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Unexpected requirement for ELMO1 in apoptotic germ cell clearance *in vivo*

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Abstract

Apoptosis and the subsequent clearance of these dying cells occur throughout development and adult life in many tissues. Failure to promptly clear apoptotic cells has been linked to many diseases¹⁻³. ELMO1 is an evolutionarily conserved cytoplasmic engulfment protein that functions downstream of the phosphatidylserine receptor BAI1, and, along with Dock180 and Rac1, promotes internalization of the dying cells⁴⁻⁷. Here, we generated ELMO1-deficient mice, and unexpectedly found them to be viable and grossly normal. However, ELMO1-deficient mice had a striking testicular pathology, with disrupted seminiferous epithelium, multi-nucleated giant cells, uncleared apoptotic germ cells, and decreased sperm output. Subsequent *in vitro* and *in vivo* analyses revealed a crucial role for ELMO1 in the phagocytic clearance of apoptotic germ cells by Sertoli cells lining the seminiferous epithelium. The engulfment receptor BAI1 and the GTPase Rac (upstream and downstream of ELMO1, respectively) were also important for Sertoli cell-mediated engulfment. Collectively, these findings uncover a selective requirement for ELMO1 in Sertoli cell-mediated removal of apoptotic germ cells and make a compelling case for a relationship between engulfment and tissue homeostasis *in vivo*.

As part of several developmental processes throughout life, we generate excess or superfluous cells; only a small subset of these cells are deemed ‘fit’ for further maturation, while the rest undergo apoptosis and are quickly cleared. Examples of such developmental processes include hematopoiesis in the bone marrow and spermatogenesis in the testes. While several mammalian signaling proteins regulating engulfment have been recently identified based on cell culture studies^{8,9}, our understanding of signaling mechanisms that regulate clearance *in vivo* in mammals is limited. To better understand the *in vivo* requirement for the engulfment protein ELMO1, we generated mice with targeted deletion of *Elmo1*. Murine ELMO1 is a 727 amino acid protein encoded by 21 exons (ref. 6 and

Author Contributions

The project was conceived and the experiments planned by J.J.L., K.S.R and M.R.E. M.R.E. generated the *Elmo1* knockout mice and performed and analysed most of the experiments in this study. S.Z. carried out the experiments using ELMO-GFP constructs. R.I.W. performed all of the IHC, Sertoli cell extraction and sperm counts. M.A.R. performed the microbubble uptake experiments. I.J.J. performed the phagocytosis assays on non-Sertoli cells. D.P. performed phagocytosis assays with Bai1 siRNA and synthesized and characterized Bai1-TSR. *C. elegans* mismigration analysis using *Elmo1* transgenes were conducted by J.M.K. J.Z. assisted with immunoblotting and qPCR studies of Sertoli cells. J.J.L. performed intratesticular injections. K.S.R., M.R.E and J.J.L. wrote the manuscript with input from the co-authors.

Supplementary Fig. S1c). We inserted *loxP* sites flanking exon 5 as it encodes a key functional region (an evolutionarily conserved Armadillo repeat¹⁰) (Supplementary Fig. S1c). Transgenic expression of ELMO1 lacking exon 5 (-exon 5) failed to rescue the distal tip cell migration defects in a *C. elegans* strain mutant for *ced-12*, the *Elmo1* orthologue (while wild-type ELMO1 was able to do so) (Supplementary Fig. S1b)⁴⁻⁷. Mice with ubiquitous deletion of *Elmo1* exon 5 in all tissues (via *Elia-Cre*), showed ~90% reduction in *Elmo1* mRNA and a complete loss of ELMO1 protein (Fig. 1b, c and Supplementary Fig. S2d). Although we were expecting embryonic lethality due to the widespread expression of ELMO1 in many cell types/tissues⁶, surprisingly, *Elmo1*^{-/-} mice were born at the predicted Mendelian ratios from *Elmo1*^{+/-} matings, without gross abnormalities (not shown). Moreover, macrophages (both resident peritoneal and bone marrow-derived) and fibroblasts from *Elmo1*^{-/-} mice engulfed apoptotic thymocytes comparable to controls (Supplementary Fig. S2a-c). This lack of an apparent defect due to loss of ELMO1 may be due in part to a compensatory upregulation of ELMO2 (Supplementary Fig S2d and data not shown). Intriguingly, Sertoli cells from *Elmo1*^{-/-} mice failed to show a compensatory increase in ELMO2 levels (Fig. 1b, c), and the testes of these mice showed a severe defect, which we pursued further.

Sertoli cells are large cells (>40 µm diameter at basal surface) that line the seminiferous tubules of the testes, playing a crucial support/nursing role throughout germ cell differentiation¹¹⁻¹³. During spermatogenesis, non-viable or genetically compromised germ cells (comprising up to 75%), die by apoptosis and are cleared by Sertoli cells^{14, 15}. However, signaling pathways regulating Sertoli cell-mediated clearance of apoptotic germ cells are not well understood. Expression of ELMO1 and DOCK180 proteins were detected in the Sertoli cells (but not germ cells) of normal mice (Fig. 1a and Supplementary Fig. S1a). ELMO1 protein was also detected in mouse Sertoli cell lines (TM4 and 15P-1) (Supplementary Fig. S1a, and not shown).

Histological examination of the testes from 6-8 week old *Elmo1*^{-/-} mice revealed a striking disruption of the normal cellular organization of the seminiferous epithelium (Fig. 1e). There was also an increase in the number of multinucleated giant cells in the *Elmo1*^{-/-} mice (Fig. 1d, e) and abundant vacuolar spaces (Fig. 1e). Giant cells arise from spermatocytes that abort meiosis and appear as a syncytium. Appearance of such giant cells is linked to defective spermatogenesis in genetically altered mice and in human pathologies linked to infertility^{16, 17}. These observations suggested that ELMO1 is required for normal homeostasis of the seminiferous epithelium in mice.

Although ~75% of germ cells die by apoptosis, very few apoptotic cells are seen in the normal testes (Fig. 2a and reference 18). *Elmo1*^{-/-} mice displayed a significantly higher number of apoptotic germ cell nuclei compared to littermate controls (Fig. 2a, b). The fraction of seminiferous tubules containing cell corpses was increased in *Elmo1*^{-/-} mice (not shown), as well as the average number of corpses per tubule (Fig. 2b), with some tubules having >20 apoptotic cells (Fig. 2a). Thus, the loss of ELMO1 appeared to significantly increase the incidence and frequency of tubules containing uncleared apoptotic cells. Although we have previously established that the majority of the apoptotic cells occurs in the early and middle stages of spermatogenesis (stages I-VIII)¹⁹, due to the severe disruption of the seminiferous epithelium in the *Elmo1*^{-/-} mice, we were unable to definitively determine the stages where the increased apoptotic nuclei are seen (Figs. 1e and 2a). It is noteworthy that there was no significant difference between the control and *Elmo1*^{-/-} mice in the total numbers of Sertoli cells isolated, their morphology or the expression of Sertoli cell markers (Figs. 2c-e, and Supplementary Figure S4a). Thus, the development and maintenance of Sertoli cell numbers appear to occur normally in mice lacking ELMO1.

We next asked whether the loss of ELMO1 affected the ability of Sertoli cells to engulf apoptotic germ cells. A technical issue in performing engulfment assays using germ cells is that Sertoli cells are also nurse cells and therefore can bind to both live and apoptotic germ cells. To distinguish bound and internalized germ cells, the targets were labeled with a pH-dependent fluorescent dye (CypHer5) that displays significantly enhanced fluorescence upon uptake and entry of apoptotic cells into the acidic environment of the phagolysosome (Fig. 2g and Supplementary Fig. S4c). Sertoli cells from *Elmo1*^{-/-} mice showed significant reduction in the internalization of apoptotic germ cells as determined by fluorescence microscopy and flow cytometry (Figs. 2e, g-h, and Supplementary Fig. S4c). Sertoli cells from *Elmo1*^{-/-} mice were also significantly impaired in their ability to engulf negatively charged 2.1 μ m carboxylate-modified beads, which can serve as surrogate apoptotic targets⁷ (Fig. 2e, f and Supplementary Fig. S4b). These data suggested that the defective engulfment of apoptotic germ cells by Sertoli cells in the absence of ELMO1 could have contributed to the phenotype of the *Elmo1*^{-/-} mice.

To directly test the requirement for ELMO1 in Sertoli cells, we crossed mice carrying a floxed *Elmo1* allele (*Elmo1*^F, Supplementary Fig. S1c) with *Amh*-Cre transgenic mice, where the Cre recombinase is expressed under control of the *anti-Müllerian hormone* promoter specific for Sertoli cells²⁰. Sertoli-specific deletion in this system was confirmed using a YFP reporter strain crossed with the *Amh*-Cre transgenic mice (Supplementary Fig. S5a, b). Moreover, *Elmo1* mRNA was specifically reduced in the Sertoli cells in the *Amh*-Cre/*Elmo1*^{F/F} mice (Fig. 3b). Analysis of the seminiferous epithelium of the *Amh*-Cre/*Elmo1*^{F/F} mice showed a significant increase in the number of uncleared apoptotic nuclei compared to control littermates (3.6-fold, $p=0.04$) (Fig. 3a, c). We also noted that the disruption of the seminiferous epithelium observed in *Elmo1*^{-/-} mice was not readily detected in the *Amh*-Cre/*Elmo1*^{F/F} mice, possibly due to residual ELMO1 expression (Fig. 3b). This less severe phenotype in the *Amh*-Cre/*Elmo1*^{F/F} context allowed us to stage the seminiferous tubules with respect to spermatogenesis; 90% of the detectable apoptotic nuclei in the *Amh*-Cre/*Elmo1*^{F/F} mice were restricted to the early and middle stages of spermatogenesis (Supplementary Fig. S5c), essentially similar to the littermate controls. To determine if loss of ELMO1 affected spermatogenesis in these mice, we quantified sperm production in the *Amh*-Cre/*Elmo1*^{F/F} mice. Compared to littermate controls, these mice displayed a reduction in mature sperm at 8 weeks of age ($n=6-8$ testis per group, $p=0.007$) (Fig. 3d). Taken together, these data suggested a specific requirement for ELMO1 in the Sertoli cell-dependent homeostatic clearance of apoptotic germ cells *in vivo*, with consequences for sperm production.

We also tested the ELMO1 signaling pathway in Sertoli cell lines *in vitro*. Overexpression of ELMO1 in the TM4 Sertoli cell line promoted phagocytosis, while expression of a previously known dominant-negative mutant of ELMO1 (T625)²¹ inhibited uptake of apoptotic targets (Fig. 3e). ELMO1 in Sertoli cells also appeared to be enriched around targets during engulfment (Supplementary Fig. S4d). Previously, ELMO1 along with DOCK180 has been shown to function as a guanine nucleotide exchange factor (GEF) for the GTPase RAC1 and thereby promote cytoskeletal rearrangement during engulfment^{6, 21}. Overexpression of wild-type ELMO1 consistently enhanced RAC-GTP levels, while the ELMO1-T625 mutant failed to enhance RAC-GTP (Fig. 3f). Furthermore, siRNA-mediated depletion of RAC1 in TM4 cells reduced the phagocytic capacity by >75% (Fig. 3g), indicating that RAC1 is necessary for Sertoli cell engulfment. Thus, ELMO1 appears to promote downstream activation of RAC1 during phagocytosis by Sertoli cells.

We next sought to gain a better understanding of how Sertoli cells recognize apoptotic germ cells and the role of known upstream engulfment receptors in signaling to ELMO1. Uptake of apoptotic targets by Sertoli cells was phosphatidylserine dependent as determined by

addition of annexin V, which masks the recognition of PtdSer on targets (data not shown). Furthermore, Sertoli cells could mediate the specific recognition and internalization of fluorescently labeled phosphatidylserine-containing bubbles, but not control bubbles containing only phosphatidylcholine (Fig. 4a). We then assessed the relative expression of three recently identified PtdSer recognition receptors *Bai1*, *Tim4*, and *Stabilin2* in primary murine Sertoli cells^{7, 22, 23}. Remarkably, *Bai1* was expressed the highest in Sertoli cells, with *Stabilin2* and *Tim4* at or below the limits of detection (Fig. 4b). Although several TIM family members (TIM-1, TIM-3 and TIM-4) have been linked to PtdSer recognition, none of these appeared to be expressed in the testes (Mammalian Reproductive Genetics database). BAI1 (brain angiogenesis inhibitor 1) is a seven-transmembrane protein belonging to the type II adhesion GPCR family, with an extended extracellular domain that can recognize PtdSer via thrombospondin type 1 repeats (TSR). BAI1 also interacts via its cytoplasmic tail with ELMO1, and can activate RAC through the ELMO1/DOCK180 complex thereby promoting apoptotic cell phagocytosis⁷. Transfection of TM4 Sertoli cells with BAI1 siRNA led to a significant reduction in engulfment (Fig. 4c, 41.3% engulfment in control siRNA treated compared to 31% in *Bai1* siRNA, n=3, $p<0.05$).

Prior to addressing the potential role of BAI1 in engulfment *in vivo*, we designed an *in vivo* model, wherein apoptotic targets can be injected into the rete testes of mice by *in vivo* micropuncture, providing a retrograde filling of the seminiferous tubules (Fig. 4d). We injected surrogate apoptotic targets (fluorescently labeled 2.1 μ m carboxylate modified beads) into the rete testis of mice, and 24-hours later prepared cryosections or squash preparations of the testes. Introduction of targets via this approach filled the seminiferous tubules on the luminal side of the testes and the beads specifically clustered/ingested along the lining of the tubule where Sertoli cells were present (Fig. 4d). We also established conditions for introducing soluble proteins/peptides into the interstitium of the testes by intratesticular injection. To address the potential role of BAI1 in engulfment by Sertoli cells *in vivo*, we used a soluble fragment containing the TSR region of BAI1 (denoted GST-BAI1-TSR)⁷ that strongly blocked the *in vitro* binding/internalization of apoptotic targets to murine Sertoli cells (Fig. 4e). We microinjected soluble BAI-TSR peptide into the interstitium of one testes and the control protein GST in the second testis of the same animal (3 mice/group). After 8 days, we analyzed the testes and made three striking observations. First, there was a marked increase in apoptotic germ cell nuclei, analogous to the genetic disruption of *Elmo1* (Fig. 1e and 4f). Second, we also saw a marked disruption of the seminiferous epithelium similar to the *Elmo1*^{-/-} mice (Fig. 1d and 4f). Third, injection of BAI1-TSR also led to reduced levels of mature sperm in the testes (Fig. 4g). Thus, the acute disruption of apoptotic cell recognition through BAI1-TSR confirms and extends the phenotype seen in mice with genetic deletion of *Elmo1* in Sertoli cells.

Collectively, these studies define a mammalian engulfment signaling pathway required for efficient clearance of apoptotic germ cells and identify a non-redundant role for the cytoplasmic engulfment protein ELMO1 and its upstream engulfment receptor BAI1 in this process^{6, 7}. Our findings also provide a formal genetic link between ELMO1 and one of the key functions of Sertoli cells, i.e. the clearance of apoptotic germ cells.

Although it has long been proposed that clearance of dying cells is a key component of organogenesis in mammals, genetic models demonstrating this have been minimal. The data presented here using conditional *Elmo1* knockout mice provides such an example. Still, our work does not exclude contribution from other signaling pathways that may operate concurrently for Sertoli cell-mediated clearance of apoptotic germ cells. Other corpse recognition mechanisms may also be operational in Sertoli-cell mediated clearance of apoptotic germ cells, and testes homeostasis, including scavenger receptors (SRB1, CD36)^{24, 25} and MER/Axl/Tyro3 family members^{26, 27}, although signaling downstream of

these receptors are not well defined. Additional PtdSer recognition/bridging molecules (such as MFG-E8) and integrins expressed in the testes may provide further redundancy in corpse clearance^{28, 29}, and whether ELMO1 may participate signaling downstream of these additional recognition mechanisms in Sertoli cells is unclear. Due to the less pronounced phenotype in the *Amh-Cre/Elmo1^{FF}* mice compared to straight *Elmo1^{-/-}* mice, it is possible that ELMO1 may also play a role in other cell types in the testes. Finally, this work reveals a novel connection between efficient corpse clearance and spermatogenesis that may open up newer avenues of investigations into immune privilege in the testes and removal of non-viable germ cells. Since the failure to promptly recognize and clear dying cells has deleterious consequences (such as autoimmunity)^{28, 30}, defining the engulfment steps during germ cell clearance could be important for disease states such as orchitis, and loss of tolerance to testicular antigens.

Methods

Antibodies and reagents

The anti-ELMO1 antibody has been described previously²¹. Anti-ELMO2 was generated by immunizing rabbits with GST-tagged full-length mouse ELMO2. Anti-ELMO1 antibody (for immunohistochemistry), Erk2, Dock180, Anti-Müllerian hormone and CathepsinL antibodies were purchased from Santa Cruz Biotechnology Inc. Other antibodies were purchased from the following sources: RAC1 (Upstate Biotechnology), F7-26 anti-ssDNA (Apostain; Alexis Biochemicals), -actin (Sigma-Aldrich), and GFP (Invitrogen). DNA oligonucleotides were obtained from Integrated DNA Technologies. Chemicals used for isolation and routine culture of Sertoli cells were purchased from Sigma-Aldrich.

Elmo1 conditional knockout mice

The *Elmo1* targeting vector was generated using an 11.5 kb *Bam*HI-*Xho*I fragment of *Elmo1* genomic DNA containing exon 5 isolated from a Sv/129 mouse strain BAC library (Incyte Genomics). The 5' and 3' homology arms were inserted into the pK11-*flr*-PgkNeo-*flr*-*loxP* plasmid³² using standard subcloning procedures. For the 5' homology arm, a 3.0 kb *Bgl*II to *Apa*I fragment was inserted 5' to the *flr*-flanked neomycin resistance cassette. The 4.3 kb *Apa*I to *Avr*II fragment containing exon 5 was inserted 3' of the neomycin cassette. A *loxP* site was introduced into the *Apa*I site 3' of the second *flr* sequence. A second *loxP* site was inserted into the *Kpn*I site 2.3 kb downstream of the first *loxP* site to flank exon 5 to allow conditional Cre-mediated deletion. The entire *Elmo1* region of the targeting construct was sequenced to ensure fidelity. IB10 ES cells were electroporated with 20 µg of *Sac*I-linearized vector and selected with 200 mg/mL Geneticin (Invitrogen) on mitomycin C-treated MEF cells. Of 224 neomycin-resistant ES clones screened, two were positive for homologous recombination as determined by PCR and Southern blotting using multiple restriction enzymes and probes (Fig. S1d and data not shown). After blastocyst injections, chimeras were bred to C57BL/6 mice. The offspring were then crossed to -actin/Flp mice (Jackson) to delete the neomycin cassette, yielding mice carrying a "floxed" *Elmo1* exon 5 allele (*Elmo1^F*, Fig. S1d). For the straight *Elmo1* knockout, mice with floxed *Elmo1* allele were crossed to *Elia*-Cre mice (Jackson) expressing Cre from the two cell stage of embryonic development to delete *Elmo1* exon 5 in all tissues³³. These mice were then crossed with C57BL/6 to remove Flp and Cre from background. Resulting mice, lacking Flp and Cre, were then intercrossed to obtain the straight knockout mice used for this study.

For Sertoli cell-specific targeting, mice expressing Cre under control of the *anti-Müllerian hormone* promoter (*Amh*-Cre) were obtained from Jackson Laboratory²⁰. Specificity of Cre-mediated deletion in Sertoli cells was tested by crossing *Amh*-Cre+ males to female *Rosa26-YFP* mice expressing EYFP in the *Rosa26* locus downstream of a *loxP*-flanked STOP

cassette³⁴. The testes of mice positive for *YFP* and *Amh-Cre* were tested for Sertoli-specific deletion of the STOP cassette (by YFP expression) by immunohistochemistry and confocal fluorescent imaging of whole-mounted seminiferous tubules (Fig. S5). For targeted deletion of *Elmo1* in Sertoli cells, *Elmo1*^{F/F} females were crossed with *Amh-Cre*⁺/*Elmo1*^{F/W} males to obtain the *Amh-Cre*⁺/*Elmo1*^{F/F} mice used in this study.

Mouse genotyping

Genotyping by Southern blot was carried out using probes generated by PCR from *Elmo1* BAC template DNA. Two probes, one 5' (SBP2) and another 3' (SBP5) of the targeting sequence were generated using the following primers: SBP2F: 5'-tct cct cct gct ttc tca ttc cgt, SBP2R: 5'-ggc ctg tta cag tta gag aga acc ca, SBP5F: 5'-tct ccc ctt tgc caa tcc cac atc, SBP5R: 5'-cag ctg cgg agg gac ttt aag gaa. Southern blotting of *Bgl*I digested of genomic DNA yielded the predicted fragments of 10 kb (WT), 4.9 kb (targeted) and 8.1 kb (*Elmo1*^{Δ5}) for both probes. Also, *Nhe*I and *Sac*I double digestion of DNA produced the predicted fragments of 10.6 kb (WT) and 8.6 kb (*Elmo1*^{Δ5}) mice by Southern blotting with SBP2.

Routine genotyping of mice was done by PCR using the following primers: *Elmo1* WT (F): acc cat atc agg ctg ccc aca taa, *Elmo1* WT (R): atg caa tgt gag cag cat tcc ctc, *Elmo1*^{Δ5} (F): gct tcc tga cgg tgg atg caa tgt, *Elmo1*^{Δ5} (R): ggc cgc gtc gac gaa gtt cct ata. For detection of WT versus floxed *Elmo1* alleles, *Elmo1* WT primers were used and yielded amplicons of 302 bp (WT) and 342 bp (floxed). For detection of WT versus exon5 deleted alleles, all four primers were used in a single PCR reaction and yielded bands of 302 bp (WT) and 338 bp (Δ5). PCR screening for *Amh-Cre* was done using the PCR primers and conditions recommended by the provider.

Sertoli cell isolation and tissue culture

For isolation of primary murine Sertoli cells according to previously published methods³⁵, 10-12 day old animals were euthanized and the testes removed and decapsulated in HBSS. The seminiferous tubules were washed once in HBSS and then gently dispersed in HBSS/0.25% trypsin/10 μg per mL DNase using fine tweezers. To this, 0.25% final soybean trypsin inhibitor (SBTI) was added and the supernatant containing interstitial cells was discarded. Dispersed tubules were then resuspended in HBSS/1 M glycine/2 mM EDTA/0.01% SBTI/10 μg per mL DNase and incubated at room temperature for 10 min and centrifuged at 1000xg for 2 min. The tubules were resuspended in HBSS/1mg per mL hyaluronidase/10 μg per mL DNase, minced with a razor and incubated for 30 min in a 37°C shaking water bath. Resulting fragments were allowed to sediment in a tube for 10 min at room temperature and the suspended material removed and Sertoli cell-enriched fragments were washed in HBSS by centrifugation. The fragments were then incubated with HBSS/1mg per mL hyaluronidase/10 μg per mL DNase and incubated for 30 min in a 37°C shaking water bath to release Sertoli cells. Cells were then washed once in HBSS and resuspended in complete Sertoli medium (F12/DMEM medium with 10% HIFBS, 1% Na pyruvate, 15 mM HEPES, 5 μg/mL transferrin, 2.5 ng/mL EGF, 10 μg/mL insulin, 1% Pen-Strep). Cells were plated on 10 cm tissue culture dishes and incubated at 34°C/5% CO₂. After 2 days, non-adherent cells were removed by washing with complete medium. Fresh complete medium was added and the adherent Sertoli cells were incubated at 34°C/5% CO₂ for 4 more days. Cells were then collected by trypsinization, and the total number of Sertoli cells were counted by Trypan exclusion on a hemacytometer (Figure 2c) and replated as indicated. This population was found to be >90% positive for Sertoli markers Cathepsin L and anti-Müllerian hormone by immunofluorescence microscopy (see Fig. 2d and Supplementary Fig. S4a).

For apoptotic germ cell preparation, testes of 8-14 week old C57BL/6 mice were prepared as above except that the non-adherent cells were collected after 18-24 hrs. This population was found to be 40-50% apoptotic by flow cytometry (Annexin V+/propidium iodide-, data not shown). TM4 and 15-P1 Sertoli cells were routinely cultured using the same conditions as primary Sertoli cells.

Phagocytosis assays (beads, cells, bubbles)

Sertoli phagocytosis assays were carried out in either complete Sertoli medium for primary Sertoli or in simplified medium (F12/DMEM, 10% HIFBS, Pen-Strep) for TM4 and 15P-1 cells. For bead engulfment, 6 μ L of red fluorescent 2.1 μ m carboxylate beads (Invitrogen) in 500 μ L medium per well were incubated for 2 hrs at 34°C/5% CO₂. For germ cell engulfment, apoptotic germ cells were labeled with 5 μ M CypHer5 dye (GE Life Sciences) in HBSS for 30 min at 37°C/5% CO₂. After washing, 5 $\times$ 10⁵ germ cells in 500 μ L engulfment medium per well were added to the Sertoli cells, centrifuged at 50xg/30 sec and incubated at 34°C/5% CO₂ for 2 hrs. Microbubble uptake was performed as previously described for a total of 30 min⁷. For analysis of all phagocytosis assays, wells were washed 5 times with cold HBSS, trypsinized, and brought to 400 μ L final volume with engulfment medium. Samples were analyzed by flow cytometry.

Immunohistochemistry

Testes were fixed in Bouin's solution, paraffin-embedded and 8 μ m cross-sections prepared. Immunodetection was carried out as previously described using the indicated antibodies¹⁸. For enumeration of apoptotic cells, Apostain labeled sections were examined under 12.5 $\times$ magnification and the total number of tubules and apoptotic cells were counted from at least two sections per testis per mouse.

Giant cell analysis

Testes were fixed in Bouin's solution and cross sections prepared and stained with hematoxylin and eosin. Giant cells were identified by their characteristic features of having multiple ($\geq$ 2) round spermatid nuclei in a syncytial body present in the lumen. For each section of testis, the total number of giant cells along with the total number of seminiferous tubules were counted and the average number of giant cells per tubule determined (Figure 1d).

Immunofluorescence microscopy

Primary murine Sertoli cells were plated in culture medium on LabTek II chamber slides (Nunc) at 3-5 $\times$ 10⁴ cells/well and cultured overnight. Cells were washed and fixed with 3.2% paraformaldehyde in PBS for 10 min at room temperature and permeabilized in 0.2% Triton X-100 in PBS for 2 min at room temperature. After blocking with 1% BSA/PBS, cells were stained with the indicated antibody (1:100) or phalloidin-Alexa488 (Invitrogen) at 1:50 for 1 h at room temperature. After washing, cells were incubated with fluorochrome-labeled secondary antibodies for 1 hr at room temperature, washed and mounted with ProLong Gold + DAPI (Invitrogen). Microscopy was performed using the Axio Imager 2 (Zeiss).

qPCR

DNAase I-treated RNA was obtained by RNeasy (Qiagen), and 50-100 ng of RNA was used as template to generate cDNA using Superscript III SuperMix (Invitrogen). qPCR was performed using gene-specific TaqMan probes on a StepOne Plus Real-Time PCR instrument (Applied Biosystems). TaqMan probes used are as follows: *mElmo1* (Mm00519109_m1), *mElmo2* (Mm01248046_m1), *mElmo3* (Mm00555221_g1), *mBai1*

(Mm00558144_m1), m *Tim4* (Mm00724709_m1), m *Stabilin2* (Mm00454684_m1), m *Gapdh* (4352339E).

Rac-GTP assay

The amount of endogenous active (GTP-bound) Rac was assayed by precipitation of cell lysates using GST-CRIB beads as previously described³⁶. Briefly, 24 hrs after plasmid transfection TM4 cells were washed twice and lysed. The cell lysates were incubated with 30 µg of GST-CRIB immobilized on beads for 1 h at 4 °C. The precipitation of RAC by GST-CRIB was detected by immunoblotting with anti-RAC1.

Transfections

For knockdown experiments, TM4 Sertoli cells were transfected with 1 µg of SmartPool siRNA (Dharmacon) using the Nucleofector Kit L with the T-30 program according to manufacturers' instructions (Amaxa). Forty-eight hours post-transfection, cells were trypsinized and replated at 5×10⁴ cells/well of a 24-well plate in triplicate and cultured overnight prior to phagocytosis assay. For overexpression experiments, TM4 cells were transfected as above using 5-15 µg of previously described ELMO1 and GFP plasmid DNA⁷ and used in assays at 24 hours post-transfection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Disrupted seminiferous tubule architecture in ELMO1-deficient mice

a, Immunolocalization of ELMO1 and DOCK180 in Sertoli cells (arrows) of adult C57BL/6 mice testes. Control, secondary antibody only. **b**, Immunoblotting of lysates from testes of adult mice with the indicated *Elmo1* genotypes. A non-specific (n.s.) band served as an internal loading control. **c**, mRNA levels of *Elmo1*, 2 and 3 in Sertoli cells relative to *Gapdh* and normalized to WT for each gene. * $p=0.01$, $n=3$ mice per group. **d**, Average number of giant cells per tubule in testes of 6-week old mice, * $p=0.03$, $n=3$ or 4 per group. **e**, H & E sections of testes of mice with the indicated *Elmo1* genotypes. Magnification, 10× (upper

panels); 25 $\times$ (*lower* panels). Arrow, a giant cell; arrowhead, vacuolar spaces in the epithelium. Error bars indicate s.e.m.

Figure 2. Requirement for ELMO1 in apoptotic cell clearance *in vivo* and *in vitro*

a, Detection of apoptotic cells in the testes of wild-type and *Elmo1*^{-/-} mice by IHC (apostain, brown) viewed under 10× (*left*) and 25× (*right*) magnification. Arrow, apoptotic cells. **b**, Average number of apoptotic nuclei per tubule. **p*=0.04, *n*=3 or 5 mice per group. **c**, Total number of Sertoli cells isolated from testes of individual mice, *n*=4. **d**, **e**, Fluorescence microscopy of WT and *Elmo1*^{-/-} Sertoli cells. **d**, Staining with antibodies against the Sertoli cell marker anti-Müllerian hormone (anti-Amh, green) or control IgG (inset). **e**, Sertoli cells were fed 2.1µm carboxylate beads (red) or apoptotic mouse spermatogenic germ cells labeled with CypHer 5 (red). Green, phalloidin staining. Arrows, cells with

engulfed targets; arrowheads, individual engulfed targets. Blue, DAPI-stained nuclei. Scale bar, 50 μ m. **f-h**, *In vitro* phagocytosis by WT and *Elmo1*^{-/-} Sertoli cells. Uptake of fluorescent 2.1 μ m carboxylate bead uptake (**f**) and CypHer 5-labeled apoptotic germ cells (**h**) was quantified by flow cytometry, *n*=4 mice per group, **p*<0.005. **g**, Representative flow cytometry histograms of data in **h**.

Figure 3. ELMO1 functions in Sertoli cell-mediated apoptotic germ cell clearance

a, Detection of apoptotic cells (apostain, brown) in testes of 6-8 week *Amh-Cre/Elmo1^{F/W}* and *Amh-Cre/Elmo1^{F/F}* mice (arrow, apoptotic cell). **b**, qPCR of *Elmo1* and *Elmo2* mRNA levels in Sertoli cells from *Amh-Cre⁺* mice with the indicated genotypes. **c**, Average number of apoptotic cells per tubule. * $p=0.04$, 4-6 mice per group. **d**, Total sperm output in 8 week *Amh-Cre⁺ Elmo1^{F/F}* mice with littermate controls. * $p=0.007$, $n=6-8$ testis per group. **e**, Phagocytosis by TM4 Sertoli cells expressing GFP, GFP-tagged wild-type or dominant-negative ELMO1 (T625) expression constructs. **f**, RAC1-GTP levels in TM4 Sertoli cells transfected with the indicated constructs. RAC1-GTP relative to actin was quantified by

densitometry. **g**, Uptake of 2.1 μ m carboxylated targets by TM4 Sertoli cells following siRNA knockdown of RAC1. *Inset*, immunoblot of lysates after RAC1 knockdown. DOCK180, loading control.

Figure 4. BAI1 participates as an engulfment receptor in Sertoli cell-mediated engulfment
a, Phosphatidylcholine (PC, blue) or phosphatidylserine (PtdSer, red) microbubble uptake by TM4 Sertoli cells. $*p < 0.05$, $n=4$. **b**, Levels of PtdSer recognition receptors by qPCR from the indicated murine tissues. # indicates mRNA below detectable level. **c**, Phagocytosis by TM4 Sertoli cells transfected with control or BAI1 siRNA. $*p < 0.05$. **d**, *Left*, schematic of microinjection into rete testis. Rete testis and efferent ducts prior to (*top right*) and 24 h after injection of fluorescent microspheres under light (*middle*) and fluorescence (*lower*) microscopy of whole mounted seminiferous. Dashed lines, tubule wall borders. Arrow, cluster of microspheres internalized by Sertoli cells. **e**, 15P-1 Sertoli cells on Matrigel

incubated with fluorescent targets along with GST or BAI1-TSR protein. **f-g**, Intra-testicular injection of GST or BAI1-TSR into 6-8 week old C57BL/6 mice. **f**, Apoptotic cells detected by IHC (apostain, brown) after 24 hr. Magnification, 12.5× (*upper* panels) and 25× (*lower* panels). Arrow, apoptotic cells. **g**, Sperm counts normalized to testis weight. $n=3$, * $p<0.05$.