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Expression of the adaptor protein m-Numb in mouse male germ cells

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Abstract

Numb is an adaptor protein that is asymmetrically inherited at mitosis and controls the fate of sibling cells in different species. The role of m-Numb (mammalian Numb) as an important cell fate-determining factor has extensively been described mostly in neural tissues, particularly in progenitor cells, in the mouse. Biochemical and genetic analyses have shown that Numb acts as an inhibitor of the Notch signaling pathway, an evolutionarily conserved pathway involved in the control of cell proliferation, differentiation, and apoptosis. In the present study, we sought to determine m-Numb distribution in germ cells in the postnatal mouse testis. We show that all four m-Numb isoforms are widely expressed during postnatal testis development. By reverse transcriptase-PCR and western blot analyses, we further identify p71 as the predominantly expressed isoform in germ cells. Moreover, we demonstrate through co-immunoprecipitation studies that m-Numb physically associates with Ap2a1, a component of the endocytotic clathrin-coated vesicles. Finally, we employed confocal immunofluorescence microscopy of whole mount seminiferous tubules and isolated germ cells to gain more insight into the subcellular localization of m-Numb. These morphological analyses confirmed m-Numb and Ap2a1 co-localization. However, we did not observe asymmetric localization of m-Numb neither in mitotic spermatogonial stem cells nor in more differentiated spermatogonial cells, suggesting that spermatogonial stem cell fate in the mouse does not rely on asymmetric partitioning of m-Numb.

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Introduction

Spermatogenesis is a cyclic process that consists of three phases: mitotic expansion of spermatogonia, meiotic division, and haploid cell differentiation that produces mature sperm. The continuous production of sperm is ensured by a small pool of spermatogonial stem cells (SSC). In rodents, it is generally assumed that, SSC undergo symmetric division either producing two new stem cells (type-As (Asingle) spermatogonia) or two interconnected cells, generated by incomplete cell cytokinesis, destined to differentiate (type-Apr (Apaired) spermatogonia). However, the possibility exists that SSC divide asymmetrically to generate one daughter SSC and one daughter cell destined to produce Apr spermatogonia. Such division would generate two single cells, morphologically alike, but with different molecular endowment (Meistrich & van Beek 1993, Watt & Hogan 2000, de Rooij 2001). Molecular mechanisms involved in the regulation of SSC (i.e. self-renewal versus differentiation) are still largely unknown, but several lines of evidence clearly indicate that glial cell line derived neurotrophic factor (GDNF) is involved in the regulation of SSC fate (Meng *et al.* 2000, Tadokoro *et al.* 2002, Yomogida *et al.* 2003). In testis, GDNF is produced by Sertoli cells, the nursing somatic cells of the seminiferous epithelium (Trupp *et al.* 1995, Meng *et al.* 2000, Tadokoro *et al.* 2002). Genes activated by GDNF pathway have recently been identified by microarray analysis performed on isolated GDNF-family receptorα-1 (GFRα-1)-positive spermatogonia, treated *in vitro* with GDNF (Hofmann *et al.* 2005). GFRα-1 is a co-receptor for GDNF expressed by type-As and type-Apr spermatogonia (von Schonfeldt *et al.* 2004, Hofmann *et al.* 2005). Interestingly, GDNF induces the expression of m-Numb and *seven in absentia* (SIAH-1), an E3-ubiqutin ligase that recognizes Numb and targets it for degradation (Susini *et al.* 2001). In *Drosophila*, Numb is an intrinsic cell-fate determinant factor, which is asymmetrically localized in mitotic stem/progenitor cells, and is segregated and asymmetrically inherited by one daughter cell (Uemura *et al.* 1989, Knoblich *et al.* 1995, Spana *et al.* 1995). Genetic and biochemical

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analyses have shown that Numb controls the fate of cell progeny by repressing the Notch pathway (Guo *et al.* 1996, Spana & Doe 1996, Wan *et al.* 2000). Numb is a scaffold protein bearing multiple protein–protein interaction regions, including an amino-terminal phosphotyrosine-binding (PTB) domain and a proline-rich carboxyl-terminal region (PRR). Numb binds to Ap2a1 (adaptor protein complex AP-2, alpha1 subunit) a subunit of the AP-2 complex, the major component of clathrin-coated endocytic vesicles (Santolini *et al.* 2000, Berdnik *et al.* 2002). Numb-mediated inhibition of Notch signaling, appears to require the *Drosophila* ortholog of Ap2a1 (alpha-adaptin), suggesting that Numb may be directly involved in targeting Notch for endocytosis (Berdnik *et al.* 2002, Le Borgne *et al.* 2005). In mammals, there are two Numb homologous, *m-numb* and *numlike* genes that are widely expressed during development and in adult tissues (Verdi *et al.* 1996, Zhong *et al.* 1996, 1997). Both gain-of-function and loss-of-function studies indicate that m-Numb and Numblike are functionally redundant and essential in vertebrate neural development (Zhong *et al.* 2000, Zilian *et al.* 2001, Petersen *et al.* 2002). The alternative splicing of *m-numb* transcripts generates at least four protein isoforms with predicted molecular masses of 65, 66, 71, and 72 kDa. The different isoforms result from the presence or the absence of amino acid inserts within the PTB domain and/or within the PRR (Dho *et al.* 1999, Verdi *et al.* 1999).

The expression pattern of m-Numb mRNA during embryogenesis and in various tissues in the adult mouse has previously been reported (Zhong *et al.* 1996). As for male germ cells, m-Numb was found to be upregulated after GDNF treatment of isolated mouse spermatogonia, both at mRNA and at protein levels (Braydich-Stolle *et al.* 2005). However, m-Numb expression profile in mouse testis and germ cells remains to be determined. In this paper, we aimed to investigate the expression of m-Numb isoforms during postnatal testis development as a first step to elucidate their physiological roles during spermatogenesis. We found that m-Numb is widely expressed both in the somatic and in the germ cell compartments of mouse testis. We also demonstrate that germ cells predominantly express the p71 m-Numb isoform. Immunocytochemical analysis revealed that m-Numb is expressed in mitotic SSC but never in an asymmetric fashion, thereby indicating that in mouse, SSC fate does not rely on asymmetric partitioning of m-Numb.

Materials and Methods

Animals and germ cells preparation

Protocols for the use of animals in these experiments were approved by the Department of Health Animal Care and Use Committee. C57BL/6 mice were purchased from Charles River Italia (Lecco, Italy) and housed in a standard animal facility with free access to food and water, in accordance with the guidelines for animal care at the University of Rome, 'La Sapienza'. To obtain adult germ cell-depleted testis, mice at 5–6 weeks of age were treated with a single i.p. injection of busulfan (Sigma; 40 mg/kg) to destroy endogenous spermatogenesis. Animals were then killed 4–8 weeks after busulfan treatment. To obtain highly purified germ cell fractions, testes from 30-day-old mice were removed, decapsulated, and digested in minimum essential medium (MEM; Gibco, Invitrogen) containing 0.45 U/ml collagenase (Serva, Italy) and 0.08 mg/ml DNase (Gibco) for 5 min under shaking at room temperature. Seminiferous tubules were collected by sedimentation and a second digestion was performed by adding fresh enzymes to the seminiferous tubules for 45 min under shaking at 32 °C. At the end of digestion, cells were recovered and cell viability evaluated by means of the dye exclusion test (0.04% Trypan blue solution). Germ cell fractions were isolated by Staput sedimentation method as previously described (Boitani *et al.* 1980). Purity of cell fractions was routinely estimated by light microscopy. Pachytene spermatocytes and round spermatids fractions were of >85% average purity. A total of 45 mice were used in three different Staput preparations. To obtain spermatogonia-enriched germ cell fraction, epithelial cell adhesion molecule (Ep-CAM)-positive cells were selected from 8-day-old testis by magnetic microbeads selection (MACS, Miltenyi Biotec; Van Der Wee *et al.* 2001). To this end, single cell suspensions were obtained as previously described (Falciatori *et al.* 2004) and the selection was performed according to the manufacturer's protocol with minor modification. Briefly, seminiferous cord cell suspensions were incubated with an anti-Ep-CAM antibody (#G8.8, Developmental Studies Hybridoma Bank, Iowa, IA, USA) on ice for 20 min. Anti-rat immunomagnetic microbeads (MACS Miltenyi Biotec 130-048-502) were added and cells selected on a column placed in the magnetic field of a MACS separator. After removal of the column from the magnetic field, the magnetically retained cells were eluted as Ep-CAM-positive selected cells. In this cell fraction, spermatogonia represented more than 85%, as judged by morphological analysis and by GATA-1 immunocytochemistry, a marker for Sertoli cell. A total of 30 mice were used in three different cell preparations.

Northern blot analysis

Total RNA was isolated from tissues and germ cell fractions with TriReagent (Sigma) according to the manufacturer's instructions, resolved by formaldehyde agarose gel electrophoresis and transferred onto a nylon membrane. Blots (25 µg/lane) were hybridized overnight at 42 °C in buffer containing radiolabeled 474 bp m-Numb cDNA (RZPD, Berlin, Germany, clone ID: IMAGp998F198840Q3), 50% formamide, 2× SSC, 1% SDS, 10% dextran sulfate, and 10 mg/ml salmon sperm

DNA. Blots were washed twice for 10 min at room temperature in 2× SSC, 0.1% SDS, twice at 65 °C in 0.1% SSC, 0.1% SDS, and then exposed to film. Densitometric analysis was performed with AIDA 2.11 software (Raytest, Straubenhardt, Germany). Experiments were repeated collectively thrice, using different RNA preparations.

RT-PCR

One microgram of total RNA isolated from 8-day-old adult and germ cell-depleted adult testis was reverse transcribed using a SuperScript II Reverse transcriptase (Life Technologies). In control samples, reverse transcriptase was omitted to monitor genomic DNA contaminations. To identify the m-Numb isoforms expressed in the testis, we employed reverse transcriptase (RT)-PCR analysis using primers that could discriminate the transcripts encoding the four isoforms. Three set of primers were designed (A, B, and C). Set A (5′-ATG AGC AAG CAG TGT TGT CCT GG-3′ and 5′-ACA GCC ATG AAA CAA TGA CAG-3′); Set B (5′-GTT CTT CAA AGG CTT TGG-3′ and 5′-ACA GCC ATG AAA CAA TGA CAG-3′); and Set C (5′-CTT GTG TTC CCA GAT CAC CAG-3′ and 5-CCG CAC ACT CTT TGA CAC TTC-3′). Primers for S26 ribosomal gene were 5′-GGC AAT GTG CAG CCC ATT CG-3′ and 5′-GGC CTC TTT ACA TGG GCT TTG-3′. PCR was performed in a volume of 50 µl containing 1 µl cDNA, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl, 50 pmol of each amplification primer, 200 µM dNTPs, and 2 U Taq DNA polymerase (Roche). The reactions were performed for 45 s at 94 °C, 40 s at 53 °C and 1 min at 72 °C for 30 cycles. Aliquot of PCR products were separated by electrophoresis on a 1.8% agarose gel and stained with ethidium bromide. Experiments were performed thrice, using different RNA preparations.

Western blot analysis

Tissues and germ cell fractions were isolated and lysed in a buffer containing 50 mM Hepes, 2 mM EGTA, 1% Triton X-100, 120 mM NaCl, 12% glycerol, 10 mM β-glycerolphosphate, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol, and protease inhibitor cocktail 1× (Sigma). Protein concentration was determined by Bradford protein assay (Bio-Rad) using gamma globulins as standards. Thirty micrograms of cleared lysates were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech). Membranes were blocked overnight in Tris-buffered saline Tween-20 (TBST) containing 5% milk powder. Rabbit anti-m-Numb (Upstate, Prodotti Gianni, Milano, Italy #07-147, diluted in the ratio of 1:500) or rabbit anti-P38 (Santa Cruz,Tebu-Bio, Magenta, Italy #sc-535, diluted in the ratio of 1:100) were diluted in TBST/BSA and were incubated with blots, 90 min at room temperature. The first antibody incubation was carried out for 90 min at room temperature. An anti-rabbit-IgGs conjugated to horseradish peroxidase (Amersham Pharmacia, Biotech #NA934, diluted in the ratio of 1:3000) was used as secondary antibody Immunostained bands were detected by the ECL chemiluminescent method (Pierce; Celbio, Pero, Italy). These experiments were collectively repeated at least thrice.

Immunoprecipitation

Samples were homogenized for protein extraction in a buffer containing 20 mM Hepes (pH 7.5), 120 mM KCl, 0.1 mM ethyleneglycol-bis(ß-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10 mM ß-glycerophosphate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 2 mmol/l phenylmethyl sulfonyl fluoride. Extracts were centrifuged for 15 min at 12 000 ***g*** at 4 °C and the supernatants were collected for immunoprecipitation experiments. Five hundred micrograms of total proteins were incubated with 1 µg rabbit anti-m-Numb (Upstate, #07-147) or mouse anti-Ap2a1 (Affinity Bioreagents, Vinci-biochem, Vinci, Italy #MA1-064) antibodies for 2 h at 4 °C under constant shaking. As controls, in parallel tubes the first antibody was omitted. Immune complex were collected by adsorption onto protein A-Sepharose or protein G-Sepharose (Sigma-Aldrich). To remove non-specifically bound materials, the Sepharose beads were washed thrice with homogenization buffer. Proteins adsorbed to the antibody–beads complex were eluted in SDS sample buffer for western blot analysis. The experiment was repeated thrice.

Immunofluorescence staining

For whole mount immunofluorescence, mouse testes were recovered, tunica removed, and the seminiferous tubules were dissected using fine forceps. The dispersed tubules were fixed in 4% paraformaldehyde at 4 °C for 90 min. Tubules were washed in PBS buffer and incubated in 1 M glycine at room temperature for 30 min. Tubules were pre-incubated in PBS containing 5% pre-immune donkey serum, 1% BSA, 0.1% Triton X-100 at room temperature for 3 h under constant shaking. Tubules were then incubated with the following primary antibodies: goat anti-m-Numb (AbCam, Cambridge, UK #ab4147), rabbit anti-GFRα-1 (Santa Cruz, #sc-10716), and rabbit anti-phosphohistone H3 (Upstate) at 4 °C for 16 h under constant shaking. After washing, tubules were incubated with donkey anti-goat Cy-3 conjugated (Jackson Immuno research Laboratories, Newmarket, UK) and donkey anti-rabbit fluorescein iso-thiocyanate (FITC)-conjugated (Jackson Laboratories). Nuclei were stained with TOTO-3 (Molecular Probes T-3604, Invitrogen). Specimens were observed with a Leica laser scanning microscope TCS SP2 and images were acquired with Leica Confocal Software

(Leica, Milano, Italy). Immunofluorescence experiments were repeated more than four times using at least two different batches of anti-m-Numb and anti-GFRα-1 antibodies.

For double immunostaining on isolated cells, tubular germ cells were obtained as described previously and spun on slides. Cells were air-dried and fixed in 4% paraformaldehyde at 4 °C for 10 min. Slides were incubated in 1 M glycine at room temperature for 10 min and pre-incubated in PBS containing 5% pre-immune donkey serum, 1% BSA, 0.1% Triton X-100 for 1 h at room temperature. Double staining was performed using goat anti-m-Numb (AbCam, #ab4147) and mouse anti-Ap2a1 (Affinity Bioreagents, #MA1-064) antibodies at room temperature for 1 h. After washing, slides were incubated with donkey anti-goat 488-conjugated antibody (Molecular Probes) and with donkey anti-mouse Cy-3 conjugated antibody (Jackson Laboratories) at room temperature for 1 h. Nuclei were stained with TOTO-3 (Molecular Probes T-3604). Specimens were observed with a Leica laser scanning microscope TCS SP2 and images were acquired with Leica Confocal Software (Leica, Germany).

Results

m-Numb mRNA expression during postnatal testis development and in germ cells

m-Numb mRNA expression pattern was investigated by northern blot analysis of total RNA from testis at different postnatal ages. In parallel, lung and brain total RNA were used as positive controls. A hybridizing band of about 4 kb, corresponding to the only transcript expressed in lung and brain, was readily detected at all ages analyzed. In addition, a larger transcript of about 7 kb was identified in all testis samples (Fig. 1A). Both transcripts showed a peak of expression in 30-day-old testis, as shown by densitometry analysis (Fig. 1B). To further analyze testicular m-Numb expression, total RNA was obtained from purified germ cell populations (Fig. 1C). Purified germ cells also expressed both transcripts, and overall m-Numb expression was higher in pachytene spermatocytes than in round spermatids. These data showed that m-Numb is expressed at defined steps of germ cell development.

Analysis of m-Numb splice variants expressed in testis

An *in silico* analysis of the *m-numb* gene was performed to obtain the organization of the coding exons (GenBank no. NC_000078). The different isoforms are generated by the alternative splicing of exon 3 and/or exon 9 as represented in Fig. 2A. The inclusion of exon 9 generates p71 and p72 (PRR^{long} isoforms) whereas its exclusion generates p65 and p66 (PRR^{short} isoforms); the inclusion or exclusion of exon 3 generates p72 and p66 (PTB^{long} isoforms), or p71 and p65 (PTB^{short} isoforms) respectively. Total RNA isolated from immature, adult and germ cell-depleted adult testis were subjected to reverse transcription followed by conventional PCR using Sets A, B, and C primers and ribosomal protein 26 (RBS26) primer set as loading control (Fig. 2B). Using the Set A primers two PCR products, corresponding to the absence or the presence of exon 3, were amplified in all the samples, even though the relative intensity of the two bands varied among samples. In adult testis, transcripts

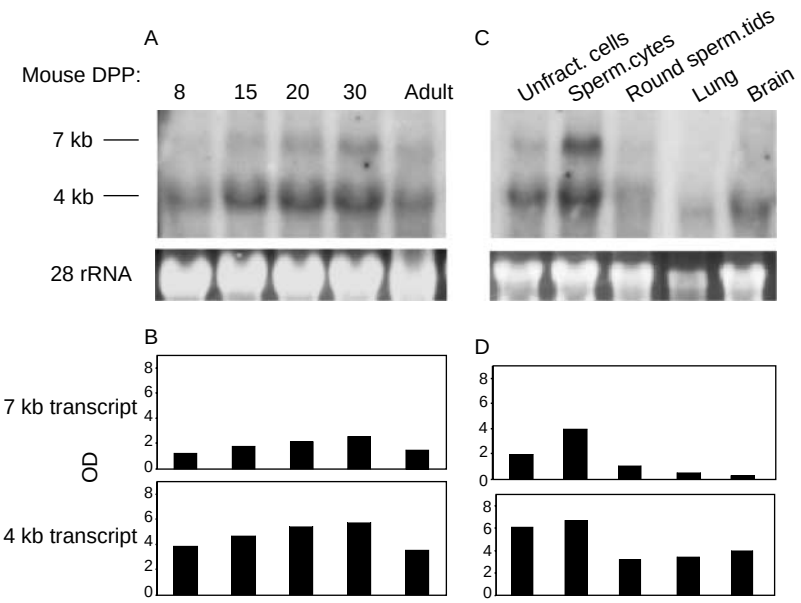

Figure 1 Northern blot analysis of m-Numb expression during postnatal testis development and in purified germ cell fractions. A representative experiment of three is shown. Total RNA was isolated from testis at the indicated *post partum* days (DPP) (A) or from purified germ cell fractions, lung and brain (C). Ethidium bromide staining of 28S rRNA served as RNA loading control. Note the 7 kb transcript present in all testis samples but not in lung and brain. Densitometric quantification of the blots from A (B) and C (D). OD values were obtained by scanning densitometry and data were normalized against 28S rRNA density values. DPP, day *post partum*.

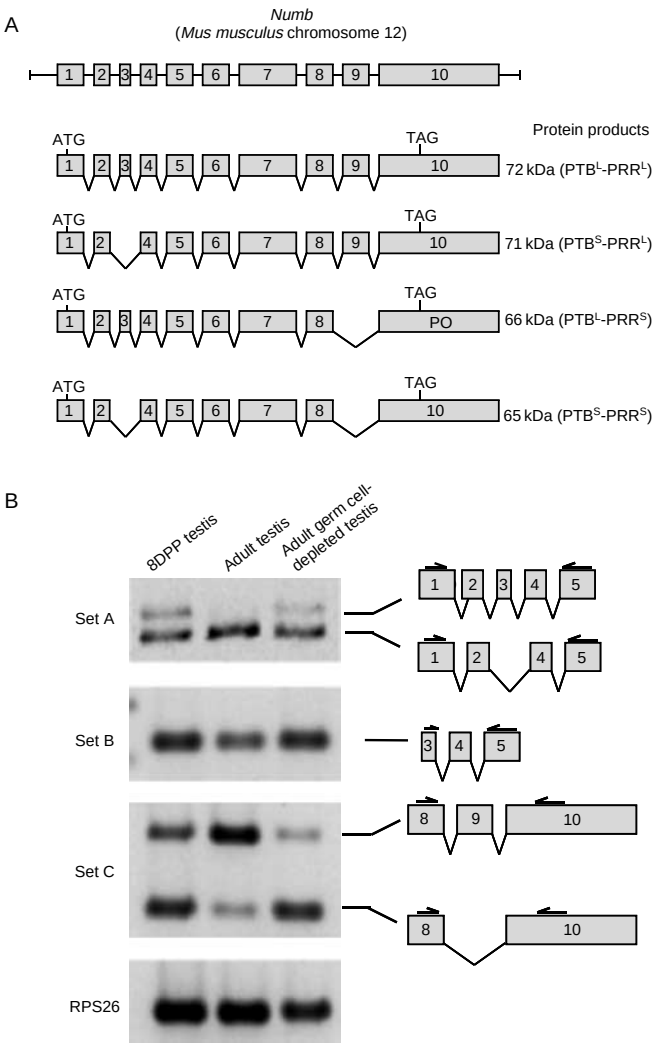

Figure 2 Identification of m-Numb splice variants expressed in testis by semi-quantitative RT-PCR. A representative experiment is shown. (A) schematic drawing of *m-numb* coding exons. At least four mRNAs are generated by the alternative splicing of exons 3 and 9. Corresponding protein variants are depicted on the right of each transcript. (B) Splice variants detection in RNAs isolated from indicated samples. Set A primers anneal to exons 1 and 5 resulting in variably migrating amplicons corresponding to the presence or the absence of exon 3. Set B includes one primer annealing directly to exon 3. Amplicon is only detected in exon 3 containing mRNAs. Set C primers anneal to exons flanking exon 9, resulting in variably migrating amplicons corresponding to the presence or the absence of exon 9. Note that in adult testis, transcripts-bearing exon 9 (set C, lane 2, upper band) were predominant and strongly reduced in germ cell-depleted adult testis (set C, lane 3). Ribosomal protein 26 (RBS26) primer set were used as loading control. DPP, day *post partum*.

without exon 3 were predominant. Amplification with Set B primers which anneal to exon 3, was in agreement with the results of using Set A primers. Finally using the Set C primers, two amplicons were identified, the slower migrating band including exon 9 and the faster migrating band excluding exon 9. In immature testis, the abundance of these two transcripts were relatively equal, whereas in the adult testis, the transcript including exon 9 was more predominantly expressed with respect to the transcript lacking exon 9. Since germ cell content is dramatically increased in adult testis, this result suggests that germ cells specifically expressed transcript for the PRR^{long} isoforms. The almost complete disappearance of the transcript including exon 9 in the germ cell-depleted testis further confirms this hypothesis. Since in adult testis, transcripts lacking exon 3 were more abundant than those bearing exon 3, this suggests that germ cells express the p71 isoform rather than p72 isoform. The ribosomal protein 26 controls demonstrated that similar amounts of total RNA from the various samples were subjected to amplification (Fig. 2B).

p71 m-Numb is expressed in germ cells

To determine the expression of the m-Numb isoforms at protein level, we analyzed testis cell extracts by western blot using a commercial antibody that recognizes all four mouse m-Numb isoforms. In a first experiment, we compared m-Numb isoforms expression pattern in different adult mouse tissues. To this end, testis extracts were analyzed in parallel to cell extracts from lung, liver, spleen, and muscle (Fig. 3A). As expected and in line with previous results in adult testis, a doublet was readily detected suggesting that in our experimental conditions, the p65/p66 as well as the p71/p72 isoforms were co-migrating (Dho *et al.* 1999). In lung, liver, and spleen only one band was identified with the same electrophoretic mobility of the lower band of the testis doublet (Fig. 3A). Next, we analyzed cell extracts isolated from testis at different postnatal ages. The results demonstrated the presence of two bands at all ages analyzed (Fig. 3B). While the intensity of the lower band did not vary among the analyzed ages, the upper band intensity paralleled the increase in testis germ cell content during this period. Strikingly in the germ cell-depleted testis only the lower band was present (Fig. 2B), further indicating that the PRR^{short} proteins (p65/p66) are the predominant isoforms expressed in the testis somatic cell compartment. Next, we analyzed cell extracts from highly purified germ cell fractions (Fig. 3C). Pachytene spermatocytes and round spermatids were isolated from adult testis while spermatogonia were obtained from 8-day *post partum* (8 DPP) testis. Germ cell fractions predominantly expressed the upper band (Fig. 3C). These results along with the RT-PCR analysis (see Fig. 2C) suggest that p71 is the probable PRR^{long} isoform expressed in germ cells.

Subcellular distribution of m-Numb in germ cells

Immunofluorescence analysis of whole mount seminiferous tubules was performed to gain more insight into the subcellular localization of m-Numb in germ cells. By laser scanning confocal microscopy, we obtained stacks

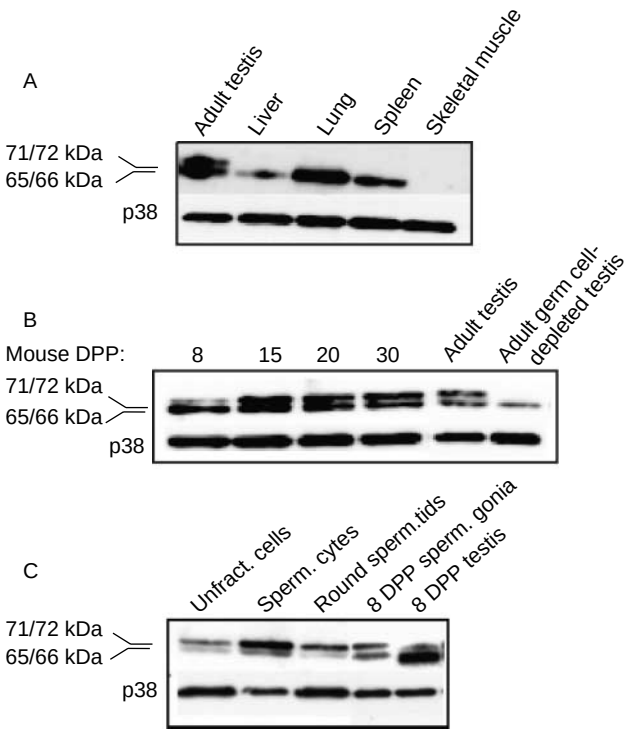

Figure 3 Western blot analysis for m-Numb expression in adult mouse tissues (A), in testis at different ages (B) and in isolated germ cell fractions (C). Thirty micrograms of proteins for each sample were subjected to SDS-PAGE, western blotted, and probed with anti-m-Numb antibody that recognize all m-Numb isoforms. Note that the p65/p66 as well as the p71/p72 isoforms were co-migrating. Germ cells predominantly expressed PRR^{long} isoform (p71) while germ cell-depleted testis expressed PRR^{short} proteins (p65/p66). P38 was used as a loading control. DPP, day *post partum*.

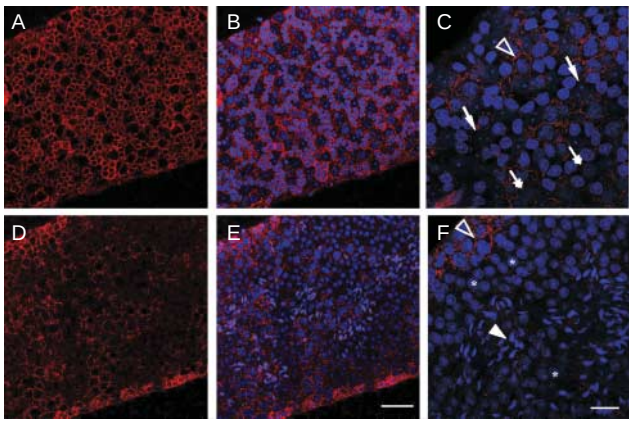

Figure 4 Expression pattern of m-Numb in adult seminiferous tubules. Tubules were stained with anti-m-Numb antibody (red color) and TOTO-3 nuclear staining (blue color-coded). Pictures are serial confocal optical slices of a representative tubule at low (A, B, D, and E) and high magnification (C and F). (A–C) basal layer of the tubule and (D–F) suprabasal layer of the tubule. Merge pictures are shown in B, C and E, F. m-Numb immunofluorescence signal was greater in primary spermatocytes (open arrowhead). Arrow: Sertoli cells; double arrow spermatogonia; open arrowhead: primary spermatocytes; asterisk: round spermatids; close arrowhead: elongating spermatids. Scale bar = 40 μm (A, B, D, and E) and 20 μm (C and F).

of longitudinal *z*-sections representing different layers of the tubules. Hence, m-Numb expression was followed in germ cell belonging to the same stage of spermatogenesis. Analysis of several seminiferous tubules indicated that m-Numb expression was not stage-specific (Fig. 4). However, by comparing staining intensity in spermatogonia and primary spermatocytes in each stage analyzed, m-Numb was clearly upregulated in the latter (Fig. 4C and F: double arrow and open arrowhead respectively). At the end of meiosis, m-Numb was downregulated in round and in elongated spermatids (Fig. 4F: asterisk and close arrowhead respectively). These data are in line with those obtained by western blot analysis, thereby validating the antibody employed for immunofluorescence analysis.

To analyze m-Numb subcellular localization in early steps of germ cells differentiation, an anti-GFRα-1 antibody was used to identify type-As and type-Apr spermatogonia (von Schonfeldt *et al.* 2004, Hofmann *et al.* 2005) in double-staining experiments (Fig. 5A–D). GFRα-1 is a glycosyl-phosphatidyl inositol-linked receptor that along with the tyrosine kinase receptor Ret binds to GDNF. In 8-day-old testis, GFRα-1 antibody clearly labeled single and paired spermatogonia and staining was localized on the cell surface and on intercellular bridges of paired cells (Fig. 5B and D). GFRα-1-positive cells were positive for m-Numb expression; in these cells, a punctuate staining located below the plasma membrane was evident. The same pattern and intensity of m-Numb staining were displayed by neighboring GFRα-1-negative spermatogonia that were recognized by nuclear morphology (Fig. 5B and D).

Localization of cell fate determinants during cell division to form apical or basal crescents of protein shows cell-cycle dependence and correlates with progression through mitosis (Prokopenko & Chia 2005). To get insight on m-Numb subcellular distribution during spermatogonial stem cell division, we performed double immunofluorescence staining on whole mount seminiferous tubules using two antibodies recognizing m-Numb and phosphohistone H3 (PH3) respectively. Phosphorylation of histone H3 is low in interphase cells and occurs almost exclusively during mitosis, becoming maximal during metaphase, thus it can be used to identify mitotic cells (Hendzel *et al.* 1997). These experiments were performed on adult testis to allow unequivocal identification of isolated mitotic SSC, since in the adult, spermatogonia are the only mitotic cell type localized at the basal layer of tubules. As expected by PH3 staining, we identified labeled spermatogonia clones of variable cell size (data not shown) as well as single-labeled cells localized at the basal layer of tubules (Fig. 5E–L). A cell was designated type-As and therefore as SSC when no other cells, labeled or with the same nuclear morphology, were present within 25 μm

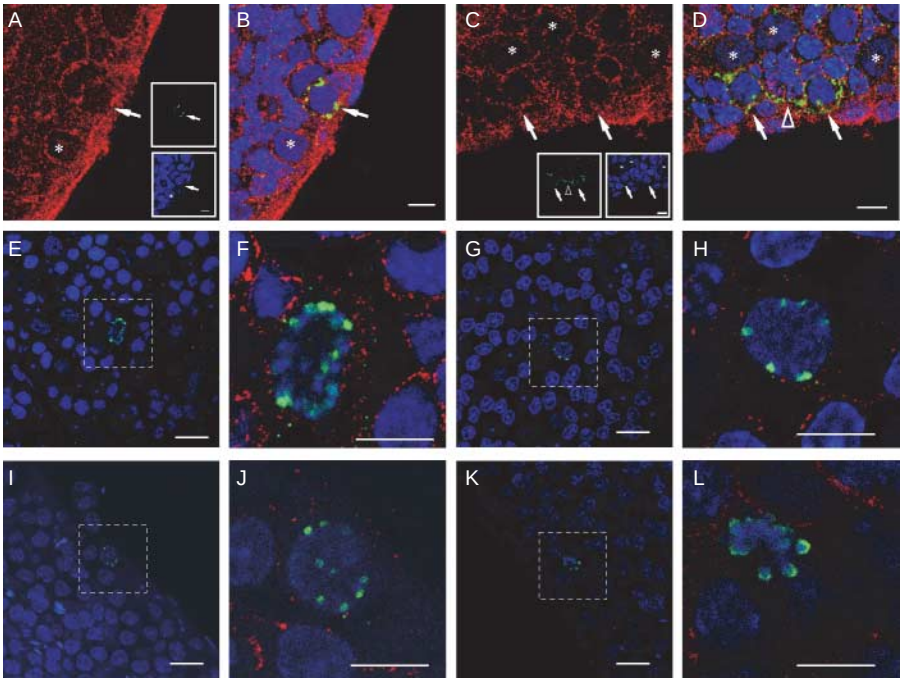

Figure 5 Localization of m-Numb in As and Apr GFRα-1-positive spermatogonia (A–D) and mitotic As spermatogonia (E–L). (A–D) Seminiferous cord isolated from 8-day-old testis were double stained with anti-m-Numb antibody (red), anti-GFRα-1 antibody (green; inset in A and C) and TOTO-3 nuclear staining (blue color-coded; inset in A and C). Merge of the pictures are shown in B and D. Both GFRα-1-positive (arrow) and negative (asterisk) spermatogonia expressed comparable amount of m-Numb. (E–L) Whole mount seminiferous tubules from adult testis were stained with anti-phosphohistone H3 (PH3) antibody to identify mitotic cells (green color), anti-m-Numb antibody (red color), and TOTO-3 for nuclear staining (blue color). Digital magnification of boxed regions in E, G, I, and K are shown in F, H, J, and L respectively. Mitotic As spermatogonia showed symmetric subcellular distribution of m-Numb. Asterisk: GFRα-1-negative spermatogonia; arrow: GFRα-1-positive spermatogonia; open arrowhead: intracellular bridges stained for GFRα-1. Scale bar = 8 μm (A and B), 8 μm (C and D), 20 μm (E, G, I, and K), and 10 μm (F, H, J, and L).

(Huckins 1971, van Keulen & de Rooij 1975). A total of 20 mitotic SSC from prophase to telophase were examined. All mitotic SCC showed a uniform m-Numb staining throughout the cytoplasm and an asymmetric m-Numb distribution (i.e. crescent-like) was never observed (Fig. 5E–L).

m-Numb and Ap2a1 interact in germ cells

Next, we examined whether an *in vivo* association between m-Numb and Ap2a1 exist in germ cells. First, m-Numb and Ap2a1 distribution were analyzed by means of double immunostaining on total germ cells obtained by enzymatic digestion of adult testis. Germ cell nuclei were stained with the fluorescent nuclear dye TOTO-3, to allow germ cell type identification. We found that Ap2a1 was widely expressed in different germ cells types where it showed a punctuate distribution just under the plasma membrane (Fig. 6A). Only a fraction of m-Numb co-localized with Ap2a1 in each cell type analyzed (i.e. spermatocytes, Fig. 6A c and f; round spermatids, Fig. 6A f; elongated spermatids, Fig. 6A i). To further investigate this interaction, cell extracts were generated from 8-day-old and adult testis and immunoprecipitated using an anti-Ap2a1 antibody or an anti-m-Numb antibody (Fig. 6B and C). At both ages analyzed, the proteins co-immunoprecipitated by both antibodies contained m-Numb as shown by immunoblotting with the anti-m-Numb antibody (Fig. 6B and C). These data demonstrate an association between m-Numb and Ap2a1 *in vivo.*

Discussion

In this study, we have analyzed m-Numb expression during murine testis postnatal development and our data collectively demonstrate that m-Numb is widely expressed in somatic cells as well as in germ cells. In mammals, four m-Numb isoforms are expressed and several lines of evidence suggest that m-Numb isoforms may have distinct functions. p65/p66 m-Numb isoforms are widely distributed in embryonic and adult tissues whereas the expression of p71/p72 isoforms is more restricted to few tissues and notably in several cell lines (Dho *et al.* 1999, Verdi *et al.* 1999). In P19 embryonic carcinoma cells, overexpression of p71/p72 isoforms (but not p65/p66 isoforms), concomitantly to retinoic acid treatment, increases BrdU incorporation compared with mock-transfected cells (Verdi *et al.* 1999). Finally, p71/p72 isoforms are predominantly expressed early during retinal development when the progenitor

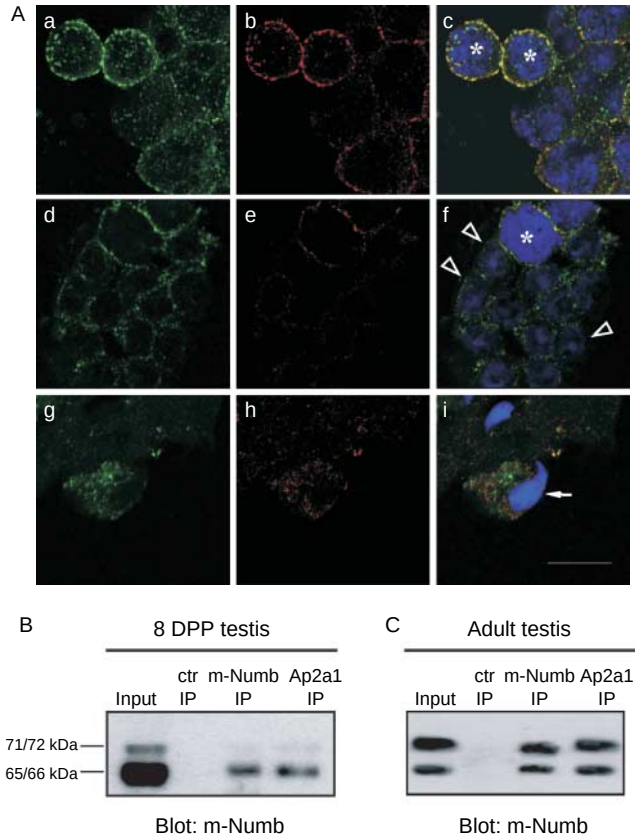

Figure 6 Physical association of m-Numb and Ap2a1 in germ cells. (A) Immunocytochemistry performed on cytospinned germ cells isolated from adult testis. Cells were double labeled with anti-Ap2a1 antibody (red color), anti-m-Numb antibody (green color), TOTO-3 nuclear staining (blue color-coded), and analyzed by confocal microscopy. Merge of the pictures are shown in c, f, and i. (B and C) Co-immunoprecipitation of m-Numb and Ap2a1 from 8 DPP (B), and adult testis (C). Lysates (500 μg) were incubated with no antibody (ctr IP), anti-m-Numb antibody (Numb IP), or anti-Ap2a1 antibody (AP2 IP). Total lysates (input: 50 μg) and immunoprecipitated proteins were subjected to SDS-PAGE, western blotted, and probed with anti-m-Numb antibody. These experiments indicate that m-Numb and Ap2a1 interact *in vivo*, both in immature and in adult testis. Asterisk, primary spermatocytes; open arrowhead, round spermatids; arrow, elongating spermatids. Scale bar = 20 μm.

population is rapidly expanding and downregulated at the time of cell differentiation (Dooley *et al.* 2003). These data have led to the hypothesis that p71/p72 isoforms (PRR^{long}) promote cell proliferation rather than cell differentiation (Dho *et al.* 1999, Verdi *et al.* 1999, Dooley *et al.* 2003). However, it is not clear yet, if the insert into the PRR domain of p71/p72 impairs Numb ability to direct cell fate choice in *Drosophila* (Verdi *et al.* 1999, Petersen *et al.* 2006, Toriya *et al.* 2006). Here, we show that among the m-Numb variants, germ cells predominantly express the p71 isoform. p71 and p72 differ in a small insert in the PTB domain (PTBi) and antibodies used in this study are not able to discriminate among them. However, semi-quantitative RT-PCR experiments suggest that p71 is predominant in germ cells, since in adult mouse testis the transcripts lacking sequences for the PTBi are more abundant than transcripts bearing the PTBi sequences. Our experiments performed on highly purified germ cell fractions, demonstrate that p71 m-Numb is expressed in the mitotic phase of spermatogenesis, is upregulated at the time of meiotic entry and downregulated during haploid germ cell differentiation (i.e. spermiogenesis). This pattern of expression suggests that in germ cells, m-Numb PPR^{long} isoforms may play additional roles besides promoting germ cell proliferation. Given the restricted expression pattern of p71 in mammalian tissues, male germ cells may represent an experimental model to exploit p71-specific cellular functions.

Numb can physically interact with Notch and inhibits its signal transduction (Frise *et al.* 1996, Guo *et al.* 1996, Spana & Doe 1996). Notch is a cell-surface receptor evolutionally conserved from invertebrates to higher vertebrates. The Notch family encodes large transmembrane receptors that interact with membrane-bound ligands encoded by the Delta/Serrate/Jagged family of genes. Upon ligand binding, Notch receptors undergo proteolytic cleavage leading to release of the Notch intracellular domain (NICD). NICD is then translocated into the nucleus where it interacts with the CBF1-SuHLag1 (CSL) family of transcription factors and activates the transcription of genes that regulate the ability of cells to respond to various proliferation, differentiation, or apoptotic cues (Artavanis-Tsakonas *et al.* 1999, Mumm & Kopan 2000, Schweisguth 2004, Radtke & Clevers 2005). The antagonism between Notch and Numb is active not only during asymmetric cell division but also in interphase cells (Petersen *et al.* 2006). Notably, m-Numb can act as an oncosuppressor in a subset of human breast cancers. Loss of Numb expression, due to its increased ubiquitination, leads to activation of Notch, which in turn is responsible for increased proliferation of tumor cells (Pece *et al.* 2004). It has been suggested that Numb could exert its inhibitory function on Notch signaling by removing the full-length and/or cleaved Notch receptor from the cell surface through endocytosis (Santolini *et al.* 2000, Berdnik *et al.* 2002, Smith *et al.* 2004). This proposal is supported by the evidence that m-Numb associates to the endocytic protein Ap2a1 in clathrin vesicles (Santolini *et al.* 2000). Additionally, in *Drosophila*, alpha-adaptin mutations mimic the loss-of-function phenotype of *numb* in sensory organ development (Berdnik *et al.* 2002). Our data demonstrate that in germ cells m-Numb interacts with the endocytic protein Ap2a1. Therefore, we suggest that one of the m-Numb functions in germ cells may be the control of Notch pathway activation. Components of the Notch pathway are widely expressed in germ cells as well as in Sertoli cells. In mouse testis, spermatogonia express all Notch receptors (Notch 1, 2, 3, and 4) and the Notch ligand

delta-1 whereas Sertoli cells express Notch 2 and the Notch-ligand Jagged-1 (Dirami *et al.* 2001, Mori *et al.* 2003, von Schonfeldt *et al.* 2004). In rat and human testis, Notch 1 and the Notch-ligand Jagged-2 are co-expressed in spermatocytes, round, and elongated spermatids (Hayashi *et al.* 2001, 2004). Taken together, these data suggest that m-Numb regulation of Notch nuclear translocation could be relevant during germ cell proliferation and differentiation.

The classic histological analysis of whole mount isolated seminiferous tubules has represented the elective methodological approach in most of the pioneering studies on spermatogonial stem cell renewal and differentiation. However, in these studies, spermatogonial cell identification had solely relied on morphological ground (i.e. nuclear morphology, stage of spermatogenesis, topographical arrangement of spermatogonia chains, etc.; Clermont & Bustos-Obregon 1968, Huckins & Kopriwa 1969, Huckins 1971, de Rooij 1973, van Keulen & de Rooij 1973). In this study, in order to gain insight in m-Numb subcellular localization in stem cells, we took advantage of this powerful methodological approach and extended it by confocal microscopic analysis of immunostained seminiferous tubules. Interphase SSC were identified as single isolated spermatogonia cells positive for GFRα-1 staining while mitotic SSC were identified as single isolated PH3-positive cells at the basal layer of adult seminiferous tubules. In our experiments, we never found asymmetric distribution (i.e. crescent-like) of m-Numb, both in interphase and in mitotic SSC, suggesting that spermatogonial stem cell fate does not rely on asymmetric partitioning of m-Numb. However, other explanations could account for observed lack of m-Numb asymmetric distribution in SSC. In this study, 20 mitotic SSC were analyzed in three independent experiments, hence if present, asymmetric distribution of m-Numb would occur in $<5\%$ (<1 out of 20) mitotic SSC. Taking into account the fact that the estimated number of SSC in adult mouse is 35 000 per testes (Tegelenbosch & de Rooij 1993) and the mitotic index in rat SSC is lower than 3.6% (reviewed by Meistrich & van Beek 1993), asymmetric m-Numb distribution in $<5\%$ mitotic SSC (which is $35\,000 \times 0.036 \times 0.05 = 63$ mitotic SSC per testis) could be rare and difficult to detect. Alternatively, our inability to observe asymmetric distribution is due to detection of all m-Numb isoforms by the antibodies used in the present study. Since western blot analysis indicated that spermatogonia cells express at least two out of the four isoforms, any isoform-specific asymmetric distribution could be masked by other symmetrically distributed isoforms. It is generally assumed that in rodents, SSC undergo symmetric division either producing two new stem cells or two interconnected cells, generated by incomplete cell cytokinesis, destined to differentiate. However, the possibility exists that SSC divide asymmetrically to generate one daughter SSC and one daughter cell destined to produce Apr spermatogonia. Such division would generate two single cells, morphologically alike, but with different molecular endowment (Meistrich & van Beek 1993, Watt & Hogan 2000, de Rooij 2001). Recently, several protocols have been developed to maintain and expand SSC *in vitro* (Nagano *et al.* 1998, 2003, Kanatsu-Shinohara *et al.* 2003, 2005, Hamra *et al.* 2004, Kubota *et al.* 2004). The possibility to study SSC division *in vitro*, under defined culture conditions, may shed light on the molecular control of SSC self-renewal and differentiation.

Our data indicate that in each seminiferous tubule analyzed, type-As and type-Apr spermatogonia (i.e. GFRα-1-positive) as well as more advanced spermatogonia (i.e. GFRα-1-negative) express comparable amount of m-Numb protein. It has recently been reported that in isolated GFRα-1-positive spermatogonia cells, the expression of m-Numb and SIAH-1 (an E3-ubiqutin ligase) are upregulated by GDNF *in vitro* (Hofmann *et al.* 2005). The induction of m-Numb transcription is paralleled by an increase in m-Numb protein content and the downregulation of Notch 1 receptor activation (Braydich-Stolle *et al.* 2005). Our data suggest that, if the induction of m-Numb expression by GDNF holds true *in vivo,* it does not translate in protein accumulation neither in target cells (single or paired type-A spermatogonia) nor in more differentiated spermatogonia. Since it has been shown that SIAH-1 binds and promotes degradation of m-Numb, it is conceivable that the concomitant upregulation of SIAH-1 by GDNF *in vivo,* may control m-Numb at posttranslational level by targeting it for ubiquitin–proteasome degradation (Susini *et al.* 2001). In this line of reasoning, it would be interesting to determine the molecular mechanisms underlining m-Numb upregulation in meiotic germ cells. In conclusion, the expression analysis of m-Numb in germ cells represents a first step to elucidate its physiological roles during spermatogenesis. Genetic and biochemical studies are warrant to clarify m-Numb functions in male germ cells.

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