Dynamic paraspeckle component localisation during spermatogenesis

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Abstract

Expression profiles and subcellular localisations of core *Drosophila* behaviour/human splicing (DBHS) proteins (PSPC1, SFPQ and NONO) and NEAT1, a long noncoding RNA (lncRNA), are investigated in developing and adult mouse testes. Core DBHS proteins are markers for the distinct subnuclear domain termed paraspeckles, while a long NEAT1 isoform scaffold facilitates paraspeckle nucleation. Paraspeckles contain many proteins (>40) and are broadly involved in RNA metabolism, including transcriptional regulation by protein sequestration, nuclear retention of A-to-I edited RNA transcripts to regulate translation and promoting survival during cellular stress. Immunohistochemistry reveals cell-specific profiles for core DBHS paraspeckle protein expression, indicating their functional diversity. PSPC1 is an androgen receptor co-activator, and it is detected in differentiating Sertoli cell nuclei from day 15 onwards, as they develop androgen responsiveness. PSPC1 is nuclear in the most mature male germ cell type present at each age, from foetal to adult life. In adult mouse testes, PSPC1 and SFPQ are present in Sertoli cells, spermatocytes and round spermatids, while the NEAT1 lncRNA appears in the punctate nuclear foci delineating paraspeckles only within Leydig cells. Identification of NEAT1 in the cytoplasm of spermatogonia and spermatocytes must reflect non-paraspeckle-related functions. NONO was absent from germ cells but nuclear in Sertoli cells. Reciprocal nuclear profiles of PSPC1 and γ -H2AX in spermatogenic cells suggest that each performs developmentally regulated roles in stress responses. These findings demonstrate paraspeckles and paraspeckle-related proteins contribute to diverse functions during testis development and spermatogenesis.

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Introduction

The nucleus contains many distinct subnuclear domains, such as the nucleolus, Cajal bodies, PML bodies and nuclear speckles, each of which performs functions without lipid bilayer membrane enclosure. One of the most recently discovered subnuclear domains is the paraspeckle (Fox *et al.* 2002). We previously demonstrated that regulated expression of certain nuclear transport machinery allows stage-specific protein access to the nucleus during spermatogenesis (reviewed in Major *et al.* 2011, Miyamoto *et al.* 2012, Loveland *et al.* 2015). A core component of the paraspeckle, paraspeckle protein 1 (PSPC1), is a developmentally regulated cargo of the nucleocytoplasmic transporter, importin α 2 (IMP α 2), in the foetal mouse testis (Major *et al.* 2015, 2017). Here we examine the expression profiles of PSPC1 and the other core-associated paraspeckle components throughout mouse spermatogenesis, to determine

whether these are also developmentally regulated and to explore the hypothesis that their coordinated synthesis would be required for paraspeckle functions.

Paraspeckles are a distinct subnuclear domain rich in RNA-binding proteins and RNA transcripts, particularly those which are A-to-I edited, coalescing to form what West *et al.* (2016) described as a ‘characteristic core-shell spheroidal’ structure when analysed using super-resolution microscopy. These are built upon the lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1), formerly known as nuclear-enriched abundant transcript 1. In addition to the three core *Drosophila* behaviour, human splicing (DBHS) paraspeckle proteins, PSPC1, SFPQ and NONO (Fox *et al.* 2002), over 60 other protein components are identified as potentially localising to paraspeckles (Naganuma *et al.* 2012, Fong *et al.* 2013). DBHS proteins have other nuclear functions outside of paraspeckles relating to RNA production and processing,

including transcriptional initiation/termination, splicing, polyadenylation and mRNA transport (Patton *et al.* 1993, Gozani *et al.* 1994, Peng *et al.* 2002, Shav-Tal & Zipori 2002, Kameoka *et al.* 2004, Kanai *et al.* 2004, Rosonina *et al.* 2005, Liang & Lutz 2006, Kaneko *et al.* 2007, Ito *et al.* 2008, Izumi *et al.* 2014, Lu & Sewer 2015). Although their roles are central to cellular functions, their contribution to spermatogenesis remains to be determined.

The lncRNA NEAT1 is a crucial paraspeckle component, serving as the nucleation scaffold which allows co-assembly of other paraspeckle components (Bond & Fox 2009, Chen & Carmichael 2009, Clemson *et al.* 2009, Sasaki *et al.* 2009, Sunwoo *et al.* 2009). The NEAT1 transcript has two isoforms that are encoded in the genome without introns; the shorter variant is termed NEAT1_1/MEN α (3.2 kb (mouse); 3.7 kb (human)), and a longer variant is NEAT1_2/MEN β (20.8 kb (mouse); 22.7 kb (human)) (Sunwoo *et al.* 2009, Naganuma & Hirose 2013). Unlike many noncoding RNAs, NEAT1_1 is highly conserved in mammals (Sunwoo *et al.* 2009). The 3' end of NEAT1_1 contains a poly(A) tail. In contrast, the NEAT1_2 transcript encodes a triple helix structure at its 3' end proposed to stabilise the transcript (Brown *et al.* 2012, Wilusz *et al.* 2012); it is initially synthesised with a tRNA-like small RNA sequence that is cleaved off by RNase P to yield the mature 3'-end (Sunwoo *et al.* 2009). Both NEAT1 transcripts can localise to paraspeckles (Souquere *et al.* 2010), but the longer NEAT1_2 transcript is essential for paraspeckle nucleation (Naganuma *et al.* 2012). The shorter NEAT1_1 transcript is also implicated in transcriptional regulation by enhancing active chromatin states of specific promoters (Chakravarty *et al.* 2014, Li *et al.* 2017).

PSPC1 is produced in two isoforms. The longer variant is highly abundant in the mammalian testis, associated with the nuclear matrix, and has proposed roles in chromatin remodelling during spermatogenesis (Myojin *et al.* 2004). PSPC1 can complex with SFPQ or NONO in an immortalised Sertoli cell line, TTE3; over-expression of each component enhanced androgen receptor-mediated translation, with PSPC1 eliciting the largest response (Kuwahara *et al.* 2006). In photobleaching experiments using HeLa cells, PSPC1 is predominantly localised to paraspeckles, but demonstrated to shuttle between paraspeckles and the nucleolus (Fox *et al.* 2002). When transcription is inhibited, PSPC1 relocates from paraspeckles to the nucleolar caps (Fox *et al.* 2002, 2005); this process also occurs naturally for a brief window during telophase, before transcription recommences (Fox *et al.* 2005). In cultured cells, PSPC1 has been implicated in the DNA damage response through regulation of the G1/S checkpoint, effectively halting the cell cycle to allow DNA repair; siRNA knockdown of PSPC1 results in G1/S checkpoint escape and increased cellular death rates (Gao *et al.* 2014). PSPC1 has also been implicated in the promotion of epithelial-to-mesenchymal transition (EMT), stemness

and metastasis through transforming growth factor β 1 (TGF- β 1) autocrine signalling and Smad2/3 target switching (Yeh *et al.* 2018).

Broad roles of SFPQ include splicing, transcription, translation, DNA repair, sister chromatid cohesion and apoptosis (see Yarosh *et al.* 2015 for review). NONO contributes to mammalian circadian rhythms (Brown *et al.* 2005) and links the circadian clock and cell cycles (Kowalska *et al.* 2013, Maier & Kramer 2013), while other DBHS proteins may serve redundant or overlapping roles at circadian-regulated genes as transcriptional cofactors (Kowalska *et al.* 2012). In addition, the DBHS proteins are each implicated in DNA double-strand break repair, a process central to spermatogenesis (Bladen *et al.* 2005, Li *et al.* 2009, 2014, Ha *et al.* 2011, Rajesh *et al.* 2011, Gao *et al.* 2014).

The functional significance of paraspeckles remains under investigation, with diverse functions now proposed and demonstrated. Paraspeckles can suppress translation by facilitating the nuclear retention of A-to-I edited RNA transcripts (Zhang & Carmichael 2001, Prasanth *et al.* 2005), regulate transcription by sequestering proteins (Hirose *et al.* 2014) and have been implicated in the first stages of miRNA biogenesis (Jiang *et al.* 2017, Krol 2017). Importantly, NEAT1 levels inversely correlate with sensitivity to stress-induced apoptosis in studies of mouse embryonic fibroblasts (Hirose *et al.* 2014) and is directly correlated with various types of cancer cells (in which it is elevated), including breast (Choudhry *et al.* 2015), cervical (Wang & Zhu 2018), colorectal (Li *et al.* 2015, Wu *et al.* 2015), glioma (He *et al.* 2016), leukaemia (Zeng *et al.* 2014, Blume *et al.* 2015), liver (Guo *et al.* 2015), lung (You *et al.* 2014, Zhao *et al.* 2014, Pan *et al.* 2015, Hu *et al.* 2016) and prostate (Chakravarty *et al.* 2014).

Despite the important roles that paraspeckles and their components play during normal development and in diseases such as cancer and amyotrophic lateral sclerosis (Nishimoto *et al.* 2013, Shelkownikova *et al.* 2014, 2018), characterisation of paraspeckle components during normal developmental processes has been limited. Given that core DBHS paraspeckle proteins are present in the adult testis (Kuwahara *et al.* 2006), with PSPC1 highly abundant (Myojin *et al.* 2004), the spatial-temporal expression patterns of these proteins and NEAT1 (Fig. 1) is interrogated in mouse testis throughout development in this study. This information sheds light on how these proteins and the formation of paraspeckles may influence spermatogenesis and the processes essential for male fertility.

Materials and methods

Animals and tissue sample preparation

Postnatal testes were obtained from Swiss and C57/BL6xCBA outbred male mice, while foetal testes and ovaries were

Figure 1 Core paraspeckle components in the mouse. (A) The core paraspeckle DBHS proteins (PSPC1, PSF and NONO) are shown with long and short variants, the conserved domains. The regions of these proteins targeted by antibodies used in this study are indicated (in red). (B) The two splice variants of NEAT1 long noncoding RNA are shown with the region used to generate a DIG-labelled riboprobe (ISH) highlighted in red, while the Stellaris probe (FISH) is a pool of fluorescently labelled probes along the length of the 5' region, shared by long (NEAT1_2) and short (NEAT1_1) transcripts (red dotted line).

collected from embryos staged by fore- and hindlimb morphology from time-mated pregnant Swiss female mice from Monash University. All investigations conformed to the NHMRC/CSIRO/AAC Code of Practice for the Care and Use of Animals for Experimental Purposes and were approved by the Monash University Standing Committee on Ethics in Animal Experimentation. Tissues for immunohistochemistry were fixed in Bouins fixative for 5 h, dehydrated through graded ethanols, paraffin embedded and sectioned (3–5 µm) using standard protocols (Loveland *et al.* 1999).

Immunohistochemistry

Immunohistochemistry with anti-PSPC1, anti-NONO and anti-SFPQ (Kuwahara *et al.* 2006) mouse monoclonal antibodies (mAbs) was performed as previously (Loveland *et al.* 1999) using 50 mM glycine for antigen retrieval (pH 3.5; >90°C for 8 min), and diluted primary antibodies (0.1% BSA/TBS) for O/N incubation at RT. Control sections lacked primary antibody. Secondary antibody incubations (1:500 in 0.1% BSA/TBS, 1 h), rabbit anti-mouse antibody (DAKO, E0354), were followed by incubation with Vectastain Elite ABC kit reagent (Vector Laboratories, distributed by Abacus Als, Waterford, Australia). Antibody binding was detected as a brown precipitate following development with 0.20 mg/mL 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Harris Haematoxylin counterstain (Sigma-Aldrich) and DPX Mountant (Sigma-Aldrich) were applied. Germ and somatic cell types were identified based on nuclear morphology and position within the developing gonad (Byskov 1986, Russell 1990).

In situ hybridisations

In situ hybridisations were performed with 300 ng of DIG-labelled NEAT1 riboprobe at 55°C as previously

(Meehan *et al.* 2000). Briefly, the 324 nt segment of mouse NEAT1 (accession numbers in Supplementary Table S1) was amplified using the primers (forward: 5'-CGCTGGGCGGACAGCTCTGG-3', reverse: 5'-ACCGGACGACCCAGGGGCAA-3'), ligated into the pGEMT Easy vector (Promega) and the DIG-labelled sense/anti-sense riboprobes generated using T7 and Sp6 RNA polymerases. After O/N hybridisation, probe binding was detected using an alkaline phosphatase-conjugated anti-DIG antibody and enzymatic conversion of the NBT/BCIP Substrate Solution (Thermo Scientific) to form an insoluble brown-purple precipitate. Sections were mounted under coverslips with GVA (Zymed-Invitrogen), and then slides were imaged using a Zeiss Axio Imager A1 fitted with a 20x objective and an AxioCam MRC5 camera (Carl Zeiss).

Northern blot

Northern blots using DIG-labelled riboprobes were performed as previously described (Hogarth *et al.* 2006). Briefly, total RNA from adult mouse testis, kidney and liver were extracted using TRIzol (Thermo Scientific) as per manufacturer's instructions and resuspended in deionised formamide. Sample concentrations were estimated with a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and 30 µg loaded per lane with a RiboRuler High Range RNA Ladder (Thermo Scientific) before electrophoretic separation through a formaldehyde agarose gel. Separated samples were transferred to Amersham Hybond XL membrane (GE Life Sciences). Membranes were blocked in Ambion ULTRAhyb (Thermo Scientific) at 68°C for 1 h, before applying DIG-labelled riboprobe (100 ng/mL, 68°C, O/N with rotary agitation). Membranes were then washed 0.1% SSC and 0.1% SDS at 68°C, and then in maleic acid buffer (0.1 M maleic acid with 0.15 M NaCl, pH 7.5, and 0.3% Tween). The bound probe was detected using an alkaline phosphatase-conjugated anti-DIG antibody (1:10,000, Roche Molecular

Biochemicals) to create a chemiluminescent signal which was captured on film after the addition of CDP-STAR substrate (Roche Molecular Biochemicals).

FISH/IF

FISH to detect NEAT1 transcript was performed with a Stellaris FISH Probe (Biosearch Technologies, Petaluma, USA) to mouse NEAT1 5' segment (labelled with Quasar 570 Dye, SMF-3009-1) as per the manufacturer's instructions on mouse testis tissue sections prepared as above or on GC-1 and GC-2 cells. GC-1 and GC-2 were grown in DMEM (supplemented with 10% (v/v) foetal calf serum, Penicillin-Streptomycin (Pen-Strep), L-Glutamine and MEM non-essential amino acids) with 5% CO₂ at 37°C on glass coverslips, and then fixed in 3.7% formaldehyde (10 min @ RT). All buffers were prepared using diethylpyrocarbonate (DEPC)-treated Milli-Q water (Merck Millipore). Cells on coverslips and tissue sections (post-rehydration) were permeabilised in 70% v/v ethanol for 1–2 h before performing FISH hybridisation for 16 h at 37°C. After washing and incubating with DAPI (4',6-diamidino-2-phenylindole, dilactate; Thermo Scientific), samples were mounted with GVA (Genemed Biotechnologies, South San Francisco, USA) or those samples undergoing subsequent immunofluorescence (for FISH/IF) were fixed with 4% paraformaldehyde (15 min @ RT). For the remaining immunofluorescence steps, PBS was used throughout. Samples were washed, blocked with 0.5% BSA and primary antibody added (mouse mAb to PSPC1; Kuwahara *et al.* 2006, 1:100 in 0.5% BSA, 1 h @ RT). Samples were washed, and secondary antibodies added (goat anti-mouse, Alexa Fluor 488, Molecular Probes – Thermo Scientific, 1:500, 0.5% BSA, 1 h @ RT). After final washes, samples were mounted with GVA (Genemed Biotechnologies) and imaged using a Leica SP5 laser scanning confocal system (DMI6000 microscope, motorised stage, 63× water/glycerol objective, Monash Micro Imaging Facility). Images were collected as Z-series and later merged to form 2D maximum intensity projections.

Co-immunofluorescence for PSPC1 and γ -H2AX was performed on rehydrated adult mouse tissue sections essentially using immunofluorescence and imaging steps described above, with minor modifications as follows. Sections were permeabilised in 0.2% Triton X-100 for 30 min prior to blocking. Primary antibodies (mouse mAb to PSPC1, 1:100, Kuwahara *et al.* 2006; rabbit anti- γ -H2AX, 1:100, AbCam, ab11174) were incubated O/N at RT and secondary antibodies (donkey anti-mouse Alexa Fluor 488, 1:500; goat anti-rabbit Alexa fluor 546, 1:500 (Molecular Probes – Thermo Scientific)) with DAPI (Thermo Scientific) were incubated for 90 min.

Western blotting

Samples were homogenised using a graded needle series in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Tween-20, 0.1% SDS, 1 mM EDTA, 50 mM Tris) with protease inhibitor cocktail (1:500, Set III, Calbiochem – Merck Millipore) at 4°C, and insoluble material was removed by centrifugation (20,000g, 20 min @ 4°C). Protein concentrations were estimated using DC Protein Quant Assay (BioRad) against a bovine serum albumin

(BSA) standard. Lysates (50 µg per lane) were mixed with 2× sample buffer, run on 12% SDS-PAGE gels with protein size standards (PageRuler Prestained Protein Ladder; Fermentas-Thermo Scientific), transferred to Amersham Hybond-C Extra nitrocellulose membrane (GE Life Sciences) and blocked in 5% skim milk/TBS (50 mM Tris, 150 mM NaCl, pH 7.5) for 1 h. Washes were with 0.1% Tween 20/TBS and incubations were RT unless otherwise stated. The membrane was rotated with an anti-PSPC1, anti-SFPQ or anti-NONO mouse mAb ((Kuwahara *et al.* 2006), 1:1000 in 5% skim milk/0.1% Tween-20/TBS, O/N, 4°C), washed, then rabbit anti-mouse IRDye800 secondary antibody (1:10000, Rockland Immunochemicals, 610431020, distributed by Jomar Life Research, Scoresby, Australia; in 5% skim milk/0.1% Tween-20/0.01% SDS/TBS) applied (1 h) and detected using the Odyssey Imaging System (LI-COR Biosciences, Lincoln, USA).

Results

PSPC1 is developmentally regulated in germ and Sertoli cells throughout testis development

Immunohistochemistry was performed to determine the cellular pattern of PSPC1 localisation in the foetal, juvenile and adult mouse testis, building on previous data (Myojin *et al.* 2004, Major *et al.* 2015). The foetal ovary was examined to determine if expression of PSPC1 in foetal germ cells was male specific or present in both sexes, as we had previously identified it in male germ cells (Major *et al.* 2015). Using the previously employed monoclonal antibody, PSPC1 protein was readily detectable in germ cell nuclei in both foetal testis and ovary at E12.5, E14.5 and E16.5 (Fig. 2), with no signal observed in any somatic cell or in the absence of primary antibody in either the ovary (Fig. 2A) or testis (Fig. 2B). In the newborn testis, at 0 days postpartum (dpp), PSPC1 was readily detectable in gonocyte nuclei, but absent from Sertoli cells (Fig. 3A). PSPC1 remained absent from Sertoli cells at 6dpp and 10dpp, but was observed in nuclei of spermatogonia as well as in the emerging spermatocyte population. PSPC1 was detected in nuclei of spermatogonia, spermatocytes and Sertoli cells at 15dpp. At 26dpp, PSPC1 protein was not detected in spermatogonia, but instead present in pachytene spermatocyte and round spermatid nuclei. The exclusion of PSPC1 from around each XY body in spermatocytes at 15 and 26dpp appeared as a white space around the blue chromatin body (Fig. 3A: day 15 'zoom'). In adult mouse testis, PSPC1 was detected in spermatocytes, round spermatids and Sertoli cells (Fig. 3B), confirming previous results (Myojin *et al.* 2004), but again excluded from spermatocyte XY body (Fig. 3B; left 'zoom'). At stage XII, when spermatocytes pass through the second meiotic division, PSPC1 was distributed throughout the cytoplasm (Fig. 3B; right 'zoom'). No cytoplasmic PSPC1 signal was detected in spermatids after the nucleus had reformed.

Figure 2 PSPC1 localises to the germ cell nucleus in foetal mouse gonads. PSPC1 immunohistochemistry on E12.5, E14.5 and E16.5 foetal ovary (A: ♀) and foetal testis (B: ♂). Control samples lacked primary antibody. Pink and blue arrowheads indicate oogonia in females and gonocytes in males, respectively; V-shape arrowheads point to metaphase cells. Scale bar equals 50µm.

Core DBHS paraspeckle components display distinct expression profiles throughout mouse testis development

The cellular profiles of the two other core DBHS paraspeckle markers, SFPQ and NONO, were also examined during mouse testis development using immunohistochemistry (Fig. 4). NONO was present in the nuclei of Sertoli cells and peritubular myoid cells at each age tested, but absent from germ cells (Fig. 4B). In contrast, SFPQ localised to spermatogonia at 5 dpp and 15 dpp, was absent from early meiotic germ cells, but detectable in pachytene spermatocytes (Fig. 4A). Nuclear SFPQ was observed in adult mouse testis spermatocytes and round spermatids, with no signal in spermatogonia. Similar to PSPC1, SFPQ appears excluded from the spermatocyte XY body in the adult. Sertoli cells were positive for SFPQ at each age examined. Both NONO and SFPQ proteins always localised to the cell nucleus and, unlike PSPC1, were also present within the nucleus of some Leydig cells, which reside in the interstitial space between the seminiferous tubules. No signal was detected in the absence of primary antibody on control sections (Fig. 4).

The lncRNA transcript NEAT1 appears within nuclear foci in adult testis Leydig cells and in immortalised germ cell lines

To ascertain if paraspeckles were present in the male germ cell subtypes in which both PSPC1 and SFPQ were detected, we next examined the long noncoding RNA NEAT1, which acts as a scaffold for paraspeckle assembly, using *in situ* hybridisation. A DIG-labelled

riboprobe designed to complement a 328nt region in the 5' end of NEAT1 (shared by NEAT1_1 and NEAT1_2 transcripts, see Fig. 1 and Supplementary Table 1, see section on supplementary data given at the end of this article) was used in an attempt to detect both short and long transcripts. Signal representing the lncRNA as an insoluble brown-purple precipitate was observed throughout the section (Fig. 5A), but was markedly strongest in the cytoplasm of spermatogonia and spermatocytes in adult testis. A Northern blot using this probe detected the shorter 3.2 kB NEAT1_1 isoform, but no discrete band was observed for the 20.8 kB NEAT1_2 transcript. The larger NEAT1_2 transcript may not be readily detectable by this probe or did not transfer to the membrane due to its larger mass. While this DIG-labelled ISH probe should pick up both short and long NEAT1 transcripts, our results suggest the DIG-labelled ISH approach was predominantly detecting the short NEAT1_1 transcript. The NEAT1_1 band detected in total testis RNA appears slightly larger than that detected in total RNA extracted from kidney or liver; however, this size offset was also observed with the 18S and 28S rRNA bands and therefore reflects a difference in the way the samples separated within the gel.

Because NEAT1 was not detected in punctuate nuclear foci as expected for paraspeckles, an additional fluorescent *in situ* hybridisation (FISH) for NEAT1 detection was performed. This employed a commercial FISH probe known to detect punctuate NEAT1 nuclear foci and targeted at the 5' segment of mouse NEAT1 that is shared by both the long and short transcripts. When hybridised with adult mouse testis sections, this probe yielded a NEAT1 signal in bright nuclear foci in Leydig cells. However, no signal above background

Figure 3 PSPC1 localises to the nucleus of germ and Sertoli cells in the postnatal mouse testis. Immunohistochemical localisation of PSPC1 in juvenile and in adult mouse testes. Control samples lacked primary antibody and are negative for signal. Yellow arrowheads: spermatogonia; green arrowheads: spermatocytes; dark blue arrowheads: round spermatids; red arrowheads: Sertoli cell nuclei; black asterisk: Sertoli cell cytoplasm; orange arrowheads: XY body; V-shape arrowhead: metaphase spermatocytes. (A) PSPC1 localisation during the first wave of spermatogenesis at ages indicated. (B) PSPC1 in the adult mouse testis. Scale bar equals 50 μ m.

levels appeared within either germ or Sertoli cells in the seminiferous tubules (Fig. 6A). In additional hybridisations examining an embryonic and postnatal testis age series, no other cell types exhibiting punctuate nuclear NEAT1 signal characteristic of paraspeckles were noted (data not shown). We also performed the same FISH method in tandem on GC-1 (Hofmann *et al.* 1992) and GC-2 (Hofmann *et al.* 1994, 1995) immortalised cell lines of germ cell origin. The presence of NEAT1 nuclear foci was confirmed within both GC-1 and GC-2 cells, suggesting both have paraspeckles (Fig. 6B). While both the ISH and FISH probes and their respective detection methods should pick up both NEAT1 transcripts our results suggest, that in our experiments, the DIG-labelled ISH approach was predominantly detecting the short NEAT1_1 transcript, while the FISH method was predominantly detecting the long NEAT1_2 transcript.

Western blots confirmed that all antibodies used to detect core DBHS paraspeckle proteins produced a single specific band of approximately the expected size

across lysates from adult mouse testis and GC-1/GC-2 cell lines (Fig. 6C). The detected band for NONO in the adult mouse testis is quite faint, in agreement with the observation that NONO is expressed within a smaller proportion of cells within the testis than PSPC1 and SFPQ (Sertoli cells only; Fig. 4). These blots demonstrate the specificity of the antibodies on adult mouse testis and validate the immunohistochemistry results (Figs 2, 3 and 4). These blots also show that in addition to NEAT1, both GC-1 and GC-2 cell lines express all three core DBHS paraspeckle proteins examined.

PSPC1 and γ -H2AX have reciprocal expression profiles in spermatogenic cells

PSPC1, SFPQ and NONO have all been implicated in DNA double-strand break repair (Bladen *et al.* 2005, Li *et al.* 2009, 2014, Ha *et al.* 2011, Rajesh *et al.* 2011, Gao *et al.* 2014, Yarosh *et al.* 2015). Mouse embryonic fibroblasts (MEFs) from NONO-deficient mice

Figure 4 SFPQ and NONO localisation differ in the mouse testis throughout development. Immunohistochemistry localisation using monoclonal antibodies for SFPQ and NONO; control (CTRL) samples lacked primary antibody. Yellow arrowheads: spermatogonia; green arrowheads: spermatocytes; dark blue arrowheads: round spermatids; red arrowheads: Sertoli cell nuclei; orange arrowheads: XY body; purple arrowheads: peritubular myoid cells; V-shape arrowheads: Leydig cells. (A) SFPQ expression at ages indicated. Scale bar equals 50 µm. (B) NONO expression at ages indicated.

upregulate PSCP1 expression to compensate for the absence of NONO (Li *et al.* 2014), and when NONO levels are reduced by siRNA knockdown, SFPQ can form a stable complex with PSCP1 that also localises to DNA damage sites (Ha *et al.* 2011). We hypothesised that since NONO was not detected in spermatogenic cells at any age, the germ cells expressing SFPQ and PSCP1, such as spermatocytes and round spermatids of the adult mouse testis, may have unique mechanisms for managing DNA double-strand breaks. Phosphorylation of histone H2AX at serine 139 to form γ -H2AX is widely considered a reliable marker of double-strand breaks (Rogakou *et al.* 1998). Many double-strand breaks are induced and then repaired as part of meiosis and

chromatin remodelling during spermatogenesis. The γ -H2AX pattern has been well documented in the testis (Hamer *et al.* 2003), and we noted that PSCP1 and SFPQ are detected in a cohort of cells/subcellular sites that are mutually exclusive from γ -H2AX during spermatogenesis. These reciprocal staining profiles are readily visualised through co-immunofluorescence for PSCP1 and γ -H2AX on adult mouse testis sections (Fig. 7). The γ -H2AX signal is present in the spermatocyte XY body, while PSCP1 is excluded from the XY body but visible across the rest of the nuclear compartment. This result suggests that PSCP1 and SFPQ may direct repair of double-strand breaks where they are present, at cellular sites that are complementary to γ -H2AX.

A ISH for NEAT1 lncRNA in Adult Mouse Testis Sections

B NEAT1 lncRNA Northern Blot

Figure 5 NEAT1 lncRNA *in situ* hybridisation with adult mouse testis sections and NEAT1 Northern Blot. (A) Localisation of NEAT1 transcript in the adult mouse testis using an anti-sense probe (i) and a control sense probe (ii). Yellow arrowheads: spermatogonia; green arrowheads: spermatocytes; dark blue arrowheads: round spermatids; red arrowheads: Sertoli cell nuclei. (B) NEAT1 Northern Blots on total RNA from extracted from testis, kidney and liver (30 µg per lane). Bands of the expected size for the shorter NEAT1_1 transcript (3.2 kb) are observed in total RNA from all samples (V-shape arrowhead) using the anti-sense probe (i) and are absent from the control sense probe (ii) blot. Size standards in kilobases (kB) indicate the RNA ladder sizes. Scale bar equals 50 µm.

A FISH/IF for NEAT1 Transcript and PSPC1 Protein in Adult Mouse Testis Tissue Sections **B** FISH for NEAT1 Transcript in GC-1 and GC-2 Cell Lines **C** Antibody Validations for PSPC1, SFPQ and NONO with Adult Mouse Testis (AdMT), GC-1 and GC-2 **Expression of other paraspeckle proteins throughout spermatogenesis**

That NEAT1 transcripts in nuclear foci, most likely corresponding to paraspeckles, were observed in interstitial Leydig cells, but not in any germ cells was somewhat surprising given that strong PSPC1 signals are found throughout the nucleoplasm and in addition, bright PSPC1 nuclear foci are often observed (Fig. 6Aiii–iv (green foci), Fig. 7, Major *et al.* 2015 and unpublished). Furthermore, the comparative expression levels of the long PSPC1 isoform protein (Fig. 1) have been shown to be many fold higher in the testis than in any other tissue examined (Myojin *et al.* 2004). Recent findings have demonstrated that many paraspeckle proteins (including PSPC1) contain prion-like domains that can facilitate the formation of hydrogels and through liquid-phase transitions are thought to contribute to paraspeckle formation (Hennig *et al.* 2015, Fox *et al.* 2018, Yamazaki *et al.* 2018). Of the seven paraspeckle proteins deemed

Figure 6 Paraspeckles in the adult mouse testis and in GC-1 and GC-2 germ cell-derived cell lines. Confocal Z-series images, visualised as maximum intensity projections, all have DAPI included as a nuclear marker and 20 µm scale bars (A and B). (A) FISH (i–ii) or sequential FISH/IF (iii–v) on adult mouse testis sections using Stellaris RNA FISH probes for NEAT1 labelled with Quasar 570 (Q570) dye. Sequential FISH/IF samples also had a PSPC1 antibody and an Alexa Fluor 488 (A488) secondary antibody applied. Dashed lines indicate the borders of adjacent seminiferous tubules, with NEAT1 signal seen as punctuate nuclear foci within the Leydig cells of the interstitial compartment between tubules. Some non-specific signal was observed, particularly within the cytoplasm of the interstitial cells, in the A488 secondary antibody only and autofluorescence control sample (2°Ab and AutoFL Ctrl; v). (B) FISH performed on GC-1 and GC-2 cell lines using Stellaris RNA FISH NEAT1 probes labelled with Quasar 570 (Q570) dye displayed punctuate nuclear NEAT1 foci, marking paraspeckles throughout the nucleus of most cells. (C) Western blots including a prestained protein ladder (λ) to indicate the approximate molecular weights in kilodaltons (kDa). Single bands of the correct approximate molecular weight were observed for PSPC1 (i), SFPQ (ii) and NONO (iii) using adult mouse testis (AdMT), GC-1 and GC-2 cell lysates (50 µg total protein per lane). The NONO band within the AdMT lysate was very faint, but comparison with the membrane incubated with secondary antibody alone (2°Ab Ctrl; iv), demonstrates a specific and clear signal for NONO in the AdMT.

to be essential to paraspeckle formation by siRNA knockdown (Naganuma *et al.* 2012, Hennig *et al.* 2015), transcripts encoding all (SFPQ, RBM14, DAZAP1, FUS, HNRNPK and HNRNPH3) except NONO have been identified in spermatocytes. This can be seen in publicly accessible microarray data (GeoProfiles; Shima *et al.* 2004, Namekawa *et al.* 2006, Barrett *et al.* 2013), with transcripts encoding many of these proteins showing peaks of expression at this stage of testis development (Fig. 8). With the exception of HNRNPK and HNRNPH3, all of these proteins have been identified to contain prion-like domains (Hennig *et al.* 2015). Overexpression of the RBM14 prion-like domain in HeLa cells can promote aggregation independent of paraspeckles (Hennig *et al.* 2015). Based on these observations, we speculate that in germ cells containing high levels of PSPC1, but undetectable NEAT1_2, the prion-like domains in PSPC1 (and potentially others such as SFPQ, RBM14, DAZAP1 and FUS) could facilitate nucleation of unique testis-specific paraspeckle-like nuclear

Figure 7 PSPC1 and γ -H2AX expression in the adult mouse testis. Confocal Z-series images, visualised as maximum intensity projections, all have DAPI included as a nuclear marker and 20 μ m scale bars. Samples had anti-PSPC1 antibody with an Alexa Fluor 488 (A488) secondary and an anti- γ -H2AX antibody with an Alexa Fluor 546 (A546) secondary applied. The secondary antibody control had no primary antibodies applied and lacks green or red signals. yellow arrowheads (spg): spermatogonia; green arrowheads (ps): pachytene spermatocytes; dark blue arrowheads (rs): round spermatids; light blue arrowheads (es): elongating spermatids; orange arrowheads (XY): XY body; V-shape arrowhead (met): metaphase spermatocytes.

structures or functional aggregates. This would explain the presence of PSPC1 punctuate nuclear foci observed in spermatocytes and spermatids. These structures may contain NEAT1_1 transcripts or rely on other RNA transcript(s) for assembly or stabilisation.

Discussion

This study extends previous reports of PSPC1 and paraspeckle proteins (Myojin *et al.* 2004, Kuwahara *et al.* 2006) to describe their localisation throughout gonadal development. Comparison of PSPC1, SFPQ, NONO and NEAT1 localisation data (summarised in Fig. 9) demonstrates that not all paraspeckle constituents are expressed in the same testicular cell types. NONO localised specifically to Sertoli and peritubular myoid cells and was undetectable in germ cells at any age, unlike PSPC1 and SFPQ, which were present in germ cells throughout testis development. In addition, SFPQ was present in Sertoli cells of the neonatal testis, whereas PSPC1 is detectable in these cells only from 15 dpp

onwards. Collectively, these data suggest that testicular cells have distinct requirements for different paraspeckle constituents and further analysis of the discrete roles of these proteins in the testis is required.

The immunohistochemistry data are consistent with dynamic roles for PSPC1 in both germ and Sertoli cells. The onset of PSPC1 expression in Sertoli cells as they become terminally differentiated matches the known transition of Sertoli cells to an androgen-responsive state (Buzek & Sanborn 1988, Zhou *et al.* 1996, Smith *et al.* 2015). Nuclear-localised PSPC1 in the foetal testis and ovary indicates a broad requirement for this protein in germ cells in the embryo. PSPC1 has been shown to regulate transcription from the androgen receptor promoter (Kuwahara *et al.* 2006), a critical control gene in Sertoli cell biology, and the longer isoform of PSPC1 is enriched in the testis (Myojin *et al.* 2004). The presence of PSPC1 in gonocytes therefore suggests it may influence androgen action in these cells (Merlet *et al.* 2007), as described in adult Sertoli cells (Kuwahara *et al.* 2006). In the early postnatal testis a function in

Figure 8 Expression profiles of paraspeckle proteins shown to be essential to paraspeckle formation by siRNA knockdown. Single probesets representing each gene were selected from each GEO dataset in a systematic manner as previously described (Major *et al.* 2011). Data are separated into lower/higher detection levels for visualisation and error bars correspond to standard error of the mean (SEM), where multiple values were present. Proteins containing known prion-like domains (Hennig *et al.* 2015) are indicated with an asterisks (*). (A) Mouse testis age series are from the GEO datasets GDS605, GDS606 and GDS607 (Shima *et al.* 2004), where progressively differentiated germ cells appear, concurrent with the first wave of spermatogenesis. Probesets shown here are as follows: PSPC1 (Pspc1 GDS605 103393_at), SFPQ (Sfpq GDS605 99621_s_at), NONO (Nono GDS605 93830_at), RBM14 (Rbm14 GDS605 100138_f_at), DAZAP1 (Dazap1 GDS606 109765_f_at), FUS (Fus GDS606 107506_at), HNRNPK (Hnrnpk GDS605 94041_at) and HNRNPH3 (Hnrnp3 GDS605 104231_at). (B) Isolated mouse germ cell types from GEO dataset GDS2390, containing Type A spermatogonia (A SpG), Type B spermatogonia (B SpG), pachytene spermatocytes (Pch SpC) and round spermatids (Rnd SpT) (Namekawa *et al.* 2006). Probesets shown here are as follows: PSPC1 (Pspc1 1423192_at), NONO (Nono 1448103_s_at), SFPQ (Sfpq 1423795_at), RBM14 (Rbm14 1436979_x_at), DAZAP1 (Dazap1 1419275_at), FUS (Fus 1451285_at), HNRNPK (Hnrnpk 1460547_a_at) and HNRNPH3 (Hnrnp3 1455491_at).

spermatogonial maturation is implied, as expression is seen solely in spermatogonia. A switch is evident by 26 dpp however, with PSPC1 now absent from mitotic germ cells, but present only in meiotic and haploid germ cells. Hence, in the developing testis, PSPC1 appears to be present as each germ cell subtype first appears. An interesting observation given PSPC1 has reported roles in the maintenance of stemness, metastasis and EMT through TGF- β 1 signalling and Smad2/3 target switching (Salvador & Gomis 2018, Yeh *et al.* 2018).

PSPC1 and SFPQ proteins are excluded from the transcriptionally inactive XY body of spermatocytes (Turner *et al.* 2004, 2006). PSPC1 is cytosolic during

metaphase/anaphase of meiosis, when no nuclear envelope is present, and this is closely followed by its translocation into the round spermatid nucleus. We interpret this as indicating PSPC1 is stable in the cytosol until the nuclear envelope reforms and then it is transported into the nucleus by IMP α 2, which is abundant at this stage (Major *et al.* 2015, 2017). PSPC1 and SFPQ are detected in a cohort of spermatogenic cells and subcellular sites that are distinct from γ -H2AX. PSPC1 has also been implicated in the DNA double-strand break repair response through regulation of the G1/S checkpoint, when it is proposed to halt the cell cycle to allow DNA repair (Gao *et al.* 2014), we postulate it could perform similar roles during meiotic divisions.

Two independent *in situ* hybridisation methods used to detect the NEAT1 lncRNAs were conducted with probes that should detect both NEAT1_1 and NEAT1_2 transcripts. However, the outcomes indicate that each method preferentially detected one of these isoforms. The fluorescence-based detection method revealed NEAT1 in punctate nuclear foci in Leydig cells and in the GC-1 and GC-2 cell lines (Fig. 6), suggesting this method and these conditions facilitated detection of the longer NEAT1_2 transcript that mediates paraspeckle nucleation. In contrast, the conventional ISH method, which relies on enzymatic production of an insoluble brown-purple precipitate, appeared to favour detection of NEAT1_1; a relatively weak signal was observed throughout the adult testis, and a distinct, stronger signal was visible in the cytoplasm of spermatogonia and spermatocytes (Fig. 5A). In agreement with this, a Northern blot using this probe detected only the shorter 3.2 kb mouse NEAT1_1 transcript (Fig. 5B). The NEAT1_1 transcript has been reported to act as a transcriptional regulator by promoting active chromatin states of specific promoters (Chakravarty *et al.* 2014, Li *et al.* 2017). While signal was not excluded from the nucleus, suggesting NEAT1 could be acting as a transcriptional regulator in these cells, the signal was greatest within the cytoplasm. SFPQ and NONO have been reported as components of a cytoplasmic RNA-transporting granule in dendrites (Kanai *et al.* 2004), but no cytoplasmic functions for NEAT1 have as yet been reported. The functional significance of cytoplasmic NEAT1 signal within male germ cells warrants further investigation.

Our observations of NEAT1 variant expression align with a report that the longer NEAT1_2 transcript is restricted to the hormone-producing Leydig and corpus luteal cells in the testes and ovaries respectively (Nakagawa & Hirose 2012), while the NEAT1_1 transcript is less restricted (Nakagawa *et al.* 2011 - Supplementary Fig. 1) Almost half of female NEAT1^{-/-} mice, which lack paraspeckles, fail to become pregnant due to severely impaired luteal tissue formation, which can be rescued by progesterone administration (Nakagawa *et al.* 2014). No phenotype for NEAT1^{-/-} male mice has been reported yet, but it would be interesting to determine if these

Figure 9 Summary of core paraspeckle component expression in the mouse testis. Expression profiles determined for paraspeckle components throughout testis development using immunohistochemistry (IHC), *in situ* hybridisation (ISH) or fluorescent *in situ* hybridisation (FISH), dashed lines indicates cells lacking detectable signal. Germ cells are shown at increasingly differentiated stages of development: spermatogonia (S), preleptotene spermatocytes (PL), zygotene spermatocytes (Z), pachytene spermatocytes (P), second meiotic metaphase (2 mm), round spermatids (R), elongating spermatids (El) and elongated spermatids (Ed). Somatic Cell types shown are Leydig, Sertoli and Peritubular Myoid (PTM) cells with nuclear (N) localisation indicated.

mice have reduced Leydig cell numbers or impaired Leydig cell function, potentially impacting androgen production and thereby masculinisation or reproductive and sexual function. Only 24% of pups from NEAT1^{-/-} mothers survive past 5 days unless placed with a foster mother, reflecting important roles in mammary gland development and function (Standaert *et al.* 2014). Other paraspeckle-related NEAT1 roles are demonstrated in adipogenesis (Gernapudi *et al.* 2016) and in muscle differentiation, as myoblasts transition to myotubes (Sunwoo *et al.* 2009). In tumours, hypoxia-inducible factor HIF-2 α can induce NEAT1 expression and paraspeckle formation, promoting cancer cell survival under hypoxic conditions (Choudhry *et al.* 2015).

The GC-1 and GC-2 cell lines were derived from testicular germ cells, corresponding to successive stages of germ cell development (GC-1: Hofmann *et al.* 1992, GC-2: Hofmann *et al.* 1994, 1995) which lacked NEAT1-positive nuclear foci (paraspeckles) and the DBHS protein NONO. Despite this, both GC-1 and GC-2 cells clearly display paraspeckles (NEAT1-positive nuclear foci; Fig. 6B) and express NONO protein (Fig. 6Ciii). The appearance of NEAT1-positive paraspeckles and NONO within both GC-1 and GC-2 cells, while they are absent from their primary cell counterparts, may arise as a consequence of their transition from primary cells to an immortalised cell line with ongoing proliferative and survival behaviour. This is in accord with observations that paraspeckles are ubiquitous across all mammalian cell lines examined to date, except embryonic stem cells (Bond & Fox 2009, Fox & Lamond 2010, Nakagawa & Hirose 2012), while *in vivo* paraspeckles are restricted to limited populations of cells within tissues (Nakagawa *et al.* 2011).

This study has carefully delineated the cellular expression profiles and subcellular localisations of the three core DBHS paraspeckles proteins throughout mouse testis development. Not all DBHS proteins were found in the same testicular cell types at the same developmental time points, suggesting that testicular cells

have distinct requirements for the different paraspeckle constituents and further analysis of the discrete roles of these in the testis is required. The lncRNA NEAT1 which acts as a structural scaffold for paraspeckles was detected throughout the testis, but evidence that long and short transcript variants are differentially expressed was provided. The potential for paraspeckle proteins to contribute to maintenance of genetic integrity in the germline was highlighted from the reciprocal expression profiles of γ -H2AX and PSPC1/SFPQ. These data provide a foundation for future studies to consider which amongst the plethora of diverse nuclear functions that DBHS proteins are being discovered to perform are relevant to spermatogenesis.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0139>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A M, C H, K L and D J designed the experiments and analyses. A M and C H implemented the experiments and analyses, with support from J Y. A M, C H and K L were primarily responsible

for writing the manuscript, with all authors making significant contributions to the final manuscript.

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