Munc13-4 functions as a Ca\(^{2+}\) sensor for homotypic secretory granule fusion to generate endosomal exocytic vacuoles

Sang Su Woo, Declan J. James, and Thomas F. J. Martin*
Department of Biochemistry, University of Wisconsin–Madison, Madison, WI 53706

ABSTRACT Munc13-4 is a Ca\(^{2+}\)-dependent SNARE (soluble N-ethylmaleimide–sensitive factor attachment protein receptor)- and phospholipid-binding protein that localizes to and primes secretory granules (SGs) for Ca\(^{2+}\)-evoked secretion in various secretory cells. Studies in mast cell–like RBL-2H3 cells provide direct evidence that Munc13-4 with its two Ca\(^{2+}\)-binding C2 domains functions as a Ca\(^{2+}\) sensor for SG exocytosis. Unexpectedly, Ca\(^{2+}\) stimulation also generated large (>2.4 μm in diameter) Munc13-4+/Rab7+/Rab11+ endosomal vacuoles. Vacuole generation involved the homotypic fusion of Munc13-4+/Rab7+ SGs, followed by a merge with Rab11+ endosomes, and depended on Ca\(^{2+}\) binding to Munc13-4. Munc13-4 promoted the Ca\(^{2+}\)-stimulated fusion of VAMP8-containing liposomes with liposomes containing exocytic or endosomal Q-SNAREs and directly interacted with late endosomal SNARE complexes. Thus Munc13-4 is a tethering/priming factor and Ca\(^{2+}\) sensor for both heterotypic SG-plasma membrane and homotypic SG-SG fusion. Total internal reflection fluorescence microscopy imaging revealed that vacuoles were exocytic and mediated secretion of β-hexosaminidase and cytokines accompanied by Munc13-4 diffusion onto the plasma membrane. The results provide new molecular insights into the mechanism of multigranular compound exocytosis commonly observed in various secretory cells.

INTRODUCTION

Trafficking of proteins in secretory and endosomal pathways is mediated by fusion machinery comprising soluble N-ethylmaleimide–sensitive factor attachment protein receptors (SNAREs), Rab, and SM (Sec 1/Munc18) family proteins acting with a diverse set of tethering/priming factors. R-SNAREs on a vesicle assemble with Q-SNAREs on a recipient compartment in a 1R:3Q ratio to form SNARE complexes that fuse membranes (Jahn and Scheller, 2006; Sudhof and Rothman, 2009; Jahn and Fasshauer, 2012). Multiple factors operate to provide donor-acceptor membrane specificity and compartment-specific fusion. SM proteins regulate SNARE complex assembly (Shen et al., 2007; Ma et al., 2011; Baker et al., 2015) along with pathway-specific tethering/priming proteins (Yu and Hughson, 2010; Ma et al., 2011; James and Martin, 2013; Hong and Lev, 2014). Tethering/priming factors are commonly direct or indirect effectors for Rab GTPases that operate in several secretory cell types (Ren et al., 2007; Lorentz et al., 2012; Marshall et al., 2015). Recently, the tethering/priming factor Munc13-4 was reported to function in endosome trafficking in addition to its known role in SG exocytosis and endosome fusion in several secretory cell types (Ren et al., 2007; Lorentz et al., 2012; Marshall et al., 2015). The results provide new molecular insights into the mechanism of multigranular compound exocytosis commonly observed in various secretory cells.
Munc13-4 in endosome fusion

FIGURE 1: Ca\(^{2+}\) sensitivity and amplitude of \(\beta\)-hexosaminidase secretion are determined by Munc13-4 C2 domains. (A) Schematic of domain structure of Munc13-4. DUF, domain of unknown function 1041; MHD, Munc13 homology domain. C2A*, C2B*, and C2A*B*, C2 domains with Ca\(^{2+}\)-binding mutations. (B) Munc13-4 immunoblotting for rMunc13-4–knockdown cells expressing wild-type (WT) or C2-domain mutant hMunc13-4 proteins. NC, sham infection. Ponceau S stain shows total protein loading of lanes. (C) Quantification of Munc13-4 immunoblotting for knockdown cells expressing WT or C2-domain mutant hMunc13-4 proteins. Values were extracted from D. (F) EC\(_{50}\) values for curves in C plotted as means ± 95% confidence interval.

and Doc2 proteins mediate the Ca\(^{2+}\)-triggered fusion of primed SGs or vesicles (Chapman, 2008; Groffen et al., 2010; Pang and Sudhof, 2010; Yao et al., 2011). Upstream of fusion, Munc13-1/2 proteins serve as Ca\(^{2+}\) sensors for tethering and priming reactions (Junge et al., 2004; Shin et al., 2010; Lipstein et al., 2012; Imig et al., 2014; Man et al., 2015). For fusion events in endosome trafficking in rest-
The present study identified a requirement for Munc13-4 in Ca\(^{2+}\)-dependent SG exocytosis and in Ca\(^{2+}\)-triggered endosome fusion in mast cell–RBL-2H3 cells. Ca\(^{2+}\)-dependent SG exocytosis required Ca\(^{2+}\)-binding residues in both C2 domains of Munc13-4. Unexpectedly, Ca\(^{2+}\) increases also triggered the assembly of large (>2.4 μm in diameter) endosomal vacuoles whose formation depended on Ca\(^{2+}\) binding to the C2 domains of Munc13-4. Ca\(^{2+}\)-triggered vacuole formation was initiated by the homotypic fusion of Munc13-4+/Rab7\(^{\ast}\) SGs, followed by fusion with Rab11\(^{\ast}\) endosomes. Munc13-4+/Rab7\(^{\ast}\)/Rab11\(^{\ast}\) vacuoles contained diverse cargo (β-hexosaminidase, cytokines) and provided a pathway for the exocytic release of these mediators. The results reveal that multiple membrane fusion events mediate assembly of multigranular compound exocytic organelles and highlight a central role for Munc13-4 in Ca\(^{2+}\)-triggered endosome fusion.

**RESULTS**

**Munc13-4 is a Ca\(^{2+}\) sensor for Ca\(^{2+}\)-evoked degranulation in RBL-2H3 cells**

Studies in permeable platelets or RBL-2H3 cells and in reconstituted SNARE-dependent liposome fusion assays suggested that Munc13-4 functions as a Ca\(^{2+}\) sensor for SG exocytosis (Ren et al., 2010; Boswell et al., 2012). To assess whether Munc13-4 functions as a Ca\(^{2+}\) sensor in mast cell–RBL-2H3 cells (Seldin et al., 1985), we conducted studies in Munc13-4-deficient cell lines established by stably expressing a lentiviral rat Munc13-4 short hairpin RNA (shRNA; Supplemental Figure S1A). Munc13-4-deficient cell lines were used to express Ca\(^{2+}\)-binding mutants of human(h) Munc13-4 harboring two D-to-N substitutions in C2 domains (C2A\(^{\ast}\), C2B\(^{\ast}\), and C2A/B\(^{\ast}\); Figure 1A). Similar replacement studies with synaptotagmin-1 or Doc2 provided evidence for a Ca\(^{2+}\) sensor role of these proteins in synaptic vesicle exocytosis because mutations shifted the Ca\(^{2+}\) sensitivity of secretion (Groffen et al., 2010; Yao et al., 2011; Sudhof, 2012). Munc13-4 depletion reduced ionomycin-stimulated β-hexosaminidase secretion by 70% compared with control cells (Supplemental Figure S1B), as previously found (Elstak et al., 2011). Stimulated β-hexosaminidase secretion was restored by the expression of wild-type hMunc13-4, which evades the shRNA for rMunc13-4 (Supplemental Figure S1B). We optimized expression of wild-type and mutant hMunc13-4 proteins to be comparable (Figure 1, B and C) because pilot studies found a nonlinear relationship between hMunc13-4 protein expression and secretory responses. Each hMunc13-4 mutant protein partially restored stimulated β-hexosaminidase secretion but with altered characteristics (Figure 1D). Maximal secretion at 10 mM [Ca\(^{2+}\)]\(_{\text{ex}}\) was reduced for C2A\(^{\ast}\) and C2A/B\(^{\ast}\) proteins compared with wild-type protein, indicating decreased efficacy. Thus the C2A domain, reported to regulate Ca\(^{2+}\)-stimulated SNARE binding (Boswell et al., 2012), controlled response amplitude. At 1 mM [Ca\(^{2+}\)]\(_{\text{ex}}\), C2A\(^{\ast}\) and C2A/B\(^{\ast}\) proteins were partially active, whereas C2A/B\(^{\ast}\) was inactive, indicating additive loss of function for each mutant C2 domain (Figure 1E). Half-maximal effective [Ca\(^{2+}\)]\(_{\text{ex1/2}}\) values were similar for C2A\(^{\ast}\) and wild-type proteins (0.58 and 0.49 mM, respectively) whereas C2B\(^{\ast}\) and C2A/B\(^{\ast}\) exhibited increased values (0.89 and 1.23 mM, respectively; Figure 1F). Thus the C2B domain, reported to mediate Ca\(^{2+}\)-dependent phospholipid binding (Boswell et al., 2012; Chica et al., 2016), conferred overall Ca\(^{2+}\) sensitivity. Combined mutations in C2A/B\(^{\ast}\) affected response amplitude and Ca\(^{2+}\) sensitivity (Figure 1, D and F). The results indicate that Munc13-4 functions as a Ca\(^{2+}\) sensor for SG exocytosis, integrating the Ca\(^{2+}\)-dependent functions of both C2 domains, similar to in vitro findings (Boswell et al., 2012).

**Munc13-4+ SGs form enlarged vacuoles upon ionomycin stimulation**

Expressed fluorescent constructs of Munc13-4 localize to SGs in RBL-2H3 cells (Neef et al., 2005; Higashio et al., 2008). We extended this to endogenous Munc13-4 in immunofluorescence studies in which Munc13-4 colocalized with vesicular monoamine transporter 2 (VMAT2; Figure 2A), the SG monoamine transporter. Munc13-4 immunofluorescence was not detected in Munc13-4–knockdown cells (Supplemental Figure S2A). Ionomycin treatment for 20 min markedly altered the localization of both Munc13-4 and VMAT2. There was extensive loss of SGs as expected for stimulated cells, but, unexpectedly, there was also the formation of large (>2.4 μm in diameter) vacuoles in which Munc13-4 and VMAT2 colocalized (Figure 2A). VMAT2 is membrane integrated, and so it is likely that a subset of SGs engaged in homotypic fusion to generate vacuoles. We further characterized membrane components on vacuoles to determine possible fusion events responsible for vacuole formation. SGs in RBL-2H3 and other hematopoietic cells acquire endosomal properties and represent a subset of Rab7+ late endosomes (Lev; Vincent-Schneider et al., 2001; Cohen et al., 2012; Luzio et al., 2014; Aouzou et al., 2014b). Rab7 colocalized with Munc13-4 on SGs in resting cells, as expected (Aouzou et al., 2012), and ionomycin treatment increased Rab7 colocalization with Munc13-4 on large vacuoles, suggesting that SGs may also fuse with additional Rab7+ LEs (Figure 2, B and C). This was also suggested by studies showing that vacuoles were LAMP2+ (Supplemental Figure S2B). Rab11, corresponding to recycling endosomes (REs), did not significantly colocalize with Munc13-4 in resting cells but localized to vacuoles with Munc13-4 in ionomycin-treated cells (Figure 2, B and C), indicating that REs participate in vacuole generation. Rab5, corresponding to early endosomes, did not significantly colocalize with Munc13-4 in resting or stimulated cells (Figure 2, B and C). The results suggest that Ca\(^{2+}\)-induced vacuoles result from a merge of Munc13-4+/Rab7+ SGs with Rab11+ REs and with additional Rab7+ LEs.

We assessed the apparent merge of Munc13-4+ membranes with Rab7+ and Rab11+ membranes by high-resolution (∼100 nm) structured illumination microscopy (SIM; Figure 2D). Munc13-4 colocalized with Rab7 and Rab11 on the vacuole in stimulated cells, indicating residence in a common membrane (Figure 2D, inset slices). High-pressure freezing-freeze-substitution electron microscopy (Murf et al., 2003) revealed that the vacuole was a single membrane-enclosed structure containing numerous 30- to 200-nm intraluminal vesicles (ILVs; Figure 2E). This indicated that vacuoles were enlarged multivesicular bodies similar to the Rab11+ vacuoles described for ionophore-treated erythroleukemia cells (Savina et al., 2005). SGs in RBL-2H3 cells contain ILVs (Vincent-Schneider et al., 2001), consistent with vacuole generation involving homotypic SG fusion.

We also assessed the distribution of R-SNAREs that might function in vacuole formation (Supplemental Figure S3, A and B). VAMP8 is a LE R-SNARE that mediates SG exocytic fusion in hematopoietic secretory cells (Paumet et al., 2000; Lippert et al., 2007; Ren et al., 2007; Lorentz et al., 2012). A fraction of VAMP8 colocalized with Munc13-4+ on SGs in resting RBL-2H3 cells and also on the vacuole in Ca\(^{2+}\)-stimulated cells. VAMP3, an R-SNARE on RE membranes (Lorentz et al., 2012), was widely distributed in the cytoplasm, and its fluorescence occasionally overlapped with that of Munc13-4 in resting cells. VAMP3 did appear to colocalize with Munc13-4 on the vacuole membrane in Ca\(^{2+}\)-stimulated cells, consistent with fusion with Rab11+ REs in vacuole generation. By contrast, VAMP7 appeared to localize within the vacuole possibly associated with ILVs. Overall the results were consistent with...
Munc13-4 was required for vacuole formation using Munc13-4–induced events that lead to vacuole formation. We determined whether Munc13-4 participates in one or more of the Ca\textsuperscript{2+}-induced membrane fusion events that lead to vacuole formation. We quantified vacuole formation by measuring the surface area of Rab27b\textsuperscript{+} vesicles (Figure 3, A and B). The largest Rab27b\textsuperscript{+} vesicles in resting wild-type cells had a mean area of 1.94 ± 1.25 μm\textsuperscript{2} (±SD), whereas 20-min ionomycin treatment increased this to 4.05 ± 3.39 μm\textsuperscript{2} (Figure 3B). Twenty-five percent of ionomycin-treated wild-type cells exhibited at least one vacuole larger than the size threshold (see legend to Figure 3B), ranging from 5 to 14 μm\textsuperscript{2}. By contrast, ionomycin treatment of Munc13-4–knockdown cells failed to stimulate vacuole formation (Figure 3, A–C, and Supplemental Figure S3E). Expression of hMunc13-4 in the knockdown cells restored vacuole formation to a level similar to that in wild-type cells (Figure 3C and Supplemental Figure S3E). Alternatively, we detected vacuole formation by the localization of enhanced green fluorescent protein (EGFP)–Rab11\textsuperscript{+} with mCherry-Rab7 on vacuoles in ionomycin-treated cells (Figure 3D). The knockdown of Munc13-4 eliminated vacuole formation (Figure 3, D and E), confirming that Munc13-4 is essential for Ca\textsuperscript{2+}-induced vacuole formation.

Ionomycin treatment in RBL-2H3 cells elevates cytoplasmic Ca\textsuperscript{2+} to levels similar to those with IgE receptor activation, although the latter elicits Ca\textsuperscript{2+} oscillations (Lin and Gilfillan, 1992; Lee and Oliver, 1995). We determined whether vacuole formation also occurs under physiological stimulation conditions of immunoglobulin E (IgE) receptor activation. The formation of Rab7\textsuperscript{+}/Rab11\textsuperscript{+} vesicles occurred with IgE-sensitized cells stimulated by antigen but not with IgE alone (Figure 3, F and G, and Supplemental Figure S3F). Similarly, treatment with thapsigargin to mobilize intracellular stored Ca\textsuperscript{2+} induced vacuole formation (Figure 3, F and G, and Supplemental Figure S3E). Similar-sized vacuoles were generated by each stimulus (Figure 3G), but the frequency of vacuole occurrence differed (Figure 3F). The results suggest that the frequency of vacuole formation depends on the magnitude and persistence of [Ca\textsuperscript{2+}]\textsuperscript{i} increases achieved for each stimulus (Lin and Gilfillan, 1992; Lee and Oliver, 1995). These studies indicate that Ca\textsuperscript{2+}-induced vacuole formation occurs under physiological stimulation conditions.
Munc13-4 is required for vacuole formation involving Ca\(^{2+}\)-dependent homotypic fusion of SGs

The preceding established that Munc13-4 was essential for Ca\(^{2+}\)-induced vacuole formation but did not determine which membrane fusion steps depended on Munc13-4. Live-cell imaging studies were used to resolve sequential fusion events in vacuole formation in ionomycin-treated cells (Figure 4A and Supplemental Video S1). Rab7\(^{+}\) SGs began forming a vacuole within 2 min after ionomycin stimulation in the cell shown (Figure 4A, inset, arrow). Subsequently Rab11\(^{+}\) REs merged with the Rab7\(^{+}\) vacuoles, with vacuole formation complete by 5–6 min in this example (Figure 4A, inset, merge). A delayed recruitment of Rab11 to Rab7\(^{+}\) vacuoles was reproducibly observed (Figure 4C), indicating that Rab7\(^{+}\) vacuoles form first and serve as acceptors for Rab11\(^{+}\) endosomes. We conducted similar studies to detect the merge of Rab11\(^{+}\) endosomes with assembling Munc13-4\(^{+}\) vacuoles (Figure 4B) and found a similar delay for the recruitment of Rab11\(^{+}\) endosomes to the forming vacuole (Figure 4C).

Similar studies in Munc13-4–knockdown cells were conducted to detect the merge of Rab11\(^{+}\) endosomes with Rab7\(^{+}\) vacuoles, but we found that Rab7\(^{+}\) vacuoles failed to form (Figure 4D and Supplemental Video S2). Rab7\(^{+}\) structures remained constant in size over a 15-min ionomycin stimulation period in the absence of Munc13-4 (Figure 4E). This indicated that Munc13-4 was required for an early step in Rab7\(^{+}\) vacuole formation. Because a subset of Rab7\(^{+}\) LEs are Munc13-4\(^{+}\) SGs, we determined whether the earlier step in vacuole formation involves the homotypic fusion of Rab7\(^{+}\)/Munc13-4\(^{+}\) SGs. Coexpression of mKate2-Munc13-4 and EGFP-Rab7 colocolated to SGs (Figure 5A, left), and ionomycin treatment promoted robust formation of Munc13-4\(^{+}\)/Rab7\(^{+}\) vacuoles (Figure 5A, right and inset, and Supplemental Video S3). Compared to Rab27b immunofluorescence for control cells, there was an increased number of vacuoles in some Munc13-4/Rab7\(^{+}\)-overexpressing cells compared with wild-type cells, but this was variable and not significant (Figure 5B). Overall these studies suggest that the Munc13-4–dependent step for Ca\(^{2+}\)-stimulated vacuole formation consists of the homotypic fusion of Munc13-4\(^{+}\)/Rab7\(^{+}\) SGs to form Munc13-4\(^{+}\)/Rab7\(^{+}\) vacuoles.

We determined whether Ca\(^{2+}\)-binding to Munc13-4 was essential for vacuole formation. Rab7\(^{+}\)-knockdown cells failed to form Rab27b\(^{+}\) vacuoles in response to ionomycin stimulation, whereas expression of hMunc13-4 fully rescued vacuole formation (Figure 5, C and D). By contrast, expression of the C2A*B\(^{+}\) hMunc13-4 mutant failed to rescue vacuole formation. Similarly, cooverexpression of EGFP-Rab7 and mKate2-hMunc13-4 rescued vacuole formation in rMunc13-4–knockdown cells (Figure 5E, top), whereas cooverexpression of EGFP-Rab7 and mKate2-C2A*B\(^{+}\) hMunc13-4 failed to rescue (Figure 5E, bottom), as quantitated in Figure 5F. These results indicate that Munc13-4 requires Ca\(^{2+}\) binding for its role in vacuole formation. Collectively, the studies of Figures 4 and 5 indicate that Munc13-4 functions in Ca\(^{2+}\)-triggered vacuole formation at the homotypic fusion of Munc13-4\(^{+}\)/Rab7\(^{+}\) SGs to form Munc13-4\(^{+}\)/Rab7\(^{+}\) vacuoles, and these intermediates fuse with Rab11\(^{+}\) REs to form mature vacuoles (Figure 5G). Munc13-4 may
We found that Ca\(^{2+}\)-activated Munc13-4 accelerates the fusion of liposomes containing VAMP8 with liposomes containing syntaxin-1/SNAP-25, the neuronal homologues of syntaxin-4/SNAP-23 (Figure 6B). The Munc13-4-stimulated FRET between liposomes was strictly dependent on Ca\(^{2+}\) (Figure 6B) and on the R- and Q-SNARE proteins (Figure 6C).

By contrast, homotypic LE fusion was reported to use VAMP8 paired with syntaxin-7, Vti1b, and syntaxin-8 (Antonin et al., 2000, 2002; Luzio et al., 2009). We found that Munc13-4 strongly promoted the fusion of VAMP8-containing liposomes with LE Q-SNARE (syntaxin-7, Vti1b, syntaxin-8) liposomes in the presence of Ca\(^{2+}\) (Figure 6A). No effects of Munc13-4 or Ca\(^{2+}\) were observed with liposomes lacking SNAREs (Figure 6C). Munc13-4 exhibited strongly Ca\(^{2+}\)-dependent binding to the Q-SNARE liposomes but did not bind liposomes lacking SNARE proteins (Figure 6, E and F). The Ca\(^{2+}\)-dependent binding of Munc13-4 was also observed for liposomes containing Vti1b alone but not to liposomes containing syntaxin-7 or syntaxin-8 (Figure 6, D and F; see Discussion). Although the fusion of liposomes reconstituted with LE SNAREs has previously been described (Shen et al., 2007; Yu et al., 2013), Ca\(^{2+}\)-bound Munc13-4 is the first regulatory factor identified that stimulates liposome fusion with reconstituted mammalian LE SNAREs. The results suggest that Munc13-4 could act as a Ca\(^{2+}\) sensor on membranes that contain LE SNARE proteins.

The association of Munc13-4 with LE SNARE complexes was confirmed by immunoprecipitation studies in RBL-2H3 cells expressing GFP-Munc13-4, which localizes normally to membranes (Figure 4). Immunoprecipitation of GFP-Munc13-4 by GFP-Trap nanobodies was highly efficient (Figure 6Ga) and found to coisolate syntaxin-7, Vti1b, syntaxin-8, and VAMP8 (Figure 6G, b–e). The retention of LE SNAREs by GFP-Munc13-4 was in each case enhanced by Ca\(^{2+}\) addition to the lysates. Each of the Munc13-4-associated LE SNARE proteins was isolated as 50-kDa or higher-molecular weight (MW) complexes that consisted of homooligomers or heterooligomers (Figure 6 legend). We recapitulated these homooligomers or heterooligomers in our SDS–PAGE system by ascribing purified LE SNARE proteins into heterotetrameric complexes that were subject to nonboiling and boiling SDS conditions shown for Vti1b (Figure 6H). These studies identifying 50-kDa or higher-MW LE SNARE complexes confirmed and extended

Figure S3, A and B; Paumet et al., 2000; Puri and Roche, 2008; Tiwari et al., 2008). For SG exocytosis, VAMP8 was reported to pair with syntaxin-4 and SNAP-23 (Paumet et al., 2000; Woska and Gillespie, 2011; Lorentz et al., 2012; Wesolowski and Paumet, 2014), and we found that Ca\(^{2+}\) promoted liposome fusion with reconstituted mammalian LE SNAREs. The results suggest that Munc13-4 could act as a Ca\(^{2+}\) sensor on membranes that contain LE SNARE proteins.

By contrast, homotypic LE fusion was reported to use VAMP8 paired with syntaxin-7, Vti1b, and syntaxin-8 (Antonin et al., 2000, 2002; Luzio et al., 2009). We found that Munc13-4 strongly promoted the fusion of VAMP8-containing liposomes with LE Q-SNARE (syntaxin-7, Vti1b, syntaxin-8) liposomes in the presence of Ca\(^{2+}\) (Figure 6A). No effects of Munc13-4 or Ca\(^{2+}\) were observed with liposomes lacking SNAREs (Figure 6C). Munc13-4 exhibited strongly Ca\(^{2+}\)-dependent binding to the Q-SNARE liposomes but did not bind liposomes lacking SNARE proteins (Figure 6, E and F). The Ca\(^{2+}\)-dependent binding of Munc13-4 was also observed for liposomes containing Vti1b alone but not to liposomes containing syntaxin-7 or syntaxin-8 (Figure 6, D and F; see Discussion). Although the fusion of liposomes reconstituted with LE SNAREs has previously been described (Shen et al., 2007; Yu et al., 2013), Ca\(^{2+}\)-bound Munc13-4 is the first regulatory factor identified that stimulates liposome fusion with reconstituted mammalian LE SNAREs. The results suggest that Munc13-4 could act as a Ca\(^{2+}\) sensor on membranes that contain LE SNARE proteins.

The association of Munc13-4 with LE SNARE complexes was confirmed by immunoprecipitation studies in RBL-2H3 cells expressing GFP-Munc13-4, which localizes normally to membranes (Figure 4). Immunoprecipitation of GFP-Munc13-4 by GFP-Trap nanobodies was highly efficient (Figure 6Ga) and found to coisolate syntaxin-7, Vti1b, syntaxin-8, and VAMP8 (Figure 6G, b–e). The retention of LE SNAREs by GFP-Munc13-4 was in each case enhanced by Ca\(^{2+}\) addition to the lysates. Each of the Munc13-4-associated LE SNARE proteins was isolated as 50-kDa or higher-molecular weight (MW) complexes that consisted of homooligomers or heterooligomers (Figure 6 legend). We recapitulated these homooligomers or heterooligomers in our SDS–PAGE system by ascribing purified LE SNARE proteins into heterotetrameric complexes that were subject to nonboiling and boiling SDS conditions shown for Vti1b (Figure 6H). These studies identifying 50-kDa or higher-MW LE SNARE complexes confirmed and extended

**Munc13-4 promotes liposome fusion with late endosomal SNAREs**

The preceding indicated that Munc13-4 and its Ca\(^{2+}\)-binding C2 domains are required for SG exocytosis and for endosomal vacuole formation in Ca\(^{2+}\)-stimulated RBL-2H3 cells. These fusion events may be mediated by VAMP8 on the SG membrane (Supplemental
the previous findings (Mascia and Langosch, 2007) that specific full-length LE SNARE proteins form very stable associations with one another (see Discussion). We conclude that Munc13-4 preferentially associates with or stabilizes LE SNARE complexes from RBL-2H3 cells.

**Ca²⁺-induced vacuoles are exocytic**

We addressed the physiological role of the Ca²⁺-induced vacuole. Mature vacuoles are of mixed endosomal origin (Rab7⁺, Rab11⁺, ILV⁺, LAMP2⁺) and contain SG constituents (Rab27b⁺, Munc13-4⁺, VMAT2⁺, β-hexosaminidase⁺). Ionomycin-stimulated β-hexosaminidase release extends over the time period of vacuole assembly, consistent with a possible exocytic role for the vacuole (Supplemental Figure S5A). This was confirmed in live-cell studies by coexpression of β-hexosaminidase (Hexβ⁺)-mCherry with EGFP-Munc13-4 (Figure 7A). Hexβ⁺mCherry localized to the lumen of a subpopulation of Munc13-4⁺ SGs in resting cells (Figure 7A, insets). Some Munc13-4⁺ SGs fused with each other upon ionomycin treatment to form a vacuole with Hexβ⁺mCherry content (Supplemental Video S4). Hexβ⁺mCherry content suddenly disappeared from the lumen of mature vacuoles within a 1-min frame, suggesting that exocytosis occurred (e.g., Figure 7A, inset 3, 18–19 min). Unit-size SGs (FWHM ≈ 0.75 μm; Figure 7A, inset 1, arrow; Grimberg et al., 2003) and intermediate-size vacuoles (FWHM ≈ 1.35 μm; Figure 7A, inset 2, arrowhead) also suddenly lost Hexβ⁺mCherry content in ionomycin-treated cells. Exocytosis from structures of various sizes is a type of Ca²⁺-triggered compound exocytosis termed multigranular, in which SGs merge with one another before their fusion with the plasma membrane (Alvarez de Toledo and Fernandez, 1990; Hartmann et al., 2003; Dvorak, 2005). It is noteworthy that vacuoles also fused with vacuoles that had undergone exocytosis (Figure 7A, inset 4), which is another form of compound exocytosis, termed sequential exocytosis (Nemoto et al., 2001; Behrendorff et al., 2011).

Simultaneous epifluorescence and total internal reflection fluorescence (TIRF) microscopy imaging confirmed that vacuoles underwent exocytosis (Figure 7B and Supplemental Videos S4 and S5). In the example shown, two large vacuoles containing Hexβ⁺mCherry were evident; one of these contacted the plasma membrane during the interval (44–48 min) shown. Contact of the vacuole with the plasma membrane was
evident at 45 min by the focal brightening of EGFP-Munc13-4 in TIRF (Figure 7B, bottom). Hexβ-mCherry was released in the next frame (Figure 7B, top). EGFP-Munc13-4 darkened at the local contact (Figure 7B, bottom) at 46 min, corresponding to fusion pore formation, and subsequently spread by diffusion onto the plasma membrane. The apparent fusion pore diameter was >500 nm and lasted for >1 min. Thus large vacuoles dock onto the plasma membrane, form a fusion pore, discharge contents, and deliver the vacuole membrane constituent Munc13-4 onto the plasma membrane. The exocytosis of a smaller vacuole (FWHM = 1.2 μm) was analyzed by TIRF at a higher frame rate to measure the diffusion of Munc13-4 onto the plasma membrane (Figure 7C and Supplemental Video S6). The vacuole approached the plasma membrane (4.8–8 s), docked (12.8–33.6 s), and opened a fusion pore at ~36 s. The diffusion coefficient for the lateral spread of EGFP-Munc13-4 of ~0.07 μm²/s (Supplemental Figure S5, B and C) was slower than lipid diffusion (~1 μm²/s) but only approximately threefold slower than VAMP2-EGFP diffusion in the exocytosis of smaller (~0.3 μm in diameter) SGs in chromaffin cells (~0.2 μm²/s; Allersma et al., 2004). The results indicate that Munc13-4 retains its membrane association before, during, and after fusion as it diffuses from the vacuole membrane onto the plasma membrane.

Ca²⁺-induced vacuoles release multiple contents

Mature vacuoles were characterized as Rab11⁺ and might receive cargo from the Golgi by the fusion of Rab11 endosomes. Cytokine trafficking in macrophages, specifically that of tumor necrosis factor α (TNFα) and IL-6, was reported to be mediated by Rab11⁺ endosomes (Manderson et al., 2007). We confirmed possible post-Golgi cytokine trafficking in Rab11⁺ endosomes for RBL-2H3 cells at early times after expression of TNFα-yellow fluorescent protein (YFP) with CFP-Rab11 or IL-6-mCherry with EGFP-Rab11, where significant colocalization in perinuclear regions was observed (Figure 8A). Munc13-4⁺ vacuoles formed after ionomycin stimulation were found to contain TNFα-YFP (Figure 8B) and IL-6-mCherry (Figure 8C). In both cases, these cargoes disappeared from vacuoles during exocytosis (marked as 0 s in Figure 8, B and C). The results indicate that cytokines represent additional cargo that can be packaged into vacuoles and discharged from them upon exocytosis.

DISCUSSION

Munc13-4 is a Ca²⁺-dependent SNARE- and phospholipid-binding protein that localizes to SGs in resting mast cells. The present work broadens Munc13-4’s cellular role as a tethering/priming factor for SG exocytosis to endosomal fusion events. With its Ca²⁺-binding C2 domains, Munc13-4 functions as a Ca²⁺ sensor that localizes to SGs in resting mast cells. The present work broadens Munc13-4’s cellular role as a tethering/priming factor for Ca²⁺-dependent fusion of liposomes containing exocytic SNARE proteins (Boswell et al., 2012; Chicka et al., 2016). These studies suggested that Munc13-4 might function as a Ca²⁺ sensor in SG exocytosis. We provide direct evidence for this in intact cells by finding that the C2A⁺ and C2B⁺ mutant Munc13-4 proteins altered the amplitude and Ca²⁺ sensitivity of Ca²⁺-induced β-hexosaminidase release, respectively. The role of both C2 domains was indicated by an additive loss of function for C2A⁺C2B⁺ Munc13-4. Munc13-4⁺ SGs were not tethered or docked at the plasma membrane in RBL-2H3 cells in our studies but translocated to the plasma membrane in Ca²⁺-stimulated cells. We suggest that Munc13-4 localized on SGs could tether SGs to the plasma membrane in stimulated cells via Ca²⁺-dependent C2B interactions with acidic phospholipids (Boswell et al., 2012; Chicka et al., 2016). Ca²⁺ binding to C2 domains could also unmask SNAP23-binding sites on Munc13-4 to initiate SNARE complex assembly for docking and priming SGs (Boswell et al., 2012; He et al., 2016).

Remarkably, Munc13-4 also functioned as a Ca²⁺ sensor to promote the de novo, Ca²⁺-triggered assembly of an endosomal vacuole. The SGs in RBL-2H3 cells and other hematopoietic cells acquire late endosomal properties and are Rab7⁺ (Vincent-Schneider et al., 2001; Benado et al., 2009; Cohen et al., 2012; Azouz et al., 2014a; Luzio et al., 2014). We found that Ca²⁺-activated Munc13-4 promoted the homotypic fusion of SGs to form Rab27β⁺/Rab7⁺/ Munc13-4⁺/VMAT2⁺/β-hexosaminidase/ILV⁺ vacuolar intermediates. Vacuoles increase in size as they mature and become Rab11⁺ from a late fusion with REs (Figure 9). Studies in cytokotoxic T-cells suggested that Munc13-4 might function to promote the fusion of Rab11⁺ with Rab7⁺ membranes (Menager et al., 2007). However, our studies were unable to determine whether a Rab11⁺ endosome merge with Rab7⁺ vacuoles required Munc13-4 because earlier fusion steps in the vacuole assembly pathway failed to occur in Munc13-4−/knockdown cells. Unlike cytokotoxic T-cells (Menager et al., 2007) or neutrophils (Johnson et al., 2016), we did not detect Munc13-4 on Rab11⁺ endosomes. The important execution point for Ca²⁺-activated Munc13-4 in the RBL-2H3 cells was at homotypic SG fusion, which initiates vacuole formation. This reveals that Munc13-4 functions in the Ca²⁺-triggered fusion of endosomal SGs in mast cells, where it serves as a Ca²⁺ sensor. The role for Munc13-4 in Ca²⁺-triggered endosome fusion characterized here is different from that in studies in cytokotoxic T-cells (Menager et al., 2007) or neutrophils (He et al., 2016), in which Munc13-4-dependent endosome fusion was observed in resting rather than Ca²⁺-stimulated cells, but local Ca²⁺ increases might activate Munc13-4 in these cases.

Munc13-4 associates with LE SNARE protein complexes

The R-SNARE VAMP8 is a common denominator for Munc13-4–dependent fusion events in RBL-2H3 cells because VAMP8 resides on SGs. For SG exocytosis, VAMP8 pairs with syntaxin-4 and SNAP-23 (Paumet et al., 2000; Lorentz et al., 2012; Wesolowski and Paumet, 2014), and we found that Ca²⁺-activated Munc13-4 accelerates fusion between VAMP8- and syntaxin1/SNAP23-containing liposomes, similar to results of a recent study using syntaxin2/SNAP23 acceptor liposomes (Chicka et al., 2016). By contrast, homotypic LE fusion uses VAMP8 paired with syntaxin-7, syntaxin-8, and Vti1b (Antonin et al., 2000, 2002). Munc13-4 promoted Ca²⁺-stimulated liposome fusion using these LE R- and Q-SNARE proteins. We also

Volume 28  March 15, 2017  799
FIGURE 6: Ca\(^{2+}\)-activated Munc13-4–dependent liposome fusion. (A) Liposome fusion was detected by dil-diD FRET between VAMP8- and syntaxin7/Vti1b/syntaxin8-containing liposomes without additions (open circles), with 1 μM Munc13-4 (gray circles), or with Munc13-4 plus 300 μM Ca\(^{2+}\) (red circles). (B) Fusion of liposomes containing VAMP8 and syntaxin1/SNAP25 was detected in the absence of additions (open circles), with 1 μM Munc13-4 (gray circles), or with Munc13-4 and 300 μM Ca\(^{2+}\) (red squares). (C) Protein-free (Pf) liposomes exhibited little fusion in the incubations conditions tested with no additions (open diamond), with 1 μM Munc13-4 (black diamonds), or with Munc13-4 and 300 μM Ca\(^{2+}\) (blue diamonds). (D, E) Munc13-4 binding to liposomes reconstituted with the indicated LE Q-SNAREs or protein free was detected in liposome flotation studies either with EGTA or Ca\(^{2+}\). Binding studies were conducted under the same conditions as in A with 1 μM Munc13-4 and EGTA or 300 μM Ca\(^{2+}\) as indicated. (F) Western blots as in D and E were quantitated and are presented as fold stimulation comparing signals with Ca\(^{2+}\) to those with EGTA (means ± SD, N = 3). Proteins used in these studies are shown in Supplemental Figure S4. (G) EGFP-Munc13-4 expressed in RBL-2H3 cells was immunoprecipitated with GFP-Trap nanobody from detergent lysates that included EGTA or CaCl\(_2\) (100 μM). Immunoprecipitates were eluted by boiling in SDS sample buffer, run on SDS–PAGE, and immunoblotted for (a) Munc13-4, (b) syntaxin7, (c) Vti1b, (d) syntaxin-8, and (e) VAMP8 as indicated. Blots were probed sequentially with several antibodies to determine whether bands were immunoreactive for different LE SNAREs or were probed individually with multiple distinct antibodies; two different antibodies detected VAMP8 in e; the results shown are representative of three independent studies for each SNARE. LE SNARE antibodies specifically recognized monomeric LE SNAREs in input lanes (syntaxin-7, 33.5 kDa; Vti1b, 26.1 kDa; syntaxin-8, 30.7 kDa; VAMP8, 13.6 kDa [not shown]). Band assignments for
found that Munc13-4 directly associates with LE SNARE protein complexes in a Ca\textsuperscript{2+}-stimulated manner. In liposome flotation studies with purified proteins, Munc13-4 exhibited Ca\textsuperscript{2+}-dependent binding to liposomes reconstituted with Vti1b or syntaxin-7/Vti1b/syntaxin-8 complexes but not with protein-free liposomes. However, immunoprecipitates of GFP-Munc13-4 in RBL-2H3 cells indicated that Munc13-4 associates with LE SNARE protein complexes, including Vti1b homodimers and homotetramers rather than with SNARE protein monomers. Vti1b exhibits strong self-associations as a transmembrane protein (Mascia and Langosch, 2007), and it is likely that the preferential binding of Munc13-4 to Vti1b and Q-SNARE-containing liposomes reflected this preference for binding to LE SNARE protein complexes. The additional LE SNARE protein complexes that coprecipitated with Munc13-4 were highly stable, ∼50-kDa heterodimeric complexes detected with multiple antibodies, which consisted of syntaxin-7/VAMP8 and Vti1b/syntaxin-8, as well as oligomeric Vti1b. These were likely generated from Munc13-4 bound from multimeric heterotetrameric LE SNARE complexes subjected to boiling and SDS–PAGE (Mascia and Langosch, 2007).

A Ca\textsuperscript{2+}-dependent association of Munc13-4 with syntaxin-7 but not Vti1b or syntaxin-8 in HEK cell lysates was recently reported (He et al., 2016). However, these studies used cooverexpression of Munc13-4 and individual LE SNARE proteins. By contrast, our studies used a GFP-Munc13-4 protein that localizes normally to membranes in RBL-2H3 cells, and the immunoprecipitates isolated endogenous LE SNARE protein complexes assigned by molecular mass and multiple antibody recognition. The results indicate that endosome docking, priming, or fusion may be driven by the Ca\textsuperscript{2+}-dependent binding of Munc13-4 to endosomal Q-SNAREs with the recruitment of VAMP8 for assembly of SNARE complexes. The liposome fusion and SNARE-binding studies presented here are important in revealing the potential for Munc13-4 to act as a Ca\textsuperscript{2+} sensor in membrane fusion events involving LE SNAREs.
In RBL-2H3 cells, Munc13-4 was associated with membranes that underwent fusion. A key element for the specificity of Munc13-4 in membrane fusion events may be its association with at least one of the fusion partner membranes. The membrane association of Munc13-4 may be mediated by Rab27b, with which it strongly colocalizes on SGs or vacuoles. However, Munc13-4 lacking a Rab27 interaction domain was reported to localize to SGs in RBL-2H3 cells (Elstak et al., 2011). This may be explained by the finding that Munc13-4 interacts with multiple Rab proteins such as Rab15, Rab11, Rab27a, Rab37, and Rab22a (Masuda et al., 2000; Shirakawa et al., 2004; Neef et al., 2005; Zografou et al., 2012; Singh et al., 2013; Higashio et al., 2016; Johnson et al., 2016). Rab27 is nonetheless required for Munc13-4 function in SG exocytosis in RBL-2H3 cells (Neef et al., 2005; Elstak et al., 2011; Singh et al., 2013). Studies have also documented Ca\(^{2+}\)-dependent interactions of soluble Munc13-4 with membranes mediated by its C-terminal C2B domain (Boswell et al., 2012; Chicka et al., 2016). Rab-bound Munc13-4 on a donor membrane could bridge to an acceptor membrane in a Ca\(^{2+}\)-stimulated cell and mediate SG docking to the plasma membrane for heterotypic fusion or to other SGs for homotypic fusion (Elstak et al., 2011; Johnson et al., 2011; Chicka et al., 2016). Thus the membrane association of Munc13-4 by a particular set of Rab GTPases may determine specificity of Munc13-4–promoted membrane fusion (Higashio et al., 2016).

**Function of exocytic vacuole and compound exocytosis**

Classically, stimulated mast cells, as well as other hematopoietic secretory cells, exocytose mediators by both simple and compound exocytosis. In RBL-2H3 cells, stimulated β-hexosaminidase secretion occurs from SGs, as well as from variable-size endosomal vacuoles (Figure 9). One mode of compound exocytosis is multigranular, in which SGs merge homotypically to form larger exocytic structures, which have been characterized in mast cells and basophils by electron microscopy (Lawson et al., 1977; Chandler and Heuser, 1980; Dvorak et al., 1981; Dvorak, 2005). However, the mechanisms responsible for multigranular compound exocytosis have not been previously elucidated. The present work indicates that Ca\(^{2+}\)-triggered multigranular compound exocytosis is mediated by Ca\(^{2+}\)-activated Munc13-4 acting to promote exocytic vacuole assembly through the homotypic fusion of SGs. That homotypic endosomal fusion may be mediated by a Rab/Munc13-4 complex could account for the finding that both Ca\(^{2+}\) and stable GTP analogues promote multigranular compound exocytosis in mast cells and eosinophils (Alvarez de Toledo and Fernandez, 1990; Hartmann et al., 2003). Multigranular compound exocytosis also occurs in many other cells triggered by high Ca\(^{2+}\), such as pancreatic β-cells, endothelial cells, neutrophils, and neurons (Lolleke et al., 2002; He et al., 2009; Valentijn et al., 2010; Hoppa et al., 2012). It will be important to assess Munc13-4’s role in these cell types.

Compound exocytic vacuoles were surprisingly complex in RBL-2H3 cells containing SG membranes and cargo as well as LE and RE membranes. Mast cells contain SGs of several types, including granules that are multivesicular endosomes (Raposo et al., 1997; Grimberg et al., 2003). As these merge to assemble an enlarged vacuole, the vacuole retains its multivesicular body characteristics (Savina et al., 2005).
Note that vacuolar surface markers used throughout these studies (Munc13-4, Rab7, Rab27b, VAMP7, VAMP8) were detected to various extents as puncta within vacuoles, consistent with their reported presence on exosomes as documented in ExoCarta (Keerthikumar et al., 2016). Invagination of the multivesicular body membrane to generate ILVs may operate basally to internalize these membrane-associated proteins.

Vacuoles in Ca$^{2+}$-stimulated RBL-2H3 cells acquired Rab11 after homotypic SG fusion events generated Munc13-4+/Rab7$^+$ precellular intermediates (Figure 4). The merge of Rab11$^+$ endosomes with the vacuole may convey Rab11 to vacuoles for vacuole exocytosis. Rab11 is involved in the exocytic fusion of REs and multivesicular bodies with the plasma membrane (Savina et al., 2005; Takahashi et al., 2012) and could activate Munc13-4 on the vacuole for vacuole exocytosis (Johnson et al., 2016). In addition, Rab11$^+$ REs deliver additional cargo from the Golgi to the vacuole. Cytokines are trafficked from the Golgi by Rab11$^+$ REs in macrophages (Manderson et al., 2007; Lacy and Stow, 2011; Stow and Murray, 2013). Although cytokine secretion from mast cells may occur largely by pathways distinct from mediator release (Blank et al., 2014), cytokines may also be delivered to multigranular vacuoles for cosecretion. We tested this concept by expressing fluorescent versions of TNFα and IL-6. At early times after transfection, a significant fraction of the newly synthesized cytokines colocalized with Rab11 transport vesicles (Figure 8A). Ionomycin stimulation of the cells resulted in TNFα delivery into the vacuole and subsequent release upon vacuole exocytosis (Figure 8B), which suggests that direct delivery of TNFα from the Golgi to the vacuoles is mediated by Rab11$^+$ REs. IL-6 also colocalized with Rab11$^+$ endosomes at early times after transfection (Figure 8A), but the delivery of IL-6 to the vacuole and its subsequent release by vacuole exocytosis in Ca$^{2+}$-stimulated cells was only detected at late times after transfection (Figure 8D), which suggests a more extended trafficking itinerary from the Golgi to vacuole for IL-6. These studies support the conclusion that vacuoles are highly heterogeneous for cargo derived from multiple trafficking pathways. Thus these organelles assembled for multigranular compound exocytosis represent a collection point for the delayed secretion of multiple constituents. Exocytic vacuoles may be polarized in mast cells for efficiently responding to localized pathogens or immune complexes, as suggested by recent studies (Carroll-Portillo et al., 2010; Jouila et al., 2015).

The Ca$^{2+}$ increases and membrane-associated Munc13-4 lead to the de novo generation of a new exocytic organelle in mast cells. Our findings provide the first molecular description of the formation of multigranular compound exocytic structures. There are apparent parallels between exocytic vacuole formation in mast cells and the generation of cytotoxic granules in T-cells, in which both are hybrid endosomal exocytic organelles. There are nonetheless differences in how the endosomal fusion machinery is used in mast cells and cytotoxic T-cells. Munc13-4 localizes to Rab11 endosomes in cytotoxic T-cells and possibly catalyzes the merge of Rab11 endosomes with Rab7+/Rab27$^+$ endosomes to generate a precursor to cytotoxic granules (Menager et al., 2007; van der Sluijs et al., 2013). By contrast, Munc13-4 localizes to the Rab7+/Rab27$^+$ vacuole during a merge with Rab11$^+$ endosomes in mast cells. Future studies will need to clarify the basis for the membrane localization of Munc13-4, which is critical for localizing its function.

**MATERIALS AND METHODS**

**Cells and materials**

RBL-2H3 cells (CRL-2256; American Type Culture Collection, Manassas, VA) were cultured in MEM supplemented with 15% fetal bovine serum (FBS) and Antibiotic-Antimycotic (Life Technologies, Carlsbad, CA). 293FT cells (Life Technologies) used for lentivirus production were cultured in DMEM supplemented with 10% FBS, Antibiotic-Antimycotic, and 500 μg/ml Genetica (Life Technologies). Cells were maintained at 37°C in a 5% CO$_2$ atmosphere. Lentiviral shRNA constructs for rat Munc13-4 designed by the TRC Consortium were purchased from Sigma-Aldrich (TRCN0000381377; St. Louis, MO) and Open Biosystems (TRCN0000027704; Lafayette, CO). pLKO.1 plasmid was purchased from Sigma-Aldrich. pMD2G and pCMVΔR8.74 constructs for lentivirus production were purchased from Addgene. pLVX-mCherry plasmid was purchased from Clontech (Mountain View, CA). To generate lentivirus, hMunc13-4 or mutant hMunc13-4 (Boswell et al., 2012), open reading frames (ORFs) were subcloned into the pLVX plasmid after removal of the mCherry ORF. The mCherry-Rab7 construct was a kind gift from A. Sorkin (University of Pittsburgh, Pittsburgh, PA), and pEGFP-Rab7 was purchased from Addgene. pmKate2-Munc13-4 and pEGFP-Munc13-4 were generated by inserting human Munc13-4 cDNA into pmKate2-C1 (Evrogen, Moscow, Russia) or pEGFP-C1 (Clontech) plasmids. Plasmids for mouse HexB-mCherry plasmid and mouse IL-6-mCherry were purchased from Genecopoeia (Rockville, MD). TNFα-YFP construct was a kind gift from M. Olzsewski (International Institute of Molecular and Cell Biology, Warsaw, Poland), and the CFP-Rab11 construct was a kind gift from M. Zerial (Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany). LAMP2-mCherry construct was a kind gift from A. Lakkaraju (University of Wisconsin–Madison, WI). Antibodies used were goat polyclonal Munc13-4 antibody (residue 967–980 amino acids; NB100-41385; used at 5–10 μg/ml for immunofluorescence) from Novus Biologicals (Littleton, CO) and rabbit polyclonal Munc13-4 antibody (full length; H00201294-D01P; used at 5–10 μg/ml for immunofluorescence) from Abnova (Taipei City, Taiwan). These are characterized for immunofluorescence in Supplemental Figure S2. Rabbit polyclonal Munc13-4 C2A antibody was generously provided by H. Horuiuchi (Tohoku University, Sendai, Japan); rabbit polyclonal VMAT-2 antibody, used at 1:1000, was generously provided by A. Ruoho (University of Wisconsin–Madison); rabbit polyclonal Rab27b antibody was purchased from Synaptic Systems (Goettingen, Germany; 168103; used at 5–10 μg/ml for immunofluorescence); sheep polyclonal syntaxin-7 antibody (AF5478; 1μg/ml for Western blotting), goat polyclonal VAMP8 antibody (AF5354; 1 μg/ml for Western blotting), and sheep polyclonal syntaxin-8 antibody (AF5448; 1 μg/ml for Western blotting) were purchased from R&D systems (Minneapolis, MN); another rabbit polyclonal antibody against VAMP8 was generously provided by G. Groblewski (University of Wisconsin–Madison; 1:3000 dilution for Western blotting); rabbit polyclonal Vit1b antibody was purchased from Sigma-Aldrich (SAB1410097; 1 μg/ml for Western blotting); and Alexa fluorophore-conjugated secondary antibodies were from Molecular Probes (Eugene, OR). Monoclonal anti-2,4-dinitrophenol (DNP) IgE, DNP–human serum albumin (HSA), ionomycin, thapsigargin, and the β-hexosaminidase substrate p-nitrophenyl N-acetyl-β-D-glucosaminide (pNAG) were from Sigma-Aldrich.

**Lentivirus transduction and generation of stable clones**

Lentiviruses were produced as in Berger et al. (2011) with modifications. 293FT cells were transfected with 6 μg of pMD2.G, 15 μg of pCMVΔR8.74, and 20 μg of shRNA plasmid per 10-cm dish by CaPO$_4$ precipitation. At 24 h after transfection, media were replaced by complete growth medium added with 11 μg/ml BSA. Supernatants were collected at 48 and 72 h after transfection. Supernatants were concentrated 200-fold by 2 h of sedimentation at 110,000 × g
and resuspended in phosphate-buffered saline (PBS) by swirling overnight. The virus solution was aliquoted for frozen storage at −80°C. Genomic RNA from the lentivirus was purified, and copy numbers of lentivirus for an aliquot were determined using Lenti-X qRT-PCR Titration Kit (Clontech) following the manufacturer’s protocol. Lentivirus infection was achieved by incubating cells with lentivirus and 10 μg/ml protamine sulfate. Munc13-4–deficient clones were selected in the presence of 0.7 μg/ml puromycin for 10–14 d with periodic medium replacement. We generated 35 stable clones, the majority of which exhibited reduced or absent Munc13-4 protein expression that correlated with reduced secretion. Results reported are representative from several stable clones. In rescue studies, a lentivirus carrying pLKO.1 vector served as a sham control. To express hMunc13-4 in knockdown (KD) cells, 48 h of infection was typically sufficient to achieve normal cell levels of expression. Ongoing suppression of endogenous Munc13-4 in rescue studies was verified by quantitative reverse transcription PCR (qRT-PCR) for the rat Munc13-4 transcript. A lentivirus that carries pLVX-mCherry served as a sham control unless there was a conflict for multichannel fluorescence microscopy. To compare C2 domain mutants with WT Munc13-4, we infected 2 × 10^4 copy of each lentivirus in 7.5 × 10^3 KD cells. To monitor variation in rescue, multiple clones were infected with hMunc13-4 lentivirus, which showed a comparable frequency of vacuole formation upon ionomycin stimulation.

qRT-PCR
Total RNAs were extracted from cells by TRizol reagent (Life Technologies) according to the manufacturer’s protocol. DNA contaminations were digested by incubation of the total RNAs with DNase I (New England Biolabs, Ipswich, MA) for 30 min at 37°C, and the enzyme was inactivated by incubation at 75°C for 10 min. RNA concentration was determined by NanoDrop (Thermo Scientific, Waltham, MA). A 1-μg amount of total RNA was subject to a reverse transcription using Affinity Script reverse transcriptase (Agilent Technologies, Santa Clara, CA) with oligo d(T) primer according to the manufacturer’s protocol. Endogenous mMunc13-4 cDNA was amplified by Brilliant II SYBR Green PCR Master Mix (Agilent Technologies) using forward primer ATCGATGCCAAGGGTGCA and reverse primer GTCAAGTCCAGTACCTGAC, and the amount of amplification was monitored by ABI7500 Fast System (Life Technologies). As a reference gene, rat β-actin cDNA was amplified and detected by the same method using forward primer AGGGCAACCGTGAAAAAGATG and reverse primer GGTACGACAGGCGATACA. Cycle threshold was determined for mMunc13-4 and β-actin from the amplification curves acquired by 7500 Software (Life Technologies). Relative expression was calculated by the EΔΔCt method using Ct values of parental RBL cell samples as controls (Pfaffl, 2001).

Secretion assays
β-Hexosaminidase was determined by its enzymatic activity for chromogenic substrate pNAG. Cells were seeded in 96-well microwell culture plates and stimulated in Tyrode’s buffer (130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5.6 mM glucose, 0.5 mg/ml bovine serum albumin [BSA], pH 7.4) with various concentration of CaCl₂ in the presence or absence of 2.5 μM ionomycin at 37°C for 20 min. Supernatants were transferred to microtiter assay plates, and the remaining cells were lysed by 1% Triton X-100 for 5 min at room temperature. Portions of supernatants and lysates were subject to incubation with 8 mM pNAG in buffer (0.1 M citrate, 0.2 M Na₂HPO₄, pH 4.5). Subsequent enzyme reactions were conducted for 1 h at 37°C. The reaction was stopped and developed by addition of glycine stop buffer (0.2 M glycine, 0.2 M NaOH, pH 10). Conversion of pNAG to yellow was read at 405 nm, with background subtraction of absorbance at 630 nm. The absorbance values of supernatant and lysate were combined for total β-hexosaminidase activity, and secretion was plotted by calculating the enzyme activity in supernatant as percentage of total activity. The Ca²⁺ dose–response curves were analyzed by nonlinear least squares fits with the model equation % secretion = % secretionₘᵢₙ + (% secretionₘᵢₙ − % secretionₘᵢₙ)/(1 + 10(logEC₅₀ − logCa²⁺) x slope) using R software. Variable slopes do not improve curve fits and therefore did not bring any significant difference to curve fitting. Consequently a slope value 1.3, which was determined for wild-type rescue samples, was applied for all the curves, with other parameters variable. Best-fitting models were visualized by Prism software (GraphPad) and the model fitting analysis was conducted through consultation with the University of Wisconsin Institute for Clinical and Translational Research (Mary Lindstrom, University of Wisconsin–Madison).

Immunoblotting
RBL cells were washed and solubilized by ice-cold 1% Triton X-100 in PBS in the presence of a protease inhibitor cocktail and 1 mM phenylmethanesulfonyl fluoride for 10 min at 4°C with gentle rocking. The lysate was cleared by centrifugation at 16,000 × g for 5 min at 4°C. SDS was added to 2% final concentration, and lysate was boiled for 5 min at 96°C. The boiled lysates were cleared by centrifugation, and protein concentration was determined by BCA Assay Kit (Pierce Chemical, Rockford, IL). Between 20 and 30 μg of lysate protein was mixed with 4× SDS sample buffer (250 mM Tris-HCl, pH 6.8, 30% glycerol, 300 mM dithiothreitol [DTT], 8% SDS, 0.02% bromophenol blue) and boiled for 5 min at 96°C before loading on 8% polyacrylamide gels. After electrophoresis (Bio-Rad, Hercules, CA), the proteins in the gel were electrotransferred to nitrocellulose membranes (0.45-μm pore; Bio-Rad). Membranes were blocked with 5% skim milk in PBST (PBS with 0.1% Triton X-100) for 1 h at room temperature and incubated with Munc13-4 antibodies diluted in 1:5000 ratio (rabbit polyclonal antibody against residues 1–262 of hMunc13-4 [Boswell et al., 2012] or goat polyclonal antibody against residues 967–980 of hMunc13-4 [Abnova]) in 5% BSA in PBST overnight. Primary antibody was detected by incubation with horseradish peroxidase–conjugated secondary antibodies for 2 h at room temperature. Blots were developed by an enhanced chemiluminescence kit (Pierce) and imaged by an ImageQuant LAS 4000 system (GE Healthcare, Pittsburgh, PA). Band intensity was quantified by image analysis using ImageJ software.

Transfection and immunofluorescence
For fluorescence microscopy, glass coverslips were coated with 0.1 mg/ml poly-d-lysine and 30 μg/ml bovine fibronectin at 37°C (Sigma-Aldrich). To express fluorescence-tagged proteins, 2 μg of DNA was mixed with RBL cells suspended in Cytomix buffer including 5 mM glutathione and 2 mM ATP in a 1-mm-gap electroporation cuvette (van den Hoff et al., 1992). The mixture was electroshocked by two consecutive 225-V pulses with 1-ms duration using an ECM830 electroporator (Harvard Apparatus, Holliston, MA). Media were replaced after 24 h, and the constructs typically expressed fluorescence proteins after 48 h of incubation. For stimulation, cells were incubated in Tyrode’s buffer with 10 mM CaCl₂ in the presence or absence of 2.5 μM ionomycin generally for 20 min. For antigen stimulation, cells were primed with 200 ng/ml anti-DNP monoclonal IgE in growth medium overnight and subsequently washed with Tyrode’s buffer and incubated with 50 ng/ml DNP-HSA in Tyrode’s buffer containing molecular biology of the cell.
10 mM CaCl$_2$ for 20 min. Alternatively, RBL cells were stimulated with 1 µM thapsigargin in 10 mM Ca$^{2+}$-containing Tyrode’s buffer. For live-cell imaging, glass-bottom culture dishes (MatTek, Ashland, MA) were coated with poly-D-lysine (0.1 mg/ml) at 37°C for 30 min, followed by coating with fibronectin (33 µg/ml) at 37°C for 30 min. For immunostaining, cells were washed and fixed in 4% formaldehyde in PBS for 30 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 20 min at room temperature. Nonspecific binding was blocked by incubating with 5% FBS or goat or donkey serum for 1 h. The cells were incubated with primary antibodies diluted in blocking solution overnight at 4°C. The cells were washed and incubated with 20 µg/ml Alexa Fluor-conjugated secondary antibodies for 1 h at room temperature. After additional washing, nuclei were stained with 2 µg/ml Hoechst 33342 for 5 min at room temperature. Cells attached to glass coverslip were buffered by Slowfade Gold solution (Life technologies) and mounted on glass slides.

**Fluorescence microscopy and image analysis** Cells were imaged by epifluorescence or TIRF illumination using a Nikon Eclipse Ti microscope controlled by NIS element software. Images were captured with an iXon Ultra EM-CCD camera through a Nikon APO 100× TIRF numerical aperture (NA) 1.49 objective. Time-lapse imaging for live cells was conducted in a humidified imaging chamber maintained at 37°C (Tokai Hit, Shizuoka-ken, Japan). Simultaneous epi-TIRF images were acquired using different Perfect Focus System offset values for epifluorescence and TIRF channels. The switching time between epifluorescence and TIRF channels was <1 s. The SIM images were acquired with a Nikon N SIM microscope (SR-APO TIRF NA 1.49 100× lens, Andor iXon 3 EM-CCD camera, and 408-, 488-, 561-, and 640-nm laser lines) using three-dimensional (3D) SIM mode and reconstructed with NIS software. Confocal images were acquired with a Nikon A1R+ confocal system using GaAsP detectors. Epifluorescence and confocal images were deconvolved, processed, and analyzed by NIS software (Nikon, Tokyo, Japan). Epifluorescence images were deconvolved by two-dimensional (2D) blind or 2D Landweber algorithm with one to five iterations. Confocal images were deconvolved by the 3D Landweber algorithm with five iterations. Deconvolution artifacts were checked by line scan analysis comparing with original images. For fluorescence overlap analysis, Manders’ overlap coefficient was calculated by JACoP plug-in in ImageJ for fluorescent pixels above threshold (Bolte and Cordelieres, 2006). Fluorescence channels were typically arranged as red fluorescence, channel 1; and green fluorescence, channel 2, in JACoP plug-in. Manders’ coefficients produced by JACoP included M1 and M2, where M1 was fraction of channel 1 overlapping with channel 2, and M2 was fraction of channel 2 overlapping with channel 1. Channel arrangements are indicated in the figure legends. Pearson’s r for Figure 8B was calculated by NIS software. For vesicle profile area determination, images were binarized by threshold function, and circular objects were detected by particle analysis function in ImageJ software. The diffusion coefficient of EGFP-Munc13-4 was calculated as in Zenisek et al. (2002) using ImageJ software. Circular regions of interest (ROIs) were drawn on the image showing fusion pore opening. The first ROI with 2-pixel diameter was located on the position of the fusion pore, and successive ROIs were drawn around the first ROI with a 2-pixel increment in diameter as concentric rings (radial sweeps). The radial sweeps were applied to images before and after fusion pore opening and mean intensities were measured. Mean intensity differences (MIDs) were calculated by subtracting mean intensity values between successive ROIs to calculate Munc13-4 fluorescence spread. The calculated MIDs were fitted into a Gaussian distribution. The Gaussian distribution of fluorescence intensity around the fusion pore and its width parameter $\sigma$ (SD) were calculated by MATLAB software (MathWorks), and Gaussian distribution curves were generated by Prism software. Squares of $\sigma$ (in micrometers) values were plotted as a function of time, yielding a linear equation ($\sigma^2 = 0.2784t + 0.3441$; $t$ in seconds), where $0.2784 \mu m^2/s = 4D$ ($D$ is the diffusion coefficient; Zenisek et al., 2002). For the figures, images were rasterized to 1000 dpi by Photoshop CS3 software (Adobe).

**Electron microscopy** RBL cells were maintained adherent on sapphire disks (3 mm in diameter, 0.05 mm in thickness) that were coated with poly-D-lysine and fibronectin. Each substrate was incubated with the disks overnight. The sapphire dishes attached with cells were placed on Parafilm M (Bemis, Oshkosh, WI) until cryofixation, which was etched by needles. The disks were dipped in a cryoprotectant 1-hexadecane before being frozen in a high-pressure freezing machine (HPM 010; Balzers). After freezing, cells were dehydrated by freeze substitution in an acetone solution containing 1% osmium and 1% water as described (McDonald et al., 2010). The frozen samples were placed at −85°C for 72 h and then moved to −20°C. Samples were incubated for 24 h at −20°C and brought to 4°C for 4 h. Finally, the samples were brought up to room temperature for 2 h and rinsed with dry acetone. Rinsed samples were embedded as follows: incubation in resin mixed of 25% Epon and 75% plastic for 1 h, 50% Epon and 50% acetone overnight, 75% Epon and 25% acetone for 1 h, and 100% Epon in vacuum for 4 h, and finally the 100% Epon was polymerized at 60°C for 48 h. The resin-containing cells were sectioned with −90-nm thickness and contrasted by aqueous 2% uranyl acetate for 5–20 min immediately before imaging under a Philips CM120 STEM system.

**GFP-Trap communoprecipitation** RBL cells were transfected with EGFP-Munc13-4 constructs. After 2 d, cells were washed with ice-cold PBS and lysed in a Tris-NaCl buffer (50 mM Tris-Cl, 300 mM NaCl, 0.5% Triton X-100, protease inhibitor cocktail, 1 mM phenylmethanesulfonyl fluoride) for 30 min at 4°C. Cell debris was sedimented by centrifugation at 14,000 rpm for 15 min at 4°C. The supernatant was adjusted to contain 0.3% Triton X-100 and then mixed with GFP-Trap M nanobody (Chromotek, Martinsried, Germany) that had been equilibrated in the lysis buffer containing 0.3% Triton X-100 and washed. CaCl$_2$ (100 µM) or ethylene glycol tetraacetic acid (EGTA; 200 µM) was added in the mixtures, which were incubated for 2 h at 4°C with rotation. After the incubation, the nanobody-lysate mixture was separated on DynaMag-2 (Invitrogen). The immune complexes were washed with the Tris-NaCl buffer containing 0.1% Triton X-100 and then washed with PBS. The immune complexes were boiled in 1× SDS-sample buffer containing 2% SDS and 75 mM DTT for 5 min at 95°C, sedimented at 5000 $\times$ g, resuspended, and separated on the magnet rack. The solutions containing the dissociated complexes were immediately subjected to SDS–PAGE electrophoresis. Munc13-4 and SNAREs in the elution were detected by Western blotting.

**SNARE and Munc13-4 protein purification** Plasmid constructs pTW34 to express rat syntaxin-1A with N-terminal histidine (His)-tagged mouse SNAP-25B were generously provided by J. E. Rothman (Yale University, New Haven, CT) and T. Weber (Mount Sinai School of Medicine, New York, NY), and exocytic neuronal SNARE proteins were purified as described (Weber et al., 1998) by nickel-nitriroacetic acid (Ni NTA; Qiagen, Hilden, Germany) chromatography. Pet28a plasmids expressing...
C-terminal His-tagged VAMP-8 and late endosome SNARE proteins syntaxin-7 and -8 and Vti1b were provided by J. Shen (University of Colorado, Boulder, CO) and purified as described (Yu et al., 2013) by Ni-NTA (Qiagen) chromatography. His$_2$-tagged human Munc13-4 protein produced in insect Sf9 cells was purified on Ni-NTA agarose (Qiagen) and further purified by Mono Q anion exchange chromatography (GE Healthcare) as previously described (Shirakawa et al., 2004).

Proteoliposome preparation
1-Palmitoyl-2-oleoyl phosphocholine (POPC), 1,2-dioleoyl phosphoserine (DOPS), 1,2-dioleoyl phosphoethanolamine (DOPE), and cholesterol were from Avanti Polar Lipids (Alabaster, AL). 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine 4-chlorobenzenesulfonate (DiD) were from Invitrogen-ThermoFisher Scientific. Lipid mixtures were spiked with 2 μl of [H]$^{3}$-diapamiltoyl PC (~2 × 10$^5$ cpm/μmol/mL; Dupont) to determine lipid recoveries and standardize fusion and liposome-binding reactions. Lipid mixtures for reconstituted liposomes mimicked late endosomal lipid composition (Yu et al., 2013). For R-SNARE reconstitution, POPC, DOPE, DOPS, cholesterol, and DiD were mixed at a 5:8:20:10:10:2% molar ratio. For Q-SNARE reconstitution, POPC, DOPE, DOPS, cholesterol, and DiI were mixed at a 5:8:20:10:10:2% molar ratio. Lipid mixtures were dried under argon gas and resuspended with SNARE proteins in elution buffer (25 mM HEPES-KOH, pH 7.4, 100 mM KCl, 500 mM imidazole-OAc, pH 7.4, and 1.0% β-octylglucoside). SNARE proteoliposomes were made by comicaellization with protein:lipid mixtures at 1:500 for neuronal or late endosomal/lysosomal Q-SNAREs and 1:200 for R-SNAREs. Detergent was removed by overnight dialysis using Slide-A-Lyzer cassettes (Thermo Scientific) against reconstitution buffer (25 mM HEPES, pH 7.4, 100 mM KCl, 10% [vol/vol] glycerol, and 1 mM DTT) containing SM-2 Biobeads at 1 g/l (Bio-Rad), and all proteoliposomes were purified by Accudenz gradient flotation and further cleaned using 7K Zeba Spin desalting columns (ThermoFisher Scientific).

FRET-based lipid mixing
The lipid-mixing assay between fluorescent donor and acceptor liposomes was performed as previously described (Boswell et al., 2012). The standard assay used 0.45 mM Q-SNARE and 0.225 mM R-SNARE liposomes in a total reaction volume of 75 μl of reconstitution buffer without glycerol supplemented with 0.1 mM EGTA. Munc13-4 protein was added to a final concentration of 1 μM. For the stimulation of Munc13-4 acceleration of SNARE-dependent liposome fusion, calcium was added at a final free concentration of 300 μM. Reactions were assembled on ice and mixed before addition to prewarmed 96-well FluoroNunc plates. Lipid mixing was observed as an increase in fluorescence resonance energy transfer from DiI to DiD labels by measuring DiD (acceptor) fluorescence at 700 ± 5 nm during DiI (donor) excitation at 514 ± 5 nm every 90 s over 2 h at 35°C using the SPECTRAMax GEMINI-XS spectrofluorometer (Molecular Devices, Sunnyvale, CA). Results are expressed as the ratio of fluorescence at time $x$/minimum fluorescence measured over the entire 2 h.

Liposome binding assay
We assessed Munc13-4 binding to protein-free and reconstituted late endosomal Q-SNARE (syntaxin8/syntaxin7/Vti1b)-containing liposomes by using buoyant density liposome flotation as previously described (Weber et al., 1998). Binding reactions with protein-free or Q-SNARE liposomes were assembled with 1 μM Munc13-4 in the absence of Ca$^{2+}$ with 0.2 mM EGTA or presence of 300 μM Ca$^{2+}$ (free concentration) and incubated at room temperature for 1 h. Lipid recovery was determined by tracer [H]$^{3}$PC lipid and matched across all binding reactions. An equal volume of 80% Accudenz was added to binding reactions for a final concentration of 40% Accudenz, transferred to 5 × 41 mm Beckman Ultra-Clear centrifuge tubes, and overlaid with 350 μl of 30% Accudenz and 30 μl of reconstitution buffer. Accudenz gradient solutions contained 0.2 mM EGTA or 300 μM Ca$^{2+}$ as appropriate. Gradients were centrifuged for 4 h at 48,000 rpm in a Beckman SW50.1 rotor with Teflon adaptors. Samples were collected from the 0/30% Accudenz interface and resolved by SDS–PAGE and Western blotting onto nitrocellulose.

Statistical analysis
All statistical significances were examined by unpaired t test ($p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

ACKNOWLEDGMENTS
We acknowledge help provided by Stephen Bruinsma in the early stages of this work. We thank Maciej Olszewski, Marino Zerial, Alexander Sorkin, Arnold Ruoho, and Aparna Lakkaraju for reagents, Ben August for support for electron microscopy imaging, and Mary Lindstrom for support on mathematical model fitting. This work was supported by National Institutes of Health grant DK025861 to T.F.J.M.

REFERENCES
Allersma MW, Wang L, Axelrod D, Holz RW (2004). Visualization of regulated exocytosis with a granule-membrane probe using total internal reflection microscopy. Mol Biol Cell 15, 4655–4668.
Alvarez de Toledo G, Fernandez JM (1990). Patch-clamp measurements reveal multimodal distribution of granule sizes in rat mast cells. J Cell Biol 110, 1033–1039.
Antonin W, Fasshauer D, Becker S, Jahn R, Schneider TR (2002). Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. Nat Struct Biol 9, 107–111.
Antonin W, Holroyd C, Tikkanen R, Honing S, Jahn R (2000). The R-SNARE endobrevin/VAMP-8 mediates homotypic fusion of early endosomes and late endosomes. Mol Mem Cell 11, 3289–3298.
Azouz NP, Hammel I, Sagi-Eisenberg R (2014a). Characterization of mast cell secretory granules and their cell biology. DNA Cell Biol 33, 647–651.
Azouz NP, Matsui T, Fukuda M, Sagi-Eisenberg R (2012). Decoding the regulation of mast cell exocytosis by networks of Rab GTPTases. J Immunol 189, 2169–2180.
Azouz NP, Zur N, Erfegan A, Ohbayashi N, Fukuda M, Amihai D, Hammel I, Rothenberg ME, Sagi-Eisenberg R (2014b). Rabs5 is a novel regulator of mast cell secretory granules: impact on size, cargo, and exocytosis. J Immunol 192, 4043–4053.
Baker RW, Hughson FM (2016). Chaperoning SNARE assembly and disassembly. Nat Rev Mol Cell Biol 17, 465–479.
Baker RW, Jeffrey PD, Zick M, Phillips BP, Wickner WT, Hughson FM (2015). A direct role for the Sec1/Munc18-family protein Vps33 as a template of exocytosis with a granule-membrane probe using total internal reflection microscopy. Mol Biol Cell 15, 4655–4668.
Behrendorff N, Dolai S, Hong W, Gaisano HY, Thorn P (2011). Vesicle-associated membrane protein 8 (VAMP8) is a SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) selectively required for sequential granule-to-granule fusion. J Biol Chem 286, 29627–29634.
Benado A, Nasagi-Atiya Y, Sagi-Eisenberg R (2009). Protein trafficking in immune cells. Immunobiology 214, 507–525.
Berger G, Durand S, Goujon C, Nguyen XN, Cordel S, Darlix JL, Cimarelli A (2011). A simple, versatile and efficient method to genetically modify immune cells. Immunobiology 214, 507–525.
Blank U, Madera-Salcedo IK, Danelli L, Claver J, Tiwari N, Sanchez-Miranda E, Vazquez-Victorio G, Ramirez-Valadez KA, Macias-Silva M, Gonzalez-Espinosa C (2014). Vesicular trafficking and signaling for cytokine and chemokine secretion in mast cells. J Immunol 5, 453.
Bolte S, Cordelieres FP (2006). A guided tour into subcellular colocalization analysis in light microscopy. J Microsc 224, 213–232.

Boswell KL, James DJ, Esquibel JM, Bruinisma S, Shirakawa R, Horiiuchi H, Martin TF (2012). Munc13-4 reconstitutes calcium-dependent SNARE-mediated membrane fusion. J Cell Biol 197, 301–312.

Brzezinska AA, Johnson JL, Munafa DB, Crozat K, Beutler B, Kiosses WB, Ellis SA, Rab27. The Rab27/SNAREs complex regulates exocytosis of neuropeptide granules. Traffic 9, 2151–2164.

Cao Q, Zhong XZ, Zou Y, Murrell-Lagardno R, Zhu MX, Dong XP (2015). Calcium release through P2X4 activates calmodulin to promote endosomal membrane fusion. J Cell Biol 209, 879–894.

Carroll-Portillo A, Spender K, Pfeffer J, Griffiths G, U H, Lidke KA, Oliver JM, Lidke DS, Thomas JL, Wilson BS, Timlin JA (2010). Formation of a novel cell synaptonemal complex. EMBO Rep 11, 2984–2994.

Colombo MI, Beron W, Stahl PD (1997). Calmodulin regulates endosome fusion and granule-granule fusion in eosinophils by Ca2+ and GTP analogs. J Biol Chem 272, 44929–44934.

Cohen R, Corwith K, Holowka D, Baird B (2012). Spatiotemporal resolution of degranulation sacs. Lab Invest 44, 174–191.

Chicka MC, Ren Q, Richards D, Hellman LM, Zhang J, Fried MG, Whiteheart SW (2016). Tethering the assembly of SNARE complexes. J Mol Biol 428, 688–692.

Chapman ER (2008). How does synaptotagmin trigger neurotransmitter release? Annu Rev Biochem 77, 613–642.

He J, Johnson JL, Monfregola J, Ramadass M, Pestonjamasp K, Napolitano Hay JC (2007). Calcium: a fundamental regulator of intracellular membrane fusion. J Cell Sci 125, 2986–2994.

Colombo MI, Beron W, Stahl PD (1997). Calmodulin regulates endosome fusion. J Biol Chem 272, 7707–7712.

Dvorak AM (2005). Ultrastructural studies of human basophils and mast cells. J Histochem Cytochem 53, 1043–1070.

Dvorak AM, Galli SJ, Morgan E, Galli AS, Hammond ME, Dvorak HF (1981). Ultrastructural studies of human basophils and mast cells. Biochem Biophys Res Commun 324, 294–301.

Dvorak AM (2005). Calcium: a fundamental regulator of intracellular membrane fusion. J Biol Chem 272, 7707–7712.

Dvorak AM, Galli SJ, Morgan E, Galli AS, Hammond ME, Dvorak HF (1981). Ultrastructural studies of human basophils and mast cells. Biochem Biophys Res Commun 324, 294–301.

Lorentz A, Baumann A, Vitte J, Blank U (2012). The SNARE machinery in exocytosis with diverse membrane trafficking pathways. Blood 118, 1570–1578.

Lipstein N, Schaks S, Dimova K, Kalkhof S, Ihling C, Kolbel K, Ashery U, Martin TF (2012). Munc13-4 reconstitutes calcium-dependent SNARE-mediated membrane fusion. J Cell Biol 209, 879–894.

Lipton U, Ferrari DM, Jahn R (2007). Role of calcium and protein kinase C in the IgE-dependent activation of phosphatidylcholine-specific phospholipase D in a mast cell (RBL 2H3) cell line. Eur J Biochem 207, 163–168.

HK, Hofmann K, Brose N (2000). Definition of Munc13-homology-domains and characterization of a novel ubiquitously expressed Munc13 isoform. Biochem J 349, 247–253.

Lacy P, Stow JL (2011). Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. Blood 118, 9–18.

Lavender A, Gallagher KM, Callebaut I, Raposo G, Certain S, Baqc D, Dumont C, Lambert N, Ouachae-Chardin M, Chedeville G, Tamary H, et al. (2003). Munc13-4 is essential for cytosolic granules fusion and is mutated in a familial hemophagocytic lymphohistiocytosis (FHL3). Cell 115, 461–473.

Glitsas G, Knecht E (2013). Ca2+/-sensor proteins in the autophagic and endocytic traffic. Curr Protein Peptide Sci 14, 97–110.

Gorth I, Mizuno K, Nakajima H, Sasaki T (2004). Involvement of Rab27b in antigen-induced histamine release from rat basophilic leukemia 2H3 cells. Biochem Biophys Res Commun 324, 294–301.

Grimes SC, Peng Z, Hammel I, Sagi-Eisenberg R (2003). Synaptotagmin III restricts motility of Rab27a-expressing vesicles to facilitate lipopolysaccharide-induced priming of exocytosis in neutrophils. J Biol Chem 286, 5647–5656.

Jain R, Fasshauer D (2012). Molecular machines governing exocytosis of synaptic vesicles. Nature 490, 201–207.

Johnson JL, He J, Ramadass M, Pestonjamasp K, Kiosses WB, Zhang J, Catz SD (2016). Munc13-4 is a Rab11-binding protein that regulates Rab11-positive vesicle trafficking and docking at the plasma membrane. J Biol Chem 291, 3423–3438.

Johnson JL, Hong K, Monfregola J, Kiosses WB, Catz SD (2011). Munc13-4 restricts motility of Rab27a-expressing vesicles to facilitate lipopolysaccharide-induced priming of exocytosis in neutrophils. J Biol Chem 286, 5647–5656.

Lee RJ, Oliver JM (1995). Roles for Ca2+ stores release and two Ca2+ influx pathways in the Fc epsilon R1-activated Ca2+ responses of RBL-2H3 mast cells. Mol Cell Biol 6, 825–839.

Lee RJ, Griffiths AM (1992). The role of calcium and protein kinase C in the IgE-dependent activation of phosphatidylcholine-specific phospholipase D in a mast cell (RBL 2H3) cell line. Eur J Biochem 207, 163–168.

Li SQ, Griffiths AM (1992). The role of calcium and protein kinase C in the IgE-dependent activation of phosphatidylcholine-specific phospholipase D in a mast cell (RBL 2H3) cell line. Eur J Biochem 207, 163–168.

Lipton U, Ferrari DM, Jahn R (2007). Endobrevin/VAMP8 mediates exocytic release of hexosaminidase from rat basophilic leukemia cells. FEBS Lett 581, 3479–3484.

Lipton U, Ferrari DM, Jahn R (2007). Endobrevin/VAMP8 mediates exocytic release of hexosaminidase from rat basophilic leukemia cells. FEBS Lett 581, 3479–3484.

Lipton U, Ferrari DM, Jahn R (2007). Endobrevin/VAMP8 mediates exocytic release of hexosaminidase from rat basophilic leukemia cells. FEBS Lett 581, 3479–3484.

Lipton U, Ferrari DM, Jahn R (2007). Endobrevin/VAMP8 mediates exocytic release of hexosaminidase from rat basophilic leukemia cells. FEBS Lett 581, 3479–3484.

Lipton U, Ferrari DM, Jahn R (2007). Endobrevin/VAMP8 mediates exocytic release of hexosaminidase from rat basophilic leukemia cells. FEBS Lett 581, 3479–3484.
Shin OH, Lu J, Rhee JS, Tomchick DR, Pang ZP, Wojcik SM, Camacho-Perez M, Brose N, Machius M, Rizo J, et al. (2010). Munc13-4 C2B domain is an activity-dependent Ca2+ regulator of synaptic exocytosis. Nat Struct Mol Biol 17, 280–288.

Shirakawa R, Higashi T, Tabuchi A, Yoshioka A, Nishioha H, Fukuda M, Kita T, Horuchi H (2004). Munc13-4 is a GT-Rab27b-binding protein regulating dense core granule secretion in platelets. J Biol Chem 279, 10730–10737.

Singh RK, Mizuno K, Wamsiecr C, Waver-Shapton ST, Recchi C, Catzd SF, Futter C, Tolmacheva T, Hume AN, Seabra MC (2013). Distinct and opposing roles for Rab27a/Miph/MyoVa and Rab27b/Munc13-4 in mast cell secretion. FEBS J 280, 892–903.

Stow JL, Murray RZ (2013). Intracellular trafficking and secretion of inflammatory cytokines. Cytokine Growth Factor Rev 24, 227–239.

Sudhof TC (2012). Calcium control of neurotransmitter release. Cold Spring Harb Perspect Biol 4, a011353.

Sudhof TC, Rothman JE (2009). Membrane fusion: grappling with SNARE and SM proteins. Science 323, 473–477.

Takahashi S, Kubo K, Wargi S, Yashabi A, Shin HW, Katoh Y, Nakayama K (2012). Rab11 regulates exocytosis of recycling vesicles at the plasma membrane. J Cell Sci 125, 4049–4057.

Thorn P, Gaisano H (2012). Molecular control of compound exocytosis: a key role for VAMP8. Commun Integr Biol 5, 61–63.

Tiwari N, Wang CC, Brochetta C, Ke G, Vito F, Qi Z, Rivera J, Soranzo MR, Zubucchi G, Hong W, Blank U (2008). VAMP-8 segregates mast cell-preformed mediator exocytosis from cytokine trafficking pathways. Blood 111, 3665–3674.

Valentijn KM, van Driek LF, Mouriuk MJ, Hendriks GJ, Arends TJ, Koster AJ, Valentijn JA (2010). Multigranular exocytosis of Weibel-Palade bodies in vascular endothelial cells. Blood 116, 1807–1816.

van den Hoff MJ, Moorman AF, Lamers WH (1992). Electrotransport in ‘intra-cellular’ buffer increases cell survival. Nucleic Acids Res 20, 2902.

van der Sluijs P, Zibouche M, van Kerkhof P (2013). Late steps in secretory lysosome exocytosis in cytotoxic lymphocytes. J Immunol 4, 359.

Vergara-Ruengi S, Martina JA, Puertollano R (2009). Identification of the penta-EF-hand protein ALG-2 as a Ca2+-dependent interactor of muco-lipin-1. J Cell Biol 284, 36357–36366.

Vincent-Schneider H, Thery C, Mazzeo D, Tenaza D, Raposo G, Bonnerot C (2001). Secretory granules of mast cells accumulate mature and immature MHC class II molecules. J Cell Sci 114, 323–334.

Wandinger-Ness A, Zerial M (2014). Rab proteins and the compartmentalization of the endosomal system. Cold Spring Harb Perspect Biol 6, a026161.

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE (1998). SNAREpins: minimal machinery for membrane fusion. Cell 92, 759–772.

Wessoloowski J, Paumet F (2013). Escherichia coli exposure inhibits exocytic SNARE-mediated membrane fusion in mast cells. Traffic 15, 516–530.

Wood SM, Meeths C, Chiang SC, Bechensteen AG, Boelens JJ, Heilmann C, Rutynowska O, Winiarski J, et al. (2009). Different NK cell-activating receptors preferentially recruit Rab27a or Munc13-4 to perforin-containing granules for cytotoxicity. Blood 114, 4117–4127.

Wosika JR, Gillespie ME (2011). Small-interfering RNA-mediated identification and regulation of the ternary SNARE complex mediating RBL-2H3 mast cell degranulation. Scand J Immunol 73, 8–17.

Yao J, Gaffaney JD, Woon SE, Chapman ER (2011). Doc2 is a Ca2+ sensor required for asynchronous neurotransmitter release. Cell 147, 665–677.

Yu H, Rathore SS, Lopez JA, Davis EM, James DE, Martin JL, Shen J (2013). Comparative studies of Munc18c and Munc18-1 reveal conserved and divergent mechanisms of Sec 1/Munc18 proteins. Proc Natl Acad Sci USA 110, E3271–E3280.

Yue Z, Griswold MJ, Gaisano H, Rhee JS (2012). VAMP8 is essential for efficient exocytosis of dense core granule vesicles at the plasma membrane. Proc Natl Acad Sci USA 110, E2371–E2380.

Yu IM, Hughson FM (2010). Tethering factors as organizers of intracellular vesicular traffic. Annu Rev Cell Dev Biol 26, 137–156.

Zenisek D, Steyer JA, Feldman ME, Almers W (2002). A membrane marker leaves synaptic vesicles in milliseconds after exocytosis in retinal bipolar cells. Neuron 35, 1085–1097.

Zografos S, Basagiannis D, Papafotika A, Shirakawa R, Horuchi H, Auerbach D, Fukuda M, Christoforidis S (2012). A complete Rab screening reveals novel insights in Weibel-Palade body exocytosis. J Cell Sci 125, 4780–4790.