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Ca$^{2+}$ releases E-Syt1 autoinhibition to couple ER-plasma membrane tethering with lipid transport

Running title: Ca$^{2+}$ releases autoinhibition of E-Syt1

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Abstract

The extended synaptotagmins (E-Syts) are endoplasmic reticulum (ER) proteins that bind the plasma membrane (PM) via C2 domains and transport lipids between them via SMP domains. E-Syt1 tethers and transports lipids in a Ca\textsuperscript{2+}-dependent manner, but the role of Ca\textsuperscript{2+} in this regulation is unclear. Of the five C2 domains of E-Syt1, only C2A and C2C contain Ca\textsuperscript{2+}-binding sites. Using liposomes-based assays, we show that Ca\textsuperscript{2+} binding to C2C promotes E-Syt1-mediated membrane tethering by releasing an inhibition that prevents C2E from interacting with PI(4,5)P\textsubscript{2}-rich membranes, as previously suggested by studies in semi-permabilized cells. Importantly, Ca\textsuperscript{2+} binding to C2A enables lipid transport by releasing a charge-based autoinhibitory interaction between this domain and the SMP domain. Supporting these results, E-Syt1 constructs defective in Ca\textsuperscript{2+} binding in either C2A or C2C failed to rescue two defects in PM lipid homeostasis observed in E-Syts KO cells, delayed diacylglycerol clearance from the PM and impaired Ca\textsuperscript{2+}-triggered phosphatidylserine scrambling. Thus, a main effect of Ca\textsuperscript{2+} on E-Syt1 is to reverse an autoinhibited state and to couple membrane tethering with lipid transport.

Key words: C2 domain/extended synaptotagmin/lipid transfer/phosphatidylserine scrambling/SMP domain
Introduction

Endoplasmic reticulum (ER)-plasma membrane (PM) contact sites represent a general feature of all eukaryotic cells (Friedman & Voeltz, 2011; Gallo et al, 2016; Saheki & De Camilli, 2017a). Their occurrence reflects the presence of proteins that tether the two membranes and mediate crosstalk between them. One such class of tethers are the extended synaptotagmins (E-Syts), resident proteins of the ER membrane that are evolutionarily conserved from unicellular organisms to all metazoans (Craxton, 2001; Craxton, 2007; Giordano et al, 2013; Levy et al, 2015; Manford et al, 2012; Perez-Sancho et al, 2015; Toulmay & Prinz, 2012). In mammals they are encoded by three different genes, E-Syt1, E-Syt2 and E-Syt3 (Min et al, 2007), which form homo- and hetero-dimers. All three E-Syts comprise an N-terminal hydrophobic hairpin through which they are anchored to the ER (Giordano et al, 2013; Saheki et al, 2016). This region is followed by a Synaptotagmin-like Mitochondrial lipid binding Protein (SMP) domain (Kopec et al, 2010; Lee & Hong, 2006; Schauder et al, 2014; Toulmay & Prinz, 2012) and multiple C2 domains, five in E-Syt1 and three in E-Syt2 and E-Syt3 (see Fig 1A) (Min et al, 2007; Saheki & De Camilli, 2017b). As shown by the crystal structure, the first two C2 domains of E-Syt2 (C2AB) are arranged in tandem (Schauder et al, 2014; Xu et al, 2014) and a similar arrangement is predicted for the C2AB domains of E-Syt1 and E-Syt3. The last C2 domain (C2E for E-Syt1 and C2C for E-Syt2 and E-Syt3) is characterized by a positively charged surface (Idevall-Hagren et al, 2015), and the additional C2 domains unique to E-Syt1 (C2CD) are similar to C2AB and most likely represent a duplication of this pair (Min et al, 2007). The recruitment of E-Syts to ER-PM contact sites requires PI(4,5)P2 in the PM and occurs constitutively in the case of E-Syt2 and E-Syt3, while it requires elevation of cytosolic Ca2+ in the case of E-Syt1 (Chang et al, 2013; Fernandez-Busnadiego et al, 2015; Giordano et al, 2013; Idevall-Hagren et al, 2015).
The defining feature of the E-Syts among proteins with multiple C2 domains is the presence of the SMP domain (Lee & Hong, 2006), a member of the TULIP domain superfamily (Kopec et al, 2010). A shared characteristic of TULIP domains, which are present both in extracellular and intracellular proteins, is the property to harbor lipids within a hydrophobic cavity and, at least in many cases, to transport them (AhYoung et al, 2015; Alva & Lupas, 2016; Jeong et al, 2016; Kopec et al, 2011; Lees et al, 2017; Liu et al, 2017; Oram et al, 2003; Qiu et al, 2007; Saheki et al, 2016; Schauder et al, 2014; Yu et al, 2016). SMP domains are typically found in intracellular proteins that act at membrane contact sites (Kornmann et al, 2009; Lees et al, 2017; Liu et al, 2017; Reinisch & De Camilli, 2016; Toulmay & Prinz, 2012). Structural and biochemical studies of the SMP domain of E-Syt2 revealed that it dimerizes to form a 90-Å-long cylinder with a deep hydrophobic groove that runs along its main axis and that contains glycerophospholipids (Schauder et al, 2014). Consistent with these properties, purified recombinant E-Syt1 transfers glycerolipids between two populations of liposomes that mimic the ER and the PM, respectively (Saheki et al, 2016; Yu et al, 2016), without selectivity for a specific headgroup (Hoglinger et al, 2017; Schauder et al, 2014).

Genome-edited cells lacking all the three E-Syts showed no major differences in the steady-state glycerolipids compositions of the PM. However, delayed clearance of the transient accumulation of diacylglycerol (DAG) at the PM produced by phospholipase C (PLC)-dependent PI(4,5)P₂ hydrolysis was observed (Saheki et al, 2016), suggesting a role of E-Syts in the homeostatic response that follows a stimulus. This phenotype was rescued by expression of E-Syt1, but not by E-Syt1 lacking the SMP domain. Additionally, rescue required an elevation in cytosolic Ca²⁺ (Saheki et al, 2016). Whether other changes at the PM occur in response to stimuli in the E-Syts KO cells remain unclear.
Of the five C2 domains in E-Syt1, two (C2A and C2C) bind Ca^{2+} (Min et al, 2007). Ca^{2+} binding to the C2C domain is responsible for ER tethering to the PM, as mutations that impair its Ca^{2+} binding were sufficient to abolish Ca^{2+}-dependent recruitment of E-Syt1 to the PM (Chang et al, 2013; Giordano et al, 2013). The mechanism involved in this effect is not fully understood yet. It may be mediated by i) a direct interaction of the C2C domain with the PI(4,5)P_{2}-rich PM (Giordano et al, 2013), ii) the release by Ca^{2+} of an inhibitory action of the C2C domain on the binding of C2E to the PI(4,5)P_{2}-rich PM (Idevall-Hagren et al, 2015) or iii) both mechanisms. In addition, mutations in the Ca^{2+}-binding sites of either the C2A or the C2C domain reduced the lipid transfer activity of E-Syt1 in vitro (Yu et al, 2016). However, the function of C2A in SMP domain-dependent lipid transfer remains elusive. Interestingly, the C2A domain of E-Syt2 and E-Syt3, two proteins whose binding to the PM is not regulated by Ca^{2+}, also contains Ca^{2+}-binding sites. A plausible scenario is that the C2A domain of the E-Syts may play a regulatory role in lipid transfer independent of tethering. Goal of this study was to test this hypothesis.

Here, we find that the Ca^{2+}-dependent properties of the C2A and C2C domains of E-Syt1 have different functions at membrane contact sites. Using a liposome-based assay, we confirm that Ca^{2+} binding to the C2C domain acts primarily by enabling the binding of the C2E domain to PI(4,5)P_{2}-rich membranes. Importantly, we show that Ca^{2+} binding to the C2A domain enables lipid transfer by E-Syt1 via the release of an autoinhibitory intramolecular interaction of this domain with the SMP domain. We also show that Ca^{2+} binding to the C2A and C2C domains play important roles in the E-Syt1-dependent regulation of lipid homeostasis at the PM in living cells.
**Results**

**C2C and C2E domains of E-Syt1 cooperate in membrane tethering.**

The contributions of individual C2 domains in E-Syt1-mediated membrane tethering were assessed using an *in vitro* liposome turbidity assay (Saheki et al, 2016). To this aim, purified cytosolic fragment of human E-Syt1, in which the N-terminal region (including the hydrophobic hairpin) was replaced by a His-tag (E-Syt1cyto, Fig 1A), were added to the mixture of two populations of liposomes. One liposome population (anchoring liposomes, ER-like in composition) comprised phosphatidylcholine (PC), a nickel-conjugated lipid [DGS-NTA(Ni)] that functions as a binding site for the His-tagged proteins, and NBD-phosphatidylethanolamine (PE). The other liposome population (target liposomes, PM-like in composition) comprised PC and two acidic phospholipids, phosphatidylserine (PS) and PI(4,5)P₂. The increase in turbidity, which reflects clustering of liposomes into larger particles, was measured as optical density at 405 nm.

Addition to the liposome mixture of E-Syt1cyto in a buffer devoid of Ca²⁺ resulted in an increase in optical density (turbidity) (Fig 1B). Upon addition of a “cocktail" of EGTA, imidazole (to disrupt the nickel His-tag interaction) and proteinase K at the end of incubation, the increase in optical density was reversed, ruling out the fusion of liposomes as the cause of the increase of optical density (Fig 1B). No change in optical density was observed in the absence of E-Syt1cyto, of DGS-NTA(Ni) in the anchoring liposomes, or of the target liposomes (Fig 1B). We conclude that although E-Syt1 is primarily diffuse throughout the ER when expressed alone, under *in vitro* conditions it can tether ER-like to PM-like liposomes even in the absence of Ca²⁺. Actually, low level of E-Syt1-dependent ER-PM contact sites can be observed at resting Ca²⁺ concentration in the cells (Fernandez-Busnadiego et al, 2015; Giordano et al, 2013).
It was shown that the C2E domain of E-Syt1 shares the properties of the C2C domains of E-Syt2 and E-Syt3, which in these two proteins mediate constitutive PI(4,5)P$_2$-dependent PM binding (Fernandez-Busnadiego et al, 2015; Giordano et al, 2013; Idevall-Hagren et al, 2015). All these three C-terminal C2 domains lack a Ca$^{2+}$-binding site and have a highly basic surface in common (Idevall-Hagren et al, 2015). Accordingly, and consistently with studies in cells (Fernandez-Busnadiego et al, 2015; Idevall-Hagren et al, 2015), a mutant E-Syt1$_{cyto}$ that lacks the C2E domain (SMP-C2ABCD, Fig 1A) failed to tether target liposomes in the absence of Ca$^{2+}$ (Fig 1B). Lack of PI(4,5)P$_2$ in the target liposomes, or replacement of the 5% PI(4,5)P$_2$ with 20% PS in these liposomes, also dramatically reduced E-Syt1$_{cyto}$-mediated Ca$^{2+}$-independent membrane tethering (Fig 1B). These results indicate that in the liposome-based system the cytosolic domain of E-Syt1 mediates tethering of the two classes of liposomes in a C2E-dependent way in the absence of Ca$^{2+}$.

We next investigated the role of Ca$^{2+}$ in liposome tethering using the same liposome-based assay. Addition of Ca$^{2+}$ (100 μM) resulted in a strong increase of the basal tethering by E-Syt1$_{cyto}$ observed in the Ca$^{2+}$-free buffer (Fig 1C). E-Syt1 contains putative Ca$^{2+}$-binding sites in its C2A and C2C domains (Giordano et al, 2013; Min et al, 2007; Xu et al, 2014). Mutations of key residues that mediate Ca$^{2+}$ binding in the C2C domain of the construct (E-Syt1$_{cyto}$ C2Cx, Fig 1A) only showed a slightly increased Ca$^{2+}$-dependent membrane tethering (Fig 1C). In contrast, mutations in the Ca$^{2+}$-binding sites in the C2A domain (E-Syt1cyto C2Ax, Fig 1A) still produced a significant increase of Ca$^{2+}$-dependent liposome tethering (Fig 1C). Both of these mutants had a similar level of Ca$^{2+}$-independent basal liposome tethering (Fig 1C). These findings were consistent with the previous reports that the C2C domain is required for Ca$^{2+}$-dependent ER-PM
tethering of E-Syt1 in intact cells (Chang et al, 2013; Giordano et al, 2013).

A lower degree of Ca\(^{2+}\)-dependent tethering occurred also with an E-Syt1\textsubscript{cyto} fragment lacking the C2E domain (SMP-C2ABCD, Fig 1A and D). This tethering did not require PI(4,5)P\(_2\) in target liposomes (Fig 1D), and occurred at a similar level for both constructs harboring Ca\(^{2+}\)-binding mutations in either the C2A domain (SMP-C2AxBCD) or the C2C domain (SMP-C2ABCxD) (Fig EV1A), although binding of SMP-C2ABCxD occurred with slower kinetics (Fig EV1B). In addition, a similar dependence on Ca\(^{2+}\) concentration [half maximal effective concentration (EC50)] was observed for the binding of SMP-C2AxBCD and SMP-C2ABCxD to membranes, as revealed by the liposome tethering assay (Fig EV1D). Given that E-Syt1\textsubscript{cyto} C2Ax showed a much stronger Ca\(^{2+}\)-dependent stimulation of tethering than E-Syt1\textsubscript{cyto} C2Cx (Fig 1C), and C2E does not bind Ca\(^{2+}\), we conclude that the increased E-Syt1\textsubscript{cyto}-mediated liposome tethering in the presence of Ca\(^{2+}\) mainly relies on a synergistic effect between Ca\(^{2+}\)-bound C2C and C2E. This most likely occurs via the release of an inhibitory action of C2C affecting C2E binding to the PI(4,5)P\(_2\)-rich membranes, since an E-Syt1 construct lacking the C2ABCD region (SMP-C2E, Fig EV1A) tethered the liposomes at a higher level than E-Syt1\textsubscript{cyto} in the absence of Ca\(^{2+}\) (Fig EV1C). This inhibition did not completely abolish the membrane binding of C2E in the absence of Ca\(^{2+}\), as C2E-dependent and Ca\(^{2+}\)-independent liposome tethering by E-Syt1\textsubscript{cyto} could be observed (Fig 1B).

To confirm that the inhibitory action of C2C on C2E is through a direct interaction, we purified C2CD and C2E, respectively, and performed cross-linking experiments. After incubation with the cross-linker BS\(^3\) [bis(sulfosuccinimidyl) suberate], a band with MW corresponding to that expected for a C2CD-C2E heterodimer appeared (Fig EV1E, red arrowhead, lane 6, 7 and 8). The heterodimer of C2CD-C2E was confirmed by mass
spectrometry analysis of the band, which revealed peptides of both C2CD and C2E, covering nearly their entire sequences (Fig EV1F). Importantly, formation of this band in response to the cross-linker was strongly reduced when the two protein fragments were incubated with both Ca2+ and liposomes, i.e. when C2CD can bind to membranes (lane 9, see right panel for quantification). The band was only weak, but this was not unexpected as in the intact protein the C2CD and the C2E domain are part of the same polypeptide and thus are in close proximity (which will increase the efficiency of heterodimer formation), while in our case they are dispersed in solution. This result strongly supports the hypothesis that C2CD inhibits the binding of C2E to a PI(4,5)P2-containing membrane through a direct interaction, which will be released by Ca2+ binding to C2C to redirect it to the membrane.

Altogether, these results demonstrate that both PI(4,5)P2 binding by C2E and Ca2+ regulation of C2C play the dominant role in the Ca2+ stimulation of E-Syt1-dependent liposome tethering.

**C2A domain of E-Syt1 participates in the Ca2+ regulation of lipid transfer in vitro.**

In view of the lack of a relevant impact of the Ca2+-binding properties of C2A on Ca2+-dependent liposome tethering by E-Syt1 (Fig 1C), we explored whether these properties play a role in lipid transfer using a Fluorescence Resonance Energy Transfer (FRET)-based assay (Saheki et al, 2016; Yu et al, 2016). The assay involves the same liposomes described above (with the addition of NBD-PE to the anchoring liposomes, henceforth defined as donor liposomes). The fluorescence of NBD, which is partially self-quenched in these liposomes, increases as a result of dequenching since NBD-PE is transferred to the target liposomes (henceforth defined as acceptor liposomes). Consistent with previous reports (Saheki et al, 2016; Yu et al, 2016), Ca2+ strongly stimulated lipid
transfer by E-Syt1cyto, as revealed by NBD-PE dequenching (Fig 2B). However, this stimulation was completely abolished by the Ca\(^{2+}\)-binding mutations in C2A (E-Syt1cyto C2Ax), in spite of very little impact of these mutations on liposome tethering (Fig 2B).

**SMP domain of E-Syt1 alone can transfer lipids in a Ca\(^{2+}\)-independent manner between tethered membranes.**

To investigate the interplay of the SMP domain and C2 domains in SMP domain-dependent lipid transfer, His-tagged SMP domain of human E-Syt1 (SMP, Fig 2A) was generated and tested for its activity in the *in vitro* lipid transfer assay. No increase in turbidity (consistent with lack of tethering) (Fig EV2A) or in lipid transfer was observed upon incubation of the SMP domain alone with donor and acceptor liposomes, even in the presence of Ca\(^{2+}\) (Fig 2C). While in principle even the SMP domain alone could mediate some lipid transfer during random encounters between liposomes, the rate of such transfer may be too low to be detected during the assay period (30 min) in the absence of tethering. However, when both SMP and a His-tagged C2ABCDE fragment of E-Syt1 (C2ABCDE, Fig 2A) were added together to the mixtures of donor and acceptor liposomes, SMP domain-dependent lipid transfer was observed in the presence of Ca\(^{2+}\) (Fig 2C), i.e. conditions under which the C2ABDCE can mediate tethering (Fig EV2A). Ca\(^{2+}\) appeared to be needed only to facilitate tethering, as a similar degree of lipid transfer by SMP domain was observed irrespective of the presence of Ca\(^{2+}\), when the two sets of liposomes were connected by another tether, His-tagged PH\(_{PLC\delta}\), which binds to PI(4,5)P\(_2\) (Garcia et al, 1995; Hammond & Balla, 2015) in the acceptor liposomes in a Ca\(^{2+}\)-independent way (Fig 2E and EV2B). These experiments further demonstrate that a key role of Ca\(^{2+}\) in E-Syt1-dependet lipid transfer is to mediate tethering. Additionally, these results also reveal that the additional importance of Ca\(^{2+}\) binding to the C2A domain in enabling lipid transfer is only manifested when the SMP
and C2A are part of the same polypeptide.

**C2A domain of E-Syt1 inhibits the activity of SMP domain in the absence of Ca²⁺ via an intramolecular interaction.**

To further analyze the property of the C2A domain of E-Syt1 in lipid transfer, His-tagged SMP-C2AB and His-tagged SMP domain were tested in parallel. When added to donor and acceptor liposomes, no transfer was observed with the SMP domain alone (see above, Fig 2C and EV3A), while the SMP-C2AB construct induced some liposome tethering and lipid transfer, but only in the presence of Ca²⁺ (Fig EV3). The lipid transfer properties of the SMP-C2AB fragment were much more pronounced, and only in the presence of Ca²⁺, when liposomes were additionally tethered by the His-tagged C2ABCDE fragment (Fig 2D). However, mutations in C2A domain that make SMP-C2AB incapable of binding Ca²⁺ (SMP-C2AxB, Fig 2A) abolished its lipid transfer activity even in the presence of Ca²⁺ and of C2ABCDE (Fig 2D). Under these conditions (presence of C2ABCDE and Ca²⁺), a robust lipid transfer was achieved by SMP alone (see above, Fig 2C and D), although it was lower than SMP-C2AB (Fig 2D), which is possibly due to a higher basal lipid transfer by SMP-C2AB compared to SMP alone in the presence of Ca²⁺ (Fig EV3A).

These findings suggested that 1) the C2A domain may block lipid transfer activity of the SMP domain via an intra- or inter-molecular autoinhibitory interaction, 2) the surface of C2A involved in this autoinhibitory interaction is not the one harboring the Ca²⁺-binding sites and 3) this inhibition is released upon binding of Ca²⁺ to the C2A domain. To test this hypothesis, we assessed lipid transfer using His-tagged PH₆PLC5 to tether donor and acceptor liposomes. In the presence of PH₆PLC5, SMP-C2AB transferred lipids in the presence of Ca²⁺, while its lipid transfer activity was barely detectable in the absence of
Ca$^{2+}$ (Fig 2E). Given that the SMP domain alone can transport lipids in Ca$^{2+}$-independent manner when liposomes are tethered by the PH$_{PLC6}$ (see above, Fig 2E), these results support the hypothesis that Ca$^{2+}$-binding to the C2A domain enhances SMP domain-dependent lipid transfer by releasing an autoinhibition.

We next tested the importance of membrane binding for the property of Ca$^{2+}$ to release the inhibitory effect of C2A on lipid transfer. Based on a.a. sequence alignment, C2A is predicted to have the same hydrophobic loop that in the C2A and C2B domain of Syt1, which participates in membrane binding (Martens et al, 2007). Introduction of mutations into bulky hydrophobic residues (V348A/L351A) of this predicted C2A loop in the SMP-C2ABCxD construct (SMP-C2AmBCxD), i.e. a construct whose binding to membranes in response to Ca$^{2+}$ is entirely dependent upon the C2A domain, abolished its Ca$^{2+}$-dependent binding to the liposomes (Fig EV1A and B). When introduced into full-length E-Syt1$_{cyto}$ (E-Syt1$_{cyto}$ C2Am, Fig 2A), the same mutations only partially impaired Ca$^{2+}$-dependent lipid transfer activity, in contrast to the complete block of activity produced by the Ca$^{2+}$ binding mutant (E-Syt1$_{cyto}$ C2Ax) (Fig 2B). These results show that Ca$^{2+}$ binding to C2A can release its inhibitory action on the SMP domain irrespective of membrane binding, but also demonstrate that Ca$^{2+}$-dependent membrane binding enhances this effect.

To gain insight into the mechanisms underlying autoinhibition, we inspected the reported crystal structure of the SMP-C2AB domains of E-Syt2 (Schauder et al, 2014), which is predicted to be very similar to the corresponding region of E-Syt1. In the crystal structure, the SMP domain forms a dimer flanked by two interfaces with the C2A domains of the monomers: an intramolecular interface and an intermolecular interface (Fig 3A). Both interfaces involve charged residues, which are conserved in E-Syt1 (Fig 3B and EV4A).
The importance of these two interfaces for the lipid transport function of E-Syt1 was assessed by mutating positively charged residues to negatively charged residues in the SMP domain. The R227E mutation in the SMP domain at the intermolecular interface (Fig 3B right) had no impact on lipid transfer ability of SMP-C2AB (Fig 3C). In contrast, the R266E/R267E double mutation in the SMP at the intramolecular interface (Fig 3B left) abolished the inhibitory effect of the C2A domain on the lipid transfer activity in the absence of Ca\(^{2+}\) when using PH\(_{PLC}\) \text{-tethered liposomes (Fig 3C). Furthermore, when the \textit{in vitro} lipid transfer assay was performed in the presence of high salt (500 mM NaCl), i.e. conditions expected to disrupt the salt bridge between the SMP and C2A, SMP-C2AB had a higher lipid transfer activity than in more physiological salt conditions (100 mM NaCl). Conversely, lipid transfer by the SMP domain alone (without the flanking C2AB domain) was the same in both conditions (Fig 3D). These results suggest that an intra-molecular salt bridge drives an autoinhibitory interaction of the C2A domain with the SMP domain.

Finally, replacing the C2AB domain pair in the SMP-C2AB construct with the C2CD domain pair of E-Syt1(SMP-C2CD, Fig EV4B) did not inhibit SMP domain-mediated lipid transfer (Fig EV4C). This was consistent with the lack of conservation between C2A and C2C in the surface of C2A that mediates the intramolecular interaction with the SMP domain (Fig EV4A). Such surface is opposite to the Ca\(^{2+}\)-binding sites, which is also the bilayer-binding surface of the C2A domain (Xu et al, 2014) (Fig EV4D). Based on crystallographic studies of the C2A domain of E-Syt2, Ca\(^{2+}\) binding to C2A results in a decrease of the negative potential of the surface of C2A that interacts with the SMP domain, most likely decreasing the strength of the interaction (Xu et al, 2014). We hypothesize that Ca\(^{2+}\) functions as a switch that shift the C2A domain from an SMP-binding state to a bilayer-binding state, thus releasing the inhibition of
SMP-mediated lipid transfer. As C2A binding to a bilayer does not require presence of acidic phospholipids (Min et al, 2007), bilayer binding of C2A domain may occur either “in cis” or “in trans”.

Collectively, these results point to occurrence of an autoinhibitory intramolecular interaction between the SMP domain and the C2A domain of E-Syt1. This autoinhibition impairs lipid transfer activity and is released by Ca²⁺.

**Role of Ca²⁺ binding to C2A and C2C in the property of E-Syt1 to clear DAG from the PM**

Having validated the importance of Ca²⁺ binding to the C2A domain of E-Syt1 in lipid transfer *in vitro*, we next tested the role of this Ca²⁺ binding on the action of E-Syt1 in living cells. A previous study (Saheki et al, 2016) showed that cells lacking the two major E-Syts (E-Syt1 and E-Syt2) (double KO, DKO) or all three E-Syts (triple KO, TKO) have a partial defect in the clearance of PLC-dependent DAG accumulation from the plasma membrane, as monitored by the fluorescence of the DAG probe C1PKC-mCherry (Codazzi et al, 2001). In WT cells overexpressing the muscarinic M1 receptor (M1R), plasma membrane DAG rapidly increases in response to the M1R agonist Oxo-M [which stimulates PLC (Horowitz et al, 2005; Suh et al, 2004; Willars et al, 1998)], and such increase is persistent in the presence of a DAG kinase inhibitor (DGKi) even after reversal of M1R activation with atropine (Fig 4A) (Saheki et al, 2016). However, excess DAG was rapidly cleared in WT cells upon the addition of ionomycin (Fig 4A), a drug that increases cytosolic Ca²⁺ (Morgan & Jacob, 1994) and thus results in the acute recruitment of E-Syt1 to the plasma membrane (Fig EV5B and C) (Saheki et al, 2016). Such clearance was not observed in E-Syts TKO cells (Fig 4A and B), and this phenotype was rescued by re-expression of EGFP-E-Syt1 (Fig 4A, B and EV5A), but not of
EGFP-E-Syt1 with mutations in the Ca\(^{2+}\)-binding sites of its C2C domain (EGFP-E-Syt1 C2Cx) (Fig 4A and B), i.e. a mutant construct that is not recruited to the PM in response to Ca\(^{2+}\) elevation (Fig EV5B and C) (Giordano et al, 2013).

Importantly, in agreement with our in vitro lipid transfer results, a construct harboring mutations in the Ca\(^{2+}\)-binding sites of the C2A domain of EGFP-E-Syt1 (EGFP-E-Syt1 C2Ax) also failed to rescue the DAG clearance defect in E-Syts TKO cells (Fig 4A, B and EV5A), although loss of Ca\(^{2+}\) binding by the C2A domain was shown not to have an obvious impact on PM recruitment upon elevation of cytosolic Ca\(^{2+}\) (Fig EV5B and C) (Chang et al, 2013). Expression of EGFP-E-Syt1 C2Am also could not rescue the DAG clearance defect in E-Syts KO cells (Fig EV5D), even if E-Syt1\(_{cyto}\) C2Am had partial lipid transfer activity in vitro (Fig 2B), demonstrating a more severe effect of this mutation in the context of a living cell.

**Ca\(^{2+}\) binding to both C2A and C2C is required for a role of E-Syt in enabling PS scrambling.**

During a systematic investigation of PM properties that may be affected in cells lacking E-Syts, we found that such cells have a defect in the externalization of PS in response to ionomycin (Fig 5A, B, EV6A and B). The increase in the surface exposure of PS, which is normally concentrated in the inner leaflet of the PM, can be measured by monitoring the recruitment to the cell surface of fluorescent annexin V [a PS binding protein (Heemskerk et al, 1997; Lhermusier et al, 2011)], which is added in the extracellular medium (Williamson et al, 1995; Zwaal et al, 2005). Conversely, loss of PS from the inner PM leaflet can be monitored lactadherin C2 domain fused with mCherry, a PS binding probe [Lact-C2-mCherry (Yeung et al, 2008)]
The ionomycin (Ca$^{2+}$)-dependent PS externalization process itself remains poorly understood. There is evidence that such scrambling can be affected by the lipid composition of the bilayer (Brown & Conboy, 2013; Contreras et al, 2010). We exploited this phenotype to further validate the physiological importance of E-Syts and of the Ca$^{2+}$-binding sites of the C2A and C2C domains of E-Syt1.

Incubation of WT cells with ionomycin induced a rapid increase in fluorescently tagged annexin V associated with the cell surface, as detected by confocal microscopy (Fig 5A and EV6A). This increase was paralleled by a decrease of the Lact-C2-mCherry signal associated with the PM, which is consistent with the scrambling of PS from the inner to the outer leaflet of the PM (Fig EV6A, C and E). In E-Syts KO cells, binding of annexin V to the cell surface was dramatically impaired (Fig 5B, D, EV6B, and G-I), and loss of Lact-C2-mCherry signal from the PM did not occur (Fig EV6B and D). Addition to cells of FM1-43, another probe that detects externalized PS and other acidic phospholipids (Zweifach, 2000), confirmed the robust defect in lipid scrambling in E-Syts KO cells (Fig EV6J). As shown by the Annexin V-Cy3 fluorescence assays, the lipid scrambling defect could be rescued by re-expression of E-Syt1 together with E-Syt2 (Fig 5C, D and Fig EV6F-H), and to a lower extent by re-expression of E-Syt1 alone (Fig 5D-F and EV6G-I), but not by an E-Syt1 construct lacking the SMP domain (EGFP-E-Syt1 ΔSMP) (Fig 5C, D and EV6F-H). Additionally, an E-Syt1 construct harboring bulky hydrophobic residues in the hydrophobic cavity of the SMP domain (V169W/L308W), and therefore defective in lipid transport (Saheki et al, 2016), had much reduced rescue activity (Fig 5E). These results confirmed that the phenotype observed was dependent upon the loss of the lipid transport activity of the E-Syts.

Importantly, E-Syt1 constructs harboring mutations that impair Ca$^{2+}$ binding to either the
C2C or to C2A domains failed to rescue PS scrambling (Fig 5C, F and EV6I), indicating that both Ca\(^{2+}\)-dependent properties of E-Syt1, recruitment to the PM and stimulation of lipid transport, are required for the rescue.

**Discussion**

Our results provide new insight into the molecular mechanisms underlying Ca\(^{2+}\)-dependent regulation of the lipid transport and membrane tethering properties of E-Syt1. We propose that these two properties are regulated by its C2A and C2C domain, i.e. its two Ca\(^{2+}\)-binding domains, respectively. C2A binds the SMP domain via a charge-based intramolecular interaction that inhibits its lipid transport function, and Ca\(^{2+}\) binding releases this inhibition. C2C mediates Ca\(^{2+}\)-regulated tethering of the ER to the PM via an action that is heavily dependent on the property of C2E to bind the PM in a PI(4,5)P\(_2\)-dependent way. Importantly, our results also show that these functions are physiologically relevant, as E-Syt1 constructs with mutations in the Ca\(^{2+}\)-binding sites of either C2 domains fail to rescue defects in lipid dynamics at the PM in E-Syts KO cells.

Using a liposome-based turbidity assays we demonstrate that although E-Syt1\(_{cyto}\)-dependent tethering is strongly stimulated by Ca\(^{2+}\), tethering occurs also in the absence of Ca\(^{2+}\), and that this tethering requires its C2E domain. This result is in agreement with the previous findings that low level of E-Syt1-dependent ER-PM contacts occurs in living cells in the absence of Ca\(^{2+}\) elevation (Fernandez-Busnadiego et al, 2015; Giordano et al, 2013) and that the C2E domain, when expressed alone, binds the PM at resting Ca\(^{2+}\) concentration (Idevall-Hagren et al, 2015). In fact, analysis of the structural homology between C2E domain of E-Syt1 and other classical C2 domains of synaptotagmins (Chapman et al, 1998; Fernandez-Chacon et al, 2001; Fukuda et al, 1995) predicts a polybasic patch at the surface of the C2E domain [i.e. a patch expected to bind
the PI(4,5)P₂-rich PM] but no Ca^{2+}-binding sites (Idevall-Hagren et al, 2015).

Our demonstration that stimulation by Ca^{2+} of E-Syt1_{cyto}-mediated liposome tethering is dependent on Ca^{2+} binding to its C2C domain is consistent with studies of overexpressed full-length E-Syt1 in living cells (Chang et al, 2013; Giordano et al, 2013). Unlike our previous conclusion that Ca^{2+}-bound C2C domain itself was responsible for binding to the PI(4,5)P₂-rich PM (Giordano et al, 2013), our current findings strongly suggest that, in addition to this function, a main action of Ca^{2+} on the C2C domain is to release its inhibitory action on the binding of C2E to membranes, corroborating a previous suggestion from studies of semi-permeabilized cells (Idevall-Hagren et al, 2015). We note that E-Syt2 and E-Syt3, which lack a central C2CD domain pair (Min et al, 2007) (see Fig 1A), but have a C-terminal C2 domain similar to the C-terminal C2 domain of E-Syt1 (C2E) (Idevall-Hagren et al, 2015), are constitutively localized at the PM even at basal Ca^{2+} concentration, further supporting an inhibitory role of C2C in C2E PM binding. Thus, we propose that the recruitment of E-Syt1 to the PM is spatially and temporally controlled by the cooperation of the C2C and C2E domains.

Primary amino acid sequence similarity suggests that the C2CD domain pair may represent a duplication of the C2AB domain pair (Min et al, 2007). However, we found that mutations in the Ca^{2+}-binding sites of C2A have very little effect on E-Syt1-mediated liposome tethering, although a low level of Ca^{2+}-dependent increase of liposomes tethering was observed when incubating liposomes with SMP-C2ABCxD or SMP-C2AB (see Fig EV1B and EV3B). This low level increase of liposome tethering is consistent with the property of the very similar C2AB domains of E-Syt2 to bind liposomes in a Ca^{2+}-dependent way (Min et al, 2007). Most likely, in the context of full-length E-Syt1, this contribution to tethering is negligible. These point mutations, however, completely
abolished the lipid transfer activity of E-Syt1, which was confirmed by 1) reduced dequenching of NBD-PE (i.e. lipid transport between the two liposomes) in the liposome-based lipid transfer assay, and 2) loss of rescue of the defects in lipid dynamics at the PM observed in E-Syts KO living cells.

An open question prior to this study was how Ca\(^{2+}\) binding to C2A contributes to the lipid transfer activity of the SMP domain. We showed here that a construct comprising the SMP domain and the flanking C2AB domain pair had negligible lipid transport activity when liposomes were tethered in the absence of Ca\(^{2+}\). However, the bilayer-anchored SMP domain alone, i.e. without flanking C2 domains, can directly transfer lipids between liposomes, although tethering of liposomes was required to make lipid transfer efficient enough to be detected. The tether did not need to be part of the SMP domain-containing construct. Additionally, the level of lipid transfer mediated by the SMP domain alone was similar in the absence or presence of Ca\(^{2+}\) when the liposomes were tethered by a Ca\(^{2+}\)-independent module (e.g. PH\(_{PLCβ}\)).

We propose that Ca\(^{2+}\)-binding to C2A enhances lipid transfer activity by releasing an intramolecular interaction with the SMP domain that keeps the SMP domain in an autoinhibited state. Inspection of the crystal structure of the Ca\(^{2+}\)-free cytosolic fragment of human E-Syt2 comprising the SMP domain and C2A and C2B (Schauder et al, 2014) supports this possibility. In the structure, which is expected to be very similar for the corresponding fragment of E-Syt1, a negatively charged surface of C2A, which is adjacent to its Ca\(^{2+}\)-binding site, interacts with positively charged residues of the SMP domain located next to the entry of the hydrophobic groove. Presence of the C2AB next to the groove may prevent the access of the SMP domain to membranes by steric hindrance (Fig 3A and B). Upon Ca\(^{2+}\) binding, the electrostatic potential of C2A becomes
neutral or positive (Xu et al, 2014), thus most likely weakening the interaction with the SMP domain and re-orienting the C2A domain for binding to the membrane (either the ER or the PM). This change, in turn, would allow the SMP domain to efficiently extract and transfer lipids (Fig 6). The importance of Ca$^{2+}$ binding to C2A for lipid transport implies that even if E-Syt2 is localized constitutively at ER-PM contacts when not heterodimerized to E-Syt1 (Giordano et al, 2013), its lipid transport activity may be Ca$^{2+}$-dependent. Thus, E-Syts may mediate lipid transport at ER-PM contacts only in response to Ca$^{2+}$ elevation, irrespective of their localization at rest.

An unexpected finding reported in this study is that absence of the E-Syts impairs Ca$^{2+}$-induced PS scrambling. The mechanisms underlying this change, which may be explained by abnormalities of the PM bilayer (Brown & Conboy, 2013; Contreras et al, 2010) as a result of the absence of the E-Syts-mediated lipid transfer between ER and PM, remain to be further explored. We believe that the E-Syts are not directly involved in PS scrambling, but that they produce this effect indirectly, for example by an effect on some properties of the PM bilayer resulting from their lipid transport properties. One reason to support this idea is that upon ionomycin addition the externalization of PS continues to increase at 15 min (Fig 5D) when EGFP-S-Syt1 presence at the PM has already returned to background levels [the signal of at the PM returns to the background level within 3 min after adding ionomycin (Fig EV5B)]. We exploited this defect of E-Syts TKO cells, as well as the previously described delay in the clearance from the PM of acutely produced DAG (Saheki et al, 2016), to assess the property of Ca$^{2+}$ binding to the C2A and C2C domain of E-Syt1 with rescue experiments. Both phenotypes could be rescued by re-expressing E-Syt1, but not E-Syt1 with mutations in the SMP domain, C2A or C2C. Thus, both the property of E-Syt1 to transfer lipids in response to Ca$^{2+}$, and the property to be recruited to the PM in response to Ca$^{2+}$ are required for the rescue. Collectively,
these findings further demonstrate the importance of both Ca$^{2+}$-bound C2 domains for the function of the E-Syts. They also support the hypothesis that the lipid transport functions of the E-Syts are involved, most likely indirectly, in enabling the PM to support PS scrambling in response to ionomycin, at least in our in vitro conditions. Thus, they provide new evidence for a role of the E-Syts in the control of lipid homeostasis at the PM. The role(s) of the E-Syts in the control of PM lipid homeostasis may be partially redundant with those of other lipid transfer proteins, given the lack of major defects in development, viability, or fertility in E-Syts KO mice (Sclip et al, 2016; Tremblay & Moss, 2016). As the E-Syts are highly conserved in evolution from unicellular organisms to mammals, their actions may become critical only under specific functional states that need to be further investigated.

In summary, we report here that elevations of cytosolic Ca$^{2+}$ not only control the localization of E-Syt1, but also enable its lipid transfer activity and that at least some of these actions are mediated by the release of autoinhibitory mechanisms. Autoinhibition by a C2 domain was reported for other proteins, e.g. Munc13 (Michelassi et al, 2017), E3 ubiquitin-protein ligase SMURF2 (Wiesner et al, 2007) and protein kinase C βII (Antal et al, 2015). Additionally, release of a C2 domain-dependent inhibitory action mediates some of the effects of Ca$^{2+}$ on synaptotagmin 1 in neurotransmitter release (Zhou et al, 2017). Thus, Ca$^{2+}$-dependent release of inhibitory interactions may be a common feature of a variety of C2 domain-containing proteins.

**Materials and Methods**

**Chemicals**

Chemicals were from the following sources: Isopropyl-β-D-thiogalactoside (IPTG) (AmericanBio), Oxo-M and atropine (Sigma-Aldrich), ionomycin (Sigma-Aldrich),
annexin V-FITC (BD and BioVision), annexin V-Cy3 (BioVision), DGK inhibitor (R 59-022, Tocris Bioscience), proteinase K (Sigma-Aldrich), FM1-43 (Molecular Probes/Life-technologies), BS\(^3\) (Thermo Fisher Sciintific). The following concentrations of chemicals are used in all of the experiments unless noted otherwise: Oxo-M, 10 \(\mu\)M; atropine, 50 \(\mu\)M; ionomycin, 2 \(\mu\)M (except in Fig EV5A and EV5D at 6 \(\mu\)M); DGK inhibitor, 50 \(\mu\)M; BS\(^3\), 500 \(\mu\)M. All lipids were obtained from Avanti Polar Lipids: 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 850375; 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 840034; L-\(\alpha\)-phosphatidylinositol- 4,5-bisphosphate [PI(4,5)P\(_2\)], 840046; 7-nitrobenzoxadiazole (NBD)-1,2-dipalmitoyl-sn-glycero-3- phosphoethanolamine (DPPE), 810144; 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino- 1-carboxypentyl) iminodiacetic acid) succinyl] [DGS-NTA(Ni)], 790404.

**Plasmids**

A plasmid encoding the M1 muscarinic acetylcholine receptor (M1R) was a kind gift from Bertil Hille (University of Washington) (Suh et al, 2004). Lact-C2-mCherry is from Sergio Grinstein (University of Toronto) (Yeung et al, 2008). C1\(_{PKC}\)-mCherry, untagged E-Syt1, Myc-E-Syt2 [Myc-E-Syt2S (Giordano et al, 2013) was used for this study], EGFP-E-Syt1, EGFP-E-Syt1 C2Cx [called mCherry-E-Syt1-mut in (Giordano et al, 2013)], EGFP-E-Syt1 \(\Delta\)SMP and EGFP-E-Syt1 V169W/L308W (EGFP-E-Syt1 SMPmut) were previously described (Giordano et al, 2013; Saheki et al, 2016). The regions coding for residues 93-1104 (E-Syt1\(_{cyt}\)), 93-941 (SMP-C2ABCD), 93-634 (SMP-C2AB), 315-1104 (C2ABCDE), 624-973 (C2CD) and 936-1104 (C2E) of human E-Syt1 were amplified by PCR and cloned using Ascl and NotI sites into the pCMV6-An-His vector (Origene) for Expi293 cell expression. SMP domain (93-327) of human E-Syt1 was cloned using Ndel and Sall sites and PH domain (11-140) of rat PLC\(\delta\)
was cloned using NheI and XhoI sites into pET-28a vector (Novagen). SMP-C2E (93-327 and 898-1104) and SMP-C2CD (93-327 and 634-973) were generated by overlap PCR and cloned into the pET-28a vector.

EGFP-E-Syt1 C2Ax (Ca^{2+}-binding deficient C2A) was generated as follows: the two aspartic acid residues at a.a. position 406 and 410, which are predicted to confer Ca^{2+}-binding properties to the C2A domain of E-Syt1 (Xu et al, 2014), were mutated to alanine using site-directed mutagenesis. The following primers were used:

E1_C2A_Mut_F,
GATTGAAGTGGAGGTGTTCGACAAGGcTCCAGATAAAGcTGACTTTCTGGGCA
GAATGAAGCTGG
E1_C2A_Mut_R,
CCAGCTTCATTCTGCCCAGAAAGTCAgCTTTATCTGGAgCCTTGTCGAACACCT
CCACTTCAATC

EGFP-E-Syt1 C2Am (membrane-binding deficient C2A) was generated as follows: the valine at a.a. position 348 and leucine at a.a. position 351 were mutated to alanine using site-directed mutagenesis. The following primers were used:

E1_C2A_Mut2_F,
GTTCCAAGGACAAATATGctAAGGGCcTcaATTGAGGGCAAGTCAGAC
E1_C2A_Mut2_R,
GTCTGACTTGCCCTCAATgCgcGCCCTTagCATATTTGTCTTTGAAC

Protein Expression and Purification
Expression in eukaryotic cells. Fragments of human E-Syt1 were expressed in Expi293 cells with an N-terminal His6-tag, as described previously (Saheki et al, 2016). Cells were harvested and lysed in buffer A [25 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1x complete EDTA-free protease inhibitor cocktail (Roche), 0.5 mM TCEP] by three freeze–thawing cycles using liquid nitrogen. The lysates were clarified by centrifugation at 17,000 x g for 30 min, and the protein was purified by a Ni-NTA column (Clontech), and was further purified by gel filtration (Superdex 200, GE Healthcare) in buffer B (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5 mM TCEP). Fractions containing E-Syt1 were pooled and concentrated to ~1 mg/ml.

Expression in bacteria. Constructs encoding E-Syt1 fragments were transformed into BL21 (DE3) RIL Codon Plus (Agilent) E. coli cells. Cells were grown in Super Broth medium at 37 °C to an OD600 of 0.6, and the expression was induced by addition of 0.5 mM IPTG for 20 h at 18 °C. The cells were harvested and lysed by sonication in buffer A. The lysates were clarified by centrifugation at 30,000 x g for 1 h, and the protein was isolated by a Ni-NTA column, and further purified by gel filtration in buffer B. Fractions containing E-Syt1 were pooled and concentrated to ~1 mg/ml.

Liposome Preparation

Anchoring/donor liposomes: 88.5:1.5:10 mole percent DOPC: NBD-DPPE: DGS-NTA(Ni) or 98.5:1.5 mole percent DOPC: NBD-DPPE.

Target/acceptor liposomes: 85:10:5 mole percent DOPC: POPS: PI(4,5)P2 or 90:10 mole percent DOPC: POPS.

Lipid mixtures were dissolved in chloroform in glass tubes and then dried under a stream of N2 gas followed by further drying in vacuum for 2 hours. The lipid films were then hydrated with buffer B. Liposomes were formed by ten freeze-thaw cycles in liquid N2
and 37 °C water and extrusion through polycarbonate filters with a pore size of 50 nm (Avanti Polar Lipids).

**Lipid Transfer Assays**

All *in vitro* lipid transfer assays were performed as previously described with slight changes (Saheki et al, 2016). In brief, the reactions were performed in 50 μL volumes. The final lipid concentration in the reaction was 0.5 mM, with donor and acceptor liposomes added at a 1:1 ratio. Reactions were initiated by the addition of protein (protein: lipid ratio 1: 1000 or 1: 400) in a 96-well plate (Corning). The fluorescence intensity of NBD was monitored with an excitation of 460 nm and emission of 538 nm every 10 sec over 30 min at 37 °C or room temperature (RT, see figure legends) by using SpectraMax M5 Microplate Reader (Molecular Devices). All data were corrected by setting the data point at 0 min to zero, and subtracting the baseline values obtained in the absence of protein. The data were expressed as a percentage of the maximum fluorescence, determined after adding 10 μL of 2.5% n-dodecyl-β-D-maltopyranoside (DDM, Avanti Polar Lipids) to the reactions after 30 min. All experiments were repeated 3 times and a representative trace is shown. Bars represent average fluorescence values from the three-pooled readings.

**Liposome Tethering Assays**

Liposome tethering assays were performed as for the lipid transfer assays, except that absorbance at 405 nm was measured to assess turbidity. Reactions were initiated by the addition of protein in a 96-well plate (Corning) using SpectraMax M5 Microplate Reader (Molecular Devices). The data were expressed as absolute absorbance values subtracted by the absorbance prior to protein addition. All experiments were repeated 3 times and a
representative trace is shown. Bars represent average 405 nm absorbance values from the three-pooled readings.

**Cross-Linking Assay**

For cross-linking with BS$_3^3$ (Thermo Fisher Scientific), 15 μM indicated proteins were incubated with or without Ca$^{2+}$ or liposomes with 500 μM BS$_3^3$ at RT for 0.5 h in 25 mM Hepes, pH 7.4, 150 mM NaCl, 0.5 mM TCEP. The reactions were quenched with 50 mM Tris-HCl, pH 8.0. All samples were analyzed by SDS/PAGE and Coomassie blue-stained gels were analyzed using Image J (NIH). The putative heterodimer band was dissected and analyzed by Mass Spectrometry by the Yale Keck Biotechnology Resource Laboratory.

**Cell culture and transfection**

HeLa cells were cultured in Dulbecco's modified essential Eagle medium (DMEM) (Life technologies) supplemented with 20% (v/v) fetal bovine serum (FBS, Life Technologies) at 37°C and 5% CO$_2$. Transfection of plasmids was carried out with Lipofectamine 2000 (Life Technologies), according to the manufacturer’s instructions. Wild-type (WT) as well as genome-edited HeLa cell lines were verified as free of mycoplasma contamination by a PCR-based method. Genome-edited cells were clonally isolated and mutations or indels were identified by sequencing. E-Syt1/2 double KO cells (DKO) were clone 6-8 and E-Syt1/2/3 triple KO cells (TKO) were clone 5 described in (Saheki et al, 2016). No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample. The same clones, either the DKO: 6-8 cell line for DKO cells or the TKO: 5 cell line for TKO cells were used for all experiments throughout this study. All cell-based experiments were repeated at least 2 times.
**Fluorescence microscopy**

For imaging experiments, cells were plated on 35mm glass bottom dishes at low density (MatTek Corp, Ashland, MA). Live cell imaging were carried out one day after transfection. Spinning disc confocal (SDC) microscopy was performed using the Improvision UltraVIEW VoX system (Perkin-Elmer) built around a Nikon Ti-E inverted microscope, equipped with PlanApo objectives (40x1.0-NA 1.0 and 60X1.49-NA) and controlled by Volocity (Improvision) software. Excitation light was provided by 488-nm/50-mW diode laser (Coherent) and 561-nm/50-mW diode laser (Cobolt), and fluorescence was detected by EM-CCD camera (C9100-50; Hamamatsu Photonics).

Total internal reflection fluorescence (TIRF) microscopy was performed on a setup built around either a Nikon TiE or Ti2 microscope equipped with 60X1.49-NA and a 100X1.49-NA objective. For most experiments, excitation light was provided by 488-nm (for GFP) and 561-nm (for mCherry) DPSS lasers coupled to the TIRF illuminator through an optic fiber. The output from the lasers was controlled by an acousto-optic tunable filter and fluorescence was detected with an EM-CCD camera (Andor iXon DU-897). Acquisition was controlled by Andor iQ software. Images were sampled at 0.20 Hz with exposure times in the 100-500 ms range.

SDC microscopy was carried out at RT and TIRF microscopy at 37°C.

**Live cell imaging**

Cells were washed twice and incubated with Ca\(^{2+}\) buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 10 mM glucose, and 2 mM CaCl\(_2\)) before imaging with either a SDC microscope or a TIRF microscope.
**PS exposure/lipid scrambling assay**

PS in the outer PM leaflet was detected using Annexin V-Cy3 (Biovision), Annexin V-FITC (BD) or FM1-43 (Molecular Probes/Life-technologies), and in the inner leaflet by expression of Lact-C2-mCherry. Cells were plated on 35mm glass bottom dishes at low density (MatTek Corp, Ashland, MA). Cells were incubated with Ca\(^{2+}\) buffer containing Annexin V-Cy3, Annexin V-FITC or FM1-43 at the indicated dilution and stimulated with 2 μM ionomycin. PS exposure was monitored with a SDC microscope at room temperature. 50 fold-dilution for Annexin V-Cy3 and 20 fold-dilution for Annexin V-FITC were used; FM1-43 was used at 3 μM. Relatively high concentration of Annexin was necessary to ensure consistent detection of PS exposure.

**Image analysis**

All fluorescent images were analyzed off-line using Image J (NIH). For the analysis of DAG levels in the PM using C1\(^{\text{PKC}}\)-mCherry, changes in PM mCherry fluorescence (TIRF miscoscopy) over time were analyzed by manually selecting large areas of the cell foot-print. Mean fluorescence intensity values of selected regions were obtained and normalized (F) to average values before stimulation after background subtraction (F0). Background signals correspond to the average fluorescent intensity of the regions where the cells are not present. Changes in Annexin V-Cy3, Annexin V-FITC, Lact-C2-mCherry or FM1-43 fluorescence over time (SDC microscopy) were analyzed by manually drawing regions at edges of cells, and peak fluorescence intensity was measured. After background subtraction, fluorescence intensities of the regions were obtained and normalized with average values before stimulation. Quantification of fluorescence changes was performed using Excel (Microsoft) and all data are presented as mean and SEM.
**Statistical Analysis**

No statistical method was used to predetermine sample size, and the experiments were not randomized for live cell imaging. Cells were pooled from 1-6 independent experiments. Comparisons of data were carried out by either the two-tailed Student’s t-test or the t-test with Bonferroni corrections for multiple comparisons as appropriate with Prism 6 (GraphPad software). Dose-response curves were fit using four-parameter logistic equations to calculate EC50 (GraphPad software).

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**Author contributions**

All authors participated in the design of experiments, data analysis and interpretation. X.B. designed and performed all of the liposome tethering and lipid transfer assays. Y.S. designed and performed all of the imaging studies in living cells. X.B., Y.S. and P.D.C. wrote the manuscript.
Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. E-Syt1 cyt specifically binds to PI(4,5)P2-containing membranes in a C2E-dependent way in the absence of Ca^{2+} and C2C, but not C2A, stimulates this
binding in the presence of Ca$^{2+}$.
A. Domain structures of E-Syts (left) and E-Syt1 constructs (right) used for the liposome tethering assays shown in the figure.

B - D. Liposomes aggregation, due to tethering of anchoring and target liposomes in the presence of E-Syt1 constructs (protein: lipid ratio 1: 1000) at RT, as assessed by increase in turbidity (OD 405 nm). Time-courses are at left and bar graphs showing quantification of OD405 increases at the end of the incubation (arrows in the left panel) are at right. (B) Effect of the lipid composition and C2E on liposome tethering by E-Syt1_cyto in the absence of Ca$^{2+}$. A cocktail of EGTA, Imidazole and Proteinase K (Cocktail) was added after 10 min. (C) Effect of the absence or presence of Ca$^{2+}$ on liposome tethering by E-Syt1_cyto with or without mutations in the Ca$^{2+}$-binding sites in C2A and C2C. (D) Effect of the absence or presence of Ca$^{2+}$ and of PI(4,5)P$_2$ in the target liposomes on liposome tethering by E-Syt1_cyto lacking the C2E domain. Mean and SD of three independent experiments. *** P<0.001; **** P<0.0001; n.s., not significant.

Figure 2. Ca$^{2+}$ binding to C2A is required for lipid transfer by the SMP domain when flanked by the C2AB domain
A. Domain structures of constructs used for the lipid transfer assays shown in the figure. PH = PH domain of rat PLCδ.

B. (left) Time-course of normalized fluorescence signals of liposomes mixtures containing 1.5% NBD-PE in the donor liposomes in the absence or presence of Ca$^{2+}$ at RT. E-Syt1 constructs were added at time 0 (protein: lipid ratio 1: 1000). (right) Quantification of the increase in NBD fluorescence at the end of the incubation (arrow in left). Mean and SD of three independent experiments. **** P<0.0001; n.s., not significant.

C - E. Lipid transfer between donor and acceptor liposomes in the presence of E-Syt1
constructs (protein: lipid ratio 1: 400) at 37 °C as assessed by dequenching of NBD-PE fluorescence. In each of the panels, time-courses are at the top and bargraphs showing quantification of NBD fluorescence at the end of the incubation (arrows in the upper panels) are at the bottom. (C) Effect of C2ABCDE-dependent liposome tethering on lipid transfer mediated by the SMP domain. (D) Effect of the Ca$^{2+}$-binding property of C2A on the lipid transfer activity of the SMP domain between C2ABCDE-tethered liposomes. (E) Effect of Ca$^{2+}$ on the lipid transfer activity of the SMP domain alone or SMP-C2AB on liposomes tethered by a PH domain. Mean and SD of three independent experiments. ** P<0.01; *** P<0.001; **** P<0.0001; n.s., not significant.

**Figure 3. C2A domain inhibits the lipid transfer activity of the SMP domain in the absence of Ca$^{2+}$ via an intramolecular interaction.**

A. Ribbon representation of the crystal structure of the SMP-C2AB of human E-Syt2 dimer in different orientations (PDB code 2DMG). One monomer is shown in regular color and the other in pale colors. The SMP domain is in yellow and the C2AB domain pairs in red. Lipid molecules are represented as stick in dark orange.

B. (left) Intramolecular interface between the SMP domain and the C2A domain. (right) Intermolecular interface between SMP domain and C2A domain.

C and D. Lipid transfer between donor and acceptor liposomes in the presence of E-Syt1 constructs (protein: lipid ratio 1: 400) at 37 °C as assessed by dequenching of NBD-PE fluorescence. In each of the panels, time-courses are at left and bar graphs showing quantification of NBD fluorescence at the end of the incubation (arrows in the left panels) are at right. (C) Effect of mutations in the SMP domain at either its intramolecular or intermolecular interface. (D) Effect of the salt concentration on the lipid transfer activity of SMP or SMP-C2AB on liposomes tethered by a PH domain. Mean and SD of three independent experiments. * P<0.05; ** P<0.01; n.s., not significant.
Figure 4. E-Syts-dependent DAG extraction from the PM in intact cells requires Ca\(^{2+}\) binding to C2 domains.

A. Time-course of normalized mCherry signal at the PM of WT and E-Syts TKO cells expressing the DAG reporter C1\(_{PKC}\)-mCherry, as assessed by TIRF microscopy, in response to the indicated compounds.

B. Quantification of F/F0 at the end of the experiment (arrow in A). Mean and SEM, n=25 cells (WT), n=28 cells (TKO), n=22 cells (TKO + EGFP-E-Syt1), n=11 cells (TKO + EGFP-E-Syt1 C2Ax), n=11 cells (TKO + EGFP-E-Syt1 C2Cx); n.s., not significant; *** P<0.001.

Figure 5. E-Syts KO cells are defective in Ca\(^{2+}\)-dependent PS scrambling.

A and B. Confocal images of WT (A) and E-Syt1/2 double KO (DKO) cells (B) before and 15 min after stimulation with 2 μM ionomycin. PS scrambling was detected with confocal microscopy by the binding to the cell surface of Cy3-labeled Annexin V (Annexin V-Cy3) present in the medium. Scale bars, 10μm.

C. Domain structures of E-Syt1 constructs used for the rescue experiments.

D - F. Normalized cell surface-associated Annexin V-Cy3 fluorescence in response to 2 μM ionomycin, as assessed by confocal microscopy, for WT and E-Syt1/2/3 triple KO (TKO) cells with and without rescue with transfected E-Syt1 constructs. Time courses are at left and bar graphs showing quantification of F/F0 at the time point (arrow in the left panels) are at right. (D) PS scrambling within the time frame indicated is abolished in E-Syts TKO cells, and rescued by expression of EGFP-E-Syt1 alone or EGFP-E-Syt1 together with Myc-E-Syt2, but not by expression of EGFP-E-Syt1 ∆SMP [mean and SEM, n=20 cells (WT), n=20 cells (TKO), n=16 cells (TKO + EGFP-E-Syt1+Myc-E-Syt2), n=24 cells (TKO + EGFP-E-Syt1), n=17 cells (TKO +...
EGFP-E-Syt1 ΔSMP); ** P<0.01, **** P<0.0001]. (E) Defective rescue of PS scrambling in E-Syts TKO cells transfected with EGFP-E-Syt1 carrying SMP domain mutations as indicated. [mean and SEM, n=20 cells (TKO), n=24 cells (TKO + EGFP-E-Syt1), n=20 cells (TKO + EGFP-E-Syt1 V169W/L308W); n.s., not significant; **** P<0.0001]. (F) Defective rescue of PS scrambling in E-Syts TKO cells transfected with E-Syt1 harboring Ca\(^{2+}\)-binding mutations in C2A or C2C [mean and SEM, n=10 cells (TKO), n=18 cells (TKO + EGFP-E-Syt1), n=15 cells (TKO + EGFP-E-Syt1 C2Ax), n=18 cells (TKO + EGFP-E-Syt1 C2Cx); n.s., not significant; **** P<0.0001].

**Figure 6. Model for Ca\(^{2+}\)-mediated releasing of an autoinhibitory conformation of E-Syt1 to couple ER-PM tethering with lipid transport.**

Ca\(^{2+}\) binding to C2A and C2C promotes their interaction with membranes: “in trans” or possibly “in cis” in the case of C2A, and “in trans” in the case of C2C. The main effects of these Ca\(^{2+}\) bindings are to release the interaction of C2A with the SMP domain that impairs lipid transport, and to enhance the binding of C2E to PI(4,5)P\(_2\) in the PM that is partially inhibited by C2C in the absence of Ca\(^{2+}\). The Ca\(^{2+}\) ions are shown as small blue circles. Double-headed red arrows indicate lipid transfer mediated by the SMP domain. Lipid transport is likely to be mediated by the shuttling of the SMP domain between the two membranes (Reinisch & De Camilli, 2016).

**Figure EV1. Ca\(^{2+}\) binding to C2C promotes E-Syt1\(_{cyto}\)-mediated liposome tethering by releasing an inhibitory action of C2C on the membrane binding of C2E.**

A. Domain structures of E-Syt1 constructs used for the liposomes tethering assays shown in (B) and (C).

B and C. Tethering of anchoring and target liposomes in the presence of E-Syt1 constructs at RT as assessed by increase in turbidity (OD 405 nm). In each of the panels,
time-courses are at left and bar graphs showing quantification of OD405 increases at the end of the incubation (arrows in the left panels) are at right. (B) Effect of the absence or presence of Ca$^{2+}$ on liposome tethering by SMP-C2AxBCD, SMP-C2ABCxD and SMP-C2AmBCxD. (C) Liposome tethering by E-Syt1_{cyto} with or without C2ABCD domains in the absence of Ca$^{2+}$. Mean and SD of three independent experiments. *** P<0.001; n.s., not significant.

D. SMP-C2ABCxD and SMP-C2AxBCD bind to liposomes in a Ca$^{2+}$-dependent manner, as revealed by liposome tethering. OD405 readings were normalized to the maximum value. Mean and SD of three independent experiments.

E. (left) Purified C2CD and C2E were incubated in the presence of the cross-linker BS$^3$ and in the absence or presence of Ca$^{2+}$ and liposomes. The left lane shows molecular weight markers, with sizes indicated in kilodaltons. The cross-linked heterodimer, as confirmed by Mass Spectrometry, is indicated by red arrowhead. (right) Normalized intensity of cross-linked heterodimer was plotted. Mean and SD of three independent experiments. ** P<0.01; n.s., not significant.

F. Sequence of C2CD and C2E used for cross-linking assay. The peptides identified by Mass Spectrometry are highlighted in red.

**Figure EV2. Both C2ABCDE and PH\textsubscript{PLCδ} can tether liposomes, although C2ABCDE, but not PH\textsubscript{PLCδ} requires Ca$^{2+}$.**

A and B. Tethering of anchoring and target liposomes in the presence of the constructs indicated (see Fig 4A, protein: lipid ratio 1: 400) at 37 °C as assessed by increase in turbidity (OD 405 nm). In each of the panels, time-courses are at left and bar graphs showing quantification of OD405 increases at the end of the incubation (arrows in the left panels) are at right. (A) Effect of the absence or presence of Ca$^{2+}$ on liposome tethering by SMP or C2ABCDE. (B) Effect of the absence or presence of Ca$^{2+}$ on liposome
tethering by $\text{PH}_{\text{PLC}6}$. Mean and SD of three independent experiments. *** $P<0.001$; **** $P<0.0001$; n.s., not significant.

Figure EV3. The partial membrane tethering activity of SMP-C2AB relative to E-Syt1$_{\text{cyto}}$ correlates with a lower $\text{Ca}^{2+}$-dependent lipid transfer activity.

A. Lipid transfer between donor and acceptor liposomes in the presence of E-Syt1 constructs (see Fig 4A, protein: lipid ratio 1: 1000) at RT as assessed by dequenching of NBD-PE fluorescence. Time-courses are at left and bar graphs showing quantification of NBD fluorescence at the end of the incubation (arrow in the left panel) are at right. Mean and SD of three independent experiments. **** $P<0.0001$; ** $P<0.01$.

B. E-Syt1$_{\text{cyto}}$ and SMP-C2AB were added to the mixture of anchoring and target liposomes (protein: lipid ratio 1: 1000) in the absence or presence of $\text{Ca}^{2+}$. Time-courses are at left and bar graphs showing quantification of OD405 increases at the end of the incubation (arrow in the left panel) are at right. Mean and SD of three independent experiments. **** $P<0.0001$.

Figure EV4. The C2CD domains pair has no direct impact on the lipid transfer activity of the SMP domain.

A. Sequence alignment of the three E-Syts from various species showing conserved amino acid residues implicated in intra-molecular (black circles) and inter-molecular (black stars) interfaces between the SMP domain and C2A domains in the crystal structure of E-Syt2. The residues of C2C corresponding to the residues of C2A involved in the SMP-C2A interaction are indicated by black squares. Note that these residues are not highly conserved.

B. Domain structures of E-Syt1 used for the lipid transfer assays shown in (C).

C. Impact of the C2AB domain pair, but not of the C2CD domain pair, on lipid transfer
mediated by the indicated proteins between PH\textsubscript{PLC\delta}-tethered donor and acceptor liposomes as determined by dequenching of NBD-PE in donor liposomes at 37 °C. Time course are shown at left and bar graphs showing quantification of % total NBD fluorescence at the end of the incubation (arrow in the left panel) are shown at right. Mean and SD of three independent experiments. ** P<0.01; n.s., not significant.

D. C2A binds Ca\textsuperscript{2+} and the SMP domain via different surfaces. The intra-molecular interface between the SMP domain (yellow) and C2A domain (red) of E-Syt2 is indicated by a blue dashed circle. The Ca\textsuperscript{2+}-binding surface of C2A is indicated by a green dashed circle. Lipid molecules are shown in orange.

**Figure EV5. EGFP-E-Syt1 C2Ax is recruited to the PM but does not rescue the defect in DAG extraction from the PM in E-Syts TKO cells.**

A. TIRF microscopy images of E-Syts TKO cells expressing WT or C2Ax mutant EGFP-E-Syt1, together with the DAG probe C1\textsubscript{PKC}-mCherry, and exposed to the indicated compounds at the indicated time. Note loss of mCherry signal in response to ionomycin in cells expressing WT E-Syt1. Scale bars, 10 μm.

B. (upper) Time-course of normalized EGFP signal at the PM of E-Syts TKO cells expressing the WT or EGFP-E-Syt1 C2Ax, as assessed by TIRF microscopy, in response to the indicated compounds. (bottom) Quantification of F/F\textsubscript{0} at 9.5 min (arrow in the upper panel). Mean and SEM, n=22 cells (EGFP-E-Syt1), n=11 cells (EGFP-E-Syt1 C2Ax), n=10 cells (EGFP-E-Syt1 C2Cx); n.s., not significant; **** P<0.0001.

C. Confocal images of E-Syts TKO cells expressing the indicated constructs before and during stimulation with 2 μM ionomycin as indicated. Scale bars, 10 μm.

D. (left) Time-course of normalized mCherry signal at the PM of E-Syts TKO cells expressing the DAG reporter C1\textsubscript{PKC}-mCherry, as assessed by TIRF microscopy, in response to the indicated compounds. (right) Quantification of F/F\textsubscript{0} at the end of the
experiment (arrow in the left panel). Mean and SEM, n=31 cells (TKO + EGFP-E-Syt1), n=35 cells (TKO + EGFP-E-Syt1 C2Ax), n=18 cells (TKO + EGFP-E-Syt1 C2Am); n.s., not significant; **** P<0.0001.

**Figure EV6. E-Syt1/2 DKO cells are defective in Ca^{2+}-dependent PS exposure.**

A - D. Confocal images of WT (A and C) and E-Syt1/2 DKO (B and D) cells incubated with FITC-labeled Annexin V (Annexin V-FITC, A and B) and expressing Lact-C2-mCherry (C and D) before and during stimulation with 2 μM ionomycin as indicated. Note the progressive accumulation of Annexin V-FITC signals (A) and decrease of Lact-C2-mCherry signals (C, insets) at the PM of WT cells, but not of DKO cells (B and D). Scale bars, 10 μm.

E. (left) Time course of normalized Lact-C2-mCherry fluorescence, as assessed by confocal microscopy, in response to 2 μM ionomycin, from WT and E-Syt1/2 DKO cells. For overexpression and rescue experiments, WT and E-Syts DKO cells were transfected with EGFP-E-Syt1 together with Myc-E-Syt2 as indicated. (right) Quantification of F/F0 at the end of the experiment (arrow in the left panel). Mean and SEM, n = 13 cells (WT), n = 13 cells (WT + EGFP-E-Syt1&Myc-E-Syt2), n = 16 cells (DKO), n = 10 cells (DKO + EGFP-E-Syt1&Myc-E-Syt2); n.s., not significant; **** P<0.0001.

F. Confocal images of E-Syt1/2 double KO (DKO) cells expressing the indicated constructs before and during stimulation with 2 μM ionomycin at the indicated time. PS scrambling was detected with confocal microscopy by the binding to the cell surface of Annexin V-Cy3 present in the medium. Note that the two EGFP-E-Syt1 constructs are recruited to the PM in response to Ca^{2+}. Scale bars, 10 μm.

G. Time course of normalized Annexin V-Cy3 fluorescence, as assessed by confocal microscopy, in response to 2 μM ionomycin, from WT and E-Syt1/2 DKO cells. For overexpression and rescue experiments, WT and E-Syts DKO cells were transfected with
either EGFP-E-Syt1 together with Myc-E-Syt2, or EGFP-E-Syt1 alone, or EGFP-E-Syt1 ΔSMP as indicated. Mean and SEM (quantification in H).
H. Quantification of F/F0 at the end of the experiment (arrow in G). Mean and SEM, n = 8 cells (WT), n = 12 cells (WT + EGFP-E-Syt1&Myc-E-Syt2), n = 14 cells (WT + EGFP-E-Syt1), n = 11 cells (DKO), n = 13 cells (DKO + EGFP-E-Syt1&Myc-E-Syt2), n = 15 cells (DKO + EGFP-E-Syt1), n = 13 cells (DKO + EGFP-E-Syt1 ΔSMP); n.s., not significant; ** P<0.01, **** P<0.0001.
I. Rescue with C2 domain Ca\(^{2+}\)-binding deficient mutants of E-Syt1. Overexpression and rescue with C2C domain mutant and C2A domain mutant are shown. Quantification of Annexin Cy3 F/F0 at the end of the experiment similar to G. Mean and SEM, n = 13 cells (WT), n = 11 cells (WT + EGFP-E-Syt1 C2Ax), n = 15 cells (WT + EGFP-E-Syt1 C2Cx), n = 12 cells (DKO), n = 10 cells (DKO + EGFP-E-Syt1), n = 15 cells (DKO + EGFP-E-Syt1 C2Ax), n = 12 cells (DKO + EGFP-E-Syt1 C2Cx); n.s., not significant; *** P<0.001.
J. Detection of PM lipid scrambling with FM1-43. Time-courses of normalized FM1-43 fluorescence, as assessed by confocal microscopy, from WT, E-Syt DKO and E-Syt TKO cells, are at left. Bar graphs showing quantification of F/F0 at the end of the experiment (arrow in left panel) are at right. Mean and SEM, n=34 cells (WT), n=28 cells (TKO), n=24 cells (DKO); **** P<0.0001.
A

Hydrophobic stretches

E-Syt1 N SMP C2A C2B C2C C2D C2E
E-Syt2 N SMP C2A C2B C2D C2E
E-Syt3 N SMP C2A C2B C2C C2D

B

Tethering (no Ca²⁺)

Cocktail

E-Syt₁_
E-Syt₁_
[30% PS and no PI(4,5)P₂]  
E-Syt₁_
[10% PS and no PI(4,5)P₂]  
E-Syt₁_
(no DGS-NTA(Ni))
E-Syt₁_
(no target liposomes)
SMP-C2ABCD  
no protein

C

Tethering

E-Syt₁_
C2Ax + Ca²⁺  
E-Syt₁_
C2Cx + Ca²⁺
E-Syt₁_
C2Ax
E-Syt₁_
C2Cx

D

Tethering

SMP-C2ABCD + Ca²⁺  
SMP-C2ABCD + Ca²⁺  
[10% PS and no PI(4,5)P₂]
SMP-C2ABCD

Fig 1
Fig 3
A  WT WT + Ionomycin
Annexin V-Cy3
E-Syt1/2 DKO E-Syt1/2 DKO + Ionomycin
Annexin V-Cy3
B
E-Syt1
E-Syt1ΔSMP
C
E-Syt1 SMPmut
F/F0 (Annexin V-Cy3)
WT TKOTKO + EGFP-
E-Syt1
& Myc-E-Syt2TKO + EGFP-
E-Syt1TKO
+ EGFP-E-Syt1ΔSMP
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 Time (min)
F/F0 (Annexin V-Cy3)
0 10 20 30 40 50
TKOTKO + EGFP-
E-Syt1TKO
+ EGFP-
E-Syt1 SMPmut
**** n.s.
F
0 5 10 15 20 25 30 F/F0 (Annexin V-Cy3)
TKO
toko
+ EGFP-E-Syt1
TKO
+ EGFP-E-Syt1 SMPmut
**** n.s.
Ionomycin
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 Time (min)
F/F0 (Annexin V-Cy3)
0 5 10 15 20 25 30
TKO
+ EGFP-E-Syt1
TKO
+ EGFP-E-Syt1 C2Ax
+ EGFP-E-Syt1 C2Cx
**** n.s.
Ionomycin
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 Time (min)
F/F0 (Annexin V-Cy3)
0 5 10 15 20 25 30
TKO
+ EGFP-E-Syt1
TKO
+ EGFP-E-Syt1 C2Ax
+ EGFP-E-Syt1 C2Cx
**** n.s.
Ionomycin
Fig 5
Fig 6

Ca²⁺ Influx

ER

And/Or

PI(4,5)P₂
PS
Ca²⁺
Fig EV2
Fig EV3
**A**

B. taurus E-Syt1 SMP 172 ...AVRGSNHQLTQTPTFTVEGELPKRDLVGVHFTQG-SKKQ1LDDVINSLQDLIDQIEVYCKFCAVGMQGLM...  
D. rerio E-Syt1 SMP 152 ...SIRATSAHLWLSTPTFVEGELPKRDLVGVHFTQG-SKKQ1LDDVINSLQDLIDQIEVYCKFCAVGMQGLM...  
H. sapiens E-Syt1 SMP 168 ...AVRGSNHQLTQTPTFTVEGELPKRDLVGVHFTQG-SKKQ1LDDVINSLQDLIDQIEVYCKFCAVGMQGLM...  
M. mulatta E-Syt1 SMP 126 ...AVRGSNHQLTQTPTFTVEGELPKRDLVGVHFTQG-SKKQ1LDDVINSLQDLIDQIEVYCKFCAVGMQGLM...  
O. cuniculus E-Syt1 SMP 155 ...AVRGSNHQLTQTPTFTVEGELPKRDLVGVHFTQG-SKKQ1LDDVINSLQDLIDQIEVYCKFCAVGMQGLM...  
X. tropicalis E-Syt1 SMP 135 ...AVRGSNHQLTQTPTFTVEGELPKRDLVGVHFTQG-SKKQ1LDDVINSLQDLIDQIEVYCKFCAVGMQGLM...  

B. taurus E-Syt1 C2A 389 ...TYEVHVEHPQIEIEVEVFDKDPDKDDFLGRMKLDVGKVLQAGVLDDWFPLQ...  
D. rerio E-Syt1 C2A 368 ...MYEVHVEHEVPGQEIEVEVFDKDPDKDDFLGRMKLDVGKVLQAGVLDDWFPLQ...  
H. sapiens E-Syt1 C2A 385 ...TYEVHVEHEVPGQEIEVEVFDKDPDKDDFLGRMKLDVGKVLQAGVLDDWFPLQ...  
M. mulatta E-Syt1 C2A 343 ...TYEVHVEHEVPGQEIEVEVFDKDPDKDDFLGRMKLDVGKVLQAGVLDDWFPLQ...  
O. cuniculus E-Syt1 C2A 372 ...TYEVHVEHEVPGQEIEVEVFDKDPDKDDFLGRMKLDVGKVLQAGVLDDWFPLQ...  
X. tropicalis E-Syt1 C2A 351 ...MYEVHVEHEVPGQEIEVEVFDKDPDKDDFLGRMKLDVGKVLQAGVLDDWFPLQ...  

B. taurus E-Syt1 C2C 706 ...IFEVVTISPQIEIEVEVFDKDLDDFLGRKSLTVTLINSGLFDEWLTLE...  
D. rerio E-Syt1 C2C 691 ...LYEVILTQIEIEVEVFDKDLDDFLGRKSLTVTLINSGLFDEWLTLE...  
M. mulatta E-Syt1 C2C 661 ...IFEVVTISPQIEIEVEVFDKDLDDFLGRKSLTVTLINSGLFDEWLTLE...  
O. cuniculus E-Syt1 C2C 690 ...IFEVVTISPQIEIEVEVFDKDLDDFLGRKSLTVTLINSGLFDEWLTLE...  
N. crassicauda E-Syt1 C2C 664 ...IFEVVTISPQIEIEVEVFDKDLDDFLGRKSLTVTLINSGLFDEWLTLE...  
X. tropicalis E-Syt1 C2C 664 ...AFEVIIIPQIEIEVEVFDKDLDDFLGRKSLTVTLINSGLFDEWLTLE...  
H. sapiens E-Syt1 C2C 713 ...IFEVVTISPQIEIEVEVFDKDLDDFLGRKSLTVTLINSGLFDEWLTLE...

**B**

**C**

Lipid transfer

**D**

C2A

SMP

**Fig EV4**
**Fig EV5**

**A**

<table>
<thead>
<tr>
<th>TIRF</th>
<th>Oxo-M</th>
<th>Atropine + DGKi</th>
</tr>
</thead>
<tbody>
<tr>
<td>TKO + EGFP-E-Syt1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TKO + EGFP-E-Syt1 C2Ax</td>
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</tr>
<tr>
<td>TKO + EGFP-E-Syt1 C2Cx</td>
<td></td>
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</tbody>
</table>

**B**

- **Oxo-M**
- **Atropine + DGKi**
- **Ionomycin**

**C**

- **E-Syts TKO**
- **Ionomycin**

**D**

- **Oxo-M**
- **Atropine + DGKi**
- **Ionomycin**
Fig EV6