Phosphatidylserine on viable sperm and phagocytic machinery in oocytes regulate mammalian fertilization

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Fertilization is essential for species survival. Although Izumo1 and Juno are critical for initial interaction between gametes, additional molecules necessary for sperm:egg fusion on both the sperm and the oocyte remain to be defined. Here, we show that phosphatidylserine (PtdSer) is exposed on the head region of viable and motile sperm, with PtdSer exposure progressively increasing during sperm transit through the epididymis. Functionally, masking phosphatidylserine on sperm via three different approaches inhibits fertilization. On the oocyte, phosphatidylserine recognition receptors BAI1, CD36, Tim-4, and Mer-TK contribute to fertilization. Further, oocytes lacking the cytoplasmic ELMO1, or functional disruption of RAC1 (both of which signal downstream of BAI1/BAI3), also affect sperm entry into oocytes. Intriguingly, mammalian sperm could fuse with skeletal myoblasts, requiring PtdSer on sperm and BAI1/3, ELMO2, RAC1 in myoblasts. Collectively, these data identify phosphatidylserine on viable sperm and PtdSer recognition receptors on oocytes as key players in sperm:egg fusion.
Sexual reproduction requires a productive fusion between the haploid male and female gametes. Prior to the fusion of the gametes, a critical step is the proper recognition between specific ligand(s) on the sperm and appropriate binding partner(s) on the egg. Recent studies both at the functional and structural levels have unequivocally established a critical role for the sperm surface protein Izumo1 and the corresponding GPI-anchored receptor Juno on the oocyte, with blocking or loss of either protein affecting fertilization. Signaling downstream of Juno in oocytes is yet to be defined, as Juno is a GPI-anchored protein. Further, 3D structure studies suggest that the Izumo1:Juno interaction is unlikely to lead to fusion and, when Izumo1 was exogenously expressed on Cos-7 cells, oocyte binding to these cells occurred but did not proceed to fusion. The tetraspanin family member CD9 on the oocyte has also been linked to mammalian fertilization. CD9 has no known ligand and it is thought to modify the membrane curvature. Interestingly, PtdSer can also be transiently exposed on viable sperm, is recognized by specific receptors located on the microvilli of the oocyte to promote sperm:egg fusion. The signaling pathway ELMO1/RAC1, downstream of the PtdSer receptors BA11/3, also participates in this event. This pathway is also conserved in the fusion of sperm with myoblasts. Taken together, our results shed light into the molecular mechanism of sperm:egg fusion.

Results

PtdSer exposed on viable sperm is required for fertilization. As part of our studies on apoptotic germ cell clearance in the testes, we noticed Annexin V staining on freshly isolated sperm from the cauda epididymis (Fig. 1a, b), suggestive of PtdSer exposure (Fig. 1c, d). Although PtdSer has been noted on sperm previously, it was considered to mark dead or non-viable sperm because PtdSer is a key eat-me signal on cells undergoing apoptosis, which facilitates recognition and uptake by phagocytes. Interestingly, PtdSer can also be transiently exposed on viable cells in certain conditions (such as activated B and T cells, myoblasts, or macrophages), and therefore, we further investigated the relevance of PtdSer exposure on the sperm.

Annexin V staining was prominently seen both on the sperm head and the midpiece, but was absent in the tail (Fig. 1c, d). During spermatogenesis, after exiting the testis, sperm transits through different segments of the epididymis: the caput, the corpus and the cauda (Fig. 1a). Classical experiments have shown that only the caudal sperm is capable of fertilization. Therefore, we assessed PtdSer exposure on sperm as it transits through the epididymis. We noticed a progressive increase in PtdSer exposure on sperm isolated from different segments of the epididymis, with the cauda epididymis, which contains the fertilization-competent sperm, displaying the highest percentage of PtdSer-positive sperm (Fig. 1e). This also indicates that PtdSer externalization is not merely an effect of sperm isolation. When we addressed whether PtdSer exposure on sperm changes with capacitation, a process known to occur in the female reproductive tract, we found a further increase in the percentage of PtdSer-positive sperm after capacitation in vitro (Supplementary Fig. 1a). The acrosome reaction is another process that occurs on sperm in the female tract. When we induced the acrosome reaction in vitro with the ionophore A23187, PtdSer continued to be detectable on sperm. Further, as Izumo1 is exposed on caudal sperm after the acrosome reaction and is a central player in fertilization, we asked whether PtdSer colocalizes with Izumo1 on sperm. Izumo1 (detected via antibody), as well as PtdSer (via Annexin V), were colocalized in the equatorial region of the sperm head (known to be involved in sperm:egg fusion) (Supplementary Fig. 2). As Annexin V can bind both PtdSer and phosphatidylethanolamine, we tested PtdSer exposure on the sperm using a second reagent. We have previously established that the soluble extracellular fragment of the PtdSer recognition receptor BA11 (BA11-TSR fused to the glutathione-S-transferase; GST) binds PtdSer but not PtdEtn; BA11-TSR (but not the control GST protein) preferentially bound to the sperm head without significant midpiece binding (Fig. 1f, g). Consistent with this observation, when we used Duramycin, which binds PtdEtn but not PtdSer, the binding was noted prominently in the midpiece with much less binding to the head (Supplementary Fig. 1b). These data suggested specific exposure of PtdSer on the heads of freshly isolated sperm from the cauda epididymis.

To address whether the sperm with PtdSer exposed on their surface are viable, we performed time-lapse microscopy, revealing that the PtdSer+ sperm were motile (Fig. 1h, and Supplementary movie 1). Further, when we analyzed the expression on the caudal sperm of cleaved caspase 3 (CC3), a marker for apoptosis, the Annexin V+ sperm were negative for CC3 (Supplementary Fig. 1c). Only after 24 h incubation ex vivo, some of the sperm showed CC3 staining (Supplementary Fig. 1d). These data suggested that PtdSer is exposed on the surface of viable and motile sperm.

We next asked whether the PtdSer exposure on sperm is functionally important for fertilization. To test this, we performed in vitro fertilization using capacitated caudal sperm and oocytes from super-ovulated C57BL/6 female mice, and quantified the emergence of two-cell embryos (see schematic in Fig. 1i). We deliberately chose three different reagents to target PtdSer, as each have unique features that are complementary, and can inform us better about the role of PtdSer during fertilization: (1) Annexin V (which has the highest affinity); (2) BA11-TSR peptide derived from the extracellular region of the PtdSer receptor BA11 fused to the GST (lower affinity than Annexin V but greater specificity for PtdSer); and (3) the soluble head group of the lipid phosphatidylserine, which has the lowest affinity of the three blocking agents but can act as a competitive inhibitor for PtdSer recognition receptors. Pleasingly, all three reagents supported the hypothesis that PtdSer on sperm contributes to fertilization. Annexin V caused >85% reduction in fertilization in three out of four independent experiments (Fig. 1j, k). Masking PtdSer on sperm with BA11-TSR also significantly reduced fertilization, and consistent with the BA11-TSR being of lower affinity than annexin V, the inhibition of fertilization with BA11-TSR was less pronounced (Fig. 1l). Addition of the soluble Phospho-L-Serine head group also resulted in significant reduction in fertilization in every experiment (Fig. 1m, n). The partial inhibition (30–40%) was expected as the monomeric soluble head groups of PtdSer have to compete against multi-valent PtdSer recognition on the sperm surface. The advantage of using the Phospho-L-serine blocking is that we could directly compare its effect against the stereoisomer Phospho-D-Serine as a control (carrying the same charge), and this did not inhibit fertilization. Of note, we confirmed that progressive motility of the sperm was not affected after masking PtdSer with Annexin V or BA11-TSR (Supplementary Fig. 1e, f). Collectively, these three approaches suggest that recognition of PtdSer on sperm (in addition to the well-described Izumo1) can contribute to in vitro fertilization.
sites, we took a simplified approach using the binding of the fluorescently labeled 2 µm carboxylate modified beads (2CMB), which are known to bind Annexin V and compete with PtdSer-exposing apoptotic cells23. Zona pellucida (ZP)-free oocytes were isolated from wild-type C57BL/6 female mice and were incubated with red-fluorescent 2CMB for 2 h. At the end of the incubation period, the oocytes were stained with CD9 antibody to identify the microvillar region that is known to interact with sperm, fixed, and analyzed by microscopy (Fig. 2a). Oocytes readily bound multiple beads, and this interaction was restricted to the microvillus region of the oocytes (Fig. 2b). Importantly, this binding was significantly decreased when the beads were pretreated with Annexin V staining.
Fig. 1 Phosphatidylserine on live sperm is important for in vitro fertilization. a Depiction of the mouse testis and epididymis. b Sperm from different regions of the epididymis were allowed to swim/disperse in TYH + BSA medium, stained with Annexin V and Hoechst, and evaluated by microscopy. c Annexin V staining of sperm. Asterisks denote sperm heads, and arrows midpiece. Scale bar, 20 μm. d Percentage of Annexin V + sperm from the caput (n = 9 mice), corpus (n = 8 mice), and cauda (n = 15 mice) epididymis, with each dot representing one mouse (six independent experiments). *p < 0.05, **p < 0.01 (one-way non parametric ANOVA was followed by Kruskal–Wallis test for multiple comparisons). e, f Sperm from cauda epididymis were incubated with 50 μg/ml GST only (e) or GST-BAI1-TSR g, washed, fixed and visualized by GST immunofluorescence. Scale bar, 20 μm. h Snap shots of a movie depicting motility of live Annexin V + (green) sperm (t: time in min). The trajectory of a single sperm is traced by a white dotted line. Scale bar, 30 μm. i Schematic of the in vitro fertilization assay: cumulus oocyte complexes isolated from wt super-ovulated females were incubated with caudal sperm previously capacitated, in the presence or absence of 10 μg/ml Annexin V, 50 μg/ml GST, or 50 μg/ml GST-BAI1-TSR. The percentage of fertilized eggs (two-cell embryos) was evaluated after 24 h. j Multiple two-cell embryos fertilization with control sperm (left panel, arrows), whereas fewer fertilized eggs were observed with Annexin V (right panel, arrows). Scale bar, 100 μm. k, l Annexin V masking of PtdSer on sperm (k, n = 4 experiments) or GST-BAI1-TSR (l, n = 4 experiments) reduces the fertilization rate. The total number of eggs analyzed is shown in parentheses. Each line represents one experiment and the matching experiments are shown (shape and color). Error bars are s.e.m. *p < 0.05, **p < 0.01 (Two-tailed unpaired Student's t test). m, n Greater unfertilized oocytes (asterisks) seen after competition with one experiment and matching experiments are shown with the same shape and color (n = 4 experiments). Error bars are s.e.m. **p < 0.01 (Two-tailed unpaired Student’s t test). Source Data are provided in the Source Data File.

In the effecrocytosis field, the PtdSer recognition by PtdSer receptors is known to include redundant mechanisms, as the charged head group of the lipid PtdSer can be recognized in a polycavalent fashion by multiple receptors to provide sufficient avidity and specificity38,40,41. Therefore, we decided to test all of the five potential PtdSer receptors detected in oocytes—CD36, BAI1, BAI3, Tim-4, and Mer-TK—via approaches that target them either singly or in combination. Interestingly, CD36 has been shown to cooperatively function with BAI1 in endothelial cells36. Therefore, to test the potential multi-pronged interaction involving both CD36 and BAI1/3, we tested the effect of antibodies targeting CD36 or BAI1/3 (via antibody that recognizes both BAI1/BAI3), either alone or combination (see schematic in Fig. 2g). Although antibodies to either BAI1/3 or CD36 alone did not inhibit fertilization (Supplementary Fig. 3d), a combination of antibodies targeting both BAI1/3 and CD36 caused a reproducible and statistically significant inhibition of fertilization in vitro (Fig. 2h, i). As a positive control, antibody to Juno could strongly inhibit fertilization (Supplementary Fig. 3i). Next, we used a more direct assay for the sperm entry into oocytes. During fertilization, the nucleus of the sperm decondenses after entry into the oocyte cytoplasm42–44. This early step of fertilization can be scored using oocytes loaded with the DNA binding dye 4’,6-diamidino-2-phenylindole (DAPI), and the appearance of DAPI-stained decondensed sperm DNA (Fig. 2k, l), suggesting that blocking three of the five PtdSer receptors (BAI1/BAI3, and CD36) expressed on oocytes can also impair fertilization in vitro, complementary to the masking of PtdSer on the sperm.

Next, we wanted to genetically test the contribution of oocyte PtdSer receptors to fertilization. Because of the extensive functional redundancy among PtdSer recognition receptors it is widely reported that single knockout of PtdSer receptors often show partial defects in apoptotic cell clearance, and defects are better revealed by deletion of more than one receptor45–48. We tested three of the PtdSer receptors expressed on oocytes using single or double knockout mice: Tim–4, BAI1, and Mer-TK. Tim–4 directly binds PtdSer while Mer-TK binds PtdSer indirectly through the bridging molecules Gas6 or Protein S [note: oocytes also express Gas639]. Mice deficient in Tim–4 alone showed a modest but statistically significant reduction in the percentage of fertilized eggs (Fig. 3b). We then tested the role of Meritk and Bait1 genetically. We performed in vitro fertilization assays with the PtdSer masking agent BAI1-TSR (Fig. 2b, c). Although PtdSer is an eat-me signal on apoptotic cells, extensive confocal sectioning of the bead-bound oocytes did not reveal obvious internalization of the beads under these conditions. These data indicated the existence of potential PtdSer-binding molecules in the microvillus region on the surface of oocytes. As masking PtdSer on sperm significantly reduced the fertilization rate, we hypothesized that the PtdSer recognition receptors on the oocytes may contribute to the steps toward sperm–egg fusion.

Several PtdSer recognition receptors with redundant functions have been identified on phagocytes to engage the PtdSer exposed on the apoptotic targets23–26. Therefore, we hypothesized that one or more such PtdSer recognition receptor(s) on the oocytes may engage the sperm during fertilization. In a previous bioinformatics analysis of oocyte genes, members of the BAI family as well as CD36 were reported to be expressed in both mouse and human oocytes27. When we assessed the mRNA expression of BAI family members and CD36, we found readily detectable expression of BAI1, BAI3, and CD36 in mouse oocytes (Fig. 2d). BAI members belong to the type II adhesion family of GPCRs (hence, also referred to as ADGBR family) with long extracellular region containing domains capable of directly binding PtdSer23,25,28–32; CD36 is a member of the scavenger receptor family, and has also been linked to the binding of PtdSer24,33–35. CD36 is also reported to function cooperatively with BAI1 on endothelial cells36. Immunofluorescence microscopy using antibodies, which recognize both BAI1 and BAI3 (referred to from here onwards as BAI1/3) or CD36, gave a prominent signal in the sperm-binding microvillus region (Fig. 2e); this staining pattern was also similar to the staining previously noted with concanavalin A37 (Fig. 2e), Juno and CD97–9 (Supplementary Fig. 3a). When we assessed the expression of other known direct PtdSer-binding receptors, we found detectable expression of the message for Timd4 but not Stab2 (Fig. 2d). Among the TAM family of receptors that can also recognize PtdSer (indirectly, via the bridging molecules Gas6 or Protein S36,38), Meritk, but not Tyro3 and Axl were noted on oocytes39. Immunohistochemistry of whole mouse ovaries revealed that BAI1 expression is detectable in oocytes from the earliest stages of folliculogenesis, with positive staining from primordial follicles through tertiary follicles (Fig. 2f). Similarly, we could readily detect staining for BAI1/3 and CD36 on human oocytes (discarded/unused oocytes acquired from clinical in vitro fertilization procedures) (Supplementary Fig. 3b). Furthermore, expression of BAI1/3 on oocytes was detectable via immunohistochemistry on tissue sections of human ovarian tissue (Supplementary Fig. 3c).
oocytes isolated either from Merk−/− or Bai1−/− mice, or mice double deficient for both Merk and Bai1. Although oocytes isolated from single deficient mice (either Merk−/− or Bai1−/−) could be fertilized similar to wild-type eggs (Fig. 3c and Supplementary Fig. 3e), the double deficient Merk−/−Bai1−/− oocytes show a significant reduction in fertilization (Fig. 3c). Collectively, these data suggest that even with the considerable redundancy among the PtdSer receptor family, a statistically significant effect can be observed in in vitro fertilization with oocytes deficient in specific PtdSer recognition receptors.
The BAII1/3-ELMO1-Rac1 signaling axis affects fertilization.

With respect to signaling downstream of PtdSer recognition receptors, CD36 has a rather short cytoplasmic tail without an obvious direct signaling, and CD36 can cooperatively signal with BAII136. Among the PtdSer receptors, signaling downstream of the BAII1 family members is one of the best characterized23,25,49. Both BAII1 and BAII3 have long cytoplasmic tails that associate with the adapter proteins ELMO1 and/or ELMO2 (depending on the cell type), with subsequent signaling (in complex Dock family proteins) and activation of the small GTPase Rac1.23,49-52. GTP-bound active Rac1 promotes actin cytoskeletal remodeling during adhesion, phagocytosis, and cell-cell fusion events (Fig. 3a). In oocytes, we detected both Elmo1 and Elmo2 mRNA, with Elmo1 expression higher than Elmo2 (Fig. 3d). At the protein level, ELMO1 expression was readily detected by immunofluorescence in isolated oocytes (Fig. 3e). To assess the importance of ELMO1 in fertilization, we crossed mice carrying floxed Elmo1 alleles (Elmo1fl/flo) with Ddx4-Cre mice, which express the Cre recombinase specifically in oocytes from the earliest stages (Fig. 3f). We super-oovulated the Ddx4-Cre/Elmo1fl/fl female mice, isolated the oocytes, and performed in vitro fertilization assays using caudal sperm from wild-type mice. We noted a significant reduction in in vitro fertilization with oocytes from Ddx4-Cre/Elmo1fl/fl mice, compared with control mice (Fig. 3g). The partial reduction is consistent with the continued expression of ELMO2, which can substitute for ELMO111. Incidentally, female nematodes lacking the ELMO homolog ced-12 have been shown to have lower fecundity, with fewer progeny produced51.

ELMO proteins (together with Dock family members) function as upstream activators of the small GTPase Rac1, which regulates actin cytoskeletal rearrangements51 (Fig. 3a). As genetically testing the requirement for Rac1 is not feasible owing to the role of Rac1 during oocyte development and other steps after the sperm entry54, we took a pharmacological approach and used the sperm DNA decondensation assay to more directly score the sperm entry into oocytes. We treated oocytes with the Rac1 inhibitor EHT-1864 (see methods) and also added EHT-1864 during co-incubation of sperm and oocytes (note that both sperm and oocytes were harvested from wild-type mice) (Fig. 3h, i). EHT-1864 caused a significant reduction in the number of oocytes with decondensed sperm DNA (Fig. 3i). Importantly, this effect did not appear to be owing to the Rac1 inhibitor affecting sperm, as sperm incubated with EHT-1864 alone under the assay conditions showed no reduction in the motility (Fig. 3k). These data suggest that oocyte BAII1/3 and CD36, as well as the ELMO–Rac1 signaling module downstream of BAII1/3 contribute to the functional steps of fertilization.

PtdSer-dependent fusion of sperm with skeletal myoblasts. Our results up to this point suggest that PtdSer on the sperm and its receptors BAII1/3, CD36, Tim-4, and Mer-TK on the oocyte can promote fusion via the ELMO–Rac1 signaling pathway. Our laboratory and others19,25,49 have previously demonstrated that PtdSer exposure on skeletal myoblasts is important for the fusion between myoblasts to form myotubes, and this occurs in a BAII1/3–ELMO–Rac1–dependent manner19,25,49,55. Intriguingly, when we examined the expression of genes linked to the sperm:egg fusion in myoblasts, we found that oocytes and myoblasts both expressed the membrane proteins CD9, CD36, BAII1, and BAII3, as well as cytoplasmic ELMO2, and Rac1 (Fig. 4a)8,25,49,56. However, whereas Juno expression was not detected in myoblasts (Fig. 4b). Therefore, we asked whether caudal sperm could fuse with skeletal myoblasts, as it has been shown with other somatic cells60,61, and whether this Juno-independent fusion was mediated by the BAII1-ELMO1-RAC1 module expressed by myoblasts.

We labeled caudal sperm with a red-fluorescent cytoplasmic dye (Calcein-AM)60, incubated them with C2C12 mouse myoblasts, and looked for myoblasts that acquire the sperm-derived Calcein-AM staining (Fig. 4c). Of note, there was no myoblast:myoblast fusion when they were in growth medium in a non-confluent state. However, some of these myoblasts are known to be poised for fusion25. Remarkably, we could readily detect transfer of sperm-derived Calcein-AM into few of the myoblasts in a quantifiable manner (Fig. 4d). To further address the fusion between sperm and skeletal myoblasts, we took three additional approaches. First, labeling sperm with another dye (DiI) produced similar results as scored by fusion with myoblasts (Supplementary Fig. 4). Second, we isolated sperm from mice expressing transgenic yellow fluorescent protein (YFP) and, after incubating them with myoblasts, we detected for YFP/GFP (green fluorescent protein) and the sperm-specific protein Izumo1 within the fusing myoblasts by immunostaining. We could readily observe the signal for YFP, and the sperm-specific protein Izumo1, in addition to the DNA from the sperm head within the myoblasts (Fig. 4e). As a third approach, we used electron microscopy to detect the presence of sperm within myoblasts. The midpieces (containing multiple mitochondria), and the tails from multiple sperm could be detected inside the cytoplasm of a myoblast (Supplementary Fig. 5c). Of note, we do not detect any obvious membrane surrounding the sperm structures, suggesting that the sperm is not contained within a phagocytic vesicle. Interestingly, aminophospholipid asymmetry on myoblasts differs from fibroblasts16, and this may, in part, explain sperm fusion with myoblasts but not fibroblasts (not shown). As C2C12 myoblasts are an immortalized cell line, we also tested whether sperm could fuse with cultured mouse primary myoblasts, which...
also express BAI1/3 and CD36 (Supplementary Fig. 6a), and this was indeed the case (Supplementary Fig. 6b, c). Further, when we incubated sperm with primary bone marrow-derived macrophages, the sperm-derived Calcein-AM was not dispersed within the cytoplasm of macrophages as was the case with myoblasts, but rather the sperm appeared to be phagocytosed by the macrophages (Supplementary Fig. 7).

We next asked whether this sperm:myoblast fusion event also depends on PtdSer exposure on the sperm and the BAI1–ELMO–RAC1 module on the myoblast. First, blocking PtdSer on the sperm (via BAI1-TSR) significantly decreased the fusion of sperm to myoblasts (Fig. 4d, f). Second, antibody-mediated blocking of BAI proteins [BAI3 is expressed at a much higher level than BAI1 in C2C12 myoblasts\(^49\)] potently blocked sperm fusion to myoblasts (Fig. 4f). Third, C2C12 myoblasts with knockdown of Elmo2 [the predominant ELMO isoform expressed in C2C12 myoblasts\(^49\)] showed a significantly reduced Calcein-AM acquisition from the labeled sperm (Fig. 4f). Fourth, the RAC1 inhibitor EHT-1864 also potently blocked the sperm:myoblast fusion (Fig. 4d, f and Supplementary Fig. 4). Consistent with the BAI1/3–ELMO–RAC1 module being involved in cytoskeletal rearrangements in cell:cell fusion during myotube...
Fig. 3 Genetic testing of PtdSer receptors and cytoplasmic signaling in oocytes. a Schematic of PtdSer receptors BAI1/3, the downstream ELMO-DOCK-RAC1 signaling pathway, and other receptors on oocytes. b, The PtdSer receptors Tim-4, BAI1 and Mer-TK participate in fertilization. ZP-intact oocytes from wt or Tim-4−/− mice b Mer-TK+/+/ BA1+/+ /+, Mer-TK−/− BA1+/+ +, or Mer-TK−/− BA1+/−/− were mixed with wt sperm, and two-cell embryos evaluated at 24 h. Fertilization index is the percentage of fertilized eggs from the experimental group divided by percentage of fertilized eggs from the control group (wt mice). Each dot represents one mouse (b, n = 6 experiments including 17 wt mice and 9 Tim-4−/− mice, c, n = 5 experiments including 15 wt mice, 6 Mer-TK−/− BA1+/+ + and 6 Mer-TK−/− BA1+/−/−, total number of eggs (parentheses). *p < 0.05 (b, two-tailed unpaired Student t test), **p < 0.01 (c one-way ANOVA followed by Dunnet’s multiple comparisons test). d Elmo1 and Elmo2 mRNA on cumulus-free Metaphase II oocytes. e Intracellular ELMO1 in isolated Metaphase II (MII) ZP-free oocytes. Scale bar, 20 μm. f Schematic for generation of oocyte-specific Elmo1-deficient mice. g Percentage of fertilized eggs after incubation of control (Elmo1+/+) or Elmo1-deficient (Ddx4-Cre/Elmo1−/−) oocytes with wt sperm (n = 6 independent experiments including 15 Elmo1+/+ mice and 12 Ddx4-Cre/Elmo1−/− mice). Each dot represents 1 or 2 pooled mice. *p < 0.05 (Two-tailed unpaired Student’s t test). h Schematic for the evaluation early sperm entry into oocytes via DAPI staining of decondensed sperm DNA. ZP-free wt oocytes were incubated with RAC1 inhibitor (EHT-1864, 80 μM) and loaded with DAPI. After several washes, sperm were added, and the presence of internalized sperm with decondensed nuclei was evaluated after 1 h. i Control oocytes displaying the decondensation of the sperm DNA incorporated into the oocyte (arrow), whereas the sperm tail has not yet been internalized. DAPI also highlights the oocyte chromosomes in anaphase II indicating the resumption of meiosis II. Scale bar, 20 μm. j Decreased percentage of oocytes with decondensed sperm DNA after RAC1 inhibition (n = 4 independent experiments). Numbers in parentheses reflect total number of eggs scored. **p < 0.01 (Two-tailed unpaired Student’s t test). k Sperm motility was not affected by RAC1 inhibition p > 0.05 (two-tailed unpaired Student’s t test). Data are presented as mean ± s.e.m. Source Data are provided in the Source Data File.
the absence of Juno. This type of combinatorial use of diverse molecules to achieve cell:cell binding/subsequent signaling is very analogous to interactions within the immune system: e.g., during T-cell interaction with an antigen-presenting cell, the central driver is the TCR:MHC/peptide interaction; yet, a host of other accessory molecules are fundamentally important for achieving specificity and optimal activation of T cells after antigen recognition. Similar to Juno (which is GPI-anchored and does not have its own signaling motif), the TCR does not have a signaling motif of its own and requires associated molecules for inducing downstream signaling and activation. Analogously, the PtdSer recognition receptors on the oocytes may work together with Juno in initiating intracellular signaling within oocytes, eventually leading to the critical step of gamete fusion. In summary, these data suggest that PtdSer on sperm and its receptors on oocytes as functional players that can work in conjunction with Izumo1 and Juno to promote sperm:egg fusion during fertilization.

Methods
Mice. C57BL/6 mice (stock 000664) and Ddx4-Cre mice (stock 006974) were purchased from the Jackson Laboratory and bred in our facilities. BAI1 deficient and Elmo1 homozygous mice were previously generated in our laboratory11. Mer-tk-deficient
Fig. 4 Spermomyoblast fusion via PtdSer and the BA13-ELMO2-RAC1 signaling axis. a Oocytes and myoblasts express similar molecules. Juno expression is readily detected on oocytes but not myoblasts. Bars represent mean ± s.e.m. c, Schematic of the spermomyoblast fusion assay. Caudal epididymal sperm were labeled with Calcein-AM (red) and co-cultured with murine C2C12 myoblasts. After 4 h, myoblasts were washed, stained with Hoechst dye (to stain nuclei) and the percentage of Calcein-AM+ myoblasts was evaluated by microscopy. d Representative images depicting myoblasts that fuse with sperm to become Calcein-AM+ (3–10% per field) under control conditions (left panel), whereas this is greatly reduced when the sperm was pretreated with BA11-TSR to mask PtdSer (middle panel) or after pretreatment of myoblasts with the RAC1 inhibitor (right panel). Scale bar, 50 μm. e Detection of sperm inside the myoblasts. YFP+ sperm were co-incubated with C2C12 myoblasts for 4 h. Myoblasts were washed, fixed, and stained by immunofluorescence with antibodies to YFP/GFP (green) and Izumo1 (pink). Phalloidin (red) and Hoechst (blue) were used to stain the actin cytoskeleton and DNA, respectively. On the left panel, the dense sperm nucleus contained within the phalloidin+ cytoplasm is shown on the cross-sectional plane. White arrows: sperm nucleus; green arrow: sperm tail; dotted line: outline of the sperm head. Scale bar: 5 μm.

mice (stock 011122) were purchased from the Jackson Laboratory and crossed with BA11 deficient mice in our facilities. Tim-4-deficient mice were kindly provided by Dr. Vijay Kuchroo (Brigham and Women’s Hospital, MA). Yellow fluorescent protein (YFP) expressing mice (stock 006148) were crossed to E2A-Cre mice (stock 003724), both from the Jackson Laboratory. All animal procedures were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of Virginia.

Sperm staining. To stain sperm with Annexin V, the caput, corpus, and cauda epididymis of adult (> 10 weeks old) male mice were dissected and the sperm were allowed to disperse for 15 min in capacitating medium (TYH + bovine serum albumin (BSA) (119 mM NaCl, 4.7 mM KCl, 1.71 mM CaCl2, 1.2 mM KH2PO4, 25.1 mM NaHCO3, 5.56 mM glucose, 0.51 mM Na pyruvate, 1% phenol red, supplemented with 4 mg/ml BSA, penicillin, and streptomycin)) for 30 min of capacitation, sperm were incubated with 10 μg/ml Annexin V (Ebisociences), 50 μg/ml GST or 50 μg/ml GST-BA11-TSR. After co-culture of oocytes and sperm in 100 μl droplets of high calcium human tubal fluid supplemented with 1 μM reduced glutathione medium for 4 h, sperm were washed several times and transferred to KSOM medium (EMD Millipore). The percentage of two-cell embryos (fertilized eggs) was determined at 24 h post insemination. Sperm motility upon incubation with control medium, Annexin V, GST, or GST-BA11-TSR was graded as progressive motility, non-progressive motility, and immotility, according to the criteria of the 5 Edition of the World Health Organization (WHO) Laboratory Manual for the Examination and Processing of Human Semen. The non-progressive motility and immotility numbers were grouped together. To test the soluble head group of PtdSer, O-Phospho-serine (Sigma) and its control O-Phospho-serine (Sigma) in IVF assays, ~ 20 ZP-free oocytes isolated from wt mice were incubated 1 × 103 capacitated sperm in 20 μl droplets for 1 h in the presence of 1 μg/ml O-Phospho-serine or o-Phospho-serine. After washes and an overnight incubation the percentage of two-cell embryos was evaluated. To test the role of PtdSer receptor recognition in the fertilization assays, BA1+/−, ELMO1−/−, Tim-4−/−, Mer−/−, BA1+/−, and wild-type male mice were super-ovulated as described above (E2A-Cre mice were inseminated with 2 × 105 capacitated sperm in 100 μl drops of TYH + BSA for 4 h. After several washes, oocytes were transferred to KSOM medium and the percentage of two-cell embryos was evaluated at 24 h post insemination.

To evaluate the role of BA11/3 and CD36 using blocking antibodies (Abs), we incubated cumulus-free ZP-intact oocytes with BA11/3 (50 μg/ml, R&D Systems, AF4996), CD36 (10 μg/ml, clone CFR D-2712, Hyclut, HM1074), Juno (10 μg/ml, clone 258A, 125102), CD9 (50 μg/ml, clone KM68, BD Biosciences, 533758) antibodies or isotype controls: mouse IgA, (10 μg/ml, Southern Biotechnologies, 0106-14) or sheep IgG, (50 μg/ml, R&D systems, 5-001-A) in TYH + BSA + 5% FBS (to avoid ZP hardening) for 1h at 37 °C. 5% CO2. After several washes in TYH + BSA (to eliminate the FBS) 25–30 oocytes were inseminated with 4 × 105 capacitated sperm in 100 μl TYH + BSA in the presence or absence of specific antibodies or isotype controls for 3 h. Oocytes were washed in TYH + BSA and transferred to KSOM for an overnight incubation at 37 °C, 5% CO2. The fertilization index was calculated as the percentage of fertilized eggs (two-cell embryos) in the experimental group divided the percentage of fertilized eggs observed in the control group. To score fertilization via the sperm DNA decondensation assay, we incubated cumulus-free ZP-intact oocytes with CD36, BA11/3, or the isotype controls as described above, and then loaded the oocytes with 10 μg/ml DAPI (BioRad) for 20 min at 37 °C, 5% CO2, and washed several times in TYH + BSA. Oocytes were then fixed in 4% paraformaldehyde and washed with 0.25% glutaraldehyde and 0.1% paraformaldehyde for 20 min at room temperature. The percentage of oocytes with DAPI+ decondensed sperm nuclei (typically 1–2/oocyte) was evaluated by microscopy. To evaluate the role of RAC1, ZP-free oocytes were prepared as described above, loaded with 10 μg/ml DAPI (BioRad) for 20 min at 37 °C, 5% CO2, and washed several times in TYH + BSA. Twenty oocytes, either untreated or incubated with RAC1 inhibitor (10 μl) for 30 min at 37 °C, 5% CO2, were loaded with 10 μg/ml DAPI (BioRad) for 20 min at 37 °C, 5% CO2. After fixation, RAC1 inhibitor was also present the fertilization steps. After three washes, oocytes were fixed in 0.25% glutaraldehyde and 0.1%
paraformaldehyde for 20 min at room temperature, washed and mounted. The percentage of oocytes with DAPI+ decondensed sperm nuclei (typically 1–2 oocytes) was evaluated by microscopy.

Staining of mouse/human eggs with antibodies. ZP-free oocytes were obtained from wild-type female mice after removal of cumulus cells and the ZP via treatment with hyaluronidase and Tyrode’s solution, respectively. Live oocytes were incubated with antibodies to BAI1/3 (R&D Systems), CD36 (BD Pharmingen, 553758), Juno (Biolegend, 123601), or both CD36 + BAI1/3 in TSX + BSA drops under paraffin oil at 37°C, 5% CO₂ for 1 h. Antibodies concentrations were the same as described above (see Methods section). For the staining of microvilli, oocytes were incubated with 50 µg/ml fluorescein isothiocyanate-Concanavalin A (Vector, FL-1001) for 5 min. Cells were fixed in 4% paraformaldehyde (PFA) + 1% BSA for 30 min at room temperature, washed and incubated with biotinylated secondary or Alexa-Fluor 488 secondary antibodies for 1 h at room temperature. After washing, cells were stained with N-Acetyl Tetrazolium (Biotin) or Streptavidin + Alexa-Fluor 647 or biotin were used. Streptavidin + Alexa-Fluor 647 or biotin were used.

Izumo1 (ProSci, 8233) diluted in 0.1% Tween-20 and 0.5% BSA. Secondary antibodies were used as described above (see Methods section). For the staining of microvilli, oocytes were incubated with 50 µg/ml fluorescein isothiocyanate-Concanavalin A (Vector, FL-1001) for 5 min. Cells were fixed in 4% paraformaldehyde (PFA) + 1% BSA for 30 min at room temperature, washed and incubated with biotinylated secondary or Alexa-Fluor 488 secondary antibodies for 1 h at room temperature. After washing, cells were stained with N-Acetyl Tetrazolium (Biotin) or Streptavidin + Alexa-Fluor 647 or biotin were used. Streptavidin + Alexa-Fluor 647 or biotin were used.

CD9 (BD Biosciences) for 30 min at 37 °C, 5% CO₂ in TYH medium. CD9 antibodies conjugated with Alexa-Fluor 488 or biotin were used. Streptavidin + Alexa-Fluor 647 or biotin were used. Streptavidin + Alexa-Fluor 647 or biotin were used.

Beads were pretreated with medium only, 50 µg/ml GST or 50 µg/ml GST-BAI1-TSR for 30 min. Oocytes were washed six times in TYH + BSA, stained with CD9 antibody for 30 min, washed, fixed in 4% PFA + 1% BSA and stained with Hoechst. The percentage of oocytes with bound beads was determined by microscopy. We scored oocytes with > 5 bound beads, as accurately counting individual beads, particularly in oocytes of the control groups that bound few or no beads was unfeasible.

Quantitative RT-PCR. Total RNA was extracted from cumulus-free ZP-intact oocytes from wild-type female mice using TriZol (Ambion) and the cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer’s instructions. qPCR for mouse Bai1, Bai2, Bai3, Tim-4, Stab2, Elmo1, Elmo2, cd36, cd52, Juno, or housekeeping gene Gapdh was performed using Taqman probes (Applied Biosystems) using StepOnePlus Real Time PCR System (ABI). Cd52 was used to determine oocyte contamination with cumulus cells.

Statistical analysis. Statistical significance was determined using GraphPad Prism 5 or 6 using unpaired Student’s two-tailed t test, one-sample t test, Mann–Whitney test, or one-way analysis of variance as according to test requirements. No inclusion/exclusion criteria were pre-established. A p value of < 0.05 (*), < 0.01 (**) or < 0.001 (***)) were considered significant.

Data availability. All the relevant data supporting the key findings of this study are available within the article and its Supplementary Information files or from the corresponding authors upon reasonable request. The source data underlying Figs. 1e, k, l, n, 2c, d, i, 1b, c, d, g, j, k, 4b, f, h, 5a, 1e, 3f, 3d, 4e, 8b are provided as a Source Data file. A reporting summary for this Article is available as a Supplementary Information file.

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References. 1. Okabe, M. Sperm-egg interaction and fertilization: past, present, and future. Biol. Reprod. 99, 134–146 (2018). 2. Bianchi, E. & Wright, G. J. Sperm meets egg: the genetics of mammalian fertilization. Annu. Rev. Genet. 50, 93–111 (2016). 3. Bianchi, E., Doe, B., Goulding, D. & Wright, G. J. Juno is the egg Izumo receptor and is essential for mammalian fertilization. Nature 508, 483–487 (2014). 4. Inoue, N., Ikawa, M., Isotani, A. & Okabe, M. The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature 434, 234–238 (2005). 5. Ohto, U. et al. Structure of Izumo1-JUNO reveals sperm-eggocyte recognition during mammalian fertilization. Nature 534, 566–569 (2016). 6. Aydin, H., Sultana, A., Li, S., Thavalingam, A. & Lee, J. E. Molecular architecture of the human sperm Izumo1 and egg JUNO fertilization complex. Nature 534, 562–565 (2016).

1. Martin, G., Sabido, O., Durand, P. & Levy, R. Phosphatidylserine expression by sperm: a marker of phosphatidylserine exposure on the surface of apoptotic germ cells in vivo. Dev. Biol. 216, 2122–2134 (2003).
2. Martin, G., Sabido, O., Durand, P. & Levy, R. Phosphatidylserine exposure of aminophospholipids and tyrosine phosphorylation in bicarbonate responsive human sperm cells. Andrology 6, 1340–1348 (2018).
3. Martin, G., Sabido, O., Durand, P. & Levy, R. Phosphatidylserine exposure in human sperm induced by calcium ionophore A23187: relationship with apoptosis, membrane scrambling and the acrosome reaction. J. Androl. 18, 393–399 (1997).
4. Banducci, A., Simoni, M., Antalikova, J., Cupperova, P. & Michalovka, K. Role of tetrascian CD9 molecule in fertilization of mammals. Physiological Res. 64, 279–293 (2015).
5. Elliott, M. R. et al. Unexpected requirement for ELMO1 in clearance of apoptotic germ cells in vivo. Nature 467, 333–337 (2010).
6. Martin, G., Sabido, O., Durand, P. & Levy, R. Phosphatidylserine externalization in human sperm induced by calcium ionophore A23187: relationship with apoptosis, membrane scrambling and the acrosome reaction. Hum. Reprod. 20, 3459–3468 (2005).
7. de Vries, K. J., Wiedmer, T., Sims, P. J. & Gadella, B. M. Caspase-independent exposure of aminophospholipids and tyrosine phosphorylation in bicarbonate responsive human sperm cells. Biol. Reprod. 66, 2128–2134 (2003).
8. Gadella, B. M. & Harrison, R. A. Capacitation induces cyclic adenosine 3’5’-monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. Biol. Reprod. 67, 340–350 (2002).
9. Hichri, R. et al. Apoptotic sperm biomarkers and the correlation between conventional sperm parameters and clinical characteristics. Andrologia 50, e21833 (2017).
10. Sessions, A. & Horwitz, A. F. Myoblast aminophospholipid asymmetry differs from that of fibroblasts. FEBS Lett. 134, 75–78 (1981).
11. Marguet, D., Luciani, M. F., Moynault, A., Williamson, P. & Chimini, G. Engagement of apoptotic cells involves the redistribution of membrane phosphatidylserine on phagocyte and prey. Nat. Cell Biol. 2, 454–456 (1999).
12. Dillon, S. R., Mancini, M., Rosen, A. & Schlissel, M. S. Annexin V binds to viable B cells and colocalizes with a marker of lipid rafts upon B cell receptor activation. J. Immunol. 164, 1322–1332 (2000).

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19. van den Eijnde, S. M. et al. Transient expression of phosphatidylserine at cell-cell contact areas is required for myotube formation. J. Cell Sci. 114, 3631–3642 (2001).

20. Hoppe, P. C. Fertilizing ability of mouse sperm from different epididymal regions and after washing and centrifugation. J. Exp. Zool. 192, 219–222 (1975).

21. Ikawa, M., Inoue, N., Benham, A. M. & Okabe, M. Fertilization: a sperm’s journey to join with the卵子 required for fertilization. J. Cell Sci. 190, 2010–2011 (2010).

22. Richard, A. S. et al. Virion-associated phosphatidylethanolamine promotes cell fusion with the卵子. Proc. Natl Acad. Sci. USA 112, 14682–14687 (2015).

23. Park, D. et al. BAI1 is an engulfment receptor for apoptotic cells upstream of the TLR4-dependent myoblast engulfment pathway. Nat. Commun. 6, 6186 (2015).

24. Nishimori, H. et al. A novel brain-specific P53-target gene, BAII, containing thrombospondin type 1 repeat inhibits experimental angiogenesis. Oncogene 15, 2145–2150 (1997).

25. Zhu, D. et al. BAII regulates spatial learning and synaptic plasticity in the hippocampus. J. Neurosci. Invest. 125, 1457–1508 (2015).

26. Bolliger, M. F., Martinelli, D. C. & Sudhof, T. C. The cell-adhesion G protein-coupled receptor BAIII is a high-affinity receptor for C1q-like proteins. Proc. Natl Acad. Sci. USA 108, 25334–25339 (2011).

27. Sigouillot, S. M. et al. The secreted protein C1QL1 and its receptor BAI3 control the noncanonical NF-κB pathway, is required for phagocytosis and cell migration. Cell 167, 114–127 (2016).

28. Hamann, J. et al. International union of basic and clinical pharmacology. XCIV. International union of pharmacology. LXXVI. Coupling G protein-coupled receptors (GPCRs) to direct myoblast fusion and ommatidial organization. Cell 167, 114–127 (2016).

29. Nishimori, H. et al. A novel brain-specific P53-target gene, BAII, containing thrombospondin type 1 repeat inhibits experimental angiogenesis. Oncogene 15, 2145–2150 (1997).

30. Tait, J. F. & Smith, C. Phosphatidylserine receptors: role of CD36 in binding of anionic phospholipid vesicles to monocytic cells. J. Biol. Chem. 274, 3048–3054 (1999).

31. Greenberg, M. E. et al. Oxidized phosphatidylserine-CD36 interactions play an essential role in macrophage-dependent phagocytosis of apoptotic cells. J. Exp. Med. 203, 2613–2625 (2006).

32. Silverstein, R. L. & Febbraio, M. CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. Sci. Signal. 2, re3 (2009).

33. Klenotic, P. A. et al. Histidine-rich glycoprotein modulates the anti-angiogenic effects of vasculostatin. Science 319, 802–807 (2008).

34. Rothlin, C. V., Carrera-Silva, E. A., Boussou, L. & Ghosh, S. TAM receptor signaling in immune homeostasis. Nature Rev. Immunol. 13, 738–749 (2013).

35. Li, E. et al. Cell fusion by tetraspanins CD9 and CD81. Biochem. Biophys. Res. Commun. 427, 750–751 (2014).

36. Segawa, K. & Nagata, S. An apoptotic ‘Eat Me’ signal: phosphatidylserine exposure. Trends Cell Biol. 25, 639–650 (2015).

37. Naito, K., Fukuda, Y. & Toyoda, Y. Effects of porcine follicular fluid on male pronucleus formation in porcine oocytes matured in vitro. Gamete Res. 21, 289–295 (1988).

38. Guan, M. et al. In vitro fertilization in mice using the MBCD-GSH protocol. Curr. Protoc. Mouse Biol. 5, 67–83 (2014).

39. Nagy, A. Manipulating the mouse embryo: a laboratory manual, Edn. 3rd. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2003).

40. Enwere, E. K. et al. TWEAK and cAP1 regulate myoblast fusion through the noncanonical NF-kappaB signaling pathway. Sci. Signal. 5, ra7 (2012).

41. Millay, D. P. et al. Myomaker is a membrane activator of myoblast fusion and muscle formation. Nature 499, 301–305 (2013).

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Author contributions

C.R. designed, performed, and analyzed most of the experiments in this study with input from K.S.R and J.L.L. W.X. performed and assisted with in vitro fertilization assays. J.S. assisted with the microscopy experiments and live imaging of sperm. K.W. performed sperm analysis and assisted with the in vitro fertilization experiments. C.S.L. performed the gene expression analysis. S.M. produced reagents and assisted in myoblast experiments and transfection of cells. S.A. assisted with the myoblast experiments. R.S. assisted with the sperm analysis and discussions. J.C.I. assisted with maintenance and genotyping of mouse colony. K.S.R, J.L.L., and C.R. wrote the manuscript with input from co-authors.
Competing interests
The authors declare no competing interests.

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