

Characterization of *rtSH3p13* gene encoding a development protein involved in vesicular traffic in spermiogenesis

Yuan QIAO¹, Ju Xiang YANG¹, Xiao Dong ZHANG¹, Yu LIU¹, Jian Chao ZHANG³, Shu Dong ZONG³, Shi Ying MIAO¹, Lin Fang WANG^{1,*}, S. S. KOIDE²

¹National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Peking Union Medical College, 5 Dong Dan San Tiao, Beijing 100005, China.

²Center for Biomedical Research, Population Council, 1230 York Avenue, New York, NY 10021, USA.

³National Research Institute for Family Planning, 12 Da Hui Ci, Hai Dian, Beijing 100081, China.

ABSTRACT

A cDNA, designated as *rtSH3p13*, was isolated from a rat testis cDNA library. It consists of 1463 bp nuclear acids, which encodes a protein of 312 amino acids and was assigned the GenBank accession number AF227439. The deduced *rtSH3p13* protein is a truncated isoform of SH3p13 as a result of mRNA alternative splicing. It is mainly expressed in the rat testis, detected in spermatid at the steps 8-19 of spermiogenesis, and found around the acrosome. During postnatal development, *rtSH3p13* appears on day 18 and reaches maximum on day 60. Further experimental results suggested that *rtSH3p13* forms a complex with activated epidermal growth factor receptor (EGFR) and interacts with synaptojanin I. Surprisingly, similar to SH3 domain, the V region of *rtSH3p13* also inhibits endocytosis in CHO cells. Our results reveal a link between an *rtSH3p13*-synaptojanin-clathrin complex-mediated formation of pits and the process of spermiogenesis.

INTRODUCTION

Spermiogenesis is the process of formation of matured elongated spermatids. During this process the germ cells possess a highly developed endocytic apparatus, which uptake proteins and other molecules to build up components of the acrosome and the flagellum. However, the mechanism of endocytosis is poorly understood in the germ cells.

Endophilins were first discovered as novel SH3 containing proteins by screening a mouse embryonic cDNA expression library using a proline-rich peptide ligand for the SH3-domain[1-3]. The rat counterparts of endophilins are also called SH3p4 (endophilin I), SH3p8 (endophilin II), and SH3p13 (endophilin III). SH3p4 is restricted to brain, SH3p8 mRNA is ubiquitously expressed but its protein is mainly detected in the brain[1, 2, 4, 5], while SH3p13 was found to express in both brain and testis, and the level in testis was much higher than that in brain. Subsequent studies suggested that SH3p4 and SH3p8 both had lysophosphatidic acid acyl transferase activity, which was essential for their functioning in synaptic-like microvesicle

formation at the plasma membrane of neuronal synapses. Although they also bind dynamin, their major binding partner is the inositol 5-phosphates, synaptojanin[1, 2, 6-11]. Both SH3p4 and SH3p8 have been identified to play a pivotal role in clathrin-mediated endocytosis[12]. In *Drosophila*, endophilin is required presynaptically at the neuromuscular junction, and its absence or mutation of endophilin dramatically impairs endocytosis *in vivo*[13, 14]. Interestingly, a recent study suggested that an endophilin-CIN85 complex was involved in the regulation of EGF receptor endocytosis[15].

We investigated the relationship between clathrin-mediated endocytosis and germ cells containing membrane pits and vesicles, which are considered to be important for germ cell division, proliferation and metabolism. We identified that *rtSH3p13*, an alternatively spliced isoform of SH3p13, was mainly expressed in specific stages of germ cells during the process of spermiogenesis. We demonstrated that this protein interacted with synaptojanin I and played an important role in the clathrin-mediated receptor endocytosis.

MATERIALS AND METHODS

Antibodies and plasmids

*Correspondence: Lin Fang WANG

Tel: +86-10-6524-0803, Fax: +86-10-6524-0529

E-mail: wanglf@ms.imicams.ac.cn

Tab 1. Primers for each domain/region of rtSH3p13.

Domain/Region	Forward primer (5'→3')	Reverse primer (5'→3')
C region	GCCTCGAG ACCATGGAAAAGAAAATAG	TA GGATCCCGTATTTCGCAGCTCTAG
V region	GCCTCGAGACCATGGCTCTTGCATCCCAAG	TAGGATCC CGGGGCTGGTCCGAGG
SH3 domain	GCCTCGAGACCATGGCACCTGCTGTCGTG	TAGGATCCCGCCGAGGTAAAGGCACAATG
C+V region	GCCTCGAG ACCATGGAAAAGAAAATAG	TAGGATCC CGGGGCTGGTCCGAGG
V+SH3 region	GCCTCGAGACCATGGCTCTTGCATCCCAAG	TAGGATCCCGCCGAGGTAAAGGCACAATG

Anti-clathrin (from rabbit and goat), anti-synaptojanin 1 (from goat), anti-EGF (from rabbit), and anti-EGFR (from rabbit) antibodies, rhodamine-labeled goat antibodies to rabbit IgG, rhodamine-labeled donkey antibodies to goat IgG, and FITC-labeled donkey antibodies to rabbit IgG were purchased from Santa Cruz Biotechnology Inc. FITC-labeled phalloidin was purchased from SIGMA. A polyclonal antibody against rtSH3p13 was developed using the fusion protein comprising of C-terminus of rtSH3p13 (V region and SH3 domain).

GFP-fusion protein encoding full-length and RFP-fusion proteins encoding C-region (1-209), V-region (210-253), SH3 domain (254-312), C+V (1-253) and V+SH3 (210-312) of *rtSH3p13* were generated using pEGFP-N1 and pDsRed1-N1 (Clontech, CA) and verified by sequencing. The primers for these constructs are as listed in Tab 1.

Cell culture and transfection

Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Twenty-four hours prior to experiments, CHO or germ cells were plated on glass coverslips, and then transiently transfected with pEGFP-N1-rtSH3p13 expression vectors using LipofectAMINE™ according to the manufacturer's instructions (Invitrogen, CA).

cDNA library Screening

ESTs derived from differential displayed mRNA of fragmentation of seminiferous tubule were used as probes to screen the rat testis λ gt10 5'-stretch cDNA library (Clontech, CA). A total of 1×10^6 clones were screened with the plaque hybridization method. Positive clones were selected and the inserts were amplified using the primers of the flank sequence of phage vectors. The amplified products were cloned into a pGEM-T Easy vector (Promega), sequenced by the ABI 377 autosequencer and submitted to GenBank.

RNA preparation and Northern blot analysis

Adult rat tissues from brain, heart, intestine, kidney, liver, lung, muscle, spleen and testis were frozen in liquid nitrogen immediately after dissection and stored at -80°C . For developmental studies, testes were collected from male mice at day 6, 18, 21, 40, and 60 of neonatal life. Total RNA was isolated and 20 μg of each sample were separated on 1% denatured agarose gel and transferred onto a positively charged nylon membrane (Boehringer Mannheim). The membrane was probed with γ - ^{32}P -labeled cDNA fragment from 1-234 nt of 5'-UTR of rtSH3p13 and then with γ - ^{32}P -labeled β -actin cDNA followed

by autoradiography.

Production of recombinant protein

Portions of the coding regions of the rtSH3p13 protein (V-SH3) corresponding to the variable region and SH3 domain of *rtSH3p13* cDNA were amplified by PCR. PCR fragments were digested by appropriate restriction enzymes and subcloned into the *Bam*HI and *Xho*I sites of pGEX-4T-3 vector that express the recombinant proteins with a glutathione S-transferase (GST) protein. The recombinant GST-V-SH3 was purified using Glutathione Sepharose 4B (Amersham Biosciences).

Immunohistochemistry

Cryostat frozen rat testis sections of 5 μm -thick were prepared according to the manufacturers' instruction (Zymed Histospain-SP, MT Rabbit ACE kit, Zymed Lab. Inc. South San Francisco). After blocking with 5% BSA in 3% H_2O_2 /PBS buffer, the testis sections were incubated with an anti-rtSH3p13 antibody (1:1000 dilution in PBS/5% BSA), or with pre-immune serum (1:1000 dilution in PBS/5% BSA) as a negative control for 1h at room temperature. After washing three times with PBS buffer, all slides were incubated with biotin-labeled anti-rabbit IgG antibody at 37°C for 30 min and then incubated with Streptavidin-coupled horseradish peroxidase at 37°C for 30 min. Sections were washed with PBS, incubated with AEC detection buffer (4 mg AEC dissolved in 1 ml dimethyl formamide and 14 ml of 0.1 M sodium acetate, pH 5.2, with 15 μl of H_2O_2), and then counterstained with Mayer's haematoxylin followed by microscopic analysis.

Preparation of testis tissue extract and germ cell extract

The separation of germ cells was performed according to Fujii *et al* [16]. Briefly, testes of adult rats were collected and the tunica albuginea was removed from each testis. The seminiferous tubules were placed in Eagle's minimal medium (pH 7.2) containing 0.02 M Hepes and 0.1% collagenase, gently unraveled with forceps, and incubated at 33°C for 30 min. After addition of PBS containing 1 mM EDTA, the tubule suspension was transferred into a conical tube and left standing for 5 min to precipitate tubule fragments. The supernatant containing Leydig cells was discarded. The tubules were reincubated in Eagle's minimal medium (pH 7.2) containing 0.02 M Hepes and 0.1% collagenase at 33°C for 15 min and, dispersed by gently pipetting in PBS containing 1 mM EDTA to remove residual Leydig cells and washed by PBS containing 1 mM EDTA. Following a 5 min's incubation, the supernatant was filtrated through a nylon

mesh and served as the germ cell fraction, which was spun at 600 g for 10 min and the cell pellet was used to prepare germ cell extracts. The frozen male rat testis and the germ cell pellet were homogenized respectively in 10 ml of homogenization buffer (20 mM Hepes, pH 7.4, 300 mM sucrose, 150 mM NaCl, 4 µg/ml of each pepstatin, aprotinin, leupeptin, and 1 mM PMSF) and spun at 2,600 rpm for 10 min at 4°C, respectively. Then Triton X-100 was added to the supernatant to a final concentration of 1%. After incubation at 4°C for 30 min, the sample was further spun for 1 h at 35,000 rpm at 4°C. The supernatant was saved as testis tissue extract and germ cell extract (1 mg/ml).

Analysis of the binding partner for rtSH3p13 by GST pull-down assay

Recombinant GST-rtSH3p13 or GST proteins were incubated with Glutathione Sepharose 4B (Pharmacia Biosciences) for 3 h at 4°C and then washed three times with PBST (1% Triton X-100 in 1×PBS). The beads were equilibrated with homogenization buffer and then added to 10 ml of germ cell extracts and incubated for 3 h at 4°C. Then the beads were boiled, separated by SDS/PAGE and transferred onto a PVDF membrane. The membrane was probed with an anti-synaptojanin I antibody and detected by BCIP/NBT reagent (Bio-Rad).

Co-immunoprecipitation assay

Anti-rtSH3p13 antibodies were incubated with 2 mg germ cell lysates and 20 µl of protein A beads at 4°C overnight, respectively. Pre-immune serum was used as a control. The protein A beads were washed three times with buffer of 20 mM Hepes, pH 7.4, 300 mM sucrose, 150 mM NaCl, 4 µg/ml of each pepstatin, aprotinin, leupeptin, and 1 mM PMSF, boiled, loaded on a 10% SDS-PAGE, and then transferred onto a PVDF membrane. The membrane was probed with an anti-synaptojanin I antibody and detected by BCIP/NBT reagent (Bio-Rad).

Detection of clathrin-mediated endocytosis in cells by fluorescence microscopy

Thirty six hours after post-transfection, the transfectants were serum starved for 6 h and then treated with EGF (100 ng/ml) on ice for 15 min. After replacing with pre-warmed medium, cells were incubated at 37°C for 30 min and then fixed with 3.7% formaldehyde in PBS at room temperature for 10 min. The fixed cells were permeabilized with PBS containing 0.1% Triton X-100 at room temperature for 10 min. After blocking with 3% BSA in PBS at 37°C for 30 min, the cells were incubated with an anti-clathrin (rabbit) or an anti-EGFR (rabbit) antibody, and then washed three times with PBS followed by an incubation with rhodamine-labeled goat antibodies to rabbit IgG. Finally, the cells were washed and mounted in 90% glycerol in PBS with 0.25% anti-fade reagent DABCO. The cells were observed under a LEICA TCS NT confocal microscope and recorded with a digital camera.

Quantification of fluorescence intensities and statistical analysis

Regional fluorescence intensities were quantified using Adobe Photoshop (v.7.0) software. To measure regional intensities, cells were selected using elliptical marquee tool. The intensity within each

cell were obtained using the histogram function for each color channel, and the resulting relative intensities of fluorescence were recorded for at least 20 cells per condition. The relative intensities of each transfected group were compared to that of the untransfected control group. The statistical significance of the analyses was evaluated using the paired Student's test.

Immunofluorescence localization

Squash preparations of rat testis were prepared according to the standard procedure. Briefly, the squash preparations were fixed with pre-chilled methanol. Following permeabilization with PBS containing 0.1% Triton X-100 at room temperature for 10 min, they were incubated with PBS buffer containing 3% BSA at 37°C for 30 min. Then the cells were incubated first with a mixture of goat polyclonal antibodies against clathrin or synaptojanin I and rabbit antiserum of rtSH3p13, and then incubated with a mixture of rhodamine-labeled donkey antibodies against goat IgG and FITC-labeled donkey antibodies to rabbit IgG. For labeling of actin in germ cells, FITC-labeled phalloidin was used according manufacturer's instruction (SIGMA) and rhodamine-labeled goat antibodies against rabbit IgG was used as secondary antibody to label rtSH3p13 with red fluorescence. The specimens were mounted with 0.25% DABCO, observed under a confocal microscopy and recorded with a digital camera.

RESULTS

Cloning and sequencing of rtSH3p13

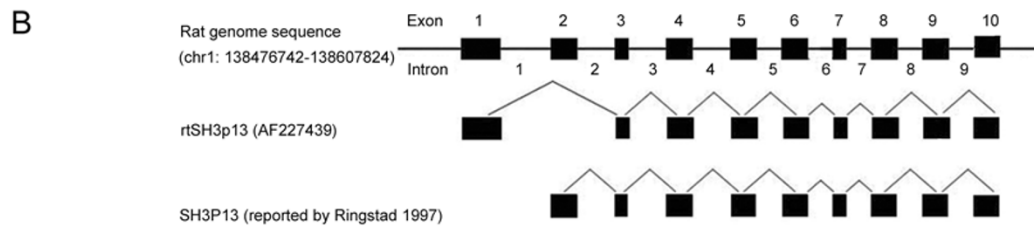
A full-length cDNA clone was isolated by screening of rat testis cDNA library. The complete cDNA of rtSH3p13 is 1463 bp, which contains an open reading frame of 936 bp with 5' and 3' noncoding regions of 456 and 67 nucleotides respectively. Protein-protein blast analysis (<http://www.ncbi.nlm.nih.gov/blast>) revealed that its coding protein is a truncated isoform of SH3p13 protein (previously isolated from rat brain cDNA library, belongs to the family of SH3p4/8/13 and contains 347 amino acids), which lacks the 35 amino acids at the N-terminal (Fig 1A). The full-length cDNA was termed rtSH3p13 (rat testis SH3p13) with GenBank accession No.AF227439. Similar to SH3p4/SH3p8/SH3p13, rtSH3p13 is composed of three regions. The N-terminal (1-209 amino acids) is predicted to form a coiled structure; the middle part (210-253 amino acids) encodes a variable region (which is diverse comparing with the part of SH3p4 and SH3p8). The C-terminal (254-312 amino acids) encodes a SH3 domain.

Splicing pattern analysis of rtSH3p13 and SH3p13

The cDNA sequence of rtSH3p13 was compared with rat genome on Genome Browser, University of California, Santa Cruz (<http://genome.ucsc.edu/cgi-bin/hgBlat>). The amino acids sequence of SH3p13 protein was reported by Ringstad *et al* in 1997[2]. However, no corresponding record comprising the complete cDNA sequence of SH3p13 could be found in Genbank. Therefore, based on

A

rtSH3p13	1		MEKKIDITSKAVAEI	15
SH3p13	1	MSVAGLKKQPHKASQLFSFKISGAEGTKLDEEFID	MEKKIDITSKAVAEI	50
mouse endophilin III	1	MSVAGLKKQPHKASQLFSEKISGAEGTKLDEEFID	MEKKIDITSKAVAEI	50
rtSH3p13	16	LSKATEYLQPNPAYRAKLGMLNTMSKLRGQVKATGYPQTEGLLGDCMLKY		65
SH3p13	51	LSKATEYLQPNPAYRAKLGMLNTMSKLRGQVKATGYPQTEGLLGDCMLKY		100
mouse endophilin III	51	LSKATEYLQPNPAYRAKLGMLNTMSKLRGQVKATGYPQTEGLLGDCMLKY		100
rtSH3p13	66	GRELGEDSAFGNSLVVGEALKLMAEVKDSLDINVKQTFIDPLQLLQDKD		115
SH3p13	101	GRELGEDSAFGNSLVVGEALKLMAEVKDSLDINVKQTFIDPLQLLQDKD		150
mouse endophilin III	101	GRELGEDSAFGNSLVVGEALKLMAEVKDSLDINVKQTFIDPLQLLQDKD		150
rtSH3p13	116	LKEIGHHLRKLKLEGRRLDYDYKRRVVGKIPPEEIRQAVEKFEESKELAERS		165
SH3p13	151	LKEIGHHLRKLKLEGRRLDYDYKRRVVGKIPPEEIRQAVEKFEESKELAERS		200
mouse endophilin III	151	LKEIGHHLRKLKLEGRRLDYDYKRRVVGKIPPEEIRQAVEKFEESKELAERS		200
rtSH3p13	166	MFNPLENDVEQVSQLAVFVEAALDYHRQSTEILQELCKRLELRITLALASCV		215
SH3p13	201	MFNPLENDVEQVSQLAVFVEAALDYHRQSTEILQELCKRLELRITLALASCV		250
mouse endophilin III	201	MFNPLENDVEQVSQLAVFVEAALDYHRQSTEILQELCKRLELRITLALASKV		250
rtSH3p13	216	FRPDYMPKPVNTSSITANGVEPSSSSKLTGTDIESDQPCCRGLYDFEPEN		265
SH3p13	251	FRPDYMPKPVNTSSITANGVEPSSSSKLTGTDIESDQPCCRGLYDFEPEN		300
mouse endophilin III	251	FRPEFMPKPVNMSITANGVGPSSSSKTFPGDTEADQPCCRGLYDFEPEN		300
rtSH3p13	266	EGELGFKEGDIIITLTNQIDENWYEGMLRGESGFFPINYVEVIVPLPR		312
SH3p13	301	EGELGFKEGDIIITLTNQIDENWYEGMLRGESGFFPINYVEVIVPLPR		347
mouse endophilin III	301	EGELGFKEGDIIITLTNQIDENWYEGMLRGESGFFPINYVEVIVPLPP		347



C

Exon 2

cggttgctatctgcgcgcgcggtgtgccagtgtagacagcgccgtggcgGTGGCCGTGG

CCGTGGAGCGCGCGGCGACCCCGGGGTGCCCGCTCCGCTCAGCGTCGCGGCCGCGTGG

CCCGGCGGAGCCCGAGACCACCCCGGGCGGGCGCCCGCGATGTCGGTGGCTGGGC

Start codon of SH3p13

TCAAGAAGCAGTTCACAAAGCCAGCCAGgtagggaggcgcgcttaggg

Exon 3

gttgtttttgcagCTGTTTAGTAAAAAATAAGTGGTGCAGAAGGAACGAAGCTAGATG

AAGAGTTTCTGGATATGAAAAAGgtaaagctgtttgaaca

Start codon of rtSH3p13

Fig 1. (A) Schematic alignment of amino acids sequences of rtSH3p13 isolated from a rat testis cDNA library and SH3p13 isolated from a rat brain cDNA library by Ringstad et al and mouse endophilin III. (B) Splicing pattern of the genome sequence coding rtSH3p13(AF227439) and SH3p13 (isolated by Ringstad *et al*). The gene encoding rtSH3p13 and SH3p13 is composed of 10 exons. Both rtSH3p13 and SH3p13 are products of alternative splicing. SH3p13 is lacking exon 1, while rtSH3p13 is lacking exon 2. (C) The alternative splicing results in different start codon of protein translation, thus produce the two isoforms: rtSH3p13 and SH3p13.

Tab 2. Locus of the exons of the gene coding rtSH3p13 and SH3p13

Exon1	Locus
Exon1	chr1: 138476746~138477133
Exon2	chr1: 138477833~138477944
Exon3	chr1: 138562105~138562173
Exon4	chr1: 138569125~138569197
Exon5	chr1: 138571411~138571554
Exon6	chr1: 138573875~138574008
Exon7	chr1: 138576518~138576676
Exon8	chr1: 138583986~138584091
Exon9	chr1: 138585113~138585222
Exon10	chr1: 138607204~138607461

the high homology between SH3p13 and mouse endophilin III (Fig 1A), the cDNA sequence of mouse endophilin III was used to BLST the rat genome. The results of the comparison indicated that the gene coding rtSH3p13 and SH3p13 is composed of 10 exons (Tab 2). Both rtSH3p13 and SH3p13 are products of alternative splicing (the detail of the splicing pattern is shown in Fig 1B), which results in different start codon of protein translation, thus produces the two isoforms: rtSH3p13 and SH3p13. SH3p13 is lacking exon 1, while rtSH3p13 is lacking exon 2 (Fig 1C).

rtSH3p13 was expressed mainly in testis and brain

The expression pattern of rtSH3p13 transcripts in eight rat tissues was determined by Northern blot analysis (Fig 2A). The result showed rtSH3p13 was most abundant in testis, could be detected in a lower level in brain tissue and no transcript was revealed in other examined tissues, which was consistent with what was reported by Ringstad *et al* [2]. Considering that the RNA preparations were obtained from adult rat, the rtSH3p13 transcripts are likely due to the male germ cell population in which more than 90% of the cells are present in the adult testis in contrast to the prepuberal animal where the somatic component is not negligible. To determine whether rtSH3p13 expression undergoes developmental regulation, RNAs isolated from testes of several day-periods after birth were analysed by Northern blot. As can be seen in Fig 2C, no rtSH3p13 transcripts were detected in the testis of 6-d-old rat; the rtSH3p13 transcripts could be detected in testes of 18, 21 and 40-d-old rat and reached the highest level in 60 d of adult rat. Analysis from the Northern blot showed that rtSH3p13 mRNA had a developmental stage-specific expression in rat testis.

In order to prepare a specific antibody against rtSH3p13,

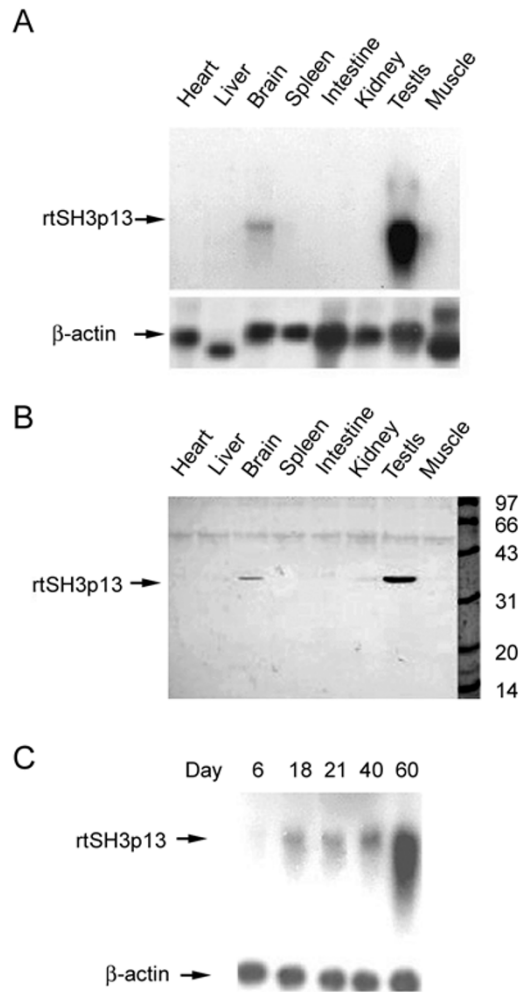


Fig 2. rtSH3p13 was mainly detected in the testis. (A) mRNA was isolated from different tissues and analyzed for expression of rtSH3p13 and β -actin mRNAs. Northern blot was performed using 5' UTR fragment (+1 to +234) as a probe. (B) Whole cell lysates prepared from various tissues were examined by Western blot to show protein expression patterns of rtSH3p13. (C) mRNA was isolated from rat testes on different days during development and then analyzed for expression of rtSH3p13 by Northern blot.

V-SH3 (Variable region plus SH3 domain of rtSH3p13) fusion protein was expressed, purified and used to immunize rabbits. After three times of immunization, high titer anti-serum was obtained. Western blot analysis showed the antibody could specifically bind to the V-SH3 fusion protein in induced pET30a (+)-V-SH3-BL21 (DE3) cell lysate (data not shown). Fifty micrograms of various tissues extracts were separated by SDS/PAGE, blotted onto a nylon membrane, and analysed with Western blot and anti-rtSH3p13 antibody. It showed that the antibody recognized a 38 kD protein in testis and brain extracts (Fig 2B), which agreed with the predicted molecular masses

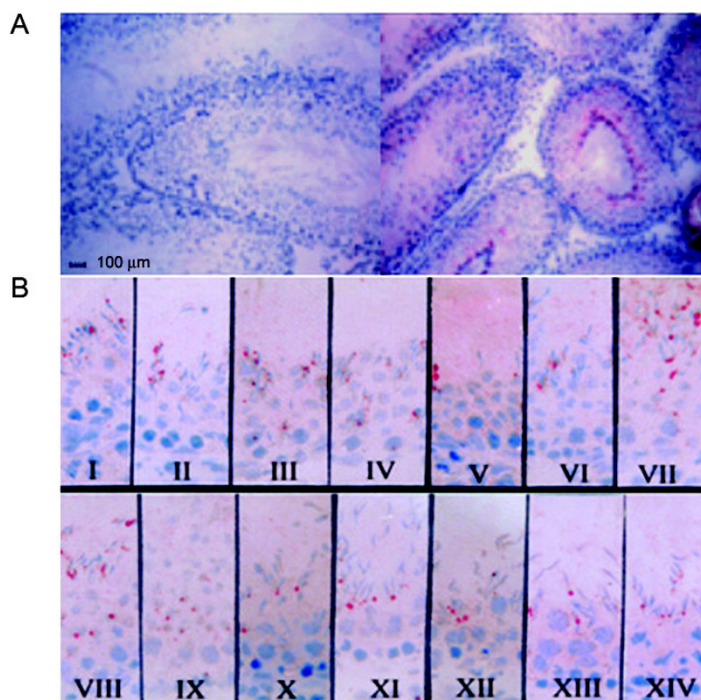


Fig 3. Involvement of rtSH3p13 in the process of rat spermatogenesis. **(A)** Immunolocalization of rtSH3p13 protein in round and elongated spermatids. Bar=100 µm. Testis sections were prepared according to manufacturer's instruction. After blocking with 5% BSA in 3% H₂O₂/PBS buffer, tissue sections were incubated with an anti-rtSH3p13 antibody. After washing with PBS, the tissue sections were incubated with Streptavidin-coupled HRP, subsequently with AEC, a substrate of horseradish-peroxidase, and then counterstained with Mayer's hematoxylin followed by microscopic analysis. (Left panel) Control preimmune serum and (right panel) anti-rtSH3p13 antibody. **(B)** Immunolocalization of rtSH3p13 in rat spermatogenic cells. Views of the 14 stages of spermatogenesis were arrayed according to the histological features. Note: staining of round spermatids at step 8, and spermatids in steps 8-19 of spermiogenesis.

of rtSH3p13 protein. From the result of Western blot, we concluded that the antibody could specifically bind to rtSH3p13 without interacting with other SH3 domain containing proteins. It can be used specifically for the localization of the rtSH3p13 protein in rat testis and advanced functional study.

Localization of rtSH3p13 protein in testis tissues by immunohistochemistry

Normal rat testis tissue sections were analysed by immunohistochemistry in order to identify the location of rtSH3p13 protein in the various testis cell types. A significant staining pattern with anti-rtSH3p13 was detected only in some round and elongated spermatids, whereas no staining was detected in spermatogonia, spermatocytes closing to basal membrane as well as in Sertoli cells and Leydig cells (Fig 3). For analysis of the specificity of the reaction, we analysed the same testis tissue sections with preimmune serum (negative control). As expected, staining with the preimmune serum did not show any significant signal. Further immunostaining investigation of multiple testis sections, it was found that positive staining appeared initially in the round spermatids at step 8, and all of the spermatids afterwards. That means in a typical spermatogenesis cycle, rtSH3p13 protein was expressed specifically in spermatids during steps 8-19, whereas no rtSH3p13 was expressed in spermatids during steps 1-7 (Fig 3). As we know that it is at these steps that rat spermatids begin

their transfiguration gradually from the round to elongated ones. The beginning of rtSH3p13 expression is concomitant with the initiation of spermatid transfiguration.

rtSH3p13 forms a complex with clathrin-synaptojanin in germ cells

Endophilin 1 and 2 have been identified to be components of clathrin-mediated endocytosis in neurons[9]. We wondered if rtSH3p13 forms a complex with clathrin-synaptojanin in testis. First, we investigated whether rtSH3p13 interacts with synaptojanin I, a key player in formation of a pit[7]. The GST-V-SH3 fusion protein was found to pull down synaptojanin I (Fig 4B). In contrast, GST alone did not pull down synaptojanin I (Fig 4B). To further examine whether the rtSH3p13 physiologically interacts with synaptojanin I in germ cells, we performed a co-immunoprecipitation assay using anti-rtSH3p13 antibodies. Consistent with the GST put-down assay, rtSH3p13 was found to co-precipitate synaptojanin I in germ cells (Fig 4C).

To localize the rtSH3p13 protein in germ cells, FITC-labeled phalloidin was used to label the actin in germ cells. Then rtSH3p13 protein expressed in germ cells was labeled by anti-rtSH3p13 antiserum and rhodamine-labeled donkey anti-goat IgG as secondary antibodies. The results indicated that rtSH3p13 was localized in acrosome region of germ cells (Fig 4D).

Finally, we investigated whether rtSH3p13 forms a com-

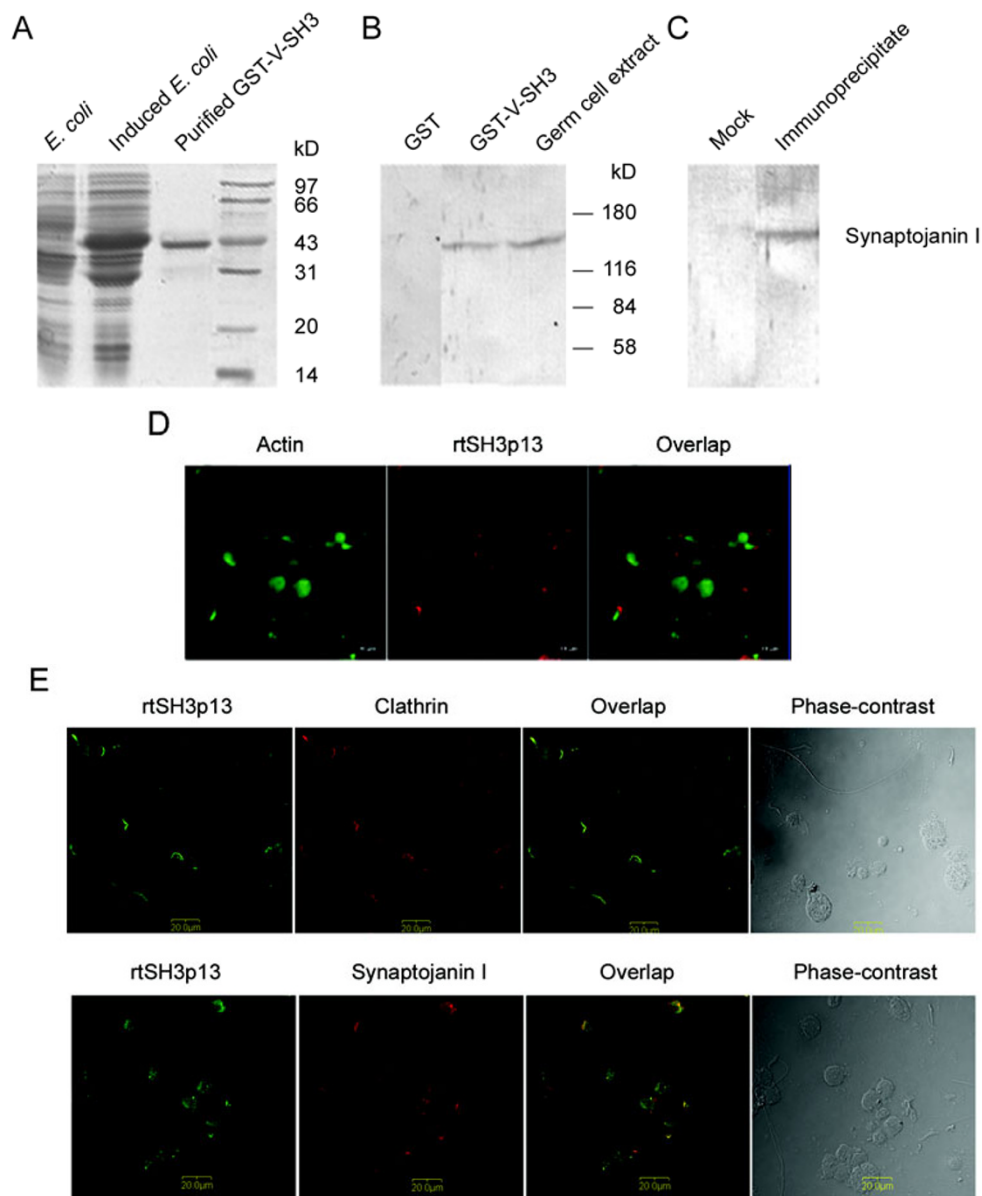


Fig 4. (A) Recombinant GST-V-SH3 was purified from bacteria via Glutathione Sepharose 4B. (B) Recombinant GST-rtSH3p13 or GST proteins were incubated with Glutathione Sepharose for 3 h at 4°C and then washed three times with PBST. Then the beads were equilibrated with homogenization buffer and then added to 10 ml of germ cell extracts. After 3 h of incubation at 4°C, the beads were washed, boiled, separated by SDS/PAGE and transferred onto a PVDF membrane. The membrane was probed with an anti-synaptojanin I antibody and detected by BCIP/NBT reagent. The GST-V-SH3 fusion protein was found to pull down synaptojanin I. In contrast, GST alone did not pull down synaptojanin I. (C) Anti-rtSH3p13 antibodies were incubated with 2 mg germ cell lysates and 20 μ l of protein A beads at 4°C overnight, respectively. Pre-immune serum was used as a control. The protein A beads were washed three times, boiled, loaded on a 10% SDS-PAGE, and then transferred on a PVDF membrane. The membrane was probed with an anti-synaptojanin I antibody and detected by BCIP/NBT reagent. rtSH3p13 was found to co-precipitate synaptojanin I in germ cells. (D) Squash preparations of rat testis were prepared according to the standard procedure. Following permeabilization with PBS containing 0.1% Triton X-100 at room temperature for 10 min, they were incubated with PBS buffer containing 3% BSA at 37°C for 30 min. To study the localization of rtSH3p13 in germ cells FITC-labeled phalloidin was used to label the actin in germ cells, rtSH3p13 protein expressed in germ cells was labeled by anti-rtSH3p13 antiserum and rhodamine-labeled goat anti-rabbit IgG used as secondary antibodies. The specimens were washed and then mounted with 0.25% DABCO. The specimens were observed under a confocal microscopy and recorded with a digital camera. The results indicated that rtSH3p13 was localized in acrosome region of germ cells. (E) To investigate whether rtSH3p13 forms a complex with clathrin and synaptojanin in testis, rat testis squash were prepared and then probed with anti-rtSH3p13, anti-clathrin or anti-synaptojanin antibodies. The specimens were observed under a confocal microscopy and recorded with a digital camera. Both rtSH3p13 and clathrin/synaptojanin were colocalized in germ cells in the squash preparation.

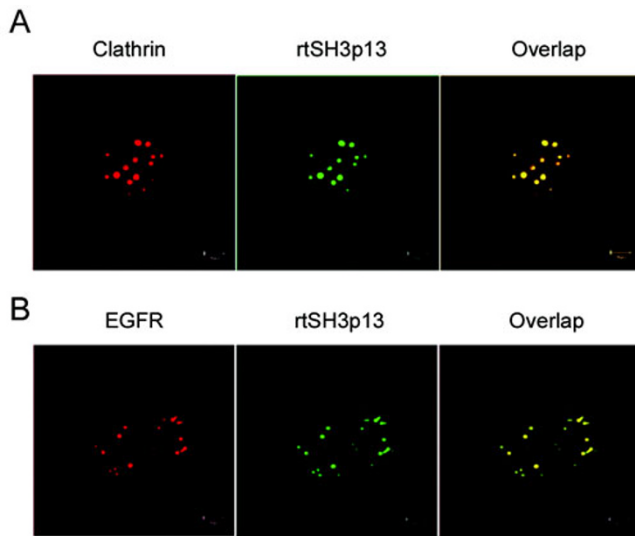


Fig 5. rtSH3p13 is required for clathrin-mediated EGFR endocytosis. Transiently transfected CHO cells expressing GFP-rtSH3p13 (green fluorescence) were starved for 6 h. Then the cells were treated with rhEGF, and fixed with 3.7% formaldehyde. The fixed cells were probed with an anti-clathrin (A) or anti EGFR antibody (B) and secondary antibodies. After washing with PBS, cells were observed under a fluorescent microscope and then recorded by a digital camera.

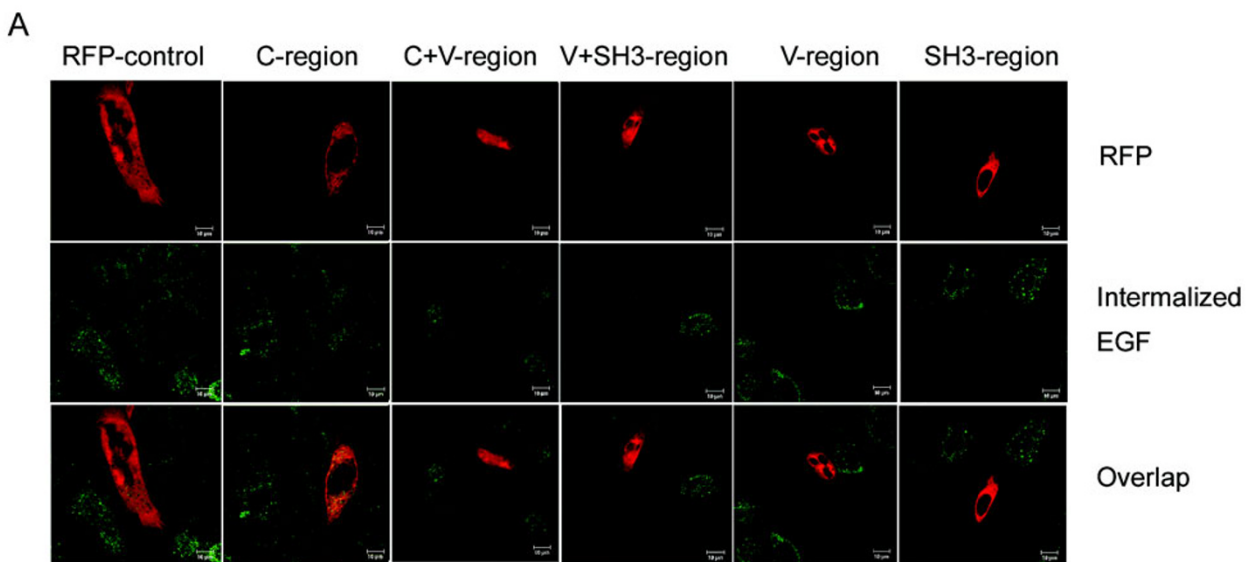


Fig 6. V region and SH3 domain inhibit EGF endocytosis in CHO cells and clathrin-coated vesicle formation in germ cells. (A) CHO cells were transfected with expression vectors RFP-rtSH3p13, RFP-C-region, RFP-C+V-region, RFP-V+SH3, RFP-V-region and RFP-SH3-domain, respectively. After 24 h, cells were starved and then treated with EGF (100 ng/ml) on ice for 15 min. After replacing with warm pre-medium, cells were incubated at 37°C for 30 min, washed intensively with PBS and then fixed followed by another incubation with antibodies. Internalization of EGF in CHO was detected using anti-EGF antibodies (small green spots) whereas RFP-fusion proteins gave red colors. (B) the statistic evaluation of relative intensities of fluorescence. The relative fluorescence intensities of each transfected group were compared to that of the untransfected control group. At least 20 cells for each condition were examined. The statistical significance of the analyses was evaluated using the paired Student's Test (*P < 0.01).

plex with clathrin and synaptojanin in testis. Rat testis squash were prepared and then probed with anti-rtSH3p13, anti-clathrin or anti-synaptojanin antibodies. Both rtSH3p13 and clathrin/synaptojanin were colocalized in elongated spermatids in the squash preparation (Fig 4E).

Taken together, these results suggest that rtSH3p13 is a component of the clathrin complex and may participate in vesicular traffic in germ cells.

rtSH3p13 is required for clathrin-synaptojanin-coated vesicle formation

To further understand the functions of rtSH3p13 in vesicle formation, we first investigated whether rtSH3p13 is required for the clathrin-mediated EGFR endocytosis in CHO cells. The GFP-rtSH3p13 encoding vector was generated and then transfected into CHO cells. After treatment with EGF, GFP-rtSH3p13 was found to be concentrated in the newly formed vesicles in cytoplasm and was co-localized with clathrin (Fig 5A), suggesting that rtSH3p13 forms a complex with clathrin. To further identify whether rtSH3p13 involves formation of EGFR-mediated endocytic vesicles in CHO cells, we performed a similar assay by using an anti-EGFR antibody to label the internalized EGFR. After treatment with EGF, both GFP-rtSH3p13 and EGFR containing endosomes formed bright spots in the cytoplasm and these two complexes co-localized properly (Fig 5B), suggesting a plausible role of rtSH3p13 in the formation of EGFR vesicles.

V region of rtSH3p13 blocks internalization of EGF

As SH3-domain gets involved in mediating the interaction of rtSH3p13 with clathrin-synaptojanin complex, we wondered whether this domain is important for rtSH3p13 in EGFR-mediated endocytosis. We found that over expression of RFP-SH3 domain of rtSH3p13 inhibited the intracellular accumulation of receptor-bound EGF (green spot) (Fig 6A) in CHO cells, further establishing the physiological relevance of interaction with rtSH3p13 in receptor-mediated endocytosis *in vivo*. Surprisingly, over expression of RFP-V region of rtSH3p13 also blocked the receptor-mediated internalization of EGF (green spot) (Fig 6A) in CHO cells. In contrast, over expression of RFP-C (coiled coil region) of rtSH3p13 only had a slight defect on the intracellular accumulation of EGF (Fig 6A). To further evaluate the importance of both V region and SH3 domain of rtSH3p13 in the receptor-mediated internalization of EGF, RFP-C+V and RFP-V+SH3 constructs were generated. However, over-expression of RFP-C+V and RFP-V+SH3 had a compromised effect on inhibition of internalization of EGF (Fig 6A). As shown in Figs 6B, the relative intensities of fluorescence in cells were quantitatively evaluated.

DISCUSSION

In neurons, clathrin-mediated endocytosis has been shown to be the central part of recycling synaptic vesicle membrane components to maintain a functional pool of secretory vesicles[9, 17]. Many protein factors that participate in clathrin-mediated synaptic vesicle recycling have been identified to interact directly or indirectly with either components of clathrin coat or the GTPase dynamin, a protein required for the maturation and scission of nascent-coated pits. Synaptojanin I is one such accessory factor. A major binding partner for synaptojanin I is the SH3 domain-containing protein SH3p4 and SH3p8[1-3, 10, 18, 19]. Both SH3p4 and SH3p8 are primarily concentrated in nerve terminals and mediate synaptic vesicle formation[2, 4, 10, 11]. Interestingly, endophilin mutations in *Drosophila* block clathrin-mediated endocytosis but not neurotransmitter release[14]. However, SH3p13 is less studied and its function is yet to be discovered.

Using an EST derived from differential displayed mRNA of fragmentation of semiferous tubule as a probe, we screened a rat testis λ gt10 5'-stretch cDNA library and identified rtSH3p13 in testis. Our analysis of the rtSH3p13 cDNA sequence indicated it is a product of alternative splicing, encoding a truncated isoform of SH3p13 identified by Ringstad *et al*[2], and contains a typical endophilin protein structure: a C-region, a V-region and a SH3 domain.

Our results also showed that rtSH3p13 was expressed mainly in testis and brain, suggesting that rtSH3p13 expression is tissue specific. Further more, in testis rtSH3p13 was found only in germ cells but not in Sertoli or Leydig cells, suggesting that its expression is also cell type specific.

Besides its tissue and cell type specificity, the expression of rtSH3p13 also displays a development-dependent manner. In the 6-day-old rat, most germ cells are immature in testis, including type A, intermediate and type B spermatogonia, the later of which is considered to commit to differentiation[20, 21]. By the day 18, preleptotene primary spermatocytes are formed and enter zygotene phase to form pachtene spermatocytes. At day 21, meiotic and postmeiotic spermatocytes and some spermatids appear in seminiferous epithilium. These spermatids undergo spermiogenesis by day 40. At day 60, the round spermatids and elongated spermatids increase significantly [20-22]. We showed that in younger rats (<18 d-old), rtSH3p13 was not detected. By the day 18, rtSH3p13 began to appear and since then it gradually increased and reached maximal level at day 60. Here we further showed that rtSH3p13 was selectively expressed in a spermatogenesis cycle.

Translational repression is a common feature of gene expression during haploid differentiation of spermatids. It has been demonstrated that, in haploid spermatids,

essentially every mRNA exhibits the evidence of translational repression[23]. rtSH3p13 appears to be such a case. The difference of rtSH3p13 mRNA levels between testis and brain shown in Fig 2A is more obvious than the difference of its protein levels between those two tissues as shown in Fig 2B, suggesting that post-transcriptional regulations are involved in rtSH3p13 translation. This hypothesis is further proved by our analysis of rtSH3p13 mRNA, which found that the mRNA of rtSH3p13 first appeared in spermatocytes in the meiotic prophase, whereas the protein appeared at step 8 spermatids and then in all of steps of spermatids (steps 9-19).

Spermatid cytoplasm contains numerous membrane pits, vesicles and membranous tubules, which are frequently anastomosed and play an important role in germ cell division, differentiation and metabolism[21, 22, 24]. Pale and dense multivesicular bodies and secondary lysosome-like structure are also evident in the cytoplasm[21, 22, 24]. However, the mechanism of formation of those vesicles or pits has not been well understood.

Our results further demonstrated that in addition to its potential role in the development of germ cells, rtSH3p13 is a component of the clathrin-coated complex. By examining rat testis squash preparations, we provided evidence that rtSH3p13 is localized in acrosome region and forms a complex with clathrin and synaptojanin I in elongated spermatids. This finding was further supported by the interaction of rtSH3p13 with synaptojanin I in germ cells. We also showed that in EGF- treated CHO cells, rtSH3p13 was concentrated in newly formed vesicles in cytoplasm and co-localized with clathrin. These results indicated that rtSH3p13 may participate in clathrin-mediated vesicular traffic in germ cells and may function in formation of acrosome.

We identified that over expression of SH3 domain of rtSH3p13 blocked the internalization of EGF in CHO cells, revealing that rtSH3p13 has a common character of SH3-domain-containing proteins that function in CCV formation[25]. Furthermore, we demonstrated that over expression of V region of rtSH3p13 blocked the accumulation of receptor-mediated EGF in CHO cells, indicating that V region plays an important role in receptor-mediated vesicle formation.

Clathrin-mediated endocytosis is very crucial for the process of internalization of receptors as well as intracellular vesicular traffic etc. It consists of sequential steps which involve the participation of many proteins such as clathrin, synaptojanin, amphiphysin, dynamin etc. In neuron, this process functions on presynaptic vesicle trafficking in nerve terminals. But the significance of clathrin-mediated endocytosis happens in sperm cells is not clear. Our study demonstrated that rtSH3p13 had an active role

in this process; this may help to elucidate the mechanism of clathrin-mediated endocytosis during spermatogenesis.

ACKNOWLEDGEMENTS

We are grateful to Dr. Chu WM for his valuable suggestion on the design of experiments. This study was supported by grants from National Natural Science Foundation of China (No. 30240019, 30300060), National High Technology Research and Development Program of China (No. 2001AA221131), Major State Basic Research Project (No. G1999055901), and State Ministry of Science and Technology Program (No. 2002BA711A01).

Received, Mar 5, 2004

Revised, May 20, 2004

Accepted, May 25, 2004

REGERENCE

- 1 Micheva KD, Kay BK, McPherson PS. Synaptojanin forms two separate complexes in the nerve terminal. Interactions with endophilin and amphiphysin. *J Biol Chem* 1997; **272**(43):27239-45.
- 2 Ringstad N, Nemoto Y, De Camilli P. The SH3p4/Sh3p8/SH3p13 protein family: binding partners for synaptojanin and dynamin via a Grb2-like Src homology 3 domain. *Proc Natl Acad Sci USA* 1997; **94**(16): 8569-74.
- 3 Sparks AB, Hoffman NG, McConnell SJ, Fowlkes DM, Kay BK. Cloning of ligand targets: systematic isolation of SH3 domain-containing proteins. *Nat Biotechnol* 1997; **14**(6):741-4.
- 4 Ringstad N, Nemoto Y, De Camilli P. Differential expression of endophilin 1 and 2 dimers at central nervous system synapses. *J Biol Chem* 2001; **276**: 40424-30.
- 5 Tang Y, Hu LA, Miller WE, et al. Identification of the endophilins (SH3p4/p8/p13) as novel binding partners for the beta1-adrenergic receptor. *Proc Natl Acad Sci USA* 1999; **96**(22): 12559-64.
- 6 Cremona O, Di Paolo G, Wenk MR, et al. Essential role of phosphoinositide metabolism in synaptic vesicle recycling. *Cell* 1999; **99**(2): 179-88.
- 7 de Heuvel E, Bell AW, Ramjaun AR, et al. Identification of the major synaptojanin-binding proteins in brain. *J Biol Chem* 1999; **272**(13): 8710-6.
- 8 Farsad K, Ringstad N, Takei K, et al. Generation of high curvature membranes mediated by direct endophilin bilayer interactions. *J Cell Biol* 2001; **155**(2): 193-200.
- 9 Hinshaw JE. Dynamin and its role in membrane fission. *Annu Rev Cell Dev Biol* 2000; **16**:483-519.
- 10 Micheva KD, Ramjaun AR, Kay BK, McPherson PS. SH3 domain-dependent interactions of endophilin with amphiphysin. *FEBS Lett* 1997b; **414**(2):308-1.
- 11 Schmidt A, Wolde M, Thiele C, et al. Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. *Nature* 1999; **401**:133-41.
- 12 Slepnev VI, De Camilli P. Accessory factors in clathrin-dependent synaptic vesicle endocytosis. *Nat Rev Neurosci* 2000; **1**(3):161-72.
- 13 Tran J, Brenner TJ, DiNardo S. Somatic control over the germline stem cell lineage during *Drosophila* spermatogenesis. *Nature* 2000; **407**(6805):754-7.

- 14 Verstreken P, Kjaerulff O, Lloyd TE, et al. Endophilin mutations block clathrin-mediated endocytosis but not neurotransmitter release. *Cell* 2002; **109(1)**:101-12.
- 15 Soubeyran P, Kowanetz K, Szymkiewicz I, Langdon WY, Dikic I. Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors. *Nature* 2002; **416(6877)**:183-7.
- 16 Fujii T, Tamura K, Copeland NG. Sperizin is a murine RING zinc-finger protein specifically expressed in haploid germ cells. *Genomics* 1999; **57**:94-101.
- 17 Jarousse N, Kelly RB. Endocytotic in synapses. *Current Opin. Cell Bio* 2001; **13**:461-9.
- 18 Giachino C, Lantelme E, Lanzetti L, et al. A novel SH3-containing human gene family preferentially expressed in the central nervous system. *Genomics* 1997; **41(3)**:427-34.
- 19 So CW, Caldas C, Liu MM, et al. EEN encodes for a member of a new family of proteins containing an Src homology 3 domain and is the third gene located on chromosome 19p13 that fuses to MLL in human leukemia. *Proc Natl Acad Sci USA* 1997; **94(6)**: 2563-8.
- 20 Clermont Y, Perey B. Quantitative study of the cell population of the seminiferous tubules in immature rats. *Am J Anat* 1957; **100**:241-66.
- 21 Clermont Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev* 1972; **52**:198-236.
- 22 Hess RA. Quantitative and qualitative characteristics of the stages and transitions in the cycle of the rat seminiferous epithelium, light microscopic observation of perfusion-fixed and plastic-embedded testes. *Biol Reprod* 1990; **43**:525-42.
- 23 Steger K. Transcriptional and translational regulation of gene expression in haploid spermatids. *Anat Embryol (Berl)* 1999; **199(6)**:471-87.
- 24 Segretain D. Endocytosis in spermatids during spermiogenesis of the mouse. *Bio cell* 1989; **67(3)**:289-98.
- 25 Simpson F, Hussain NK, Qualmann B, et al. SH3-domain-containing proteins function at distinct steps in clathrin-coated vesicle formation. *Nat Cell Biol* 1999; **1(2)**:119-24.