Université de Montréal

A Novel Purification Method for Binder of SPerm Proteins and characterization of the Protein Interaction Network of BSPH1

Une nouvelle méthode de purification pour les protéines Binder of Sperm (BSP) et réseau d'interaction protéique de BSPH1

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Résumé

Les protéines Binder of Sperm (BSP) appartiennent à une superfamille de protéines exprimées dans le système reproducteur masculin, plus particulièrement dans les vésicules séminales chez les ongulés, et dans l'épididyme chez l'humain et la souris. Jusqu'à présent, des rôles variés chez différentes espèces ont été démontrés pour les protéines BSP, tels que dans la motilité et la capacitation chez le bovin. Cependant, leur rôle demeure élusif chez d'autres mammifères comme la souris et l'humain. Des études *in vivo* récentes ont démontré que la délétion des gènes *Bsph1* et *Bsph2* chez la souris n'a aucune conséquence sur la fertilité, et n'induit aucune anomalie au niveau de l'appareil reproducteur masculin. Afin d'élucider le rôle spécifique de la protéine BSP chez l'humain (BSPH1), nous avons d'abord développé une méthode de purification efficace permettant d'obtenir la protéine BSPH1 fonctionnelle car ces protéines ne sont présentes qu'en quantité infime dans l'épididyme humain. Suite, a la purification de BSPH1, j'ai réalisé des expériences *in vitro* et cherché à identifier son réseau d'interaction protéique. Il a été démontré que les protéines BSP interagissent avec des groupes pseudo-choline tels que le diéthylaminométhyle par affinité plutôt que par des interactions ioniques. Le diéthylaminoéthyle est chargé positivement et par conséquence, est un échangeur d'anions faible, mais les BSP interagissent avec affinité à la résine. Cette étude présente également une nouvelle méthode de purification rapide et peu coûteuse, qui fournit des protéines BSP recombinantes de grande pureté qui peuvent être utilisées pour étudier leurs rôles dans la fécondation chez les mammifères. Nous avons montré que la pré-incubation des ovocytes avec la protéine BSPH1 recombinante peut diminuer le taux de fécondation de manière dose-dépendante. Les spermatozoïdes ont également été pré-incubés avec un anticorps anti-BSPH1 et ont montré une diminution du taux de fécondation. Pour identifier le réseau d'interaction protéique de BSPH1, j'ai utilisé la méthode « Proximity-dependent biotin identification » (BioID) couplée à la spectrométrie de masse. Les résultats de la spectrométrie de masse ont démontré une interaction entre BSPH1 et toutes les sous-unités du complexe CCT / TRIC (Chaperonin containing tailless complex polypeptide 1 (CCT) ou tailless complex polypeptide 1 ring complex (TRiC)). Ce complexe interagit avec un autre complexe appelé BBSome (Bardet–Bied syndrome complex), qui joue un

rôle important dans le transport de protéines à travers les cils primaires. BSPH1 a également interagi avec un grand nombre de protéines de la famille CEP (centrosome-associated proteins), importantes dans la formation des cils primaires par les microtubules et de la maturation du centrosome, qui soutiennent le rôle de BSPH1 dans les cils primaires.

Dans l'ensemble, cette étude démontre que BSPH1 pourrait avoir un nouveau rôle en tant que chaperonne, à travers les cils primaires dans les cellules qui l'expriment dans l'appareil reproducteur masculin.

Mots clés: Infertilité masculine, Purification des protéines, Chromatographie d'affinité, protéines BSP, Protéines épididymaires, Identification de la biotine dépendante de la proximité

Abstract

Binder of SPerm (BSP) proteins belong to a superfamily of proteins expressed in the male reproductive tract, particularly in seminal vesicles of ungulates (e.g., bovine, ram) and in the epididymis of humans and mice. So far, BSP proteins have been shown to play different roles in different species such as in motility and capacitation in bovine; however, their role remains unclear in other mammals. For instance, depletion of *Bsph1*/*Bsph2* in mice had no effect on fertility. In order to elucidate the specific role of BSP protein in humans (BSPH1), I sought to investigate a purification method to produce functional human BSP protein, as these proteins are only present in minute amounts in the human epididymis. Following purification of BSPH1, I carried out *in vitro* experiments and sought to identify its protein interaction network. BSP proteins have been shown to interact with pseudo-choline groups such as diethylaminomethyl through affinity rather than ionic interactions. Diethylaminoethyl is positively charged and therefore is a weak anion exchanger, but BSPs interact through affinity to this resin. This study presents a new, rapid and cost-effective purification method that provides recombinant BSP proteins of a high purity level, which can be used to study their roles in mammalian fertilization. We showed that pre-incubation of oocytes with recombinant BSPH1 can decrease fertilization rate in a dose-dependant manner. Sperm were also preincubated with anti-BSPH1 antibody and showed a decrease in fertilization rate. Secondly, I used BioID (proximity-dependent biotin identification), coupled with mass spectrometry to identify the protein-protein interaction network of BSPH1 by proximity labeling. Mass spectrometry results showed an interaction between BSPH1 and all subunits of the CCT/TRIC complex (Chaperonin containing tailless complex polypeptide 1 (CCT) or tailless complex polypeptide 1 ring complex (TRiC). This complex interacts with another complex called BBSome (Bardet–Biedl syndrome complex), which plays a role in protein trafficking through primary cilium. I also identified BBS proteins, as well as other proteins, that interact with the BBSome complex and regulate protein trafficking in the cilia.

BSPH1 also interacted with a large number of CEP (centrosome-associated proteins) family proteins, important in the formation of primary cilium through microtubules and centrosome maturation, which further support the potential implication of BSPH1 with the primary cilia.

Overall, this study demonstrates that BSPH1 may have a new role as a chaperone involved in protein trafficking through the primary cilia in cells that express it in the male reproductive system

Keywords: Male infertility, Protein purification, Affinity chromatography, Binder of sperm protein Epididymal proteins, proximity-dependent biotin Identification

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List of abbreviations

2H: 2-Hybrid

AEX: Anion-exchange

AlphaScreen: Amplified luminescent proximity homogeneous assay screen

AMP: Adenosine monophosphate

ApoA-I: Apolipoprotein A-I

ARIC: Acrosome reaction ionophore challenge

ATP: Adenosine triphosphate

BCA: Bicinchoninic acid

BioID: Proximity-dependent biotin Identification

BioSITe: Biotinylation site identification technology

BirA: Biotin ligase

BLI: BioLayer interferometry

B-PER: Bacterial protein extraction reagent

BSA: Bovine serum albumin

BSP: Binder of Sperm

BSPH: Binder of sperm homologue

CA: Carbonic anhydrase

CALR: Calreticulin

CASA: Computer-assisted semen analysis

CD: Circular dichroism spectroscopy

CEX: Cation-exchange

CLU: Clusterin

CM: Carboxymethyl

CRISP: Cysteine-rich secretory protein

Cryo-EM: Cryo-electron microscopy

DEAE: Diethylaminoethyl

DNA: Deoxyribonucleic acid

Dox: Doxycycline

E. coli: Escherichia coli

EDTA: Ethylenediaminetetraacetic acid

ELIZA: Enzyme-linked immunosorbent assay

Fn2: Fibronectin type 2 domain

FRET: Förster resonance energy transfer

FSH: Follicle-stimulating hormone

GAG: Glycosaminoglycan

GnRH: Gonadotropin-releasing hormone

GST: Glutathione S-transferase

HDL: High-density lipoproteins

His: Histidine

HPLC: High-pressure liquid chromatography

ID: Identification

IEXC: Ion exchange chromatography

IgG: Immunoglobulin G

IMAC: Immobilized metal affinity chromatography

IPTG: Isopropyl-D-thiogalactopyranoside

ITC: Isothermal titration calorimetry

IVF: In vitro fertilization

KDa: Kilodalton

LB: Luria-Bertani

LDL: low-density lipoprotein

LH: luteinizing hormone

LOCI: Luminescent oxygen channeling assay

MBP: Maltose binding protein

MWCO: Molecular weight cut off

Ni: Nickel

NMR: Nuclear magnetic resonance spectroscopy

PC: Phosphatidylcholine

PDL: Proximity-dependent labeling

pH: Potential of hydrogen

PLA2: Phospholipase A2

PPI: Protein-protein interactions

Prss37: Serine protease 37

PVDF: Polyvinylidene fluoride

RIFS: Reflectometric interference spectroscopy

ROA: Raman optical activity spectroscopy

ROS: Reactive oxygen species

SAXS: Small Angle X-ray Scattering

SCD: Sperm chromatin dispersion

SCSA: Sperm chromatin structure assay

SDS PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEC: Size exclusion chromatography

SPAM1: Sperm adhesion molecule 1

SPR: Surface plasmon resonance spectroscopy

TCA: Trichloroacetic acid

Tet: tetracycline

TEX101: Testis expressed 101

Tris-HCL: (hydroxymethyl) aminomethane.Tris-hydrochloride

Trx: Thioredoxin

UV: Ultraviolet

WHO: World Health Organization

ZP: Zona pellucida

To my mother who was always there

To my father who always encouraged me to go on adventures, especially this one

To my siblings for all their courage in every aspect of life

To my cat although he cannot appreciate it

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Chapter 1 – Introduction

In this chapter, I will discuss fertilization and infertility with a focus on male infertility, its genetic causes, sperm morphology, spermatogenesis and sperm maturation.

1.1. Fertilization

Fertilization is the process that requires the combination of a sperm and an oocyte to create a diploid zygote. Fertilization occurs in the ampulla segment of the oviduct. Sperm-egg interaction and compartmentalization is depicted in Figure 1.

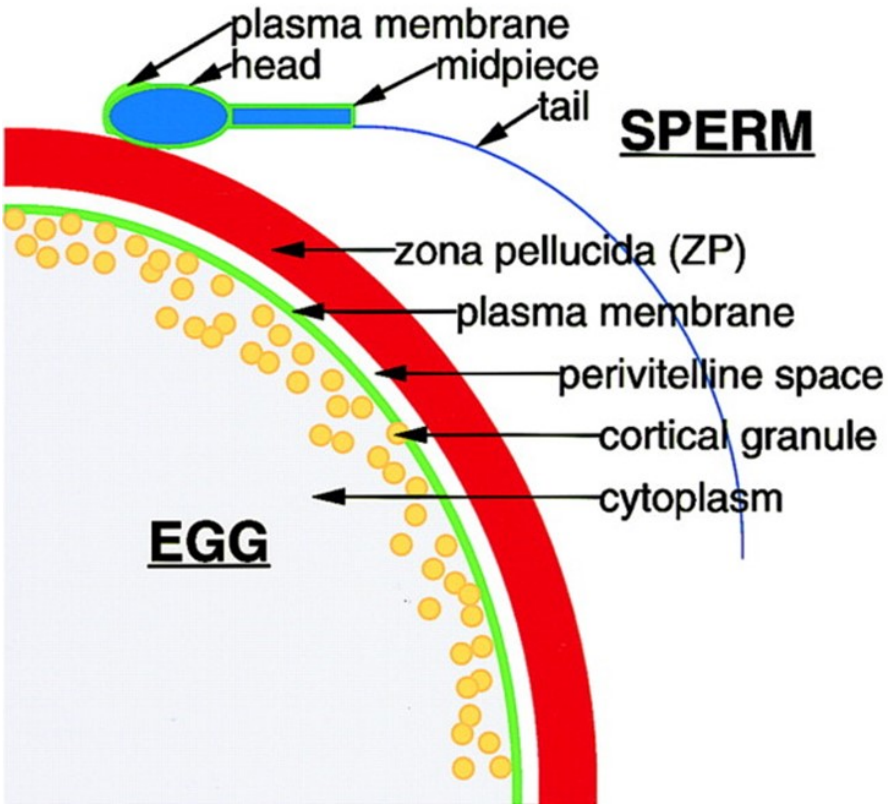

Figure 1. – Figure 1. Overview of sperm-egg interaction and compartmentalization

1.2. Infertility

According to the World Health Organization (WHO), infertility is defined as a couple's inability to conceive after one year of regular unprotected intercourse [1]. When there is no pregnancy with

the same partner, it is called primary infertility or secondary when there has been a previous pregnancy [2].

1.2.1. Male infertility

Infertility affects 15% of the general population [3] and affects their quality of life in many aspects such as psychological stress and general health. Infertility is associated with global public health as it affects more than one person and influences their welfare. A large proportion of research on infertility has been focused on female aspects, however, male infertility is responsible for half of the infertility cases [4], indicating the importance of this work presented in this thesis. Deficiency in spermatogenesis, sperm transportation, and sperm delivery are the leading causes of male infertility [10]. To date, the most common assessment for male infertility is a semen analysis test; however, normal results from standard semen analysis does not guarantee a future healthy child. Furthermore, an abnormal semen analysis test does not indicate infertility [5]. Several studies have linked poor sperm quality to lifestyle, weight, alcohol usage, smoking, diabetes, and hormonal diseases for instance. In many infertility cases, the precise reason remains unclear; hence, it is called idiopathic infertility, which could be congenital or acquired. About 15% of idiopathic male infertility relates to genetic causes [6], including chromosomal aberrations, Y chromosome microdeletions and point mutations. Over 3000 genes are implicated in spermatogenesis; however, their exact functions in this process are not all defined. Next generation sequencing advancements helped to partially unwind this dilemma by finding gene mutations in the corresponding patient's group [2]. Moreover, the implication of alterations in epigenetic modifications and their association with male infertility has been reported in several studies [7]. Genetically, microdeletions in the azoospermia factor (AZF) region on the Y chromosome are recognized as the most recurrent genetic abnormalities in male infertility, with some of these deletions impairing spermatogenesis [8]. A number of autosomal genes mutations have been identified as underlying factors of male infertility, such as SYCP3, KLHL10, SPATA16 and SEPT12, which are associated with azoospermia, oligozoospermia, globozoospermia and oligoasthenozoospermia, respectively [9].

1.2.2. Clinical assessment of male infertility

The primary evaluation of male infertility is restricted to physical examination and semen analysis, which consists of series of tests that diagnose the quality and quantity of sperm (e.g., morphology,

motility, viability) as well as seminal plasma. Other parameters such as pH, fructose concentration, and agglutination are assessed in semen. Conventional semen parameters are illustrated in table 1. Nonetheless, when there are no obvious complications in these parameters, in-depth semen analyses can be performed for further clarification (e.g., DNA damage, reactive oxygen species, binding assays, sperm chromatin structure assay) [11].

Table 1. Word health organization (WHO) reference value for semen analysis [11]

Volume	2 mL or more
pH	7.0–8.0
Sperm concentration	15 million or more/mL
Total No. of spermatozoa	39 million or more spermatozoa/ejaculate
Motility	40% or more progressive motility or 32% (a+b) (within 1 hour after ejaculation)
Morphology	4.0% or more (normal forms)
Viability	58% or more live spermatozoa
Leukocytes (10^6/mL)	<1.0
Mixed antiglobulin reaction	Less than 50% spermatozoa with adherent particle

Source: WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 5th edition. Geneva (Switzerland): World Health Organization; 2010.

1.2.2.1. Sperm morphology

Sperm cells consist of three major sections: head, neck and tail. Any irregular manifestation (head, midpiece or tail abnormality) in each of these parts can lead to a decrease in semen quality. Several articles show the correlation between abnormal sperm phenotype and poor *in vitro* fertilization (IVF) outcomes due to reduced fertilizing ability. Morphological defects, such as globozoospermia (round head) or stunted tail (short tail sperm), could result in male infertility [12, 13]. A schematic of mammalian spermatozoa is presented in Figure 2.

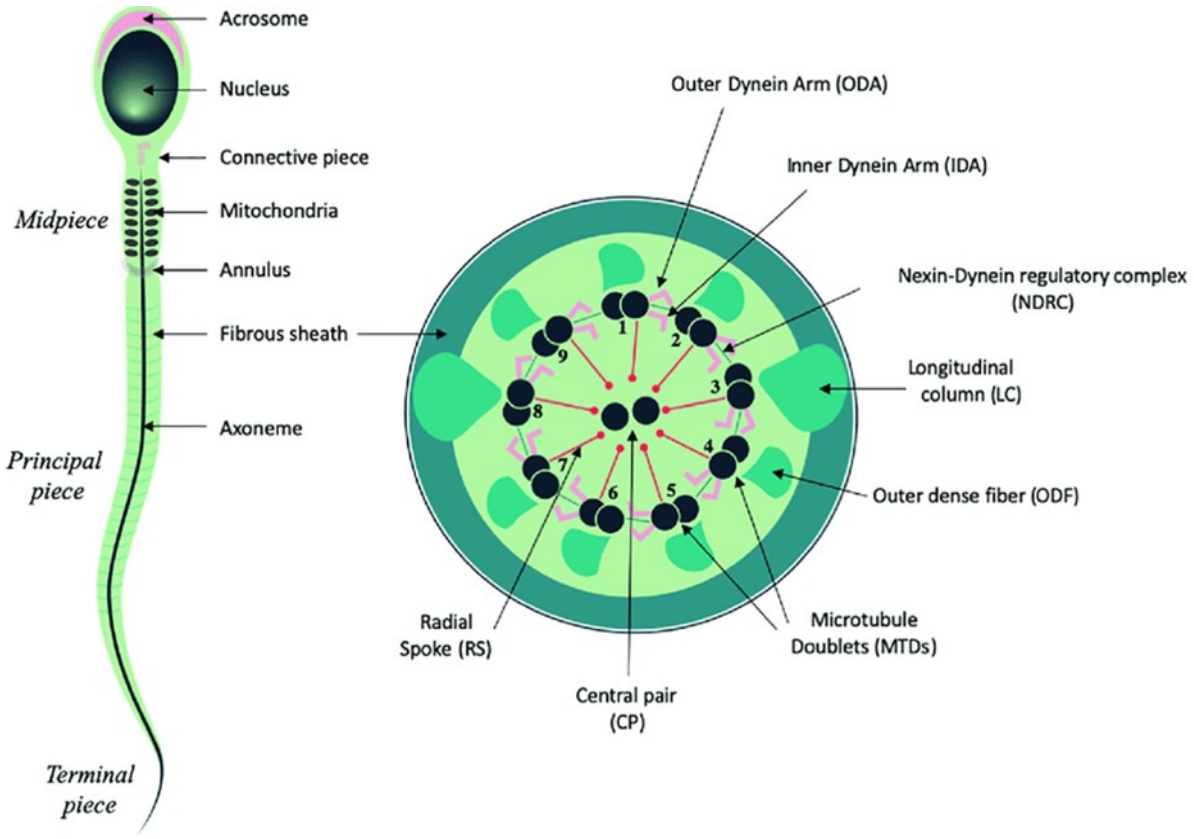

Figure 2. – Mammalian spermatozoa representation [14]

1.2.2.2. Sperm motility

Sperm cells generate different motility patterns, such as linear progressive motility and hyperactivation. Computer-assisted semen analysis (CASA) was designed to generate a digital image by measuring the movement of the sperm head. Hyperactivation is another form of sperm movement, which is the hallmark of capacitated spermatozoa and aids spermatozoa to penetrate the zona pellucida. It is defined by the high amplitude and asymmetrical movement of flagella [11]. The main reason for disabled motility is the presence of reactive oxygen species (ROS) in seminal plasma [15]. Sperm with DNA fragmentation have a negative correlation with sperm motility, but also with concentration and normal morphology [16]. In addition, aging reduces sperm motility and is associated with reduced fertility [17].

1.2.2.3. Acrosomal integrity

The acrosome is the large enzymatic vacuole present over the head of the sperm. It contains several hydrolytic enzymes that, when secreted, enable the sperm to penetrate the zona pellucida of the egg [18]. Many studies showed that there is a correlation between reduced numbers of

acrosome reacted spermatozoa and male infertility [11]. The acrosome status is another criterion used to assess male fertility and can be evaluated by inducing the acrosome reaction with the ionophore challenge test (ARIC). In the ARIC test, calcium ionophore A23187, a mobile ion-carrier, is used to elevate intracellular Ca2+ and induce the acrosome reaction in intact sperm cells, after which one can determine levels of complete or incomplete acrosome reaction [11].

1.2.2.4. Reactive oxygen species

In the male reproductive system, there should be a balance between ROS production and antioxidant scavengers. Previous studies showed that a low concentration of ROS is essential for sperm capacitation (detailed in section 2.5), which is the second step in the sperm maturation process. Elevated intracellular levels of ROS generate oxidative stress, which produces free radicals that can attack the DNA molecule and impact sperm DNA integrity by causing breaks in the DNA strands. Oxidative stress can also impact sperm motility and cause membrane lipid peroxidation, which is associated with loss of sperm membrane function and integrity [11]. Chemiluminescence assays are used to measure ROS activity in semen. However, the accuracy of this method for the measurement of different types ROS such as superoxide anions or hydrogen peroxide is unclear [11]. Another way that high levels of ROS could lead to infertility is by triggering the ROS-induced imbalance of male reproductive hormonal profiles through effects on the hypothalamus-pituitary-gonadal (HPG) axis [19].

1.2.2.5. Sperm DNA damage

In males, research studies showed that approximately 40% of idiopathic infertility is related to chromatin fragmentation [21]; which can originate from various events. As mentioned above, oxidative stress can cause DNA damage and could lead to subfertility [21]. DNA damage in spermatozoa can also occur via errors in meiotic recombination, infection and xenobiotic exposure [22]. Unfortunately, there are few repair mechanisms in the cell to counteract the effects of DNA damage. To detect sperm DNA damage, methods such as the sperm chromatin structure assay (SCSA), sperm chromatin dispersion (SCD) or halo test, tunel assay, comet assay, and sperm aneuploidy screening are usually used [23]. Patients with a 30% DNA fragmentation index are considered infertile. Although both IVF and spontaneous pregnancies can proceed in the presence of high levels of DNA damage in sperm, this significantly increases the risk of miscarriage [24]. Sperm DNA is highly condensed in a chromatin format by protamines, which are positively charged DNA-binding proteins. Although less abundant, nucleosomal histone proteins are present in sperm

chromatin and the DNA associated with this structure is more vulnerable than protamine to oxidative stress [24]. Importantly, regions of the sperm genome composed of histones overlap genes that are essential for development (e.g., imprinting genes) [24].

2. Overview of the male reproductive tract

The reproductive tract consists of primary organs (testis) and secondary organs (epididymis, prostate gland, seminal vesicles, bulbourethral glands, vas deferens and penis) (Figure 3). Reproductive organs originate from the embryonic mesoderm and the male reproductive organs develop from the Wolffian duct [25].

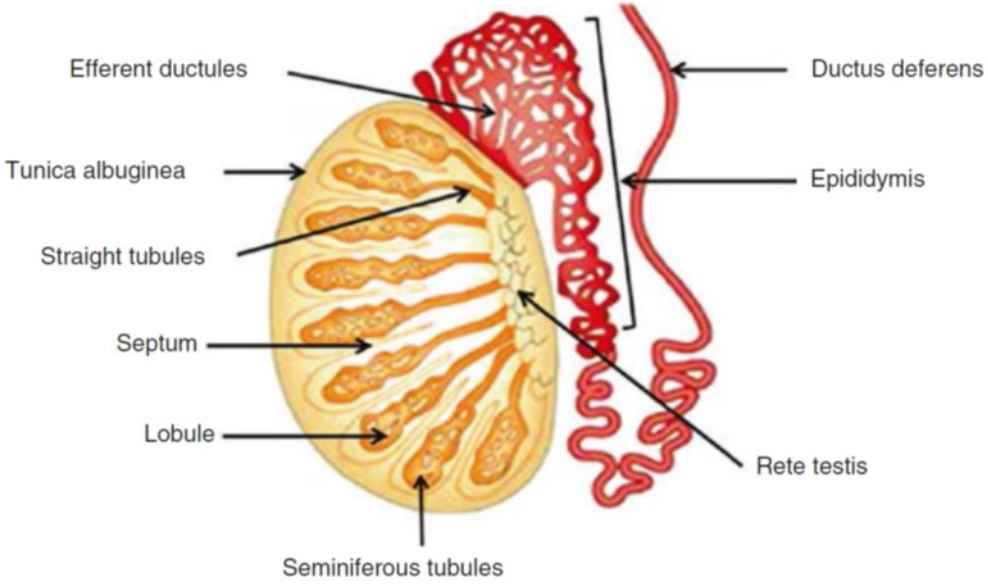

Figure 3. – Schematic representation of testis, epididymis and vas deferens [25]

2.1. Germ cells and spermatogenesis

Spermatogenesis is the process through which sperm cells are produced from spermatogonia stem cells in the seminiferous tubules. The entire spermatogenesis process takes about 64 days in humans and 34 days in mice [25]. The goal of spermatogenesis is to transfer genetic heritage to the next generation by producing unique and highly differentiated cells. During spermatogenesis, approximately 20 to 30 million sperm per milliliter of semen are produced. Spermatogenesis is controlled by over 3000 genes that are mostly located on autosomal chromosomes, with only a few on the Y chromosome. We know that follicle-stimulating hormone (FSH) and testosterone are responsible for the initiation of spermatogenesis during young adulthood [26]. In the seminiferous tubules of the testis, spermatogonia stem cells (germ cells) divide mitotically and provide two

types of cells, type A for restoring stem cell lineage, and type B for cells that differentiate into primary spermatocytes. B cells divide into two secondary spermatocytes (Meiosis I). Eventually, secondary spermatocytes convert to haploid spermatocytes by Meiosis II. During spermatogenesis, sertoli cells (columnar shape) provide structural and nutritional support for germ cells as indicated in Figure 4 [27]. Sertoli cells are also implicated in the phagocytosis and ingestion of residual bodies and the release of spermatids during spermiation. The Sertoli cells are numerically stable and unable to proliferate.

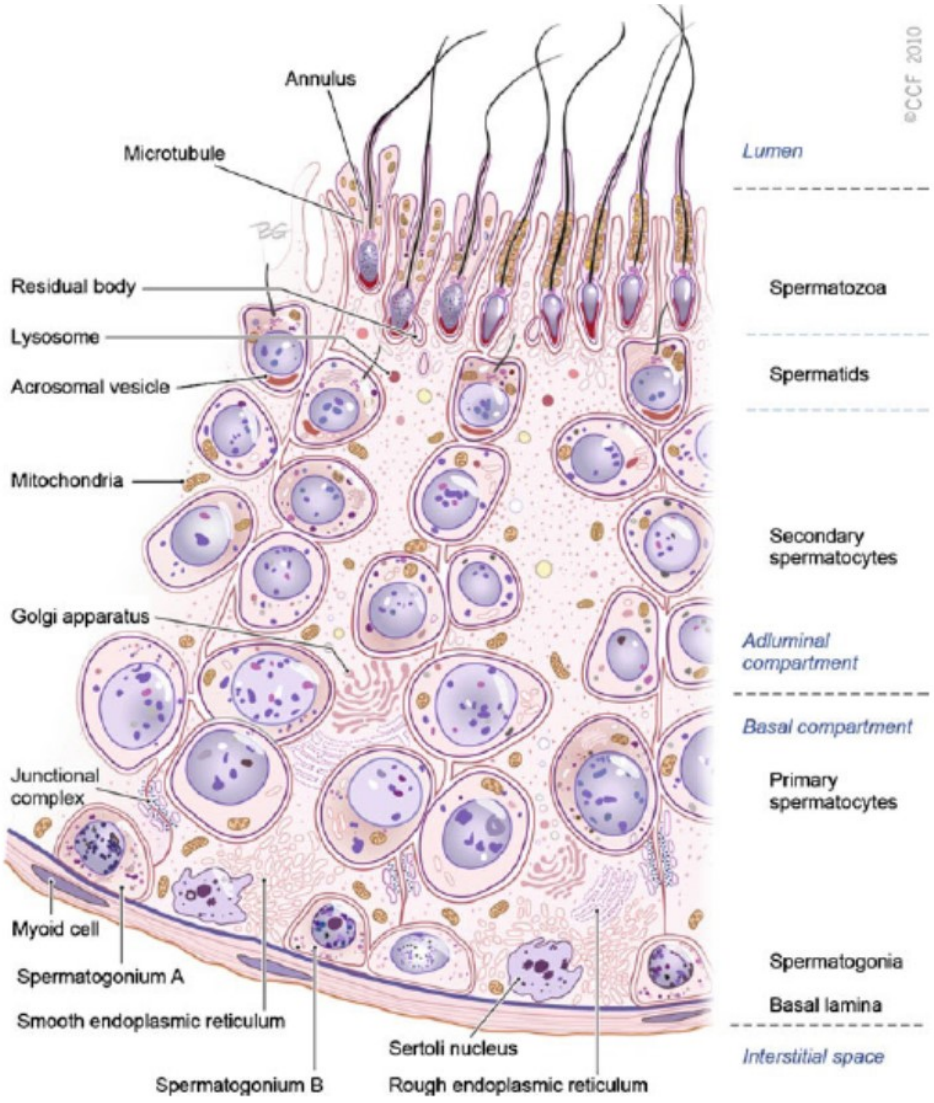

Figure 4. – Overview of the process of Spermatogenesis [28].

Spermatogonia undergo mitosis to create primary spermatocytes, or meiosis to become secondary spermatocytes. Then, a second meiosis converts secondary spermatocytes to spermatids, which will become spermatozoa following the process of spermiogenesis.

2.2. Hormonal regulation of spermatogenesis

Normal spermatogenesis is under the control of the hypothalamic gonadotropin-releasing hormone (GnRH), which affects the secretion of Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior part of the pituitary gland. LH stimulates testosterone production from the interstitial cells of the testes (Leydig cells), while FSH stimulates Sertoli cells and regulates their proliferation as is shown in Figure 5 [29,30]. The secretion of FSH and LH is controlled by negative feedback. Sertoli cells are nursing cells and provide nutrients for germ cells. They also produce inhibin protein when the quantity of sperm is high, which has a negative effect on GnRH/FSH secretion. Likewise, testosterone has the same effect on GnRH/LH when the concentration in blood is too high [31]. Testosterone is one form of androgen in seminiferous tubules, without which spermatogenesis would not be continued after meiosis. The absence of androgen in the testis would lead to loss of blood-testis barrier cohesion, and mature spermatids could not develop their final elongated shape [32]. Testosterone is also essential for the maintenance of spermatogenesis [33]. Testosterone can also be converted into dihydrotestosterone, which is a more active form of androgen [34]. The androgen receptor consists of a variable N-terminal domain, DNA binding domain, ligand binding domain and C-terminal domain. The androgen receptor is mapped on the X chromosome and is present in a variety of tissues such as bone, muscle and prostate [34].

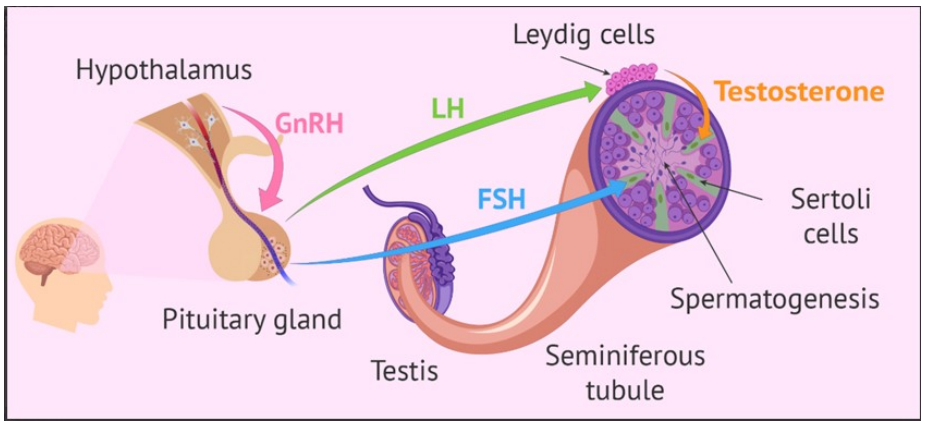

Figure 5. – Hormonal regulation of spermatogenesis [35].

The gonadotropin-releasing hormone affects the anterior pituitary gland, which releases LH and FSH. These hormones affect different cells in the testis and regulate spermatogenesis as well as secondary sexual characteristics.

2.3. Seminal plasma

The seminal plasma acts as a vehicle to simultaneously carry, protect and nourish sperm cells. The accessory glands (i.e., seminal vesicles, prostate gland, and the bulbourethral glands) are responsible for the production of the seminal plasma [36]. The seminal plasma components include DNA, RNA, microRNA, lipids and proteins, as well as different ions such as zinc, which can act either and an inhibitor or cofactor of many enzymes implicated in sperm coagulation and liquefaction. Moreover, the seminal plasma contains proteins (membrane proteins) that originated from the spermatozoa, such as TEX101 [37]. Defects in TEX101 were shown to be related to spermatogenic problems such as azoospermia and male infertility [38]. Proteomic studies using seminal plasma from fertile versus infertile groups revealed different protein expression patterns between groups. For instance, the seminal plasma from asthenozoospermic (reduced sperm motility) patients showed differential expression of proteins required for sperm motility such as AKAP4, GAPDHS and ODF2 [39,40].

In addition to the direct impact of the seminal plasma on sperm, this fluid has also been shown to stimulate the immune system of the female reproductive tract. The concentration of some proteins such as interleukins, cytokines and prostaglandins, is higher in seminal plasma than in blood, suggesting a possible role of seminal plasma in regulating the female immune system [41]. Other signs of paternal impacts on maternal immunity are the presence of antigens that are carried by sperm cells or expressed on leucocytes. For example, it has been shown that mouse sperm can express class I and class II MHC (major histocompatibility complex) [42]. In addition, some antigens are common to sperm and seminal plasma as well as to trophoblasts cells such as MHC class I and CD46, which suggest the importance of paternal immune cells in the occurrence of normal implantation (embryo attachment to the uterus) [43].

The uterus produces myeloid cells, specifically neutrophils and macrophages, when exposed to seminal plasma. These immune cells can produce antigens called matrix metalloproteinase (MMPs), which can alter the uterus environment by rebuilding extracellular matrix and new blood vessels and consequently, provide a unique surrounding for embryo implantation [44].

We know that sperm from epididymis or washed ejaculated sperm are sufficient for fertilization but might compromise outcomes due to the role of the seminal plasma in the uterine immune system [45]. Studies have shown that the seminal plasma can induce the expression of a series of

genes related to leukocytes and cytokines, and immune tolerance in the human cervix [46]. It was also shown *in vitro* that decidualization (endometrium changes for the acceptance of the embryo) speeds up in the presence of seminal plasma [47].

Interestingly, the seminal plasma can also act on the maternal immune response to the fetus and influence outcomes such as implantation or miscarriage. For example, one component of the seminal plasma, TGFβ, has three isoforms that are present in seminal plasma and responsible for proliferation and differentiation of different immune cells and induction of immune tolerance via Treg cells (regulatory T cells that regulate other immune cells) [48]. Prostaglandins are another component of the seminal plasma that are secreted from the seminal vesicles and are important for induction and control of inflammation during pregnancy [49]. Furthermore, CD38 (glycoprotein appeared on the immune cells) is an important component of seminal plasma involved in fetomaternal tolerance. Kim et al showed that CD38 regulates immune balance through the increased number of Treg and Dendritic cells [50].

Additionally, growth factors such as VEGF, EGF and FGF are also components of seminal plasma. Many growth factor receptors are present in the uterine epithelium, supporting the fact that seminal plasma can contribute to the vascularization and decidualization of the endometrium [51]. The balance between seminal plasma factors and their interactions with endometrial cells is represented in Figure 6.

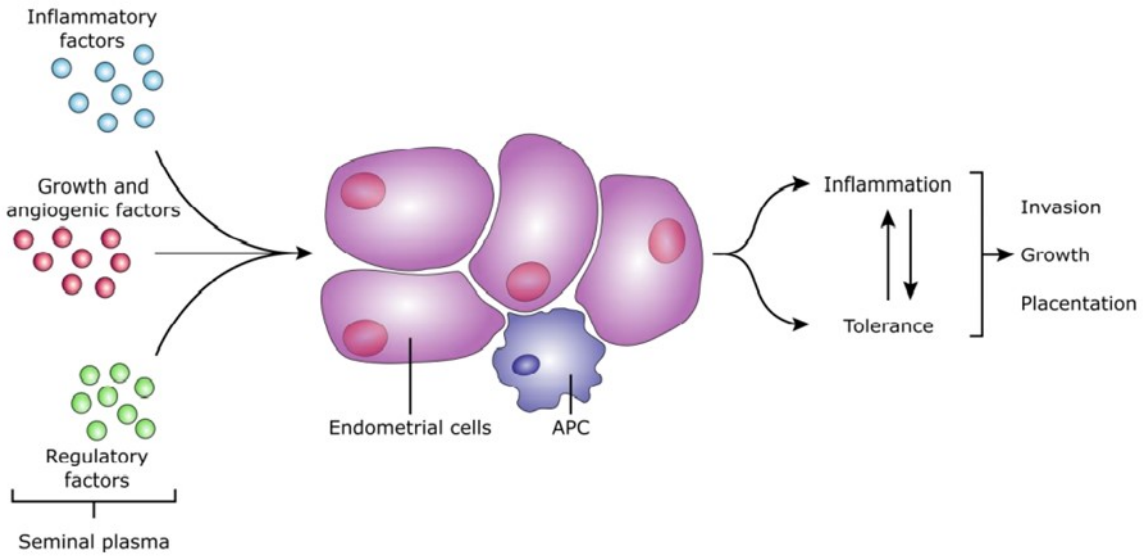

Figure 6. – Schematic representation of the balance between pro-inflammatory factors and tolerance inducing factors of seminal plasma encountering the female reproductive tract [52]

2.4. Seminal plasma and implantation

Implantation involves a form of maternal tolerance that allows a growing embryo to be accepted in the early stages of pregnancy (the blastocyst will attach to the uterus wall in order to receive nutrition and grow).

The conceptus (embryo and the extraembryonic layers) can produce and release cytokines and activate pathways related to implantation, including signalling by interleukins, VEGF and TGFB. These pathways are upregulated in normal pregnancies; however, studies have shown that some of these pathways are supressed in patients having undergone recurrent abortions.

Many factors can affect implantation, especially when assisted reproductive techniques are involved. It has been shown that the presence of seminal plasma can improve implantation rates following IVF treatments [53]. Studies show that the concentrations of proinflammatory cytokines are altered in the seminal plasma of partners of patients who experience recurrent miscarriages. For instance, the concentration of IL-1β is significantly reduced, while concentrations of interferon gamma (IFNY) is increased in the seminal plasma of these men [54]. It is not clear exactly which of the seminal plasma components are involved in implantation directly; however, some studies demonstrated that IL-18 promotes induction of IFNY and has a negative effect on successful pregnancy after IVF [55].

2.5. Seminal plasma and pregnancy complications

Preeclampsia is defined as high blood pressure after 20 weeks of pregnancy, accompanied by the presence of proteins in the urine due to the damage to organs such as kidneys [56]. The chance of having preeclampsia is higher in the first pregnancy. Studies have linked the risk of preeclampsia occurrence with the duration of vaginal exposure to seminal plasma [57]. Kho et al., also showed that women whose children had low birth weights had less cumulative exposure to seminal plasma [58]. Together these results suggest that the seminal plasma could play a role beyond the initial stages of pregnancy. Such studies also support the idea that exposure to partner antigens in seminal plasma would lead to fewer pregnancy complications.

2.6. Sperm and Seminal plasma RNAs

We know that sperm cells are transcriptionally and translationally inert and that most of the cytoplasmic content in RNA is removed during spermiogenesis. However, the entire population of RNA is not removed from sperm cells [59]. The amount of RNA, including coding and noncoding RNA, in a spermatozoa is extremely low, in the order of magnitude of 10 to 50 fg in human [60]. Recent studies demonstrated that sperm RNAs contribute to the early development of the embryo [59]. Seminal plasma, however, contains various types of RNA that originate from different sources such as prostate, epididymis and seminal vesicles. Most of these RNAs are released from exosomes and play an essential role in intracellular communications [61]. Communication between paternal RNAs and the male and female reproductive tracts is presented in Figure 7.

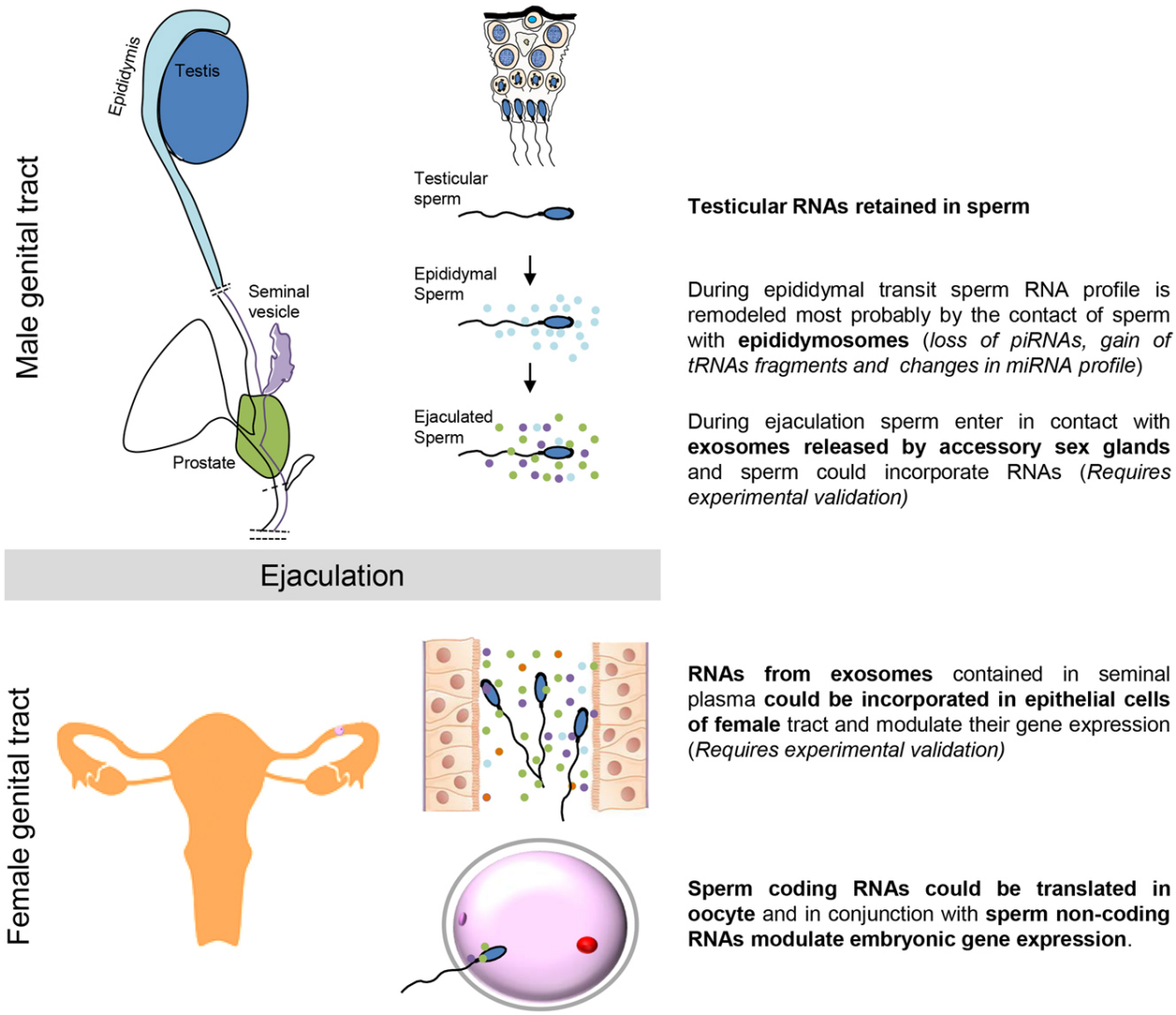

Figure 7. – Schematic presentation of paternal RNAs in the male and female reproductive tract [59].

2.7. Epididymis

2.7.1. Anatomy of the epididymis

The epididymis connects the testicles to the vas deferens. This narrow and convoluted tube is the longest in the mammalian species (6 meters in human). The epididymis is subdivided into three segments: proximal caput, corpus, and cauda [62]. It contributes to the transport, concentration, maturation and storage or spermatozoa. The sperm cells travel from the caput to the cauda in about 2 to 4 days in human. The epididymis is divided into segments, the number of which differs between species (e.g., 10 in mouse, 19 in rat) [63]. The epididymis consists of different types of

cells, which include the principal, basal, apical, narrow, dendritic and clear cells (depicted in Figure 8 [68]). Principal cells are the most abundant and constitute up to 80% of the total epididymal cell population. They are responsible for the majority of proteins secreted into the epididymal lumen. Basal cells represent 10 to 20% of the epididymal cell population, and their function remains unclear. Apical cells represent 5% of the cell population and are responsible for the luminal pH (alkaline) through production of carbonic anhydrase (CA) family enzymes. These narrow cells are involved in pH control and degradation of some proteins within their lysozymes. The apical cells and the narrow cells are different from each other in their distribution and structure. Both apical cells and narrow cells are found only in the proximal segment of the epididymis, where the sperm gain motility and fertilizing ability. Apical cells are more abundant in the initial segment, whereas narrow cells are restricted to the intermediate segment of the epididymis. The presence of CA II (enzyme that regulates homeostasis and reversible regulation of CO_2 to HCO_3) in narrow cells, and not apical cells, was first revealed by immunohistochemistry and suggested their capability of regulating the luminal pH [64]. In addition, the presence of cathepsin D and beta-hexosaminidase A (proteases) in both apical and narrow cells suggest that they might be involved in the removal of some proteins from epididymal lumen to lysosomes [64]. Hermo et al showed that CA II, III, IV, XII, and XIV were present in the rat epididymis, suggesting a potential role of CA in the regulation of luminal pH. CA III is expressed in principal cells, whereas it is documented that apical cells express CA IV [65], suggesting their specific ways in the regulation of luminal pH in different cells. The different isoforms of CA are distinct in their efficiency. For example, CA II is more efficient than CA III.

Clear cells comprise up to 5% of the epithelium and are distributed equally in the different epididymal segments and unreactive to different type of CA. Halo cells which originate from immune cells such as B and T lymphocytes, express CA III. Lastly, epididymal dendritic cells are responsible for the immunological tolerance of epididymal tissue and considered as the guardians of the epididymis. They can stimulate T cells and produce different cytokines [66]. They also recognize atypical sperm cells and induce immune balance [67].

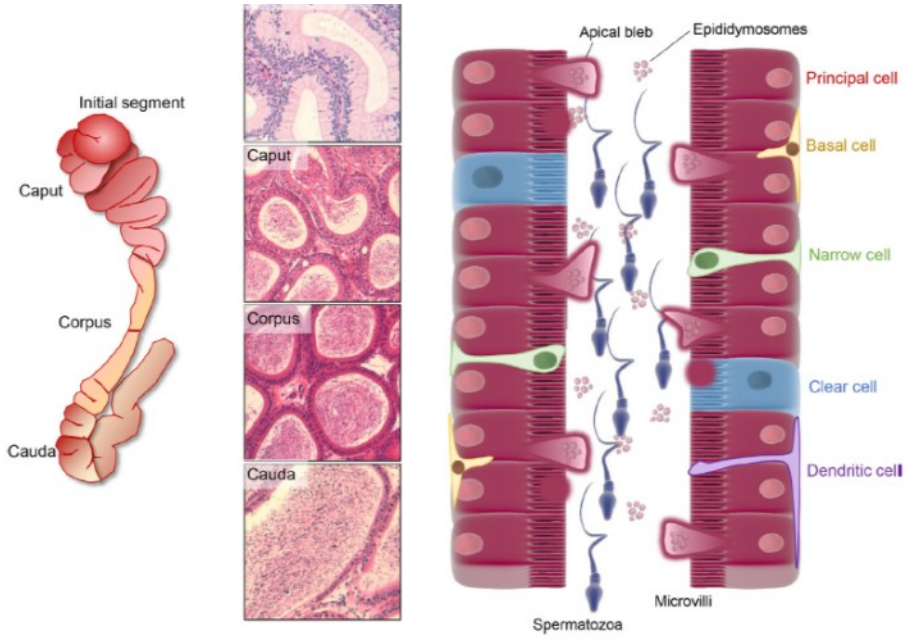

Figure 8. – Anatomy of the epididymis with the major cell types [69].

The Epididymis consists of different cells such as apical, basal, principal, clear and dendritic cells.

2.7.2. Epididymal epithelial secretions

There are two pathways of protein secretion in the epididymis: merocrine and apocrine secretion. The merocrine pathway is the primary method of protein excretion in principal cells to constitute the luminal milieu. It has been shown that the merocrine pathway facilitates protein secretion via the formation of vesicles that pass through the Golgi apparatus and are labeled with a signal peptide to be exocytosed. The apocrine pathway, as a complementary transportation system, works with epididymosomes (small membrane bound vesicles) [69]. Apocrine secretion occurs via the invagination of the plasma membrane, which creates a vesicle. Therefore, a portion of the cytoplasm is lost.

2.7.3. Sperm transportation

The human epididymal tube (6 meters) is relatively short compared to that of domestic animals (up to 50 meters), but it still takes 2 to 4 days for human sperm to transit the entire distance [70]. Movement of sperm through the epididymis is controlled by different sources such as contractions of the tunica albuginea of the testis and hydrostatic pressures produced by seminal plasma fluid [71]. Once ejaculated in the female reproductive tract, sperm transportation through the cervix takes 20 minutes to reach the uterus. Sperm swimming is considered to be a slow transportation

process, as such sperm transport mainly relies on uterine muscle movement, which is considered as fast movement [72].

2.8. Sperm maturation steps

2.8.1. Epididymal Maturation

For proper maturation of sperm, both phenotypical and biochemical events that alter protein expression and enzyme concentrations need to occur in precise segments of the epididymis. Interestingly, sperm maturation happens without gene transcription or protein translation in the actual sperm cell. The most important events include water reabsorption, as well as changes of the ion, microRNA and protein concentrations in the luminal fluid of the epididymis due to up- or down-regulation of gene expression in the cells composing the different segments. For instance, microRNA expression in mouse epididymis is constantly up and down regulated from caput to cauda epididymis. Mir-204 and mir196-b-5p are the examples of this turnover [69, 73]. The most abundant epididymal protein is clusterin (CLU), which originates from Sertoli cells [74]. Clusterin proteins are extracellular chaperones believed to be important in sperm maturation [75]. Sperm reaches its full functional maturity in the proximal segment of the epididymis, where it gains the ability to move, before being stored in the distal segment.

2.8.2. Epididymosomes and sperm maturation

Epididymosomes are small membrane bound vesicles that are secreted from the epididymal epithelium and have a substantial impact on sperm maturation by releasing new proteins and non-coding RNA in different segments of epididymis [76]. The protein composition of epididymosomes can vary depending on the epididymal segments, pH, temperature and zinc concentration [77]. Some of these proteins are like integral membrane proteins, and others resemble surface proteins [77]. Some epididymosomal proteins can have glycosylphosphatidylinositol anchors, such as HE5 (CD52) (human epididymis-specific Protein 5) in humans, and SPAM1 (sperm adhesion molecule 1) and hyaluronidase in mice, which are believed to play a role in sperm membrane organization [78].

The functions of epididymosomal proteins have yet to be clarified; however, the importance and role of a number of them has been determined. For instance, P26h/P25b (plasma membrane 26 hamster and plasma membrane 25 bovine respectively) plays a key role in zona pellucida binding

ability, whereas HE5/CD52 is implicated in immunological fertility, and ubiquitin plays a role in the removal of abnormal spermatozoa from epididymis [78]. Epididymosomes are also rich in sphingomyelin and cholesterol, which are two components of lipid rafts. Some epididymosomal proteins are found only in raft microdomains (insoluble fraction), such as P25b in bovine, and some are characterized in triton soluble segments such as aldose reductase. In addition, epididymosome associated proteins lack N-terminal signal peptides; therefore, they cannot migrate to the endoplasmic reticulum. They must be produced in the cytoplasm and transported to the epididymis via apocrine secretion [77].

2.8.3. Capacitation

Sperm capacitation was reported independently by Austin and Chang 70 years ago [79]. Sperm capacitation is the second part of the maturation process and happens in the female reproductive tract. Without sperm capacitation, fertilization would not occur.

Capacitation and acrosome reaction of sperm are two fundamental events that lead to fertilization of the oocyte. It is accompanied by several changes in sperm such as protein kinase phosphorylation, changes in membrane lipids and phospholipids and intracellular calcium uptake [80]. There are two capacitation events, fast and slow. The fast events are accompanied by the activation of the flagellum right after the sperm is released from the epididymis, while the slow events consist of the stimulation and invigoration of protein kinase A and tyrosine phosphorylation. Cholesterol removal by serum albumin and binder of sperm proteins (BSPs) are landmarks of slow events of capacitation that occur in the female genital tract [79].

Cholesterol efflux is the hallmark of capacitation. Cholesterol is described as a decapacitating factor because the removal of sterols like cholesterol by albumin or high-density lipoproteins (HDL) is considered the initiation of capacitation. Phospholipase A is responsible for the esterification of cholesterol, and lysophospholipids are the by-product of this enzymatic activity, which enhances membrane permeability to calcium [81].

2.8.4. Tyrosine phosphorylation

Tyrosine phosphorylation is another hallmark of capacitation, and it has been shown to occur in bovine, mouse, and hamster [81–83]. Phosphorylation is a post-translational modification that can take place on serine/threonine residues. It has been shown that the flagellum is the most significant sperm compartment that encounters tyrosine phosphorylation, and this modification is

required for sperm hyperactivation "(i.e., strong and non linear movement of sperm)" and cumulus cell "(i.e., outer cell layer of the oocyte)" penetration [84]. For instance, HSP-90 is a chaperone protein found on mouse sperm that gains tyrosine phosphorylation during the capacitation process [85].

2.9. Epididymal proteins

The generation of knock out mice opened new avenues to investigate the importance of epididymal proteins. Such an approach was used to define the role of CRISP (cysteine rich secretory protein) family proteins, a group of abundant epididymal glycoproteins. Removing (knock-out) CRISP1 in mice revealed that this protein was implicated in the sperm-to-zona binding and gamete fusion; whereas, a similar approach for CRISP4 showed that the protein is required for zona penetration and sperm-egg fusion. Mice with double deletion of CRISP1/CRISP4 displayed severe male fertility defects [86, 87]. As indicated in Figure 9, CRISP family proteins are implicated in a different stage of sperm functions. Another example of epididymal proteins is Sperm Adhesion Molecule 1 (SPAM1), also called PH-20, which is expressed in the testis and epididymis and has at least three roles demonstrated so far; hyaluronidase activity for cumulus penetration, and zona binding and acrosome reaction through calcium pathways [88].

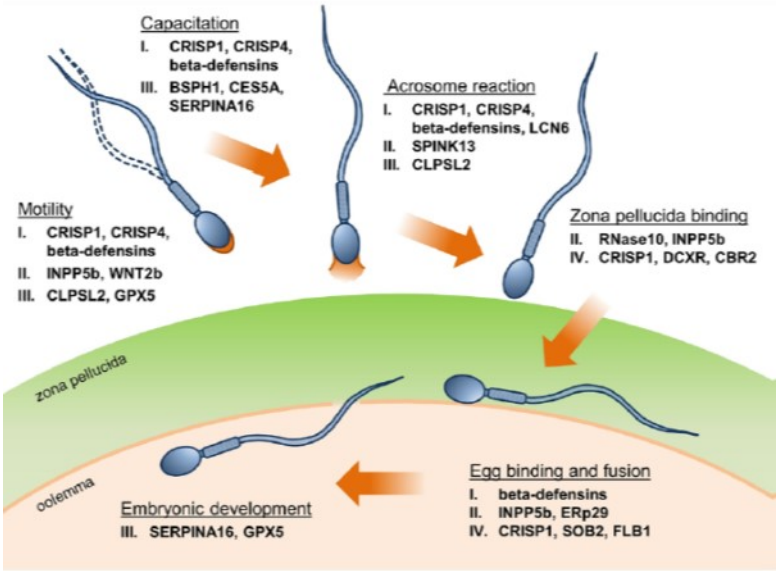

Figure 9. – Importance of epididymal proteins in the fertilizing ability of spermatozoa [89].

2.10. Primary cilia

Almost all cells have primary cilia (non-motile cilia) (i.e. cellular antenna) that control cell migration, differentiation, polarity, proliferation, cell signalling and homeostasis [90]. There are two types of cilia: motile and non-motile (Figure 10). Their structures are composed of microtubules that build up axoneme structures with an extension from the basal body and bordered by invagination of the plasma membrane [91]. The sperm flagellum is a solitary motile cilium (SMC). There are also various types of cilia in the male reproductive tract. Multiple motile cilia (MMC) are found in the efferent ductules, small tubules that connect the rete testis with the head of the epididymis. Non-motile primary cilia are also present in testis, epididymis and prostate [92]. Moreover, primary cilia are found to be associated with the basal cells of the epididymis [90].

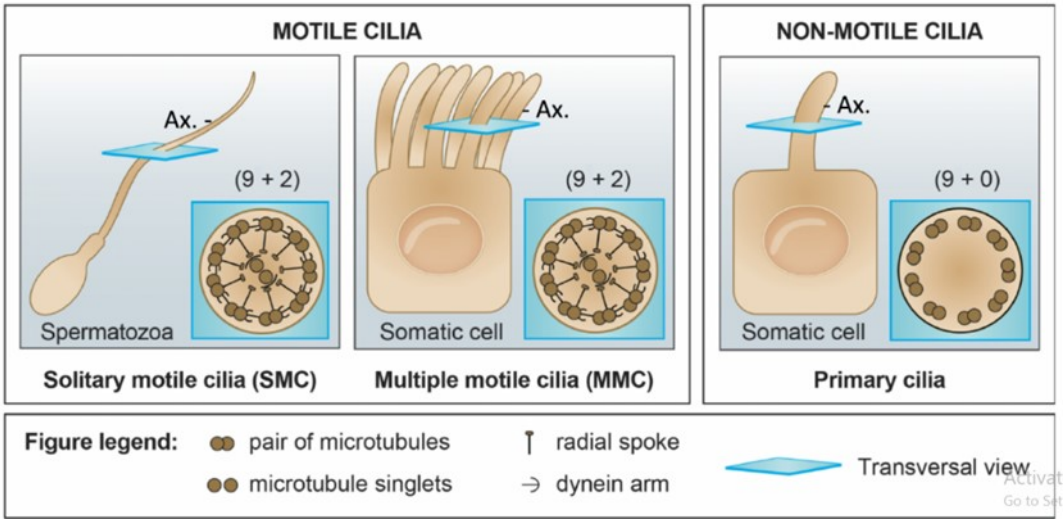

Figure 10. – Schematic representation of cilia [92].

The cilia are the invagination of the plasma membrane connected to the basal body, which consist of centrioles. This structure is important during mitotic division. In addition, the cilia are made up of axoneme (microtubule-based structure that shape the center of the cilium), which have a bidirectional flow of ciliary proteins and complexes. The cilia do not have ribosomes and depend on cytoplasmic produced proteins to grow. The movement of proteins from the basal body to the ciliary tip is called anterograde movement, and heterotrimeric motor kinesin-2 is responsible for this movement. The ciliary proteins retrograde from ciliary tips to the cells by means of dynein motors [93]. The primary role of cilia is related to the sense of smell as it has been shown that they play a role in olfaction and photoreceptions. Primary cilia impairment could lead to ciliopathies, which are multi-phenotype disorders [94]. Their importance is extended in Bardet-Biedl syndrome,

which is a multi-phenotype disorder. Patients with BBS are unable to smell and demonstrate retinal impairment. They also show other disabilities such as polydactyly, obesity and kidney disorders.

It has been proposed that hedgehog (Hh) and wingless-related integrin (Wnt) pathways are interrelated in primary cilia [95, 96]. This hypothesis comes from a study that showed that mutations in the gene regulating intraflagellar transport can cause defects in Hh signalling. Hedgehog signalling modulates cell proliferation and differentiation, and disturbance in Hh signalling can lead to cancer and embryo development defects [97].

Smo and patched proteins, which both are transmembrane proteins present in primary cilia, are regulators of hedgehog signalling pathways. Upon responding to Hh ligands on the patched receptor, Smo is transported to the primary cilia and patched is released from the membrane to the cytoplasm, which triggers downstream activation of the Gli transcription factor. This means that activation or repression of hedgehog signalling is totally dependent on primary cilia. It was also shown that the absence of primary cilia in mice and xenopus leads to the impairment of hedgehog signalling pathways [98].

Furthermore, the primary cilia also activate Wnt signalling. In contrast with hedgehog signalling, Wnt can activate several downstream signalling pathways that include β-catenin (canonical) or without β-catenin (non-canonical). The Dishevelled (Dsh) protein is important for both pathways; cytoplasmic Dsh is essential in the canonical pathway whereas plasma membrane Dsh is important in the non-canonical pathway [99]. A schematic explanation of hedgehog signalling through primary cilia is presented in figure 11.

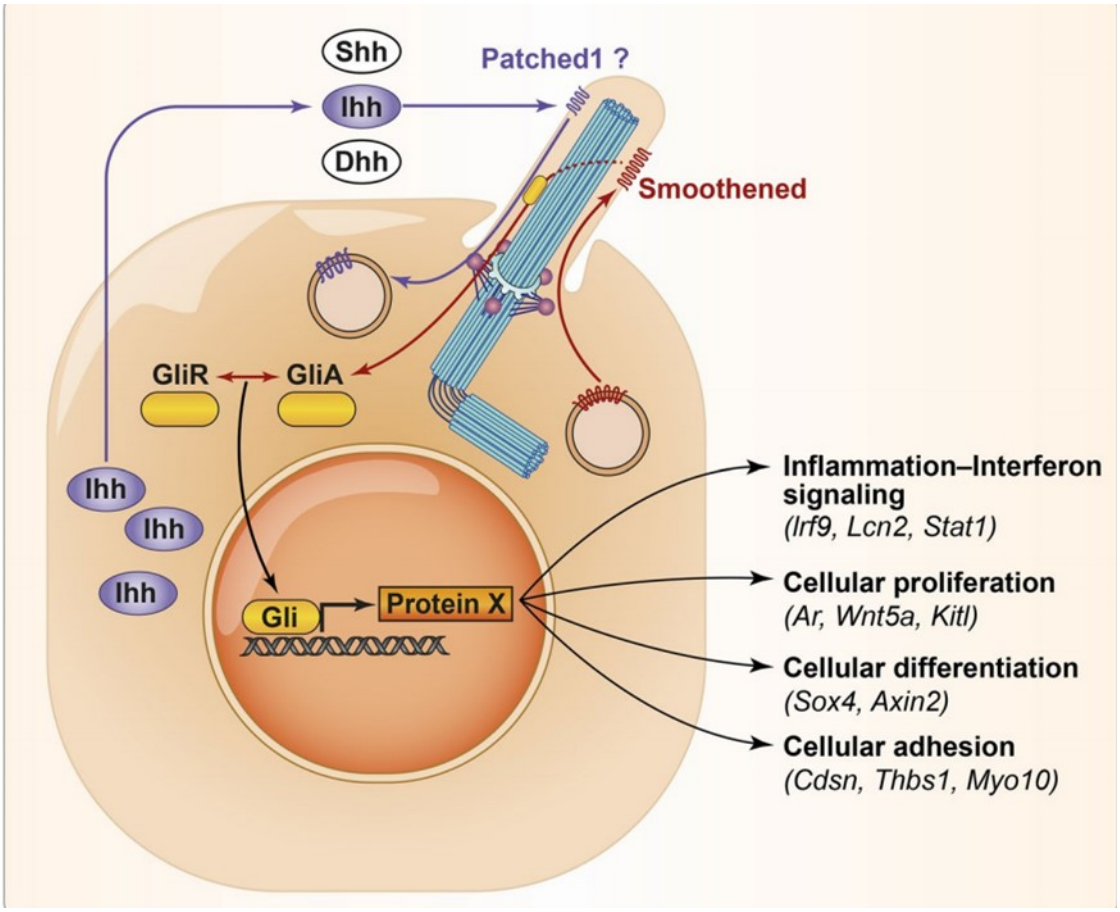

Figure 11. – Activation of hedgehog signalling through primary cilia.

In the presence of Hh ligand, the patched 1 receptor transfers to the cytoplasm, which triggers recruitment of the Smo receptor from the plasma membrane to the cytoplasm. This promotes the activation of the Gli transcription factor, relocation to the nucleus and downstream gene activation [92].

Another study by Girardet et al. demonstrated that when the primary cilia growth is inhibited by ciliobrevin D in the mouse epididymis, as well as *in vitro* study in DC2 cells, the expression level of proteins such as pathed 1 and smoothened also decrease, indicating regulation of the Hh signalling pathway by primary cilia [92]. In the same study, Hh was shown to regulate cellular proliferation, differentiation as well as immune response [92]. Moreover, primary cilia are important in other pathways such as Notch and growth factor signaling pathways [100,101].

Autophagy is also one of the homeostasis processes that can be regulated by the primary cilia in both selective and non-selective ways. For instance, the microtubule-associated protein 1A/1B-

light chain 3 (LC3) protein, which is a marker of the autophagy pathway, interacts with ciliary proteins such as pericentriolar material 1 (PCM1) and Oral-facial-digital syndrome 1 (OFD1). In addition, cilia formation and disassembly are regulated by complex autophagy pathways [102].

2.11. Ciliopathies

The primary cilia are about 5-10 µm long and can identify any perturbations in the environment, chemical or growth factors. Because of the presence of so many Ions channels in their structure, the primary cilia are important for calcium efflux and cell homeostasis [103].

Due to the near ubiquity of the primary cilia and their importance in organ development and homeostasis, defects in their specific proteins could lead to diseases in one or several organs. Disease related to primary cilia are considered as heterogeneous recessive disorders. Some of these ciliopathy syndromes include, Beside Bardet Biedl Syndrome (BBS), Meckel-Gruber Syndrome (MKS), Joubert Syndrome (JBTS), Alstrom Syndrome (AS), Nephronophthisis (NPHP) and Ellis-van Creveld Syndrome (EVS) [103].

2.12. Different roles of primary cilia

The primary cilia also exist in metazoans such as fruit flies and C. elegans, though limited to certain cells. Protozoa and unicellular eukaryotes also use cilia for different purposes such as sensing the environment and photoreception [98]. To be noted not all animals can be used as models to study cilia. For instance, yeast or Arabidopsis are depleted of primary cilia.

Another role of the primary cilia is to regulate cell cycles. The primary cilia disassemble before mitosis, and the basal body must reshape to the centriole. AURKA is the basal body associated protein that phosphorylates histone deacetylase. Blockage of this protein's activation could prevent cells from entering the s phase [104].

Since primary cilia are invaginations of the plasma membrane, they are important for cell shape and cytoskeletal formation. Although they are made of microfilaments, the ciliary spike has a different composition, including clathrins and actin, which contributes to ciliary stability [105].

The significance of primary cilia is explicit and therefore any mutation in genes that encode primary cilia or protein degradation of their cargo trafficking may result in serious disease.

3. Binder of sperm proteins

In this section I discuss of binder of sperm proteins and their known role in mammalian fertilization.

3.1. Binder of sperm protein (BSP)

The human BSP gene is mapped on the big arm of chromosome 19 (19q13.33). The final mRNA is composed of 7 exons, which encodes 132 amino acid with a molecular mass of 15693 Da (Protein Accession: Q075Z2) [106]. BSP is a secretory protein expressed only in the epididymis, with a trace in testis, therefore it is considered as tissue specific. In mice, there are two isoforms, *Bsph1* and *Bsph2*, which are located on chromosome 7 and the proteins have 16.2 KDa and made up five exons. Mouse BSP proteins are also exclusively expressed in the epididymis, in small quantities [107]. BSP proteins are expressed in the seminal vesicles in ungulates; however, in human and mice, they are expressed in the epididymis in very small quantities [110]. BSPs from different species with their specific characteristics are presented in Table 2.

Table 2. Comparison of BSP proteins in different species

	Place of expression	Invitro studies	Concentration	Properties	References
Human	Epididymis	Promote capacitation	Low	unknown	G. Plante et al, 2014
Mouse	Epididymis	Promote capacitation	Low	unknown	G. Plante et al, 2012
Bovine	Seminal vesicle	Promote capacitation	High	Sperm motility Sperm reservoir chaperonin	I. Therien et al, 2001
Boar	Epididymis	Does not Promote capacitation	Low	unknown	M. Lusignan et al, 2007
Rabbit	Epididymis	unknown	Low	unknown	B. Nixon et al, 2008

BSPs are small proteins that share a similar structure in across species including ram, goat, stallion, bison, rat, rabbit and human. These proteins are composed of an N-terminal domain and two fibronectin type-II domains (Fn2), linked by seven amino acids linker (each domain has two disulfide bonds) with a short C-terminal domain. Their N-terminal domain varies from one species to the other. The BSP structure is presented in Figure 12. The N-terminal domain of the rabbit protein consists of 380 amino acids, which is longer compared to other species [108]. One of the landmarks of BSP proteins is the C-X-F-P-F motif (X = valine or nonpolar residue), which is present

at the first cysteine of the Fn2 domain [109]. A post translational modification (O-glycosylation which helps cell adherence) is present in the N-terminal domain of some species, such as bovine. However, there is no O-glycosylation in humans and mice [109].

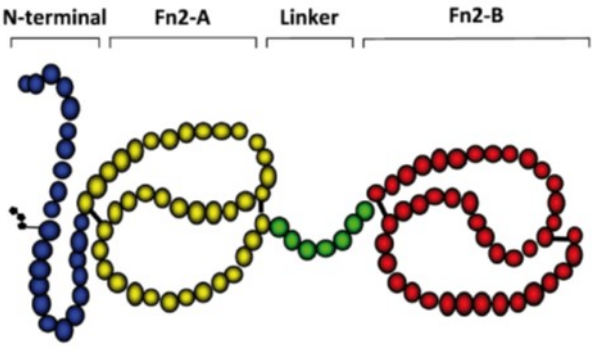

Figure 12. – Schematic presentation of the structure of the bovine BSP1 protein [108].

3.2. Binding properties of BSPs

BSPs have a propensity to bind to different ligands such as gelatin, heparin, phosphatidylcholine (PC), liposomes, low-density lipoprotein (LDL) and Diethylaminoethyl (DEAE) through their conserved regions (Fn2A and Fn2B). The use of recombinant proteins confirmed these binding studies as is shown in Table 3 [111].

Table 3. Interaction partners of epididymal BSP proteins [112]

		Gelatin	LDL	PC	Heparin	Chondroitin Sulfate B	Spermatozoa
Human	BSPH1	+	+	++	++	–	++
Mouse	BSPH1	+		++	++	–	++
	BSPH2	+			++	–	++
Rabbit	BSP1						++

+ or ++, binding; –, no binding; LDL: Low-density lipoproteins; PC: phosphatidylcholine.

3.3. Sperm motility

BSP1 in bovine can remarkably increase the motility of ejaculated spermatozoa in a dose-dependent manner, suggesting that this protein has a significant impact on sperm motility in this species [113]. In other species such as rabbit, BSP is localized only on the sperm flagellum. Since BSP is expressed in the corpus segment of the rabbit epididymis, it is added to sperm after they

gain progressive motility. Therefore, it does not seem to be implicated in motility in the rabbit species [114].

3.4. Sperm reservoir in the female reproductive tract

Sperm migration into the uterus is accompanied by the formation of a sperm reservoir in the initial part of the oviduct. This refers to the trapping of sperm by some carbohydrates of uterine epithelium before fertilization [115]. The ampulla–isthmus is considered as the location of the reservoir in mammalian species [116]. The sperm reservoir exists for at least three logical reasons. First, it diminishes the number of sperm that reach the ampulla (site of fertilization), therefore avoiding polyspermy (too many sperm penetrating the oocyte at the same time). Second, it allows the selection of the best candidate for oocyte fertilization. Third, it gives enough time for the sperm to be capacitated and hyperactivated, which prevents premature capacitation [116]. Some studies demonstrated a possible role for Bovine BSP in the formation of the sperm reservoir through their interaction with the oviductal epithelium that contains annexins (soluble, hydrophilic proteins that bind to phospholipids in a calcium-dependant manner) and were proposed to act as a BSP receptor that connects sperm to the uterus [117,118].

3.5. BSP impact on sperm cryopreservation

The procedure of storing and preserving sperm by freezing is called sperm cryopreservation, a process that can have a detrimental impact on sperm viability and quality. It is essential for those who want to undergo medical procedures such as radiotherapy or chemotherapy to undergo sperm cryopreservation in advance if they wish to have children in the future [119].

Seminal plasma secreted from the accessory glands and seminiferous tubules act as a carrier for sperm [120]. This fluid has supporting elements such as fructose, Ca+, alkaline buffer, and several proteins that maintain the viability of sperm. On the other hand, seminal plasma also contains detrimental components for sperm membrane integrity, such as decapacitation factors (cholesterol). These are proteins that can interact with cholesterol and phospholipids, such as BSP proteins. Decapacitation is the reversible process of capacitation, which inhibits premature capacitation. Decapacitation factors are sperm surface proteins that regulate the ability of sperm to fertilize an oocyte. Seminal plasm also contains inhibitors of motility (e.g., semenogelin). Therefore, the seminal plasma contains various components that can affect sperm preservation during the cryopreservation process. To remove the detrimental effects of BSP proteins,

cryopreservation protocols have used egg yolk and milk in their buffer. Because they bind to BSPs and prevent them from binding to the sperm membrane and promoting efflux [121]. However, when egg yolk is used as an extender, its properties can restore sperm competency. It has been shown that LDL (low-density lipoprotein) from egg yolk has a better effect than whole egg yolk [122]. In egg yolk, BSP protein interacts with LDL and this interaction prevents cholesterol efflux; therefore, allowing the sperm membrane to remain intact. When milk is used, proteins such as casein micelles, α-lactalbumin, and β-globulin have the same effect and can interact with BSPs and maintain sperm plasma membrane integrity. These interactions (BSP with LDL) are fast and stable even after the freeze-thawing of seminal plasma [123].

3.6. Localization of BSPs

In terms of their localization on the sperm membrane, BSP proteins can have different localization patterns depending on the species. According to Plante et al., 2014, localization patterns are different in mice and humans based on the sperm capacitation state. Mouse BSPH1 and BSPH2 were found on the anterior and post acrosomal regions of uncapacitated sperm and relocate to the equatorial segment following capacitation. In humans, BSPH1 is mostly localized around the neck and equatorial segment of the sperm head [111].

3.7. Sperm capacitation

Seminal plasma plays an essential role in the function and survival of sperm. One crucial factor of seminal plasma is cholesterol, which is present in high quantity and is known as an inhibitor of capacitation. This was tested by Cross (1996) by purifying seminal plasma using different chromatography steps [124]. Capacitation occurs with calcium loading in sperm, increasing the intracellular pH and protein tyrosine phosphorylation, and finally leads to acrosome reaction [125].

In addition to these factors, the female reproductive tract also contains sterol sulfatase, whose activity is higher in the endometrium compared to the oviduct. It is suggested that sperm membrane sterols could act as a ligand for sterol sulfatase activity of the uterus. Therefore, the female reproductive tract would help the transition of sperm cholesterol [126]. In bovine species, *in vitro* studies have shown that BSP proteins can promote cholesterol efflux in a dose and time-dependent manner; therefore, it is believed that BSP proteins participate in the alterations of sperm membrane that occur during capacitation [127]. BSP proteins in the epididymis cannot

capacitate sperm in the absence of high-density lipoproteins /glycosaminoglycans (HDL/GAGs) and until it reaches the uterus and encounters HDL/GAG and creates a cholesterol efflux.

In vitro studies demonstrated that human recombinant-BSPH1 (recombinant-BSPH1) promotes human sperm capacitation in a dose-dependent manner. However, BSPH1 has no effect on tyrosine phosphorylation or sperm motility parameters [128]. These results were consistent with previous studies in mice where they showed that mouse BSPH2 is non-essential for fertility [129], likely due to genetic redundancy (more than one gene is responsible for the effect) [130]. Recent studies showed that a double knock out mice model (mouse-*Bsph1/Bsph2*) displayed normal fertility, albeit a significant age dependent increase in the weights of male pups spanning days 6 and 21, and 6 weeks of age [131].

3.7.1. Role of BSPs in capacitation

One alteration that occurs during capacitation is a change in the net negative charge of the sperm membrane, which goes from significantly high levels during epididymal transit to low levels during capacitation. Epididymal maturation increases the negative charge of the sperm surface due to the absorbance of sulphoglycerolipids and sialoglycoproteins. However, during capacitation, the negative surface between the plasma membrane and the outer acrosomal membranes decreases due to the removal of sialic acid and sulfate residues [132].

The cholesterol and phospholipid ratio constantly changes during sperm maturation, and it decreases during capacitation [133]. GAGs can promote sperm capacitation in bovine [134]. In addition, HDL present in follicular fluid and whose concentration increases during the estrous cycle as well as during the follicle maturation phase can interact with BSP and alter the lipid composition of the bovine sperm membrane leading to capacitation [135].

Before capacitation, during sperm transport in the epididymis and particularly in the cauda segment, BSPs cover the sperm membrane and inhibit the membrane modifications that lead to capacitation. They also maintain sperm motility during epididymal storage [136]. Bovine BSPs, specifically BSP1, act as binding sites on the sperm membrane for choline phospholipids [137]. In addition, BSP1 prevents premature acrosome reaction by inhibiting protein kinase C activity [138]. Comparatively, BSPs in ungulates constitute around sixty percent of total proteins of seminal fluid and originate from seminal vesicles, whereas BSP proteins in humans and mice are found in low

concentrations and originate from the epididymis [139]. The proposed mechanism of BSP-potentiated sperm capacitation is shown in Figure 13.

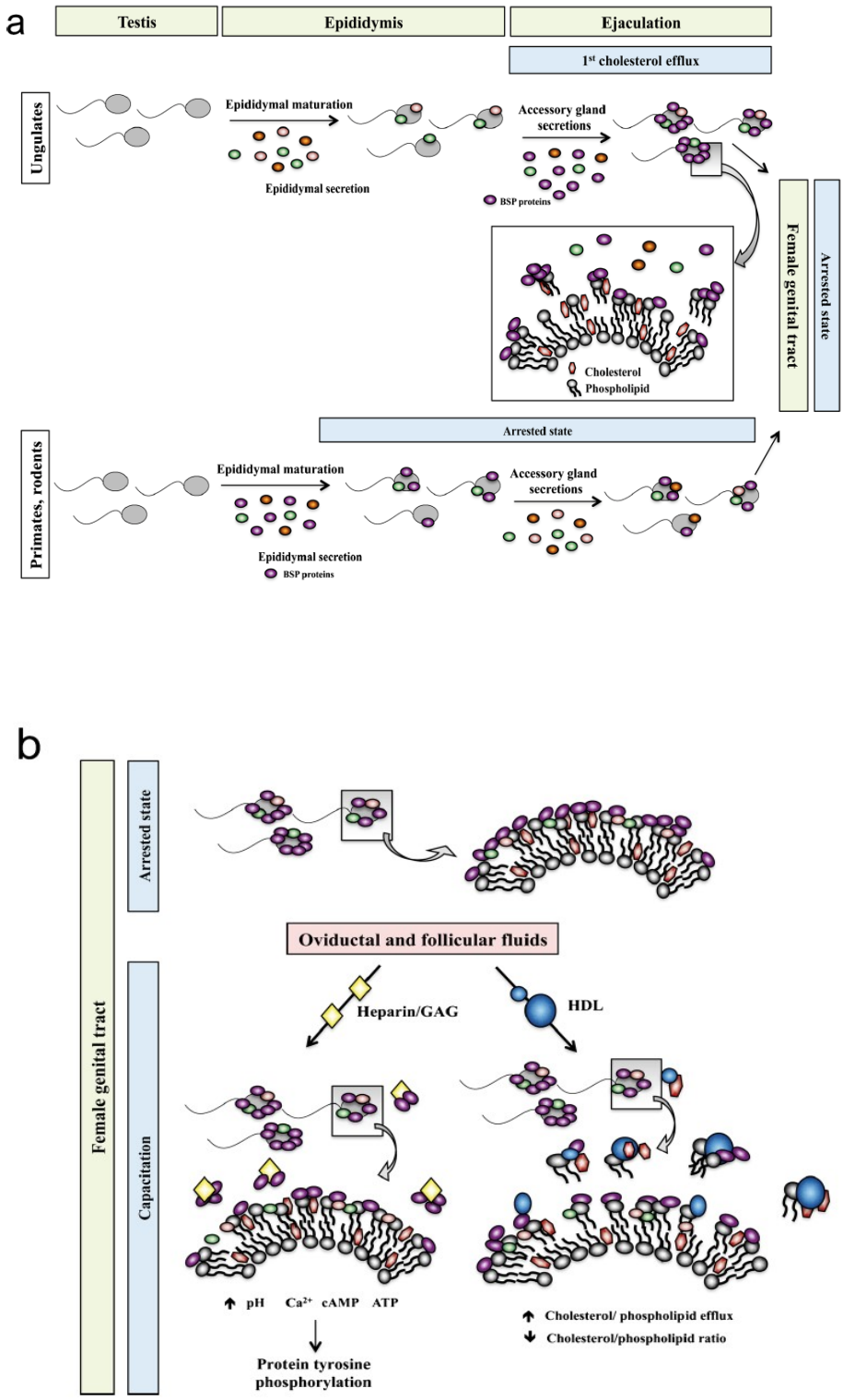

Figure 13. – Mechanism of BSP-induced sperm capacitation [108].

A) BSPs cover sperm membrane during epididymal transit and inhibit premature sperm capacitation. B). In the HDL-induced mechanism, cholesterol efflux happens when BSP proteins interact with HDL, a component of follicular fluid; however, heparin-induced capacitation occurs through protein tyrosine phosphorylation.

Lane et al. showed that bovine anti- BSP antibodies could inhibit capacitation induced by heparin in a dose-dependent manner [140]. The same type of experiment was carried out to demonstrate the essential role of HDL in capacitation, but no inhibition was observed, suggesting that high concentrations of HDL or low concentrations of anti-BSP antibodies were used in the experiment that HDL could not inhibit the capacitation. Since tyrosine phosphorylation is one of the hallmark events of capacitation, phosphorylation was also evaluated in the presence of heparin and HDL. An increase in tyrosine phosphorylation was observed with heparin-induced capacitation, while phosphorylation levels did not change with HDL [140]. According to the literature, tyrosine phosphorylation that occurs during capacitation is a slow event, which takes two to four hours. However, in other cell signalling pathways (e.g., cell proliferation, adhesion or migration), tyrosine phosphorylation occurs relatively rapidly, taking up to thirty minutes. Therefore, the mechanism of HDL-induced capacitation is different from heparin-induced capacitation and is independent of tyrosine phosphorylation.

3.8. BSP proteins and sperm-egg interactions

During fertilization, sperm-egg interaction is a species-specific phenomenon that starts with the release of chemoattractant by the egg. Most molecular aspects of sperm-egg interaction and fertilization are still unclear. Recently, a critical sperm surface protein (Izumo) was identified and shown to interact with the Juno receptor on the egg. This interaction is conserved among mammals and Juno knock out mice are infertile [141].

The oocyte has multiple layers such as corona radiata, perivitelline space, vitelline membrane and zona pellucida. Sperm-zona pellucida binding is one of the first steps in sperm-egg fusion. The zona pellucida is the glycoprotein layer of mammalian oocyte and comprises four major proteins: ZP1, ZP2, ZP3, and ZP4. They are considered as sperm receptors that bind to the capacitated sperm and trigger the acrosome reaction [142]. Zona pellucida interacts with protein from seminal plasma. Interestingly, Liberda et al. showed, using an enzyme-linked binding assay (ELBA), that BSP1 (PDC109) and BSP5 in bovine have an affinity towards mannose rich residue of zona pellucida saccharides [143].

Recently, a study by Hamed et al. displayed the possible role of recombinant mouse BSPH1 in sperm-egg interaction using zona intact oocyte. Fertilization was assessed using the IVF method, and when oocytes were pre-incubated with different concentrations of recombinant mouse-BSPH1, a dose-dependent inhibition of fertilization was observed [144].

4. Protein-protein interactions

In this section, I will discuss protein-protein interactions and some techniques used to investigate these interactions, specifically, proximity-dependent biotin identification (BioID), which I applied in my project.

4.1. Protein-protein interactions

Protein-protein interactions (PPI) are defined as physical contact between proteins in live cells. This physical contact and the interaction of proteins with other elements should be excluded while it is being synthesized or during folding as these represent interactions with ribosomes or folding chaperones and not the mature protein's actual interaction network. Moreover, these interactions can be transient or permanent since living cells are constantly undergoing turnover at different stages of life [165]. When analysing the PPI context, two types of data collection can be considered: binary and co-complex. Binary is a result of direct physical interactions, while co-complex quantifies physical interactions in a complex. The latter measures both direct and indirect interactions [165]. There are various available databases for these data, with some based on experimental data and others on predictions, as illustrated in Table 4.

Table 4. The main databases in the PPI [165]

Acronym	Database Full Name and URL	PPI Sources	Type of MI	Species	*n* Proteins (Dec. 2009)	*n* Interactions (Dec. 2009)
Primary Databases: PPI experimental data (curated from specific SSc & LSc published studies)						
BIND	Biomolecular Interaction Network Database, http://bond.unleashedinformatics.com/	Ssc & Lsc published studies (literature-curated)	PPIs & others	All	[31,972]	[58,266]
BioGRID	Biological General Repository for Interaction Datasets, http://www.thebiogrid.org/	Ssc & Lsc published studies (literature-curated)	PPIs & others	All	[28,717]	[108,691]
DIP	Database of Interacting Proteins, http://dip.doe-mbi.ucla.edu/dip/	Ssc & Lsc published studies (literature-curated)	Only PPIs	All	20,728	57,683
HPRD	Human Protein Reference Database, http://www.hprd.org/	Ssc & Lsc published studies (literature-curated)	Only PPIs	Human	27,081	38,806
IntAct	IntAct Molecular Interaction Database, http://www.ebi.ac.uk/intact/	Ssc & Lsc published studies (literature-curated)	PPIs & others	All	[60,504]	[202,826]
MINT	Molecular INTeraction database, http://mint.bio.uniroma2.it/mint/	Ssc & Lsc published studies (literature-curated)	Only PPIs	All	30,089	83,744
MIPS-MPact	MIPS protein interaction resource on yeast, http://mips.gsf.de/genre/proj/mpact/	Derived from CYGD	Only PPIs	Yeast	1,500	4,300
MIPS-MPPI	MIPS Mammalian Protein-Protein Interaction Database, http://mips.gsf.de/proj/ppi	Ssc published studies (literature-curated)	Only PPIs	Mammalian	982	937
Meta-Databases: PPI experimental data (integrated and unified from different public repositories)						
APID	Agile Protein Interaction DataAnalyzer, http://bioinfow.dep.usal.es/apid/	BIND, BioGRID, DIP, HPRD, IntAct, MINT	Only PPIs	All	56,460	322,579
MPIDB	The Microbial Protein Interaction Database, http://www.jcvi.org/mpidb/	BIND, DIP, IntAct, MINT, other sets (exp & lit.-curated)	Only PPIs	Microbial	7,810	24,295
PINA	Protein Interaction Network Analysis platform, http://csbi.ltdk.helsinki.fi/pina/	BioGRID, DIP, HPRD, IntAct, MINT, MPact	Only PPIs	All	[?]	188,823
Prediction Databases: PPI experimental and predicted data ("functional interactions", i.e., interactions *lato sensu* derived from different types of data)						
MiMI	Michigan Molecular Interactions, http://mimi.ncibi.org/MimiWeb/	BIND, BioGRID, DIP, HPRD, IntAct, & nonPPI data	PPIs & others	All	[45,452]	[391,386]
PIPs	Human PPI Prediction database, http://www.compbio.dundee.ac.uk/www-pips/	BIND, DIP, HPRD, OPHID, & nonPPI data	PPIs & others	Human	[?]	[37,606]
OPHID	Online Predicted Human Interaction Database, http://ophid.utoronto.ca/	BIND, BioGRID, HPRD, IntAct, MINT, MPact, & nonPPI data	PPIs & others	Human	[?]	[424,066]
STRING	Known and Predicted Protein-Protein Interactions, http://string.embl.de/	BIND, BioGRID, DIP, HPRD, IntAct, MINT, & nonPPI data	PPIs & others	All	[2,590,259]	[88,633,860]
UniHI	Unified Human Interactome, http://www.mdc-berlin.de/unihi/	BIND, BioGRID, DIP, HPRD, IntAct, MINT, & nonPPI data	PPIs & others	Human	[22,307]	[200,473]

A large number of alternative approaches have been developed over the last few decades to rectify current problems in the field of protein-protein interaction. These problems include capturing PPI

in live, intact cells and collecting these data, which has been made possible by proximity labeling techniques. In addition, new PPI prediction methods are able to predict PPI more accurately with higher confidence scores and less false positives when compared to previous databases.

It is noteworthy that the number of proteins is larger than the number of genes due to splicing and post-transcriptional modifications. Therefore, for many proteins, the diversity and combinations in complexes have yet to be investigated. For such investigations, we can use various methods such as pull-down assays, two-hybrid assays, gel filtration chromatography, isothermal titration calorimetry (ITC), Förster resonance energy transfer (FRET), luminescent oxygen channeling assay (LOCI), reflectometric interference spectroscopy (RIFS), surface plasmon resonance spectroscopy (SPR), circular dichroism spectroscopy (CD), Raman optical activity spectroscopy (ROA), small-angle x-ray scattering (SAXS), nuclear magnetic resonance spectroscopy (NMR), cryo-electron microscopy (Cryo-EM) and proximity-dependent labeling (PDL) [166]. Lastly, but importantly, I used Proximity-dependent biotin identification (BioID) and pull-down assays for my experiments, which I briefly explain below. I used the BioID assay because it is a powerful technique to study PPI in the live cells and overcome challenges related to studying insoluble proteins or membrane-associated proteins as well as transient protein interaction. Moreover, biotin is non-toxic to cells. Pull-down assays were also used as validation studies.

4.2. Pull-down assay

A pull-down assay is an *in-vitro* affinity purification method using a specific matrix to immobilize the target protein on its surface. To do so, the protein of interest is labeled with a specific tag and can be captured on the solid phase while it is in the complex. The elution fraction can be analyzed in order to find the protein partners using western blot, mass spectroscopy or protein sequencing [166].

4.3. Proximity-dependent labeling

Proximity-dependent biotin identification is a PPI technique that employs biotinylation of proteins in a live cell system. This method can screen weak or transient protein interactions [167]. In addition, it has the advantage of studying insoluble proteins, such as membrane proteins. BioID can be used to study various protein-protein interactions in various contexts, such as in centrosomes [168], in different signalling pathways [169], or cell-cell adhesion [170].

The BioID technique originates from the DamID (DNA adenine methyltransferase identification) method, in which Dam methylase is fused to the target protein to evaluate DNA-protein interactions. In BioID, the biotin ligase enzyme (BirA; 35 KDa) is fused to the target protein in a viral vector. In the cell, the tagging radius of BioID is about 10 nm [168]. Owing to the size of the BirA protein in BioID, protein localization might be impaired. To overcome this, an improved and more efficient approached (BioID2) was developed using a smaller ligase that originates from *Aquifex aeolicus* [168]. An overview of the BioID method is shown in Figure 14. Briefly, a biotin ligase is fused with the target protein and allows biotinylation of adjacent proteins upon the addition of biotin to the media for a determined incubation period (e.g., 24 hours). The lysine residues of adjacent proteins will be biotinylated via a permanent covalent bond [171]. Cells are then lysed, washed and the proteins are pulled down using streptavidin to capture the biotinylated protein complexes. Finally, mass spectrometry is used to identify the biotinylated proteins.

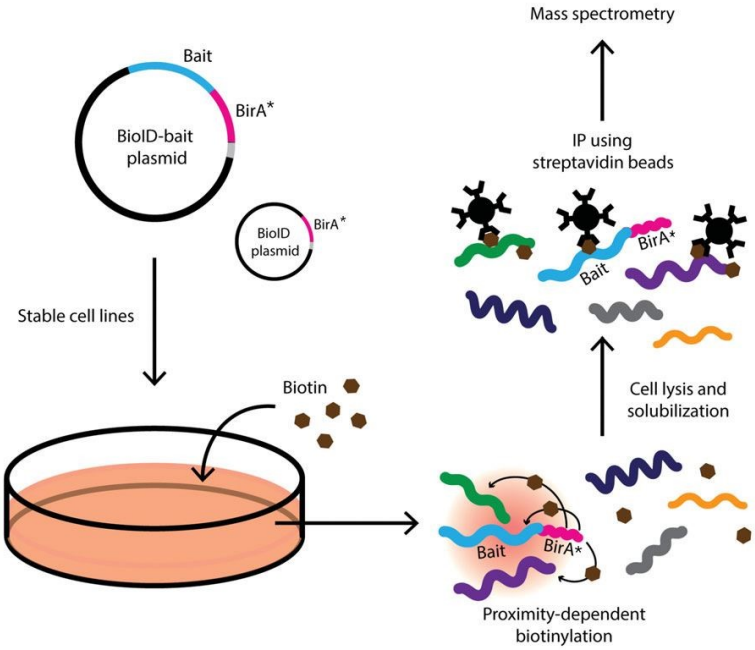

Figure 14. – BioID Flowchart [172]

One of the advantages of BioID is the strong bonding between streptavidin and biotin that enables harsh washing in the following pull-down assay to remove non-specific interactions. Moreover, biotinylation of vicinal proteins allows access to the topography of proteins present in the target cell line. BioID can also be used to study RNA binding proteins [173]. Another application is split BioID, in which two inactive parts of the enzyme become one active fragment when there is an interaction between two specific proteins [174]. Furthermore, in order to be more specific in the

study of adjacent biotinylated protein, Biotinylation Site Identification Technology (BioSITe) was developed using antibodies [175].

4.4. Mechanism of Biotinylation

The Biotinylation process is composed of two steps; first, the BirA enzyme catalyzes the conjugation of biotin to adenosine triphosphate (ATP) to produce biotinoyl-5'-adenosine monophosphate (AMP), then the activated BirA can react with a specific lysine residue in interacting proteins to form an amide bond [176]. An overall summary of the BioID mechanism is illustrated in Figure 15.

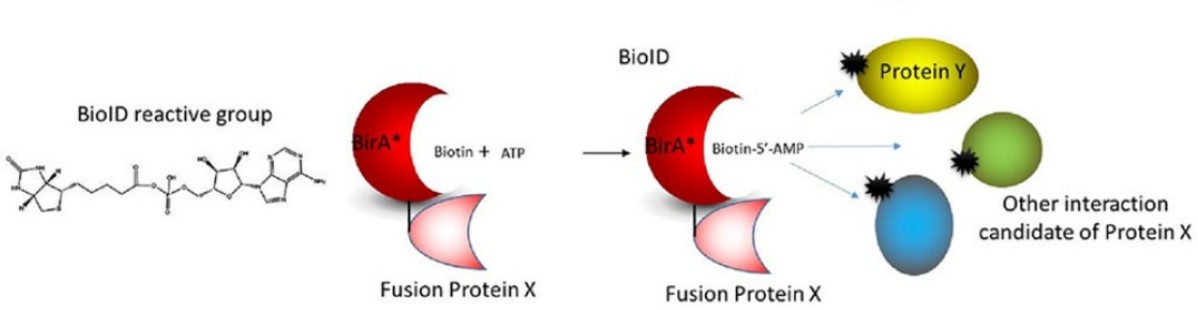

Figure 15. – BioID mechanism [177]

4.5. BioID workflow

There are some major steps that must be considered in the BioID experimental design, such as the design of the vector, selection of cell lines and validation of results.

4.5.1. Design of the BioID vector

One primary challenge in this field is the blueprint of the candidate vector. Care should be taken to design a vector for the BioID construct because it should resemble the native protein, which is expressed endogenously. An appropriate selectable marker (e.g., puromycin, blasticidin), along with the appropriate tag system (e.g., Flag, His, HA-tag), must be chosen. N- or C-terminal orientation of the fusion enzyme (BirA) regarding the protein of interest is an essential part of the design in order not to miss particular protein-protein interactions. The size and type of the vector are also important because they might affect downstream cloning. The vector could have inducible

elements such as the tet (tetracycline) system, which allows precise and reversible expression of the target protein [171,178].

4.5.2 Tet system

Many regulatory systems control eukaryotic gene expression, such as temperature, heavy metal ions and oxygen [179]. The TET system is particularly efficient and was developed around 30 years ago by Gossen et al [180]. It is used for specific transient gene expression and can be employed both in-vivo and in-vitro. Briefly, the tet-on system allows activation of gene expression by adding tetracycline or doxycycline (dox; a tetracycline derivative). On the other hand, in the tet-off system, gene expression is silenced by the addition of dox or tet [181]. Controlling specific gene expression is very important since overexpression can change protein localization. A schematic explanation of the tet system is presented in Figure 16.

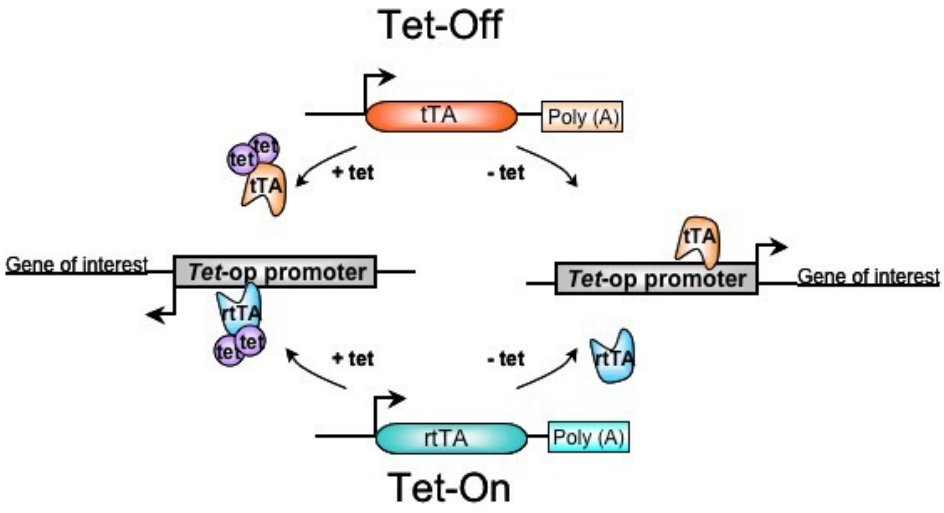

Figure 16. – Tet system [182].

The Tet off system deactivates gene expression after the addition of tetracycline and the Tet on system activates gene expression after the addition of tetracycline

4.5.3. Selection of cell line

After successfully cloning the target gene inside an appropriate vector, it will be expressed in a suitable cell line to study PPI. The cell line should be easily transfectable and allow to be used in the downstream pull-down assay. The cells should be easy to grow and manipulate. For example, in my studies, I used Hela cells, which are easy to handle and easy to infect.

4.5.4. Doxycycline and biotin induction

In BioID experiments, after the induction of gene expression with doxycycline and addition of biotin for the biotinylation process, cells are harvested for downstream experiments. Biotinylated proteins are recovered using a streptavidin pulldown approach. Since biotin molecules are small, stable and have an affinity to streptavidin via a strong non-covalent bond, streptavidin pulldown approaches are very robust.

Eventually, protein complexes captured on the streptavidin beads are sent for mass spectrometry (SCIEX Triple TOF) analysis for identification, which is based on digestion by a hydrolytic enzyme such as trypsin and peptide generation coverage. Finally, the result is presented as protein IDs and number of hits.

4.5.5. Validation of BioID results

Once potential interaction partners of proteins of interest are identified by BioID, one can confirm these results using BioID-mass spectrometry independent approaches. Validation of mass spectrometry results by pulldown assays and western blots or co-immunoprecipitation can be used to determine if the identified interaction was accurate. To do so, after the expression of the target protein, the protein is immunoprecipitated using a specific tag (e.g., FLAG) and probed with a specific antibody using the western blot technique. Alternatively, the candidate protein can be cloned in the expression vector and co-transfected along with the target protein, after which it can be co-immunoprecipitated in both orientations and probed with antibodies in a western blot.

5. Thesis Rationale

Binder of SPerm (BSP) proteins are male tissue-specific proteins expressed either in the seminal vesicles or the epididymis. BSPs originating from seminal vesicles are believed to be essential for sperm function and fertility. On the contrary, BSPs originating from the epididymis are suggested to play other roles than fertility (mouse & rabbit). For example, double knockout mice generated for (Bsph1/Bsph2) showed normal fertility. Surprisingly, the weight of double knockout male pups increased in a time-dependant manner (days 6 and 21, as well as 6 weeks of age) compared to the wild type. In other species such as rabbit, BSPs only exist on the sperm flagellum, indicating a role in motility. However, BSPs are expressed in the corpus segment of the epididymis, after obtaining progressive motility; therefore, it does not seem to be implicated in motility in this species regardless of its localization [1]. Studies on other epididymal BSP proteins are still lacking.

Therefore, evolutionary comparison studies are needed to compare BSPs that originate from seminal vesicles with those secreted by the epididymis. It seems that BSPs originating from seminal vesicles are essential for fertilization, whereas epididymal BSP proteins may play other functions than in fertility. The goal of my first aim is to provide a method to purify recombinant BSPs for downstream analysis. This is important due to the low expression of BSPs in the epididymis, which prevents isolation of the native protein in sufficient quantity. My second aim is to detect proteins or complexes with which BSPH1 interacts, in order to infer its possible localization and function.

The focus of this research has been on epididymal protein; human BSPH1. In view of our previous research on epididymal BSPs, namely, the results of our studies with knock out mice and in the rabbit species, I expected to identify potential functional roles for these tissue-specific proteins that differ from a direct role in fertility. In this study, I explored the protein interaction network of BSPH1 using the BioID technique in order to improve our understanding of their biological role.

6. Hypothesis & objectives

6.1. Hypothesis and Objective 1

BSP proteins bind to phosphatidylcholine; more specifically, they interact with cho

line moieties. Therefore, we hypothesized that BSP proteins interact with pseudocholine moieties such as diethylaminoethyl (DEAE) groups. To test this, we purified the recombinant binder of sperm proteins (human and mouse) on the diethylaminoethyl (DEAE) column. Additionally, we verified whether the affinity purified proteins promoted sperm binding to oocytes in *in vitro fertilization* assays and whether anti-BSP antibodies inhibited sperm-egg interaction.

6.2. Hypothesis and Objective 2

In vitro studies suggest a role for BSPH1 in sperm capacitation. However, double knockout mice generated for (*Bsph1/Bsph2*) showed normal fertility. Therefore, we hypothesized that the function of BSPH1 may not be limited to fertility. In order to identify other possible roles of BSPH1, we employed Proximity-Dependent Biotinylation (BioID) to characterize the protein interaction network of the human BSPH1 protein.

Chapter 2 – Article 1

Title: Novel affinity chromatography method for the efficient purification of recombinant Binder of SPerm homolog proteins

Summary of Paper 1:

The manuscript entitled “Novel Affinity Chromatography Method for the Efficient Purification of Recombinant Binder of SPerm Homolog Proteins” describes an efficient method to purify Binder of SPerm proteins based on their interaction with pseudo-choline groups such as DEAE through affinity rather than ionic interactions.

Diethylaminoethyl is used in ion-exchange chromatography. It is a weak anion exchanger (positively charged), thus negatively charged proteins bind to it and can be eluted using a high concentration of salts such as 1-2 M sodium chloride. However, BSP proteins interact with the DEAE matrix through affinity and not ionic interactions. This implies that BSP proteins cannot be eluted with a high concentration of salts, whereas other proteins are washed away with 1 M NaCl. Eventually, target proteins (BSPs) can be eluted using 8 M urea (a denaturing agent) or choline chloride (a specific eluent).

Authors contributions:

Authors contributed to the design and execution of experiments, data collection and analysis, interpretation of results, drafting of the article, revisions and final approval for submission. This article was published on September 3, 2020.

Novel Affinity Chromatography Method for the Efficient Purification of Recombinant Binder of SPerm Homolog Proteins

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Running title: Novel purification method for Binder of SPerm proteins

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Non-standard abbreviations:

(ApoA-I)	Apolipoprotein A-I
(BCA)	Bicinchoninic acid assay
(B-PER)	Bacterial protein extraction reagent
BSP	Binder of sperm
DEAE	Diethylaminoethyl
E. coli	Escherichia coli
(HDL)	High-density lipoproteins
(IMAC)	Immobilized metal affinity chromatography
(IPTG)	Isopropyl- □-D-thiogalactopyranoside
(IVF)	In vitro fertilization
(LB)	Luria-Bertani
(MWCO)	Molecular weight cut off
(PLA2)	Phospholipase A2
(PVDF)	Immobilon-P Polyvinylidene difluoride
(TCA)	Trichloroacetic acid
(Trx)	Thioredoxin tag

Abstract

In mammalian species, a family of proteins named the Binder of SPerm proteins, which are expressed in the male reproductive tract, have been shown to play a role in epididymal sperm maturation and sperm capacitation. Recently, one homolog from human and two homologs from mouse were characterized. In order to further investigate the biochemical activity of these proteins, efficient purification procedures are required to isolate the proteins. Since these proteins are produced in very minute quantities, we exploited the high capacity of Escherichia coli to produce larger quantities of recombinant proteins that were subsequently purified using affinity chromatography on a diethylaminoethyl-Sephadex A-25 column. Binder of sperm proteins have been shown to interact with pseudo-choline groups such as diethylaminoethyl through affinity rather than ionic interactions. The aim of the current study was to develop a novel method for purifying these recombinant proteins, produced in Escherichia coli cells. Diethylaminoethyl is positively charged and is a weak anion exchanger, but binder of sperm proteins interact with affinity to this resin. This study presents a new, rapid, and cost-effective purification method that provides with an exceptional purity level, which can be used to study their roles in mammalian fertilization.

Keywords: Affinity purification; Binder of SPerm proteins; Diethylaminoethyl; Epididymal proteins; Ionic interactions

Introduction

Binder of SPerm proteins are highly conserved in mammalian species. These low molecular weight proteins are expressed exclusively in the male reproductive tract [1–3]. In the last decade, one BSP homolog (BSPH1, 16 kDa) in human and two in mouse (BSPH1, 12.6 kDa and BSPH2, 14.4 kDa) were identified [4]. The human and rodent BSP proteins are expressed in low quantities in the epididymis, unlike ungulate BSPs, which are expressed in large quantities by the seminal vesicles then incorporated into the seminal plasma upon ejaculation. They contain a variable N-terminal domain, two tandemly-arranged fibronectin type-II domains, and have additional 2-3 amino acids in their C-terminal tail [5]. Previous studies did not document any glycosylation of human and mouse BSP proteins. However, extensive and systematic post-translational modification studies are still lacking [2]. It is well documented that BSPs potentiate the in vitro capacitation of mouse, human as well as bovine sperm [6–8]. BSP proteins interact with sperm membrane lipids such as phosphatidylcholine and sphingomyelin, leading to sperm membrane rearrangements (both lipids and proteins), which leads to sperm capacitation. This process is also accompanied by sperm hyper- activation and exposure of sperm surface receptors through which egg binding occurs [9].

Over the years, other groups suggested additional roles for BSP proteins in different species, such as regulation of sperm cell volume, molecular chaperone activity, and formation of the oviductal sperm reservoir through interaction with epithelial cells [9, 10, 11]. However, additional investigations are still necessary to further understand the roles of BSP proteins in human and mouse fertility. To perform in vitro analyses, procedures must be developed to allow the efficient purification of recombinant BSP proteins.

Various studies by our group and others have shown that BSP proteins interact with several biomolecules such as phospholipase A2 (PLA2), calmodulin, annexins, heparin, or glycosaminoglycans, high-density lipoproteins (HDL), apolipoprotein A-I (apoA-I) and gelatin/collagen [12,13]. The affinity of BSP proteins for gelatin, heparin, and glycosaminoglycans has been used for the purification of BSP proteins from seminal fluids of animal sources [14–17]. However, these affinity methods are not useful for the purification of recombinant BSP proteins expressed in bacterial cells in view of the fact that the reagents and/or solutions used in protein extraction from bacterial cells would interfere in the purification.

In addition to interaction with the above biomolecules, BSP proteins bind to phosphatidylcholine; more specifically, they interact with choline moieties as well as pseudo-choline moieties such as diethylaminoethyl (DEAE) groups (Figure 1) [18,19]. Furthermore, matrices coupled with p-aminophenyl phosphorylcholine or DEAE groups (Figure 1) can bind BSP proteins with high affinity and matrices bound-BSP proteins could be eluted using urea, or sodium thiocyanate [18,19]. The latter is known to disrupt hydrophobic interactions suggesting that interaction between choline and BSPs could be hydrophobic. However, the elution could also be achieved using choline chloride (specific agent), indicating the fact that the interaction between BSPs and choline is structure-based and could also involve hydrophobic interaction. Furthermore, BSPs bind to DEAE groups even in the presence of high salt concentrations, such as 1 M NaCl, indicating the significance of hydrophobic interactions over ionic. Based on the above facts, the mechanism of interactions between BSPs and the DEAE matrix appears to be as shown in Figure 1. We capitalized on this biochemical affinity and developed a novel and more efficient purification method for recombinant BSP proteins from bacterial sources.

Materials and methods

Reagents

Ampicillin was purchased from Millipore Sigma (St. Louis, Missouri, United States). Isopropyl--D-thiogalactopyranoside (IPTG) was from Invitrogen (Carlsbad, CA, USA). Origami B (DE3) pLysS cells were from Novagen (La Jolla, CA, USA). Bacterial protein extraction reagent (B-PER) was from Thermo Fischer Scientific (Rockford, IL, USA). DEAE-Sephadex A-25 was purchased from GE healthcare life sciences (Mississauga, ON, Canada). Immobilon-P Polyvinylidene difluoride (PVDF) membranes were purchased from (Millipore, Nepean, ON, Canada). Protein content was estimated using the BCA method [20].

Protein expression and purification

Production of human and mouse recombinant BSPH1 and BSPH2 proteins in E. coli was performed as described recently [21]. In brief, Bacteria were transformed with the plasmid vector and grown in Luria-Bertani (LB) medium with 100 µg/ml ampicillin. Cells were incubated at 37°C with shaking at 250 rpm for 5 hours to reach an optical density of 0.5-0.8 at 600 nm. IPTG (1 mM) was added to the media and cells were incubated for 20 hours at room temperature (22-23°C) with shaking at 250 rpm. Cell suspensions were centrifuged at 6000g for 10 min at 4°C, then resuspended with 2X binding buffer (100 mM Tris, 10 mM EDTA, 150 mM NaCl; 2 ml buffer/gram of cell pellet) and frozen at -20°C until downstream purification. After thawing, the cell suspension B-PER was added (4 ml of per gram of cell pellet), incubated with mixing on a rotator (Fischer Scientific) for 20 minutes, and then subjected to sonication with the amplitude of 50% for ten cycles of 10 seconds on ice. Urea (6 M) was added to cell lysates to denature and solubilize proteins and centrifuged at 20,000g for 30 minutes at 4°C. The supernatant was filtered through an acrodisc filter (1.2 µm) using a syringe, and the filtrate was diluted four times to reach a final concentration of 1.5 M urea before loading onto a DEAE column as described below.

Affinity chromatography

All protein purifications were carried out at room temperature. One gram of DEAE-Sephadex A-25 was allowed to swell in 50 ml of 1X binding buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl) for two days, then packed into a glass column (1 x 10 cm). The column was equilibrated with a 1X binding buffer with 10-bed volumes at a flow rate of 25-30 ml/h. Cell extracts containing 1.5 M urea from previous steps were loaded onto the DEAE-column. The column was then washed with approximately 30-bed volumes of 1 M NaCl to suppress ionic interactions and to remove contaminants until the optical density of the eluates reached zero. Unabsorbed fractions were concentrated to 2-3 ml using an ultrafiltration cell equipped with a 3000-Da molecular weight cut off (MWCO) membrane and using nitrogen gas pressure around 30-50 psi, for subsequent analysis.

Adsorbed proteins were eluted with elution buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 8 M urea) at a flow rate of 30 mL/hour. The presence of protein in the different fractions was monitored by determining the absorbance at 280 nm. Protein-containing fractions were pooled and concentrated to 2-3 mL using Amicon ultrafiltration membranes with a 3000-Da MWCO. Concentrated proteins were desalted using a gel filtration column (Sephadex G-25; 1.5 cm × 30 cm) with 50 mM ammonium bicarbonate, then lyophilized and stored at -20°C for further analysis.

Protein electrophoresis and western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) in 18% gels was conducted based on Laemmli [22] using the Mini Protean 3 apparatus (Bio-Rad). Gels were either stained with Coomassie Brilliant Blue R-250 or transferred electrophoretically to PVDF membranes (Bio-Rad, Mississauga, ON, Canada). Immunoblotting was performed using either His-Probe mouse monoclonal antibodies, sc-8036 (Santa Cruz, Santa Cruz, CA, USA) (1:1,000) or affinity-purified antibodies against (His)6-tagged human-recombinant BSPH1 (1:3,000) [21]. Goat anti-mouse IgG (1:3,000) or goat anti-rabbit IgG (1:10,000) were used respectively as secondary antibodies (Bio-Rad, Mississauga, ON, Canada). To detect mouse BSPH1/2 proteins, polyclonal antibodies against the 15-amino acid peptide corresponding to the C terminus of murine BSPH1 (SLTPNYNKDQVWKYC) were used. Details of the production and

characterization of these polyclonal antibodies were described previously [6]. Mouse BSPH1 polyclonal antibodies were used at a dilution of (1:1,000), with goat anti-rabbit antibodies as a secondary antibody (1:10,000) [17,5].

The Western Lightning Chemiluminescence Reagent kit (Perkin–Elmer, Boston, MA, USA) and Fuji LAS-3000 image analyzer (Fujifilm; Stamford, CT, USA) were used to reveal blots. To assess the expression and purity of target protein (human BSPH1 samples only), we performed trichloroacetic acid (TCA) precipitation of proteins and analyzed them by 18% SDS-PAGE using Coomassie Blue staining. To aid protein precipitation, 5 µl of a 1 µg/µl Bovine Serum Albumin solution was added to each 40 µl aliquot of the pooled column fractions, and then 15 µl of cold 60% TCA was added to the samples (final concentration of TCA was 15%) to precipitate proteins. TCA precipitation was done for human samples only to concentrate protein samples and to remove contaminants such as salts. Mouse BSPH1 and BSPH2 column fractions were loaded on gels directly, as described below. Ten µl of the sample from each fraction was added to 10 µl of 2X Laemmli buffer and heated at 95– 100°C for 10 min before loading onto the gel.

Results

Previously, immobilized metal affinity chromatography (IMAC) was used for the purification of recombinant BSP proteins. This method suffers from a plethora of pitfalls, IMAC being frequently accompanied by contamination and needing several steps to obtain pure proteins, which might increase the cost of downstream analysis [23]. Notwithstanding these limitations, IMAC is frequently used by academics as well as industries to purify proteins for different research purposes in terms of versatility. The focus of the current research was to develop a purification method for proteins of the BSP superfamily that is more efficient than existing methods in terms of yield, activity, and purity of recombinant proteins.

Purification and characterization of human recombinant binder of sperm homolog

Figure 2 illustrates the fractionation of human rec-BSPH1 on a DEAE-Sephadex column. The E. coli cell lysates containing recombinant BSPH1 proteins were passed through the column, and all flow-through was collected as a pooled fraction (Total OD: 456 and Cell extract volume:104 ml). The column was then washed with a wash buffer to remove all proteins by

ionic interactions. The wash fractions were collected and pooled and mixed with the flow-through fraction (together designated as the unabsorbed fraction) and preserved for subsequent analysis. Adsorbed proteins were then eluted using elution buffer containing a high urea concentration. As seen in Fig. 2A, a significant amount of protein was eluted in 8 M urea (the peak fraction absorbance at 280 nm was > 0.9). The eluted protein fractions were pooled, concentrated to about 2 ml, and desalted on a Sephadex G-25 column using 0.05 M ammonium bicarbonate as an elution buffer.

Figure 2B-E shows the results of the purification of human rec-BSPH1 using our method. Samples from the different fractions: cell pellet, cell lysate or supernatant, unabsorbed (flow-through), wash (E1) and eluted (E2) fractions were analyzed by SDS page and Coomassie Blue staining, and western blotting.

As shown in Fig. 2B, there is a major band at ⍰ 32 kDa in the first three fractions (pellet, supernatant, and unabsorbed), which is the expected molecular weight of human rec-BSPH1, around 10 µg proteins were loaded in each lane. Following overnight destaining of the Coomassie Blue stained polyacrylamide gel, a 32-kDa protein band was revealed in fraction E2 (Fig. 2C). There were several nonspecific bands in other fractions (Fig. 2B) when compared to fraction E2 (Fig. 2C), which shows a single band, indicating the purity of the protein. As shown in Fig. 2D, one major band was observed around 32 kDa in the fraction E2 eluted with 8 M urea, which is consistent with previous results using anti-rec-BSPH1 antibodies [8]. To confirm that the band indeed corresponds to rec-BSPH1, western blotting was performed on the same fraction using the His-Probe monoclonal antibodies revealing similar results (Fig. 2E).

This result also was confirmed by (LC-MS/MS). Human BSPH1 sequences were identified, as shown in Fig. 2F. Peptide sequences identified by LC-MS/MS are underlined. The rectangle on the gel in Fig. 2C represents the band that was cut out of the gel and subjected to LC-MS/MS analysis.

Purification and characterization of mouse recombinant binder of sperm homolog 1.

A similar approach was used for the purification mouse rec-BSPH1. The input, corresponding to the bacterial cell lysate, was passed through the DEAE-Sephadex column with the same conditions as mentioned previously (Total OD: 410 and Cell extract volume: 96 ml). There was a

slight difference in the elution pattern of mouse rec-BSPH1 as compared with human rec-BSPH1, which is depicted in Fig. 3A. In addition, there was a more significant peak following elution with 8 M urea (eluted fractions displayed higher absorbance at 280 nm),which can be explained by the higher expression level of the mouse rec-BSPH1 protein in comparison to human rec-BSPH1 in the Origami B (DE3) pLysS bacterial cells. To verify the expression and purity of mouse recombinant-BSPH1, the column fractions were processed as described for human rec-BSPH1 and analyzed by SDS-PAGE, followed by Coomassie Blue staining. As depicted in Fig. 3B, there was a single band of ~32 kDa in the lane corresponding to the fraction eluted with 8 M urea.
In the flow-through or unabsorbed fraction (Fig. 3B; Un), a faint band at ≈ 32 kDa can be seen, which could be due to the saturation of the column's binding capacity. The presence of mouse rec-BSPH1 was verified by western blot with two antibodies (His-tag and anti-mouse- BSPH1). As shown in Fig. 3C, the presence of mouse rec-BSPH1, was revealed in all column fractions with His-Probe antibodies. These results were also confirmed using polyclonal antibodies against mouse rec-BSPH1, where we can see that the target protein is clearly recognized in all fractions except wash fraction E1, as is shown in Figs. 3D-E.

Purification and characterization of mouse recombinant binder of sperm homolog 2

An advantage of this method is that one can use similar approaches for the three recombinant BSP proteins (one human and two mouse proteins). The type-2 structures, the conserved regions in BSPs that are responsible for their binding properties and affinity for DEAE. Thus, the bacterial cell lysate containing mouse rec-BSPH2 was similarly passed through the DEAE-Sephadex column (Total OD: 560 and Cell extract volume: 112). After washing the column with wash buffer, mouse rec -BSPH2 proteins were eluted using elution buffer containing 8 M urea. The elution pattern was almost the same as for mouse rec-BSPH1, with higher absorbance of the eluted fractions than was observed for human rec-BSPH1.

Coomassie Blue staining of the different column fractions following mouse rec-BSPH2 is shown in Fig. 4B. To verify the presence of mouse rec-BSPH2, a Western blot was performed using anti-His probe antibodies and is presented in Fig. 4C. Less BSPH2 protein is seen in the unabsorbed fraction, which is in agreement with the higher yield of mouse rec- BSPH2. Results of mouse rec-BSPH2 purification on a DEAE-Sephadex column showed a band at ≈ 32 kDa in all fractions, which is the protein's expected molecular weight. Our optimized purification

protocol resulted in a high yield of purified mouse rec-BSP protein in a single step. The summary of the purification of human and mouse BSP proteins is shown in Table 1.

Discussion

In the history of recombinant protein purification, there has been a shift towards affinity or tag-based purification. Purification of recombinant proteins produced in bacterial host cells has generated considerable interest not only in terms of their fast growth rate and low cost but also their ease of transformation. In a previous study [21], researchers showed that in addition to using a thioredoxin tag (Trx) to enhance protein solubility and increase in the probability of correct folding, glutathione-S-transferase and histidine tags were also tried for purification purposes. The major drawbacks of the His-tag approach are nonspecific adsorption and low yield of purified protein. Another bottleneck of this approach is that histidine-rich proteins from the host bacterial cell in the protein mixture can compete with the His-tagged target protein for binding to the column, leading to contamination of the target protein [23]. Eluting heavy metals (Hg2+ > Cu2+ > Cd2+ > Co2+) along with the desired protein is another concern due to toxicity, as these metals could impact target protein activity for subsequent in vitro biochemical assays. BSP proteins contain four disulfide bridges; therefore, correct folding and translocation from cytoplasm to periplasm would be challenging due to the potential mispairing of cysteines in the formation of these disulfide bridges, which can also cause aggregation [24]. During protein folding, disulfide bridges can be found in three different states: oxidized, reduced, and isomerized, and changing between these states depends on an enzyme for electron transfer [25]. Furthermore, due to the presence of many different reductases, the cytoplasm of E. coli, this environment is not suitable for the proper formation of disulfide bridges. Therefore, recombinant proteins have to be transferred to the periplasm space to allow proper bonding [26]. We applied two strategies to overcome this obstacle: the first and the most important was the addition of a thioredoxin tag to increase protein solubility, which has been shown to lead to correct protein conformation and function [27]. The second was the addition of urea to the cell lysate to denature and enhance the solubility of proteins, followed by a decrease in urea concentration to refold the target protein to the correct final state prior to loading onto the DEAE column. In addition, the thioredoxin tag was previously shown to enhance the solubility of recombinant BSP proteins expressed in

E. coli [21]. As anticipated, our experiments showed that solubility was improved when we applied these two strategies. Taking everything into account, this purification method is superior to other methods of BSP protein purification, such as gelatin- or heparin-agarose chromatography methods [15, 16, 28]. Our method, based on the high affinity of BSP proteins for pseudo-choline groups, is easy to optimize for efficient and rapid purification of recombinant BSP proteins. However, the basic molecular mechanism involved in BSP protein interaction with choline and pseudo-choline groups has yet to be elucidated, but current evidence indicates it is mainly structure-dependent and possibly involves hydrophobic interactions (Fig. 1). In this study, binding and elution buffers have a pH, 7.5. Recombinant mouse BSPH1, mouse BSPH2, and human BSPH1 have an isoelectric point of 5.54, 5.94, and 6.13, respectively. At the buffer pH of 7.5 (above pI), BSPs possess negative surface charges, which are expected to interact with DEAE by ionic interactions. However, high salt concentration (1M NaCl) in the washing buffer is ineffective in detaching proteins from the column. Consequently, BSPs' interaction with the DEAE is structure-based, and only chaotropic or denaturing agents can disrupt these interactions Desnoyers et al. [18,19] provided evidence that the BSP binding sites on the sperm membranes are, in fact, choline-phospholipids (such as phosphatidylcholine, phosphatidylcholine, plasmalogen, and sphingomyelin). The characteristics of the phospholipid-binding sites for BSPs on sperm could help elucidate the role of BSPs in capacitation and fertilization or in some unknown functions. Several affinity purification methods have been exploited for the purification of proteins from the BSP superfamily, which all require a higher number of steps and present more challenges in obtaining relatively pure target protein when compared to the method we present here [29]. Our study provides a method to obtain highly pure recombinant BSP proteins from the bacterial cell lysate following DEAE-Sephadex chromatography, made possible because of the unique interaction of BSP proteins with DEAE pseudocholine groups (Fig. 1). The affinity of BSP proteins with choline and pseudocholine groups has a biological role in mediating the interaction between BSP proteins and the sperm membrane [18, 19,30]. The various BSP homologs from human and mouse were expressed using similar experimental conditions, yet protein yield was higher for mouse BSPH2 compared to mouse and human BSPH1. Increasing the column bed volume could help to obtain a better recovery of purified protein [31]. Another key element to note in this study is the retaining of His-tag in order to use the anti-His antibody for optimization of purification method since it was difficult to provide a general

protocol to optimize the purification of our target protein. Interestingly, disruption of the affinity between DEAE groups and rec-BSP proteins can be achieved by using either urea or choline chloride. 8 M urea disrupts hydrophobic interactions and hydrogen bonds and easily elute BSPs from DEAE column. In the final step, urea was removed by size exclusion chromatography using ammonium bicarbonate solution for elution (desalting). Finally, active recombinant protein powder could be obtained by lyophilization of the eluted fractions. Whereas choline chloride binds to the recombinant proteins, and therefore, it is not suitable for downstream studies. Elimination of choline would require additional steps (dialysis against 6-8 M urea, desalting, and then lyophilization).

In contrast to previous purification techniques, the advantages of DEAE method are the simplicity of the methodology, the high binding capacity of the column, and its stability in a broad range of ionic strength and pH conditions compared to other matrices. In order to confirm the functionality of BSP proteins following the purification procedure, sperm-egg interaction assays were performed in vitro using recombinant mouse BSPH1 [32]. Results demonstrated that the recombinant purified mouse BSPH1 inhibited fertilization when it was preincubated with oocytes, suggesting a possible receptor on the egg surface. Fertilization rates were assessed using an in vitro fertilization assay (IVF) [32].

Concluding remarks

We described a novel technique based on the affinity of the BSP proteins to pseudo-choline groups that can be applied for the purification of recombinant BSP family proteins and could potentially be useful in experiments to purify native BSP protein from human seminal fluid in order to investigate the roles of BSPs proteins in sperm maturation process such as motility and capacitation as well as sperm-egg interactions. It also has biomarker potential in reproductive medicine; the concentration of this protein can be measured in seminal plasma of infertile versus fertile men as demonstrated with TEX101 protein [33].

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Conflict of interests

The authors declare no conflicts of interest.

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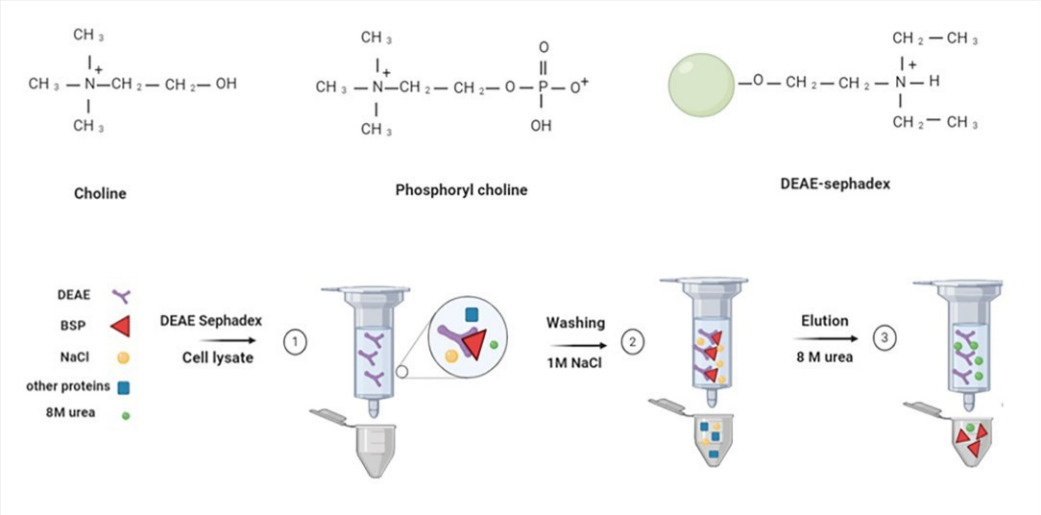

Figure 1. Schematic representation of affinity interaction of BSP proteins with DEAE-Sephadex. DEAE Sephadex A-25 is a weak anion exchanger, the interaction between BSPs and DEAE is not ionic, but is based on the molecular affinity of BSP proteins for the DEAE groups.

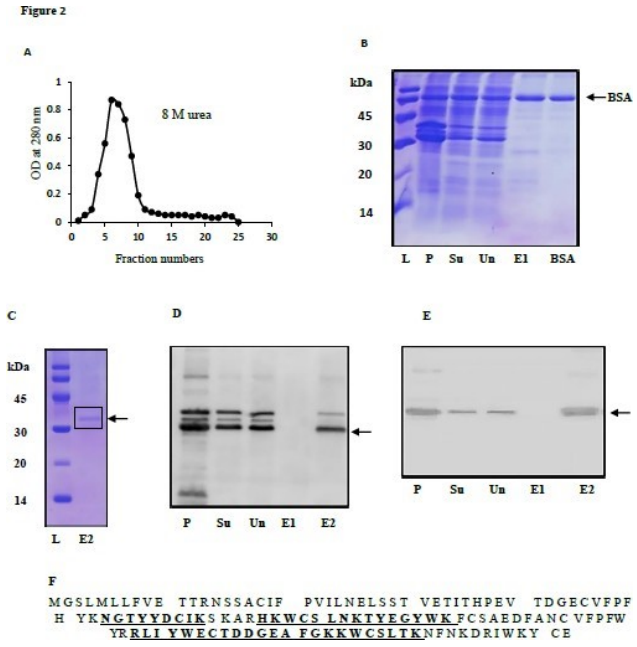

Figure 2. Purification and characterization of human recombinant binder of sperm homolog Experimental details are in the Materials and Methods section. A) Elution pattern of bound proteins following loading of human rec-BSPH1-expressing bacterial cell lysates on a DEAE-Sephadex column. Affinity bound rec-BSHPH1 was eluted with Tris-buffer containing 8 M

urea. B) SDS-PAGE pattern of the different column fractions. Proteins were stained with Coomassie Blue: L, molecular weight ladder; P, pellet; Su, supernatant (cell lysate); Un, Unabsorbed; E1, Eluate with 1 M NaCl; and 5 □g BSA was added to aid protein precipitation to all samples. C) Coomassie Blue staining of purified Human recombinant BSPH1 (fraction E2) on DEAE column g protein as loaded). D) Immunoblot of the different column fractions following DEAE-Sephadex chromatography using anti-rec-BSPH1 antibodies. P, pellet; Su, supernatant; Un, Unabsorbed; E1, Eluate with 1 M NaCl and E2, Eluate with 8 M urea. E) Immunoblots of the different column fractions following DEAE- Sephadex chromatography using anti-His antibodies. Samples were prepared and loaded on the gels in exactly the same manner as in Fig. 2D. F) LC-MS/MS analysis of the extracted 32-kDa band. Identified amino acids are in bold and underlined. The arrow at 32 kDa indicates the position of human rec-BSPH1.

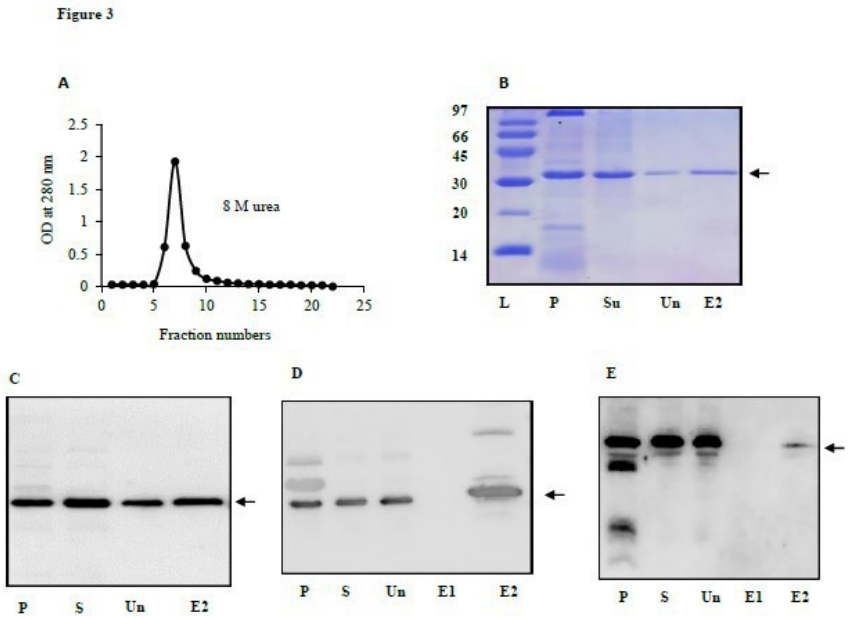

Figure 3. Purification and characterization of mouse recombinant binder of sperm homolog 1.

Experimental details are in the Materials and Methods section. A) Elution pattern of bound proteins following loading of mouse rec-BSPH1-expressing bacterial cell lysates on a DEAE- Sephadex column. Affinity bound rec-BSHPH1 was eluted with Tris-buffer containing 8 M urea. B) SDS-PAGE pattern of the different column fractions. Proteins were stained with Coomassie Blue: L, ladder; P, pellet; Su, supernatant; Un, unabsorbed and E2, Eluted fraction (8 M urea). C) Immunoblot pattern of various fractions of chromatography using anti-His antibody: P, pellet; S, supernatant; Un, unabsorbed; E2, eluted with buffer containing 8 M urea. D) Anti-His probe immunoblots of the different column fractions following DEAE- Sephadex chromatography. P, pellet; Su, supernatant; Un, unabsorbed; E1, Eluate with 1 M NaCl; E2, Eluate with 8 M urea. E) Immunoblot of column fractions using anti-mouse rec- BSPH1. Samples were prepared and loaded on the gels in exactly the same manner as in Fig 3D. The arrow at 32 kDa indicates the position of mouse rec-BSPH1.

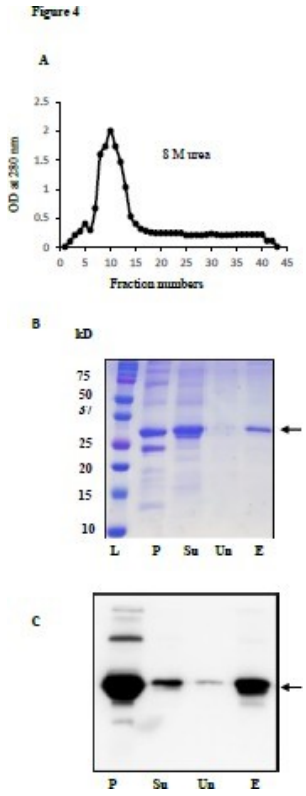

Figure 4. Purification and characterization of mouse recombinant binder of sperm homolog

Experimental details are in the Materials and Methods section. A) Elution pattern of bound proteins with Tris-buffer containing 8 M urea following loading of mouse rec-BSPH2-expressing bacterial cell lysates on a DEAE-Sephadex column. Affinity bound rec-BSHPH2 was eluted. B) SDS-PAGE pattern of the different purification fractions. Proteins in the 15 % polyacrylamide gels were visualized using Coomassie Blue stain: L, ladder; P, pellet; S, supernatant; Un, unabsorbed; E, Eluate with 8 M urea. C) Immunoblot of the various chromatography fractions using anti-His antibodies. Samples were prepared and loaded on the gels in exactly the same manner as in Fig 4B. The arrow at 32 kDa indicates the position of human rec-BSPH2.

Table. Summary of purification of human and mouse BSP proteins

	Human BSPH1	Mouse BSPH1	Mouse BSPH2
Wet cell pellet in 1 L (g)	6.5	6	7
Cell extract (ml)	104	96	112
Total OD (280 nm) of input	456	410	570
OD (280 nm) of DEAE pooled fraction elution with 8 M urea	4.3	3.5	10.6
OD (280 nm) of G-25 Sephadex pooled fraction eluted with 0.05 M Ammonium bicarbonate	1.6	1.6	5.2
Final Yield – lyophilized Powder (mg)	1.5	1.5	4.5

The initial wet cell weight was between 6 to 7 grams.

The protein concentration was assessed by absorbance measured at 280 nm.

Final weight of the protein was after desalting on Sephadex G-25 column and lyophilization.

Chapter 3 – Article 2

Title: Role of Binder of SPerm homolog 1 (BSPH1) protein in mouse sperm-egg interaction and fertilization

Summary of paper 2:

In mice, the Binder of Sperm Homolog 1 protein is exclusively expressed in the epididymis. BSP proteins play a role in the membrane modification events that occur during sperm capacitation. In the current study, we investigated the role of mouse recombinant BSP homolog 1 (rec-BSPH1) in sperm-egg inter-action. Mouse oocytes were co-incubated with different concentrations of rec-BSPH1 or control proteins and then inseminated with sperm. To establish whether rec-BSPH1 interfered with *in vitro* fertilization of mouse oocytes, rec-BSPH1 binding to egg and sperm was first tested using an immunodetection assay. In separate experiments, sperm were immuno-neutralized by anti-rec-BSPH1 antibodies to indirectly verify the implication of BSPH1 in sperm-egg interaction and fertilization. The study revealed a dose-dependent inhibition of fertilization when oocytes were pre-incubated with rec-BSPH1. Moreover, sperm immuno-neutralization with anti-rec-BSPH1 antibodies led to dramatic motility changes, followed by compromised fertilization. In view of these results, we conclude that BSPH1 could be a marker of sperm fertility and thus an eventual target for male contraceptive development.

Authors contributions:

Hamed Heidari-Vala as a first author contributed to the conception and design of experiments as well acquisition of data and drafting of the manuscript.

Samin Sabouhi Zarafshan contributed to the expression and purification of three recombinant proteins (human and mouse rec-BSPH1, and mouse BSPH1), and provided related data presented in the supplementary section.

Bruno Prud'homme provided technical assistance.

Abdullah Alnoman contributed to the technique development of some experiments.

Puttaswamy Manjunath as a corresponding author, ensured integrity and accuracy of data, manuscript revision and final approval.

Role of Binder of SPerm homolog 1 (BSPH1) protein in mouse sperm-egg interaction and fertilization

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ABSTRACT

In mice, the Binder of Sperm Homolog 1 protein is exclusively expressed in the epididymis, similarly to its human counterpart. Previous studies with mouse and human BSPH1 revealed that BSP proteins play a role in the membrane modification events that occur during sperm capacitation. In the current study, we investigated the role of mouse recombinant BSP homolog 1 (rec-BSPH1) in sperm-egg interaction. Mouse rec-BSPH1 was produced by transforming E. coli with a pET32a vector carrying BSPH1 cDNA and purified using immobilized metal (Ni2+) affinity chromatography. Mouse oocytes were co-incubated with different concentrations of rec-BSPH1 or control proteins and then inseminated with sperm. To establish whether rec-BSPH1 interfered with *in vitro* fertilization of mouse oocytes, rec-BSPH1 binding to egg and sperm was first tested using an immunodetection assay. In separate experiments, sperm were immuno-neutralized by anti-rec-BSPH1 antibodies to indirectly verify the implication of BSPH1 in sperm-egg interaction and fertilization. The study revealed a dose-dependent inhibition of fertilization when oocytes were pre-incubated with rec-BSPH1. Moreover, sperm immuno-neutralization with anti-rec-BSPH1 antibodies led to dramatic motility changes, followed by compromised fertilization. In view of these results, we conclude that BSPH1 could be a marker of sperm fertility and thus an eventual target for male contraceptive development.

INTRODUCTION

Sperm are specialized cells whose primary function is to carry paternal genetic material to an egg. As such, these haploid cells have lost many of the properties that are typically implicated in gene expression in somatic, diploid cells. Due to this unique state, the protein expression machinery of sperm is compromised upon spermiogenesis [1-4]. Once the post-testicular journey has started, sperm surface modifications begin to occur that prepare the sperm for fertilization [5]. Such membrane remodeling events are triggered by the epididymal secretome, accessory gland fluids within epididymal transit as well as fluids secreted in the oviduct, and they collectively enable sperm to fertilize the oocyte [6-12]. Binder of SPerm (BSP) proteins are secreted in the epididymis and interact with phosphocholine moieties of the sperm membrane, triggering cholesterol/phospholipid efflux. After these alterations in membrane fluidity, sperm capacitation occurs while sperm are exposed to capacitating factors in the female reproductive tract [13, 14]. Besides their role in capacitation, BSP proteins have been suggested to have a chaperone-like activity, to be a cell viability marker as well as to be involved in the formation of the oviductal sperm reservoir and, more recently, in sperm-egg interaction [15-17]. Ever since the BSP family of proteins was first discovered in bovine seminal plasma [18], several other mammals have also been shown to express homologs of these proteins with molecular weights varying from 15 to 30 kDa [17]. BSP family members contain a variable N-terminal domain followed by two tandemly arranged fibronectin type II (Fn2) domains separated by a short linker sequence, and in some cases a short C-terminal domain [19]. Sequencing analysis showed considerable conserved motifs throughout the members of the BSP family, most commonly in the region of the Fn2 domains (Fn2-A and Fn2-B). This particular structure is implicated in the biological functions of BSP proteins [17, 19]. Besides ungulate BSP proteins, a role for murine Binder of SPerm Homolog 1 (BSPH1) in sperm membrane stabilization (decapacitation) and destabilization (capacitation) was recently proposed. BSP proteins are secreted by the epididymis and they bind to sperm membrane phospholipids, thereby preventing the free movement of lipids. This stabilization of the sperm membrane structure, or decapacitation, protects the membrane from untimely destabilization, in

order to prevent premature capacitation. Upon ejaculation, sperm pass through the oviduct where high density lipoproteins (HDL) and glycosaminoglycans (GAGs) interact with sperm-bound BSP proteins, which destabilizes the membrane by removing BSPs as well as phospholipids and cholesterol [14, 20].

The multistep capacitation process includes several biochemical changes, including an increase in ion permeability and intracellular pH, which initiates an intracellular molecular signaling cascade resulting in protein kinase activation and protein tyrosine phosphorylation and, ultimately, leads to the acrosome reaction. Only capacitated sperm can undergo the acrosome reaction [21-25]. In addition to undergoing their own maturation, sperm must additionally travel through the cumulus cells surrounding the oocyte, through the zona pellucida and the oolemma, where they come in contact with many different molecules, before fertilization takes place. Until now, BSP proteins were best known for their interaction with molecules that modulate capacitation; however, a potential role for BSP proteins during the fertilization process has never been investigated. Our studies indicate that BSPH1 protein retains its localization on the sperm surface beyond capacitation, ascribing a possible role in sperm-egg interaction [13, 20, 26]. The aim of the present study was to elucidate the contribution of BSPH1 to sperm-egg interaction in the mouse model.

MATERIALS AND METHODS

Animals

Health-certified CD-1 (ICR) mice (male: ≥8 weeks; female: 5-8 weeks) were purchased from Charles River Laboratories (Kingston, NY, USA) and were accommodated in the animal carefacility of the Maisonneuve-Rosemont Hospital Research Center. Animals had ad libitum access to filtered water and food, under temperature-controlled (22 ± 1°C), light-controlled (a light cycle of 12 h light: 12 h dark) and specific pathogen-free (SPF) environment at least 3-5 days before experimentation. Animal protocols were approved by the Maisonneuve-Rosemont Hospital ethics committee and experimental work was carried out according to the guidelines of the Canadian Council of Animal Care.

Recombinant protein expression and purification

The recombinant BSPH1 protein was expressed as previously described [14, 27]. In brief, E.coli OrigamiB (DE3) pLysS transformed with the pET32a vector carrying the His-tagged BSPH1 cDNA construct were grown under IPTG-induction to reach $O.D_{600nm}$ ~ 0.5-0.8. Cell suspensions were then centrifuged at 6,000 ×*g* for 10 min at 4°C, resuspended in 4X binding buffer (2 M NaCl, 80 mM Tris-HCl, 20 mM imidazole, pH 7.4) and stored at -20°C for downstream analysis. Once the cell suspension was thawed, an equal volume of bacterial protein extraction reagent (B-PER) was added, the mixture was incubated with rotation for 20 minutes and then cells were subjected to sonication with an amplitude of 50% (five cycles of 10 sec on ice and 1 min off) (Branson Digital 450 Sonifier). Urea was added to achieve a final concentration of 6 M, the solution was kept mixing for an hour, and cell extracts were finally centrifuged at 20,000 ×g for 30 min at 4°C.

Cell lysate supernatant was filtered through Acrodisc® (1.2 µm) and then subjected to chromatography on a Ni2+ charged His-Bind® resin (Novagen, MilliporeSigma, La Jolla, CA), column, where gradual on-column refolding was performed using a urea gradient (6 M to 0 M). The column was then washed with 20 mM imidazole to remove unspecific interactions and BSPH1 proteins were eluted using elution buffer (500 mM NaCl, 20 mM Tris-HCl and 200 mM imidazole, pH 7.4). The absorbance of eluted fractions was monitored at 280 nm, after which selected protein fractions from the chromatography were pooled and concentrated to 2 ml using an

ultrafiltration technique. Protein was desalted on a gel filtration column (Sephadex G-25, 1.5 x 24 cm), which was pre-equilibrated with 0.05 mM ammonium bicarbonate. The desalted protein fractions were pooled, lyophilized and stored at 4°C.

Electrophoresis and Western blotting

SDS-PAGE was performed according to the Laemmli method [28] on 15% polyacrylamide gels using the Mini-Protean apparatus (Bio-Rad; Mississauga, ON, Canada). Two gels were run for each sample under the same conditions; the first was stained with Coomassie Brilliant Blue R-250 (Bio-Rad), and proteins in the other gel were transferred electrophoretically to Immobilon-P PVDF membranes (Bio-Rad) overnight in a cold room. Immunodetection was performed using either His-Probe monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or affinity-purified antibody against (His)6-tagged recombinant BSPH1 (anti-rec-BSPH1) (produced by Medimabs, Montréal, QC, Canada), at a concentration of 1:1000 and 1:500, respectively. Goat anti-mouse IgG-HRP (1:3000) or goat anti-rabbit IgG-HRP (1:10 000) were used as secondary antibodies. The bands were revealed using a chemiluminescence reagent (Perkin-Elmer, Boston, MA) and a Fuji LAS-3000 image analyzer (Fujifilm; Stamford, CT). To verify protein loading, membranes were stained with a solution of 0.5% Amido Black 10B (Bio-Rad).

Superovulation and egg retrieval

This part of experiment was mostly adapted from Byers et al study [29]. Nonetheless some modifications were inevitable to set for used strain (CD1). In order to get MII oocytes, 5-8 week-old females were subjected to superovulation via intraperitoneal injection of 9 IU PMSG (≥1,000 IU/mg; Sigma-Aldrich) and 9 IU hCG (Sigma-Aldrich) 48 h apart. Mice were then euthanized 16 h post-hCG, and both oviducts were cut out and placed in M2 medium (Millipore). Oviducts were transferred to a new dish, where swollen ampullae were nicked with dissection forceps under a stereo microscope. The released cumulus-oocyte complexes (COCs) were then treated with 3-5 µl hyaluronidase (10 mg/ml) (from bovine testes; Sigma-Aldrich) in M2 medium for 2-3 min. Upon cumulus cell removal, eggs were washed sequentially 2 times with M2 medium and 3 times with HTF medium, which were previously equilibrated in 5% CO2 and humidified

atmosphere at 37°C. To obtain zona-free oocytes, eggs were treated with Tyrode's solution (Sigma-Aldrich) for 30 seconds to digest the zona pellucida (ZP).

Immunolocalization of rec-BSPH1 on oocytes

The immunolocalization method was adapted from published protocols [30-32]. Ten to 20 oocytes were treated with 300 µg/mL rec-BSPH1 protein in PBS containing 3% bovine serum albumin (BSA), or with PBS-3% BSA alone, for 1 h at room temperature. Eggs were then washed five times in PBS containing 3% BSA, fixed in 4% paraformaldehyde for 30 min at room temperature then washed another five times. Afterwards, eggs were incubated with either anti-rec-BSPH1 polyclonal antibody (1:100), anti-BSPH1 15-mer (1:100), corresponding to the 15 C-terminal amino acids of BSPH1 [26], monoclonal His-probe (1:50), or normal rabbit serum IgG (NRS-IgG)as a control (1:100) in PBS containing 3% BSA for 1 h at 37°C with 5% CO2. To remove excess antibodies, eggs were washed five times with PBS containing 3% BSA and then incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG or FITC-labeled goat anti-mouse IgG at a dilution of 1:200, in PBS containing 3% BSA for 1 h at 37°C with 5% CO2. To counterstain the nucleus, eggs were soaked for 5-10 minutes at room temperature in 10 µg/ml DAPI in PBS containing 3% BSA and then washed five times before mounting with slow fade medium on the stage of an Olympus IX73P1F (Olympus Corp., Japan) inverted microscope. Images were acquired using a monochromatic Peltier cooled 1.4 megapixel CCD Olympus XM10 (Olympus Corp., Japan) camera controlled by the Olympus cellSens software.

Sperm retrieval and preparation

CD-1 male mice (≥8 weeks) were sacrificed by cervical dislocation and then dissected to remove both the cauda epididymis and vas deferens while trying to avoid adipose tissue and vascular debris. Tissues were minced (four to five times) in 300 µl of equilibrated (5% CO2 and humidified atmosphere, 37°C) HTF medium and left at 37°C for 10 min to allow motile sperm to swim out. Sperm suspensions were gently removed and placed into tubes (45º-inclined) containing 1 ml equilibrated HTF at 37°C for 90 min, where motile sperm were allowed to swim up and capacitate. From the upper layer, 10 µl were removed to measure sperm concentration.

Immunolocalization of recombinant and native BSPH1 on sperm

This protocol was mostly adapted from Plante et al [14, 26]. 2×106 sperm were incubated for 1 h with 30 µg/ml rec-BSPH1 or without protein (control), in PBS or HTF medium, respectively, for uncapacitated and capacitated sperm treatment. To induce the acrosome reaction, 200 µl of capacitated sperm were incubated for an additional 30 min with 5 µM calcium ionophore A23187 (Sigma-Aldrich). Sperm suspensions were fixed with 4% paraformaldehyde for 30 min at room temperature and washed 3 times (2 min; 8,000 ×g) with PBS containing 1% BSA. 15 µl were smeared on Poly-L-lysine coated slides (FisherScientific, Ottawa, ON, Canada) and then allowed to dry. Sperm fixed on the poly-L-lysine slides were permeabilized for 10 minutes with PBS containing 0.1% TritonX-100 and 0.2% paraformaldehyde, washed three times with PBS, and incubated for 1 h at room temperature in PBS-1% BSA (or 30 min in PBS-3% BSA) to avoid nonspecific binding. Slides were then incubated for 1 h at room temperature with anti-rec-BSPH1 antibody (1:50) or NRS-IgG (1:50) in PBS containing 0.1% BSA. Excess antibodies were removed by washing three times with PBS containing 1% BSA, and then slides were incubated for 1 h at room temperature with FITC-conjugated goat anti-rabbit IgG (1:100) in PBS-0.1% BSA. After washing thoroughly, slides were counterstained with Propidium Iodide (Thermo Fisher Scientific) for 5-10 min. Finally, slides were washed, air dried and mounted with Permount (Fisher Chemical) or slowfade mounting medium (Thermo Fisher Scientific). Observations were made using a fluorescence microscope and images were captured as mentioned previously. To immunolocalize native BSPH1 on sperm, an experiment was adapted from Herrero et al. and Plante et al. [14, 31]. Approximately 30,000 capacitated or uncapacitated sperm were smeared on Poly-L-lysine slides and air-dried. Sperm were then fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized for 10 minutes with PBS containing 0.1% TritonX-100 and 0.2 % paraformaldehyde. After 3 washes, slides were blocked in PBS containing 1% or 3% BSA, for 1 h and 30 min, respectively at room temperature. Slides were incubated with anti-rec-BSPH1 antibody (1:25) or NRS-IgG (1:50) for 1 h at room temperature and washed 3 times before incubation with FITC-conjugated goat anti-rabbit IgG at a dilution of 1:50. Subsequent steps were performed as described above for rec-BSPH1.

In vitro fertilization assay

Eggs pre-incubated with rec-BSPH1

Oocytes were placed in 100 µl of equilibrated HTF and incubated for 1 h without or with 300 µg/ml rec-BSPH1, as well as with same concentration of ovalbumin or thioredoxin (TRX) as controls. Eggs retrieved from COCs were washed and approximately 10 oocytes (either zona-intact or zona-free) were placed in 100 µl of equilibrated HTF. Eggs were incubated with different concentrations of mouse rec-BSPH1 (1, 3, 10 and 30 µg/ml). Following 1 h incubation at 37°C with 5% CO2, eggs were washed twice in M2 and HTF, respectively. Finally, oocytes were gently transferred to new dishes containing an equilibrated HTF drop (100 µl) and then inseminated with capacitated sperm following a ratio of 1000 sperm per egg. After 18-20 hours of incubation, dishes were observed and the fertilization rate was recorded using an inverted phase-contrast microscope (Olympus 1X73). Fertilization was analyzed by the assessment of pronuclei or cleavage. The fertilization rate was expressed as the percentage of eggs fertilized against the total number of eggs inseminated. A total of 40-50 oocytes were counted for each protein concentration. In some experiments, eggs were washed 4-5 hours post-insemination and moved to new equilibrated HTF medium to obtain clearer images and two cell embryos.

Sperm pre-incubated (treatment) with anti-BSPH1

To further investigate the effect of BSPH1 on sperm-egg interaction, we used antibodies to block native BSPH1. After 1 h of sperm swim-up in capacitation medium (HTF), motile sperm from the upper layer were counted and distributed in control and treatment dishes. Sperm were then incubated with anti-BSPH1 antibodies (1:100, 1:50 and 1:25 dilutions) or NRS-IgG (1:50) for 30-45 min at 37°C. Immediately before insemination, sperm motility was measured using a Sperm Class Analyzer (SCA) system (Microptic, Barcelona, Spain) and fresh eggs were inseminated as mentioned previously and assessed as described above.

Statistical Analysis

Data are presented as the mean ± SEM. Differences were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test using GraphPad Prism 5. A P value of < 0.05 was considered significant.

RESULTS

Characterization of recombinant BSPH1

The purity of every batch of recombinant protein isolated by immobilized metal ion affinity chromatography was assessed by SDS-PAGE (Fig. 1A), and immunodetection using the His-probe antibody (Fig. 1B) and anti-recBSPH1 antibodies (Fig. 1C). After confirming the purity of the recombinant protein by SDS-PAGE and immunoblot analysis, a number of functional assays were conducted.

Potential BSPH1 binding sites on the oocyte surface

The specific localization of rec-BSPH1 on the egg surface was examined by immunofluorescence staining of eggs that had been incubated in the presence or absence of rec-BSPH1. Eggs were incubated in the presence or absence rec-BSPH1, fixed and then incubated with either anti-BSPH1, anti-15mer or anti-His probe primary antibodies, or with NRS-IgG as a control (substituted primary antibody). The middle panel of Figure 2 shows the immunofluorescence images obtained after incubation of eggs with FITC-conjugated secondary antibodies. Regardless of background noise, no significant fluorescence signals were observed in zona-intact oocytes immunostained with either polyclonal antibodies (anti-rec-BSPH1 and anti-15mer) or with monoclonal anti-His probe, when compared to oocytes incubated in the absence of rec-BSPH1 or with NRS-IgG (Fig. 2). Eggs were counterstained with DAPI (Fig. 2, left panel) and signals for egg nuclei were observed, confirming the presence of eggs on the slides. To examine whether there is specific binding of rec-BSPH1 beyond the zona pellucida, the same experiment was repeated using zona- free eggs. As shown in Figure 3, no considerable difference in the immunofluorescence signal was observed in zona-free eggs incubated with rec-BSPH1 compared to controls. None of the eggs (zona-intact and zona-free) incubated with recombinant protein revealed any clear immunofluorescence signal, suggesting that rec-BSPH1 does not bind to the mouse egg surface.

Inhibition of fertilization following egg pre-incubation with rec-BSPH1

As this is the first study to examine the role of rec-BSPH1 in mouse sperm-egg interaction, we attempted to verify whether blocking rec-BSPH1 binding to the egg would compromise the success of IVF. Complementary to the immunofluorescence experiments shown in Figure 2, this assay aimed to verify whether oocyte fertilization by mouse sperm was affected when oocytes were pre- incubated with rec-BSPH1 prior to conducting the fertilization experiment. We hypothesized that following pre-incubation of oocytes with rec-BSPH1, all assumptive BSPH1 binding sites on the egg surface would be occupied, thus inhibiting native mouse sperm BSPH1 from binding. To verify this, zona-intact eggs were incubated for 1h in the presence or absence (medium alone) of rec-BSPH1, ovalbumin (non-specific protein control), or TRX-His-S (control to eliminate the possible tag effect) before washing and insemination with capacitated mouse sperm. As shown in Figure 4A and B, zona-intact eggs pre-incubated with rec-BSPH1 showed a significantly (≥50%, $P < 0,001$) lower rate of fertilization compared to controls. Such an inhibition indicated that sperm-egg interaction is significantly compromised following pre-incubation of eggs with rec-BSPH1. This inhibition was also observed for zona-free eggs (data not shown). Using similar methods, experiments were performed to reveal if the inhibitory effect of protein was dose-dependent. As seen in Figure 4B and C, there was a significant difference in fertilization rates between eggs pre-incubated with control proteins and with 1 µg/ml ($P <0,05$), 3 µg/ml ($P <0,01$), 10 µg/ml or 30 µg/ml ($P < 0,001$) rec-BSPH1.

Localization of recombinant and native BSPH1 on mouse sperm

Although the concentration of native sperm-bound BSPH1 protein and of BSPH1 in mouse seminal plasma is not yet known, previous studies on epididymal mouse sperm and tissue extracts suggest that these concentrations are quite low [14]. In order to confirm the immunolocalization of BSPH1 on mouse sperm (previously shown in Plante et al, 2012) and to ensure that there were no differences in the results obtained with our different batches of polyclonal anti-rec-BSPH1 antibodies, we localized native and rec-BSPH1on mouse sperm by immunofluorescence using these antibodies.

As shown in Figure 5, for the detection of native BSPH1 a weak signal was visualized on the sperm neck and mid-piece when anti-rec-BSPH1 was used as the primary antibody, compared to NRS-IgG treated samples and controls without any primary antibody. When uncapacitated sperm were pre-incubated with rec-BSPH1 prior to immunodetection, a stronger fluorescence signal was observed in the mid-piece than what was observed for immunodetection of the native protein. In addition, as shown in the merged panel, weak fluorescence was visualized on the anterior acrosomal segment (Fig.5; white asterisk). In capacitated sperm pre-incubated with rec-BSPH1, we observed the localization of rec-BSPH1on the mid-piece and over the post-acrosomal and equatorial segments, whereas no signal was observed in control groups.

Effect of sperm pre-incubation with anti-rec-BSPH1 antibodies on in vitro fertilization

To test if the immuno-neutralization of native BSPH1 affects sperm-egg interaction during IVF, eggs were inseminated with motile sperm that had been pre-incubated with different concentrations of anti-rec-BSPH1 antibodies (1:25, 1:50 and 1:100 dilutions) for 30 min. The IVF results showed that pre-incubation of sperm with anti-rec-BSPH1 at a dilution of 1:25 could inhibit the fertilization rate by over 85%, while lower concentrations of antibody caused an inhibition of 50-60% (Fig. 6A). On the other hand, sperm pre-incubated with NRS-IgG (1:50) displayed a slightly lower fertilization rate (≈ 20%) compared to control (without antibody). However, the difference was not statistically significant. We also examined the effect of anti-rec-BSPH1 antibodies on mouse sperm motility. Sperm incubated with NRS-IgG showed a small decrease in progressive motility when compared to control (without antibodies) after 90 min, though this decrease was not statistically significant. However, a dose-dependent decrease of the percentage of motile sperm (progressive motility) in anti-rec-BSHP1 antibody-treated samples was observed (Fig. 6B). Additionally, the decrease in motility observed in sperm treated with anti-rec-BSPH1 at a 1:25 dilution was significantly different when compared to other treatments. In general, sperm treated with anti-rec-BSPH1 antibodies showed a tendency of agglutination. As expected, this tendency was more pronounced with higher antibody concentrations (data not shown). Interestingly, sperm appear to overcome agglutination by swimming to the peripheral area of the drop.

DISCUSSION

Fertilization, the end-point of sexual reproduction, is made possible thanks to the mutual interactions between sperm and egg, which can occur either naturally or in vitro. A few specific molecules are thought to be implicated in this process from both sides. Thus, the absence or malfunction of any of these molecules has the potential to disrupt fertility. Defects in these molecules could therefore be responsible for unexplained phenotypes of infertility [33]. Previous studies by ours and other groups have shown that the Binder of SPerm (BSP) protein family is implicated in capacitation through biochemical changes in the sperm membrane. The immunolocalization of BSPH1 to different segments of mouse sperm led us to further characterize the involvement of this protein during in vitro fertilization. Since native BSP proteins are expressed in fairly low amounts in the mouse and human male reproductive tract [14, 26], the production and purification of recombinant BSPH1 protein was a necessary. All protein batches were characterized by electrophoresis and immunoblotting to ensure that there were no inter-batch differences prior to using recombinant proteins for functional assays. To our knowledge, this is the first report of a potential implication of BSPH1 in mouse sperm-egg interaction. Our IVF results revealed that pre-incubation of oocytes with rec-BSPH1 (300 µg/ml) could diminish the fertilization rate, while pre-incubation with ovalbumin or thioredoxin tag (Trx-His- S) did not lead to any significant difference when compared to control (without protein). Subsequent experiments showed a negative correlation between fertilization rates and rec-BSPH1 protein concentration used for egg pre-incubation. This inhibition of fertilization in eggs pre-incubated with rec-BSPH1 protein was dose-dependent. Interestingly, both patterns (Fig. 4A and 4B) show almost the same inhibition level at rec-BSPH1 concentrations of 10, 30 and 300 µg/ml, suggesting that a possible saturation limit was attained. As the majority of proteins known to mediate sperm-egg interaction follow a key–lock system [34], this inhibition was expected due to a presumed antagonistic effect of rec-BSPH1 on potential mouse egg BSPH1 receptors. Moreover, the specific binding of rec-BSPH1 to binding sites on the egg surface would hinder the binding of native sperm-bound BSPH1 to the egg, thus disrupting effective interaction and leading to reduced fertilization rates. Alternatively, it is possible that there is no specific

receptor for BSPH1 on the egg surface. As shown in Figures 2 and 3, no specific rec-BSPH1 immunofluorescence signal was observed either on zona-intact or on zona-free eggs following the pre-incubation of eggs with rec-BSPH1. Although some studies in the bovine species indicated that BSP proteins can bind to isolated ZP due to the saccharide content of ZP glycoproteins [35, 36], no evidence has shown such binding of BSP proteins to mouse oocytes. Our findings from immunolocalization features elicit different assumptions. First, the fertilization inhibition caused by the pre-incubation of eggs with rec- BSPH1 does not depend directly on specific binding of native sperm-bound BSPH1 to the oocyte. Second, even if rec-BSPH1 binds to the egg through a specific receptor, potential conformational changes induced upon protein-receptor interaction could conceal the bound protein from immunoreaction with anti-rec-BSPH1 antibodies and the monoclonal anti-His probe, thus preventing immunolocalization and fluorescence signal. In addition, the presence of BSA, which plays a crucial role in the fertilization medium [37, 38] in which the eggs were incubated, brings about another presumption. Apart from a contribution to sperm capacitation, serum albumin has been implicated as a transporter to enhance cholesterol efflux from different cells [39], as was shown to play a role in mouse oocyte cholesterol depletion by methyl-β-cyclodextrin (MβCD) [40]. In this latter case, cholesterol removal caused a decrease in the in vitro fertilization rate was observed, suggesting that the disorganization of oocyte membrane rafts can affect the rate and quality of fertilization [40]. However, although BSPH1 probably has no direct implication in BSA-induced capacitation [20], it may have a synergistic effect on BSA-induced cholesterol removal. A possible hypothesis is that the co-incubation of eggs with BSA and rec-BSPH1 could induce cholesterol efflux from the egg membrane followed by premature membrane reorganization and compromised sperm-egg interaction. Consistent with the findings of Buschiazzo et al, with MβCD and BSA, we observed a dose-dependent and synergistic fertilization inhibition effect with BSA and BSPH1. Taken together, these findings have the potential to shed new light onto the binding behavior of BSPH1. Since previous studies showed that BSPH1 binds to the equatorial segment of capacitated sperm [14, 41] and that the equatorial segment has been shown to be involved in sperm-egg interaction and fusion [42], we investigated whether BSPH1 could be involved in functions other than capacitation. For this reason, we aimed to measure the potential

effect of BSPH1 immuno-neutralization on sperm fertilizing ability. As shown in Figure 5, in partial agreement with Plante et al (2012), native and recombinant BSPH1 display the same localization pattern, albeit with differences in signal intensity. When detecting native BSPH1, the low fluorescence intensity observed in the sperm mid-piece can be ascribed to the low concentrations of this protein in murine sperm [14, 41] (Fig. 5). A stronger signal was visualized on the mid-piece of uncapacitated sperm that had been incubated with rec-BSPH1 compared to controls. The mid-piece region of spermatozoa is mostly associated with sperm motility. Thus, it is possible that BSPH1 protein may modulate sperm motility through the calcium ATPase, as is the case for bovine BSP1 [43, 44]. In addition, the binding of rec-BSPH1 on both the post-acrosomal and equatorial segments of capacitated sperm, as shown by a strong immunofluorescence signal in Fig. 5, consistent with Plante et al (2012, 2014), suggests a role beyond modulating motility [14, 41]. To further characterize the potential role of BSPH1 in sperm-egg interaction, sperm were incubated with anti-rec-BSPH1 antibodies in order to examine the effect of blocking BSPH1 activity on various parameters and functions. Using a SCA, we took motility images of sperm exposed to anti-rec-BSPH1 antibodies. These images revealed sperm agglutination, consistent with what Nixon et al (2008) reported for the effect of anti-BSP1 antibodies on rabbit sperm motility [45]. Furthermore, this effect was also reflected in motility parameters where anti-rec- BSPH1 dilution 1:25 renders most sperm immotile. IVF results followed this observation, with fertilization failure in many replicates when eggs were inseminated with sperm that had been exposed to anti-rec-BSPH1 antibodies. Previous studies indicated that BSPH1 could modulate sperm capacitation similarly to its counterparts in other species [41, 46]. Our findings show that sperm incubated with anti-rec-BSPH1 antibodies had difficulties to fertilize eggs, even though the most motile sperm had been chosen for insemination, suggesting that something else in the downstream processes leading to fertilization could also be affected, in addition to motility. Sperm hyperactivation is an increase in the amplitude of flagellar whip-like motion, which happens in a timely manner with capacitation [12, 47]. Although hyperactivation is triggered by tyrosine phosphorylation, in which rec-BSPH1 has been shown to have no direct effect in human sperm [13], BSPH1 neutralization could potentially diminish hyperactivation and compromise the fertilization

rate. This is consistent with results by Plante and Manjunath (2015), which showed an inhibitory effect of BSPH1 blockage on HDL-induced capacitation and tyrosine phosphorylation of mouse sperm [20]. In the current study, the BSA present in the incubation medium promoted capacitation, which led sperm to become hyperactivated and eventually undergo the acrosome reaction. Since specific cholesterol binding sites have not yet been identified for albumin [48], which is a low-affinity, high-capacity cholesterol transporter, the presence of a modulator molecule such as BSPH1 is plausible. Our results suggest that BSPH1 neutralization over the mid-piece and head region can interrupt the function of BSA as a main capacitating factor in vitro. This interruption likely led to aberrant Ca2+ store mobilization, and to a failure to induce hyperactivated motility and/or the acrosome reaction. We suggest that when BSPH1 and cholesterol are retained over the mid-piece (e.g., following neutralization), capacitation would not be complete, thus precluding the triggering of lateral motility and hyperactivation [47]. Regarding IVF results and sperm neutralization BSP proteins seem to increase the affinity of albumin for cholesterol, especially when there is no alternative sterol acceptor. Such a contribution of BSPH1 to BSA-induced capacitation is suggested by our sperm motility analyses and in the reduced fertilization rates observed when high concentrations of anti-rec-BSPH1 antibodies likely prevented BSA from binding BSPH1 and fully driving capacitation. Amongst sperm-specific molecules, those for which antibodies display effective inhibition of sperm-egg interaction in vitro could bring us new insights towards the development of non- hormonal contraceptives. Taken together, our results showed that BSPH1-immunoblocked sperm seem to be affected during the fertilization process, at least *in vitro*. These effects could be attenuated *in vivo* because of the presence of capacitating factors other than albumin.

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CONFLICT OF INTEREST

The authors have no conflict of interest

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LEGENDS

Figure 1. Characterization of purified rec-BSPH1. 20 μg of protein was analyzed by electrophoresis in 15% SDS-PAGE gels subjected to (A) Coomassie staining and (B) western blotting using monoclonal His-probe antibodies (1:1000). Two different protein batches (SS121 and SS122) were tested. (C) Western blotting of the SS121 protein batch using polyclonal anti-rec-BSPH1 antibodies (1:500). Bands around 32 kDa correspond to the recombinant BSPH1 protein. Std, molecular weight standard.

Figure 2. Immunolocalization of rec-BSPH1 on zona-intact eggs. Eggs were incubated with or without rec-BSPH1 (300 μg/mL), washed and then incubated with (A) anti-15mer antibodies, (B) anti-rec-BSPH1 antibodies or (C) anti-His-probe antibodies. NRS-IgG was used as a control in these experiments. For all samples, eggs were counterstained with DAPI to localize nuclei. Original objective magnification was ×40, scale bar =20 μm (A) and ×60, scale bar =10 μm (B and C). As image acquisition parameters were kept consistent for all samples of a same experiment, all images shown together in an individual panel can be compared with each other but not with next panel, which represents a different experiment.

Figure 3. Immunolocalization of rec-BSPH1 on zona-free egg. Eggs were treated with Tyrode's solution for 30 sec, incubated with or without rec-BSPH1 (300 μg/mL), washed and then incubated with (A) anti- 15mer or (B) anti-rec-BSPH1 antibodies. For all samples, eggs were counterstained with DAPI to localize nuclei. Original objective magnification was ×40, scale bar =20 μm.

Figure 4. In vitro fertilization assay following pre-incubation of eggs with rec-BSPH1. (A) IVF with zona-intact eggs shows a significant inhibition of the fertilization rate when eggs were pre-incubated with rec-BSPH1 protein (300 μg/ml) protein (***P< 0.001). As expected, control proteins (Ovalbumin and TRX) had no significant effect. (B) Dose-dependent inhibition of fertilization of zona-intact eggs pre-incubated with mouse rec-BSPH1. Differences were significant compared to control (*P< 0.05; **P< 0.01; *** P< 0.001). As noticed by looking at both graphs A and B, there is no significant difference in inhibition level caused by 10 and 30 μg/ml of mBSPH1 (Fig 4B) and that of 300 μg/ml (Fig 4A) (**C**) Microscopy images showing the fertilization

state in the absence (control) or presence of recombinant BSPH1. Fertilized, unfertilized and degenerated oocytes are shown by black, white and grey arrowheads, respectively.

Figure 5. Immunolocalization of native and rec-BSPH1 on cauda epididymal sperm.

Sperm were washed and then incubated with or without rec-BSPH1 (30 µg/ml) in PBS or TF,corresponding to uncapacitated and capacitated sperm. For immunodetection of native BSPH1, sperm were incubated without recombinant proteins (medium alone; PBS). All samples were then incubated with anti-rec-BSPH1 antibodies, except for a control that was incubated with NRS-IgG. For all samples, sperm were counterstained with propidium iodide (PI) to localize nuclei in the sperm heads. White asterisk shows a weak fluorescent signal in anterior acrosomal segment. Scale bar =10 µm. White arrows in second last panel of figure 5 show mid-piece extremities.

Figure 6. Effect of anti-rec-BSPH1 antibodies on in vitro fertilization and mouse sperm motility.

Cauda epididymal mouse sperm were incubated for 1 h in capacitating medium (HTF), then incubated with different dilutions of anti-rec-BSPH1 antibodies (1:25, 1:50, 1:100) for 30 min. (**A**) Fertilization rate (%) results are shown for each group from five independent experiments (~ 50 eggs). Significant differences between means compared with control (sperm alone) or NRS- IgG (1:50) are shown by different letters. (**B**) Sperm motility and kinematic parameters were assessed by loading 3 µl of sperm suspension onto a prewarmed 20-µm Leja Chamber slide on the warm stage of a Nikon Eclipse 50i microscope connected to the SCA system. All parameters were measured for approximately 200 spermatozoa.

Figure 1

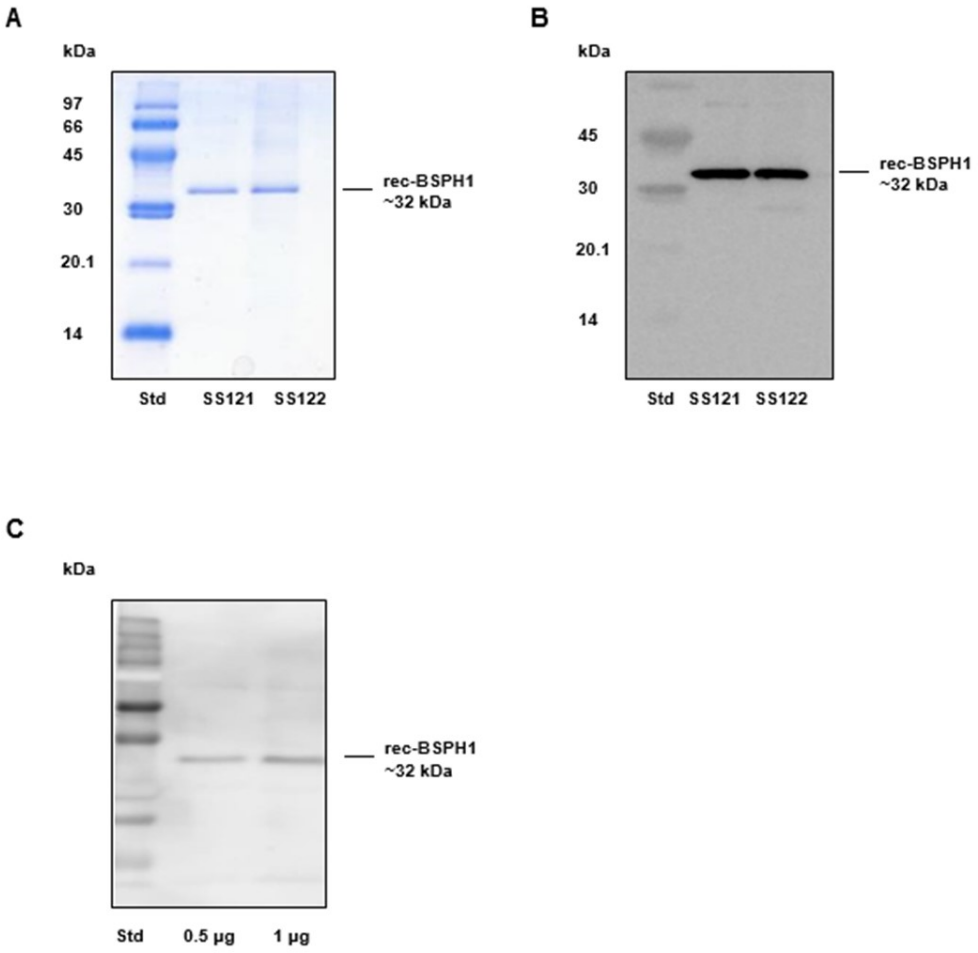
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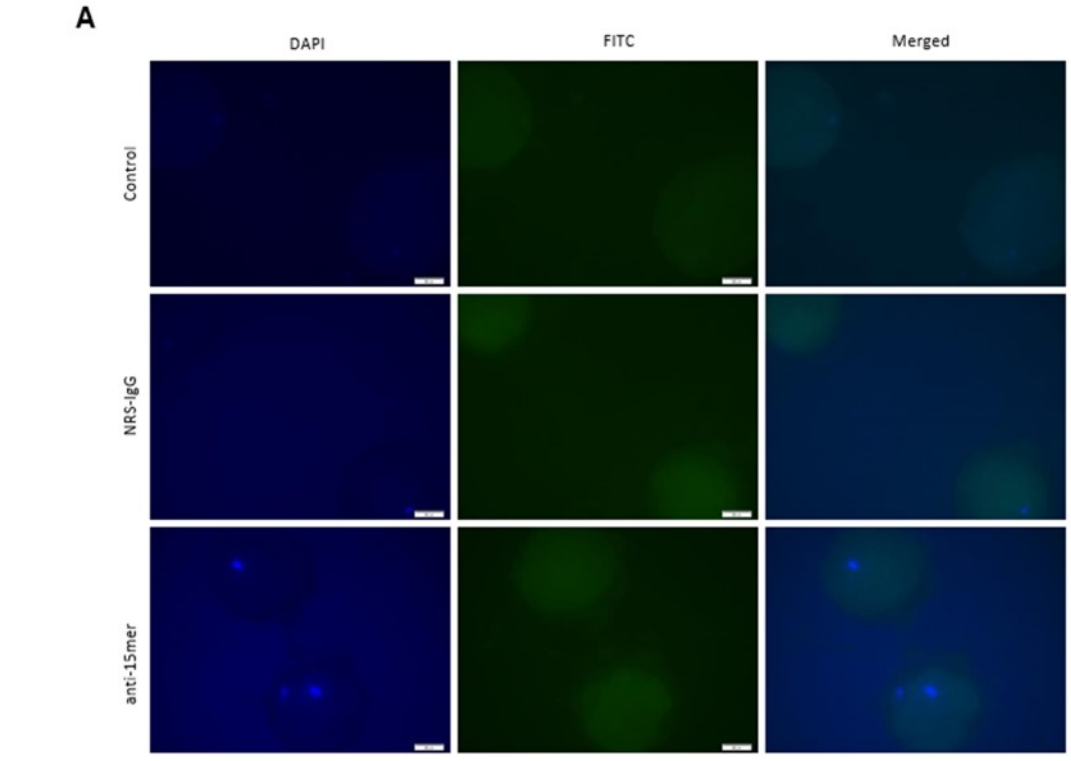
Figure 2 A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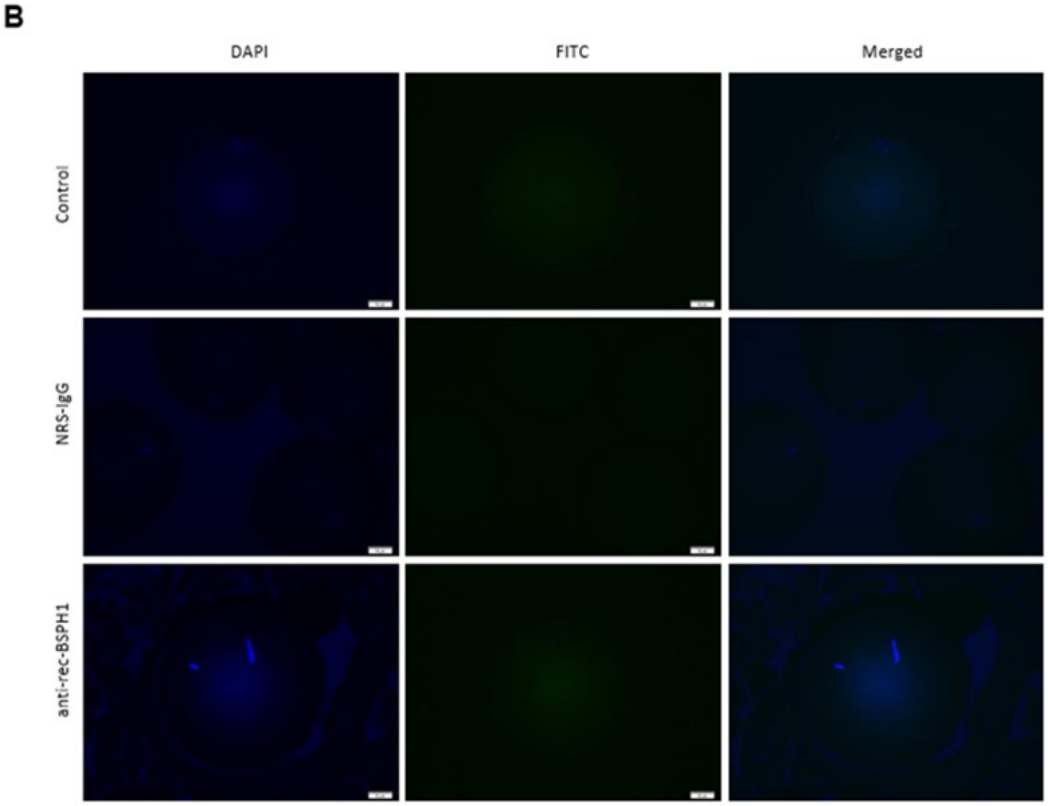
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DAPI
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Control
NRS-IgG
anti-rec-BSPH1

Figure 2 C

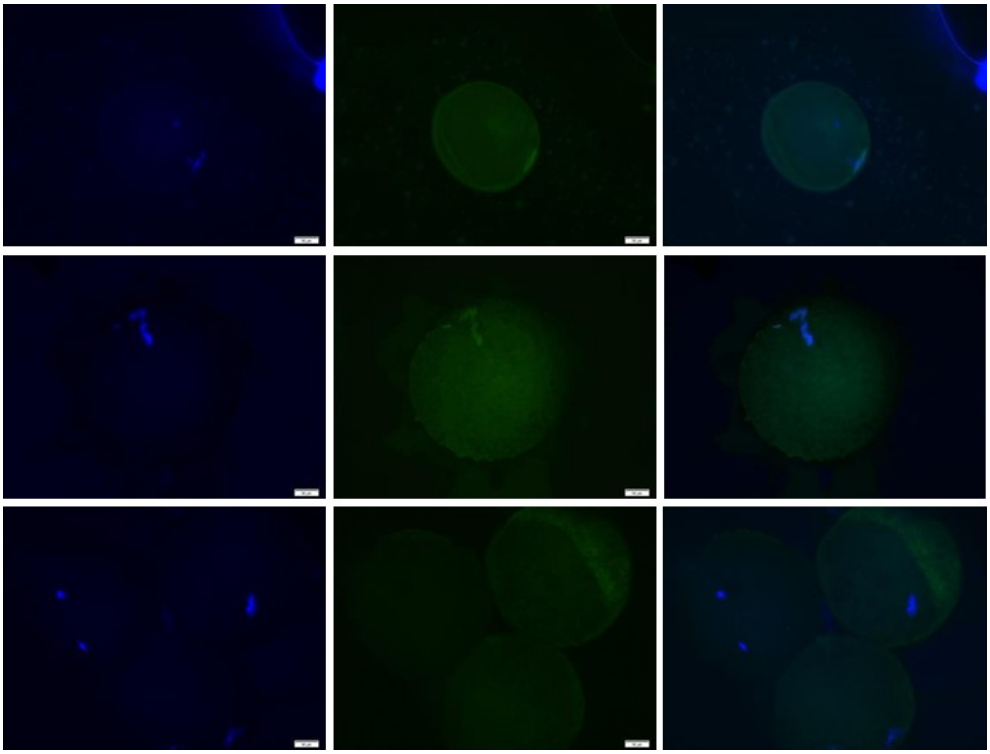


Figure 3 A

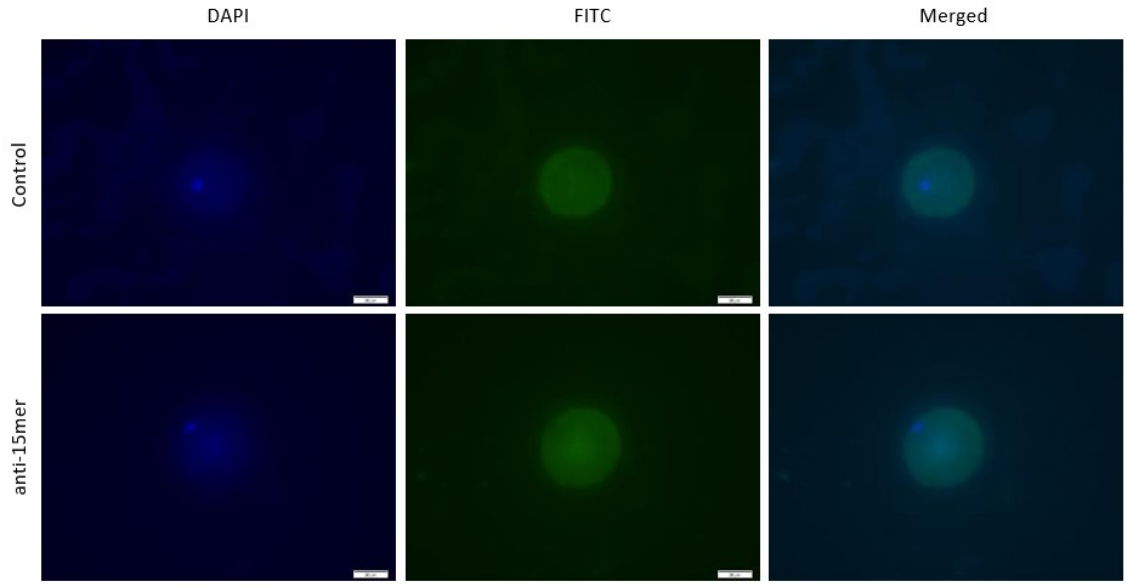


Figure 3 B

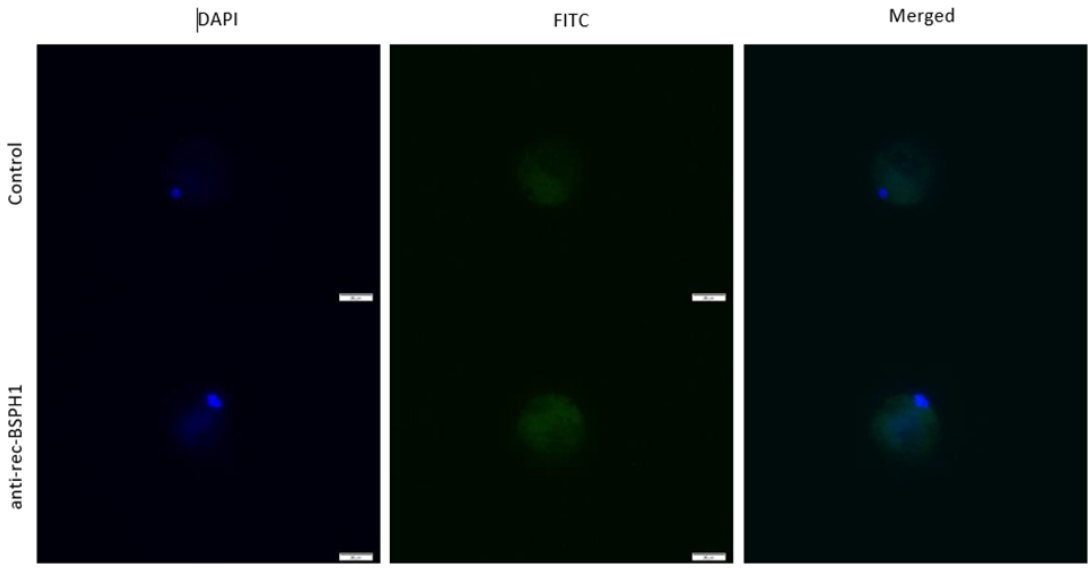


Figure 4

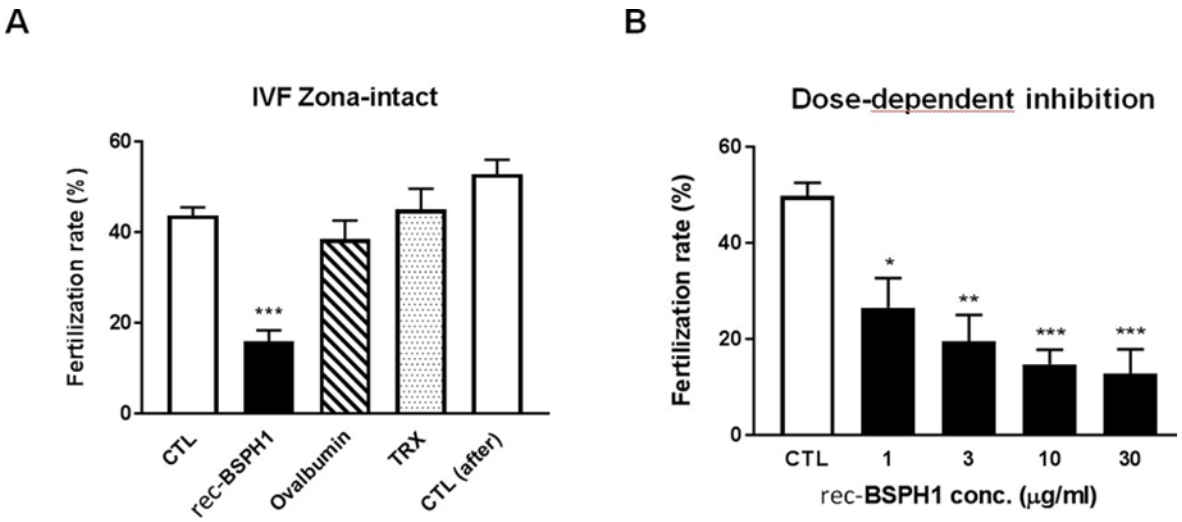


Figure 4 C

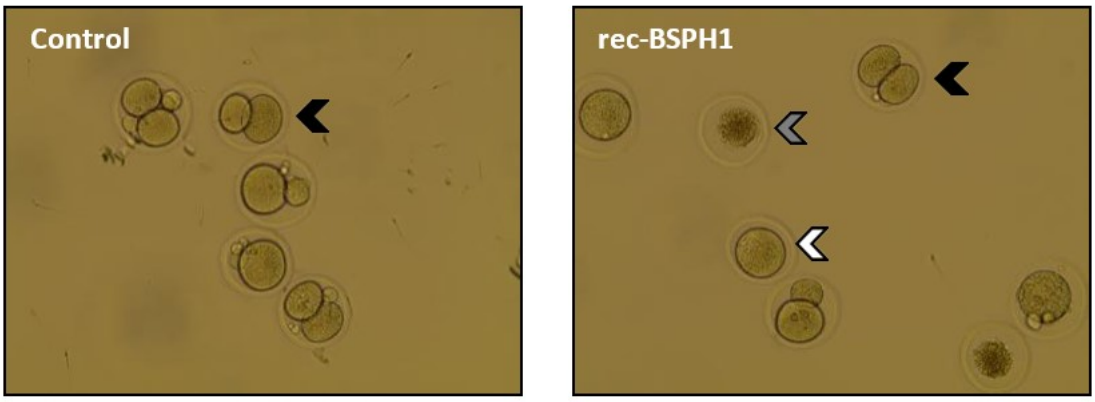


Figure 5

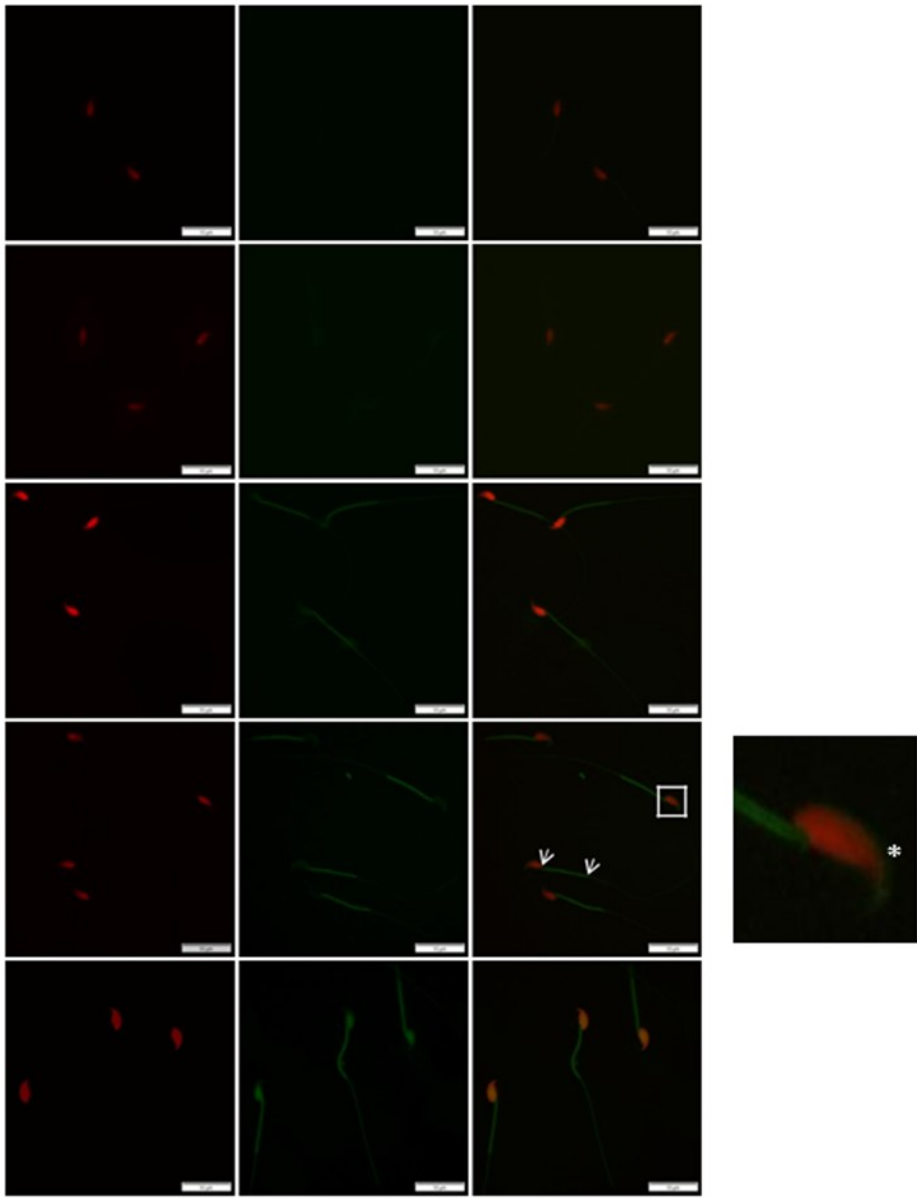
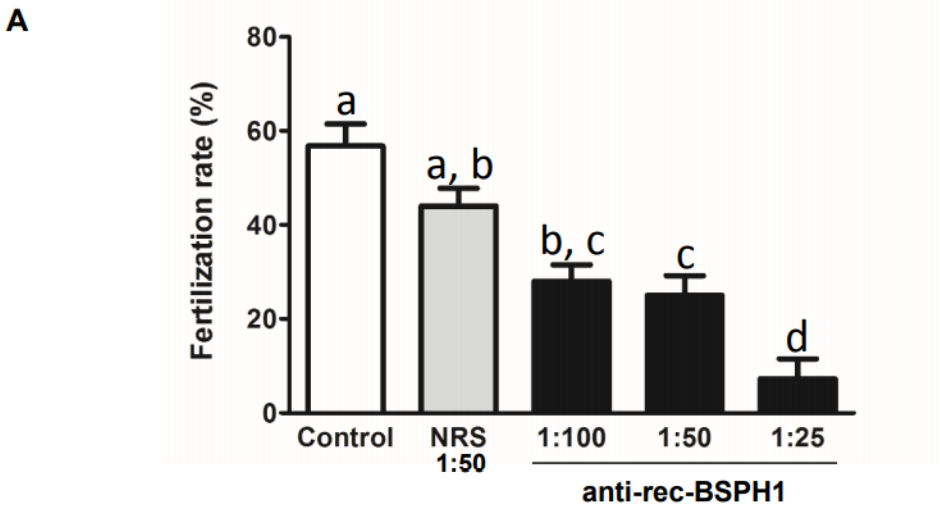
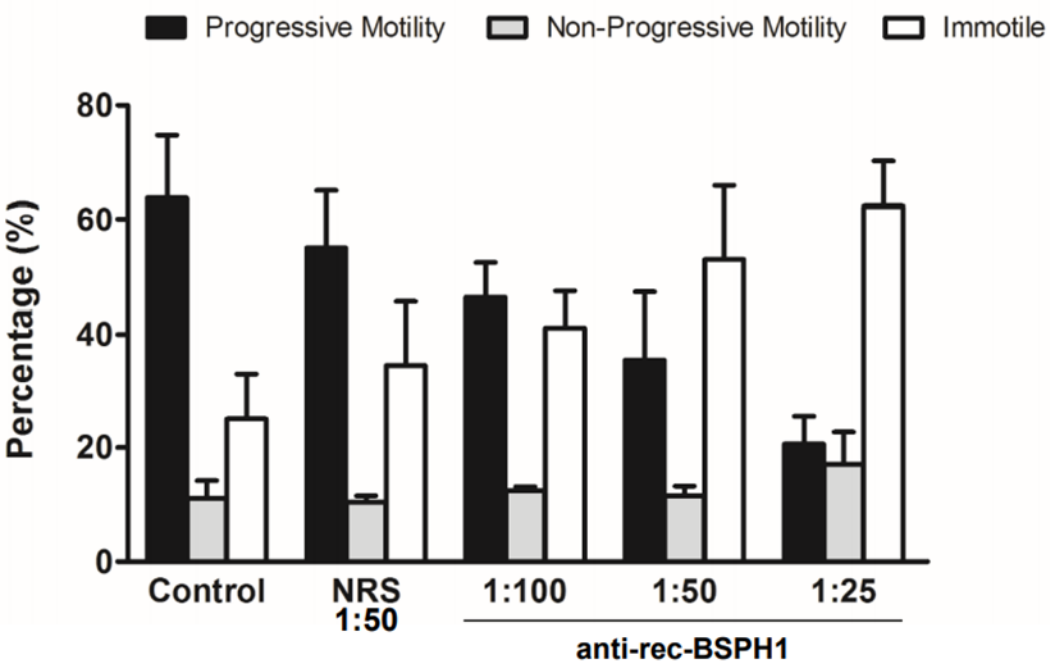


Figure 6


A
Fertilization rate (%)
80
60
40
20
0
a
a, b
b, c
c
d
Control
NRS 1:50
1:100
1:50
1:25
anti-rec-BSPH1


Progressive Motility
Non-Progressive Motility
Immotile
Percentage (%)
80
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20
0
Control
NRS 1:50
1:100
1:50
1:25
anti-rec-BSPH1

Chapter 4 – Article 3

Title: Identification of novel protein interaction partners for human binder of sperm protein

Summary of paper 3:

Binder of SPerm (BSP) proteins are male tissue-specific proteins (expressed in either the seminal vesicles or epididymis). BSP proteins were previously believed to be essential for sperm function and fertility; however, recent evidence suggests that BSPs might be dispensable for fertility in mice. The focus of this research was on the human epididymal protein BSP homolog 1 (BSPH1). BSPH1 was shown to interact with a few partners such as collagen, heparin as well as phosphatidylcholine. In order to further expand our understanding of the molecular functions of BSPH1, we set out to determine the protein interaction network of BSPH1 using the BioID technique. Proximity labeling coupled to mass spectrometry has significant advantages for the study of insoluble and membrane bound proteins and can detect weak or transient interactions compare to the conventional methods. Our BioID experiments identified number of cilia-associated proteins such as the CCT/TRIC complex, centrosomal proteins (CEP), BBS11/TRIM32 and BBS17/LZTFL1, to name a few. Cilia are microtubule based, sensory structures that are divided into two categories (motile and non-motile), each having their own role. Non-motile cilia (primary cilia) are present in almost all organelles. Primary cilia in the male reproductive system are present in the testis, epididymis and prostate, and are involved in cellular proliferation and adhesion through the hedgehog signalling pathway. On the other hand, motile cilia are present in the efferent ductulus of the male reproductive system and are responsible for inhibition of sperm aggregation. Altogether, further experiments need to be done to confirm the possible role of BSPH1 in relation to primary cilia.

Author contributions:

Samin Sabouhi Zarafshan as first author was responsible for data gathering and drafting the article.

Frédérick A. Mallette as corresponding author ensured integrity and accuracy of data as well as revision for final approval.

Serge McGraw as co-corresponding author contributed to the revision of the article and preparation of the final draft.

Identification of novel protein interaction partners for human binder of sperm protein

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Abstract

Binder of sperm (BSP) proteins are present in all-male mammalian species. They are expressed in the seminal vesicles and epididymis and share a common structure (consisting of two fibronectin type-II domains). BSPs also share other features such as binding to choline phospholipids, which are the major lipids of the sperm membrane. In several species, BSPs have been shown to have a positive impact on *in-vitro* sperm capacitation, sperm motility and viability. They are also thought to play a role in forming the oviductal sperm reservoir and play a role in sperm-egg interaction. However, recent BSP gene knock out studies in mouse indicated no effect on fertility. In order to reveal potential novel functions of the human Binder of SPerm Protein (BSPH1), we used proximity labeling coupled to mass spectrometry to identify its protein-protein interaction network. Identified proteins with a significant false discovery rate (FDR <0.02) include all subunits of the chaperonin containing tailless complex polypeptide 1 (CCT) or tailless complex polypeptide 1 ring complex (CCT/TRiC). It is known that CCT/TRiC interacts with the BBSome complex and mediates its assembly. The BBSome complex is made up of eight BBS proteins, and is involved in protein trafficking and primary cilium biogenesis. As shown in our results, BBS11/TRIM32 and BBS17/LZTFL1 are other BBS proteins that interact with BSPH1. The literature shows that BBS17 interacts with the BBSome complex and regulates protein trafficking in cilia. Additional evidence that links BSPH1 to primary cilia and their chaperone-like activity is the emerging number of centrosomal proteins identified which are important for the formation of primary cilia through microtubule and centrosome maturation. Our results suggest a potential role for BSPH1 as a chaperonin protein that interacts with a number of primary cilia proteins.

Keywords: BioID, epididymal protein, Binder of SPerm proteins, primary cilia, CCT/TRIC, BBSome

Introduction

The mammalian epididymis is a unique environment in which sperm mature, acquire the ability to be motile, and are stored. Epididymal proteins are essential for the first sperm maturation step, which is accompanied by sperm motility [1]. The epididymis is a highly specialized organ, consisting of three different segments named the caput, corpus and cauda [1]. Since sperm are transcriptionally and translationally inactive, they rely on proteins expressed and secreted by the epididymis for their maturation, which occurs during their transit through this organ [1]. One of these epididymal proteins called Binder of SPerm proteins (BSP) is part of a family that is ubiquitously expressed among mammals. BSPs are small, acidic proteins sharing a common structure consisting of two fibronectin type-II domains conserved among vertebrates [2], separated by a seven amino acid linker. There are two homologs in mouse (Bsph1, Bsph2) and only one homolog has been identified in humans (BSPH1) [3]. BSPs interact with phosphatidylcholine in the sperm membrane and have been shown to promote sperm membrane cholesterol efflux and modifications [4]. To date, their impact during *in vitro* sperm capacitation, motility, and formation of the oviductal sperm reservoir have been investigated [5–8]. For instance, bovine BSP1 has been demonstrated to increase sperm motility *in vitro* in a dose-dependent manner [6]. Nevertheless, the exact role of BSP proteins in fertility remains unclear, as *Bsph1/2* double knock out mice remain fertile [9]. In order to elucidate potential novel roles of human BSPH1, we sought to identify its protein-protein interaction network using proximity-dependent biotin identification (BioID). The BioID approach, a widely used proximity labeling method, allows the detection of weak and transient interactions and provides a history of protein interactions over a specific period of time in living cells [10]. BioID utilizes a biotin ligase fused to the target protein to biotinylate proximal proteins upon addition of biotin to live cells. The biotinylated proteins can be separated from the cell lysate using streptavidin pull-down, and then identified using mass spectrometry. BirA (biotin ligase)-dependent labeling of interacting proteins is efficient within a 10 nm radius [11]. Another advantage of this technique is the ability to study insoluble proteins as well as membrane proteins, which can be difficult to achieve using conventional approaches. Using BioID, we successfully uncovered the BSPH1 interactome in HeLa cells. We observed all subunits of the TCP1 ring complex (TRIC) in the mass spectrometry analysis

of BSPH1 interacting proteins. CCT/TRIC is a hetero-oligomeric complex that displays chaperone activity, promoting the correct folding of proteins [12]. Besides its chaperone activity, TRIC has an additional role in the formation of tubulin/actin microfilament polymerization in cytoskeleton process [12]. The BBSome protein complex has been shown to interact with TRIC for its assembly [13]. In fact, BBS6, BBS10, and BBS12 form a complex with CCT/TRIC, which together are essential for protein trafficking in cilia [14]. Within the epididymis, important structures called primary cilia play critical roles during development (before puberty) as well as in adulthood. This microtubule-based structure is formed in the shape of an antenna that can receive signals from the extracellular matrix and respond through intraflagellar protein trafficking to the destination (cytoplasm) and the other way around [15]. Seo et al., showed that the CCT/TRIC complex knockdown in zebrafish led to Bardet-Biedl syndrome (BBS) [14]. Bardet-Biedl syndrome (BBS) is, a rare autosomal recessive syndrome that is associated with more than 21 loci, and 25 percent of patients are idiopathic [16]. Individuals suffering from Bardet-Biedl syndrome have polydactyly, obesity, and retinal dystrophy, and its frequency is 1:160,000 in Northern Europe [17,18]. From the other perspective, CCT/TRIC and heat shock proteins (HSP) have also been suggested to play a role in sperm-egg interaction [19]. CCT/TRIC is present on the proximal part of the sperm head regardless of capacitation state and interacts with HSP proteins, specifically HSPE1. The elevation of) CCT/TRIC amount on the sperm head during capacitation supports its indirect role in sperm-egg interaction [19].

There are two types of cilia in the epididymis: motile cilia, which ensure the movement of fluid, and non-motile or primary cilia. Recently, the role of primary cilia as sensory organelles that mediate signal transduction at embryogenesis as well as during adulthood was demonstrated. For instance, the Wingless and Int-1 (Wnt), and sonic hedgehog (SHH) pathways are notable in cilia, among others [20]. Interestingly, cilia do not have any ribosomes; therefore, the source of cilia proteins should be the cytoplasm, with protein trafficking happening through intraflagellar transport (IFT) from cytoplasm to the extra cellular matrix and the other way around [20]. This suggests that the function of primary cilia is in the interactions between cytoplasmic proteins. Laura Girardet et al. demonstrated that although all types of hedgehog ligands are present in epididymal epithelial cells, only Indian hedgehog (IHH) can be secreted [21]. Briefly, when the IHH

agonist is present, patch1 is activated and translocated out of primary cilia. Recruitment of smoothened (SMO) occurs in the primary cilia, which triggers activation of the Gli transcription factor and downstream gene regulation [21].

Our findings potentially linking BSPH1 to primary cilia could lead to the identification of novel molecular roles for BSPs proteins in the epididymis, and a better understanding of the male reproductive tract.

MATERIAL AND METHODS

Plasmids and reagents

PSTV2-N term, PSTV2-C term and PSTV2-N-BirA-GFP were kindly provided by Dr. Anne-Claude Gingras. pLenti CMV rtTA3 Blast (W75691), PSPAX2 (12260), PMD2.G (12259) and pENTR were from Addgene (Watertown, Massachusetts, USA).

HeLa and 293T cells were purchased from the American Type Culture Collection (ATCC). 10% heat-inactivated fetal bovine serum (SH3039603) was from GE healthcare Hyclone, Canada and DMEM from Gibco, Thermofisher Scientific (Waltham, Massachusetts, USA). Trypsin-EDTA (0.25%) was purchased from Life technology (25200114).

Lipofectamine and OPTI-MEM were purchased from Invitrogen. Protease inhibitor mix and phosphatase inhibitors were obtained from Roche. All primers were purchased from Invitrogen. Primary antibodies: anti-FlagM2 antibody was from (Sigma-Aldrich; F7425) and used at a 1:1000 dilution, HRP-Conjugated Streptavidin was obtained from Thermofisher Scientific and used at a dilution of 1:5000, and anti-tubulin, used at a 1:5000 dilution, was from Sigma-Aldrich (B-5-1-2; T51680). CCT5 antibody was purchased from Life technology (11603-1-AP) and CCT8 antibody was purchased from Novus biological (NBP1-56608).

Alexa Fluor 488 and 594 goat anti-mouse secondary antibody (dilution of 1:1,000) were from ThermoFisher Scientific (A11008, A11030). HRP-conjugated goat anti-mouse antibody was from Cell Signaling (7076S). DAPI (4,6-diamidino-2-phenylindole) was purchased from Invitrogen. Images were detected using Olympus BX53 fluorescence, and Image J was used for recoloring of

the pictures. PVDF membranes for Western blot were from Bio-Rad (1620177). Polyethylenimine, Linear, MW 25000, Transfection Grade (PEI 25K) from Polysciences (23966-1). Paraformaldehyde solution 4% in PBS was from Santa Cruz (sc-281692).

BSPH1 gene blocks were purchased from Integrated DNA Technologies (IDT). LR clonase II enzyme mix was from Invitrogen (11791020). Restriction enzymes were from New England Biolabs. VECTASHIELD Antifade mounting medium for fluorescence was from Vector Laboratories.

Gateway cloning

BSPH1 gene blocks and corresponding primers were used to amplify products with sticky ends using NotI and AscI restriction enzymes (primers are listed in Table 1). Corresponding sites were cut on pstv2 vectors as well as PCR products. The presence of BSPH1 in the expression clone (PENTR) was confirmed by sequencing. We used pstv2-BirA-Flag as a gateway destination vector [22]. LR (attl and attR site recombination) reaction was done between entry vectors and destination vectors; after sequencing confirmation, validated clones were used to generate a stable cell line.

Cell culture and lentiviral production and infections

All HeLa or 293T cell lines were cultured with Dulbecco's modified Eagle medium (DMEM) with 10% FBS and 1% penicillin/streptomycin in the incubator at 37°C and 5% CO_2. Briefly, lentivirus particles were produced using 293T cells using 6 μg DNA of each BioID constructs or rtTA as well as 3.75 μg of psPAX2 packaging plasmid and 1.25 μg pMD2.G envelope plasmid with 25 μL of Lipofectamine 2000 reagent in 1 mL of optimum medium. Medium was changed 24h post-transduction, and virus particles were harvested 48 hours post-transduction. The viral soup was filtered through 0.45 μm filters and either stored in -80^{o}C or along with 8 μg/mL polybrene, and 10% FBS were added to the Hela cells. HeLa cells were first infected with the rtTA lentivirus to produce a Tet-on stable cell line and selected using blasticidin 10μg/mL.

BioID

The BioID protocol was adapted from the previously described method [22]. Briefly, cells were seeded in 15 cm plates to be confluent at 75% on the next day and were induced with 0.5 μg/mL

doxycycline (Dox) and 50 µM biotin for 24h. Cells were then washed twice with cold PBS and harvested. Cells were flash-frozen on dry ice and stored at -80˚C until the next step. In this step, cells were lysed either to check protein expression using western blot, or for affinity purification (AP).

Affinity purification

We adapted the protocol from Samavachi et al. [23]. Briefly, cells were resuspended using modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.5 mM EDTA, 1 mM $MgCl_2$, 1% NP40, 0.1% SDS, 0.4% sodium deoxycholate, 1 mM PMSF and 1x Protease Inhibitor). The cell suspensions were then sonicated for three cycles of 5s with 30% amplitude. 250 U of Ribonuclease and 10 µg of RNase A were added, and the suspensions were incubated at 4°C for 15 min. The suspensions were then centrifuged at 15000 x g for 15 min. Then, streptavidin agarose beads were washed in RIPA buffer, and 25 µL of the agarose beads was incubated with each sample for 3h at 4°C. This was followed by a one-time wash with 2% SDS buffer (25 mm Tris-HCl, pH 7.4, 2% SDS), twice with RIPA buffer, once with TNTE buffer (50 mm Tris-HCl, pH 7.4, 150 mm NaCl, 1 mm EDTA, 0.1% NP40) and four times with 50 mM ammonium bicarbonate (ABC buffer). Trypsin (1 µg) was then added to the samples for digestion, and incubated overnight at 37°C, followed by three additional hours of digestion with 0.5 µg Trypsin. Subsequently, proteins were resuspended with 50% formic acid and stored at -80^{o}C until further analysis.

Immunofluorescence

Immunofluorescence was performed as described previously [24]. Briefly, cells were seeded on glass coverslips in 6-well dishes, then fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.2% Triton X-100 in Phosphate-buffered saline (PBS) comprising 3% bovine serum albumin (BSA). Cells were blocked in PBS containing 3% BSA for 60 minutes to minimize unspecific binding of primary antibodies. Samples were incubated with the following: Anti-Flag-M2 at a dilution of 1:1000, and streptavidin at a dilution of 1:2000, Alexa Fluor 488 (1:1000), Alexa Fluor 546-conjugated goat anti-mouse secondary antibodies (1:1000), followed by 4,6-diamidino-2-phenylindole (DAPI) staining. Coverslips were mounted using mounting media, and images were taken using an Olympus BX53 fluorescence microscope then analyzed using ImageJ software.

Western blot

Cells were lysed in lysis buffer containing 10 mM Tris pH 7.4, 120 mM NaCl, 0.5% Triton, protease inhibitors, and phosphatase inhibitor. The suspension was then sonicated on ice three times for 10s, followed by centrifugation at 15,000 x g. Protein concentrations were assessed using the Pierce BCA Protein Assay Kit. Cell extracts were separated on SDS-PAGE and transferred to PVDF membranes. Primary antibodies were used as follows: α-tubulin (1:5000 dilution) and Flag-M2 (1:1000 dilution). Streptavidin-HRP was used with at 1:5000 dilution. HRP-conjugated goat anti-mouse was used as a secondary antibody with a dilution of 1:3000. Corresponding proteins were detected using enhanced chemiluminescence (ECL) and analyzed using the Fuji LAS-3000 image analyzer.

Results

Generation and expression of BirA-BSPH1

BioID constructs for the analysis of the BSPH1 interactome were generated using Gateway cloning. Two different fusion proteins of BSPH1-BirA were generated (C-terminal BirA, N-terminal BirA) in order to cover all interactions and to limit the effect of the steric hindrance of the BirA-tag in the analyses [25]. Next, these constructs were lentivirally transduced into HeLa cells (Figure 1). The BioID study relied on Tet-inducible expression of BSPH1-BirA to control the expression level and timing of induction. As a control, we used a GFP-BirA-flag vector as well as non-infected cells. The biotinylation pattern was observed when probed with a streptavidin antibody (Figure. 1A). Cells that were not induced by doxycycline were also used as a negative control. Upon induction with doxycycline, several bands were detected on SDS-page using streptavidin-HRP, indicating efficient BirA-mediated biotinylation, as well as endogenous biotinylation of proteins such as carboxylases [26].

The induction of the BirA-tagged proteins was also confirmed using anti-flag antibody (Figure. 1B), where bands around 40 KDa correspond to the approximate molecular weight of BirA-BSPH1 fusion proteins, while the 52 KDa bands represent the BirA-GFP protein. Tubulin was used as a loading control (Figure. 1C).

To further confirm the expression of the different BioID constructs, we performed immunofluorescence experiments. HeLa cells stably expressing BioID fusion proteins were stained for biotin using streptavidin (594) and Flag (488), and DAPI was used for nuclei staining. The localization pattern with anti-Flag antibody seems to be different in both constructs due to the N and C pattern of the constructs that can infer their localization.

Identification of the BSPH1 interactome using BioID

Two replicates for each condition (BSPH1-BirA, BirA-BSPH1 and GFP-BirA) were sent to the mass spectrometry. Mass spectrometry (MS) data was retrieved from the SCIEX Triple TOF apparatus. MS identified a total of 3433 proteins. However, the number of identified peptides exhibiting significant FDR (0, 0.01, and 0.02) reached 3277 candidates (Table 2). In order to analyse how these results can be categorized into functional groups, we utilized String and Reactome analyses. Our results highlighted the group of interactions with ciliary proteins with high spectral count in both N-terminal and C-terminal fused BSPH1-BirA constructs when compared to GFP-BirA controls, suggesting their association with BSPH1.

First, String is utilized to predict enriched subsets of interacting protein networks. (https://string-db.org/ version 11.0). The String protein-protein interaction enrichment p-value was < 1.0e-16, saint data (significance analysis of interactome) was shown in Table 2 . Enrichment indicates that the proteins are biologically linked as a group. String analysis showed a number of biological processes in gene ontology, such as cellular catabolic process and cellular component organization or biogenesis, which had the lowest false discovery rate. Moreover, String analysis identified other functional enrichments such as the VCP-NSFL1C complex. NSFL1C is one of the VCP adaptors and its presence in regard to ciliogenesis has been documented [27]. Additionally, depletion of VCP impairs the intraflagellar transport B (IFT-B) complex and affects protein trafficking consequently [27].

In addition, String analysis showed a relationship between the BSPH1 protein interaction network and the cell cycle (M phase, Mitotic anaphase, and separation of sister chromatids).

Using the open-access Reactome database (http://www.reactome.org) [28], a pathway analysis tool to support protein-protein network data analysis, we identified a list of candidates related to cilia biogenesis with emphasis on primary cilia (Table 3). The primary cilia are signal transduction mediators for a number of pathways, including SHH and Wnt [29]. Cilia include the microfilament structure of the axoneme, a basal body and a plasma membrane. They also have a distinct form of protein and lipids in the ciliary membrane versus the plasma membrane [169]. Primary cilia are dynamic structures and their turnover relies on proteins such as the tubulin family, which make the structure component of primary cilia [31]. Various members of the tubulin family were identified as BSPH1-interacting proteins, such as TUBB, TUBB6, TUBB2A and TUBB4A (Table 3). The *TUBB* gene translates into beta tubulin, which can make a dimer with alpha tubulin and establish the microtubule structure. Microtubules are the major constituents of primary cilia, therefore Tubb family proteins are important for primary cilia biogenesis [32].

In addition, the five subunits of the CCT/TRIC complex were recovered as BSPH1-interacting partners (Table 3). CCT is a hetero-oligomeric complex of about 900 kDa, which uses tubulin and actin as substrates for microtubule polymerization [33]. CCT/TRIC is known as a chaperonin complex, which is essential for assembly of the BBSome complex in primary cilia [34]. The BBSome is a complex containing eight subunits, which is important in cilia homeostasis [35]. Mutations in this complex result in Bardet–Biedl syndrome (BBS) [35]. Moreover, the BBSome complex is involved in the identification of proteins through G-proteins coupled receptors and can act as an adaptor for their transportation in the primary cilia [35].

An earlier study in tetrahymena demonstrated that CCT subunits colocalize with tubulin in cilia and are important for cilia biogenesis [36,37]. Interestingly, knocking out CCTα and CCTY in tetrahymena leads to failure in ciliogenesis resulting in cell death [36]. In view of the evidence from this 2010 study, CCT could also be implicated in tubulin turnover and cilia biogenesis in response to ciliary impairment [33]. Interestingly, Reactome analysis revealed another potential interacting partner of BSPH1, LZTFL1 (BBS17), which is a member of the Bardet–Biedl syndrome (BBS) protein family. LZTFL1 (BBS17) modulates BBSome trafficking in the primary cilia [38]. Contribution of LZTFL1 (BBS17) to energy homeostasis and regulation of leptin signalling in the

hypothalamus has been documented [38]. The leptin signalling pathway modulates food intake and disruption in this signalling pathway could lead to weight gain or obesity [38].

Moreover, numerous centrosomal proteins such as CEP131, CEP152 and CEP72 were identified among BSPH1-interacting proteins (Table 3). Centrosomal proteins participate in cilia biogenesis and are essential for engaging BBS proteins in the cilia [39]. The major satellite protein called PCM1 is interacts with many protein scaffolds and complexes. PCM1 also interacts with CEP72 and CEP131. CEP131 is considered as a basal body constituent of primary cilia. Recently, a study by Stapels et al. demonstrated that CEP is essential for genome stability. CEP131 depletion can lead to decreased cell proliferation and increased DNA damage after mitosis [40]. Moreover, CEP72 is another centrosomal protein required for translocation of the BBSome complex to cilia [39].

Ciliary proteins are important due to the wide range of syndromes, named ciliopathies, and multi-phenotypic disorders (obesity, infertility, polydactyly, etc.) that have been linked to defects in primary cilia [20].

A similar pattern of results was obtained using the interactive heatmap and dot blot viewer. The analysis was done using a prohits-viz online source [41]. BioID results associated to ciliogenesis that have significant FDR are represented using dark round circles for both BSPH1 constructs (fused with BirA at N-ter or C-ter) (Figure. 3). Therefore, CCT/TRIC subunits were analyzed in subsequent validation studies. These results suggest that members of the TUBB family and the CCT/TRIC complex are important candidates to reveal the function of the BSPH1 in relation to the primary cilia.

BioID spatial proximity labeling results related to primary cilia are listed in Table 4, with references to published articles identifying their biological roles. It is notable that a group of centriolar proteins as well as satellite proteins are present in our data, highlighting that BSPH1 may have a role in ciliogenesis.

Gene ontology enrichment analysis

Gene ontology (GO) describes relationships between genes/proteins with respect to their molecular functions, cellular component as well as the biological processes they are involved in. We used the GOrilla online tool [42] to perform GO on the BSPH1 interaction network identified using BioID.

GO analysis revealed cytoskeletal fiber enrichment genes consisting of three categories: microfilaments, microtubules, and intermediate filaments (Table 5). These cytoskeletal fibers provide a scaffold to support cell shapes or cilia extensions [43], further suggesting a possible role for BSPH1 in relation to cilia.

Validation of the BSPH1 protein interactions identified by BioID

The BioID approach generates enormous amounts of data that must be carefully analyzed and validated using different methods. We first sought to validate the interaction of CCT/TRIC with BSPH1 because of their potential to interact with the BBSome complex. Using streptavidin pull-down, we confirmed the interaction of CCT5 with BSPH1 (Figure. 4), as was observed using BioID. The CCT5 antibody labeled a band around 60 kDa both in the input and BSPH1 pulldown, but not in the control pulldown. Expression of flag-BirA fusion proteins was validated using anti-Flag antibody, showing a 42-kDa band for BSPH1 fusion constructs, and 52 kDa for the GFP construct. Tubulin was used as a loading control. In addition, streptavidin pulldown upon induction of BSPH1-BirA did not show any interaction for alpha tubulin, as expected based on BioID results.

As further confirmation of our results, we validated the interaction of another subunit of the CCT/TRIC complex with BSPH1, CCT8, using two independent replicates (Figure. 5). Probing with anti-CCT8 antibody revealed a signal around 60 kDa in the input lanes as well as in the BSPH1-BirA samples. BirA-BSPH1 fusion proteins were revealed using anti-Flag antibody with a corresponding molecular weight of 42 kDa, while GFP-BirA fusions were observed around 52 kDa. Thus, we confirmed the interaction of BSPH1 with CCT5 and CCT8, which are key regulators of BBSome assembly [35].

Proposed model for potential novel functions of BSPH1

To date, the exact roles of BSPH1 remain unclear. However*, in vitro* studies showed a dose-dependent increase of sperm capacitation by BSPH1 [44]. Based on our preliminary results, we speculate that BSPH1 could also be involved in protein trafficking through primary cilia in the epididymis. From this standpoint, we presented a putative model of BSPH1 interacting partners through its potential physiological role in primary cilia (Figure. 6).

Discussion

The list of interacting partners for BSPH1 identified using proximity labeling BioID suggest some potential novel functions for this epididymal protein. First, we identified a significant enrichment for the different subunits of the CCT/TRIC complex, comprising CCT5, CCT8, CCT6A and CCT2, when compared to GFP-BirA controls. CCT/TRIC is primarily defined as a chaperonin system essential for protein folding [45], but also mediating disassembly of the mitotic checkpoint complexes (MCC) [46]. Impairment of the CCT/TRIC complex could lead to cancer or neurodegenerative diseases (upregulation of CCT/TRIC subunits) [47]. This complex, which has a molecular weight around 1000 kDa, consists of eight subunits per ring in which each has a specific role in the proper folding of proteins [48]. Each subunit has three domains: equatorial (E; ATP binding site), apical (A; Interaction site), and intermediate (I), the latter of which connects the two other domains [47]. The CCT/ TRIC complex plays critical functions in different biological processes including autophagy [49], proteostatic control of telomerase [50], myeloid leukemia [51], TFIID assembly [52], and cilia biogenesis [53].

The CCT/TRIC complex is crucial for cilia assembly [36,54]. Specifically, it interacts with the BBSome complex, which is important in protein trafficking through primary cilia [34]. BBS6, BBS10 and BBS12 interact with TRIC and form the BBSome complex [14]. Mutations in the BBSome or CCT/TRIC complex are associated with Bardet-Biedl syndrome, which can lead to obesity, polydactyly and retina dysfunctions [14,35]. As an example, BBS7 knockout mice recapitulate BBS phenotypes such as obesity and male infertility due to a defect in the sperm flagellum [55].

Additionally, BBS11/TRIM32 and BBS17/LZTFL1 are other BBS proteins that interact with BSPH1. BBS17 interacts with the BBSome complex and regulates protein trafficking in cilia [56]. BBS17/LZTFL1 is also implicated in the leptin pathway and regulates food intake [38]. LZTFL1

knock out mice showed obesity and leptin resistance. Interestingly, longer cilia were observed in the fibroblast cells of LZTFL1-null animals compared to wild type counterparts [38]. Similarly, Marzieh Eskandari et al. showed that *bsph1/bsph2* double knock out male pups gained weight in a time dependant manner compared to controls. However, no studies on primary cilia were conducted in these mice [57].

According to our BioID results, BSPH1 interacts with numerous centrosomal proteins (CEP), including CEP131, CEP85, CEP152, CEP72 and CEP295, which are involved in the formation of cilia [58–61]. A mutation in these proteins (CEP64, CEP290 and CEP141) could lead to ciliopathies [62]. Truncated CEP19 is correlated with BBS and morbid obesity, as well as spermatogenic failure. CEP19 interacts with CEP350 in early ciliogenesis, and the interaction of CEP350 with BSPH1 was shown in our MS data [63]. CEP131 is conserved in mammals and drosophila and has a conserved role in ciliogenesis, as revealed by knockdown approaches [59]. CEP295 is essential for the conversion of centriole to centrosome during mitosis [60]. CEP72 has been shown to be essential for the localization of BBS4 to the primary cilia [39]. CEP152 has been shown to be indispensable for the centriole duplications [61].

Here, we highlighted the potential interaction between the CCT/TRIC complex and BSPH1. Interestingly, the CCT/TRIC complex is present on the initial segment of the sperm head regardless of the capacitation state [19]. The CCT/TRIC complex works in parallel with HSPD1 and HSPE1 at the time of capacitation and during sperm-egg interaction [19]. In the same study, incubation of sperm with antibodies against subunits of the CCT/TRIC complex caused partial inhibition of sperm-egg interaction in sperm-zona assays due to the inability to suppress complete adhesion. Therefore, an indirect regulation of sperm-egg interaction by the CCT/TRIC complex has been postulated [19].

We exploited a novel approach involving proximity-dependent labeling to uncover the BSPH1 interactome. We uncovered an interaction of BSPH1 with the CCT/TRIC complex, which could have an indirect impact on sperm-egg interaction. Second, we observed the interaction of BSPH1 with CCT/TRIC and BBS proteins such as LZTFL1 and a number of centrosomal proteins suggesting its involvement in protein trafficking through primary cilia [64].

The primary cilia in the epididymis play critical functions. It has been suggested that primary cilia are elongated from basal cells in the adult epididymis [65], and are important for both the development and homeostasis of the male reproductive system [66]. Primary cilia are present in some undifferentiated cells (Leydig and Sertoli cells) before puberty and disappear in adult cells, suggesting their possible role in cell differentiation [67]. In addition, primary cilia are considered as signaling hubs that transfer signals from extracellular matrix to the cytoplasm and conversely both during embryogenesis and adulthood through Hedgehog, Wnt or calcium signaling depending on cellular conditions [66]. Girardet et al. examined canonical Hh pathways with either agonist (SAG) or inhibitor (Cyclo) in the DC2 mouse cell line, which was under low-serum media to induce primary cilia. They demonstrated that the GLI3 transcription factor was regulated upon treatment with SAG and reduced to its suppressed form GLI3R in the DC2 cell line [21]. Moreover, gene expression profiles upon treatment with SAG demonstrated the upregulation of genes involved in immunity and defense systems. In the same study, in order to demonstrate the regulation of Hh pathway through primary cilia, DC2 cells were treated with ciliobrevin D, which is a cytoplasmic dynein inhibitor. Results showed smaller primary cilia length both *in vivo* and *in vitro*, accompanied with downregulation of genes affected by Hh signaling such as *Patched1* and *Smoothened* [21].

Given these points, further experiments need to be done to confirm the possible role of BSPH1 in relation to primary cilia.

It is important to take additional steps to further elucidate the function of BSPH1. First and foremost, and important experiment will be to observe the phenotype of epididymal primary cilia in the bsph1/bsph2 double knock out mice in order to see any perturbations in the number or elongation of this organelle.

Another interesting future study would be an *in vivo* BioID study to see the consequences of BSPH1 overexpression in a relevant mouse model.

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Conflict of interest

The authors declare no conflicts of interest.

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Table 1. Summary of primers used in cloning

BSPH1 oligos to clone in pENTR	Sequences
Bsph1nostop-NotI-For	5'-AAATATGCGGCCGCCATGGGCTCCCTGATGCTTC -3'
Bsph1nostop-AscI-Rev	5'-TTGGCGCGCCCTTCACAGTATTTCCAAATTCGGTCC -3'
Bsph1nostart-NotI-For	5'-AAATATGCGGCCGCCGGCTCCCTGATGCTTCTC -3'
Bsph1nostart-AscI-Rev	5'-TTGGCGCGCCCTCATTCACAGTATTTCCAAATTCGG -3'

Table 2. Saint analyzed BioID result for BSPH1 in Hela Cells

Prey gene	Saint score	FDR
ASS1	0.98	0.01
ETFA	0.96	0
MTR	1	0.01
OCRL	0.91	0
PEX1	0.99	0
APRT	1	0
GNAS	1	0.02
POLR2A	0.86	0.01
POLR2B	0.97	0
RPL27A	1	0
ATP5PD	0.99	0
AK4	1	0
DDX19B	0.98	0
VPS13A	1	0
RRM1	1	0
INF2	1	0
WIPI2	0.98	0
GTF3C2	0.98	0
UNC45A	0.99	0.01
ABCE1	0.97	0.01
CYLD	0.98	0
TAX1BP1	0.97	0
USP5	0.99	0.02
IMMT	0.83	0
GTF3C5	1	0.01
MOV10	0.92	0
ECD	1	0
EFTUD2	1	0
TRAFD1	1	0
PSMA1	1	0
TRAPPC9	1	0.02
COPS7A	0.76	0
PNPLA6	0.99	0.01
UBXN6	0.96	0
HPD	1	0
AAAS	1	0
PFDN6	1	0
CEP152	1	0
HSDL2	1	0
PSMC5	0.97	0

UCHL5	1	0
NDUFS1	1	0
ENO1	1	0
PPP6R2	1	0
POLD1	1	0
FAAP20	1	0
CUL3	1	0
DYNC1H1	1	0
ECH1	1	0.01
EIF2S3	0.96	0.02
DNAJA1	0.86	0
AARS1	1	0
ATP5F1B	1	0.01
ATP6V1A	0.98	0.01
CSNK1E	0.95	0.01
DDB1	0.96	0
KPNA2	1	0.02
MTAP	0.79	0
NUP88	1	0.02
PHB	0.76	0.01
PIK3C3	0.93	0
PSMA5	1	0
PSMC3	1	0
PSMD2	1	0
PSMD3	1	0
PSMD8	1	0
FSCN1	1	0
SNRPC	1	0.02
SNX2	0.83	0
TTC1	1	0
UBA1	1	0
VBP1	1	0
OFD1	1	0.01
EDF1	0.97	0
USP13	1	0
EIF2S1	1	0
CAD	1	0
FARSA	1	0.01
FLOT2	0.97	0
PDCD5	1	0
DDX1	1	0
PPID	1	0
MVP	1	0
PDCL	0.97	0

LAMA1	1	0
FLOT1	0.99	0
PSMD14	1	0
STUB1	1	0
DNAJA2	1	0
NBR1	1	0
MTHFD1	1	0
CAPZA1	1	0.02
SMC1A	0.77	0
IPO7	1	0.01
PRPF8	0.89	0
TBL3	1	0
CCT8	1	0
HSPH1	1	0
RUVBL2	0.99	0
PRDX3	1	0
COPS5	1	0
PYCR1	1	0
SKIV2L	0.99	0
DNAJB4	1	0
FAF1	1	0
DDX20	1	0.01
CCT5	0.97	0
PFAS	1	0.01
SF3B3	0.92	0
ZC3H7A	1	0
RTCB	0.97	0
CORO1C	1	0.02
PRPF19	0.79	0
PIK3R4	0.99	0
TTI1	0.99	0
KNTC1	0.97	0
USP34	0.99	0.01
TOMM70	0.88	0
TBC1D4	0.99	0.01
RNF10	0.96	0
ANKLE2	0.97	0
CLUH	1	0
NUP160	1	0
TMEM131	1	0
POLR1A	1	0
KIFBP	1	0
CPSF3	1	0
TRMT112	0.98	0

PIH1D1	0.97	0
PARVA	1	0
NANS	1	0
UBR4	1	0
SIPA1L2	0.98	0
DOCK6	1	0
VPS11	1	0
RIC8A	0.98	0
EGLN1	1	0.01
MTARC1	0.96	0
EPS8L2	1	0
CEP85	0.98	0
SPATA5L1	1	0.01
ESRP2	0.93	0
VCPIP1	1	0
TANC2	1	0
HUWE1	1	0.01
GRWD1	0.97	0
PARP10	1	0
USP54	0.99	0
PATL1	1	0
TDRD9	1	0.02
CCDC50	0.76	0
TMCO4	1	0
MIA3	1	0
P00761	1	0.02
ALDOA	0.84	0
PFKM	1	0
PFKL	1	0.02
SMN1	0.85	0
RPS6KA1	1	0.01
PDE8B	0.96	0
IKBKG	0.99	0.02
ITPR1	0.87	0.02
CRYZ	0.76	0
TOM1	1	0
PHB2	1	0
GPN1	1	0
EIF4ENIF1	1	0
CCT7	1	0
PEG10	1	0
LDHB	1	0
LDHA	1	0
SH3GLB1	1	0

RNF213	1	0
CSNK1A1	1	0
HSP90B1	0.99	0
AK2	1	0
CCT6A	1	0
FLII	1	0.01
GAPDH	0.97	0
HSPE1	1	0
PFDN5	1	0
PGAM1	1	0
PSMD1	0.98	0
PSMD12	1	0
PTPN11	1	0
RBMS1	1	0
TPM2	1	0
DNAJC7	1	0
CDK1	1	0
SQSTM1	1	0.01
HSD17B10	0.94	0
CLTC	1	0
HSPA1L	1	0
DSTN	1	0
TUBA1B	0.99	0
RGPD6	1	0
CCT2	1	0
IGF2BP3	1	0.02
STIP1	0.86	0
ATE1	1	0.01
VCP	0.96	0
BRCA1	1	0.01
PRDX5	0.92	0
CEP131	1	0.01
CDK5RAP2	0.95	0
PPM1A	1	0
MYL6	1	0.02
GOLPH3	0.86	0
TCP1	0.99	0
SYNM	1	0.01
NUP155	0.94	0
TUBB	1	0
TUBB6	0.98	0.01
EPPK1	0.87	0
RAB9A	1	0
GBF1	0.97	0

RAD23B	0.99	0
TARS1	0.98	0.02
MYCBP2	0.78	0
RPAP1	1	0
TNRC6A	0.97	0
ABCD1	1	0
DENND4A	0.97	0
ARHGEF10	0.97	0.01
HERC1	0.96	0
USP9X	1	0.01
BORCS7	0.96	0
IGBP1	0.99	0
PFDN2	1	0
ATAD3A	1	0
PLAA	1	0
PDCL3	0.97	0
ENTR1	1	0
PPA2	0.97	0
PDE8A	1	0
MTARC2	0.97	0
PI4KA	0.97	0.01
PRSS1	0.96	0
TCF25	1	0.01
USP25	0.96	0.01
TIMM50	0.96	0.01
FPGS	0.96	0
TBCE	1	0.01
EDC3	0.96	0
RBM34	1	0.01
PPP6R3	0.96	0.02
XIAP	0.83	0
RBM28	0.97	0
FAM193A	0.99	0
IARS1	1	0.01
MAGEB2	0.95	0
ABCD3	0.98	0
BCAS2	0.99	0
TAB1	1	0.02
POLR3C	0.76	0.01
GMEB2	0.96	0
LARP4B	0.99	0
GEMIN4	0.97	0
UBXN1	1	0
BCCIP	0.97	0

SMG9	0.98	0.01
TANGO6	0.96	0
CEP295	1	0
GDI2	0.97	0
CAMK2B	0.97	0
CCT6B	1	0
PJA2	1	0.01
TUBB2A	0.96	0
POLR1C	1	0
GRAMD2B	0.97	0.01
GDA	0.96	0
DCTN2	1	0
ECHDC1	0.97	0.01
UBP1	0.96	0.01
DHX30	0.96	0.01
SLC12A4	0.89	0
ASNS	0.98	0.01
GSR	0.96	0.01
ALDH9A1	0.96	0.01
ILK	0.96	0
RPS21	0.99	0
CDC16	0.99	0.01
TRIM32	0.96	0
HCCS	0.97	0
NUDCD1	0.97	0
POLR1B	0.99	0
GSTK1	1	0
EIF2B3	0.97	0.01
PRELID3B	0.96	0
PSMB3	1	0.01
RAB27B	0.96	0
BAG2	0.98	0
TTC4	1	0.01
COPB2	0.96	0
IDE	0.99	0
PDIA3	0.97	0
MDH2	1	0
POLR3A	0.97	0
KEAP1	1	0
TBK1	1	0.01
UTP25	0.96	0
WASHC4	1	0
TELO2	1	0
TOMM22	1	0.01

LZTFL1	0.96	0
CCDC14	1	0
NUP85	1	0
WDR82	1	0
FAM83D	1	0
MRPL43	0.98	0
PPP1R15B	1	0.01
CMBL	0.96	0
PDHA1	0.97	0
DHFR	1	0
MCMBP	0.97	0
CAPN2	0.98	0
KDM4A	1	0
RAB1A	1	0
OSBPL10	0.99	0
SEC24A	0.97	0
TUBB4A	0.97	0.01
SNU13	0.95	0.01
STIL	0.96	0.01
DCAF7	0.96	0.01
BOP1	0.96	0.01
DNAJC11	0.96	0.01
NME1	0.96	0
IRAK1	0.97	0
PDLIM7	0.98	0
PRKAA1	0.98	0.01
KLHL13	0.96	0
PRKAG1	0.98	0
NCKAP5L	0.97	0.01
ANKRD40	0.96	0.01
CCDC18	0.96	0
CBS	1	0
PPIH	0.98	0
BUB3	0.97	0
EFL1	0.97	0.01
POLR3B	0.96	0
ZWINT	0.98	0.01
VDAC1	0.96	0
ASS1	1	0
HADHA	1	0
MTR	1	0
OCRL	1	0
PEX5	1	0
PEX1	0.99	0

APRT	1	0
POLR2A	1	0
POLR2B	1	0
PRIM2	1	0.01
RPL27A	0.89	0
RPL29	0.98	0
TIMM50	0.97	0
ATP5PD	0.98	0
ACTR2	1	0
ZWINT	1	0
PKP4	1	0
CACYBP	0.99	0
FAM83B	1	0
VPS13A	1	0
FPGS	1	0
HAX1	1	0
RRM1	0.99	0
PLAA	1	0
CPT1A	1	0
WIPI2	0.99	0.01
UBA52	0.96	0
USP14	1	0
BTF3	1	0.01
NCKAP5L	0.93	0
PPAN-P2RY11	1	0
ABCE1	0.99	0
TKT	1	0
TBCE	1	0
RABEP1	1	0
WDR62	1	0.02
USP5	0.81	0.01
TRIM32	0.96	0
IMMT	1	0
NACA	1	0
GTF3C5	1	0
MOV10	0.99	0
PHLDB2	1	0
FNDC3B	1	0
EIF2AK2	1	0
EDC3	1	0
EFTUD2	1	0
TRAFD1	1	0
PSMA1	1	0
DENND4A	1	0

GPN1	1	0
SRP54	1	0
POLR3B	1	0
RBM34	1	0
PNPLA6	1	0
RBM28	0.98	0
ATAD3A	0.97	0.01
UBXN6	0.96	0
HPD	1	0
AAAS	1	0
LDHB	1	0
ASNS	1	0
HADH	0.98	0
MARF1	0.98	0.01
PFDN6	0.88	0
SECISBP2L	1	0.01
UCHL5	0.96	0
CNOT2	1	0.02
ENO1	0.84	0
RBM15	1	0.01
PPP6R2	0.96	0
CAPG	0.99	0.01
MPP5	0.96	0
POLD1	1	0
FAAP20	1	0
DDX6	1	0.02
PSMD11	0.83	0
CSTF2	0.98	0.02
DYNC1H1	0.86	0
ECH1	1	0
EIF2S3	0.98	0
DNAJA1	1	0
HSPB1	1	0.01
EIF3E	0.98	0
AARS1	1	0.02
ATP5F1B	0.79	0
ATP6V1A	0.99	0
CSNK1E	1	0.01
DDB1	0.97	0
HSPD1	1	0.01
KPNA2	0.96	0
MTAP	0.99	0
NUP88	0.98	0
PHB	1	0.01

PIK3C2B	0.94	0.01
PIK3C3	0.97	0
POLA2	1	0
PSMA2	0.97	0
PSMA5	0.99	0
PSMC3	1	0
PSMD2	1	0
PSMD3	1	0
PSMD4	1	0
PSMD8	1	0
FSCN1	1	0
SNRPC	1	0.02
SNX2	0.87	0
TTC1	1	0
UBA1	1	0
OFD1	1	0
EIF3F	1	0
EIF3H	0.99	0
EDF1	1	0
GMPS	1	0.01
HERC1	0.95	0
USP13	1	0
EIF2S1	1	0
CAD	1	0
FARSA	1	0
FLOT2	1	0
KIF11	1	0.01
RAB7A	0.96	0
PDCD5	0.99	0
HGS	1	0
DLG5	1	0
TXNL1	1	0
DDX1	1	0
MVP	1	0
ALDOC	1	0.01
PDCL	0.96	0
LAMA1	1	0
MPHOSPH10	1	0.01
PSMD14	0.93	0
STUB1	1	0
BCAS2	1	0
DNAJA2	1	0
NBR1	1	0
MTHFD1	1	0

PCNT	1	0
TAB1	1	0
CAPZA1	1	0.01
PCM1	0.96	0
SMC1A	1	0
RAN	1	0.02
EMG1	0.78	0
TADA3	0.99	0
PRPF8	0.98	0
TBL3	1	0
PSMC4	1	0
CCT8	1	0
DDX18	1	0
PRDX3	0.98	0
ASCC3	1	0.01
COPS5	0.97	0
PYCR1	1	0
SKIV2L	1	0
DNAJB4	1	0
FAF1	1	0
PKP3	1	0
DDX20	1	0
ATF6	1	0
CCT5	1	0
GTF3C4	1	0.02
CAVIN1	0.83	0
GMEB2	1	0
PFAS	1	0
PFDN2	0.99	0
SF3B3	1	0
NOB1	1	0
RTCB	1	0
TNRC6A	1	0
PRPF19	1	0
LATS2	1	0
PIK3R4	1	0
UBXN4	1	0
ARHGEF10	0.99	0
TTI1	0.97	0
CEP350	1	0.02
TBC1D4	0.77	0
SEC16A	0.98	0
RNF10	0.99	0
PPP6R1	0.98	0.02

SIPA1L3	0.86	0.02
TAB2	0.87	0
N4BP3	1	0.01
ANKLE2	0.96	0
LARP4B	0.99	0
SIK2	1	0.02
BOP1	0.86	0
DNMBP	1	0.01
NUP160	0.87	0
WASHC4	1	0
SYNM	1	0
TMEM131	0.98	0
POLR1A	1	0
RPAP1	1	0
UBXN1	0.98	0
UBR5	1	0
TELO2	1	0
CPSF3	1	0
BIRC6	1	0
CENPF	1	0
TRMT112	1	0.01
MTARC2	0.96	0
NPLOC4	1	0
CEP72	1	0
NANS	1	0
PUS7	1	0
SMG9	1	0
UBFD1	0.97	0
UBR4	1	0.01
SIPA1L2	0.96	0
VPS11	1	0
BCORL1	1	0
EGLN1	0.97	0
UBE2O	0.97	0.01
MTARC1	0.88	0
CEP85	1	0
YTHDC2	1	0.01
SPATS2	0.95	0
SPATA5L1	1	0.01
PDCL3	0.89	0
NOL9	1	0.01
VCPIP1	0.93	0
TANC2	1	0
KIF18A	1	0.01

HUWE1	0.96	0.01
GRWD1	0.99	0
CCNB1	1	0.01
EIF2A	0.96	0.02
MED10	0.77	0
PURB	0.97	0
RAVER1	1	0.01
DTX3L	0.92	0
WDR36	0.98	0.02
SPICE1	0.86	0
USP54	0.98	0
PATL1	1	0
N4BP1	1	0
TDRD9	0.98	0.01
FAM83H	0.93	0
MIA3	1	0
POLR1C	1	0
CCDC18	1	0
P00761	1	0
ABCD1	0.98	0
ALDOA	1	0
APC	1	0
KRT17	0.99	0.01
SMN1	0.88	0
GNAS	1	0
WASHC2C	0.98	0
PICALM	1	0
CUL4A	0.98	0.02
PDE8B	0.83	0
AP2B1	1	0.01
GON4L	0.93	0
DCUN1D4	0.98	0.02
STK26	0.81	0
NFKB2	1	0
OCIAD1	1	0
TAX1BP1	1	0
PXN	1	0.02
IKBKG	0.79	0.02
ITPR1	0.9	0
CRYZ	0.98	0
TOM1	1	0
ECD	1	0
MADD	1	0.01
PHB2	0.94	0

MYL12B	1	0
SLC12A4	1	0
TBC1D15	1	0.01
MYO19	0.94	0
PPP6R3	0.98	0.02
EIF4ENIF1	0.88	0
CCT7	1	0
SSX2IP	1	0
LAS1L	1	0.01
UBAP1	0.95	0
RPS6KA3	1	0
PEG10	0.98	0
SH3GLB1	1	0
NSFL1C	1	0
PDE8A	1	0
RELA	1	0.02
TNIP1	0.88	0
EPS15L1	1	0.02
TARS1	0.86	0.01
NOL10	0.93	0
CSNK1A1	1	0
DOCK7	1	0
AK2	0.99	0
CCT6A	1	0
CDK1	1	0
EPS15	1	0
FLII	1	0
GAPDH	1	0
HSPE1	1	0
PFDN5	1	0
PPP1CB	1	0
PSMC1	1	0
PSMC5	1	0
PSMD1	1	0
RBM4B	1	0
RBM14	1	0
RBMS1	0.99	0
TPT1	1	0
DNAJC7	0.99	0
VBP1	1	0
VDAC1	1	0
KRT7	1	0
KRT80	1	0.01
STAM	0.93	0.01

AURKA	0.92	0
URI1	1	0
SQSTM1	1	0.02
WARS1	0.84	0.02
HSD17B10	0.78	0
FXR2	1	0.02
FXR1	0.84	0
FMR1	0.98	0
PDLIM7	1	0
DSTN	1	0
GOLGA3	1	0.02
MAT2A	0.82	0
CCT3	0.98	0.02
TUBA1B	0.82	0
TUBA4A	1	0
RGPD6	1	0
CCT2	1	0
IGF2BP1	1	0.01
IGF2BP3	0.89	0.01
STIP1	0.9	0
VCP	1	0
GTF3C3	1	0.02
PRDX5	0.87	0
PJA2	1	0
CEP131	1	0
RAB10	1	0.02
OSBPL10	0.83	0
CDK5RAP2	1	0
KIAA1217	1	0
MYL6	1	0
NMT1	0.99	0.02
GOLPH3	0.85	0
TNS1	1	0
ESRP2	1	0
TCP1	0.99	0
PI4KA	1	0
SMG7	0.97	0
CCDC50	1	0
TUBB6	1	0.01
TUBB2A	0.94	0
ERC1	1	0.01
EPPK1	0.93	0
PLCB3	1	0
UHRF1	0.97	0

DCAF7	0.97	0
POLR3A	1	0
KIAA0100	1	0.01
HNRNPDL	0.96	0.01
ARF1	0.96	0.01
DCTN1	0.96	0.01
SLC2A1	0.97	0.01
ZC3H7A	0.96	0
HEATR1	1	0
NUP85	1	0
JAK1	1	0
ABCD3	1	0
C2CD3	0.98	0.01
PACS1	0.96	0
CPEB4	0.97	0.01
PSMD12	0.96	0
ACTN4	1	0
ANAPC5	0.98	0.01
TTK	0.96	0.02
IGBP1	0.85	0
DMXL1	1	0.01
ADRM1	0.96	0.01
CEP295	0.96	0
KDM4A	1	0
SRCAP	0.97	0
ILK	0.98	0
CLUH	1	0
SIPA1L1	1	0
DDAH1	1	0
PLRG1	0.98	0
TTC4	1	0
PASK	0.97	0
CNDP2	0.98	0.01
CEP152	0.96	0
NDUFS1	1	0.01
CYLD	0.96	0
ETFA	1	0
DIAPH3	1	0.02
UBP1	0.76	0
NAF1	1	0.01
AFAP1	0.96	0
RUSC2	0.98	0
CPSF7	1	0
GSTK1	1	0

WDR46	1	0
XIAP	1	0
HSDL2	0.98	0
EIF3L	1	0
FAM193A	0.99	0
PSMC6	1	0
SNRPG	0.98	0
DRG1	0.99	0
ACTL6A	1	0
BUB1	0.98	0.02
COPB2	0.85	0
TXNDC9	1	0
FLOT1	1	0
PRKAA1	1	0
PARP4	1	0
POLR3C	1	0.01
MAGED2	0.96	0
USP34	1	0.02
MYCBP2	0.77	0
GEMIN4	1	0
CDKAL1	1	0
PIH1D1	0.98	0
WDR11	1	0
PPP1R15B	1	0
TAB3	0.99	0
PPP1CA	0.98	0.01
LDHA	0.95	0
CCT6B	1	0
CLTC	1	0
C2CD2	1	0
DDX52	1	0
HNRNPH3	0.99	0
TMCO4	0.99	0
KPNA1	0.97	0
RPS5	0.98	0
STK3	0.98	0.01
MCM3	0.96	0
KEAP1	0.98	0
RABGEF1	1	0
RPAP2	1	0.01
SNU13	0.96	0
VRK2	0.98	0.01
EPN1	0.96	0
CNST	0.97	0.01

EEF1AKNMT	0.96	0
ACTL8	0.97	0
PLEKHG1	1	0
CCZ1	0.98	0
USP9X	1	0
ZEB1	1	0.01
GDI2	0.96	0
UTP18	1	0
UBXN2B	1	0.01
PSAT1	0.96	0
HPRT1	0.97	0
PFKM	1	0
UBE3A	1	0
PRIM1	0.97	0
ATP5F1C	0.97	0
RPS7	0.98	0
RPS10	0.99	0
AK4	0.97	0
HAUS8	0.98	0
RPSA	1	0
RPS21	0.98	0.01
GOPC	0.96	0
GTF3C2	0.98	0
EFL1	0.98	0.01
CRTC3	0.96	0.01
TYMS	0.96	0
PAICS	1	0
MID1IP1	1	0
NRDC	0.98	0
ADSS2	1	0
POLR1B	1	0
OBSL1	1	0
BUB1B	1	0.02
PFKP	0.77	0
USP15	1	0
MCMBP	0.98	0.01
PRELID3B	0.96	0
CNOT10	1	0
DCTN2	1	0
KHDRBS1	1	0
DRG2	0.97	0
EEF1G	0.99	0.02
IPO5	0.84	0
OSBP	1	0

PRKCI	1	0
PSMB1	0.99	0
PSMB7	1	0
RARS1	1	0
YARS1	0.97	0
PDXK	1	0
BAG2	0.97	0
ACSL4	1	0
NFKBIE	1	0
CCNB2	1	0.01
PFN1	0.96	0
ARPC3	0.98	0
IDH1	0.97	0
PPIH	0.97	0
BRAP	0.97	0
METAP2	0.97	0
TBK1	1	0
USP25	1	0
NDUFA8	1	0
UTP25	1	0
FIG4	1	0.01
TCF25	0.96	0
FAM98A	1	0.01
POMP	0.96	0
PELO	0.99	0
MPP6	0.99	0
CNOT11	0.98	0
WDR55	1	0
YTHDF1	0.97	0.01
WDR6	0.93	0
DNAJC11	0.98	0
PARVA	1	0
UBA6	1	0.01
STOX2	0.96	0.01
XPO5	0.96	0
CCDC14	1	0
ILRUN	1	0.01
SIK3	0.96	0
TRABD	0.98	0.01
WDR82	0.96	0
APOL2	1	0
TRIM41	0.98	0
CFAP36	1	0
NUP43	0.97	0

PDHA1	0.97	0
PGK1	0.98	0
HSD17B4	0.98	0.01
DHFR	0.96	0
ECHDC1	1	0
PSMA4	1	0
ATXN3	1	0
AZI2	0.98	0
RAB9A	0.97	0
ARPC4	1	0
PRKAG1	1	0
GDA	0.98	0
RNF213	1	0
CAPN2	1	0
DHPS	1	0
MAGEB2	0.99	0
MAPK4	1	0
IRAK1	1	0
PSMB2	0.97	0
RAD23B	1	0
MAP3K7	0.98	0
RAB1A	1	0
RAB14	1	0
TRIP13	1	0
PPA2	0.98	0
RPL36A	1	0
DNAJB6	0.99	0
GRSF1	0.97	0
NT5C2	0.97	0
ARMC6	1	0
FBXO41	0.97	0
ATF6B	0.97	0
PDIA6	0.98	0.01
GRAMD2B	0.96	0
TOM1L2	1	0
BAP1	0.97	0.01
MALT1	0.96	0
KLHL13	0.98	0
CIP2A	0.97	0
AMOTL1	1	0
ANKRD28	0.97	0
BCCIP		0
GBF1	1	0
NRAS	0.97	0.01

FANCD2	0.97	0
WDR1	0.96	0
RRS1	0.97	0
BRAT1	0.97	0.01
PLK1	0.97	0.01
BTF3L4	0.96	0.01
DCAF1	0.96	0.01
PPID	0.96	0

Table 3. Reactome analysis related to cilia biogenesis

Input	UniProt Id	Input	UniProt Id	Input	UniProt Id
ARF1	P18085	C2CD3	Q4AC94	CCT2	P78371
CCT3	P49368	CCT5	P48643	CCT8	P50990
CDK1	P06493	CDK5RAP2	Q96SN8	CEP131	Q9UPN4
CEP152	O94986	CEP72	Q9P209	CSNK1E	P49674
DCTN1	Q14203-2	DCTN2	Q13561	DYNC1H1	Q14204
GBF1	Q92538	HAUS8	Q9BT25	LZTFL1	Q9NQ48
OFD1	O75665	PCM1	Q15154	PCNT	O95613
PLK1	P53350	SEC16A	Q9NRR6	TCF25	Q13509
TCP1	P17987	TUBA1B	P68363, Q9H853	TUBA4A	P68366
TUBB	P07437	TUBB2A	Q13885, Q9BVA1	TUBB4A	P04350
TUBB6	Q9BUF5				

Table 4. Summary of the genes related to primary cilia and their identified roles

Gene name	Identified role	FDR	Reference
BBS11/TRIM32	Bardet-Biedl Syndrome 11	0	Piya Sen Gupta et al, 2009
BBS17/LZTFL1	This protein interacts with Bardet-Biedl Syndrome (BBS) proteins and, through its interaction with BBSome complexes, regulates protein trafficking to the ciliary membrane	0	JiangsongJiang et al, 2016
PCM1	Major protein of the pericentriolar satellite/BBSome may transiently interact with PCM1	0	Maxim A. X. Tollenaere et al, 2015
PSMD2	Regulates proteasome activity at the basal body of the cilium	0	Christoph Gerhardt et al, 2016
PSMD1	Proteasome subunits	0	Christoph Gerhardt et al, 2016
AURKA	May play a role in cilium turnover and risk of obesity	0	Jarema J. Malicki et al, 2017
HSP40 family	Involved in cilia regeneration in sea urchin	0	Megan S. Kane et al, 2017
CFAP36/ CCDC104	Cilia and flagella associated proteins	0	David K. Breslow et al, 2019
OFD1	Centriole and centriolar satellite/involved in the biogenesis of the cilium	0	Veena Singla et al, 2010
CEP 350/85/97/192/170/131/295/55/72	Centrosomal proteins involved in centriole biogenesis	0,0.01,0.02	Ambuj Kumar et al, 2013
TTUBB/TUBB6/TUBB2A/TUBB4A/TUBA4A/TUBA1B	Structural component of microtubules	0,0.01	Wolfgang Baehr et al, 2018
CCT complex: TCP1/CCT2/CCT3/CCT4/CCT5/CCT6A/CCT7/CCT8	This complex may play a role in the assembly of the BBSome/ A complex involved in ciliogenesis and regulations of transports vesicles to the cilia	0	Satyabrata Sinha et al, 2014
VCP	Inhibition of vcp lead to shorter cilia	0	Malavika Raman et al, 2015

Table 5. Gene ontology enrichment analysis (Gorilla)

Gene ontology analysis	P value scale
Supramolecular complex, supramolecular polymer, supramolecular fiber, extracellular organelle, exosome and vesicles, polymeric cytoskeletal fiber, intermediate filament	$<10^{-9}$
Cytoskeletal fiber, vacuolar lumen, vesicle, non-membrane bounded organelle, extracellular region, membrane, azurophilic granule	$<10^{-7}$ to 10^{-9}
Membrane enclosed lumen, protein containing complex, proteasome regulatory particles, chaperon complex, cell junction, secretory granule lumen, Ribonucleoprotein complex, aggresome, anchoring junctions, adherens junction	$<10^{-5}$ to 10^{-7}
Cytosleleton, microtubules, intermediated filament cytoskeleton, proteasome accessory complex, Zona pellucida receptor complex, peptidase complex	$<10^{-3}$ to 10^{-5}
Nuclear part, catalytic complex, cytoplasmic vesicle part	$<10^{-3}$

Figure legends:

Figure 1. Stable expression of BioID constructs with (D)+ and without (D)- doxycycline and in the presence of biotin (B)+, in Hela cells. 0.5 µg/ml doxycycline was used for induction. A). Biotinylated proteins were probed with Streptavidin-HRP with 1:5000 dilution. B). The BioID constructs were detected with an anti-flag antibody in the presence of doxycycline with a dilution of 1:1000. C). Anti-tubulin antibody with a dilution of 1:5000

Figure 2. Immunofluorescence (IF) staining of Hela cells expressing of BioID constructs with (D)+ and without (D)- doxycycline and in the presence of biotin (B)+. Streptavidin 568 (red), Flag M2 (green), Dapi (blue).

Figure 3. Interactive heat map and dot blot viewer analysis for proteins related to ciliogenesis (A&B). Relative abundance will increase with a bigger and darker round circle.

Figure 4. Western blot validation of BioID results. A). CCT5 subunit of TRIC complex validated using the CCT5 antibody with a dilution of 1:500. B). Anti-flag M2 antibody with a dilution of 1:1000. C. Anti-tubulin antibody with a dilution of 1:5000.

Figure 5. Western blot validation of BioID results. A). CCT8 subunit of TRIC complex validated using CCT8 antibody with a dilution of 1:500. B). Anti-flag M2 antibody with a dilution of 1:1000. C. Anti-tubulin antibody with a dilution of 1:5000.

Figure 6. Schematic model of BSPH1 and its interacting partners in its potential physiological role in primary cilia. The interaction of BSPH1 with the CCT/TRIC complex as well as with the TUBB family are illustrated.

Figure 1

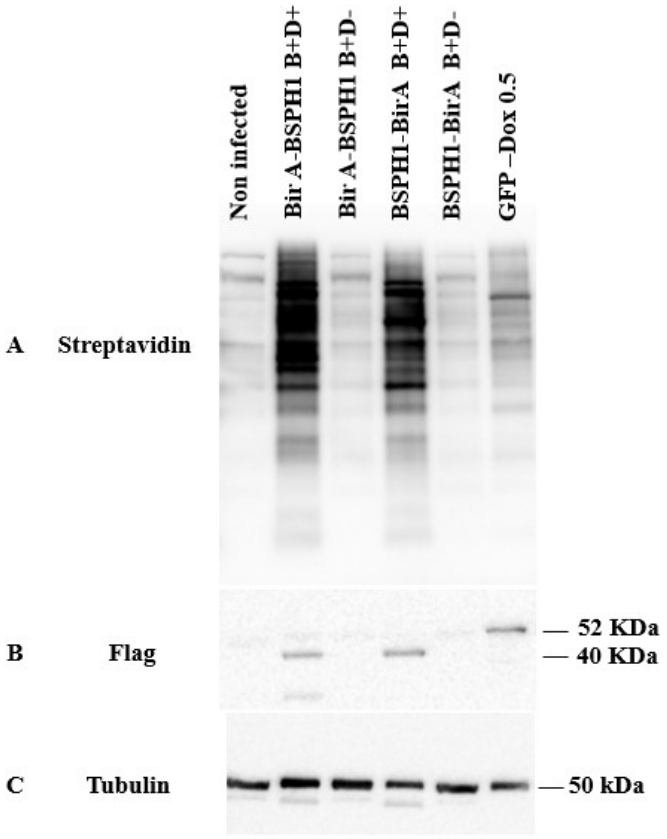

Non infected
Bir A-BSPH1 B+D+
Bir A-BSPH1 B+D-
BSPH1-BirA B+D+
BSPH1-BirA B+D-
GFP –Dox 0.5
A Streptavidin
B Flag
C Tubulin
52 KDa
40 KDa
50 kDa

Figure 2

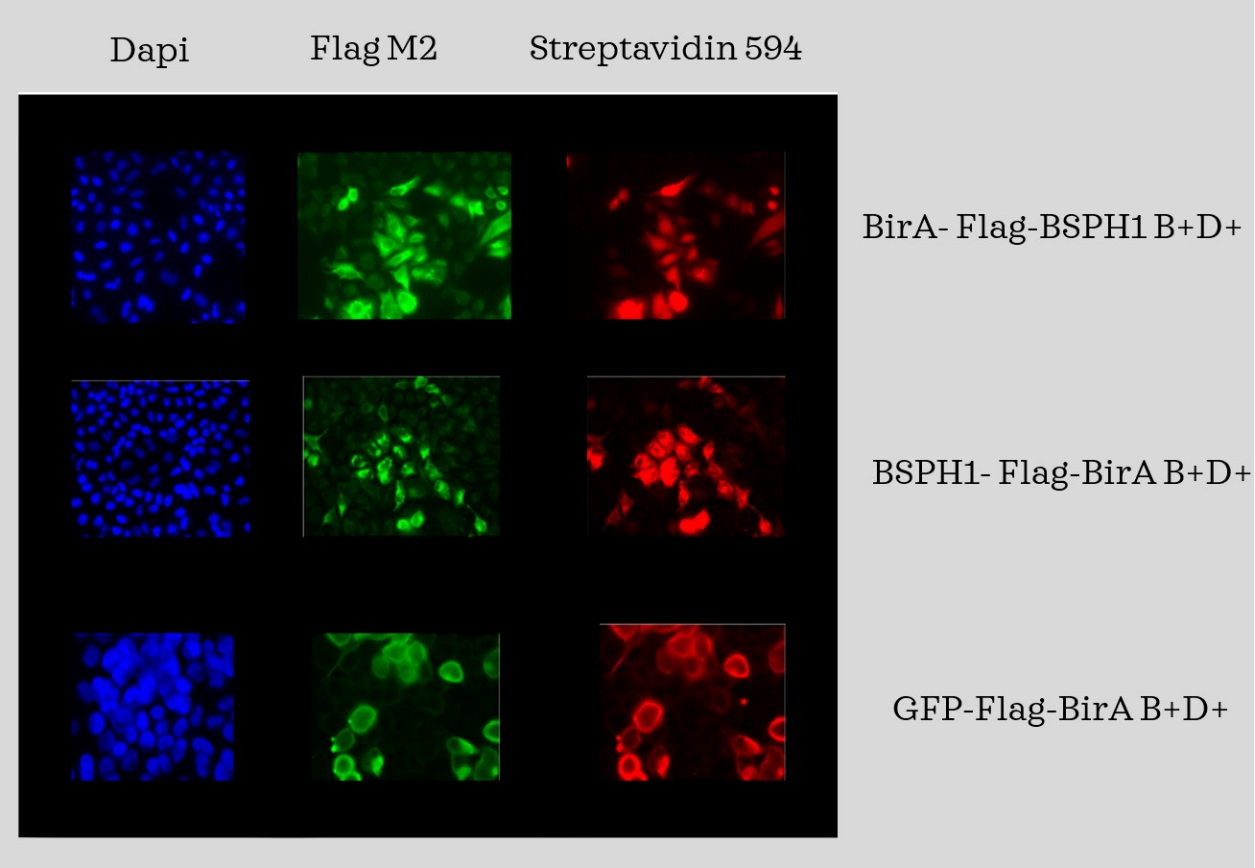

Dapi
Flag M2
Streptavidin 594
BirA- Flag-BSPH1 B+D+
BSPH1- Flag-BirA B+D+
GFP-Flag-BirA B+D+

Figure 3

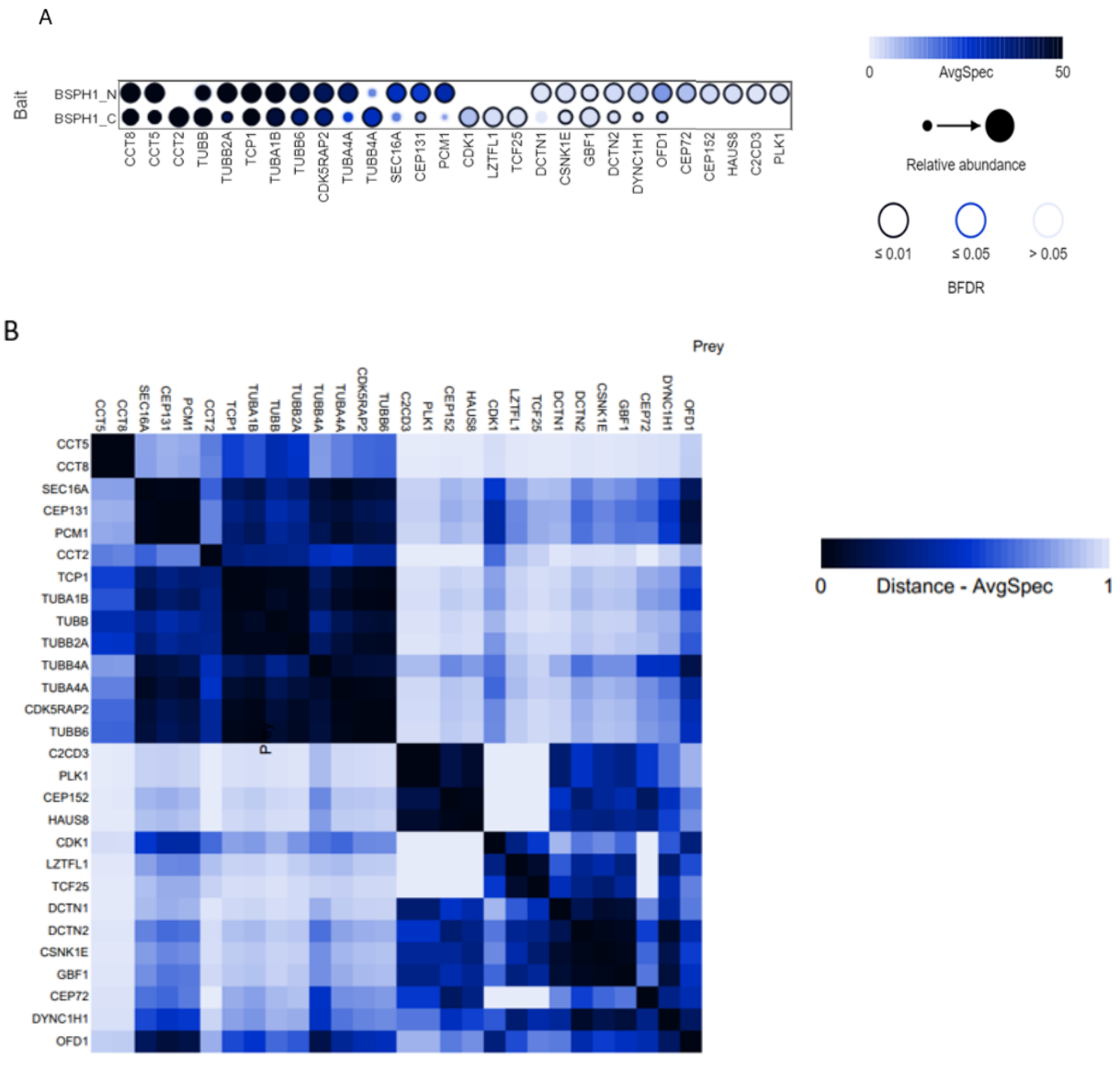

A
Bait
BSPH1_N
BSPH1_C
CCT8
CCT5
CCT2
TUBB
TUBB2A
TCP1
TUBA1B
TUBB6
CDK5RAP2
TUBA4A
TUBB4A
SEC16A
CEP131
PCM1
CDK1
LZTFL1
TCF25
DCTN1
CSNK1E
GBF1
DCTN2
DYNC1H1
OFD1
CEP72
CEP152
HAUS8
C2CD3
PLK1
0
AvgSpec
50
Relative abundance
≤ 0.01
≤ 0.05
> 0.05
BFDR
B
Prey
Bait
0
Distance - AvgSpec
1

Figure 4

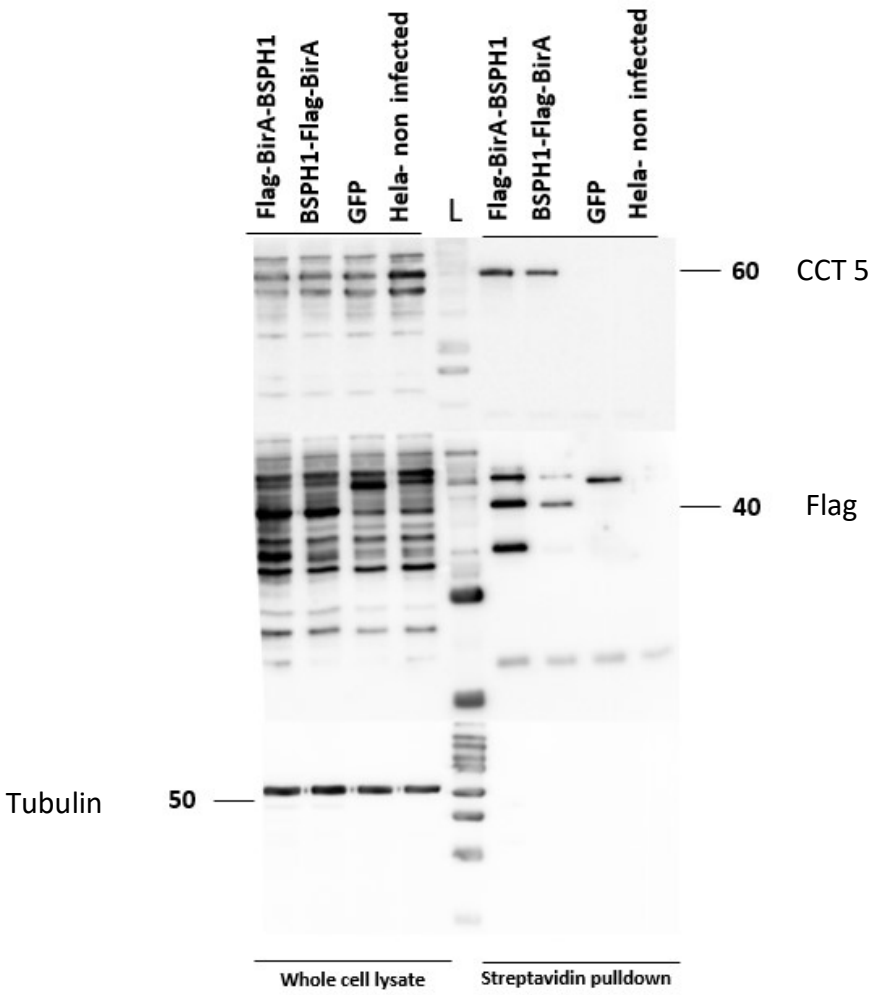

Flag-BirA-BSPH1
BSPH1-Flag-BirA
GFP
Hela- non infected
L
Flag-BirA-BSPH1
BSPH1-Flag-BirA
GFP
Hela- non infected
60
CCT 5
40
Flag
Tubulin
50
Whole cell lysate
Streptavidin pulldown

Figure 5

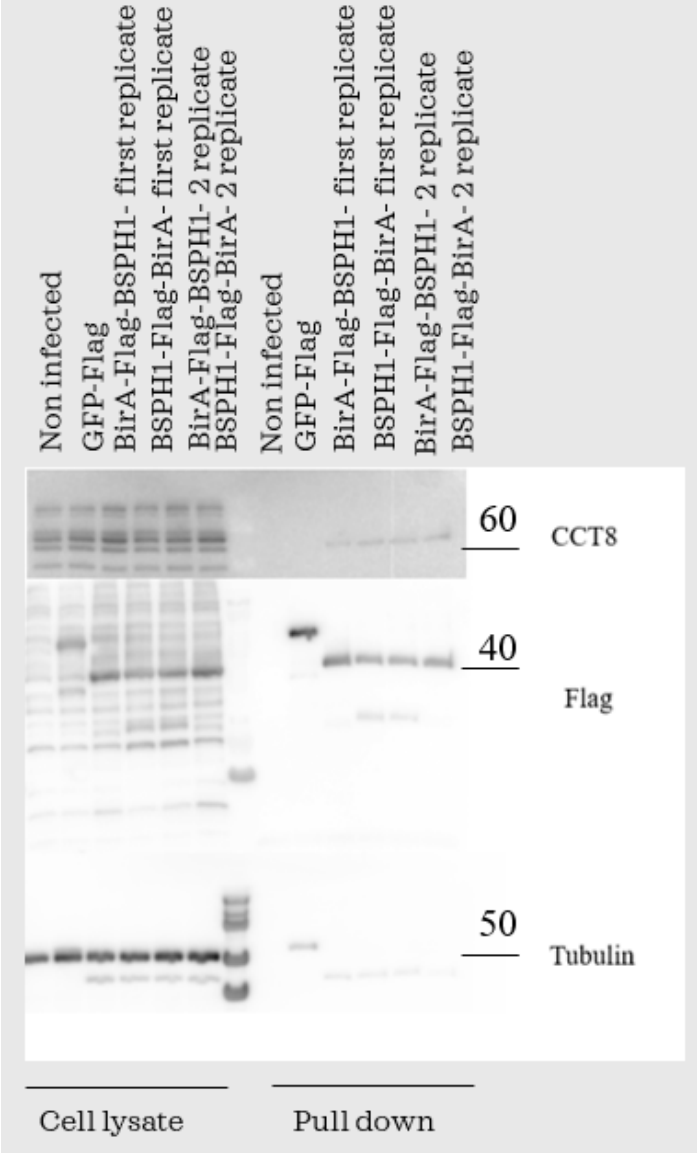
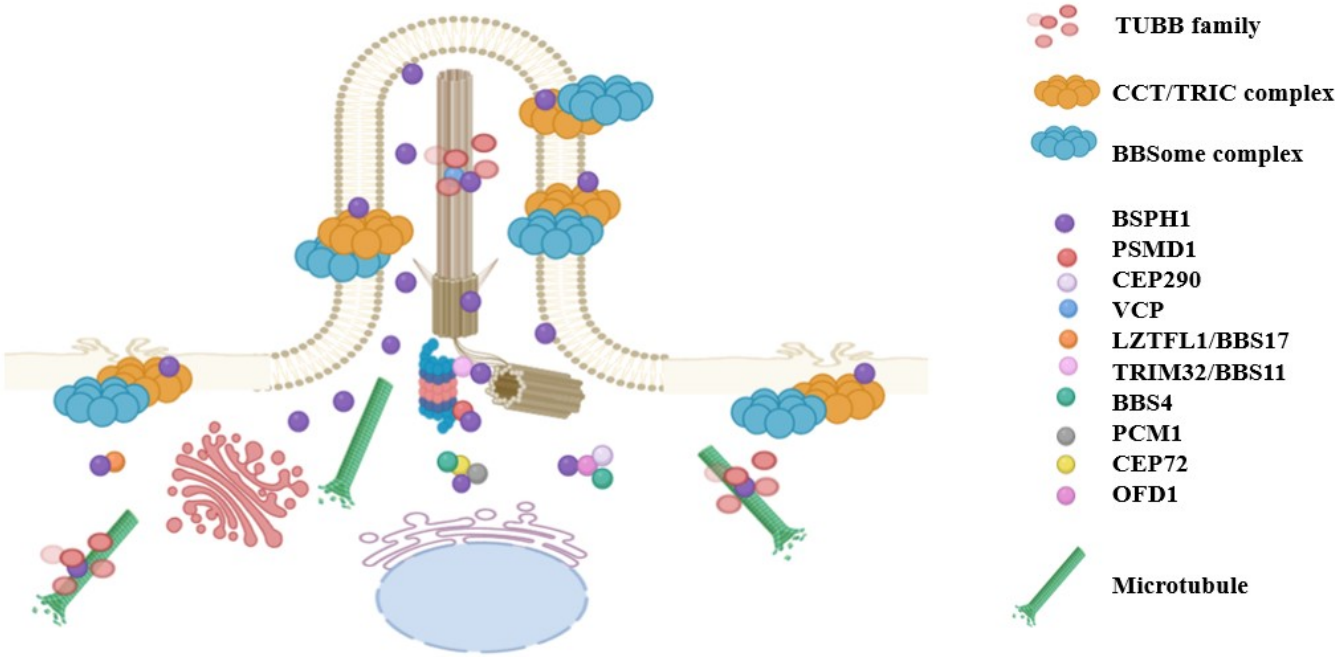

Non infected
GFP-Flag
BirA-Flag-BSPH1- first replicate
BSPH1-Flag-BirA- first replicate
BirA-Flag-BSPH1- 2 replicate
BSPH1-Flag-BirA- 2 replicate
Non infected
GFP-Flag
BirA-Flag-BSPH1- first replicate
BSPH1-Flag-BirA- first replicate
BirA-Flag-BSPH1- 2 replicate
BSPH1-Flag-BirA- 2 replicate
60
CCT8
40
Flag
50
Tubulin
Cell lysate
Pull down

Figure 6


TUBB family
CCT/TRIC complex
BBSome complex
BSPH1
PSMD1
CEP290
VCP
LZTFL1/BBS17
TRIM32/BBS11
BBS4
PCM1
CEP72
OFD1
Microtubule

Chapter 5 – General Discussion and Conclusions

The ultimate phase of oogenesis and spermatogenesis is to prepare highly specialized cells for fertilization. Only a few of these peculiar cells will remain in the site of fertilization (ampulla). In humans, approximately one oocyte and 100 sperm are candidates, for a limited time, to become part of the future offspring. Interspecies fertilization is uncommon in mammals due to Zona pellucida barriers. However, there are some exceptions, such as mule [209]. Zona pellucida glycoproteins comprise complex polysaccharides that bring the absence of interspecies fertilization. The first step in fertilization is the maturation of the sperm and oocyte. Oocytes are arrested in metaphase II before fertilization [210]. On their side, sperm also need to undergo two specific maturation steps: epididymal maturation and capacitation. During epididymal transit, which takes one to two weeks, lipids and protein rearrangements of the sperm membrane occur and are accompanied with acquisition of motility [211]. Epididymal maturation involves many proteins originating from the epididymis and testis. The epididymis is segmented into four regions, but according to Domeniconi et al., it is a highly specialized segment that can be considered a single organ, which evolved from the Wolffian duct [212]. Hox genes are considered essential in the segmentation of Wolffian ducts. The initial segment of the epididymis is different in its anatomy of vascularization compared to the rest of the epididymis, making it unique [212]. In humans, BSP proteins are expressed in the initial segment and caput of the epididymis and are among the important components of the epididymal milieu. However, the expression profiles of BSP proteins are different according to species. While in boar and ram, BSP expression occurs in the last part of the epididymis (cauda), rabbit BSPs are expressed in the middle (corpus) of the epididymis tube [108]. It is documented that the initial segment of the epididymis secretes proteins involved in sperm membrane modifications [213]. Sperm chromatin condensation is another element of epididymal maturation, which is the result of the oxidation of some groups (cysteine-thiol groups to disulfide bonds) in protamine [213]. Several studies showed that a number of proteins are present in different concentrations in the epididymis of individual species. Albumin, clusterin, NCP2 and lactoferrin are abundant epididymal proteins in human [214]. Interestingly, Wang et al. demonstrated the existence of 4675 different proteins in sperm using

mass spectrographic analysis, testifying to the complex composition of human semen [214]. Since some of these proteins are present in very low concentration, having access to purified recombinant proteins to study their function is an asset.

5.1 Exploiting unique affinities to purify epididymal proteins

In order to identify the technique best suited to purify BSP proteins, the first step is to determine their unique characteristics. BSPs are a family of small acidic proteins containing disulfide bridges, of which some are glycosylated, and some are not. Their isoelectric points range from 3.6 to 5.2. However, recombinant proteins have a higher pI due to their tags. Most studies to date by Manjunath and co-workers were conducted using bovine BSPs. BSPs consist of two Fn2 domains that are conserved in vertebrates. It is believed that these domains evolved from kringle domains, which are also present in invertebrates [215], arthropods and nematodes [216]. Fn2 domains were first characterized in fibronectin (blood plasma and extracellular matrix protein) and in seminal plasma proteins [217]. The core tryptophan residue is conserved in Fn2 and Kringle domains (composed of 80 amino acids and specific structure, consist of 3-loops and 3- disulfide bridges) indicating the essential role of tryptophan for the function of domains during evolution [215]. Fibronectin proteins share some common binding features with BSPs, such as binding to collagen (type I, II, IV, V) and heparin [218]. BSPs have some specific binding properties, such as affinity to choline phospholipids, which is the major lipid of the sperm membrane [165]. BSP1 also shows a weaker interaction with other type of lipids such as phosphatidylserine and phosphatidylglycerol [219]. The interaction of BSP1 with choline phospholipids but not with ethylamine suggests that the phosphate group is not crucial for the binding affinity of BSP1 to choline phospholipids. However, this characteristic is different in other BSPs. BSP5 is another form of BSP in bovine, which was shown to have strong affinity to choline phospholipids and a broader range of affinity to other lipids, such as phosphatidic acid, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine and cardiolipin [165]. Muller et al. showed that the interaction of BSP1 with sperm membrane choline phospholipids is a very fast process that changes the fluidity of the membrane [220]. In addition, BSP1 has a specific affinity to carbohydrates, and this interaction may be involved in the formation of the sperm reservoir in the uterine epithelium, a process mediated by L-fucose and D-mannose [221]. The sperm

reservoir has a pivotal role in the maintenance of sperm viability and motility [136]. Bovine BSP1 localizes to the midpiece of sperm, indicating a possible role in motility mediated by calcium ATPase activity, as suggested by some studies [222–224]. The interaction of BSP1 with choline phospholipids of the sperm membrane led to the proposal of a mechanism for a second maturation step of spermatozoa (capacitation) [225,226]. It has been shown that cholesterol is a membrane fluidity regulator that bobs up and down between phospholipids. Membrane fluidity is essential for the diffusion of lipids and proteins across the membrane and cross talk between cells and the environment. The proposed mechanism for the role of BSP1 in capacitation consists of two phases. There is a first cholesterol efflux that occurs at the time of ejaculation, and a second cholestrol efflux that occurs the female genital tract, where cholesterol acts as a molecular docking unit that interacts with BSPs via choline phospholipids [227]. Cholesterol efflux is accompanied by an increase in intracellular calcium and conversion of sperm membrane phospholipids to lysophospholipids, which prepares sperm membrane receptors for fusion to the oocyte. The interaction of BSP proteins with phosphorylcholine is calcium independent [228]. Knowing this characteristic of BSP proteins, I used the pseudo choline moiety to test their interaction and use as a possible purification tool. Desnoyers et al. used *p*-aminophenyl phosphoryl choline-agarose for purification of BSP proteins in 1993. Traditionally, it is used for purification of C-reactive proteins (CRP). In the 1993 study, washing the column using high salt concentrations or changing the pH from acidic to basic conditions did not elute the BSP proteins from the column, suggesting that the interaction of BSPs with the column was not ionic but structural. BSP proteins were eluted using urea, choline chloride or sodium thiocyanate [228]. Quaternary methyl ammonium (QMA) resin, which is a silica-based polymer, is also used to purify BSP proteins because it is an anion exchanger and structural analogue of choline [228]. Diethylaminoethyl (DEAE) is another choline analogue which has the same function as QMA. BSPs cannot be eluted using high salt concentrations, low acidic or high basic pH conditions or chelating agents such as EDTA. Therefore, the interaction is not ionic. These results also indicate that the phosphate group is not important for the interaction given that DEAE does not have a phosphate group. If no ionic, the binding affinity of BSPs to choline phospholipid could be structural (mediated through the Fn2 domains) which are conserved region of BSPs. Based on my recent

results using recombinant proteins, these interactions cannot be both ionic and affinity-based due to strong binding of BSPs to the column in the presence of high salts (1 M NaCl). Bhanu P et al. demonstrated that substitution of alanin with tryptophan in Fn2 domains of BSPs changes not only binding affinity but also chaperone-like activity. The three tryptophane residues in BSPs (W47, W93, and W106) seem to be evolutionary conserved and carry the binding characteristics of BSPs [215]. In the same study, fluorescence studies and erythrocyte lysis assays showed that these residues are essential for lipid binding affinity [215]. If not structure-based, the interaction of BSPs with DEAE could be based on secondary bonds (van der waals or hydrogen bonds), which can be disrupted with 7-8 M urea. There are other ways to purify BSP proteins but the balance between purity and the amount of purified protein is important. The efficiency of this method is over 90 percent given its time effectiveness (few steps) and the high purity of the target proteins.

One example of this method is the purification of insulin on DEAE sephadex [229]. Insulin can be eluted from DEAE sephadex in the absence of 8 M urea but with a high salt concentration; however, in the presence of 8 M urea, separation can be achieved with low salt concentrations. This suggests that the interaction of insulin with DEAE resin is not only ionic [229]. The resolution of purified protein increases in the presence of 8 M urea and might be due to a change in the isoelectric point of the protein. Khademi et al. showed that urea can enhance resolution and separation efficiency of proteins that are being eluted from an anion exchange chromatography column [230]. This effect might be due to the chaotropic (ability to change water structure) characteristic of urea, which can interact with both water and the target protein [230]. Another study by Angarita et al. showed the purification of Apo-A1 using DEAE resin and urea. In this study, urea forced the disassociation of the protein complexes, specially endotoxins that were trapped inside of the Apo-A1 protein, which is very important for therapeutic applications [231].

Understanding these unique interactions helps us to understand the function of BSP proteins because of their interactions with membrane lipids. It can also opens the door to a better understanding of pathogens (viruses) that can interact with the lipid content of the cell membrane and get the key to the cell city.

When pathogens such as viruses enter host cells, they need to be attached to receptors or directly to the membrane in ordr to enter their genomic material. In this procedure, viruses interact with specific lipids that can be different based on the strains. For example, rhabdovirus vesicular stomatitis virus (VSV) enters cells thanks to their interactions with phosphatidylserine [232].

The analogue of bovine BSP1 exists in the equine species, (previously called the horse seminal plasma protein 1/2 or HSP-1/2), with some differences in the N-terminal domain. The equine BSP1 protein consist of 121 amino acids. Analysis of its sequence also shows the conserved tryptophan residue. The equine BSP1 can also interact with the lipid bilayer, though with less avidity compared to bovine BSP1. It can modify membrane rigidity and promote aggregation [233]. Studies showed that equine BSP1 was attached to sperm in the epididymis, unlike bovine BSP1. In addition, there are cholesterol vesicles in stallion seminal plasma (prostasomes), which control membrane fluidity and cholosterol efflux [234]. Many studies suggested a chaperone-like activity of bovine and equine BSPs, through protecting other proteins from heat or change of pH and oxidative stress. However, the interaction of these proteins with choline phospholipids decreased their chaperone activity in a dose-dependant manner [235,236].

Based on the above, I developed a methodology for the purification of recombinant BSP proteins in humans and mice. Many attempts have been made previously to purify BSP proteins with different techniques. Since BSP proteins interact with choline and pseudocholine groups, I used DEAE Sephadex for their purification. 8 M urea was used as an elution buffer. Urea was initially added to the mixture to increase the solubility of the proteins and disrupt the formation of inclusion bodies. 8 M urea eliminates hydrophobic and hydrogen bonds and efficiently elutes BSPs from the DEAE column. Choline chloride or sodium thiocyanate also could be used as an elution buffer. However, choline choloride permanently changes the stability of proteins; thus, it is not useful if the proteins are to be used in downstream studies. The purification procedure was the same for the human and mouse recombinant proteins. The only difference was the higher expression level of the recombinant mouse-BSPH2 compared to mouse-BSPH1 and human-BSPH1 under the same purification conditions in bacteria.

Studies to determine the exact mechanism of BSP protein interaction with choline phospholipids have been insufficient and inconclusive. A better understanding the lipid-protein interactions in the cell membrane and changes occuring in the lipid membrane upon lipid-protein interaction can help us understand disease mechanisms that are caused by alterations in membrane lipid or protein compositions [237].

5.2 Role of mouse binder of sperm protein and sperm-egg interaction

The aim of this study was to elucidate the contribution of mouse BSPH1 in sperm-egg interaction and fertilization using affinity-purified mouse BSPH1 and BSPH2 proteins.

Studies showed that mouse-BSPH1 localizes to the equatorial segment of the sperm head, supporting the idea that mouse-BSPH1 could be implicated in sperm-egg interaction [128,129]. Native mouse-BSPH1 was detected on the midpiece of sperm with a low intensity of fluorescence due to low concentrations of native protein, though a stronger signal was observed when recombinant mouse-BSPH1 was incubated with uncapacitated sperm [144]. An emerging high intensity signal of mouse-BSPH1 on the post acrosomal section of capacitated sperm seem to be important beyond *in vitro* capacitation (sperm-egg interaction). In order to investigate this approach, IVF experiments were conducted to verify whether rec-BSPH1 could block the oocyte surface and inhibit the fertilization process. Results showed that the fertilization rate decreased by about 50% in zona-intact as well as zona-free conditions when compared to oocytes incubated with ovalbumin. These results indicated a possible inhibition of the BSPH1 receptor on the oocyte surface by recombinant BSPH1, which hampered the interaction of native sperm-bound BSPH1 with corresponding receptors on the oocyte surface. This speculation is consistent with the bovine BSP study that showed binding of BSPs to zona pellucida carbohydrates [143]. In another experiment, sperm were preincubated with anti-BSPH1 antibodies at different dilutions in order to immunosuppress native BSPH1, followed by IVF. Results showed that the fertilization rate decreased by 85% with a dilution of 1:25, suggesting *in vitro* immuno-suppression of sperm that compromised fertilization could be an *in vitro* marker of fertility. Moreover, sperm treated with anti-BSPH1 antibody showed decreased progressive motility at the same dilution. Sperm treated with anti-BSPH1 antibody also showed propensity to agglutination. Nevertheless, the most motile

sperm were chosen for IVF, and fertilization rates diminished when sperm were preincubated with anti-mouse-BSPH1 antibody, which is consistent with previous work [238]. Plante et al. demonstrated that antibody blockage of native mouse-BSPH1 can affect HDL induced capacitation as well as the tyrosine phosphorylation that typically accompanies capacitation [238]. One hypothesis regarding the blockage of native BSPH1 with antibody and the resulting decreased IVF rate is the presence of BSA in the media, which can interact with BSPH1 on the sperm head. Upon blockage of BSPH1 with antibody, this interaction would have been lost, resulting in reduced acrosome reaction and reduced hyperactivation.

5.3 The unique protein interaction network of the human binder of sperm protein

The central dogma of biology describes the one way and irreversible direction of genetic material. The information carried in the nucleotide elements of DNA are transcribed to RNA in the nucleus. Subsequently, the RNA sequence is converted to amino acids in the cytoplasm through a process called translation. Then, the synthesized proteins must be modified to become functionally active, and translocated to their proper location. Post-translational modifications such as glycosylation, phosphorylation or ubiquitination, to name a few, are needed for the proper function of proteins. Post-translational modifications of proteins can be reversible, based on their functions and interactions. Protein-protein interactions (PPI) is a fundamental biological process for cellular function. Identification of PPI can lead to a better understanding of disease mechanisms and their possible treatments. Abnormal PPI may lead to impairments in different steps of the reproductive process, such as sperm maturation, sperm egg interaction and fertilization. Therefore, understanding PPI networks can help us establish fundamental mechanisms of fertility, causes of infertility as well as aid in the treatment of infertility. PPI can be obligate (permanent interaction) or nonobligate (transient interaction). There are heterooligomeric PPI as well as homooligomeric PPI. It is known that 80 percent of a proteins' function occurs through groups or complexes. Therefore, revealing the hidden functions of a target protein can be achieved by studying the proteins that interact with it [239]. PPI can change the target protein's specificity, as well as activate or suppress the target proteins' functions, or change its affinity to a specific subunit [240].

Different techniques such as BioID coupled mass spectrometry and chip-based methods can increase our knowledge of PPI networks.

PPI studies can be divided into three different categories; *in vivo, in vitro* and *in silica* studies. The yeast two-hybrid (Y2H) method is used in *in vivo* PPI studies [241] and the tap tagging system coupled mass spectrometry is used in *in vitro* studies. Computational tools to predict PPI such as Coev2Net and TSEMA have been developed in recent years [242]. I applied the BioID technique *in vitro* to study the PPI of the human BSPH1 protein in the Hela cell line. Proximity-dependent biotin identification was first introduced by Kyle J Roux in 2012 [243]. The BioID method coupled to mass spectrometry identified BSPH1 partners and a total of 3433 interacting proteins.

Recently, double knock out mice (BSPH1, BSPH2) were shown to be fertile [130,131], which suggests that in male mice, the function of BSPs could be compensated by other proteins that work in parallel. Interestingly, the male pups' weight increased remarkably in a time-dependant manner (days 6 and 21 as well as 6 weeks). Conversely, the weight of female pups remained unchanged during the same time course. This result also suggested that the BSPs expression are specific to males. Eskandari et al. showed that an 81-Kb sequence between the BSPH1 and BSPH2 genes was also removed in the process of deletion and apparently, these 81 kb contained an androgen receptor binding site. Androgens are steroid hormones that regulates male sex characteristics through changing to testosterone, and can trigger weight gain [244]. Studies showed that a slight increase in testosterone could result in weight gain in prepubertal children [244].

Difference in the location of expression and quantity of protein secreted may play a role in the functions of BSPs in different species. They represent 60 percent of seminal plasma in bovine, where they are expressed by seminal vesicles, compared to low quantities founds in mice and humans, which may indicate a change of their role during evolution.

BioID results brought us to focus on sperm-egg recognition and interaction due to the identification of chaperones such as heat shock proteins as well as almost all subunits of the CCT/TRIC complex , which had previously been studied by other groups [181,245]. Given that sperm are transcriptionally, and translationally inactive, post-translational modifications are an

essential part of sperm capacitation and sperm-egg interaction events. The role of tyrosine phosphorylation and glycosylation in sperm membrane remodeling has been extensively documented [246].

This knowledge comes from the work of Dun et al., which stated that the CCT/TRIC complex modulates sperm-egg interaction [181]. In 2011, Dun et al. found two complexes on mouse sperm that bind to zona pellucida proteins. They used mass spectrometry to identify the subunits of this complex [181]. Results showed that the CCT/TRIC complex on the sperm head interacts with the zona pellucida regardless of capacitation state. In addition, CCT8, CCT2 and CCT6A were shown to be localized on the preacrosomal segment of sperm [181]. In the same study, they concluded that the CCT/TRIC complex on the sperm head interacts with the zona pellucida indirectly. Through incubation of sperm with antibodies against some subunits of this complex and performing zona binding assays, they proposed indirect zona binding of the CCT/TRIC complex due to their inability to suppress complete adhesion. They identified 8 subunits for this complex [181].

It has been shown that CCT/TRIC interacts with ZPBP2 and ZP3R on the zona pellucida. While anti-ZPBP2 antibodies did not remove binding, a combination of anti-ZPB2 and anti-ZP3R antibodies abolished binding. Interestingly, the highest expression level of CCT/TRIC complex was seen in the mammalian testis, where it might be involved in spermiogenesis [201]. Soues et al. demonstrated that the CCT/TRIC complex is associated with centrosomes and microtubules in sperm cells, which is consistent with our results [201]. Moreover, they demonstrated that the CCT/TRIC complex is associated with heterochromatin not only in germ cells but also in somatic cells [201]. Identifying CCT on the sperm head during spermiogenesis suggests that CCT might have specific role in spermatogenesis [201].

CCT/TRIC cooperates in protein folding and initially prevents aggregation. It is also involved in assemblies of microtubules along with tubulin and actin [247]. CCT5, CCT8 CCT6a and CCT2 scored very high in our mass spectrometry results, supporting the hypothesis that BSPH1 could act as a chaperone along with the CCT/TRIC complex. Alternatively, one of the subunits of this complex might interact with BSPH1 at the time of capacitation or before and mediate sperm-egg interaction indirectly.

The exact mechanism of sperm-zona interaction is still unclear and the subject of much debate [248]. Calmegin (CLGN) is a testis-specific protein with chaperone activity. It has been shown that CLGN null male mice are infertile due to the inability of their sperm to adhere to the zona pellucida, the outer layer of the oocyte [249], suggesting that Calmegin mediates sperm-egg interactions and as a chaperon interact with other sperm surface proteins. On the oocyte side, three proteins named ZP1, ZP2 and ZP3 are responsible for sperm-egg adhesion in humans and mice [250]. In addition to Calmegin, heat shock proteins HSPD1, HSP90 and HSP90B1 are present during capacitation and act as tyrosine phosphorylated chaperones [245]. Other HSP proteins that work closely with HSPD1 are HSPE1 and HSP10, which are responsible for correct protein folding and essential for mitochondrial biogenesis, in addition to a documented role in apoptosis and cell signalling pathways [251]. HSPE1 is expressed in sperm, colocalizes with HSPD1 during capacitation and appears on the front part of the sperm head (acrosome pouch), indicating its role in the initiation of sperm-egg interactions [245]. Moreover, the exocytotic release of acrosomal enzymes is accompanied by a loss of HSPE1, and its expression is elevated in capacitated sperm compared to uncapacitated sperm [245]. Overall, studies suggest that these chaperons have the potential to increase sperm-egg interaction [252].

Our mass spectrometry results prompted us to look more closely into primary cilia. We also researched the role of primary cilia in cells via the CCT/TRIC complex and their impact on bardet-biedl syndrome (BBS, OMIM 209900). To explain this correlation, we need to explain the function of primary cilia in eukaryotic cells. The function of non-motile primary cilia in epithelial cells remained unknown until findings showed a correlation of mammalian hedgehog (Hh) signalling with protein transport via primary cilia. These studies come from the gene sets involved in the regular function of primary cilia and Hh signalling [253]. The Hh signalling pathway is essential in organ development and its disruption has been studied in many types of cancer. The correct regulation of Hh signalling is linked to primary cilia and perturbation of these regulations is linked to specific syndromes [254]. Set of proteins that are important in drosophila are also implicated in mammalian Hh signalling. There are three types of Hh proteins; Sonic SHH, Indian IHH and Desert DHH. DHH is limited to the gonads, unlike SHH and IHH, and its regulation may occur through primary cilia [255]. SHH is essential in neurodevelopment in vertebrates. Studies have

shown that mutations in primary cilia genes would change Hh patterns [254]. Protein trafficking is essential for Hh signalling pathways. For instance, assembly of a membrane protein called SMO in primary cilia happens in the reflection of Hh presence. Overall, many proteins are needed to assemble the structure of primary cilia and the Hh signalling pathway has a broad range of effects on proteins that contribute to the formation of cilia.

Another point of view comes from studies that showed the relationship between cilia dysfunction and syndromes such as ciliopathies, Bardet–Biedl syndrome and Alström syndrome, and their phenotype of obesity [256]. Bardet–Biedl syndrome is characterized by other symptoms such as polydactyly and kidney failure, as well as retinitis pigmentosa. To better understand this syndrome, we need to know the genes involved and their protein interaction network. 19 genes are associated with this syndrome, and 7 of these genes form a complex called the BBSome, which is involved in primary cilia formation [257]. BBS6, 10 and 12 interact with the CCT/TRIC complex, of which the subunits were identified in our mass spectrometry results. Understanding the interactions and functions of each subunit in relation to the CCT/TRIC chaperonin complex can also shed light on the function of BSPs. BSP knock out mice demonstrated increased weight in male offspring, which is a similar phenotype as that observed with BBS patients. Moreover, the eight subunits of the CCT/TRIC complex were identified in our mass spectrometry results. This complex interacts with the BBSome, which contains Bardet-Biedl syndrome proteins. PSMD1, another gene identified in our mass spectrometry results, is independently associated with Bardet-Biedl syndrome. CCT/TRIC is a hetro-oligomeric complex with low amino acid conservation between the eight subunits [201] indicating that each subunit can have a different role. Interestingly, in another study, BBS6, 10 and 12 were shown to interact with the CCT/TRIC complex and be involved in the formation of BBSome in primary cilia [191]. Importantly, the chaperonin activity of the CCT complex is crucial for assembly of the BBSome complex as well as for vesicle delivery and protein trafficking to primary cilia [191]. It has been shown in a number of studies that many subunits of the CCT complex are essential for cilia assembly [202,203]. Another set of important proteins are TuBB family proteins. TTUBB, TUBB6, TUBB2A, TUBB4A were also present in our mass spectrometry data with significant FDR. The TUBB gene is translated into to beta tubulin, which can form a dimer with alpha tubulin and establish the microtubule

structure.Microtubules are the major constituents of primary cilia; therefore, the Tubb family is important in primary cilia biogenesis. CCT could also be implicated in tubulin turnover and cilia biogenesis in response to ciliary impairment [202].

Another striking data element from our mass spectrometry results is the emerging number of centrosomal proteins (CEP). CEP such as CEP131,170,192 are usually necessary for the formation of cilia/flagella and mutations in these proteins could result in ciliopathies such as BBS. Interestingly, CEP 19 is associated with morbid obesity, spermatogenic failure and BBS. Although CEP 19 did not score in our mass spectrometry data, it cooperates with CEP350 in early ciliogenesis [179]. CEP 350 was detected in our mass spectrometry data.

CEP131 is conserved in mammals and drosophila and has a preserved role in ciliogenesis. CEP131 knockout mice were infertile due to not being able to establish the ciliary structure of the sperm tail [258]. In addition, CEP 295 was found to be essential for centriole to centrosome conversion, and is enhanced in the daughter centriole [259]. Moreover, it has been shown that CEP72 is essential for the correct localization of BBS4 in primary cilia [260]. Furthermore, CEP152 has been shown to be indispensable for centriole duplication [261].

Beside CEPs, other proteins such as PCM1 (pericentriolar material) were present in our mass spectrometry results, and are essential for cilia biogenesis, regulation and OFD1 degradation, which is necessary for assembly of primary cilia [102].

Zhang et al. demonstrate that BBS7 knockout mice present the BBS phenotype such as obesity and male infertility, the latter of which is due to a defect in the sperm flagellum [262]. Although we did not see male infertility or subfertility in BSPH1/BSPH2 knock out mice, we observed an increase in the weight of male offspring. As indicated in literature, one of the most common phenotypes of this syndrome is obesity. Our previous observation of weight increase in double knock out mice suggests a possible role of BSP proteins in metabolism [131]. Moreover, the genes related to BBS are localized on different chromosomes and 25 percent of BBS patients are idiopathic. Therefore, additional genes are involved in the formation of this multi-phenotypic syndrome.

Our studies provide additional support for previous research (knock out mice) that epididymal BSPs might not only be involved in fertility but also have other functions. This is in agreement with *in vitro* and *in vivo* studies. As predicted, we were anticipating to identify potential other functions for BSPH1, such as homeostasis through primary cilia. We are aware that our research may have some limitations and require additional studies, such as *in vivo* BioID experiments or more validation experiments. The apparent lack of correlated functions between seminal vesicle BSPs and epididymal BSPs may be attributed to evolutionary enforcement. Taken together, these results open a new avenue for a role of epididymal BSPs in other areas, specifically metabolism. They may also contribute to rare syndromes such as BBS, for which 25% of the cases are idiopathic.

5.4. Perspective

The exact role of epididymal BSP proteins has yet to be confirmed through further experiments to identify specific mechanisms that require BSPH1. Knock out studies in mice should be continued with more statistically significant numbers and for more generations. In addition, measuring leptin, glucose, and insulin levels in knock out versus controls, as well as observing obesity and type II diabetes would be essential. Immunohistochemical staining of epididymal primary cilia could confirm whether gene knockout affects the number, elongation, and orientation of primary cilia in this organ. *In vivo* BioID might provide a better confirmation of the involvement of BSPH1 in cilia protein trafficking and its possible relation to ciliopathies.

Moreover, endeavors in the field of cilia biogenesis and ciliopathies must be pursued at different levels from *in vitro* to translational research, in order for researchers to identify therapeutic options.

Appendix

In this section, I will discuss in more details the purification of recombinant proteins and the common purification techniques that I used during my PhD, such as different types of chromatography (affinity, Ion exchange, size exclusion) as well as dialysis and ultrafiltration methods.

Prokaryotes and production of recombinant proteins

Since prokaryotes do not have a nucleus, transcription and translation processes occur at the same time. Eukaryotes are more complicated, and transcription occurs inside the nucleus, wheras translation occurs in the cytoplasm using ribosomes. Moreover, prokaryotes do not have introns in their genes, unlike eukaryotes. The prokaryote genome expresses about 2000 proteins; however, the eukaryotic genome is significantly larger and more complicated. Prokaryotic DNA is single stranded, circular and devoid of histones, compared to eukaryotes whose DNA is paired, linear and associated with histones. Because of prokaryote characteristics (fast growth and inexpensive), they are used for the production of recombinant proteins (*Escherichia coli*) [263,264].

Amino acids characteristics

Proteins are made from building blocks called amino acids. Proteins have both positive and negative charges (ampholytes); in other words, when they dissolve in water, they can be either acidic or basic. The isoelectric point of proteins defines the pH at which the net charge of the protein is neutral, which depends on the amino acid composition of the protein. The net charge of a protein affects its aggregation, solubility and purification characteristics [265]. Some amino acids have a negative charge, such as aspartic acid and glutamic acid, whereas others have positive charge, such as lysine, histidine and arginine. Charged groups are mostly distributed all over the protein surface and play essential roles in protein biochemistry, such as receptor-ligand bonds, enzyme-substrate catalytic activity, and protein conformation (folding), to name a few [265].

Recombinant protein expression

A combination of different DNA sequences to form a new gene that is cloned into an expression vector will result in the production of recombinant proteins. Recombinant proteins can have therapeutic value and clinical applications from the production of vaccines to cancer treatment.

The first step of recombinant protein expression is cloning the target cDNA into the right vector, followed by induction of expression in bacterial cells, such as *E. coli*. Usually, *E. coli* is the first option because of its fast growth and low-priced culture for proteins that are not post translationally modified. *E. coli* strains can be engineered, such as BL21 (DE3) strain, to have specific characteristics such as disabled proteases and an adaptive lac promoter. In the production of recombinant protein, the insertion of a small protein or peptide tag in the design of the expression clone is a commonly used to facilitate purification steps and protein solubility.

Protein solubility

One of the main difficulties in protein expression is protein aggregation. To overcome such an obstacle in protein expression, the addition of various fusion tags (e.g., Glutathione S-transferase, thioredoxin (TRX), Maltose binding protein (MBP)) can be used [266]. Several factors affect protein solubility, such as molecular weight, amino acid composition, and hydrophobic or hydrophilic groups. For instance, proteins with negative charges are more soluble above their isoelectric point [267]. Low concentration of salts or chaotropic elements such as urea can disrupt secondary structure (denaturation) and enhance solubility; hydrogen bonds can also improve protein solubility. Protein aggregation can also be affected by hydrophobic interactions. For instance, guanidine hydrochloride is a more robust chaotropic component, which is used to solubilize inclusion bodies (aggregated forms of recombinant proteins). Another example is guanidine thiocyanate, which is another chaotropic element that is stronger than guanidine hydrochloride, but more expensive.

Chromatography

The term chromatography means drawing of different colors since it was used for the first time to designate the separation of plant pigments. Chromatography is one of the protein separation techniques, and it consists of a mobile phase and a stationary phase. The mobile phase can be a gas or liquid, while the stationary phase can be liquid (e.g., ink) or solid (e.g., paper). If the mobile phase is liquid, it is called liquid chromatography. If the mobile phase is gas, it is called gas chromatography. Chromatography has some advantages, such as separating a broad range of molecules from atom size to large elements. Moreover, chromatography can also provide separation along with identification and purification of more than one element. There are different chromatography types, such as column chromatography, paper chromatography, thin-layer chromatography, and high-pressure liquid chromatography (HPLC), to name a few [268]. In addition to chromatography applications in biochemistry, it is a useful method in medicine as well. Using paper chromatography to identify specific amino acids related to a metabolic disorder and using gas chromatography coupled to mass spectrometry to measure steroid hormones in blood or urine are examples of chromatography applications in clinical research [269]. Overall, column chromatography is considered not only a method for separation but also to purify different elements such as proteins, lipids, or carbohydrates.

Ion Exchange Chromatography

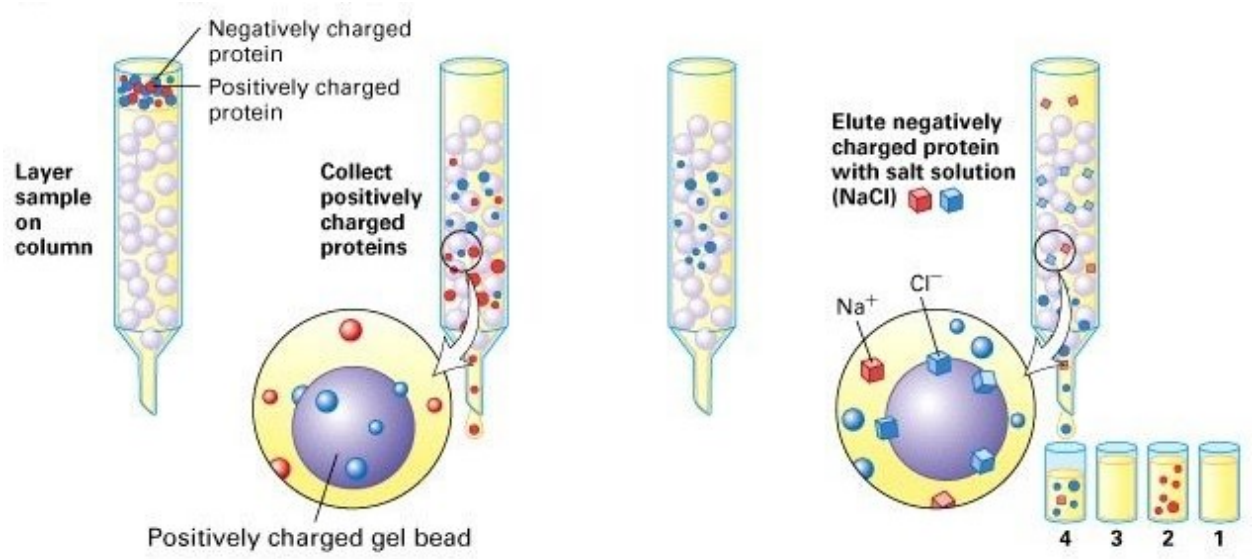


Figure 17. Ion exchange chromatography

The basis of ion exchange chromatography (IEC) is the ionic interactions between a charged group of the target protein and the opposite charge of the matrix. IEC is traditionally accompanied with a salt or pH gradient. It is also an alternative for the separation of protein isoforms [270]. There are two kinds of IEC: anion-exchange (AEX), which contains positively charged groups, and cation-exchange (CEX), which contain negatively charged groups in their resins. With an increasing gradient of salt concentration (NaCl), proteins start to elute, with weakly interacting proteins eluting first (usually unwanted proteins). Both CEX and AEX have weak and strong resin exchangers. For instance, diethylaminomethyl (DEAE) is a weak anion exchanger, and it is positively charged. Quaternary resin is considered as a strong anion-exchanger, and it preserves its ionic strength through different pH ranges. On the other hand, sulfonic acid is a strong cation-exchanger, and carboxymethyl (CM) is a weak cation-exchanger. IEC is one of the fundamental methods used in therapeutics because of its non-denaturing condition. A schematic overview of IEC is represented in Figure 17.

Affinity Chromatography

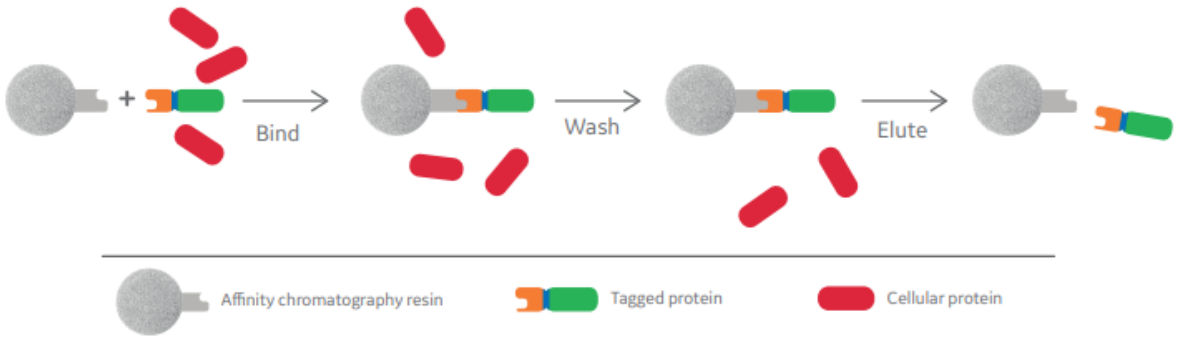


Figure 18. Schematic presentation of tagged protein purification [271]

Affinity chromatography was first described by Cuatrecasas, Wilchek and Anfinsen in 1968 for enzyme purification [272]. Affinity chromatography is a type of liquid chromatography that favors biological interaction properties for purification [273]. Affinity chromatography has many clinical research applications. For instance, the Boronate affinity chromatography technique (Based on

phenyl-boronic acid) in is used in diabetes mellitus management and lectin affinity chromatography is used for purification of isoenzymes. Other affinity chromatography applications are antibody binding affinity (immunoaffinity) such as protein A and G resin, which facilitated the study of the immunoglobulins in different species [273]. Other applications of affinity chromatography are purification of DNA binding proteins to study histones or transcriptional cofactors in the replication or transcriptional context, since they all share the common characteristic of binding to DNA. These are divided into specific, nonspecific, single and double DNA strand affinity chromatography. In addition, heparin-agarose and phosphocellulose are widely used for the purification of transcription factors [274]. RNA binding proteins also can be purified using RNA affinity chromatography [275]. Overall, DNA affinity chromatography is a significant part of genetic studies since it allows identification and purification of numerous elements such as polymerases, restriction enzymes and transcription factors, to name a few. a schematic explanation of affinity chromatography is represented in Figure 18.

Immobilized metal affinity chromatography (IMAC)

IMAC is based on the natural affinity of histidine or cysteine amino acids of proteins to metal ions such as copper, nickel and cobalt [276]. The metal is fixed to the solid phase (e.g., agarose beads) and the protein is usually tagged with histidine amino acids. It was first used in 1975 by Porath for fractionation of human proteins [277]. In addition to purification, IMAC has the advantage of concentrating diluted samples. IMAC also has a high capacity for protein adsorption without changing protein conformation. Moreover, it is in accordance with a broad range of buffers as well as chaotropic agents.

Affinity tags

Affinity tags are not only useful for purification but also for solubility of proteins in biological mixtures. Affinity tags increase the stability of proteins by diminishing the chance of degradation. Polyhistidine, Maltose Binding Protein, Calmodulin Binding Peptide, intein-Chitin Binding Domain, and Streptavidin-Biotin are some of the most used affinity tags [278]. Some tags also have protease recognition sites to be cleaved after their duty is done (Sumo or Flag). The binding of

affinity tags to IMAC resin can be covalent or noncovalent. For instance, glutathione-s transferase (GST) binds non-covalently to the affinity matrix.

Size Exclusion Chromatography

Size exclusion chromatography (SEC), sieve chromatography, gel filtration, or gel-permeation chromatography can separate particles based on their molecular size. Size exclusion matrices can be dextran/agarose or polymers. SEC has some important features such as column bed, void volume and pore volume. Void volume is the volume of the mobile phase, and the column bed is the volume of the solid portion of the column. The broad implication of SEC is that agarose beads have a porous structure with slightly different sizes, which allows separation of particles based on their size properties. Large particles pass the agarose pores faster than small particles as they are excluded from the pores, which face more challenges between bead pores. Proteins tend to have a spherical shape, while DNA and carbohydrates are linear molecules. Therefore, DNA and carbohydrates will be eluted from the column earlier than proteins of the same size, and then salts elute eventually.

Desalting

One of the essential applications of gel filtration is desalting or salting out the purified proteins. In the desalting technique, small pore beads are usually selected for the fast purification of peptides, while larger ones are used for protein complexes. Desalting is typically the last purification step, and the matrix retains the biological activity of the samples since there is no interaction between the sample and the beads. A schematic explanation of desalting is presented in Figure 19.

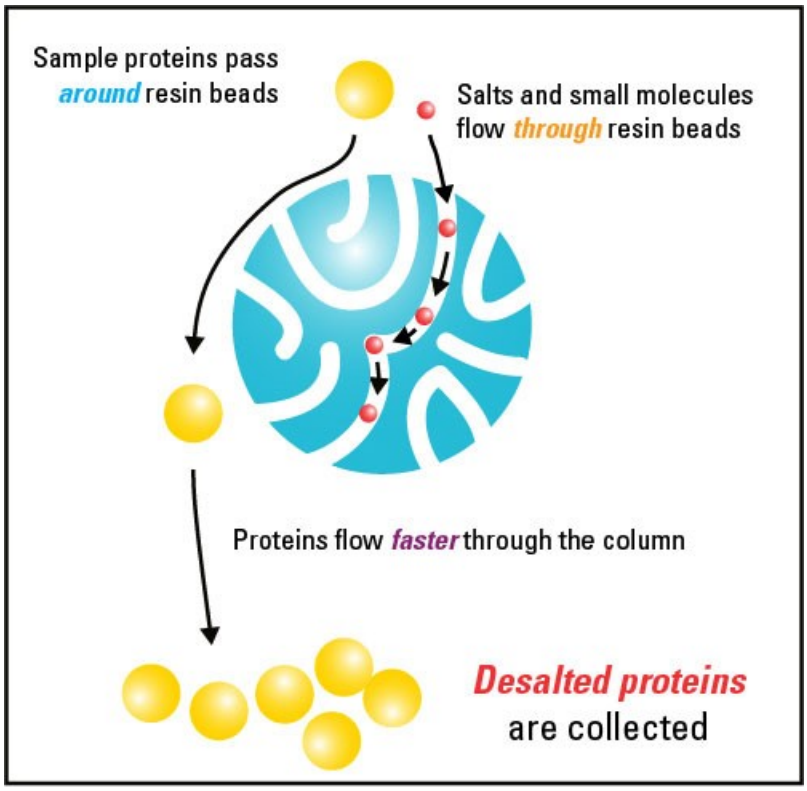


Figure 19. Schematic explanation of desalting [279]

Dialysis

Dialysis is another form of separation that utilizes a semipermeable membrane. It is useful to separate proteins from salts or from reducing agents such as β-mercaptoethanol. It is often performed overnight, utilizing large volumes for the buffer exchange. The larger molecules cannot enter the small pores of the membrane while the smaller ones (salts) diffuse through the pores. Dialysis is usually accompanied with several buffer exchange and is usually performed at room temperature because higher temperatures speed up the diffusion process. If the protein concentration is high or the molecular weight is low, it will substantially increase diffusion speed. It is essential to choose the best way to remove contaminants as some techniques could change downstream results. One example is the removal of EDTA, which is a usual component in protein purification procedures to inhibit protease activity. Andreia Mónico et al. showed that ultrafiltration (a type of dialysis) is a better procedure to remove EDTA from the sample than dialysis, which was confirmed by NMR [280]. Ultrafiltration can not only be used as a purifying device but also as a protein concentrator. Hydrostatic pressure against the filter (different pore

sizes) with a specific molecular weight cut off (MWCO) leads to separation of the proteins from contaminants. MWCO is the molecular weight at which 90 percent of the proteins are retained by the membrane.

Protein concentration and purification assessment

The purity and quality of proteins are essential for downstream biological studies. Care should be taken to avoid the aggregation and misfolding of proteins in the process of purification. Storage temperature, pH of buffers, and salt concentration should be optimized to have the best results. There are different assays to determine total protein concentrations such as the Lowry assay, bicinchoninic acid assay (BCA), Bradford assay, and (ultraviolet-visible) spectrophotometry [281]. Gel electrophoresis (SDS-PAGE) followed by Coomassie Blue Staining, silver staining or zinc-reverse staining is a common way to verify protein size and degradation (integrity) or smeared bands [282]. Western blots are normally utilized to determine protein specificity using related antibodies. These techniques are fast and compatible with mass spectrometry. Eventually, mass spectrometry can precisely identify the amino acids of purified proteins as well as post-translational modifications.

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