CHAPTER I

Immunochemical Characterization of Liprin α3
INTRODUCTION
The acrosome formation

The acrosome is an organelle that develops over the anterior part of head in the spermatozoa. It is a cap-like structure derived from Golgi apparatus. The acrosome formation is completed during testicular development. Acrosome contains hydrolytic enzymes like Hyaluronidase and Acrosin. In humans, rhesus monkeys, rat and mice, the synthesis of acrosome-specific proteins, like Proacrosin and Acrogranin, begins at the Pachytene spermatocyte stage (Anakwe et al., 1991, Bermudez et al., 1994) and proteins packed in vesicles called Proacrosomal granules (PAGs) are transported to developing acrosome. These granules do not proceed to the plasma membrane but instead remain on the boundaries of the Golgi apparatus until the completion of meiosis. Therefore, newly formed round spermatids contain these vesicles and vesicle associated proteins (Moreno et al., 2000b, Bermudez et al., 1994, Escalier et al., 1991).

It has been shown that in rhesus monkeys and mice, Syntaxin and the v-SNARE, VAMP envelop the PAGs of step-1 and step-2 spermatids (Moreno et al., 2000a, Ramalho-Santos et al., 2001). At later steps of differentiation, VAMP and Syntaxin localize in the acrosomal vesicle and remain there until the mature sperm is formed. A differential expression of t- or v-SNAREs in PAGs of Pachytene spermatocytes and early spermatids may control the initial steps of acrosome formation. The process of acrosome biogenesis shares many characteristics with the formation of secretory granules in neuroendocrine cells and acrosome reaction shares similarities with secretory exocytosis at synapse (Tooze, 1998).

Shaping of the Acrosome: Role of Membrane Trafficking and the Golgi Apparatus

As represented in Fig. 7, during steps 4–7 in the mouse or equivalent steps in other species, the acrosomal vesicle grows and then flattens over the spermatid nucleus, covering up to two-thirds of the total nuclear surface (Burgos and Fawcett, 1955, Moreno et al., 2000b). Electron microscopy data have suggested that the acrosomal vesicle’s growth in volume is mainly due to the continuous fusion of Golgi-derived vesicles (Susi et al., 1971, Sutovsky et al., 1999). In guinea pigs many convolutions and blebs on the
acrosomal membrane have been interpreted as proof of vesicle fusion (Burgos et al., 1986).

If the spermatids use this mechanism to complete acrosome formation, some Golgi proteins should be present in the acrosome. Immunofluorescence and immunogold studies carried out with probes against Golgi proteins, such as Golgin95/GM130, Giantin, Mannosidase II, and beta-COP, revealed a horseshoe-shaped Golgi apparatus, with the concave side facing the acrosome, in steps 2–7 of rhesus monkey, mouse, and bull spermatids (Moreno et al., 1998, Moreno et al., 2000, Moreno et al., 2000b). But not in late elongating spermatids or mature sperm in mouse, bull, or rhesus monkey (Moreno et al., 1998, Moreno et al., 2000, Ramalho-Santos et al., 2001, Moreno et al., 2000b). The membrane fluidity may help to remodel the acrosomal membrane and may somehow participate in the shaping of the acrosome. This suggests that vesicular traffic machinery is involved in a membrane remodelling process of the acrosome (Moreno et al., 2000b). The acrosome of mature sperm contains both unique acrosomal enzymes and common enzymes of lysosomal origin (Tulsiani et al., 1998). Their presence in the acrosome may reflect a low efficiency of the spermatid sorting machinery, and the SNARE and Rab families of proteins could regulate this process. Thus it is found that, Syntaxin, VAMP, and NSF are present in the acrosomal vesicle throughout acrosome biogenesis (Moreno et al., 2000, Ramalho-Santos et al., 2000, Ramalho-Santos et al., 2001, Moreno et al., 2000b).

**Acrosome**

Acrosome formed by the process described above, seems to be a distinct and complex new vesicle, with a unique identity which is different from any organelle-vesicle in other exocytic or endocytic systems. In mouse spermatids at step 7 (and equivalent step in other species), the acrosome-nucleus complex moves towards the cell periphery and remain attached to the plasma membrane. As a result of this process, the acrosome-nucleus complex rotates so that the acrosome faces the basal membrane rather than the lumen of the seminiferous tubule. This process generates two clear domains in the acrosome membrane that will be of prime importance during the AR. The part of the acrosome membrane lining the nuclear surface is the inner acrosomal membrane (IAM),
and that attached to the plasma membrane is the outer acrosomal membrane (OAM). The OAM fuses with the plasma membrane during the AR in capacitated sperm, but the IAM remains intact even after the sperm fuses with the oocyte plasma membrane as illustrated in figure 8 (Barros et al., 1996, Barros et al., 1967). Thus, these two membrane domains are functionally, and probably molecularly, different. There is no information about the genesis of these differences and how they are maintained over time, but they probably arise during spermiogenesis. One hypothesis is that by step 7 the domains of the acrosomal membrane are already set up and the OAM has SNARE proteins that guide the acrosomal-nucleus complex in its route through the plasma membrane. In this way, proteins such as VAMP, Syntaxin, or Rab could specify the target membrane of the acrosomal-nucleus complex (Ramahlo-santos et al., 2002). The same proteins may have some role during the next step in the biology of the sperm, the AR (Ramahlo-santos et al., 2002).

**Proteins of membrane trafficking**

Important regulators of membrane trafficking are the members of the Rab family of small GTPases (Martinez and Goud, 1998). These proteins play a crucial role in fusion of transport vesicles with their appropriate targets (Somsel et al., 2000, Pfeffer 1999). Rab6 has been found mainly on the Golgi apparatus and as a faint acrosomal label in mouse developing spermatids (Ramalho-Santos et al., 2001). However, rab5-positive vesicles are found in close proximity to the acrosome, in step 4 and step 5 spermatids, and rabaptin-5, a downstream effector for rab5 also has a similar pattern of distribution (Ramalho-Santos et al., 2001). Rab7 was also found to be associated with the acrosomal vesicle in both mouse and bull developing spermatids (Ramalho-Santos and Moreno, 2001). Rab3A thought to modulate the AR and was detected only in the acrosome of epididymal sperm and not in earlier steps of differentiation (Ramalho-Santos et al., 2001, Ramalho-Santos and Moreno, 2001). The calcium-dependent fusogenic activity is controlled by Synaptotagmins (Martinez et al., 2000). Synaptotagmins have been found in both human and mouse sperm, although the isoform present in each species seems to be different (Ramalho-Santos et al., 2000, Hutt et al., 2002, Michaut et al., 2001). Synaptotagmin and Rab3A were reported to interact with RIM (Rab Interacting
Figure 7 Different steps of acrosome development in round spermatid
(Ramalho-santos et al., 2002)

Figure 8 Membranes of Sperm head and process of acrosome reaction.
(A) PM: Plasma Membrane, OAM: Outer Acrosomal Membrane, IAM: Inner Acrosomal Membrane, N: Nucleus. (B) During acrosome reaction OAM fuses with overlaying PM at multiple points thereby releasing acrosomal content (C) Membrane after acrosome reaction EQ: Equatorial Segment (Yoshida et al., 2010)
Molecule) in a calcium dependent manner. However RIM interacts with MUNC13, RIM-BP and Liprins in a constitutive manner (Schoch et al., 2002).

**Liprin α proteins**

The Liprin-α proteins were first identified by their interaction with the LAR-RPTPs (LAR family of Receptor Protein Tyrosine Phosphatases) (Serra-Pages et al., 1995). In vertebrates, four Liprin-α genes are present, Liprin-α1, α2, α3 and α4, whereas *Caenorhabditis elegans* and *Drosophila* have only a single Liprin-α gene: *syd-2* (synapse-defective-2) and *Dliprin/liprin- α* respectively. Liprin-α2 and Liprin-α3 are expressed exclusively in mammalian brain, while Liprin-α1 and Liprin-α4 are also found in non-neuronal tissue (Serra-Pages et al., 1998). Liprin-αs are well conserved, with ~50% amino acid identity between human Liprin-α1 and worm SYD-2 (Spangler and Hoogenraad, 2007). This family of proteins is characterized by N-terminal coiled-coil region that mediates homo- and hetero-multimerization and three SAM (sterile-α-motif) domains making up the LH (Liprin homology) region that binds to LAR-RPTP (Pulido et al., 1995).

**Acrosome reaction**

A mammalian spermatozoon is characterized by two functional parts, both perfectly adapted to perform a number of necessary functions before the sperm can carry out its main challenge, the fertilization of an egg.

After encountering the egg, a series of functionally coupled events are initiated, which comprise reactions mainly mediated by the sperm head, like recognition and binding of the extracellular glycoprotein coat surrounding the egg, sperm-oocyte fusion and the fusion of their nuclei. However, freshly ejaculated sperm are not immediately competent to carry out their main task but have to gain this potency during a series of spatial reorganization and biochemical modification processes, collectively termed “Capacitation”.

This event leads to a “zipper-like” formation of multiple fusion pores which propagate to the posterior part of the acrosome and hence achieve the required entire dispersal of
acrosomal content. The complete fusion of the apical part of the outer acrosomal membrane to plasma membrane is also necessary to assure exposure of the inner acrosomal membrane in order to fuse with the oocyte's plasma membrane.

Studies indicate that acrosomal exocytosis is mediated by molecular mechanisms that are homologous to the secretion of a vesicle at axon endings in neuronal cells. In spite of many similarities AR shows striking difference such as (1) sperm contain a single secretory vesicle; (2) there are multiple fusion points between acrosomal membrane and the overlaying plasma membrane; (3) exocytosis leads to a vesiculation and total membrane loss; and (4) the AR involves no membrane recycling (Michaut et al., 2001). And, there are conspicuous similarities as depicted in Fig. 9. Like Synapse, acrosomal membrane is also special types of membranes and has been compared in different ways. Schuel and Burkman (2005) compared synapse and acrosome exocytosis based on the presence of receptors for neurotransmitters and psychoactive drugs, and they found that these receptors directly affect the sperm function like acrosome reaction. Other investigators compared membrane proteins like t-SNARE, v-SNARE involved in acrosomal exocytosis and neurotransmitter release (Zitranski et al., 2010; Tomes et al., 2002). Fusion of acrosomal membrane is mediated by many membrane associated proteins and involvement of proteins such as Synaptotagmin, Synaptobrevin, SNAP-25 and Rab3a has been reported in acrosome exocytosis (Iida et al., 1999; Katafuchi et al., 2000; Ramalho-santos et al., 2000). Rab3a brings about its action through its interacting molecule RIM which is a known interacting partner of Liprin α3 at synapse (Fig. 9) (Wang et al., 1997; Schoch et al., 2002). These reports indicate that acrosomal exocytosis is mediated by molecular mechanisms that are homologous to the secretion of a vesicle at axon endings in neuronal cells. Proteins like MUNC, Kinesin motors, SNARE proteins, previously considered to be as brain or neuron specific, have now been show to carry out similar function in sperm (Tomes et al., 2002; Bello et al., 2012). Presence of RIM in sperm and its implication in acrosome reaction has been recently reported (Bello et al., 2012).

In case of acrosome, proteins like Rab3 (Iida et al., 1999), SNARE proteins like SNAP-25, Syntaxin, Syntobrevin (Tomes et al., 2002, Michaut et al., 2001 Roggero et al.,
Figure 9 Similarities in the interactome involved in vesicle fusion at the synapse and acrosome reaction. (Mittelstaedt et al., 2010)
2007, De Blas et al., 2005), RIM and MUNC (Bello et al., 2012), actin cyto-architecture change (Brener et al., 2003) and calcium channel VDCC (Florman et al., 1992) have already been reported to be present and implicated in acrosome reaction. However, the presence and implication of Liprin α3 in sperm remains to be elucidated.
MATERIALS & METHODS
Materials

Nitrocellulose membranes (Hybond-C Extra) and ECL Plus™ Western blotting detection reagent, Percoll were obtained from GE Health Care (Buckinghamshire, UK). Protein molecular weight markers from Fermentas (Maryland, USA), Dulbecco's Modified Eagle Medium (DMEM) was procured from Gibco, Delafield's hematoxylin, xylene, paraffin, Hydrogen peroxide (H₂O₂) were procured from Qualigens (Mumbai, India), Chemicals for preparation of Phosphate Buffer Saline (PBS), Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE), Western blotting reagents, urea were purchased SRL India Ltd (Mumbai, India), poly-L-lysine, paraformaldehyde (PFA), 3,3'-diaminobenzidine (DAB), were obtained from Sigma (Steinheim, Germany), Collagenase and DNase I was procured from Sigma (St. Louis, USA), Non-fat dry milk powder (NFDM) was procured from Anikspray (Mumbai, India). Bovine Serum Albumin Fraction V (BSA) was obtained from USB (Cleveland, OH, USA), Complete protease inhibitor cocktail and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Roche (Mannheim, Germany). Trizol was procured from Invitrogen (CA, USA) and Im PromII™ reverse transcription kit was procured from Promega (Madison, WI, USA), Kodak X-ray films purchased from Asset healthcare (Silvassa, India), Tamoxifen citrate tablets were purchased from (Lyka Lab, Mumbai, India).

Antibodies

Polyclonal antibody to Liprin α3 protein (ppfia3), monoclonal antibodies to beta actin were procured from Sigma (St Louis, MO, USA), anti gm130 antibody was purchased from BD Biosciences (California, USA) and HRP and FITC labeled secondary antibody, normal swine sera were from Dako (Glostrup, Denmark). Alexa fluor antibodies were procured from Invitrogen (Oregon, USA).

Animals

Adult inbred randomly selected male Holtzman (HM) rats and adult male Swiss mice used in this study were maintained at a temperature of 22-23°C, humidity of 50-55% and a cycle of 14h light 10h dark with food and water available ad libitum. Male HM rats of age day 5, 10, 20, 30, 40, 50 and 60 were used for ontogeny studies. All animal care
practices and experimental procedures complied with the guidelines of the Care and Prevention Society against Cruelty of Experimental Animals (CPCSEA) and were approved by the Institutional Animal Ethics Committee (IAEC).

Methods

Preparation of reagents:

**General buffers**

**0.1M Phosphate buffered saline (pH 7.4):**

Sodium dihydrogen orthophosphate (NaH$_2$PO$_4$.2H$_2$O) : 2.370 g
Disodium hydrogen orthophosphate (Na$_2$HPO$_4$.2H$_2$O) : 11.536 g
Sodium chloride : 6.800 g
Distilled water : 800 ml

**Lysis buffer:**

SDS : 0.5 g
Distilled Water : 50 ml
Protease inhibitor : 1 tablet

**SDS-PAGE Reagents:**

**30% Acrylamide solution:**

Acrylamide : 29.2 g
N, N'-Methylene bis-acrylamide : 0.8 g
Distilled water : 100 ml

Filtered through 3µm filters in amber colored bottle and stored at 4°C.

**Resolving gel buffer (pH 8.8):**
1.5 M Tris base : 18.16 g

pH adjusted to 8.8 with 1N HCl

Volume made up to 100 ml with distilled water.

**Stacking buffer (pH 6.8):**

1.0 M Tris base : 12.12 g

pH adjusted to 6.8 with 1N HCl

Volume made up to 100 ml with distilled water.

**10% SDS solution:**

10 g of sodium dodecyl sulfate (SDS) dissolved in 100 ml of distilled water.

**10 % Ammonium per Sulphate (APS) solution**

100 mg of APS dissolved in 1 ml of distilled water (Freshly prepared).

**N, N, N', N'- tetramethylenediame (TEMED)**

**Sample buffer (Laemmli buffer):**

10 % SDS : 5.0 ml

Glycerol : 5.0 ml

0.5 M Tris base pH 6.8 : 5.0 ml

β-mercaptoethanol : 0.5 ml

Distilled water : 8.5 ml

Pinch of Bromophenol Blue

**Electrode buffer (pH 8.8):**

Glycine : 14.40 g
Tris base : 3.03 g
SDS : 1.00 g
Distilled water : 1000 ml

Transfer buffer:
Glycine : 14.40 g
Tris Base : 3.03 g
Distilled water : 800 ml
Methanol : 200 ml

Preparation of resolving and stacking gel:

<table>
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<th>Reagents</th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>10 % gel</td>
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</tr>
<tr>
<td>30 % acrylamide solution</td>
<td>3.300 ml</td>
<td>0.330 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>4.000 ml</td>
<td>1.400 ml</td>
</tr>
<tr>
<td>Resolving gel buffer (pH 8.8)</td>
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<td>0.250 ml</td>
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<tr>
<td>(Stacking gel Buffer pH 6.8)</td>
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</tr>
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<td>10 % SDS</td>
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<td>0.020 ml</td>
</tr>
<tr>
<td>10 % APS</td>
<td>0.100 ml</td>
<td>0.020 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004 ml</td>
<td>0.002 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>10.000 ml</td>
<td>2.000 ml</td>
</tr>
</tbody>
</table>
Stripping buffer

Glycine : 1.5 g
Distilled water : 100 ml
pH : 2.5 (1N HCl)

Ponceau S stain:

Ponceau S stain : 0.5 mg
Glacial acetic acid : 1 ml
Distilled water : 99 ml

Immunohistochemistry reagents:

Bouin's fixative:

Saturated picric acid : 75 ml
30 % formaldehyde : 25 ml
Glacial Acetic Acid : 5 ml

Tamoxifen treatment

- Tamoxifen was prepared by dissolving tamoxifen citrate tablet containing 10 mg in water by sonication.
- We administered Tamoxifen to adult male rats orally between 9.00 h–11.00 h via a rat feeding tube.
- The concentration used was 0.4 mg/kg/day for 5 days a week for 90 days. Vehicle control male rats were administered water only.

PHTPP and DPN treatment

- PHTPP and DPN were prepared in DMSO, and administered to Holtzman mature male rats
• PHTPP was administered subcutaneously at 0.08mg/kg/day for 60 days, and DPN was administered subcutaneously at 0.1 mg/kg/day for 60 days.

• Vehicle control male rats were administered DMSO only

**Human sperm sample**

We collected human semen sample from participants attending the infertility clinic at the Institute. Ethical approval was obtained from the Human Ethics Committee for clinical studies of the Institute. Samples were analyzed as per the guideline set by WHO. Samples with normal parameters were included in this study.

**Sperm preparation**

• Sperm were processed for Western blot and indirect immunofluorescence (IIF) experiments as described earlier (Wakle et al., 2005). Briefly, four mice or rats were sacrificed and epididymis was excised and excess of fat pads and connective tissues were removed.

• Caput, corpus and cauda epididymides were transferred to petri plate containing 0.01M PBS pH 7.4. Seven to eight radial cuts were given to the tissue to release spermatozoa. Petri plate was gently shaken for 4-5 min at 37°C.

• Suspension containing sperm was separated and centrifuged at 800 x g for 20 min at 4°C. Purity was checked under phase contrast microscopy. The pellet was subjected to three washes with 0.01M PBS.

• For immunofluorescence studies sperm were fixed in 4% PFA fixative at 4°C for 10 min. Excess fixative was removed by giving three washes of 0.01M PBS. The sperm was then suspended in PBS at a concentration of 1 X 10^5 and were smeared on poly L-lysine coated glass slides. Slides were stored at -80°C till further use.

• Unfixed rat sperm population from all regions of epididymis was treated with 6M urea as per the protocol described by the Escalier et al (1997). Briefly, sperm population was air dried onto glass slide and it was treated with 6M urea solution for 30 min at 4°C. Excess of solution was then washed off by three washes of 0.01M PBS. Smears were fixed as per the above mentioned protocol. Slides were then used for indirect immunofluorescence.
**Testicular cell preparation**

- Testes were detunicated and tubules were minced in pre-warmed DMEM. Supernatant was collected, centrifuged at 800xg for 10-15 min and washed.
- Resultant pellet was either fixed in 4% PFA at 4°C for 10 min or used for protein extraction. Excess of fixative was removed by giving three washes of 0.01M PBS and pellets were suspended in PBS and were smeared on poly L-lysine coated glass slides.
- Fixed testicular cells were permeabilized as and when mentioned by treating first with 4M NaCl for 5 min and then with 0.1% Triton X-100 for 5 min. Excess of detergent was washed off by rinsing slides for 3 times in 0.01M PBS. Slides were stored at -80°C till further use.

**Isolation of Spermatogenic cells**

Spermatogenic cells were isolated from adult rat testes using method described by Moreno et al, 2006 with some modifications. Briefly, the testes were dissected into a petri dish with DMEM. Seminiferous tubules showing dark lumen were considered as the site of sperm release, which was cut into small pieces (this tubule contains step 19 and step 7 spermatids) under stereomicroscope (Discovery V8 SteREO, Carl Zeiss, Göttingen, Germany). Collagenase treatment was given as follow:

1. Incubate the tubules with Collagenase 2mg/ml + 10μg/ml of DNase I in DMEM F-12 for 30 min at 37 °C with occasional gentle pipetting.
2. Allow the tubules to settle down and pipette out supernatant containing Leydig cells
3. Repeat the procedure for one more time
4. Tubules were then treated twice with DMEM F-12 + 2 mg/ml of Collagenase + 1 mg/ml Hyaluronidase type II for 20-30 min on shaking water bath at 37 °C.
5. Allow it to settle down
6. Remove the supernatant and add 1ml of DMEM F-12 with 2mg/ml collagenase + 10μg/ml DNase I + 1 mg/ml Hyaluronidase type III for additional 20-30 min.
7. Centrifuge for 10 min at 500 x g and collect the released Spermatogenic cells and wash the cells twice in DMEM and smear it onto poly-L-lysine-coated glass slides.

**Testicular sperm isolation by Percoll gradient centrifugation**

- Testicular sperm were isolated as described previously (Suryawanshi et al., 2011). Briefly, testes were dissected and minced in 0.01M PBS, resulting cell mixture was centrifuged and the pellet was washed twice and re-suspended in 0.01M PBS.
- A discontinuous Percoll gradient was prepared in a 15 ml falcon tube by adding 2 ml of Percoll of each concentration i.e. 45% (bottom of the tube), 30%, 25%, 18%, and 10% (Top most). As illustrated in figure 10.
- 2 ml of re-suspended testicular pellet was layered on this and centrifuged at 600 x g for 25 min at 4°C.
- After centrifugation, spermatozoa cell population was recovered from the layer formed at the junction of sample and 10% Percoll.
- Purity of the population was checked under phase contrast microscope. Testicular sperm fractions with more than 97% purity were used for SDS PAGE and Western blotting. If the purity was less than 97%, the fractions were discarded.

**Human sperm sample preparation**

- Human sperm were processed as described earlier (Khan et al., 2011). Semen sample of 150 μl was layered on the bottom of a Falcon tube containing 1.5 ml washing medium (DMEM) and incubated at 37 °C for 30 min.
- After 30 min, the upper part (1 ml) of the supernatant was aspirated. Supernatant was spun at 800 x g for 10 min, and pellet was reconstituted in medium and used either for protein extraction or fixed in PFA as mentioned above and used for Indirect Immunofluorescence (IIF).
Isotonic Percoll solution (90%) (Stock) = 90 ml Percoll + 10 ml of (filtered) 10X PBS

<table>
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<tr>
<td>30</td>
<td>3.33 + 6.67</td>
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<tr>
<td>25</td>
<td>2.77 + 7.23</td>
<td>10.0</td>
</tr>
<tr>
<td>18</td>
<td>2.0 + 8.0</td>
<td>10.0</td>
</tr>
<tr>
<td>10</td>
<td>1.11 + 8.89</td>
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</table>

Fig 10 Preparation of different gradients of Percoll for centrifugation
**Protein preparation**

- Protein extraction was done from testis, epididymis (from different age groups) and somatic tissues like liver, lung, spleen, kidney, intestine, brain, muscle, heart, thymus as per the protocol described (Wakle et al., 2005).
- Briefly, tissues were excised carefully and excess of fat and connective tissues were removed and homogenized at 4°C in lysis buffer (1% SDS and protease inhibitor cocktail tablet prepared in distilled water).
- Testicular cell preparation, sperm pellet from rat, mouse and human and somatic tissue homogenate were incubated at 4°C overnight in lysis buffer. Next day pellets were sonicated (Ralsonics Ultrasonic Processor, Model RP-120-122, India) at 100% output for 1 min bursts at 30 seconds intervals for total 5 min.
- The pellet was then centrifuged at 10,000 x g for 30 min at 4°C. Supernatant was collected and protein estimation was done by using modified Brad ford protocol. Protein extracts were then aliquoted and stored at -80°C till further use.

**Protein Estimation**

1. 0.5 mg/ml BSA stock (USB corporation)
2. Distilled water - 10ml
3. Bradford’s solution
   - CBB G250 10mg
   - Distilled water (MQ) 85ml
   - Ethanol 5ml
   - Ortho-Phosphoric acid 10ml
4. Distilled water
Method:

<table>
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<th>Tube No.</th>
<th>Concentration (μg/system)</th>
<th>BSA (μl)</th>
<th>Distilled Water (μl)</th>
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<tr>
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<td>40</td>
<td>80</td>
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</tr>
<tr>
<td>7</td>
<td>50</td>
<td>100</td>
<td>--</td>
</tr>
<tr>
<td>Sample lysates</td>
<td>Suitable dilutions</td>
<td>Total Volume 100</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Protocol for protein estimation by Bradford’s method (1976)

1. The standard dilutions were prepared as given in the above table.
2. 1ml Bradford’s reagent was added to each tube while vortexing.
3. Developed color was measured at 595nm using UV/Vis spectrophotometer.
4. Samples were diluted and protein concentration was calculated per microliter of sample used.

**SDS PAGE and Western blotting**

- Proteins were denatured in Laemmli loading buffer at 95 °C for 5-7 min.
Forty microgram of protein of different tissues were separated by electrophoresing at 80 V for 3 h as described by Laemmli (1970) on 10% polyacrylamide gel using an electrophoresis apparatus (BioRad Laboratories; Richmond, CA) (Laemmli et al., 1970).

Separated proteins were then transferred to nitrocellulose membrane under cooling conditions according to the procedure described by Towbin et al (1979) at 100 V for 1 h.

Transferred proteins were visualized using Ponceau S (0.1% Ponceau S in 0.5% acetic acid). Membrane was distained by washing with 0.01M PBS and processed for immunodetection as described earlier (Wakle et al., 2005).

Briefly, non-specific sites on the membrane were blocked with 5 % (w/v) NFDM-PBS for 1 h at room temperature. Anti Liprin α3 antibody was added at a dilution of 1 in 500 in 0.01M PBS and incubated at 4°C overnight.

A membrane with no primary antibody or Normal IgG served as a ‘Negative Control’.

On the following day membrane was washed thrice with 0.1 % Tween 20 in PBS and then incubated with 1 in 3000 dilution of Swine anti rabbit HRP secondary antibody for 1 h at room temperature. Membrane was washed as described above.

Detection was done using Enhanced Chemiluminescence plus (ECL) kit. Developed blot were recorded on blue base X-ray sheets.

Blots were stripped as per the protocol mentioned below.

Blots were washed excessively with 0.1% PBS-Tween -20 thrice for 10 min each. Blots were then incubated with stripping buffer at 80 °C for 10-20 min. Blots were allowed to cool and then washed with 0.1M PBS twice to remove excess of stripping solution.

Blots were then blocked with 5% NFDM-PBS for 1 h at room temperature and probed with beta actin at 1 in 3000 dilution at 4 °C overnight next day blots were washed as mentioned above and then probed with rabbit anti mouse HRP at 1 in 3000 dilution in 1% NFDM for 1 h at room temperature.

Blots were washed and developed as mentioned above to check the protein load. Each Western blotting experiment was repeated thrice.
Detection of Liprin α3 at transcript level

We extracted RNA from brain, testis and epididymal tissue by using Trizol method as described below:

**Homogenization:**

- Tissue (50 -100 mg) + 1 ml Trizol volume should be sufficient to dip tissue fragment.

- Homogenize the tissue in homogenizer. Place the tubes on ice while homogenizing to maintain temperature at 4 °C.

- Incubate homogenized sample for 5 min on ice to permit complete dissociation of nucleoprotein complex.

- Remove insoluble material from homogenate by spinning the homogenate at 12,000 x g for 10 min at 4 °C

  - Collect supernatant in a fresh tube.

**Phase separation:**

- Add 0.2ml of chilled chloroform per ml Trizol and cap it.

- Shake tubes gently for 1 min and incubate them at for 2-3 min on ice.

- Spin the tubes at 12000xg for 10 min at 4 °C.

**RNA precipitation:**

- Transfer the RNA containing aqueous phase to a fresh tube.

- Add 0.5ml of chilled isopropyl alcohol to precipitate RNA

- Incubate the sample on ice for 10 min

- RNA can be stored at this step. Put the tubes at -20 °C for overnight.

- Or pellet the RNA by spinning the tubes at 12000 g for 10 min at 4 °C.

**RNA Wash:**

- Remove supernatant and wash the pellet once with 1 ml of chilled 75% alcohol
• Gently mix the sample and centrifuge at 7500xg for 5 min at 4 °C.

• Discard the supernatant and invert the tubes on tissue paper. Keep at room temperature for drying for 15-20 min.

Dissolve RNA:

• Dissolve RNA in RNase free water for 10 min at 50-55 °C.

• Quantitate RNA by taking ratio A260/A280.

• DNA interferes and affects the quality of the RNA. DNase treatment is required.

**DNase Treatment**

• 1μg of RNA per 10 μl of reaction + 1 μl of DNase enzyme (1mg/ml)

• Incubate tubes at 37 °C for 15 min

• Heat deactivation of enzyme was done at 70 °C for 5 min

**Reverse Transcription**

cDNA was synthesized using Im Prom II reverse transcription kit. The procedure followed was as follows:

**RNA mixture**

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<th>Volume</th>
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<tbody>
<tr>
<td>RNA</td>
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</tr>
<tr>
<td>Random Primers</td>
<td>1 μl</td>
</tr>
<tr>
<td>RNase free water</td>
<td>3 μl</td>
</tr>
<tr>
<td>Total Volume</td>
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Heat the tube for 5 min at 70 °C and snap chill on ice

**RT system**

<table>
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</thead>
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<td>5X reaction buffer</td>
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<tr>
<td>MgCl₂ (1.5 mM)</td>
<td>1.2 μl</td>
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<tr>
<td>dNTP</td>
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</table>
Prom II Reverse transcriptase Enzyme 1.0 µl
Nuclease free water 7.8 µl
Total Volume 15.0 µl

"No RT control" contains no "reverse transcriptase enzyme"

Add RNA Mixture and RT system so total volume becomes 20.0 µl

And set a program on thermal cycling machine as follows

**Anneal:** 25 °C for 5 min

**Extension:** 40 °C for 60 min

**Inactivation:** 70 °C for 15 min

*Polymerase Chain reaction*

Resulting cDNA was then used for polymerase chain reaction using ABM master mix with primer set forward 5' GCCAACTCAAGATGGTGAGCA 3', reverse 5' GCACACTAGCAGGAGTAAGTC 3'

2X Master Mix 12.5 µl
Forward Primer 1.0 µl
Reverse Primer 1.0 µl
cDNA Template 3.0 µl
Nuclease free water 7.5 µl
Total Volume 25.0 µl

Program was set on thermal cycler as follows

- Head denaturation 95 °C for 5 min
- 35 cycles of
  - 95 °C for 1 min
  - 64 °C for 1 min
  - 72 °C for 1 min
- And additional final extension of 10 min at 72 °C
- Cool down to 4 °C

Agarose electrophoresis was carried out using 1% Agarose in 1X TAE (Tris, Acetic acid and EDTA) at 100 V for 45 min.

**Tissue processing and sectioning**

We perfused mature rats with Bouin’s fixative as per the protocol (D’Souza et al 2009). Testis, epididymis were excised and then dehydrated through serial grades of alcohol as mentioned below:

- 10% Alcohol for 10-20 min
- 30% Alcohol for 10-20 min
- 50% Alcohol for 10-20 min
- 70% Alcohol for 10-20 min
- 80% Alcohol for 10-20 min
- 90% Alcohol for 10-20 min
- 100% Alcohol for 10-20 min
- Xylol (Xylene + Alcohol 1:1) for 20-30 min
- Xylene for 20-30 min till tissue clears
- Tissues were then embedded in paraffin and blocks were made.
- Five micron sections were cut on a microtome (RM 2125RT; Leica, Wetzlar, Germany) and placed on 30% alcohol so that section doesn’t get folded, then it was floated in a water bath at 55-55 °C so that paraffin section is properly get unfolded and tissue gets properly spread. It was then taken and placed on glass slides precoated with poly L-lysine.

**Immunohistochemistry (IHC)**

- Immunohistochemical analysis was performed as described earlier (Wakle et al., 2005). Briefly, paraffin sections were deparaffinized at 56 °C for 10-15 min and then in xylene for 30 min.
- Endogenous peroxidase activity was quenched with methanol containing 0.03% H₂O₂ for 30 min and rehydrated through a graded series of alcohol solutions.
Sections were then blocked with 1.5% (v/v) normal swine sera for 1 hour at room temperature.

Anti Liprin α3 antibody was used at a dilution 1 in 150. Slides were then incubated overnight in moist chamber at 4°C.

On the following day, slides were washed thrice with 0.01M PBS for 5 min each. They were then incubated for 1 h with 1 in 100 diluted swine anti-rabbit secondary HRP antibody at room temperature in a humid chamber.

Slides were washed as mentioned before and the immune-peroxidase color reaction was developed using 3,3′-diaminobenzidine (DAB) substrate solution, which was prepared by adding 0.03% H₂O₂ to 0.1% solution of DAB in PBS and color was developed in the dark for not more than 1 min.

The reaction was terminated by immersing the slides in distilled water for 5 min and then counterstained for 30 seconds using Delafield's Hematoxylin. The sections were allowed to differentiate under running tap water for 15 min, dehydrated through a series of alcohol grades, cleared in xylene, and mounted in DPX mounting medium.

The section incubated with only PBS or normal IgG served as ‘Negative Control’. Slides were examined on a Zeiss Axioscope microscope (Carl Zeiss Inc.; San Marcos, CA). Each set of experiment was repeated thrice.

**Indirect Immunofluorescence (IIF)**

Indirect Immunofluorescence was performed on urea treated and untreated rat epididymal sperm, mouse epididymal sperm and on spermatozoa obtained from human ejaculates as per the protocol described earlier (Wakle et al., 2005).

Briefly, smears were blocked in PBS containing 2% (w/v) BSA fraction V at room temperature for 1 h.

This was followed by incubation with a 1 in 100 dilution of anti Liprin α3 antibody at 4°C overnight in a moist chamber.

A slide with no primary antibody or normal rabbit IgG served as a ‘Negative Control’. Next day slides were washed thrice for 5 min in 0.01M PBS. Swine
anti-rabbit FITC conjugated secondary antibody at a 1 in 100 dilution and counter
stained with DAPI (300nM).

- After 1 h slides were washed as described above. Slides were mounted in prolong
gold antifade and were observed under confocal fluorescent microscope (LSM
510 MEA Zeiss, Jena, Germany). Each set of experiments were performed thrice.

**IIF on different Spermatogenic cells**

- Smear of Spermatogenic cells isolated from digested tubules were permeabilized
with 0.1% Triton X-100 for 5-10 min at room temperature (RT).
- Nonspecific sites were then blocked by 1% BSA for 1 h at RT. Smear was the
incubated with anti Liprin α3 and anti gm130 diluted 1 in 100 at 4°C overnight.
- Next day, slides were washed thrice with 0.1M PBS. Alexa fluor goat anti rabbit
488 and Alexa fluor goat anti mouse 594 was used at 1 in 100 dilution along with
counter stain DAPI for 1 h at room temperature.
- Slides were washed again as described above. Slides were then mounted in
prolong gold antifade and were observed under confocal fluorescent microscope
(LSM 510 MEA Zeiss, Jena, Germany).
- Slide with no primary antibody served as ‘Negative Control’.

**Sequential treatment of caudal sperm proteins**

- We performed sequential extraction on caudal epididymal sperm as per the
protocol described by Syntin and Cornwall. (1999).
- Briefly, spermatozoa were collected from rat caudal epididymis in 0.01M PBS.
Sperm were pelleted at 800 x g resulting supernatant was collected and pellet was
divided in four parts. The protocol is represented pictorially (Figure 11)
- Sperm pellets were then sequentially treated with PBS containing increasing
concentrations of sodium chloride (150mM, 1M, 2M and 4M). Subsequently,
sperm pellets were treated with 0.1% Triton X-100 prepared in 0.01M PBS.
- All treatments were carried out at room temperature for 20 min with gentle
mixing. After each treatment sperm suspension was centrifuged at 800 x g for 10
min.
Rat Caudal Sperm pellet

Normal buffered Saline 0.0 M PBS

Centrifuge at 800g for 10 min

20 min at Room temperature with gentle mixing

Recover Pellet

Resuspend pellet in PBS containing 1M NaCl

Centrifuge at 800g for 10 min

20 min at Room temperature with gentle mixing

Recover Pellet

Resuspend pellet in PBS containing 2M NaCl

Centrifuge at 800g for 10 min

20 min at Room temperature with gentle mixing

Recover Pellet

Resuspend pellet in PBS containing 4M NaCl

Centrifuge at 800g for 10 min

20 min at Room temperature with gentle mixing

Recover Pellet

Resuspend pellet in 0.1M PBS containing 0.1% Triton X-100

Centrifuge at 800g for 10 min

20 min at Room temperature with gentle mixing

Pellet

Pellets and Vacuum dried supernatants are suspended in Lysis buffer

Heat at 95 °C for 5 minutes, Centrifuge at 800 g for 20 min at 4 °C, Load supernatants and perform SDSPAGE

Fig. 11 Sequential extraction of proteins from Rat caudal sperm
Supernatants obtained after each treatment were dialyzed overnight against distilled water, lyophilized and re-suspended in Laemmli buffer. Pellets obtained after each treatment were subjected to 1% SDS protein extraction as mentioned previously, all samples were heat denatured at 95°C for 5 min in Laemmli buffer and subjected to SDS-PAGE and immunoblotting as mentioned earlier. The experiment was performed thrice.

Blot with no primary antibody served as ‘Negative Control’.

**In silico estrogen responsive elements (ERE) analysis**

- We carried out *in silico* ERE analysis for Liprin α3 gene using MatInspector Genomatix v2.4 Software GmbH, München (Germany). The promoter region from 3,000 bp upstream to 500 bp downstream from the transcription start sites (TSS sites) was submitted for analysis.

**Hormonal Regulation**

- Tamoxifen, PHPP and DPN treated and vehicle control animals were sacrificed and testes were removed and detunicated and tubules were minced in 0.01M PBS and subjected to protein extraction as mentioned before.
- Protein lysate was then used for Western blotting as mentioned before.
- Densitometric analysis was done using Chemi genius GelDoc system from Syngene (India).
- Beta actin was used as a housekeeping protein and values obtained were normalized with beta actin values.
- Student’s t-test was applied to determine the statistical significance.

**Isolation of epididymosomes**

- Epididymosomes were collected as per the procedure described by Légaré et al (1999). Briefly, epididymal fluid was collected by teasing whole epididymal tissue in PBS. The suspension was centrifuged at 5000 x g for 20 min.

TKS 9306
• The supernatant was recovered and divided into two tubes. One tube was considered as epididymal fluid with epididymosomes and was lyophilized and heat denatured in Laemmli buffer.

• Second tube was subjected to ultracentrifugation at 100,000 x g for 2 h at 4°C. The resulting pellet comprised of the pure epididymosomes while the supernatant fluid devoid of epididymosomes was lyophilized and heat denatured using Laemmli buffer.

• All the samples were processed for SDS-PAGE and Western blot analysis as described before.
RESULTS
Western blot analysis of Liprin α3

To detect the presence of Liprin α3, Western blot analysis of total testicular cell extract, percoll isolated testicular sperm, epididymal sperm and tissue protein was carried out. The cognate protein was identified at 133kDa in the lane of testicular cell extract, epididymal tissue and sperm protein as represented in fig 12a, lanes 1, 3, 4, 5, 6, 7, 8. Percoll isolated testicular sperm protein did not show the presence of Liprin α3. (Fig 12a, lane 2). Protein from somatic tissues such as liver, muscle, intestine, spleen, kidney, lung, heart, thymus and brain were used for Western analysis and is represented in fig 12b. Liprin α3 was detected at 133 kDa in intestine, lungs and brain (Fig 12b lane 3, 6 and 9). Blots stripped and probed with beta actin antibody, which served as a loading control, showed the actin band at 45 kDa as represented in lower panel of Fig 12. Blots probed with secondary antibody alone or normal rabbit IgG which served as negative control did not show any reactivity (Fig 12 a1 and b1).

The Western blot analysis indicates that, the protein is present in tissue and sperm of all the regions of epididymis, testicular cell extract as well as in somatic tissues like brain, intestine and lungs.

Detection of Liprin α3 at transcript level

To check the expression of Liprin α3 at transcript level RT-PCR was performed on brain, testis and epididymal tissues and is shown in Fig 13. An expected product of 610 bp size was observed in all three tissues indicating that no different forms are present in testis and epididymis. Respective beta actin lanes show equal load. Negative Control with No template Control did not show band.

Immunohistochemical localization of Liprin α3

After detecting Liprin α3 at protein and at transcript level, it was essential to identify the specific cell types in testis and epididymis expressing Liprin α3 and towards this IHC was done. In the testis, it was found in Stage XIII and XIV (Fig. 14 C). Cytoplasmic expression of protein can be seen in Pachytenes and Diplotenes as indicated by arrows. The staining was also observed in round spermatids stage I-V (Fig. 14 A, B);
Figure 12a Western blot analysis of Liprin α3 protein in rat tissue and sperm. Testicular tissue (lane 1), isolated testicular sperm (lane 2), caput sperm (lane 3), corpus sperm (lane 4), caudal sperm (lane 5), caput tissue (lane 6), corpus tissue (lane 7) and cauda tissue (lane 8). An arrow indicates band of interest at 133 kDa. Corresponding beta actin panels (below panels) show equal protein load.

Figure 12 a1 represents respective negative control and shows no specific bands.
Figure 12b. Western blot analysis of Liprin α3 in rat somatic tissues. Liver (lane 1), muscles (lane 2), intestine (lane 3), spleen (lane 4), kidney (lane 5), lungs (lane 6), heart (lane 7), thymus (lane 8) and brain (lane 9). Arrows indicate specific of Liprin α3 at 133 kDa. Corresponding beta actin panels (below panels) show equal protein load. Figure 12b1 represents respective negative control indicating no bands
Figure 13 Expression of Liprin α3 in different tissues by RT PCR.
Testis (lane 1), Epididymis (lane 2), Brain (lane 3), Respective Negative control (lane 4, 5, 6). Arrows indicate respective band size. Lower panel represents respective beta actin controls.
Figure 14 Immunohistochemical localization of Liprin α3 protein in rat testicular and epididymal section. (A) and (B) stage I-VII, arrows indicate cytoplasmic localization in round spermatids (R). (C) Stage XIII and XIV, arrows indicate cytoplasmic localization in dividing cells of stage XIV, Pachytene cells (P) and in stage XIII Diplotenes (D). An arrow indicates localization in epididymal epithelial cells and luminal sperm (LS) (Panel D). A1, B1, C1 and D1 represent negative controls of testis and epididymis probed with normal rabbit IgG. Bar represents 25 μm.
however no reactivity was seen in elongating spermatid and sperm. Immunohistochemical localization of the protein in epididymis is represented in Fig 14 D. Positive brown staining can be seen in epithelial cells and luminal sperm (LS) in all the regions of the epididymis (representative picture shown) as indicated by arrow. No reactivity was seen in tissue sections incubated with normal IgG (panel A1, B1, C1 and D1).

*Immunofluorescent localization of Liprin α3 in epididymal sperm*

Localization of Liprin α3 on sperm from all the three regions of epididymis and on urea treated epididymal sperm is presented in Fig 15a. Panel A, C, E depicts Liprin α3 localization on equatorial and acrosomal region of caput, corpus and cauda epididymal sperm. No change in localization pattern can be seen following treatment with 6M Urca (Fig 16 B, D, F). Normal IgG controls did not show any fluorescent staining (Fig. 15b).

*Localization of Liprin α3 during acrosome formation*

We carried out IIF on Spermatogenic cells; localization of Liprin α3 is represented in Fig 16a. Panel A, represents round spermatid showing localization of Liprin α3 in a developing acrosome. Panel B represents localization of Golgi apparatus marker gm130. Liprin α3 co-localizes with Golgi marker gm130 (panel C). The extent of overlap is represented in panel D. Liprin α3 localization can be seen in elongating spermatid, where we could see a different localization pattern of protein (panel E). Testicular sperm showed acrosomal as well as equatorial localization as shown in panel F. However, no staining was observed in testicular sperm and in negative controls (Fig 16b).

*Sequential extraction of Liprin α3*

To check the extent of association of Liprin α3 with sperm surface, caudal sperm were sequentially treated with isotonic and hypertonic salt solution, Liprin α3 was found to be absent in the supernatants of PBS containing 150 mM, 1M, 2M and 4M NaCl (Fig 17, lanes 6-9) indicating the absence of a loosely attached form of protein with outer plasma membrane. It was seen that the supernatant following treatment with Triton X-
Figure 15a Indirect immunofluorescent localization of Liprin α3 on rat epididymal sperm. Arrows indicate localization in the acrosome and equatorial region. Localization in the acrosome and equatorial region was seen in untreated caput, corpus and caudal sperm (Panel A, C, E) and urea treated caput, corpus, cauda epididymal sperm Panel B, D, F respectively. Insets represent respective DIC. Magnification 100X, bar represents 5 µm.
Figure 15b Negative Control for Indirect immunofluorescent localization of Liprin α3 on rat epididymal sperm. Sperm were probed with normal rabbit IgG/secondary antibody only. Urea untreated (A) and Urea treated (B). Magnification 100X, bar represents 5 μm.
Figure 16a Indirect immunofluorescent localization of Liprin α3 on rat spermatogenic cells showing crescent shaped acrosome in step 7 spermatid panel A (green), Golgi marker gm130 panel B (red), co-localization of Golgi and Liprin α3 panel C (yellow), Scatter plot showing the coefficient of co-localization is represented in panel D. Localization pattern of Liprin α3 during acrosome shaping, elongating spermatid (panel E) and permeabilized testicular spermatozoa (panel F). Magnification 100X, bar represents 5 μm.
Figure 16b Negative control for Indirect immunofluorescent localization of Liprin α3 on rat spermatogenic cells. Cells were incubated with normal IgG/secondary antibody only. Negative control did not show any reactivity. Step 7 spermatid (A), Elongating spermatid (B) and Testicular spermatozoa (C)
100 showed a band at 130kDa (lane 10, indicated by an arrow) indicating the presence of tightly bound form of Liprin α3. It was observed that all the pellet fractions post salt and detergent treatment showed band at 133kDa, (Fig. 17, lane 1-5). Pellet post Triton treatment still showed the reactivity indicating that it requires further stringent extraction procedure to extract all acrosomal protein. Buffer alone or normal IgG which served as negative controls did not show any reactivity.

*Liprin α3 is present in epididymosomes*

Epididymosomes are secreted by epididymal epithelial cells and are involved in transfer of certain proteins on sperm surface. To investigate the presence of Liprin α3 in epididymosomes Western analysis was performed and results are represented in Fig 18. Epididymal fluid (lane 1) and purified epididymosomes (lane 3) showed the presence of Liprin α3. However, fluid devoid of epididymosomes did not show any reactivity (lane 2) indicating the association of Liprin α3 with epididymosomes. Respective lanes incubated with normal rabbit IgG did not any show reactivity.

*Developmental expression pattern of Liprin α3*

In order to understand the developmental regulation of Liprin α3 expression, we analyzed rat testis and epididymis during postnatal development by Western blot. The protein was detected at day 20 although at very low levels in the testis (Fig 19a, lane 3) while in the epididymis it could be seen on day 40 (Fig 19b, lane 5). There was a gradual increase of the protein expression after postnatal day 20 in the testis and day 40 in the epididymis and the expression reached the highest level on postnatal day 60 as shown in Fig 20. Blots stripped and probed with beta actin antibody shows equal protein load. Negative control with no primary antibody as well as normal IgG did not show any reactivity (Fig 19 a1 and 19 b1).

*Immunohistochemical localization of protein expression in rat testis and epididymis from different age groups*

In the immunohistochemical studies with testis and epididymal sections, similar observations were made and are represented in Fig 20 and 21 respectively. The testicular
Figure 17 Western blot analysis of rat caudal sperm following sequential treatment with hypertonic solution and detergent. Lanes 1-5 represent pellets from PBS, 1M NaCl, 2M NaCl, 4M NaCl and 0.1% Triton X-100 treated sperm respectively and lanes 6-10 represent supernatants from PBS, 1M NaCl, 2M NaCl, 4M NaCl and 0.1% Triton X-100 treated sperm respectively. Please note lane number 10, (supernatant following 0.1% Triton X-100 treatment) shows a band at 130. Negative control, buffer only did not show any reactivity.

Figure 18 Western blot analysis showing presence of Liprin α3 in epididymosomes. Epididymal fluid with epididymosomes, (lane 1), epididymal fluid devoid of epididymosomes shows no reactivity (lane 2), epididymosomes (lane 3). Blots probed with normal rabbit IgG served as a negative control.
Figure 19 Western blot analysis showing developmental expression of Liprin α3 in rat testis (a) and epididymis (b). Tissue proteins from day 5, 10, 20, 30, 40, 50, 60 old animals are shown in lanes 1, 2, 3, 4, 5, 6, 7 respectively. Protein expression can be seen at day 20 in testis and in epididymis at day 40. Corresponding lower panels below panels a and b are beta actin controls indicating equal load. Respective negative control did not show any reactivity (Fig 19 a1 and b1)
Figure 20 Immunohistochemical analysis of Liprin α3 in rat testis from different age groups. (A) Post natal Day 5, (B) Day 10, (C) Day 20, (D) Day 30, (E) Day 40, (F) Day 50, (G) Day 60. Arrows indicate the localization of protein. Corresponding negative controls (A1, B1, C1, D1, E1, F1, G1) shows no reactivity. Bar represents 25 μm.
Figure 21 Immunohistochemical analysis of Liprin α3 in rat epididymis from different age groups. (A) Post natal Day 5, (B) Day 10, (C) Day 20 (D) Day 30 (E) Day 40 (F) Day 50 (G) Day 60. Arrows indicate the localization of protein. Corresponding negative controls (A1, B1, C1, D1, E1, F1, G1) shows no reactivity. Bar represents 25 μm.
tissue showed expression from day 20 (figure 20, panel C) with positive staining seen in the early pachytenes. On postnatal day 30 and 40 (figure 20, panel D, E), Liprin α3 protein expression dominated in Pachytenes, Diplotenes and in most meiotically dividing round cells. In the epididymal sections the positive staining was seen from day 30 (figure 21, panel D) onwards in the cytoplasm of the cylindrical shaped principal cells of the epididymal epithelium as shown by arrows in the panel. On postnatal day 60 (figure 21, panel G), luminal sperm of the cauda epididymis showed expression (panel G). Sections of testis and epididymis incubated with normal IgG controls did not show any positive staining.

*In silico estrogen responsive elements (ERE) analysis*

Several sperm proteins are known to be hormonally regulated. To check if Liprin α3 is under hormonal regulation, *in silico* analysis was performed using MatInspector Genomatix v2.4 Software. It was observed that three putative estrogen response elements are present in Liprin α3 gene as shown in figure 22a, which indicates that the protein expression is likely to be under estrogen regulation.

*Hormonal regulation of Liprin α3*

To verify the *in silico* results we further investigated Liprin α3 expression in rat testis. Protein from tamoxifen treated and vehicle control rat testis were analyzed by Western blot and the results are represented in fig 22b which show that the protein is under regulation of estrogen. Densitometric analysis revealed two fold reduction in the expression of Liprin α3 in tamoxifen treated testis, which is statistically significant (fig 22c).

*Liprin α3 is under estrogen regulation*

To validate further we used ER β antagonist (PHTPP) and ER β agonist (DPN) treated rat model. Testicular proteins were analysed for the protein levels of Liprin α3 in these treatment models. As represented in Fig. 23, a decrease in the expression of Liprin α3 in presence of ER β antagonist was seen. The densitometric analysis confirms two fold decrease in the testicular Liprin α3 expression. However, an increase in expression was
Figure 22a

**GENE ID: 140591**  
**Gene Size: 1-28842**

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Figure 22a In *silico* analysis of Liprin α3 gene for the presence of Estrogen Responsive Elements (EREs) (a), Blue region is putative promoter region, red is ATG site, yellow represents EREs. The details of EREs are summarized in the table.
Figure 22b Western blot of Liprin α3 Control (lane 1) versus tamoxifen treated (lane 2) rat testis. Respective beta actin control represents equal protein load.

(c) Densitometric analysis of Liprin α3 expression Decreased expression of protein in presence of Tamoxifen. Values were normalized with beta actin. Asterisk indicates statistically significant reduced expression (p < 0.05) of Liprin α3 in tamoxifen treated rat testis.
Figure 23 Western analysis of testicular protein from PHTPP and DPN treated rats. (a) Western analysis on PHTPP treated rat testis, a decrease in protein expression (lane 2) can be seen as compare to control (lane 1). The corresponding densitometric analysis revealed a significant two fold decrease in the expression in presence of ERβ antagonist (p<0.01). (b) Western analysis on DPN treated rat testis, an increase in protein expression (lane 2) can be seen as compare to control (lane 1). The corresponding densitometric analysis revealed a significant five fold increase in the expression in presence of ERβ antagonist (p<0.05).
observed in presence of ER β agonist. A five fold increase in the expression of Liprin α3 in presence of DPN.

Conservation of protein expression

Functionally important proteins are conserved across species. To determine the protein expression of Liprin α3 in different species Western blot and indirect immunofluorescence was performed on mouse and human sperm and the results are depicted in fig 24a and 24b respectively. Western blot analysis showed species specific known variation in molecular weight which was 100 kDa in mouse sperm, it was found to be approximately 100kDa (Fig 24a, lane 1). In human sperm it was found to be 130 kDa (Fig24a, lane 2). Corresponding lanes probed with beta actin antibody represent equal protein load.

Fluorescent images are represented in Fig 24b panel i and ii. It was interesting to note that both mouse sperm and human also showed equatorial as well as anterior acrosomal staining (Fig 24b panel i & ii respectively). IgG controls did not show any specific reactivity by both Indirect immunofluorescence and Western analysis.
Figure 24 Conservation of protein Liprin α3 across species. (a) Western blot using sperm protein from mouse cauda (lane 1) and human sperm (lane 2). Lower panel represents beta actin controls. Negative control did not show reactivity (b) Immunofluorescent localization of Liprin α3 on mouse caudal sperm (i) and human sperm (ii). Bar indicates 10μm.
DISCUSSION
Membrane fusion is a key event in many biological processes. These processes are controlled by various fusogenic agents. Recent observations indicate that an "acrosomal synapse" is formed by lipid raft-derived membrane milieu and multidomain scaffolding proteins. The term "Acrosomal synapse" was coined by Zitranski et al. (2010). Substantial data exist which indicates many similarities in acrosome reaction and fusion of a vesicle at synapse. Acrosome synapse seems to be responsible for ensuring the attachment of the huge acrosomal vesicle to the overlying plasma membrane, as well as for preventing an accidental spontaneous loss of the single acrosome. It has been a topic of discussion that an apparent arbitrary expression of neuronal genes during sperm cell development occurs as a by-product of the changes taking place in chromatin structure that take place during repackaging of DNA into the condensed nuclei of mature spermatozoa. Gene products like cadherin, t-snare, v-snare, calcium sensing Synaptotagmin, and arc were originally thought to be brain-specific and were later found to be expressed in testis as well. (Mayorga et al., 2007, Hecht 1995, Maier 2002). It is now evident that membrane fusion process happening at synapse or at acrosome is driven by common set of proteins. The presence and participation of SNARE, SNAP-25, RIM in sperm acrosome fusion or acrosome reaction underlines the same.

Liprin α3 is one such protein reported in brain and neuronal tissue. Liprin α family is known to be involved in vesicle transport across the synapse to conduct a signal. The Liprins were discovered in a pull down assay with LAR and therefore named as LAR Interacting Protein 1 (LIP.1) (Serra-Pagès et al., 1995). It is discovered that Liprin α interacts with LAR through its C-terminal Liprin’s homology (LH) domain (Serra-Pages et al 1998). Both Liprins and LAR are known to be required for cargo transport and membrane fusion events at synapse (Miller et al., 2005, Zhen et al., 1999). Thus, Liprin α3 is one such protein reported to be present at synapse in brain, neuronal tissue. In the present study we report for the first time its presence in both testis and epididymis using immunohistochemical (IHC), IIF and Western analysis.

Liprin α3 was identified in a sperm proteomics study carried out by our group (Suryawanshi et al., 2011). Further analysis by Western blotting confirms its presence in testis and epididymis, thus validating proteomics findings. Transcript analysis performed indicates that Liprin α3 transcript is present in brain, testis and epididymis. Immuno-
histachemical localization was performed on rat testis to stage the expression of Liprin α3 during spermatogenesis. Liprin α3 expression can be seen in the cytoplasm of Pachytene, Diplotenes and round spermatids in testicular sections. Several acrosomal proteins such as Syntaxin have been reported to be synthesized in cytoplasm and then assembled in the acrosome (Moreno et al 2006). It is known that synthesis of Acrogranin starts as early as pachytnene spermatocyte during cell differentiation. The diffuse pattern of Acrogranin was seen in the entire cytoplasm presumably because of the presence of nascent Acrogranin in a rough Endoplasmic reticulum (Anakwe and Gerton, 1990). A similar phenomenon was noticed in case of Liprin α3 in the present study. The observed cytoplasmic staining of Liprin α3 in histochemistry indicates the probable presence of nascent protein. However, the presence of Liprin α3 was not evident in elongating spermatids in IHC. We therefore used sensitive technique like Indirect Immunofluorescence for detection. IIF clearly showed the presence of protein in acrosomal region of elongating and testicular spermatozoa. This indicates that Liprin α3 is an acrosomal protein.

In spermatogenesis spermatid undergoes major morphogenetic changes includes acrosome formation and development of flagellum. During the process of acrosome formation, developing acrosome shows different crescent shapes. The proteins associated with this process also exhibit similar localization pattern over the developing acrosome in this process as shown in fig 25.

**Figure 25 Acrosome biogenesis.** AV: acrosomal Vesicle, NE: Nuclear Envelope (Ramalho-santos et al., 2002)
Figure 26 Testis frozen section stained with Golgi marker GM 130 (Anti GM130-FITC, Green), polyclonal anti KIF1C (Texas Res, Red) and nuclear counterstain DAPI (Blue). Arrow indicates an overlap between to dyes (Yellow) indicating Golgi based vesicle transport in spermatid. (Yang and Sparry, 2003)
As depicted in Fig. 25, acrosome originates from Golgi and flattens over nucleus thus appears to be a crescent shape. This process can be seen in stage I, II, III of spermatogenesis and steps 5 to 7 of spermiogenesis in round spermatid. During this process or stages Golgi apparatus constantly secretes vesicles to form proacrosome. These vesicles need to be transported from Golgi to the developing acrosome. This transport of vesicles from Golgi to acrosome is based on kinesin motors. Yang and Sparry (2003) found that kinesin motor KIFC1 is actively involved in the process of vesicle trafficking from Golgi to acrosome and thus participates in acrosome biogenesis. Co-localization of Golgi marker GM130 and KIFC1 performed on testis frozen section shows overlapping existence over developing acrosome, exhibiting typical pattern of acrosome formation (Fig 26). Liprin α proteins are known interacting partner of kinesin motor protein. Shin et al (2003) described interaction of Liprin α with neuron specific kinase KIF1A. This observation led us to speculate the probable role of Liprin α3 in acrosome biogenesis. To ascertain whether Liprin α3 indeed showed hallmark pattern of acrosome biogenesis, we carried out IIF on testicular Spermatogenic cells which revealed that Liprin α3 not only shows pattern of acrosome formation but also co-localizes with Golgi marker GM130 (Fig. 16a A to C), indicating its probable role in vesicle transport along with Golgi during acrosome biogenesis.

Indirect Immunofluorescence shows Liprin α3 localization on acrosome of elongating acrosome and on anterior region of mature sperm. The anterior localization of Liprin α3 was seen in rat mouse and human sperm. This conserved nature of expression suggests Liprin α3 is functionally important. Also, proteins involved in membrane fusion are reported to be present on the anterior region. SNARE proteins like Syntaxin and VAMP/Synptobrevin are reported to be present at equatorial segment and involved in membrane fusion (Ramalho-Santos et al., 2000). During acrosome formation, acrosome membrane forms distinct three regions. One side which is in close proximity is termed as IAM (Intra Acrosomal Membrane), the other facing the overlying plasma membrane is OAM (Outer Acrosomal Membrane), and the region where acrosomal vesicle folds is called as equatorial segment of acrosome (As depicted in fig 8). Unchanged equatorial localization of Liprin α3 confirms that it is an acrosomal protein. In concordance with this observation the anterior localization of Liprin α3 indicates its contribution towards
acrosome fusion. To confirm acrosomal origin of Liprin α3 we carried out sequential extraction using a published protocol (Syntin and Cornwall, 1999). We were able to distinguish between intra acrosomal and membrane associated proteins. Liprin α3 reactivity in sperm supernatant treated with 0.1% Triton X-100 confirming that no loosely bound form of Liprin α3 is associated with sperm and Liprin α3 is an intra acrosomal protein.

The spermatogenesis is a well regulated process. It is known that many sperm proteins are developmentally regulated and under rigorous hormonal regulation. During the development, the expression of estrogen receptors starts in Pachytene spermatocytes (van Pelt et al., 1999). The expression pattern of Liprin α3 as seen in the present study overlaps with the expression of estrogen and androgen receptor by day 20 in rat testis (Majo et al., 1999). This led us to speculate the probable involvement of hormones in expression of Liprin α3. Our findings suggest that, progressive increase in the protein expression during the pre-pubertal period does not depend exclusively on higher androgen levels, which are triggered at puberty and therefore would probably require the participation of low levels of androgen and other factors including hormones like estrogen. In another study conducted on breast cancer cell line, shows proteins like Synaptotagmin 1, Syntaxin 1, RAB3A, SNAP25, Synaptobrevin 2 involved in membrane fusion at synapse are under regulation of estrogen (Wright et al., 2009). Interestingly, according to the proteomic study conducted on breast cancer tissues, Liprin α proteins are differentially expressed in Estrogen Receptor α- (ER-/−) tissues, which indicates Liprin α proteins may be under the regulation of estrogen (Rezaul et al., 2010). To verify the direct correlation of estrogen and vesicle trafficking component the effects of estrogen on breast cancer line were checked, and it was observed that Liprin α4 (PPFIA4), rab27b (RAB27B) genes are under regulation of estrogen (Wright et al., 2009). In silico analysis of Liprin α3 carrioud out by us revealed the presence of Estrogen Response Elements (ERE) and absence of Androgen response elements (ARE) upstream to the initiation codon of Liprin α3 indicating the role of estrogen in protein expression. However these in silico results need to be validated using in vivo system. To confirm the same, we used two animal models. We checked the expression profile of Liprin α3 in the presence of estrogen agonist and an estrogen antagonist.
Tamoxifen is a known and well-studied estrogen antagonist. It has been reported that Tamoxifen is a selective estrogen receptor modulator (SERM), can act as an agonist or an antagonist depending on the dose used, target organ, sex and species (MacGregor et al., 1998). Studies by Pathak et al. (2010) have demonstrated its anti-estrogenic effect in rat testis. Hence, we used the tamoxifen treated rat model to validate the in silico results. The protein from tamoxifen treated rat testis was used for western analysis. We observed that in testis Liprin α3 is down regulated post tamoxifen treatment, suggesting Liprin α3 protein is under the estrogen regulation. To validate the estrogen involvement further, we used two rat models. We used well known estrogen receptor antagonist, 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) and estrogen agonist, 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN) in our study. PHTPP is a known inhibitor of ERβ activity. It is a selective estrogen ERβ receptor antagonist that displays 36-fold selectivity over ERα. It exhibits full antagonism at ERβ in a co-transfection assay in human endometrial cancer cells (HEC-1), with minimal effects on ERα. In presence of this ERβ inhibitor we found that there is a twofold decrease in the expression of Liprin α3 in rat testis. In contrast, in the presence of ERβ agonist (DPN) we could see an increase in Liprin α3 expression. DPN is highly potent estrogen ERβ receptor agonist with a 70-fold selectivity over ERα. These results confirm that protein is under estrogen regulation and is regulated via ERβ.

Spermatogenesis produces millions of sperm; however, these are functionally immature. Epididymal transit confirms functional maturity. During this sojourn existing proteins are modified and/or new proteins are acquired or synthesized in epididymal epithelial cells. These proteins are transported to sperm via prostatic like particles. There are substantial evidences shows that epididymal epithelial cells secrete prostatic like particles called “Epididymosomes” (Girouard et al., 2011, Frenette et al., 2010, Sullivan et al., 2007). Proteins are transported to sperm surface through epididymosome based transport. Secretion of these vesicles from epididymal epithelial cells involves membrane fusion, so that vesicles are released outside into the lumen. The presence of proteins like SNAREs on epididymosomes confirm that presence of membrane docking proteins is a prerequisite for membrane fusion and vesicle exocytosis (Girouard et al., 2011). The presence of Liprin α3 on epididymosomes indicates its
probable role in vesicle transport mediated through membrane fusion.

Formation of RIM/MUNC/Rab3A/ Liprin α complex is a critical step in synaptic vesicle docking in the neurons (Andrews-Zwilling et al., 2006). On the basis of our results and reported function of Liprin α3 in literature, its presence on sperm acrosome indicates its likely involvement in membrane fusion during acrosome reaction. It has been recently reported that outer acrosome membrane fusion with plasma membrane is primed by Rab3a and its interacting molecule RIM (Bello et al., 2012). The association of RIM and Liprin α proteins has been demonstrated in a study conducted on brain tissues and it was observed that Liprin α proteins interact with RIM thorough its coiled coil domain in calcium dependent manner (Ko et al., 2003). Of the molecules described in synaptic vesicle docking, RIM/MUNC/Rab3A complex has been shown to participate in acrosomal exocytosis and play a central role in membrane docking (Bello et al., 2012). Interestingly, we feel that the presence of Liprin α3 on the acrosome region of sperm as shown by us could be the fourth partner in the reported complex. In conclusion we can therefore state that, the identification of Liprin α3 has led to identification of one more member of this complex which has an important role in acrosome reaction. Similarities between acrosomal fusion and synapse fusion strengthen our belief that Liprin α3 protein is conserved scaffolding protein involved in acrosome reaction.