Zygote

cambridge.org/zyg

EH domain binding protein 1-like 1 (EHBP1L1), a protein with calponin homology domain, is expressed in the rat testis*

Massimo Venditti[1], Aldo Donizetti[2], Francesco Aniello[2] and Sergio Minucci[1]

[1]Dipartimento di Medicina Sperimentale, Sez. Fisiologia Umana e Funzioni Biologiche Integrate 'F. Bottazzi', Università degli Studi della Campania 'Luigi Vanvitelli' via Costantinopoli, 16-80138 – Napoli, Italy and [2]Dipartimento di Biologia, Università di Napoli 'Federico II, via Cinthia', 21-80126 – Napoli, Italy

Research Article

Cite this article: Venditti M *et al*. (2020) EH domain binding protein 1-like 1 (EHBP1L1), a protein with calponin homology domain, is expressed in the rat testis. *Zygote*. **28**: 441–446. doi: 10.1017/S0967199420000301

Received: 6 April 2020
Accepted: 23 May 2020
First published online: 29 July 2020

Keywords:
Actin; Ehbp1l1; Meiosis; Spermatogenesis; Testis

Authors for correspondence:
Sergio Minucci. Dipartimento di Medicina Sperimentale, Sez. Fisiologia Umana e Funzioni Biologiche Integrate 'F. Bottazzi', Università degli Studi della Campania 'Luigi Vanvitelli' via Costantinopoli, 16-80138 – Napoli, Italy. Tel: +39081-5665829.
E-mail: sergio.minucci@unicampania.it.
Francesco Aniello. Dipartimento di Biologia, Università di Napoli 'Federico II, via Cinthia', 21-80126 – Napoli, Italy. Tel: +39081-2535014.
E-mail: faniello@unina.it.

*In memory of our friend, Professor Anna Cardone.

Summary

In this paper, with the aim to find new genes involved in mammalian spermatogenesis, we isolated, for the first time in the rat testis, a partial cDNA clone that encoded EH domain binding protein 1-like 1 (Ehbp1l1), a protein that has a single calponin homology domain (CH). Bioinformatic analysis showed that EHBP1l1 contains three domains: the N-terminal C2-like, the CH and the C-terminal bivalent Mical/EHBP Rab binding (bMERB) domains, which are evolutionarily conserved in vertebrates. We found that Ehbp1l1 mRNA was expressed in several rat tissues, including the liver, intestine, kidney and also in the testis during its development, with a higher level in testis from 12-month-old animals. Interestingly, *in situ* hybridization experiments revealed that Ehbp1l1 is specifically expressed by types I and II spermatocytes, this result was validated by RT-PCR performed on total RNA obtained from enriched fractions of different testicular cell types. As EHBP1l1 has been described as linked to vesicular transport to the actin cytoskeleton and as an effector of the small GTPase Rab8, we hypothesized that it could participate both in cytoskeletal remodelling and in the regulation of vesicle sorting from the *trans*-Golgi network to the apical plasma membrane. Our findings provide a better understand of the molecular mechanisms of the differentiation process of spermatogenesis; Ehbp1l1 may also be used as a new marker of testicular activity.

Introduction

Mammalian spermatogenesis is a complex multistage process in which immature germ cells undergo division, meiosis and differentiation to form round spermatids. Finally, after a series of biochemical and morphological changes, spermatids elongate and then functional spermatozoa are generated in the testis (Senoo *et al.*, 2002; McLean *et al.*, 2003; Dunleavy *et al.*, 2019)

Spermatogenesis is a well coordinated developmental programme in which all the various steps are defined by cell type- and stage-specific induction or repression of the expression of specific genes (Zhao and Garbers, 2002; Chu and Shakes, 2013; Pariante *et al.*, 2016a; Chemek *et al.*, 2018; Venditti and Minucci 2017, 2019).

The process of spermatogenesis is under a genetic and molecular programme (Sassone-Corsi, 2002; de Mateo and Sassone-Corsi, 2014) and requires precise sequential expression of many genes that are unique to spermatogenesis (Escalier, 2001; Ergoli *et al.*, 2020). In addition, accurate transcriptional and post-transcriptional regulation is necessary to support the highly coordinated expression of specific genes for each step of spermatogenesis (Zhou *et al.*, 2019).

Given this background, the identification and characterization of spermatogenic-related genes is an important contribution to understanding the mechanisms of both spermatogenesis and human reproduction (Guo *et al.*, 2010; Santillo *et al.*, 2019; Venditti *et al.*, 2020). In this study, an attempt was made to isolate genes involved in rat spermatogenesis and to use them as new markers for true testicular activity. In this context, we isolated, for the first time in rat testis, a partial cDNA clone for EH domain binding protein 1-like 1 (Ehbp1l1; previously named Tangerin) that had a single calponin homology domain (CH) and two transmembrane domains (Chauhan *et al.*, 2002; Friedberg, 2010). These proteins have been described to link vesicular transport to the actin cytoskeleton (Guilherme *et al.*, 2004; Shi *et al.*, 2010) as an effector of the small GTPase Rab8 (Nakajo *et al.*, 2016; Eguchi *et al.*, 2018).

Calponin is an actin filament-associated regulatory protein expressed in smooth muscle and many types of non-muscle cells (Liu and Jin, 2016). *In vitro* studies have demonstrated that calponin binds actin (Takahashi *et al.*, 1988a; Singh *et al.*, 2014) and cross-links microfilaments (Leinweber *et al.*, 2000). In addition, calponin interaction with many other cytoskeleton and related proteins have been described to date (Takahashi *et al.*, 1988b; Childs *et al.*, 1992; Fujii and Koizumi, 1999; Fujii *et al.*, 2000; Szymanski, 2004; Wu and Jin, 2008).

CAMBRIDGE UNIVERSITY PRESS

Interestingly, the CH domain is a specific structural feature that has been related to actin binding; it is composed of about 120 residues located on the N-terminal side of calponin (Rozenblum and Gimona, 2008; Singh *et al.*, 2014). The CH domain has been found in both signalling and cytoskeleton-related proteins (Leinweber *et al.*, 1999; Orlova *et al.*, 2001; Ishisaki *et al.*, 2001; Sjöblom *et al.*, 2008). Moreover, in most of these proteins the specific actin-binding region consists of two CH domains in tandem (Galkin *et al.*, 2003; 2010), however the actin-binding capacity of the single CH domain is still controversial (Gimona and Mital, 1998; Gimona and Winder, 1998; Stradal *et al.*, 1998). In this paper, to expand current knowledge on the molecular mechanisms underlying the differentiation processes of germ cells into mature spermatozoa, we characterized Ehbp1l1 expression and localization in adult rat testis and also during testicular development.

Materials and methods

Animals and tissue collection

Male Wistar rats (*Rattus norvegicus*) were housed under defined conditions (12D:12L) and were fed with standard food and provided with water *ad libitum*. Animals were sacrificed at several stages of the development: infant–prepuberal period (14 and 30 days old), adult (3 and 6 months old) and 12 months old (old; three animal/each time). All the animals were killed by decapitation under ketamine anaesthesia (100 mg/kg i.p.) in accordance with local and national guidelines covering experimental animals. Testis were collected from rat of all stages, several tissues (lung, spleen, kidney, liver, small intestine, seminal vesicles, and muscle and one testes) were dissected from 3-month-old rats, tissues were frozen quickly by immersion in liquid nitrogen, and stored at −80°C until RNA extraction. In addition, the other 3-month-old testis was fixed in Bouin's fluid for histological analysis. Finally, to isolate germinal cells by centrifugal elutriation, an additional two 3-month-old rats were sacrificed and the testes were used (see below).

Cloning of Ehbp1l1 *cDNA by RT-PCR*

Total RNA from tissues and testis collected at different stages of development was isolated using TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA). The quantity (ng/ml) and purity (260/280 and 260/230 ratios) of total RNAs were assessed using a NanoDrop 2000 spectrophotometer (Thermo, Waltham, MA, USA). To remove potential contamination of genomic DNA, RNA aliquots (10 µg) were treated with 2U DNase I (Amersham Bioscience) according to the manufacturer's recommendations (Falvo *et al.*, 2018). First-strand cDNA was synthesized using 5 µg of total pooled RNA, the reverse transcription reaction was conducted in 20 µl of total volume reaction according to the manufacturer's recommendations (Venditti *et al.*, 2018a, 2019). As a negative control, the same reaction was carried out on a pool of RNA without using the reverse transcriptase enzyme (RT−).

Based on the published mRNA sequence of *Rattus norvegicus Ehbp1l1* variant 2 (NCBI data bank accession NM_001129997.1), polymerase chain reaction (PCR) was performed using the following primers: Ehbp1l1 For: 5′-GCAACAGAAAGCAGAGAGGG-3′ and Ehbp1l1 Rev: 5′-ATGTAAAATAGCACAGAAGGCCA-3′. An appropriate region of *Rattus norvegicus* β-actin mRNA (Act; NCBI GenBank accession no. NM_031144.3) was amplified with specific oligonucleotide primers (Act For: 5′-CTCTTCCAGCCTTCCTTCCT-3′; Act Rev: 5′-CTGCTTGCTGATCCACATC-3′) and used as a control. PCR amplification was carried out for 35 cycles with denaturing at 94°C for 45 s, annealing at 58°C for 45 s and extension at 72°C for 45 s, followed by a final extension at 72°C for 5 min. Amplification products were electrophoresed on 1.2% agarose gel and visualized with ethidium bromide staining and ultraviolet (UV) light. The expected DNA amplicons for variant 2 were 382 bp for Ehbp1l1, and 300 bp for β-actin. The amplicons were purified using the QIAGEN gel extraction kit (QIAGEN, Hilden, Germany) and were cloned into a pGEM-T Easy Vector and sequenced on both strands to confirm specificity, in accordance with the manufacturer's instruction. Nucleotide sequences were compared with the NCBI GenBank database (www.ncbi.nlm.nih.gov) to confirm the specificity of PCR products.

In situ hybridization

For *in situ* hybridization, randomly chosen testis sections from 3-month-old animals (7 sections/animal) were treated following the same conditions as previously described (Pariante *et al.*, 2016b; Venditti *et al.*, 2018b) using a plasmid containing Ehbp1l1 cDNA linearized with either *Xho*I or *Bam*HI enzymes to produce a template for an antisense or sense probe, using T7 or T3 RNA polymerases, respectively. The sense (control) and antisense cRNA probes were prepared by *in vitro* transcription with DIG-uridine triphosphate (UTP) (Roche Diagnostics) as recommended by the manufacturer.

Isolation of germinal cells by centrifugal elutriation

For isolation of germinal cells, we followed the protocol of Quesada *et al.* (1996). Testes from two 3-month-old rats were decapsulated, resuspended in 10 ml of Dulbecco's minimal essential medium (DMEM) and seminiferous tubules, free of interstitial tissues, were obtained using collagenase treatment (0.25 mg/ml). Seminiferous tubules were then incubated at 37°C for 60 min in DMEM containing 0.25 mg/ml collagenase, 0.075 mg/ml DNase I, and 0.5% bovine serum albumin (BSA). After incubation, the cell suspension was centrifuged for 10 min at 1200 *g*. Aliquots of the pellet were complexed with propidium iodide and subjected to cytofluorimetry analysis in a Becton–Dickinson cytofluorimeter. Total germinal cells were resuspended in DMEM, in the presence of 0.1 mg/ml DNase I and 0.5% BSA, and separated into fractions enriched in various cell types using centrifugal elutriation, as described by Meistrich (1977). A cell suspension of 10 ml (180–220 × 10^6 cells) was loaded into a JE-6 Beckman elutriator rotor and separation was performed at 3000–2000 rpm and flow rates of 13–40 ml/min. The buffer employed was PBS containing 0.5% BSA; several 50 ml fractions were collected. Aliquots of the pellet of single fractions were complexed with propidium iodide and subjected to cytofluorimetry analysis in a Becton–Dickinson cytofluorimeter.

Results and Discussion

Correct male gamete formation requires the precise coordination of expression of many genes that harmonize the delicate cell cycle events as well as the differentiation mechanisms underlying spermatozoa production (Lin *et al.*, 2017). As most of these genes remains to be identified, an attempt to characterize those specific to spermatogenesis in the rat testis was made. Here we isolated a

https://doi.org/10.1017/S0967199420000301 Published online by Cambridge University Press

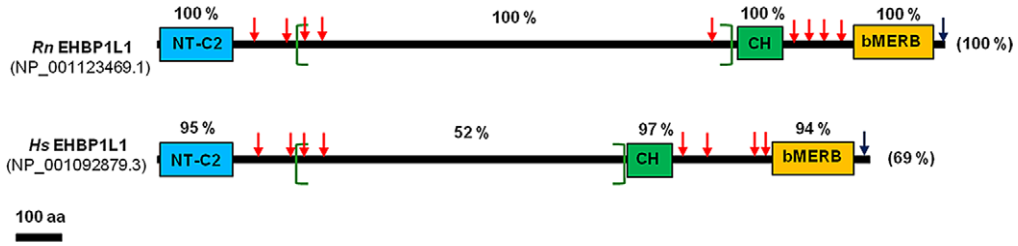

Figure 1. Schematic representation of domain architecture of rat and human EHBP1L1. The single domains or the entire amino acid sequence were aligned using Clustal Omega software with the default parameters to find the per cent identity. Black brackets indicate the overall per cent identity between rat and human proteins. Green brackets identify the amino acid region encoded by the long exon of rat and human transcript variants (NM_001129997.1 and NM_001099409.3, respectively). Red arrows indicate the proline-rich (PxxP) motifs. The black arrow indicates the C-terminal prenylation motif (CaaX-box). NT-C2, N-terminal C2 domain; CH, calponin homology domain, bMERB, Mical/EHBP Rab binding domain.

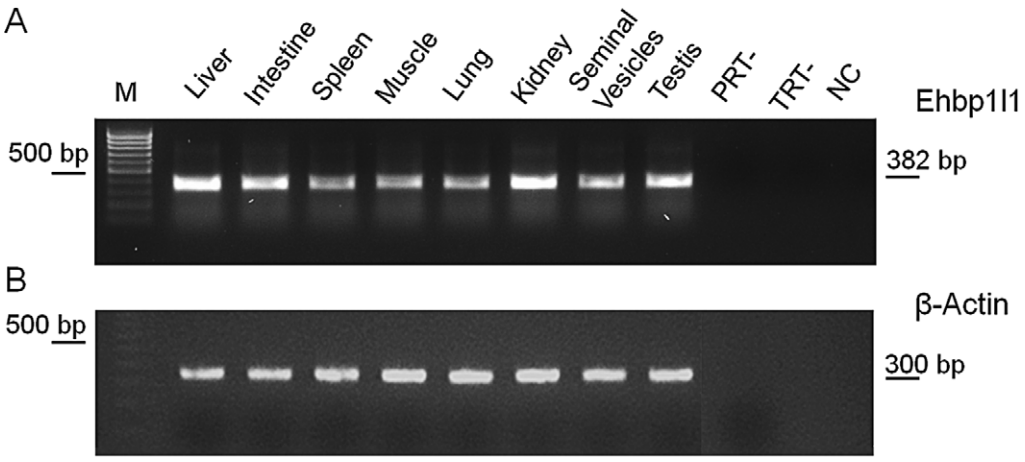

Figure 2. Expression of *Ehbp1l1* mRNA in adult rat tissues. Agarose gel electrophoresis of RT-PCR products performed on total extracts of liver, intestine, spleen, muscles, lung, kidney, seminal vesicles and testis. *Ehbp1l1* is expressed in all analyzed tissues. Quality of the cDNA samples was checked by amplifying a fragment of housekeeping gene rat β-actin mRNA. M: molecular weight marker (GeneRuler Express DNA Ladder). nc: negative, no-cDNA control. PRT−: pool of negative control of the RT reaction, performed by omitting reverse transcriptase. TRT−: testis-specific negative control of the RT reaction, performed as for PRT−.

partial cDNA clone encoding for a fragment of about 120 amino acids of EH domain binding protein 1-like 1 (Ehbp1l1, previously named Tangerin). This protein contained a CH domain (CH) and two transmembrane domains (Chauhan *et al.*, 2002; Friedberg, 2010). The first identified protein of this family was calponin (Takahashi *et al.*, 1988a), which is involved directly in the regulation of actomyosin interactions in several tissues, most prominently in smooth muscle contraction/relaxation cycles and in neuronal outgrowth (Burgstaller and Gimona, 2004).

Analysis of the NCBI database revealed that the rat *Ehbp1l1* gene includes different transcriptional isoforms. The longest transcript variant (NM_001129997.1) is characterized by a long exon of 2689 bp in length, also found in the corresponding human transcript (NM_001099409.3) and encodes for a protein that contains three well known domains: the N-terminal C2-like domain (NT-C2), which binds phosphatidylserine and phosphatidylethanolamine (Lemmon, 2008), the CH domain and the C-terminal bivalent Mical/EHBP Rab binding (bMERB) domain (Fig. 1). The evolutionary conservation of these three domains is evidenced by the per cent identity obtained when comparing the corresponding EHBP1L1 sequences of rat and human (Fig. 1). In particular, the per cent identity of the three domains was higher compared with the per cent identity of the overall corresponding sequences (Fig. 1). Rat and human EHBP1L1 proteins also shared many proline-rich (PxxP) motifs (PR), known to be involved in SH3 protein binding (Kaneko *et al.*, 2008), such as Bin1 and amphiphysin1 and dynamin1 (Nakajo *et al.*, 2016). As evidenced in Fig. 1, these motifs were clustered mainly in two regions, one region was just downstream of the NT-C2 domain, and the second region was included between the CH and bMERB domains (Fig. 1). In addition, we also evaluated the presence of the C-terminal prenylation motif (CaaX-box) in the mammalian proteins; this box is probably needed for EHBP1l1 prenylation and delivery to the plasma membrane (Gao *et al.*, 2009). As shown in Fig. 1, both rat and human EHBP1L1 share this motif at their C-terminal ends. Interestingly, rat and human EHBP1L1 proteins bear a central region that is responsible for their sequence diversity. This amino acid sequence is encoded in the vast majority by the amino acid sequence of the longest exon. In fact, the per cent identify corresponding to this amino acid region for the rat and human proteins was 52% (Fig.1).

As we first isolated the Ehbp1l1 clone from rat testis, to verify if Ehbp1l1 mRNA is distributed ubiquitously, its presence was assessed using PCR analysis on 3-month-old rat tissues (Fig. 2). Preliminarily, the primers were tested for their sequence specificity using an amplification reaction carried out on cDNA from 14-day-old Wistar testis rats. The PCR was analysed by gel electrophoresis and the resulting amplicon was purified, subcloned, and sequenced to confirm reaction specificity (data not shown). The analysis confirmed that Ehbp1l1 was expressed in all analyzed tissues, with

https://doi.org/10.1017/S0967199420000301 Published online by Cambridge University Press

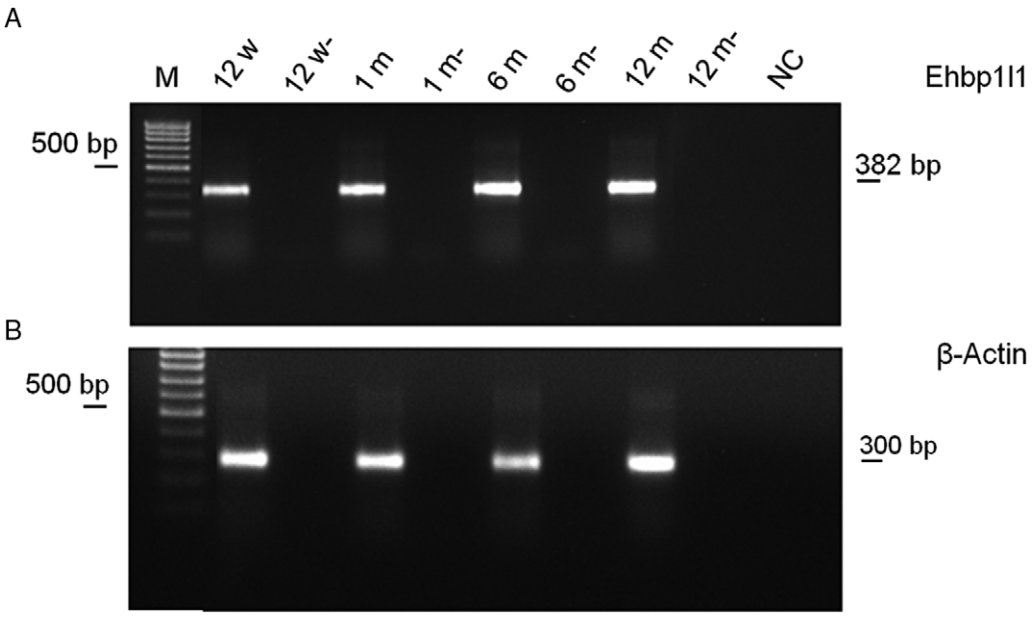

Figure 3. Expression of *Ehbp1l1* mRNA in rat testis development. Agarose gel electrophoresis of RT-PCR products performed on total extracts from 12 week, and 1-, 3-, 6- and 12-month-old rat testis. *Ehbp1l1* was expressed in all analyzed tissues. Quality of cDNA samples was checked by amplifying a fragment of the housekeeping rat β-Actin mRNA. M: molecular weight marker (GeneRuler Express DNA Ladder). nc: negative, no-cDNA control. All the RT− controls were negative.

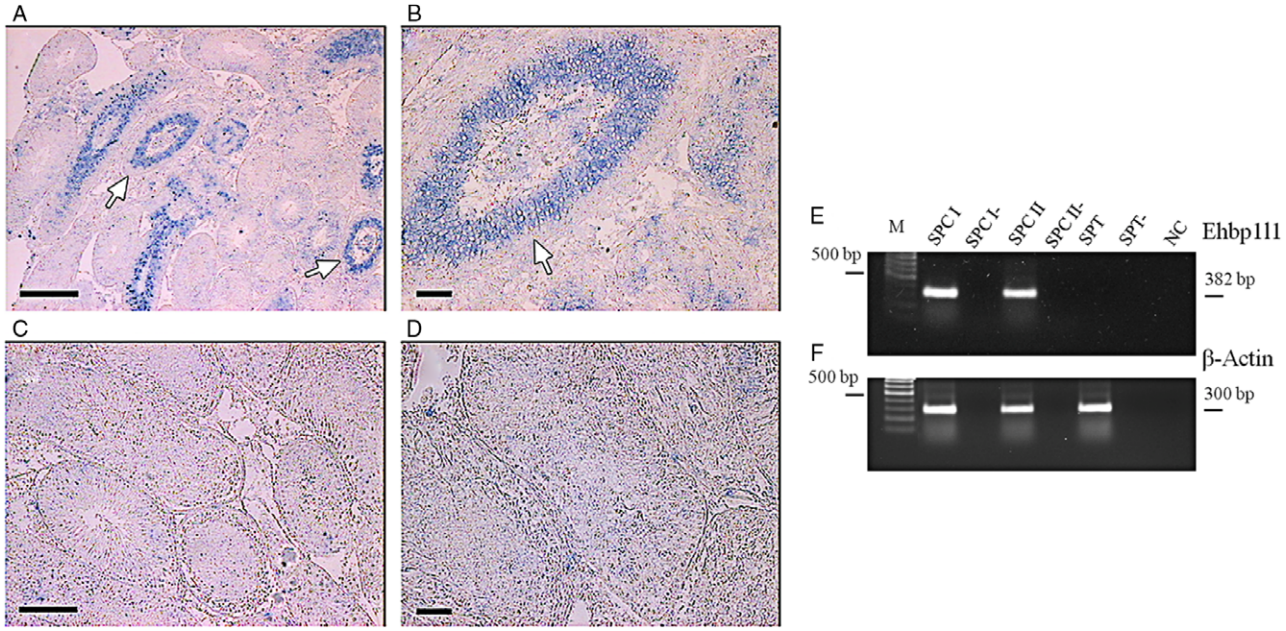

Figure 4. Localization of Ehbp1l1 mRNA in the testis of adult rat and expression analysis on isolated germ cells. (*A*–*D*) Sections were treated with antisense probe (*A*); high magnification in (*B*); or sense probe as a negative control (*C*, *D*). Blue staining indicates positive cells, which include meiotic types I and II SPC. All the other cell types were negative. Scale bars represent 20 μm in (*A*, *C*), 5 μm in (*B*, *D*). (*E*, *F*) Agarose gel electrophoresis of RT-PCR products performed on total extracts from type I SPC, type II SPC and SPT isolated from adult rat testis. *Ehbp1l1* is expressed in types I and II SPC and not in SPC. Quality of the cDNA samples was checked by amplifying a fragment of the housekeeping gene rat β-actin mRNA. M: molecular weight marker (GeneRuler Express DNA Ladder). nc: negative, no-cDNA control. All samples for the RT− controls were negative.

a highest levels in the liver, small intestine, kidney and testis (Fig. 2A). As our interest was specifically for gonads, we performed an expression pattern analysis using a RT-PCR assay at different time points of testis development. As reported in the Fig. 3(A), Ehbp1l1 mRNA was revealed at each stage of testis maturation, showing a higher level at the end of the analyzed period (12 months) compared with the earlier stages. These data indicated that Ehbp1l1 is required for all the rat reproductive period. In both experiments, quality control of cDNA was performed using specific primers for β-actin mRNA (Figs 2B and 3B), while, as expected, no amplification bands were detected in the negative controls (RT−).

To determine which testis cell types expressed the *Ehbp1l1* gene, we performed *in situ* hybridization experiments. In particular, we

https://doi.org/10.1017/S0967199420000301 Published online by Cambridge University Press

used a DIG-labelled antisense RNA probe to mark Ehbp1l1-expressing cells (Fig. 4A, B) and the corresponding DIG-labelled sense RNA probe as the experimental control to evaluate the specificity of the revealed hybridization signals (Fig. 4C, D). An *in situ* hybridization assay was carried out on 3-month-old testes, the youngest but completely sexually mature of the previously analyzed stages. Interestingly, as showed in Fig. 4A, B, a strong signal was detected only in the primary and secondary spermatocytes (SPC, arrows) while it was completely absent in spermatogonia, spermatids (SPT), spermatozoa, as well as in somatic cells (i.e. Sertoli and Leydig cells).

As well known, SPCs (and their female counterpart) are the only cells that undergo meiosis division to form type I SPC and type II SPC. Interestingly, proteins containing CH domains have been associated with cell division, and in particular to binding the actin filaments that are required for the assembly and dynamics of the contractile actomyosin ring (Shannon and Li, 1999) and, in some cases, the domains were specific for meiotic division (Mintz *et al.*, 1999; Connolly *et al.*, 2014). Therefore Ehbp1l1, thanks to its CH domain, could participate in the cytoskeletal remodelling, necessary for a proper cell division.

Moreover, it is possible to hypothesize also an additional role for Ehbp1l1 in these phases: via the proline-rich domain, the relative protein is able to interact with Rab8, Bin1/amphiphysin II, and dynamin to form a multiprotein complex, which regulates the transport of protein cargos from the *trans*-Golgi network (TGN) to the apical plasma membrane (Nakajo *et al.*, 2016; Ang *et al.*, 2004). During SPT differentiation, one of the key aspects is acrosome formation, a membrane-bound organelle that releases hydrolytic enzymes to facilitate sperm penetration through the zona pellucida of the oocyte (O'Donnell, 2015). Its biosynthesis begins in round SPT soon after meiosis, with coated vesicles growing from the TGN sorted gradually to the nuclear membrane (Ventela *et al.*, 2000; Kierszenbaum *et al.*, 2011). It has to be highlighted that, in mammals, during spermiogenesis a progressive transcriptional inactivation occurs (Kierszenbaum *et al.*, 2011) and, immediately before this, two significant bursts of RNA synthesis happen in midpachytene SPC and in round SPT, respectively, therefore supporting the translation of proteins incorporated in the components of differentiating SPT (Monesi, 1965; Monesi *et al.*, 1978). As we already characterized the prothymosin-alpha mRNA/protein system, which showed a comparable temporal expression pattern (Ferrara *et al.*, 2010), we hypothesized that expression of the Ehbp1l1 transcript starts in types I and II SPC, and that the expression of the protein would probably take place in differentiating SPT, to sustain the vesicle trafficking from TGN (Ventela *et al.*, 2000; Kierszenbaum *et al.*, 2011).

To further validate these results, we performed an elutriation experiment on testes of two adult rats (3 months old) to separate the different classes of testis cells. We obtained enriched fractions of three different cell types from which total RNA was extracted and used for RT-PCR. As expected, we observed a specific Ehbp1l1 amplification band of the expected size only in types I and II SPC fractions, but not in the SPT fraction (Fig.4E). As mentioned previously, quality control of cDNA was performed using specific primers for β-actin mRNA (Fig. 4F) and no amplification bands were detected in the negative controls (RT−).

In conclusion, from our data, we could hypothesize a role for Ehbp1l1 during rat spermatogenesis. Given the presence of specific domains in the protein sequence, which allow EHBP1l1 to participate both in cytoskeletal remodelling and regulation of vesicle sorting from the TGN to the apical plasma membrane, and given the specific expression of this mRNA in the rat meiotic cells, its possible role during the differentiation phases of spermiogenesis could be hypothesized.

Acknowledgements. In memory of our friend, Prof. Anna Cardone.

Financial support. The authors received financial support for research, authorship, and publication of this article from the Department of Experimental Medicine, University of Campania 'Luigi Vanvitelli' (2018), Italy.

Conflict of interest. None

Ethical standards. The experimental protocol and animal housing conditions were in accordance with the Italian guidelines (D. Lvo 116/92) and authorized by the local Animal Care Committee (Servizio veterinario ASL 44, Prot. Vet. 22/95).

References

Ang AL, Taguchi T, Francis S, Fölsch H, Murrells LJ, Pypaert M, Warren G and Mellman I (2004). Recycling endosomes can serve as intermediates during transport from the Golgi to the plasma membrane of MDCK cells. *J Cell Biol* **167**, 531–43.

Burgstaller G and Gimona M (2004). Actin cytoskeleton remodelling via local inhibition of contractility at discrete microdomains. *J Cell Sci* **117**, 223–31.

Chauhan BK, Reed NA, Zhang W, Duncan MK, Kilimann MW and Cvekl A (2002). Identification of genes downstream of Pax6 in the mouse lens using cDNA microarrays. *J Biol Chem* **277**, 11539–48.

Chemek M, Venditti M, Boughamoura S, Mimouna SB, Messaoudi I and Minucci S (2018). Involvement of testicular DAAM1 expression in zinc protection against cadmium-induced male rat reproductive toxicity. *J Cell Physiol* **233**, 630–40.

Childs TJ, Watson MH, Novy RE, Lin JJ and Mak AS (1992). Calponin and tropomyosin interactions. *Biochim Biophys Acta* **1121**, 41–6.

Chu DS and Shakes DC (2013). *Spermatogenesis. Adv Exp Med Biol* **757**, 171–203.

Connolly AA, Osterberg V, Christensen S, Price M, Lu C, Chicas-Cruz K, Lockery S, Mains PE and Bowerman B (2014). *Caenorhabditis elegans* oocyte meiotic spindle pole assembly requires microtubule severing and the calponin homology domain protein ASPM-1. *Mol Biol Cell* **25**, 1298–311.

de Mateo S and Sassone-Corsi P (2014). Regulation of spermatogenesis by small non-coding RNAs: role of the germ granule. *Semin Cell Dev Biol* **29**, 84–92.

Dunleavy JEM, O'Bryan M, Stanton PG and O'Donnell L (2019). The cytoskeleton in spermatogenesis. *Reproduction* **157**, 53–72.

Eguchi T, Kuwahara T, Sakurai M, Komori T, Fujimoto T, Ito G, Yoshimura SI, Harada A, Fukuda M, Koike M and Iwatsubo T (2018). LRRK2 and its substrate Rab GTPases are sequentially targeted onto stressed lysosomes and maintain their homeostasis. *Proc Natl Acad Sci USA* **115**, E9115–24.

Ergoli M, Venditti M, Picillo E, Minucci S and Politano L (2020). Study of expression of genes potentially responsible for reduced fitness in patients with Myotonic dystrophy type 1 and identification of new biomarkers of testicular function. *Mol Reprod Dev* **87**, 45–52.

Escalier D (2001). Impact of genetic engineering on the understanding of spermatogenesis. *Hum Reprod Update* **7**, 191–210.

Falvo S, Chieffi Baccari G, Spaziano G, Venditti M, Rosati L, Di Fiore MM and Santillo A (2018). StAR protein and steroidogenic enzyme expressions in the rat Harderian gland. *C R Biol* **341**, 160–166.

Ferrara D, Izzo G, Pariante P, Donizetti A, d'Istria M, Aniello F and Minucci S (2010). Expression of prothymosin alpha in meiotic and post-meiotic germ cells during the first wave of rat spermatogenesis. *J Cell Physiol* **224**, 362–8.

Friedberg F (2010). Singlet CH domain containing human multidomain proteins: an inventory. *Mol Biol Rep* **37**, 1531–9.

Fujii T and Koizumi Y (1999). Identification of the binding region of basic calponin on alpha and beta tubulins. *J Biochem* **125**, 869–75.

Fujii T, Takagi H, Arimoto M, Ootani H and Ueeda T (2000). Bundle formation of smooth muscle desmin intermediate filaments by calponin and its binding site on the desmin molecule. *J Biochem* **127**, 457–65.

Galkin VE, Orlova A, VanLoock MS and Egelman EH (2003). Do the utrophin tandem calponin homology domains bind F-actin in a compact or extended conformation? *J Mol Biol* **331**, 967–72.

https://doi.org/10.1017/S0967199420000301 Published online by Cambridge University Press

Galkin VE, Orlova A, Salmazo A, Djinović-Carugo K and Egelman EH (2010). Opening of tandem calponin homology domains regulates their affinity for F-actin. *Nat Struct Mol Biol* **17**, 614–6.

Gao J, Liao J and Yang GY (2009). CAAX-box protein, prenylation process and carcinogenesis. *Am J Transl Res* **1**, 312–25.

Gimona M and Mital R (1998). The single CH domain of calponin is neither sufficient nor necessary for F-actin binding. *J Cell Sci* **111**, 1813–21.

Gimona M and Winder SJ (1998). Single calponin homology domains are not actin-binding domains. *Curr Biol* **8**, R674–5.

Guilherme A, Soriano NA, Bose S, Holik J, Bose A, Pomerleau DP, Furcinitti P, Leszyk J, Corvera S and Czech MP (2004). EHD2 and the novel EH domain binding protein EHBP1 couple endocytosis to the actin cytoskeleton. *J Biol Chem* **279**, 10593–605.

Guo X, Shen J, Xia Z, Zhang R, Zhang P, Zhao C, Xing J, Chen L, Chen W, Lin M *et al.* (2010). Proteomic analysis of proteins involved in spermiogenesis in mouse. *J Proteome Res* **9**, 1246–56.

Ishisaki Z, Takaishi M, Furuta I and Huh N (2001). Calmin, a protein with calponin homology and transmembrane domains expressed in maturing spermatogenic cells. *Genomics* **74**, 172–9.

Kaneko T, Li L and Li SS (2008). The SH3 domain–a family of versatile peptide- and protein-recognition module. *Front Biosci* **13**, 4938–52.

Kierszenbaum AL, Rivkin E and Tres LL (2011). Cytoskeletal track selection during cargo transport in spermatids is relevant to male fertility. *Spermatogenesis* **1**, 221–30.

Leinweber BD, Leavis PC, Grabarek Z, Wang CL and Morgan KG (1999). Extracellular regulated kinase (ERK). interaction with actin and the calponin homology (CH). domain of actin-binding proteins. *Biochem J* **344**, 117–23.

Leinweber B, Parissenti AM, Gallant C, Gangopadhya SS, Kirwan-Rhude A, Leavis PC and Morgan KG (2000). Regulation of protein kinase C by the cytoskeletal protein calponin. *J Biol Chem* **275**, 40329–36.

Lemmon MA (2008). Membrane recognition by phospholipid-binding domains. *Nat Rev Mol Cell Biol* **9**, 99–111.

Lin YH, Ke CC, Wang YY, Chen MF, Chen TM, Ku WC, Chiang HS and Yeh CH (2017). RAB10 interacts with the male germ cell-specific GTPase-activating protein during mammalian spermiogenesis. *Int J Mol Sci* **18**, pii: E97.

Liu R and Jin JP (2016). Calponin isoforms CNN1, CNN2 and CNN3, regulators for actin cytoskeleton functions in smooth muscle and non-muscle cells. *Gene* **585**, 143–53.

McLean DJ, Friel PJ, Johnston DS and Griswold MD (2003). Characterization of spermatogonial stem cell maturation and differentiation in neonatal mice. *Biol Reprod* **69**, 2085–91.

Meistrich ML (1977). Separation of spermatogenic cells and nuclei from rodent testes. *Methods Cell Biol* **15**, 15–54.

Mintz L, Galperin E, Pasmanik-Chor M, Tulzinsky S, Bromberg Y, Kozak CA, Joyner A, Fein A and Horowitz M (1999). EHD1–an EH-domain-containing protein with a specific expression pattern. *Genomics* **59**, 66–76.

Monesi V (1965). Synthetic activities during spermatogenesis in the mouse RNA and protein. *Exp Cell Res* **39**, 197–224.

Monesi V, Geremia R, D'Agostino A and Boitani C (1978). Biochemistry of male germ cell differentiation in mammals: RNA synthesis in meiotic and postmeiotic cells. *Curr Top Dev Biol* **12**, 11–36.

Nakajo A, Yoshimura S, Togawa H, Kunii M, Iwano T, Izumi A, Noguchi Y, Watanabe A, Goto A, Sato T and Harada A (2016). EHBP1L1 coordinates Rab8 and Bin1 to regulate apical-directed transport in polarized epithelial cells. *J Cell Biol* **212**, 297–306.

O'Donnell L (2015). Mechanisms of spermiogenesis and spermiation and how they are disturbed. *Spermatogenesis* **26**, e979623.

Orlova A, Rybakova IN, Prochniewicz E, Thomas DD, Ervasti JM and Egelman EH (2001). Binding of dystrophin's tandem calponin homology domain to F-actin is modulated by actin's structure. *Biophys J* **80**, 1926–31.

Pariante P, Dotolo R, Venditti M, Ferrara D, Donizetti A, Aniello F and Minucci S (2016a). First evidence of DAAM1 localization during the post-natal development of rat testis and in mammalian sperm. *J Cell Physiol* **231**, 2172–84.

Pariante P, Dotolo R, Venditti M, Ferrara D, Donizetti A, Aniello F and Minucci S (2016b). Prothymosin alpha expression and localization during the spermatogenesis of *Danio rerio*. *Zygote* **24**, 583–93.

Quesada P, Atorino L, Cardone A, Ciarcia G and Farina B (1996). Poly (ADPribosyl).ation system in rat germinal cells at different stages of differentiation. *Exp Cell Res* **226**, 183–90.

Rozenblum GT and Gimona M (2008). Calponins: adaptable modular regulators of the actin cytoskeleton. *Int J Biochem Cell Biol* **40**, 1990–5.

Santillo A, Venditti M, Minucci S, Chieffi Baccari G, Falvo S, Rosati L and Di Fiore MM (2019). d-Asp upregulates PREP and GluA2/3 expressions and induces p-ERK1/2 and p-Akt in rat testis. *Reproduction* **158**, 357–67.

Sassone-Corsi P (2002). Unique chromatin remodeling and transcriptional regulation in spermatogenesis. *Science* **296**, 2176–8.

Senoo M, Hoshino S, Mochida N, Matsumura Y and Habu S (2002). Identification of a novel protein p59(scr)., which is expressed at specific stages of mouse spermatogenesis. *Biochem Biophys Res Commun* **292**, 992–8.

Shannon KB and Li R (1999). The multiple roles of Cyk1p in the assembly and function of the actomyosin ring in budding yeast. *Mol Biol Cell* **10**, 283–96.

Shi A, Chen CC, Banerjee R, Glodowski D, Audhya A, Rongo C and Grant BD (2010). EHBP-1 functions with RAB-10 during endocytic recycling in *Caenorhabditis elegans*. *Mol Biol Cell* **21**, 2930–43.

Singh SM, Bandi S, Winder SJ and Mallela KM (2014). The actin binding affinity of the utrophin tandem calponin-homology domain is primarily determined by its N-terminal domain. *Biochemistry* **53**, 1801–9.

Sjöblom B, Ylänne J and Djinović-Carugo K (2008). Novel structural insights into F-actin-binding and novel functions of calponin homology domains. *Curr Opin Struct Biol* **18**, 702–8.

Stradal T, Kranewitter W, Winder SJ and Gimona M (1998). CH domains revisited. *FEBS Lett* **431**, 134–7.

Szymanski PT (2004). Calponin (CaP). as a latch-bridge protein–a new concept in regulation of contractility in smooth muscles. *J Muscle Res Cell Motil* **25**, 7–19.

Takahashi K, Abe M, Hiwada K and Kokubu T (1988a). A novel troponin T-like protein (calponin). in vascular smooth muscle: interaction with tropomyosin paracrystals. *J Hypertens Suppl.* **6**, S40–3.

Takahashi K, Hiwada K and Kokubu T (1988b). Vascular smooth muscle calponin. A novel troponin T-like protein. *Hypertension* **11**, 620–6.

Venditti M and Minucci S (2017). Prothymosin alpha expression in the vertebrate testis: a comparative review. *Zygote* **25**, 760–70.

Venditti M and Minucci S (2019). Subcellular localization of prolyl endopeptidase during the first wave of rat spermatogenesis and in rat and human sperm. *J Histochem Cytochem* **67**, 229–243.

Venditti M, Fasano C, Santillo A, Aniello F and Minucci S (2018a). First evidence of DAAM1 localization in mouse seminal vesicles and its possible involvement during regulated exocytosis. *C R Biol* **341**, 228–34.

Venditti M, Donizetti A, Fiengo M, Fasano C, Santillo A, Aniello F and Minucci S (2018b). Temporal and spatial expression of insulin-like peptide (insl5a and insl5b). paralog genes during the embryogenesis of *Danio rerio*. *J Exp Zool B Mol Dev Evol* **330**, 33–40.

Venditti M, Aniello F, Santillo A and Minucci S (2019). Study on PREP localization in mouse seminal vesicles and its possible involvement during regulated exocytosis. *Zygote* **27**, 160–5.

Venditti M, Fasano C, Minucci S, Serino I, Sinisi AA, Dale B and Di Matteo L (2020). DAAM1 and PREP are involved in human spermatogenesis. *Reprod Fertil Dev* **32**, 484–94.

Ventela S, Mulari M, Okabe M, Tanaka H, Nishimune Y, Toppari J and Parvinen M (2000). Regulation of acrosome formation in mice expressing green fluorescent protein as a marker. *Tissue Cell* **35**, 501–7.

Wu KC and Jin JP (2008). Calponin in non-muscle cells. *Cell Biochem Biophys* **52**, 139–48.

Zhao GQ and Garbers DL (2002). Male germ cell specification and differentiation. *Dev Cell* **2**, 537–47.

Zhou F, Chen W, Jiang Y and He Z (2019). Regulation of long non-coding RNAs and circular RNAs in spermatogonial stem cells. *Reproduction* **158**, 15–25.