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ORIGINAL ARTICLE

Sperm Biology

Characterization of the protein expression and localization of hnRNP family members during murine spermatogenesis

Xiao-Li Wang^{1,*}, Jin-Mei Li^{1,*}, Shui-Qiao Yuan^{1,2,3}

Mammalian testis exhibits remarkably high transcriptome complexity, and spermatogenesis undergoes two periods of transcriptional cessation. These make the RNA-binding proteins (RBPs) the utmost importance during male germ cell development. Heterogeneous nuclear ribonucleoproteins (hnRNPs) are a large family of RBPs implicated in many steps of RNA processing; however, their roles in spermatogenesis are largely unknown. Here, we investigated the expression pattern of 12 hnRNP family members in mouse testes and found that most detected members are highly expressed in the testis. Furthermore, we found that most of the detected hnRNP proteins (hnRNPD, hnRNPK, hnRNQP, hnRNPU, and hnRNPU1) display the highest signals in the nuclei of pachytene spermatocytes, round spermatids, and Sertoli cells, whereas hnRNPE1 exclusively concentrates in the manchette of elongating spermatids. The expression of these hnRNP proteins showed both similarities and specificity, suggesting their diverse roles in spermatogenesis.

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Keywords: gene expression; heterogeneous nuclear ribonucleoproteins; mice; Sertoli cells; spermatogenesis

INTRODUCTION

Spermatogenesis is a tightly regulated complex and a cyclic developmental process by which spermatogonia generate the mature spermatozoa. Mammalian testes contain remarkably more widespread transcriptomes than other organs (brain, cerebellum, heart, kidney, and liver), mainly due to substantially diverse transcriptomes in meiotic spermatocytes and postmeiotic round spermatids.^{1,2} In addition, male germ cells are characterized by complex alternative splicing patterns, and more enormous larger proportions of protein-coding genes present splice variants in germ cells, especially in spermatocytes/spermatids, than in somatic cells and Sertoli cells.¹ Moreover, during their differentiation, male germ cells experience two periods of transcriptional quiescence and chromatin inaccessibility when coordinating the accurate temporal and spatial expression of genes required for unceasing development. The first transcriptional quiescence period occurs in early meiotic cells (leptotene and zygotene spermatocytes), and the cell undergoes DNA repair by crossing over to resolve homologous chromosome recombination.^{3,4} The second phase of transcriptional cessation is during the transition from elongating spermatids to mature spermatozoa, histone-to-protamine replacement, and chromatin compaction into the head region of the spermatozoon.^{5–7} Transcriptome complexity and the unique transcriptional dynamics, primarily regulated by RNA-binding proteins (RBPs) during spermatogenesis, make RBPs the utmost importance.

RBPs constitute a large class of proteins in cells capable of assembling with double- or single-stranded (ss) RNA to form ribonucleoproteins (RNPs). They manipulate the function and fate of RNAs at all stages, including alternative splicing, RNA modification, nuclear export, localization, stability, and translation efficiency.^{8,9} Notably, RBPs localize in both the nucleus and cytoplasm; some RBPs can shuttle between the nucleus and cytoplasm. Heterogeneous nuclear ribonucleoproteins (hnRNPs) represent a large subclass of RBPs, which are mainly localized in the nucleus and act as critical proteins in cellular nucleic acid metabolism, including transcriptional and translational regulation, alternative splicing, and mRNA stabilization.¹⁰ The number of hnRNP families was originally 24 from hnRNPA1 to hnRNPU but has continued to grow, since some previously well-known proteins were later regarded as hnRNPs.¹¹

Structurally, hnRNPs contain several unique RNA-binding domains (RBDs). The most common domain is the RNA recognition motif, which is responsible for RNA-binding specificity;^{12,13} the RGG RNA-binding domain is composed of Arg-Gly-Gly repeats responsible for homologous and heterologous interactions with other hnRNPs;^{14,15} the KH domain (hnRNP K homology domain) is composed of three-stranded beta-sheets packed against three alpha-helices and is capable of binding with both ssDNA and ssRNA;^{16–18} and in addition, auxiliary domains, such as proline-, glycine- or acid-rich domains, are also present in the hnRNP family.¹⁸ Based on the diverse domains, hnRNPs

¹Institute of Reproductive Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China; ²Laboratory of Animal Center, Huazhong University of Science and Technology, Wuhan 430030, China; ³Shenzhen Huazhong University of Science and Technology Research Institute, Shenzhen 518057, China.

*These authors contributed equally to this work.

Correspondence: Dr. SQ Yuan (shuiqiaoyuan@hust.edu.cn)

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can form dynamic complexes with RNA and other RBPs and regulate cell fate owing to constant remodeling of the RNA-protein complex compositions.^{11,19} Within the cell, hnRNP proteins function at all levels of RNA metabolism by assembling on nascent and processed mRNAs; therefore, hnRNPs are capable of controlling gene regulation at the posttranscriptional levels in both health and disease.

RNA processing in the male germline is critical from spermatogonia to sperm, and many RBPs have been demonstrated to be essential during spermatogenesis.²⁰ However, the expression pattern and localization of hnRNP proteins during spermatogenesis are largely unknown. Here, we detected the protein expression levels of several hnRNP members in multiple mouse organs and showed that most of them are highly expressed in testes. Further investigation of hnRNP members in developing testes revealed that most of them are ubiquitously expressed in the nuclei of spermatogenic cells, including spermatogonia and meiotic cells until round spermatids, as well as Sertoli cells. Provocatively, most hnRNP proteins showed substantially stronger signals in mid- and late-pachytene spermatocytes, round spermatids, and Sertoli cells than those in other germ cell types, potentially reflecting specific functional requirements of hnRNP proteins during male germ cell development.

MATERIALS AND METHODS

Animals and ethics statement

C57BL/6J mice (*Mus musculus*) were obtained from the Laboratory Animal Center at Huazhong University of Science and Technology (Wuhan, China). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Huazhong University of Science and Technology (approval No. S2795). All experiments with mice were conducted ethically according to the Guide for the Care and Use of Laboratory Animal.

Western blot (WB)

WB analyses were conducted as described previously with slight modifications.²¹ Briefly, the indicated samples were lysed in radioimmunoprecipitation assay (RIPA) buffer (Cat# CW2333S, CWBIO, Taizhou, China). The protein concentration was detected by the BCA kit (P0012S, Beyotime, Shanghai, China). After being denatured with 5× sodium dodecyl sulfate (SDS) loading buffer (P0015L, Beyotime) at 98°C for 10 min, the proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene difluoride (PVDF) membranes (#1620184, Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% skimmed milk (BS102, Biosharp, Hefei, China) in Tris-buffered saline-Tween (TBST) for 1 h before incubation overnight with the specific primary antibodies. Then, the membranes were washed with TBST and incubated with the appropriate secondary horseradish peroxidase (HRP)-conjugated antibodies for 1 h. Clarity Western ECL Substrate (1705061, Bio-Rad) was used for chemiluminescence detection and was photographed using the ChemiDoc XRS+ system (Bio-Rad).

Immunofluorescent (IMF) staining

IMF staining of testicular cryosections was conducted as described previously with slight modifications.²¹ In brief, testes were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, followed by dehydration in increasing sucrose gradient solution (5%, 10%, 15%, and 20% for 30 min, respectively). Then, they were embedded in Tissue-Tek optimal cutting temperature (4583, SAKURA, Torrance, CA, USA) compound and sectioned at 5 μm thick. After boiling for antigen retrieval for 15 min in citrate buffer (pH = 6.0), the slides were blocked

with blocking solution (containing 3% normal goat serum and 3% fetal bovine serum in 1% bovine serum albumin) for 1 h. The sections were stained with the appropriate primary antibodies overnight at 4°C and secondary antibodies for 1 h at room temperature (RT). Laser confocal scanning images were captured using a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan).

Meiotic chromosome spreads assay

Chromosome nuclear spread was performed as described previously with slight modifications.²² Briefly, testes were dissected from mice quickly, and the tunica albuginea was removed. Then, seminiferous tubules were incubated in hypotonic buffer (30 mmol l⁻¹ Tris, 50 mmol l⁻¹ sucrose, 17 mmol l⁻¹ trisodium citrate dihydrate, 5 mmol l⁻¹ ethylenediaminetetraacetic acid [EDTA], 0.5 mmol l⁻¹ dithiothreitol [DTT], and 0.5 mmol l⁻¹ phenylmethylsulfonyl fluoride [PMSF], pH = 8.2) for 1.5 h and were cut into pieces in 100 mmol l⁻¹ sucrose. Then, the cells were spread to a thin cell layer on glass slides coated with freshly 2% paraformaldehyde solution containing 0.15% Triton X-100. After being fixed for 2 h in a moist chamber at RT, the slides were washed with 0.4% Photo-Flo (1464510, Kodak, Rochester, NY, USA). The spread chromosome slides were dried completely at RT and then blocked with blocking solution for 1 h for immunostaining.

Antibodies

Primary antibodies specific to hnRNPD (12770-1-AP, 1:1000 for WB, 1:200 for IMF), hnRNPF (67701-1-Ig, 1:1000 for WB), hnRNPK (11426-1-AP, 1:1000 for WB, 1:200 for IMF), hnRNPP2 (FUS: fused in sarcoma; 11570-1-AP, 1:1000 for WB), hnRNPU1 (110578-1-AP, 1:1000 for WB, 1:200 for IMF), α-Tubulin (66031-1-Ig, 1:200 for IMF), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 60004-1-Ig, 1:5000 for WB) were purchased from ProteinTech (Wuhan, China). Anti-hnRNPA2B1 antibody (sc-374053, 1:1000 for WB), anti-hnRNPU (sc-32315, 1:1000 for WB, 1:100 for IMF), and anti-synaptonemal complex protein 3 (SYCP3; sc-74569, 1:100 for IMF) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Primary antibodies specific to hnRNPE1/PCBP1 (A1044, 1:1000 for WB, 1:200 for IMF), hnRNPE2/PCBP2 (A2531, 1:1000 for WB), hnRNPH1 (A5924, 1:1000 for WB), hnRNPM (A6937, 1:1000 for WB), and hnRNPNQ (A9609, 1:1000 for WB, 1:200 for IMF) were purchased from Abclonal Technology (Wuhan, China). Anti-γ-H2A.X antibody (ab26350, 1:200 for IMF) was purchased from Abcam (Shanghai, China). Secondary antibodies specific to HRP goat antirabbit IgG (A21020-1, 1:10 000 for WB), HRP goat antimouse IgG (A21010-1, 1:10 000 for WB), DyLight 488 goat antimouse IgG (A23210, 1:500 for IMF), and DyLight 594 goat antirabbit IgG (A23420, 1:500 for IMF) were purchased from Abbkine (Shanghai, China). Secondary antibodies specific to Alexa Fluor™ 488 goat antirabbit (H+L; A32731, 1:500 for IMF) and Alexa Fluor™ 594 goat antimouse (H+L; A-11005, 1:500 for IMF) were purchased from Invitrogen (Camarillo, TX, USA).

RESULTS

Protein expression of hnRNP families in multiple tissues and postnatal mouse testes

To investigate the spatial protein expression of hnRNP families in different mouse tissues, we collected multiple tissues, including the brain, heart, liver, stomach, lung, kidney, ovary, and testis. WB was performed to detect the protein levels of 12 hnRNP family members using specific commercial antibodies, including hnRNPA2B1, hnRNPD, hnRNPE1, hnRNPE2, hnRNPF, hnRNPH1, hnRNPK, hnRNPP2 (FUS), hnRNPNQ, hnRNPM, hnRNPU, and hnRNPU1.

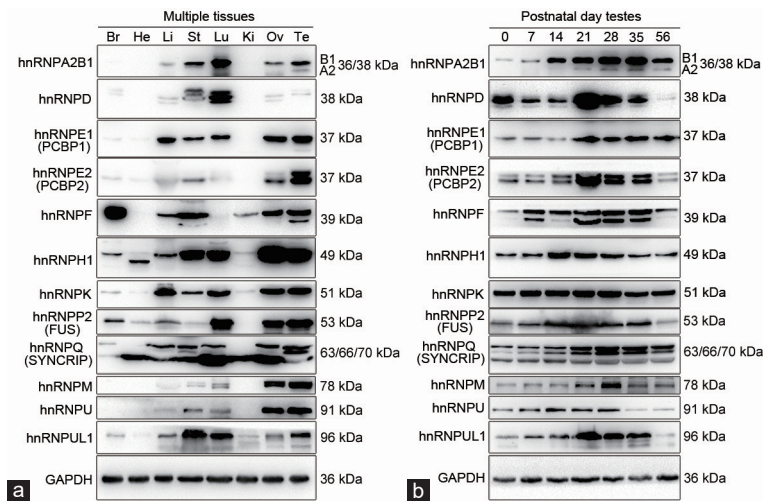


Figure 1: Western blot analyses of the protein expression levels of 12 hnRNP family proteins in (a) multiple mouse tissues and (b) postnatal developing testes. GAPDH was used as a loading control. Br: brain; He: heart; Li: liver; St: stomach; Lu: lung; Ki: kidney; Ov: ovary; Te: testes; hnRNP: heterogeneous nuclear ribonucleoprotein; hnRNPU1: heterogeneous nuclear ribonucleoprotein U-like (hnRNPU1) protein 1; FUS: fused in sarcoma; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; B1: one isoform of hnRNA2B1; A2: the other isoform of hnRNA2B1.

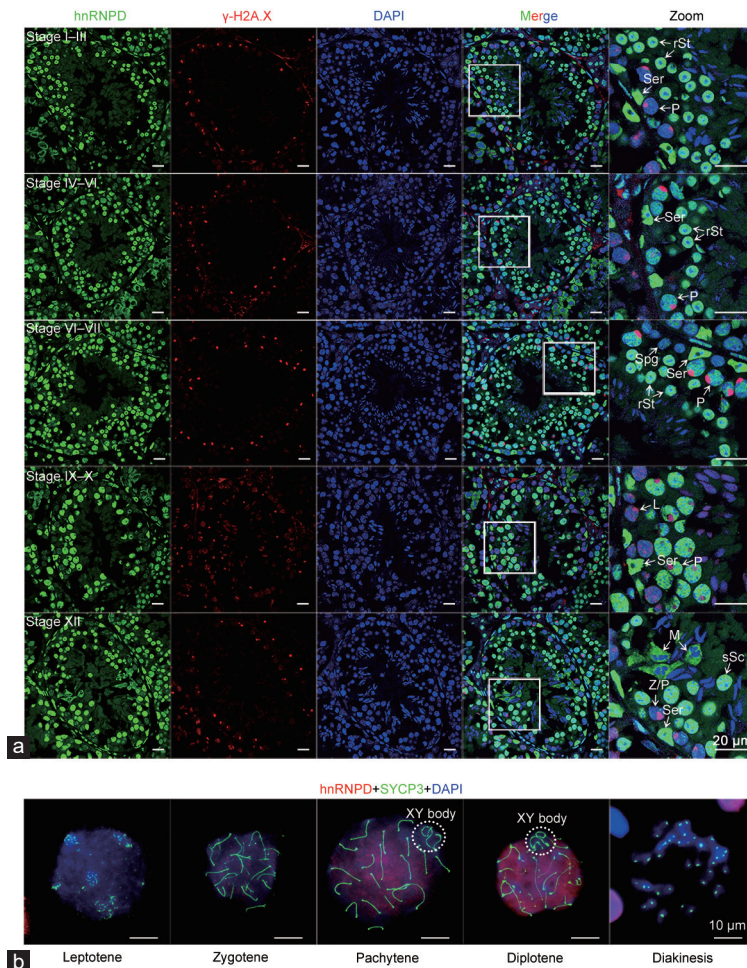


Figure 2: Spatiotemporal localization of hnRNPD in adult mouse testicular cells. (a) Immunostaining of hnRNPD (green) and γ -H2A.X (a marker for DNA double-strand breaks, red) on wild-type (WT) adult testis sections in different stages of seminiferous tubules is shown. Scale bars = 20 μ m. (b) Chromosomes spread from different subtypes of WT spermatocytes immunostained with hnRNPD (red) and SYCP3 (a marker for lateral element of synaptic complex, green) are shown. Scale bars = 10 μ m. Spg: spermatogonia; L: leptotene spermatocytes; Z: zygotene spermatocytes; P: pachytene spermatocytes; rSt: round spermatids; M: meiotic divisions; sSc: secondary spermatocytes; Ser: Sertoli cells; hnRNP: heterogeneous nuclear ribonucleoprotein; DAPI: 4',6-diamidino-2-phenylindole; SYCP3: synaptonemal complex protein 3.

Generally, except for hnRNPD, all the other detected proteins showed relatively high expression in the reproductive system, including the testes and ovaries (**Figure 1a**). Interestingly, several proteins displayed two or three bands, including hnRNPA2B1, hnRNPD, hnRNPE2, hnRNPF, and hnRNPPQ. In addition, all of the detected hnRNP family proteins were continuously expressed in postnatal developing testes from postnatal P0 to adulthood (**Figure 1b**).

Notably, although these detected hnRNP members are highly expressed in testes, they showed distinct expression patterns in postnatal developmental testes. Individually, hnRNPA2B1 is most expressed in the lung, followed by the testis, stomach, ovary, and liver, and rarely in the brain, heart, and kidney. hnRNPA2B1 exhibits two bands corresponding to the 36 kDa (A2) and 38 kDa (B1) isoforms separately, especially in the testis. Furthermore, hnRNPA2B1 is continuously expressed in the postnatal testis from P0 to adulthood, with a greatly increased expression level at P14. hnRNPD bears three bands and is highly expressed in the lung followed by the stomach, with weak expression in the brain, liver, ovary, and testis, rarely in the heart and kidney. Interestingly, in postnatal testes, the middle band of hnRNPD is the highest enriched, and the expression level showed two peaks at P0 and P21 separately, whereas the lowest expression in adult testis, suggesting its crucial role in the development of pro-spermatogonia and completion of meiosis during the first wave of spermatogenesis. Both hnRNPE1 and hnRNPE2 are highly expressed in the testis and ovary, with an obvious increase in P21 testis; specifically, hnRNPE2 exhibits a decreased expression at P56 testis, whereas hnRNPE1 displayed comparable protein levels from P21 to P56 testes. hnRNPF showed the highest protein levels in the brain, followed by the testis, ovary, and stomach, and two bands were present in the testis. In postnatal developing testes, hnRNPF showed an expression burst in P7 and a decrease in P56 testes, especially for the lower band.

hnRNPH1 is most highly expressed in the ovary and testis, followed by the stomach and lung; interestingly, it specifically displays a lower band in the lung, suggesting a distinct isoform in the lung. hnRNPH1 increases in the P14 testis but gradually decreases until the P56 testis. hnRNPK is highly expressed in the liver, lung, ovary, and testis and is stably expressed in postnatal developmental testis, which is different from the expression pattern of other detected hnRNP family proteins. hnRNPP2 is highly expressed in the lung, ovary, and testis and showed an increase in P14 but a decrease in P56 testis. hnRNPPQ displayed three bands in multiple tissues and showed a gradual increase in postnatal developmental testis. Both hnRNPM and hnRNPU exhibited the highest protein levels in the ovary and testis; however, in postnatal developmental testis, hnRNPM showed a peak in P28 testis, whereas hnRNPU displayed gradually increased protein level from P0 to P14 and remained stable until P28, then a decrease of expression from P35 to P56 testis. Heterogeneous nuclear ribonucleoprotein U-like (hnRNPUL) protein 1 (hnRNPUL1) is highly expressed in the stomach, lung, and testis and showed an increase in P21 but a decrease in P56 testis. Together, these data indicate that the members of the hnRNP family are highly expressed in the testis and may play critical roles during spermatogenesis and male germ cell development.

Localization of hnRNP family proteins during adult spermatogenesis

The high expression of the members of the hnRNP family in the testes motivated us to further explore their cell type-specific expression patterns during spermatogenesis, including hnRNPD, hnRNPE1, hnRNPK, hnRNPPQ, hnRNPU, and hnRNPUL1. Seminiferous tubules of the adult wild-type (WT) testes were coimmunostained with specific targeting antibodies and γ -H2A.X (a marker for DNA double-strand breaks) or SYCP3 (a marker for lateral element of synaptic complex), which is exclusively expressed in primary spermatocytes during meiosis and is also a hallmark of spermatogenesis.²³

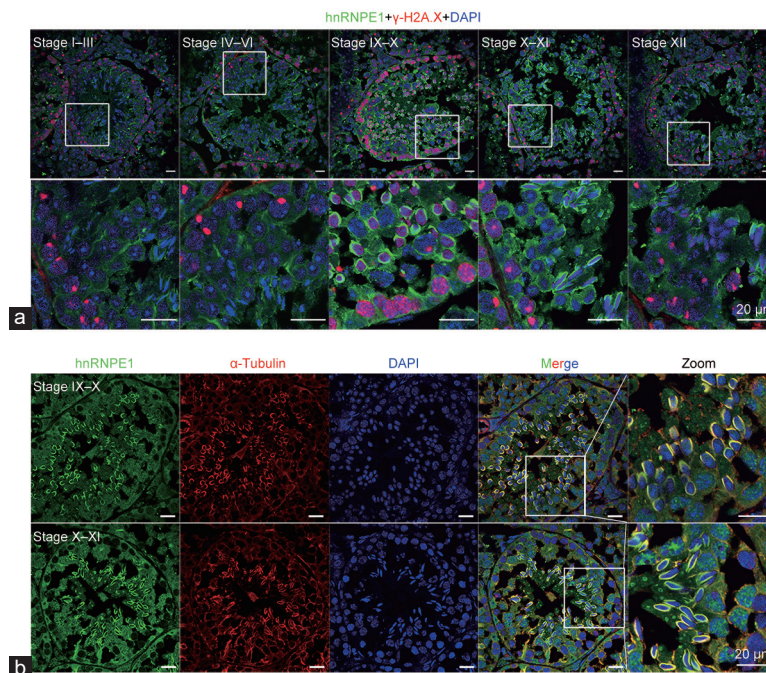


Figure 3: Spatiotemporal localization of hnRNPE1 in adult mouse testicular cells. (a) Immunostaining of hnRNPE1 (green) and γ -H2A.X (red) on wild-type (WT) adult testis sections in different stages of seminiferous tubules is shown. Scale bars = 20 μ m. (b) Immunofluorescence of hnRNPE1 (green) and α -tubulin (a manchette marker, red) on WT adult testis sections in different stages of seminiferous tubules is shown. Scale bars = 20 μ m. hnRNP: heterogeneous nuclear ribonucleoprotein; DAPI: 4',6-diamidino-2-phenylindole.

Localization of hnRNPD in testicular cells

The results showed that the hnRNPD appeared faintly in early pachytene spermatocytes but highly in round spermatids of stage I–III seminiferous tubules, and gradually increased in pachytene spermatocytes and persistently high levels in round spermatids afterward. Meanwhile, hnRNPD is continuously expressed in Sertoli cells in all different stages. Furthermore, hnRNPD showed weak expression in spermatogonia, leptotene spermatocytes, and zygotene spermatocytes (**Figure 2a**). Consistently, immunostaining of the spread nuclei showed that the hnRNPD signal was distributed uniformly throughout the nucleus, which was weak in leptotene and zygotene spermatocytes but much stronger in pachytene and diplotene spermatocytes (**Figure 2b**). Interestingly, hnRNPD was found to be excluded from the XY body formed in the pachytene and diplotene stages (**Figure 2b**).

Localization of hnRNPE1 in testicular cells

Interestingly, unlike the other detected hnRNP family members, hnRNPE1 showed a distinct localization, which was concentrated in the manchette of elongating spermatids during the elongation/condensation process (**Figure 3a**) and colocalized with α -tubulin,

a manchette marker (**Figure 3b**). Manchette is a transient skirt-like microtubular platform in elongating spermatids and is essential for nuclear shaping and sperm tail formation.²⁴ Manchette exhibits a precise developmental timeline, which first appears in step 7 spermatids (which appear in stage VII seminiferous tubules) with the assembly of short microtubules around the nucleus and then quickly forms a microtubular platform that projects from the perinuclear ring surrounding the nucleus into the spermatid cytoplasm in step 8 spermatids (which appear in stage VIII seminiferous tubules). Manchette keeps associated with the spermatid nucleus during the nuclear remodeling phase until it is disassembled around steps 13–14 (which appear in stage I–III seminiferous tubules) in advance of the sperm tail midpiece formation via an unknown mechanism.²⁵ The specific location of hnRNPE1 suggests its unique function during spermiogenesis.

Localization of hnRNPK in testicular cells

hnRNPK was previously found to be distributed in spermatogonia, spermatocytes, Sertoli cells, Leydig cells, and myoid cells in porcine testis.²⁶ In this study, we examined its expression pattern in mouse testis and found that hnRNPK is predominantly expressed in the nuclei of

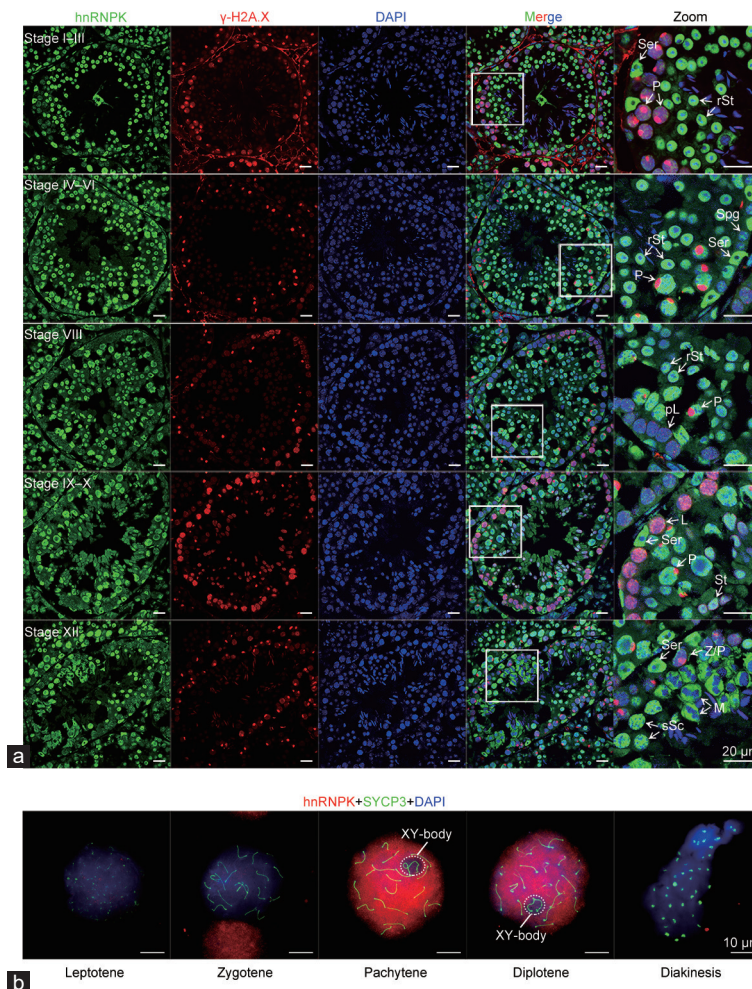


Figure 4: Spatiotemporal localization of hnRNPK in adult mouse testicular cells. (a) Immunostaining of hnRNPK (green) and γ -H2A.X (red) on wild-type (WT) adult testis sections in different stages of seminiferous tubules is shown. Scale bars = 20 μ m. (b) Chromosomes spread from different subtypes of WT spermatocytes immunostained with hnRNPK (red) and SYCP3 (green) are shown. Scale bars = 10 μ m. Spg: spermatogonia; pL: preleptotene spermatocytes; L: leptotene spermatocytes; Z: zygotene spermatocytes; P: pachytene spermatocytes; rSt: round spermatids; M: meiotic divisions; sSc: secondary spermatocytes; Ser: Sertoli cells; hnRNP: heterogeneous nuclear ribonucleoprotein; DAPI: 4',6-diamidino-2-phenylindole; SYCP3: synaptonemal complex protein 3.

zygotene spermatocytes and pachytene spermatocytes and at much higher levels in mid- and late-pachytene spermatocytes to postmeiotic round spermatids (**Figure 4a**). In round spermatids, hnRNPK signals persisted until steps 9–10 spermatids (**Figure 4a**), which is consistent with recent literature.²⁷ In addition, hnRNPK also showed strong signals in the nuclei of Sertoli cells (**Figure 4a**). Consistently, analysis of spermatocyte nuclear spreads showed faint signals in leptotene and zygotene spermatocytes and strong signals in pachytene and diplotene spermatocytes (**Figure 4b**). Of note, hnRNPK was also found to be excluded from the XY body formed in the pachytene and diplotene stages (**Figure 4b**), which is similar to the expression pattern of hnRNPD (**Figure 2b**).

Localization of hnRNPQ in testicular cells

Similar to hnRNPD and hnRNPK, hnRNPQ was remarkably highly expressed in pachytene spermatocytes, round spermatids, and Sertoli cells and relatively weakly expressed in spermatogonia, leptotene spermatocytes, and zygotene spermatocytes

(**Figure 5a**). In contrast, hnRNPQ showed granular signals in round spermatocytes, and costaining of hnRNPQ with PNA (an acrosome marker) revealed that the granular signals of hnRNPQ were not colocalized with the acrosome (**Figure 5b**). Subsequently, analyses through spermatocyte nuclear spreads confirmed the strong expression of hnRNPQ in the nuclei of meiotic spermatocytes but also excluded it from the XY body formed in the pachytene and diplotene stages (**Figure 5c**). Curiously, hnRNPQ exhibited signals in structures much like centrioles in pachytene and diplotene spermatocytes (**Figure 5c**, as arrowheads indicate), suggesting the potential critical function of hnRNPQ during germ cell differentiation.

Localization of hnRNPU and hnRNPU1 in testicular cells

Similar to hnRNPD, hnRNPK, and hnRNPQ, both hnRNPU and hnRNPU1 showed much stronger signals in pachytene spermatocytes, round spermatids, and Sertoli cells (**Figure 6a** and **6b**), which is in agreement with our previous report.²⁸ Notably, hnRNPU is

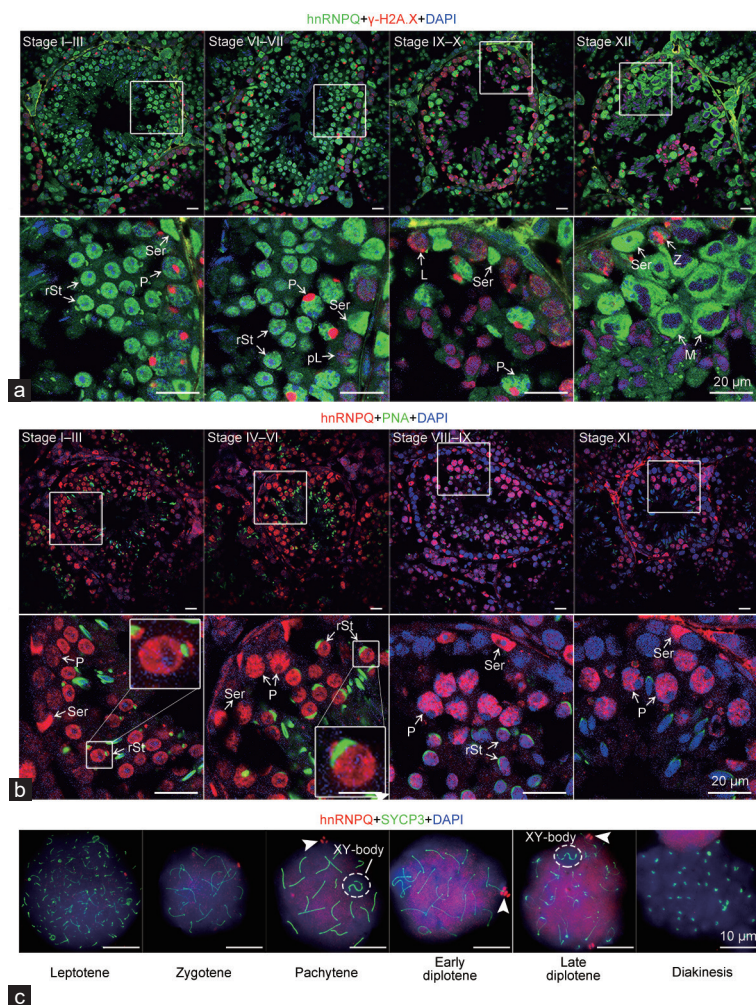


Figure 5: Spatiotemporal localization of hnRNPQ in adult mouse testicular cells. (a) Immunostaining of hnRNPQ (green) and γ -H2A.X (red) on wild-type (WT) adult testis sections in different stages of seminiferous tubules is shown. Scale bars = 20 μ m. (b) Immunostaining of hnRNPQ (red) and PNA (an acrosome marker, green) on WT adult testis sections in different stages of seminiferous tubules is shown. Scale bars = 20 μ m. (c) Chromosomes spread from different subtypes of WT spermatocytes immunostained with hnRNPQ (red) and SYCP3 (green) and DAPI (blue) are shown. Arrowheads indicate a centriole-like structure. Scale bars = 10 μ m. pL: preleptotene spermatocytes; L: leptotene spermatocytes; Z: zygotene spermatocytes; P: pachytene spermatocytes; rSt: round spermatids; M: meiotic divisions; Ser: Sertoli cells; hnRNP: heterogeneous nuclear ribonucleoprotein; DAPI: 4',6-diamidino-2-phenylindole; SYCP3: synaptonemal complex protein 3.

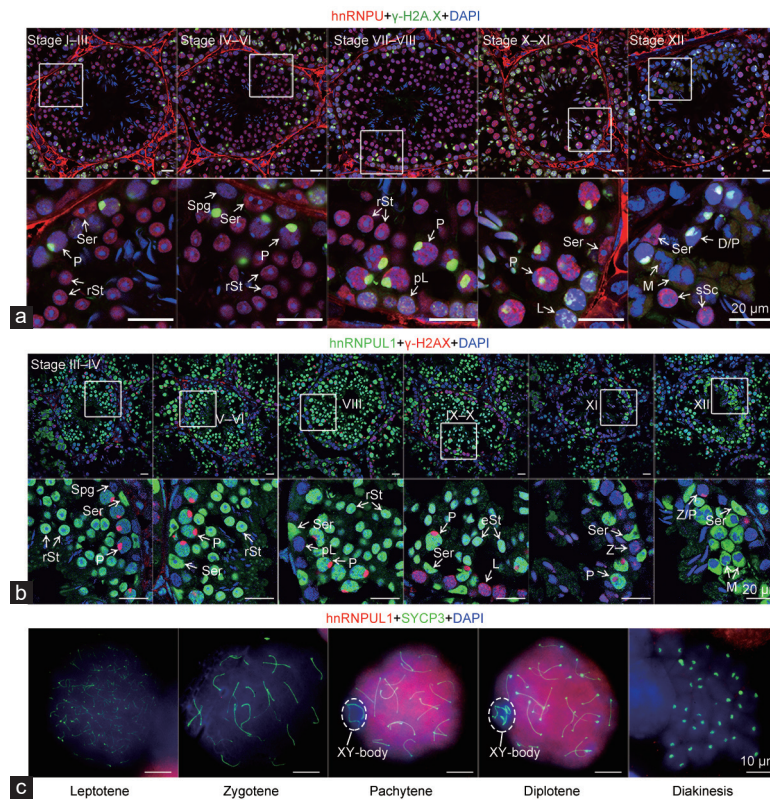


Figure 6: Spatiotemporal localization of hnRNP and hnRNPUL1 in adult mouse testicular cells. (a) Immunostaining of hnRNP (red) and γ -H2A.X (green) on wild-type (WT) adult testis sections in different stages of seminiferous tubules is shown. Scale bars = 20 μ m. (b) Immunostaining of hnRNPUL1 (green) and γ -H2A.X (red) on WT adult testis sections in different stages of seminiferous tubules is shown. Scale bars = 20 μ m. (c) Chromosomes spread from different subtypes of WT spermatocytes immunostained with hnRNPUL1 (red) and SYCP3 (green) are shown. Scale bars = 10 μ m. Spg: spermatogonia; pL: preleptotene spermatocytes; L: leptotene spermatocytes; Z: zygotene spermatocytes; P: pachytene spermatocytes; D: diplotene spermatocytes; rSt: round spermatids; M: meiotic divisions; sSc: secondary spermatocytes; Ser: Sertoli cells; hnRNPUL1: heterogeneous nuclear ribonucleoprotein U-like (hnRNPUL) protein 1; hnRNP: heterogeneous nuclear ribonucleoprotein; DAPI: 4',6-diamidino-2-phenylindole; SYCP3: synaptonemal complex protein 3.

also expressed in spermatogonia and other meiotic spermatocytes (leptotene and zygotene spermatocytes). In contrast, hnRNPUL1 is nearly indiscernible in spermatogonia, leptotene spermatocytes, and zygotene spermatocyte (Figure 6b and 6c). Consistently, analyses through spermatocyte nuclear spreads confirmed the strong expression of hnRNPUL1 in the nuclei of pachytene and diplotene spermatocytes but were also excluded from the XY body formed in the pachytene and diplotene stages (Figure 6c). hnRNPUL1 was reported to be associated with the DNA double-strand break (DSB) sensor complex MRE11-RAD50-NBS1 (MRN), thereby regulating DSB repair.²⁹ Whether hnRNPUL1 regulates DSB repair in meiotic processes and the specific role of hnRNPUL1 during spermatogenesis need to be further elucidated.

DISCUSSION

In the present work, we characterized the expression pattern of several hnRNP family proteins in adult male germ cells. Almost all the selected proteins were highly expressed in the male reproductive system, suggesting their significant function in male germ cell development. Interestingly, each hnRNP family protein showed a distinct expression pattern in postnatal developing testes. For example, some proteins exhibited high expression at specific time points, such as hnRNPD, whereas some showed continuous expression levels, such as hnRNPK, indicating the specific requirement and different functions of hnRNP family proteins at different stages during spermatogenesis.

Through IMF of the seminiferous tubule, most detected hnRNP family proteins showed high distribution in the nucleus of pachytene spermatocytes, round spermatids, and Sertoli cells. Since pachytene spermatocytes and round spermatids harbor high transcriptional activity and complex transcriptomes,¹ the high expression of hnRNP family proteins in these germ cells provides significant clues to their functional relevance during spermatogenesis. Notably, most of the hnRNP family proteins were found to be highly expressed in both germ cells and Sertoli cells, suggesting their roles in the communication between Sertoli cells and germ cells. Furthermore, some hnRNP family proteins showed a specific cellular location, indicating their unique function in regulating specific organelles during spermatogenesis. For instance, hnRNPE1 is located in the manchette, which is only present during spermatid elongation.³⁰

The fact that hnRNP family members ubiquitously regulate transcripts throughout their life cycle and are highly expressed in spermatogenic cells aroused a series of scientific questions: (1) What is their specific function in a different type of germ cell? (2) Is the function dynamically changed? (3) How do hnRNP family proteins selectively bind their targets in male germ cells? (4) Are there any germ cell-specific targets during spermatogenesis? and (5) How do hnRNP family proteins regulate RNA targets, and what cellular goals will they ultimately achieve? Our recent study revealed the essential role of hnRNP proteins in spermatogenesis and male fertility, such as hnRNPUL1, in regulating neonatal Sertoli cell and prepubertal testicular development.²⁸ In addition, Xu *et al.*²⁷ found that conditional knockout

of *Hnrnpk* in mouse male germ cells caused male infertility during to meiosis arrest at the pachytene stage. Clearly, we need to approach the many hnRNP family proteins involved in spermatogenesis with a broad open mind, ready to discover their common and specific functions in male germ cell development.

AUTHOR CONTRIBUTIONS

SQY and XLW conceived and designed the research. XLW and JML performed the bench experiments and data analyses. XLW wrote the manuscript. SQY revised the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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