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Original Article

Human RING finger protein ZNF645 is a novel testis-specific E3 ubiquitin ligase

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Abstract

A large number of testis-specific genes are involved in the complex process of mammalian spermatogenesis. Identification of these genes and their roles is important for understanding the mechanisms underlying spermatogenesis. Here we report on a novel human RING finger protein, ZNF645, which contains a C3HC4 RING finger domain, a C2H2 zinc-finger domain, and a proline-rich region, indicating that it has a structure similar to that of the c-Cbl-like protein Hakai. ZNF645 was exclusively expressed in normal human testicular tissue. Immunohistochemical analysis confirmed that ZNF645 protein was present in spermatocytes, round and elongated spermatids, and Leydig cells. Immunofluorescence staining of mature sperms further showed that the ZNF645 protein was localized over the postacrosomal perinuclear theca region and the entire length of sperm tail. An *in vitro* ubiquitination assay indicated that the RING finger domain of the ZNF645 protein had E3 ubiquitin ligase activity. Therefore, we suggest that ZNF645 might act as an E3 ubiquitin-protein ligase and play a role in human sperm production and quality control.

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Keywords: E3 ubiquitin ligase, RING finger protein, spermatogenesis, ZNF645

1 Introduction

Mammalian spermatozoan development is a complex process that involves the proliferation and differentiation of spermatogonia, the meiosis of spermatocytes, and drastic morphological changes that occur during the progression from round spermatids to mature sperm [1]. Complete understanding of mammalian spermatogenesis is not easy because we have still not identified the structure or function of many of the genes that are involved in this process. However, it has been hypothesized that more than 2 300 genes in mice—about 4% of the mouse genome—are specifically expressed in the testis [2]. Identification of these genes and their biological function would be a considerable challenge, but it would be very helpful in elucidating the molecular process of spermatogenesis and thereby identifying a vast number of candidates for contraceptive targeting [3].

Zinc-finger proteins constitute the most abundant protein super-family in the mammalian genome and they are involved in a variety of cellular activities, such as development, differentiation and tumour suppression. It has been reported that there are 46 conserved zinc-finger domains in the 1 573 zinc-finger-containing proteins, as listed in the InterPro database [4]. The five most frequent domains are the C2H2, PHD finger, KRAB-box, LIM and RING finger domains. RING finger proteins are characterized by the presence of a cysteine-rich domain (CX2CX[9–39]CX[1–3]HX[2–3]CX2CX[4–48]CX2C) and are involved in many biological processes, including signal transduction, transcriptional regulation, apoptosis and ubiquitination [5–7].

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As one of the most versatile cellular regulatory mechanisms, ubiquitination is involved in many physiological and pathological events, including spermatogenesis, fertilization and sperm quality control [8, 9]. Ubiquitination involves a cascade of highly specific enzymes, such as E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin-protein ligase [10]. The E3 ubiquitin-protein ligase plays a key role in determining the specificity and timing of the ubiquitination of substrates and the functional alteration or degradation of the targeted protein [11, 12].

Until now, only a few RING finger proteins with E3 ubiquitin ligase activity have been reported to be involved in mammalian spermatogenesis. For example, RNF4 has been reported to modulate both steroid-receptor-dependent and basal transcription, as well as to interact with a variety of nuclear proteins involved in cell growth regulation [13, 14]. MEX (MEKK1-related protein X), a testis-specific E3 ubiquitin ligase, promotes death receptor-induced apoptosis [15]. A third protein, the mouse protein Rnf133, may play a role in sperm maturation [16]. Here we describe a novel RING finger protein, ZNF645, which is expressed specifically in human testis and sperm and has E3 ubiquitin ligase activity.

2 Materials and methods

2.1 Human samples

Normal human testicular and lung tissue were taken from a body donor died in an accident from West China Hospital, Sichuan University, Chengdu, China. The semen was obtained from a normal healthy donor, and informed consent for the samples was obtained from both a relative of the body donor and the semen donor. The present study was authorized by the Ethical Committee of West China Hospital, Sichuan University, China.

2.2 Analysis of the expression pattern of ZNF645

The First-Strand cDNA Kit, which contained the cDNA preparations from 16 different human tissues (heart, liver, brain, lung, kidney, spleen, pancreas, thymus, small intestine, colon, testis, ovary, prostate, placenta, skeletal muscle and peripheral leukocytes), was purchased from Clontech (Mountain View, CA, USA). The primers used to specifically amplify the *ZNF645* gene were 5′-AGTATGTCCGCGCTGTCGTTAT-3′ (forward) and 5′-TGTGGCTGATTATGTTGCTCTTGT-3′ (reverse). A 980-bp fragment of *G3PDH* was co-amplified as an internal control with the primers 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ (forward) and 5′-CATGTGGGCCATGAGGTCCACCAC-3′ (reverse). The polymerase chain reaction (PCR) mixture was first heated at 94ºC for 1 min. Next, 30 PCR cycles were carried out using the following parameters: denaturing at 94ºC for 30 s, annealing at 65ºC for 30 s and extension at 72ºC for 1 min. The reaction was completed with a final extension step that was performed at 72ºC for 10 min. The PCR products were then separated using 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

2.3 Immunoblot analysis of the ZNF645 protein

The rabbit anti-ZNF645 polyclonal antibody (cat. No. ARP36191) was purchased from Aviva Systems Biology Company (San Diego, CA, USA). Human testicular and lung tissues as well as cultured cells from various cell lines (including HEK293T, HeLa, A549 and HepG2) were homogenized on ice in RIPA lysis buffer with 1 μL per mL protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The extraction of sperm protein and its fractionation were performed as described previously [17]. Protein samples were then subjected to SDS-PAGE and the protein bands were electroblotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) and then blocked with 5% nonfat milk for 1 h. Next, the filters were incubated with rabbit anti-ZNF645 polyclonal antibody (1:1 000 dilution) for 1 h and then washed three times in PBS-T for 10 min each and incubated with goat-anti-rabbit second antibody conjugated to horseradish peroxidase (1:10 000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After additional four washing steps, the reactive bands were visualized with an immunostaining kit (Millipore) and exposed to X-ray films (Kodak, Xiamen, China).

RNF141 antibody, which is targeted against proteins that are specifically present in the acrosome and tail fractions of sperm, was used to confirm that there was no cross-contamination between the theca, acrosome and tail fractions. RNF141 has been reported to be specifically present in the acrosome and tail of mouse sperm acrosome [18]. In addition, in our previous experiment, we demonstrated the same localization in human sperm (unpublished data).

2.4 Immunohistochemical analysis of ZNF645 in human testis

Immunohistochemical analysis of ZNF645 in human testis was performed as described previously [17].

Briefly, 5-μm human testis sections were deparaffinized in xylene and progressively rehydrated in a graded series of ethanol/water mixtures. Antigens were retrieved with 10 mmol L^{-1} sodium citrate, and 0.3% H_2O_2 in methanol was used to destroy any endogenous peroxidase activity that was present. After they were washed in PBS and blocked with 1% goat serum, the sections were incubated with the rabbit anti-ZNF645 polyclonal primary antibody (1:500 dilution) at 4ºC overnight, rinsed in 1 × PBS and stained with an ABC detection kit (Pierce, Rockford, IL, USA). Sections were counterstained with hematoxylin. Preimmune rabbit serum was used as the primary antibody for the negative controls. Spermatozoa immunoreactivity was also investigated using the same methods.

2.5 Sperm immunofluorescence staining

Semen was allowed to liquefy for 30 min, then the sperm were washed three times in 1× PBS (pH 7.4), air-dried onto microscope slides, permeabilized with cold methanol for 15 min, and air-dried again as previously described [18, 19]. Then the slides were covered with 10% goat serum for 30 min to block nonspecific antibody binding and stained with 1:500 rabbit anti-ZNF645 primary antibody in blocking buffer for 2 h in a humidified chamber at room temperature. After being washed three times with PBS, the slides were stained with 1:100 goat anti-rabbit IgG fluorescein isothiocyanate-conjugated antibody (Sigma) for 1 h. Sperm nuclei were then stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Preimmune rabbit serum was used as the primary antibody in the negative control group. The mounted slides were examined under an Olympus IX71 fluorescent microscope (Olympus, Tokyo, Japan).

2.6 Analysis of the full-length cDNA sequence of the ZNF645 gene

Rapid amplification of the 5′ and 3′ cDNA ends (RACE) of the *ZNF645* gene was performed using the SMART RACE cDNA amplification kit (Clontech) using the following primers: 5GSP: 5′-CTCAGCACAGGATAACGACAGCGCGGAC-3′ and 3GSP: 5′-ACCTTGTCCACCAACGCGGAGTCCACCT-3′. The PCR products were recovered using the QIAEX Gel Extraction Kit (Qiagen, Hamburg, Germany), cloned into the pMDT20 Vector (Takara, Dalian, China) and sequenced in Invitrogen (Shanghai, China). The whole cDNA sequence of the *ZNF645* gene was assembled after removing the overlapping sequences. The deduced amino acid (aa) sequence was searched against the InterPro database (http://www.ebi.ac.uk/Tools/InterProScan/) to identify possible functional domains. The aa sequences alignment of human ZNF645 and its homologues was performed with the EBI ClustalW Web tool (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

2.7 Recombinant protein expression and in vitro ubiquitination assay

A partial *ZNF645* coding region containing a C3HC4 motif was amplified using the *Eco*RI or *Xho*I restriction site-containing primers 5′- ATGAATTCGGACATTAAGATAAACATCA -3′ (forward) and 5′-ATCTCGAGTTAACCTCGTTTATGCGCCTCA-3′ (reverse). The E2 ubiquitin-conjugating enzyme UBC4 coding sequence was also amplified using the primers 5′-CGAATTCGGCTCTGAAGAGAATCCAC-3′ (forward) and 5′-AGCGGCCGCTTACAT CGCATACTTCTG-3′ (reverse). Next, the PCR product was subcloned into the prokaryotic expression vector pGEX-5X-3. The glutathione-*S*-transferase (GST) fused proteins GST-ZNF645 and GST-UBC4 were expressed in *Escherichia coli* BL21(DE3) and purified with glutathione-sepharose 4B (GE Health, Sweden) according to the manufacturer's instructions. The GST-c-Cbl fused protein was used as a positive control for E3 ubiquitin ligase and produced as previously described [20].

E1 ubiquitin-activating enzyme and FLAG-tagged ubiquitin were purchased from Sigma Company. Ubiquitination and GST-ZNF645 fused protein activity assays were carried out with a solution containing 100 ng E1, 100 ng GST-UBC4 fused protein, 5 μg GST and GST-fused proteins, 2 μg Flag-tagged ubiquitin, and 2 mmol L^{-1} ATP in a buffer containing 50 mmol L^{-1} Tris-HCl (pH 7.5), 2.5 mmol L^{-1} $MgCl_2$ and 0.5 mmol L^{-1} DTT. After incubation at 30ºC for 90 min, the reaction was stopped with 5 × SDS sample buffer containing 5% β-mercaptoethanol. The results of the FLAG-tagged ubiquitin assay were detected by Western blot analysis.

3 Results

3.1 Expression pattern of the ZNF645 gene

In our search for testis-specific genes, a gene named *ZNF645* was found in the NCBI Unigene database (http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?UG

ID=149614&TAXID=9606&SEARCH=ZNF645). It had been isolated from human testis (FLJ25735 fis, clone TST05676; Genbank accession No. AK098601) as part of the Japanese NEDO human cDNA sequencing project. To investigate the expression profile of the gene in humans, we carried out PCR amplification of the gene in 16 human tissue and cell line cDNA pools. The results showed that the expression of *ZNF645* mRNA was restricted to the testes of normal human adults (Figure 1A). In addition, the Western blot analysis of whole protein extracted from normal human testis, lung tissue and multiple cell lines confirmed that ZNF645 was expressed only in human testes as a specific 49-kDa protein product (Figure 1B), not in normal human lung tissue and the HEK293T, HeLa, A549 or HepG2 cell lines (Figure 1C).

3.2 Cellular and subcellular localization of ZNF645 protein in human testis

The immunohistochemical analysis of the cell-type distribution of the ZNF645 protein showed that it was present mainly in the cytoplasm of spermatocytes, round spermatids and Leydig cells (Figure 2). To determine whether ejected sperm contain the ZNF645 protein and to determine its localization in these ejected sperm, indirect immunofluorescence staining and immunoreactivity studies were carried out on spermatozoa (Figure 3). The results showed that in mature spermatozoa, ZNF645 was observed only over the postacrosomal perinuclear theca region and the entire length of the sperm tail (Figures 3A, C and H). The rabbit preimmune serum was used as the negative control and no signal was detected over human sperm (Figures 3D, F and G). Next we extracted the sperm protein fraction, which included the plasma membrane, cytoplasm, acrosome, tail, theca and nuclear proteins. After performing the immunoblot analysis on these proteins using the rabbit anti-ZNF645 antibody, we found that ZNF645 was present only in the protein fractions derived from sperm theca and tail (Figure 4A), which again con-

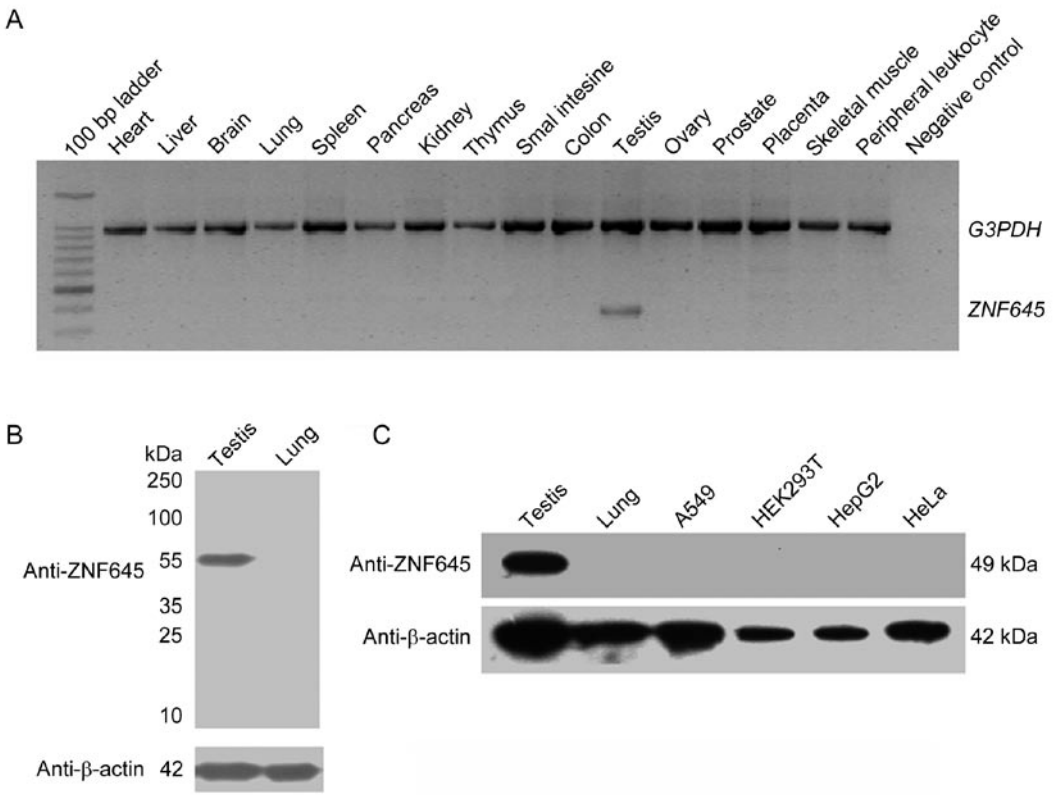

Figure 1. Expression pattern of *ZNF645* gene in various human tissues and cells. (A): Of the multiple human tissue samples that were tested, *ZNF645* mRNA expression was detected only in human testis by polymerase chain reaction analysis. The integrity of the examined cDNA was assessed by measuring the mRNA expression of *G3PDH*. (B): A single 49-kDa band of ZNF645 protein was detected in normal human testis by Western blot analysis using rabbit anti-ZNF645 polyclonal antibody as the primary antibody. (C): ZNF645 protein was present only in normal human testis.

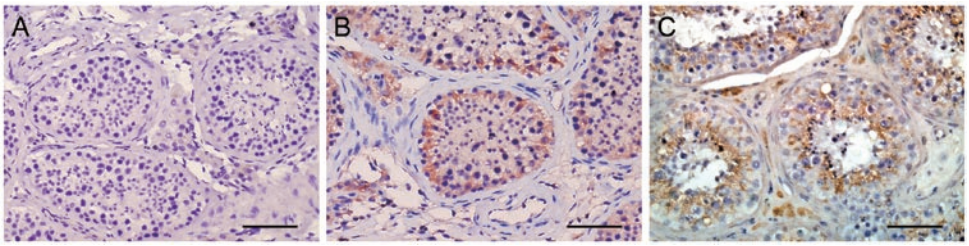

Figure 2. Immunohistochemical localization of ZNF645 in normal human testis. The presence of ZNF645 protein was revealed by brown staining. (A): Negative control with preimmune rabbit serum; (B): ZNF645 protein was present in the cytoplasm of spermatocytes and Leydig cells of stages II–IV; (C): ZNF645 protein was present in spermatocytes, round spermatids and Leydig cells of stages VII–VIII. Bars = 50 μm.

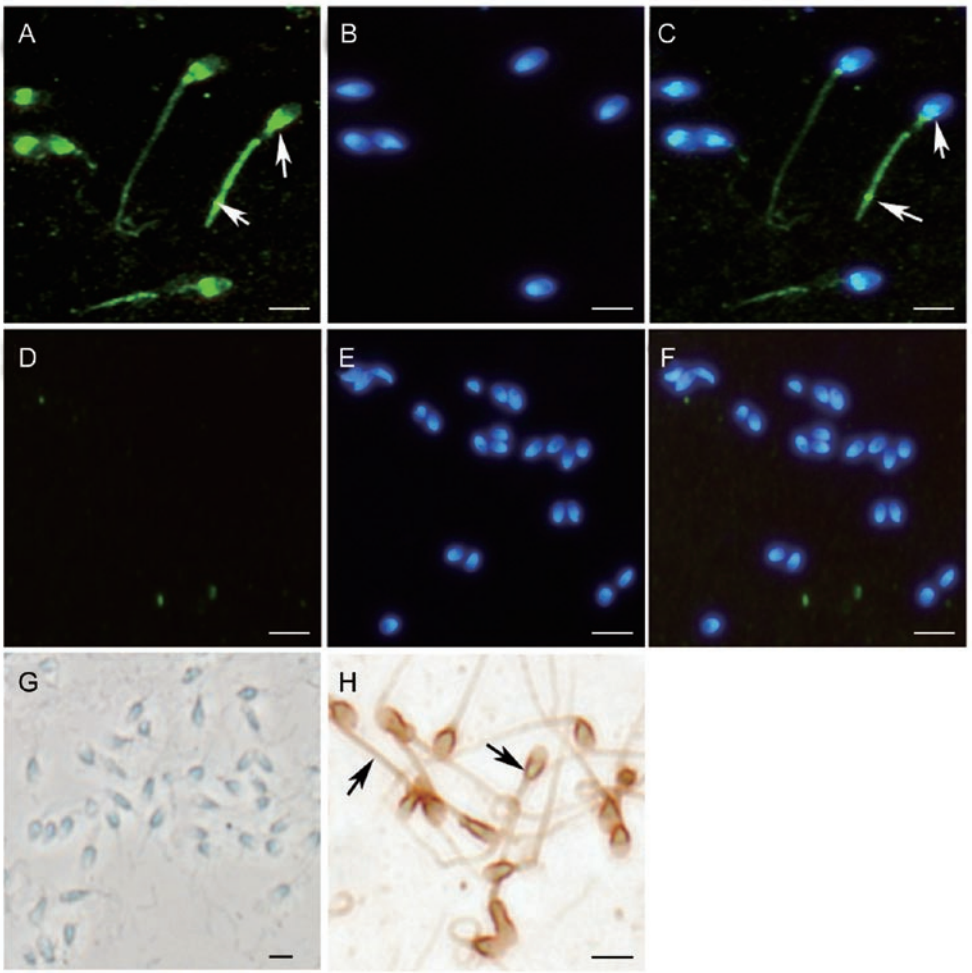

Figure 3. ZNF645 protein localizes over the postacrosomal perinuclear theca region and tail region of human sperm. (A)–(C), (H): Sperm incubated with rabbit anti-ZNF645 polyclonal antibody. (D)–(G): Sperm incubated with preimmune rabbit serum as a negative control. (A, D) Indirect immunofluorescence microscopy image (green). (B), (E): Sperm nuclei stained with DAPI (blue). (C), (F): Merged images of A and B, and D and E, respectively. (G), (H): DAB staining indicating ZNF645 immunoreactivity on human sperm. The arrows indicate the domain-specific location of the ZNF645 protein on human sperm. Bars = 5 μm.

firmed the above-mentioned localization analysis. We also performed immunoanalysis with anti-Rnf141 antibody to confirm that the theca protein was not contaminated by tail protein and found that the Rnf141 signal was distributed in the human sperm acrosome and tail, but not in the theca (Figure 4B).

3.3 Structural features of the gene ZNF645

By performing rapid amplification of 5′ and 3′ cDNA ends (RACE) of the *ZNF645* gene, we obtained its full-length cDNA sequence (Genbank accession No. GQ355336), which was 29 nt longer than that of the *ZNF645* cDNA clone (FLJ25735 fis) in the 5′ upstream transcription region. The *ZNF645* gene contains a 1 275-nt (nucleotide) open reading frame, with the start codon ATG at position 80 and the stop cordon TAA at position 1 357. It encodes a polypeptide of 425 aa (Supplementary Figure 1). When searched against the InterPro database with the deduced aa sequence, a C3HC4-type RING finger domain was found at the N-terminus from aa residues 57 to 97, along with a C2H2 zinc-finger domain (aa residues 112–138) and a proline-rich region (aa residues 254–343).

In searching the homologues of ZNF645, we observed that it had 52% identity with the human and mouse c-Cbl-like protein Hakai [21] and that they both contained the conserved C3HC4 RING finger domain, C2H2 zinc-finger domain and proline-rich region in the same order (Figure 5). The fact that they possessed such parallel primary structures suggested that they may have similar functions.

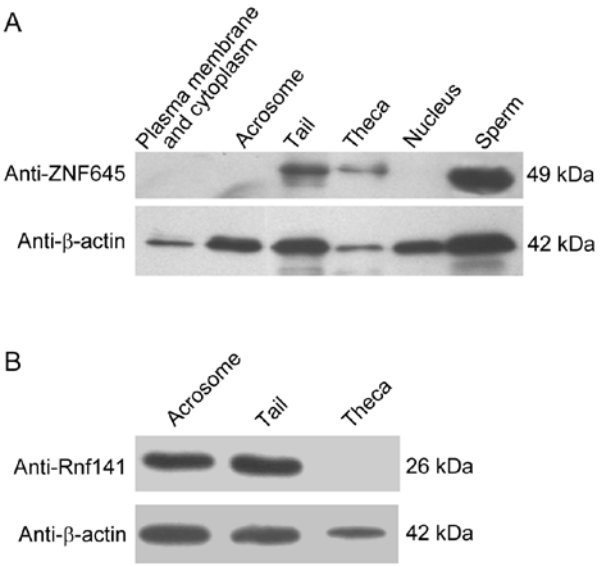

Figure 4. Immunodetection of ZNF645 protein in the different protein fractions of human semen. (A): ZNF645 was detected only in the theca and tail protein fractions of human sperm; (B): Rnf141 was observed only in the acrosome and tail protein fractions of human sperm.

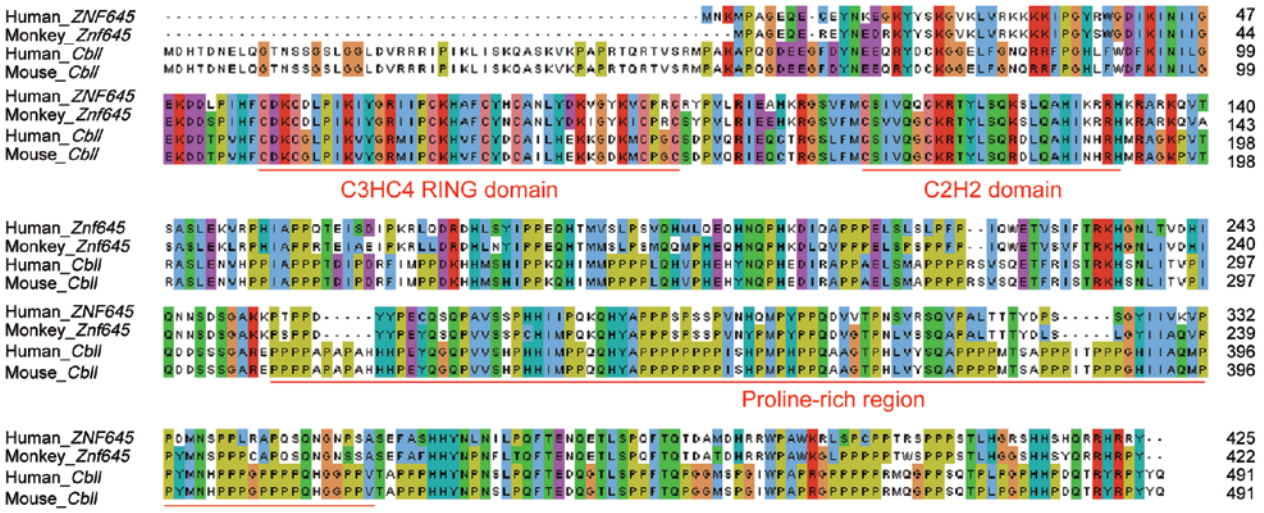

Figure 5. Alignment of the amino-acid sequences of ZNF645 and its homologues. Sequence alignment of human ZNF645 with monkey Znf645 (Q4R361, 85% identity), human c-Cbl-like protein (Human_Cbll, B7ZM03, 52% identity) and mouse c-Cbl-like protein (Q9JIY2-2, 52% identity) was performed with the EBI ClustalW web tool (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The conserved C3HC4-type RING domain, C2H2 zinc-finger domain and proline-rich region are underlined in red.

3.4 ZNF645 is an E3 ubiquitin ligase

To determine whether the ZNF645 RING finger protein had intrinsic E3 ubiquitin ligase activity like its homologue, Hakai, we generated a GST fusion protein, GST-ZNF645, which encompassed aa residues 39–109 with the entire RING domain of ZNF645; a GST-UBC4 (as an E2 ubiquitin-conjugating enzyme); and a GST-c-Cbl (as a positive control of E3 ubiquitin ligase) (Figure 6A). Then an *in vitro* ubiquitination assay was carried out in which the GST-ZNF645 and GST-c-Cbl fusion proteins were used as ubiquitin E3 ligases. As shown in Figure 6B, polyubiquitin chains were observed only in the presence of the GST-ZNF645 and GST-c-Cbl fusion proteins, indicating that the ZNF645 RING finger domain does have E3 ubiquitin ligase activity.

4 Discussion

It is well known that the testis possesses a large number of tissue-specific genes termed "Chauvinist

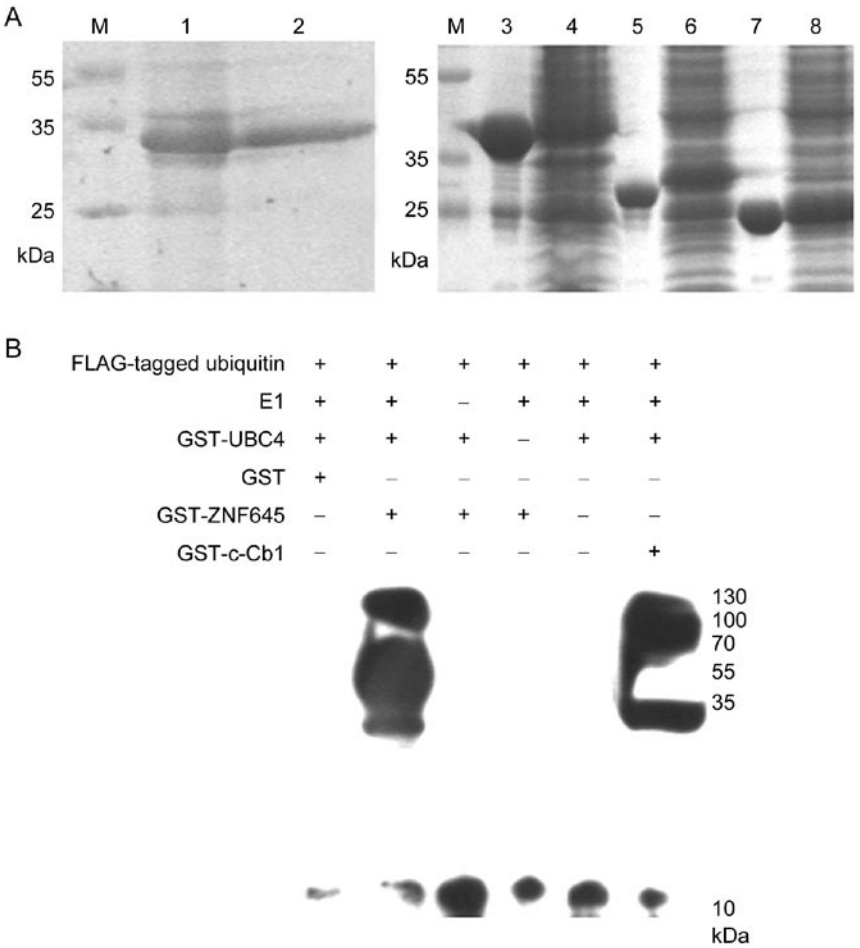

Figure 6. SDS-PAGE analysis of the expression and purification products of GST, GST-ZNF645, GST-UBC4 and GST-c-Cbl fused proteins (A) and the ubiquitination of GST-ZNF645 and GST-c-Cbl in the *in vitro* ubiquitination assay (B). M: Protein ladder; Lane 1: Soluble protein extract containing induced GST-ZNF645; Lane 2: purified GST-ZNF645 fused protein; Lane 3: purified GST-UBC4 fused protein; Lane 4: soluble protein extract containing induced GST-UBC4 fused protein; Lane 5: purified GST-c-Cbl fused protein; Lane 6: soluble protein extract containing induced GST-c-Cbl fused protein; Lane 7: purified GST protein; Lane 8: soluble protein extract containing GST protein. Polyubiquitin chains were only observed in the presence of the GST-ZNF645 and GST-c-Cbl (positive control) fusion proteins. E1: E1 ubiquitin-activating enzyme; GST-UBC4: purified GST fused UBC4 protein, an E2 ubiquitin-conjugating enzyme; GST: purified GST protein, a negative control; GST-ZNF645: purified GST fused ZNF645 protein, a putative E3 ubiquitin-protein ligase; GST-c-Cbl: purifed GST fused c-Cbl protein, an E3 ubiquitin-protein ligase.

genes", which either are abundantly transcribed in the testis or have their transcripts post-transcriptionally processed in a testis-specific manner [2, 22]. Identification and functional study of these genes would greatly help us improve our understanding of spermatogenesis. In this study, we definitively identified a novel testis-specific RING finger protein, ZNF645. It is expressed in the cytoplasm of spermatocytes, spermatids and Leydig cells, which indicates that it may have an important role in sperm production. The fact that ZNF645 was also found to be present over the postacromal perinuclear theca region and in tails of ejected sperm suggests that it may have a necessary role in controlling the quality of sperm during fertilization.

More than 30 RING finger proteins have been reported to be involved in spermatogenesis in rats, mice and humans [23], and only few of them have been identified to be E3 ubiquitin ligases. E3 ubiquitin ligases are responsible for the specific recognition of the multitude of substrates of the ubiquitin system and are the least-defined components of the pathway [10]. In most cases, the RING finger domain serves as a scaffold that brings the E2 ubiquitin-conjugating enzyme and the substrate into close proximity such that the activated ubiquitin moiety from the E2 ubiquitin-conjugating enzyme can be efficiently transferred to the substrate. This, in turn, leads the substrate to be ubiquitinated and further degraded by the 26S proteasome complex [24]. In the present study, we sought to determine whether ZNF645 had E3 ubiquitin ligase activity in an assay in which UBC4, an E2 ubiquitin-conjugating enzyme, was also used. ZNF645 is homologous with the c-Cbl-like protein Hakai, which is the cellular homologue of the v-Cbl transforming gene of the Cas NS-1 murine leukaemia virus [25, 26]. Hakai has been shown to induce the ubiquitination and endocytosis of the E-cadherin complex and thereby modulate cell adhesion as well as to have a crucial role in various cellular processes, including tumorigenesis and embryonic development [21, 27, 28]. Whether ZNF645 functions similarly to Hakai during spermatogenesis needs to be further studied and is a topic of some interest because it has a primary aa sequence that is similar to that of the Hakai protein. In addition, our preliminary experiments showed that ZNF645 was also expressed in some human lung cancer tissues (data not shown), which indicated that ZNF645 may also play a role in tumorigenesis. All of these observations have provided us with important information that we will use in our ongoing in-depth functional studies of the ZNF645 protein.

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Appendix

Supplementary Figure 1. Nucleotide and deduced aa sequences of human *ZNF645* gene. The deduced aa sequences of human *ZNF645* ORFs are shown beneath the DNA sequences. The numbers in the left and right margins indicate the nt and aa positions, respectively. The predicated ring finger C3HC4 motif is boxed, Zinc finger C2H2 motif is shadowed, and the proline-rich region is indicated in the shadowed boxes. The primers of human *ZNF645* gene for the construction of GST-ZNF645 RING fused protein are underlined and the PCR primers for human *ZNF645* gene expression analysis are double underlined. The 29 italic letters are nt longer than the sequence of ZNF645 cDNA clone (FLJ25735 fis). The tailing signal AATAAA is underlined and shaded. The nt sequence of this gene has been deposited in Genbank under the Acession No. GQ355336.