Dear Editor,

The acrosome reaction (AR) in mammalian spermatozoa is a prerequisite for successful fertilization, because it leads to the release of hydrolytic enzymes from the acrosomal vesicle along with the exposure of the oocyte-recognition protein Izumo on the sperm surface (Bianchi et al., 2014). AR mainly follows the conserved principles of calcium-regulated exocytosis in neurons/neurosecretory cells, which is reflected by initial vesicle docking/priming steps and calcium-triggered SNARE-mediated membrane fusion (Tsai et al., 2012). However, there are some unique features of AR whose underlying molecular mechanisms are largely unknown: The acrosome is a single, huge vesicle whose exocytosis is realized by multipoint fusions of the outer acrosomal and sperm plasma membrane (Tsai et al., 2012). In addition, stimulating sperm with the oocyte’s Zona pellucida (ZP) leads to a zipper-like progression of fusion pore formation that starts at the posterior acrosomal region and consistently proceeds in an anterograde direction, whereas the fusion is initiated at random sites following calcium ionophore treatment (Buffone et al., 2009). This directed propagation requires extraordinary temporal and spatial orchestration of individual pore-forming events. Remarkably, individual exocytotic events at neuronal synaptic junctions also require tight coordination, which is accomplished by a protein network termed cytomatrix of the active zone (CAZ), consisting of the multi-domain CAZ-proteins Munc13, RIM, RIM-BP, Liprin-α, ELKS/ERC, Piccolo/Aczonin, and Bassoon (Sudhof, 2012). Thus, we hypothesized that a CAZ-like network could also coordinate the success of sperm AR.

First, expression of the giant proteins Bassoon and Piccolo/Aczonin was examined in murine spermatozoa by immunostaining, revealing that both proteins localize to the acrosomal region (Figure 1A). To analyze their possible function in either spontaneous or calcium-induced AR, epitope-specific antibodies were introduced into sperm by the established streptolysin-O (SLO) permeabilization method (Bello et al., 2012). Quantification of AR revealed that the anti-Bassoon antibody neither affected spontaneous AR nor calcium-stimulated acrosomal exocytosis index (AEI) (Figure 1B and Supplementary Figure S2A). In contrast, inhibition of Piccolo/Aczonin resulted in a strong decrease of calcium-stimulated AEI (Figure 1B), whereas spontaneous AR was not significantly influenced (Supplementary Figure S2A).

CAZ-proteins exhibit an extraordinary isoform/splicing variability resulting in subtypes lacking distinct interacting domains (Sudhof, 2012). Since Munc13 and RIM recently have been identified in human sperm (Bello et al., 2012), it was essential to elucidate whether they are also present in sperm of other mammalian species and to additionally unravel the exact isoform(s) expressed in spermatozoa. Immunoblot analysis of rat (Figure 1E [spermatozoa, P2]) and mouse (data not shown) sperm membranes revealed only minor immunoreactivity for the Munc13-1 isoform, whereas strong ubMunc13-2-immunolabeling was detected. This is supported by mass spectrometry (MS) analyses of rat testis (Supplementary Table S1 and Figure S4B), which unequivocally identified ubMunc13-2-derived peptides. Immunostaining confirmed the acrosomal localization of Munc13 in murine (Figure 1C and D) and rat spermatozoa (Supplementary Figure S1). The same acrosomal localization was observed for the prevailing sperm RIM-isoform RIM2 (Figure 1H; Bello et al., 2012). Immunoblot analyses performed to further differentiate between subtypes of RIM2 revealed that the full-length α-isofrom RIM2α is more abundant than the RIM2β-protein in rat (Figure 1G [spermatozoa, P2]) and mouse (data not shown) sperm membranes. Since a capacitation-dependent membrane raft clustering of AR-controlling SNARE-proteins has recently been observed (Tsai et al., 2012), an association of the two identified CAZ-proteins ubMunc13-2 and RIM2 with sperm-derived detergent-resistant membranes (DRMs) was examined. Figure 1E and G documents that ubMunc13-2 and RIM2 indeed co-migrated with Caveolin-1-containing membrane rafts in rat sperm DRMs. The functional impact of distinct Munc13 and RIM binding modules for AR was assessed using domain-specific antibodies as selective inhibitory tools. Whereas none of the anti-Munc13 antibodies detecting either N- or C-terminal regions of Munc13 nor any of the employed anti-RIM antibodies recognizing either N-terminal or different central domains of RIM-proteins altered spontaneous AR (Supplementary Figure S2B and C), a strong decrease of AEI was detected for all analyzed anti-Munc13 and anti-RIM antibodies (Figure 1F and I).

The ability of RIM-proteins to integrate different sub-steps of synaptic vesicle release through their diverse interacting
Figure 1 Functional relevance of Piccolo/Aczonin, Munc13, and RIM2α for the sperm acrosome reaction. (A) Bassoon and Piccolo/Aczonin are both localized in the acrosomal region of murine sperm. (B) Antibodies selective for Piccolo/Aczonin inhibit calcium-induced AR, which is restored by peptide block, whereas antibodies recognizing Bassoon have no effect. (C) Murine spermatozoa display acrosomal Munc13-immunolabeling, which is prevented by peptide block. (D) Acrosomal localization of Munc13 is confirmed in co-staining experiments with the acrosomal marker PNA. (E) UbMunc13-2 represents the major Munc13-isoform in spermatozoa and is associated to DRMs. (F) Antibodies recognizing either N- or C-terminus of Munc13 prevent calcium-induced AR. (G) RIM2α immunoreactivity is enriched in the caveolae-containing DRM fraction in sperm. (H) RIM2 expression in murine sperm is restricted to the acrosomal region. (I) Domain-specific anti-RIM antibodies, detecting either the zinc finger-motif of RIM1α and RIM2α, the PDZ domain primarily of RIM2α and RIM2β or part of the proline-rich region of RIM2α and RIM2β, all inhibit calcium-induced AR. (J) Potentiation of AR by A23187 is slightly, but not significantly (n.s.), higher in wild-type (+/+) than in RIM2α-deficient
partners (Sudhof, 2012; Supplementary Figure S5A) was addressed by identifying RIM2-binding partners using tandem MS analyses of testicular RIM2-co-immunoprecipitates (Supplementary Figure S3). As expected, a possible transient complex of RIM2, Rab3, and Epac was not detected in testis, since it requires activation by GTP and/or cAMP (Branham et al., 2009). However, ubMunc13-2 and the CAZ-component ELKS/ERC2 were found to bind endogenous RIM2 (Supplementary Table S1), which actually provides the first evidence of ELKS/ERC2 expression in testis. Moreover, additional pull-down studies revealed that the proline-rich region of RIM2 precipitated RIM-BP3 (Supplementary Figure S5), the major testicular isoform of the RIM-BP-family of CAZ-proteins, which in complex with RIM-proteins are able to recruit voltage-gated calcium channels (VGCC) into the vicinity of synaptic vesicles (Sudhof, 2012).

The particular significance of the full-length RIM2α-isoform for successful AR was examined using sperm of RIM2α-deficient mice (Schoch et al., 2006), which do not show any obvious morphological alterations (Supplementary Figure S7). When analyzing the impact of successful RIM2α-deletion (Supplementary Figure S6) on spontaneous AR, a consistent increase in exocytosis over time was observed for both wild-type and RIM2α-deficient sperm (Supplementary Figure S8). However, RIM2α-knockout sperm exhibited a generally higher disposition to undergo spontaneous AR (Supplementary Figure S8), suggesting that an alternate mechanism or remaining RIM2β- and RIM1-isoforms can compensate for the lack of RIM2α in capacitation-independent spontaneous AR (Schoch et al., 2006). Next, AR incidence was comparatively examined for wild-type and RIM2α-deficient sperm either by directly increasing cytosolic calcium with the ionophore A23187 (Tsai et al., 2012) or by activation of the signal transduction pathway induced by solubilized ZP (Gadella, 2012). Figure 1 documents that AR potentiation by A23187 was slightly, but not significantly, lower in RIM2α-deficient compared with wild-type sperm. However, by quantifying the potency of ZP to drive AR, a 45% reduction of AR-potentiation was observed upon RIM2α deletion (Figure 1K).

Taken together, the presented results indicate that the CAZ-proteins RIM2, ubMunc13-2, and Piccolo/Acanzon play a critical role in regulating calcium-mediated AR in rodent spermatooza. With respect to their functions at the presynaptic active zone (Sudhof, 2012), it is tempting to speculate that they form the molecular backbone of a protein network between the two fusing membranes, which could be responsible for a physical interaction of the multiple SNARE complexes including their regulatory players (e.g. Complexins) and/or for an additional recruitment of these complexes to specialized membrane raft microenvironments (Tsai et al., 2012). However, one key result of our study is that RIM2α deletion led to a significant inhibition of AR induction by solubilized ZP, whereas providing calcium ad libitum reverses the AR impairment. Since inhibition of multiple RIM isoforms always led to a significant reduction of calcium-induced AEI (Figure 1), one may suggest a functional compensation of RIM2α by remaining sperm RIM2β- and RIM1-isoforms (Supplementary Figure S6; Bello et al., 2012) that could also account for the weak reproductive phenotype of RIM2α-deficient mice (Schoch et al., 2006). However, RIM1α/β and RIM2β are not able to fully compensate for the functional relevance of RIM2α for ZP-triggered AR. Remarkably, ionomycin-induced AR was found to initiate at random sites of the acrosome, whereas ZP stimulates AR in an orderly manner with strict anterograde propagation of membrane fusion (Buffone et al., 2009). Thus, we suggest that the specific function of RIM2α encompasses ZP-dependent signaling and/or an involvement in the calcium control of fusion. In this context, it is essential to mention that the special task of RIM at the active zone is to control positional priming, which is dependent on the ability of RIM to target vesicles into the vicinity of VGCCs (Sudhof, 2012). Thus, it is conceivable that RIM2α in sperm is also responsible for positioning calcium channels in close proximity to the ‘ready to fuse’ SNARE complexes, thereby linking an increase in cytosolic calcium to the zipper-like progression of ZP-induced multiple fusion pore formation (Buffone et al., 2009) and subsequent exposure of the Izumo protein (Bianchi et al., 2014). Remarkably, interaction between Izumo and its oocyte counterpart Juno is characterized by low binding affinity, suggesting that stable gamete pairing may require multiple Juno and Izumo molecules to achieve an adequate binding avidity (Bianchi et al., 2014). Thus, it is tempting to speculate that a potential RIM2-based CAZ-like protein meshwork, presenting the molecular fundament for membrane fenestration, might not only be essential for ensuring efficient release of the acrosomal content but also for increasing the number of accessible Izumo molecules to allow multiple Izumo–Juno pairing.

— sperm. (K) ZP induction of AR is significantly reduced upon RIM2α deletion in non-permeabilized sperm. A, D, and H show that no labeling is observed in control samples lacking primary antibody incubation. Dotted lines trace the acrosome [ac] and nucleus [nu]. Results presented in B, F, and I are mean acrosomal exocytosis indices (AEI) ± SEM of 7, 11, and 8 independent experiments, respectively. Results presented in J and K are mean values of 11 wild-type (J and K), 9 (J) and 8 (K) knockout sperm preparations. Unless specified otherwise, results are representative of at least three independent experiments. For calculation of AEI, potentiation of AR, and actual AR percentages of control samples, see Supplementary Methods and Figure S2.

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