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3 **Stage-specific expression of the Atce1/Tisp40 α isoform of CREB3L4 in mouse spermatids ¹**

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5 Running head: CREB3L4 isoforms in mouse spermatids

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1 **ABSTRACT**

2

3 The maturation of haploid spermatids into spermatozoa relies on the timely production of
4 proteins required for spermatid differentiation. The mammalian CREB3L4 (cAMP responsive
5 element binding protein 3-like 4) gene encodes a bZIP transcription factor that associates with
6 the membrane of the endoplasmic reticulum. CREB3L4 is presumed to play an important role in
7 protein maturation via its involvement in the cellular response to endoplasmic reticulum stress.
8 In mice, the *Creb3l4* gene gives rise to two distinct classes of mRNAs through the use of
9 alternate promoters. Transcripts that initiate upstream of the first coding exon encode a 370-
10 amino acid protein designated Tisp40 β whereas transcripts that initiate downstream of the first
11 coding exon encode Atce1/Tisp40 α , a truncated (315 aa) form of Tisp40 β . In the mouse testis,
12 *Creb3l4* transcripts are known to be expressed exclusively in postmeiotic spermatids but the
13 presence of CREB3L4 protein in spermatids has not been formally demonstrated. We produced
14 an antibody directed against the carboxy terminus of mouse CREB3L4 and used it in
15 immunostaining experiments to document that CREB3L4 protein accumulates in post-meiotic
16 spermatids in a stage-specific manner. Moreover, we show that Atce1/Tisp40 α is the major form
17 of CREB3L4 in mouse testis. These findings suggest that testis-specific isoforms of *Creb3l4*
18 could play an important role in spermatid differentiation.

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20 Key words: AbZIP, endoplasmic reticulum, unfolded protein response

1 INTRODUCTION

2

3 Spermogenesis is the phase of male germ cell development during which spermatids mature
4 into spermatozoa. During this phase, haploid round spermatids that are embedded in the
5 epithelium of the mammalian seminiferous tubule undergo a fascinating series of profound
6 genetic and morphological changes. These changes include nuclear condensation, acrosome
7 formation, a reduction in cytoplasmic volume, and the formation of a flagellum to impart
8 motility.

9

10 The molecular processes that result in the formation of mature spermatozoa are not fully
11 understood but they ultimately rely on the timely production of proteins that are involved in
12 spermatid differentiation. One transcription factor that has been shown to play a crucial role in
13 spermiogenesis is the bZIP (basic region-leucine zipper) transcription factor CREM (cAMP-
14 response-element modulator). Inactivation of the *Crem* gene in mice causes an arrest of
15 spermatogenesis at the round spermatid stage and, consequently, infertility (Blendy et al, 1996;
16 Nantel et al, 1996). Infertility in these mice has been attributed to the failure of the germ cells to
17 express CREM-responsive genes, such as the protamines that replace histones to allow DNA
18 compaction (Kimmins et al, 2004).

19

20 The early phases of spermiogenesis are characterized by intense transcriptional activity
21 (Eddy, 2002) which implies that the endoplasmic reticulum (ER) must function optimally to
22 ensure that the required proteins are properly synthesized and processed. An important
23 regulatory mechanism implicated in the maintenance of ER function is the “unfolded protein

1 response (UPR)” (a.k.a. “ER stress response”), an adaptive response that serves to re-establish
2 normal ER function following disruptions such as the accumulation of unfolded or misfolded
3 proteins (Zhang and Kaufman, 2004). This is achieved by promoting the degradation of
4 misfolded proteins, by attenuating protein translation to reduce the amount of nascent proteins,
5 and by increasing the production of the ER chaperones that are required to ensure the proper
6 processing of nascent proteins.

7
8 bZIP transcription factors that associate with the ER serve an important role in the UPR by
9 activating the transcription of genes that code for ER proteins, such as chaperones (Zhang and
10 Kaufman, 2004). In order to do so, ER bZIP proteins must first transit through the Golgi
11 apparatus where they are processed by proteases that separate their amino-terminal transcription
12 factor domains from their carboxy-terminal membrane-associated regulatory domains. The
13 transcription factor domains of ER bZIP proteins then travel to the nucleus to regulate gene
14 expression. CREB3L4 (CRE binding protein 3-like 4) is an ER-associated bZIP protein that was
15 independently isolated as A1bZIP (Androgen-Induced bZIP) in human prostate cancer cells (Qi
16 et al, 2002), as Tisp40 (Transcript induced in spermiogenesis 40), a spermatid-specific transcript
17 in mouse (Fujii et al, 2002), and as Atce1 (Attaching to CRE-like 1), a protein identified in a
18 two-hybrid screen for proteins that interact with Tctex2 (t-complex-associated testis expressed
19 3), a protein implicated in fertility (Stelzer and Don, 2002). Although the role of CREB3L4 in
20 ER function has not been fully characterized, the studies conducted to date have established that
21 CREB3L4 localizes to the ER, can be processed by Golgi proteases and that its nuclear form can
22 activate gene expression via regulatory elements that are common to genes induced during the
23 UPR (Nagamori et al, 2005).

1
2 In humans, CREB3L4 is most abundant in prostate (Qi et al, 2002) but standard as well as
3 quantitative RT-PCR assays have revealed the presence of appreciable amounts of CREB3L4
4 mRNA in testis (Cao et al, 2002; Cunha et al, 2005). In the mouse, on the other hand, CREB3L4
5 mRNA is most abundant in testis (Nagamori et al, 2005) and CREB3L4 transcription is up-
6 regulated during mouse spermiogenesis (Fujii et al, 2002). Together these observations support
7 the concept that CREB3L4 could potentially play an important role in assuring ER function
8 during spermiogenesis. However, it is important to note that the *Creb3l4* gene encodes at least
9 two distinct transcripts that initiate from different transcription start sites (Fig. 1). The first
10 transcript to be identified in mouse testis initiates in intron three of the gene and contains eight of
11 the nine coding exons (Nagamori et al, 2005; Stelzer and Don, 2002). This mRNA codes for a
12 315-residue protein designated Atce1/Tisp40 α which contains codons 56 to 370 of the larger
13 Tisp40 β protein. Transcripts that encode Tisp40 β initiate upstream of exon 1 or 2 and contain all
14 nine coding exons of the gene.

15
16 The discovery of two distinct CREB3L4 isoforms in mouse is significant because Tisp40 β is
17 a potent transcriptional activator whereas Atce1/Tisp40 α is not (Nagamori et al, 2005). In order
18 to understand the role of CREB3L4 in mammalian reproduction, it is therefore of paramount
19 importance to determine precisely which CREB3L4 isoform is present in testis. In this report,
20 immunostaining experiments performed using an antibody raised against the carboxy terminal
21 portion of mouse CREB3L4 documented that the CREB3L4 protein is expressed in a stage-
22 specific manner in differentiating spermatids. Moreover, we found that the most abundant forms
23 of CREB3L4 present in mouse testis lack the amino terminal activation domain of mouse

1 CREB3L4. These findings have important implications for future studies of CREB3L4 in
2 reproduction.

3

4 **MATERIALS AND METHODS**

5

6 *Animals*

7

8 The use of animals was approved by our institutional animal care committee. The animals
9 used in this study (male C57BL/6 mice and New Zealand rabbits) were housed under standard
10 conditions in a CCAC (Canadian Council on Animal Care)- and AAALAC (Association for
11 Assessment and Accreditation of Laboratory Animal Care)-certified animal facility. Mice were
12 anesthetized prior to tissue collection.

13

14 *Antibody Production and Purification*

15

16 A peptide corresponding to amino acids 354-367 (KARPPGQIRGMVHT) of mouse
17 CREB3L4 (GenBank Accession No. NM_030080) was synthesized in our facility and
18 conjugated to KLH via a cysteine residue appended to its amino terminus. This particular peptide
19 was selected because its sequence is not present in other proteins of the mouse CREB3 family
20 and it did not match any other known mouse protein in the GenBank database. Antibodies were
21 produced by injecting the conjugated peptide to three rabbits using standard procedures.
22 Antiserum from rabbit number 1462 (AB1462) was selected on the basis of its performance in
23 immunoblotting experiments and subsequently purified by affinity chromatography after

1 immobilizing the immunogenic peptide to SulfoLink Coupling Gel (Pierce Biotechnology,
2 Rockford, IL). All the immunostaining and immunoblotting experiments presented in this report
3 were performed using affinity-purified AB1462 antibody.
4

5 *Mouse Tissue Preparation*

6

7 For immunostaining experiments, four three month-old and two six week-old male mice
8 were perfused through the left ventricle with 10% buffered formalin for 15 min. Following
9 perfusion, the testes were collected and immersed in the same fixative for 24 hr, and then
10 embedded in paraffin blocks. Testes used for immunoblotting were collected from sexually
11 mature male mice, snap-frozen in liquid nitrogen and homogenized using a tissue grinder in lysis
12 buffer (6 M urea, 20 mM Tris pH 6.8, 1% SDS) supplemented with protease inhibitors (Roche
13 Applied Science, Indianapolis, IN). Protein extracts were stored at -80°C prior to use.
14

15 *Immunostaining*

16

17 Paraffin sections (4 μm) were deparaffinized in toluene and rehydrated through ethanol.
18 Endogenous peroxidase activity was eliminated by preincubation in 3% H_2O_2 in methanol for 30
19 min. A microwave retrieval technique using citrate buffer was applied (Tacha and Chen, 1994)
20 and non-specific binding was blocked using 10% (v/v) goat serum diluted in Dako antibody
21 diluent (DakoCytomation California, Carpinteria, CA). The sections were incubated with
22 antibody 1462 (diluted 1:1000) for 75 min at room temperature, washed in PBS buffer, and
23 incubated with biotinylated anti-rabbit secondary antibody for 10 min and then with streptavidin-

1 peroxidase for another 10 min. Under microscope monitoring, diaminobenzidine was used as the
2 chromogen to visualize the biotin/streptavidin-peroxidase complexes. Counterstaining was
3 performed using #2 Gill's hematoxylin. As a negative control, an excess (5-fold) of the synthetic
4 peptide was co-incubated with the primary antibody for 3 h at room temperature.

5

6 *Expression Vectors*

7

8 The full-length mouse CREB3L4 open reading frame (ORF) was isolated from mouse
9 prostate RNA by reverse transcription-polymerase chain reaction (PCR) amplification. The PCR
10 product was then cloned in frame with sequences encoding a C-terminal haemagglutinin (HA)
11 epitope in a modified pcDNA3 (Invitrogen, Carlsbad, CA) expression plasmid. The recombinant
12 protein contains amino acids 1-370 of CREB3L4 (GenBank accession No. NM_030080)
13 followed by amino acids SRGP and the HA epitope (YPYDVDPDYASL). This plasmid was then
14 used as a template for PCR amplification to generate plasmids producing an untagged form of
15 Tisp40 β as well as HA-tagged and untagged forms of Atce1/Tisp40 α (mouse CREB3L4 codons
16 56-370). Tisp40 α HA also contains the SRGPYPYDVDPDYASL extension at its C terminus. The
17 sequences of all PCR products and cloning junctions were verified using an automated
18 sequencer. Plasmid DNA for transfection experiments was purified by gravity-flow anion
19 exchange (Qiagen, Valencia, CA).

20

1 *Transient Transfections and Immunoblotting*

2

3 Mouse CREB3L4 expression plasmids were transfected into human kidney 293 cells using
4 ExGen 500 transfection reagent (Fermentas Life Sciences, Hanover, MD). The cells were
5 harvested 48 hr post-transfection and whole-cell extracts were prepared in lysis buffer (see
6 above). Protein extracts from mouse testis (30 µg/lane) and transfected 293 cells (4 µg/lane) were
7 immobilized on nitrocellulose following electrophoresis through 10% denaturing polyacrylamide
8 gels. The blots were pre-incubated for 1 hr at room temperature in TBS (0.9% NaCl, 10 mM
9 Tris-HCl, pH 8.0) containing 5% (w/v) powder milk and then incubated overnight at 4 °C in
10 fresh TBS/milk containing a 1:1000 dilution of AB1462. The blots were washed in TBS
11 containing 0.05% Tween-20 and 0.05% NP-40 (four times 15 min at room temperature) and then
12 incubated for 1 hr at room temperature in TBS/milk containing a 1:10,000 dilution of
13 peroxidase-conjugated AffiniPure goat antirabbit IgG (Jackson ImmunoResearch Laboratories,
14 West Grove, PA). After another series of washes, antigen-antibody complexes were revealed
15 using the Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, Wellesley, MA).

16

17 *Deglycosylation Experiments*

18

19 Extracts from mouse testis and from transfected 293 cells prepared in lysis buffer were
20 denatured in 1X Glycoprotein denaturing buffer (5% (w/v) SDS, 0.4 M DTT) at 100°C for 10
21 min. Denatured testis (30 µg) and 293 cell (4 µg) extracts were then incubated in 50 mM sodium
22 citrate buffer alone or buffer containing 1000 units of Endoglycosidase H (New England
23 BioLabs, Ipswich, MA) for 16 hr at 37°C prior to electrophoresis.

1 RESULTS

2

3 Newly synthesized ER-bound bZIP transcription factors such as CREB3L4 initially localize
4 to the ER and only translocate to the nucleus following removal of their C-terminal regulatory
5 domains. To determine the distribution and cellular localization of unprocessed CREB3L4
6 proteins in mouse testis we required an antibody that recognizes the C-terminal domain of mouse
7 CREB3L4. We therefore generated polyclonal antibodies against a 14-residue C-terminal peptide
8 of mouse CREB3L4 (aa 354-367) in rabbits (Fig. 1). This antibody would be expected to detect
9 both Tisp40 β and Atce1/Tisp40 α in immunostaining experiments but should be able to
10 discriminate between the two polypeptides in immunoblotting experiments.

11

12 Testes were collected from sexually mature three-month old C57BL/6 mice and cross-
13 sections were processed for immunostaining using affinity-purified antiserum number 1462
14 (AB1462). As shown in Fig. 2, AB1462 stained the epithelium of the seminiferous tubules
15 whereas interstitial cells and Leydig cells were unlabelled. The staining reaction was completely
16 abolished when an excess of the immunogenic peptide was used to immunoabsorb the antiserum.
17 Close examination of stained seminiferous tubules revealed that only the post-meiotic cells
18 (round or elongated spermatids) were labelled whereas spermatocytes, spermatogonia and Sertoli
19 cells were not labelled. Interestingly, some seminiferous tubules displayed little or no staining
20 whereas other tubules showed a strong staining reaction.

21

22 The staining pattern observed with AB1462 in mouse testis strongly suggested that the
23 abundance of CREB3L4 might fluctuate as a function of mouse spermatid development. To

1 quantitatively assess differences in CREB3L4 abundance during the cycle of the seminiferous
2 epithelium, we staged the seminiferous tubules present on the cross-section shown in Fig. 2 and
3 captured high magnification photomicrographs of representative tubules. These photographs
4 were then arranged in sequence according to the mouse testis epithelial cycle as presented in Fig.
5 3 (Russell et al, 1990). The first observation we made was that round and elongating spermatids
6 displayed exclusively cytoplasmic staining, thereby indicating that newly synthesized CREB3L4
7 localizes to the cytoplasm of post-meiotic germ cells. Interestingly, the intensity of the
8 cytoplasmic staining reaction varied markedly in both round and elongated spermatids at
9 different stages of spermatogenesis.

10

11 At stage I of the cycle, a weak staining reaction was detectable in step 1 round spermatids as
12 well as in step 13 elongated spermatids (Fig. 3a). At stage III of the cycle, a staining reaction of
13 similar intensity was observed in step 2-3 round spermatids whereas step 14 elongated
14 spermatids displayed a reduction in staining intensity (Fig. 3b). By stage IV of the cycle, step 15
15 elongated spermatids showed practically no staining. In contrast, the staining of step 4 round
16 spermatids was much more pronounced than that observed in earlier steps (Fig. 3c). The intensity
17 of the labelling of round spermatids increased progressively through stage VI (Fig. 3d) to
18 achieve maximal intensity at stage VII in step 7 round spermatids (Fig. 3e). At this point of the
19 cycle, step 16 spermatids were unlabelled. A strong labelling reaction persisted in step 9
20 elongating spermatids (Fig. 3f) but its intensity decreased progressively thereafter through steps
21 10 and 11 (Fig. 3g, h). At stage XII, the labelling of step 12 spermatids was comparable to that
22 observed at step 13 of stage I (Fig. 3i). This stage-specific staining pattern was observed in all

1 four 3-month old male mice examined, as well as in six-week old C57BL/6 mice (data not
2 shown).

3
4 To verify if both Tisp40 β and Atce1/Tisp40 α are present in the testes of sexually mature
5 mice we performed immunoblotting experiments using AB1462. Two abundant polypeptides
6 with apparent molecular weights of 42 and 36 kDa, hereafter referred to as p42 and p36, were
7 detected in mouse testes (Fig. 4A). The apparent molecular weights of these proteins are similar
8 to the 44 and 38 kDa polypeptides that were previously detected in mouse testis extracts using a
9 polyclonal antibody raised against the N-terminal portion common to Tisp40 α and Tisp40 β
10 (Nagamori et al, 2005). Based on the electrophoretic mobility of *in vitro*-synthesized Tisp40
11 proteins, Nagamori et al. deduced that the 44 and 38 kDa polypeptides correspond to Tisp40 β
12 and Tisp40 α , respectively. However, the 42-44 kDa polypeptide is much more abundant than the
13 36-38 kDa polypeptide in testis extracts, which is inconsistent with the observation that the
14 Tisp40 β mRNA is less abundant than the Tisp40 α mRNA (Nagamori et al, 2005). Moreover,
15 Nagamori et al. also showed that recombinant Tisp40 proteins are glycosylated when they are
16 transiently produced in human HeLa cells, suggesting that p42-44 and p36-38 might correspond
17 to differentially glycosylated forms of the same polypeptide.

18 In an attempt to resolve this issue we transiently produced HA epitope-tagged versions of
19 Tisp40 β and Atce1/Tisp40 α in human 293 cells and compared their apparent molecular weights
20 to those of p42 and p36. As shown in Fig. 4A, the apparent molecular weight of Tisp40 β HA was
21 considerably greater than that of p42. In addition, transiently expressed Tisp40 β HA also
22 generated polypeptides with apparent molecular weights similar to those observed in cells
23 transfected with Tisp40 α or Tisp40 α HA. It is possible that these could result from internal

1 initiation or from degradation. Most importantly, the high molecular weight forms observed with
2 Tisp40 β HA were not present in cells transfected with Tisp40 α HA or in testis. The apparent
3 molecular weight of HA-tagged Atce1/Tisp40 α was only slightly greater than that of p42,
4 suggesting that p42 might correspond to Atce1/Tisp40 α . In agreement with this prediction, a
5 recombinant untagged form of Atce1/Tisp40 α co-migrated precisely with p42. Interestingly,
6 transiently expressed Tisp40 α also produced a 36 kDa polypeptide.

7 The results of these immunoblotting experiments suggested that Atce1/Tisp40 α could give
8 rise to the 42 and 36 kDa proteins present in mouse testis but they did not clarify the nature of
9 these polypeptides. The Tisp40 α mRNA contains two internal methionines (see Fig. 1) which
10 could potentially give rise to a 36 kDa protein. We therefore inactivated methionine codons 123
11 and 156 to determine if internal initiation could explain the production of p36 in cells transfected
12 with Tisp40 α mRNA but the mutated expression plasmid still produced p42 and p36 (data not
13 shown).

14 To determine if p42 corresponds to a glycosylated form of p36, we incubated testis extracts
15 as well as extracts of 293 cells expressing untagged recombinant Tisp40 α or Tisp40 β with
16 endoglycosidase H. As shown in Fig. 4B, transiently expressed Tisp40 β produced a migration
17 pattern distinct from that observed in testis extracts whereas the migration pattern obtained with
18 transiently expressed Tisp40 α was similar to that seen in testis. Addition of endoglycosidase H to
19 testis extracts converted p42 to p36, indicating that p42 is indeed a glycosylated form of p36.
20 Endoglycosidase H also converted the 42 kDa protein produced by Tisp40 α to the 36 kDa form.
21 In cells producing Tisp40 β , endoglycosidase H reduced the abundance of high molecular weight
22 forms. The lower molecular weight polypeptides seen in cells transfected with Tisp40 β mRNA
23 could correspond to degradation products or the products of internal initiation (this has not been

1 investigated further). In fact, lysates programmed with Tisp40 β mRNA give rise to a polypeptide
2 that co-migrates with *in vitro*-translated Tisp40 α (Nagamori et al, 2005). Whether this occurs in
3 vivo remains to be determined. Taken together, these results indicate that Tisp40 α is the major
4 CREB3L4 protein product in mouse testis and that Tisp40 α exists as an abundant glycosylated
5 form and a less abundant unglycosylated form.

6

7 **DISCUSSION**

8

9 This study of CREB3L4 proteins in mouse testis confirmed and extended previous
10 observations regarding CREB3L4 expression in mouse testis and, importantly, revealed crucial
11 new information regarding the identity of testis CREB3L4 proteins. These findings should have a
12 determining influence on the orientation of future studies that will address the role of CREB3L4
13 in reproduction.

14

15 Previous studies employing *in situ* hybridization detected CREB3L4 mRNA exclusively in
16 spermatids (Nagamori et al, 2005; Stelzer and Don, 2002). However, the distribution and relative
17 abundance of CREB3L4 protein(s) in mouse testis had not yet been examined. The
18 immunostaining data presented here confirm that CREB3L4 proteins are produced in
19 differentiating spermatids and provide direct evidence that the abundance of CREB3L4 proteins
20 varies during spermatid differentiation. The abundance of CREB3L4 increases gradually through
21 steps 1-6, peaks at step 7, and decreases thereafter through step 12. During spermiogenesis,
22 transcription occurs until the midpoint of the postmeiotic phase i.e. step 7-8 (Eddy, 2002). Thus,
23 the presence of CREB3L4 coincides with a phase during which the ER would be solicited to

1 process a large number of proteins. The fact that CREB3L4 is not detected in spermatocytes and
2 in elongated spermatids indicates that the protein is dispensable at these stages of male germ cell
3 differentiation.

4
5 In view of the fact that genes can give rise to multiple isoforms, some of which are endowed
6 with unique functional properties, it is important that we know the tissue-specific distribution of
7 such isoforms. The presently available antibodies do not allow us to distinguish between the two
8 Tisp40 isoforms. We therefore used transiently expressed recombinant Tisp40 proteins combined
9 with deglycosylation experiments to determine, with a reasonable degree of certainty, that the
10 315-amino acid Atce1/Tisp40 α protein is the major CREB3L4 protein produced in mouse testis
11 and that it exists as glycosylated and unglycosylated forms. The observation that Atce1/Tisp40 α ,
12 rather than Tisp40 β , is detected in mouse testis is consistent with the fact that several genes
13 encode testis-specific transcripts which result from the use of alternative promoters (Kleene,
14 2001; Sassone-Corsi, 2002).

15
16 Our findings appear to contradict a recent study which concluded that both Tisp40 β and
17 Tisp40 α are present in testis (Nagamori et al, 2005). Although Nagamori et al. demonstrated that
18 Tisp40 proteins are glycosylated when they are expressed in HeLa cells, they did not specifically
19 examine the glycosylation state of endogenous Tisp40 proteins in testis. Because the testis
20 proteins detected by Nagamori et al. are indistinguishable in size from p42 and p36 reported in
21 this study, we would anticipate that their p44 is a glycosylated form of p38. Interestingly,
22 Nagamori et al. recently reported that Creb3l4 is a target of CREM and that CREM activates
23 transcription of the Tisp40 α mRNA via response elements located between the transcription start

1 sites of the Tisp40 β and Tisp40 α mRNAs (Nagamori et al, 2006). In their assays, CREM did not
2 activate the promoter region located upstream of the Tisp40 β mRNA. Taken together, these data
3 from H. Nojima's laboratory combined with those reported here support the conclusion that
4 Tisp40 α is the major CREB3L4 protein in testis.

5
6 The realization that Tisp40 β is not produced in differentiating spermatids has important
7 implications regarding the role of CREB3L4 in male reproduction. Indeed, Atce1/Tisp40 α lacks
8 a large portion of the transcription activation domain present in Tisp40 β and a recent report
9 demonstrated that Tisp40 β could activate transcription via an unfolded protein response element
10 whereas Tisp40 α failed to activate transcription via this same element (Nagamori et al, 2005).
11 Thus, one might be tempted to conclude that Atce1/Tisp40 α (and p36) actually serves as a
12 transcriptional repressor, possibly by interfering with the activity of other bZIP transcription
13 factors. On the other hand, it is also possible that Atce1/Tisp40 α could regulate gene expression
14 via other unidentified response elements, or that it could only act as a transcriptional activator
15 when dimerized with another bZIP protein.

16
17 Hiroshi Nojima's laboratory recently reported that the nuclear form of Tisp40 α is capable of
18 dimerizing with CREM and that this interaction augments the interaction between CREM and a
19 CRE (Nagamori et al, 2006). Such an association seems somewhat counterintuitive as a bZIP
20 protein that is implicated in ER stress would not be expected to contribute to the activity of a
21 transcription factor whose actions could ultimately stress the ER. Possibly, the CREM-Tisp40 α
22 dimer regulates a subset of genes that serve to protect the ER and/or the CREM-Tisp40 α dimer
23 fulfills a specialized function in spermatids. In fact, H. Nojima's laboratory discovered that the

1 CREM-Tisp40 α dimer recruits a histone chaperone. The functional significance of this
2 interaction remains to be determined.

3
4 The importance of CREB3L4 in mammalian reproduction is supported by a recent report
5 which described alterations in the reproductive system of male mice in which the *Creb3l4* gene
6 was inactivated by replacement with a green fluorescent protein (Adham et al, 2005). Although
7 the resulting mice were fertile, inactivation of *Creb3l4* resulted in a significant reduction in the
8 number of spermatozoa in the epididymis. This reduction was attributed to increased apoptosis of
9 haploid spermatids, which is consistent with a protective role of CREB3L4 in ER function.

10
11 In summary, the data presented herein indicate that the Atce1/Tisp40 α isoform of CREB3L4
12 is expressed in a stage-specific manner during spermatid differentiation in mice. Future studies
13 focussing on the regulation and action of this testis-specific CREB3L4 variant will allow us to
14 better understand the function of this potentially important gene in mammalian reproduction.

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2

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1 **FIGURE LEGENDS**

2

3 **Figure 1**

4 Structure of the *Creb3l4* gene and encoded polypeptides. The *Creb3l4* gene on mouse
5 chromosome 3 is depicted as a solid line. The two 5' non-coding exons are represented as white
6 vertical rectangles whereas the nine coding exons are shown as black rectangles. The
7 approximate positions of the transcription start sites of mRNAs that encode the Tisp40 β and
8 Atce1/Tisp40 α proteins are indicated by arrows above and below the gene, respectively. The two
9 proteins are depicted as rectangles which are further subdivided to illustrate the contribution of
10 each coding exon to the protein sequence. The first complete codon encoded by each exon is
11 indicated within the Tisp40 β diagram (these have been omitted from the Atce1 diagram for
12 simplicity). The positions of the bZIP domain and that of the peptide used to immunize rabbits
13 are shown above the Tisp40 β diagram. Note that the first AUG of the Atce1/Tisp40 α cDNA
14 corresponds to Met 56 of Tisp40 β . The GenBank accession numbers for the *Creb3l4* gene, the
15 full-length mouse CREB3L4 protein, and the Atce1 mRNA are NC_000069, NM_030080 and
16 AF287260, respectively.

17

18 **Figure 2**

19 Localization of CREB3L4 protein in mouse testis. a) Paraffin-embedded cross-section of mouse
20 testis immunostained with an affinity-purified rabbit polyclonal antibody (AB1462) directed
21 against the C-terminus of mouse CREB3L4. Seminiferous tubules at stages IV, IX and X of the
22 epithelial cycle are labelled. b) A consecutive serial section was processed as in 'a' except that

1 an excess of the immunogenic peptide was used to immunoabsorb the antiserum. Scale bar = 40
2 μm .

3

4 Figure 3

5 Stage-specific expression of CREB3L4 during spermiogenesis. a) Representative cross-sections
6 of seminiferous tubules at Stage I (a), III (b), IV (c), VI (d), VII (e), IX (f), X (g), XI (h) and XII
7 (i) of spermiogenesis were immunostained with affinity-purified AB1462. The cytoplasmic
8 staining pattern is best seen in panel e (Stage VII) in which the pale bluish nuclei of step 7
9 spermatids are surrounded by a dark brown staining reaction. The seminiferous tubules shown
10 here were selected from the same testis cross-section. Scale bar = 30 μm . b) Schematic
11 representation of CREB3L4 expression in mouse spermatids. The stages of the seminiferous
12 epithelium cycle are identified by Roman numerals whereas the steps of spermatid development
13 are indicated by Arabic numerals (modified from Russell et al, 1990). The color reflects the
14 intensity of the immunostaining reaction.

15

16 Figure 4

17 Characterization of glycosylated and non-glycosylated forms of Atce1/Tisp40 α in mouse testis.

18 a) Extracts prepared from mouse testis (lane 5), untransfected human kidney 293 cells (lane 1),
19 and from 293 cells transiently transfected with plasmids encoding HA-tagged Tisp40 β (lane 2),
20 HA-tagged Tisp40 α (lane 3) or untagged Tisp40 α (lane 4) were probed with AB1462. The
21 positions of the 42 and 36 kDa polypeptides are indicated. b) Testis extracts (lanes 6-7) and
22 extracts of 293 cells transiently transfected with plasmids encoding untagged forms of Tisp40 β
23 (lanes 2-3) and Tisp40 α (lanes 4-5) were incubated in the absence (-) or presence (+) of

1 endoglycosidase H and then analyzed by immunoblotting using AB1462. Untransfected 293 cell
2 extracts are in lane 1. The immunoblots presented here are representative of independent
3 experiments performed using extracts from different mouse testes and independent transfections.

BZIP (K195 – L256)

KARPPGQIRGMVHT
(aa 354-367)

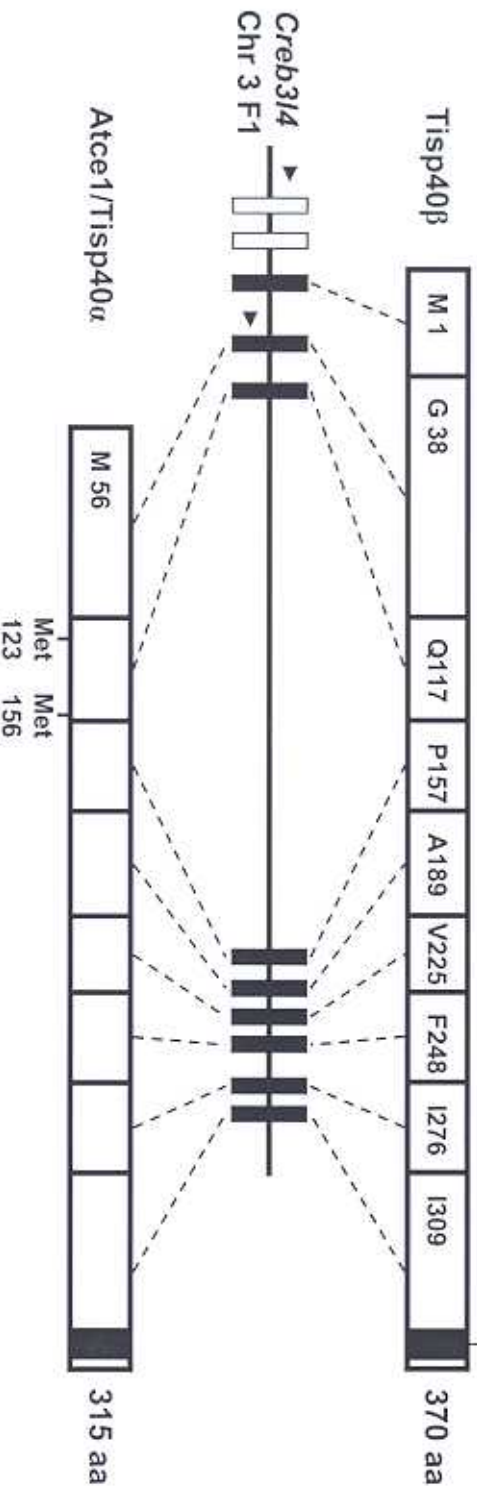


Figure 2

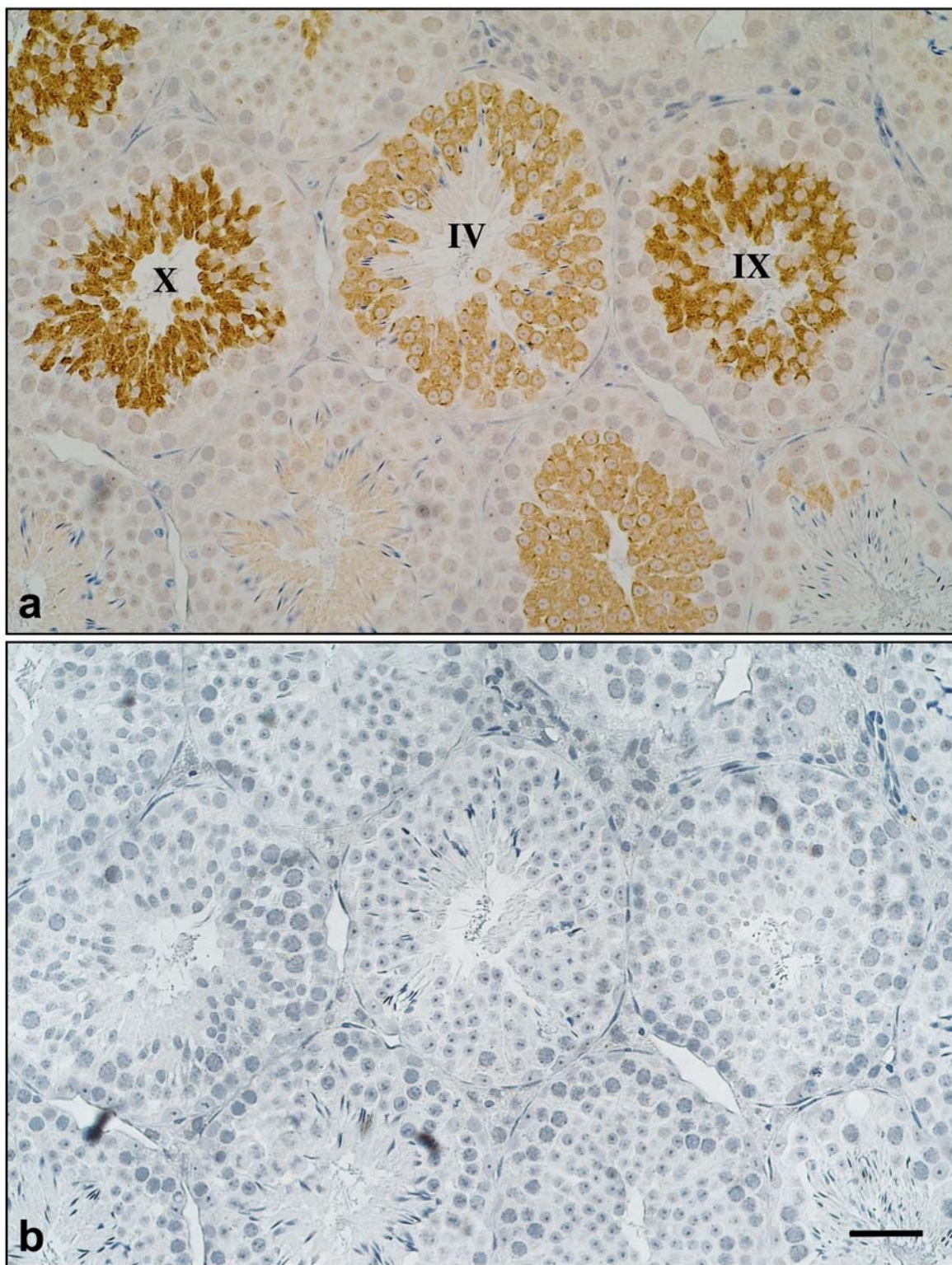
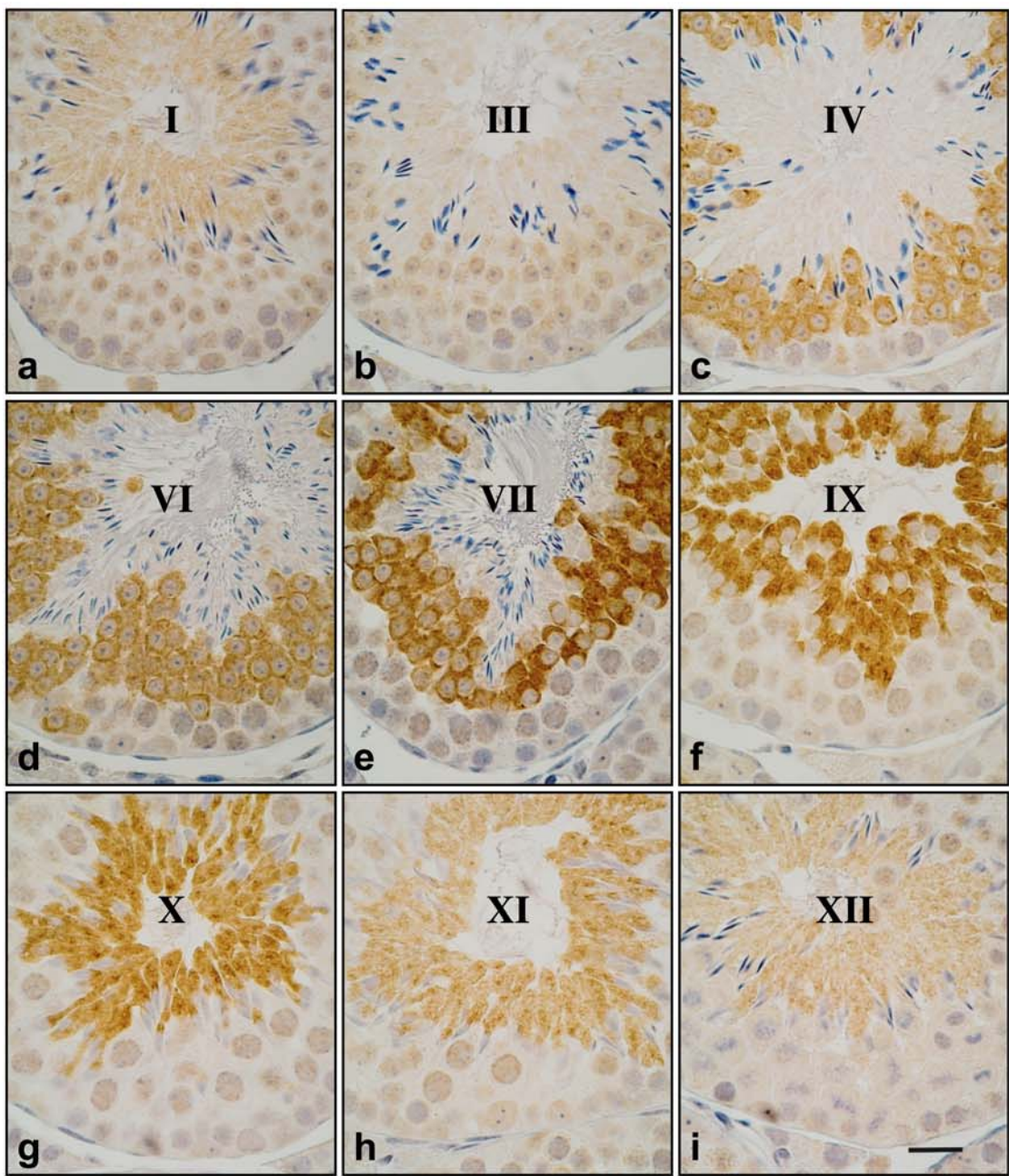


Figure 3

a



b

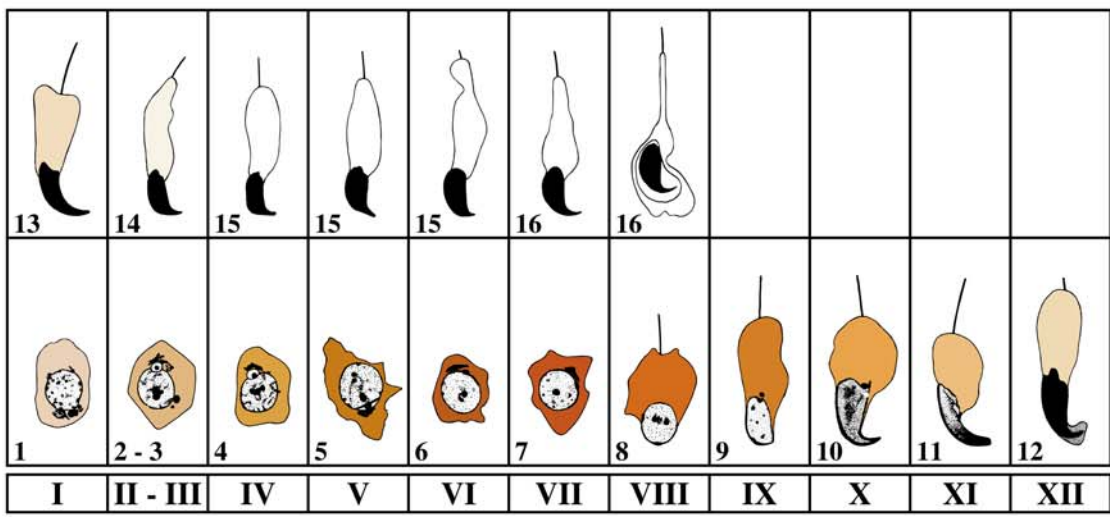
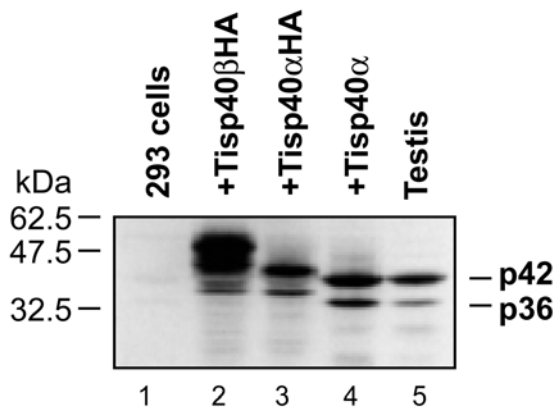


Figure 4

a



b

