

**Characterization and elucidating the functional role of uroplakins
in the male reproductive tract**

*Thesis submitted to the University of Hyderabad for the award of
Doctor of Philosophy in the Department of Animal Biology*

By

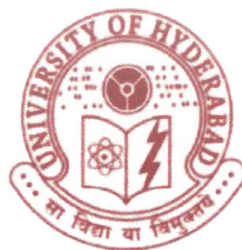
Suresh Babu Munipalli

(Reg. No. 14LAPH10)



**Department of Animal Biology
School of Life Sciences
University of Hyderabad
Hyderabad - 500 046
Telangana, India**

JULY-2022



University of Hyderabad
School of Life Sciences
Department of Animal Biology

DECLARATION

I, **Suresh Babu Munipalli**, hereby declare that this thesis entitled **“Characterization and elucidating the functional role of uroplakins in the male reproductive tract”** submitted by me under the guidance and supervision of **Prof. Suresh Yenugu** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Prof. Suresh Yenugu

(Supervisor)

Dr. SURESH YENUGU
Professor
Department of Animal Biology
School of Life Sciences
University of Hyderabad
Hyderabad-500046. (TS), INDIA

M. Suresh Babu .

Suresh Babu Munipalli

(Reg.No.14LAPH10)



University of Hyderabad

School of Life Sciences

Department of Animal Biology

CERTIFICATE

This is to certify that this thesis entitled **“Characterization and elucidating the functional role of uroplakins in the male reproductive tract”** is a record of bonafide work done by **Mr. Suresh Babu Munipalli**, a research scholar for Ph.D. programme in the Department of Animal Biology, University of Hyderabad under my guidance and supervision for a full period prescribed under Ph.D. ordinances of this University. The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma. We recommend his thesis entitled for submission for the degree of Doctor of Philosophy of the University.


Prof. Suresh Yenugu

Supervisor

Dr. SURESH YENUGU
Professor
Department of Animal Biology
School of Life Sciences
University of Hyderabad
Hyderabad-500046. (TS), INDIA


Head

Department of Animal Biology

अध्यक्ष / HEAD

जंतु जैविकी विभाग

Department of Animal Biology


Dean

School of Life Sciences

DEAN

School of Life Sciences
University of Hyderabad
Hyderabad-500 046.



University of Hyderabad
School of Life Sciences
Department of Animal Biology

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This is to certify that this thesis entitled “**Characterization and elucidating the functional role of uroplakins in the male reproductive tract**” is a record of bonafide work done by **Mr. Suresh Babu Munipalli**, a research scholar for Ph.D. programme in the Department of Animal Biology, School of Life Sciences, University of Hyderabad under my guidance and supervision. This thesis is free from plagiarism and has not been submitted in part or in full to this or any other University or institution for the award of any degree or diploma. Parts of the thesis have been:

A. Published in the following journal:

1. **Babu Munipalli, S.** and S. Yenugu, *Uroplakin expression in the male reproductive tract of rat. General and Comparative Endocrinology*, 2019. 281: p. 153-163. (<https://pubmed.ncbi.nlm.nih.gov/31181195/>).

B. Presented in the following conferences:

1. **Suresh Babu Munipalli**, & Suresh Yenugu. Characterization of Uroplakin expression in the male reproductive tract. Poster presentation in “International Symposium on Comparative Endocrinology and Integrative Physiology (CEIP 2015)”, Thiruvananthapuram, Kerala.
2. **Suresh Babu Munipalli**, & Suresh Yenugu. Characterization of Uroplakin expression in the male reproductive tract. Poster presentation in “International Symposium on Integrative Physiology and Comparative Endocrinology (ISIPCE-2016)”, Banaras Hindu University, Varanasi.

Further, the student has passed the following courses towards the fulfillment of the coursework requirement for Ph.D.

Course Code	Name	Credits	Pass/Fail
AS 801	Analytical Techniques	4	Pass
AS 802	Research Ethics, Data Analysis and Biostatistics	3	Pass
AS 803	Lab Work and Seminar	5	Pass



Prof. Suresh Yenugu

Supervisor

Dr. SURESH YENUGU
Professor
Department of Animal Biology
School of Life Sciences
University of Hyderabad
Hyderabad-500046. (TS), INDIA



Head 20.7.22

Department of Animal Biology

अध्यक्ष / HEAD
जंतु जैविकी विभाग
Department of Animal Biology



Dean
School of Life Sciences
DEAN
School of Life Sciences
University of Hyderabad
Hyderabad-500 046.

Acknowledgements

I express my sincere gratitude to my teacher and research supervisor, Prof. Suresh Yenugu for his overall guidance and suggestions throughout the period of my work. As a supervisor, he has given full freedom to execute the experiments with critical suggestions. I express my deepest gratitude for his magnanimous and kind heartedness. In addition to research, I am very grateful to him for his kind help even in many situations of my personal life across this period.

I would like to thank my doctoral committee members Prof. Aparna Dutta Gupta, Prof. B. Senthilkumaran and Prof. Naresh Babu V Sepuri, for their insightful comments and suggestions for this study.

I sincerely acknowledge the Head of Department- Current and previous heads for providing equipment in good condition and facilities to carry out this work. I sincerely acknowledge the dean of the school of Lifesciences - Current and previous deans for allowing me to use school facilities to carry out this work.

I thank animal house In-charge Prof. K. Arun Kumar, for allowing me to use animal facilities to carry out this work. I thank all the animal house staff for their kind help throughout this period.

I thank FACS In-charge/s Dr. Radheshyam Mourya, and Dr. Roy Kamati for allowing me to use FACS facilities.

I express my sincere thanks to all my teachers at PG level, at UG level, Inter, high school and primary school.

I thank RGNF (JRF and SRF) for providing financial assistance to carry out this work. Financial support from UoH, CSIR, ICMR, IOE, DBT and DST to the laboratory is highly acknowledged.

I am grateful to my laboratory colleagues Aisha Jamil, Mounika Marri and Priyanka Patra for their help cooperation, and support in all respects during my work. I would like to thank my laboratory seniors Dr. Bernali Biswas, Dr. Madhu Babu Golla, Dr. Rajesh Anjireddy, Dr. G. Narmadha and P. Lavanya, Dr. Anandha Rao, Dr. Sangeeta for their help and efforts to establish the lab. I thank laboratory helper Narasihanna for his timely help.

I thank to late Prof. Aparna Dutta Gupta, and Prof. B. Senthilkumaran for my expertise in Wax sectioning and H and E staining.

I thank Prof. Arun Kumar for allowing me to use the animal house facility and for helping me whenever I need.

I thank the NIAB Animal house facility for maintaining Knockout mice and taught me animal handling and mating study.

I thank Dr. Aurélie Jory for generated Upk1a heterozygous mice and special thanks to National Center for Biological Sciences (NCBS).

I thank Dr. Nirmalya Ganguli for teaching me about testicular microinjection.

I Thank Dr. Radheshyam Maurya and Mr. Prince Sebastian for FACS assistance and analysis.

I thank Dr. Prasad Tammineni , Deepti madam, and Nalini madam for the high-resolution imaging.

I thank Dr. J. Madhupraksh for teaching me protein modelling.

I thank Dr. Sireesh Kumar for the infection model generation.

I thank Mr. Mahesh and Mr. Sridhar for the Histopathology study.

I special thanks to the Novel gene Technology team Bipin Sir, Abhishek sir, Naveen, and Aman, for the transcriptome studies.

I am incredibly thankful to Dr. Gurranna and Dr. Jyothi Chaitanya Pagadala for their continuous support.

I am highly thankful to all my friends at the University of Hyderabad.

I am highly thankful to all juniors in the life science for their help.

I am so fortunate to having a lovable and fabulous sister Sujatha, and my brother Ravi Kumar for their constant encouragement, support, and cooperation across this journey and I would have not reached to this level without them.

Thanks to the almighty for everything!

FINALLY, I THANK MY EVER-LOVING PARENTS, BULLEIAH AND NIRMALA

Suresh Babu Munipalli...

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Abbreviations

UPKs	: Uroplakins
PGC	: Primordial germ cells
SSCs	: spermatogonial stem cells
PTM	: Peritubular myoid
LH	: luteinizing hormone
FSH	: Follicle-Stimulating Hormone
TP1	: Transition protein 1
SPAG11A	: Sperm-associated antigen 11A
AUM	: Asymmetric unit membranes
VUR	: Vesicoureteral reflux
ER	: Endoplasmic reticulum
UPEC	: Uropathogenic <i>Escherichia coli</i> ,
PBS	: Phosphate buffered saline
SOPMA	: Self-Optimized Prediction Method with Alignment
HEK293	: Human embryonic kidney 293 cells
LC540	: Rat Leydig cell line - 540
RCE	: Rat caput epididymal cell line
PATE	: Prostate and Testis expressed
LYZL	: Lysozyme like
PFA	: Paraformaldehyde
CDK7	: Cyclin-dependent kinase 7
PPIs	: Protein-protein interactions
HE4	: Epididymis protein 4
CFTR	: Cystic fibrosis transmembrane conductance regulator

FGF	: fibroblast growth factor
Y2H	: Yeast two hybrid screening
DNA-BD	: DNA-binding domain
DNA-AD	: DNA – Activation domain
RGN	: Regucalcin
PSMB1	: Proteasome subunit beta 1
TEX44	: Testis expressed 44
SNRPN	: Small nuclear ribonucleoprotein polypeptide N
RFP	: Red fluorescent protein
GFP	: Green fluorescent protein

Title

**Characterization and elucidating the functional role of
uroplakins in the male reproductive tract**

Introduction

Male reproductive system

The male reproductive system combines testes, epididymides, vas deferens, and ejaculatory duct and also includes accessory sex glands (prostate, bulbourethral and seminal vesicles). A pictorial representation of a typical organization of the male reproductive organization is presented in Figure 1.

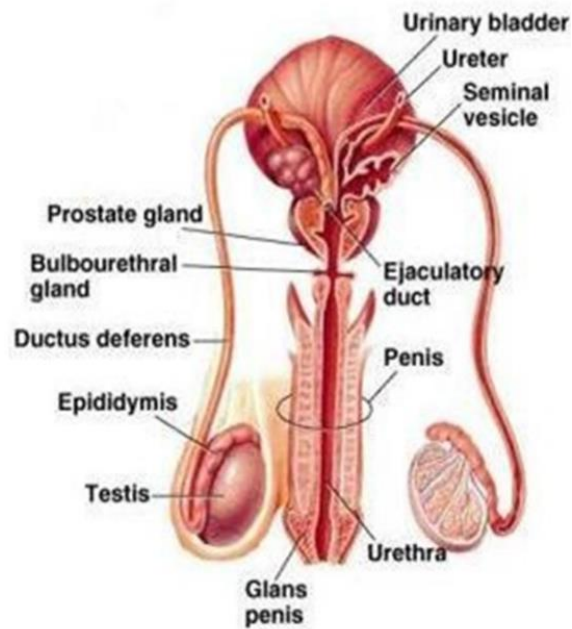


Figure 1. Male reproductive system

Testes

Oval-shaped structures in the scrotal sac (a bag-like structure) just behind the penis. Tunica albuginea (a fibrous capsule) sheaths both the testes. The testis is partitioned into 200–400 wedge-shaped lobes by the fibrous tissue of the tunica albuginea. Three to ten coiled tubules, or seminiferous tubules, are present within each lobe. Testis acts as both exocrine and endocrine glands. Leydig cells of the testis produce testosterone, a factor crucial for spermatogenesis and other physiological functions (bone mass, fat distribution, sex drive muscle size, strength, etc.) The exocrine function of the testis is to produce spermatozoa in the seminiferous tubules.

Epididymis

A highly intricate tubular organ, called the epididymis, is trisected into three portions i.e caput, corpus, and cauda. The sperm passes from the testes to the vas deferens through the epididymis. The immotile testicular sperm attain motility and fertilizing ability when they cross the epididymal regions. The broad portion of the epididymis is represented by the caput, which is located over the testes posteriorly and nourishes the sperm till they start to undergo maturation. Sperm start to become more motile in the corpus epididymis. After development, sperm are stored in the cauda until ejaculation.

Vas deferens

A long muscular tube beginning from the epididymis that extends across the pelvic cavity and ends right outside the bladder. For ejaculation, the vas deferens facilitates the movement of mature sperm to the urethra.

Ejaculatory duct

The ejaculatory duct transports sperm into the urethra with the prostate's essential secretions and supplements for sperm activity.

Seminal vesicles

The two seminal vesicles produce the fluidic compositions of the semen. An estimated 50 to 80 percent of semen's volume is made up of the liquid produced by the seminal vesicles. Seminal fluid contains ingredients intended to keep sperm viable after entering the vagina. Energy-producing fructose aid in sperm motility and prostaglandins prevent an immune response by the tissues of the female genital tract. The seminal fluid alkalinity aids in neutralizing the acidic environment in the female genital tract. Various clotting factors aid in the longevity of sperm.

Prostate gland

The prostate gland is positioned immediately beneath the bladder. The principal purpose of the prostate is to furnish the fluid for sperm transport and nourishment. Enzymes, zinc, and citric acid found in prostatic fluid make semen a perfect environment for sperm cells to live.

Bulbourethral gland:

The bulbourethral glands are a pair of pea-shaped exocrine glands, also known like Cowper's gland, are located posterolateral to the membranous urethra. By secreting lubricating mucus, they add to the final amount of semen.

Spermatogenesis

The primary function of the male reproductive system is the production of sperm (spermatogenesis) in the seminiferous tubules of the testis. This process starts with differentiating primordial germ cells (PGC) into spermatids through mitotic and meiotic divisions. For instance, in humans, the primordial germ cell is the main undifferentiated stem cell that distinguishes connecting gametes, spermatozoa, or oocytes. (Nikolic, Volarevic, Armstrong, Lako, & Stojkovic, 2016).

The multiplication and development of spermatogonial stem cells (SSCs), which come from PGCs in the early embryo, maintain the production of mammalian sperm (Ohta, Wakayama, & Nishimune, 2004). Spermatogonial stem cells (SSCs), which can self-renew and produce daughter cells to bear terminally differentiated cells, or spermatozoa, are necessary for the lifelong maintenance of spermatogenesis (Figure 2). Spermatogenesis occurs in the seminiferous tubules and constitutes three main cell types: germ cells, Sertoli cells, and peritubular myoid (PTM) cells. The crucial primary role played by these cells is to provide niche for SSCs (Richardson, Kleinman, & Dym, 1995). Leydig cells in the interstitial space of the testis secrete testosterone in response to stimulation by luteinizing hormone (LH). In spermatogenesis (a process tightly controlled by testosterone), diploid spermatogonia undergo mitosis until they start to transform into gametes. These gametes eventually become primary spermatocytes, which divide once during the initial meiotic phase to produce two haploid secondary spermatocytes. After a second meiotic division, the secondary spermatocytes both resulted in two spermatids, which will eventually mature into sperm. The immature sperm will enter the epididymal caput, and maturation is facilitated by the epididymal secretions. The matured sperm is stored in epididymal cauda.

Spermatogenesis

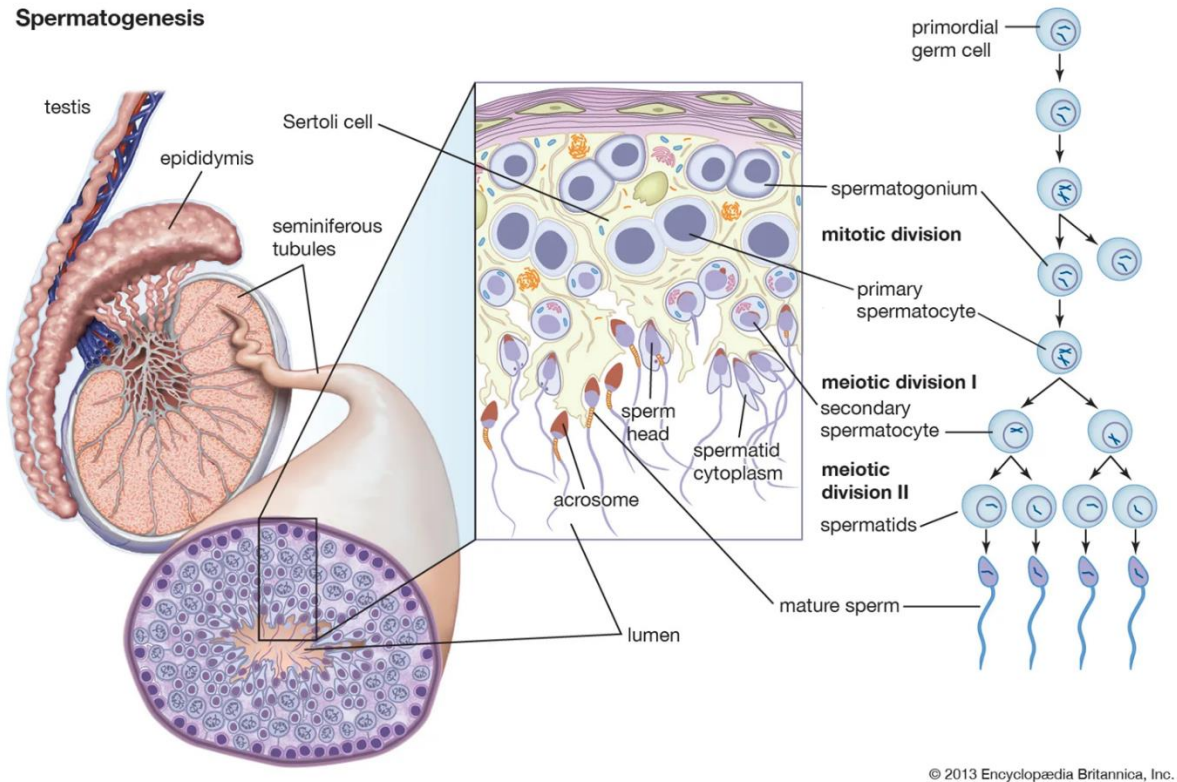


Figure 2. Representation of the Spermatogenesis

Factors involved in spermatogenesis and maturation

Spermatogenesis initially starts from neonatal development through testis and epididymis. In this sequential development process, several factors help form sperm and its maturation. In the period of neonatal development, the progenitor cells convert into gonocytes in the presence of SCF/cKit, which is crucial for maintaining primordial germ cells. Sertoli cells also express SCF when stimulated by Follicle-Stimulating Hormone (FSH). (Rossi, Sette, Dolci, & Geremia, 2000). The pluripotent nature of the PGC is supported by the expression of the *Stella* and *Oct4* and alternative pluripotent genes in germ cells; and repression of *Hoxb1*, *Hoxa1*, *Evx1*, and *Lim1*, homeobox genes that negatively regulate somatic cell differentiation (Saitou *et al.*, 2003; Yeom *et al.*, 1996). The PGCs multiply and move through the mesentery and endoderm of the hindgut before migrating bilaterally to the genital ridges. Meiosis begins in females or mitotic arrest in the case of males and initiates differentiation into each oocyte or spermatozoa. (Molyneaux, Stallock, Schaible, & Wylie, 2001). Postmigration, PGCs are

distinguished by the expression of various RNA binding proteins, namely, *MVH*, *DAZL*, and *NANOS3*. In the female XX embryo, the PGCs constantly proliferate and consequently enter prophase I of meiosis. XY PGCs undergo mitotic arrest and entry into the genital ridges, where they persist in the quiescent G 0/G 1 cell cycle phase during the embryonic period. (McLaren, 2001). PGCs, which move from the embryonic ectoderm postnatally, give rise to SSCs. The transcription factor-encoding genes *Bcl6b*, *Etv5*, and *Lhx1*, as well as the site factor of glial cell line-derived neurotrophic factor (GDNF), significantly impact the self-renewal of rodent SSCs. (Oatley & Brinster, 2008).

During sperm production in the testis, i.e., spermatogonial stage to spermatids, factors that define chromatin modeling, transcription, and RNA binding are involved. Numerous transcription elements involved in spermatogenesis, including heat shock factor 2 (HSF2) and OVOL are implicated in germ cells. Sertoli cells express WT1, RHOX5, SOX8, and FOXI1, contributing to sperm maturation in the epididymis (Bettegowda & Wilkinson, 2010). At the chromatin level, two of the four canonical core histones, H2A, H2B, H3, and H4 (that make up nucleosomes in male germ cells) form octamers to regulate gene expression and fold the DNA (Talbert & Henikoff, 2010). Non-histone proteins like transition protein 1 (TP1) and transition protein 2 (TP2) that are arginine and lysine-rich with an excellent affinity for DNA play a crucial role in the control of chromatin structure of spermatids. The protein BRDT, found in the testis, is implicated by pachytene spermatocytes. Shortened BRDT lacking bromodomain exhibits problems in post-meiotic male germ cells (Shang, Nickerson, Wen, Wang, & Wolgemuth, 2007).

One of the families of RNA-binding proteins (RBPs) found in germ cells is the Y-box protein family, which includes the variants MSY1, MSY2, and MSY4. The role of MSY2 transcription factor promotes the transcription of testis-specific genes. Also, it functions as a structural constituent of messenger ribonucleoproteins (mRNPs) in the cytoplasm of maturing germ cells (Gu et al., 1998). Human testis expresses YBX2, also referred to as "Contrin," the homologue of MSY2. Alterations in the YBX2 gene result in altered protamine expression, which in turn causes aberrant spermatogenesis and infertility in humans.(Hammoud, Emery, Dunn, Weiss, & Carrell, 2009; Yang, Morales, Medvedev, Schultz, & Hecht, 2007)

The three parts that made up the epididymis were the proximal caput, the extended corpus, and the expanded distal cauda. The immature sperm that the testis produces will go into the

epididymis, where maturation will occur. The epididymal caput produces intraluminal components that are necessary for sperm motility and fertilization.(Sullivan & Micusset, 2016). The expression of the sperm-associated antigen 11A (SPAG11A), which is androgen-dependent and plays a function in sperm maturation, is restricted to the caput epididymis's main cells in mice.(Pujianto, Loanda, Sari, Midoen, & Socharso, 2013).

Transmembrane proteins in male reproduction

Transmembrane proteins are the cell surface receptors involved in the signal transduction process crucial for gametogenesis and fertilization. The ion channel receptors, namely, calcium channels regulate calcium levels for sperm function and fertilization (Darszon, Nishigaki, Beltran, & Trevino, 2011). The male germ cells express ryanodine receptors (RyRs); and the mobilization of calcium through RyR channels regulates the maturation of male germ cells (Chiarella, Puglisi, Sorrentino, Boitani, & Stefanini, 2004). Similarly, enzyme-linked receptors, such as c-Kit, have a pivotal role in the differentiation of gonocytes and testis maturation (Prabhu *et al.*, 2006). When spermatogonia mature into spermatozoa, differential gene expression and cell-to-cell communication are moderated by the endocrine stimuli resulting from FSH/LH and testosterone. The FSHR, a transmembrane G-protein receptor on Sertoli cells, drives spermatogenesis (Oduwale, Peltoketo, & Huhtaniemi, 2018).

Tetraspanins (also known as tetraspan or TM4SF) are a broad family of integral membrane proteins, and 33 groups make up this superfamily in humans. The homologs of human tetraspanins, which are found in all eukaryotic species, are broadly distributed in cells and tissues. They are involved in various processes, including differentiation, adhesion, motility, cell activation, and proliferation (Maecker, Todd, & Levy, 1997). Tetraspanin family members include membrane protein CD9. Despite CD9's wide tissue distribution, animals lacking CD9 mainly exhibited female infertility caused by a problem with the sperm-egg fusion process(Kaji, Oda, Miyazaki, & Kudo, 2002). The tetraspanin family proteins CD9, CD63, CD81, and CD151 are crucial for immunity, cell-to-cell communication, and fertilization (Desalle, Chicote, Sun, & Garcia-Espana, 2014). The Uroplakin1a (UPK1a) and Uroplakin1b (UPK1b) also belong to 20th and 21st tetraspanin families, present on mammalian bladder epithelium (urothelium)(Jenkins & Woolf, 2007).

Uroplakins (UPKs)

Uroplakins (UPKs) are integral membrane proteins that fall under one of two families: The four-transmembrane domain tetraspanin (TSPAN or TM4SF) family, which includes UPK1a (UPIa) and UPK1b (UPIb) (Yu, Lin, Wu, & Sun, 1994); and the single-spanning transmembrane uroplakins, which include UPK2a, UPK2b, UPK3a (UPIIIa, UPK3A), UPK3b (UPIIIb, UPK3B), UPK3c and UPK3d. UPK2b is predominant in non-mammalian vertebrates, UPK3c exists in mammals, reptiles and birds, while UPK3d is dominant in bony fish (Garcia-Espana *et al.*, 2006; Lin, Wu, Kreibich, & Sun, 1994; Wu *et al.*, 1994). The UPKs are primarily involved in forming urothelial plaques by forming unique structures called asymmetric unit membranes (AUM). UPK1a and UPK1b (both 27 kDa), UPK2 (15 kDa), and UPK3 (47 kDa) are some of the critical integral membrane proteins found in the apical surface of mammalian urinary bladder epithelium (Ryan *et al.*, 1993). In forming urothelial plaques, UPK1a mainly interacts with UPK2, while UPK1b specifically interacts with UPK3a to produce the heterotetramer (Figure 3). Ninety percent of the urothelial apical surface is made up of hexagonally packed, two-dimensional crystals made of urothelial plaque. (Matuszewski *et al.*, 2016). (Figure 3). Excess uroplakins are drawn into the cell once the bladder is emptied, which reduces the amount of uroplakin-covered surface. In reaction to the increasing capacity of the bladder filling the bladder with urine, uroplakins return to the urothelial surface (Lewis, 2000).

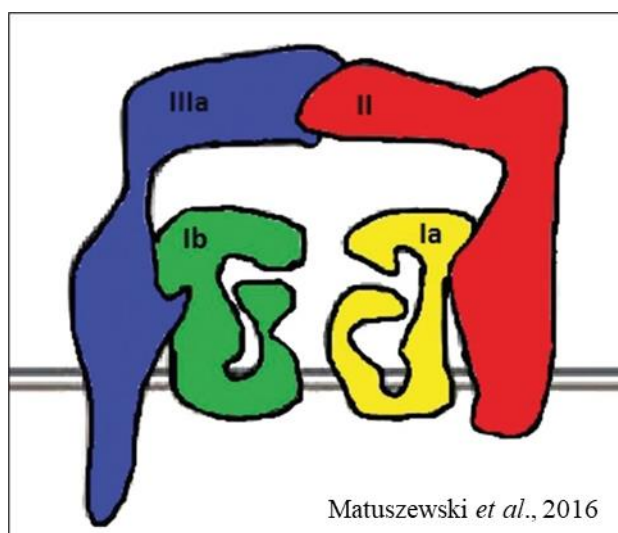


Figure 3: Schematic representation of uroplakin heterotetramer that contributes to the formation of urothelial plaques.

Urothelial plaques maintain a persistent permeability barrier to preserve the blood from toxic elements of the urinary tract (Negrete, Lavelle, Berg, Lewis, & Zeidel, 1996). In *Upk2* or *Upk3a* null-mutant mice, the heterodimeric partner of the specific protein expressed by each mutant gene is disrupted, which causes abnormal plaque formation (Hu *et al.*, 2000; Lewis, 2000). Symptoms include severe vesicoureteral reflux (VUR), reduced renal function, and hydronephrosis in mice lacking UPK2, UPK3a, or both (Hu *et al.*, 2000; Kong *et al.*, 2004). The initial and most crucial step in the infection of the urinary tract and bladder by uropathogenic *E. coli* (UPEC) bacteria. The bacteria adhere to the urothelial surface through particular connections between the UPEC FimH and the mannosylated glycoprotein on the urothelial surface (Ofek, Hasty, Abraham, & Sharon, 2000). The primary mannosylated glycoprotein (UP1a/1b) on the urothelial surface can interact with type 1-fimbriated *E. coli* (Wu, Sun, & Medina, 1996). The UPK1a acts as a receptor for the type-1 fimbriated *E. coli*, which causes urinary tract infection (Zhou *et al.*, 2001).

Recent investigations have also reported the role of uroplakins in reproductive physiology. The distribution of uroplakins was consistent in the *Xenopus laevis* bladder, fat cells, oocytes, zebrafish pronephric tubules, and various mammalian nonurothelial organs and malignancies (Garcia-Espana *et al.*, 2006; Mitra *et al.*, 2012). In *Xenopus laevis*, Upk3a and Upk1b function as receptors when sperm and eggs are in contact (Mahbub Hasan *et al.*, 2014). Upk3b is expressed in the epididymis and the ovarian follicle in a time-dependent manner (Kuriyama, Tamiya, & Tanaka, 2017). Double-knockout *Upk2/3a* mice had a reduced litter size. The Uroplakins are also found in subcellular localization in the oocyte of multivesicular bodies such as the endoplasmic reticulum (ER), Golgi bodies, and late endosomes (Liao *et al.*, 2018).

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Chapter - 1

In silico and in vivo Characterization of Rat Uroplakins

1.1 Introduction

Uroplakins (UPKs) are a group of proteins that form the major constituents of urothelial plaques. In the mammals, five UPKs, namely, UPK1a, UPK1b, UPK2, UPK3a and UPK3b (a minor isoform of UPK3a) are characterized. Besides these, UPK2b and UPK3c and UPK3d were identified in other species (Desalle *et al.*, 2014, Garcia-Espana *et al.*, 2006, Wu *et al.*, 1994). Mammalian UPKs are divided into two groups. UPK1a and UPK1b belong to the tetraspanin family, whereas UPK2 and UPK3 are grouped under the monospanin family (Yu *et al.*, 1994, Lin *et al.*, 1994, Wu and Sun 1993). The former family of proteins span the plasma membrane four times whereas the later span the plasma membrane only once. The plaques formed by UPKs generates an asymmetric unit membrane (AUM), which in turn functions to provide permeability barrier and structural stability to the urothelium. The formation of plaques involves specific interaction between the UPKs. UPK1a interacts with UPK2 whereas UPK1b interacts with UPK3a (Wu *et al.*, 1994, Hu *et al.*, 2005, Tu *et al.*, 2006, Tu *et al.*, 2002).

The functional significance of UPKs vary across the species. In mammals, though their major function is important for development, differentiation and homeostasis of the urothelium (Arpenter *et al.*, 2016), in the *Xenopus* oocytes UPK3a and UPK1b form a complex to mediate sperm-egg interaction and fertilization (Sakakibara *et al.*, 2005, Hasan *et al.*, 2011, Mahbub Hasan *et al.*, 2005). In the zebrafish, UPK3a related protein contributes to epithelial cell polarization and morphogenesis of pronephric tubules (Mahbub Hasan *et al.*, 2005). A recent study indicates that the UPK2/3 proteins are related to phosphotyrosine phosphatases and thus may have a role in cellular signalling (Chicote *et al.*, 2017). UPK knock out mice display renal failure and high rates of mortality (Kong *et al.*, 2004, Hu *et al.*, 2000). Mutations in the UPK genes resulted in renal hypo dysplasia, adysplasia and renal failure in humans (Jenkins *et al.*, 2005, Schonfelder *et al.*, 2006). Further, UPKs serve as anchors for Uropathogenic *E. coli* (UPEC) during infections (Wu *et al.*, 2009). They allow binding of the type-1 fimbriae expressing UPEC strains and facilitate their binding to the urothelial surface and triggering a cascade of events that favor bacterial infection and migration not only in the bladder, but also in the upper urinary tract organs such as urethra and renal pelvic urothelia (Wu *et al.*, 1996, Mulvey *et al.*, 1998, Martinez *et al.*, 2000). Altered levels of UPKs in urothelial carcinomas are considered as useful markers for the diagnosis,

detection and prediction of urothelial carcinomas (Wu *et al.*, 2009, Huang *et al.*, 2007, Zupancic *et al.*, 2011).

Because of the tetraspanin nature of UPKs, they are predicted to play a key role in a variety of functions such as cell migration, immune signaling, membrane architecture and infection (Hemler 2003, Levy and Shoham 2005). The presence of UPKs in respiratory, ocular and other epithelial cell types indicate a wider role for these proteins beyond the urothelial functions (Adachi *et al.*, 2000, Olsburgh *et al.*, 2003). The spatiotemporal expression of UPK3b in the epididymal and testicular sperm and ovarian follicles implicates them in gametogenesis and the development of gamete delivery organs (Kuriyama *et al.*, 2017). Recently it was demonstrated that mouse UPKs are expressed in nonurothelial lineages (Liao *et al.*, 2018). All the known uroplakins were found to be expressed on the oocytes and associated with multivesicular bodies. Further, they were localized on the hook of the sperm. *In vitro* assays demonstrated reduced fertilization rates in mice eggs under conditions of knock down or antibody protection. Further, UPKII/IIIa double knock out mice had smaller litter size suggesting are important in reproduction (Liao *et al.*, 2018). Though UPKs have been well characterized in the urinary tract, their role in other organ systems are not well studied. The organs of male reproductive system has many anatomical similarities with the urinary system and both these are subjected to infections by UPEC, the bacterial species that expresses the type 1 fimbriae. In light of these similarities and the scarce of studies on the role of UPKs in other organ systems, the aim of this study was to analyze the expression pattern of UPK gene and their protein products in the male reproductive system of rat and to study their possible functional role during endotoxin challenge was analyzed. We observed that UPKs are abundantly expressed in the male reproductive tract of rat and their expression altered during endotoxin challenge both *in vitro* and *in vivo*.

1.2. Materials and methods

1.2.1. *In silico* analyses

The rat genome available at UCSC Genome Browser rat assembly RGSC6/rn6 was used to download all the known *Upk* gene sequences. Various properties of the genes and their proteins were predicted using *in silico* tools (Table 1). Based on the genome assemblies deposited in Ensembl, gene neighborhood analyses was carried out. Self-Optimized Prediction Method with

Alignment (SOPMA) was used to predict the percentage of all the secondary structures (α -helix, β -turn, extended strand and random coil) (Geourjon and Deléage 1995, Frishman and Argos 1995).

Table 1. Computational tools used for the *in silico* analyses.

Analysis	Tool used	Website
Sequence retrieval	UCSC Genome Browser assembly	https://genome.ucsc.edu/
Similarity search	NCBI BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Multiple sequence alignment	T-COFFEE	http://tcoffee.crg.cat/
Pairwise alignment	CLUSTALW	http://www.genome.jp/tools/clustalw/
General properties	EXPASY server	http://expasy.org/proteomics
Post translational modifications	Sequence manipulation suite	http://www.bioinformatics.org/sms2/
Genomic neighbourhood	Ensembl	http://asia.ensembl.org/index.html
Secondary structure prediction	SOPMA	https://npsa-prabi.ibcp.fr/NPSA/npsa_sopma.html
Molecular modelling	GPCR-I-TASSER	https://zhanglab.ccmb.med.umich.edu/GPCR-I-TASSER/
Structure validation	PROCHECK	https://www.ebi.ac.uk/thornton-rv/software/PROCHECK/

1.2.2. Molecular modelling

Amino acid sequences of UPK1A, UPK1B, UPK2, UPK3A and UPK3B were obtained from NCBI database and submitted to the GPCR-ITASSER server (<https://zhanglab.ccmb.med.umich.edu/GPCR-ITASSER/>) in the FASTA format. The server usually employs a hybrid approach that integrates experimental mutagenesis data with *ab initio* transmembrane helix assembly simulations to construct the final 3Dmodel of target proteins (Zhang *et al.*, 2015). Models with high confidence score (C-score) were selected, which is typically

in the range of (-5 to 2), where a C-score of a higher value signifies a model with a higher confidence and vice-versa. Further, the structural validation was performed using PROCHECK (Laskowski *et al.*, 1993) and Ramachandran plot analysis (Ramachandran *et al.*, 1963). Models with 90% and above residues in the most favored or allowed or additionally allowed regions were considered for further analysis. PyMOL was used for visualization of the selected models, structural comparison and picture representation.

1.2.3. Animals and tissue collection

Wistar rats were purchased from National Center for Laboratory Animals, National Institute of Nutrition, Hyderabad, India. The rats were housed at animal facility maintained at ambient temperature (25 °C) with free access to food and water and were daily monitored for their well-being. For collection of tissues, the animals were terminated in a carbondioxide containing jar. All procedures involving animals were conducted using the guidelines for the care and use of laboratory animals to minimize suffering and this study was specifically approved by the Institutional Animal Ethics Committee of University of Hyderabad (IAEC/UH/151/2017/01/SY/P7).

To determine the overall tissue distribution patter of *Upks*, brain, heart, lungs, liver, kidney, spleen, ovary, uterus, cervix, bladder, caput (without the initial segment and corpus), cauda, testis, seminal vesicles and prostate were collected from adult Wistar rats (aged 90 days; n=5 each of male and female). Epididymides and testes obtained from 1- to 60-day old Wistar rats (n=5 for each age group) were used to study the developmental regulation. The tissues were placed in PBS, removed any excess fat and snap frozen in liquid nitrogen and stored at -80 °C until use. For collection of spermatozoa, cauda epididymides from adult Wistar rats were placed in PBS and squeezed gently with forceps. The spermatozoa were resuspended in PBS for further processing.

1.2.4. Polymerase chain reaction (PCR)

Total RNA isolated from different tissues using commercially available kits (Qiagen) was subjected to DNase digestion to remove any contaminated DNA. 2 µg of total RNA was reverse transcribed and the expression of *Upks* in these tissues was carried out using exon spanning gene specific primers (Table 2) in a thermal cycler (cycling conditions: 94 °C for 1 min followed by 30 cycles at 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec, and with a final round of extension at 72 °C for 10 min). No reverse transcriptase and no template controls were included to confirm that the amplification is specific. The PCR amplicons were electrophoresed on 2% agarose gels and the images captured using a gel documentation system. The identity of the PCR amplicons was confirmed by sequencing through a commercial source (Bioserve Technologies Hyderabad, India). For quantification of gene expression, real time PCR was carried out using SYBR master mix kit (Applied Biosystems, Warrington, UK) in a thermal cycler (Applied Biosystems) using standard conditions.

Table 2. Gene specific primers used in this study.

Gene name	Primer direction	Primer sequence (5'-->3')	Length (bp)	GC (%)	Tm (°C)	Amplicon size (bp)
<i>Upk1a</i>	Forward	CTCCTGCATCACATCCTACACC	22	55	56.7	187
	Reverse	GTAATTCACCCAGTCCATGG	20	50	51.8	
<i>Upk1b</i>	Forward	GCAGATGCTGATGAGGTATC	20	50	51.8	184
	Reverse	GTAGTCAGCATCGCTATTC	19	47	48.8	
<i>Upk2</i>	Forward	GAGCCAATGACAGCAAAGTG	20	50	59.4	153
	Reverse	ATTTGGTTCCTGGTGTGAGG	20	50	59.8	
<i>Upk3a</i>	Forward	GAAGCCTCTGTGCATGTTCG	20	55	53.8	203
	Reverse	CACAAGGGGTCAGGTCAAAGG	21	57	56.3	
<i>Upk3b</i>	Forward	CTGAGTCTAGACCTGATTCCTAC	24	50	57.4	198
	Reverse	CTTAGCAGCGGTCTGTGGGTTCTG	24	58	60.8	
<i>Gapdh</i>	Forward	CCAATGTATCCGTTGTGGATCT	22	45	60.1	157
	Reverse	GAGTTGCTGTTGAAGTCACAG	21	48	59.5	

1.2.5. Immunoblotting

Urinary bladder, caput, cauda, testes seminal vesicle and prostate tissues of 90day old Wistar rats were homogenated in RIPA buffer (25mM Tris-HCL, pH 7.6; 150mM NaCl; 1% each of NP-40, sodium deoxycholate and sodium dodecyl sulfate) and centrifuged at 12,857g for 10 min. The protein in the supernatant was quantified by Lowry's method. Total protein (100 µg) was electrophoresed on 15% SDS PAGE and transferred to nitrocellulose membrane (Hybond ECLTM; GE Healthcare, Little Chalfont UK) in a transfer buffer (25mM Tris; 192mM Glycine, 20% methanol (v/v); pH 8.3) with a constant voltage of 25 V for 16 h. 5% skim milk was used to block the membrane for 2 h at room temperature followed by probing (for 1 h) with primary antibody against the respective UPK protein. (UPK1A (Cat # ab183503) or UPK1B (Cat# ab185970) raised in rabbit (Abcam Biotechnology, Cambridge, UK) or UPK2 (Cat# sc-15178) raised in goat (Santa Cruz Biotechnology, Dallas, USA) or UPK3B (Cat# sc-165867) raised in goat (Santa Cruz). The blots were then washed thrice with TBS (Tris-buffered saline) and TBS-T (Tris-buffered saline, 0.1% Tween-20) followed by incubation with anti-rabbit secondary antibody (Cat# 65120) raised in goat or anti-goat secondary antibody (Cat# 611620) raised in rabbit (Invitrogen, Carlsbad, USA). After the incubation the membranes were washed thrice with TBS and TBS-T each for 10 min. At the end of washing, the membrane was developed using enhanced chemiluminescence kit (GE Healthcare, Buckinghamshire, UK).

1.2.6. Immunofluorescence

Epididymides (caput, cauda) and testes obtained from adult Wistar rats were fixed in Bouin's solution and 4% paraformaldehyde solution respectively and embedded in paraffin. Five micron sections (obtained by serial sectioning) placed on a glass slide were kept on a platform preheated to 60 °C for 5 min followed by washings with xylene, gradient alcohol (70–100%) and PBS for 10 min each. Slides were placed in a container filled with 10mM citrate buffer, pH 6.5 maintained at 60C for 12 min (for antigen retrieval) and subjected to permeabilization with PBS containing 1% triton X-100 (PBST) for 15 min. 10% goat serum was then added and incubated for 45 min to allow blocking. Sections were incubated for one hour with the respective primary antibody as indicated in the immunoblotting section above. The primary antibody was washed off thoroughly and the sections incubated with anti-rabbit (Cat# 656111; Invitrogen) or anti-goat (Cat# sc-2777; Santa Cruz) -FITC-labelled secondary antibody. Nucleus was stained with 4', 6-

diamidino-2-phenylindole (DAPI; Sigma Aldrich, St. Louis, USA) followed by addition of mounting medium and covered with cover slip. Multiple images (around 10) for each sample were recorded by taken in a trinocular fluorescence microscope with excitation and emission at 495 nm and 519 nm respectively. In the case of spermatozoa, smears on glass slides were prepared, air dried and fixed with 4% paraformaldehyde. They were then permeabilized with PBST, blocked with 10% goat serum and processed in a similar way as that of tissue sections.

To determine the specificity of antibodies, appropriate controls were included. Since UPKs are difficult to express using recombinant technology, pcDNA vector that encodes either one of the UPK proteins was transfected into HEK cells and incubated with primary and FITC labelled secondary antibody (positive control). Untransfected HEK cells incubated with primary and FITC-labelled secondary antibody served as negative control. Further, another negative control to test the nonspecific binding of secondary antibody was also included by incubating the tissue sections with only FITC-labelled antibody.

1.2.7. Treatment with LPS *in vitro* and *in vivo*

Rat testicular carcinoma cells (LC540) were obtained from National Centre for Cell Science, Pune, India and were maintained in EMEM medium (Sigma Aldrich, St. Louis, USA) containing 5% FCS at 37 °C. The rat caput immortalized cell lines (RCE) were a kind gift from Dr. Daniel Cyr, University of Quebec, Canada. They were maintained in DMEM medium containing 5% FBS at 32 °C. 1×10^6 cells were plated in each well of a 24 well plate (Sigma Aldrich, St. Louis, USA) and allowed to adhere. Following replacement of culture medium, they were treated with 100 ng/ml *E. coli* lipopolysaccharide (LPS; Sigma Aldrich, St. Louis, USA) and incubated up to 24 h. The treatment dose and time points were chosen basing on our previous studies (Biswas and Yenugu 2013). Cells were collected at different time points (0, 3, 6 12 and 24 h after treatment) for RNA isolation. Since LPS was dissolved in phosphate buffered saline (PBS), pH 7.4, control cells were treated with PBS. The experiments were conducted three times in triplicates. To determine the effect of LPS on *Upk* gene expression, adult male Wistar rats (n=5 for each group) were treated intra peritoneally with 1 mg/ kg body weight of *E. coli* LPS and sacrificed at different time points (0, 6, 12 and 24 h after LPS injection). The dosage of LPS was based on our previous studies (Biswas and Yenugu 2011). Control animals (n=5) received PBS equivalent to the volume that was used for LPS treatment. Caput, cauda, testis, seminal vesicle and prostate

were collected at each of these time points. They were then snap frozen in liquid nitrogen and stored at -80°C until further use.

1.2.8. Statistical analyses

Statistical analyses were performed using one-way ANOVA (multiple comparison; Holm-Sidak test) and Student's t-test available in Sigma Plot software 12.5, Build 12.5.0.38 (SPSS Inc., Chicago, IL, USA). Values shown are Mean $\pm$ S.D. * $p < 0.05$ denotes statistical significance.

1.3. Results

1.3.1. *In silico* analyses

To gain insight into the role of UPKs in the male reproductive system, we initiated *in silico* studies to gather first-hand information on the characteristic features of their genes and proteins. A thorough search in the rat genome indicated that the gene sequence of *Upk1a*, *Upk1b*, *Upk2* and *Upk3a* (NM_001108911.1, NM_001024253.1, NM_001109523.1 and NM_001130507.1 respectively) are already available in the GenBank. Besides these, alternate transcripts for *Upk1a* (XM_017589474.1; XM_008759176.2; XM_017589475.1) and *Upk1b* (XM_006248367.2; XM_006248368.3) were found to be predicted. Besides the reported *Upk* gene sequences, identification and characterization of the alternate transcripts is an important aspect in studying the functional characterization. Hence, using cDNA from rat urinary bladder, the amplicons corresponding to *Upk1a* alternate transcript 2 (XM_008759176.2), *Upk1b* alternate transcript 1 (XM_006248367.2) were amplified and sequenced. Rat *Upk3b* is not yet reported. Two alternate transcripts (XM_341056.8 and XM_006249208.3) are predicted. To characterize rat *Upk3b* further, we amplified XM_341056.8. The sequence obtained was submitted to GenBank and were assigned the accession numbers KU310911 (*Upk1a* alternate transcript 2), KU310910 (*Upk1b* alternate transcript 1) and KU310912.1 (*Upk3b* alternate transcript 1). For practical purposes, in this manuscript, we labelled the *Upk1a* alternate transcript 2, *Upk1b* alternate transcript 1 and *Upk3b* alternate transcript 1 as *Upk1a*, *Upk1b* and *Upk3b* respectively. We observed that *Upk3b* gene was located at chromosome 12q.12 (Figure. 1), whereas *Upk1a*, *Upk1b*, *Upk2* and *Upk3a* genes were localized to the chromosomal positions 1q21, 11q21, 8q22 and 7q34 respectively (as per the information available at NCBI). The coding region of the mRNA and the corresponding amino acid sequence of *Upk1a*, *Upk1b* and *Upk3b* characterized in this study are shown in Figures.

2 and 3. The general characteristic features of all the rat *Upk* genes and their protein products were compiled based on the information available in the rat genome at NCBI and are given in Table 3.

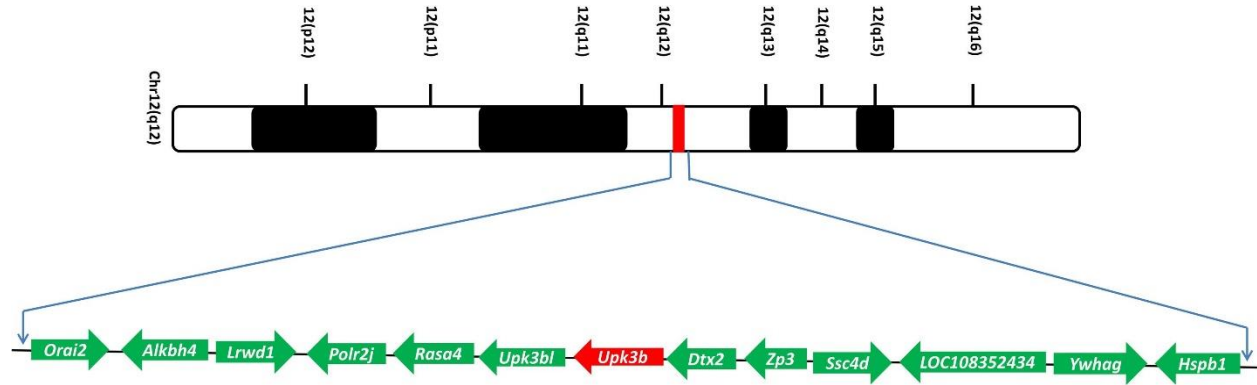


Figure 1. Genomic localization of rat *Upk3b* gene. Arrows indicate direction of transcription. Positions were taken from UCSC Genome Browser rat assembly RGSC6/rn6. Distance between genes is not to scale. Rat *Upk3b* sequences was submitted to GenBank and assigned the accession number KU310912.1. *Upk3b* gene is indicated in red color for better representation.

<i>Upk1a</i> (alternate transcript 2)	<i>Upk1b</i> (alternate transcript 1)
atggcttctacagcgacagagggagagaaggggtctcctgtgggtgtggggctgctggctc M A S T A T E G E K G S P V V V G L L V gtgggcaacatcattatctgtctcagggcctggcctgttgcggagacagtgtgggtg V G N I I I L L S G L A L F A E T V W V acggctgaccagtagctgtgtaccctgatggcgctcctcaggcaaggacgactcttc T A D Q Y R V Y P L M G V S G K D D V F gccggtgctggatgccatctctcggcctctcctctcgtgggtggcagctttggt A G A W I A I F C G F S F F V V A S F G gtaggagctgcgctctgtcgtcgtcggtacatgatcctcagctatctgctgctgact V G A A L C R R R Y M I L T Y L L L M L atagctacatcttcagtggtgctcctcgtcgtacacatcctacacccagcgactacatg I V Y I F E C A S C I T S Y T H R D Y M gtgtccaacccatccctgataaccaagcaaatgttgacctctacagtcgggacaccgac V S N P S L I T K Q M L T F Y S A D T D caggggcaagagctaacccgctcctgggacccgatcatgattgagcaagagtgctgcggc Q G Q E L T R L W D R I M I E Q E C C G acatctggcccatggactgggtgaattacacatcagccttcaggggcagccacccggaa T S G P M D W V N Y T S A F R A A T P E gtgggtgtccctgggccccactgtgctgctgcgggactgggaactttattccatcaat V V F P W P P L C C R R T G N F I P I N gaagatggctgccgagtaggacacatcctgtttaccaagggtgtgcttcgaacac E D G C R V G H M D Y L F T K G C F E H atcagcatgcccattgacagctacacgtggggcatatcgtggtttgcttggcattcttg I S H A I D S Y T W G I S W F G F A I L atgtggactctccctgtgatgatgatgccatgtattttacaccattctctga M W T L P V M M I A M Y F Y T I L -	atgctgtggagtgagctctgtgcagcatcagggaagagacggcagagaagaccaggcagcca M L W S E S V Q H Q E E T A E E T R Q P gtccagtgaggaaatcctgaaatggcgaagacgattccaccgttcgttgccttcagggc V Q W E I L K M A K D D S T V R C F Q G ctgtgatttttggacacgtgattgtgtgtgtgtggatcgccctgacagcagagtgt L L I F G H V I V G M C G I A L T A E C atcttctgtatctgacaaacacagcctttatcactcttgaagccaccaacaacgat I F F V S D Q H S L Y P L L E A T N N D gatattcttggggcagcttgtagagcatgttttgggacatcgtcctcttctgcttct D I F G A A W I G M F V G I C L F C L S gttctagccatagtaggaattatgaagtccaacaggaaatcctcttggcgtacttctac V L A I V G I M K S N R K I L L A Y F I atgatgtttatagtgatgtgtgtttgaagtggcgtcttgcacacagcagccacacaaga M M F I V Y G F E V A S C I T A A T Q R gactttttcagaccaaccccttctcgaagcagatgctgatgaggtatcaaaacaacagc D F F T T N L F L K Q M L M R Y Q N N S cccccaacaaatgacgacaaatggaagaacagctatgtcaccaagacctgggtaggctc P P T N D D K W K N S Y V T K T W D R L atgctgcaggaccactgctgtgggttaaacggctcgtcagactggcagaaataacacct M L Q D H C C G V N G P S D W Q K Y T S gccttcgagtgaggagaatagcgtgctgactaccctggcctcggcagtgctgtgctatg A F R V E N S D A D Y P W P R Q C C V M gacaagcttcaagagccccctcaactggagcgttgaactcggagtgctgttattac D K L Q E P L N L D A C K L G V P G Y Y cacagtcagggtgttatgagctaatctctggaccaatggatcgccacgctggggagtt H S Q G C Y E L I S G P M D R H A W G V gcctgtttggtattgtattctctgctggactcttgggttctctgggacacatgttc A W F G F A I L C W T F W V L L G T M F tactggagcagaattgagtactaa Y W S R I E Y -

Figure 2. Rat *Upk1a* and *Upk1b* mRNA coding region and the predicted protein sequence. Amino acids are indicated in single letters. The rat *Upk1a* and *Upk1b* cDNA sequence was submitted to Genbank and was assigned the accession number KU310911 and KU310910 respectively.

***Upk3b* (alternate transcript 1)**

```
atggagttcaccgcatgcccctcaccccttgcccctgttctcccgggtacttatgtgg
M E F T R M R P H P W P L L L P V L M W
cttcccaaagcctgagcttagacctgattccctacacgccgcagataactgccaggac
L P Q S L S L D L I P Y T P Q I T A R D
ctgggaggggaaggtcacagccactacgttctctctggagcagcctcggtgcgtctttgat
L G G K V T A T T F S L E Q P R C V F D
gagcacgcctcaagtaatgacaccatctggctagtgggtggctttcagcaatgcctccagg
E H A S S N D T I W L V V A F S N A S R
gactttcagaaccacagaccgctgctaagatcccgaccttcccacagctgctgactgac
D F Q N P Q T A A K I P T F P Q L L T D
ggccactatgatgacattaccctgtccctggatcagctgccatgtgaggacctgaccggat
G H Y M T L P L S L D Q L P C E D L T G
ggcagtgagggtgttcaggtgcttcgggtgggcaatgacttcggctgttaccagcgaccc
G S G G V Q V L R V G N D F G C Y Q R P
tattgcaacgctccccctcccagccagggcccttacagtgtaagttccttgtaatggat
Y C N A P L P S Q G P Y S V K F L V M D
gctggtggcccccaggctgaaacgaagtgggtctaattccatttttctccaccaaggg
A G G P P K A E T K W S N P I F L H Q G
aagaacccaacgccattgacacatggcctggctcggcggagcggctgtatgatcgtcata
K N P N A I D T W P G R R S G C M I V I
acttctatccttccggctcctggccggcctcttgctcctggctttcctggcagcctccact
T S I L P V L A G L L L L A F L A A S T
acgcgtttctccagcctgtggtggcctgaggaagcccctgagcagctgcggattggctcc
T R F S S L W W P E E A P E Q L R I G S
ttcatgggaaaacgttacatgactcaccatatccccccagcgaggctgccacactgccg
F M G K R Y M T H H I P P S E A A T L P
gtgggctgcgagcctggcctggacccccttcccagttctcagcccatag
V G C E P G L D P L P S L S P -
```

Figure 3. Rat *Upk3b* mRNA coding region and the predicted protein sequence. Amino acids are indicated in single letters. The rat *Upk3b* cDNA sequence was submitted to Genbank and was assigned the accession number KU310912.1.

Table 3. General predicted and deduced features of rat uroplakin genes and their encoded proteins

Attribute		<i>Upk1a</i>	<i>Upk1b</i>	<i>Upk2</i>	<i>Upk3a</i>	<i>Upk3b</i>
Chromosome		1q21	11q21	8q22	7q34	12q12
Gene accession number		NC_005100.4	NC_005110.4	NC_005107.4	NC_005106.4	NC_005111.4
Gene length (bp)		282763074	90463843	133307652	145729302	52716770
Total number of exons		6	8	5	6	7
mRNA accession number		KU310911	KU310910	NM_001109523.1	NM_001130507.1	KU310912.1
Length of mRNA (nt)		1498	1980	822	1026	1158
Coding sequence (CDS)		215..988	181..1044	61..615	29..892	182..1009
Protein accession number		AMB20857.1	AMB20856.1	NP_001102993.1	NP_001123979.1	AMB43178
Number of amino acids		257	287	184	287	275
Molecular Weight (kDa)		28.90	33.10	19.5	30.19	30.22
Localization		transmembrane	transmembrane	transmembrane	transmembrane	transmembrane
Isoelectric point (pI)		5.14	5.22	10.28	5.54	6.05
O-glycosylation sites		171,172,178,193	160, 200, 207	NIL	32,80,81,86,87,88,92,97,188,192,268	254,258
GRAVY index		0.500	0.0700	0.519	0.071	-0.064
Phosphorylation sites	Serine	12,54,115,162, 227	33, 65, 69, 110, 160, 171, 200, 207, 242, 283	3,31,33,37,43,68,88,102,118,126,129,130,164	27,56,69,72,76,80,86,115,183,192,205,217,239,240,248,265,268,282,	24,51,64,65,109,122,148,153,194,214,269
	Threonine	4, 6, 133, 139, 178, 214	13, 34, 138, 176, 278	4,9,97,110,113,133,137,160	38,39,87,88,92,108,123,160,180,188,244,245,251	37,87,99,169,243
	Tyrosine	119, 228	172, 198, 211, 239	115,116	100,132,166,266,	103,141,241,
Signal peptide (amino acid range)		No	No	Yes (1-25)	Yes (1-18)	Yes (1-26)
Disulfide bonds		69-86, 107-158, 110-189, 159-217, 192-204	37-133, 52-217, 60-218, 95-232, 98-186, 187-245	51-80	12-142, 15-47, 110-152	57 - 136, 115 - 142, 196 - 263

Depending on their organization in the plasma membrane, UPK1a and UPK1b belong to tetraspanin family, whereas the other UPKs are included in the monospanin family. Analyzing sequence similarity among a group of proteins provides information on the possible functional roles. Such an analyses is lacking for the rat UPK proteins. To determine the similarity among the rat UPKs, their sequences were aligned using T-coffee program (Figures. 4 and 5). Among UPK1A and UPK1B, the sequence similarity was about 53% (Table 4). The similarity score of UPK2 with UPK3A and UPK3B was 23 and 24 respectively. UPK3A and UPK3B had a similarity score of 39 (Table 4). The rat, mouse and human UPK sequences were aligned to determine the homology of

each of the UPK among these species (Figures 6). Rat UPK1A displayed 98 and 70% similarity with the mouse and human counterparts respectively (Table 4). Similarly, UPK1B, UPK2 and UPK3A and UPK3B displayed a very high similarity with the mouse and human counterparts. (Table 4).

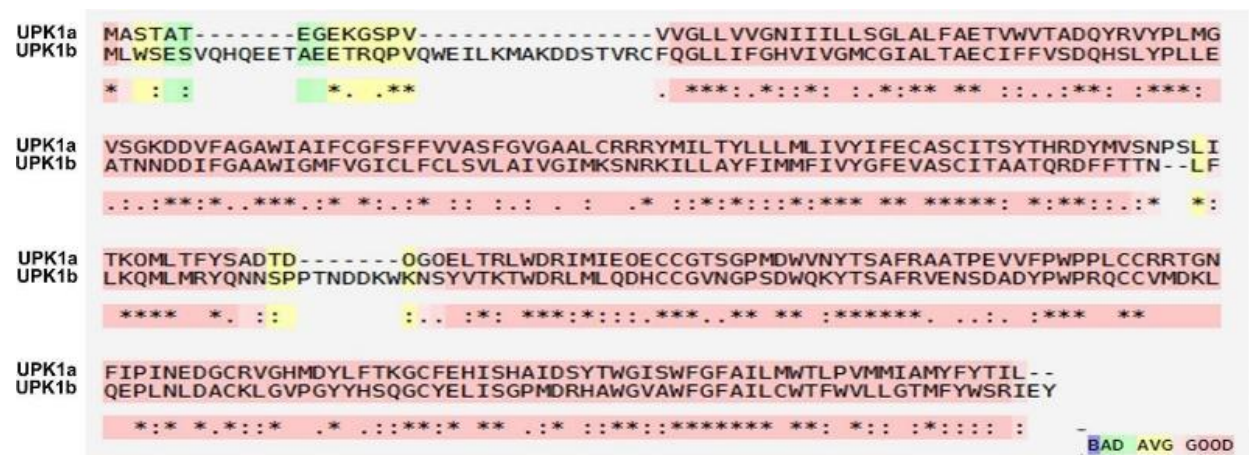


Figure 4. Multiple sequence alignment of UPK1a and UPK1b proteins. Alignment of rat UPK1a and UPK1b protein sequences. Identical and similar amino acids are indicated by (*) and (: and.) respectively. Color coding indicates agreement between all the various pairwise structural alignments. Pink brick regions are in perfect agreement across all the methods, while blue regions have a very poor agreement.

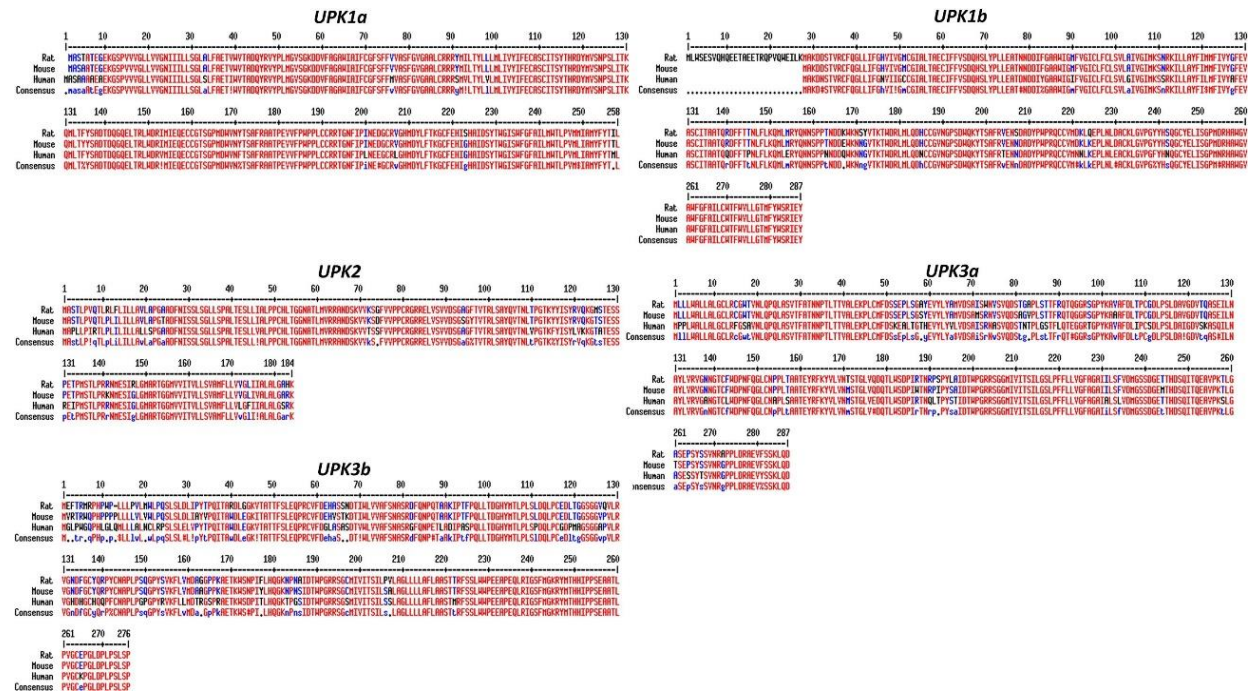


Figure 6. Alignment of rat, mouse and human UPK proteins. Amino acids indicated in blue indicate a match among two of the three species, whereas those indicated in black indicate a mismatch in one of the species or in all three species. A dot in the consensus sequence indicates a mismatch (amino acids in black color font) in all the three species.

Gene expression is influenced by the nature of genes present in its vicinity and this can be determined by neighbourhood analyses. *Upk* genes are located on different chromosomes and such an analyses to determine the nature of genes that flank them in the rat and the comparison with the mouse and human genomes is not reported. We show that *Psenen*, *U2af114*, *Igflr1*, *Zptb32*, *Cox6b1* and *Rbm42* genes were found to be commonly present in the rat, mouse and human chromosomal regions where *Upk1a* was located (Figure 7). In the case of *Upk1b*, the genes *Igs11*, *B4galt4*, *Tmem39a* and *Poglut1* were present in the neighbourhood region in all the three species (Figure 7). Genes that were commonly present in the neighbourhood of *Upk2* were *Slc37a4*, *Trappc4*, *Bcl9l* and *Cxcr5* (Figure 8). *Smc1b*, *Ribc2*, *Fam118a* and *Fbln1* were the genes commonly present in the *Upk3a* neighbourhood in all the three species (Figure 8). Genes that were commonly present in the neighbourhood of *Upk3b* were *Dtx2*, *Zp3*, *Ssc4d*, *Ywhag* and *Hspb1* (Figure 8).

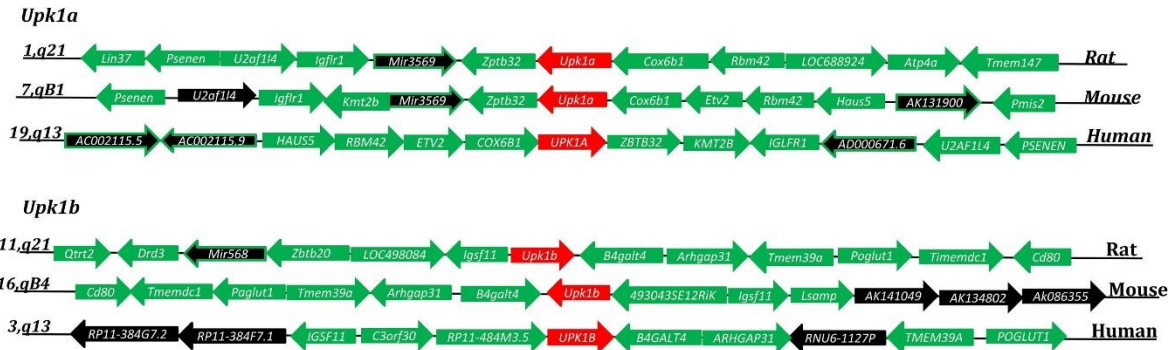


Figure 7. Genomic neighborhood analysis of rat, mouse and human Upk1a and UPk1b genes. Green arrows with names represent the genes and directions of the arrow represent the direction of sense strand. Red arrow indicates Upk gene. Black arrows indicate noncoding/pseudo/hypothetical genes. Alpha numerals on the left of each row indicates chromosomal region. The order of chromosomal regions is rat, mouse and human.

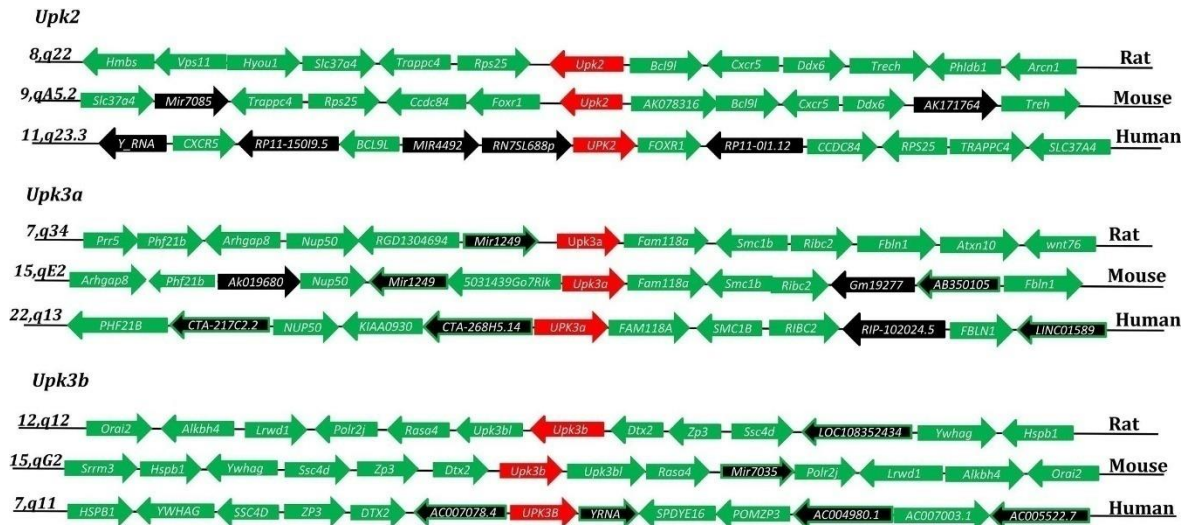


Figure 8. Genomic neighborhood analysis of rat, mouse and human Upk2, Upk3a and Upk3b genes. Green arrows with names represent the genes and directions of the arrow represent the direction of sense strand. Red arrow indicates Upk gene. Black arrows indicate noncoding/pseudo/hypothetical genes. Alpha numerals on the left of each row indicates chromosomal region. The order of chromosomal regions is rat, mouse and human.

Though the three dimensional structures for UPKs in some species are reported, such an attempt was not made for rat UPKs. Because of the differences in the amino acid sequence homology of these proteins among the species, the three dimensional structure of rat UPKs may

vary. Hence, to gain further insight into the structural aspects of UPKs, their three dimensional structure was obtained (Figure 9). The templates used for generating the three dimensional structures of UPKs varied significantly (Table 5). Ramachandran plots generated for each of the UPKs indicated that majority of the amino acids are in the allowed regions and the structures predicted are valid (Table 5). UPK1a and UPK1b that belong to tetraspanin family display more of alpha-helix, whereas UPK2, UPK3a and UPK3b that belong to monospanin family tend to have more random coil (Figure 9). To correlate the observations made in the three dimensional structure, the percentage of different structural features were determined by Self-Optimized Prediction Method with Alignment (SOPMA). UPK1a was found to have 40.07, 23.34, 8.17 and 28.40% of alpha-helix, extended strand, beta-turn and random coil respectively (Table 6). UPK2 also showed such a similar distribution wherein the percentage of alpha-helix, extended strand, beta-turn and random coil were 42.85, 18.11, 7.31 and 31.70 respectively (Table 6). It appears that UPK1a and UPK1b have higher content of alpha-helix followed by random coil, extended strand and beta-turn. In UPK2, the percentage of alpha-helix, extended strand, beta-helix and random coil content was 32.06, 22.28, 10.32 and 35.32 (Table 6). UPK3a is predicted to have 19.51, 26.82, 10.45 and 43.20 percent of alpha-helix, extended strand, beta-helix and random coil (Table 6). UPK3b contains 12.72, 22.18, 5.45 and 59.63 percentage of alpha-helix, extended strand, beta-helix and random coil respectively (Table 6). From these analyses it appears that the percentage of random coil was higher followed by extended strand, alpha helix and beta-turn in the case of members belonging to monospanin family (UPK2, UPK3a and UPK3b).

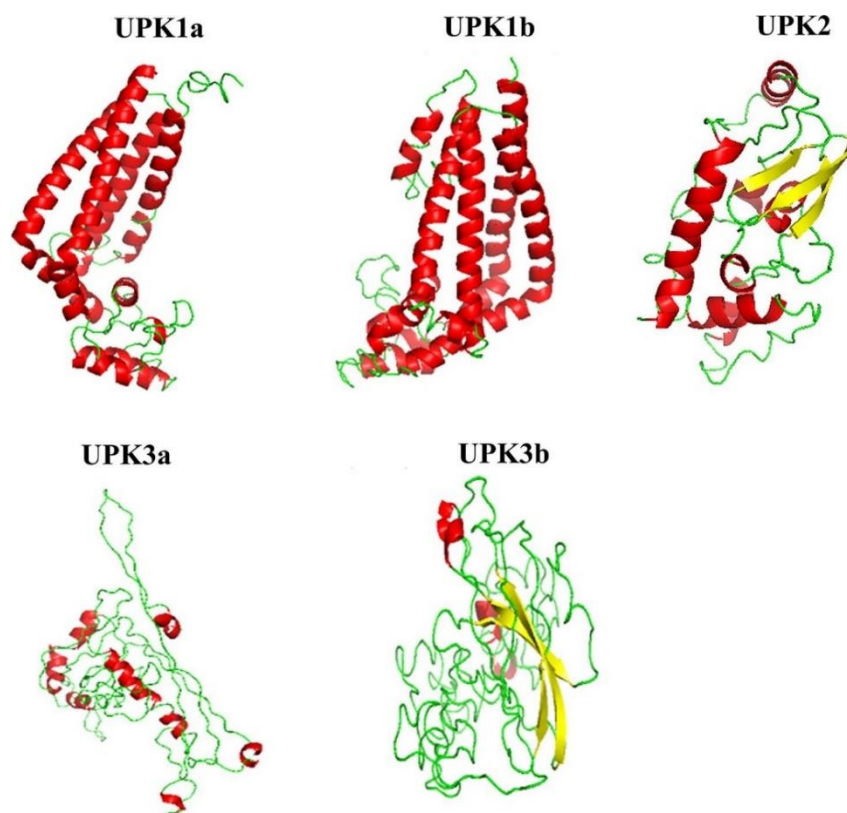


Figure 9. Three dimensional structure of rat UPK proteins. Amino acid sequences of UPK1a, UPK1b, UPK2, UPK3a and UPK3b were submitted to the GPCR-I-TASSER server and models with high confidence score (C-score; (−5 to 2) were selected. Structural validation was performed using PROCHECK and Ramachandran plot analysis and models with 90% and above residues in the most favored or allowed or additionally allowed regions were considered for further analysis. PyMOL based visualization showing β - sheets in yellow, helices in red and loop regions in green.

Table 5. *Ramachandran plot analysis for the 3D structure of rat uroplakins.*

Name of the model	UPK1a	UPK1b	UPK2	UPK3a	UPK3b
Crystal ID of template used for generating 3D model	CD81, 3RKO	CD81, 3QNQ	5L75, 5FMV, 5L75, 5C17, 4I18, 5N8P, 2PFF, 4KG7, 2COV	4BML, 5E7L, 4NL6, 2EDY, 5GAO, 4MGU, 4LDU, 2EYZ, 1TIA, 3L5H	5E7L, 5C2V, 5E52, 4LDU, 4LDV, 2EDY, 2EYZ, 4NL6, 1TIA
Residues in most favored regions, %	174, 77.3%	183, 71.2%	98, 62.8%	129, 53.1%	92, 41.4%
Residues in additional allowed regions, %	39, 17.3%	53, 20.6%	51, 32.7%	95, 39.1%	83, 37.4%
Residues in generously allowed regions, %	7, 3.1%	15, 5.8%	3, 1.9%	10, 4.1%	28, 12.6%
Residues in disallowed regions, %	5, 2.2%	6, 2.3%	4, 2.6%	9, 3.7%	19, 8.6%
Number of non-glycine and non-proline residues	225	257	156	243	222
Number of end-residues (excl. Gly and Pro)	2	2	2	2	1
Number of glycine residues	20	18	15	22	20
Number of proline residues	10	10	11	20	32
Total number of residues	257	287	184	287	275

Table 6. *Secondary structure prediction by Self-Optimized Prediction Method with Alignment (SOPMA)*

Protein name	Alpha helix (%)	Extended strand (%)	Beta turn (%)	Random coil (%)
UPK1a	40.07	23.34	8.17	28.40
UPK1b	42.85	18.11	7.31	31.70
UPK2	32.06	22.28	10.32	35.32
UPK3a	19.51	26.82	10.45	43.20
UPK3b	12.72	22.18	5.45	59.63

1.3.2. Uroplakin expression in the rat

Analyzing *Upk* gene and protein expression pattern in the male reproductive tract and their possible contribution to this organ system has gained importance in the recent past. In this study, to gain an in depth understanding of *Upk* expression in the male reproductive system of rats, their mRNA expression pattern was analyzed using PCR. *Upk1a* and *Upk1b* were expressed in the caput, cauda, testis, seminal vesicle and prostate (Figure 10A). *Upk2* was predominantly expressed in the testis with lower level of expression in the caput, cauda, seminal vesicle and prostate. *Upk3a* mRNA was not detected in all the male reproductive tract tissues. *Upk3b* expression was evident in the caput, cauda, testis and seminal vesicle with no expression in the prostate (Figure 10A). All *Upks* were expressed abundantly in the bladder, which was used as a positive control. Western blotting indicated the expression of UPK1a, UPK1b and UPK2 and UPK3b proteins in the caput, cauda, testis, seminal vesicle and prostate (Figure 10B).

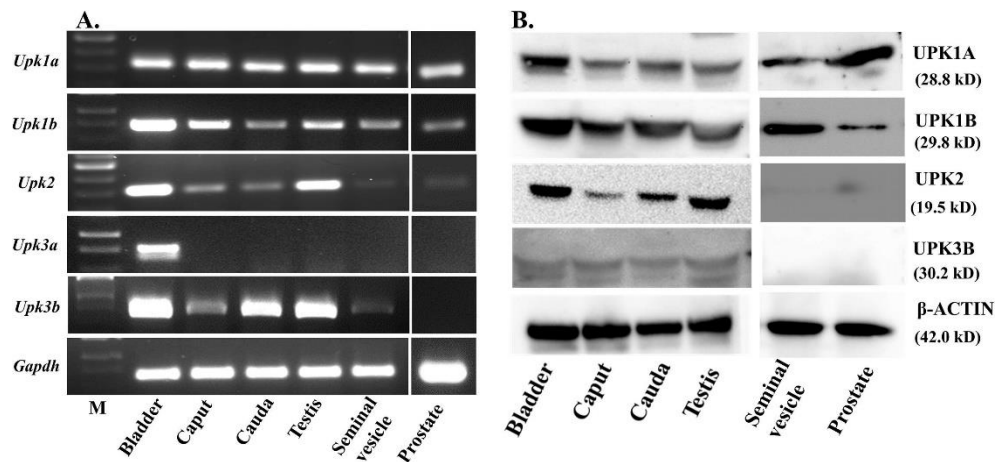


Figure 10. Expression of *Upk* mRNA and protein in the rat male reproductive tissues. (A) *Upk* mRNA expression. Total RNA isolated from bladder, caput, cauda, testis, seminal vesicle and prostate was reverse transcribed and the cDNA obtained was used as template for amplifying the *Upk1a*, *Upk1b*, *Upk2*, *Upk3a* and *Upk3b* by gene specific PCR. *Gapdh* expression was analyzed to serve as internal control. (B) UPK protein expression. 100 µg of the protein obtained from bladder, caput, cauda and testis homogenates were electrophoresed on 15% SDS-PAGE and transferred to nitrocellulose membrane. The immunoblots were probed with UPK1a or UPK1b antibody (raised in rabbit) or UPK2 antibody (raised in goat) or UPK3b antibody (raised in goat); followed by incubation with labelled anti-rabbit secondary antibody raised in goat or anti-goat secondary antibody raised in rabbit.

To further confirm whether *Upks* are male reproductive specific, their expression was analyzed in other tissues of male rats and in the reproductive tissues of female rats. *Upk1a*, *Upk1b*, *Upk2* and *Upk3b* were found to be expressed in all the tissues analyzed, whereas *Upk3a* was restricted to lung, kidney and cervix (Figure 11).

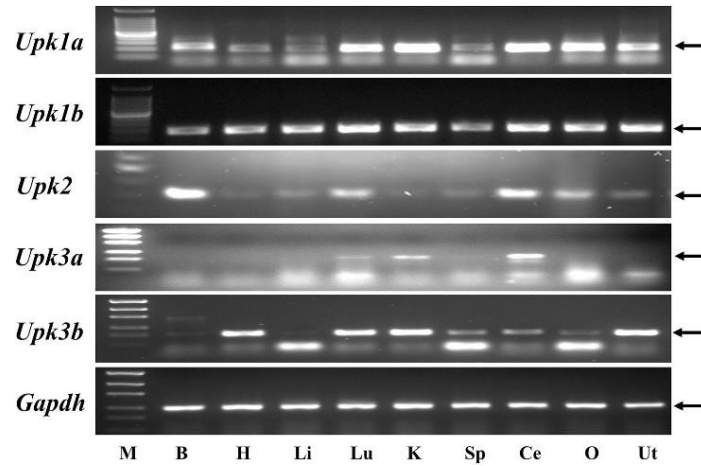


Figure 11. Tissue distribution of *Upks*. Total RNA isolated from Brain (B), Heart (H), Lungs (Lu), Liver (Li), Kidney (K), Spleen (Sp), Cervix (Ce), Ovary (O) and Uterus (Ut) was reverse transcribed and the cDNA obtained was used as template for amplifying the *Upk1a*, *Upk1b*, *Upk2*, *Upk3a* and *Upk3b* by gene specific PCR. *Gapdh* expression was analyzed to serve as internal control. M indicates DNA ladder. Arrow indicates the amplicon of interest.

Since gene regulation in the testis and epididymis is highly regulated by androgens during development, *Upk* mRNA expression pattern in the epididymis and testis during different stages of development was analyzed (Figure 12). In the epididymis, *Upk1a* and *Upk2* expression was evident from 20 days of postnatal development, whereas *Upk1b* and *Upk3b* were detected in all the stages. *Upk3a* was not detected at all the stages analyzed (Figure 12). In the testis, except for *Upk3a*, other *Upks* were found to be expressed at all the developmental stages analyzed (Figure 12).

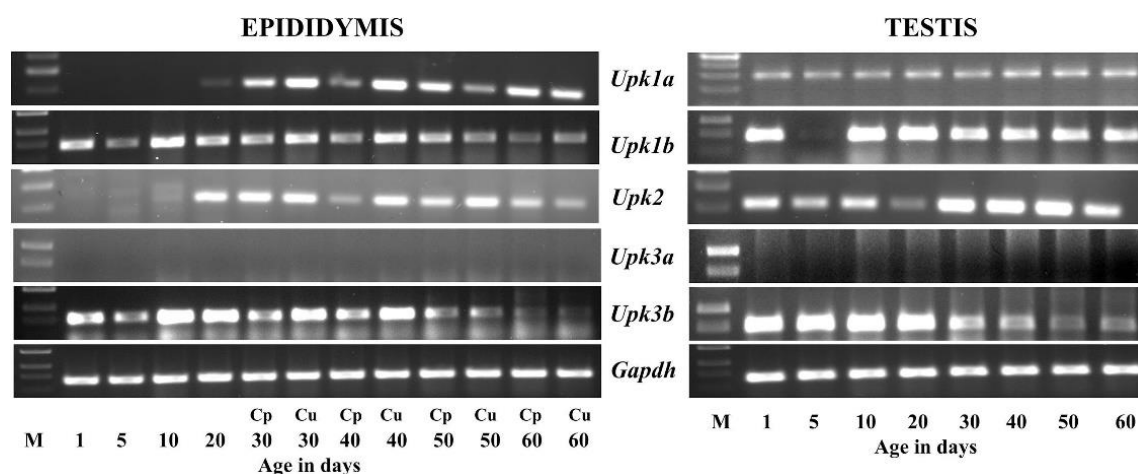


Figure 12. Developmental regulation of *Upk* genes in epididymis and testes. RNA from epididymis and testes collected from 1, 5, 10, 20, 30, 40, 50 and 60 day old rats was reverse transcribed and the cDNA obtained was used as template for amplifying the *Upk1a*, *Upk1b*, *Upk2*, *Upk3a* and *Upk3b* by gene specific PCR. *Gapdh* expression was analyzed to serve as internal control. Cp- caput, Cu-cauda.

To determine if UPKs may have a role in male reproductive physiology, sperm function and fertilization, we examined their localization in the caput, cauda, testis and male gametes by immunofluorescence microscopy using a trinocular fluorescent microscope. Prior to the localization analyses in the tissues, the specificity of the antibodies were tested by including positive and negative controls. We put our best efforts for recombinant expression of UPK proteins to be used in peptide controls. However, we met with little success. Alternatively, HEK cells were transfected with pcDNA vector encoding either one of the UPK proteins and incubated with the respective primary and secondary antibodies (positive control). Further, untransfected HEK cells were incubated with primary and secondary antibody served as negative controls. HEK cells transfected with the vector encoding the UPK protein showed abundant fluorescence (Figure 13), whereas such a fluorescence was not observed in untransfected cells (Figure 14). Further, we also checked for the non-specific binding of the secondary antibody by incubating the tissue sections only with FITC-labelled secondary antibody. The negative controls (incubated only with the secondary antibody) did not shown any fluorescence (Figure 15). These observations suggest that the antibodies used were specific to the respective UPK protein.

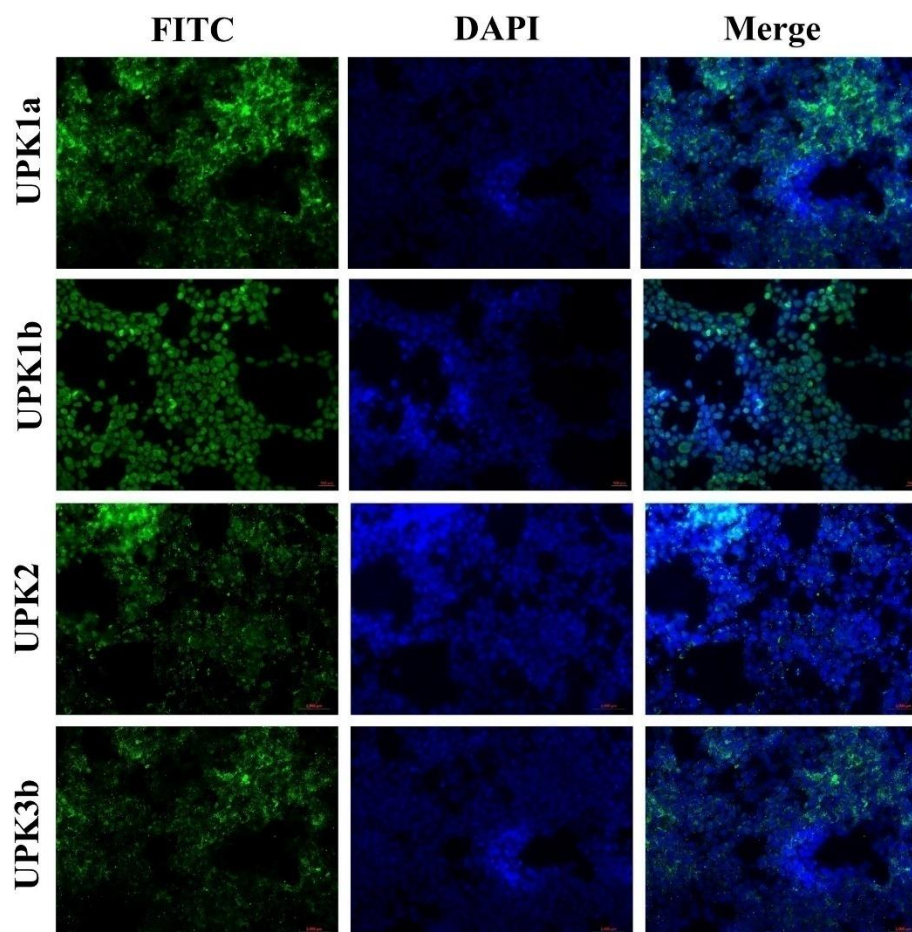


Figure 13. UPK antibody specificity in positive controls. HEK cells were transfected with pCDNA vector encoding UPK1a or UPK1b or UPK2 or UPK3b. 24 hours after transfection, the cells were fixed on glass cover slips. They were incubated with UPK1a or UPK1b antibody (raised in rabbit) or UPK2 antibody (raised in goat) or UPK3b antibody (raised in goat); followed by incubation with FITC labelled anti-rabbit secondary antibody raised in goat or anti-goat secondary antibody raised in rabbit. Nucleus was stained with 4', 6-diamidino-2-phenylindole and images taken using fluorescence microscope with excitation and emission at 495 nm and 519 nm respectively.

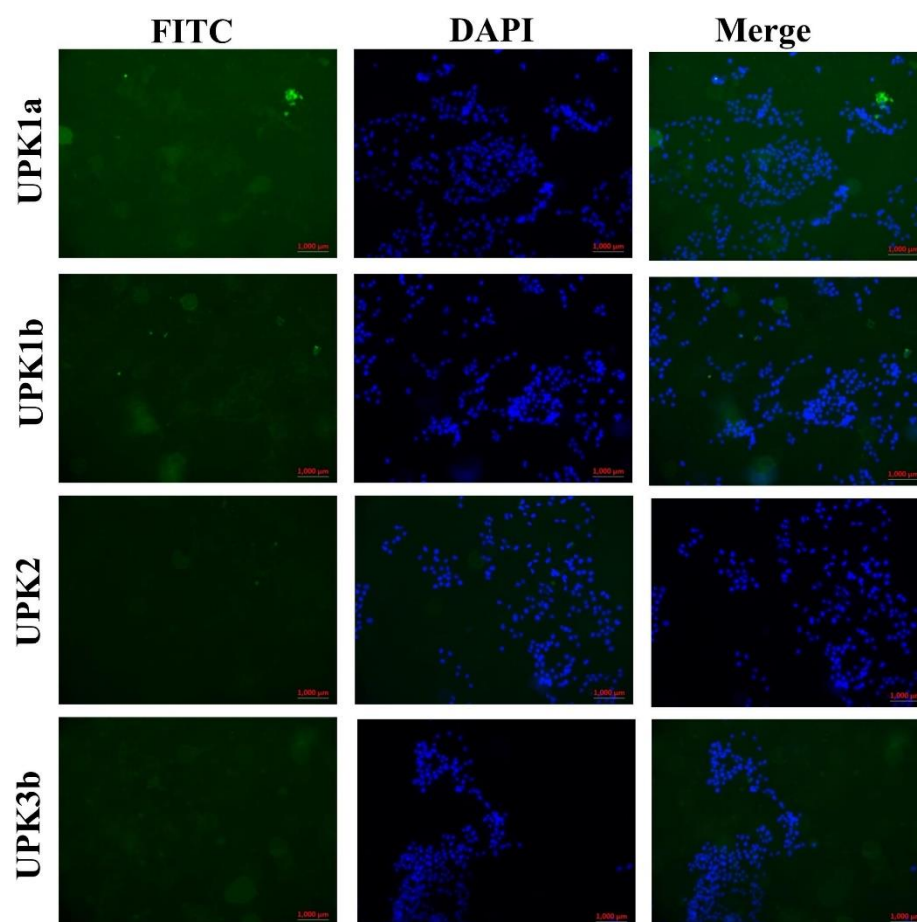


Figure 14. UPK antibody specificity in negative controls. Untransfected HEK cells were incubated with UPK1a or UPK1b antibody (raised in rabbit) or UPK2 antibody (raised in goat) or UPK3b antibody (raised in goat); followed by incubation with FITC labelled anti-rabbit secondary antibody raised in goat or anti-goat secondary antibody raised in rabbit. Nucleus was stained with 4', 6-diamidino-2-phenylindole and images taken using fluorescence microscope with excitation and emission at 495 nm and 519 nm respectively.

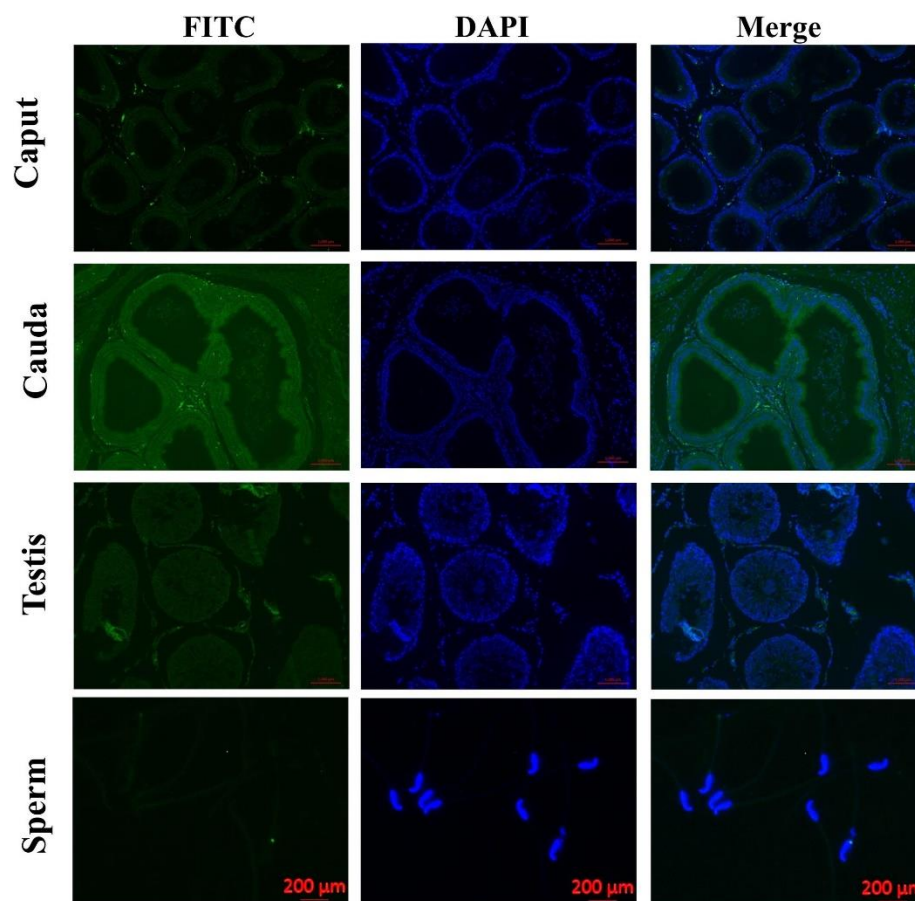


Figure 15. Immunofluorescence detection in negative control. Testis, Caput, Cauda and Spermatozoa were incubated with FITC labelled anti-rabbit secondary antibody raised in goat or anti-goat secondary antibody raised in rabbit. Nucleus was stained with 4', 6-diamidino-2-phenylindole and images taken using fluorescence microscope with excitation and emission at 495 nm and 519 nm respectively.

UPK1a was localized in majority of the cells that are closer to the basement in the caput and cauda. In the testis, the localization was observed throughout the seminiferous tubules and also in the interstitial cells. Further, it was localized in all the regions of the sperm (Figure 16). Similar staining pattern was observed for UPK1b (Figure 17), UPK2 (Figure 18) and UPK3b (Figure 19). In the sperm, all the UPKs were localized in the head, mid piece and tail regions (Figures 16–19).

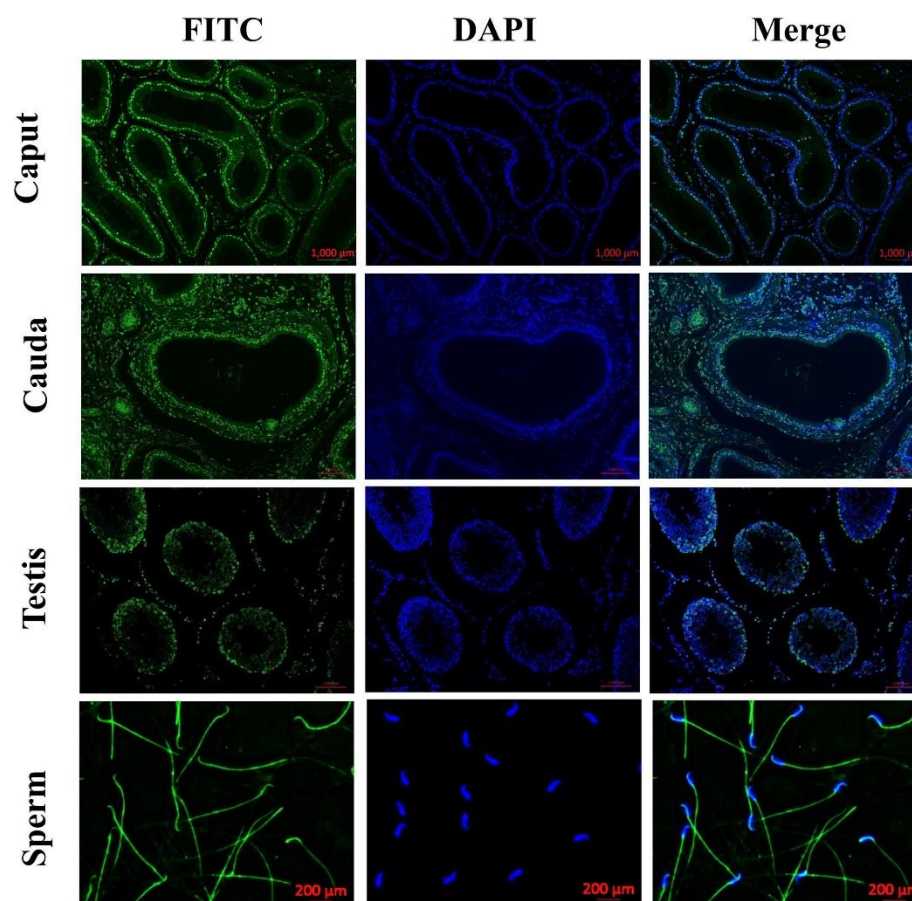


Figure 16. Immunolocalization of UPK1a. Serial sections (5 μm thickness) of the rat testes and epididymides and smears of spermatozoa were fixed and incubated for one hour with UPK1a antibody (raised in rabbit) followed by incubation with FITC labelled anti-rabbit secondary antibody raised in goat. Nucleus was stained with 4', 6-diamidino-2-phenylindole and images taken using fluorescence microscope with excitation and emission at 495 nm and 519 nm respectively. Magnification: 10X.

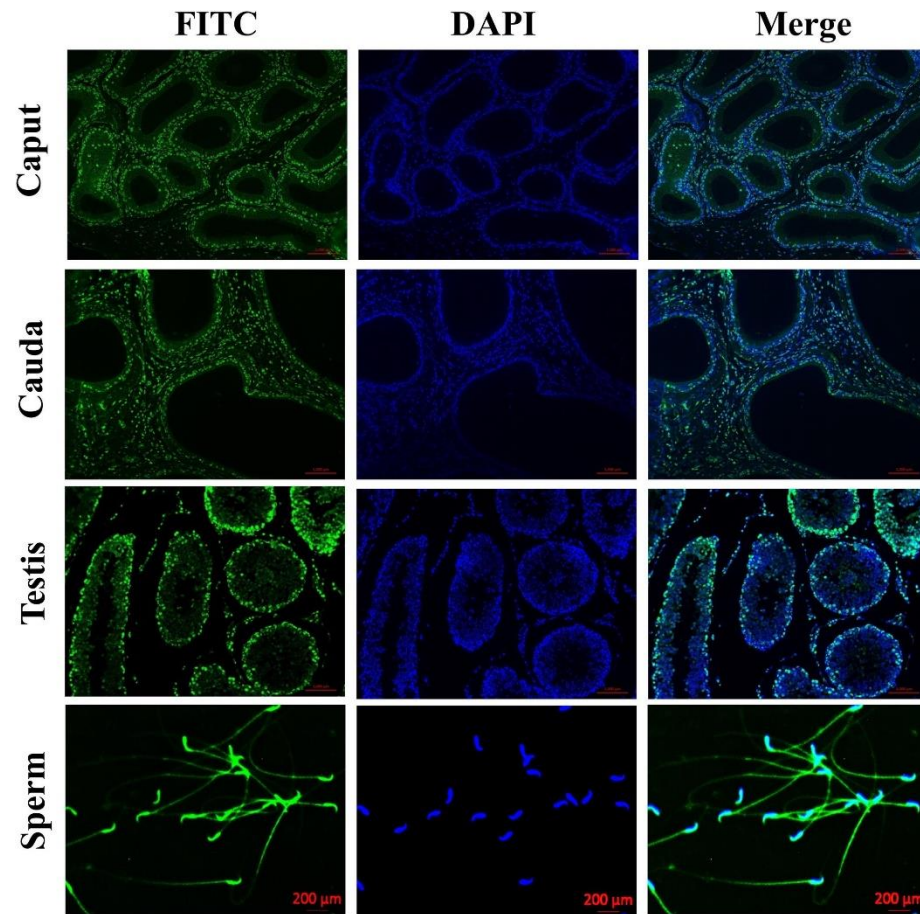


Figure 17. Immunolocalization of UPK1b. Serial sections (5 μm thickness) of the rat testes and epididymides and smears of spermatozoa were fixed and incubated for one hour with UPK1b antibody (raised in rabbit) followed by incubation with FITC labelled anti-rabbit secondary antibody raised in goat. Nucleus was stained with 4', 6-diamidino-2-phenylindole and images taken using fluorescence microscope with excitation and emission at 495 nm and 519 nm respectively. Magnification: 10X.

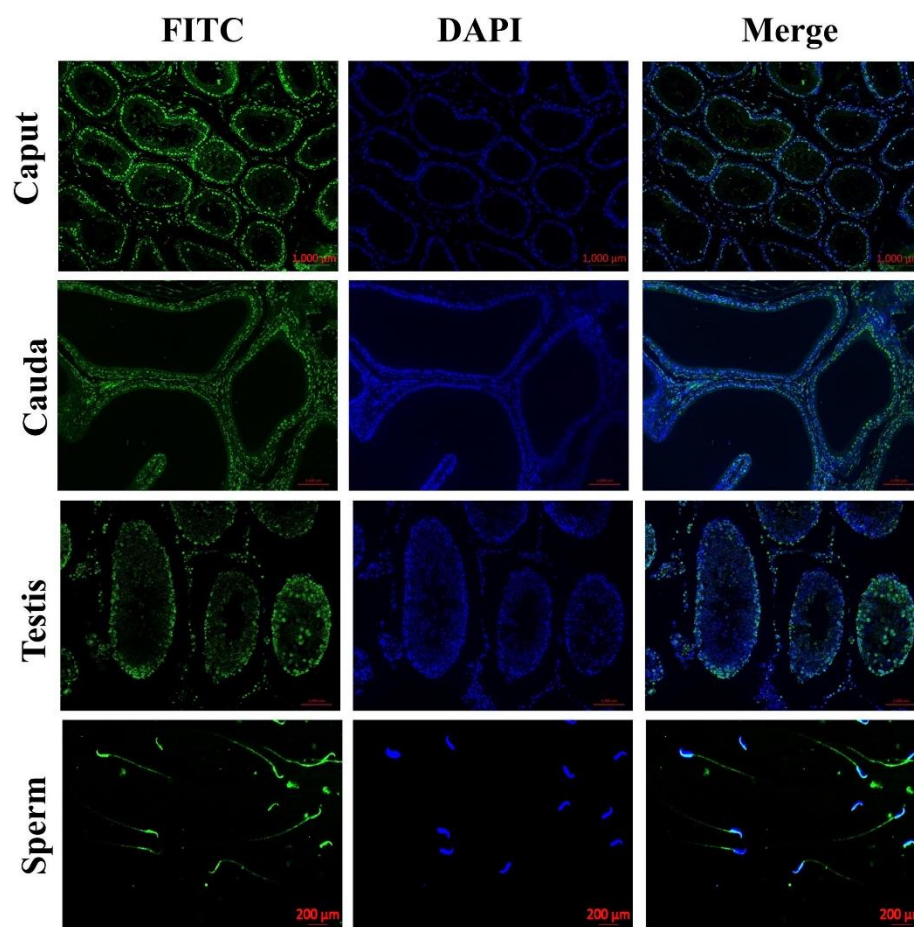


Figure 18. Immunolocalization of UPK2. Serial sections (5 μm thickness) of the rat testes and epididymides and smears of spermatozoa were fixed and incubated for one hour with UPK2 antibody (raised in goat) followed by incubation with FITC labelled anti-goat secondary antibody raised in rabbit. Nucleus was stained with 4', 6-diamidino-2-phenylindole and images taken using fluorescence microscope with excitation and emission at 495 nm and 519 nm respectively. Magnification: 10X.

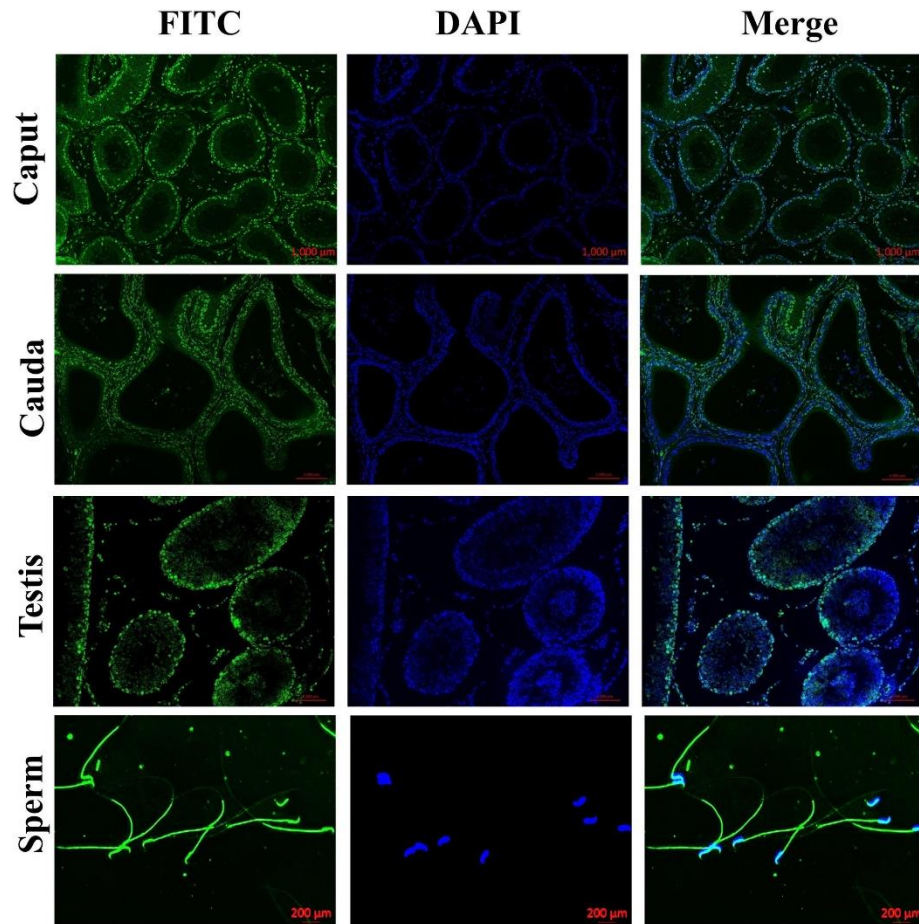


Figure 19. Immunolocalization of UPK3b. Serial sections (5 μm thickness) of the rat testes and epididymides and smears of spermatozoa were fixed and incubated for one hour with UPK3b antibody (raised in goat) followed by incubation with FITC labelled anti-goat secondary antibody raised in rabbit. Nucleus was stained with 4', 6-diamidino-2-phenylindole and images taken using fluorescence microscope with excitation and emission at 495 nm and 519 nm respectively. Magnification: 10 $\times$.

1.3.3. Modulation of *Upk* gene expression by LPS

Male reproductive tract proteins are known to have roles in innate immunity besides sperm function. Since we observed that *Upks* are abundantly expressed in the male reproductive tract tissues, we anticipate that they may have additional role in innate immune responses and this could be a novel function of UPKs that has not been reported till now. In order to understand the possible functional role of UPKs in the male reproductive system immune responses, we analyzed the dynamics of their mRNA levels during endotoxin challenge using *in vitro* and *in vivo* model

systems. Since *Upk1a*, *Upk1b* and *Upk3b* were expressed in LC540 and RCE cells, further analyses were carried out on only these genes. In LC540 cells, *Upk1a* expression increased significantly up to 12 h after treatment, whereas such an increased expression of *Upk1b* was observed only up to 3 h. However, *Upk3b* expression was decreased 24 h after LPS challenge (Figure 20). In the RCE cells, *Upk1a*, *Upk1b* and *Upk3b* expression increased significantly up to 6 h after LPS treatment followed by a decline below the control levels at the later time points (Figure 20).

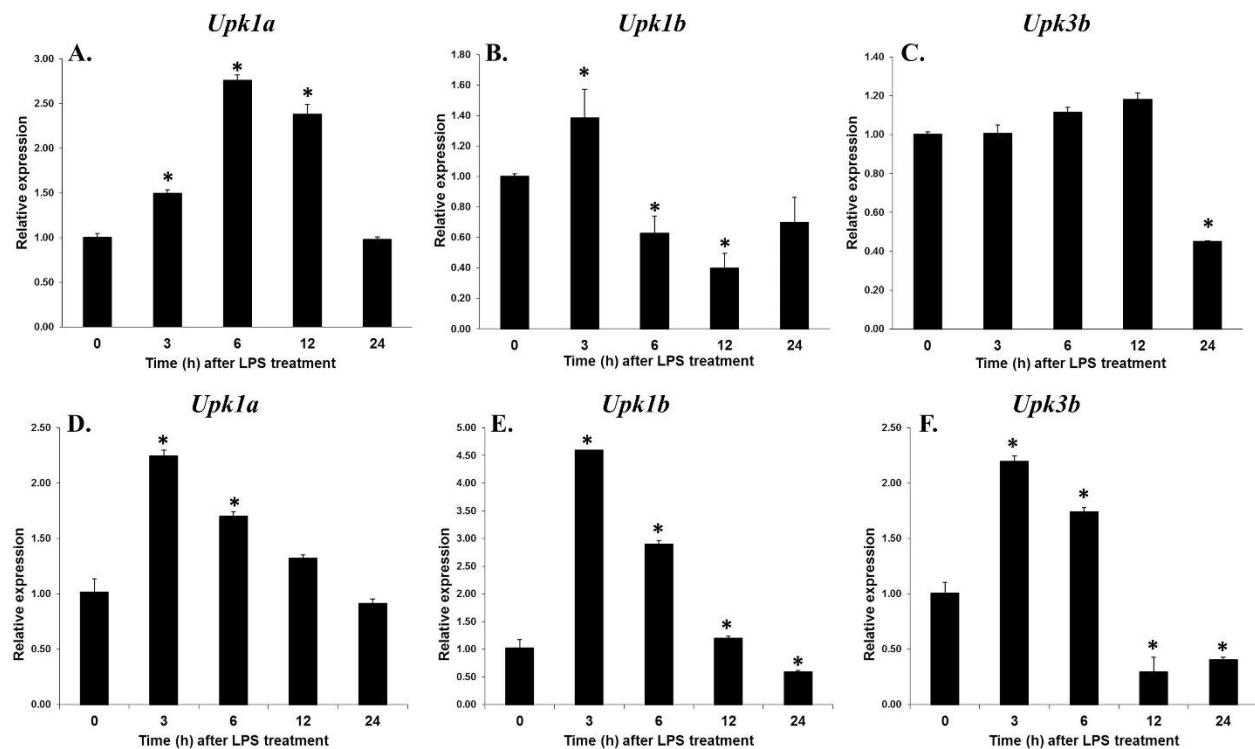


Figure 20. Immunolocalization of UPK3b. 1×10^6 cells were plated in each well of a 24 well plate and treated with 100 ng/ml LPS. RNA isolated from cells were collected at 3, 6, 12 and 24 h after LPS treatment was reverse transcribed and the resulting cDNA was used to analyze *Upk1a*, *Upk1b* and *Upk3a* gene expression by real time PCR. A-C, LC540 cells; D-F, RCE cells. Values shown are Mean $\pm$ S.D. * denotes $p < 0.05$ compared to 0 h control.

The effect of endotoxin challenge was also evaluated *in vivo* using rat as the model system. In the caput obtained from rats challenged with LPS, the mRNA levels of *Upk1a*, *Upk2* were not altered; whereas, a time dependent significant decline in *Upk1b* and *Upk3b* was observed (Figure 21). In the cauda, the mRNA levels of all the *Upks* were found to be significantly decreased following LPS administration (Figure 21). Testicular *Upk1a* and *Upk1b* expression was decreased at all the time points after LPS treatment. *Upk2* though increased at the 3 h time point was found

to be decreased at the later time points. *Upk3b* expression remained unchanged. (Figure 21). *Upk1a*, *Upk1b*, *Upk2* mRNA levels were significantly reduced in the seminal vesicles. *Upk3b* mRNA levels were down regulated at 6 and 24 h after LPS challenge, (Figure 21). In the prostate, though *Upk1a* and *Upk2* mRNA levels increased in a time dependent manner after LPS challenge up to 12 h followed by a decline, the mRNA levels of *Upk1b* were significantly reduced at all the time points analyzed (Figure 21).

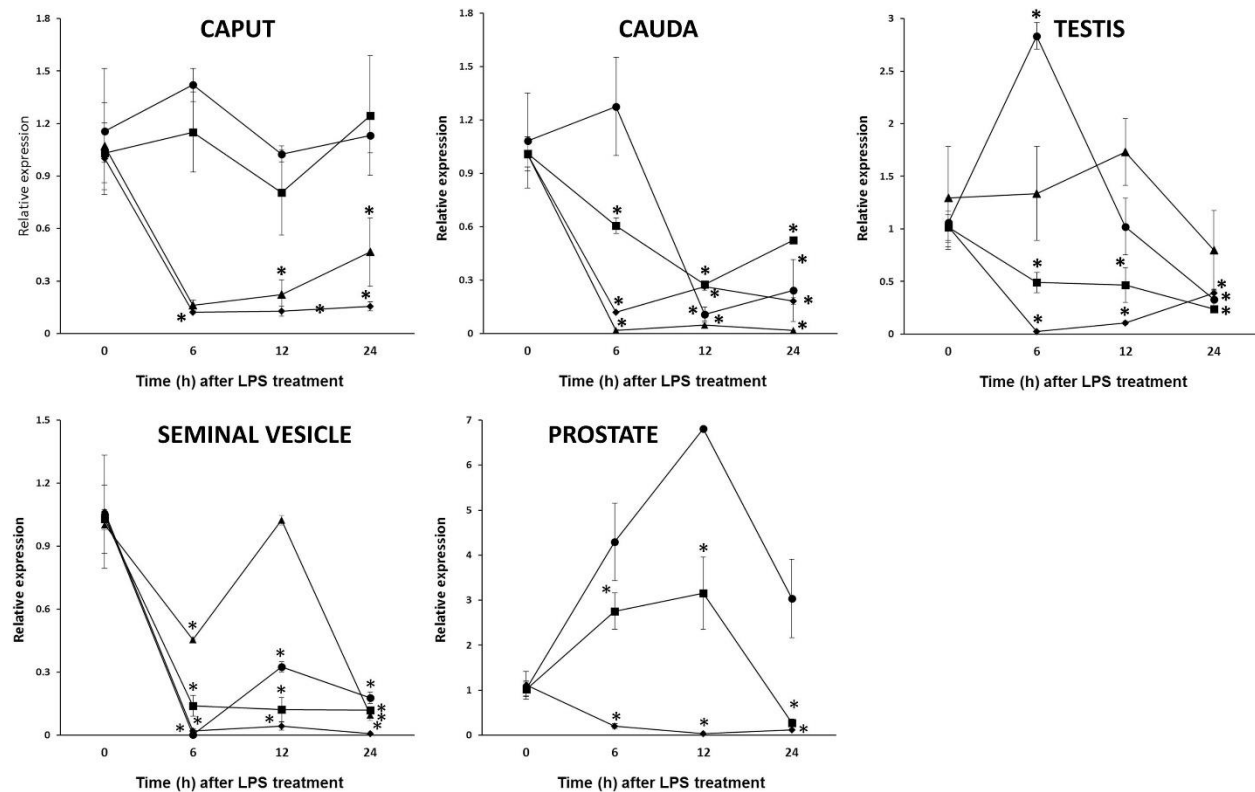


Figure 21. *Upk* gene expression in the male reproductive tract upon endotoxin challenge. Rats were challenged with a single intraperitoneal dose (1 mg / kg body weight) of LPS. Caput, cauda, testis, seminal vesicle and prostate were collected at 0, 6, 12 and 24 h after LPS treatment. RNA isolated from these tissues was reverse transcribed and the resulting cDNA was used for analyzing *Upk* gene expression by real time PCR. ■ – *Upk1a*; ♦ – *Upk1b*; ● – *Upk2*; ▲ – *Upk3b*. Values shown are Mean $\pm$ S.D. * denotes $p < 0.05$ compared to 0 h control.

1.4. Discussion

UPKs are implicated to play a crucial role in urothelial physiology and pathogenesis (Carpenter *et al.*, 2016, Wu *et al.*, 2009, Liao *et al.*, 2018, Zupancic and Romih 2013). They have been projected as key factors to indicate urothelial infections and cancers of the urothelial system (Lee 2011). However, their expression beyond the urothelial systems was proposed (Lee 2011). *Upk* expression was reported in the human female genital tract (Shapiro *et al.*, 2000) and may have a role in the developmental process (Cunha *et al.*, 2017) and pathophysiology (Ogawa *et al.*, 1999). Reduced *Upk* gene expression is associated with poor prognosis in colorectal and adenocarcinoma patients (He *et al.*, 2014, Zheng *et al.*, 2014). UPKs were implicated to be crucial in the fertilization process (Liao *et al.*, 2018). Analyzing the role of UPKs in other organ system has been an emerging area of research in the recent past. This study aims at investigating the possible functions of UPKs in the male reproductive system using rat as the model system. The rationale is based on the fact that male reproductive system and urothelium are of same embryonic origin and thus UPKs may play a crucial role in many cellular and tissue processes.

UPKs first emerged in cartilaginous fish, which could be a common ancestor of vertebrates (Garcia-Espana *et al.*, 2006). Over a period of time UPKs were identified in many species and are found to be highly conserved and have common evolutionary ancestors (Desalle *et al.*, 2014, Wu *et al.*, 1994). Recently three new UPKs were identified and this demonstrates the continued interest to study the role of UPKs in mammalian physiology (Desalle *et al.*, 2014). Rat genome wide search indicated the presence of five *Upk* genes, namely, *Upk1a*, *Upk1b*, *Upk2*, *Upk3a* and *Upk3b*, of which *Upk3b* sequence is predicted. Sequence analyses revealed that the rat *Upk3b* is located on chromosome 12. Similar to the rat, the mouse and human *Upks* identified till date are located on different chromosomes, suggesting that they are not clustered though they contribute to the common function. We identified and reported the rat *Upk3b* gene and this is a novel aspect of this study. *Upk3b* gene contains seven exons and is transcribed to an 1158 nucleotide mRNA that encodes a protein with 275 amino acids. Rat UPK3b contains a single transmembrane domain similar to that of mouse and human monospanins. However, its sequence similarity with UPK2 and UPK3a was found to be only 14 and 28 percent respectively. Rat UPK3b shows 92 and 82 percent homology with its mouse and human counterparts respectively. Though the rat UPK3b is classified as a monospanin and displays similarities to that of human in terms of localization of the

gene on a separate chromosome and the predicted function in urothelium, sequence homology reveals that it appears to be closely related only to the mouse UPK3b. Gene neighborhood analyses for rat *Upk* genes indicated similarities in the nature of genes present in the vicinity of *Upk* genes of mouse and human. A number of genes were commonly present in the vicinity of *Upks* in all the three species. It is possible that the location of *Upk* genes in relation to the other genes is conserved through evolutionary process across the species. Three dimensional structure analyses of rat UPKs indicate that UPK1a and UPK1b (tetraspanin family members) and UPK2, UPK3a and UPK3b (monospanin family members) are similar in appearance. The high content of alpha-helix in the tetraspanin family members and that of random coil in the monospanin family members appears to correlate with the number of spans they make in the plasma membrane. The 3D structure of UPKs proposed in this study are indicative and whether the proposed structures can fit into the existing models of asymmetric unit membrane particles needs to be validated using advanced methods such as nuclear magnetic resonance spectroscopy. Information presented on the sequence similarity among rat UPKs and between UPKs of different species, genome neighborhood and three dimensional structure are relatively less reported for UPKs of rat and other species; and thus contributes to the further understanding of the functional role of these proteins.

Though, UPKs were predicted to be expressed in other tissues (Lee 2011), their presence and importance in the male reproductive tract is gaining importance in the recent years. The importance of UPKs in fertilization was recently demonstrated in the mouse (Liao *et al.*, 2018). In the rat model system, characterization of UPKs still remains to be explored. Determination of expression pattern and functional role of UPKs may vary from species to species and hence this study aimed to understand the contribution of UPKs in the rat male reproductive tract. *Upk1a*, *Upk1b*, *Upk2* and *Upk3b* mRNA was detected in the caput, cauda, testis, seminal vesicle and prostate, whereas *Upk3a* was not detected in any of the tissues. Presence of UPK1a, UPK1b, UPK2 and UPK3b in the caput, cauda and testis as detected by immunoblotting further confirmed the expression of UPKs in male reproductive tract. The expression of *Upk* mRNAs in other tissues of the rat suggest that these genes are ubiquitously expressed and may have functions beyond the urinary system. In the rat, UPK expression in the urethra and vaginal introitus was associated with the developmental process (Cunha *et al.*, 2017). Such a correlation between *Upk* gene expression and postnatal development was not studied in the male reproductive tract. Developmental

regulation of the male reproductive tract in many species is governed by dynamic changes in gene expression pattern, which in turn is determined by the fluctuating levels of androgens (Rodriguez *et al.*, 2001, Harris and Bartke 1974). Our results indicate that, in the epididymis, *Upk1b* and *Upk3b* are constitutively expressed starting from early stages of development, whereas *Upk1a* and *Upk2* are detected at later stages. In the testis, except for *Upk3a*, all other *Upks* seem to be expressed in all the stages of development. It appears that some *Upks* are essential at all stages of development whereas some are governed by androgen levels. Testosterone levels vary greatly during the developmental process. A steady increase in testosterone levels occurs in the rete testis of 30–130 day old rats (Harris and Bartke 1974, Harris and Bartke 1981). Androgen levels in the epididymis of rat decline from birth until 20 days and a normal level of 10 ng/g tissue (35 nM) is maintained until approximately 40 days after which, the levels begin to increase to that of the adult, between 15 and 20 ng/g (Charest *et al.*, 1989). Serum testosterone levels in the young rat remain low and do not begin to increase to adult levels until 35–40 days of age (Nayfeh *et al.*, 1966). It can be hypothesized that the androgens may influence the expression of *Upk* genes directly by binding to their promoter elements or through modulation of other transcription factors. Immunofluorescence microscopy revealed the localization of UPKs in the rat male reproductive tract tissues and on the spermatozoa. We previously demonstrated the localization of proteins that play an important role in spermatogenesis and sperm maturation. They were found to be abundant in the epithelial lining of caput, cauda and testis and on spermatozoa (Rajesh and Yenugu 2012, Rajesh and Yenugu 2015, Narmadha *et al.*, 2011, Narmadha and Yenugu 2016). In this study, we show that UPKs are predominantly localized in the epithelial lining of caput, cauda and testis, suggesting that they may play a crucial role in spermatogenesis and sperm maturation. A recent study reported the localization of UPK1b in the apical region of the cauda epididymis (Liao *et al.*, 2018). Since the tetraspanin UPKs are expected to be localized in the vesicles, they should be detected in the apical regions. However, the localization pattern we demonstrate is different from that is reported by Liao *et al.* This could be due to variation in the species being studied. Further, imaging using high resolution microscopy are warranted to demonstrate the subcellular localization of UPKs in the tissues analysed in this study. All the UPKs analyzed were localized throughout the sperm. Liao *et al.* reported that all UPK proteins were localized only to the head region of the mouse sperm (Liao *et al.*, 2018). The pattern of localization of UPKs i.e. throughout the rat sperm could be due to species variation. The presence of these proteins on the sperm indicates their possible role in

many functions such as capacitation, acrosome reaction, sperm egg recognition and fertilization. Since the urothelial and reproductive tract tissues originate from the same embryonic origin, this could be an important reason for the presence of UPKs in both these organ systems. The exact role of UPKs in the male reproductive tract and their contribution to sperm function needs further investigation.

Proteins in the male reproductive tract have been demonstrated to have functions beyond spermatogenesis and sperm maturation. Ample evidence exists that some of them play a crucial role in innate immunity. For example, members belonging to defensin, Sperm Associated Antigen 11 (SPAG11), Prostate and Testis expressed (PATE), Lysozyme like (LYZL) families were found to be altered in response to endotoxin challenge or in model systems that mimic an infection (Biswas and Yenugu 2011, Rajesh and Yenugu 2012, Narmadha *et al.*, 2011). Their expression was epigenetically regulated (Biswas and Yenugu 2014). Since UPKs were found to be expressed abundantly in the male reproductive tract, it is possible that they may also contribute to innate immunity. Implicating UPKs for their multiple roles in the same organ system has not been demonstrated earlier and our approach in this direction is novel. We observed that *Upk* expression was generally down regulated when challenged with LPS both *in vitro* and *in vivo*. It is well established that Uropathogenic bacteria use UPKs as an advantage to infect the urothelial tissues (Mulvey *et al.*, 1998, Martinez *et al.*, 2000). UPK3a signaling is very crucial for bladder response during bacterial infection (Thumbikat *et al.*, 2009). Presence of surfactant D protein acted as a competitor to FimH (fimbrial tip-positioned adhesive protein) of UPEC because of its ability to bind to UPK1a and thereby reducing the available levels of UPK1a and thus the bacteria induced inflammation (Kurimura *et al.*, 2012). In view of the above evidences, it is possible that UPKs may have a significant role during endotoxin or bacterial induced responses in the male reproductive system. Since LPS is a component of the capsule of Uropathogenic bacteria, the down regulation of *Upk* mRNAs in the male reproductive tract tissues during LPS challenge could be a protective mechanism. It is possible that down regulation of UPKs may result in non-availability of UPKs for Uropathogenic bacterial binding and thereby lowering the infection severity. We previously demonstrated that LPS induced changes in the defensin expression involved TLR (toll-like receptor) mediated NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) activation and epigenetic changes such as DNA methylation and histone acetylation / methylation

(Biswas and Yenugu 2014, Biswas *et al.*, 2015). UP1a promoter methylation is implicated in bladder carcinoma (Cunha *et al.*, 2017), suggesting that the promoters of *Upk*s could be modulated by epigenetic changes under altered physiological conditions. It would be very interesting to study the molecular mechanisms (signaling pathways and epigenetic changes) by which LPS modulates *Upk* gene expression and also the interaction of Uropathogenic bacteria with UPKs of male reproductive system.

We conclude that UPKs are abundantly expressed in the male reproductive tract of rat and may contribute to the general physiology of this organ system, spermatogenesis, sperm function and immune responses.

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Chapter-2

Functional characterization of Uroplakin1a (*Upk1a*) using knock-out model

2.1. Introduction

The barrier permeability and structural stability of urothelium in the bladder is conferred by uroplakins (UPKs), which form plaques to generate an asymmetric unit membrane (AUM). Depending on the number of times they span the plasma membrane, two classes of them have been identified, namely monospanins and tetraspanins. UPKs identified in different species are UPK1a, UPK1b, UPK2, UPK2b, UPK3a, UPK3b (a slight isoform of UPK3a), UPK3c and UPK3d (Desalle, Chicote, Sun, & Garcia-Espana, 2014; Garcia-Espana *et al.*, 2006; X. R. Wu *et al.*, 1994). The plaque formation stems out of specific interaction among the UPKs (UPK1a interacts with UPK2, while UPK1b interconnects with UPK3a) (C. C. Hu *et al.*, 2005; Tu, Kong, Sun, & Kreibich, 2006; Tu, Sun, & Kreibich, 2002; X. R. Wu *et al.*, 1994). Besides their primary role in protecting the urothelium, they are implicated in a variety of physiological and pathological processes. Elevated levels of UPKs in benign urological diseases (Szymańska, Matuszewski, Dembowski, & Piwowar, 2021), implication of Upk2a as a potential biomarker for the detection of lung carcinoma (Leivo, Tacha, & Hansel, 2021; J. Zhu *et al.*, 2021), up regulation of *Upk1a* and anti-sense RNA to *Upk1a* in lung cancer (Byun, Choi, Jeong, Yoon, & Baek, 2020), promotion of glycolysis and proliferation in liver cancer (Song *et al.*, 2020) and Upk1b as a novel prognostic marker for gastric cancer (Z. Zhu *et al.*, 2020) are some of the roles of UPKs reported in the last two years.

The significance of UPKs in male reproductive physiology is gaining importance. UPK3a and UPK11b forms a complex to mediate sperm-egg interaction and fertilization in *Xenopus* oocytes (Hasan, Fukami, & Sato, 2011; Mahbub Hasan *et al.*, 2005; Sakakibara *et al.*, 2005). The time and location dependent expression of UPK3b in the sperm, epididymis and ovarian follicles (Kuriyama, Tamiya, & Tanaka, 2017) their expression in oocytes and association with multivesicular bodies and reduced fecundity of UPII/IIIa knock out provides further evidence on their role in reproductive physiology (Liao, Chang, Liang, Chung, Wei, Nguyen, Zhou, Talebian, Krey, Deng, Wong, Chicote, Grifo, Keefe, Shapiro, Lepor, Wu, DeSalle, Garcia-Espana, *et al.*, 2018). The sperm-egg membrane fusion signal is mediated by the oocyte/egg membrane microdomain (MD)-associated uroplakin III-Src system to kick start the embryonic and zygotic development *Xenopus laevis* (Sato & Tokmakov, 2019). We demonstrated the expression of

Upk1a, *Upk1b*, *Upk2* and *Upk3b* mRNA and protein in the male reproductive tract of rat (Babu Munipalli & Yenugu, 2019). During uropathogenic *E. coli* (UPEC) infection, UPKs serve as anchors and this is mediated by the binding of the type-1 fimbriae to initiate a series of events that favour bacterial infection in a variety of tissues (Martinez, Mulvey, Schilling, Pinkner, & Hultgren, 2000; Mulvey *et al.*, 1998; X. R. Wu, Kong, Pellicer, Kreibich, & Sun, 2009; X. R. Wu, Sun, & Medina, 1996). We also demonstrated that the expression of UPKs in the epididymis, testis seminal vesicle and prostate was altered under conditions of endotoxin challenge (Babu Munipalli & Yenugu, 2019). However, in depth studies that explored the involvement of UPKs at the molecular level in the male reproductive tract are not yet reported. In order to further understand the functional significance of UPKs in male reproductive tract, we generated *Upk1a* knockout mice and analyzed their fecundity, sperm function and responsiveness of the male reproductive tract tissues to UPEC infection. Further, the testicular transcriptome profile of *Upk1a* knockout mice was obtained by next generation sequencing. We report that the *Upk1a* male mice are sub fertile with reduced sperm count, displayed normal sperm function (capacitation and acrosome reaction) and lacked the ability to clear the UPEC efficiently. Transcriptome analyses of the testis of knockout mice revealed that genes associated with a wide variety of biological, cellular and molecular function were differentially expressed, indicating the multifunctional roles of UPKs in testicular function.

2.2. Materials and methods

2.2.1. Generation of *Upk1a* knockout mice

Upk1a knockout mice (**B6NJ: C57BL/6N***JNcbs-Upk1A^{em2MGEF}*) generated by using Cas9 / sgRNA, at Mouse Genome Engineering Facility, NCBS (National Centre for Biological Sciences), Bangalore, India. The strategy used for generation of the knockout mice is depicted (Figure 1). Specific guide RNAs were designed to target the mouse *Upk1a* gene (NM_026815.2) to facilitate deletion of the region between 392 to 557 (166 bp) that spans a part of intron 1 and exon 2 and part of intron 2 (Figure 2). The 5' → 3' sequence of the guide RNAs used are: sgRNA#34 (on plus strand): 5'- TTTGGTGAGACGCGGAATAA [TGG-PAM] -3' and sgRNA#38 (on minus strand): 5'- TACTTCAGACCCCATCTACC [AGG-PAM] -3'. B6/NJNcbs mice zygotes were microinjected in the pronuclear region with a solution containing Cas9 protein

(50 ng/μl final), sgRNA#34 (12.5 ng/μl final) and sgRNA#38 (12.5 ng/μl final). Resulting founders were back crossed twice with wild type mice (B6/NJNcbs). After two successive B6/NJNcbs backcrosses, the animals obtained were heterozygote *Upk1a*^{+/-} N2 mice. Homozygote (*Upk1a*^{-/-}) knockout animals that had a deleted sequence (part of intron 1 and exon 2 and part of intron 2) were generated by crossing *Upk1a*^{+/-} N2 with *Upk1a*^{+/-} N2. DNA of male and female mice from tail regions were subjected to genomic PCR to confirm the deletion using PCR primers that flanked the targeted region (Figure 2). The PCR amplicons (wild type and *Upk1a*^{-/-} 536 and 360 bp respectively) were sequenced. Knockout mice used for further studies were obtained from the same founder and age matched.

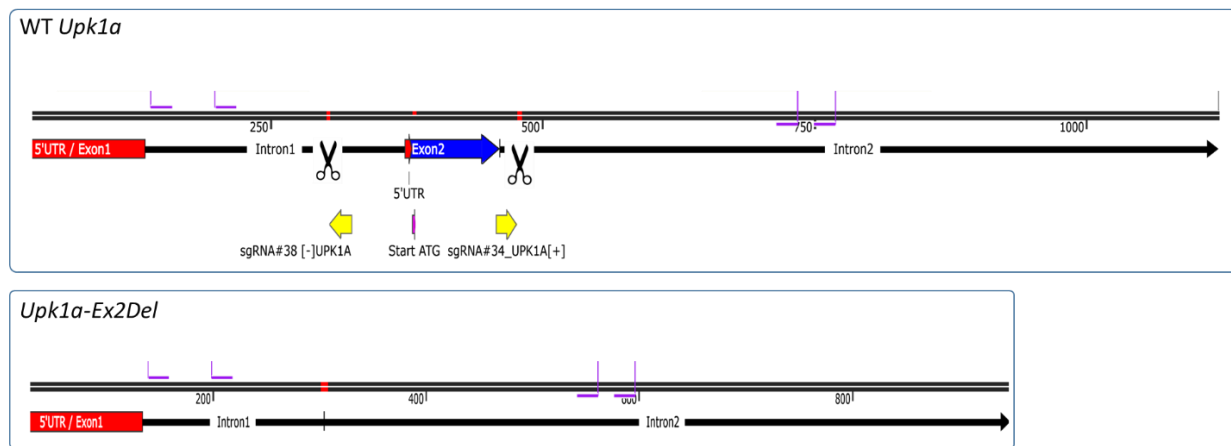


Figure 1: Strategy to generate *Upk1a* knockout mice. Upper panel shows the gene structure of mouse *Upk1a* (5'UTR, exon1, intron 1, exon2 and intron 2). Scissors indicate the targeted regions to generate the knockout mice. sgRNA#38 and sgRNA34 were used to target the minus and plus strands. FP and RP are genotyping primers. Lower panel indicates the structure of exon1 deleted *Upk1a* gene mediated by CRISPR-Cas9 editing.

B6NJ: C57BL/6NJNcbs-Upk1A^{em2MGEF}

AGATCACCTTGCCCCAGTGGCCAGTCAGGAGCACAATCCTGAATTTTCAGGTCTGTAAATCACTTGAGTGTCAAGGAG
CTCATAAAAATCTATCATGGTCAGTCTGCTGAACCTCCTGCCCCCTCCTCCTGAGACTGGCTCAAGGAGGGGTTTG
GGCATATATAGGGAGCTGCTGTAGCTGGGGCTGACCAGAGAGGCTTCTGTAGAGAAGGTGAGGAGGTGTTCTGGGGG
TTGACAGGGCAAGGGGCTGGGGAGGGCAACAGCTGCCCTGCAGT**TAGAGGCTGCTCGGCTTTTCTTCTTCTGTAGAA**

(FP)

GGCTCACTGCTAAGCTGTTGGACTCTGGGCATATGGACACCAGCCTGGGCTCTGCAGCTCATGAGCATCTCCT**GGTA**
GATGGGGTCTGAAGTAGACCCTGCCTCCTGCCCTCCTAACTTCTGCCCTGCTTCTGTGTCCCCAGCAGGAAGATGGC

sgRNA#38 [-]UPK1A

TTCTGCAGCGACAGAGGGAGAGAAGGGGTCTCCTGTGGTGGTGGGGCTGCTGGTCGTGGGCAACATCATTATTTGG
TGAGACGCGGAATAATGGGAGCGGGAAAGTGTGGTGGGGAGGTGGCAGCTGAGATCCCAGGTCTATGAGCTGCAGGC

sgRNA#34 _UPK1A[+]

TCTAGTCCCAGATCCTAAAGGGGATCCCAGCTACCTGTTCTTACAGAGGGAGCAACCTGTTCTCTGGGAATCCTTCT
TGTGGATCATGTAGATTATCCACAACCTCAGGGGAAACGCTTGAGAGACAAGAGTGGTAAACACACACACACACACA
CACACACACACACAGCCGT**AATCGCAGCTTTTGAAGGCT**GAGGCCAGAGAATAATGGCTGTGAGTCTGAAGCCA

(RP)

ATCTAACTATAGTGTGTGAAGTTCTGTTTAGAGAGAGGAAGGGGGAGGGGGAGGGGGAGGGGGAGGGGGAGGGAGAG
GAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGAGAGAGAAGAGAAGAGAAGAGAAGAGAA
GAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGAGAAGGGAAGAGAAGAGAAGTTGAGAATTAGTG
TCAAGAGAATCTGTCAAGTATCAAGTACTGGAACCTACTCTGTAGACCAGGTTGGCCTCGAACTCAGAGATCCTCCT
GCCTCTGCCTCTGCCCCTGGGTTCTAGGATTAAAGGTGTGC

Figure 2. Gene sequence of mouse *Upk1a*. Exon 1 and Exon 2 sequences are indicated in red colored font, while the red colored and underlined sequence is the CDS. 5'UTR and intronic sequences are in black colored font. Yellow highlighted sequences are the targets for the guide RNAs. The 166 bp region targeted for deletion (392 to 557) indicated in bold font lies within the yellow highlighted sequences. Grey highlighted sequences are identified to design forward primer (FP) and reverse primer (RP) for genotyping. The expected amplicon size for wild type and knockout is 536 and 370 bp respectively.

2.2.2. RT-PCR

RNA was isolated to generate cDNA by using commercially available kits (Promega) and PCR analyses carried out with *Upk1a* gene specific primers. The typical condition for a PCR reaction was: 94°C for 1 min; 30-32 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec and the termination stage of extension at 72°C for 10 min. Electrophoresed the PCR amplicons on 2% agarose gels. Using SYBR master mix kit (Applied Biosystems, Warrington, UK), real-time PCR was performed with standard cycling conditions by in a thermal cycler (Applied

Biosystems). For calculating the fold change in expression, *Gapdh* levels were measured to serve as internal control.

2.2.3. Assessment of fecundity

Wild type and *Upk1a*^{-/-} mice (aged 90 days; n = 6 for each group) were subjected to natural mating to assess fecundity. Each male mice was housed with two female mice and monitored for detection of pregnancy. Further females were separated and noted the litter sizes.

2.2.4. Assessment of capacitation and acrosome reaction

Capacitation and acrosome reaction in the spermatozoa was measured as reported previously (Rajesh & Yenugu, 2015, 2017). Filipin, the cholesterol-binding dye (Cat no. F9765, Sigma Aldrich, USA), was used to assess capacitation. During capacitation, cholesterol loss is evident, which can be monitored using a specific fluorescent dye, filipin. Capacitated spermatozoa display reduced fluorescence intensity compared to their uncapacitated counterparts (Bou Khalil *et al.*, 2006). Spermatozoa (from mice aged 90 days) incubated with 25 μ M filipin for 1 hr and extensively washed with PBS were permitted to capacitate in M2 medium for 4 hours. Uncapacitated spermatozoa were also included in the assay. A flow cytometer (BD Biosciences) was used to measure the fluorescence with excitation (340 nm) and emission wavelengths (425 nm).

The acrosome reaction is characterized by an enhancement in the levels of intracellular calcium concentration ($[Ca^{2+}]_i$). The same was assessed using Fluo-3-AM (a calcium-binding fluorescent dye). To initiate an acrosome reaction, the ionophore, A23187 (10 μ M; (Sigma Aldrich, USA) was added to spermatozoa (from mice aged 90 days), and put the reaction in the incubator should be maintained at 37°C, 5% CO₂ and 95% air about one hour. After the incubation, Fluo 3-AM (10 mM) was added, and the fluorescence intensity was measured in a flow cytometer with excitation and emission wavelengths set at 488 nm and 515–540 nm, respectively. Fluorescence intensity was measured for 10,000 individual sperm cells. Linear and logarithmic modes were used to collect Forward scatter (FSC) and side scatters (SSC). The intensity of fluorescence of capacitated / acrosome reacted spermatozoa is demonstrated as percent relative to the fluorescence intensity noticed in the uncapacitated / acrosome intact spermatozoa.

2.2.5. Histological evaluation

Different tissues of wild-type and *Upk1a* knockout mice (aged 90 days) were placed for 24 hr in 4% paraformaldehyde (PFA) and washed with 70% ethanol. Sequential tissue dehydration was carried out with graded ethanol (80, 90, and 100%) and isopropanol overnight at 60°C. The processed tissues were embedded in paraffin wax, and five-micron sections were prepared for histological staining. Xylene-based deparaffinization and rehydration in graded ethanol (100, 90, 80, 70, and 50 %) were carried out, followed by staining for 10 mins duration with Harris hematoxylin solution. Consequently, sections were handled by washing with distilled water in 1% hydrochloric acid for 30 sec for differentiation and immersion in 0.2% ammonia water for 1 min. Staining was also carried out for 1 min with 0.2% eosin Y solution. The sections were then dehydrated by placing them consecutively in 50, 70, 80, 90, and 100% alcohol. Washed with xylene and mounted using a xylene-based mounting medium. For histopathological evaluation in multiple fields images Captured for each section was conducted by a board-certified histopathologist. The histopathologist reported histopathological changes as per the established principles (KN, AK, & DK, 2013; R., 2013).

2.2.6. UPEC infection and assessment

Briefly, 30,000 UPEC suspended in 100 µl PBS was injected intraperitoneally into wild-type and knockout mice (aged 90 days), and the animals were constantly monitored. Animals were sacrificed 1, 3, and 5 days after injection. Caput, cauda, testis, seminal vesicle, prostate, bladder, liver, and kidney were collected and homogenized in sterile PBS. To resolve the number of UPEC present in the tissues. Real-time PCR was carried out for papC, a UPEC-specific gene. Initially, serial dilutions (1:10 to 1: 1,00,00,000 containing 33000000 to 33 *E. coli*) of UPEC were prepared, and 10 µl of each was subjected to real-time PCR. A graph plotted with Ct value and *E. coli* number on the X and Y axes, respectively. The bacterial count in each tissue was determined by correlating the Ct value on the graph.

2.2.7. Gene chip hybridization, data collection, and enrichment analysis.

The quality of RNA isolated from the testis of wild-type and knockout mice (aged 90 days) was checked with Agilent TapeStation system, and RNA-seq was carried out using Illumina HiSeq platform. FastQC and MultiQC software are used for the data quality check (de Sena Brandine & Smith, 2019). The data was checked for base call quality distribution, % bases above Q20,

Q30, %GC, and sequencing adapter contamination. All the samples passed the QC threshold (Q30>90%). Raw sequence reads were processed to remove adapter sequences and low-quality bases using fastp(S. Chen, Zhou, Chen, & Gu, 2018). QC passed reads were mapped onto *Mus musculus* (GCF_000001635.27_GRCm39_genomic.fna) reference genome using STAR v2 aligner (Dobin *et al.*, 2013). On average, 99.86% of the reads aligned onto the reference genome. Gene level expression values were obtained as reading counts using feature Counts software (Liao, Smyth, & Shi, 2014). Differential expression analysis was carried out using the DESeq2 (Love, Huber, & Anders, 2014). The read counts were normalized (variance stabilized normalized counts) using DESeq2, and differential enrichment analysis was performed. Genes with absolute log2 fold change ≥ 1 and ≤ -1 with a p-value ≤ 0.05 were considered significant. The genes that displayed unique differential expression were used for Gene Ontology (GO) and trail improvement analysis by DAVID software.

2.2.8. Statistical analyses

Holm-Sidak test is used for statistical analyses using Sigma Plot software (SPSS Inc., Chicago, IL, USA). The values revealed are Mean $\pm$ S.D. * indicates $p < 0.05$ compared with the respective control.

2.2.9. Ethics approval statement

The *Upk1a*^{-/-} mice were generated under the NCBS-IAEC project (No. #AJ-1/2015(R1-E). The University of Hyderabad Institutional Animal Ethics Committee approved the animal studies (UH/IAEC/SY/2021-1/20)

2.3. Results

2.3.1. Genotypic characterization of knockout mice

Heterozygote *Upk1a*^{+/-} N2 mice obtained from NCBS, Bangalore were crossed to generate *Upk1a*^{-/-} mice. Genotyping was performed using primers that are flanked on either sides of the targeted sequence of *Upk1a* gene. An amplicon with a size of 536 bp was obtained by genomic PCR with DNA from wild type animals, whereas the amplicon size was 370 bp in *Upk1a*^{-/-} mice (Figure 3A). In the heterozygote mice, two PCR amplicons of sizes 536 bp and 370 bp were observed. The effect of *Upk1a* gene knockout on the expression of its mRNA was analyzed by RT-PCR in the bladder. *Upk1a* mRNA expression was completely absent in the knockout mice as demonstrated by PCR analyses (Figure 3B).

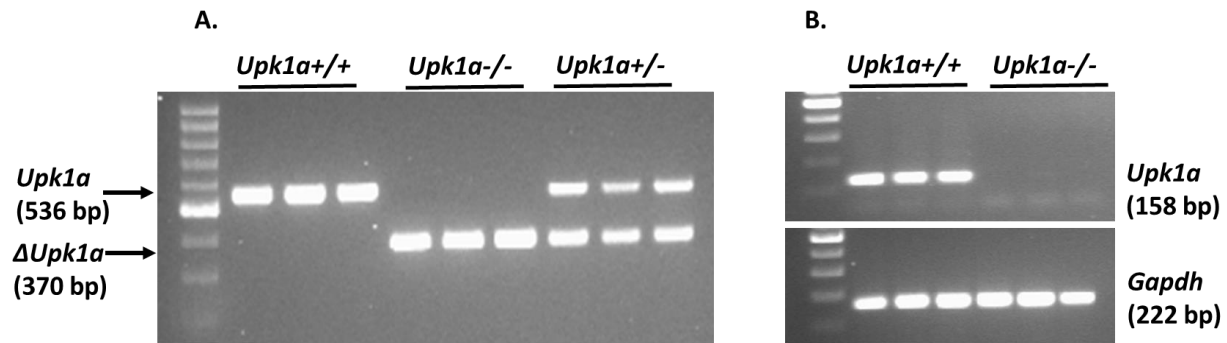


Figure 3. Genotyping and *Upk1a* gene expression. **(A).** Genotyping of wild type, *Upk1a* heterozygous and *Upk1a* knockout mice. DNA was isolated from the tail piece and subjected to genomic PCR using primers that flank the regions of the targeted region. Wild type amplicon size: 536; Knockout amplicon size: 370 bp. **(B).** *Upk1a* mRNA expression in bladder of wild type and knockout mice. RNA isolated from the bladder was reverse transcribed and *Upk1* mRNA expression was assessed by PCR using gene specific primers.

2.3.2. Body and organ weights

There was no difference in the average body weight of wild type and knockout male mice (Table 1). However, significant increase in the relative organ weight of bladder, kidney and prostate was evident. On the contrary significant decrease was observed for caput epididymis and testis. Pictures of the organs that showed changes in the size / weight are presented in figure 4.

Table 1. *Relative organ weights of wild type and knockout mice.*

Tissues	Wild type	Knockout
Bladder	0.0136 ± 0.00019	0.0028 ± 0.00103*
Caput	0.0012 ± 0.00007	0.0009 ± 0.00012*
Testis	0.0041 ± 0.00012	0.0031 ± 0.00015*
Prostate	0.00088 ± 0.00007	0.0018 ± 0.00056*
Seminal vesicle	0.0119 ± 0.00145	0.0134 ± 0.00161
Pancreas	0.007 ± 0.00057	0.0066 ± 0.00078
Spleen	0.0029 ± 0.00022	0.0031 ± 0.00025
Kidney	0.0078 ± 0.00024	0.0434 ± 0.00304*
Liver	0.0482 ± 0.0060	0.0080 ± 0.00612
Lung	0.0093 0.00103	0.0059 ± 0.00209
Heart	0.00631 0.00062	0.0124 ± 0.00094
Brain	0.0177 0.00027	0.0183 ± 0.00205

* Indicates $p < 0.05$.

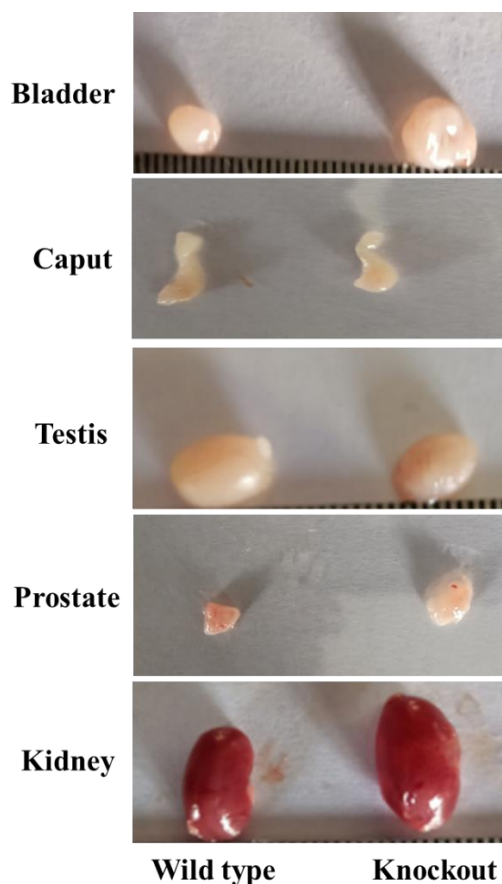


Figure 4. Representative images of organs that displayed change in weight. Bladder, caput, testis, prostate and kidney were taken out from wild type and *Upk1a* knockout mice and photographed.

2.3.3. Fecundity, sperm count and sperm function

The effect of *Upk1a* mRNA ablation on the male reproductive function was evaluated in terms of fecundity, sperm count and sperm function. Fecundity (litter size obtained after natural mating) of *Upk1a* knockout male mice was significantly decreased when compared to their wild type counterparts (Figure 5). The average number of pups in ten litters each for wild type and knock mice was 9.81 ± 1.55 and that of *Upk1a* knockout mice was 6.1 ± 1.28 . Concomitant with the decreased litter size, sperm count was also found to significantly less in the *Upk1a* knockout mice (Figure 5). The sperm count (in millions; mean $\pm$ S.D.) in the wild type and *Upk1a* knockout mice was 16.32 ± 1.66 and 12.27 ± 0.67 respectively. Sperm function (capacitation and acrosome reaction) was assessed to determine whether this could also contributed to the decreased litter size.

In the wild type capacitated sperm, a decrease in fluorescence intensity was observed, indicating the occurrence of capacitation (Figure 6). In the sperm of knockout mice, there was no significant decrease in fluorescence intensity, indicating that spermatozoa of knockout mice failed to undergo capacitation. In the sperm of wild type mice increased fluorescence of Fluo-3AM was evident, indicating the occurrence of acrosome reaction (Figure 6). Similar increase in the fluorescence intensity was also observed in the sperm of knockout mice, suggesting that acrosome reaction may not be affected in *Upk1a* ablated mice.

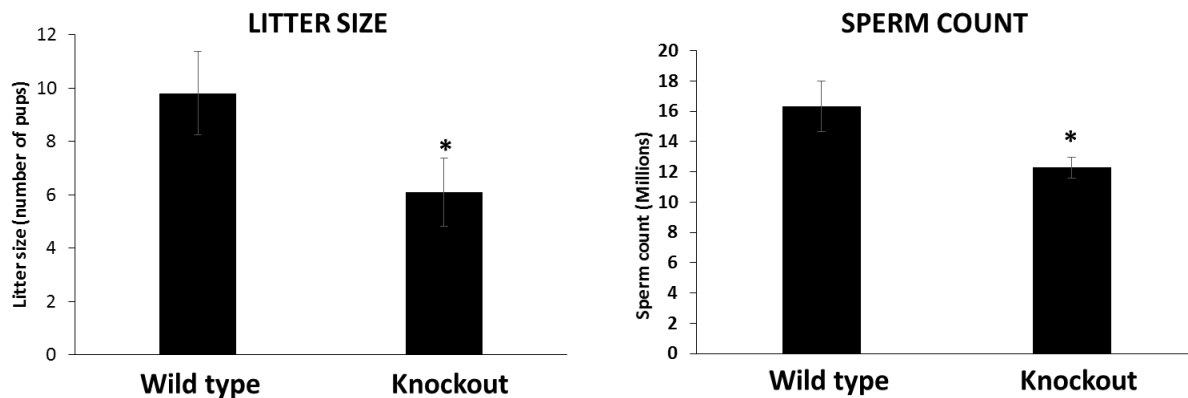


Figure 5. Fecundity in wild type and *Upk1a* knockout mice. **A.** Litter size. Adult wild type and knockout mice ($n=6$ each) were mated with females of proven fertility for a week. The number of pups born were noted. **B.** Caudal spermatozoa were collected in phosphate buffered saline (pH 7.0) and counted in a hemocytometer. Values expressed are mean $\pm$ S.D. * indicates $p < 0.05$ compared to the wild type control.

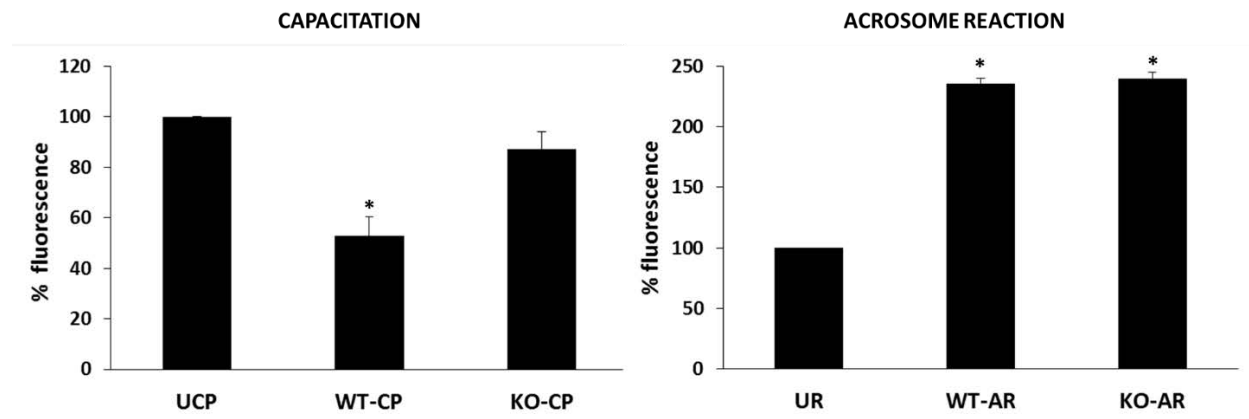


Figure 6. Sperm function in wild type and *Upk1a* knockout mice. **(A).** Capacitation. Caudal sperm were incubated with the cholesterol binding dye fillipin ($25 \mu\text{M}$) and allowed to capacitate. Fluorescence intensity was measured in a flow cytometer with excitation and emission wavelengths at 340 and 425 nm respectively. **(B).** Acrosome reaction. Spermatozoa collected from the cauda were subjected to spontaneous acrosome reaction using the ionophore (A23187; $10 \mu\text{M}$). They were then stained with Fluo 3-AM (10 mM) and the fluorescence intensity measured in a flow

cytometer with excitation and emission wavelengths set at 488 nm and 515–540 nm respectively. The fluorescence intensity of capacitated / acrosome reacted spermatozoa is expressed as percent relative to the fluorescence intensity observed in the uncapacitated / acrosome intact spermatozoa. UCP – uncapacitated; WT-CP – wild type capacitated; KO-CP – knockout capacitated; UR – unreacted; WT-AR – wild type acrosome reacted; KO-AR – knockout acrosome reacted; Values expressed are mean $\pm$ S.D. * indicates $p < 0.05$ compared to UCP / UR.

2.3.4. Histopathology

Anatomical changes that may occur in the *Upk1a* knockout mice was evaluated by subjecting different tissues to histopathological evaluation (Table 2 / Figure 7, 8, 9 and 10). Moderate inflammation in tubules along with mild hyperplasia of tubular epithelial cells was observed in the caput epididymis (Figure 7). In the cauda epididymis severe ductular atrophy characterized by mucosal epithelial cells surrounded by dense thickened fibrosis / connective tissue was observed (Figure 7). Testes were characterized with foci of seminiferous tubular edema, mild degenerative changes, mild hyperplasia of Leydig cells and mild edema / accumulation of fluids in between tubules (Figure 7). Prostate was characterized with severe atrophy of tubular glands in which massive proliferation of fibrous/ connective tissue surrounding the mucosal epithelial of tubular glands and atrophy of mucosal epithelial cells /glands with infiltration of multinucleated giant cells (Figure 7). Foci of mild degenerative changes in mucosal epithelial cells was evident in the seminal vesicle (Figure 8). In the bladder, severe mucosal transitional epithelial (urothelium) hyperplasia and severe sub mucosal fibrosis; accumulation of inflammatory exudate with dead debris and inflammatory cells in lumen was evident (Figure 8). In the kidney, atrophy of glomerulus, severe tubular nephritis in associated with tubular/ interstitial inflammation, degeneration and fibrosis, while some of the tubules were in dysplastic or pre neoplastic stages (Figure 8). Liver was characterized by mild foci of infiltration of inflammatory cells in centrilobular region, whereas normal portal region with portal vein, bile duct and hepatic artery was observed (Figure 8). Lungs displayed moderate alveolar/ interstitial edema with inflammation and fibrosis (Figure 9). The brain was characterized with multiple foci of apoptotic neurons while morphology of frontal cortex, hippocampus and cerebral cortex was normal (Figure 9). Though normal morphology of acinar cells was observed in the pancreas, atrophy of islets was evident (Figure 9). Normal morphology of the heart and spleen was evident (Figure 10).

Table 2. *Histopathological changes in wild type and Upk1a knock out mice*

Tissue	Wild type	Upk1a knockout
Caput	Normal morphology	Moderate inflammation in tubules [red arrow] along with mild hyperplasia of tubular epithelial cells [green arrow]
Cauda	Normal morphology	Severe ductular atrophy characterized by mucosal epithelial cells surrounded by dense thickened fibrosis / connective tissue.
Testis	Mild interstitial edema/ accumulation of fluids in between seminiferous tubules. Mild proliferation of Leydig cells.	Foci of seminiferous tubular edema and mild degenerative changes. Mild hyperplasia of Leydig cells. Mild edema / accumulation of fluids in between tubules
Seminal vesicle	Normal morphology	Foci of mild degenerative changes in mucosal epithelial cells.
Prostate	Normal morphology of mucosal glands.	Severe atrophy of tubular glands in which massive proliferation of fibrous/ connective tissue surrounding the mucosal epithelial of tubular glands. Atrophy of mucosal epithelial cells /glands with infiltration of multinucleated giant cells.
Bladder	Moderate sub mucosal inflammation and fibrosis.	Severe mucosal transitional epithelial [urothelial] hyperplasia [Red arrow] and severe sub mucosal fibrosis [green arrow]. Accumulation of inflammatory exudate with dead debris and inflammatory cells in lumen.
Pancreas	Normal morphology of islets.	Normal morphology of acinar cells of pancreas. Atrophy of islets of pancreas with apoptosis of beta cells.
Spleen	Normal morphology of lymphatic follicles	Normal morphology of lymphatic follicles were observed
Kidney	Normal morphology.	Atrophy of glomerulus, severe tubular nephritis is associated with tubular/ interstitial inflammation, degeneration and fibrosis [Red arrow]. Some of the tubules are in dysplastic or pre neoplastic stages [green arrow].
Liver	Normal morphology.	Mild foci of infiltration of inflammatory cells found in centri lobular region. Normal portal region with portal vein, bile duct and hepatic artery.
Heart	Normal morphology.	Normal morphology
Lungs	Normal morphology.	Moderate alveolar/ interstitial edemawith inflammation and fibrosis.
Brain	Normal morphology.	Multiple foci of apoptotic neurons. Normal morphology of frontal cortex, hippocampus and cerebral cortex.

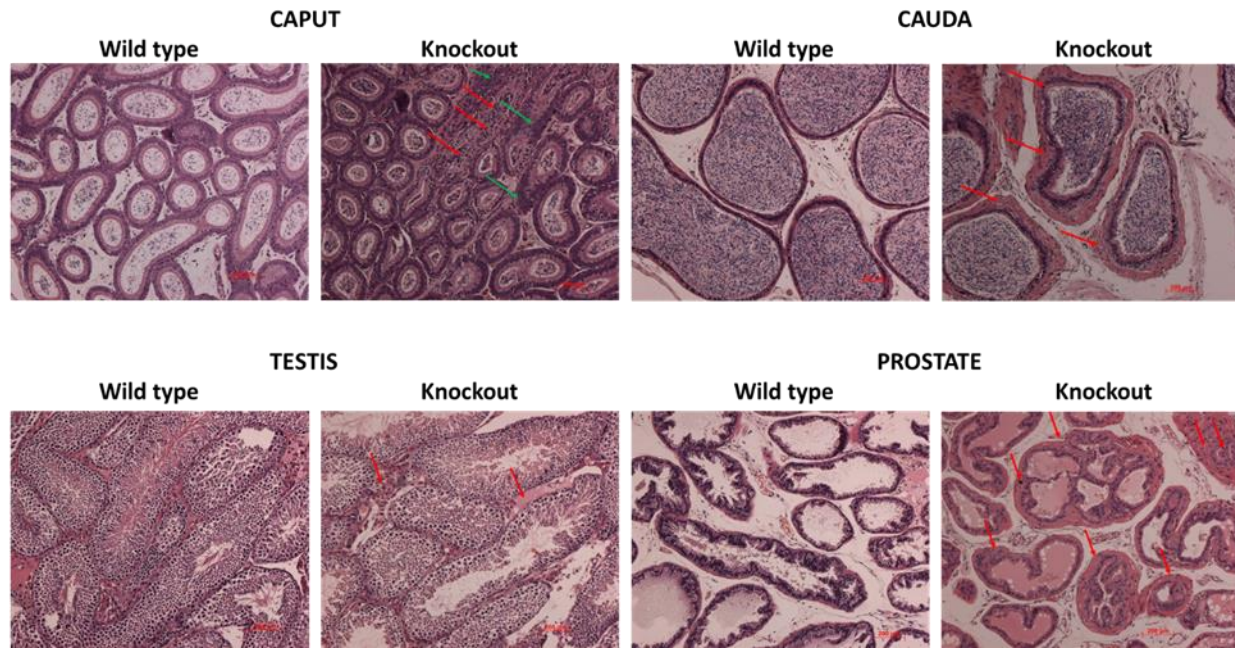


Figure 7. Histopathological analyses of caput, cauda, testis and prostate in wild type and *Upk1a* knockout mice. Five micron sections of the tissues were made and stained with Hematoxylin and Eosin and observed for histopathological changes under a light microscope (magnification: 200 μm). Arrows indicate the histological damage. **Caput:** Moderate inflammation in tubules [red arrow], mild hyperplasia of tubular epithelial cells [green arrow]. **Cauda:** Severe ductular atrophy and dense thickened fibrosis / connective tissue. **Testis:** Seminiferous tubular edema, mild hyperplasia of Leydig cells and accumulation of fluids in between tubules. **Prostate:** Severe atrophy of tubular glands, massive proliferation of fibrous/ connective tissue, atrophy of mucosal epithelial cells /glands and infiltration of multinucleated giant cells.

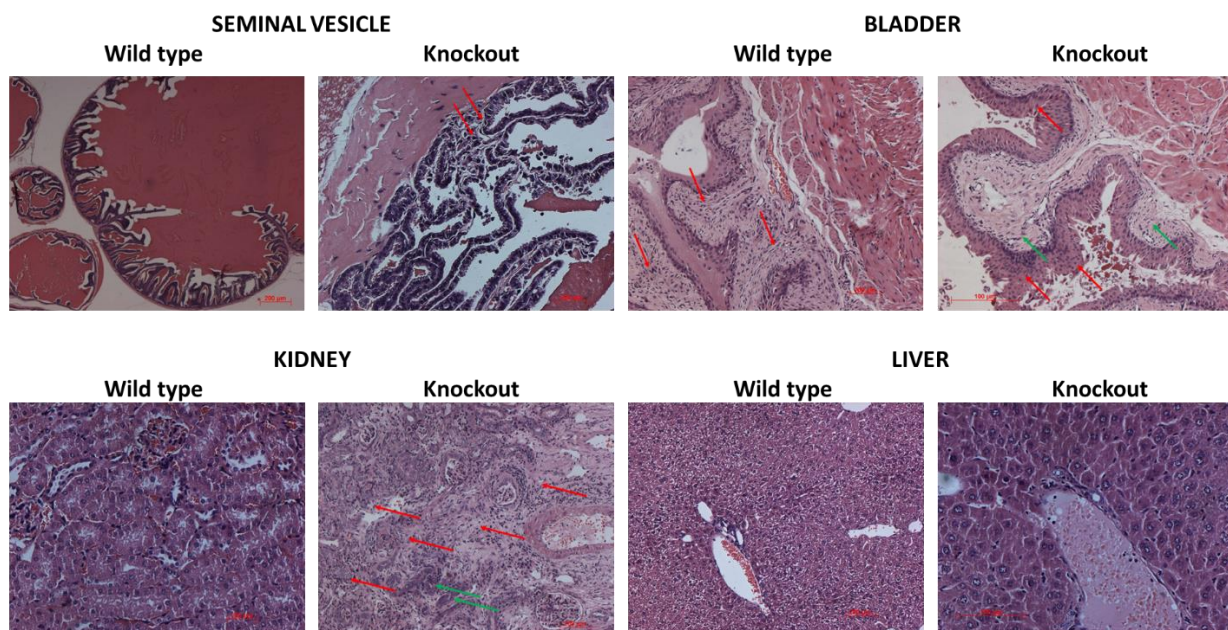


Figure 8. Histopathological analyses of seminal vesicle, bladder, kidney and liver in wild type and *Upk1a* knockout mice. Five micron sections of the tissues were made and stained with Hematoxylin and Eosin and observed for histopathological changes under a light microscope (magnification: 200 μ m; bladder and liver of knockout 100 μ m). Arrows indicate the histological damage. **Seminal Vesicle:** Foci of mild degenerative changes in mucosal epithelial cells. **Bladder:** Severe mucosal transitional epithelial [urothelial] hyperplasia [Red arrow] and severe sub mucosal fibrosis [green arrow]. Accumulation of inflammatory exudate and inflammatory cells in lumen. **Kidney:** Atrophy of glomerulus, severe tubular nephritis and tubular/ interstitial inflammation, degeneration and fibrosis [Red arrow]. Dysplastic or pre neoplastic stages of tubules [green arrow]. **Liver:** Infiltration of inflammatory cells in centri lobular region.

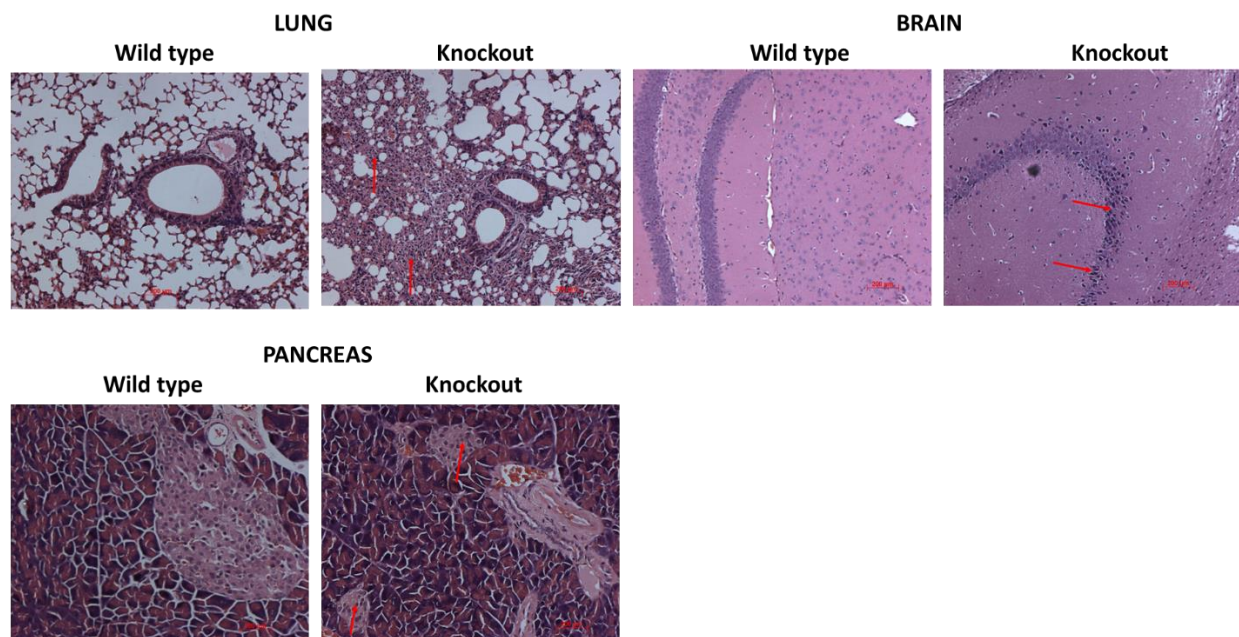


Figure 9. Histopathological analyses of lung, brain and pancreas in wild type and *Upk1a* knockout mice. Five micron sections of the tissues were made and stained with Hematoxylin and Eosin and observed for histopathological changes under a light microscope (magnification: 200 μ m). Arrows indicate the histological damage. **Lung:** Moderate alveolar/ interstitial edema with inflammation and fibrosis. **Brain:** Multiple foci of apoptotic neurons. **Pancreas:** Atrophy of islets of pancreas with apoptosis of beta cells.

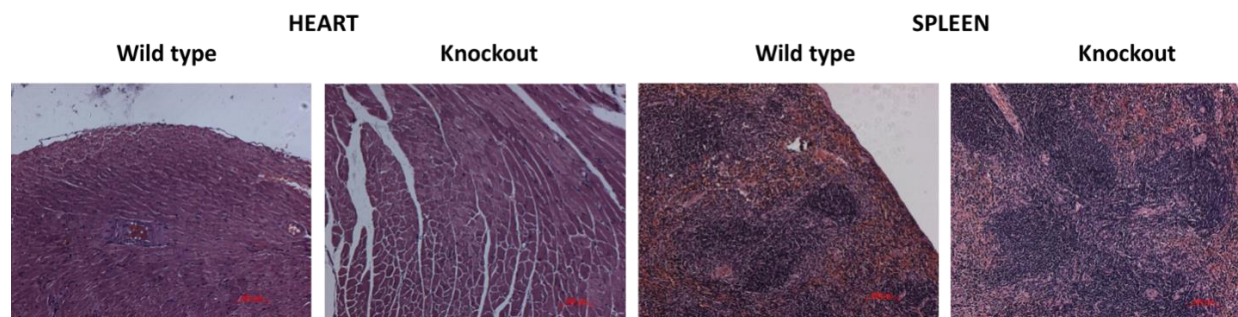


Figure 10. Histopathological analyses of heart and spleen in wild type and *Upk1a* knockout mice. Five micron sections of the tissues were made and stained with Hematoxylin and Eosin and observed for histopathological changes under a light microscope (magnification: 200 μ m).

2.3.5. UPEC infection and bacterial clearance

Since UPK1A is a crucial component of the plaques that protect the urothelium, we analyzed the effect of ablation of this protein on UPEC infection and time dependent efficacy of clearance of the bacterial cells. After day 1 of infection, no significant difference in the number of UPEC was observed in the caput, cauda, testes, seminal vesicle, prostate, bladder, kidney and liver of *Upk1a* knockout mice, when compared to the wild type (Figures 11 and 12). The number of UPEC continued to remain significantly high in all the tissues of knockout mice at 3 days after infection, when compared to the tissues of wild type mice. Similar trend was observed at the 5 day time point (Figures 11 and 12).

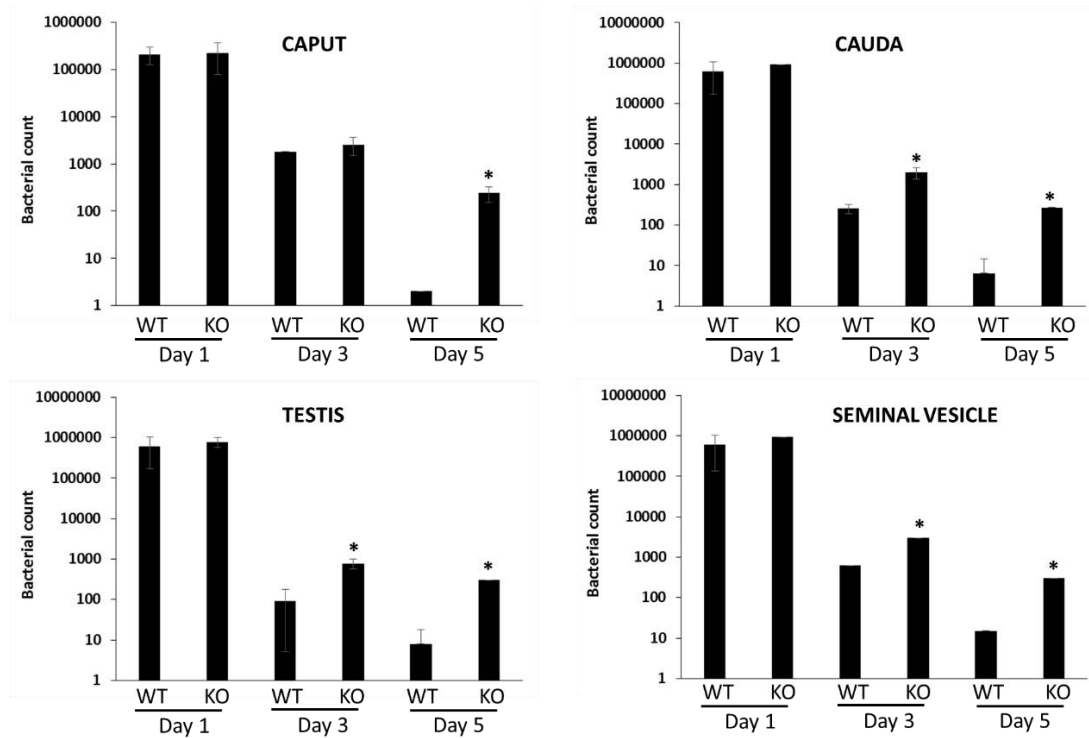


Figure 11. Bacterial clearance in UPEC injected wild type and *Upk1a* knockout mice. Adult wild type and *Upk1a* knockout mice were intraperitoneally injected with 30,000 cfu of UPEC suspended in 100 ul PBS. Caput, cauda, testis and seminal vesicle were collected at 1, 3 and 5 days after injection. Real time PCR was performed for the UPEC specific gene *papC* to determine the number of UPEC present in the tissues. * indicates $p < 0.05$ compared to the wild type at each time point.

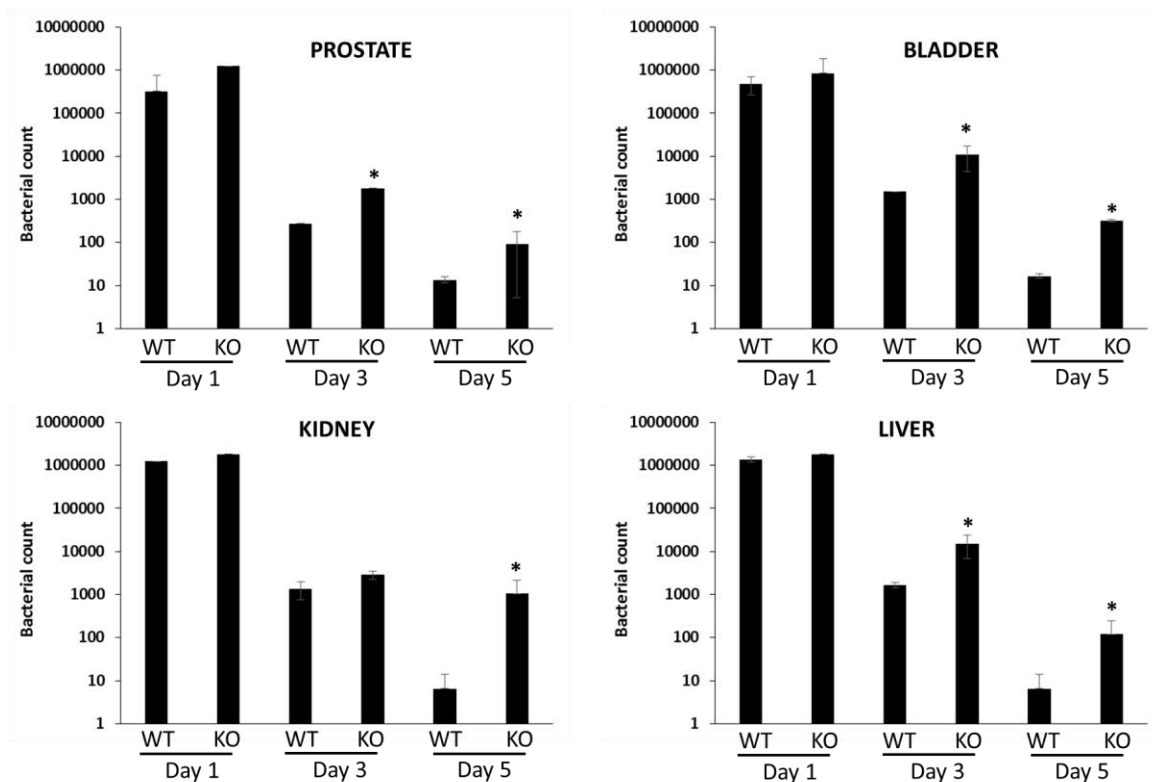


Figure 12. Bacterial clearance in UPEC injected wild type and *Upk1a* knockout mice. Adult wild type and *Upk1a* knockout mice were intraperitoneally injected with 30,000 cfu of UPEC suspended in 100 ul PBS. Prostate, bladder, kidney and liver were collected at 1, 3 and 5 days after injection. Real time PCR was performed for the UPEC specific gene *papC* to determine the number of UPEC present in the tissues. * indicates $p < 0.05$ compared to the wild type at each time point.

2.3.6. Transcriptome analyses

Since the reduced fecundity observed in knockout mice was found to be concomitant with reduced sperm count, we analyzed the differential global transcriptome changes in the testis of these mice in comparison to the wild type mice. Details of the different types of genes that are differentially expressed are presented (Table 3). Among the 34503 genes analyzed, 148 of them were differentially expressed (67 up regulated and 81 down regulated). The list of up and down regulated genes are provided in [supplementary table 1 and 2](#) respectively (***Please click on the link provided to access supplementary data***). Surprisingly, we observed that 1128 genes that are expressed in the testis of wild type mice were completely absent in the knockout mice ([Supplementary table 3](#)). On the contrary, 2330 genes were found to be expressed only in the testis

of knockout mice, but absent in the wild type ([supplementary table 4](#)). Gene ontology using DAVID software was evaluated to understand the possible physiological processes that could be affected by the genes that are unique in the testis of wild type and knockout mice ([supplementary tables 5 and 6](#)). The processes in which at least 20 genes were implicated are listed (Tables 4 and 5). The set of genes that were absent in the testis of *Upk1a* knockout mice (uniquely expressed in wild type) primarily affected the integral components of membrane (plasma membrane), G-protein receptor activity and signaling, olfactory receptor activity and perception of smell and in organization of extracellular space / region (Table 4). The set of genes that were uniquely expressed in *Upk1a* knockout mice (absent in wild type mice) predominantly affected the integral components of membrane (plasma membrane), G-protein receptor activity and signaling, olfactory receptor activity and perception of smell, organization of extracellular space / region, immune and inflammatory responses to pathogens (Table 5). We also conducted gene ontology analyses for genes that were differentially expressed ([Supplementary table 7](#)). Interestingly, we observed that genes involved in spermatid development, meiotic cell cycle, regulation of gene expression and synaptonemal complex were differentially regulated (Table 6).

Table 3. Distribution of genes in the testis of *Upk1a* knockout vs wild type Mice

	Coding	Noncoding (lncRNA, snoRNA, snRNA)	Unassigned	sRNA	Ribosomal	tRNA	Others	TOTAL
Genes analyzed (in number)	19421	4934	8499	0	2	0	1647	34503
Differentially expressed	58	30	52	0	0	0	8	148
Up regulated*	29	13	21	0	0	0	4	67
Down regulated [#]	29	17	31	0	0	0	4	81

*Log2FC > 1.0 and p < 0.05; [#]Log2FC < -1.0 and p < 0.05;

Table 4. Processes influenced by genes that are absent in the testis of *Upk1a* knockout mice (unique to wild type)

Category	ID and Description	Count
CC	GO:0016021- Integral component of membrane	173
CC	GO:0005886- Plasma membrane	153
MF	GO:0004930- G-protein coupled receptor activity	122
BP	GO:0007186- G-protein coupled receptor signalling pathway	104
MF	GO:0004984- Olfactory receptor activity	91
BP	GO:0007608- Sensory perception of smell	84
CC	GO:0005615- Extracellular space	57
CC	GO:0005576- Extracellular region	49
CC	GO:0005887- Integral component of plasma membrane	40
BP	GO:0007165- Signal transduction	31
MF	GO:0005549- Odorant binding	31

Table 5. Processes influenced by genes that are uniquely expressed in the testis of *Upk1a* knockout mice (absent in wild type)

Category	ID and Description	Count
CC	GO:0016021- Integral component of membrane	309
CC	GO:0005886- Plasma membrane	258
BP	GO:0007186- G-protein coupled receptor signalling pathway	159
MF	GO:0004930- G-protein coupled receptor activity	157
CC	GO:0005615- Extracellular space	147
BP	GO:0007608- Sensory perception of smell	114
MF	GO:0004984- Olfactory receptor activity	114
CC	GO:0005576- Extracellular region	102
BP	GO:0007165- Signal transduction	56
CC	GO:0005887- integral component of plasma membrane	54
CC	GO:0009897- Eexternal side of plasma membrane	42
BP	GO:0045087- Innate immune response	41
BP	GO:0006955- Immune response	35
MF	GO:0005549- Odorant binding	35
BP	GO:1990830- Cellular response to leukemia inhibitory factor	34
BP	GO:0042742- Defense response to bacterium	31
BP	GO:0019236- Response to pheromone	30
MF	GO:0016503- Pheromone receptor activity	30
BP	GO:0071222- Cellular response to lipopolysaccharide	23
MF	GO:0005125- Cytokine activity	22

BP	GO:0006954- Inflammatory response	21
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Table 6. Processes influenced by genes that are differentially expressed in the testis of *Upk1a* knockout vs wild type mice.

Category	ID and Description	Count
BP	GO:0007186- G-protein coupled receptor signalling pathway	9
MF	GO:0004930- G-protein coupled receptor activity	9
BP	GO:0007286- Spermatid development	5
BP	GO:0051321- Meiotic cell cycle	5
BP	GO:0010468- Regulation of gene expression	5
CC	GO:0000795- Synaptonemal complex	5

2.4. Discussion

UPKs initially thought to play a critical role in protecting the urothelium have increasingly been reported for their multifunctional role in many normal physiological processes besides their implication in pathological conditions. The functional role of UPKs have been evaluated in transgenic animal model systems. *Upk2* and *Upk3a* knockout mice exhibit significant alterations in bladder function (Aboushwareb *et al.*, 2009). Abnormal targeting and defective glycosylation of *Upk1b* was reported in *Upk3* knockout mice (P. Hu *et al.*, 2000). Using *Upk1b* knock mice, the importance of this protein in urinary tract development, urothelial differentiation and homeostasis was demonstrated (Carpenter *et al.*, 2016). In terms of reproductive function, *Upk2/3a* double knockout mice were sub fertile (Liao, Chang, Liang, Chung, Wei, Nguyen, Zhou, Talebian, Krey, Deng, Wong, Chicote, Grifo, Keefe, Shapiro, Lepor, Wu, DeSalle, Garcia-España, *et al.*, 2018). In view the limited information on the role of UPKs in male reproductive function, we used *Upk1a* knockout mice to gain further insights.

The relative organ weights of caput epididymis and testis were significantly reduced in the *Upk1a* knockout mice indicates the importance of this protein in developmental processes of these organs. Alterations in the maturation of gametes and gamete delivery organs and the implication of *Upk3b* was demonstrated (Kuriyama *et al.*, 2017). The decreased weight of epididymis and testis could have contributed to the decreased fecundity observed in this study. On the other hand, significant increase in the relative organ weight of bladder, kidney and prostate was observed.

UPKs have been implicated in a variety of cancers (Matuszewski *et al.*, 2016). The increase in the weight of bladder, kidney and prostate could be due to the loss of controlling mechanisms of *Upk1a* both at developmental and in adult stages. However, in depth investigations are required to determine the role of *Upk1a* in controlling the mechanisms that govern organ development and cellular homeostasis. Histopathological analyses revealed inflammation, hypertrophy and degenerative changes in the epididymis, testis, prostate and seminal vesicle of *Upk1a* knockout mice. Such histopathological changes in the male reproductive tract tissues implicated with loss of *Upk1a* were not reported previously. It is possible that the histological damages in the male reproductive tract tissues could have contributed to deficiency in spermatogenesis (due to altered composition of seminal secretions of seminal vesicles and prostate) thereby resulting in reduced fecundity observed in this study. Anatomical damages were also observed in the bladder, kidney, liver, lungs and brain. Limited information is available on the histopathological changes that occur under conditions of *Upk* knockdown. Loss of *Upk3* was associated with urothelial barrier defect (Zwaans *et al.*, 2021). Accelerated renal parenchymal loss was evident in *Upk1a* knockout mice (Jackson *et al.*, 2018). The damage to liver, lung and brain observed in the *Upk1a* knockout mice indicate a far reaching role of this protein in multiple organ systems. Further studies are warranted to decipher the physiological, cellular and molecular aspects in these organs in *Upk1a* knockout mice.

The role of UPKs in mammalian reproductive physiology is not well reported. In this study, the male reproductive function in terms of fecundity and spermatogenesis appear to be hampered in *Upk1a* knockout mice, though capacitation and acrosome reaction was not affected. Reduced fertilization rates and decreased fecundity was previously reported in *Upk3a* knockout mice (Liao, Chang, Liang, Chung, Wei, Nguyen, Zhou, Talebian, Krey, Deng, Wong, Chicote, Grifo, Keefe, Shapiro, Lepor, Wu, DeSalle, Garcia-Espana, *et al.*, 2018). It is established that the average litter size of C57BL/6J mice strain is 5 to 11 and it depended on the maternal weight gain (Finlay, Liu, Ermel, & Adamson, 2015). Our results indicate that the average litter size of wild type mice was 9.81 ± 1.55 and that *Upk3a* knockout mice was 6.1 ± 1.28 and the difference was statistically significant. It appears that the knockout mice are producing average litter size. However, the average maternal weight gain was not noted in this study. Thus, with the current results with us, it appears to be ambiguous whether the *Upk1a* knockout mice show reduced fertility. We demonstrated the localization of UPKs in male reproductive tract and on spermatozoa

(Babu Munipalli & Yenugu, 2019) and predicted a crucial role for these proteins in this organ system. Results of this study provided evidence that *Upk1a* may govern events in spermatogenesis; and it would be interesting to explore further studies in this direction. It is a very well established fact that *Upk1a* acts as an anchor for UPEC adherence to urothelium. The binding of UPKs to the type I pili of the bacteria induces signaling in bladder cells and play a vital role in urinary tract infections (Thumbikat, Berry, Schaeffer, & Klumpp, 2009; Thumbikat, Berry, Zhou, *et al.*, 2009). In this study, we observed that the number of UPEC continued to remain higher in *Upk1a* knockout mice up to 5 days after challenge, when compared to wild type mice. The absence of *Upk1a* does not allow binding of UPEC to urothelial cells thereby lacking initiate signaling cascades for innate responses to clear the bacterium. Further, a variety of factors such as cytokines play an important role in bacterial clearance (Muñoz-Carrillo *et al.*, 2018). In the transcriptome analyses of this study, we observed that immune and inflammatory responses to pathogens were also affected due to differential expression of genes in *Upk1a* knockout mice. It is possible that the key factors of inflammation, especially cytokines may have a role in the decreased bacterial clearance in this study. Studies directed to evaluate the role of cytokines in bacterial clearance in *Upk1a* knockout mice are warranted.

Extensive transcriptome analysis under conditions of *Upk* gene ablation are not yet reported. Since reduced fecundity was associated with compromised spermatogenesis in this study, we conducted high throughput transcriptome analyses using RNA isolated from the testis. Though differential expression of many genes was observed, an interesting aspect is the unique expression of 2330 genes and absence of 1128 genes in the testis of *Upk1a* knockout mice. Gene ontology analyses indicated that these genes are involved in integral components of membrane (plasma membrane), G-protein receptor activity and signaling, olfactory receptor activity and perception of smell, organization of extracellular space / region, immune and inflammatory responses to pathogens. It is well established that UPKs are trans membrane proteins and form an integral part of the urothelium not only in the bladder but in other urinary tract tissues (Matuszewski *et al.*, 2016). The perturbations in the processes related to plasma membrane, G-protein signaling and receptor activity are in line with the aspects of UPK localization and function. The implication of odorant receptors in sperm chemotaxis is gaining importance (Ali *et al.*, 2021). It is possible that an interplay may exist between *Upk1a* and olfactory receptors. Significant reduction in the levels of UPK3a was associated with inflammation in observed in patients with chronic spinal cord Injury

and recurrent urinary tract infections (S. Y. Wu *et al.*, 2022). Inflammation in bladder tissues after irradiation in mice was associated with decreased UPK3 expression (Zwaans *et al.*, 2021). The differential expression of genes that play role in inflammation due to *Upk1a* knock down reinforces the role of UPKs in many disease processes. In the set of genes that were differentially expressed in the testis of *Upk1a* knockout vs wild type mice, processes that belonged to spermatid development, meiotic cell cycle, regulation of gene expression and synaptonemal complex were affected; all of which are crucial in spermatogenesis. Knockdown of cyclin-dependent kinase 7 (CDK7) resulted in failure of meiosis initiation, DNA repair and synaptonemal complex formation in the spermatogonia of mice (X. Chen *et al.*, 2021). Similarly, Transactive response DNA-binding protein of 43 kDa (TDP-43) knockout mice displayed synaptonemal defects and failure of meiosis in spermatocytes (Thumbikat, Berry, Schaeffer, *et al.*, 2009). Thus, the differential expression of genes that govern meiosis and synaptonemal complex formation in *Upk1a* knockout mice indicates the role of this gene in spermatogenesis and this could be one of the reasons for the decreased sperm count observed in this study. We observed that large number of genes were exclusively expressed in the testis of knockout mice and at the same time many genes that were expressed in the testes of wild type mice were absent in the knockout mice. Gene ontology analyses revealed that some of the pathways were commonly affected in both these conditions i.e. genes specifically expressed in either WT or KO mice testis. However, the number of genes that are involved vary a lot in each of the pathway under these two different conditions. As a matter of caution, it is to be stated that whether the enrichment or absence of these genes in the knockout mice will affect the pathways in an enhanced or downgraded responses needs in depth investigation.

In conclusion, we report that *Upk1a* knockout mice are sub-fertile with compromised sperm count, and displayed impaired capacitation but normal acrosome reaction. Anatomical damage to the male reproductive tissues (caput, cauda, testis, prostate and seminal vesicle) as well as non-reproductive tissues (bladder, pancreas, kidney, liver and brain) were evident, suggesting that this gene plays role in the functioning of multiple organs. The lower efficiency of bacterial clearance in the tissues of *Upk1a* mice indicates the failure of initiating signaling mechanisms of the urothelium to sense and clear the invading pathogens. Differential expression of genes involved in a variety of physiological process due to *Upk1a* ablation further reinforces the crucial role of this gene in normal and disease processes.

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Chapter-3

Evaluating the functional role of Uroplakin1a at the molecular level using Yeast Two Hybrid Screening

3.1. Introduction

Protein-protein interactions (PPIs) are crucial in many cellular and sub-cellular processes, such as signaling, ion transport, metabolite trafficking, protein folding, etc. Determination of PPIs is initially accomplished by *in silico* methods, which involves phylogenetic profiling, identifying structural patterns and homologous pairs, intracellular localization, and post-translational modifications (Barh *et al.*, 2014). The information thus obtained is deposited in PPI databases generated by large-scale integration efforts, including curating the large number of research papers and reports and creating a controlled vocabulary for describing PPI experiments (Kotlyar, Pastrello, Rossos, & Jurisica, 2018). Mutations in the amino acid sequence of the proteins affect PPIs. Identification of such mutations by computational approaches is very important in understanding the consequences effected by the PPIs in the biological system (DK, P, Uppin, S, & CPD, 2018). Further, PPIs have been implicated in a variety of diseases and validated as potential drug targets. For example, the inhibitors of PPIs are developed as therapeutic targets for the treatment of cancer (Hardcastle, 2016).

Production and maturation of male germ cells involve the interaction of a diverse nature of proteins to mediate structural, biochemical and molecular events that are crucial in this process. The etiology of spermatogenic failure involves Testis-Specific Y-Centric Protein-Protein Interaction Network (Ansari-Pour, Razaghi-Moghadam, Barneh, & Jafari, 2016). Human testis-specific protein TEX101, interactome indicated the interactions between proteins that are essential for spermatogenesis and seminal plasma composition (Schiza *et al.*, 2018). Similarly, the amyloid precursor protein interaction network in human testis entails a large number of sentinel proteins crucial for male reproduction (Silva *et al.*, 2015). A combination of RNA and protein profiling data when combined with network interactions identified a large set of genes associated with spermatogenesis in humans and mice (Petit *et al.*, 2015). Interaction of epididymal proteins with sperm proteins has been an active area of study for many decades and continues to be an active area of investigation. For example, rat specific epididymal proteins interaction with sperm was demonstrated in 1980 (Adrina, Fernanda, Gonzalez, Lucrecia, & Jorge, 1980) Interaction of proteins of epididymal origin with spermatozoa.). The sperm proteins are extensively modified due to interaction with the epididymal luminal proteins and these interactions are crucial for sperm function and fertilization (Björkgren & Sipilä, 2019). The protein interactome of Human

Epididymis protein 4 (HE4) plays a crucial role in fertilization, specifically in sperm maturation, motility and capacitation (Kant *et al.*, 2019). The interaction of EPPIN, an epididymis specific protein with proteins in the seminal fluid presented the diversity of PPIs of different organs (Mariani *et al.*, 2020). The interaction of cystic fibrosis transmembrane conductance regulator (CFTR) with fibroblast growth factor (FGF) and luteinizing hormone (LH) are implicated in azoospermia and epididymal maldevelopment caused by cryptorchidism (Hadziselimovic, Verkauskas, & Stadler, 2022).

In the previous chapters, we demonstrated that UPKs are abundantly expressed in the male reproductive tract tissues and on sperm. Ablation of Upk1a by knockout strategy resulted in subfertility in the mice. Further, their expression was modulated during endotoxin / bacterial challenge. It is thus pertinent that these proteins may govern a variety of physiological, cellular, biochemical and molecular processes. The possibility of UPK interaction with proteins that are involved in male gametogenesis and sperm function is worth investigating. Hence, in this part of the study, we report the interacting partners of UPK1A and UPK2 using yeast two hybrid screening technique.

3.2. Materials and methods

3.2.1. Yeast two hybrid screening

The yeast two hybrid screening (Y2H) was performed using Matchmaker Gold Yeast Two-Hybrid System (Takara Cat. No. 630489) to identify the possible interacting partners of UPK1A. It is based on the principle that when the DNA binding domain and activation domain that are expressed as fusion proteins with the bait and prey proteins respectively are in close proximity, transcription of ADE2 and HIS occurs which allows the survival of the yeast on synthetically defined medium that is devoid of certain amino acids (Figure 1).

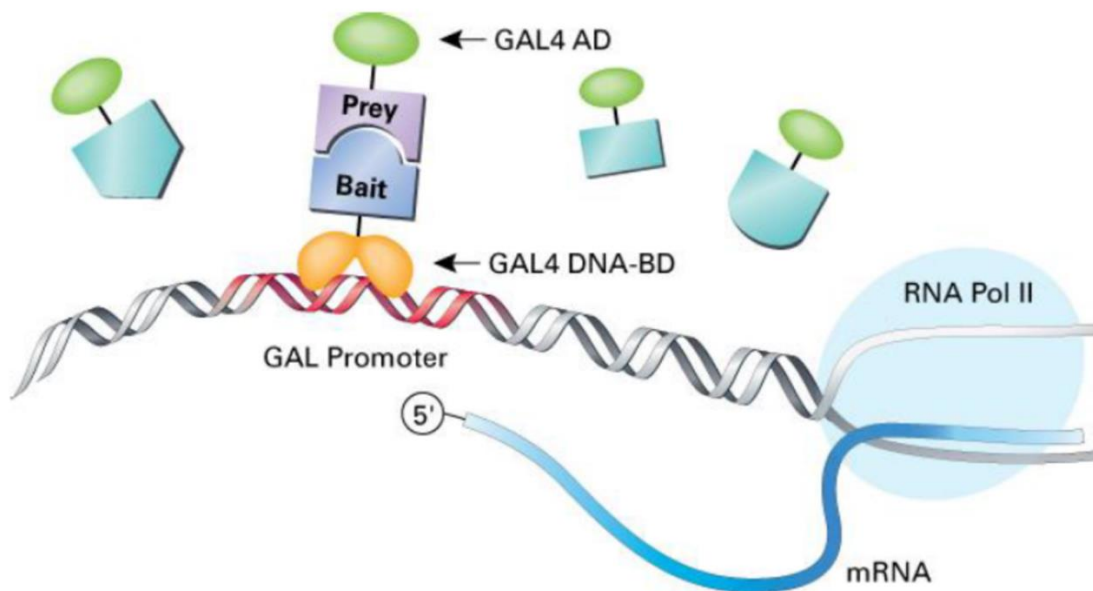


Figure 1. Principle of the yeast two hybrid screening.

The entire procedure consisted of the following basic steps (Figure 2)

- Validation of control plasmids
- Auto-activation and toxicity determination
- Identification of UPK1A interacting partners in the testis cDNA library
- Confirmation of interaction between the partners

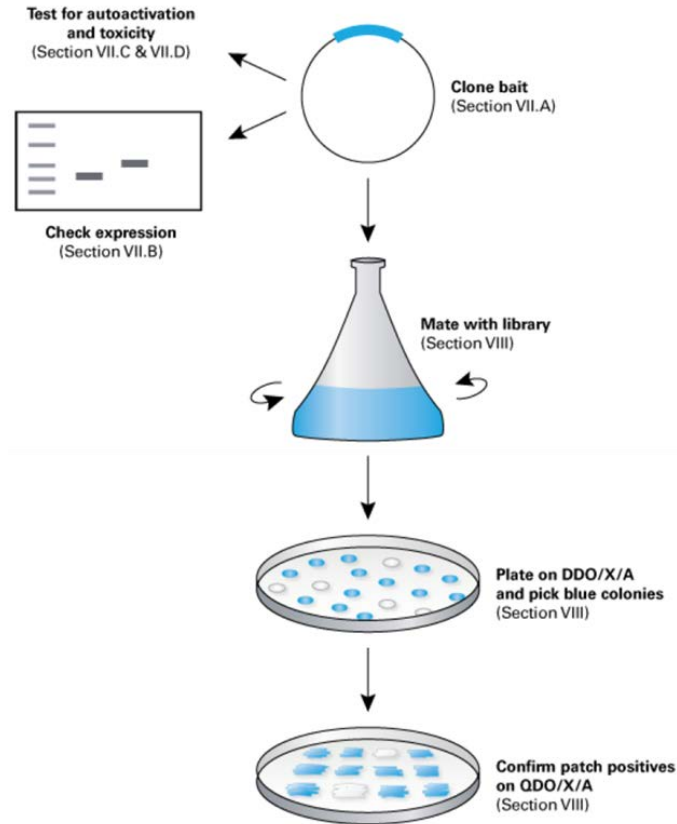


Figure 2. Over view of the yeast 2 hybrid screening procedure.

3.2.2. Performing control experiments for validation

Yeast strains and plasmids

- Y2H Gold - *Saccharomyces cerevisiae* strain Y2H Gold detects interactions between the proteins through four reporter gene expressions (*AUR1-C*, *ADE2*, *HIS3*, and *MEL1*).
- Y187 - *Saccharomyces cerevisiae* strain Y187 for prey vector construct transformation and library preparation.
- pGBKT7-BD – Vector that encodes the Gal4 DNA binding domain.
- pGADT7-AD – Vector that encodes the DNA activation domain.
- pGBKT7-53 – Gal4 DNA-BD fused with murine p53
- pGBKT7-Lam – Vector that encodes lamin linked with the DNA binding domain
- pGADT7-T – Vector that encodes large T-antigen SV40 linked with the activation domain.

Preparation of yeast culture medium and plates

A mixture of yeast extract, peptone, and dextrose was mixed to prepare YPD media essential for the growth of *Saccharomyces cerevisiae* strains (Y2H Gold and Y187 strains). The synthetically defined (SD) medium is frequently used to cultivate *S. cerevisiae* that satisfies all yeast cells' nutritional requirements supplemented with carbon and nitrogen. Minimum medium is prepared by adding essential amino acids to the SD medium. Which plasmids and activated reporters are chosen will depend on the specific minimum medium that is used. Leucine and tryptophan are left out of the formulation (or "Dropped Out"), which is why the medium is known as SD/-Trp/-Leu dropout. The SD/-Trp/-Lue dropout medium is employed for choosing pGBKT7, the bait, and pGADT7, the prey plasmids. The other essential amino acids, such as glycine and aspartic acid are present in the medium. Since the plasmids encode genes for biosynthesis of tryptophan, and leucine, the cells carrying Matchmaker bait and prey plasmids can thrive. As part of the two-hybrid assay's confirmation step, the SD/-Trp/-Lue/-His/-Ade dropout nourishment is used to choose the bait and prey plasmids to activate the Gal-responsive HIS3 and ADE2 genes. The colonies that develop on it likewise produce proteins that interact to trigger HIS3 and ADE2 and contain both bait and prey plasmids. After the two-hybrid screen, this medium is used to verify interactions.

Culture of yeast strains and transformation with control plasmids

As an initial step, the yeast strains Y2H Gold and Y187 were grown on YPD plates for use in further experiments (Figure 3). The yeast strains were then transformed with control plasmids (pGBKT7-53, pGBKT7-Lam and pGADT7-T) to test their ability to grow on selection media plates (SD/-Trp and SD/-Lue). The transformation procedure, in brief, is the method followed for transforming the control plasmids into competent yeast cells by polyethylene glycol (PEG)/lithium acetate (LiAc) (Takara. Yeastmaker™ Yeast Transformation System Cat. Nos. 630439). 5×10^6 cells from the overnight incubated primary culture were added to 250 ml of YPD broth and incubated for 4-5 hours at 30°C with constant rotation. 1×10^8 cells from the secondary culture were used for small-scale transformation. The cell pellet was resuspended with 500 µl of PEG/LiAc. The carrier DNA 50 µg and 100 ng plasmid DNA were added to the competence cells. Briefly, vortexed cells were incubated for 40 minutes at 42°C, centrifuged at 13,000 rpm for 15

sec, resuspended in 200 µl of sterile water and plated the cell suspension on selective dropout media.

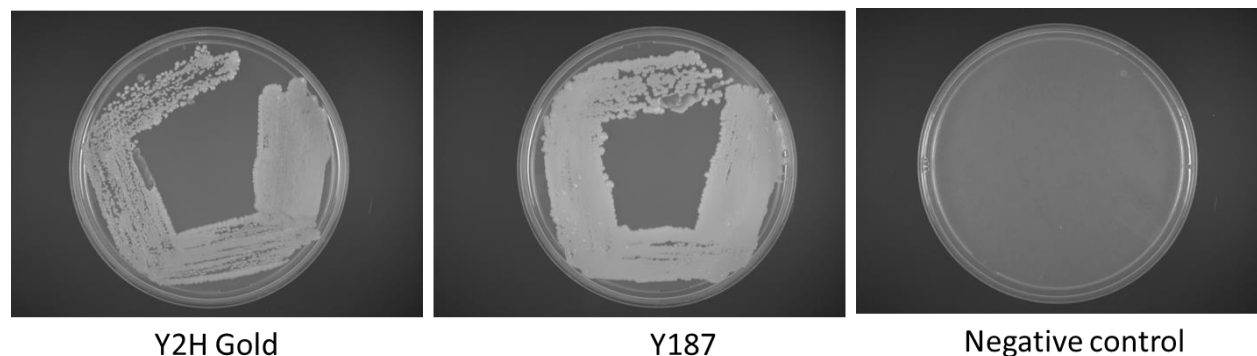


Figure 3. Representative SD agar plates showing the growth of Y2H Gold and Y187. Negative control was included to detect the possible non-specific growth.

Y2H Gold and Y187(pre-y strain) transformed with the control plasmids displayed growth on SD/-Trp and SD/-Lue agar plates (Figure 4). Colonies that appeared on the SD/-Trp and SD/-Lue plates were streaked onto the fresh plate to confirm their growth (Figure 4). Since the yeast transformed with control plasmids showed growth on selection media, the next step was to perform mating of Y2H Gold transformed pGBKT7-53 with prey strain transformed pGADT7 to demonstrate a positive interaction. Similarly, mating was also conducted between Y2H Gold transformed with pGBKT7-Lam and prey strain transformed with pGADT7-T interacts negatively. Subsequent cross mating among these two conjugants were plated on 2X YPD agar plates. Growth was observed for the conjugants both in the positive and negative interaction experiments (Figure 5). Yeast colonies from the 2X YPD plates were then streaked on to quadruple dropout (SD/-Trp/-Lue/-His/-Ade) agar plates. Growth was observed in conjugants resulting from the mating of Y2H Gold transformed pGBKT7-53 with prey strain transformed pGADT7-T, indicating a positive interaction (Figure 6). Growth was not observed in conjugants that were derived from Y2H Gold transformed with pGBKT7-Lam and prey strain transformed with pGADT7-T mating, indicating a negative interaction. Thus, the authenticity of the plasmids, mating and interaction was established (Figure 6).

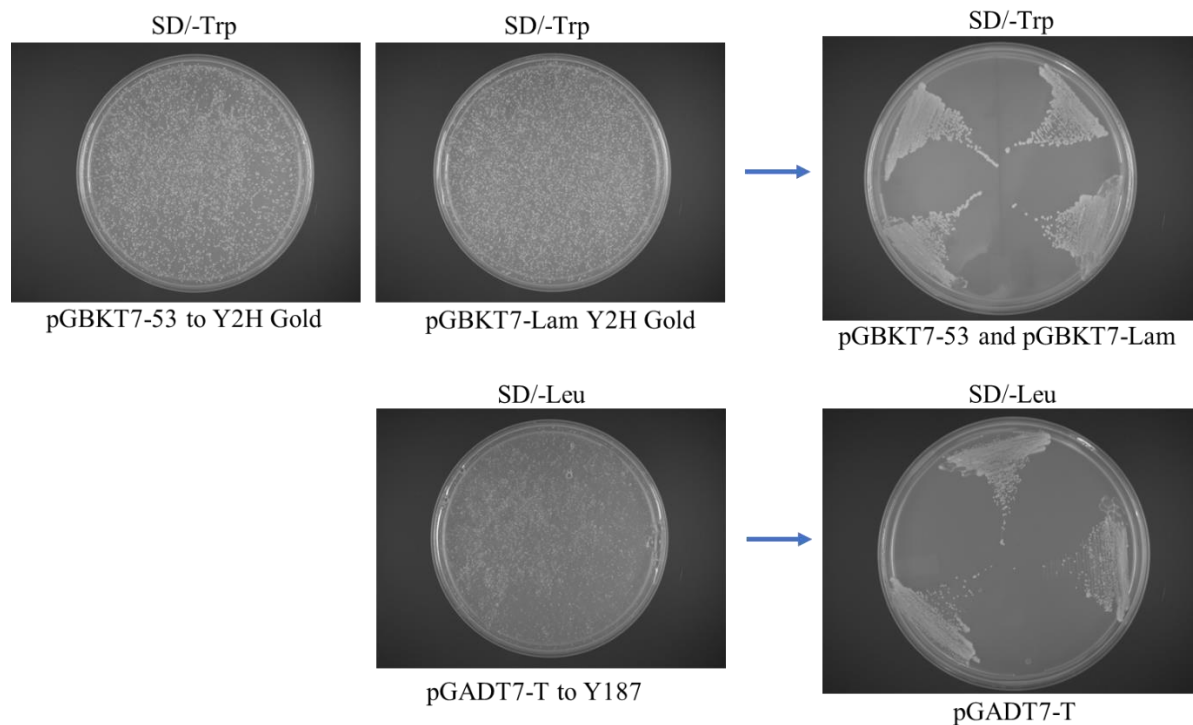


Figure 4. Representative SD/-Trp and SD/-Leu showing the growth of Y2H Gold and Y187 transformed with pGBKT7-53, pGBKT7-Lam and pGADT7 control experiments plasmids. Negative control was included to detect the possible non-specific growth. Colonies that appeared on SD/-Trp and SD/-Leu plates were streaked on to fresh SD/-Trp and SD/-Leu plates. Representative SD/-Trp and SD/-Leu agar plates showing the growth of Y2H Gold and Y187 transformed with control plasmids. Negative control was included to detect the possible non-specific growth.

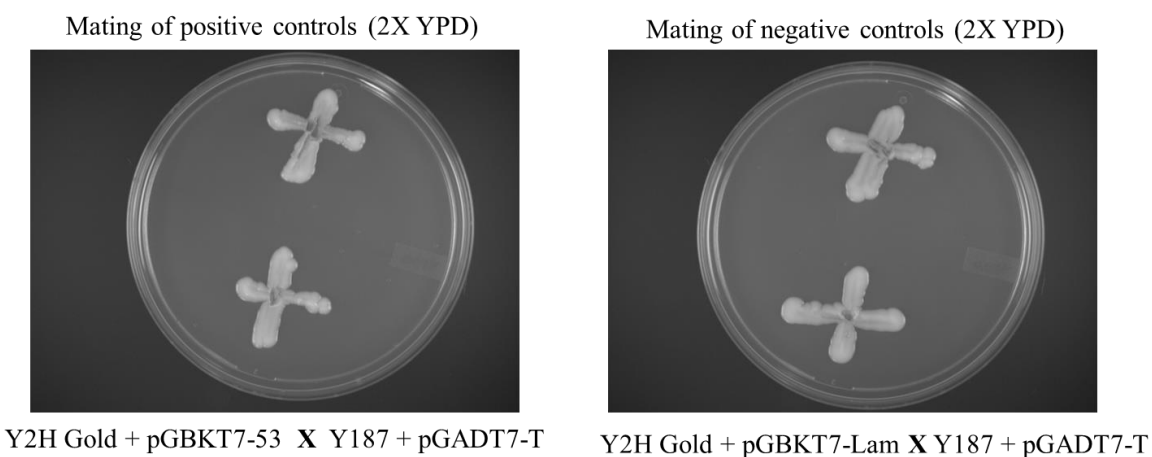


Figure 5. Mating of positive controls. Y2H Gold and Y187 were transformed with pGBKT7-53 and pGADT7-T, respectively. The two strains were cross-mated, and the conjugants streaked on 2X YPD agar plates. **Mating of**

negative controls. Y2H Gold and Y187 were transformed with pGBKT7-Lam and pGADT7-T, respectively. The two strains were cross-mated, and the conjugants streaked on 2X YPD agar plates. Representative 2X YPD plates showing the growth of conjugants.

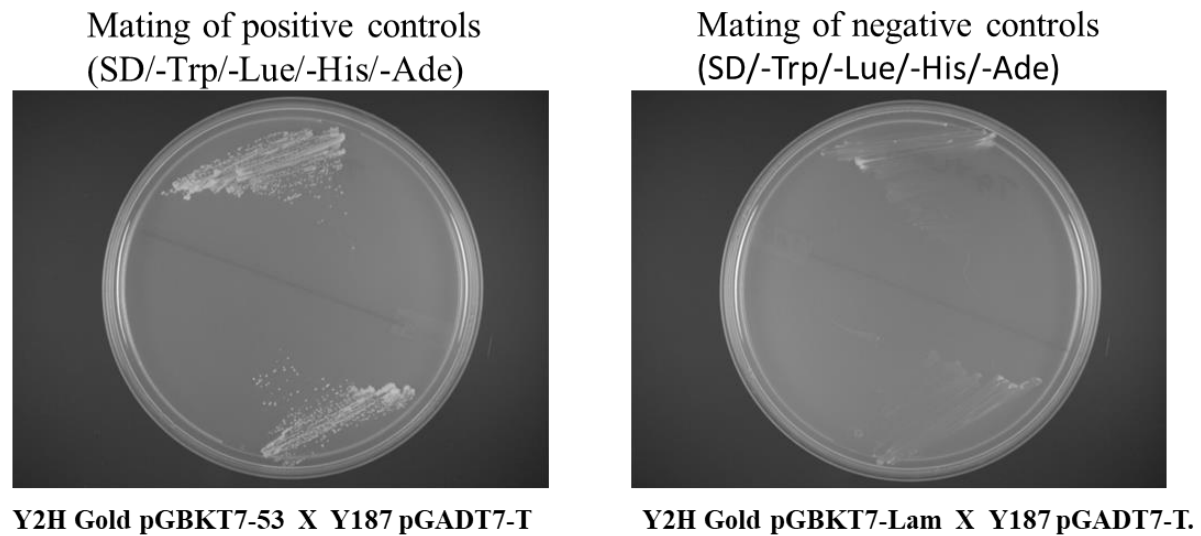


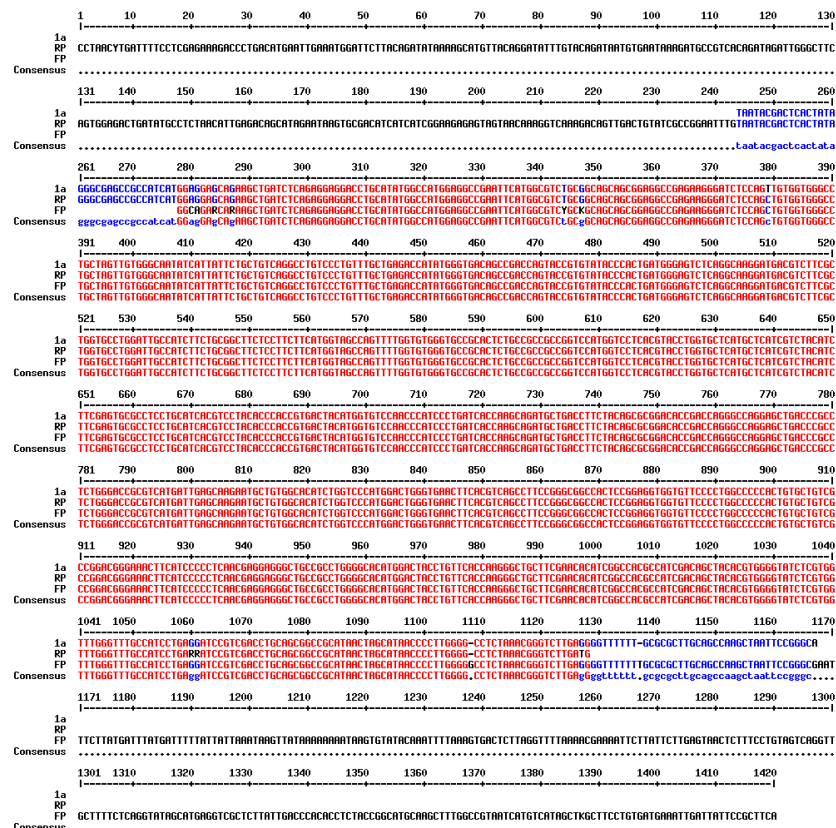
Figure 6. Mating of positive controls. Y2H Gold and Y187 were transformed with pGBKT7-53 and pGADT7-T, respectively. The two strains were mated, and the conjugants that showed growth on 2X YPD plates were then streaked on quadruple dropout (SD/-Trp/-Lue/-His/-Ade) agar plates. Mating of negative controls. Y2H Gold and Y187 were transformed with pGBKT7-Lam and pGADT7-T respectively. The two strains were mated, and the conjugants that showed growth on 2X YPD plates were then streaked on quadruple dropout (SD/-Trp/-Lue/-His/-Ade) agar plates. Representative SD agar plates show the growth of conjugants.

3.2.3. Cloning and testing the bait for auto-activation and toxicity

To determine the interaction partners of UPK1a and UPK2, primers were designed to amplify their coding sequence (Table 1). Using human testis cDNA, UPK1a was amplified. It was then cloned into pGBKT7 vector between the BamH1 and EcoR1 restriction digestion sites. The plasmid (pGBKT7-UPK1A) was then sequenced for the presence of the cloned fragment and proper orientation (Figure 7). Y2H Gold yeast were transformed with pGBKT7-UPK1A and pGBKT7-UPK2. Auto activation test was performed by plating the transformed with pGBKT7-UPK1A and pGBKT7-UPK2 plasmids on the SD/-Trp and SD/-Lue Plate. No growth was observed on SD/-Lue, indicating no auto-activation for these genes (Figure 8). Further, toxicity test was

Table 1. Primers used for cloning Upk1a and Upk2

Name	Sequence 5'-3'	RE site	Length (bp)	GC (%)	Tm ⁰ C	Size (bp)
H UPK1A pGBKT7 FP	gccgaattcATGGCGTCTGCGGCAGCAGC	EcoRI	29	66	68.6	840
H UPK1A pGBKT7 RP	ggcggatccTCAGGATGGCAAACCCAAACC	BamHI	30	60	67.1	
H UPK2 pGBKT7 FP	gccgaattcGACTTCAACATCTCAAGCCT	EcoRI	29	48	61.5	498
H UPK2 pGBKT7 RP	ggcggatccTTACTTGCGGGAGCCCAGTG	BamHI	29	66	68.6	



98



Figure 8. Autoactivation and toxicity tests. For determination of autoactivation, Y2H Gold yeast transformed with pGBKT7-UPK1A and pGBKT7-UPK2 were plated onto SD/-Trp and SD/-Lue agar plate. To determine toxicity, Y2H Gold yeast transformed with pGBKT7-UPK1A, pGBKT7-UPK2 and pGBKT7 empty vector were streaked on the SD/-Trp plate and growth was observed.

3.2.4. Identification of UPK1A / UPK2 interacting partners in the testis cDNA library

Y2H Gold yeast transformed with pGBKT7-UPK1A or pGBKT7-UPK2 were mated with Y187 yeast transformed with human testis cDNA library (Takara, Cat. No. 630470) were plated on SD/-Trp/-Lue agar plates. Resulting colonies were handpicked and streaked onto fresh SD/-Trp/-Lue agar plate. Representation of the pattern of streaking is shown in Figure 9. Colonies that showed growth on SD/-Trp/-Lue plates were then streaked onto double (SD/-Trp/-Leu), triple (SD/-Trp/-Leu/-His) and quadruple (SD/-Trp/-Leu/-His/-Ade) dropout agar plates. The number of colonies picked for UPK1A and UPK2 are shown in tables 2 and 5. Colonies that showed growth on quadruple dropout plates were selected and colony PCR performed. The amplicon was then sequenced and the identity of the interacting partner was determined by using BLAST tool available at NCBI.

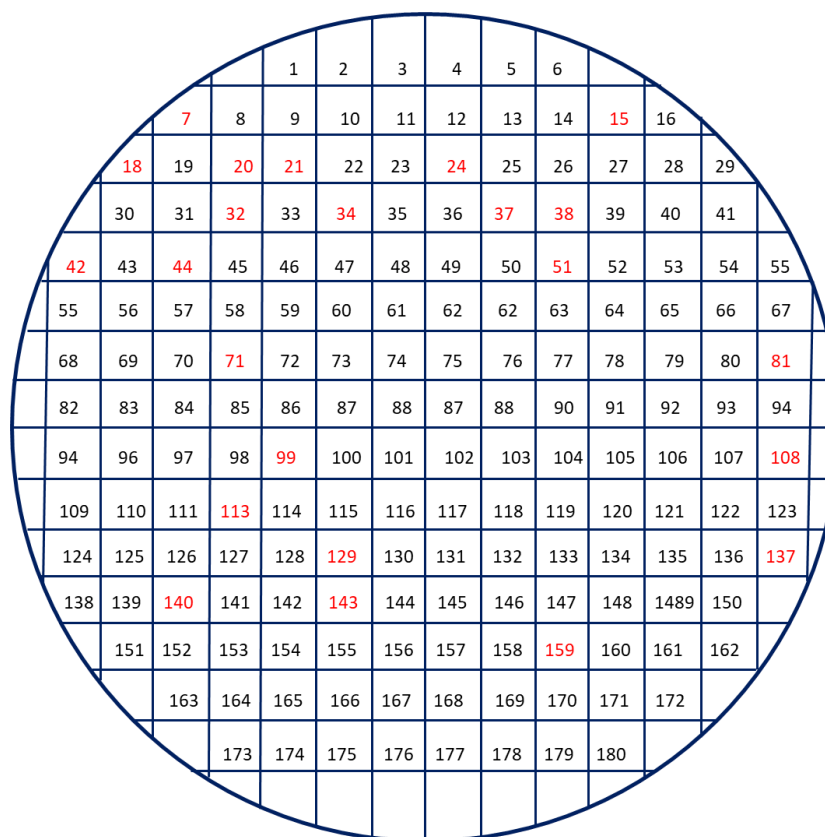


Figure 9. Representative diagram of the pattern of streaking of colonies on SD/-Trp/-Lue agar plate.

3.2.5. Confirmation of interaction

To confirm the interaction between human UPK1A and the possible partners, mammalian cell culture system was used. Upk1a was cloned immediately next to pDsRed2, a red fluorescent protein of pDsRed2-N1 vector, such that the expression of UPK1a-DsRed2 fusion protein is detected by the red color under a fluorescence microscope. Similarly, the interacting partner of UPK1a or UPK2 is cloned immediately next to EGFP, a green fluorescent protein, of pEGFP-N1 vector, such that the expression of interacting partner-EGFP fusion protein is detected by the green color under a fluorescence microscope. The cloned plasmids (3 ug) mentioned above mixed with P3000™ Reagent 6ul, Lipofectamine™ 3000 Reagent 6ul (Invitrogen Cat, No. L3000008) 250 ul of Opti-MEM (Gibco™, Cat. No. 11058021) were added to a culture of 1×10^6 HEK adherent cells in a 60mm plate under Opti-MEM. The complete medium was than replaced after 5 hours. Twenty-four hours after the transfection, the cells were observed under a fluorescence microscope

(Lawrence & Mayo) with the excitation and emission set to 561 nm and 580 to 620nm band pass respectively to visualize the expression of UPK1A-DsRed2 (red color) and the interacting partner-EGFP with the excitation and emission 488nm and 500 to 550 band pass (green color). Further, to determine the probability of co-localization / interaction of UPK1A and its interacting partner, HEK cells expressing these proteins were subjected to high resolution microscopy (Leica TCS SP8 Laser Scanning confocal Microscope). Further analyzes these images with Leica, Software: LAS X Version 3.5.7.23225 and Fiji software 1.0.

3.3. Results

To determine the various molecular factors that may interact or assist UPK1a for its role in male reproductive tract, we directed our studies to determine the possible interaction factors using yeast two hybrid screening. We used the Matchmaker *GAL4* two-hybrid assay. In this assay, a bait protein is articulated as a fusion to the Gal4 DNA-binding domain (DNA-BD), and the libraries of prey proteins are expressed as fusions to the Gal4 activation domain. In the Matchmaker Gold Yeast Two-Hybrid System, when bait and library fusion proteins interact, the DNA-BD and AD are brought into proximity to activate the transcription of four independent reporter genes (*AUR1-C*, *ADE2*, *HIS3*, and *MEL1*).

3.3.1. Identifying the interaction partners of UPK1A and UPK2

Conjugants of Y2H Gold strain transformed with pGBKT7-UPK1A or pGBKT7-UPK2 and mated with Y187 strain transformed with testis cDNA library when plated on double drop out plates (SD/-Trp/-Leu) formed colonies. The colonies that appeared on double drop out SD-agar plates were then sequentially transferred to triple (SD/-Trp/-Lue/-His) and quadruple dropout (SD/-Trp/-Lue/-His/-Ade) agar plates. The number of colonies transferred from each of the plates are presented in table 2. The colonies that showed growth on the quadruple dropout plate were selected and yeast colony PCR was performed. The resulting amplicons were sequenced to know the identity of the interacting partner. From the sequencing results, we identified 36 possible interacting partners of UPK1A as shown in table 3. Among the 36 possible interaction partners identified, for a few of them, the coding region appears to be interacting with UPK1A (Table 4). We noticed that the sequence obtained for Regucalcin (RGN) and Proteasome subunit beta 1

(PSMB1) was completely matching with their full coding region and this region appears to interact with UPK1A. Hence, these two proteins were selected for further analyses.

Table 2. Details of colonies picked to identify the interacting partners of UPK1A

SD Plate	Colonies picked up (n)	Colonies observed (n)
SD/-Trp/-Lue (mated culture spreading)	--	So many
SD/-Trp/-Lue	1600	1600
SD/-Trp/-Lue/-His	1600	550
SD/-Trp/-Lue/-His/-Ade	550	200

Table 3. Possible interacting partners of UPK1A identified by colony PCR with Testicular cDNA librariy of *Homo sapiens*

Colony No	UPK1A interacting Sequence matched mRNAs
34	Pecanex 1 (PCNX1)
51	Guanosine monophosphate reductase (GMPR)
99,3,113	Helicase for meiosis 1 (HFM1)
129	Tubulin alpha 3c (TUBA3C)
159,3, 3	Threonyl-tRNA synthetase 2, mitochondrial (TARS2)
78	Long intergenic non-protein coding RNA 608 (LINC00608)
81	chromosome 8 clone RP11-300I14 map q24.13
121	Immunoglobulin lambda gene locus DNA, clone:288A10
127	Mitochondrial ribosomal protein L13 (MRPL13)
1	Collagen type XI alpha 1 chain (COL11A1)
3, 46	Chromosome 17, clone RP11-676J12
4	Y-chromosome specific repetitive DNA family (DYZ1)
5	Similar to ribosomal protein S8, clone IMAGE:3447074
11	Regucalcin (RGN), transcript variant 4
15	SET binding factor 2 (SBF2), RefSeqGene (LRG_267)
19	Acetyl-CoA carboxylase alpha (ACACA)
23	Methyltransferase like 26 (METTL26)
24	BAC clone RP11-323K15 from 7
25	S-transferase mu 3 (GSTM3)

26, 3	Structural maintenance of chromosomes 4 (SMC4)
33	Coiled-coil domain containing 87 (CCDC87)
35	cDNA clone IMAGE:4826441
36	Ornithine aminotransferase (OAT)
37	Zinc finger protein 17 (ZNF17)
39	TAR (HIV-1) RNA binding protein 1 (TARBP1)
40	Acetoacetyl-CoA synthetase (AACS)
42	Component of oligomeric golgi complex 7 (COG7)
47	Maestro heat like repeat family member 7 (MROH7)
51	Chromosome 5 clone CH17-477F8
52	Regulator of G protein signaling 22 (RGS22)
56	Striatin 4 (STRN4)
66	Proteasome subunit beta 1 (PSMB1)
66 2 nd	Clone HTL-S-45 testicular secretory protein Li 45 mRNA
88	SURP and G-patch domain containing 2 (SUGP2)
106	Cytoplasmic polyadenylation element binding protein 2 (CPEB2)
108	Tubulin alpha 3c (TUBA3C)

Table 4. Possible interacting partners in which the coding sequence may interact with UPK1A

Gene Name	3'UTR	CDS	3' that matches with known sequence UTR	CDS that matches with known sequence	No of Nucleotides	No of Amino acids	No of amino acids matched
Regucalcin (RGN)	1-990	991-1674	No	1045-1674	630	227	19-227(210)
Glutathione S-transferase mu 3 (GSTM3)	1-310	311-988	169-310	311-988	820	225	No
Ornithine aminotransferase (OAT)	1-253	254-1573	No	1202-1573	372	439	124
TAR (HIV-1) RNA binding protein 1 (TARBP1)	1-76	77-4866	No	4276-4866	591	1621	1425-1621(197)
Regulator of G protein signaling 22 (RGS22)	1-128	129-3923	101-128	129-182, 468-723	338	1264	1-18, 114-199(104)
Striatin 4 (STRN4)	1-33	34-2316	No	1647-2316	670	760	539-760 (220)
Proteasome subunit beta 1 (PSMB1)	1-65	66-791	28-65	66-791	764	241	241
SURP and G-patch domain containing 2 (SUGP2)	1-92	93-3341	No	1748-2587	840	1082	553-831

Cytoplasmic polyadenylation element binding protein 2 (CPEB2)	1-193	194-3193	No	3068-3193	126	999	959-999 (41)
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In the case of UPK2A, the number of colonies that appeared on double drop out (SD/-Trp,-Lue) were sequentially transferred to triple (SD/-Trp/-Lue/-His) and quadruple dropout (SD/-Trp/-Tyr/-His/-Ade) agar plates is shown in table 5. Basing on the sequence of the amplicons obtained by colony PCR, 21 possible interacting partners were identified (Table 6). Of these 21, for a few of them, the coding region appears to be interacting with UPK2 (Table 7). The complete coding region of testis expressed 44 (TEX44) and small nuclear ribonucleoprotein polypeptide N (SNRPN) were found to be interacting with UPK2A (Table 7).

Table 5. Details of colonies picked to identify the interacting partners of UPK2

SD Plate	Colonies picked up (n)	Colonies observed (n)
SD/-Trp -Lue (mated culture spreading)	---	So many
SD/-Trp-Lue	600	600
SD/-Trp,-Lue-His	600	150
SD/-Trp-Lue-His-Ade	150	90

Table 6. Possible interacting partners of UPK2 identified by colony PCR with Testicular cDNA librariy of *Homo sapiens*

Col No	Name of the Gene interacting partner for the UPK2
2	Premelanosome protein (PMEL)
6	Nischarin (NISCH)
8, 16, 55, 62,	Chromosome 17 open reading frame 80 (C17orf80)
15, 24,	Pseudopodium enriched atypical kinase 1 (PEAK1)
18	General transcription factor Ili pseudogene 20 (GTF2IP20),
19	Premelanosome protein (PMEL)
25	Signal recognition particle 68 (SRP68)
27	Pre-mRNA processing factor 6 (PRPF6)
29	Transformer 2 beta homolog (TRA2B)
32	Transmembrane p24 trafficking protein 10 (TMED10)
51	BAC clone RP11-33B1 from 4

67	Centromere protein U (CENPU)
73	Transmembrane p24 trafficking protein 10 (TMED10)
76	Sidekick cell adhesion molecule 1 (SDK1)
82	Integrin subunit beta 1 (ITGB1)
84	Testis expressed 44 (TEX44)
85	Solute carrier family 30 member 1 (SLC30A1)
87	Small nuclear ribonucleoprotein polypeptide N (SNRPN),
91	Signal recognition particle 68 (SRP68)
97	Mucin like 3 (MUCL3)
98	Arginine-glutamic acid dipeptide repeats (RERE)

Table 7. Possible interacting partners in which the coding sequence of may interact with UPK2

Gene Name	3'UTR	CDS	5'UTR	3' that matches with known sequence UTR	CDS that matches with known sequence	No of Nucleotides Matched	No of Amino acids	No of amino acids matched
Premelanosome protein (PMEL)	1-86	87-2072	2072-2195	No	1350-2072	723	661	422-661 (240)
Nischarin (NISCH)	1-38	39-4553	4554-5139	No	1739-2302	563	1504	567-754 (188)
Homo sapiens chromosome 17 open reading frame 80 (C17orf80)	1-273	274-1995	1996-3624	No	540-1511	972	609	111-434 (324)
Homo sapiens pseudopodium enriched atypical kinase 1 (PEAK1)	1-576	577-5817	5818-19319		3817-4653	837	1746	1081-1359 (279)
Homo sapiens general transcription factor Ili pseudogene 20 (GTF2IP20), non-coding RNA	2947	No						
Homo sapiens signal recognition particle 68 (SRP68), transcript variant 1	1-26	27-1910	1911-2831	No	861-1796	936	627	279-590 (312)
Homo sapiens pre-mRNA processing factor 6 (PRPF6)	1-114	115-2940	2941-3047	No	1751-2778	1029	941	546-888 (343)
Homo sapiens transformer 2 beta homolog (TRA2B)	1-114	115-1021	1022-4178	No	398-985	588	288	82-277 (196)
Homo sapiens transmembrane p24 trafficking protein 10 (TMED10)	1-33	34-694	695-4109	No	316-693+	378	219	95-219 (125)

Homo sapiens BAC clone RP11-33B1 from 4	130879	No						
Homo sapiens centromere protein U (CENPU)	1-34	35-1291	1292-1494	12-34	34-880	846	418	1-282 (282)
Homo sapiens sidekick cell adhesion molecule 1 (SDK1)	1-335	336..6977	6977-10593	No	4690-5756	1066		
Homo sapiens integrin subunit beta 1 (ITGB1)	1-86	87-2483	2484-3735	No	1909-2483	576	798	599-789 (191)
Homo sapiens testis expressed 44 (TEX44)	1-88	89-1276	1277-1420	1-88	89-1033	945	395	1-315 (315)
Homo sapiens solute carrier family 30 member 1 (SLC30A1)	1-549	550-2073	2074-5893	No	1161-2073	912	507	205-507 (303)
Homo sapiens small nuclear ribonucleoprotein polypeptide N (SNRPN)	1-560	561-1283	1284-1562	94-560	561-1052	492	240	1-164 (164)
Mucin-like protein 3 precursor	1-27	28-4209	4210-5813	No	3286-4209	924	1393	1087-1393 (307)
Homo sapiens arginine-glutamic acid dipeptide repeats (RERE)	1-625	626-5326	5327-8009	No	641-1750	1110	1566	6-375 (370)

3.3.2. Confirmation of the interaction of UPK1A with RGN and PSMB1

To further confirm the interaction of UPK1A with RGN we cloned these genes in two different fusion fluorescent-tagged vectors. The UPK1A was cloned such that it is expressed as a fusion protein with red fluorescent protein (RFP) whereas RGN or PSMB1 as a fusion protein with green fluorescent protein (GFP). HEK cells were co-transfected with plasmids that express UPK1A-RFP and RGN-EGFP displayed red and green fluorescence indicating the expression of these two fusion proteins (Figure 10). Overlay of images taken at higher magnification displayed the presence of red and green fluorescence in the same region of cells (Figure 11), indicating that UPK1A and RGN may be interacting. To gather further evidence on the co-localization / interaction, high-resolution imaging was performed. Co-localization of UPK1A and RGN was also evident in the images obtained by high resolution microscope (Figure 12). To obtain a quantitative assessment of the extent of localization / interaction, Pearson's correlation for multiple images was obtained (Table 8). From the Pearson's Correlation values it appears that the colocalization / interaction rate between UPK1A and RGN was around 57%.

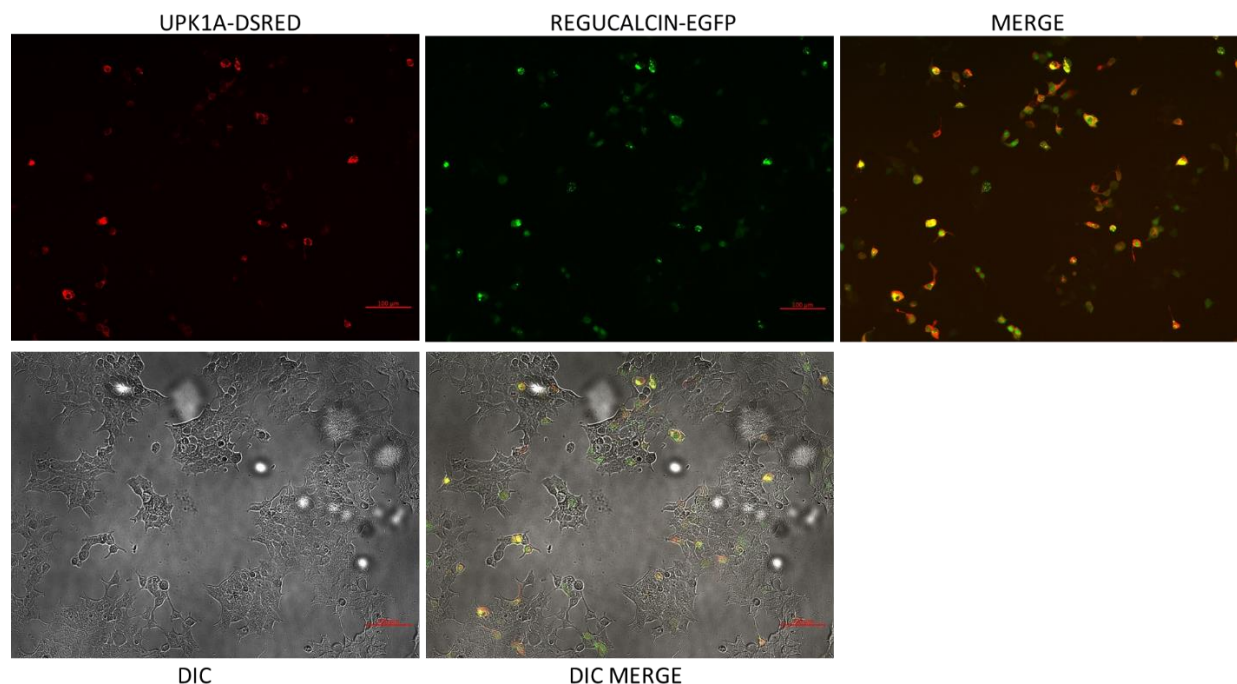


Figure 10. Localization of UPK1A and RGN in HEK cells. HEK cells were co-transfected with plasmids that express UPK1A-RFP and RGN-EGFP as fusion proteins. Red and green colour indicates the expression of UPK1A and RGN respectively. Scale bar: 100 μm .

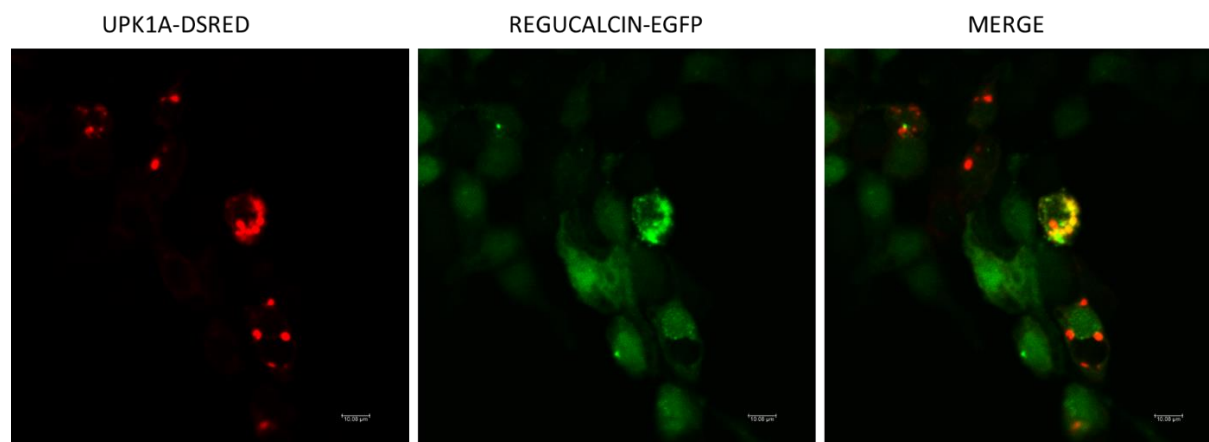


Figure 11. Localization of UPK1A and RGN in HEK cells at higher magnification. HEK cells were co-transfected with plasmids that express UPK1A-RFP and RGN-EGFP as fusion proteins. Red and green colour indicates the expression of UPK1A and RGN respectively. Scale bar: 100 μm .

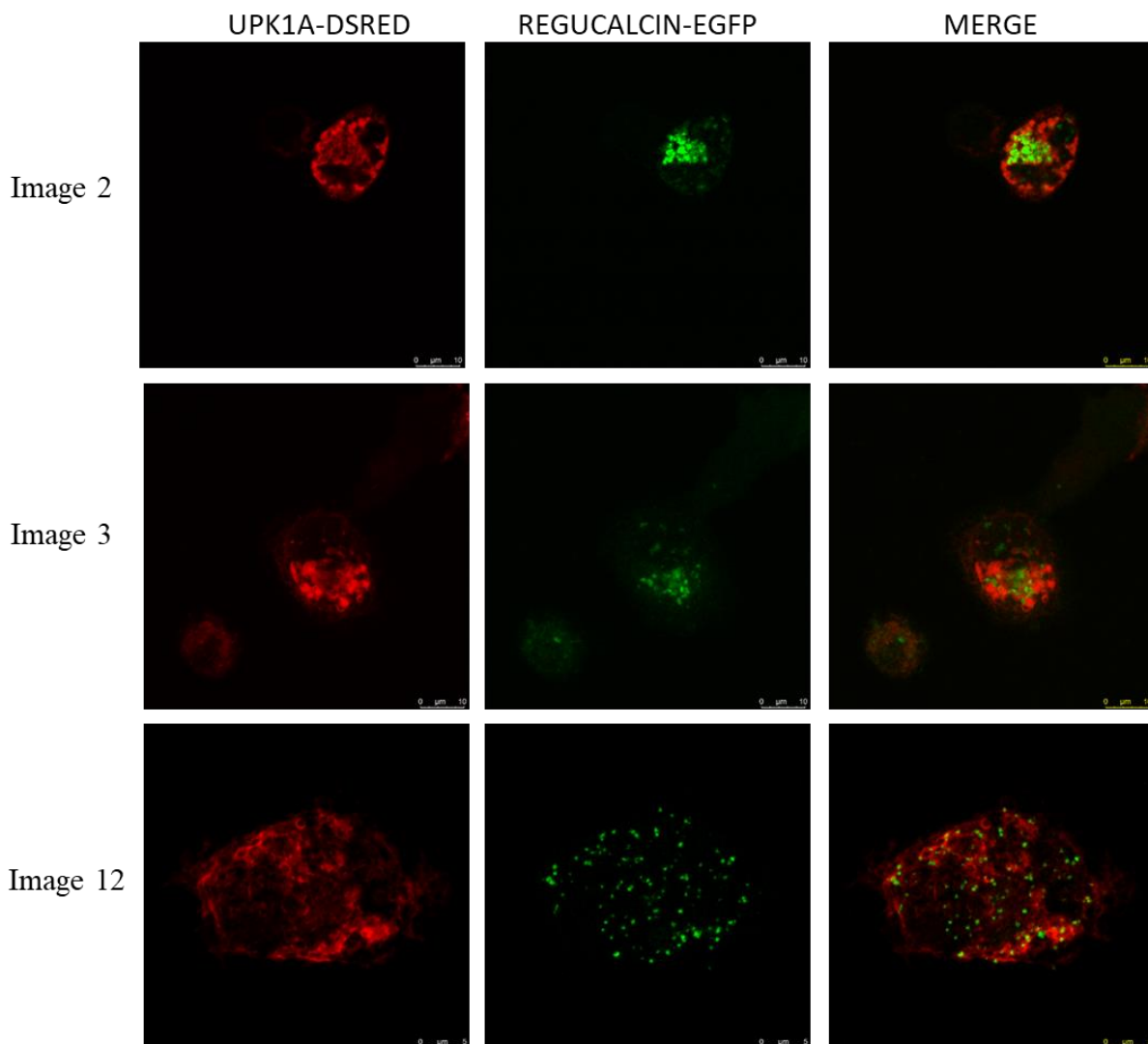


Figure 12. Localization of UPK1A and RGN by high resolution imaging. HEK cells were co-transfected with plasmids that express UPK1A-RFP and RGN-EGFP as fusion proteins. Red and green colour indicates the expression of UPK1A and RGN respectively. Scale bar: For Image 2 and 3, 10 μ m, For Image 12, 5 μ m.

Table 8. Pearson's correlation values for the localization / interaction between UPK1A and RGN.

Image Name	Pearson's Correlation	Overlap Coefficient	Colocalization Rate (%)
2	0.542	0.5819	46.07%
3	0.5513	0.6589	54.02%
5	0.2296	0.4273	15.96%
6	0.7246	0.7492	39.77%

7	0.4667	0.6498	77.04%
8	0.4294	0.6284	63.26%
9	0.5026	0.5787	56.83%
10	0.3182	0.6134	36.27%
11	0.6312	0.7543	87.08%
12	0.5786	0.7516	77.84%
13	0.4373	0.7034	71.98%
Average	0.491954545	0.645172727	56.92%

On the same lines, co-transfection of HEK cells with plasmids that express UPK1A-RFP and PSMB1-EGFP allowed the expression of these two fusion proteins, as indicated by the red and green fluorescence (Figure 13). High resolution microscopy based images also confirmed the co-localization of UPK1A-RFP and PSMB1-EGFP in HEK cells (Figure 14). The extent of colocalization / interaction between UPK1A and PMSB1 was approximately 40% as determined from the Pearson's Correlation values (Table 9).

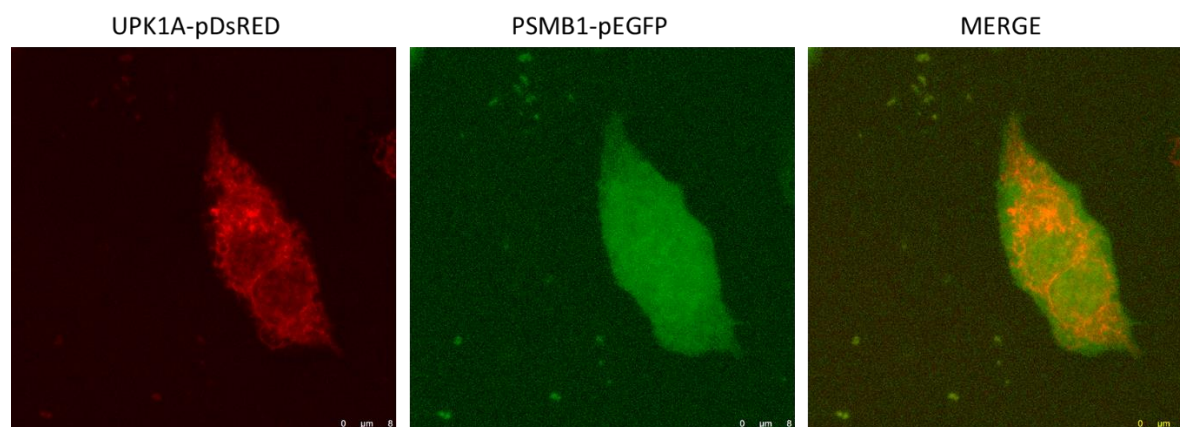


Figure 13. Localization of UPK1A and PSMB1 in HEK cells at higher magnification. HEK cells were co-transfected with plasmids that express UPK1A-RFP and PSMB1-EGFP as fusion proteins. Red and green colour indicates the expression of UPK1A and RGN respectively. Scale bar: 8 μ m.

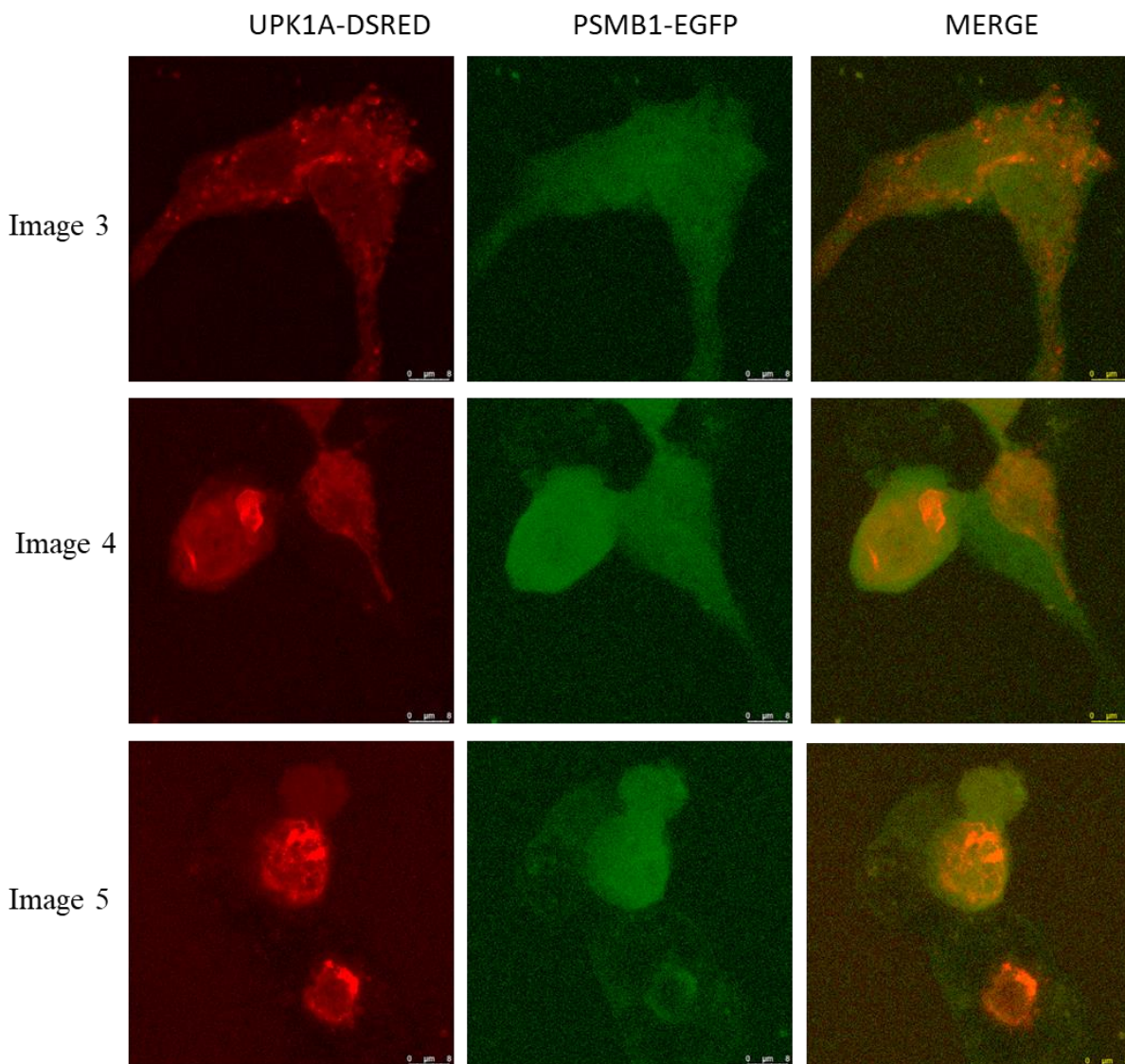


Figure 14. Localization of UPK1A and PMSB1 by high resolution imaging. HEK cells were co-transfected with plasmids that express UPK1A-RFP and PMSB1-EGFP as fusion proteins. Red and green colour indicates the expression of UPK1A and RGN respectively. Scale bar: 8 μ m.

Table 9. *Pearson's correlation values for the localization / interaction between UPK1A and PMSB1.*

Image Name	Pearson's Correlation	Overlap Coefficient	Colocalization Rate
3	0.2986	0.5247	51.06%
4	0.3747	0.5909	55.53%
5	0.2681	0.5107	43.03%
6	0.3582	0.6584	39.27%
7	0.338	0.6088	72.46%
8	0.5009	0.6755	72.04%
9	0.4123	0.6668	67.57%
10	0.4331	0.7169	81.15%
11	0.4322	0.6533	60.55%
Average	0.379566667	0.622888889	60.30%

3.4. Discussion

Cell surface receptors (transmembrane proteins) play role in communication between the cells and biological functions, mainly involved in signal transduction. Their role in the male reproductive physiology, especially during gametogenesis, maturation and fertilization are reported. The ion channel linked receptors, namely, Calcium (Ca²⁺) channels participate in fertilization (Darszon, Nishigaki, Beltran, & Treviño, 2011). The enzyme-linked receptor, c-kit (CD117) type-III receptor tyrosine kinase is involved in proliferation of progenitor cells to gonocytes (Prabhu et al., 2006). Similarly, the G protein-coupled receptor, FSHR, present on Sertoli cells contributes to spermatogenesis (Oduwole, Peltoketo, & Huhtaniemi, 2018). CD9, a tetraspanin, plays an important role in sperm-egg interaction (Jégou *et al.*, 2011). Uroplakins, being transmembrane proteins (monospanins and tetraspanins) are thought to primarily contribute to the urothelial integrity. However, their role as signaling molecules either individually or by interacting with other proteins are not explored. Hence, in this part of the study, we explored the possible interacting partners of UPK1A and UPK2 to determine whether proteins that are crucial to male gametogenesis, maturation and fertilization act in conjunction with Uroplakins.

Results of the yeast two hybrid analyses backed with fluorescence microscopy indicated a strong interaction between UPK1A and regucalcin (RGN), a highly conserved, calcium-binding protein that is preferentially expressed in the liver, kidney and other tissues. It plays an important role in calcium homeostasis and its multifunctional role in the regulation of cell functions in liver, kidney cortex, heart and brain is well studied. It primarily is involved in promotion of urinary calcium transport in the epithelial cells of kidney cortex (Yamaguchi, 2015). Interaction of UPK1A with RGN may have effects on calcium homeostasis and this probably indicates a novel role for UPK1A in the urothelium. Further, RGN contributes to the promotion of dormancy of prostate cancer (Sharma et al., 2021). UPK1A interaction with RGN and its implication on prostate cancer pathogenesis is a new area to be investigated. On the other hand, Ca^{2+} homeostasis in spermatogenesis and sperm function is crucial and its disruption causes male infertility (Laurentino *et al.*, 2012). RGN is widely distributed in the male reproductive tract and exerts a suppressive effect on in vitro sperm capacitation (Pillai et al., 2017). UPK1A knockout caused subfertility in this study indicating a possible interaction between UPK1A and RGN is required for normal spermatogenesis needs to be addressed.

We also report the interaction of UPK1A with proteasome subunit beta type-1 (PSMB1) using yeast two hybrid screening and high resolution fluorescence microscopy. The non-catalytic component of the 20S core proteasome complex is involved in the proteolytic degradation of most intracellular proteins. PSMB1 recognizes degradable proteins, for protein quality control purpose or key regulatory protein components for dynamic biological processes. Compromised proteasome complex assembly and function leads to reduced proteolytic activities and the accumulation of damaged or misfolded protein species; leading to neurodegenerative, cardiovascular diseases, inflammatory responses and autoimmune diseases, and systemic DNA damage responses leading to malignancies. The role of PSMB family members in male reproductive physiology are reported. Meiosis I progression in spermatogenesis requires a type of testis-specific 20S core proteasome (PSMA8) (Q. Zhang, Ji, Busayavalasa, Shao, & Yu, 2019). The proteasome subunit $\alpha 4$ s is essential for formation of spermatoproteasomes and histone degradation during meiotic DNA repair in spermatocytes (Z. H. Zhang *et al.*, 2021). Further, proteolysis required in spermatogenesis mediated by 20S-PA200 complex (UniProt, 2022). RhoS (a new member of Rho GTPases family specific to sperm) associates with PSMB5, a catalytic subunit of the proteasome, in a series of stage-specific spermatogenic cells (N. Zhang *et al.*, 2010). Though the role of PSMB1 family

members in male reproductive physiology is reported by few studies, the role of PSMB1 in spermatogenesis, maturation and fertilization remains unexplored. The implication of UPK1A interaction with PSMB1 on the functioning of spermatoproteasome will be an interesting aspect to study further. In conclusion, we report the possible interaction of UPK1A with RGN and PSMB1 by yeast two hybrid screening and by high resolution microscopy. The implications of these interactions in the male reproductive physiology, especially in gametogenesis, sperm maturation and fertilization remains to be investigated further in detail.

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SUMMARY

Though UPKs have been well characterized in the urinary tract, their role in other organ systems is not well studied. The organs of the male reproductive system have many anatomical similarities with the urinary system and both these are subjected to infections by UPEC, the bacterial species that express the type 1 fimbriae. In light of these similarities and the scarcity of studies on the role of UPKs in other organ systems, it is important to analyze the expression pattern of *Upk* genes and their protein products in the male reproductive system and their possible functional role in male reproduction and innate immunity. Further, in-depth studies that explored the involvement of UPKs at the molecular level in the male reproductive tract are not yet reported. To explore such functional roles, the common practice is to use gene knockout mice. Thus, we generated *Upk1a* knockout mice and analyzed their fecundity, sperm function and responsiveness of the male reproductive tract tissues to UPEC infection. The physiological processes in an organ system are governed by complex interactions of genes and the absence of a specific gene may affect the normal function. Hence, it is important to determine the testicular transcriptome profile of *Upk1a* knockout mice to assess the global impact of under conditions of *Upk1a* gene ablation. Protein-protein interactions are crucial in many cellular and sub-cellular processes, such as signaling, ion transport, metabolite trafficking, protein folding, etc. The sperm proteins are extensively modified due to interaction with the epididymal luminal proteins and these interactions are crucial for sperm function and fertilization. Since UPKs are known to be expressed in the male reproductive tract tissues and on sperm, it is worth analyzing the possible interaction partners of UPKs and their relevance to sperm function. To accomplish this, yeast two-hybrid (Y2H) screening is the standard protocol. Thus, the interaction partners of UPKs were determined by Y2H.

Objective 1: *In silico* and *in vivo* characterization of rat Uroplakins

To the best of our knowledge, for the first time, we report the expression of UPKs in the male reproductive system. *Upk1a*, *Upk1b*, *Upk2*, and *Upk3b* mRNA and their corresponding proteins were abundantly expressed in the caput, cauda, testis, seminal vesicles, and the prostate. Their expression was not developmentally regulated. UPK protein expression was also localized on the spermatozoa, suggesting a role for these proteins in sperm function. In the rat testicular and epididymal cell lines, *Upk* mRNA levels increased in response to lipopolysaccharide challenge.

However, in the caput, cauda, testes, seminal vesicle, and prostate obtained from LPS-treated rats, *Upk* mRNA expression was significantly reduced. Results of this study indicate a role for UPKs in male reproductive physiology and innate immune responses.

Objective 2: Functional characterization of Uroplakin1a (*Upk1a*) using knock-out model

We report that *Upk1a* knockout mice are sub-fertile with compromised sperm count, but with normal sperm function (capacitation and acrosome reaction). Anatomical damage to the male reproductive tissues (caput, cauda, testis, prostate, and seminal vesicle) as well as non-reproductive tissues (bladder, pancreas, kidney, liver, and brain) were evident, suggesting that this gene plays role in the functioning of multiple organs. The lower efficiency of bacterial clearance in the tissues of *Upk1a* knock-out mice indicates the failure of initiating signaling mechanisms of the urothelium to sense and clear the invading pathogens. Differential expression of genes involved in a variety of physiological processes due to *Upk1a* ablation further reinforces the crucial role of this gene in normal and disease processes.

Objective 3: Evaluating the functional role of Uroplakin1a at the molecular level using Yeast Two-Hybrid Screening

Yeast two-hybrid screening was conducted to determine the interaction partners of Uroplakins (UPK1A and UPK2). 37 and 21 positive colonies were obtained on a quadruple plates for screening for UPK1A and UPK2. RGN and PSMB1 were identified as potential interaction partners and the co-localization was evident with immunofluorescence microscopy, high-resolution microscopy, and Pearson's coefficient. The role of regucalcin in sperm calcium dynamics and sperm capacitation is reported (39,40). Since UPK1A knockout caused subfertility in this study; a possible interaction between UPK1A and RGN is required for normal spermatogenesis needs to be addressed. The role of PSMBs in the meiotic progression during spermatogenesis and the formation of spermatoproteasomes is reported (41,42). The implication of UPK1A interaction with PSMB1 on the functioning of spermatoproteasome will be an interesting aspect to study further.

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Uroplakin expression in the male reproductive tract of rat

Suresh Babu Munipalli, Suresh Yenugu*

Department of Animal Biology, University of Hyderabad, Hyderabad 500046, India

ARTICLE INFO

Keywords:

Uroplakins
Epididymis
Testis
Sperm
Lipopolysaccharide

ABSTRACT

Uroplakins (UPKs) play an important role in the normal and pathophysiology of the urothelium. They protect the urothelium and play a crucial role during urothelial infections by Uropathogenic *E. coli*. However, their functions beyond this organ system remain unexplored. A wide variety of proteins secreted in the male reproductive tract tissues contribute to spermatogenesis, sperm maturation, fertilization and innate immunity. However, the presence of UPKs and their possible contribution to the male reproductive tract physiology is not yet reported. Hence, in this study, we characterized UPKs in the male reproductive tract of rats. To the best of our knowledge, for the first time, we report the expression of UPKs in the male reproductive system. *Upk1a*, *Upk1b*, *Upk2* and *Upk3b* mRNA and their corresponding proteins were abundantly expressed in the caput, cauda, testis, seminal vesicles and the prostate. Their expression was not developmentally regulated. UPK protein expression was also localized on the spermatozoa, suggesting a role for these proteins in sperm function. To study the role of UPKs in innate immunity, *Upk* mRNA expression in response to endotoxin challenge was evaluated *in vitro* and *in vivo*. In the rat testicular and epididymal cell lines, *Upk* mRNA levels increased in response to lipopolysaccharide challenge. However, in the caput, cauda, testes, seminal vesicle and prostate obtained from LPS treated rats, *Upk* mRNA expression was significantly reduced. Results of this study indicate a role for UPKs in male reproductive physiology and innate immune responses.

1. Introduction

Uroplakins (UPKs) are a group of proteins that form the major constituents of urothelial plaques. In the mammals, five UPKs, namely, UPK1a, UPK1b, UPK2, UPK3a and UPK3b (a minor isoform of UPK3a) are characterized. Besides these, UPK2b and UPK3c and UPK3d were identified in other species (Desalle et al., 2014, Garcia-Espana et al., 2006, Wu et al., 1994). Mammalian UPKs are divided into two groups. UPK1a and UPK1b belong to the tetraspanin family, whereas UPK2 and UPK3 are grouped under the monospanin family (Yu et al., 1994, Lin et al., 1994, Wu and Sun 1993). The former family of proteins span the plasma membrane four times whereas the later span the plasma membrane only once. The plaques formed by UPKs generates an asymmetric unit membrane (AUM), which in turn functions to provide permeability barrier and structural stability to the urothelium. The formation of plaques involves specific interaction between the UPKs. UPK1a interacts with UPK2 whereas UPK1b interacts with UPK3a (Wu et al., 1994, Hu et al., 2005, Tu et al., 2006, Tu et al., 2002).

The functional significance of UPKs vary across the species. In mammals, though their major function is important for development, differentiation and homeostasis of the urothelium (Carpenter et al.,

2016), in the *Xenopus* oocytes UPK3a and UPK1b form a complex to mediate sperm-egg interaction and fertilization (Sakakibara et al., 2005, Hasan et al., 2011, Mahbub Hasan et al., 2005). In the zebrafish, UPK3a related protein contributes to epithelial cell polarization and morphogenesis of pronephric tubules (Mahbub Hasan et al., 2005). A recent study indicates that the UPK2/3 proteins are related to phosphotyrosine phosphatases and thus may have a role in cellular signaling (Chicote et al., 2017). UPK knock out mice display renal failure and high rates of mortality (Kong et al., 2004, Hu et al., 2000). Mutations in the UPK genes resulted in renal hypo dysplasia, adysplasia and renal failure in humans (Jenkins et al., 2005, Schonfelder et al., 2006). Further, UPKs serve as anchors for Uropathogenic *E. coli* (UPEC) during infections (Wu et al., 2009). They allow binding of the type-1 fimbriae expressing UPEC strains and facilitate their binding to the urothelial surface and triggering a cascade of events that favor bacterial infection and migration not only in the bladder, but also in the upper urinary tract organs such as urethra and renal pelvic urothelia (Wu et al., 1996, Mulvey et al., 1998, Martinez et al., 2000). Altered levels of UPKs in urothelial carcinomas are considered as useful markers for the diagnosis, detection and prediction of urothelial carcinomas (Wu et al., 2009, Huang et al., 2007, Zupancic et al., 2011).

* Corresponding author at: Department of Animal Biology, University of Hyderabad, P.O. Central University, Hyderabad 500046, India.

E-mail addresses: ysnaidu@yahoo.com, sureshsl@uohyd.ernet.in (S. Yenugu).

<https://doi.org/10.1016/j.ygcen.2019.06.003>

Received 26 February 2019; Received in revised form 29 May 2019; Accepted 6 June 2019

Available online 07 June 2019

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ORIGINAL ARTICLE

Tlr1-13, *Nod1/2* and antimicrobial gene expression in the epididymis and testis of rats with alloxan-induced diabetes

Suresh Babu Munipalli | Marri Reddy Mounika | Jamil Aisha | Suresh Yenugu

Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad, India

CorrespondenceSuresh Yenugu, Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad – 500046, India.
Emails: ysnaidu@yahoo.com; sureshsl@uohyd.ernet.in**Funding information**

Indian Council of Medical Research, Grant/Award Number: 61/6/2011/BMS

Abstract

Pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and nuclear oligomerization domain (NOD) receptors along with antimicrobial proteins and peptides (AMPs) are crucial for innate immunity. The pathology of insulin-dependent diabetes mellitus is associated with the disrupted expression of TLRs, NODs and AMPs in the kidney, lungs and other organs. However, such a relation in the male reproductive tract is not yet investigated. In this study, we analysed the expression pattern of *Tlr1-13*, *Nod1/2* receptors and AMPs (β -defensins and defensin-like proteins of the Sperm-Associated Antigen 11 (*Spag11*) family) in the male reproductive tissues (caput, cauda and testis) obtained from diabetic or insulin-treated diabetic or untreated control rats. Alterations in the expression pattern of *Tlr1-13*, *Nod 1/ 2*, *Defb1*, 2, 21, 24, 27, 30 and *Spag11a/ c/ t* were observed under diabetic conditions. Administration of insulin to diabetic rats could modulate the expression pattern of only some these genes. Results of our study indicate perturbed gene expression profile of *Tlrs*, *Nod1/2*, *Defbs* and *Spag11* isoforms in the epididymis and testis of diabetic rats, and this could be one of the important reasons for the increased risk of infections in the male genital tract.

KEYWORDS

antimicrobial, defensin, diabetes, toll-like

1 | INTRODUCTION

Innate immune system confers immediate defence strategies against pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular pattern molecules (DAMPs), and this is accomplished through the recruitment of pattern recognition receptors (PRRs) and production of antimicrobial molecules. PRRs involved are toll-like receptors 1-13 (TLRs 1-13), nuclear oligomerization domain 1 and 2 (*NOD1* and *NOD2*) receptors, RIG-I-like receptors, AIM2-like receptors and the receptor for advanced glycation end products (RAGE; Tang, Kang, Coyne, Zeh, & Lotze, 2012). Signalling pathways effected by these receptors promote the production of antimicrobial proteins and peptides, and these processes are well characterised in reproductive and in other organ systems (Mukherjee, Karmakar, & Babu, 2016). We and other groups demonstrated the abundant expression

of *Tlr1-13* in the male reproductive tract of different species (Biswas et al., 2009; Biswas & Yenugu, 2011; Liman, Alan, & Apaydin, 2019; Palladino, Johnson, Gupta, Chapman, & Ojha, 2007; Palladino, Savarese, Chapman, Dughi, & Plaska, 2008; Pioli et al., 2004). The general gene expression pattern and LPS/uropathogenic *Escherichia coli*-mediated induction of rat *Defbs* and DEFB-like proteins and peptides belonging to the Sperm-Associated Antigen 11 (SPAG11) family were reported in the male reproductive system (Biswas & Yenugu, 2011, 2013, 2014; Yenugu, Chintalgattu, et al., 2006; Yenugu, Hamil, French, & Hall, 2006). The involvement of TLR4 in modulating *Defb* and *Spag11* gene expression via the NF- κ B pathway and epigenetic mechanisms was reported (Biswas & Yenugu, 2013, 2014).

Disruption in the innate immune signalling cascades and the subsequent defects in the expression of antimicrobial peptides are a hallmark of autoimmune diseases, atherosclerosis and diabetes mellitus

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