Identification of distinct cytotoxic granules as the origin of supramolecular attack particles in T lymphocytes

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Cytotoxic T lymphocytes (CTL) kill malignant and infected cells through the directed release of cytotoxic proteins into the immunological synapse (IS). The cytotoxic protein granzyme B (GzmB) is released in its soluble form or in supramolecular attack particles (SMAP). We utilize synaptobrevin2-mRFP knock-in mice to isolate fusogenic cytotoxic granules in an unbiased manner and visualize them alone or in degranulating CTLs. We identified two classes of fusion-competent granules, single core granules (SCG) and multi core granules (MCG), with different diameter, morphology and protein composition. Functional analyses demonstrate that both classes of granules fuse with the plasma membrane at the IS. SCG fusion releases soluble GzmB. MCGs can be labelled with the SMAP marker thrombospondin-1 and their fusion releases intact SMAPs. We propose that CTLs use SCG fusion to fill the synaptic cleft with active cytotoxic proteins instantly and parallel MCG fusion to deliver latent SMAPs for delayed killing of refractory targets.

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CYTOTOXIC T LYMPHOCYTES (CTL) FIGHT AGAINST PATHOGENS AND CANCER BY ERADICATING INFECTED OR TRANSFORMED CELLS, REFERRED TO COLLECTIVELY AS TARGET CELLS. TARGET CELL KILLING OCCURS AT A SPECIALIZED CONTACT ZONE CALLED THE IMMUNOLOGICAL SYNAPSE (IS), BY THE REGULATED EXOCYTOSIS OF CYTOTOXIC GRANULES (CG)\(^1\)-\(^3\). CGS CONTAIN PERFORIN-1 (Prf1) AND GRANULE B (GzmB) AND THE MEMBRANE ANCHORED FAS-LIGAND (FasL) THAT induce target cell death by apoptosis and necrosis\(^4\)-\(^5\). The fusion of CGs with the plasma membrane at the IS is mediated by a specific SNARE complex consisting of the t-SNAREs Syntaxin11 and SNAP-23 on the plasma membrane and a v-SNARE on the CG membrane\(^6\)-\(^7\). The v-SNARE of humanne CTLs is VAMP27 and that of mouse CTLs is VAMP2 (aka synaptobrevin2, Syb2)\(^8\)-\(^9\).

CGs are lysosome-related cell-type specific organelles, which share features with endosomes and lysosomes\(^10\)-\(^11\). The proteoglycan serglycin (Srgn) is required to form dense cores, named for their appearance in transmission electron microscopy, and enables retention of GzmB in CGs through its binding to dozens of GzmB molecules\(^12\)-\(^13\). Serglycin-deficient CGs display defective CG granulation, but only show mild defects in their killing capacity\(^14\)-\(^16\), indicating the existence of alternative killing pathways. Numerous morphological studies point to a heterogeneity of Prf1- and GzmB-containing organelles\(^17\)-\(^21\). This heterogeneity may indicate intermediate forms during biogenesis or it may indicate different functional CG types.

CTLs release Prf1 and GzmB in two forms\(^22\). Prf1 and GzmB disperse rapidly as soluble proteins that are immediately available to interact with the target cells, but are restricted to the IS to provide specificity\(^23\). Recent evidence indicates that about half of the released Prf1 and GzmB are found in supramolecular attack particles (SMAPs), which have a core of cytotoxic proteins and a shell of thrombospondin-1 (TSP-1)\(^22\). SMAPs remain active for hours and thus may act in a different time frame and outside of the IS\(^24\). The source of SMAPs within cytotoxic cells is unknown.

Here, we employ a synaptobrevin2-mRFP knock-in mouse\(^8\) in an unbiased approach to purify mature, fusion-competent CGs from mouse CTLs. Using density gradient centrifugation followed by immuno-isolation and mass spectrometry we find two classes of fusion-competent CGs. Transmission and scanning electron microscopy analysis discerned different morphologies, with one CG class containing a single dense core and uniform diameter (single core granule, SCG) and the other class containing multiple cores and varying diameters (multi core granule, MCG). Mass spectrometry of purified granules shows that both granules contain large amounts of GzmB. However, they differ in that SCGs are lysosomal-like, containing cathepsins, while MCGs are more heterogeneous and contain endosomal-like proteins such as Rabs. Analyses by super-resolution and total internal reflection fluorescence microscopy demonstrates that both classes of CGs fuse at the IS and that MCGs are the source of SMAPs. Our results show an additional type of cytotoxic granule nearly 40 years after the discovery of SCG.

**Results**

**CTLs from synaptobrevin2-mRFP knock-in mice as source for fusion-competent cytotoxic granules.** We previously generated a knock-in mouse in which a monomeric red fluorescent protein is fused to the C-terminus of the v-SNARE Syb2 (Syb2 KI)\(^8\). The CGs from Syb2 KI CTLs are labeled with mRFP, polarize to the IS upon target cell contact (Fig. 1a) and co-localize with GzmB (Fig. 1b). Because Syb2 is required to mediate the fusion of CG at the IS\(^8\), CGs derived from the Syb2 KI mouse provide a unique source for the selective purification of fusion-competent CGs.

We isolated CD8\(^+\) T cells from Syb2 KI mice, activated them for 3 days and assessed their phenotype by flow cytometry. We obtained 85% viable effector cells, of which all were Syb2-mRFP\(^+\) (Supplementary Fig. 1a, b), 97% were CD44\(^++\), 41.0% were CD62L\(^-\) and 89% of CD25\(^+\) activated cells (Supplementary Fig. 1c-e). After verifying the high yield of CTLs, we used nitrogen cavitation to crack the cells open, removed the cell debris by low-speed centrifugation, and fractionated the supernatant by ultracentrifugation on a discontinuous sucrose density gradient (Fig. 1c). Fluorescently labeled particles were concentrated at the interfaces between 1.0 and 1.2 M, and 1.2 and 1.4 M sucrose named fractions 6 and 8, respectively (Fig. 1c bottom). These fractions were enriched with the CG protein GzmB (Fig. 1d) and contained intact CGs (Fig. 1e).

For further purification, we incubated the granule-enriched gradient fractions 6 and 8 with magnetic beads containing an immobilized monoclonal antibody specific for Syb2 (Fig. 2a). Western blot analysis of the immuno-isolated material showed an enrichment of not only Syb2 but also the CG content proteins GzmB (Fig. 2b) and Prf1 (Supplementary Fig. 2a). On the other hand, contaminating membranes such as plasma membrane fragments, identified by Na\(^+/\)K\(-\)ATPase, remained in both supernatants (Fig. 2b). In order to confirm that the purified granules were still intact after immuno-isolation we crossed the Syb2 KI mice with our recently reported GzmB-mTFF knock-in (GzmB KI) mice\(^25\) and repeated the entire procedure of ultracentrifugation and immuno-isolation with CTLs derived from these double KI (Syb2/GzmB DKI) mice. Confocal microscopy showed a strong co-localization of both fluorophores (Fig. 2c). Object based co-localization algorithm determined that 66.1 ± 0.6% Syb2 mRFP\(^+\) spots co-localized with GzmB-mTFF and that 83.3 ± 0.5% mTFF\(^+\) spots co-localized with mRFP (n = 3, mean ± SD). This demonstrated that the GzmB was still contained within the granules (Fig. 2c). Correlative light and electron microscopy (CLEM) of the immuno-precipitates of fractions 6 (IP6) and 8 (IP8) further confirmed the co-localization of Syb2 and GzmB and the integrity of the granule membrane (Fig. 2d).

CTLs contain two classes of exocytosis-competent granules with different morphology. We next visualized the morphology of the granules contained in IP6 and IP8 by electron microscopy. Scanning electron microscopy in secondary electron mode (Fig. 3a, left) and back-scattered electron mode (Fig. 3a, right) showed that granules from both IP6 had a round, smooth appearance on the Dynabeads, with granules from IP6 being slightly larger in diameter than granules from IP8. Transmission electron microscopy on these IP samples fixed by high-pressure freezing showed that IP8 contained a homogeneous population of granules with a single dense core occupying the entire lumen of the granule (Fig. 3b, lower row), reminiscent of the classical CGs described in the literature\(^11\)-\(^20\). In contrast, granules from IP6 were quite variable in their size and composition, containing two or more particles with dense core-like structures and several other less electron dense particles (Fig. 3b, upper row; Supplementary Fig. 3a). Based on their appearance in electron microscopy we refer to granules from IP6 as multi core granules (MCGs) and to granules from IP8 as single core granules (SCGs) (Fig. 3c).

Quantitative analysis of granule diameter yielded an average diameter of 364 ± 12 nm (mean ± sem; N\(_{IP6} = 3\), n\(_{granules} = 118\) for MCGs and 293 ± 8 nm for SCGs (mean ± sem; N\(_{IP8} = 3\), n\(_{granules} = 85\); Fig. 3c). As shown in Fig. 3b the morphological appearance varied particularly for MCGs from IP6. Therefore, we further analyzed the different observed variants. While more than 80% of granules from IP8 contained a single dense core, almost 60% of granules from IP6 contained multiple dense cores and more than 20% contained several vesicles surrounded by bilayer membranes (Fig. 3d). We also noticed that several particles...
Nitrogen Cavitation

Transmission electron microscopy (TEM) images of sucrose gradient fractions 6 and 8. N\text{gradients} = 1; n\text{images} = 2. d Quantitative western blot for sucrose fractions 1-10 (0.7 µg protein per lane, fraction 10 with max. volume of 30 µl) probed against proteins that reside on CGs (Syb2-mRFP and GzmB), plasma membrane (Na+/K+-ATPase), mitochondria (SDHA; technical replicate) and cytoskeleton (β-actin (reprobed) and α-tubulin). N\text{gradients} = 3.

Fig. 1 Cytotoxic granules (CGs) of CTLs from synaptobrevin2-mRFP knock-in (Syb2 KI) mice can be visualized after subcellular fractionation. a Confocal time-lapse images of a representative activated cytotoxic T lymphocytes (CTLs) derived from Syb2 KI mice in contact with a target cell. Endogenously labeled CGs (red, left) polarized to the immunological synapse (IS) upon T cell-target cell contact (middle) and lead to target cell death (right). N\text{images} = 6; n\text{cells} = 31. b Representative structured illumination microscopy (SIM) images of a CTL isolated from Syb2 KI mouse with mRFP (magenta) labeled CGs. Cells were stained with an anti-granzyme B (GzmB) antibody coupled to Alexa647 (green). N\text{patches} = 1; n\text{images} = 33. c Flow chart for CG isolation procedure based on discontinuous sucrose density gradient centrifugation. Ten 1 ml fractions (1-10) were collected from the gradient and pelleted on glass coverslips. Confocal images of the fractions are shown below. N\text{gradients} = 1; n\text{images} = 2. e Transmission electron microscopy (TEM) images of sucrose gradient fractions 6 and 8. N\text{gradients} = 3; n\text{images} = 20 per fraction. Source data are provided as a Source data file.

Multi core and single core granules have different protein compositions. To learn about the composition of MCGs and SCGs, we subjected IP6 and IP8 to mass spectrometry analysis. Because naïve CD8+ T cells do not contain CGs, we used immuno-precipitates of naïve CD8+ T cells as negative control (Supplementary Fig. 2b, c). Label-free quantification (LFQ) of five and three biological and technical replicates of IP6 and IP8, respectively, showed a very high reproducibility (Supplementary Fig. 5a, b). For MCG we identified a total of 212 proteins, while for SCG a total of 384 proteins could be detected. Interestingly, only 156 proteins were found to be present in MCG and SCG (Fig. 4a, Supplementary Data 1). Both granule types contained GzmB and Syb2-mRFP confirming their cytotoxic and exocytotic competence. We also found LAMP-1 and almost all the subunits of vesicular H\textsuperscript{+}-ATPase (Fig. 4b, Supplementary Fig. 5c, d). The latter is required to generate the granule’s intra-vesicular acidic environment\textsuperscript{27}. This was in a good agreement with similar labeling of MCGs and SCGs with LysoTracker (Supplementary Fig. 6). Prf1 was not significantly enriched nevertheless, three peptides of Prf1 were detected in 2 out of 6 measurements in IP8 (7% coverage). The presence of Prf1 was further verified by
immunoblotting in IP6 and IP8 (Supplementary Fig. 2a). The SCG fraction was lysosomal-like with cathepsins as a marker while the MCG fraction contained a number of recycling plasma membrane proteins and a specific subset of Rab GTPases associated with endosomal-like compartments (Fig. 4b, Supplementary Fig. 5c, d). Notably, MCG also contained proteins previously associated with SMAPs by mass spectrometry analysis (e.g., RPL12, HSP90b1) (Fig. 4b). While released SMAPs contained few membrane proteins, the MCGs naturally contain many membrane proteins associated with their limiting membrane and intraluminal vesicle membranes. Sunburst plots of gene ontology analysis for both fractions showed clear differences in putative subcellular localization and biological function (Fig. 4c, d). These data indicate that these two granule types represent distinct steady state compartment, rather than different stages of a single maturation pathway.

Supramolecular attack particles are released from multi core granules. After identifying putative SMAPs in MCGs, we examined whether we could visualize their release. For that purpose, we seeded CTLs derived from wild-type and GzmB KI mice for 90 min on supported lipid bilayer (SLB) containing anti-CD3 antibody, thus stimulating SMAP release. CTLs were then fixed in place on the SLB and stained with anti-GzmB antibody. Structured illumination microscopy (SIM) indicated the presence of GzmB+ particles on the SLB associated with and away from cells (Fig. 5a, arrows). This is consistent with SMAPs being released by CTLs that subsequently migrated away from the initial attachment site, leaving SMAPs behind. Because TSP-1 is a component of SMAP’s shell we sought to provide additional evidence for SMAPs in MCGs by expressing TSP-1-GFPspark together with GzmB-mCherry fusion constructs in CTLs. SIM images of fixed, double-transfected CTLs clearly demonstrated a partial co-localization of TSP-1 and GzmB defining the MCG compartments, whereas granules that are only GzmB+ are SCGs (Fig. 5b). Furthermore, we observed that TSP-1 was largely enriched in isolated MCG populations rather than in SCG (Supplementary Fig. 7). This data strengthens our findings that MCGs and SCGs carry distinct cargos. Additionally, we took advantage of the fact that wheat germ agglutinin (WGA) specifically binds to glycoproteins present in SMAPs. CTLs from GzmB KI were pre-incubated with WGA-Alexa647 for 90 min to allow uptake in SMAPs and seeded on poly-ornithine or anti-CD3 coated coverslips, for resting and activated CTLs, respectively. We found that about 60% of granules were MCGs co-labeled with WGA and GzmB, 20% were SCGs only containing GzmB, while the rest were small single WGA-positive organelles distributed throughout the cell cytoplasm and at the IS (Fig. 5d). The size of these organelles was 410 ± 7 nm, while the MCGs were 322 ± 7 nm in diameter (Fig. 5e, mean ± sem; n = 275 for MCG and n = 156 for SCG in 20 cells). Note that the size distribution of MCGs and SCGs corresponded closely to the size distribution measured on IP6 and IP8 by EM (Fig. 3c). To investigate the secreted SMAPs we seeded CTLs labeled with WGA on SLBs. After additional 90 min, cells were washed away and the remaining SMAPs were stained with anti-TSP-1 antibody. The representative SIM images showed co-localization of GzmB, TSP-1 and WGA, which formed a ring around the GzmB and TSP-1 fluorescent signal (Fig. 5c). To verify their morphology in intact cells, we performed CLEM experiments on CTLs which had been co-transfected with GzmB-mCherry and TSP-1-GFPspark and incubated with WGA. The organelle in the electron micrograph correlating to fluorescent puncta in all SIM channels had indeed a similar morphology as isolated MCGs shown in Fig. 3b (Fig. 5f, right row). We also found granules, which contained only GzmB and were smaller with a more uniform contents, resembling SCGs. Furthermore, we detected smaller organelles containing only TSP-1. These data further establish that SMAPs are stored in MCGs.

To evaluate the potential killing efficiency of both granules we measured the amount of GzmB content. For that we visualized intact granules with STED microscopy from fraction 6 and 8 that were settled on glass coverslips. This allowed us to clearly resolve 2.5 ± 0.2 (n = 52) individual SMAPs per MCGs (Fig. 6a). Interestingly, it also showed that GzmB is not evenly distributed in the dense core of SCGs. Overall the GzmB fluorescence was 40% weaker and more diffuse in SCGs than in MCGs (p < 0.001, n = 52 and 44 for MCG and SCG, respectively; Fig. 6b). The fraction of the granule volume occupied by GzmB particles was slightly but not significantly higher in SCGs as compared to MCGs. Therefore, the overall concentration of GzmB, which is directly related to the GzmB fluorescence intensity in the entire granule volume, is higher in MCGs than in SCGs by 20%...
To visualize fusion of SCG and MCG in real time we performed TIRF microscopy on CTLs seeded on SLBs. We again used CTLs derived from the GzmB KI mouse to endogenously label both granule classes and pre-incubated them for 90 min with WGA to label SMAPs. We frequently identified single CTLs releasing both SCGs and MCGs (Fig. 7a, c), with SCGs characterized by being GzmB-positive and WGA-negative (SCG fusion event at 39.2 s of Fig. 7a; Supplementary Movie 3) and MCG by being double-positive for GzmB and WGA (fusion event at 78.4 s of Fig. 7a). The MCG fusion events displayed partial dissipation of TFP from the fusion sites, which might represent the release of a small amount of the cleaved TFP from GzmB25 (Fig. 7b). Nevertheless, significant TFP puncta persisted at these sites, which we interpret as SMAPs (Fig. 7a; Supplementary Movie 3). Interestingly, MCG fusion event generated multiple SMAPs that became more distinct over time (see framed inset Fig. 7a). To verify this finding we co-transfected wild-type CTLs with GzmB-mCherry and TSP-1-GFPspark and followed SMAP release by TIRF microscopy. We again observed the simultaneous release of co-localizing GzmB and TSP-1 in several, closely
clumped puncta (Fig. 7d, Supplementary Movie 4). The most likely explanation for this phenomenon would be the release of several SMAPs from a single MCG, which then remain attached at the SLB membrane as individual entities. We further assessed the cytotoxic effect of MCGs and SCGs on target cells. For this we centrifuged for 1 h either fraction 6 or 8 down on poly-ornithine coated glass coverslip at high speed to partially crack open the granules. Then we seeded P815 target cells on them and fixed and immuno-stained them with anti-cleaved caspase3 antibody, which is an early marker for apoptosis. Already after 6 h we observed that either SCG or MCG containing fractions were able to induce cleaved caspase3 expression in target cells (Fig. 7e, f). In long term (19 h), MCGs were more efficient to kill target cells as SCGs under our experimental conditions, most likely due to the concentrated amount of cytotoxic proteins stored in SMAPs (Fig. 6b). Hence, we clearly demonstrated that SMAPs are being stored in cytotoxic MCGs that are released upon IS formation.

**Discussion**

We have isolated fusion-competent Syb2 positive granules from mouse CTLs by ultracentrifugation and immuno-isolation and performed a detailed analysis of the CGs by mass spectrometry, super-resolution microscopy, live TIRF microscopy and electron microscopy. Besides the expected SCG we now report a distinctive class of fusion-competent granules with distinct morphology and protein composition, which we refer to as MCG. Interestingly, we found similar classes of granules in human NK cell, which have potential use for cancer therapy Supplementary Fig. 4). MCGs contain distinct SMAPs and/or several bilayer membrane-based vesicles, and they fuse at the IS in parallel to SCG. Thus, we have identified the source of recently described SMAPs, opening avenues for future immuno-therapeutic approaches targeted at MCGs and optimization of NK- and CTL-mediated killing.

A wealth of studies over the past decades have investigated the mechanism by which CTLs kill their targets. The generally
accepted picture is that upon T cell receptor mediated recognition CGs are being brought to the target cell interface, where they fuse and release their toxic content. Death is caused by two pathways, the Prf1/Gzm pathway and the Fas/Fas-ligand pathway, which both induce apoptosis and/or necrosis in the target cell. Because proteins from both pathways have been found on classical CGs with a single dense core, it was assumed that exocytosis of one class of CGs is responsible for target cell death. That simplistic view has been challenged by CG composition analysis and the report of cytotoxic exosomes released by NK cells, and CTLs. Bulk analysis of these exosome preparations suggested that FasL, GzmB, and Prf1 were present along with tetraspanins. These particles dispayed cytotoxic activity against tumors in vivo.

Fig. 5 Multi core granules contain supramolecular attack particles (SMAPs). a SIM images of WT (left) and GzmB KI (right) cells on supported lipid bilayers (SLB) represented in 3D (top) and 2D (bottom). Cells were stimulated on anti-CD3 antibody coated lipids for 90 min and stained with anti-GzmB (red) antibody after fixation. White arrows point to secreted SMAPs. Nmouse = 1; nimages = 3 and 6 for WT and GzmB KI, respectively. b SIM images of one resting WT CTL expressing GzmB-mCherry and Thrombospondin 1 (TSP-1)-GFPspark seeded on poly-ornithine coated coverslip. Nmouse = 1; ncells = 14. c SIM images of secreted SMAPs on SLB from GzmB KI CTLs pre-incubated with wheat germ agglutinin (WGA)-647 to label intracellular, glycoprotein-rich compartments. Secreted SMAPs were stained with anti-TSP-1 antibody. Nmouse = 1, nimages = 16. d Analysis of SCG and MCG percentage in resting cells (left) and stimulated cells at the IS (right). To trigger synapse formation CTLs were seeded on anti-CD3 coated coverslips. GzmB single positive granules were identified as SCGs, while GzmB and WGA double positive granules as MCGs. Black lines represent mean ± sem; ncells = 16; entire cells and ncells = 35; IS. Student’s t-test was used to compare values; *** Two-tailed P-value < 0.001. e Line profile (top) of one representative MCG (left) and SCG (right) acquired with SIM to depict granule diameter measurement method. Inset: SCG contained only GzmB (green) while the MCG contained WGA-647 and GzmB (yellow). Scale bar, 0.2 μm. Bottom scatter dot plot of granule diameter measured as the full width at half maximum in the line plots. Black line represents mean and gray line median. Nmouse = 3; ncells = 20; ngranules = 275 and 156 for MCG and SCG, respectively. Mann–Whitney U test was used to compare values; ***p < 0.001. f CLEM images of a representative stimulated CTL as in (b) pre-incubated with WGA-647 and stained with DAPI. Shown are SIM images and its corresponding TEM overlaid image. In the enlarged TEM image, arrows point to different fluorescent proteins using the color-code of the upper panels. White arrow indicate the MCG marked in all three channels. Nmouse = 1; ncells = 18. Source data are provided as a Source data file.
It would be interesting to further explore whether this function is also preserved in MCGs when they differentiate from SCGs, as suggested by the presence of facilitating factors such as Munc13-4. Concerning the biogenesis, the differences in protein composition between MCGs and SCGs (Fig. 4) indicate that both granules have distinct maturation routes leading to two parallel secretory pathways. It will be interesting to study whether human genetic defects like Chediak-Higashi38,39 and Hermansky-Pudlak40,41 syndromes, which are instrumental in understanding the biogenesis of SCGs, also affect the biogenesis of MCGs.

Fig. 6 MCG contain more Gzmb than SCG. a 2DSTED single plane images of individual granules from fraction 6 (left) and 8 (right), that were isolated from Syb2 KI mice, and were gently seeded on glass coverslips to ensure their integrity. Granules were stained with anti-GzmB (top, green) and anti-RFP with STED compatible secondary antibody to visualize Syb2 (middle, red). The bottom images show the merged channels. No deconvolution was applied on the images in order to preserve the diffuse GzmB staining visible especially in SCGs. b Analysis of GzmB content of the granules depicted in (a) shown as scatter dot plots superimposed with a box plot, in which the center black line is the median and the white line the average. The boundaries of the box represent the 25th to 75th percentiles, while the whiskers are 10th/90th percentiles. Left, mean fluorescence intensity of the GzmB particles. Center, mean fluorescence of GzmB spots related to the entire volume of the granule measured on the Syb2 channel. Right, fractional GzmB volume over the entire volume the granule given in percent. N_{granules} = 52 and 44 from fraction 6 (MCGs) and 8 (SCGs) respectively. **p = 0.006, ***p < 0.001 computed with the Mann-Whitney U test. Source data are provided as a Source data file.

fractions from NK cells contain FasL-positive exosomes, but that Prf1 and GzmB may be contained in co-purified SMAPs due to their similar size and density22,24. SMAPs consist of a core of Prf1 and GzmB surrounded by a glycoprotein shell and lack a limiting lipid bilayer22. They were also shown to be able to kill target cells, and again no SMAPs have been found within single dense core granules. The new MCGs we identify in this work now resolves these issues and most likely represent the source of exosomes identified through 14-3-3-ε38, and SMAPs containing Prf1 and GzmB.

We envision MCGs as multi-purpose organelles containing SMAPs, exosomes and probably also cytokines that can fuse in parallel with SCGs at the IS (Fig. 8). MCGs as well as SCGs can kill target cells (Fig. 7e, f), and MCGs appear to contain more Gzmb than SCGs (Fig. 6b). It is tempting to speculate that SCGs and MCGs might function under different processes in the attack of CTLs against target cells. While SCGs might act to rapidly kill a relatively small number of target cells, MCG secretion gains relevance as a second line of attack for targets that resist the soluble cytotoxic proteins. The extra release of packaged lytic molecules might then have a better chance to deliver sufficient Gzms to the cytoplasm of the target cell. In addition to cytolytic killing, GzmB has been shown to play an important function in CTL transmigration through parenchymal tissue46. It would be interesting to further explore whether this function is also supported by GzmB released from MCGs. Furthermore, the observed heterogeneity in MCG morphology and composition (Figs. 3 and 4) may enable CTLs to further fine-tune their killing efficiency depending on which individual MCG is exocytosed (see Fig. 8 right for an example). Thus, the co-existence of functionally different lytic granules in individual CTLs might contribute to the heterogeneity of CTL responses and, in turn, to the robustness of their effector function37–39. In this context it will be interesting to investigate the mechanisms of MCG biogenesis and fusion in the future. While our data clearly show that fusion of MCG is a SNARE-dependent process8, the efficiency of fusion might be regulated by intracellular factors such as local calcium concentration, phosphorylation status of involved proteins or presence of facilitating factors such as Munc13-4.

Methods

Mice. Synaptobrevin2-mRFP knock-in (Syb2 KI) and granzyme B-mTFP knock-in (GzmB KI) mice were generated as described previously23,37. Syb2 KI and GzmB KI crossed mice (Syb2/GzmB DKO) were used in experiment shown in Fig. 2. Transgenic mice and wildtype mice used in this study were all in C57BL/6N background. Additionally, all the animals used for experiments were at the age of 15–22 week-old and of both sexes. Animals were kept under the housing conditions of 22 °C room temperature, 50–60% humidity and 12 h dark/light cycles. All experimental procedures were approved and performed according to the regulations by the state of Saarland (Landesamt für Verbraucherschutz, AZ.: 2.4.1.1).

Cell culture. Splenocytes were isolated from 15–22 week-old mice as described before47. Briefly, naïve CD8 T cells were positively isolated from splenocytes using Dynabeads FlowComp Mouse CD8+ kit (Invitrogen) as described by the manufacturer. The isolated naïve CD8+ T cells were stimulated with anti-CD3/anti-CD28 activator beads (1:0.6 ratio) and cultured for 3 days at 37 °C with 5% CO2. Cells were cultured at a density of ~13 × 10^6 cells/ml in 775 culture flasks with IMDM medium (Invitrogen) containing 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin (Invitrogen), 30 U/ml recombinant IL-2 (Gibco) and 50 µM 2-mercaptoethanol. These activated effector CTLs were used for organole isolation and mass spectrometry analysis. Naïve CD8+ T cells were collected immediately after CD8 positive cell isolation from splenocytes without any stimulation as a control. For functional assay shown in Fig. 5 and Fig. 7, effector cells were cultured 5 days before the experiments. P815 target cells (ATCC TIB64, directly purchased from DSMZ, #ACC1) were cultured in RPMI medium (Invitrogen) containing 10%
FCS, 50 U/ml penicillin, 50μg/ml streptomycin (Invitrogen), and 10 mM HEPES (Invitrogen) at 37 °C with 5% CO₂.

NK92 cell line (ATCC CRL-2407, kind gift from Prof. Borrow, University Oxford) were cultured in complete NK cell medium ((RPMI-1640 (Sigma Aldrich; #R8758), 10% FBS (Thermo Fisher Scientific; #A3160801), 100 U/ml penicillin, 100 μg/ml streptomycin (Thermo Fisher Scientific; #15140122)) supplemented with 100 units/mL of recombinant human IL-2 (PeproTech; #200-02). Cells were cultured every three days and kept at 37 °C in humidi fied incubator with 5% CO₂.

Plasmids and antibodies. Human TSP1-GFPSpark was cloned in the pMAX vector by digesting the Thrombospondin-1/TSP1 cDNA ORF Clone, Human, C-GFPSpark tag from SinoBiological (cat # HG10508-ACG) with KpnI and XbaI FastDigest Restriction Enzymes (Thermo Scientifi c). The same was performed for pMAX vector, and both the vector and insert were ligated using T4 DNA ligase (Promega). The GzmB-mCherry construct was generated by fusing mCherry at C-terminus of mouse Gzmb gene. Additional GGSGGSGGS linker sequence was inserted between GzmB and mCherry. GzmB-linker-mCherry was then cloned into

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Fig. 7 Both classes of CGs, MCG and SCG, fuse at the IS and are killing competent. a Total internal reflection fluorescence microscopy (TIRFM) snapshot images of GzmB KI (green) CTL stained with WGA-647 (magenta) on supported lipid bilayer. White solid arrows point to one MCG fusion event and white open arrows point to one MCG fusion event over time. Blue arrows indicate the time of fusion. Pictures in frame show the MCG before and after fusion. Images were recorded at 5 Hz. Nmouse = 3, ncells = 16. b Representative granule secretion profile of SCG and MCG from the cell shown in (a). The loss of fluorescence indicates the release of solubilized GzmB and the remaining/increased WGA signal indicates the secreted SMAPs on the supported lipid bilayer. c Percent of secreted MCG and SCG from experiments as shown in (a). Nmouse = 3, ncells = 25. d TIRFM snapshot images of a WT CTL expressing GzmB-mCherry (red) and TSP-1-GFPspark (green) on glass coverslip coated with an anti-CD3 antibody. White arrows point to one MCG secretion event, blue arrows indicate the time of secretion. Frame shows the MCG before and after fusion. Images were recorded at 10 Hz. Nmouse = 2, ncells = 17. e Target cell killing assay. Confocal images of B16 target cells seeded coverslips covered with MCG (fraction 6) and SCG (fraction 8). Cells were stained with anti-cleaved caspase3 antibody (green) to determine cell apoptosis at two different time points. Cells seeded on poly-ornithine alone were used as control. DAPI staining (blue) was used to count the cells. f Time dependent analysis of the killing assay is shown as a violin plot with superimposed box plot and outliers. The boundaries of the box represent the 25th to 75th percentiles, while the whiskers are 10th/90th percentiles. The white dot corresponds to the median. Nmouse = 1, ncells = 300. N corresponds to the number of independent experiments and analyzed cells, respectively. One-way ANOVA on rank was used to compare values within time point group; "p < 0.001. Source data are provided as a Source data file. Scale bar in framed pictures are 0.5 μm.

Fig. 8 MCG as a distinct class of cytotoxic granules for SMAP release. Proposed model of a cytotoxic T lymphocytes that contains two classes of CGs (MCG and SCG), both of which can fuse in parallel at the immunological synapse (left). While the SCG content diffuses rapidly after release, MCGs deposit supramolecular attack particles (SMAPs) that retain their integrity in the synaptic cleft. The latter is illustrated in the exemplary electron micrograph on the right.

pMAX vector25. The antibodies used in this work are described in detail in Table 1. All the antibodies except anti-Sec61β antibody are commercially available and are well-established and shown to be valid. The validation of the in-house generated antibody anti-Sec61β antibody is shown in the Source Data file (Supplementary Fig. 2a).

Flow cytometry. For flow cytometry analysis, 0.2–0.5 x 10⁶ CD8⁺ T cells were resuspended in D-PBS (Gibco) and incubated in the dark for 30 min on ice with cell surface specific FITC-, APC or PE-conjugated antibodies against CD44, CD62L and CD25 (Table 1). The viable T lymphocytes were gated based on their size and granularity. Effector Syb2 KI cells were gated using the mRFP signal compared to the baseline signal of wild type cells. The fluorescence signal of antibody-stained cells were further analyzed by subtracting mRFP signal in unstained Syb2 KI cells. Finally, the immune cell subsets were analyzed according to the antibody labeling. Data were acquired by using a BD FACSAria III analyzer (BD Biosciences) with BD FACSDiva software 6.0. The data were analyzed with FlowJo v10.7 software.

Cell homogenization and subcellular fractionation. 0.8–1.2 x 10⁶ activated CD8⁺ T cells from one Syb2 KI mouse, 0.7–0.8 x 10⁶ unstimulated naive CD8⁺ T cells from four to six Syb2 KI mice or 0.8 x 10⁶ activated CD8⁺ T cells from one syb2 KI/GzmB DKI mouse were harvested and washed once in buffer (Invitrogen, PBS with 0.1% BSA and 2 mM EDTA) before resuspension in 2 ml of homogenization buffer (300 mM sucrose, 10 mM HEPES (pH 7.3), 5 mM EDTA (pH 8.0) supplemented with protease inhibitors (3 mM Pefabloc, 10 μM E64 and 10 μg Pepstatin A). The cell suspension was transferred into a pre-chilled cell disruption bomb (Parr 1019HC T304 SS) connected to a nitrogen source. After 25 min emulsification at 800 psi the nitrogen pressure was released and the cell homogenate was collected. The cell lysate was centrifuged for 10 min at 1000 x g at 4 °C to pellet unbroken cells, partially disrupted cells and nuclei. The resulting post-nuclear supernatant was layered on top of a discontinuous sucrose density gradient with 0.8, 1.0, 1.2, 1.4, and 1.6 M sucrose in 10 mM HEPES with 5 mM EDTA with protease inhibitors as described before (pH 7.3), 2 ml for each fraction. After ultracentrifugation at 100,000g for 90 min at 4 °C in a SW40Ti rotor (Beckman), 1.2 ml fractions were collected from the top of the gradient and supplemented with fresh protease inhibitors. 61 μl of each fraction was added to 16 μl of 4x Lithium dodecyl sulfate (LDS) sample-buffer (Serva) and 50 mM DTT and heated at 96 °C for 7 min prior to SDS-PAGE and immunoblotting.

Western blot. Sucrose fractions were separated by SDS-PAGE on precast 10% Bis-Tris gels with MES running buffer or 4–12% gradient Bis-Tris gels with MOPS running buffer (NuPage, Invitrogen). Proteins were then transferred to 0.2 μm pore-size nitrocellulose membrane and blocked with 5% non-fat dry milk powder in TBS buffer containing 20 mM Tris, 0.15 M NaCl, and 0.05% Tween 20, pH 7.4 for 2 h at 20 ± 2 °C. For quantitative western blots protein concentrations in sucrose fractions were measured using Pierce660 reagent (Sigma Aldrich). The immunoblots were analyzed with anti-alpha 1 Na⁺/K⁺-ATPase, anti-SHDA, anti-alpha tubulin, anti-perforin, anti-β actin, anti-synaptobrevin2, anti-RFP, anti-gramyne B, anti-RPS10, anti-Sc61β, NTAf1 and horseradish peroxidase (HRP)-conjugated goat anti-mouse (H + L or light chain for immunoprecipitation) or anti-rabbit secondary antibodies (F(ab)², heavy or light chain) (Table 1). For reproping western blot membranes were stripped in stripping solution (Invitrogen) for 5–15 min at 20 ± 2 °C. Finally, the blot was developed using enhanced chemiluminescence reagents (SuperSignal West Dura Chemiluminescent Substrate; Thermo Fisher Scientific) and imaged by gel documentation (FluorChem M system, ProteinSimple).

Immunoprecipitation. Dynabeads Protein G magnetic beads (Invitrogen) were washed twice in PBS before use and 15 μl Dynabeads were incubated with 5 μg anti-Syb2 antibody (Table 1) and rotated for 40 min at 20 ± 2 °C. Syb2-mRFP positive fractions 6 and 8 were diluted 1:1 in 320 mM KCl, 10 mM HEPES, 5 mM EDTA solution (pH 7.3), added to the antibody-conjugated Dynabeads and rotated at 20 ± 2 °C overnight. After five washing steps with 500 μl 160 mM KCl buffer the Dynabeads were collected for western blot, LSM, TEM, SEM analysis or mass spectrometry. For western blot analysis 61 μl supernatant was mixed with 16 μl 2x LDS buffer and 50 mM DTT, heated at 96 °C for 5 min and stored at −20 °C. For mass spectrometry Dynabeads were mixed with 18 μl 2x LDS and 2 μl DTT (1 M), heated at 96 °C for 10 min and kept at −80 °C.

Total internal reflection fluorescence (TIRF) microscopy. The TIRFM setup (Visitron Systems GmbH) was based on an IX83 (Olympus) equipped with a UAPON100XOTIRF NA 1.49 objective (Olympus), solid-state excitation lasers at 561 nm, 488 nm, 647 nm and 436 nm, an ALAS illumination control system (Roper Scientific, S.A.S), an evolve-EM 515 camera (Photometrics) and a filter cube including Semrock (Rochester) EF444/520/590/Di01 dichroic and FF-1001-5645/637/623 emission filters. The setup was controlled by Visiview software (version 4.0.0.11, Visi-tron GmbH). For TIRFM, day 5 bead activated CTLs isolated from GzmB KI or WT mice were used. 2 h before the experiment, beads and IL-2 were removed from CTLs, 3 x 10⁶ cells were resuspended in 30 ml of extracellular buffer (10 mM glucose, 5 mM HEPES, 155 mM NaCl, 4.5 mM KCl and 2 mM MgCl₂) and allowed to settle for 1–2 min on anti-CD3ε antibody (30 μg/ml, Table 1) coated coverslips or supported lipid bilayer. Cells were then perfused with extracellular buffer containing calcium (10 mM glucose, 5 mM HEPES, 140 mM NaCl, 4.5 mM KCl, 2 mM MgCl₂ and 10 mM CaCl₂) to stimulate CG secretion. In order to distinguish SCG and MCG, cells were co-labeled with GzmB and SMAP markers, WGA or TSP-1. To label SMAPs in T cells, cells were either transfected with SMAP marker TSP-1-GFPspark construct or stained with
**Table 1 Antibodies used in this study.**

| Antibody anti- | Supplier | Identifier | Source | Dilution | Application |
|---------------|----------|------------|--------|----------|-------------|
| β-Actin       | Sigma-Aldrich | AC-15 | Mouse | 1:5000 | WB |
| CD3ε         | BD Pharmingen | 145-2C11 | Mouse | 3:100 | Glass coating |
| Cleaved Caspase-3 (Asp175) | Cell Signaling | 96615 | Rabbit | 1:200 | ICC |
| CD25-FITC    | BD Pharmingen | 7D4 | Rat | 1:200 | FACS |
| CD44-APC     | eBioscience | IM7 | Rat | 1:200 | FACS |
| CD62L-FITC   | BD Pharmingen | MEL-14 | Rat | 1:200 | FACS |
| Granzyme B   | Cell Signaling | 42755 | Rabbit | 1:5000 | WB |
| Granzyme B   | Invitrogen | GB11 | Mouse | 1:200 | ICC |
| Granzyme B+  | Biologend | GB11 | Mouse | 1:100 | ICC |
| Perforin-1   | Invitrogen | PA1-22489 | Rabbit | 1:5000 | WB |
| Rabbit STAR 580 | abberior | ST580-1002 | Goat | 1:1000 | WB |
| RFP          | Genway Biotech | GWB-3B-397 | Rabbit | 1:5000 | WB |
| RPS10        | Abcam | EPR8545 | Rabbit | 1:10,000 | WB |
| SDHA         | Abcam | 2E3GC12FB2 AE2 | Mouse | 1:2000 | WB |
| Synaptobrevin2 | Hommadmade human aa2-10 | 69.1 | Rabbit | 1:5000 | WB |
| Thrombospondin-1 | Cell Signaling | D7ESF | Rabbit | 1:100 | ICC |
| α-Tubulin    | Abcam | ab4074 | Rabbit | 1:5000 | WB |
| Mouse IgG, (H + L), HRP | Invitrogen | 32420 | Goat | 1:1000 | WB |
| Mouse light chain, HRP | Merck | AP202P | Goat | 1:1000 | WB |
| Rabbit heavy chain HRP | Abcam | 2A9 | Mouse | 1:5000 | WB |
| Rabbit IgG, F(ab')2, HRP | Merck | A0132P | Goat | 1:10,000 | WB |
| Rabbit light chain HRP | Merck | MAB201P | Mouse | 1:5000 | WB |

WGA-Alexa647 (Thermo Fisher Scientific, W32466). For WGA staining, GzmB KI cells were pre-incubated with 1 µg/ml WGA-Alexa647 for 1.5 h at 37 °C allowing WGA being endocyotically for secretion analysis. For another independent labeling, WT cells were co-transfected TSP-1-GFPspark and GzmB-mCherry plasmids. Cells were recorded for 7 min at 20 ± 2 °C. 488 nm, 561 nm, and 647 nm excitation laser were used to visualize mTFP/GFP, GzmB-mCherry and WGA-Alexa647. The images were taken with an acquisition frequency of 5 Hz and exposure time of 30 ms for GzmB-KI and WGA recording and an acquisition frequency of 10 Hz and exposure time of 100 ms for TSP-1-GFPspark and GzmB-mCherry recording. Images and time-lapse series were analyzed using ImageJ or the Fiji package of ImageJ. GOG secretion analysis was performed using ImageJ with the plugin Time Series Analyzer. A change of fluorescence within 200 ms with accompanied diffusion cloud in GzmB channel was defined as fusion.

Supported lipid bilayers (SLB). SLB were prepared for TIRF imaging to visualize granule secretion at immunological synapse and secreted SMAPs attached to the SLB after degranulation. SLBs were prepared as previously described48,49. Briefly, to prepare a clean glass chamber for SLB, the glass coverslips pre-washed with acid piranha and a plasma cleaner were mounted on sticky-Slide VI0.4 (Ibidi) to form 6 well channels. Small unilamellar liposomes were prepared using 18:1 DGS-NTA(Ni) (790404C-AVL, Avanti Polar Lipids), 18:1 Biotinyl Cap (870282C-AVL, Avanti Polar Lipids) and 18:1 DGS-NTA (790404C-AVL, Avanti Polar Lipids). SLB were allowed to form by incubating 50 µl of liposome suspension per 11 cm coverslip. The solution was coated on 12.5 mm glass coverslips overnight at 4 °C for organelle temperature control. Images were acquired as z-stacks over time. The total pixel, threshold was adjusted independently for each channel.

**Evaluation of MCG and SCG cytotoxicity.** MCGs (fraction 6) and SCGs (fraction 8) were isolated from smooth gradient fractionation as described above. After dilution in 160 mM NaCl buffer with 10 mM HEPES (pH 7.3) another step of centrifugation was done for 60 min at 15,000 x g at 4 °C in a SW41Ti rotor (Beckmann) on gelatin-coated coverslips50. For quality control 500 µl of sucrose fraction 6 and 8 of Syb2/GzmB DKI CTLs were pooled and centrifuged on coverslips for fluorescence analysis. The images were acquired with a 63x Plan Apochromat objective (NA 1.4; Zeiss) with laser excitation at 561 nm for Syb2 Ki signal and 488 nm for GzmB KI signal. The maximum projection images are shown. Object based co-localization analysis was performed with DiAna plugin of image51. Individual fluorescent spots were identified with the iterative segmentation procedure using the following settings: step value 100, size: 15-700 pixel, threshold was adjusted independently for each channel.

**Confocal microscopy.** The killing of target cells by CTLs was visualized by confocal microscopy (LSM 780, Zeiss). Briefly, day 5 effector Syb2 Ki CTLs (1 x 10^5) were loaded with 10 mM HEPEs and 10 µg/ml anti-C3dε antibody (Table 1) to promote T cell to form contacts with target cells. Live imaging was performed under 37 °C temperature condition. Images were acquired as z-stacks over time. The total thickness of the stack was 8 µm while distance between individual slices was 1 µm. To evaluate pH value in MCG, day 4 GzmB KI CTLs were first loaded with 2.5 µg/ml WGA-Alexa647 for 30 min and added additional 100 nM LysoTracker Red DND-99 (Thermo Fisher Scientific) in culture medium for another 1 h, allowing dyes to label MCG and acid compartment respectively. After staining, cells were washed twice with culture medium before imaging.

Structured illumination microscopy (SIM). T cells were fixed in ice-cold 2% PFA in Dulbecco’s PBS (Thermo Fisher Scientific) for 20 min. For staining, cells were permeabilized with 0.1% TritonX-100 in Dulbecco’s PBS (permeabilizing solution) for another 20 min followed by 30 min blocking in solution containing 2% BSA prepared in permeabilizing solution. Cells were stained with either Alexa 647 conjugated anti-GzmB (Biolegend) or anti-thrombospondin-1 antibodies (Table 1). Finally, cells were mounted with Mowiol based mounting medium and observed at SIM microscope (Zeiss Elyra PS.3.). Images for correlative fluorescence and electron microscopy (CLEM) of organelles bound to antibody-conjugated Dynabeads Protein G were acquired with excitation light of 488 and 561 nm wavelengths. Almost the entire field of view of a
200-mesh grid (around 90 µm²) could be observed with a ×63 Plan-Apochromat objective by SIM, allowing a perfect orientation relative to the grid bars. After adjusting the highest and lowest focus planes for z-stack analysis in brightfield, the images were recorded with a step size of 100 nm to scan the organelle-Dynabead complexes.

Fluorescent images for CLEM of SMAP containing cells were excited with 405, 488, 561, and 647 nm wavelengths to visualize (DAPI, TSP-1-GFPspark, GzmB-mCherry and WGA-647), respectively. The DAPI image was recorded to identify both the nucleus of the CTL and the image plane. The images were acquired using microscope control software (version 7.0).

**Electron microscopy.** Sucrose fraction 6 and 8 were diluted in D-PBS to a final sucrose concentration of 0.8 M and 10 µl of each fraction were dropped on a polioform-coated 200 mesh copper grid (Plano) and incubated for 30 min. After fixation with 2% paraformaldehyde and 1% glutaraldehyde, samples were contrasted with Uranyl Resin (Electron Microscopy Sciences)56. Electron micrographs were obtained using a Tecnai G2 12 TWIN (Thermo Fisher Scientific). After immunoprecipitation the complex of organelle and Dynabeads Protein G fraction 6 and 8 were suspended in 2% gelatin in PBS-buffer, pipetted into membrane carriers (1.5 mm x 1.0 ml) and vitrified in a high pressure-freezing system (EM FACT2; Leica). Freeze-substitution and embedding in Epon was done as described previously57. Anti-rabbit-STAR580 and anti-mouse-STARred were used as secondary antibodies. The images were acquired using ImageJ or FIJI version 15 and above (National Institute of Health). Statistical tests are indicated in each figure legend. Unless mentioned otherwise, statistical tests are indicated in each figure legend. Unless mentioned otherwise, statistical tests are indicated in each figure legend.

**Cryo-soft X-ray tomography (CSXT).** NK-92 Cells were plunge frozen on carbon coated electron microscopy grids (Ted Pella, Inc. Redding, CA). AAM, Laboratories equipment Ltd, Reading, UK; #G255). Tilt series were collected on the Xrdia Ultra-XRM S220c X-ray microscope (Zeiss) at the B24 beamline of the Diamond synchrotron with a PxiTix-XXO:1024B CCD camera (Teddley Princeton Instruments, Birmingham, UK) and a 40 nm zone plate with X-rays of 500 eV. Tilt series were collected from −60° to +60° with an increment of 0.5°. X-ray tomograms were reconstructed using eMotic, part of the IMOD package58. Post-processing was performed with ImageJ (National Institute of Health).

**Imaging data analysis and statistical analysis.** All images were analyzed with image J or FIJI version 15 and above (National Institute of Health). Granule sizes and their contents from different imaging techniques were analyzed with Igor Pro 6.04 or SigmaPlot 14.0. All statistical tests were performed with Igor or SigmaPlot and data are represented as mean ± SEM (or SD when specified). The statistical tests were selected in a high-throughput and unbiased way. Mann–Whitney U test was used for two-group comparison. All p-values were calculated with two-tailed statistical tests and 95% confidence intervals. *p < 0.01, **p < 0.001. In the box plots, the boundaries of the box represent the 25th to 75th percentile, a black line within the box marks the median, while the white line extends to the means. Whiskers above and below the box indicate the 10th and 90th percentiles, respectively.

**Sample preparation of immuno-isolated vesicles for mass spectrometry-based proteomics.** Proteins of immuno-isolated samples were resolved on NuPAGE 4–12% Bis-Tris gradient gels (Thermo Fisher Scientific), stained with 12
InstantBlue Coomassie Protein Stain (Expedeon) and subjected to in-gel digestion with trypsin as described. Briefly, entire gel lanes were cut into 23 pieces of 1 mm²; these were dried and reconstituted in solution containing 2% [v/v] acetonitrile and 0.05% [v/v] trifluoroacetic acid and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS).

LC-MS/MS analysis. Peptides derived from three (IPs) and five (IPs) biological replicates were analyzed as technical duplicates on Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific), coupled to a Dionex UltiMate 3000 UHPLC system (Thermo Scientific) equipped with an in-house-packed C18 column (RepriOfil-Pur 120 C18-AQ, 1.9 µm pore size, 75 µm inner diameter, 30 cm length, Dr. Maisch GmbH). Samples were separated applying the following gradient: mobile phase A consisted of 0.1% formic acid [v/v], mobile phase B of 80% acetonitrile/0.08% formic acid [v/v]. The gradient started at 5% B, increasing to 10% B within 3 min, followed by 10–45% of B within 33 min, then keeping mobile phase B constant at 90% for 6 min. After each gradient, the column was again equilibrated to 5% B for 6 min. The flow rate was set to 300 nl/min. Eluting peptides were desalted using a data-dependent top-30-acquisition methods. MS1 spectra were acquired with a resolution of 60,000 in the Orbitrap covering a mass range of 350–1600 m/z. Injection time was set to 50 ms and automatic gain control (AGC) target to 1 x 10^5. Dynamic exclusion covered 25 s.

Precursor ion charge state screening was enabled, all unassigned charge states were rejected. Spectra with a charge state of 2–7 were included in the MS2 spectra which were recorded with a resolution of 15,000 in the Orbitrap, injection time was set to 60 ms, AGC target to 1 x 10^5 and the isolation window to 1.6 m/z. Fragmentation was enforced by higher-energy collisional dissociation (HCD) at 30%.

Mass spectrometry data analysis and visualization. Raw files were processed by MaxQuant (MQ) software (version 1.6.0.1) and its built-in Andromeda peptide search engine with the following settings to identify proteins: trypsin/P was used as digested enzyme with maximal two missed cleavage sites; tandem mass spectrometry (MS/MS) spectra were searched against a customized version of the April 2016 release of the UniProt complete Mus musculus proteome sequence database, in which the sequence of synaptobrevin2 (VAMP2) was completely replaced with sequence of C-terminally mRFP-tagged Syb2 protein; carbamidomethylated cysteines were set as fixed and oxidation of methionine as variable modification (maximal allowed number of modifications per peptide was set to five); maximum false discovery rate was set to 0.01 both on peptide and protein levels. Further parameters were used as set by the default settings.

MaxQuant’s label-free quantification (LFQ) algorithm (MaxLFQ) was applied to calculate LFQ ratios. LFQ intensities thresholded to allow the relative comparison of protein amounts across samples. The default settings (MaxQuant version 1.6.0.1) for LFQ intensity calculations were applied, LFQ minimum ratio count of two for each pairwise comparison step was required, as well as unique and razor peptides were considered for the quantification.

To interpret the protein quantitation and co-enrichment data, analysis was performed by using the software platform Perseus (version 1.6.2.2). ProteinGroups.txt file and the corresponding intensity matrices (LFQ intensities) were loaded into Perseus and the results cleaned for reverse hits, contaminants and identified only by site. Positive intensity values were logarithmized (log2). Using the ‘categorical annotation rows’ option, biological and technical replicates for the corresponding sample points (affinity purified gradient fraction 6 and 8 from stimulated and naive (control) CTL) were set equal, (three and five biological replicates for fractions 8 and 6, respectively, were each measured as technical duplicates). Only proteins identified in at least 50% of biological replicates and value filter set to 50% in at least one group were taken under consideration for the significance analysis. Signals, that were originally zero prior to conversion to log2, were imputed with random numbers from a normal distribution (total matrix mode), whose mean and standard deviation were chosen to simulate low abundance values (width = 0.3; shift = 0.15). The significance of co-enriched proteins was determined by a volcano plot-based approach combining two sample t-test-derived p values with (fraction # vs control) ratio information.

Significance lines in the volcano plot corresponding to a given FDR were determined by a permutation-based method. Threshold (ε FDR) and SO (ε cut-off) values were set equal to 0.05 (%5) and 0.1, respectively. The resulting data tables were exported and subjected to further analysis in Excel 2016 (Microsoft Office Package 2016). Categorical annotation by biological function and/or subcellular localization was performed manually for all proteins that were identified as significantly enriched in each of the two Syb2 positive fractions. Protein annotations were summarized in annotation matrix tables within Excel. Each annotation unit was based only on published scientific literature available on NCBI’s website (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/) and UniProt (https://www.uniprot.org/) for the categorical annotation of proteins with enzymatic function, the enzyme nomenclature database provided by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) was used as a source (https://www.qmul.ac.uk/sbs/iubmb/enzyme/). Sunburst diagrams were created by Excel 2016 software (Microsoft Office). The diagrams represented hierarchically the correlation between numeric and categorical variables generated during the protein annotations. To modify graphically the color representation and to add categorical labels, sunburst diagrams were imported as vector files into Adobe Illustrator CS5. Percentage of proteins in the main categories were calculated based on the total number of significantly enriched proteins for each fraction sample. Proportional Venn diagrams were generated using BioVenn web-based application. Color adaptations of the BioVenn diagrams were undertaken using Adobe Illustrator CS5.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Mass spectrometry data are available in Supplementary Data 1. The mass spectrometry raw and MaxQuant output files were deposited to the ProteinXchange Consortium (www.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD025655. Databases are available under the same dataset identifier. All original data sets (western blots, electron micrographs, CSXT images, immunofluorescent images) are available for inspection in the Source Data file have been deposited in ZENODO repository server (https://zenodo.org/) with the dataset identifier 10.5281/zenodo.575216. The accession code will be provided by the corresponding author upon reasonable request. All other data are provided in the article and its supplementary files or from the corresponding author upon reasonable request. Source data are provided with this paper.

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