deficiency. This selective impairment by HUFA deficiency is similar to the phenotype caused by the loss of endosomal recycling molecules, suggesting that HUFAs may play a critical function in this recycling process.

Methods
Animal and diet
All animal work was approved by the University of Illinois Institutional Animal Care and Use Committee. C57BL/6J wild type mice (+/+) and homozygous Fads2-null (-/-) males with C57BL/6J background were used for the study. Animals were fed AIN93G diet (containing sufficient linoleic and α-linolenic acid, but not preformed n-3 or n-6 HUFAs) from weaning to 16 weeks of age. All mice were singly caged from weaning until sacrifice.

Testis preparation
Mice were euthanized by CO₂ inhalation. Testis were then excised and submerged in cold 4% methanol-free formaldehyde (Thermo Scientific) for an 18 hour fixation at 4°C, then testis were transferred to cold 70% ethanol prior to paraffin embedding. Sections were cut at 5 µm and dried overnight for immunostaining.

Confocal immunofluorescence
For immunostaining, sections were deparaffinized, rehydrated and antigen retrieval was carried out using a 45 min incubation in 10 mM sodium citrate buffer at 98°C. Samples were then permeabilized with 1% Triton-X, blocked for 1 h with 5% normal goat serum (Jackson Immuno) and incubated with primary antibodies overnight at 4°C. After washing, secondary antibodies were incubated for 2 h at room temperature, and finally samples were counterstained for 30 min. with Hoechst (Invitrogen; 3.5 µg/ml). Sections were mounted in Prolong Gold (Invitrogen) and cured for 24 h at room temperature. Cover glass was then sealed with top coat nail polish and sections were held at 4°C until analysis. All comparisons between groups were made after carefully matching seminiferous tubules based on their stage in the cycle of the seminiferous epithelium (Hess and Renato de Franca, 2008). The following primary antibody dilutions were used: nectin-2 (sc-14799; Santa Cruz, goat polyclonal; 1:50), nectin-3 (sc-14806; goat polyclonal; 1:50), actin (ab3280; Abcam, mouse monoclonal; 1:200), integrin α1 (sc-9936; goat polyclonal; 1:50), N-cadherin (Abcam 5D5; mouse monoclonal; 1:200). The following
secondary antibodies were used, all at a 1:200 dilution: donkey anti-goat 488 (sc-362255), goat anti-mouse 647 (Invitrogen).

Sequential double staining was carried out using primary antibodies from different hosts with an additional serum blocking step following the first secondary antibody incubation. Immunostaining was visualized with a Zeiss LSM 700 laser scanning confocal microscope. Laser power, gain and offset were kept constant across samples. Imaris and Zen 2011 software was used for analysis.

**Electron Microscopy**

*Fads2* -/- and *Fads2* +/+ males fed AIN93G until 16 weeks of age were perfused as described previously (Roqueta-Rivera et al., 2011). Briefly, mice were injected with heparin 15 min prior to pentobarbital anesthesia, followed by PBS and 5% glutaraldehyde perfusion through the left ventricle. Testis were post fixed in osmium tetroxide/potassium ferrocyanide, dehydrated and embedded in epoxy-resin. Section were cut at 1µm and stained with toluidine blue, cut into 70 nm sections with an ultramicrotome and imaged with a Hitachi H600 electron microscope.

**Biotin Injection**

Blood-testis barrier permeability was assessed using the previously described method (Bi et al., 2013). Briefly, three *Fads* +/+ and three *Fads* -/- were anesthetized by isoflurane gas, delivered by a precision vaporizer at 2-5% to effect. Testis were exposed through a small incision in the scrotal layer and 20 µl of biotin tracer (EZ-Link Sulfo-NHS-LC-Biotin (Fisher Scientific; 21336)) at 10 mg/ml in sterile PBS was injected using a 25 µl Hamilton syringe directly into the interstitium of one testis. After 30 min under anesthesia, mice were euthanized by cervical dislocation, and testis were dissected out and fixed in 4% paraformaldehyde for 18 h at 4°C, as above. Testis were embedded in paraffin, sectioned at 5 µm, deparaffinized, rehydrated and subjected to antigen retrieval as above. Tissue slices were then incubated with FITC-conjugated streptavidin (Invitrogen) at 1:500 for 30min., counterstained with Hoechst at 3.5 µg/ml for 15 min and mounted in Prolong Gold. FITC and Hoechst were imaged with a Zeiss LSM 700 laser scanning confocal microscope.
Fig. 2. Nectin-2 localizes normally at Sertoli-Sertoli, but not Sertoli-spermatid adhesions under HUFA deficiency. Nectin-2 (green) distribution in the seminiferous epithelium at multiple stages in the cycle of the seminiferous epithelium is visualized by immunofluorescence. A) In both wildtype (wt) and knockout (ko), nectin-2 localizes at Sertoli-Sertoli contacts (white arrows) near the basement membrane, but in the ko, localization is lost at Sertoli-spermatid contacts (red arrows), which organize in apical regions of Sertoli cells. DNA shown in blue. Scale bar=10 µm. B) Nectin-2 (green) localization adjacent to representative wt and ko spermatids at sequential steps of spermiogenesis. In wt, a robust perinuclear organization is established between steps 7-9 and maintained through step 14. In ko, nectin-2 fails to organize in this perinuclear region during any step of spermiogenesis. Scale bar=5 µm. (C) Maximum intensity projections from deconvolved 3D images of step 9 spermatids showing nectin-2 (green). In wt, nectin-2 organizes into caps that cover the anterior end of each spermatid at the sites of Sertoli-spermatid contact. In ko, nectin-2 organization at spermatids is completely lost. Scale bar=3 µm.
Fig. 3. Nectin-3 is expressed, but fails to distribute to the anterior poles of elongating spermatids under HUFA deficiency. A) Nectin-3 (green) localization in representative wt and ko spermatids at sequential steps of spermiogenesis. In both wt and ko, expression is upregulated at step 9, but only in the wt is nectin-3 localized at both anterior regions (red arrows) and caudal regions (white arrows) of spermatids. In the ko, nectin-3 distribution to the anterior poles of spermatids is lost (red arrows). Scale bar=5 μm. B) Maximum intensity projections from deconvolved 3D images of step 9 spermatids showing nectin-3 (green) localization. In the wt, nectin-3 organizes into caps at the anterior poles (red arrows) and into sheet-like patterns along the caudal regions of nuclear elongation. In the ko, only the caudal distribution is established, and nectin-3 is absent from anterior poles (red arrows). Scale bar=3 μm.
Fig. 4. Sertoli cells initially landmark Sertoli-spermatid interface with actin cap assembly under HUFA deficiency. A) Actin (gold), visualized by immunofluorescence, at step 9 of spermiogenesis (Stage IX of cycle of the seminiferous epithelium). In both wt and ko, actin organizes in arcs adjacent to nuclei of spermatids (red arrows) and surrounding leptotene spermatocytes at the basement membrane (white arrows). The asterisk (*) in the Stage IX tubule of the ko indicates step 16 spermatids that are abnormally retained in seminiferous tubules past spermiation. B) Actin localization at step 14 of spermiogenesis (Stage II-III of cycle of the seminiferous epithelium). In the ko, actin organization at spermatids is lost by step 14 of spermiogenesis (red arrows), whereas localization near the basement membrane remains undisturbed as it covers the top of intermediate spermatogonia (white arrows). Scale bar=10 µm.
Fig. 5. Cell adhesion molecules (CAMs) fail to colocalize with actin caps at sites of Sertoli-spermatid contact under HUFA deficiency. A) Nectin-2 (green), actin (red) and their colocalization (yellow) adjacent to step 9 spermatids, visualized by sequential double stain immunofluorescence. In wt Sertoli cells adjacent to step 9 spermatids, nectin-2 is predominantly distributed at the apical Ectoplasmic Specialization (ES), as defined by actin caps. In the ko, nectin-2 shows no preferential distribution to the apical ES, despite the successful assembly of actin caps. Scale bar=10 µm. B) Integrin β1 (green) visualized with actin (red) in Sertoli cells adjacent to step 11 spermatids. In the wt, integrin β1 organizes in puncta that colocalize with actin caps. In the ko, no colocalization is seen. Scale bar=5 µm. C) Nectin-3 (green), actin (red) and their colocalization (yellow) at step 9 spermatids visualized by sequential double stain immunofluorescence. In wt step 9 spermatids, nectin-3 colocalizes with actin caps at the anterior poles. In ko step 9 spermatids, nectin-3 shows no distribution to anterior poles, despite the successful assembly of actin caps and spermatid expression of nectin-3. Scale bar=5 µm.
Fig. 6. The apical ES ultrastructure is normal, but spermatid and Sertoli PMs dissociate under HUFA deficiency. Structure of the apical ectoplasmic specialization (ES), which consists of the Sertoli cell plasma membrane (PM), actin filament plaques and endoplasmic reticulum (ER) cisternae. A) The apical ES is similar in wt and ko at steps 8-9 of spermiogenesis. However, in the wt, Sertoli and spermatid plasma membranes (PM) are closely apposed along the entire length of the apical ES; whereas in the ko, the spermatid PM appears ruffled and there are multiple points of separation (asterisks) between spermatid and Sertoli cell PMs. (Apx, acroplaxome; NDL, nuclear dense lamina; Acr, acrosome). Scale bar=100 nm.
Fig. 6. (continued) The apical ES ultrastructure is normal, but spermatid and Sertoli PMs dissociate under HUFA deficiency. B) At steps 10-11 in the wt, actin filaments have organized into bundles (white arrowheads) arranged in between the ER cisternae and Sertoli PM throughout the apical ES. In the ko, there is minimal bundling of actin filaments (white arrowheads) and there are large stretches where no actin is found (black arrowheads). Scale bar=1 µm. C) At step 12 in the wt, puncta-like actin bundles are regularly spaced along the entire length of the apical ES (white arrowheads), and Sertoli-spermatid plasma membranes (PM) are tightly apposed across the same distance. By step 12 in the ko, substantial Sertoli-spermatid PM separation (double-sided arrow) is seen, in addition to multiple points of more moderate bilayer separation (asterisks). In the ko, no puncta-like actin bundles are found and several stretches of apical ES show no actin (black arrowheads); the nucleus also fails to elongate properly and an aberrant protrusion of the perinuclear ring can be seen. Scale bar=1 µm.
Fig. 7. Dynamic restructuring of cell adhesion molecules (CAMs) at the blood testis barrier (BTB) is unaffected by HUFA deficiency. A) N-Cadherin localization at the BTB in sequential stages of spermatogenesis. In both wt and ko at stages III-V of the cycle of the seminiferous epithelium (left column), N-Cadherin organizes apically to spermatogonia (sp) and basally to early pachytene spermatocytes (asterisks). In both wt and ko at stages VI-VII (middle column), N-Cadherin organizes both apically and basally to preleptotene spermatocytes (ps). By stages IX-X (right column), N-Cadherin organizes basally to leptotene spermatocytes (LS), in both wt and ko. Scale bar=5 µm. B) Nectin-2 (green), N-Cadherin (red) and their colocalization (yellow) visualized with sequential double staining immunofluorescence. In both wt and ko, nectin-2 organizes properly at Sertoli-Sertoli contacts, as defined by N-Cadherin localization. Scale bar=5 µm.
Fig. 8. The morphology of the basal ectoplasmic specialization (ES) is unaffected by HUFA deficiency. The actin plaques and the endoplasmic reticulum (ER) cisternae characteristic of the basal ES organize similarly in the wt and ko. Adjacent Sertoli plasma membranes (PM) are tightly apposed along the entire length of the basal ES, where actin filaments have organized into bundles (white arrowheads) arranged in between cisternae of ER and Sertoli cell PMs. Scale bar=500nm
Fig. 9. The blood-testis barrier (BTB) is not permeable to biotin under HUFA deficiency. 
Cross sections of wt and ko seminiferous tubules from testis fixed 30 min following an intratesticular biotin injection. Biotin is shown in green and DNA is shown in blue. At Stage III-IV, injected biotin diffuses into the seminiferous epithelium to surround intermediate spermatogonia (In) and early B spermatogonia (b), germ cells that have not yet migrated through the BTB. Biotin does not surround pachytene spermatocytes (P), round spermatids (rs), or elongated spermatids (es), germ cells that have already migrated through the BTB. At Stage VII-VIII, injected biotin consistently surrounds preleptotene spermatocytes (pL), which have not yet migrated through the BTB, but did not diffuse into the adluminal compartment of the seminiferous tubule. Scale bar=10 µm.
Fig. 10. A proposed model for the selective disruption of nectin-2 localization. A) In the HUFA sufficient wt, nectin-2 distribution to Sertoli-Sertoli adhesions at the basolateral plasma membrane domain likely follows a ubiquitous biosynthetic route: from the endoplasmic reticulum to the Golgi apparatus, sorted into vesicles at the trans-Golgi network (TGN), and delivered directly to the plasma membrane (green arrows). However, for distribution to Sertoli-spermatid adhesions at apical membrane domains, nectin-2 may require rerouting through multiple endosomal compartments (purple arrows), as suggested by (Guttman et al., 2004; Young et al., 2009; Young et al., 2012). B) In the HUFA deficient ko, selective loss of nectin-2 localization at apical Sertoli-spermatid adhesions may be due to defects in endosomal redistribution, leading to the premature release of round spermatids and a severe disruption in the morphogenesis required for proper spermatozoa formation.
References


Chapter 4: Dietary DHA fully restores proper spermatid development and retention in seminiferous tubules of D6D-/-

Abstract
Acrosome biogenesis and Sertoli-spermatid adhesion formation are critical developments of round spermatid maturation into spermatozoa. These two processes were recently demonstrated to be dependent on sufficient testis abundance of the highly unsaturated fatty acids (HUFAs) docosahexaenoic acid (DHA) and arachidonic acid (ARA), HUFAs enriched in the phospholipids of testis tissue in humans and mice. Here we report that disruptions in the redistribution of Trans-Golgi network (TGN) vesicular trafficking protein VAMP4 and TGN vesicle tethering protein golgin-97 are induced by HUFA deficiency, and we demonstrate their restoration with dietary supplementation of either ARA or DHA. Analysis of the molecular integrity of Sertoli-spermatid contacts revealed that either ARA or DHA dietary supplementation was capable of promoting normal Sertoli cell distribution of the critical Sertoli-spermatid adhesion protein nectin-2. However, a more functional assessment of Sertoli-spermatid adhesion integrity – the histological assessment of sloughed round spermatids in the epididymis and retained elongating spermatids in seminiferous tubules – demonstrated that only dietary DHA supplementation fully restored Sertoli-spermatid adhesion, while ARA supplementation was less effective. These findings identify new roles for vesicle trafficking proteins in acrosome biogenesis and provide potential mechanistic functions for HUFAs that are enriched in the testis.

Introduction
Although an insufficiency in omega-3 fatty acids in humans has been linked to a wide variety of health concerns including Alzheimer’s disease (Lukiw et al., 2005), coronary heart disease (Ramsden et al., 2013), sub-optimal fetal development (Rogers et al, 2013) and male infertility (Zalata et al., 1998), mechanistic functions specific to omega-3 HUFAs have been poorly defined. Male fertility was recently shown to be dependent on testis tissue enrichment of the omega-3 highly unsaturated fatty acid (HUFA) docosahexaenoic acid (DHA), whereas the omega-6 HUFA arachidonic acid was shown to be dispensable, with sufficient testis DHA (Stroud et al., 2009; Roqueta-Rivera et al., 2010; Roqueta-Rivera et al., 2011). This HUFA-
deficient male infertility phenotype was characterized by a failure of acrosome biogenesis and the abnormal appearance of round spermatids in the epididymis.

The acrosome is classically described as an organelle derived from small vesicles of Golgi origin which are released from the TGN beginning at the onset of Golgi Phase in round spermatids (Leblond and Clermont 1952). The extensive involvement of the Golgi apparatus during acrosome formation has been confirmed primarily by ultrastructural analysis of transmission electron microscopy as well as periodic acid Schiff staining of glycoproteins of Golgi origin (Hermo et al. 2010), and this level of analysis has been used to classify four sequential phases of spermiogenesis, defined by morphological criteria. The first phase, “Golgi phase,” consists of transformations that occur at or adjacent to the Golgi, including the appearance of small translucent vesicles which coalesce to form the more electron-dense proacrosomal granules. These proacrosomal granules eventually fuse to form the acrosomal granule (Leblond and Clermont 1952; Hermo et al. 2010) which tethers tightly to the nuclear envelope by directly interacting with a testis specific cytoskeletal structure, the acroplaxome (Kierszenbaum et al. 2003). In the second phase, “Cap Phase,” the acrosomal granule transforms from a spherically shaped organelle to more of a flattened sacculus that spreads over a majority of the anterior region of the nucleus (Hermo et al. 2010).

Although the small translucent vesicles that fuse to form the developing acrosome have been classically described as originating directly from the Golgi apparatus, being released from the TGN at the onset of Golgi Phase in round spermatids (Leblond and Clermont, 1952; Kang-Decker et al., 2001), indirect routes of vesicular trafficking involving movement through endosomal compartments have recently been proposed to play critical roles in acrosome formation (Berruti et al. 2010). Vesicle-associated membrane protein 4 (VAMP4) is a membrane fusion protein of the SNARE family that has been localized primarily in the trans-Golgi network region in several cell types (Steegmaier et al. 1999, and Feldmann et al. 2009), including Golgi-phase round spermatids (Guo et al. 2010). In round spermatids, VAMP4 is thought to play a critical role in the fusion of proacrosomal vesicles to support proper acrosome development, based on an ultrastructural analysis of mouse testis following an in vivo RNA-mediated knockdown of VAMP4 (Guo et al. 2010). The golgin protein golgin-97, a resident protein of the Trans-Golgi network thought to contribute to the molecular stability of the Golgi
apparatus during periods of dynamic biogenesis, has been shown to tether retrograde transport vesicles moving from endosomes to the TGN (Jing et al. 2010). Although golgin-97 is known to be expressed in spermatids (Hermo et al., 2010), its function remains unknown.

Sertoli cells, the epithelial cells of the testis, regulate spermatid retention in the seminiferous epithelium during their maturation by establishing adherens-like cell-cell adhesions with spermatids. These cell adhesion complexes, which are unique to the testis, are known as apical ectoplasmic specializations (Vogl et al., 2008; Cheng and Mruk 2010). These structures serve to tether spermatids directly to Sertoli cells beginning at the onset of spermatid elongation in spermiogenesis, and the cell adhesion molecules nectin-2 and nectin-3 are critical components of this adhesion complex (Takai et al., 2008). Following the successful completion of testis spermatid maturation (spermiogenesis), the apical ectoplasmic specialization, that serves to adhere morphologically mature spermatozoa to Sertoli cells, is actively disassembled by the proteolytic action of matrix metalloproteinases (Siu and Chang, 2004) and Sertoli cell invagination of Sertoli-spermatid adhesion complexes (Young et al., 2012).

The aim of this study was to characterize the failure of acrosome biogenesis under HUFA deficient male infertility at the level of post-Golgi protein trafficking, and to assess the effectiveness of dietary HUFA supplementation in restoring the integrity of Sertoli-spermatid adhesions at molecular and functional levels.
Results

Glycosylated proteins of maturing spermatids fail to distribute properly under HUFA deficiency

To follow the distribution of glycosylated proteins destined for acrosomal delivery during spermiogenesis, the peanut agglutinin lectin PNA was used to stain intact testis tissue slices from D6D wild type (wt) animals and D6D knockout (ko) animals at sequential stages in the cycle of the seminiferous epithelium with fluorescent confocal microscopy. As PNA is specific for terminal beta-galactose residues, round spermatids at the Golgi phase (or stage III in the cycle of the seminiferous epithelium) displayed PNA staining in one discrete perinuclear puncta, characteristic of Golgi positioning, in both wt and ko spermatids (Fig.1A, Stage III, red arrows). Moving into cap phase (stage VII in the cycle of the seminiferous epithelium), the PNA stain is redistributed in wt spermatids to organize into a perinuclear arc (Fig.1A wt stage VII; red arrows, and Fig.11B wt), a formation consistent with the shape of the developing acrosome. In the ko, however, PNA staining does not demonstrate this redistribution; rather, it remains concentrated in discrete puncta similar to its distribution in Golgi phase spermatids (Fig.11A, ko stage VII, red arrows; and Fig.11B, ko). Differential interference contrast (DIC) of spermatids at the initiation of elongation (step 8 of spermiogenesis) shows how nuclei have migrated to the plasma membrane at anterior poles of spermatids and cytosolic organelles migrated have to regions caudal to this anterior pole (Fig.11B). PNA staining in these step 8 spermatids shows how in the ko, PNA-positive puncta have migrated to a region of spermatids consistent with the migration pattern of the Golgi apparatus during spermatid elongation, whereas the migrating Golgi apparatus in wt spermatids is free of PNA stain (Fig.11B). These findings indicate that an abnormal accumulation of glycosylated proteins in the Golgi region of spermatids occurs during the stages of acrosome formation under HUFA deficiency.

Abnormal Golgi accumulation of TGN vesicle trafficking proteins coincides with a failure of acrosome biogenesis under HUFA deficiency

We next aimed to more precisely define the organellar localization of PNA-stained proteins in HUFA-deficient spermatids in a stage-specific fashion by probing for the Golgi resident and TGN vesicle fusion protein VAMP4. By using immunofluorescent staining of intact testis tissue slices, we imaged seminiferous tubules at multiple stages throughout the cycle of the
seminiferous epithelium, as defined by classical morphological criteria. We show that at the Golgi phase (stage II-III), VAMP4 localization in wt spermatids is restricted to one, discrete perinuclear puncta (Fig. 12A, white arrows), and that this pattern of localization is found in ko spermatids as well (Fig. 12D, white arrows). Acrosomal resident proteins in Golgi phase spermatids, labeled with the peanut agglutinin lectin PNA, are shown to be localized entirely within this VAMP4-positive puncta in both the wt (Fig. 12C, white arrows) and ko (Fig. 12F, white arrows).

Moving into cap phase (stage VI-VII), distribution of VAMP4 in spermatids has changed dramatically in the wt, and now organizes as two distinct structures (Fig. 12G, white arrows), and the PNA-labeled resident proteins of the acrosome have been redistributed from one discrete puncta to a new discrete structure – the perinuclear cap of the developing acrosome (Fig. 12H). The sequential double staining of VAMP4 and PNA reveals a consistent relationship, with predictable regions of colocalization and mutually exclusive protein distribution in wt cap phase spermatid. VAMP4 colocalizes with PNA at the bulbous center of the cap-like structures (Fig. 12I, white arrowheads), but does not colocalize with PNA along the thinner edges of the PNA-stained acrosome. These PNA-only regions of the acrosome appear on either side of the rounded center where colocalization with VAMP4 occurs, or as uninterrupted arcs in spermatids where the cross section of the tissue slice has failed to cut through this VAMP4-positive core. There is also a region of VAMP4 staining in wt cap phase spermatids where no PNA is found (Fig. 12I, white arrows), but which is organized directly adjacent to the developing PNA-stained acrosome. In ko spermatids at the cap phase, no reorganization of the vesicle fusion protein VAMP4 is found (Fig. 12J, white arrowheads). Although there is an increased VAMP4 signal dispersed throughout the cytosol, the prominent staining in one, discrete perinuclear puncta remains (Fig. 12J, white arrowheads). The PNA stain in ko spermatids at the cap phase shows no arc-like organization and instead has clustered into multiple puncta that primarily colocalize with the single VAMP4 puncta (Fig. 12L, white arrowheads). Three dimensional images (composites of ~12 serial cross sections imaged every 300nm of depth in the z-axis) highlight the areas of colocalization between VAMP4 and PNA that are found in both wt and ko, as well as areas of mutually exclusive staining found only in the wt (Fig. 13).
These results demonstrate that, during the transition from Golgi phase to cap phase, acrosomal resident proteins are redistributed in the wt from a VAMP4-positive compartment (Fig. 12C, white arrows) to a separate compartment – the wide stretching, arc-like acrosome organelle, that is largely absent of VAMP4, although a central region of this organelle displays strong colocalization of VAMP4 and PNA (Fig. 12I). At the same time, a VAMP4-positive compartment that contains no PNA stain appears in the region immediately adjacent to the growing acrosome (Fig. 12I, white arrows). In spermatids of ko mice, this striking redistribution of proteins does not occur; rather both VAMP4 and PNA appear to accumulate in the Golgi apparatus, where they resided during Golgi phase (Fig. 12F and L).

Elongated step 14 or 16 spermatids, shown in wt seminiferous tubule cross sections at Golgi (Stage II-III) and cap phase (Stage VI-VII), respectively, display only PNA staining, with VAMP4 entirely absent from these more mature spermatids (Fig. 12B). This clearly demonstrates that VAMP4 distribution to the developing acrosome is transient, and any functional influence of VAMP4 is restricted to the formation of the acrosome, as opposed to the maintenance, or later activity, of this organelle.

We next probed for the TGN and recycling endosomal tethering factor golgin-97, in Golgi and cap phase spermatids. In both wt and ko spermatids at the Golgi phase (Stage II-III), golgin-97, like VAMP4, is confined to one perinuclear puncta (Fig. 14A and C, white arrowheads). In cap phase, the HUFA deficient spermatids of the ko do not shown any deviation from this Golgi phase localization pattern, as golgin-97 remains localized exclusively in one discrete globular unit (Fig. 14D, white arrowheads). In the HUFA sufficient wt, however, the golgin-97 has now organized into two distinct patterns within spermatids. There is one globular puncta (Fig. 14B, white arrowheads), consistent with the TGN localization pattern at Golgi phase, and there is also an additional arc-like distribution (Fig. 14C, white arrows), consistent with the cap-like organization of the newly forming acrosome.

**The Trans-Golgi Network interacts with proacrosomal dense-core vesicles in wt but not HUFA-deficient ko spermatids**

As both the TGN vesicle fusion protein VAMP4 and the TGN vesicle tethering factor golgin-97 demonstrated abnormal accumulations in the Golgi of HUFA-deficient spermatids during
acrosome formations, we next assessed the TEM ultrastructure of the TGN at early stages of Golgi and cap phase spermatids.

We found that in the HUFA sufficient wt, saccular and tubulovesicular extensions of the TGN, peeling off from the Golgi stacks, appear to tether to the newly forming dense-core proacrosomal vesicles and the proacrosomal granule formed in the wt (Fig. 15A and B). Although smaller dense-core proacrosomal vesicles form in the ko, we found these structures to interact minimally with saccular elements of the TGN (Fig. 15C and D). Further, regions of TGN and proacrosomal granule lipid bilayer association in the wt (Fig. 15B) are found directly adjacent to regions of the proacrosomal granule bilayer that show extensive membrane ruffling, characteristic of sites of vesicle budding and fusion. This proximity of ruffled acrosomal membranes to regions where tubulovesicular structures of the TGN align with the growing acrosome might indicate a role for these TGN extensions in directing the traffic of proacrosomal vesicles, perhaps by docking vesicles or promoting their fusion with the acrosome.

Taken together, these results suggest that the regulated redistribution of both golgin-97 and VAMP4 out of the Golgi stacks, possibly into prolonged tubulovesicular extensions that align against the developing acrosome, is a critical development of acrosomal protein delivery and acrosome formation. We show here that these molecular and structural developments are disrupted under HUFA deficiency.

**Either ARA or DHA supplementation restores acrosome formation and TGN trafficking protein redistribution in D6D -/- mice**

After highlighting the nature of TGN disruption in maturing spermatids under HUFA deficiency, we next sought to evaluate the effectiveness of dietary HUFA supplementation in resolving the abnormal Golgi accumulation of TGN vesicle trafficking proteins. We restored ko testis HUFA tissue levels with either the omega-6 HUFA arachidonic acid (ARA) or the omega-3 HUFA docosahexaenoic acid (DHA) by feeding ko animals a diet supplemented with these select fatty acids at 0.2% (w/w). PNA staining of testis from ARA-fed ko mice revealed a restoration of PNA distribution in cap phase spermatids (Fig. 16A) and elongating spermatids (Fig. 16B). Feeding the omega-3 HUFA DHA similarly restored PNA distribution to the arc-like distribution of the developing acrosome in both cap phase (Fig. 16C) and elongating spermatids (Fig. 16D).
Likewise, VAMP4 localization shifted from the single perinuclear puncta found in Golgi phase spermatids (Fig. 17A) to an arc-like distribution in cap phase spermatids of both ARA and DHA-fed ko mice (Fig. 17B), similar to wt VAMP4 redistribution.

**Supplementation with dietary DHA, but not ARA, restores both elongated spermatid retention and spermiation in D6D +/- mice**

Although dietary supplementation with either the omega-6 ARA or the omega-3 DHA to D6D ko mice restored the TGN protein movement of acrosomal development and Sertoli cell nectin-2 distribution to the Sertoli-spermatid interface, histological assessments of seminiferous tubules and epididymis indicated a more complete restoration of Sertoli-spermatid adhesion integrity with dietary DHA supplementation (Figs 18-20).

Analysis of seminiferous tubules immediately preceding and immediately following spermiation revealed defects in spermatid retention and spermatozoa release in ko mice not supplemented with dietary DHA (Figs 18,19). Although the average number of round spermatids in tubules at stage VII in the cycle of the seminiferous epithelium is relatively similar between all groups (data not shown), the number of retained elongated spermatids is reduced in ko animals not supplemented with dietary DHA at 0.2% (Fig. 18A and B). Following spermiation, in stage IX seminiferous tubules, ko animals not supplemented with dietary DHA contain both abnormally retained spermatozoa and a reduced number of elongating spermatids (Fig. 19A and B), suggestive of defects in both round spermatid adhesion and elongated spermatozoa release in the absence of sufficient DHA enrichment.

Tissue slices from fixed cauda epididymis were analyzed from wt mice and ko mice separated into five dietary treatment groups: non-supplemented, 0.1% ARA (w/w) supplemented, 0.2% ARA supplemented, 0.1% DHA supplemented, and 0.2% DHA supplemented. Multiple cross sections of epididymal areas were examined for the presence of round spermatids. As compared to the epididymis of wt, the epididymis from ko animals either not supplemented with HUFAs, or supplemented with ARA, was found to contain an abundance of round, immature spermatids (Fig. 20A). Although dietary supplementation of ko mice with DHA at 0.1% (w/w) did not reduce the appearance of round spermatids in the epididymis, dietary supplementation of
ko mice with 0.2% DHA reduced round spermatid presence in the epididymis to a level significantly lower than non-supplemented wt animals (Fig. 20B).
Discussion
This investigation into the dysfunctions of HUFA-deficient male infertility identified new molecular mediators of acrosome biogenesis and defined the functional significance of dietary HUFAs on round spermatid retention in the seminiferous epithelium and demonstrated their influence on the regulated redistribution of TGN proteins during acrosome biogenesis. The abnormal retention of both resident proteins of the acrosome and post-Golgi vesicular trafficking proteins within the Golgi of round spermatids from HUFA-deficient D6D ko mice indicates that testis HUFA enrichment is critical for the regulated redistribution of proacrosomal proteins out of the Golgi. The restoration of acrosome biogenesis and vesicular trafficking protein movement by dietary supplementation of either ARA or DHA HUFAs underscores the importance of dietary HUFA consumption for the maintenance of male fertility. On the other hand, the fact that the abnormal appearance of immature, round spermatids in the epididymis was only corrected completely when the omega-3 DHA was supplemented in the diet, and not when the omega-6 HUFA ARA was supplemented, suggests that DHA is the more critical of the two testis enriched HUFAs for the production of mature spermatozoa.

Our interpretation that a disruption in the regulated redistribution of post-Golgi vesicle trafficking proteins VAMP4 and golgin-97 may lead to a failure of acrosome biogenesis is consistent with recent reports on the molecular nature of acrosome biogenesis.

Vesicle-associated membrane protein 4 (VAMP4), a membrane fusion protein of the SNARE family that localizes primarily at the trans-Golgi network, is a critical mediator of acrosome biogenesis. Immunofluorescence was used to localize VAMP4 at the acrosome of elongating spermatids, and following siRNA knockdown of VAMP4 in mouse testis, an arrest of acrosome biogenesis and apparent failure of proacrosomal granule fusion was demonstrated (Guo et al. 2010.) That knockdown of VAMP4 prevented spermatids from progressing beyond the early stages of acrosome development – proacrosomal vesicle fusion into a proacrosomal granule – suggests that VAMP4 exerts its influence during the vesicle fusion events that establish acrosome formation at its dense-core granule phase. This critical role for the TGN vesicle fusion protein VAMP4 in the early stages of acrosome biogenesis would be consistent with proposed functions for VAMP4 in other cell types. Although the function of VAMP4 in intracellular trafficking is commonly described as directing endosome-to-TGN traffic, there is evidence for its
role in regulating biogenesis of secretory granules or other short-lived and highly responsive intracellular compartments, not unlike the acrosome. In acinar cells of the parotid gland, specialized secretory granules in the early stages of their formation contain high levels of VAMP4, but following their maturation into high density granules VAMP2 is much more abundant (Katsumata et al. 2007), supportive of a role for VAMP4 in, specifically, the early stages of specialized granule assembly. Likewise, our findings that VAMP4 colocalization with acrosomal resident proteins is robust in the Golgi phase, and restricted to the granule region of the developing acrosome in early cap phase, and then gone entirely in the more mature acrosomal phase, suggests that VAMP4 exerts its influence in, specifically, the early stages of acrosomal granule assembly. In adipocytes, VAMP4 was shown to direct intracellular trafficking of the insulin responsive glucose transporter GLUT4 to an insulin sensitive intracellular compartment when VAMP4 knockdown with siRNA treatment disrupted the assembly of this compartment and prevented insulin-mediated GLUT4 translocation to the plasma membrane (Williams and Pessin 2008). This finding demonstrated a role for VAMP4 in redirecting proteins of Golgi origin from a constitutive pathway to a compartment of regulated release. As the acrosome is in essence a secretory compartment that only fuses with the plasma membrane to release its contents upon direct stimulation (a process termed the acrosome reaction), our finding that VAMP4 demonstrates dramatic relocalization from the TGN to the acrosome suggests this fusion protein similarly redirects protein flow to a compartment of regulated release in spermatids.

Our finding that the Golgi tether golgin-97 may also play a significant role in acrosome biogenesis is consistent, primarily, with evidence of the function of this protein demonstrated in cell culture. Golgins are long, coiled-coil molecules with curved membrane binding affinity at the N-terminus, by way of an amphipathic lipid packing sensor (ALPS) motif, and adaptor binding affinity at the C-terminus, by way of a GRIP-related Arf binding (GRAB) domain (Drin et al. 2008). By simultaneously binding to adaptor protein-containing regions of the Golgi apparatus and with lipid vesicles of a narrow range of size, golgins can serve to promote the tethering of vesicles destined for fusion with the Golgi. The golgin protein Golgin-97 has been shown to tether retrograde transport vesicles moving from endosomes to the TGN (Jing et al. 2010) and also to participate in the trafficking of the adhesion molecule E-cadherin in post-Golgi
tubulovesicular carriers of the recycling endosome (Lock et al. 2005). Golgin-97 has also been shown to move between the cytosol and the cytosolic side of the TGN, localizing primarily along tubular membrane extensions of the TGN (Hesse et al., 2013). Our finding that the newly forming acrosomal granule interacts directly with TGN saccular elements peeling off of the cis-face of the Golgi stacks might be explained by this role for golgin-97 in tethering vesicles along the tubular or saccular extensions of the TGN. The tethering factor Golgin-97 has been shown to tether retrograde transport vesicles moving from recycling endosomes to the TGN, a trafficking route shown to promote the TGN localization of VAMP4 (Jing et al. 2010). Our finding that golgin-97 displays a pattern of intracellular redistribution similar to VAMP4 at the cap phase of spermiogenesis suggests that this tethering factor carries out this function in spermatids as well, serving to tether incoming VAMP4-containing vesicles to the developing acrosome. The role for golgin-97 in tethering vesicles of recycling endosome origin, taken together with the acrosomal redistribution we demonstrate in this study, further supports the idea that acrosomal biogenesis is dependent on vesicle movement from endosomes in addition to vesicles released directly from the TGN. Although golgin-97 has previously been shown only to localize at the Golgi in spermatids (Ramalho-Santos et al., 2001), the functional analysis of a similar golgin, TMF/ARA160, revealed a critical role for this protein in spermatid acrosome formation. TMF/ARA160, like golgin-97, serves as a tethering factor for retrograde transport to the Golgi (Yamane et al., 2007), and male mice deficient in TMF/ARA160 were infertile, with spermatids showing a failure to develop acrosomes during the Golgi phase of spermiogenesis (Lerer-Goldshtein et al. 2010). This further demonstrates the essentiality of Golgi retrograde tethering factors during acrosome biogenesis.

Our finding that, only by supplementing D6D ko mice with dietary DHA were we able to fully prevent the abnormal appearance of round, immature spermatids in the epididymis is consistent with previous assessments of HUFA deficient male mice. When epididymal sperm counts were evaluated in D6D ko males supplemented with Rab11 0.2% ARA or 0.2% DHA and compared to wt or heterozygous animals being fed a diet without preformed HUFAs, dietary DHA increased sperm counts to levels significantly higher than did dietary ARA (Roqueta-Rivera et al., 2010). In fact, dietary DHA was capable of increasing sperm counts in the D6D ko to levels that were significantly higher than the wt animals fed a diet without preformed HUFAs.
(Roqueta-Rivera et al., 2010). Our finding here, that providing dietary supplementation of DHA to D6D ko mice can completely prevent the premature sloughing of round spermatids into the epididymis, may also explain this role for DHA in restoring sperm counts in D6D ko mice.

The increased number of mature spermatozoa and reduced number of prematurely released round spermatids found in the epididymis of DHA fed D6D ko mice might be explained by a restoration in Sertoli-spermatid adhesion integrity. Critical Sertoli-spermatid cell-cell adhesion molecules nectin-2 and nectin-3 were recently found to be selectively mislocalized in HUFA-deficient males, showing pattern of distribution as cellular regions distant from sites of Sertoli-spermatid contact (Abbott et al., under review). Although we found here that dietary supplementation with either ARA or DHA was capable of restoring Sertoli cell nectin-2 distribution to sites of Sertoli-spermatid contact, the quantitative assessment of immature spermatids released into the epididymis suggests that DHA more completely restores the integrity of these adhesions, or the efficiency of their formation.

In conclusion, this study demonstrated essential roles for testis HUFAs as regulators of germ cell adhesion integrity and vesicular trafficking protein distribution. The restoration of acrosome biogenesis, vesicle trafficking protein and Sertoli-spermatid adhesion protein localization by either ARA or DHA, taken together with complete restoration of immature spermatid retention in the seminiferous epithelium by DHA only, and not ARA, suggests that dietary DHA intake, sufficient to maintain testis enrichment, is critical for the healthy production of spermatozoa and maintenance of male fertility.

Methods
Animals and diet
All animal work was approved by the University of Illinois Institutional Animal Care and Use Committee. C57BL/6J wild type mice (+/+) and homozygous Fads2-null (-/-) males with C57BL/6J background were used for the study. Animals were fed AIN93G diet (containing sufficient linoleic and α-linolenic acid, but not preformed n-3 or n-6 HUFAs) from weaning to 16 weeks of age. For dietary supplementation, homozygous Fads2-null (-/-) males were fed AIN93G supplemented either ARA or DHA at 0.2% (w/w) as previously described (Roqueta-
Rivera et al., 2010), or supplemented with either ARA or DHA at 0.1% (w/w) from weaning until sacrifice at 16 weeks of age. All mice were singly caged after weaning.

**Testis preparation**
Mice were euthanized by CO₂ inhalation. Testis were then excised and submerged in cold 4% methanol-free formaldehyde (Thermo Scientific) for an 18 hour fixation at 4°C, then testis were transferred to cold 70% ethanol prior to paraffin embedding. Sections were cut at 5µm and dried overnight for immunostaining.

**Histology**
Tissue sections were stained with periodic acid Schiff (PAS) stain and H&E. The stage in the cycle of the seminiferous epithelium was determined for individual tubules according to classical morphological criteria, as previously described (Hess and Renato de Franca, 2008). Multiple cauda epididymis cross sections and seminiferous tubule cross sections at each stage in the cycle of the seminiferous epithelium were used to determine the mean germ cell/unit area from each dietary treatment group (n=3). Statistical analysis was conducted using one-way ANOVA and Student’s t-test. Data are presented as mean ± SD; P < 0.05 was considered statistically significant.

**Confocal immunofluorescence**
For immunostaining, sections were deparaffinized, rehydrated and antigen retrieval was carried out using a 45 min incubation in 10 mM sodium citrate buffer at 98°C. Samples were then permeabilized with 1% Triton-X, blocked for 1 h with 5% normal goat serum (Jackson Immuno) and incubated with primary antibodies overnight at 4°C. After washing, secondary antibodies were incubated for 2 h at room temperature, and finally samples were counterstained for 30 min. with Hoechst (Invitrogen; 3.5 µg/ml) and Lectin PNA for 30min (L-32458, Invitrogen; 1:200). Sections were mounted in Prolong Gold (Invitrogen) and cured for 24 h at room temperature. Cover glass was then sealed with top coat nail polish and sections were held at 4°C until analysis. All comparisons between groups were made after carefully matching seminiferous tubules based on their stage in the cycle of the seminiferous epithelium (Hess and Renato de Franca, 2008). The following primary antibody dilutions were used: VAMP4 (sc-134557; Santa Cruz, rabbit polyclonal; 1:50), golgin-97 (rabbit polyclonal, 1:200) nectin-2 (sc-14799; Santa Cruz, goat polyclonal; 1:50). The following secondary antibodies were used, all at a 1:200 dilution: goat anti-rabbit 488 (Invitrogen), donkey anti-goat 488 (sc-362255). Immunostaining
was visualized with a Zeiss LSM 700 laser scanning confocal microscope. Laser power, gain and offset were kept constant across samples. Imaris and Zen 2011 software was used for analysis.

**Electron Microscopy**

_Fads2_-/- and _Fads2_ +/- males fed AIN93G until 16 weeks of age were perfused as described previously (Roqueta-Rivera et al., 2011). Briefly, mice were injected with heparin 15 min prior to pentobarbital anesthesia, followed by PBS and 5% glutaraldehyde perfusion through the left ventricle. Testis were post fixed in osmium tetroxide/potassium ferrocyanide, dehydrated and embedded in epoxy-resin. Section were cut at 1µm and stained with toluidine blue, cut into 70 nm sections with an ultramicrotome and imaged with a Hitachi H600 electron microscope.
Fig. 11. Glycosylated proteins of maturing spermatids fail to distribute properly under HUFA deficiency. A) Seminiferous tubule cross sections of wt and ko mice at stage III and VII in the cycle of the seminiferous epithelium labeled with the lectin PNA (gold) and Hoechst (blue). Red arrows indicate round spermatids and green arrows indicate elongating spermatids. Scale bar=10µm. B) Magnification of individual spermatids from wt and ko stage VIII seminiferous tubules stained with PNA and Hoechst, also showing differential interference contrast. Scale bar=3µm.
Fig. 12. VAMP4 is abnormally retained in the Golgi under HUFA deficiency. Immunofluorescent staining of VAMP4 (green) and PNA (red) and their colocalization (yellow) from seminiferous tubule cross sections of fixed testis tissue slices. Stage III tubules of wt (A-C) and ko (D-F) with white arrows indicating regions of round spermatids with VAMP4 localization. Stage VII tubules of wt (G-I), white arrows indicate the two distinct localizations of VAMP4 (G). In the Stage VII wt merge image (I) white arrowheads indicate regions of VAMP4 and PNA colocalization and white arrows indicate regions of VAMP4 without PNA colocalization. In the Stage VII ko (J-L), white arrowheads indicate VAMP4 puncta in round spermatids. Scale bar=5μm.
Fig. 13. VAMP4 redistribution in cap phase is disrupted under HUFA deficiency. Immunofluorescent staining of VAMP4 (green) and PNA (red) and their colocalization (yellow) in wt and ko cap phase spermatids. The three dimensional image is reconstituted from ~12 serial cross sections imaged every 300nm of depth in the z-axis. Scale bar=2µm.
Fig. 14. Vesicle tether factor golgin-97 remains accumulated in the Golgi under HUFA deficiency. Immunofluorescent staining of golgin-97 (green) in seminiferous tubules of wt and ko mice at stages II-III and V-VI in the cycle of the seminiferous epithelium. In both wt at stage II-III (A) and ko (C), Golgi distribution of golgin-97 in round spermatids is indicated by white arrowheads. In stage V-VI in the wt (B), white arrowheads indicate globular distribution of golgin-97 in round spermatids and white arrows indicate the arc-like distribution found only in the wt at cap spermatids. Round spermatids from the ko in Stage V-VI (D) show round spermatids with punctate distribution of golgin-97 only (white arrowheads). rs=round spermatids, es=elongated spermatids, PS=pachytene spermatocytes. Scale bar=5 µm.
Fig. 15. The Trans-Golgi network interacts directly with the acrosomal granule. Transmission electron microscopy of Golgi phase spermatids from wt (A,B) and ko (C,D). Magnification of boxes from A and C are shown in B and D, respectively. Trans-Golgi network (TGN) and dense core granules (DCG). Bar=400nm (A,C); Bar=200nm (B,D).
Fig. 16. Glycosylated protein distribution to the acrosome is restored with dietary supplementation of either ARA or DHA. PNA-stained (gold) cross sections of seminiferous tubules from ko animals fed dietary ARA (A,B) or DHA (C,D). In stage V-VI, both cap phase round spermatids and more mature elongating spermatids display arc-like distributions from the PNA stain (A,C). At Stage IX, PNA stain is shown at anterior poles on spermatids at the onset of elongation (B,D). Scale bar=10µm.
Fig. 17. Cap phase redistribution of VAMP4 is restored by dietary supplementation with either ARA or DHA. VAMP4 (green) at round spermatids (pink arrows) of Stage III tubules (A) display a punctate distribution in all groups. In Stage VI (B), VAMP4 distribution in spermatids (arrows) is restored in ko mice fed ARA or DHA, but not in non-supplemented ko mice. sp=round spermatids, sc=pachytene spermatocytes. Scale bar=10μm.
Fig. 18. Premature spermatid sloughing from seminiferous tubules is prevented with dietary DHA. A) Stage VII seminiferous tubules cross sections show the reduced number of retained elongated spermatids in all groups except wt and DHA supplemented ko. Scale bar=20µm. B) The number of retained elongated spermatids present in Stage VII seminiferous tubules, expressed as mean spermatids present per square µm of Stage VII seminiferous epithelium. For each dietary treatment group, n=3. Statistical analysis was conducted using one-way ANOVA and Student’s t-test. Data are presented as mean ± SD; P < 0.05 was considered statistically significant.
Fig. 19. Abnormal retention of step 16 spermatids in seminiferous tubules is prevented with dietary DHA. A) Stage VIII cross sections capture the process of mature spermatozoa release and Stage IX shows the abnormal retention of step 16 spermatozoa in all ko groups, with the exception of 0.2% DHA-supplemented ko mice. Scale bar=20µm. B) The number of step 16 spermatozoa present in Stage IX seminiferous tubules, expressed as mean spermatozoa present per square µm. For each dietary treatment group, n=3. Statistical analysis was conducted using one-way ANOVA and Student’s t-test. Data are presented as mean ± SD; P < 0.05 was considered statistically significant.
Fig. 20. Premature spermatid sloughing into the epididymis is prevented with dietary DHA. A) Cauda epididymis images demonstrate the presence of round spermatids in all groups, with the exception of wt and 0.2% DHA supplemented ko animals. Scale bar=20µm. B) Fixed tissue slices of the cauda epididymis were examined for the presence of round spermatid, recorded as the total number of immature, round spermatids per square µm and expressed here as the fold change as compared to wt. Data are presented as mean ± SD; P < 0.05 was considered statistically significant.
References


Chapter 5: Summary and future research

Summary of Major Findings
The work presented here demonstrates critical functions for DHA and ARA, the HUFAs enriched in testis, in promoting both acrosome biogenesis and the assembly of Sertoli-spermatid adhesion complexes. In contrast to the previously described wide cytosolic dispersion of the acrosomal protein acrosin and successful release of small translucent vesicles from the cis-face of the Golgi apparatus during spermatid maturation in D6D ko mice, we show here an abnormal Golgi accumulation of the intracellular trafficking regulators VAMP4 and golgin-97. Additionally, glycosylated proteins labeled with the lectin PNA appear to be abnormally retained in the Golgi along with VAMP4, which coincides with a failure of acrosome biogenesis under HUFA deficiency. Taken together, these findings suggest that the HUFAs ARA and DHA may exert their influence on acrosome biogenesis by promoting specialized modes of vesicular trafficking and secretory granule biogenesis.

We show here that the previously reported reduction in sperm count and abnormal appearance of immature spermatids in the epididymis of D6D ko males, coincides with a selective impairment of Sertoli-spermatid adhesions, but not Sertoli-Sertoli adhesions, under HUFA deficiency. Although Sertoli-spermatid adhesion protein localization was restored by either ARA or DHA, a complete restoration of immature spermatid retention in the seminiferous epithelium by DHA only, and not ARA, suggests that dietary DHA intake, sufficient to maintain testis enrichment, is critical for the efficient assembly of Sertoli-spermatid adhesion complexes required to maintain normal sperm counts. Additionally, the selective nature of adhesion protein mislocalization found in the testis under HUFA deficiency indicates that the construction of Sertoli-Sertoli adhesions is regulated differentially from the assembly of Sertoli-spermatid adhesions, and may depend on adhesion protein movement through distinct trafficking itineraries.

Implications and Future Work
The work presented here is consistent with multiple levels of human evidence supporting the connection between HUFAs and human fertility. As idiopathic male infertility has been characterized in part by sperm with reduced enrichment in DHA, there is the potential that dietary supplementation with DHA could be utilized as an approach to support or improve sperm health in men.
It is also critical to note that HUFAs are highly enriched in the brain as well, and that roles for the nectin adhesion molecules described here are also known to serve critical functions at synapses in the brain. Heterotypic adhesions between different nectin proteins have been shown to support interactions between adherent pre- and post-synaptic neurons in the brain, epithelial layers of the eye, and also between sensory neurons and their support cells in the inner ear. It is possible that the functional interaction between nectins and HUFAs suggested by this work to occur in the testis may exist in these other cell types where HUFAs are similarly enriched.

Lastly, dietary reference intakes (DRIs) for essential fatty acids do not currently include intake recommendations for HUFAs. Although the omega-6 linoleic acid is widely available in the typical western diet, typical intake of either the 18-carbon omega-3 alpha-linolenic acid or the omega-3 HUFA DHA is substantially lower. In light of recent associations found connecting higher DHA or fish consumption to a reduction in the risk of a variety of poor health outcomes (e.g. CHD, Alzheimer’s disease, and sub-optimal fetal development), there is tremendous interest in establishing a DRI specifically for DHA. The critical functions for testis DHA enrichment in prevention of premature spermatid release shown here, indicate that human semen samples can be utilized for the development of this DHA DRI. As other tissues preferentially enriched with DHA (i.e. brain, retina) are essentially inaccessible to researchers, semen samples may be the ideal tissue to determine a highly precise measure of dietary DHA sufficiency. There is a need to focus further work on translating the testis dysfunctions, described here with D6D ko mice, to a semen phenotype that can be evaluated in humans.