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# The sperm equatorial segment: an organizing center for sperm protein relocalization and facilitation of fertilization

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# The sperm equatorial segment: an organizing center for sperm protein relocalization and facilitation of fertilization

**By**

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## List of Abbreviations

α- alpha

Ac- the sperm acrosome

$BS^3$ – Bis (sulfosuccinimidyl) suberate

BSA- bovine serum albumin

CEC- the sperm cauda epididymal content

CRISP- cysteine-rich secretory protein

DTT- dithiothreitol

EGTA- ethylene glycol tetra acetic acid

EqS- the sperm equatorial segment

EqSS- the sperm equatorial subsegment

F-actin- filamentous actin

FITC- fluorescein isothiocyyanate

HSM- human sperm media

IACUC- Institutional Animal Care and Use Committee

Pac- the sperm postacrosome

PBS- phosphate buffered saline

PBS-T- phosphate buffered saline Tween 20

PNA-TRITC- peanut agglutinin-tetramethyl rhodamine-5-isothiocyanate

RT- room temperature

S2- signature 2 sequence

SDS- sodium dodecyl sulphate

TBS-Tris buffered saline

WH- Whitten's HEPES-buffered solution

WHO – World Health Organization

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## Abstract

The equatorial segment (the EqS) of mammalian sperm has been reported to be a site for sperm-egg fusion initiation. The subdomain, called the equatorial subsegment (the EqSS), within the EqS has been identified in sperm of many species; however, there is no clear report showing the existence of this subdoman in human sperm. The EqS has been hypothesized to be an organizing center for assembly of molecular complexes required for gamete interaction and fusion. Several sperm proteins have been reported to relocate to the EqS to make this region fusion competent. However, molecular mechanisms that regulate relocalization of sperm proteins have not been clearly elucidated. The current study aimed to understand mechanisms underlying the relocalization of sperm proteins during the acrosome reaction. Based on the previous studies in our laboratory, sperm-associated α-L-fucosidase was detected to relocalize from the anterior of the acrosome to the EqS of human sperm after the acrosome reaction. In conjunction with the study in our laboratory aimed to investigate roles of this enzyme during fertilization by using mice as a model of study, the current project first refined the subcellular localization of membrane-associated α-L-fucosidase in mouse sperm before and after the acrosome reaction using immunolocalization and fluorescence microscopy. The experiments revealed that membrane-associated α-L-fucosidase was originally localized throughout the anterior of the acrosomal region; however, the enzyme could be detected only when mouse sperm were permeabilized. These observations suggest that the enzyme may reside in the acrosomal membranes, and that α-L-fucosidase

progressively migrates from the acrosomal membranes to the plasma membrane over the EqS by the influence of lateral diffusion. CRISP2 protein was another human sperm protein used as a marker to study mechanisms of protein relocalization during the acrosome reaction. We found that Latrunculin A could not reduce the presence of CRISP2 at the EqS of acrosome-reacted human sperm, indicating that actin polymerization is not involved in facilitating CRISP2 relocalization during the acrosome reaction. Results also revealed that CRISP2 is a component of the acrosomal matrix. During the acrosome reaction, but not capacitation, CRISP2 was released into the media and re-associated with the EqS in a dose-dependent manner. These results suggest that the released CRISP2 can reassociate with the EqS during the acrosome reaction. The stability of CRISP2 at the EqS of acrosome-reacted human sperm was also studied. The association stability was very strong since high ionic strength solution, mild sonication, reducing agents, and non-ionic detergent with low concentration could not dissociate CRISP2 from the EqS of acrosome-reacted human sperm. However, a low concentration with a short period of incubation in the anionic detergent, SDS, could extensively remove CRISP2 from the EqS, but could not extract the CRISP2 population at the neck of sperm cells. Interestingly, EGTA caused redistribution of CRISP2 over the sperm head. Data suggest that stabilization of CRISP2 at the EqS of human sperm depends on calcium ions. This project also investigated the oocyte binding ability of acrosomal CRISP2 by incubating zona-intact and zona-free hamster eggs with the purified biotinylated human acrosomal CRISP2. CRISP2 bound to the plasma membrane of the oocyte but not to the zona pellucida, suggesting the presence of conserved

complementary sites of CRISP2 on hamster oocyte plasma membrane. These observations raise the possibility that CRISP2 at the EqS may help modify the EqS plasma membrane to make this domain fusion competent or act as an adhesion molecule for sperm-egg interaction and fusion.

Taken together, the results revealed that human sperm use different mechanisms to facilitate the relocalization of sperm proteins from other regions to the EqS during the acrosome reaction. Membrane proteins like α-L-fucosidase seem to migrate laterally in the plasma membrane whereas soluble proteins stored in the acrosomal cap like CRISP2 are released and reassociate with the plasma membrane of the EqS. The reassociation of this protein may facilitate sperm-egg fusion since acrosomal CRISP2 can bind to the targets on the oolemma of hamster oocytes.

# Chapter 1

## Introduction

The origin of life starts when the smallest cell in the body, a sperm cell, meets and unites with the largest cell in the body, an oocyte. This step is called “fertilization”. Fertilization seems to be an easy step where sperm and egg interact and fuse to combine paternal and maternal genetic materials. In fact, fertilization is a delicate and complicated event. Sperm must undergo a complex series of physiological and biochemical changes in the cell and on the surface to gain fertilization competence. Our laboratory is interested in understanding molecular mechanisms or processes underlying fertilization. New basic insights into fertilization may have applications in development of novel contraceptive stretagies, such as immunocontraception. Also, new discovery can lead to development of a standard diagnosis for male infertility. Since sperm cells are transcriptionally inactive, they cannot synthesize new proteins. Moreover, sperm cells lack the ER and the Golgi apparatus which are required for post-translational modifications of proteins being transported to the plasma membrane or secreted to the extracellular environment. All sperm proteins need to be sequestered in specific compartments and are exposed to function at the specific time during fertilization. The following sections explain the organization of mammalian sperm structure, relocalization of sperm proteins to different surface domains, and possible mechanisms of sperm relocalization. Also, α-L-fucosidase and CRISP2 proteins are focused since they were used in the current study as a model for studying mechanisms underlying sperm protein relocalization.

## 1.1 Differentiation and subcellular structure of mammalian sperm

Mammalian sperm are highly specialized cells. They need to undergo a complex series of differentiating processes to gain fertilization competence. During spermiogenesis, round spermatids differentiate to the elongated testicular sperm. These sperm further undergo post-testicular differentiation and maturation in the epididymes. During the transit of the sperm cell through the epididymes, the plasma membrane changes by the release, modification and adsorption of proteins and lipids. The role of these surface alterations is not fully understood. In most of mammalian species, sperm cells are fully matured when they reach the cauda epididymis (Eddy et al., 1994). Sperm cells continue modification in the female reproductive tract, where capacitation is induced to produce sperm fully able to fertilize an oocyte (Visconti and Kopf, 1998). Capacitation is known to be induced by cholesterol efflux and consequent molecular events involving lipid rearrangements in the sperm plasma membrane that are coupled to changes in ion influxes and to several signaling cascades (Visconti et al., 1995a; Visconti et al., 1995b; Visconti et al., 1999).

Mammalian spermatozoa are polarized cells that show obvious compartmentalization of the membrane system which is organized into functional domains. Each domain shows special arrangement of distinct lipid compositions and segregation of certain proteins. In intact sperm, the plasma membrane on the head encloses the anterior acrosome, the equatorial segment, and the postacrosomal region. In the tail, the membrane covers the anterior part of the flagellum (midpiece), containing the mitochondria, and the posterior portion of the

tail (principal piece), containing the fibrous sheath surrounding the outer dense fibers (Figure 1.1) (Downing Meisner et al., 2005).

### 1.2 The sperm equatorial segment and equatorial subsegment (EqS, EqSS)

Since sperm cells are transcriptionally inactive and cannot synthesize any proteins, sperm cells have developed an alternative strategy of storing proteins until they are needed by dividing the sperm head into different compartments. This strategy provides a mechanism by which sperm cells can sequester proteins until they are needed. The process of sequential exposure of sperm proteins must be accurate to ensure successful fertilization. The acrosome (Ac), the equatorial segment (EqS), and the postacrosome (PAc) have distinct subcellular structures and proteins. A study in ram spermatozoa by atomic force microscopy (AFM) illustrated unique sperm surface topology of each domain that is distinguishable from that of other domains (Figure 1.2) (Ellis et al., 2002).

During capacitation and the acrosome reaction, different proteins become exposed as the membrane is modified. Several studies showed that compartmentalization of the acrosomal matrix is significant for precisely regulating the release of acrosomal contents during the course of acrosomal exocytosis (Kim et al., 2001; Kim and Gerton, 2003). These studies revealed that soluble acrosomal materials are rapidly released during the early stages of the acrosome reaction, while acrosomal matrix proteins remain associated with the sperm for prolonged periods of time following the induction of acrosomal

exocytosis. These findings suggest that transitional acrosomal intermediates may have significant functions in the fertilization process.

Mammalian sperm undergo the acrosome reaction when they bind to the zona pellucida. This process must be completed before sperm enter the zona pellucida (Austin and Bishop, 1958; Yanagimachi, 1988b). Therefore, the sperm that have penetrated the zona pellucida to contact the oolemma must be acrosome-reacted. If we inseminate intact sperm with zona-free oocytes, sperm may bind firmly to the oolemma but never fuse with it. In contrast, acrosome-reacted sperm bind and fuse immediately with the oolemma under the same conditions (Yanagimachi and Noda, 1970; Yanagimachi, 1988a).

Different domains within the sperm head play significant roles at different steps of fertilization. The EqS has been of interest for long time because it is considered to be important for fertilization. The study in 1970 using electron microscopy revealed the existence of the EqS and its ultrastructural changes in the hamster sperm head during fertilization. This study suggested that the EqS is of great functional importance because it 1) remains intact after the acrosome reaction, 2) lies beneath the plasma membrane domain involved in egg membrane fusion, and 3) is the site where sperm nuclear envelope breakdown occurs (Yanagimachi and Noda, 1970). Surprisingly, studies in aspects concerning the EqS are not so advanced even though this structure had been described for long time.

The EqS becomes noticeable when round spermatids differentiate to the elongated testicular sperm and the size and shape of this compartment are

species-specific (Phillips, 1977). The EqS has been demonstrated to locate at the posterior of the acrosomal region (Downing Meisner et al., 2005). Electron microscopy studies of surface replicas of sperm heads of eight mammalian species showed that the sperm EqS is composed of hexagonally packed particles with 170 angstrom-space (Phillips, 1977). Freeze-fracture studies (Friend and Fawcett, 1974; Suzuki, 1981) and atomic force microscopy (AFM) (Allen et al., 1995; Ellis et al., 2002) showed that the structural features of the EqS are distinguishable from those of the plasma membrane overlying the Ac and PAc domains. The EqS has a relatively smooth surface compared to the rough, uneven surface of the post-acrosomal area. A sub compartment named the equatorial subsegment (EqSS) was also visualized as a semicircular structure within the EqS with a coarse, uneven surface. At the boundary with the EqS, an abrupt change occurs with a clearly defined line (in boar and bull) or necklace of rectangular depressions (in ram) between the two domains. The EqSS was reported to change its topography during the acrosome reaction (Ellis et al., 2002; Jones et al., 2007). The changes that occur within the EqS and the EqSS surface may be very significant for preparing sperm for fertilization.

In mammals, the initiation of fusion starts between the oocyte plasma membrane and the sperm EqS plasma membrane. The EqS is always exposed to the extracellular environment before and after the acrosome reaction. Therefore, the EqS must undergo a physiological change at some time during or after capacitation and the acrosome reaction to become fusion competent. During capacitation, the plasma membrane changes its properties, including increase of membrane permeability and fluidity to prepare sperm for the acrosome reaction.

The plasma membrane and the outer acrosomal membrane over the anterior of the acrosomal area undergo vesicularization to allow acrosomal components to be released to the extracellular environment. The plasma membrane and the outer acrosomal membrane over the EqS region do not undergo vesicularization during the acrosome reaction. There must be important mechanisms that are responsible for stabilizing and maintaining the integrity of the EqS, and the machanisms are still unknown. However, the lack of membrane vesicularization of the EqS does not mean that there are no biochemical and physiological changes within the EqS during the acrosome reaction since the plasma membrane overlying the EqS becomes fusogenic only after the acrosome reaction has completely occurred (Allen and Green, 1995; Takano et al., 1993; Yanagimachi, 1994).

The sperm EqSS, has been hypothesized to be an organizing center for assembly of molecular complexes that initiate fusion competence (Jones et al., 2008). It has been reported that the EqS is a site for a restriction of a variety of surface antigens, ion channels (Lee et al., 1984; Saling et al., 1985; Myles and Primakoff, 1984; Toshimori et al., 1998; Luconi et al., 1998; Benoff, 1999; Hao et al., 2004), and intracellular components (Camatini et al., 1986a b; Camatini and Casale, 1987; Feinberg et al., 1991; Howes et al., 2001; Wolkowicz et al., 2003; Kamaruddin et al., 2004; Mitchell et al., 2007). These proteins may be significant for facilitating sperm-egg fusion.

### 1.3 Relocalization of sperm proteins to the EqS

The functional significance of the EqS to fertilization is strengthened by recent observations showing that several proteins, necessary for mammalian sperm-oocyte membrane fusion, relocalize from other regions to the EqS during the acrosome reaction. It has been reported that IZUMO1, an acrosomal membrane protein proven to be crucial for sperm-egg fusion, relocates from the anterior of the acrosome of mouse and human sperm to the site(s) where fusion will take place, including the EqS (Inoue et al., 2011). Another sperm protein, equatorin, has been reported to move from the peripheral region of the anterior acrosome of mouse sperm to the plasma membrane overlying the EqS during the advanced and final stages of the acrosome reaction (Yoshida et al., 2010). Recently, it has also been demonstrated that tetraspanin family protein CD9 in mouse sperm undergoes unique relocalization which has a similar localization pattern to that of equatorin (Ito et al., 2010). Other sperm membrane-associated proteins have been reported to relocalize from the acrosomal region to the plasma membrane overlying the EqS. Human SED1 protein [Secreted protein containing two $NH_2$-terminal notch-like epidermal growth factor (EGF) repeats and two COOH-terminal Discoidin/F5/8 complement domains] is a sperm-associated protein that facilitates sperm adhesion to the egg zona pellucida (Shur et al., 2004; Shur et al., 2006). SED1 is secreted from the initial segment of the epididymis, where it binds to the sperm plasma membrane overlying the acrosome and during the acrosome reaction, SED1 relocalizes to the EqS plasma membrane (Copland et al., 2009). The sperm membrane-associated α-L-fucosidase has also been reported to relocalize from the acrosomal region to the EqS of human sperm

during the acrosome reaction (Venditti et al., 2007). Moreover, the results from my study published in Phopin et al. (2012) showed that the structurally bound α-L-fucosidase becomes progressively restricted to, and localized within, the sperm EqS of acrosome-reacted mouse sperm. It has been hypothesized that α-L-fucosidase acts as an integral component of the complex EqS and is positioned for possible engagement during sperm–oocyte interactions and/or triggering of processes of early embryogenesis. A similar model has been reported by Cohen et al. (2007) for another sperm protein, protein DE, a member of the CRISP family. This bound protein DE migrates to the EqS as the acrosome reaction occurs, and participates in sperm–egg fusion (Cohen et al., 2007). Several other investigators have shown that certain proteins that may function in sperm–egg interaction or fusion appear at the EqS or PAc during the acrosome reaction. These proteins include DE (Rochwerger and Cuasnicu, 1992), the PH-20 antigen (Myles and Primakoff, 1984), and N-cadherin (Marín-Briggiler et al., 2010). However, molecular mechanisms that regulate the translocation of the sperm proteins from the acrosome to the EqS during the acrosome reaction are largely unknown.

### 1.4 Possible mechanisms regulating sperm protein translocation to the EqS

Several models have been proposed to explain the dynamic movement of sperm proteins from original regions to the EqS. These models include lateral diffusion, involvement of cytoskeletal proteins, and reassociation.

### 1.4.1 *Lateral diffusion*

Capacitation activates sperm surface alterations (Yanagimachi, 1994). These surface changes involve removal of the extracellular coating at the sperm plasma membrane, including decapacitation factors, and the absorption of sperm activation factors. Moreover, the albumin-mediated removal of cholesterol causes the sperm plasma membrane to exhibit changes in its lipid composition and fluidity. The changes in the sperm plasma membrane fluidity can be monitored using specific stains such as chlortetracycline (Mattioli et al., 1996), merocyanin (Harrison et al., 1996), or filipin (Suzuki-Toyota et al., 2000). The progress of capacitation and the alteration of specific lipid staining patterns correspond to the increased level of tyrosine phosphorylation in the tail of sperm which causes hyperactivated motility (de Vries et al., 2003).

The lateral polarity of the sperm plasma membrane during the acrosome reaction has been reported by a study where a fluorescent glycolipid was incorporated into the plasma membrane of living sperm (Gadella et al., 1994; Gadella et al., 1995). The approach used in this study is to incorporate the two fluorescent tagged galactolipids: galactose (3-sulphate)-β1-1′[(Nlissamine rhodaminyl)-12-aminododecanoyl]-sphingosine (SGalCer(C12-LRh) and galactose-β1-1′[(N-lissamine rhodaminyl)-12-aminododecanoyl]-sphingosine (GalCer(C12-LRh) into the lipid bilayer of sperm plasma membranes and follow their lateral distribution within the membrane. The changing patterns of fluorescence indicated that the migration of glycolipids from the apical domain to the EqS during *in vitro* capacitation and zona binding occurs prior to the acrosome reaction (Figure 1.3).

A hypothesis published by B. M. Gadella et al. (1991) predicts that the glycolipid seminolipid (SGalAAG) prevents fusion of the plasma membrane with intracellular membranes because studies found that SGalAAG is distributed over the acrosome of intact sperm (B. M. Gadella et al., 1991, 1994). This hypothesis was supported by physicochemical data indicating that sulphogalactolipids stabilize the lamellar phase of lipid bilayers (Wolf et al., 1986). Another study reported that incubation of spermatozoa with arylsulphatase A (a seminal plasma enzyme that desulphates SGalAAG on intact spermatozoa) causes a migration of glycolipids from apical to EqS of uncapacitated sperm cells (B. M. Gadella et al., 1994) and triggers the degenerative acrosome reaction (Gadella et al., 1991). A similar observation in rearrangement of transmembrane proteins in the boar sperm head plasma membrane during capacitation was reported (Aguas and da Silva, 1989). The demonstration of lateral movement and efflux of glycolipids from the apical to the EqS of the plasma membrane implies that sperm may use this strategy to facilitate the movement of particular sperm membrane proteins from other regions to the EqS during capacitation and the acrosome reaction.

### 1.4.2 *Involvement of actin cytoskeleton*

In somatic cells, actin is a key cytoskeletal protein that plays significant roles in regulating cell shape, migration, and interaction with extracellular matrices (Cooper, 1991). Actin has been demonstrated in male germ cells from various mammalian species (Vogl, 1989). In spermatogenic cells, actin is primarily localized in the subacrosomal space between the nucleus and the developing

acrosome of spermatids (Vogl, 1989). The actin present in spermatids is believed to play a role in the determination of cell shape and for shaping the nucleus during spermiogenesis (Oko, 1998). Since the amount and subcellular distribution of actin appears to differ between species, it has been suggested that the function of actin in sperm may be species-specific (Flaherty et al., 1986); (Lora-Lamia et al., 1986). The structure, location, and function of actin filaments in mature sperm remain largely unknown. However, filamentous (F)-actin has been described in mammalian species (Flaherty, 1987); (Breed and Leigh, 1991); (Moreno-Fierros et al., 1992); (Vogl et al., 1993); (de las Heras et al., 1997). In human sperm, F-actin can be found in different compartments including the acrosomal space, the EqS, the post acrosomal regions, and the tail (Clarke et al., 1982); (Ochs and Wolf, 1985; Virtanen et al., 1984); (Fouquet and Kann, 1992). Studies in different mammalian species including hamster, guinea pig, rabbit, boar, bull, and human revealed the presence of actin in the acrosomal region (Talbot and Kleve, 1978); (Camatini et al., 1986); (Flaherty et al., 1988); (Olson and Winfrey, 1991); (Moreno-Fierros et al., 1992), supporting the possible role in sperm capacitation and the acrosome reaction. During capacitation, actin monomers polymerize to form F-actin. However, during the acrosome reaction, F-actin is depolymerized to facilitate fusion between the plasma membrane and the outer acrosomal membrane of the sperm for the acrosomal exocytosis (Brener et al., 2003). A number of studies in different species including boar, guinea pig, mouse, bull, ram, and humans (Brener et al., 2003) showed that actin polymerization occurs during capacitation. Polymerization of G-actin to F-actin during capacitation has been proved to be responsible for the translocation of

phospholipase C from the cytosol to the sperm plasma membrane and the formation of F-actin during capacitation is known to be dependent on protein kinase A activation, protein tyrosine phosphorylation, and phospholipase D (Breitbart et al., 2005). Several actin-binding proteins have been reported to be involved in the dynamics of actin polymerization in sperm (Schafer and Cooper, 1995). These include calicin (von Bülow et al., 1995), the capping proteins CPb3 (von Bülow et al., 1997) and CPa3 (Tanaka et al., 1994), destrin, thymosin b10, testis-specific actin capping protein (Howes et al., 2001), gelsolin (de las Heras et al., 1997), scinderin (Pelletier et al., 1999), and the actin-related proteins Arp-T1and T2 (Heid et al., 2002). Although actin polymerization during capacitation is widely studied, the dynamics of actin polymerization during the acrosome reaction are poorly understood. A study in guinea pig sperm showed that the relative concentration of F-actin at the EqS and the PAc increased during the acrosome reaction and the formation of F-actin was inhibited by Cytochalasin D treatment which resulted in the impaired fertilization. Moreover, it has been revealed that actin polymerization in the EqS and the postacrosomal regions of guinea pig sperm during the acrosome reaction is regulated by G proteins and the formation of F-actin is necessary for sperm incorporation into the egg cytoplasm (Delgado-Buenrostro et al., 2005). Actin polymerization may be involved in the more subtle mechanisms during the acrosome reaction. Actin dynamics may accompany some of the membrane reorganization events. Moreover, actin dynamics may be crucial for the redistribution of sperm membrane proteins and the maintenance of surface proteins important in sperm-egg recognition involving specific regions of the sperm membrane. A recent

study in knockout mice demonstrated that relocalization of IZUMO1 (a crucial protein important for sperm-egg fusion) from the acrosomal compartment to other regions, including the EqS and the PAc, during the acrosome reaction is regulated by the testis-specific serine kinase 6 (TSSK6) (Sosnik et al., 2009). Interestingly, this study also found that actin polymerization in the postacrosomal region after the acrosome reaction was inhibited in *TSSK6*-null mice. This finding suggests a possible involvement of the actin cytoskeleton in relocalization of sperm membrane proteins during the acrosome reaction and that TSSK6 is needed for the regulation of actin dynamics.

### 1.4.3 *Reassociation of acrosomal proteins*

During the acrosome reaction, the outer acrosomal membrane and the plasma membrane of mammalian sperm undergo vesicularization to release acrosomal components to the extracellular environment. Many sperm membrane proteins localized in the inner and outer acrosomal membrane are bound to the hybrid vesicles formed between the outer acrosomal membrane and the plasma membrane during vesicularization. It has been reported that several intracellular sperm proteins relocate (via the hybrid vesicle reassociation) to the surface overlying the EqS. A study in guinea pig sperm showed that during the acrosome reaction, G11 antigen is relocated to the EqS accompanying hybrid vesicles (Allen and Green, 1995). A recent study in mouse sperm suggests that Equatorin may be relocalized to the plasma membrane over the EqS by this mechanism (Manandhar and Toshimori, 2001).

For soluble acrosomal proteins, it has been proposed that the enzymes released from the acrosome during the acrosome reaction may activate latent fusion proteins, stimulate migration of fusion protein, and remove steric and charge barriers to membrane apposition (Cowan et al., 1991). Acrosin is an acrosomal protease that was observed to be released during the acrosome reaction and reassociates to the EqS of hamster sperm to modify the plasma membrane overlying this compartment to make sperm competent for fusion (Takano et al., 1993). Another study reported that acrosin epitopes have been localized to the plasma membrane of the EqS and post-acrosomal region of the sperm head (as well as on the tail) of acrosome-reacted rabbit spermatozoa (Richardson et al., 1991). Therefore, some sperm proteins may use this strategy to bind to the EqS during the acrosome reaction.

### 1.5 Sperm associated α-L-fucosidase

Sperm associated α-L-fucosidases have been hypothesized to be involved in fertilization. The presence of this enzyme on mammalian sperm in different species suggests significant roles in gamete interaction. Mammalian sperm associated α-L-fucosidases have been of interest in our laboratory. This enzyme has been identified and characterized in humans (Alhadeff et al., 1999; Khunsook et al., 2003). Previous studies in the laboratory found evidence suggesting roles of sperm associated α-L-fucosidases in reproductive processes. In a study using a Syrian hamster model system, sperm associated α-L-fucosidase was suggested to be involved during sperm-egg fusion (Venditti et al.,

2010). In 2012, Phopin et al. reported that anti-fucosidase antibody and purified human liver α-L-fucosidase significantly inhibit sperm-egg zona binding, membrane binding, or fusion and penetration, and that the active site of the enzyme, at least in mice, is not involved in sperm-egg intereaction (Phopin et al., 2013).

Moreover, a previous study in the laboratory showed that sperm associated α-L-fucosidases were relatively enriched within the EqS of human sperm only after the acrosome reaction (Venditti et al., 2007). When mouse sperm were used as a model for studying roles of sperm associated α-L-fucosidases, we also observed the relocalization of this enzyme to the EqS of mouse sperm after the acrosome reaction (Phopin et al., 2012). The presence of this enzyme at the EqS of human and mouse sperm after the acrosome reaction supports the notion that the fucosidase has a role in the intimate species signature interactions between sperm and oocyte and the mechanisms that facilitate the relocalization of the enzyme are not known yet. Therefore, studying the relocalization behavior of sperm associated α-L-fucosidases would allow us to understand more about mechanisms underlying sperm protein movement during the acrosome reaction.

### 1.6 Cysteine-rich secretory protein 2

Mammalian cysteine-rich secretory protein 2 (CRISP2 or TPX1) has been originally reported to be testis specific (Kasahara et al., 1987a, b; Kasahara et al., 1989). However, a study in human sperm found that CRISP2 is also

observed in the epididymis (Krätzschmar et al., 1996). Many studies have reported that CRISP2 can be found in sperm of various mammalian species including humans (Busso et al., 2007) (Busso et al., 2005). These reports also suggest that CRISP2 expressed in the male reproductive tract is a multifunctional protein that plays roles in different stages of sperm development and/or fertilization. Structural analysis of several CRISP family members, including CRISP2, demonstrated that CRISPs are modular proteins and they share a similar molecular structure. All proteins in this family contain a motif of 16 absolutely conserved cysteines that folds into two domains: an N-terminal CRISP, antigen 5 and Pr-1 (CAP) domain that contains six conserved cysteine residues, and the smaller C-terminal CRISP (or cysteine-rich) domain that contains 10 conserved cysteine residues (Gibbs et al., 2008). Within the CRISP domain, it is composed of two regions: a hinge and an ion channel regulatory (ICR) region. Several studies revealed that all cysteines are involved in intramolecular disulfide bond formation (Eberspaecher et al., 1995; Guo et al., 2005; Shikamoto et al., 2005; Wang et al., 1993). The rat *CRISP2* gene has been cloned (Maeda et al., 1998), and the encoded CRISP2 protein has been revealed to be produced and secreted from spermatogenic cells at various differentiation stages where it mediates the specific adhesion of spermatogenic cells with Sertoli cells (Maeda et al., 1999). In 2006 Gibbs at al. suggested that CRISP2 is involved in capacitation by regulating ion channel activity and calcium fluxes.

In mature sperm, CRISP2 was found to be a component of the guinea pig acrosome (Hardy et al., 1988). Also, in rat sperm, CRISP2 was reported to exist as a component of the acrosome and the outer dense fibers of the tail (O'Bryan

et al., 1998); O'Bryan et al., 2001). Moreover, in mouse (Busso et al., 2007) and human sperm (Busso et al., 2005), CRISP2 is reported to be an intra-acrosomal protein. The possible role of CRISP2 in fertilization has been studied in mouse. Busso et al. (2007) demonstrated that mouse CRISP2 remains associated in the EqS of the head after the acrosome reaction. This group also suggested that sperm CRISP2 is involved in sperm-egg fusion via a specific region in the CAP domain. In guinea pig sperm, it has been reported that autoantigen 1 in the acrosome is the homologue of mouse and human TPX1 and is a member of the cysteine-rich secretory protein (CRISP) family (Foster and Gerton, 1996). CRISP2 in guinea pig sperm was found to be a soluble component of the acrosome which is released during the acrosome reaction (Kim et al., 2001; Kim and Gerton, 2003). In humans, sperm CRISP2 has been reported to be associated with the EqS after the acrosome reaction and plays role in sperm-egg fusion (Busso et al., 2005). However, the source of CRISP2 that is found at the EqS of the acrosome-reacted human sperm and mechanisms underlying the relocalization of this protein to the EqS are largely unknown.

Human sperm CRISP2 protein is not cleaved during the acrosome reaction, indicating that proteolytic modifications do not play roles in promoting the fusion ability of CRISP2 (Busso et al., 2005). Particularly, structure-function studies revealed that the Signature 2 (S2) sequence, an evolutionarily conserved motif in the PR-1 domain of the CRISP family, is important for binding to the oocyte and this sequence is extremely conserved in CRISP1 and CRISP2 of rodent species (Ellerman et al., 2006).

Although it has been suggested in many species (Hardy et al., 1988; O'Bryan et al., 1998; O'Bryan et al., 2001) that CRISP2 is a component of the acrosome, there is no clear evidence showing that CRISP2 is compartmentalized in the acrosome of human sperm. Moreover, CRISP2 was found at the EqS of human sperm only after the acrosome reaction (Busso et al., 2005). However, molecular mechanisms that regulate the translocation of human sperm CRISP2 from the acrosome to the EqS during the acrosome reaction are largely unknown.

### 1.7 Hypotheses

The goals of my research were to examine the overarching hypothesis that the EqS is an organizing center for the relocalization of sperm proteins crucial for sperm-egg interaction and fusion. This current project was designed to test the following 10 specific hypotheses. 1) Human sperm contain the equatorial subsegment which is enriched in tyrosine phosphorylated proteins. 2) The localization patterns of α-L-fucosidase in the mouse sperm membrane system differs between intact, permeabilized, capacitated, and acrosome-reacted sperm cells. 3) Mouse sperm membrane-associated α-L-fucosidase gradually moves toward the EqS as the acrosome reaction proceeds. 4) The localization patterns of CRISP2 protein in human sperm differs between intact, permeabilized, capacitated, and acrosome-reacted sperm cells. 5) Human sperm CRISP2 relocalizes to the EqS during the acrosome reaction with the involvement of filamentous actin. 6) Human sperm CRISP2 detected at the anterior acrosomal region of permeabilized sperm cells is a component of the acrosomal vesicle. 7)

Acrosomal CRISP2 protein is released during the acrosome reaction. 8) The released acrosomal CRISP2 binds to the EqS of acrosome-reacted human sperm. 9) The association stabilization at the EqS of acrosome-reacted human sperm is strong. 10) The released human sperm acrosomal CRISP2 binds to complementary sites on the plasma membrane of hamster eggs.

How does the relocalization of sperm proteins to the EqS occur in mammalian sperm cells during the acrosome reaction? This important question is the major focus of this thesis. New basic insights into molecular mechanisms regulating sperm protein relocalization may have applications in development of new contraceptive stretegies or a new standard diagnosis for male infertility. Several methods were used to test the above hypotheses. They include immunofluorescence study, Western blot analysis, *in vitro* biotinylation, affinity bead protein purification, and protein binding assays.

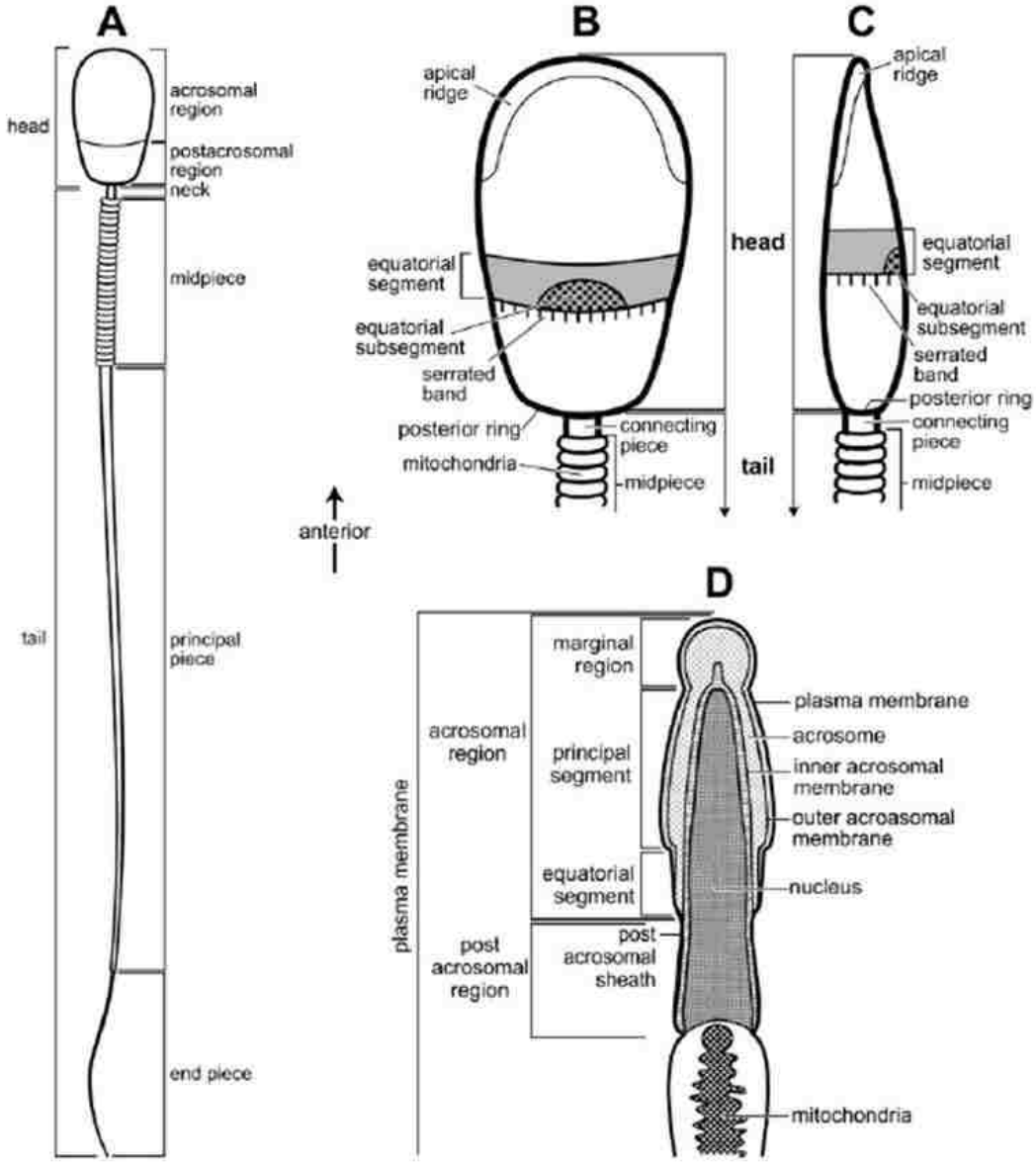



**Figure 1.1. Generalized drawings of a mammalian sperm head with the intact plasma membrane (which surrounds the entire sperm cell) (Downing Meisner et al., 2005)**. **A** Entire sperm, **B** Broad sperm head (flat side), **C** Lateral side of the sperm head, **D** Longitudinal section of the sperm head.

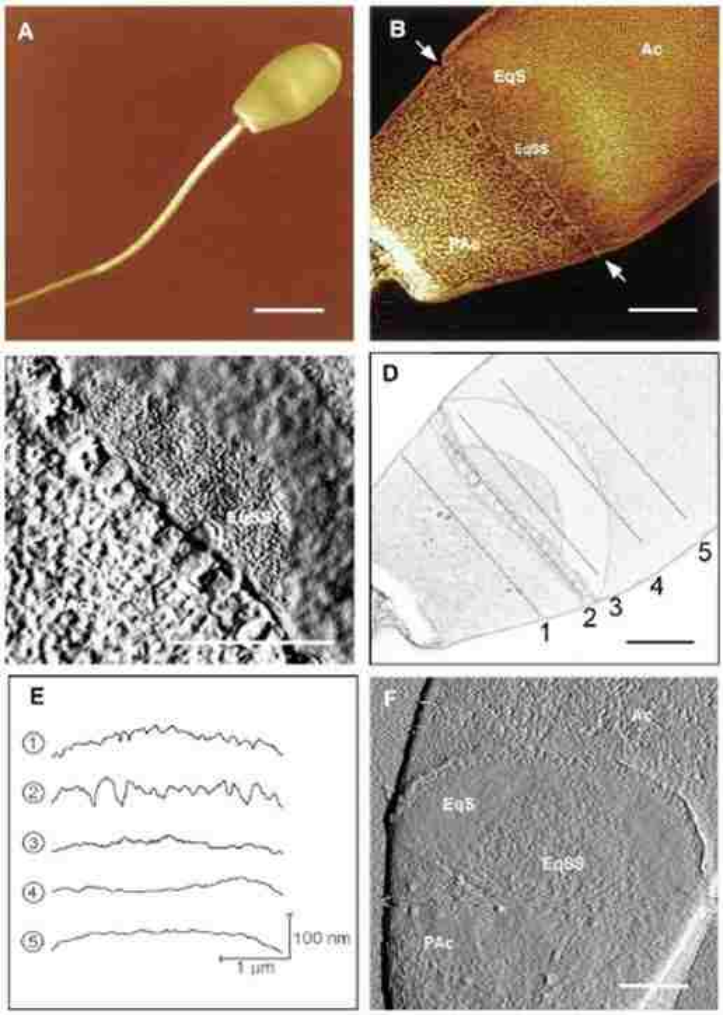



**Figure 1.2. AFM image of ejaculated ram spermatozoa (Ellis et al., 2002). A** A low-magnification scan (contact mode) of a single ram spermatozoan. **B** A higher magnification scan of the central head region of the spermatozoan shown in (A). Arrows indicate the position of the necklace of rectangular pits that demarcate the boundary between the postacrosmal (PAc) region and the EqS. Note the less wrinkled surface of the acrosomal (Ac) plasma membrane relative to that on the PAc region. The EqS is relatively smooth except for a crescent-shaped area adjacent to the necklace. **C** Higher magnification of the crescent-shaped area (known as the EqSS) within the EqS. Again note the difference in surface topography between the EqSS and EqS and the necklace of rectangular pits. **D** Tracing of the image in (B) to illustrate the position of the line profiles shown in (E). **E** surface corrugations in profile 5 are noticeably less than in profiles 1 and 2. The deepest depressions are found in profile 2. **F** AFM scan of a spermatozoan under buffer. The same surface topography and regional characteristics are present as in (B) with the EqSS very prominent. Bar, 5 µm in (A) and 1µm in (B), (C), (D), and (F). Note that (A) and (B) have been colorized to improve contrast.

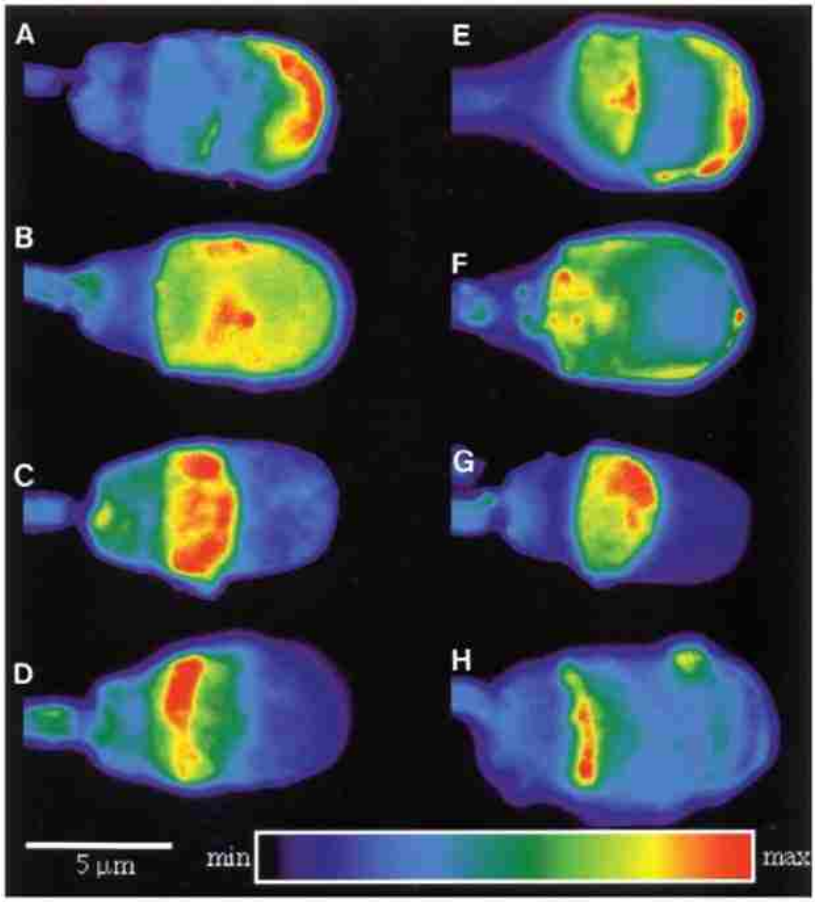



**Figure 1.3. Lateral distribution of fluorescent glycolipids after their incorporation into the plasma membrane on sperm cells with intact acrosomes and plasma membranes (Gadella et al., 1995).** Lateral distribution of SGalCer(C12-LRh) (**A-D**) and GalCer(C12-LRh) (**E-H**) after their incorporation into the sperm plasma membrane. The sperm heads were oriented with their flat surface perpendicular to the optical axis of the microscope. (**A**) The distribution of SGalCer(C12-LRh) in the plasma membrane of a freshly ejaculated sperm cell. (**B-D**) Subsequent stages of migration of SGalCer(C12-LRh) during capacitation in vitro with HBSS containing 2 mM $CaCl_2$. (**E**) The distribution of GalCer(C12-LRh) in the sperm plasma membrane of a freshly ejaculated sperm cell. (**F-H**) Subsequent stages of redistribution of GalCer(C12-LRh) during capacitation (conditions as above). The micrographs were taken after a capacitation period of: (**B,F**) 30 minutes; (**C,G**) 90 minutes; (**D,H**) 4 hours. The fluorescence intensity bar represents a linear scale of digitized fluorescence intensities in the images. In all cases the maximum/minimum (background) fluorescence intensity ratio was approximately 25.

# Chapter 2

# Materials and Methods

## Part I. Visualization of sperm surface domains and evaluation of the acrosome status in human and mouse sperm

### *Human sperm*

### 2.1 Human sperm collection

Human semen specimens were obtained from healthy, male donors over 18 years of age with informed, written consent, in accord with protocols approved by the Lehigh University Institutional Review Board. The semen quality, including morphology, motility, and numbers of sperm, was observed according to World Health Organization (WHO, 1999) criteria.

### 2.2 Human sperm washing

All human semen specimens were washed with Percoll® gradient fractionation as described by (Suarez, 1986) et al. (1986). Briefly, sperm cells were washed and enriched by centrifugation through a Percoll® (Sigma, St. Louis, MO, USA) gradient (isotonic 47.5% over 95% Percoll®) for 25 minutes at 500 × g at room temperature (RT). Top and interface layers were removed and human sperm

media (HSM), containing 117 mM NaCl, 0.3 mM $NaPO_4$, 8.6 mM KCl, 2.5 mM $CaCl_2$, 0.49 mM $MgCl_2$, 2 mM glucose, 19 mM sodium lactate, 25 mM $NaHCO_3$, 0.25 mM sodium pyruvate, pH 7.4 and 280 miliosmolar along with penicillin (5000 I.U./mL) and streptomycin (5000 µg/mL) to inhibit bacterial growth, was added to the remaining volume to yield a final volume of 10 mL, and the sample was centrifuged for 10 minutes, 500 × g at RT. The supernatant was discarded and the resulting sperm pellet was collected. The final pellet was resuspended in HSM to a final concentration of 20-40 million cells per mL.

### 2.3 Human sperm capacitation

After sperm washing, 500 µL of Percoll® washed sperm was transferred to a 35 mm Petri dish and capacitated in HSM, supplemented with 3.5% bovine serum albumin (BSA) (Sigma) for four hours, 37°C, 5% $CO_2$. Following capacitation, cells were washed in 10 mL HSM by centrifugation for 5 minutes, 500 × g and resuspended in HSM with the final concentration of 20-40 million cells/mL. Hypermotility and tyrosine phosphorylation at the tail of human sperm detected with 4G10 antibody were monitored to indicate level of sperm capacitation (Carrera et al., 1996; Visconti et al., 1995a; Visconti et al., 1995b; Visconti and Kopf, 1998). The conditions used to induce human sperm capacitation can allow human sperm to undergo complete capacitation since they have been tested in our laboratory, and approximately 10-20% of human sperm can undergo the spontaneous acrosome reaction after 4 hours of capacitation. Moreover, capacitated human sperm can penetrate zona-free hamster oocytes.

### 2.4 Induction of human sperm acrosome reaction

Following capacitation, capacitated cells were treated with 0.01mM of calcium ionophore A23187 (Molecular Probes, OR, USA) for 1 hour, 37°C, 5% $CO_2$. Following incubation, cells were washed by adding HSM to bring the volume to 10 mL and centrifuged at 500 × g for 5 minutes. Then the pellet was resuspended in HSM to make a concentration of 20-40 million cells/mL.

***Mouse sperm***

### 2.5 Housing of animals

All experiments were conducted according to the approved Lehigh University, Institutional Animal Care and Use Committee (IACUC) (Protocol No. 90). Adult (8 to 10 weeks) CF1 male mice were obtained from Charles River Laboratories (Wilmington, MA, USA), housed individually, and allowed free access to food and water. The animals were kept on a 14:10 light:dark cycle, with lights on at 8:00 AM and off at 10:00 PM.

### 2.6 Mouse sperm collection

Two male mice were sacrificed under $CO_2$ sedation and asphyxiation according to IACUC approved protocols. Fresh cauda epididymes were retrieved by postmortem dissection, the connective tissue and remaining blood vessels were removed. Cauda epididymes were placed in an organ culture dish containing 1

mL of Whitten's HEPES-buffered solution (WH) containing 100 mM NaCl, 4.7 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 5.5 mM glucose, 1 mM pyruvic acid, 4.8 mM L(+)-lactic acid hemicalcium salt in 20 mM Hepes, pH 7.3. WH medium was removed. After rinsing for additional two times, one milliliter of WH medium was added to the dish and mouse cauda epididymes were cut around 5 times and incubated for 15 minutes at 37°C, 5% $CO_2$ to release cauda fluid and sperm [the cauda epididymal contents (CEC)]. Then CEC was washed by Percoll® gradient and resuspended as described in section 2.2.

### 2.7 Mouse sperm capacitation

One hundred microliters of Percoll® washed mouse sperm were capacitated in an organ culture dish containing 100 µL of 3% BSA in WH medium to make 1.5% final concentration of BSA/WH solution. The sperm suspension was gently mixed and incubated for 1 hour at 37°C, 5% $CO_2$. Then 10 mL of WH medium was added and the sperm suspension was washed by centrifugation for 10 minutes, 500 × g at RT. The supernatant was discarded and the resulting mouse sperm pellet was collected. The final pellet was resuspended in WH medium to a final concentration of 20-40 million cells per mL. Hypermotility of mouse sperm together with tyrosine phosphorylated proteins on the tail, stained with 4G10 antibody(Ficarro et al., 2003; Visconti et al., 1995a; Visconti et al., 1995b; Visconti et al., 1999)(Ficarro et al., 2003; Visconti et al., 1995a; Visconti et al., 1995b; Visconti et al., 1999)(Ficarro et al., 2003; Visconti et al., 1995a; Visconti et al., 1995b; Visconti et al., 1999)(Ficarro et al., 2003; Visconti et al., 1995a; Visconti et

al., 1995b; Visconti et al., 1999)(Ficarro et al., 2003; Visconti et al., 1995a; Visconti et al., 1995b; Visconti et al., 1999), were monitored to indicate level of sperm capacitation.

### 2.8 Induction of mouse sperm acrosome reaction

One hundred ninety eight microliters of capacitated mouse sperm was incubated in an organ culture dish with WH medium, containing 2 µL of 1mM A23187 ionophore to make a final 0.01 mM ionophore solution, for 60 minutes at 37°C, 5% $CO_2$. Then the mouse sperm suspension was washed by centrifugation for 5 minutes, 500 × g at RT. For studying dynamic movement of α-L-fucosidase, ionophore-treated mouse sperm were harvested at different time points (0, 15, 30, 45, 60, and 120 minutes) during the acrosome reaction. The percentage of acrosome-reacted sperm was scored for a minimum of 200 cells following PNA-TRITC staining. The experiment was repeated three times.

### 2.9 Preparation of sperm sample slides

Ten microliters of Percoll® washed human or mouse sperm with the final concentration of $10^4$ cells per mL was placed onto Esco Fluoro glass slides (Erie Scientific, Oak Ridge, NJ, USA) and air-dried at RT for 3 hours. Sample slides were fixed with 4% (W/V) paraformaldehyde dissolved in PBS for 30 minutes at RT. Then, sample slides were washed three times with PBS for 15 minutes.

## 2.10 Evaluation of the acrosome status of intact, capacitated, and ionophore-induced human and mouse sperm

Sample slides of intact, capacitated, and ionophore-induced sperm were prepared as described in section 2.9. Peanut agglutinin (PNA) from *Arachis hypogaea* binds the carbohydrate sequence Gal-β(1-3)-GalNAc on the outer acrosomal membrane of mammalian sperm; therefore, it is used to determine the status of the acrosome. Fluorescent PNA used in this study is PNA-TRITC (Sigma). One hundred microliters of 20 µg/mL of PNA-TRITC was added onto each slide and incubated for one hour at RT in a dark moisture box. After three washes with PBS and one wash with distilled water, all sample slides were mounted with 10 µL of VECTASHIELD® mounting media (Vector Laboratories, Burlingame, CA, USA) and covered with No.1 coverslips. Sample slides were viewed using Nikon eclipse TE 2000-U fluorescence microscope (Nikon, Tokyo, Japan), and micrographs were captured with a SPOT RT KE camera. Sample slides were viewed and scored under a fluorescence microscope (Mortimer et al., 1987). Briefly, sperm that have completed the acrosome reaction would show loss of PNA staining at the acrosome but still present the signal at the EqS of human and mouse sperm whereas intact, mildly permeabilized, and capacitated human sperm would show strong PNA staining at the acrosome.

## Part II. Detection of the EqSS in human sperm

### 2.11 Strong permeabilization of human sperm

Sample slides of washed human sperm were prepared as described in section 2.9. Then, sample slides were incubated with cold absolute methanol for 10 minutes at -20ºC and immediately immersed in cold acetone for 30 seconds at -20ºC. Sample slides were washed three times in PBS for 15 minutes.

### 2.12 Indirect immunofluorescence for tyrosine phosphorylated proteins in intact, permeabilized, capacitated, and acrosome-reacted human sperm

Sample slides of paraformaldehyde-fixed intact, methanol/acetone-permeabilized, capacitated, and ionophore-induced human sperm were incubated with 100 µl of 5 µg/mL primary antibody mouse-anti-tyrosine phosphorylated protein monoclonal antibody clone 4G10 (4G10 antibody) (Millipore, MA, USA) overnight at 4°C in a moisture box. For the negative control group, some sample slides were incubated with PBS under the same conditions. After three washes with PBS for 15 minutes, all sample slides were incubated with 100 µL (1 µg/mL) of secondary antibody goat-anti-mouse conjugated with fluorescein isothiocyanate (FITC) (KPL, Gaithersburg, MD, USA). After washing three times with PBS, sample slides were counterstained for 30 minutes at RT in a moisture box with 100 µL of PBS containing a nuclear staining dye [1 µg/mL Hoechst 33342 (Molecular Probe)] and 20 µg/mL of PNA-TRITC. After three

washes with PBS and one wash with distilled water, all sample slides were mounted and visualized as described in section 2.10.

## Part III. Progressive relocalization of α-L-fucosidase in mouse sperm during the acrosome reaction

### 2.13 Partial permeabilization of mouse sperm

Percoll® washed mouse sperm were diluted in 1 mL of PBS to make the final concentration of $10^4$ cells/mL. Then mouse sperm suspensions were frozen at -80°C for 30 minutes and thawed at 37°C.

### 2.14 Immunolocalization of α-L-fucosidase in washed, permeabilized, capacitated, and acrosome-reacted mouse sperm

Sperm sample slides were prepared as described in section 2.9. Percoll® washed, partially permeabilized, capacitated, or ionophore-treated mouse sperm sample slides were fixed with 4% paraformaldehyde for 15 minutes at RT, then washed with PBS for three times. These sperm samples were incubated overnight at 4°C with 25 μg/mL of rabbit anti-FUCA1 polyclonal antibody (Abcam, Cambridge, MA), washed with PBS for three times, and incubated with PBS containing 1 μg/mL of FITC-conjugated goat anti-rabbit antibody (KPL, Gaithersburg, MD). Sperm samples slides were counterstained with 20 μg/mL of

PNA-TRITC, and sperm nuclei were stained with 1 μg/mL of Hoechst 33342 for 30 minutes at RT as in section 2.12. After washing three times with PBS and one time with distilled water, sperm samples slides were mounted and visualized as described in section 2.10. Different localization patterns of α-L-fucosidase on mouse sperm at different time points during the acrosome reaction were quantified. The experiment was repeated three times and at least 200 cells were counted per trial.

## Part IV. Subcellular localization of cysteine-rich secretory protein 2 (CRISP2) in washed, permeabilized, capacitated, and acrosome-reacted human sperm cells

### 2.15 Indirect immunofluorescence of CRISP2 in washed, permeabilized, capacitated, and acrosome-reacted human sperm

Sperm sample slides were prepared as described in section 2.9. 200 μL of 4% paraformaldehyde was added onto sample slides and the samples were incubated at RT for 15 minutes for fixing. After three PBS washes, some sample slides were permeabilized with 0.5% Triton X-100 in PBS for 5 minutes at RT, then washed three times, 5 minutes each with PBS. Percoll® washed (intact), Triton X-100 permeabilized, capacitated, and ionophore-treated human sperm sample slides were incubated with 5 μg/mL of rabbit anti-human CRISP2 antibody (Sigma) at 4°C overnight, washed again three times with PBS, and

incubated with 1 μg/mL of goat anti-rabbit IgG-FITC for 1 hour at RT. After three additional PBS washes, sample slides were counterstained with PBS containing 1 μg/mL of Hoechst 33342 and 20 μg/mL of PNA-TRITC for 30 minutes at RT. The capacitated cells were incubated with 4G10 antibody for an additional 1 hour before Hoechst 33342 and PNA-TRITC counterstaining to determine capacitation-associated protein tyrosine phosphorylation (Carrera et al., 1996; Visconti et al., 1995a; Visconti et al., 1995b; Visconti and Kopf, 1998). After three final PBS washes, sample slides were mounted and visualized as described in section 2.10.

## Part V. Detection of F-actin in human sperm and determination of its functional role in relocalization of CRISP2 to the EqS during the acrosome reaction

### 2.16 Staining of F-actin in the head of human sperm

Washed human sperm were induced to undergo the acrosome reaction by ionophore as described in section 2.4 with or without the presence of 25 μM of F-actin inhibitor, Latrunculin A (Enzo Life Sciences, Farmingdale, NY, USA) (Latrunculin A binds to actin monomers and inhibits polymerization of F-actin). Some washed sperm were permeabilized with 0.5%Triton X-100 as described in section 2.15. Then sperm sample slides were prepared as described in section 2.9. After paraformaldehyde fixing and washing, sample slides were incubated

with 1:1000 of Phalloidin-TRITC (Sigma) in PBS for 1 hour. After three PBS washes and one wash with distilled water, sample slides were mounted and visualized by fluorescence microscopy as described in section 2.10.

### 2.17 Inhibitor treatment and coimmunolocalization of CRISP2 and PNA-TRITC in acrosome-reacted human sperm

To test whether F-actin is involved in the relocalization of CRISP2 to the EqS during the acrosome reaction, Latrunculin A was used to inhibit F-actin formation during the acrosome reaction. Percoll®-washed human sperm were capacitated and induced to undergo the acrosome reaction (as in section 2.3 and 2.4, respectively) in the presence of varied concentrations of Latrunculin A (0, 25, 50, and 100 μM) throughout the course of capacitation and the acrosome reaction. The ionophore-treated human sperm were then washed three times and subjected to indirect immunofluorescence with anti-CRISP2 antibody and PNA-TRITC. The percent of CRISP2/PNA-TRITC positive at the EqS was quantified in the control group and Latrunculin A-treated groups. The experiment was repeated three times and at least 200 cells were counted per trial.

## Part VI. Determination of CRISP2 as a component of the acrosomal cap of human sperm

### 2.18 pH-dependent solubilization of human sperm acrosomal matrix

Sperm cells were divided into two identical groups ($10^7$ cells per group). The first group of sperm cells was incubated with HSM pH 4 for 30 minutes at RT and the second group was incubated with HSM pH 11 at RT for the same period of time. Then, the cell suspensions were centrifuged at 500 × g to separate supernatants and sperm pellets. The collected supernatants were centrifuged again at 10,000 × g at 4°C for 10 minutes. The sperm pellets were washed with 10 mL of HSM and centrifuged at 500 × g for 10 minutes.

### 2.19 SDS-PAGE and Western blot analysis

Supernatants and pellets obtained from section 2.18 were subject to SDS-PAGE and Western blot analysis. Briefly, all supernatants and pellets were diluted in reducing Laemmli buffer (Sigma). The samples were heated at 98˚C for 10 minutes and separated in a 12% gel by SDS-PAGE and electro-blotted onto PVDF membranes (Amersham Pharmacia, Buckinghamshire, UK). Membranes were blocked with blocking solution [5% skimmed milk powder in TBS-T (pH 7.6) containing 0.02 mol/L Tris-HCl, 0.137mol/L NaCl, and 0.1% Tween 20], for 1 hour at RT and then incubated with 1 μg/mL of anti-CRISP2 antibody overnight at 4˚C. After washing three times in TBS-T for 15 minutes, membranes were

incubated with 1:5000 of biotinylated goat anti-rabbit IgG (KPL) for 1 hour at RT, washed three times in TBS-T for 15 minutes, and incubated with a preformed avidin and biotinylated horseradish peroxidase macromolecular complex (Thermo Scientific, Rockford, IL, USA) for 30 minutes at RT. Membranes were washed three times with TBS-T for 15 minutes, and immune complexes were detected using ECL Western Blotting Detection System (GE Healthcare Ltd, Buckinghamshire, UK).

## Part VII. Release of CRISP2 from the acrosome of human sperm during the acrosome reaction

### 2.20 Detection of CRISP2 in the supernatants of the ionophore-treated human sperm

Percoll® washed sperm were divided into three groups ($10^7$ cells each). The control washed sperm pellet was incubated with 100 µL of HSM pH 7.4 at 37°C, 5% $CO_2$ throughout the course of capacitation and the acrosome reaction (5 hours). The second group was capacitated as described in section 2.3. The last group was capacitated, and the pellet was washed again by adding 5 mL of HSM pH 7.4 and recentrifuged at 500 × g for 5 minutes at RT. The resulting pellet was reconstituted to bring to the final volume of 100 µL, and ionophore A23187 was added to the solution with the final concentration of 0.01mM to induce the acrosome reaction as described in section 2.4. Then, all sperm suspensions

were incubated for 60 minutes, 37°C, 5% $CO_2$. Intact, capacitated, and ionophore-treated sperm suspensions were centrifuged at 500 × g for 5 minutes at RT to separate sperm pellets and supernatants. After centrifugation, supernatants were collected and centrifuged at 10,000 × g for 10 minutes at 4°C to remove insoluble materials. The sperm pellets from each group were washed by adding 5 mL of HSM and recentrifuged at 500 × g for 5 minutes at RT and then reconstituted with 100 µL of HSM. All samples (supernatants and pellets of intact, capacitated, and ionophore-treated groups) were subject to SDS-PAGE and Western blot analysis using anti-CRISP2 antibody as described in section 2.19.

## Part VIII. Reassociation of CRISP2 at the EqS of human sperm during the acrosome reaction

### 2.21 Isolation of human sperm acrosomal CRISP2 released during the acrosome reaction

Sperm samples from three different donors were combined ($10^9$ cells) and subject to the acrosome reaction as described in section 2.4. After the acrosome reaction, the supernatant (1 mL) was collected by centrifugation at 10,000 × g 4°C for 30 minutes. The protein quantity was measured using the BioRad protein assay kit (BioRad, Hercules, CA, USA). For CRISP2 purification, 50 µL (6 mg) of Dynabeads® protein G (Invitrogen, Grand I Island, NY) was transferred to a 1.5

mL centrifuge tube. Prior to incubation with the primary antibody, Dynabeads® protein G was washed three times with PBS pH 7.4 containing 0.02% of Tween-20 (PBST). Next, 40 µg of rabbit anti-CRISP2 antibody was incubated with Dynabead-protein G for 10 minutes at RT with gentle rotation. Then the bead-G protein-antibody complex was covalently linked using a crosslinking reagent, $BS^3$ [5 mM (Bis[sulfosuccinimidyl] suberate)] (Invitrogen), dissolved in conjugation buffer (20 mM sodium phosphate, 0.15 M NaCl, pH 7.4) for 30 minutes at RT with rotation to prevent co-elution of anti-CRISP2 antibody. After washing the bead-G protein-antibody complex three times with PBST, the ionophore-treated supernatant was incubated with the bead-protein G-anti CRISP2 antibody complex for 1 hour at RT. The supernatant was removed and the beads were washed by placing the tube on the magnet, removing old buffer, and adding new PBST three times. After removing all PBST, the beads were transferred to a new tube and 200 µl of elution buffer (50 mM glycine pH 2.8) was added to the beads, and incubation was performed with gentle agitation for 2 minutes at RT. Then the beads were discarded by replacing the tube on the magnet and the eluent was collected. The pH of the eluent was adjusted to neutral with 1 M Tris-HCl pH 7.5.

### 2.22 *In vitro* biotinylation of human sperm CRISP2

Purified human acrosomal CRISP2 protein was subject to *in vitro* biotinylation. Briefly, 2.5 µL of EZ-Link Sulfo-NHS-Biotin (sulfosuccinimidobiotin) (Thermo Scientific) stock solution (10 mM) was added to the purified human acrosomal CRISP2 protein solution (200 µL) to provide a 50-fold molar excess of biotin

reagent. The solution was incubated for 2 hours on ice. Then, non-reacted biotinylation reagents were quenched with 50 mM Tris (pH 8.0). The solution was then subject to bead purification again to eliminate the contaminants. The purified biotinylated human sperm acrosomal CRISP2 was finally eluted with 50 µL of elution buffer and the pH was adjusted to neutral by 1 M Tris-HCl, pH 7.5.

### 2.23 Silver staining and Western blot analysis

To test the purity of the purified biotinylated human sperm acrosomal CRISP2 after purification by bead-anti-CRISP2 antibody complex, I performed silver staining to compare the presence of proteins in the supernatants before and after purification. Briefly, 50 µg of the supernatant prior to the purification and 2 µg of the eluent were subject to SDS-PAGE. After gel electrophoresis, the gel was fixed with a fixing solution (40% ethanol, 10% acetic acid, and 50% $H_2O$) for 1 hour at RT. After washing with several changes of distilled water, the gel was sensitized in 0.02% sodium thiosulfate solution for 1 minute and washed with distilled water three times for 15 minutes. Then the gel was incubated with cold silver nitrate solution (0.1% $AgNO_3$, 0.02% formaldehyde) for 20 minutes at 4˚C, washed with distilled water for one minute, developed in 3% sodium carbonate solution (3% $Na_2CO_3$, 0.05% formaldehyde), and terminated in 5% acetic acid for 5 minutes at RT. Western blotting using anti-CRISP2 antibody as described in section 2.15 was also performed to verify the existence of human acrosomal CRISP2 after purification.

## 2.24 Binding assay of CRISP2 at the EqS of acrosome-reacted human sperm

Human sperm were capacitated for 4 hours and the the acrosome reaction was induced as described in section 2.3 and 2.4, respectively. The purified biotinylated human acrosomal CRISP2 at 6.25 µg/mL was incubated with $10^7$ cells/mL of ionophore-treated human sperm at the beginning and throughout the entire course of the acrosome reaction (1 hour). For the control group, the same volume of the elution buffer pH 7.4 was added into the medium. After 1 hour of the incubation at 37°C, 5% $CO_2$, sperm samples were washed by centrifugation at 500 × g for 5 minutes at RT. Then, sperm sample slides were prepared and fixed as described in section 2.9 and 2.10, respectively. After washing three times with PBS, sperm sample slides were incubated with 5 µg/mL of streptavidin-FITC (BioLegend, San Diego, CA, USA) for 1 hour, washed three times with PBS, mounted, and observed under a fluorescence microscope as described in section 2.10. The percent of biotinylated CRISP2 at the EqS of acrosome-reacted human sperm cells was calculated and compared to that of the control group. The experiment was repeated three times and at least 200 cells were count per trial.

In some experiments a reduced concentration of capacitated human sperm cells ($10^4$ cells/mL) was incubated with different concentrations (0, 6.25, 12.50, and 31.25 µg/mL) of the purified biotinylated human acrosomal CRISP2 at the beginning and throughout the entire course of the acrosome reaction before staining with streptavidin-FITC.

## Part IX. Association stability of CRISP2 at the EqS of acrosome-reacted human sperm

### 2.25 Treatments of acrosome-reacted human sperm

Human sperm were induced to undergo the acrosome reaction as described in section 2.4. Ionophore-treated human sperm were incubated with different agents, including 1 M NaCl for 30 minutes, 5 mM DTT (Dithiothreitol) for 30 minutes, 0.5% Triton X-100 for 30 minutes, 30 mM EGTA for 30 minutes, 0.1% SDS for 1 minute. All of these treatments were done at RT and all agents were dissolved in HSM. For mild sonication, human sperm suspension (in HSM) was sonicated on ice (10 seconds pulse on and 10 seconds pulse off) for 3 times by using a Model 60 Sonic Dismembrator set at an intensity of 3 (Fisher Scientific, Pittsburgh, PA). Then all sperm samples were evaluated by immunofluorescence using anti-CRISP2 antibody as described in section 2.15.

## Part X. Binding of human acrosomal CRISP2 to the plasma membrane of hamster oocytes

### 2.26 Removing the zona pellucida of hamster oocytes

Hamster oocytes were purchased from EmbryoTech Laboratories Inc (Haverhill, MA, USA) and thawed according to the manufacturer's instructions. The zona

pellucida of the oocytes was removed by incubating with 0.1% trypsin (Sigma) for 2-3 minutes at RT with monitoring under the dissecting microscope to avoid under or over treatment (Yanagimachi et al., 1976). The zona-free oocytes were washed three times by transferring oocytes into 400 µl fresh new drops of M2 medium (Sigma).

### 2.27 Staining hamster oocytes with the purified biotinylated human sperm acrosomal CRISP2 protein

To evaluate whether hamster oocytes contain complementary targets of human sperm acrosomal CRISP2, hamster oocytes were incubated with 25 µg/mL of the purified biotinylated human sperm acrosomal CRISP2 protein for 1 hour at 37°C, 5% $CO_2$. The oocytes were then washed with PBS containing 4 mg/mL BSA (PBS-BSA) for 15 minutes by changing to several PBS-BSA droplets and fixed with a 100 µL droplet of 4% paraformaldehyde containing 4 mg/mL bovine serum albumin (BSA) for 15 minutes and then washed three times with PBS-BSA for 15 minutes. Then the oocytes were incubated with 5 µg/mL of streptavidin-FITC for 1 hour at RT. After washing three times with PBS-BSA for 15 minutes, the nuclei of the oocytes were stained with 1 µg/mL of Hoechst 33342 for 30 minutes at RT. After washing three times with PBS-BSA for 15 minutes, the oocytes were transferred to a glass slide in a 10 µL drop of VECTASHIELD® mounting media. A cover slip supported by a mixture of petroleum jelly and mineral oil (4:1) was gently placed on top of the drop to flatten the oocytes (Bean et al., 1991). Fluorescence microscopy was performed as described in section 2.10. Zona-free

hamster oocytes were also stained with the purified biotinylated human sperm acrosomal CRISP2 and streptavidin-FITC. For comparison, some hamster oocytes were subject to indirect immunofluorescence using anti-human CRISP2 antibody as described in section 2.15.

# Chapter 3

## Results-I

## Subcellular domains on human and mouse sperm, and progressive relocalization of α-L-fucosidase to the EqS of mouse sperm during the acrosome reaction

### 3.1 Visualization of domains on sperm head surface and evaluation of the acrosome status

The use of lectins as probes of mammalian sperm surface has been previously studied (Koehler, 1981) and it has been suggested that peanut agglutinin can be used to study the localized specialization of highly differentiated cell membrane and as a marker for acrosome status (Mortimer et al., 1987). Peanut agglutinin is among those lectins that can be used to visualize the acrosomal area of mammalian sperm (Mortimer et al., 1987). Peanut agglutinin (PNA) is a plant lectin protein derived from *Arachis hypogaea* and it specifically binds a particular disaccharide sequence (Gal-β(1-3)-GalNAc) on the surface of sperm cells.

Therefore, this study used a fluorescent PNA (PNA-TRITC, PNA conjugated with tetramethyl rhodamine isothiocyanate) to visualize surface domains, including the anterior of the acrosome and the EqS of human and mouse sperm. For human sperm, when washed nonpermeabilized sperm cells were stained with PNA-TRITC, the entire acrosomal regions, including the anterior acrosome and the

EqS, were strongly stained (Figure 3.1). The PNA signal is very specific to the acrosome with no staining at other regions on the sperm surface.

When human sperm cells were induced to undergo capacitation, the PNA signal could still be seen throughout the entire acrosome of most cells (Figure 3.2). However, the strength of the signal was weaker than that of intact human sperm. Results show that some of capacitated human sperm cells had undergone spontaneous acrosome reaction as indicated by the PNA staining still detected at the EqS with the concomitance of the loss of the PNA signal from the anterior of the acrosome (Figure 3.2, arrow head). However, when the PNA staining patterns were counted, there was only 10 to 20 percent of the capacitated population that had undergone spontaneous acrosome reaction (data not shown). The level of capacitation was determined by the observation of sperm hypermotility together with staining for tyrosine phosphorylated proteins at the tail of sperm cells using anti-tyrosine phosphorylated protein antibody (4G10 monoclonal antibody). The percent of human sperm with hypermotility was around 98% (data not shown) and this number was consistent with that of 4G10 staining at the tail of these sperm cells.

When human sperm were induced to undergo the acrosome reaction by using calcium ionophore A23187 for 60 minutes, the vast majority of human sperm cells showed loss of PNA staining at the anterior of the acrosome with the signal still detectable at the EqS (Figure 3.3, arrow head). A small population of human sperm cells showed intact acrosome staining (Figure 3.3, arrow). The percent of

the acrosome reaction was around 70 to 75 percent after 60 minutes of the acrosome reaction (data not shown).

In the following investigation, involving the relocalization of α-L-fucosidase during the acrosome reaction, the acrosome status of mouse sperm was also evaluated by PNA staining. In this study, intact/nonpermeabilized, capacitated, and ionophore-induced mouse sperm were stained with PNA-TRITC. The level of capacitation was also evaluated by criteria described for human sperm. Data showed that the detected PNA signal is restricted at the marginal surface of the anterior of the acrosome of intact mouse sperm (Figure 3.4, intact). The staining did not stain surface areas beyond the anterior acrosome. In other words, the EqS of intact mouse sperm is not stained by PNA-TRITC. This is different from PNA-TRITC staining of intact human sperm where the entire acrosome, including the EqS, is recognized by PNA.

Capacitated mouse sperm showed a similar PNA-TRITC staining pattern as seen in intact mouse sperm (Figure 3.4, capacitated). However, like in capacitated human sperm, there was a small population of capacitated mouse sperm that had undergone spontaneous acrosome reaction as judged by the PNA signal detected in the EqS of mouse sperm (Figure 3.4, capacitated, arrow head). The EqS of mouse sperm is a large proportion when compared to the anterior of the acrosome. When compared to mouse sperm, the area of the EqS of human sperm is smaller than that of the anterior of the acrosome. When mouse sperm were treated with ionophore, a major population of mouse sperm showed staining at the EqS (Figure 3.4, acrosome-reacted, arrow).

### 3.2 Determination of the presence of the EqSS in human sperm

Besides the anterior acrosome and the EqS, there are reports revealing the presence of another subcellular domain on mammalian sperm. The EqSS was originally identified by atomic force microscopy as a discrete region within the EqS of Artiodactyl spermatozoa (Downing Meisner et al., 2005). Moreover, a study in 2006 revealed that the EqSS of boar, ram, and bull sperm is enriched in tyrosine phosphorylated proteins and also showed preliminary evidence for the presence of the EqSS in mouse and rat sperm (Jones et al., 2008). This study used antibody against tyrosine phosphorylated proteins (4G10 antibody) to visualize the EqSS. Surprisingly, there is no report, either by electron microscopy or 4G10 staining, about the existence of the EqSS in human sperm. Based on the conservation of the EqSS in various mammalian species, I hypothesized that the EqSS exists in human sperm. Therefore, anti-tyrosine phosphorylated protein monoclonal antibody (4G10) was used in this current project to discover the EqSS of human sperm.

Immunofluorescence showed that incubation of intact unpermeabilized human sperm (more than 95% viability) with 4G10 monoclonal antibody showed negative staining on the human sperm head (Figure 3.5A). PNA-TRITC staining indicated that the acrosome of human sperm is intact. However, when human sperm were fixed and permeabilized with absolute methanol and acetone, positive staining of tyrosine phosphorylated proteins was observed and the staining pattern was seen as a puncta-like signal within the EqS domain as stained by PNA-TRITC (Figure. 3.5B). PNA-TRITC staining indicated that

methanol/acetone permeabilization completely solubilized the membrane system over the anterior of the acrosome, but the membrane overlying the EqS of most human sperm cells was resistant to this permeabilization condition (Figure. 3.5B, PNA-TRITC). However, some sperm cells showed a faint staining or loss of PNA staining at the EqS with the detectable signal of tyrosine phosphorylated proteins (Figure. 3.5B, merge).

Based on the fact that capacitation and the acrosome reaction can naturally permeabilize sperm cells, I hypothesized that capacitated and acrosome-reacted human sperm cells present similar 4G10 staining as seen in permeabilized human sperm. When human sperm were induced to undergo capacitation and stained with 4G10 monoclonal antibody, the signal could be detected strongly in the tail as expected. Interestingly, tyrosine phosphorylation was also detected in either the entire acrosome or in the EqS with the dispersed signal remaining in the anterior of the acrosome (Figure 3.6). PNA-TRITC staining indicated that the acrosome of capacitated human sperm is intact as expected (Figure 3.6, PNA-TRITC).

When human sperm cells were induced to undergo the acrosome reaction for short period of time (30 minutes) and stained with 4G10 antibody, most sperm cells that were undergoing the early acrosome reaction showed strong 4G10 staining throughout the acrosome or punctate staining dispersed throughout the acrosome, including the EqSS (Figure 3.7). PNA staining indicated that sperm cells did not completely undergo the acrosome reaction; in fact, they were in the early stages of the acrosome reaction as judged by faint and diffuse PNA signal

in the anterior of the acrosome (Figure 3.7, PNA-TRITC). Surprisingly, tyrosine phosphorylation at the tail of 30 minute ionophore-treated human sperm cells disappeared, but the signal at the neck of the sperm could still be observed (Figure 3.7).

When the acrosome reaction of human sperm was induced for 60 minutes, most cells showed loss of 4G10 in the head, but strong staining was seen again in the tail of completely acrosome-reacted cells (Figure 3.8). However, there was a small population of sperm cells showing tyrosine phosphorylation in the EqS and the acrosomal area near the EqS (Figure 3.8). PNA staining indicated that most cells had undergone the complete acrosome reaction as judged by the PNA signal at the EqS without remaining signal detectable in the anterior of the acrosome (Figure 3.8, PNA-TRITC).

### 3.3 Localization of α-L-fucosidase in cauda epididimal mouse sperm

According to the discovery in our laboratory that α-L-fucosidase reappeared at the EqS of human sperm after the acrosome reaction (Venditti et al., 2007), we continued investigating this phenomenon in mouse sperm since the activity, distritbution, stability, and of roles of α-L-fucosidase were studied in mice (Phopin et al., 2012; Phopin et al., 2013).

The localization of α-L-fucosidase in mouse sperm was investigated by comparing immunofluorescence patterns of mouse sperm before and after permeabilization. For permeabilization, many conditions, including using

methanol, different concentrations of Triton X-100, and mild sonication, have been tried for mouse sperm cells and none of these methods provided successful staining for mouse sperm α-L-fucosidase (data not shown). The study indicated that mouse sperm α-L-fucosidase is sensitive to regular permeabilizations. Many studies reported that freezing and thawing sperm cells from cryopreservation can result in sperm membrane disruption (Check et al., 1991; Hammadeh et al., 1999; Lin et al., 1998). Additionally, the freeze and thaw protocol for permeabilization was reported for studying localization of tetraspanin CD9 in mouse sperm (Ito et al., 2010). Therefore, in this current study, mouse sperm were partially permeabilized by one round of freezing and thawing before performing immunofluorescence using anti-fucosidase antibody. The results showed that anti-FUCA1 antibody could not stain intact sperm (Fig. 3.9A) whereas permeabilized sperm were found to be positive to the antibody with the signal at the anterior of the acrosome (Fig. 3.9B). After ionophore treatment, α-L-fucosidase was detected at the EqS of acrosome-reacted mouse sperm with the signal evenly distributed throughout the EqS (Fig. 3.9C).

### 3.4 Relocalization of α-L-fucosidase from the anterior of the acrosome to the EqS of mouse sperm during the acrosome reaction

To analyze the localization behavior of sperm α-L-fucosidase during the acrosome reaction, anti-FUCA1 antibody was used to trace α-L-fucosidase within the sperm head at each time point during the artificial acrosome reaction. Intact sperm (without induction of the acrosome reaction) were negative to an anti-

FUCA1 antibody (Figure 3.10A) as previously shown in Figure 3.9A. At the initial stage of the acrosome reaction (Figure 3.10B), α-L-fucosidase was found predominantly at the anterior of acrosomal region. At the early stage (Figure 3.10C), α-L-fucosidase completely filled in the entire area of the anterior of the acrosome. Moreover, at this stage, the enzyme began to appear in the EqS. At the advanced stage (Figure 3.10D), almost all of α-L-fucosidase localized into the EqS with the remaining signal still detected at the anterior of the acrosome. At the final stage (Figure 3.10E), the immunostaining signal was evenly distributed throughout the entire EqS. FUCA1-immunostaining pattern changes at every stage of the observed acrosome-reacted sperm were similar to those of membrane-bound fluorescent lectin PNA-TRITC. The percentage of acrosome-reacted sperm with each of the four of FUCA1-immunostaining patterns at each time point during the artificial acrosome reaction was also evaluated. These results are summarized in Figure 3.11. It was found that at 0 minutes, the majority of sperm were in an initial stage (48%) and the percentage gradually decreased to 5.2% at 120 minutes. The percentage of sperm with an early stage pattern was highest at 30 minutes (40.4%) and gradually decreased to 18.2% at 120 minutes. The percentage of sperm with an advanced stage pattern was low at the beginning of the acrosome reaction and gradually increased to reach the maximum (40.7%) at 60 minutes. The percentage of sperm with a final stage signal was dramatically increased after 30 minutes to reach the highest point (42.3%) at 120 minutes. The experiment was performed three times, 200 human sperm cells were counted per each trial, and the data were presented as mean±S.M.E.

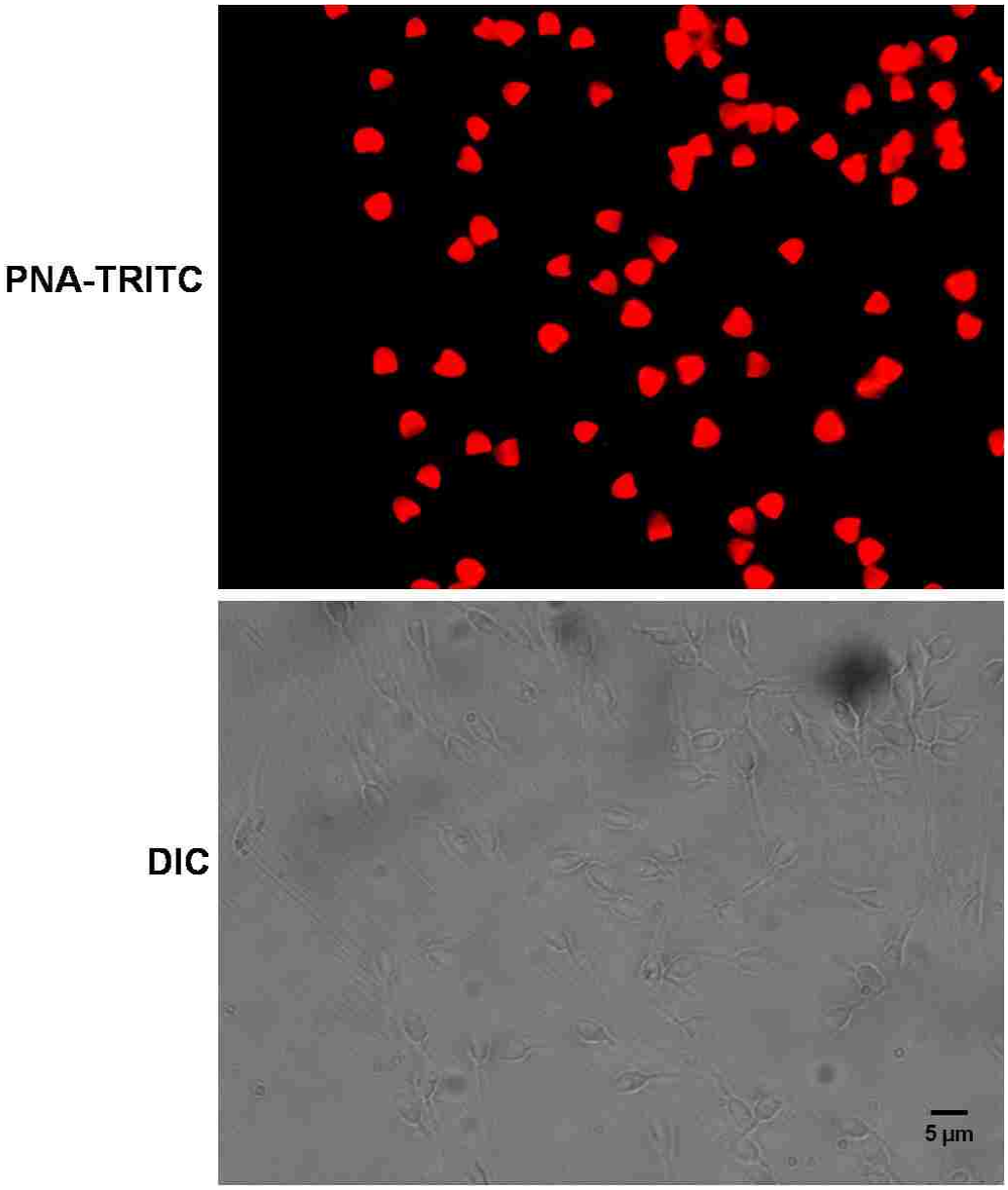



**Figure 3.1. Determination of the acrosome status in nonpermeabilized human sperm (intact) using PNA-TRITC staining**. DIC shows the whole sperm structure. Data are representative of 3 trials (200 cells/trial).

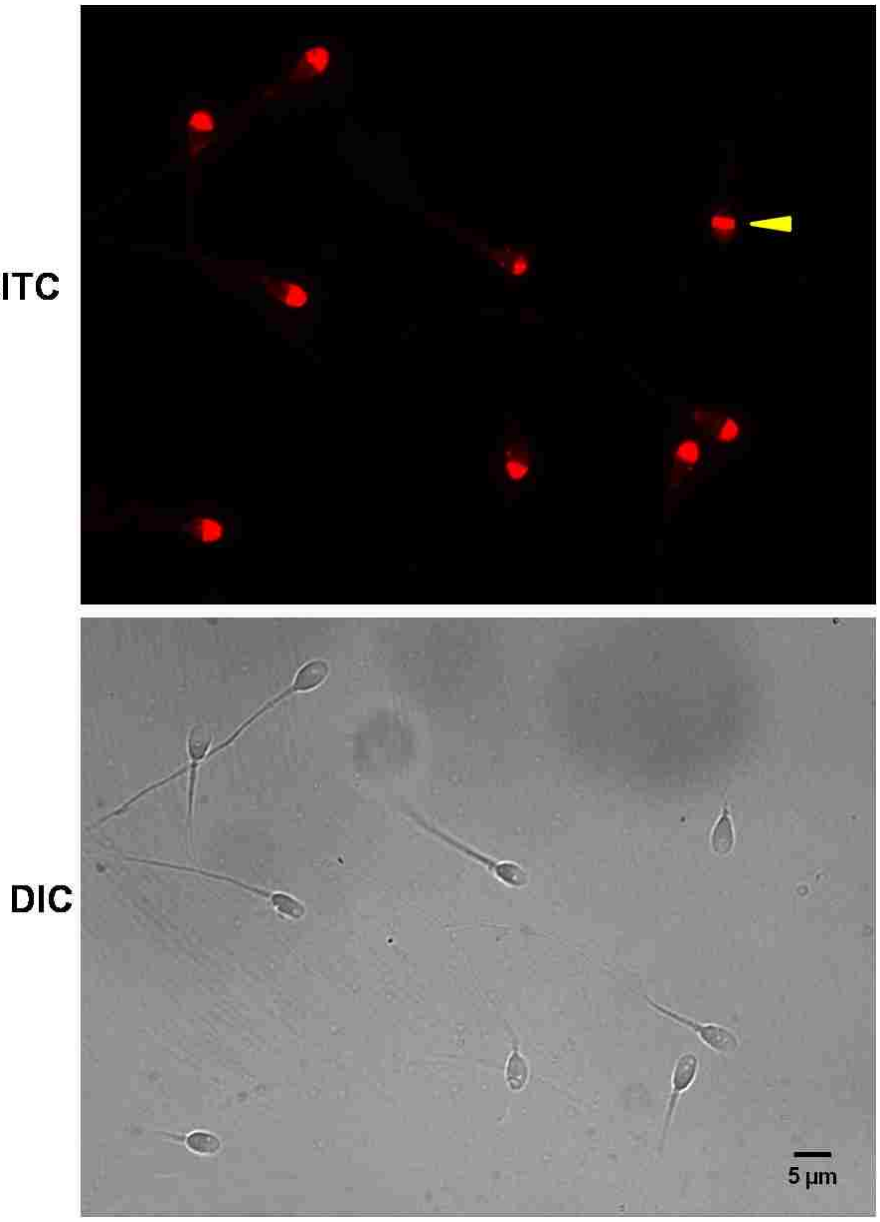



**Figure 3.2. Determination of the acrosome status in capacitated human sperm using PNA-TRITC staining**. DIC shows the whole sperm structure. An arrow head indicates a sperm cell that had undergone spontaneous acrosome reaction with PNA signal at the EqS. Data are representative of 3 trials (200 cells/trial).

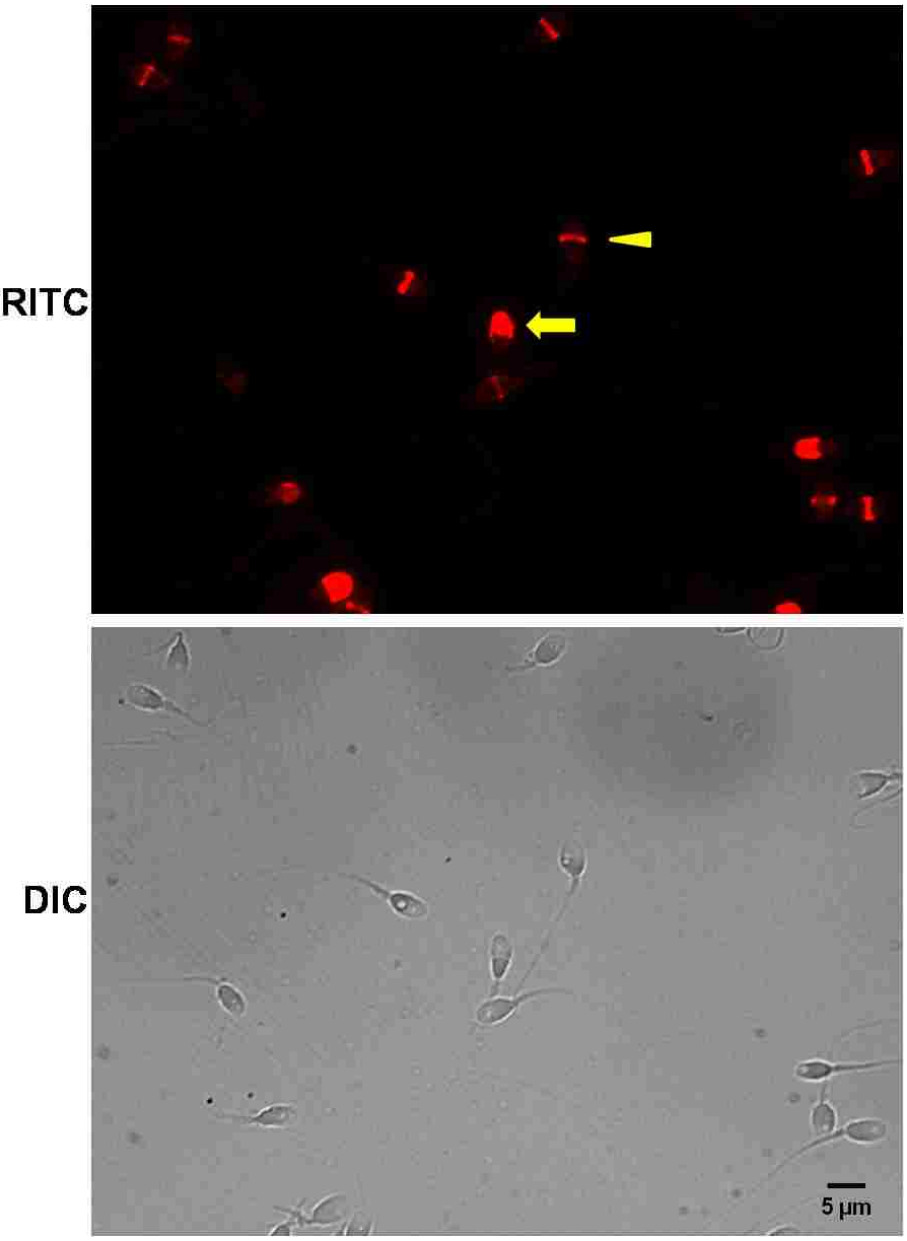



**Figure 3.3. Determination of the acrosome status in ionophore-treated human sperm using PNA-TRITC staining**. DIC shows the whole sperm structure. An arrow head indicates a sperm cell that had undergone the complete acrosome reaction with PNA signal at the EqS. An arrow indicates a sperm with acrosome intact after ionophore treatment. Data are representative of 3 trials (200 cells/trial).

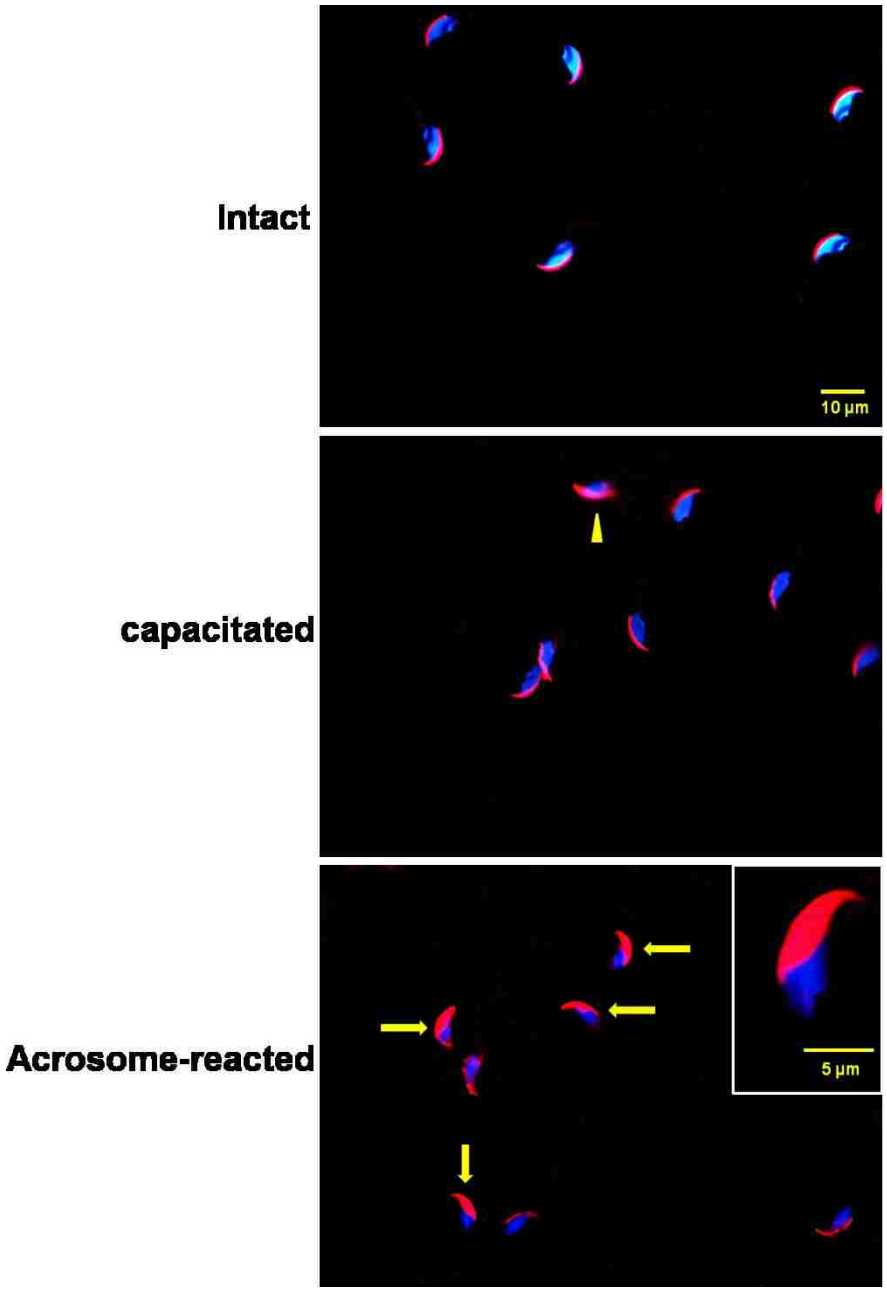



**Figure 3.4. Determination of the acrosome status in intact, capacitated, and acrosome-reacted mouse sperm using PNA-TRITC staining**. Nuclei were counterstained with Hoechst 33342 (blue). An arrow head indicates a sperm cell that had undergone spontaneous acrosome reaction in capacitated mouse sperm. Arrows indicate ionophore-treated mouse sperm that had undergone the complete acrosome reaction. Data are representative of 3 trials (200 cells/trial).

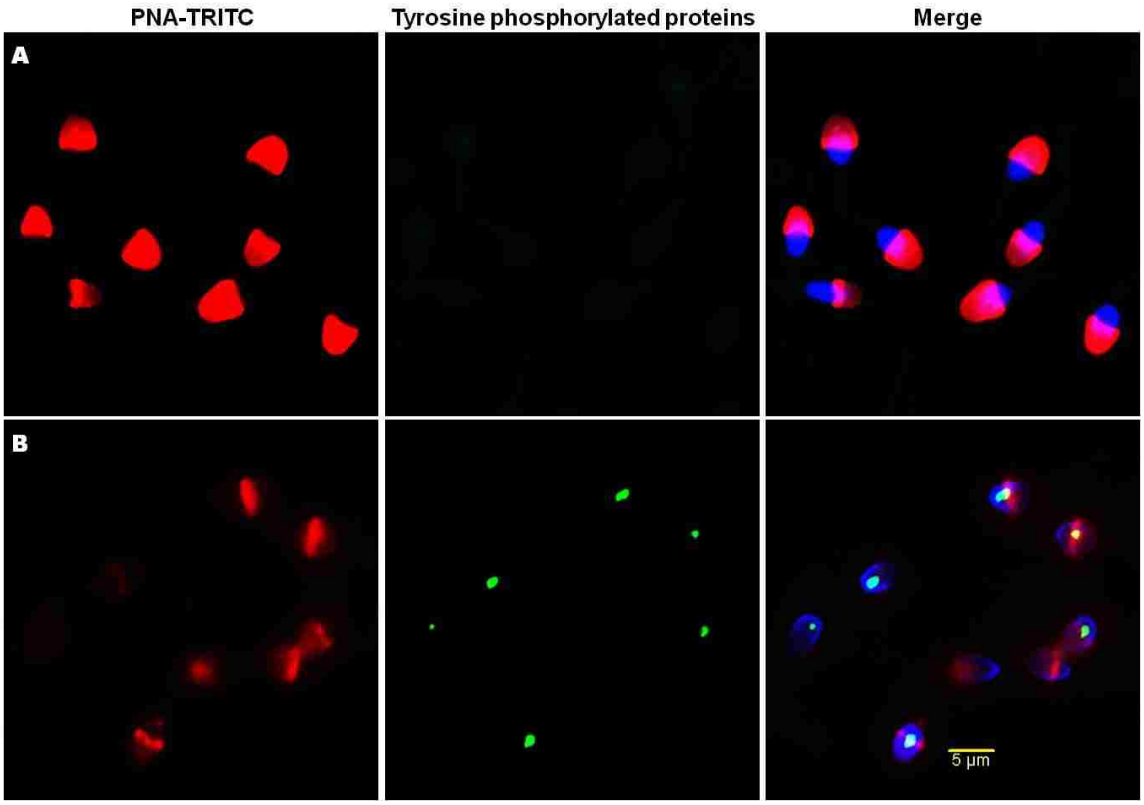



**Figure 3.5. The existence of the EqSS in human sperm cells.** Immunolocalization of tyrosine phosphorylated proteins in human sperm using 4G10 monoclonal antibody. (A) Washed (intact) human sperm, (B) Methanol/acetone-permeabilized human sperm were labeled with PNA-TRITC (red) and 4G10 antibody (green). Nuclei were counterstained with Hoechst 33342 (blue). Data are representative of 3 trials (200 cells/trial).

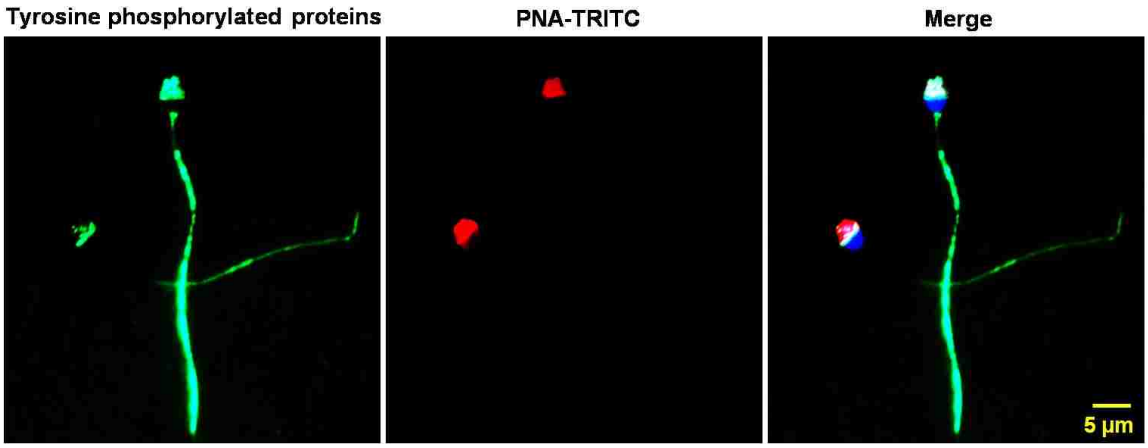



**Figure 3.6. Immunolocalization of tyrosine phosphorylated proteins in capacitated human sperm.** Anti-tyrosine phosphorylated proteins (4G10) monoclonal antibody (green), PNA-TRITC (red), Nuclei (blue) stained with Hoechst 33342. Data are representative of 3 trials (200 cells/trial).

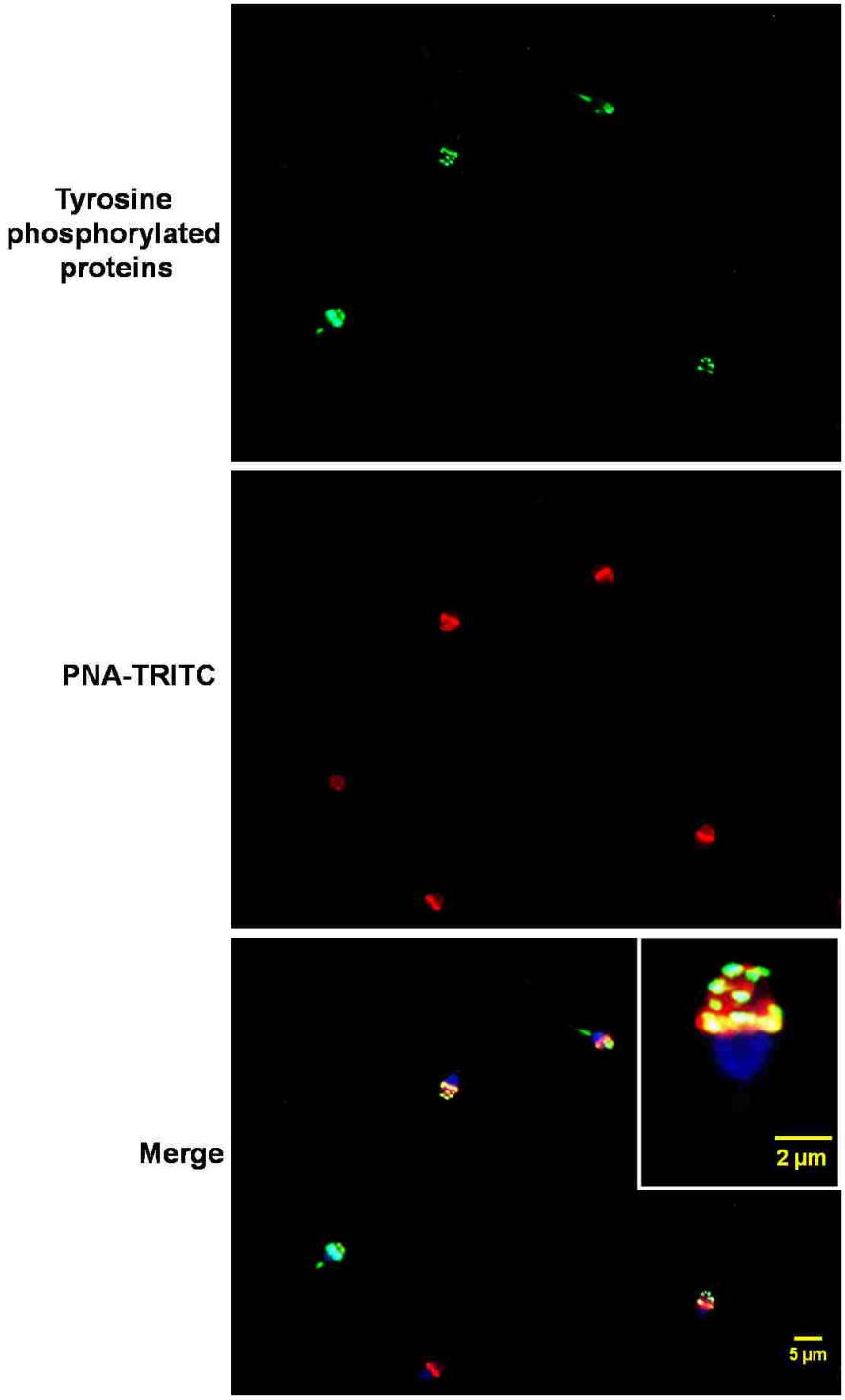



**Figure 3.7. Immunolocalization of tyrosine phosphorylated proteins in 30 min ionophore-treated human sperm**. Anti-tyrosine phosphorylated protein (4G10) monoclonal antibody (green), PNA-TRITC (red). Nuclei (blue) stained with Hoechst 33342. Data are representative of 3 trials (200 cells/trial).

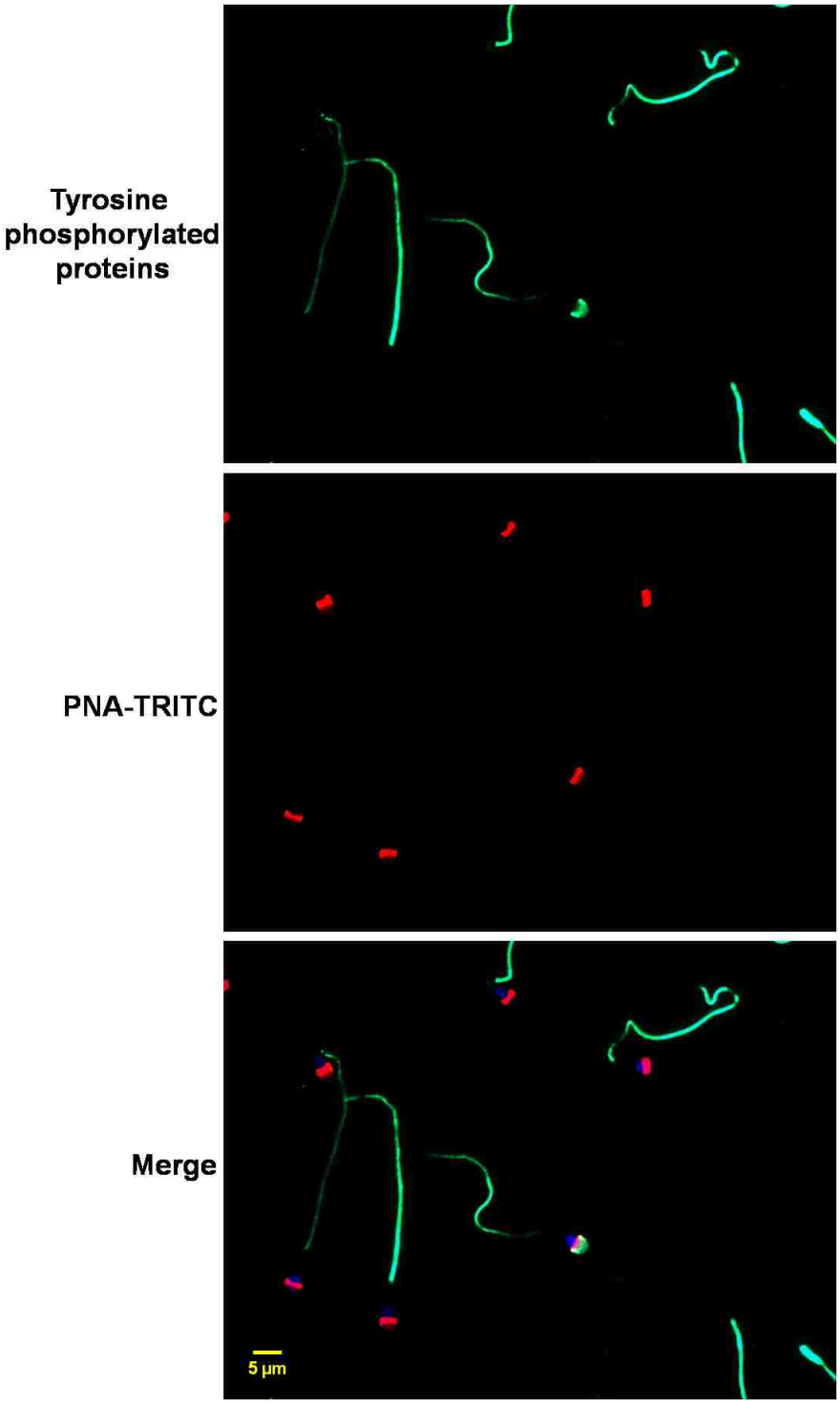



**Figure 3.8. Immunolocalization of tyrosine phosphorylated proteins in 60 min ionophore-treated human sperm**. Anti-tyrosine phosphorylated protein (4G10) monoclonal antibody (green), PNA-TRITC (red). Nuclei (blue) stained with Hoechst 33342. Data are representative of 3 trials (200 cells/trial).

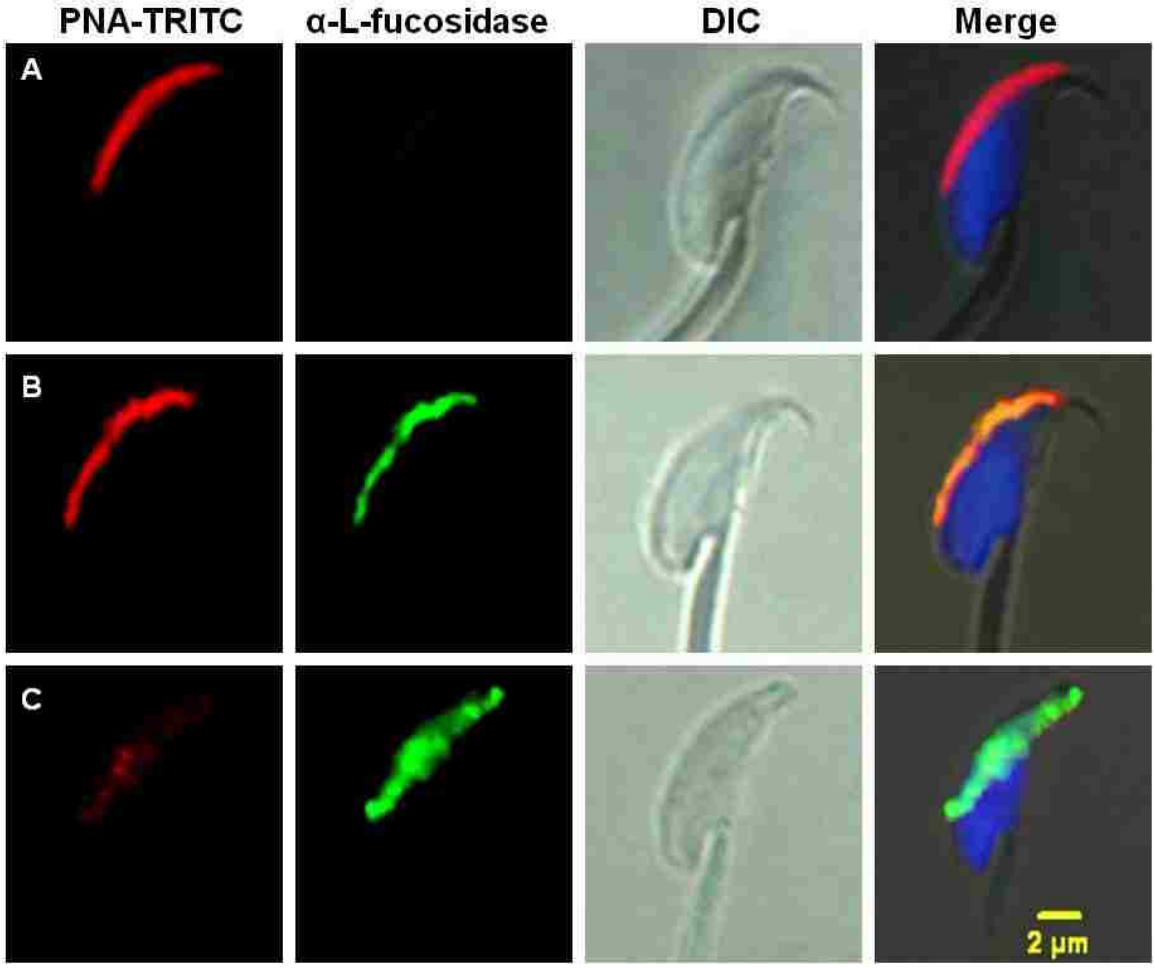



**Figure 3.9. Immunolocalization of α-L-fucosidase in mouse cauda epididymal sperm.** Mouse cauda epididymal sperm cells were stained with anti-FUCA1 antibody (green) and PNA-TRITC (red), and nuclei were counterstained with Hoechst 33342 (blue). (A) Washed (intact) sperm**.** (B) Partially permeabilized sperm by freezing at -80°C followed by thawing at 37°C. (C) Acrosome-reacted sperm.

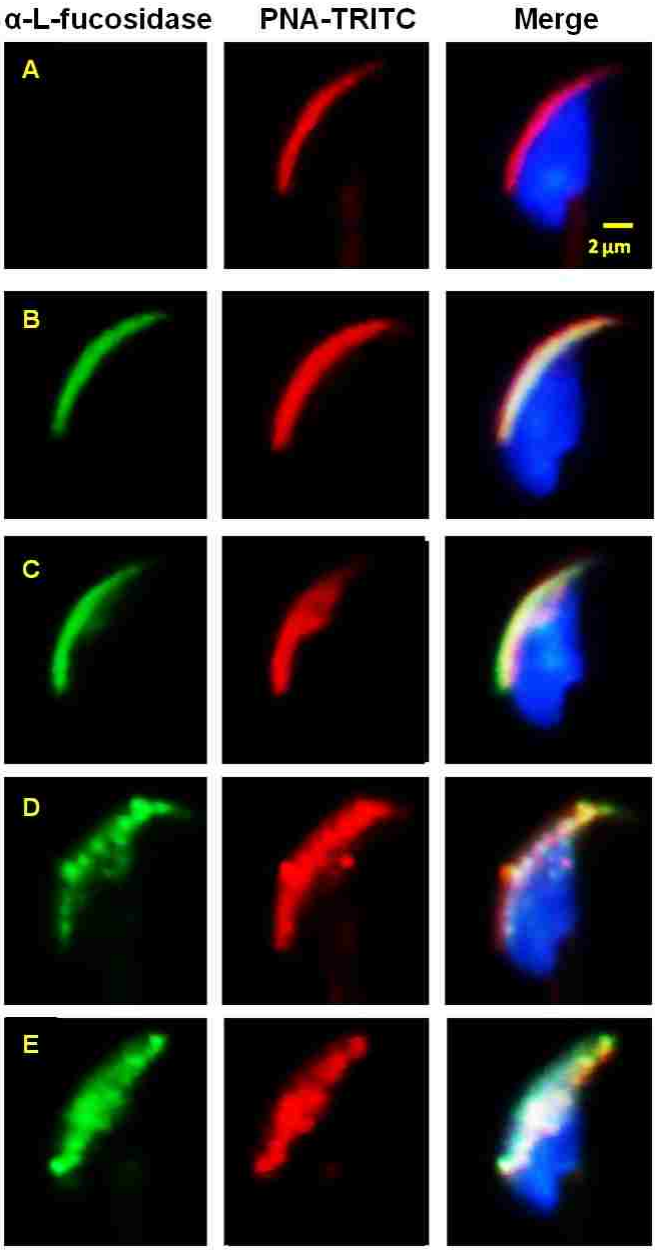



**Figure 3.10. Relocalization of α-L-fucosidase in mouse cauda epididymal sperm during the acrosome reaction.** Mouse cauda epididymal sperm were stained with anti-FUCA1 antibody (green) and PNA-TRITC (red), and nuclei were counterstained with Hoechst 33342 (blue). (A) Washed (intact) sperm. (B-E) Acrosome-reacted sperm. (B) Initial stage of the acrosome reaction. (C) Early stage of the acrosome reaction. (D) Advanced stage of the acrosome reaction. (E) Final stage of the acrosome reaction.

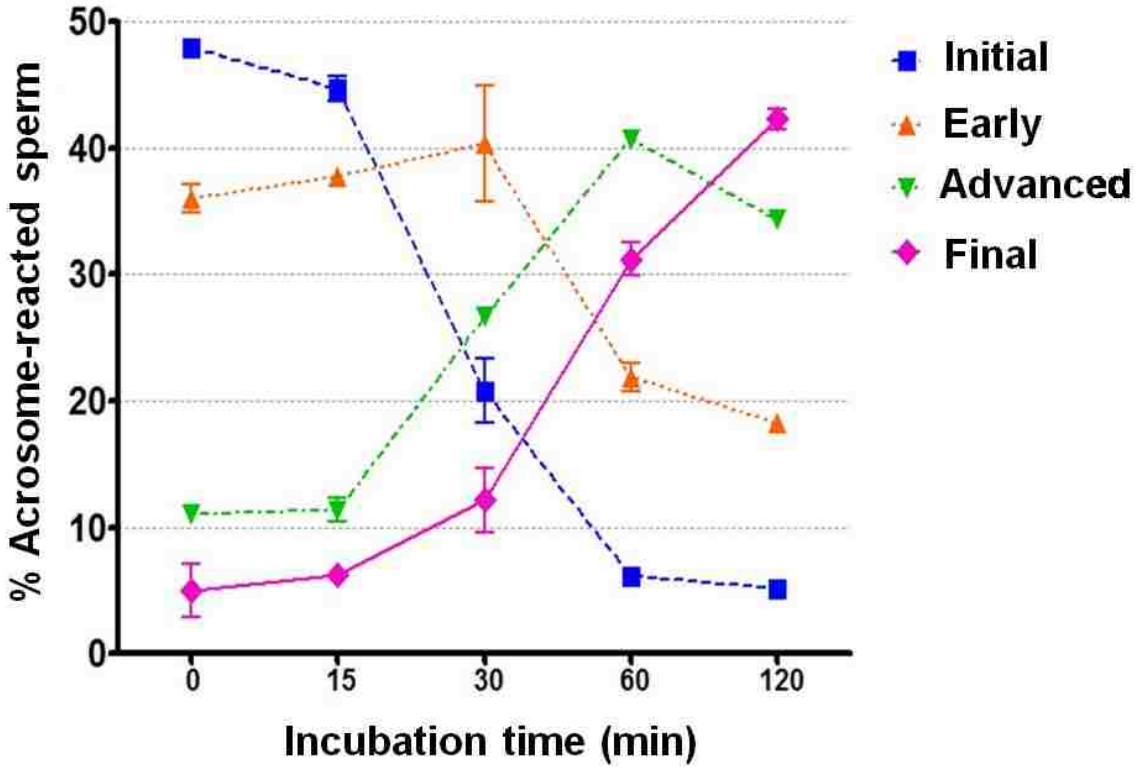



**Figure 3.11. Line graph showing the progression of α-L-fucosidase on mouse sperm during the acrosome reaction.** The percentage of α-L-fucosidase and PNA-TRITC positive acrosome–reacted sperm at each time point was calculated and presented as mean±S.M.E. The experiment was done 3 times and 200 cells were counted per trial.

# Chapter 4

# Results-II

## Subcellular localization and relocalization of cysteine-rich secretory protein 2 on human sperm

### 4.1 Localization of CRISP2 on human sperm

In addition to the observation in human and mouse sperm that sperm associated α-L-fucosidases relocalize to the EqS after the acrosome reaction for the possibility that this enzyme may help make the plasma membrane over the sperm EqS competent for sperm-egg interaction and fusion, I continued investigating the relocalization behavior during the acrosome reaction of other sperm proteins in which the roles in sperm-egg interaction or fusion are strongly suggested. CRISP2 is one of the sperm proteins with suggested roles in sperm-egg fusion and reported to be associated at the sperm EqS of different species (Busso et al., 2005; Busso et al., 2007). Therefore, this study uses CRISP2 as a model to investigate relocalization behavior of human sperm protein to the EqS and mechanisms responsible for facilitating sperm protein relocalization.

Results from indirect immunolocalization showed that intact unpermeabilized human sperm were negative to anti-CRISP2 antibody (Figure 4.1A). However, anti-CRISP2 antibody stained permeabilized human sperm with a strong signal

that was restricted within the anterior of the acrosomal compartment and some staining at the neck of the sperm (Figure 4.1B). Following ionophore treatment to induce the acrosome reaction, the positive CRISP2 signal at the acrosome disappeared, but became prominent at the sperm EqS (Figure 4.1C). Capacitated sperm were also positive to anti-CRISP2 antibody with the signal restricted to the anterior of the acrosome. CRISP2 positive capacitated cells showed no staining at the EqS (Figure 4.1D).

### 4.2 F-actin and the relocalization of CRISP2 during the acrosome reaction

Based on results from immunolocalization of CRISP2 on human sperm before and after the acrosome reaction, I hypothesized that CRISP2 present at the EqS of acrosome-reacted human sperm was gradually translocated from the anterior acrosome to the EqS by the influence of actin polymerization during the acrosome reaction.

To test the hypothesis that CRISP2 relocalization was dependent on actin polymerization, the presence of F-actin must be seen in acrosome-reacted human sperm. Permeabilized and acrosome-reacted human sperm (incubated with or without Latrunculin A) were stained using phalloidin-TRITC. The results showed that F-actin was faintly detected in the head of permeabilized human sperm (Figure 4.2A) whereas actin polymerization in the EqS and the post-acrosomal area were strongly detected in acrosome-reacted human sperm (Figure 4.2B). However, when Latrunculin A was present during the acrosome

reaction induction, F-actin formation was completely inhibited in acrosome-reacted human sperm (Figure 4.2C).

The ability of Latrunculin A to decrease CRISP2 appearance in the EqS was also determined. Acrosome-reacted human sperm showed normal staining of CRISP2 at the EqS (Figure 4.3A). Latruculin A at 25 μM could successfully inhibit the polymerization of F-actin. However, the signal of CRISP2 still appeared at the EqS of acrosome-reacted cells that were treated with 25 μM of Latrunculin A during the acrosome reaction (Figure. 4.3B). PNA-TRITC staining, used to monitor the acrosome status, showed the signal at the EqS of acrosome-reacted sperm and the PNA-TRITC signal colocalized with the signal of CRISP2 in acrosome-reacted human sperm that were incubated with or without Latrunculin A (Figure. 4.3A-B, CRISP-2/PNA-TRITC). The percent of CRISP2 signal at the EqS of acrosome-reacted human sperm that were treated with Latrunculin A was not different from that of the untreated group as shown in Figure 4.4. The experiment was performed three times, two hundred acrosome-reacted human sperm cells were counted per each trial, and the data were presented as mean±S.M.E.

### 4.3 Determination of CRISP2 as a component of the acrosomal cap of human sperm

According to studies in other species that showed that CRISP2 is an intraacrosomal protein (Foster and Gerton, 1996; Kim et al., 2001; Kim and

Gerton, 2003), it is reasonable to hypothesize that human CRISP2 is also an intraacrosomal protein.

The stabilization of the sperm acrosomal matrix is dependent on pH. Low pH does not affect the integrity of the matrix whereas high pH activates the endogenous proteolytic activity which then destabilizes the structure of the acrosomal matrix (Huang et al., 1985; Nakanishi et al., 2001). Based on this fact, I determined whether CRISP2 is stored in the acrosomal cap as a soluble component by Western blot analysis of collected pellets and supernatants of human sperm, incubated in HSM pH 4 and HSM pH 11, with anti-CRISP2 antibody. The results showed that after incubation with HSM pH 4, CRISP2 protein was detected in the pellet but not in the supernatant. However, the supernatant from cells that were incubated with HSM pH 11 showed a strong immunoreactive band of CRISP2, but the cell pellet was negative to anti-CRISP2 antibody (Figure 4.5).

### 4.4 Release of CRISP2 from the acrosome of human sperm during the acrosome reaction

To test the hypothesis that human acrosomal CRISP2 is released during the acrosome reaction as reported in other species (Kim et al., 2001; Kim and Gerton, 2003). Human sperm were induced to undergo the acrosome reaction by ionophore treatment, and then the sperm pellets and supernatants were collected to determine the presence of CRISP2 by Western blot analysis. The supernatant

of the ionophore-treated group showed an immunoreactive band of CRISP2 at the molecular size of 25 kDa. However, no CRISP2 band was observed in the supernatant of the fresh sperm (Figure 4.6). Sperm pellets from the control group (fresh sperm incubated in HSM pH 7.4 throughout the entire period of capacitation and the acrosome reaction) and the ionophore-treated group showed strong immunoreactive bands of CRISP2 (Figure 4.6).

Moreover, when sperm samples were collected at different time points during the acrosome reaction, soluble CRISP2 in the supernatants of the ionophore-treated sperm samples could be detected with an apparent linear increase over time (Figure 4.7A). The cell-associated population of CRISP2 could also be detected by Western blot (Figure 4.7A). The number of sperm used at different time points during the acrosome reaction was equal ($8\times10^7$ cells) in each group as confirmed by anti tubulin antibody (Figure 4.7A). The increase of CRISP2 protein in the supernatants of the ionophore-treated cells at different time points was related to the percent of the acrosome reaction evaluated by PNA-TRITC staining (as shown in Figure 3, PNA-TRITC). At 0, 15, 30, and 60 minutes of the ionophore induction, the percent of the acrosome reaction was 0.5, 27, 30, and 75 percent, respectively (Figure 4.7B). Immunofluorescence was also performed to detect the presence of CRISP2 within the sperm head of the ionophore-treated sperm at different time points during the acrosome reaction. The results showed that the percent of CRISP2 and PNA-TRITC positive signals at the EqS of sperm cells increased over time. This increase reached the maximum value of about 67% after 60 minutes of the acrosome reaction (Figure 4.8). In contrast, the population of sperm that was CRISP2 and PNA-TRITC positive at the acrosome was

highest (96%) at 0 min of ionophore treatment and gradually decreased to about 28% after 60 minutes of the acrosome reaction (Figure 4.8). The experiment was performed three times, two hundred human sperm cells were counted per each trial, and the data were presented as mean±S.M.E.

In order to rule out the possibility that CRISP2 may be released during capacitation, sperm cells were capacitated and collected at different time points (0, 1, and 4 hours). The supernatants and cell pellets of the samples were separated and subject to Western blot analysis. Results showed strong immunoreactive bands of CRISP2 at around 25 kDa, remaining with the cells at every time point of collection (Figure 4.9A). However, no positive immunoreactive band in the supernatants of capacitated samples was detected (Figure 4.9A). The cell numbers collected at different time points during capacitation were approximately equal as judged by anti-tubulin antibody (Figure 4.9A). The capacitated cells at 4 hours of the incubation were collected and stained with anti-tyrosine phosphorylated protein antibody (4G10) to verify the level of capacitation. The level of capacitation was around 98% as judged by the positive signal at the tail of the sperm (Figure 4.9B).

### 4.5 Isolation of human sperm acrosomal CRISP2 protein

SDS-PAGE and silver nitrate staining showed that the supernatant from ionophore-treated sperm contained a lot of protein, including proteins at the molecular size of 25 kDa (Figure 4.10 lane 1). However, after affinity purification by Protein G bead-anti-CRISP2 antibody complex described in methods, only a single band at approximately 25 kDa was observed (Figure 4.10 lane 2).

Moreover, when Western blot analysis was performed by using anti-CRISP2 antibody, a single immunoreactive band was observed with the molecular mass ratio of 25 kDa in the supernatant (lane 3) or in the purified sample (lane 4) (Figure 4.10).

### 4.6 Reassociation of human sperm acrosomal CRISP2 to the EqS of acrosome-reacted human sperm

To test whether CRISP2 protein released from human sperm acrosome during the acrosome reaction can reassociate with the EqS of acrosome-reacted sperm, ionophore-treated sperm were incubated with 6.25 µg/mL of the purified biotinylated human sperm acrosomal CRISP2 and the appearance of CRISP2 on the sperm with streptavidin-FITC was detected. A population of acrosome-reacted cells showed strong CRISP2 signal at the EqS and some cells showed a dot-like signal at the marginal area of the EqS (Figure 4.11B). None of the acrosome-reacted sperm incubated with elution buffer alone and stained with streptavidin-FITC showed fluorescent positive signal on the sperm (Figure 4.11A), indicating that the streptavidin-FITC bound specifically to sperm with bound biotinylated CRISP2. In this study 49% of acrosome-reacted sperm showed a strong signal at the EqS (Figure 4.12A). When the concentration of the purified biotinylated human sperm acrosomal CRISP2 was fixed at 6.25 µg/mL, but the number of sperm cells was diluted from $10^7$ to $10^4$ cells/mL, the percent of cells with CRISP2 at the EqS shifted to 57% (Figure 4.12B). When the concentration of the purified biotinylated human sperm acrosomal CRISP2 were

increased to 12.5 and 31.25 µg/mL, the percent of CRISP2 at the EqS of the acrosome-reacted cells reached 63 and 86%, respectively (Figure 4.12B). Fiugure 4.12 C shows an increase in biotinylated CRISP2 binding at the EqS of acrosome-reacted human sperm.

### 4.7 Association stability of CRISP2 at the EqS of acrosome-reacted human sperm

Based on the above results showing that CRISP2 is a soluble component of the acrosomal cap, released during the acrosome reaction, and reassociated with the EqS of the acrosome reacted human sperm, I hypothesized that CRISP2 association at the EqS is strongly stabilized if it plays roles as an adhesion molecule for facilitating sperm-egg membrane fusion which normally requires the moderate durability of fusion proteins at the site of fusion. Therefore, the stability of CRISP2 protein at the EqS of the acrosome-reacted human sperm cells was tested against the effect of different chaotropic agents and mechanical force. To test the association stability of CRISP2 at the EqS of acrosome-reacted sperm, ionophore-treated human sperm were exposed to rigorous extraction conditions. The control group which was acrosome-reacted cells stained with anti-CRISP2 antibody showed around 80% of CRISP2 staining at the EqS of human sperm. High ionic strength solution (1M NaCl) was used to determine whether CRISP2 associates with target molecules on the plasma membrane over the EqS by

electrostatic interactions. Results showed that CRISP2 signal was strongly detectable at the EqS and no different staining pattern was observed between the control group and NaCl-treated acrosome-reacted human sperm (Figure 4.13).

Since CRISP2 protein contains many cysteine residues within the structure and may form inter-molecular disulfide bonds with target molecules on the EqS plasma membrane during the acrosome reaction, a reducing agent DTT was used to destroy disulfide bond formations, but this treatment also did not reduce CRISP2 staining at the EqS (Figure 4.14). Mechanical force generated by mild sonication was also used to extract CRISP2 from the EqS. Although the level of sonication used was mild, the mechanical force was strong enough to dissociate sperm heads and tails but did not disrupt the morphology of the head (Figure 4.15, DIC). Results from immunofluorescence revealed that mechanical force generated by sonication could not significantly extract CRISP2 from the EqS (Figure 4.15). Due to the fact that the signal sequence at the N-terminus of CRISP2, although short, contains hydrophobic residues, this protein might associate with the plasma membrane overlying the EqS by hydrophobic interactions. Therefore, in this study Triton X-100 and SDS were used to solubilize the sperm plasma membrane. Data showed that Triton X-100 at the concentration of 0.5% for 30 minutes was not strong enough to solubilize CRISP2 from the EqS of human sperm (Figure 4.16). Again, the morphology of acrosome-reacted human sperm after Triton X-100 treatment was intact when compared to that of the control acrosome-reacted sperm (Figure 4.16, DIC). However, when 0.1% SDS was used for 1 minute, results showed that SDS

could completely extract CRISP2 protein from the EqS of acrosome-reacted human sperm with the remaining population detectable at the neck (Figure 4.17). The signal of CRISP2 at the neck was stronger than that when we stained permeabilized or acrosome-reacted human sperm (Figure 4.17).

Finally, since CRISP2 has been suggested to regulate some ion channels' activity and thereby may regulate calcium fluxes during sperm capacitation (Gibbs et al., 2006), calcium ion-dependent stabilization of CRISP2 at the EqS was examined to determine the involvement of calcium ions in stabilization of CRISP2 at the EqS. We incubated acrosome-reacted human sperm with 30 mM EGTA for 30 minutes to chelate calcium ions in the media. Interestingly, results demonstrated that treatment of acrosome-reacted human sperm with EGTA did not cause loss of CRISP2 from the sperm, but the signal of CRISP2 was detected throughout the entire area on sperm head, including the anterior of the acrosome, the EqS, and the postacrosomal (Figure 4.18). The signal of CRISP2 at the neck was still seen as a dot-like staining which was not different from that of the control sperm staining (Figure 4.18). Although CRISP2 protein showed a redistribution pattern after EGTA treatment, the morphology of acrosome-reacted human sperm was seen to be well preserved when compared to the control sperm cells (Figure 4.18, DIC).

### 4.8 Human sperm acrosomal CRISP2 binds to the plasma membrane of hamster oocytes

Since there are many reports showing the suggested roles of CRISP2 during sperm-egg fusion (as discussed in Chapter 1), it is reasonable to hypothesize that CRISP2 released from the acrosome of human sperm during the acrosome reaction can bind to the oocyte. Moreover, we have evidence that the acrosomal CRISP2 can associate with the EqS of the acrosome-reacted cells; therefore, proof of the binding ability of acrosomal CRISP2 to the oocyte would provide more evidence to strengthen the hypothesis that CRISP2 at the EqS of acrosome-reacted human sperm is involved in sperm-egg fusion. In this study, we used human sperm-hamster oocyte as a model to investigate the binding ability of sperm acrosomal CRISP2.

To determine whether hamster oocytes contain a homologue of human CRISP2 protein, we stained the oocytes with anti-human CRISP2 antibody. The results showed that anti-human CRISP2 antibody gave a negative signal on the oocytes (Figure 4.19A). To further examine whether hamster oocytes contain complementary binding sites for human sperm acrosomal CRISP2, zona-intact and zona-free hamster oocytes were incubated with the purified biotinylated human sperm acrosomal CRISP2 and CRISP2 was detected with anti-human CRISP2 antibody and streptavidin-FITC. Results showed a strong colocalization signal of CRISP2 detected with anti-human CRISP2 antibody or streptavidin-FITC on the plasma membrane of the oocytes but not on the zona pellucida, a clear circular structure surrounding the stained oocytes (Figure 4.19B). Zona-free

oocytes also showed a strong signal around the plasma membrane of the oocyte (Figure 4.20B). Zona-free oocytes treated with elution buffer and stained with streptavidin-FITC showed no signal on the surface of the oocyte (Figure 4.20A).

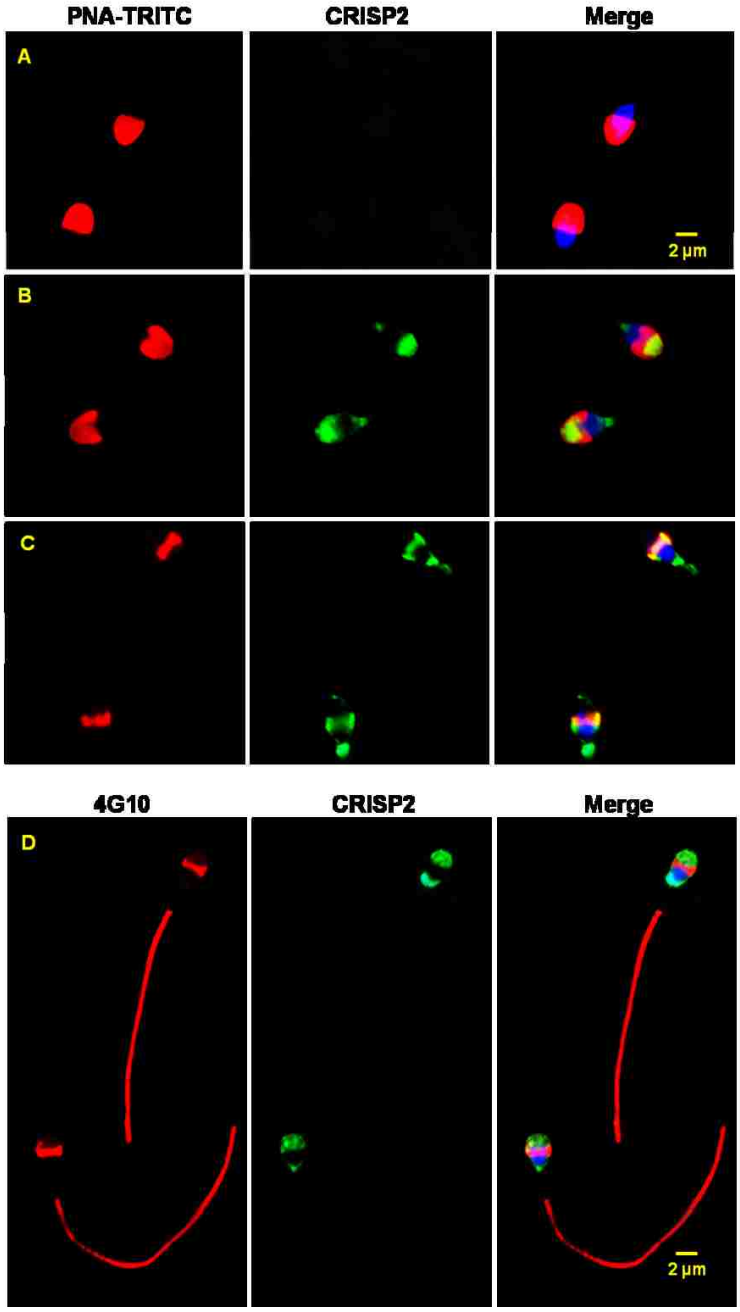



**Figure 4.1. localization of CRISP2 in human sperm cells.** (A) Washed (intact) human sperm. (B) 0.5% Triton X-100-permeabilized sperm. (C) Acrosome-reacted sperm. (D) Capacitated sperm. A-C Human sperm were stained with anti-CRISP2 antibody (green) and PNA-TRITC (red). D Human sperm were stained with anti-CRISP2 antibody (green) and anti-tyrosine phosphorylated protein antibody (4G10) (red). Nuclei of human sperm were counterstained with Hoechst 33342 (blue).

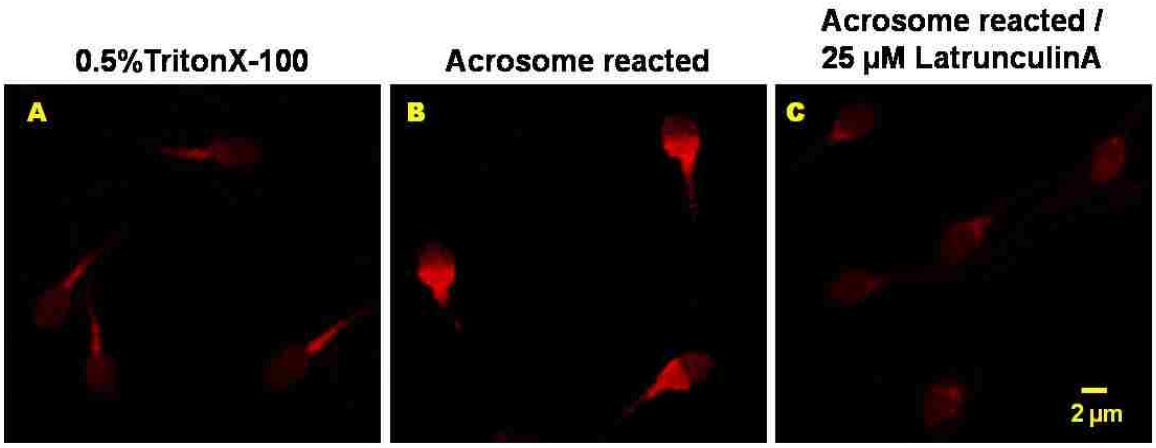



**Figure 4.2. Inhibition of actin polymerization in acrosome-reacted human sperm. A** F-actin staining with phalloidin; (A) 0.5% Triton X-100-permeabilized sperm, (B) Acrosome-reacted sperm, (C) Acrosome-reacted sperm with the presence of 25 µM Latrunculin A. Data are representative of 3 trials (200 cells/trial).

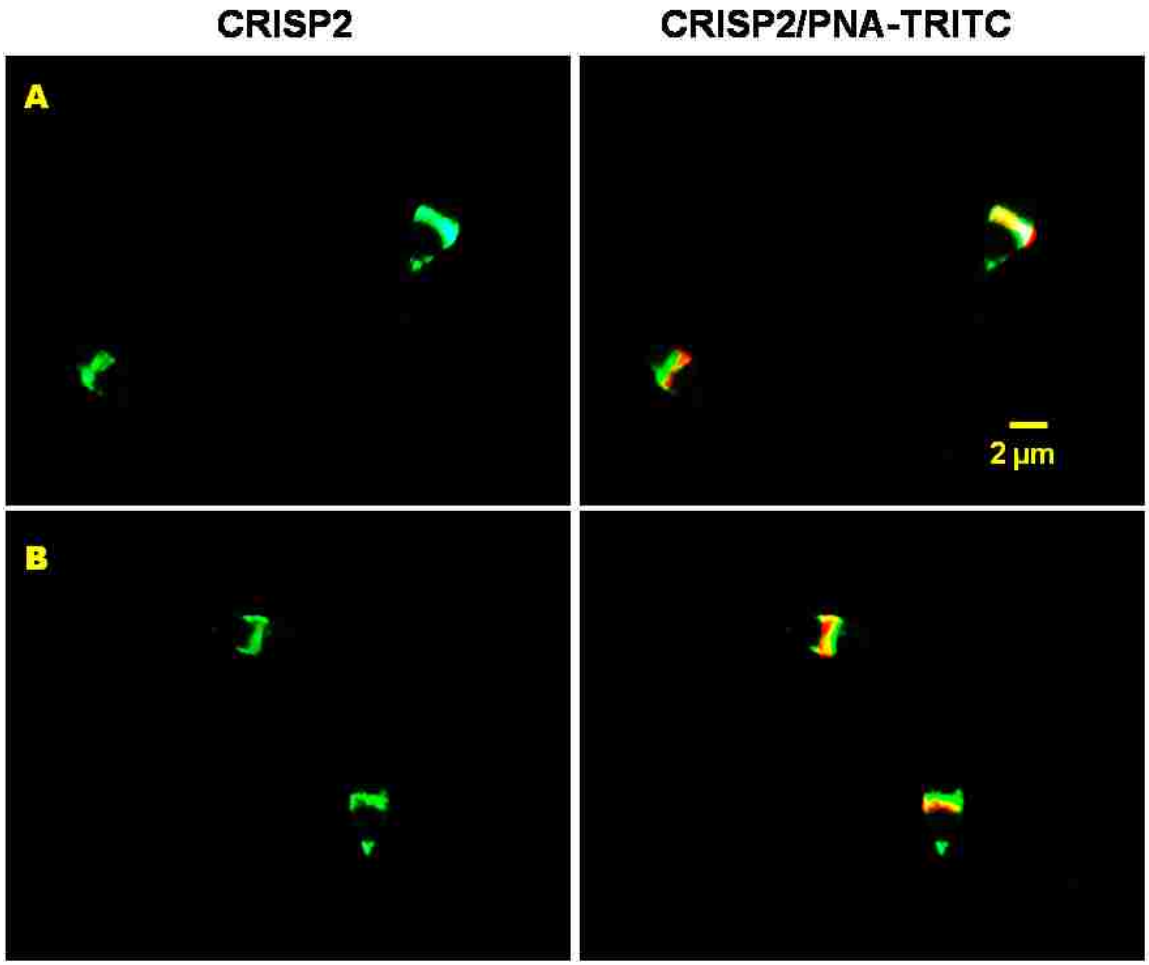



**Figure 4.3. Actin polymerization is not involved in relocalization of CRISP2 during the acrosome reaction.** Indirect immunofluorescence with anti-CRISP2 antibody (green) and PNA-TRITC (red) of acrosome-reacted human sperm; (A) Acrosome-reacted sperm (control), (B) Acrosome-reacted sperm with the presence of 25 µM Latrunculin A. Data are representative of 3 trials (200 cells/trial).

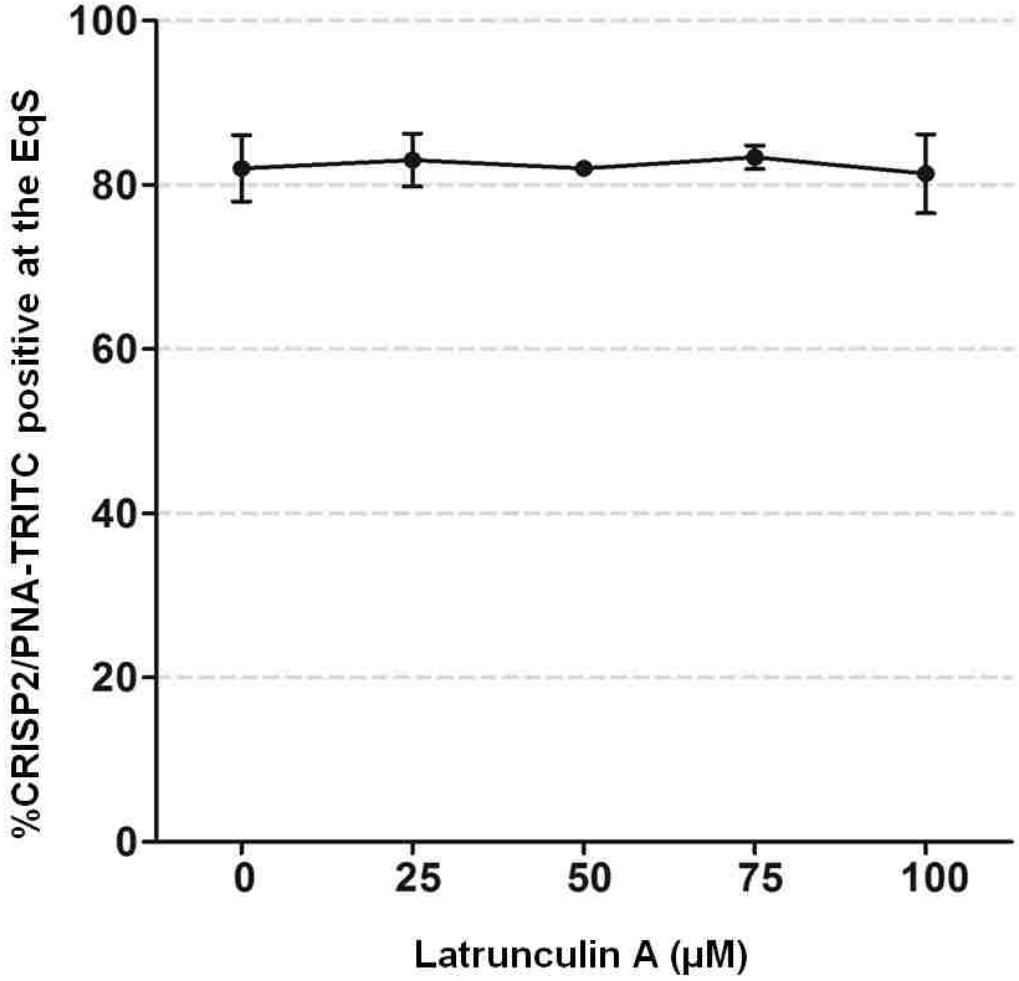



**Figure 4.4. Line graph presenting percent of CRISP2/PNA-TRITC at the EqS of acrosome-reacted human sperm in the presence of different concentrations of Latrunculin A**. Results represent the mean ± S.M.E. The experiment was done three times and 200 acrosome-reacted cells were counted per trial.

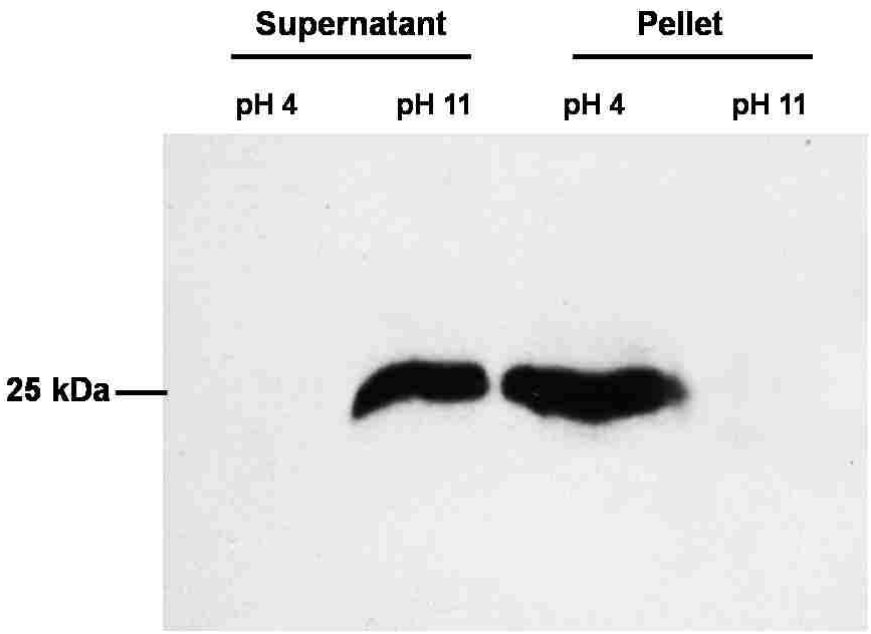



**Figure 4.5. CRISP2 is a component of acrosomal cap.** Western blot of supernatants and pellets after incubation with HSM pH 4 and HSM pH 11.

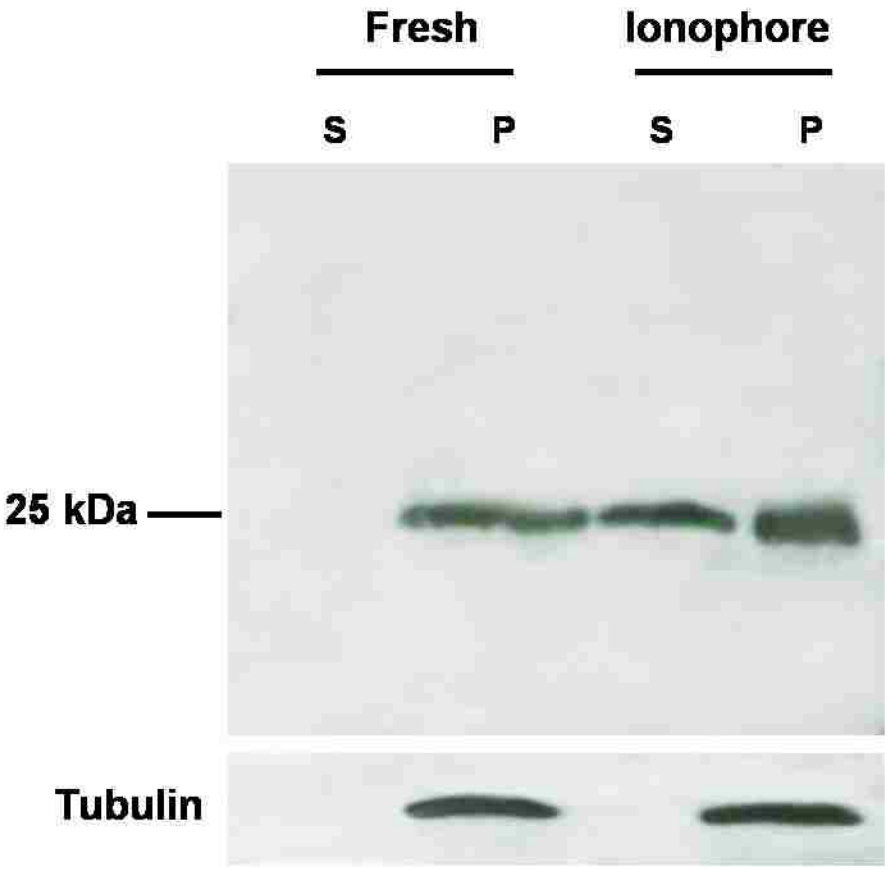
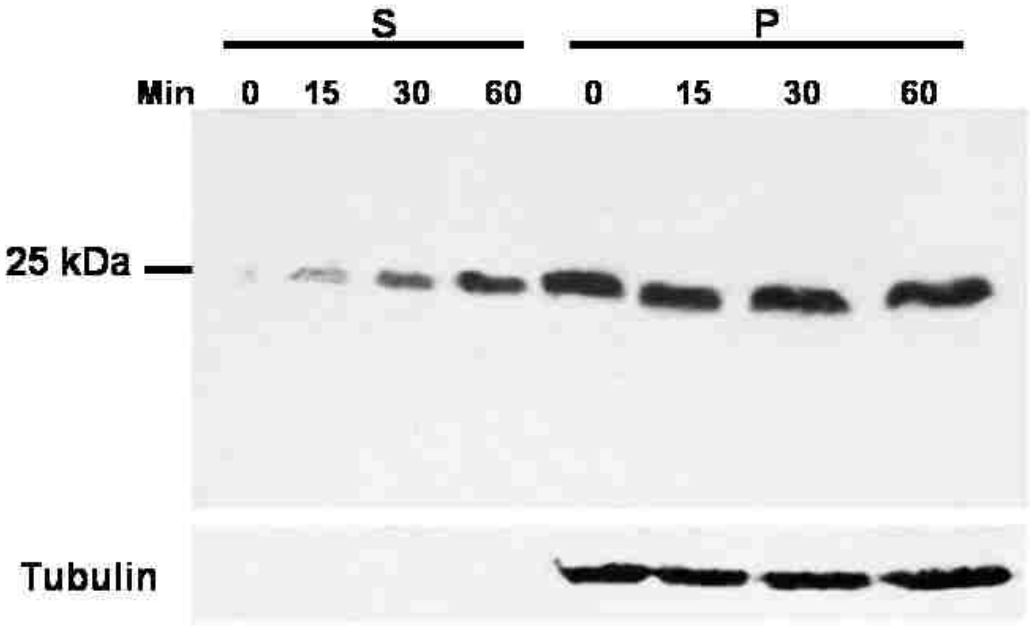



**Figure 4.6. Release of CRISP2 during the acrosome reaction.** Western blot of supernatants (S) and pellets (P) of fresh and ionophore-treated human sperm. Anti-α tubulin antibody was used as an internal control.

A




B

| Min | % acrosome reaction |
|---|---|
| 0 | 0.5 |
| 15 | 27 |
| 30 | 30 |
| 60 | 75 |

**Figure 4.7. Sequential release of CRISP2 during the acrosome reaction. A** Western blot of supernatants (S) and pellets (P) at different time points (0, 15, 30, 60 minutes) during the acrosome reaction. **B** Percent of acrosome-reacted cells at different time points during the acrosome reaction.

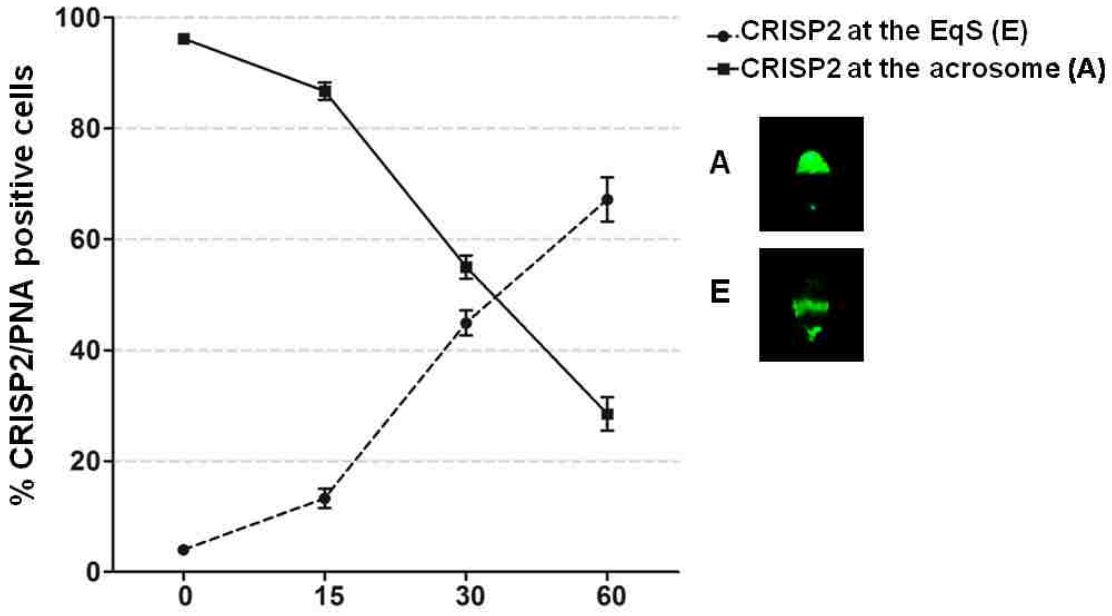



**Figure 4.8. Immunofluorescence of CRISP2 at the acrosome and the EqS of acrosome-reacted sperm at different time points during the acrosome reaction**. Percent of CRISP2/PNA-TRITC positive cells shown with the mean ± S.E.M. Data are representative of 3 trials (200 cells/trial).

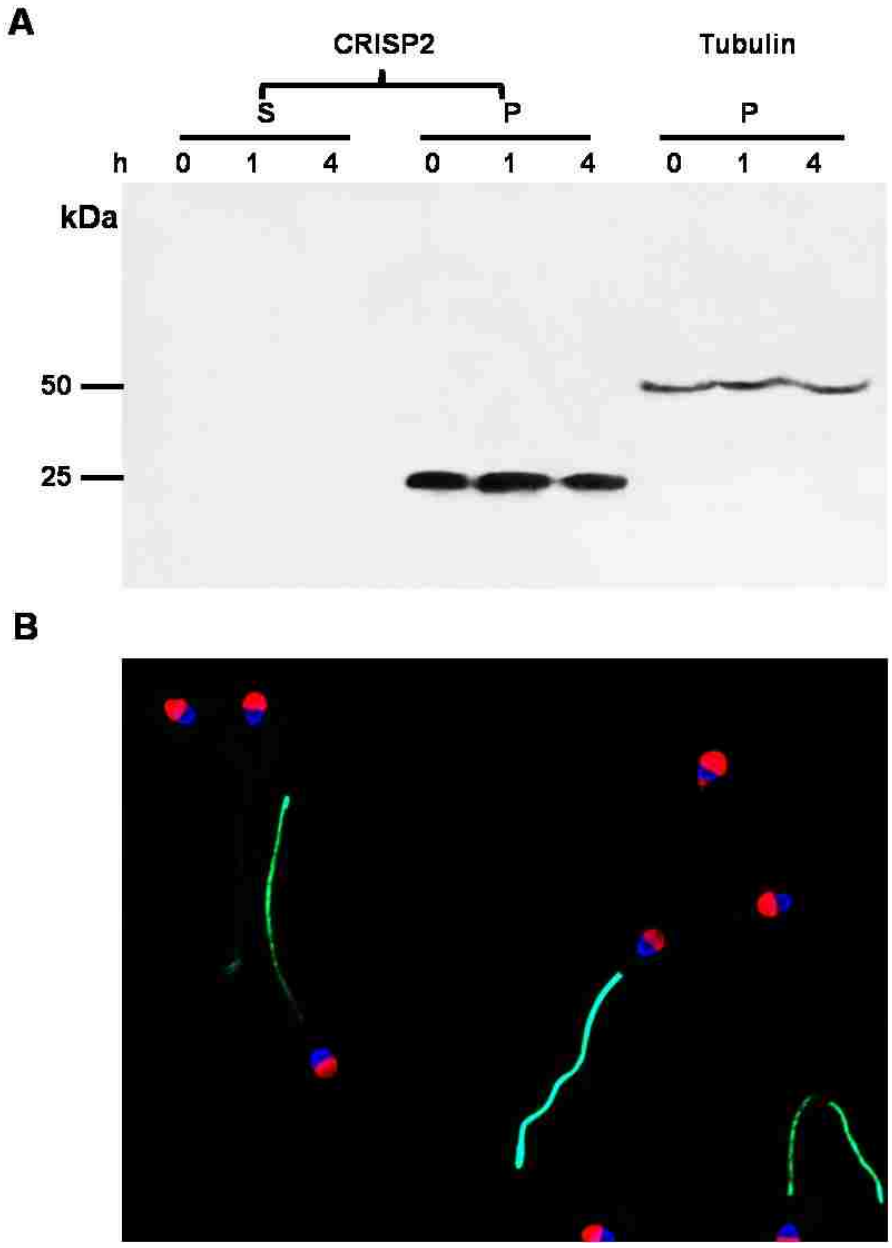



**Figure 4.9. CRISP2 detection in capacitated human sperm at different time points.** **A** Western blot of supernatants (S) and pellets (P) at different time points (0, 1, and 4 hours) during capacitation. **B** Immunofluorescence for tyrosine posphorylated proteins in the tail and the EqS of human sperm detected with 4G10 monoclonal antibody (green), counterstained with PNA-TRITC and Hoechst 33342 (blue) for nuclei.

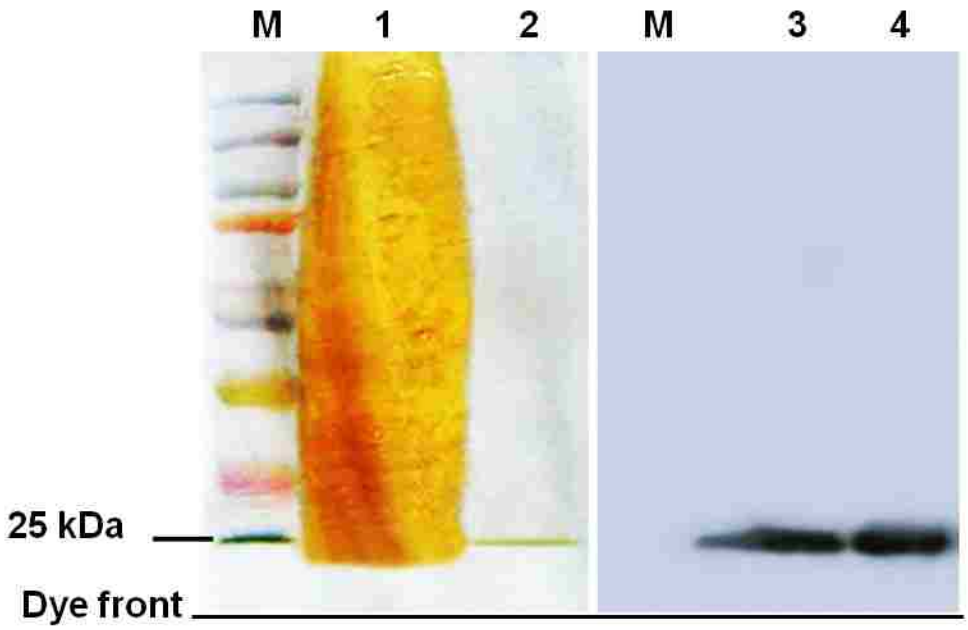



**Figure 4.10. Isolation of human sperm acrosomal CRISP2.** Silver staining (1, 2) and Western blot (3, 4) of ionophore-treated supernatant before and after bead purification; M = protein marker, 1, 3 = supernatants from ionophore-treated sperm sample before bead purification (50 µg protein), 2, 4 = supernatants from ionophore-treated sperm sample after 1 hour of bead purification (2 µg protein).

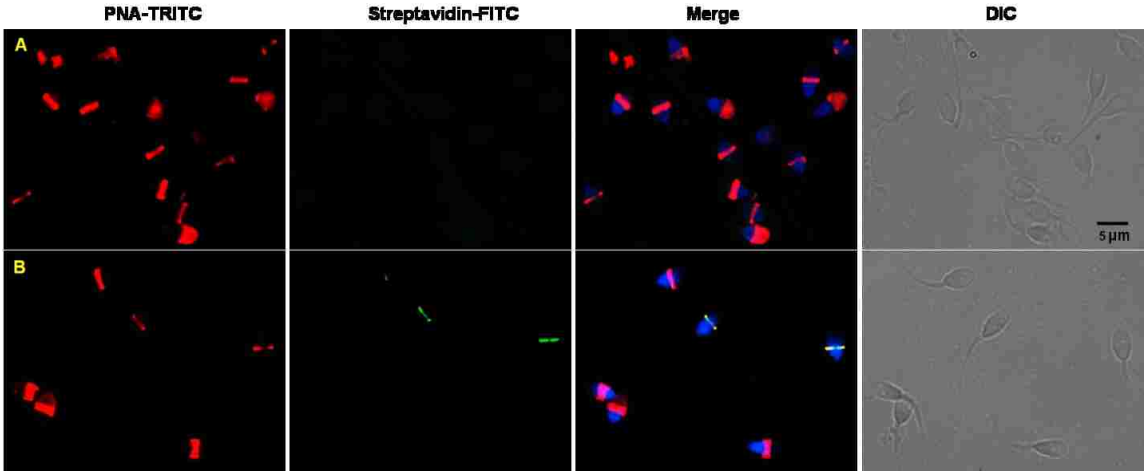



**Figure 4.11. Reassociation of CRISP2 at the EqS of acrosome-reacted sperm**. CRISP2 binding assay**;** (A) Acrosome-reacted human sperm stained with PNA-TRITC (red) and streptavidin-FITC (green), (B) acrosome-reacted sperm pre-incubated with 6.25 µg/mL of the purified biotinylated human sperm acrosomal CRISP-2 and stained with PNA-TRITC and streptavidin-FITC. Nuclei (blue) were stained with Hoechst 33342. Scale bar = 2 µm. Data are representative of 3 trials (200 cells/trial).

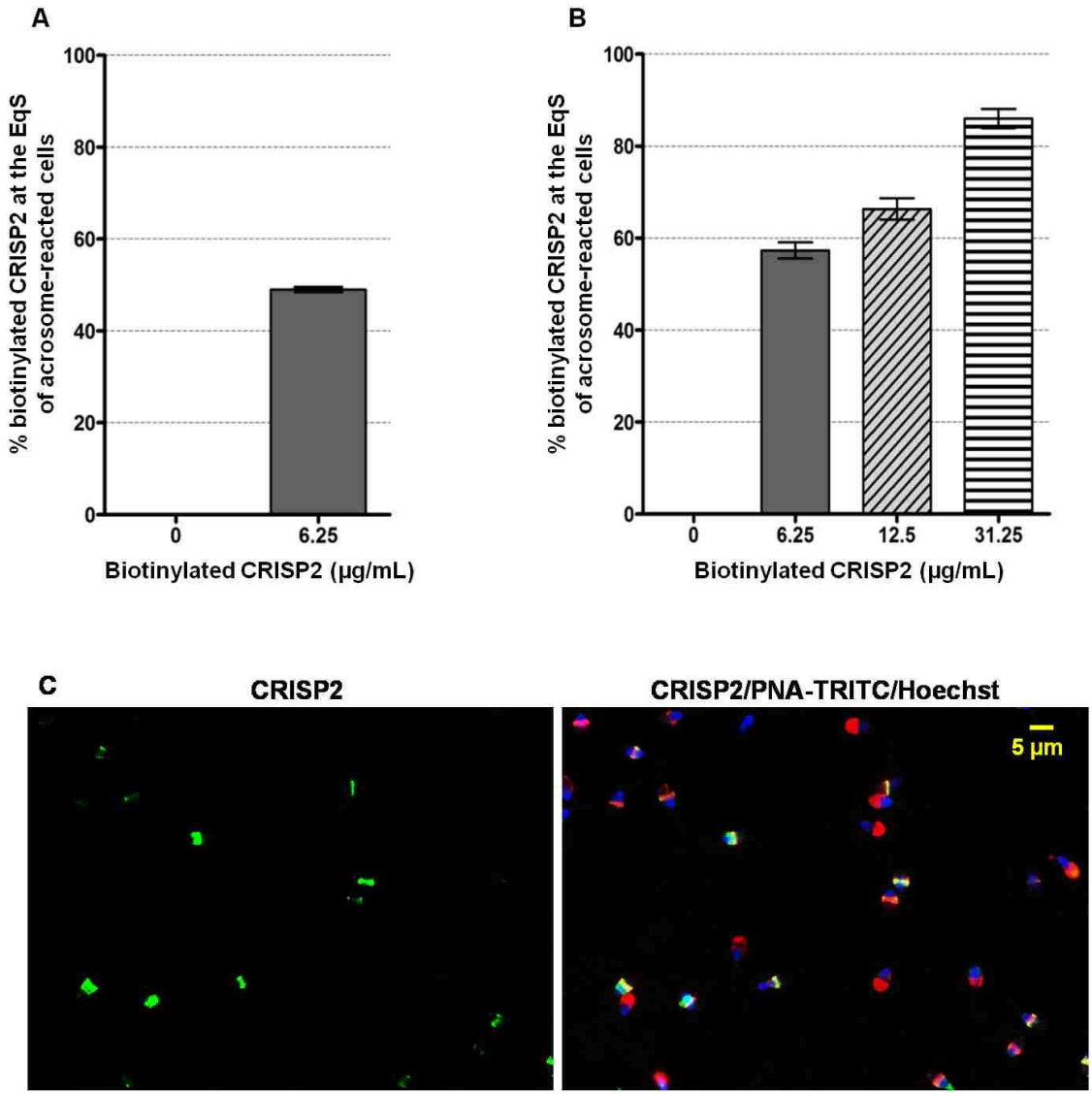



**Figure 4.12. Percent of CRISP2 positive signal at the EqS of the acrosome-reacted sperm. A** Percent of CRISP2 positive signal at the EqS of the acrosome-reacted sperm after incubating sperm ($10^7$ cells/mL in 200 µL solution) with 6.25 µg/mL of biotinylated CRISP-2 protein. **B** Percent of CRISP2 positive signal at the EqS of the acrosome-reacted sperm after incubating sperm ($10^4$ cells/mL in 200 µL solution) with different concentrations of biotinylated CRISP2 protein. **C** Binding of biotinylated CRISP2 at the EqS of acrosome-reacted human stained with streptavidin-FITC (green) and counterstained with PNA-TRITC (red). Nuclei (blue) were stained with Hoechst 33342. The experiment was repeated three times and 200 acrosome-reacted cells were counted per trial.

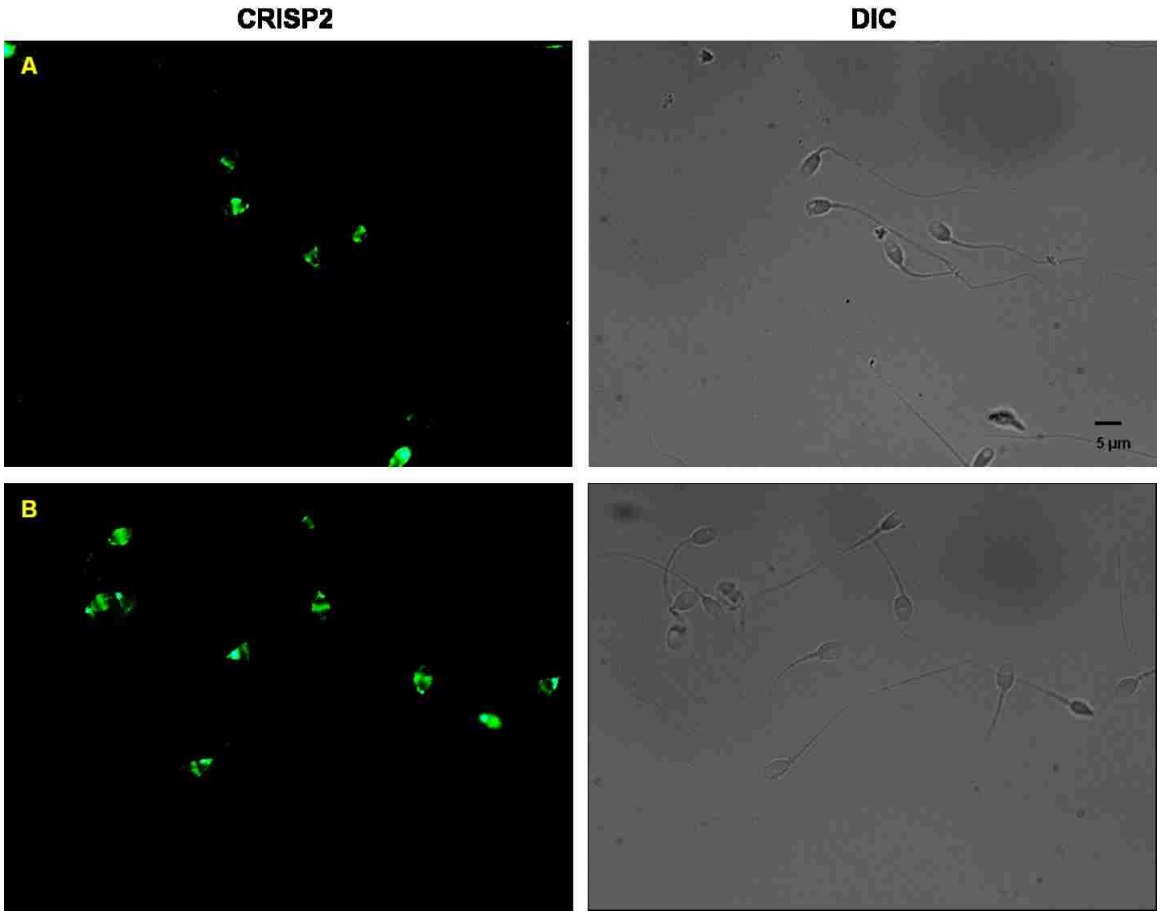



**Figure 4.13. Association stability of CRISP2 at the EqS of acrosome-reacted sperm treated with NaCl**. **A** Acrosome-reacted human sperm. **B** Acrosome-reacted human sperm treated with 1 M NaCl for 30 minutes. Data are representative of 3 trials (200 cells/trial).

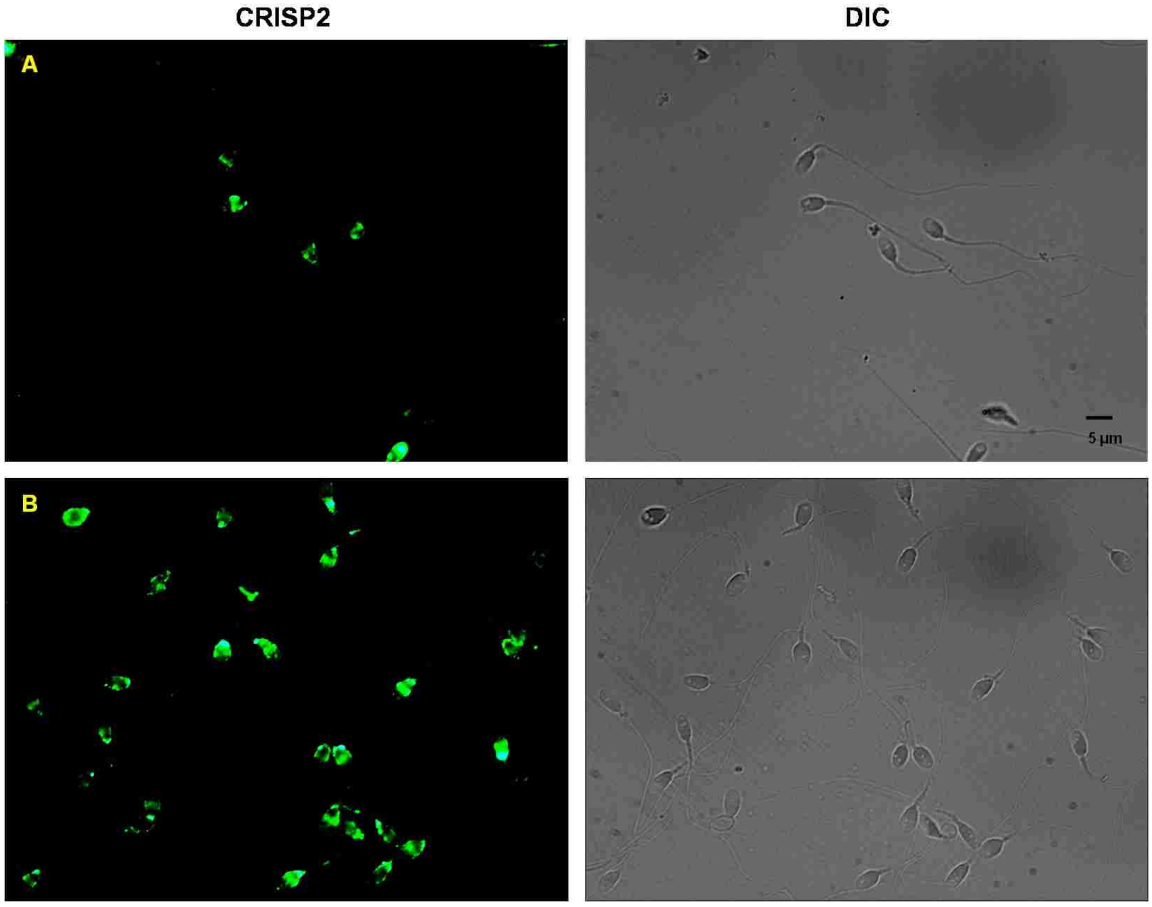



**Figure 4.14. Association stability of CRISP2 at the EqS of acrosome-reacted sperm treated with DTT**. **A** Acrosome-reacted human sperm. **B** Acrosome-reacted human sperm treated with 5 mM DTT for 30 minutes. Data are representative of 3 trials (200 cells/trial).

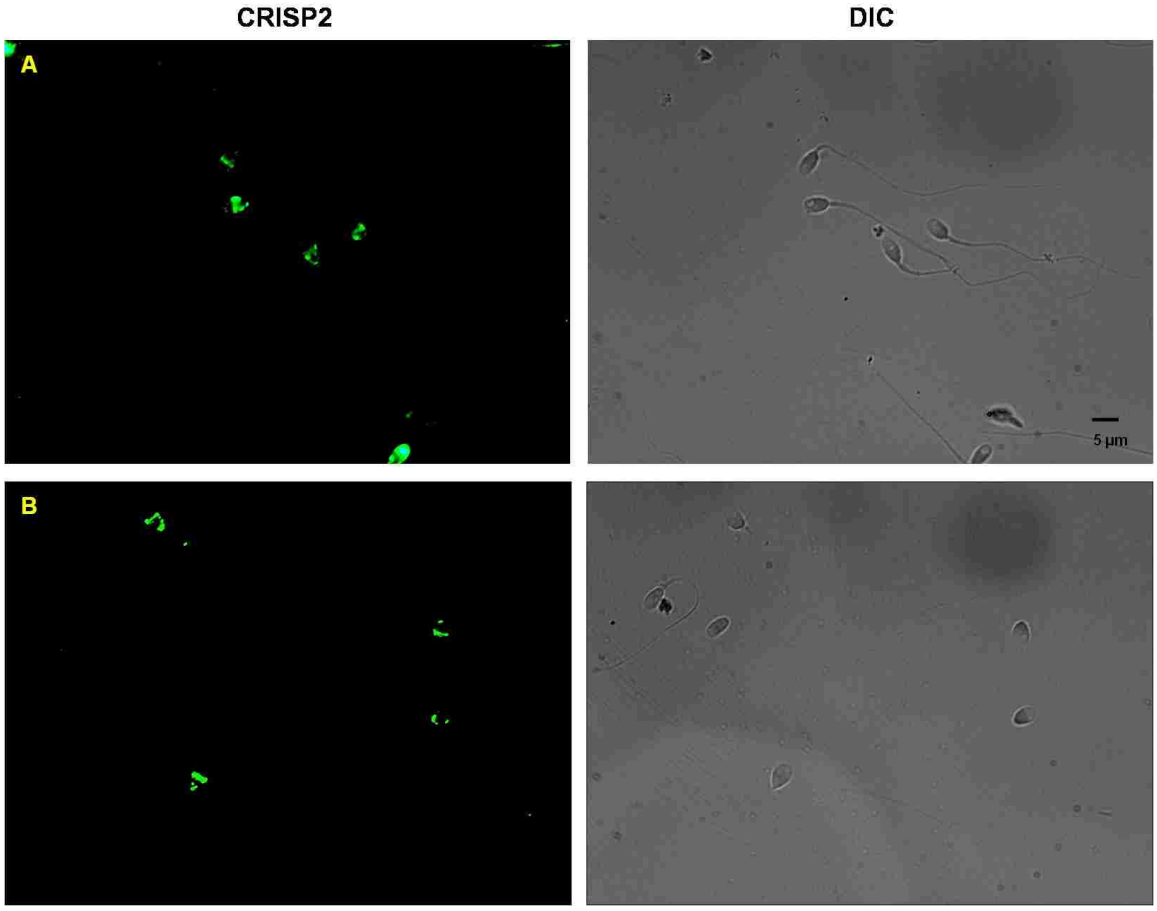



**Figure 4.15. Association stability of CRISP2 at the EqS of acrosome-reacted sperm after mild sonication**. **A** Acrosome-reacted human sperm. **B** Acrosome-reacted human sperm with mild sonication set at intensity 3, 10 seconds, three times. Data are representative of 3 trials (200 cells/trial).

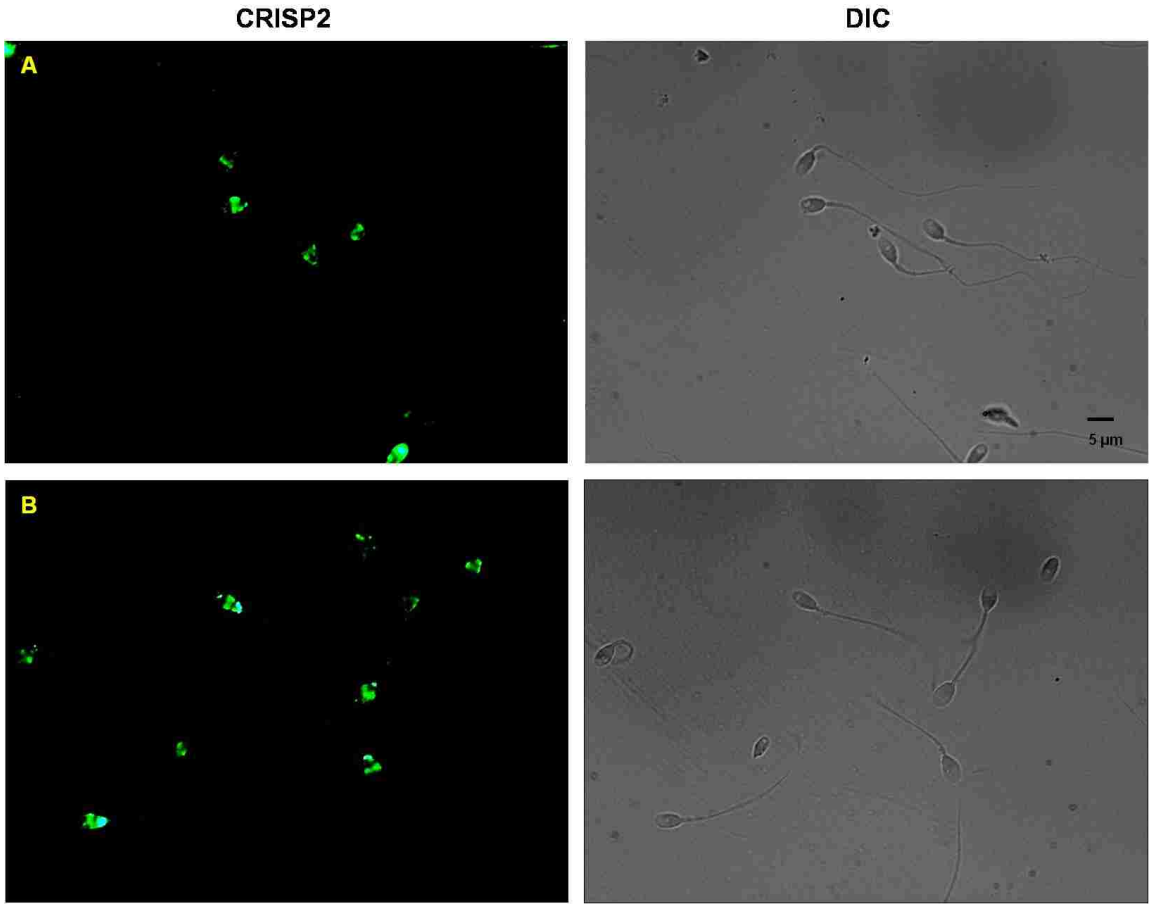



**Figure 4.16. Association stability of CRISP2 at the EqS of acrosome-reacted sperm treated with Triton X-100**. **A** Acrosome-reacted human sperm. **B** Acrosome-reacted human sperm treated with 0.5% Triton X-100 for 30 minutes. Data are representative of 3 trials (200 cells/trial).

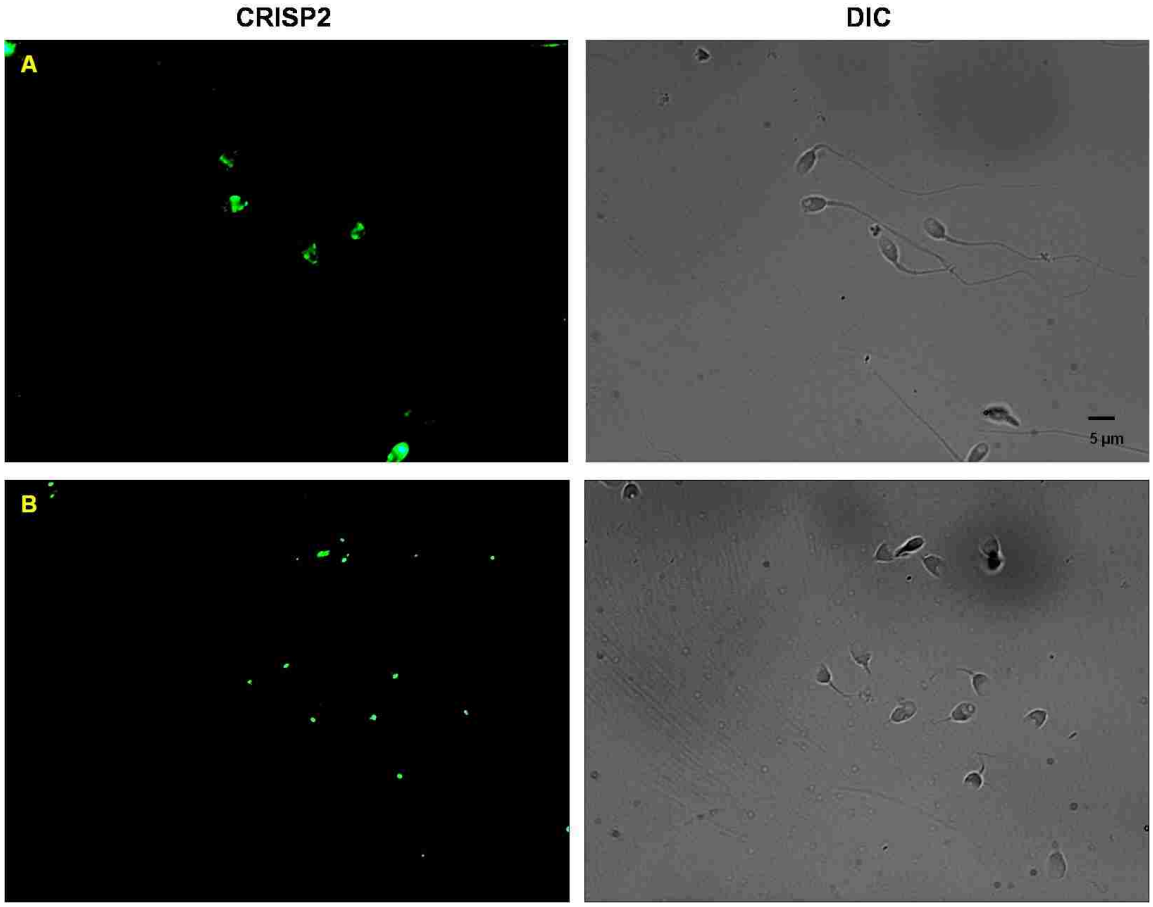



**Figure 4.17. Association stability of CRISP2 at the EqS of acrosome-reacted sperm treated with SDS**. **A** Acrosome-reacted human sperm. **B** Acrosome-reacted human sperm treated with 0.1% SDS for 1 minute. Data are representative of 3 trials (200 cells/trial).

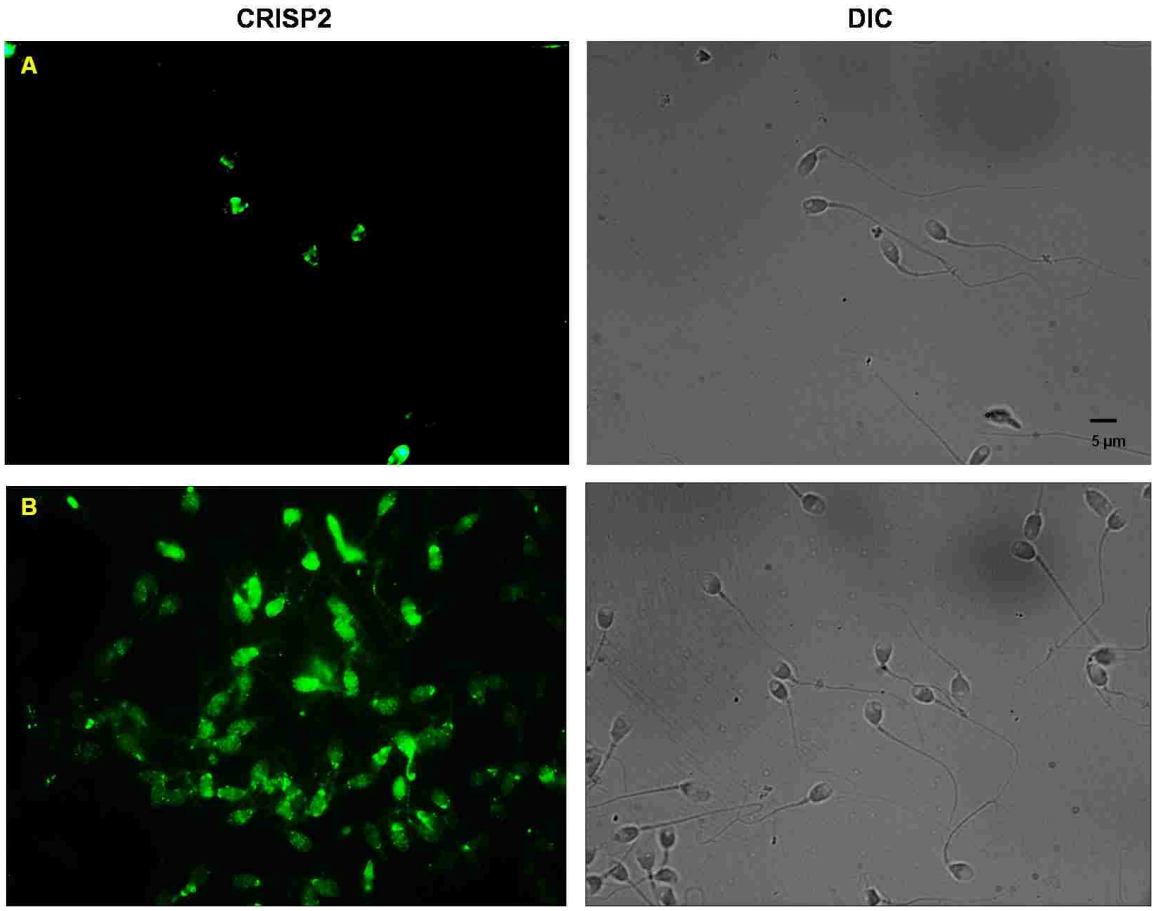



**Figure 4.18. Association stability of CRISP2 at the EqS of acrosome-reacted sperm treated with EGTA**. **A** Acrosome-reacted human sperm. **B** Acrosome-reacted human sperm treated with 30 mM EGTA for 30 minutes. Data are representative of 3 trials (200 cells/trial).

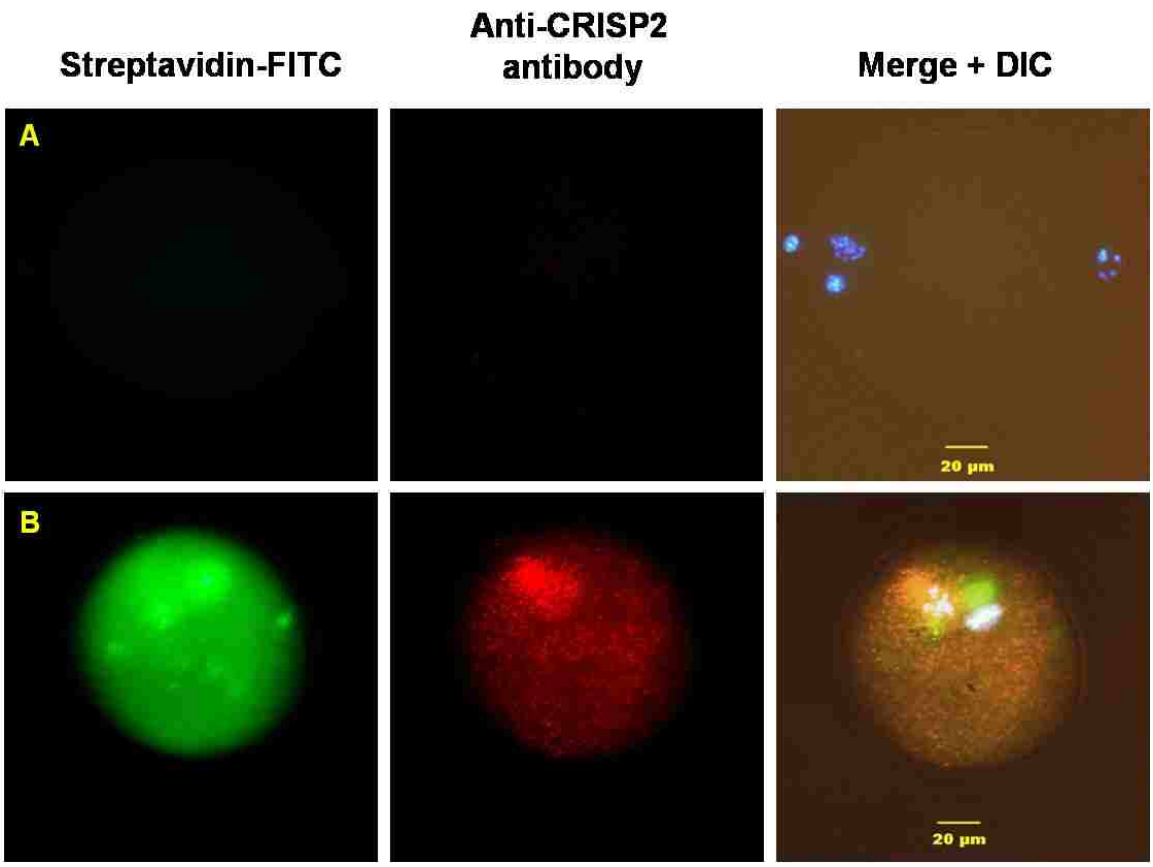



**Figure 4.19. Binding of human sperm acrosomal CRISP2 to the plasma membrane of zona-intact hamster oocytes.** A zona-intact hamster oocytes stained with steptavidin-FITC and anti-human CRISP2 antibody (with anti-rabbit IgG-TRITC antibody, (B) zona-intact hamster oocytes preincubated with 25 µg/mL of the purified biotinylated human sperm acrosomal CRISP2 and stained with streptavidin-FITC and anti-human CRISP2 antibody. Nuclei of the oocyte were stained with Hoechst 33342 (blue). Data are representative of 3 trials (10 oocytes/trial).

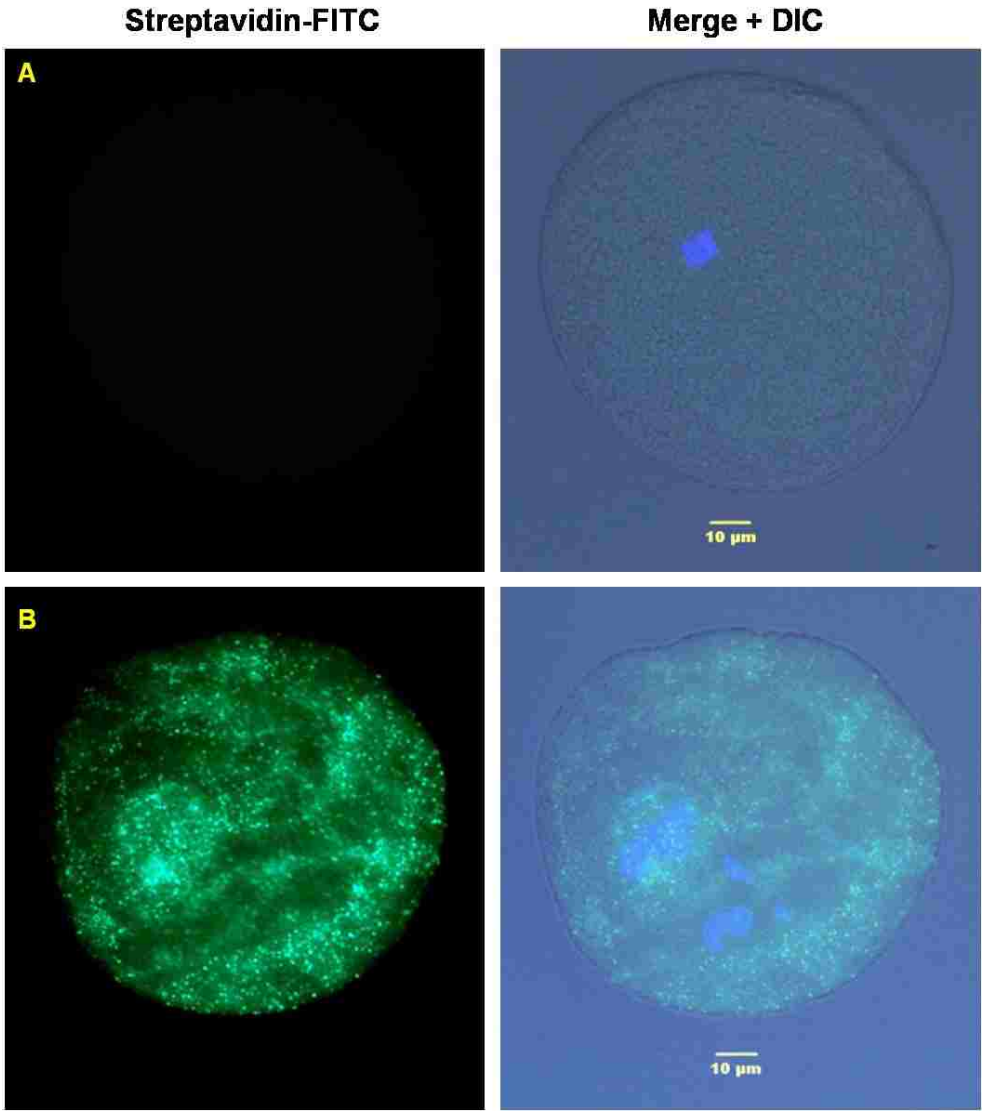



**Figure 4.20. Binding of human sperm acrosomal CRISP2 to the plasma membrane of zona-free hamster oocytes. A** zona-free hamster oocytes stained with steptavidin-FITC, **B** zona-free hamster oocytes preincubated with the purified biotinylated human sperm acrosomal CRISP2 and stained with streptavidin-FITC. Nuclei of the oocyte were stained with Hoechst 33342 (blue). Data are representative of 3 trials (10 oocytes/trial).

# Chapter 5

## Discussion

Mammalian sperm are highly specialized cells with extreme differentiation and membrane organization into different domains and subdomains, including the anterior acrosome, the EqS, the postacrosomal area, the midpiece, and the tail. Each domain contains specific molecules confined to the domain and the membrane barriers prevent free movement on molecules by lateral diffusion across different domains (James et al., 2004). However, the composition of sperm membranes and the restriction of molecules on specific domains are not fixed. The dynamic changes, both physiological and biochemical, in the sperm membrane system must occur in order to make sperm competent for progressive steps in fertilization. During mammalian sperm capacitation, membrane fluidity and permeability increase significantly (Buffone et al., 2009a; Buffone et al., 2009b; Flesch and Gadella, 2000; Martínez and Morros, 1996), and specific glycolipids relocalize from the anterior of the acrosome to the EqS and the postacrosomal area (Gadella et al., 1994; Gadella et al., 1995).

The EqS of mammalian sperm has been identified over 40 years ago, beginning with the discovery that this domain is responsible for initiating sperm-egg fusion (Yanagimachi and Noda, 1970). A subdomain, named the EqSS, has been reported in many species and this subdomain is thought to be an organizing center for assembly of molecular complexes required for sperm-egg fusion (Jones et al., 2008). However, there is no study reporting the presence of the EqSS in human sperm. Several sperm proteins have been reported to relocalize

on the plasma membrane overlying the EqS during the acrosome reaction (Copland et al., 2009; Inoue et al., 2011; Ito et al., 2010; Shur et al., 2004; Shur et al., 2006; Yoshida et al., 2010). Also, α-L-fucisidase has been reported by our laboratory to localize to the EqS of acrosome-reacted human sperm (Venditti et al., 2007). It is logical to envision that relocalization of sperm proteins to the EqS is crucial. Nevertheless, evidence explaining mechanisms that regulate the relocalization of sperm proteins during the acrosome reaction is limited.

This current study provides more evidence on mammalian sperm surface subdomains, relocalization of membrane-associated and soluble sperm proteins, and molecular mechanisms underlying sperm protein relocalization during the acrosome reaction. Data on the possible relevance in fertilization of sperm protein at the EqS are also presented in this dissertation.

### 5.1 Visualization of domains on sperm head surface and evaluation of the acrosome status

In the current project we utilized a plant lectin, PNA, derived from *Arachis hypogaea* to characterize sperm surface domains, including the anterior acrosome and the EqS, of human and mouse sperm. When PNA-TRITC was used to stain intact human sperm, the signal was detected throughout the entire acrosome, including the EqS (Figure 3.1). The evenly distributed signal over the acrosome and the EqS is an indicator that the membrane system over the acrosome is intact. Interestingly, when the same staining was performed in intact

mouse sperm, the only region that was fluorescent was the marginal area of the anterior acrosome (Figure 3.4, intact). There was only negative staining seen at the EqS or other regions over the mouse sperm head. The difference in PNA staining may be due to the unique organization of the sperm membrane system which may be species-specific. This statement is reasonable since we observed that the morphology, including sizes and shapes, of sperm is distinct between human and mouse. Moreover, the sizes and shapes of the sperm domains, including the anterior acrosome and the EqS are different between these two species.

When human and mouse sperm cells were capacitated, the PNA signal observed was similar to that of the intact sperm with staining patterns specific to particular species as described for intact human and mouse sperm PNA staining (Figure 3.2 for human sperm and Figure 3.4, capacitated for mouse sperm). This observation indicates that capcitation does not result in loss of Gal-β(1-3)-GalNAc disaccharide on the surface of sperm cells even though the membrane fluidity and permeability are normally increased during capacitation. In other words, increased membrane fluidity and permeability during capacitation does not cause migration of certain molecules, with Gal-β(1-3)-GalNAc attached, to the EqS. It means that during capacitation, there must be a mechanism responsible for stabilizing surface molecules in place. However, we observed a small population of capacitated sperm that had undergone spontaneous acrosome reaction during capacitation (Figure 3.2 and Figure 3.4, arrow heads). In human sperm, an indicator of the complete acrosome reaction is a sharp PNA band detected at the center of human sperm heads (Figure 3.3, arrow head).

This domain is in fact the EqS. In capacitated human sperm, the percent of capacitated population that showed the PNA-TRITC staining at the EqS was approximately 10 to 20 percent (data not shown). Although the percent of spontaneous acrosome reaction was small, this number indicates that the capacitation condition used was optimal for preparing human sperm cells for the acrosome reaction. A similar observation that the small population of capacitated mouse sperm had undergone spontaneous acrosome reaction was seen (Figure 3.4, capacitated). The staining pattern of PNA that indicates spontaneous acrosome reaction in mouse sperm was the PNA signal in the EqS which is a large area at the center of the mouse sperm head (Figure 3.4, capacitated)..

When human sperm were incubated with calcium ionophore to induce the acrosome reaction, the vast majority of cells showed PNA staining at the EqS. There was a small number of sperm that showed intact acrosomes. The percent of the acrosome-reacted human sperm cells was around 70 to 75 percent (data not shown). A similar observation was seen in mouse sperm that were induced for the acrosome reaction. The percent of acrosome-reacted mouse sperm was about the same as reported in humans (data not shown).

The observation that acrosome-reacted human sperm showed loss of PNA staining at the anterior acrosome, but showed the signal at the EqS can be explained by the fact that during the acrosome reaction the outer acrosomal membrane and the plasma membrane over the anterior of the acrosome fuse together for acrosomal exocytosis. This event results in the complete loss of the outer acrosomal membrane and the plasma membrane over this region.

However, the membranes overlying the EqS are not fused during the acrosome reaction. Therefore, the PNA signal previously observed in intact human sperm can still be seen after the acrosome reaction. In addition to the loss of sperm membranes during acrosomal exocytosis, it is possible that certain molecules, with bound Gal-β(1-3)-GalNAc disaccharides, migrate to the EqS under the influence of lateral distribution and become restricted at this particular domain thereafter. For mouse sperm, we did not observe the original confinement of Gal-β(1-3)-GalNAc disaccharide at the surface of intact (or permeabilized, data not shown) mouse sperm. Therefore, the likely explanation for the observation of PNA staining at the EqS of acrosome-reacted mouse sperm is that certain molecules, with bound Gal-β(1-3)-GalNAc disaccharide, migrate to the EqS during the acrosome reaction.

In this study, subcellular domains of human and mouse sperm, before and after the acrosome reaction, can be distinguished by PNA-TRITC staining. This staining can be used to evaluate the status of the acrosome and visualize the EqS of human and mouse sperm. However, a more precise characterization of subdomains of sperm cannot be visualized by PNA staining. The following section describes another staining strategy used for visualizing more precisely a well defined subdomain on human sperm.

### 5.2 The existence of the EqSS in human sperm

It is well known that the EqS on the head of mammalian sperm is a very crucial domain for initiating sperm-egg fusion. This domain is not a homogeneous

mixture of lipid compositions and proteins. Instead, it is a very complex area which requires distinct changes (in both physiological and biochemical modifications) before it becomes fusion competent. Previous studies reported the existence of a subdomain with unique features embedded within the EqS. This subdomain is called the equatorial subsegment (EqSS). It is puzzling why the EqSS on mammalian sperm has not been observed and identified for so long. Many studies in a wide range of species (Allen et al., 1995; Allen and Green, 1995; Ellis et al., 2002; Friend and Fawcett, 1974; Phillips, 1977; Suzuki, 1981; Yanagimachi, 1994) by early high-resolution microscopy, including TEM, scanning EM, and FF-EM, did not mention this subdomain. A study by(Jones et al., 2008) has provided information on the morphology, post-testicular development, and composition of the EqSS in boar, bull, and ram spermatozoa along with preliminary evidence for its presence in mouse and rat spermatozoa. However, the presence of the EqSS in human sperm has not yet been revealed. Data presented in this dissertation provide additional evidence for the EqSS in human spermatozoa. Since the sensitivity and specificity of staining of the sperm EqSS with anti-tyrosine phosphorylated protein monoclonal antibody clone 4G10 in other species have been reported in previous studies, human sperm were stained with 4G10 monoclonal antibody to determine the presence of the EqSS. Results from immunofluorescence using 4G10 antibody showed strong staining of tyrosine phosphorylated proteins in the center of the human sperm head (Figure 3.5B). Specifically, the staining pattern resembled a puncta-like stain detected at the center of the EqS (Figure 3.5B). The EqS was stained with PNA which binds strongly to specific sugar moieties over the acrosome of intact/fresh

human sperm and remains tightly associated with the EqS after strong permeabilization or the acrosome reaction. The dot-like region within the EqS is likely to be the EqSS because the staining pattern was similar to that reported in other species (Jones et al., 2008) even though the size and shape of the staining are different in different species. Incubation of freshly washed human spermatozoa (99% motility) with 4G10 antibody stained no cells on the anterior acrosomal and equatorial segment domains (Figure 3.5A). However, the presence of tyrosine phosphorylated proteins within the EqS could be observed in human sperm that were fixed and permeabilized with absolute methanol and acetone, respectively. The observation that the staining of tyrosine phosphorylated proteins in the human sperm EqSS can be seen only after permeabilization indicates that tyrosine phosphorylated proteins are localized intracellularly. Immunogold labeling of ram spermatozoa with 4G10 monoclonal antibody showed that tyrosine phosphorylated proteins localize to the outer acrosomal membrane within the confines of the EqSS as determined by AFM (Jones et al., 2008). It is reasonable to presume that this fact also applies to human sperm which will explain why detection of tyrosine phosphorylated proteins in the EqSS of fresh human sperm requires permeabilization. However, 4G10 immunogold labeling of human sperm will provide clear visualization of the EqSS needed to ensure this presumption.

The presence of tyrosine phosphorylated proteins strongly suggests that the EqSS should be a site of active protein kinases. However, the possibility that specific proteins may be tyrosine-phosphorylated at other regions and translocated to the EqSS during sperm maturation cannot be excluded. The most

interesting question is how tyrosine phosphorylated proteins are retained within the EqSS and do not disperse throughout the membrane system. Although it is believed that molecular barriers exist within the sperm head to play roles in preventing free movement of molecules between domains, this fact awaits further investigation.

In 2008, it was reported that the major tyrosine phosphorylated proteins in the EqSS of ram and boar sperm heads were identified as orthologs of human SPACA1 (Jones et al., 2008). Human SPACA1 is a testis-specific transmembrane glycoprotein that localizes to the EqS and/or the inner acrosomal membrane throughout the whole acrosome (Hao et al., 2002).

The detection of tyrosine phosphorylated proteins in the center of the EqS of human sperm strongly suggests the existence of the EqSS in human sperm. This observation is reasonable because the same observation can be seen in sperm in different mammalian species ranging from rodent to bovine, indicating the conserved nature of this particular domain in mammalian sperm. Conservation of the EqSS in different species of mammals suggests the potential significance of this domain in fertilization. Therefore, the EqSS merits further investigation as the potential site for sperm-egg interaction or the specialized region where membrane fusion is initiated between sperm and egg at fertilization.

In addition to staining for tyrosine phosphorylated proteins in permeabilized human sperm, we also included the staining in capacitated and acrosome-reacted human sperm. We originally expected to see tyrosine phosphorylation in the tail of capacitated sperm as reported (Ficarro et al., 2003; Visconti et al.,

1995a; Visconti et al., 1995b) and in the EqS and the EqSS. Results showed tyrosine phosphorylation in the tail of capacitated sperm as expected and also in the EqS of most cells. Also, some cells showed tyrosine phosphorylation over the entire acrosome. These observations, showing that during capacitation many proteins on the sperm surface are tyrosine phosphorylated, indicate the need of tyrosine phosphorylation for promoting the full function of certain proteins. When tyrosine phosphorylated proteins were detected in acrosome-reacted sperm harvested 30 minutes after ionophore treatment, most cells presented the punctate signal over the anterior acrosome and the EqS, and the signal at the EqSS was overwhelmed by that of the EqS. Some cells presented even distribution of tyrosine phosphorylated proteins over the acrosome. When the acrosome status was evaluated by PNA staining, we found that most human sperm cells were in the early stages of the acrosome reaction as judged by a faint diffused PNA signal that remained detectable in the anterior acrosome. This indicates that human sperm had not undergone the complete acrosome reaction. Data suggest that tyrosine phosphorylation on the human sperm head occurs extensively during early stages of the acrosome reaction. However, the signal at the tail disappeared. Moreover, when a longer incubation with ionophore was performed, the signal at the tail of acrosome-reacted human sperm could be seen again, but the signal at the head of most sperm cells significantly reduced. Some cells still showed the signal at the EqS with the remaining signal detectable in the posterior of the acrosome (the pre-EqS). Results indicate that the acrosome status in individual cells varies under identical conditions. The observation of the appearance and disappearance of tyrosine phosphorylated

proteins detected in human sperm cells induced to undergo the acrosome reaction at two different time points can be explained by the possibility that there is a feedback mechanism that controls the phosphorylation status on human sperm during capacitation and the acrosome reacton. The likely explanation is the involvement of tyrosine-specific phosphatases that dephosphorylate certain proteins at a specific time.

### 5.3 Subcellular localization of α-L-fucosidase in mouse sperm cells

Since most of the previous work in our laboratory focused on roles of α-L-fucosidase in fertilization and a study in human sperm showed that α-L-fucosidase relocalizes to the EqS after the acrosome reaction (Venditti et al., 2007), it is interesting to investigate whether the same phenomenon can be observed in other species. An attempt to explore more about the EqS and EqSS is challenging and worth investigating. Therefore, to gain a better understanding about the roles of the sperm EqS and what mechanisms sperm use to reorganize specific proteins on this domain to make sperm competent for fusion, α-L-fucosidase was the first focus because this protein has been of interest in the laboratory for its roles in fertilization. Although the roles of α-L-fucosidase have not been clearly elucidated in human, the roles of this protein in hamster (Phopin et al., 2012; Venditti et al., 2005; Venditti et al., 2007; Venditti et al., 2010) and mouse (Phopin et al., 2013) have been investigated in the laboratory. It has been reported that α-L-fucosidase was found predominantly over the anterior acrosome of permeabilized or capacitated human sperm and was detectable at

the EqS after the acrosome reaction (Venditti and Bean, 2009). What mechanisms are responsible for the reappearance of the enzyme at the EqS is not known yet. This dissertation presents additional evidence in a mouse model supporting the phenomenon discovered in human sperm.

In this study, the presence of α-L-fucosidase was detected in fresh, permeabilized, and acrosome-reacted mouse sperm. Data showed that α-L-fucosidase cannot be detectable in fresh mouse sperm. However, mild permeabilization of mouse sperm by one round of freezing and thawing showed a positive staining of α-L-fucosidase over the anterior of the acrosomal area and the staining did not expand into the EqS. These results indicate that α-L-fucosidase is originally localized underneath the plasma membrane. In other words, α-L-fucosidase is an intracellular protein that may reside in the acrosomal cap of the acrosome or associate with the outer or inner acrosomal membranes. The possibility that this protein is a component of the acrosome is less likely since results from my Western blot analyses that have been published recently (Phopin et al., 2012) suggested that there are at least two populations of α-L-fucosidase that are associated with the acrosomal membrane of mouse sperm. One population is tightly associated with the acrosomal membrane whereas the other population loosely binds to the acrosomal membrane. The results clearly showed that cell-bound α-L-fucosidases in mouse sperm are composed of at least two isoforms with the molecular mass ratios of 56 and 49 kDa. This study also revealed that capacitation of mouse sperm unmasks a cryptic store of α-L-fucosidase from within the cells by observing a significant increase in activity in the supernatant of sperm cells that have undergone capacitation. However, α-L-

fucosidase activity associated with capacitated and acrosome-reacted mouse sperm cells was persistent and stable through multiple incubations and manipulations with an insignificant amount of this enzyme released during the acrosome reaction. These observations indicate that α-L-fucosidase is less likely to be a component of the acrosomal cap of mouse sperm. Moreover, this dissertation presents the discovery that α-L-fucosidase reappears at the EqS of mouse sperm after the acrosome reaction. This study suggests that there are at least two distinct populations of mouse sperm-associated α-L-fucosidase. The first population is a pool of the enzyme, primarily within the acrosomal area which is liberated during capacitation. This enzyme may act as a decapacitating factor that is normally released during capacitation to facilitate progression of sperm through the female reproductive tract during natural reproductive processes. The second population of the enzyme that is tightly associated with the acrosomal membranes is not readily extractable due to its intimate association with other structures within the sperm. During the morphological reorganization that takes place during capacitation and the acrosome reaction, these structurally bound fucosidase molecules become restricted to, and localized within, the sperm EqS. However, the molecular mechanisms responsible for the relocalization of the enzyme have not been elucidated. I hypothesized that the population of α-L-fucosidase that tightly associates with the sperm acrosomal membrane migrates to the EqS during the acrosome reaction by lateral movement of lipids during the acrosome reaction where the plasma membrane and the outer acrosomal membrane are fused.

## 5.4 Dynamic relocalization of mouse sperm membrane-associated α-L-fucosidase during the acrosome reaction

Based on results from immunolocalization studies of fucosidase on mouse sperm that showed the restriction of the enzyme at the EqS only after the acrosome reaction together with the hypothesis that the population of α-L-fucosidase tightly associated with the sperm acrosomal membranes migrates to the EqS during the acrosome reaction, we designed this study to monitor the dynamic movement of α-L-fucosidase on the head of mouse sperm over the course of the acrosome reaction. Mouse sperm are a good model for studying dynamic changes of certain membrane-associated molecules because the surface over the head of the cell is very large compared to that of human sperm (Figure 3.4 and 3.3, respectively). The surface of human sperm head is much less than that of mouse sperm and this limits the capability for observing dynamic changes of surface molecules that occur in a very delicate fashion.

Therefore, in this current project mouse sperm samples were collected at different time points during the acrosome reaction for immunofluorescence analyses. Results from immunofluorescence studies showed five unique staining patterns of fucosidase on the mouse sperm head which include negative staining, the signal at the marginal area of the anterior acrosome, the signal at the anterior acrosome with a faint and diffused staining in the EqS, the punctuated signal at the EqS with some space, and the even signal throughout the entire EqS (Figure 3.10A-E, respectively). These observations are consistent with those in PNA staining used for monitoring the acrosome status. Results

clearly suggest that the observed fucosidase movement follows the stages of the acrosome reaction. Therefore, the patterns of fucosidase on mouse sperm during the acrosome reaction were classified into five categories which are nonacrosome-reacted sperm, initial acrosome-reacted sperm, early acrosome-reacted sperm, advanced acrosome-reacted sperm, and final acrosome-reacted sperm. The percent of sperm presenting initial and early fucosidase staining patterns decreased over the course of the acrosome reaction whereas the percent of sperm presenting advanced and final fucosidase staining patterns increased over the course of the acrosome reaction. These results show that during the acrosome reaction, α-L-fucosidase progressively moves from the acrosomal region toward the EqS, suggesting that the acrosome reaction induces relocalization of this enzyme to the new region. The specific mechanisms responsible for the movement of this enzyme during the acrosome reaction are not clearly elucidated. Exocytosis of the acrosomal membrane during the acrosome reaction might expose a certain isoform of α-L-fucosidase, originally stored and tightly associated with the outer acrosomal membrane, to the plasma membrane overlying the EqS by lateral diffusion. In 1995 Gadella et al. studied polarity migration behavior of glycolipids in the plasma membrane of boar sperm head. In this study, a fluorescent sulphogalactolipid analogue, galactose (3-sulphate)-β1-1' [(N-lissamine rhodaminyl)-12-aminododecanoyl]-sphingosine, and the desulphated derivative galactose- β1-1' [(N-lissamine rhodaminyl)-12-aminododecanoyl]-sphingosine were incorporated into the plasma membrane of living boar spermatozoa and lateral distribution of these fluorescent glycolipids over the sperm head was observed. They found that the

fluorescent lipids were enriched in the apical region of acrosomal area of freshly ejaculated sperm cells. However, after *in vitro* capacitation with 2 mM $CaCl_2$ or with 4% (w/v) bovine serum albumin, the lipids redistributed to the EqS. Also, the presence of fluorescent lipids was observed at the EqS of sperm after binding to the zona pellucida, indicating that the migration of glycolipids from the apical to the EqS during *in vitro* capacitation and zona binding occurs prior to the acrosome reaction. The movement of glycolipids seminolipid (SGalAAG) and desulphoseminolipid (GalAAG) to the EqS is believed to be a prerequisite for the acrosome reaction. It has been hypothesized that SGalAAG prevents fusion of the plasma membrane with intracellular membranes by stabilizing the lamellar phase of lipid bilayers (Gadella et al., 1991; Gadella et al., 1994). Therefore, the migration of SGalAAG from the apical and pre-equatorial subdomains may destabilize the regions, and thereby facilitate fusions between the sperm plasma membrane and the outer acrosomal membrane that eventually lead to acrosomal exocytosis. Studies in our laboratory showing that membrane-associated α-L-fucosidase on human sperm (Venditti et al., 2007) and mouse sperm (Phopin et al., 2012) redistributed from the apical surface of the acrosomal region to the EqS after the acrosome reaction raise the possibility that sperm membrane-associated α-L-fucosidase is originally stabilized at the intracellular anterior acrosomal region by specific glycolipids, including SGalAAG, and redistribute to the EqS by lateral diffusion following the movement of glycolipids to participate in the formation of molecular complexes required for sperm-egg fusion. Membrane-associated α-L-fucosidase is not the only sperm surface protein reported to relocalize to the EqS during the acrosome reaction. A similar model has been

reported by (Cohen et al., 2007) for protein DE, a member of the CRISP family, that segregates to two populations within sperm. One of them is loosely bound to sperm and released from the sperm cells during capacitation, acting as a ‘‘decapacitating factor.’’ A second population of sperm-associated protein DE becomes an integral membrane protein that remains tightly associated with sperm after capacitation. This bound protein DE migrates to the EqS as the acrosome reaction occurs, and participates in sperm–egg fusion (Cohen et al., 2007). The same explanation for the relocalization of sperm membrane-associated α-L-fucosidase where lateral diffusion provides a strong driving force for the movement of the enzyme may also be applicable for protein DE.

Sperm membrane-associated α-L-fucosidase has been studied in our laboratory for a long time to provide a better understanding of its roles in fertilization. The relocalization of this enzyme to the EqS has been hypothesized to participate in the formation of molecular complexes that are crucial for fertilization. Although the specific molecular mechanisms that facilitate the movement of this enzyme toward the EqS during the acrosome reaction have not been clearly elucidated, results from recent studies in human and mouse sperm suggest the possible influence of lateral diffusion that occurs during capacitation and the acrosome reaction because α-L-fucosidase has been proven to be associated with human sperm membranes and at least one population of the enzyme has been suggested to be tightly-bound to mouse sperm cells. Therefore, a model explaning lateral diffusion of mouse sperm membrane-associated α-L-fucosidase to the EqS during the acrosome reaction was proposed and presented in Figure 5.1.

The mechanism of reassociation of hybrid vesicles, formed between the outer acrosomal membrane and the plasma membrane during the acrosomal exocytosis, to the EqS cannot be excluded. The hybrid vesicles may contain remnants of α-L-fucosidase and convey this enzyme to the EqS via fusion to the EqS plasma membrane. However, results from enzyme assays showed that when compared to the supernatant obtained from mouse sperm capacitation, there was very low fucosidase activity in the supernatant obtained from mouse sperm samples induced for the acrosome reaction (Phopin et al., 2012). It probably indicates that the vast majority of loosely-bound fucosidase is released from mouse sperm during capacitation, but the tightly-bound population is not easily dissociated from the sperm membrane. Therefore, the mechanism of reassociation of this enzyme to the EqS via hybrid vesicle fusion is less likely to be possible. To better discriminate among these possibilities, immunogold labeling and electron microscopy would be the best way to provide clear visualization for α-L-fucosidase relocalization. Since the study using a mouse model was terminated after a previous graduate student Kamonrat Phopin had finished her experimental work, many interesting additional studies, including other mechanisms responsible for relocalization of α-L-fucosidase in mouse sperm such as involvement of F-actin and the association stability of this enzyme at the EqS of mouse sperm, were also terminated. Future investigations in these research aspects, when a mouse model is available, would provide more evidence to clarify among these hypotheses.

### 5.5 Subcellular localization of CRISP2 in human sperm cells

In addition to α-L-fucosidase, this study focuses on another sperm protein, CRISP2, because its role in sperm-egg fusion has been strongly suggested in rodents. In order to study relocalization of this protein, the subcellular localization of CRISP2 in washed/intact, permeabilized, capacitated, and acrosome-reacted human cells was visualized by indirect immunofluorescence using anti-human CRISP2 antibody. PNA-TRITC was used to monitor the acrosome status, and anti-tyrosine phosphorylated protein monoclonal antibody antibody (4G10 antibody) was used to visualize staining at the tail of human sperm as an indicator of capacitation. Results showed that unpermeabilized human sperm were negative to CRISP2 staining. Therefore, CRISP2 is not localized on the sperm plasma membrane. Triton X-100-permeabilized cells were positive with a strong signal restricted to the acrosomal region. This observation indicates that CRISP2 protein is an intracellular protein. However, the specific location of CRISP2 is not known yet. CRISP2 may localize at the outer or inner acrosomal membranes beneath the plasma membrane or may reside in the acrosome as a soluble component, as reported in guinea pig sperm (Kim et al., 2001; Kim and Gerton, 2003), as well as a component associated with the acrosomal matrix. Capacitated human sperm cells also showed CRISP2 staining restricted to the anterior of the acrosomal area. Interestingly, after the acrosome reaction, the positive signal for CRISP2 was clearly seen at the EqS of cells that had undergone the complete acrosome reaction. These observations support the hypothesis, suggested by (Busso et al., 2005), that human sperm head CRISP2 is an intra-acrosomal protein. The signal of CRISP2 observed in the anterior of

the acrosome of capacitated sperm suggests that during capacitation, the antibody gained ability to access its antigen due to the increase in membrane fluidity and permeability (Buffone et al., 2009a; Flesch and Gadella, 2000; Martínez and Morros, 1996). The signals of CRISP2 observed in the permeabilized and capacitated groups did not extend beyond the anterior of the acrosome, indicating that CRISP2 is not originally localized at the membrane system overlying the EqS. However, after the complete acrosome reaction CRISP2 specifically appeared at the EqS of human sperm, concomitant with the complete loss of CRISP2 signal at the anterior acrosome. These observations strongly suggest that CRISP2 relocalizes to the EqS during the acrosome reaction. A puncta-like CRISP-2 signal could also be detected at the connecting region between the head and the midpiece. In addition to CRISP2 staining in the head and the midpiece of human sperm cells, there was a weak labeling observed in the tail of these sperm, reflecting the presence of the protein in the outer dense fibres of the tail as reported for the rat (O'Bryan et al., 1998; O'Bryan et al., 2001). Studies by another group (Busso et al., 2005) revealed that CRISP2 does not undergo proteolitic cleavage or additional modifications after capacitation and the acrosome reaction. It means that the structure of CRISP2 remains intact as its original form throughout the entire maturation process and the acrosome reaction.

Why CRISP2 reappears at the EqS after the acrosome reaction is an interesting question. When considering the importance of EqS as a place for sperm-egg fusion initiation, it is reasonable to hypothesize that CRISP2 may be involved in making the EqS competent for sperm-egg fusion. In 2005 the potential relevance

of CRISP2 for human gamete interaction was examined by studying the inhibitory effect of anti-TPX1 antibody (anti-CRISP2 antibody) on the ability of human sperm to penetrate zona-free hamster oocytes (Busso et al., 2005). The study found that insemination of gametes with the presence of the antibody significantly decreased the percentage of penetrated oocytes in a dose-dependent manner. The authors speculated that CRISP2 present at the EqS of acrosome-reacted human sperm could mediate gamete fusion by interacting with complementary sites on the oocyte surface. In the discussion of this report (Busso et al., 2005) they claimed that mouse recombinant CRISP2 bound to the surface and inhibited penetration of zona-free rat oocytes. However, results for this particular experiment were not published. Based on these studies, the presence of CRISP2 at the EqS seems to be important for sperm-egg fusion. However, although the possible population of CRISP2 that may play roles in sperm-egg fusion has been suggested to be enriched at the EqS of acrosome-reacted sperm, the molecular mechanisms that facilitate the enrichment of CRISP2 at the EqS during the acrosome reaction still remain undiscovered. Investigation into the understanding of how CRISP2 relocalizes to the EqS during the acrosome reaction is explored in this study using different approaches as discussed in the following sections.

### 5.6 Actin polymerization is not involved in relocalization of CRISP2 during the acrosome reaction

Several possibilities could account for relocalization of CRISP2 during the acrosome reaction. One of these possibilities is the involvement of intracellular

cytoskeletal proteins. A recent study in mouse sperm revealed the involvement of filamentous actin in redistribution of IZUMO 1 protein which is a crucial candidate with known function in gamete fusion. Since CRISP2 has also been suggested to be a candidate important for sperm-egg fusion, it is logical to hypothesize that F-actin may facilitate the movement of CRISP2 during the acrosome reaction. Actin has been demonstrated in male germ cells from various mammalian species (Vogl, 1989). Moreover, F-actin has also been described in the sperm of mammalian species (Breed and Leigh, 1991; de las Heras et al., 1997; Flaherty, 1987; Moreno-Fierros et al., 1992; Vogl, 1989; Vogl et al., 1993). In human sperm, F-actin can be found in different compartments including the acrosomal space, the EqS, the post acrosomal regions, and the tail (Clarke et al., 1982; Fouquet and Kann, 1992; Ochs and Wolf, 1985; Virtanen et al., 1984). A recent study in knockout mice demonstrated that relocalization of IZUMO1 from the anterior of the acrosome to other regions, including the EqS and the postacrosomal region during the acrosome reaction is regulated by the testis-specific serine kinase 6 (TSSK6) (Sosnik et al., 2009). This study also found that actin polymerization in the postacrosomal region after the acrosome reaction was inhibited in TSSK6-null mice, suggesting a possible involvement of the actin cytoskeleton in relocalization of sperm membrane proteins during the acrosome reaction. The study herein tested the hypothesis that actin dynamics are responsible for CRISP2 relocalization during the acrosome reaction. The study was designed to use an inhibitor of F-actin polymerization, Latrunculin A, to inhibit F-actin formation during the acrosome reaction, and the changes in patterns of CRISP2 localization were observed. The results showed that when

Latrunculin A was used, the formation of F-actin during the acrosome reaction was completely inhibited, but we observed no inhibition of CRISP2 relocalization to the EqS, indicating that actin polymerization is not involved in the redistribution of CRISP2 to the EqS during the acrosome reaction.

CRISP2 might also reach the plasma membrane over the EqS by migration from the acrosomal membranes during the acrosome reaction by lateral diffusion as described for fucosidase. However, this mechanism is less likely to be possible because of the fact that CRISP2 does not contain hydrophobic domains in the mature protein (Kasahara et al., 1989), which does not support the insertion of CRISP2 into the sperm acrosomal membranes. Therefore, another possibility is that CRISP2 protein is a soluble protein residing in the acrosomal cap of human sperm.

### 5.7 CRISP2 is a component of the acrosomal cap and is released during the acrosome reaction

The observation in this study from immunoflorescence and a previous report (Busso et al., 2005) showing that CRISP2 signal disappears from the anterior of the acrosome after the acrosome reaction together with the soluble nature of CRISP2 reported for other species (Hardy et al., 1991; Kim et al., 2001) raise the possibility that human sperm CRISP2 is originally stored in the acrosomal vesicle and released during the acrosome reaction. Important soluble proteins including hyaluronidase, dipeptidyl peptidase, and CRISP2 have been reported to be

constituents of the soluble compartment of the acrosome of guinea pig sperm and these enzymes are released during the acrosome reaction (Hardy et al., 1991). Therefore, it is reasonable to hypothesize that CRISP2 is stored in the acrosomal cap and released during the acrosome reaction.

To test the hypothesis, sperm cells were induced to undergo the acrosome reaction, and then the supernatants and pellets were collected to assay the presence of CRISP2 by Western blot analysis. The pellets of the control and the ionophore-treated groups showed CRISP2 bands at approximately 25 kDa, revealing the cell-associated population. A strong immunoreactive band of CRISP2 was found in the supernatant of a 60 minutes ionophore-treated group whereas the supernatant of fresh sperm (the control group) incubated for the same period of time throughout the course of capacitation and the acrosome reaction showed no CRISP2 band. Also, sperm samples were collected at different time points during the acrosome reaction and subjected to Western blot analysis. The results show that CRISP2 protein in the supernatant of ionophore-treated cells increased over time and this increase was correlated with the increased percent of the acrosome reaction. These results indicate that CRISP2 is sequentially released into the supernatant during the acrosome reaction. Capacitation for a long period of time (4 hours) did not induce CRISP2 release, indicating that CRISP2 release really occurs during the acrosome reaction. This observation provides evidence supporting that the human sperm head CRISP2 is primarily a component of the acrosomal cap. This finding is supported by a study in guinea pig sperm that revealed that CRISP2 is a soluble protein that is rapidly released during the early state of the acrosome reaction (Kim et al., 2001). The

sperm acrosome is compartmentalized into different compartments which contribute to differential release of acrosomal components during the acrosome reaction (Hardy et al., 1991; Kim and Gerton, 2003). The integrity of the acrosomal matrix largely depends on pH. Low pH does not affect the integrity of the matrix whereas high pH activates the endogenous proteolytic activity which then destabilizes the structure of the acrosomal matrix (Huang et al., 1985; Nakanishi et al., 2001). Results demonstrated that human sperm head CRISP2 was well preserved within the sperm acrosome in an acidic environment (pH 4). On the contrary, a high pH buffer can completely solubilize CRISP2 from the cells. Taken together, all results support that CRISP2 is a soluble component of the human sperm acrosomal cap which is released during the acrosome reaction. This observation raises the possibility that CRISP2 appearing at the EqS of human sperm after the acrosome reaction may come from the population stored within the acrosomal cap.

### 5.8 Reassociation of acrosomal CRISP2 at the EqS of acrosome-reacted sperm

The current study hypothesizes that the released CRISP2 reassociates with the EqS membrane during the course of the acrosome reaction. This idea is supported by a study that demonstrated that testicular CRISP2 is produced and secreted from spermatogenic cells at various differentiation stages, and reassociates with the plasma membrane of spermatogenic cells to facilitate the specific adhesion of spermatogenic cells with Sertoli cells (Maeda et al., 1999).

Based on this hypothesis, the current study was designed to prove the reassociation behavior of this protein in mature sperm undergoing the acrosome reaction. Since I proved that this protein is released into the media during the acrosome reaction, CRISP2 was collected from the ionophore-treated supernatant, purified using affinity bead purification, and subjected to *in vitro* biotinylation. The results from silver nitrate staining and Western blot analysis showed that CRISP2 was successfully isolated from the acrosomal vesicle of human sperm.

To determine whether the purified biotinylated human sperm acrosomal CRISP2 can bind to the EqS of human sperm during the acrosome reaction, the ionophore-treated human sperm were incubated with the purified biotinylated CRISP2. Results showed that 49% (or only around half of acrosome-reacted sperm population) of acrosome-reacted cells specifically bound CRISP2, detected with streptavidin-FITC, at the EqS. This might be explained by the fact that the stage of the acrosome reaction in each individual sperm is not equal. Although PNA-TRITC showed the loss of acrosomal signal and strong staining at the EqS, the subtle changes on the EqS membrane necessary for CRISP2 binding may not be fully ready in all sperm. Moreover, it was hypothesized that during the acrosome reaction, the native CRISP2 protein released into the supernatant competes with the biotinylated CRISP2 to bind to the EqS of acrosome-reacted sperm. To test the latter hypothesis, a decreased number of acrosome-reacted sperm was incubated with increased concentrations of biotinylated CRISP2. When the concentration of the purified biotinylated human sperm acrosomal CRISP2 was increased, the percent of sperm with CRISP2

appearing at the EqS of acrosome-reacted sperm increased in a dose-dependent manner. This finding suggests that CRISP2 released from the acrosome of human sperm during the acrosome reaction can bind to the EqS. Therefore, the likely explanation for the appearance of CRISP2 at the EqS is that CRISP2 is derived from the population stored in the acrosomal cap. For comparison, when intact or capacitated sperm cells were incubated with the purified biotinylated human sperm acrosomal CRISP2 for the same period of time, negative staining at the EqS or at other regions within the sperm head was found. This observation indicates that the binding ability of CRISP2 to the EqS of acrosome-reacted sperm is very specific to the EqS and significantly depends upon the modifications of the membrane overlying the EqS during the acrosome reaction. When sperm bind to the zona pellucida of the oocyte, the acrosome reaction is initiated. As the acrosome reaction proceeds, acrosomal contents, including CRISP2, are released to facilitate penetration of sperm through the zona pellucida. During sperm-zona penetration the sequentially released acrosomal contents, including CRISP2 are concentrated in the specific site of the zona pellucida matrix; therefore, CRISP2 may gradually bathe the plasma membrane of the EqS facilitating reassociation.

The EqS of mammalian sperm has been revealed to be a site for the initiation of sperm-egg fusion (Bedford et al., 1979; Yanagimachi, 1988). The plasma membrane of the EqS of intact, capacitated, and acrosome-reacted sperm is exposed to the extracellular environment at all times, but only the EqS of acrosome-reacted sperm can fuse with the oolemma. Therefore, to make the EqS fusion-competent at the right time, the plasma membrane overlying the EqS

must undergo physiological changes during the acrosome reaction. It has been thought that major factors responsible for these changes come from acrosomal components differentially released during the acrosome reaction. In 1992 (Díaz-Pérez and Meizel, 1992)suggested that the acrosomal phosphoramidon-inhibitable metalloendoprotease released during the acrosome reaction functions to increase the fusibility of the EqS. This hypothesis was supported by (Takano et al., 1993) with the finding that the enzymes released from the acrosome during the acrosome reaction may activate latent fusion proteins, stimulate migration of fusion proteins (Cowan et al., 1991), and/or remove steric and charge barriers to membrane apposition. Acrosin is another acrosomal protein released during the acrosome reaction and it has been proven to be essential for modification of the EqS. The epitopes of Acrosin have been localized to the plasma membrane of the EqS and post-acrosomal region of acrosome-reacted rabbit sperm (Richardson et al., 1991). Acrosin has been suggested to play significant roles in altering biophysical and biochemical characteristics of the plasma membrane over the EqS to make this compartment competent to fuse with the oolemma (Takano et al., 1993). The released acrosomal proteases including metalloendoprotease and Acrosin may not act alone. Their full functions may require the involvement of other acrosomal components. A study in 2003 by (Milne et al., 2003) reported that the cone snail CAP protein, TEX31, which is structurally related to CRISP2 protein, is a site-specific protease. Taken together, the observations that acrosomal CRISP2 reassociates to the EqS after the acrosome reaction suggest that acrosomal CRISP2 may act in concert with other acrosomal components at the EqS to make the plasma membrane at this region

fusible. Another possible function of this protein at the EqS is to act as an adhesion molecule to facilitate sperm-egg fusion. A reassociation model explaning CRISP2 association with the EqS during the acrosome reaction was created and presented in Figure 5.2.

## 5.9 Association stability of CRISP2 at the EqS of acrosome-reacted human sperm

Since CRISP2 at the EqS has been hypothesized to have importance for sperm-egg fusion, its association to the sperm membrane is significant for stabilizing the interaction with its complementary molecules on the oocyte surface. Therefore, the stability of CRISP2 binding to the EqS of acrosome-reacted sperm was studied. Results showed that a high ionic strength solution, a reducing agent, a mild non-ionic detergent, and mild sonication could not dislodge bound CRISP2 from the EqS. Only a strong detergent SDS could extract CRISP2 from the EqS, but this detergent could not completely dissociate CRISP2 population at the neck of the sperm. This observation suggests that CRISP2 stabilization at the EqS of acrosome-reacted sperm is very strong as would be expected if it plays a role as an adhesive molecule between sperm and egg during the fusion process. Interestingly, sperm treated with EGTA showed a diffused signal of CRISP2 over the head of sperm, indicating that the EqS of the sperm is destabilized when $Ca^{2+}$ in the medium is depleted, resulting in CRISP2 protein redistribution to other regions, and indicating that stabilization of CRISP2 at the EqS depends on $Ca^{2+}$. This suggestion is supported by a study using fluorescently tagged

liposomes consisting of negatively charged phospholipids which could bind specifically to the EqS of human sperm only after the acrosome reaction (Arts et al., 1994). The study found that sperm treated with EDTA caused fluorescence to spread from the EqS towards other membrane domains. Moreover, pretreatment of acrosome-reacted spermatozoa with EDTA for 10 minutes before the addition of liposomes inhibited the fusogenic activity of the EqS. Arts and colleagues suggested that the lipid-diffusion barrier is destabilized when $Ca^{2+}$ is depleted. However, how calcium ions are involved in EqS stabilization is not well understood. In sperm cells, it has been reported that the Crisp domain of CRISP2 protein can regulate ion channel activity, specifically $Ca^{2+}$ fluxes, suggesting its roles during sperm capacitation (Gibbs et al., 2006). It is possible that calcium depletion affects the functions of particular surface molecules that, with the presence of $Ca^{2+}$, maintain the integrity of the lipid-diffusion barrier in the EqS. Calcium-dependent functions have been observed in other cell types. In epithelial cells tight junctions prevent lipid diffusion between the basolateral and apical membrane domains and calcium depletion causes tight junctions to open, resulting in redistribution of fluorescently tagged liposomes from the apical surface to the basolateral membrane domain (van Meer et al., 1986). Intracellular molecules with functions depending on calcium cannot be excluded. Since the major source of calcium for intracellular activities of sperm cells comes from the extracellular environment, the lack of $Ca^{2+}$ in the media may result in inadequate $Ca^{2+}$ import and consequently inactivate molecules that are responsible for maintaining the integrity of the EqS.

## 5.10 Binding of human sperm acrosomal CRISP2 to the plasma membrane of zona-intact and zona-free hamster oocytes

The current study showing that CRISP2 reassociates very strongly with the EqS of human sperm during the acrosome reaction raises the possibility that this protein may function as an adhesion molecule to facilitate sperm-egg fusion. To test this hypothesis, the binding ability of the acrosomal CRISP2 to hamster oocytes was investigated in this study. The study was designed to answer whether CRISP2 can bind to hamster oocytes and what specific parts of the oocyte CRISP2 can bind. Therefore, in this project a study of CRISP2 binding was performed in both zona-intact and zona-free hamster oocytes. Results showed that the purified biotinylated human sperm acrosomal CRISP2 could bind to the plasma membrane of hamster oocytes but not the zona pellucida. The binding of CRISP2 around the plasma membrane of zona-intact hamster oocytes detected with streptavidin-FITC was consistent with the CRISP2 staining detected with anti-human CRISP2 antibody as we observed colocalization of streptavidin-FITC and anti-human CRISP2 antibody (TRITC). Data indicate that CRISP2 derived from the acrosomal vesicle can bind to the EqS and the oolemma of hamster oocytes. These observations suggest that the population of CRISP2 at the EqS can bind to the oolemma of hamster oocytes. Although this study did not investigate whether anti-CRISP2 antibody reduces the number of sperm tightly bound to the plasma membrane of hamster oocytes or number of penetrated sperm (fusion), it was studied in the human sperm/zona-free hamster oocyte model (Busso et al., 2005). This study reported that the presence of anti-TPX1 antibody (anti-CRISP2 antibody) during gamete co-incubation produced a

significant and dose-dependent inhibition in the percentage of penetrated zona-free hamster oocytes without affecting sperm motility, the acrosome reaction, or sperm binding to the oolemma. This study is consistent with previously published work in mice and rats (Busso et al., 2007; Ellerman et al., 2006). Therefore, it is reasonable to propose that human sperm acrosomal CRISP2 stored in the acrosomal vesicle, released into the extracellular environment, and reassociated with the EqS may bind to the complementary sites on the plasma membrane of the oocyte and facilitate sperm-egg fusion. Further attempts to isolate and identify the specific targets of CRISP2 on the plasma membrane of human or hamster oocytes would clarify this proposed model and provide more understanding in mammalian fertilization.

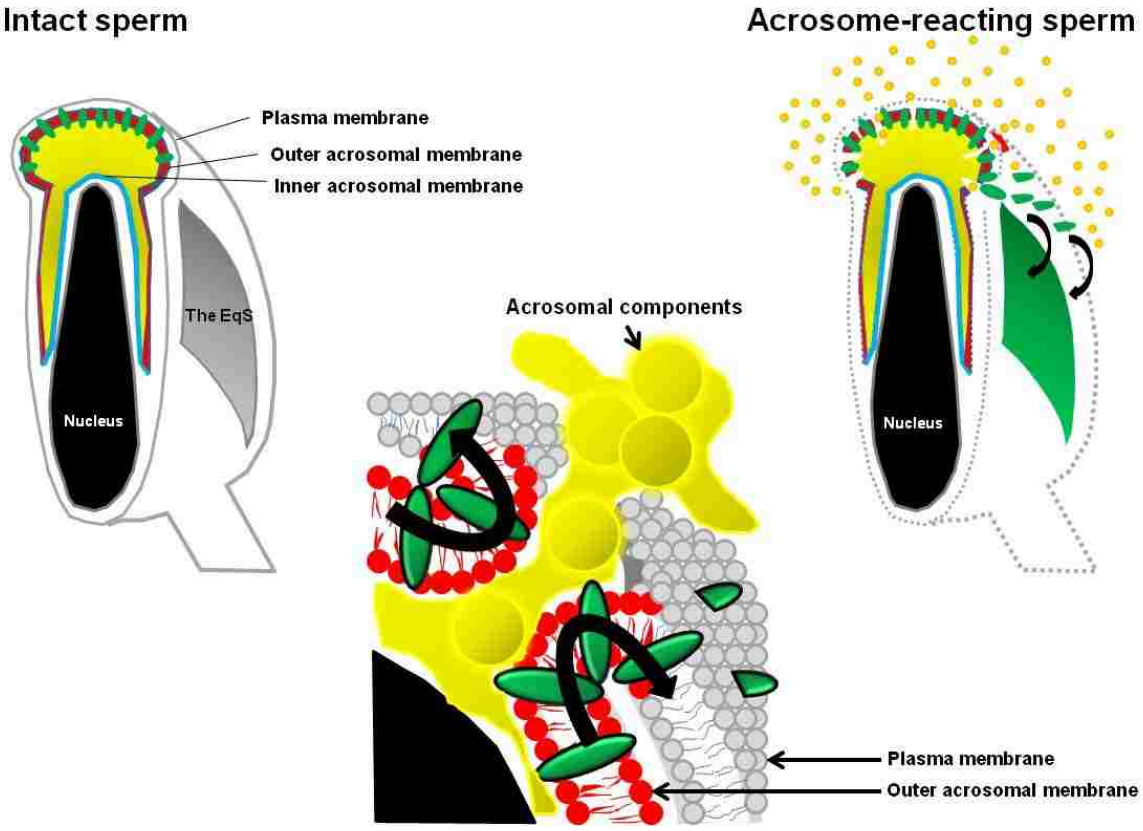



**Figure 5.1 A lateral diffusion model for relocalization of α-L-fucosidase to the EqS of mouse sperm during the acrosome reaction.** In intact mouse sperm, α-L-fucosidase (green) is originally localized in the outer acrosomal membrane (red). During the acrosomal exocytosis, where the plasma membrane and the outer acrosomal membrane fuse together, α-L-fucosidase migrates to the plasma membrane overlying the EqS through lateral diffusion. The sperm nucleus is shown in black. The plasma membrane is shown in grey. The inner acrosomal membrane is shown in light blue. The acrosomal components are shown in yellow.

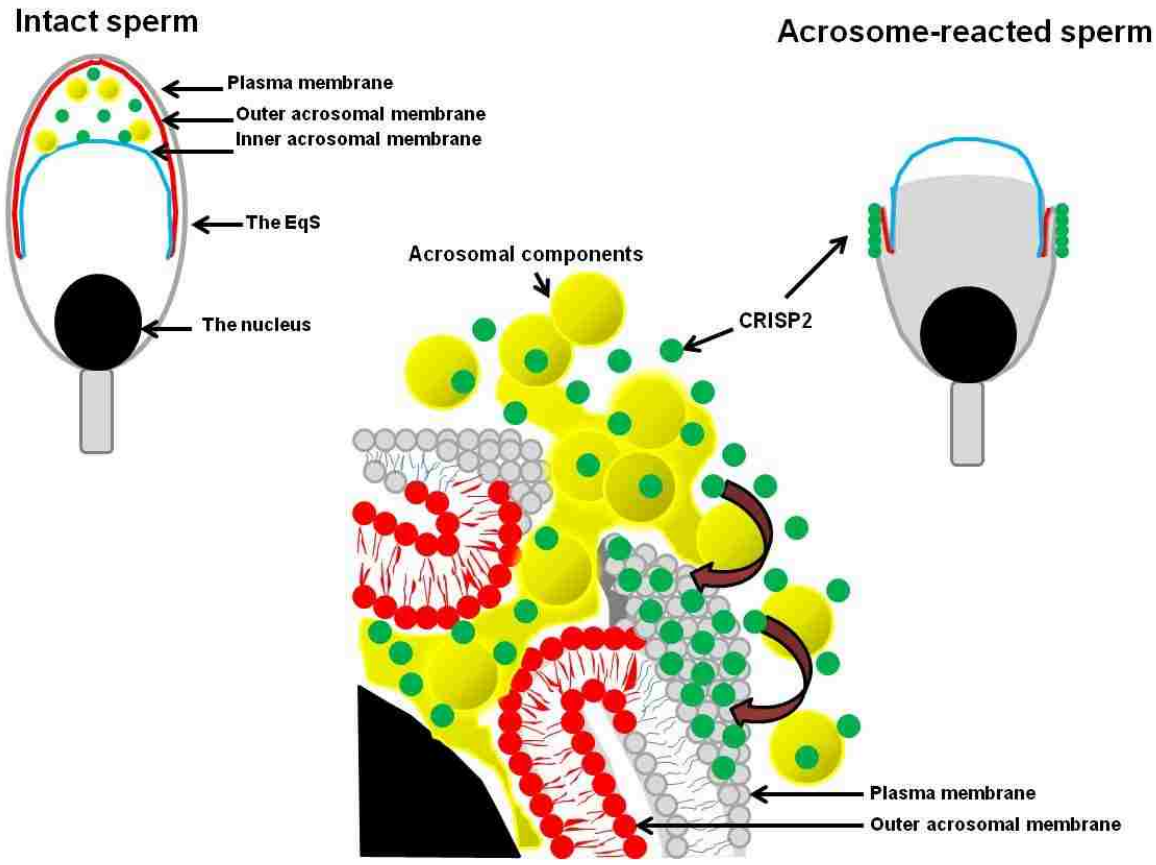



**Figure 5.2 A reassociation model for relocalization of CRISP2 to the EqS of acrosome-reacted human sperm during the acrosome reaction.** In intact human sperm, CRISP2 (green) is originally localized in the acrosomal cap, an area between the outer (red) and the inner (light blue) acrosomal membranes. During the acrosomal exocytosis, where the plasma membrane and the outer acrosomal membrane fuse together, CRISP2 is released and reassociates with the plasma membrane overlying the EqS. The sperm nucleus is shown in black, the plasma membrane is shown in grey, and the acrosomal components are shown in yellow.

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# Curriculum Vitae

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## EDUCATION

2008-2013 Ph.D. in Cell and Molecular Biology, Lehigh University

**Dissertation Title**: *The sperm equatorial segment: an organizing center for sperm protein relocalization and facilitation of fertilization*

**Dissertation Advisor**: Professor Linda J. Lowe-Krentz

2002-2004 B.S., Biology with honors, Khon Kaen University, Thailand

## ACADEMIC HONORS AND AWARDS

2008-2013 Full Scholarship from the Royal Thai Government for pursuing a Doctoral Degree in the United States of America

2011 Trainee Award from the American Society of Andrology

2004 Outstanding Undergraduate Research Project Presentation Award, Department of Biology, Faculty of Sciences, Khon Kaen University, Thailand

**RESEARCH EXPERIENCES AND EMPLOYMENT**

2008-2012 **Graduate Research Fellow:** Lehigh University, Biological Sciences Department:

**Mentor:** Professor Linda J. Lowe-Krentz

- Investigated molecular mechanisms regulating smooth muscle cell-specific gene expression: post translational modifications of E twenty-six (ETS)-like transcription factor 1(Elk-1)

  - *Cell culture and biochemical techniques*

- Studied effect of cGMP-dependent protein kinase (PKG) knock down on heparin signaling,

  - *Using small RNA interfering (siRNA) and PKG inhibitors*

- Investigated molecular mechanisms underlying the relocalization of human sperm proteins to the sperm equatorial segment during the acrosome reaction

  - *Using techniques in biochemistry, molecular and cell biology: protein purification, in vitro biotinylation, indirect immunofluorescence, Western blot analysis*

**Mentor:** Professor Barry S. Bean

- Studied mechanisms of relocalization of sperm cysteine rich secretory protein 2 (CRISP2) during the acrosome reaction

- Studied roles of mouse sperm membrane associated alpha-L-fucosidase in fertilization

  - *Using enzyme assay, in vitro fertilization (IVF), and Intracytoplasmic Sperm Injection (ICSI)*

2005-2008 **Employed Researcher:** Chiang Mai University, Department of Clinical Immunology, Faculty of Associated Medical Sciences, Thailand

**Mentor**: Associate Professor. Dr. Chatchai Tayapiwatana

- Production of Phage displaying leukocyte surface molecule for ligand tracing

  - *Molecular cloning/Recombinant DNA technology*
  - *Phage Display technique*

- Development of single chain variable fragment (scFv) intrabody for cancer therapies

  - *Functional assay for various scFv$_s$*
  - *Adenoviral transduction to transfer therapeutic genes into target cells*

- Production of recombinant proteins for antibody production

  - *Gene cloning and expression in E.coli*
  - *Recombinant protein production and purification*
  - *Animal immunization and screening of antibodies*

2000-2004 **Undergraduate Research Project Fellow:** Khon Kaen University, Department of Biology, Faculty of Sciences, Thailand

**Advisor**: Dr.Sumpars Khunsook

- Characterized alpha-L fucosidase in *Bos indicus* semen to study roles of this enzyme in the reproductive system.

  - *Using basic biochemical and molecular biology techniques including enzyme isolation, purification from sperm plasma membrane, chromatography, protein separation (SDS-PAGE), and Western blot analysis*

## INVITED PRESENTATIONS AND SEMINARS

February 2013 Graduate student poster presentation, Department of Biological Sciences, Lehigh University

April 2011 Annual meeting poster presentation, American Society of Andrology, Montreal, Quebec, Canada

March 2011 Graduate student/Postdoc presentation, Department of Biological Sciences, Lehigh University

October 2011 Open house poster presentation, Department of Biological Sciences, Lehigh University

October 2010 Open house poster presentation, Department of Biological Sciences, Lehigh University

October 2009 Open house poster presentation, Department of Biological Sciences, Lehigh University

## PUBLICATIONS

1. **Nimlamool W,** Bean BS, Lowe-Krentz LJ (2013). Human sperm CRISP2 is released from the acrosome during the acrosome reaction and reassociates to the equatorial segment. Manuscript resubmitted to *Molecular Reproduction and Development*
2. Phopin K, **Nimlamool W,** Lowe-Krentz LJ, Douglass E, Taroni JN, Bean BS (2013) Roles of mouse sperm-associated alpha-L-fucosidases in fertilization. *Mol Reprod Dev* DOI 10.1002/mrd.22164
3. Published abstract: **Nimlamool W**, Slee JB, Lowe-Krentz LJ (2013). Fluorescent imaging allows identification of organelle specific signaling in individual vascular smooth muscle cells. ASBMB annual meeting.
4. Phopin K, **Nimlamool W**, Bartlett MJ, Bean BS (2012) Distribution, crypticity, stability, and localization of α-L-fucosidase of mouse cauda epididymal sperm. *Mol Reprod Dev* 79:208-217
5. Published abstract: **Nimlamool W**, Phopin K, Bean BS (2011) Dynamic relocalization of alpha-L-fucosidase of mouse sperm during spermatogenesis and the acrosome reaction. American Society of Andrology 36th annual meeting. *Journal of Andrology*

## REFERENCES

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