**ADP-ribosylation factor-like 3, a manchette-associated protein, is essential for mouse spermiogenesis**

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**Abstract:** During spermiogenesis, the manchette is an important structure for sperm head and tail formation. However, the mechanisms responsible for this process are poorly understood. In a previous study, we established a comparative proteome profile for mouse testis during the first wave of spermatogenesis, and provided lists of proteins of potential importance in the regulation of male fertility and infertility. Here we have selected Arl3, one of these interesting proteins, and investigated its expression and function during spermiogenesis. Western blotting was used to determine Arl3 expression levels in mice at different time points from birth to adulthood. The results show Arl3 was expressed from birth, and the expression level increased significantly from Week 4, when mouse spermiogenesis begins. Immunohistochemistry and indirect immunofluorescence were used to investigate the Arl3 expression during sperm development, and the intracellular localization of Arl3 in more detail. In elongating spermatids from steps 8 to 15, Arl3 was localized to the posterior section of the head, in a similar pattern to the manchette. The Arl3 signal was colocalized during spermiogenesis with α-tubulin, a marker for the manchette. To investigate the possible functional role of Arl3, mouse testes were injected with small interfering RNA (siRNA) against Arl3, or control siRNA. Western blotting showed a 60% reduction in the Arl3 expression after 72 h, and a significant increase in sperm abnormalities after 3 weeks compared with the negative control. In conclusion, we propose that Arl3 is a novel manchette-related protein with an important role in spermiogenesis.

**Key words:** Arl3 / manchette / RNAi / spermiogenesis

**Introduction**

Spermatogenesis is a biological process that involves successive mitotic, meiotic and post-meiotic phases. Meiosis is unique to gamete formation, whereas the post-meiotic phase, known as spermatogenesis, only occurs during male germ cell development (Wolgemuth et al., 1995; Eddy, 2002). During spermiogenesis, the morphogenetic process, shaping and condensation of the nucleus as well as the formation of the acrosome and the tail take place. These dramatic changes require a stringent, well-coordinated and unique system that regulates specific patterns of gene and protein expression (Krausz and Sassone-Corsi, 2005; Tanaka and Baba, 2005).

The manchette, one of the important structures in spermiogenesis, is a transient construct that emerges between steps 8 and 16 and encircles part of the nuclei of elongating spermatids in mice. The timing of manchette development is very precise: it appears during early elongation and disappears as the processes of elongation and condensation of the spermatid nucleus approach completion (Kierszenbaum, 2002; Toshimori and Ito, 2003; Matsuoka et al., 2008). Components of the manchette have been identified, including many microtubular proteins, such as microtubule-dependent motor proteins and microtubule-associated proteins (Kierszenbaum and Tres, 2002; Yamaguchi et al., 2004; Akhmanova et al., 2007). Several findings suggest that the manchette may serve to convey molecules necessary for spermatid nuclear condensation and tail formation (Kierszenbaum et al., 2002; Tovich et al., 2004). However, the detailed mechanism and the important molecules involved in this process remain to be elucidated.

In a previous study, we established a comparative proteome profile for mouse testis during the first wave of spermatogenesis and...
identified 362 differential protein spots corresponding to 257 different proteins involved in the initiation of mouse spermatogenesis (Huang et al., 2008; Huang and Sha, 2011). Several proteins from this list were chosen for further investigation and ADP-ribosylation factor-like 3 (Arl3) was one of these interesting proteins. Arl3 is a Ras-related small GTP-binding protein. Like other ARF family members, Arl3 binds guanine nucleotides, but it lacks ARF activity (Memon, 2004; Cavenagh et al., 1994).

The aim of this study was to investigate the levels of expression and the localization of Arl3 at different time points during spermiogenesis and to determine whether this protein plays a functional role in sperm development.

### Materials and Methods

#### Animals

Male CD-1 mice (aged 0 weeks, 1 week, 10 days, 2, 3, 4 weeks and adult) were obtained from the animal center of Nanjing Medical University (Nanjing, Jiangsu, China). All experiments requiring the use of animals were approved by the ethical board of Nanjing Medical University.

#### Sample preparation and protein extraction

Testes obtained from male CD-1 mice were solubilized in lysis buffer (7 M urea, 2 M thiourea, 4% [w/v] CHAPS, 2% [w/v] DTT) in the presence of 1% (w/v) protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL).

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**Figure 1** Western blotting of Arl3 in mouse testis at different developmental stages. The upper panel indicates Arl3 as a dot in the 2DE gel. The middle panel shows western blotting of mouse testis with the anti-Arl3 antibody at the same time points. The lower panel shows the control blots using antibody to β-tubulin.

**Figure 2** Localization of Arl3 in adult mouse testis by immunohistochemical analysis. Magnification is ×200 in (A), ×400 in (B) and (C). (A) Arl3 was highly expressed in the seminiferous tubules. (B) Arl3 was strongly expressed from late stage of pachytene spermatocytes to elongating spermatids in adult mouse testis. At Stage I of spermatogenic cycle, Arl3 was expressed in round spermatids (arrows) and in elongating spermatids (asterisks), but not expressed in early stage of panchytene spermatocyte. At Stage I, Arl3 was expressed in the late stage of pachyte seprmatocyte (arrowheads). (C) Specific staining was not present in tissue incubated with normal rabbit IgG. Scale bars are all 10 μm.
USA), then homogenized and sonicated. The mixture was placed on a shaker at 4°C for 1 h, and the insoluble matter was removed by centrifugation at 40,000 g and 4°C for 1 h. The protein concentration in each sample was determined by the Bradford method, using bovine serum albumin as the standard.

Western blotting

Samples containing 100 μg of protein were electrophoresed on a 12% SDS polyacrylamide gel and transferred onto a PVDF membrane (GE Healthcare, Pittsburgh, PA, USA). Membranes were blocked in TBS containing 5% non-fat milk powder for 1 h and then incubated overnight with anti-Arl3 (1:1000; No: 10961-1-AP; Proteintech, Chicago, IL, USA) or anti-β-tubulin antibody (1:2000; No: ab6046; Abcam, Cambridge, MA, USA) diluted in TBS containing 5% non-fat milk powder. Anti-β-tubulin antibody was used as a loading control. Membranes were washed and then incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1000; Beijing ZhongShan Biotechnology Co., Beijing, China). Specific proteins were detected using an ECL kit and AlphaImagerTM (GE Healthcare).

Immunohistochemistry

Bouin’s solution-fixed, paraffin-embedded sections from mouse testis were immunostained as described previously (Zhang et al., 2007). In brief, after quenching the endogenous peroxidase activity, sections were blocked using blocking serum and then incubated overnight at 4°C with anti-Arl3 antibody (1:100). Sections were then incubated with HRP-conjugated secondary antibody (Beijing ZhongShan Biotechnology, Beijing, China). Immunoreactive sites were visualized in brown using diaminobenzidine and mounted for bright field microscopy (Axioskop 2 plus; Zeiss, Oberkochen, Germany). To confirm the specificity of the anti-Arl3 antibody, negative controls were processed in an identical manner, except that the primary antibody was replaced by normal IgG (ab27478; Abcam, Cambridge, MA, USA).

Indirect immunofluorescence

Testis tissue was embedded in optimal cutting temperature (OCT) compound, cryosectioned into 5-μm-thick sections using a Leica Model CM1900 cryostat (Leica Microsystems, Wetzlar, Germany), mounted on slides and left to dry before fixation. Single cell suspensions prepared from testes using a Medimachine (Becton Dickinson, NJ, USA) were air dried onto slides. The prepared slides were fixed with 4% formaldehyde in PBS for 30 min, washed three times with PBS for 5 min each time and then blocked with goat serum (Beijing ZhongShan Biotechnology) for 2 h at room temperature. Following incubation with primary antibodies (Arl3, 1:100) overnight at 4°C, cells were incubated with FITC-labeled secondary antibody (Beijing ZhongShan Biotechnology) at a 1:1000 dilution for 1 h at room temperature. For negative controls, the primary antibody was replaced with normal rabbit IgG. The nucleus was stained with DAPI. Figure 3 shows the localization of Arl3 in adult mouse testis by indirect immunofluorescence. Magnification is ×400 in (A) and (C), ×1000 in (B). (A) Arl3 was expressed from primary spermatocyte to elongating spermatid. Arrows (in B Merge) indicate Arl3 was distributed as irregular punctate signal in the post-nucleus area in elongating spermatids. White boxed area was highlighted to show the localization. (C) Specific staining was not present in tissue incubated with normal rabbit IgG. Scale bars are all 10 μm.

Figure 3 Localization of Arl3 in adult mouse testis by indirect immunofluorescence. Magnification is ×400 in (A) and (C), ×1000 in (B). (A) Arl3 was expressed from primary spermatocyte to elongating spermatid. Arrows (in B Merge) indicate Arl3 was distributed as irregular punctate signal in the post-nucleus area in elongating spermatids. White boxed area was highlighted to show the localization. (C) Specific staining was not present in tissue incubated with normal rabbit IgG. Scale bars are all 10 μm.
Figure 4  Distribution of Arl3 at each stage of germ cell development, from spermatocyte to sperm. Magnification is ×1000. Indirect immunofluorescence shows Arl3 was expressed as punctate signal in pachytene spermatocytes. In round spermatids, the signal was still in the cytoplasm, with a similar distribution to the centrosomes. In elongating spermatids at Steps 8–15, Arl3 was located in the posterior head of spermatids, consistent with the involvement in the development of the spermatid tail. In the sperm from the caudal epididymis, Arl3 was located in the axon of the sperm tail.
5 μg/ml Hoechst H33342 (Sigma, St. Louis, MO, USA) for 30 s or 5 μg/ml propidium iodide (PI; Sigma, St. Louis, MO, USA) for 5 min. For step 13 spermatids, six different planes were scanned using a CarlZeiss LSM710.

Intratesticular injection of siRNA against Arl3

Double-stranded Arl3 Stealth™ siRNAs against Arl3 mRNA (Invitrogen, Grand Island, NY, USA; Catalog Nos MSS226063, MSS226065 and MSS226067) were diluted to a final concentration of 20 μM and stored at −20°C. The intratesticular injection was similar to that previously described (Lu et al., 2006; Guo et al., 2010). In brief, 3-week-old male mice were anesthetized with sodium pentobarbital and testes were exteriorized through a midline abdominal incision ~5-mm long. Approximately 3–5 μl of individual siRNA mixed with indicator (0.4% trypan blue) was injected into the seminiferous tubules via the rete testis. Control testes were injected with negative control siRNA (Invitrogen; No. 12935-400). Following injection, the testes were replaced into the abdomen and the incisions were sutured. The mice recovered and were raised for another 3 weeks. Western blotting was used to assay the relative changes in protein expression between groups 72 h after injection and verify the efficiency of the three siRNAs against Arl3. Subsequent in vivo RNAi experiments were performed under the same conditions.

Results

Expression of Arl3 during the first wave of mouse spermatogenesis

In a previous study, we constructed the proteome profile that is functional during the first wave of mouse spermatogenesis (Huang et al., 2008). Arl3 appeared at birth as a spot in the 2DE gels and its expression level increased gradually with the development of the testis (Fig. 1). To verify these results, we used mouse testis protein from the same time points as in the proteome profile (0, 1, 2, 3, 4 weeks and adulthood) to perform western blotting. The results showed that the 20 kDa Arl3 protein was weakly expressed from birth to Day 21, with a significant increase in the expression on Day 28 which was maintained until adulthood (Fig. 1).

Location of Arl3 in adult mouse testis

The results of immunohistochemistry showed that Arl3 was strongly expressed at all germ cell types from the pachytene spermatocyte to the elongating spermatids in adult mouse testis (Fig. 2A and B).
In order to clearly demonstrate the location of Arl3, indirect immunofluorescence was performed in adult mouse testis, and it gave similar results to the immunohistochemistry. In particular in elongating spermatids, Arl3 was distributed in the post-nuclear region and in these spermatids close to the lumen, the strong signal appeared as linear streaks consistent with localization to the spermatid tails (Fig.3).

Distribution of Arl3 during spermatogenesis
Further analysis to assess the localization of Arl3 by light microscope, we prepared preparations of the major germ cell types. The results showed that Arl3 expression was detected as a punctate signal in pachytene spermatocytes, and in round spermatids the signal was still distributed in the cytoplasm, in a similar pattern to the centrosomes. However, during spermiogenesis, we found the Arl3 signal was located in the posterior head of the elongating spermatid and was consistent with the development of the spermatid tail (Fig. 4). The distribution was similar to the manchette structure. In the sperm from the caudal epididymis, Arl3 was located in the axoneme of the sperm tail (Fig. 4).

Arl3 is a manchette-associated protein
From the above results, we predicted that Arl3 was located in the manchette. To verify this hypothesis, we conducted the following two experiments. First, we captured spermatids at step 13 to scan the positive signal under a CarlZeiss LSM710 and analyzed the results in six different planes. The results showed the Arl3 signal was located in the distal region of the nucleus (Fig. 5), consistent with the location of the manchette. We then used α-tubulin as a marker for the manchette to compare its location with that of Arl3. The results showed that in spermatids from step 10 to 15, the signals of Arl3 and α-tubulin were overlapping, suggesting that Arl3 was located in the manchette (Fig. 6).

Deformity of sperm after interference with Arl3 during spermiogenesis
The manchette is an essential structure during spermiogenesis and so we predict that Arl3 must play an important role during this process. To confirm this, we carried out in vivo RNAi interference, using double-stranded Arl3 Stealth™ siRNAs. As described in our previous study, mouse spermiogenesis begins 3 weeks after birth (Huang et al., 2008). So we used 3-week-old mice for injection. Injected siRNA materials reached a~40% of the seminiferous tubules. Western blotting demonstrated that Arl3 proteins were significantly suppressed in the testis 72 h after injection with the siRNA MSS226067 (designated as R7; Fig. 7).
Three weeks after intratesticular injection of siRNA against Arl3, sperm from the caudal epididymis were collected for morphological analysis. Two students counted the rate of sperm abnormality with double blind and the variability and the details of the parameters measured were shown in Table I. The results showed that nearly 27% of sperm from testes treated with R7 siRNA against Arl3 were abnormal, compared with 15% in the negative control group \((P < 0.05)\). Sperm with abnormal tail was also significantly increased in the experimental group. Although the rate of sperm with abnormal head had no statistical significance compared with the control group, the tendency was increasing (Fig. 8A). We observed that a large number of sperm from the caudal epididymis in mice treated with R7 SiRNA against Arl3 had an abnormal head shape, a lasso-like coiled tail or were decapitated (Fig. 8B). In sperm from the negative control group, these abnormalities were barely detectable.

**Discussion**

Arl3 was first cloned from human and rat tissues (Cavenagh et al., 1994). Eight years later, the function of the small G protein-encoding \(LdARL-3A\) gene isolated from \(Leishmania donovani\), a homolog of the human \(ARL-3\) gene, was reported (Cuvillier et al., 2000). When a constitutively ‘active’ form of \(LdARL-3A\) was overexpressed, the promastigotes were immobile with a very short flagellum, and the length of the flagellum was inversely proportional to mutant protein expression (Cuvillier et al., 2000). Further studies reported that Arl3 \((-/-)\) mice were born at a sub-Mendelian ratio and failed to thrive (Schrick et al., 2006). These mice exhibited abnormal development of renal, hepatic and pancreatic epithelial tubule structures. Moreover, mice lacking Arl3 exhibited photoreceptor degeneration. These results were the first to show Arl3 in a ciliary disease affecting the kidney, biliary tract, pancreas and retina. Although they did not observe the sperm of Arl3 \((-/-)\) mice, we predict that these mice might exhibit abnormal sperm morphology because the sperm tail is a ciliary structure.

In our study, Arl3 was expressed weakly during the first 3 weeks after birth, and highly expressed from 4 weeks until adulthood. It is known that at 4 weeks, during the first wave of spermatogenesis, spermiogenesis begins with the sperm tail or flagellum and head develops. We therefore initially predicted that Arl3 was involved in mouse spermiogenesis. Further investigation of the detailed distribution patterns of Arl3 expression at different stages during spermatocyte development and spermiogenesis, as described in the results section, were consistent with its involvement in spermiation. The introduction of siRNA into germ cells degrades the target mRNA and stops the cell transcribing new mRNA. The levels of protein expressed by the target gene can therefore be suppressed for a relatively long period.

**Table I** The variability and the details of the parameters measured with double-blind method during sperm abnormality rate analysis.

<table>
<thead>
<tr>
<th>Group</th>
<th>Head abnormality (mean ± SD)</th>
<th>Tail abnormality (mean ± SD)</th>
<th>Total abnormality (mean ± SD)</th>
<th>Data sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neg (%)</td>
<td>9.04 ± 2.3</td>
<td>6.5 ± 0.99</td>
<td>15.54 ± 1.34</td>
<td>From Min</td>
</tr>
<tr>
<td>9.95 ± 2.61</td>
<td>3.72 ± 1.23</td>
<td>13.68 ± 1.43</td>
<td>From Bo</td>
<td></td>
</tr>
<tr>
<td>9.50 ± 2.45</td>
<td>5.11 ± 1.11</td>
<td>14.61 ± 1.39</td>
<td>Combined</td>
<td></td>
</tr>
<tr>
<td>R7 (%)</td>
<td>14.13 ± 4.24</td>
<td>12.06 ± 0.52*</td>
<td>26.19 ± 4.74*</td>
<td>From Min</td>
</tr>
<tr>
<td>14.25 ± 6.53</td>
<td>14.15 ± 1.33*</td>
<td>28.40 ± 5.19*</td>
<td>From Bo</td>
<td></td>
</tr>
<tr>
<td>14.19 ± 5.33</td>
<td>13.10 ± 0.41*</td>
<td>27.30 ± 4.92*</td>
<td>Combined</td>
<td></td>
</tr>
</tbody>
</table>

*\(P < 0.05\).*
long period of time (Aigner, 2006). Phenotypic analysis of sperm from the first wave of spermatogenesis showed that the rate of sperm abnormality, particularly an abnormal head shape, a lasso-like coiled tail or decapitation, was significantly different between the Arl3 siRNA injection group and the control group. This phenotype was very similar to that seen after loss or deletion of many manchette-associated proteins. The Hook1 gene is predominantly expressed in the manchette, and the loss of Hook1 function causes the azh phenotype: abnormal head shape and abnormal bending and looping of the sperm tail (Cole et al., 1988; Mendoza-Lujambio et al., 2002). Katanin p80 is also a manchette-associated protein. Perturbed katanin p80 function results in male sterility characterized by sperm with an abnormal head shape (O’Donnell et al., 2012). RIM-BP3 protein is associated with the manchette, and targeted deletion of the RIM-BP3 gene also resulted in male infertility owing to abnormal sperm heads, characterized by a deformed nucleus and a detached acrosome (Zhou et al., 2009).

During spermiogenesis, the manchette appears at the time when the spermatid nucleus begins to elongate, and disappears as elongation and condensation approach completion. The elongated manchette is thought to be used for temporary storage and transport of structural and signaling proteins (Rivkin et al., 1997; Kierszenbaum, 2001). This sorting mechanism is described as intramanchette transport (IMT) (Kierszenbaum, 2002). Research to date indicates that IMT cannot only transport cargo proteins to the developing sperm tail for use in tail formation, but also transports cargo proteins that are involved in spermatid nuclear condensation (Burfeind and Hoyer-Fender, 1991; Tres and Kierszenbaum, 1996; Brohmann et al., 1997; Kierszenbaum et al., 2002). The manchette is therefore a very important structure for sperm head and tail formation.

In this study, we present preliminary results indicating that mouse Arl3 is a novel manchette-associated protein. However, the in-depth function of Arl3 needs further exploration. In future studies we will explore the protein interactions of Arl3 during manchette development, and further germ cell-specific knockout of Arl3 will be conducted to clarify the molecular mechanism of Arl3 function during mouse spermiogenesis.

Authors’ roles
Y.Q. and M.J. are the co-authors. They contributed to acquisition of data, initial analysis of data. Y.Y. and Y.B. helped Qi and Jiang to do experiments. B.Z. helped count rate of abnormal sperm in the revision. X.G. helped in analysis of data. X.H. contributed to interpretation of data, draft the article and revise it critically for important intellectual content. Z.Z. and J.S. contributed to conception and design of the whole experiment.

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Conflict of interest

None declared.

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