Synaptotagmin 5 regulates calcium-dependent Weibel-Palade body exocytosis in human endothelial cells

Camille Lenzi[1], Jennifer Stevens[2]*, Daniel Osborn[1], Matthew J. Hannah[3], Ruben Bierings[4] and Tom Carter[1]

[1] Molecular and Clinical Sciences Research Institute, St George's, University of London, SW18 ORE, [2]MRC National Institute for Medical Research, London, NW7 1AA,[3]Microbiology Services Colindale, Public Health England, London, NW9 5EQ.[4]Plasma Proteins, Sanquin Research and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands.

Current address:

*Kings College London medical school

Corresponding Author: Prof. T. Carter,
Molecular and Clinical Sciences Research Institute,
St George's University,
London,
SW17 0RE
tcarter@sgul.ac.uk

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Summary statement: How elevations of intracellular free Ca^{2+} concentration are detected by Weibel-Palade bodies (WPBs) remains unclear. Here we show that synaptotagamin 5 is a WPB-associated Ca^{2+}-sensor regulating exocytosis.

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Abstract

Elevations of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) are a potent trigger for Weibel-Palade body (WPB) exocytosis and secretion of Von Willebrand factor (VWF) from endothelial cells, however, the identity of WPB-associated Ca^{2+}-sensors involved in transducing acute increases in $[Ca^{2+}]_i$ into granule exocytosis remain unknown. Here we show that synaptotagmin 5 (SYT5) is expressed in human umbilical vein endothelial cells (HUVEC) and is recruited to WPBs to regulate Ca^{2+}-driven WPB exocytosis. Western blot analysis of HUVEC identified SYT5 protein, and exogenously expressed SYT5-mEGFP localized almost exclusively to WPBs. shRNA-mediated knockdown of endogenous SYT5 reduced the rate and extent of histamine-evoked WPB exocytosis and reduced secretion of the WPB cargo VWF-propeptide (VWFpp). The shSYT5-mediated reduction in histamine-evoked WPB exocytosis was prevented by expression of shRNA-resistant SYT5-mCherry. Overexpression of SYT5-EGFP increased the rate and extent of histamine-evoked WPB exocytosis, and increased secretion of VWFpp. Expression of a Ca^{2+}-binding defective SYT5 mutant (SYT5-Asp197Ser-EGFP) mimicked depletion of endogenous SYT5. We identify SYT5 as a WPB-associated Ca^{2+} sensor regulating Ca^{2+}-dependent secretion of stored mediators from vascular endothelial cells.

Introduction

Endothelial cells store von Willebrand factor (VWF) and a complex mixture of inflammatory mediators, vasoactive peptides and regulators of tissue growth in special secretory granules called Weibel-Palade bodies (WPBs) (Knipe et al., 2010; Schillemans et al., 2018a; van Breevoort et al., 2012). WPB cargo molecules act together at sites of vessel injury to reduce blood loss, control infection and aid in tissue repair, but have also been implicated in various disease states (see discussion). WPBs undergo different modes of exocytosis resulting in rapid (subsecond) cargo release (Babich et al., 2008; Conte et al., 2015), selective cargo secretion (Babich et al., 2008; Nightingale et al., 2018), compound/cumulative exocytosis (Kiskin et al., 2014; Valentijn et al., 2011) as well as slower forms of cargo release (2-10 seconds) requiring post-fusion recruitment of actomyosin to the WPB (Nightingale et al., 2011). Physiological and pathological mediators can trigger VWF secretion through several intracellular signalling pathways (Huang et al., 2012; Lowenstein et al., 2005; Schillemans et al., 2018a), however, sustained elevations of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) constitute a particularly potent trigger (Birch et al., 1994; Zupancic et al., 2002). Surprisingly, little is known about how increases in $[Ca^{2+}]_i$ are sensed and transduced into WPB exocytosis. Early studies identified a role for calmodulin (CaM) in Ca^{2+}-driven VWF secretion (Birch et al., 1992). Ca^{2+}-CaM binds the guanine nucleotide exchange factor RalGDS which activates the small GTPase RalA (Rondaij et al., 2008). RalA binds components of the exocyst complex that is involved in vesicle-plasma membrane docking, but also stimulates phospholipase D1 (PLD1) activity through activation of ADP-ribosylation factor 6 (Arf6) (Vitale et al., 2005). The latter is important for Ca^{2+}-driven VWF secretion (Disse et al., 2009), and together these processes provide a mechanism to generate domains at the plasma membrane that directly, or through recruitment of adapter proteins, promote WPB docking and fusion. Annexin A2 (AnxA2) in complex with the Ca^{2+} binding protein S100A10 may represent one such adapter complex (Brandherm et al., 2013; Chehab et al., 2017; Gerke, 2016). Cytosolic AnxA2 is recruited to the plasma membrane by acidic phospholipids, such as phosphatidic acid, where

it promotes further phospholipid clustering (Gerke, 2016). Importantly, S100A10 can bind the WPB-Rab27A associated effector Munc13-4 (Chehab et al., 2017) providing a molecular scaffold linking the WPB to the plasma membrane. WPBs may also engage the plasma membrane through a Rab27A-Slp4a-syntaxin binding protein 1 (STXBP1) complex (Bierings et al., 2012; van Breevoort et al., 2014). Once close to the plasma membrane, WPB-fusion is driven by SNARE proteins (see (Schillemans et al., 2018b; van Breevoort et al., 2014) and references therein) and is, almost universally, regulated by one or more vesicle-associated Ca^{2+}-sensors (Südhof, 2014). The nature of the WPB-associated Ca^{2+}-sensors that regulate WPB fusion remain unknown. The best characterised family of vesicle-associated Ca^{2+}-sensors are the syntaptotagmins (SYTs) (Chapman, 2008; Südhof, 2014).There are 17 mammalian SYT isoforms (Craxton, 2010) and all share a common basic structure consisting of a short highly-variable N-terminal region, a transmembrane domain, a linker region, and two C-terminal C2A and C2B domains that mediate Ca^{2+}-dependent binding to phospholipids (Pang and Südhof, 2010). The properties of Ca^{2+}-dependent phospholipid binding/dissociation and capacity to drive membrane fusion vary between the different SYT family members (Bai et al., 2004; Davis et al., 1999; Hui et al., 2005), and studies indicate that multiple SYT isoforms (both Ca^{2+}-dependent and -independent (von Poser et al., 1997)) contribute to fine tuning vesicle fusion kinetics to the specific needs of the cell (Luo and Sudhof, 2017; Rao et al., 2017; Robinson et al., 2002). Here we show that SYT5 is expressed in human umbilical vein endothelial cells (HUVEC) and is recruited to WPBs where it regulates Ca^{2+}-driven WPB exocytosis.

Results and Discussion

SYT5 is expressed in HUVEC and localises to WPBs

Western blot analysis showed SYT5 is expressed in HUVEC (Fig.1A). Our commercial antibody to SYT5 did not recognise SYT5 by immunocytochemistry so instead we expressed SYT5-mEGFP and analysed the subcellular localisation by counter-staining with antibodies to different subcellular compartments. SYT5-mEGFP co-localised almost exclusively with WPBs (Fig.1B and Fig. S1) and was detected on perinuclear TGN46-positive WPBs indicating that SYT5 is incorporated into the WPB during its formation (Fig. S1A). Overlap analysis of EGFP (green) and WPB-VWF (red) signals in dual labelled images gave Manders' colocalization Coefficients, M1 (green overlap with red) and M2 (red overlap with green) of 0.998±0.0007 (s.e.m.) and 0.838±0.025 respectively (n=10 cells). The molecular basis for SYT5 trafficking to secretory granules, remains unclear, although studies of other SYT's (e.g. SYT1 and SYT7) show the N-terminal regions and palmitoylation of cysteine residues within the linker between the transmembrane and C2A domains play important roles in directing these SYTs to their target membranes (Han et al., 2004; Kang et al., 2004). Because SYT1 is reported to localise to pseudo-WPBs in AtT20 cells (Blagoveshchenskaya et al., 2002), we also analysed this SYT in HUVEC. Although SYT1 protein was detected (Fig. S2A), SYT1-EGFP localised to the plasma membrane and not WPBs (Fig. S2B-C). shRNA depletion of endogenous SYT1 mRNA had no significant effect on VWFpp secretion (Fig. S2D) indicating SYT1 does not regulate WPB exocytosis. Having established that SYT5 can be recruited to WPBs we next determined if it might play a role in regulating Ca^{2+}-dependent hormone-evoked WPB exocytosis.

Depletion of SYT5 modulates histamine-evoked VWFpp secretion and WPB exocytosis

We depleted endogenous SYT5 by shRNA using a lentiviral vector with a puromycin selection cassette that allowed duel expression and selection of cells expressing both the shSYT5 (or shcontrol (shCTRL)) and VWFpp-EGFP, enabling fluorescent labelling of WPBs in transduced cells. Transduced cells were directly monitored for fluorescent WPB exocytosis evoked by the physiological Ca^{2+}-dependent secretagogue histamine (Erent et al., 2007; Hamilton and Sims, 1987; Lorenzi et al., 2008). shSYT5 treatment reduced SYT5 mRNA by 73% (Fig. 2A) and by >65% at the protein level (Fig. 2B). Comparison of changes in $[Ca^{2+}]_i$ during histamine (100 µM) stimulation of Fura-2 loaded HUVEC in shCTRL or shSYT5 transduced cells showed no effect of shSYT5 treatment (Fig. 2C upper panel), however in the same experiments the kinetics and extent of fluorescent WPB exocytosis was significantly altered in shSYT5 transduced cells (Fig. 2C lower panel). There was a significant reduction in the mean maximal rate of WPB exocytosis response to histamine (shCTRL; 2.2 ± 0.4 WPBs/second, n=35 cells, shSYT5; 1.3 ± 0.1 WPBs/second, n=46 cells, P=0.043, t-test) and a reduction in the fraction of fluorescent WPBs that underwent exocytosis (shCTRL; black trace, 30.8 ± 2.1%, 527 fusion events, n=35 cells, shSYT5; grey trace,18.1 ± 1.3%, 507 fusion events, n=46 cells, P<0.0001, t-test). SYT5 depletion had no effect on WPB movements close to the plasma membrane or on the fraction of WPBs showing restricted movements (Fig. S3A). Over expression of SYT5-mCherry containing 7 silent mutations in the region targeted by shSYT5 (SYT5-mCherry (7sm)), labelled WPBs and prevented inhibition of WPB exocytosis in shSYT5 treated cells (Fig. S4). Consistent with direct analysis of WPB exocytosis we found that histamine-evoked VWFpp secretion was reduced in shSYT5 treated cells (Fig.2D). At 100 µM histamine the reduction in secretion was ~40% compared to shCTRL, similar to the ~40% reduction in WPB exocytosis observed directly by live cell imaging.

SYT5 overexpression increases histamine-evoked VWFpp secretion and WPB exocytosis

We next examined the effect of SYT5-mEGFP overexpression on WPB exocytosis and VWFpp secretion. Overexpressed SYT5-mEGFP labels WPBs exclusively allowing us to directly visualize the organelles and their exocytosis by monitoring changes in WPB morphology and the abrupt loss of WPB SYT5-EGFP fluorescence, as described previously for other WPB membrane proteins (Knipe et al., 2010). SYT5-mEGFP overexpression was compared to data from VWFpp-EGFP expressing HUVEC as control and the data (Fig. 3) is presented in the same way as in Fig. 2C. Histamine evoked identical increases in $[Ca^{2+}]_i$ in SYT5-EGFP (grey) and the control VWFpp-EGFP (black) expressing HUVEC (Fig. 3A upper panels). However, in SYT5-mEGFP expressing cells there was a significant increase in the mean maximal rate of WPB exocytosis from 2.1±0.31 WPBs/second (VWFpp-EGF; n=30) to 5.9±2.4 WPBs/second (SYT5-EGFP, n=18 cells, P=0.049 t-test) and a significant increase in the fraction of fluorescent WPBs that underwent exocytosis (VWFpp-EGFP; black trace, 28.0±1.5%, 517 fusion events, n=30 cells, SYT5-EGFP; grey trace, 36.7±1.63%, 512 fusion events, n=18 cells, P<0.0004, t-test). Consistent with live imaging data SYT5-mEGFP overexpression significantly increased histamine-evoked VWFpp secretion (Fig. 3B). No effect of SYT5 overexpression was found on WPB movements or on the fraction of WPBs showing restricted motion close to the plasma membrane (Fig. S3B) indicating that SYT5 does not contribute to WPB immobilisation at the plasma membrane.

Ca^{2+}-independent SYT5 mutant decreases histamine-evoked WPB exocytosis

To confirm that SYT5 function depends on its ability to sense Ca^{2+} we mutated the third aspartate residue of the Ca^{2+}-binding motif of the C2A domain of SYT5-mEGFP to a serine to generate the calcium-insensitive mutant SYT5-Asp197Ser-mEGFP (von Poser et al., 1997) (Fig. 4A) and overexpressed this in HUVEC using Nucleofection™. SYT5-Asp197Ser-mEGFP localised to WPBs (Fig. 4B; Manders' Colocalization Coefficients, M1 and M2 of 0.993±0.0007 and 0.920±0.013 (n=10 cells). Analysis of WPB exocytosis revealed a dominant negative

effect of SYT5-Asp197Ser-mEGFP on a back ground of WT SYT5. SYT5-Asp197Ser-mEGFP significantly reduced both the mean maximal rate of WPB exocytosis from 3.1±0.53 WPBs/second (VWFpp-EGF; n=17) to 1.4±0.29 WPBs/second (SYT5-Asp197Ser-mEGFP, n= 25 cells) (P=0.0046, t-test) and the fraction of fluorescent WPBs that underwent exocytosis (VWFpp-EGFP; black trace, 38.9±3.5%, 532 fusion events, n=17 cells, SYT5-Asp197Ser-mEGFP; grey trace, 23.1±1.5%, 422 fusion events, n=27 cells, P<0.0001, t-test) (Fig. 4C).

Our results provide evidence for a role for SYT5 in regulating Ca^{2+}-dependent WPB exocytosis. SYT5 was the first non-neuronal member of the SYT family to be described (Hudson and Birnbaum, 1995, Craxton and Geodert, 1995) and has been implicated in regulating Ca^{2+}-driven exocytosis in neuronal, endocrine and neuroendocrine cell types (Birch et al., 1992; Fukuda et al., 2002; Gut et al., 2001; Iezzi et al., 2004; Lynch and Martin, 2007; Roper et al., 2015; Saegusa et al., 2002; Xu et al., 2007) as well as pH regulation of phagosomes and phagocytosis in macrophages (Vinet et al., 2008; Vinet et al., 2009). SYT5 has a lower Ca^{2+}-affinity for phospholipid or SNARE protein interactions compared to the other main SYT reported to function in endocrine and neuroendocrine cell types, SYT7 (Chieregatti et al., 2004; Gustavsson et al., 2009; Hui et al., 2005; Iezzi et al., 2004; Schonn et al., 2008; Sugita et al., 2001). The higher Ca^{2+}-affinity of SYT7 is thought to underlie its role in asynchronous neurotransmitter release at low $[Ca^{2+}]_i$ (Bacaj et al., 2013; Luo and Sudhof, 2017; Weber et al., 2014), the sensitivity of SYT7-containing dense core vesicles of chromaffin cells to low $[Ca^{2+}]_i$ and weak stimulation (Rao et al., 2017), and in vesicle replenishment and release during insulin secretion as $[Ca^{2+}]_i$ declines to low levels (Dolai et al., 2016). However, the ability to sense low $[Ca^{2+}]_i$ during weak stimulation is not a prominent feature of endothelial WPBs. The rate of WPB exocytosis under resting conditions is very low (Erent et al., 2007) and WPB exocytosis is largely insensitive to small increases in $[Ca^{2+}]_i$ during weak stimulation (Birch et al., 1994; Erent et al., 2007). Studies in permeabilized or whole cell patch-clamped endothelial cells show a supra micromolar $[Ca^{2+}]$ threshold for activation of WPB exocytosis (Frearson et al., 1995; Zupancic et al., 2002), and a requirement for sustained high (5-30µM) $[Ca^{2+}]_i$ to drive strong exocytosis and VWF secretion (Birch et al., 1994; Birch et al., 1992;

Carter and Ogden, 1994). Such high $[Ca^{2+}]_i$ are achieved during stimulation with physiological agonists or cell injury that occur at wound sites (Carter and Ogden, 1994; Zupancic et al., 2002). SYT5 with its lower Ca^{2+}-affinity for phospholipid and SNARE protein interactions may help to limit WPB exocytosis during weak cell activation. This is potentially important because WPBs store high molecular weight forms of VWF that are potent at capturing platelets to the vessel wall, a process vital during primary haemostasis at wound sites (Sadler, 1998), but potentially hazardous if released inappropriately. Elevated VWF is a risk factor for coronary heart disease, ischemic stroke and sudden death (van Schie et al., 2011; Wieberdink et al., 2010). WPBs also contain and co-release inflammatory mediators (P-selectin and chemokines) and tissue growth regulators (IGFBP7, Ang2) many of which have been linked to the aetiology of vascular disease (Papadopoulou et al., 2008). Thus, the involvement of a lower affinity SYT and a requirement for larger prolonged increases in $[Ca^{2+}]_i$ may minimise the risk of unwanted WPB exocytosis under normal conditions where endothelial cells may experience intermittent low level activation.

The nature of the molecular interactions between SYT5 and WPB SNAREs remain to be determined. Endothelial cells utilize at least two distinct SNARE complexes to regulate WPB exocytosis, one comprising syntaxin 4:SNAP23:VAMP3 and a second complex comprising syntaxin-3:SNAP23:VAMP8 (Fu et al., 2005; Matsushita et al., 2003; Schillemans et al., 2018b; van Breevoort et al., 2014; Zhu et al., 2015; Zhu et al., 2014), and each complex may play a role in specific modes of WPB exocytosis (Schillemans et al., 2018b). SYT-SNARE interactions have been extensively studied for neuronal SYT1, which binds both the neuronal SNAP (SNAP-25) and syntaxin (syntaxin 1) in heterodimers or fully assembled SNARE complexes (reviewed in (Chapman, 2008)). SYT5 binds poorly to the WPB SNAP, SNAP23 (Chieregatti et al., 2004), and although the SYT5 C2AB domains can bind syntaxin 1 (Li et al., 1995) it remains to be established if endothelial syntaxins implicated in WPB exocytosis bind SYT5. WPB exocytosis and VWFpp secretion was significantly reduced but not abolished upon depletion of endogenous SYT5. This is most likely due to the incomplete (~65%) depletion of SYT5 in these experiments, but may also reflect the involvement of other SYT

isoforms in the Ca^{2+}-sensing mechanism and/or of the cytosolic Ca^{2+}-sensors CaM and the AnxA2/S100A10 complex described in the introduction. AnxA2-phospholipid interactions are typically low affinity and fit well with a general requirement for high $[Ca^{2+}]_i$ for WPB exocytosis. The ability of S100A10 to bind WPB-associated Munc13-4 (Chehab et al., 2017) and of AnxA2 to bind SNAP-23 (Wang et al., 2007) indicate that WPB exocytosis is likely coordinated by a complex network of Ca^{2+}-sensors ensuring that WPB exocytosis only occurs when needed.

Methods

Tissue culture, VWF and VWFpp ELISA assays, antibodies, and reagents

Primary HUVECs tested for contamination were purchased from PromoCell GmbH (Heidelberg, Germany) and cultured as previously described (Hannah et al., 2005). Human Embryonic Kidney-293 (HEK-293) cells were cultured in Minimal Essential Medium (MEM) Alpha Medium 1x (Invitrogen) supplemented with 10% fetal calf serum (Biosera, Ringmer, UK) and 50 µg/ml gentamycin (Invitrogen) at 37°C, 5% CO_2 as previously described (Kiskin et al., 2010). Secreted VWF propolypeptide (VWFpp) was assayed by specific ELISA as previously described (Hewlett et al., 2011). Primary antibodies (Abs) along with the dilutions for immunofluorescence or western blotting are given in Supplementary Table S1. All reagents were from Sigma-Aldrich unless otherwise stated. Fura-2/AM was from Invitrogen.

DNA Constructs, Site Directed Mutagenesis, Lentiviral Production and Transfection

The VWF propeptide fused to enhanced green fluorescent protein (VWFpp-EGFP) has been described previously (Hannah et al., 2005). A mEGFP fusion protein of human SYT5 was made using the ligation independent cloning (LIC) approach as previously described (Bierings et al., 2012) using the primers in Supplementary Table S2. SYT1-EGFP was constructed by amplification of SYT1 from HUVEC cDNA using SYT1 specific primers that are flanked by HindIII (forward: 5'-AGT TTAAGCTTATGGTGAGCGA-3'; HindIII in bold) and AgeI sites (reverse; 5'-TAAAACCGGTCCCT TCTTGACGGC-3'; AgeI in bold), respectively. The 1289 bp amplicon was digested with HindIII and AgeI and was cloned in frame with EGFP between

the HindIII and AgeI sites in EGFP-N1 (BD Biosciences Clontech, Saint-Germain-en-Laye, France). pSYT5-mCherry was made by transferring mCherry as an NheI/AgeI fragment from mCherry-N1 LIC vector (Bierings et al., 2012) to NheI/AgeI digested SYT5-mEGFP. To make a shSYT5 resistant SYT5-mCherry construct we used site directed mutagenesis on pSYT5-mCherry to generate seven silent mutations along the shRNA target site (g518a, t521c, c524t, a527c, c530t, g533a, g536a). The genetic changes were introduced using Agilent's QuikChange II site directed mutagenesis kit, forward primer 5'-aggaagtgaaggggctgggccaaagctatatcgataaagtacagccagaagtagaggagctgg-3' and reverse primer 5'-ccagctcctctacttctggctgtactttatcgatatagctttggcccagccccttcacttcct-3'. Correct integration of the desired mutations were confirmed using a CMV promoter targeting primer (5'-CAACGGGACTTTCCAAAATG-3') and Sanger sequencing (Source Bioscience Ltd). The SYT5 calcium-insensitive mutant (SYT5-Asp197Ser-EGFP) with the aspartate residue at position 197 (third aspartate in the Ca^{2+}-binding motif of the C2A domain) mutated to a serine residue (von Poser et al., 1997) was made by site-directed mutagenesis with the QuickChange® method (Agilent Technologies UK Limited, Cheshire, UK) using primers 5'-GGTCATGGCGGTGTAC**AGC**TTCGACCGCTTCTCT-3' (forward) and 5'-AGAGAAGCGGTCGAA**GCT**GTACACCGCCATGACC-3' (reverse) (mutated bases in bold). SYT5-mEGFP was transferred to a lentiviral vector by cloning a 2304 bp NdeI/AscI fragment or a 2598 bp NdeI/NotI fragment, respectively, into NdeI/AscI or NdeI/NotI digested LVX-mEGFP-LIC (van Breevoort et al., 2014).

SYT5 shRNA was obtained from the MISSION® shRNA library developed by TRC at the Broad Institute of MIT and Harvard and distributed by Sigma-Aldrich (Supplementary Table S3). Lentiviral vectors for transfection of SYT5 specific shRNA or mEGFP tagged SYT5 constructs were made as follows. LKO.1-puro-CMV-TagRFP-U6-shC002 (Sigma), a puromycin selectable lentiviral vector expressing MISSION® library shRNAs from the U6 promoter and TagRFP from the CMV promoter, was cut with NheI and PstI to replace TagRFP with a NheI-SalI-BclI-FseI-XmaI-PacI-PstI linker formed by annealing olligos RBNL204 (5'-

CTAGCGTCGACTGATCAGGCCGGCCCCCGGGTTAA TTAACTGCA-3') and RBNL205 (5'-GTTAATTAACCCGGGGGCCGGCCTGATCAGTCGACG-3'), yielding LKO.1-puro-CMV-linker-U6-shC002. LKO.1-puro-CMV-mEGFP-U6-shC002, which simultaneously expresses mEGFP and shRNAs from the CMV and U6 promoter respectively, was constructed by amplifying mEGFP from mEGFP-LIC (Bierings et al., 2012) with RBNL232 (5'-TATATGATCACTATGGTGAGCAAGGGCGAGGAGCTGTTC-3') and RBNL222 (5'-ATATGGCCG GCCTTACTTGTACAGCTCGTCCATGCCG-3'). The 744 bp amplicon was digested with BclI and FseI and cloned between the BclI and FseI sites in LKO.1-puro-CMV-linker-U6-shC002. LKO.1-puro-CMV-VWFpp-mEGFP-U6-shC002, fluorescently labelling WPBs by expression of VWFpp-mEGFP and simultaneously knocking down target SYT using shRNA from the MISSION® library, was constructed by amplifying VWFpp-EGFP (Hannah et al., 2005) with RBNL214 (5'-TATAGCTAGCGCCACCATGATTCCTGCCAGATTTGCCGGGG-3') and RBNL222. The 3058 bp amplicon was digested with NheI and FseI and cloned between the NheI and FseI sites in LKO.1-puro-CMV-linker-U6-shC002. Clone TRCN0000000959 targeting SYT5 had lost the EcoRI site. Primers were designed to amplify the shRNA cassette flanked by a 5' SphI and a 3' EcoRI site (forward: 5'-TCGT**GCATGC**CGATTG GTGGAAGTAAGG-3', SphI restriction site in bold; reverse: 5'-GCCT**GAATTC**AAAAACCAGAGTT ACATAGACAAGGTC-3', EcoRI restriction site in bold). The 2064 bp amplicon was digested with SphI and EcoRI and cloned between SphI- and EcoRI-sites in LKO.1-puro-CMV-mEGFP and LKO.1-puro-CMV-VWFpp-mEGFP vectors. The constructs were sequence verified. Lentiviral plasmids were produced in Stbl3 bacteria.

Lentiviral transduction and transfection by Nucleofection

Lentiviral production in HEK293T cells and lentiviral transductions of HUVECs were performed essentially as described previously (van Breevoort et al., 2014). pMD2-G (Addgene plasmid #12259), pRSV-Rev (Addgene plasmid #12253) and pMDLg/pRRE (Addgene plasmid #12251) helper plasmids were gifts from Didier Trono (Dull et al., 1998). Lentiviraly transduced endothelial cells were selected using puromycin treatment (0.5 µg/ml for 48 hours). After 48 hours incubation, transduced HUVEC were passaged in 6-well plates, 24-well plates or 35 mm poly-D-lysine coated glass bottom culture dishes (MatTeK, Ashland, MA) depending on the experiment conditions. ELISA, qPCR, WB or live cell imaging were performed on the transduced HUVEC when confluent. For conventional transfection of HUVEC the Amaxa Nucleofection™ system was used with HUVEC OLD Nucleofector™ Solution containing 2-4 µg of target DNA and programme U-01 according to the manufacturer's instructions (Lonza Biologics Plc, Slough, UK). Cells were used for experiments 48 hours following transfection. HEK cell transfection by Nucleofection™ was identical to that for HUVECs with the exceptions that MEM Alpha medium was used in place of HGM, Cell Line Nucleofector™ Solution V was used in place of HUVEC OLD Nucleofector™ Solution, Nucleofection programme was Q-01.

RT-PCR and quantitative PCR analysis

RNA was extracted using RNeasy Mini Kit (QIAGEN). The quantity and purity of extracted RNA was determined by measuring its absorbance at 280 and 260 nm using a Nandrop-1000® device (Thermo Scientific, Denmark). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ThermoFisher Scientific). Briefly, 1 µg of RNA was added to the reaction mix, and cDNA was synthesized using one cycle of heating to 55°C for 20 minutes following by an increase to 94°C for two minutes. Subsequent PCR amplification of HUVEC cDNA was achieved using 40 cycles of denaturation (94°C for 15 seconds), followed by annealing (55°C for 30 seconds) and extension (72°C for 1 minute). PCR was performed using a Mastercycler® machine (eppendorf, Stevenage).The products of

PCR were run on a 1.5% agarose gel and visualized by ethidium-bromide staining. All bands were sequenced verified (GATC Biotech, Cologne, Germany).

Immunocytochemistry and Immunoblotting

For immunocytochemistry HUVEC or HEK were grown on 9mm glass coverslips and immunostaining and confocal fluorescence imaging of fixed cells were performed as previously described[3]. The intensity measurements, exposures at each wavelength were first set to ensure no detector saturation on the brightest sample and then kept constant for all images. Images were prepared in Adobe Photoshop CS6. Immunoblotting was carried out as previously described (Bierings et al., 2012).

Live cell imaging, vesicle tracking, confocal imaging of fixed cells and fluorescence overlap analysis.

Exocytosis of VWFpp-EGFP-containing and EGFP-SYT5 associated WPBs were determined as previously described (Erent et al., 2007; Knipe et al., 2010). The moment of fusion of EGFP-SYT5 containing WPBs was determined by an abrupt decrease in WPB fluorescence on fusion as previously described (Knipe et al., 2010). Automatic tracking of WPB movements was carried out as previously described (Conte et al., 2016). Image data were acquired at 10 frames/second in Winfluor (http://spider.science.strath.ac.uk/sipbs/software_imaging.htm), exported as raw format to GMimPro/Motility freeware software (Dr Gregory Mashanov, Francis Crick Institute Mill Hill Laboratory, London, www.mashanov.uk). The automatic single particle tracking (ASPT) module in GMimPro (Mashanov and Molloy, 2007) was used to track the X,Y position in time of individual WPBs expressing VWFpp-EGFP or SYT5-EGFP, yielding maximum velocities and maximum displacements. ASPT settings were FWHM500nm, R7, L20, Q25 and C5000. The time, X and Y positions for WPBs for individual cells were exported in text file format for subsequent analysis of mean squared displacement (MSD) in Matlab using custom written functions. MDS plots were fitted as previously described and the proportion of WPBs showing subdiffusive/restricted diffusion and their corresponding cage

radii determined (Conte et al., 2016). Confocal images for fixed cells were taken at room temperature using either Leica SP2 or SP8 confocal microscopes (Mannheim, Germany) equipped with 40x, 63x and 100x objectives (HCX PL APO40x 1.2 NA, PLAPO 63x/1.40, PLAPO100x 1.4NA) or a BioRad Radiance 2100 confocal running LaserSharp 2000 software and equipped with a Nikon 60x and x100 PLAPO 1.40 NA objectives. Dual color images were acquired sequentially with pinhole setting Airy 1, image size 1024x1024 and frame averaging over 6-12 scans. To determine the fractional overlap of green (EGFP) with red (VWF) signals (Manders' Colocalization Coefficient M1) or vici versa (Manders' Colocalization Coefficient M2) in images of HUVEC expressing SYT5-GFP or SYT5 SYT5-Asp197Ser-mEGFP and stained for endogenous VWF, we used the ImageJ plugin JACoP that implements the Manders' Colocalization Coefficient with the Costes method for automatically estimating threshold values for identifying background levels (Costes et al., 2004), as reviewed in (Dunn et al., 2011).

Statistical analysis

Data were plotted in Origin 2017 or GraphPad Prism Version 7.02. Statistical analysis was by nonparametric *t* test (except where indicated) using GraphPad Prism Version 7.02. Significance values are shown on the Figures or in Figure legends. Data are shown as mean±SEM.

Authorship

CL, JS, RB, TC performed research and analyzed data; MJH, RB and DO contributed vital reagents and expertise; TC designed the research; TC and CL wrote the paper.

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Figures

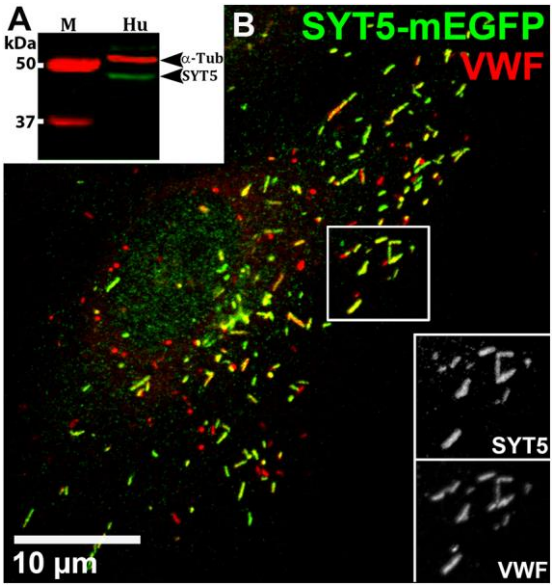

Figure 1. SYT5 is expressed in HUVEC and recruited to WPBs. (**A**) Representative western blot of HUVEC (Hu) lysate probed with the rabbit SYT5 primary antibody (abcam ab116452, 1:200). Marker sizes (M) are indicated. α-tubulin was used as loading control. The strong band at approximately 48 kDa represents SYT5 protein, confirmed by depletion after shSYT5 treatment (Figure 2). (**B**) Confocal fluorescence image of a HUVEC 48 hours after Nucleofection™ with SYT5-mEGFP. Cells were immuno-labelled with antibodies to GFP (sheep; green) and VWF (rabbit; red). Scale bar is 10µm. Inset panels (greyscale) here and below are from regions indicated by white boxes. Manders' Colocalization Coefficients for the fractional overlap of EGFP signal with that of the WPB-VWF signal (Manders' Coefficient M1) was 0.998±0.0007 (s.e.m.) and for WPB-VWF signal overlapping the EGFP signal (Manders' Coefficient M2) was 0.838±0.025 (n=10 cells). Confocal fluorescence images here and in all subsequent figures were taken at room temperature.

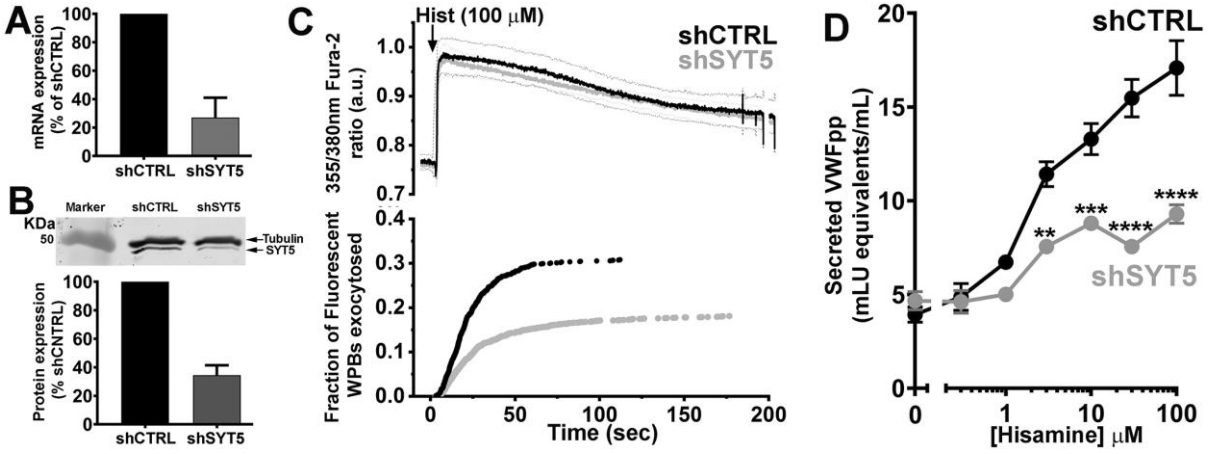

Figure 2. SYT5 depletion reduces WPB exocytosis and VWFpp secretion (**A**) Quantification of shRNA mediated SYT mRNA depletion after lentiviral transduction. Data is normalized to shCTRL (mean±s.e.m. of 4 independent experiments). (**B**) **top;** western blot showing SYT5 depletion by shRNA and **bottom**; quantification of SYT5 depletion (mean±s.e.m of 4 independent experiments). SYT5 was detected using a rabbit SYT5 primary antibody (abcam ab ab116452, 1:200), α-tubulin was used as loading control. (**C**) top panel shows the mean 355nm/380nm Fura-2 fluorescence ratio recorded in shCTRL (black, n=12 cells) or shSYT5 (grey, n=12 cells) treated HUVEC expressing VWFpp-EGFP and stimulated with histamine (100μM, arrow). Here and in subsequent Figures thin dashed lines show the ±95% confidence limits for the mean fluorescence ratios (black; shCTRL, grey; shSYT5). Lower panel in **A** shows the cumulative plot of histamine-evoked WPB fusion times scaled by the mean fraction of WPBs that underwent exocytosis. (**D**) Shows histamine (0.3-100μM)-evoked VWFpp secretion from HUVEC following lentiviral transduction with shCTRL (black) or shSYT5 (grey). Data are mean±s.e.m of 4 independent experiments, each carried out in triplicate. *P≤0.5, ** P≤ 0.01, *** P≤ 0.001, **** P≤ 0.0001, t-test.

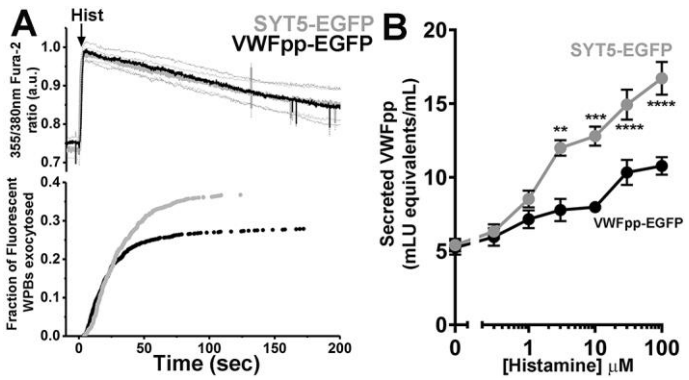

Figure 3. SYT5 overexpression increases WPB exocytosis and VWFpp secretion. A. top panel shows the mean 355nm/380nm Fura-2 fluorescence ratio recorded in VWFpp-EGFP (black, n=12 cells) or SYT5-EGFP (grey, n=12 cells) treated HUVEC expressing VWFpp-EGFP and stimulated with histamine (100μM, arrow). Lower panel in B shows cumulative plots of histamine-evoked WPB fusion times scaled by the mean fraction of WPBs that underwent exocytosis. **B.** Histamine (0.3-100μM)-evoked VWFpp secretion from HUVEC expressing VWFpp-EGFP (black) or SYT5-EGFP (grey) after lentiviral transduction. Data is mean±s.e.m of 3 independent experiments, each carried out in triplicate. *P≤0.5, ** P≤ 0.01, *** P≤ 0.001, **** P≤ 0.0001, t-test.

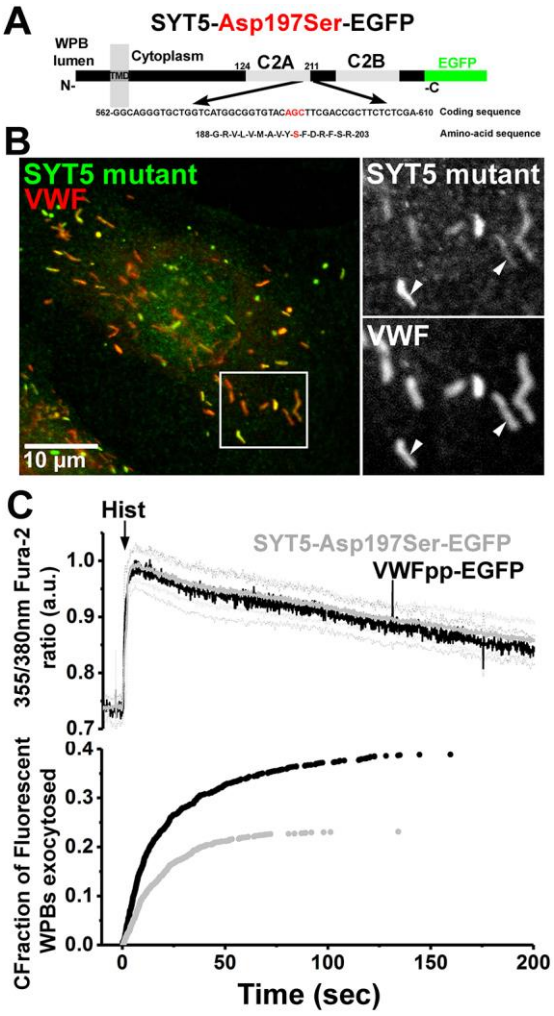

Figure 4. Ca^{2+}-independent SYT5 mutant decreases WPB exocytosis. (A) Cartoon showing the point mutation, Asp197Ser, in the C2A domain of SYT5-EGFP generating the calcium-insensitive mutant. (**B)** Fluorescence image of a HUVEC expressing SYT5-Asp197Ser-EGFP (48 hours post-transfection) and immuno-labelled for GFP (green; rabbit Ab) and endogenous VWF (red, sheep Ab). Arrows in grayscale images show the localization of SYT5-Asp197Ser-EGFP to WPBs. Manders' Colocalization Coefficients for the fractional overlap of EGFP signal with that of the WPB-VWF signal (Manders' Coefficient M1) were 0.993±0.0007, and for WPB-VWF signal overlapping the EGFP signal (Manders' Coefficient M2) 0.920±0.013 (n=10 cells). (**C)** top panel shows the mean 355nm/380nm Fura-2 fluorescence ratio recorded in HUVEC expressing VWFpp-EGFP (black, n=12 cells) or SYT5-Asp197Ser-EGFP (grey, n=12 cells) stimulated with histamine (100µM, arrow). Lower panel in Aiii shows the cumulative plot of histamine-evoked WPB fusion times scaled by the mean fraction of WPBs that underwent exocytosis.

Supplementary Materials

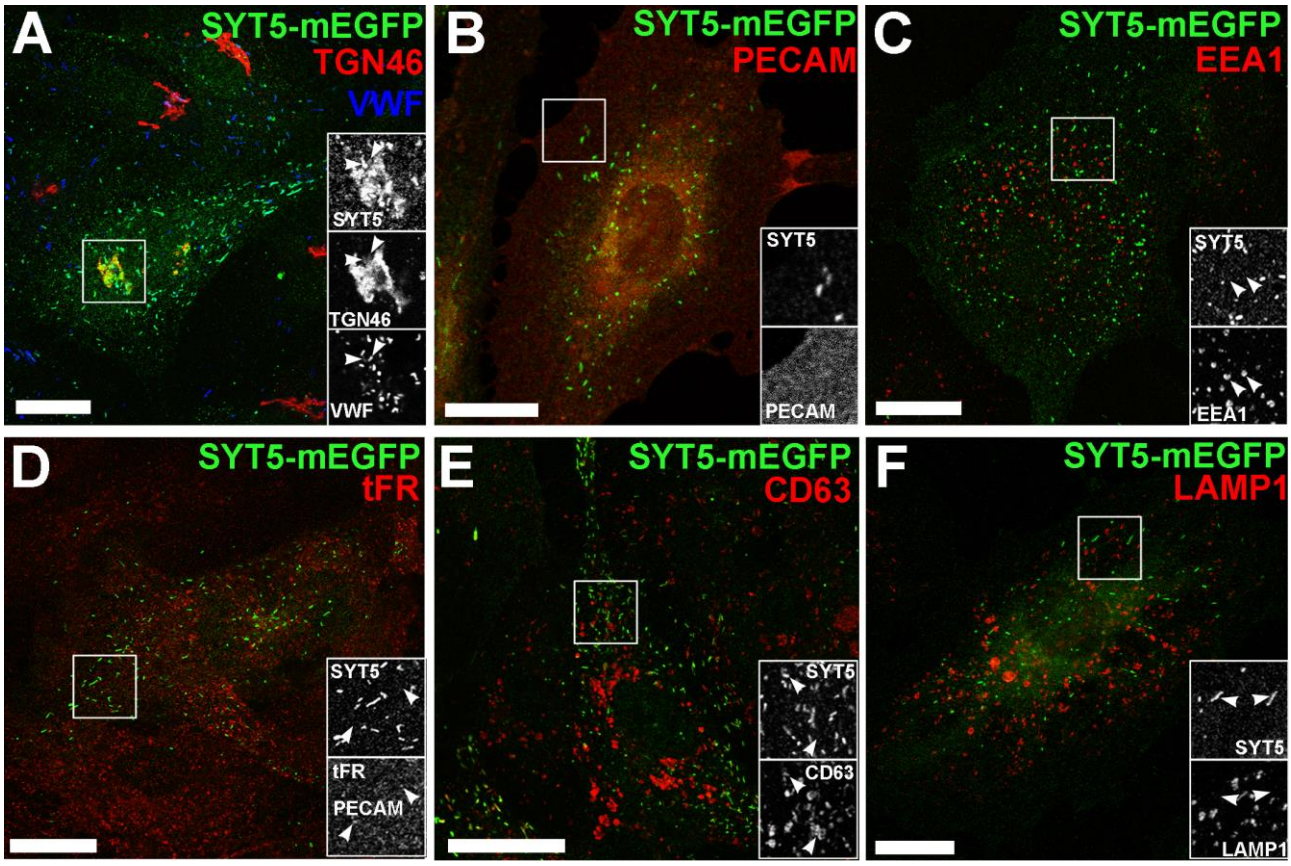

Figure S1. Subcellular distribution of SYT5-mEGFP. Confocal fluorescence images of single HUVEC 24 hours after Nucleofection™ with SYT5-mEGFP, and immuno-labeled with specific antibodies to GFP (sheep; green), VWF (top left panel only, rabbit; blue) and (A) TGN46, (B) PECAM, (C) tFR, (D) EEA1, (E) CD63 or (F) LAMP1 as indicated (red). Scale bars are 10μm. Inset panels show in greyscale regions indicated by white box.

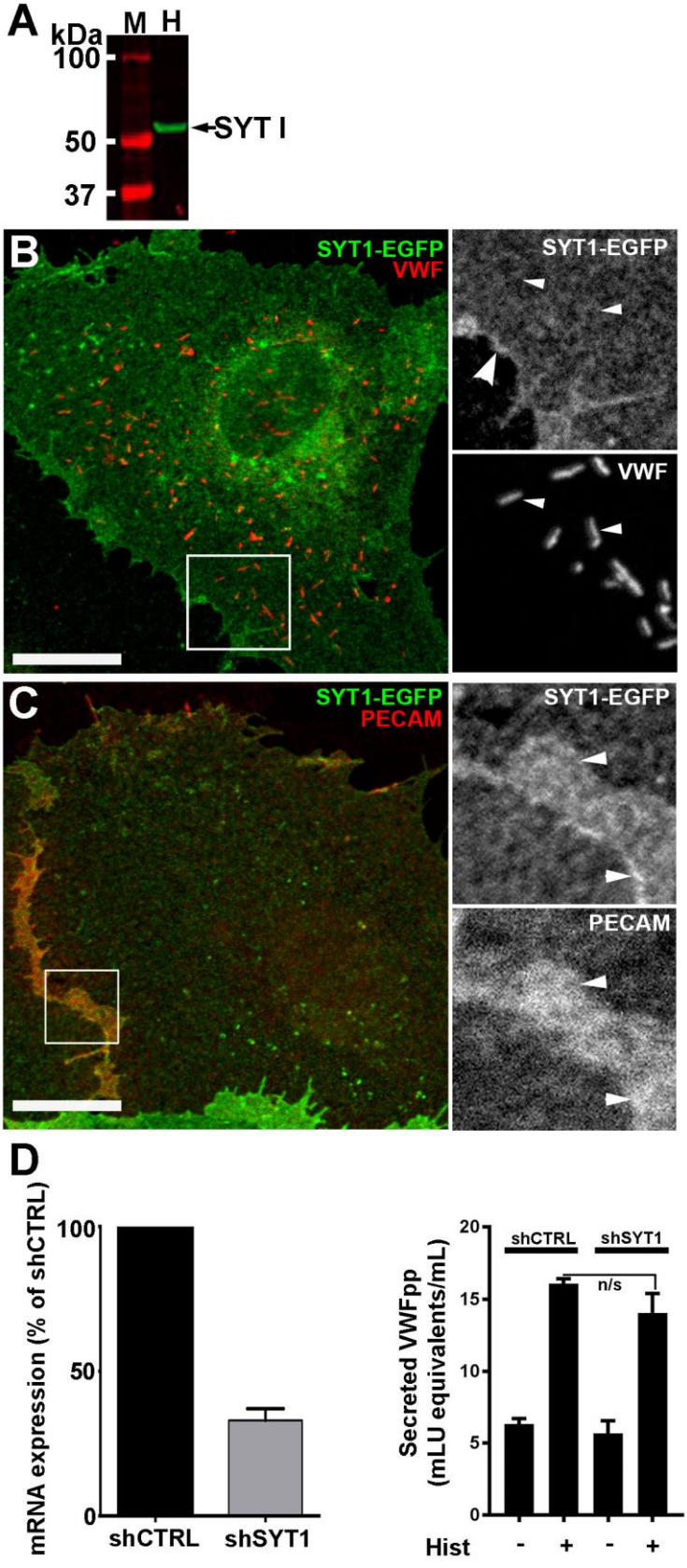

Figure S2 SYT1 is expressed in HUVEC but is not recruited to WPBs. (A) representative Western blot produced by probing HUVEC (H) lysate with the SYT1 primary antibody at a concentration of 1:200. The sizes of the marker (M) are shown on the left. A strong band at approximately 55 kDa represents SYT1 protein. (**B, C)**, Confocal fluorescence images of HUVEC 24 hours after Nucleofection™ with SYT1-mEGFP, and immuno-labeled with specific antibodies to (**B**) GFP (sheep; green), VWF (rabbit; red) and (**C**) GFP (sheep; green), PECAM (mouse; red) Scale bars are 10μm. Inset panels show in greyscale regions indicated by white box. (**D**) left; Quantification of shSYT1 mediated SYT1 mRNA depletion. Data is normalized to shCTRL and is mean±SEM of 3 independent experiments carried out in duplicate. Right; Histamine (100 μM) evoked VWFpp secretion in shCTRL or shSYT1 treated HUVEC. Experiment shown is mean ±sem and is representative of 3 independent experiments each carried out in triplicate.

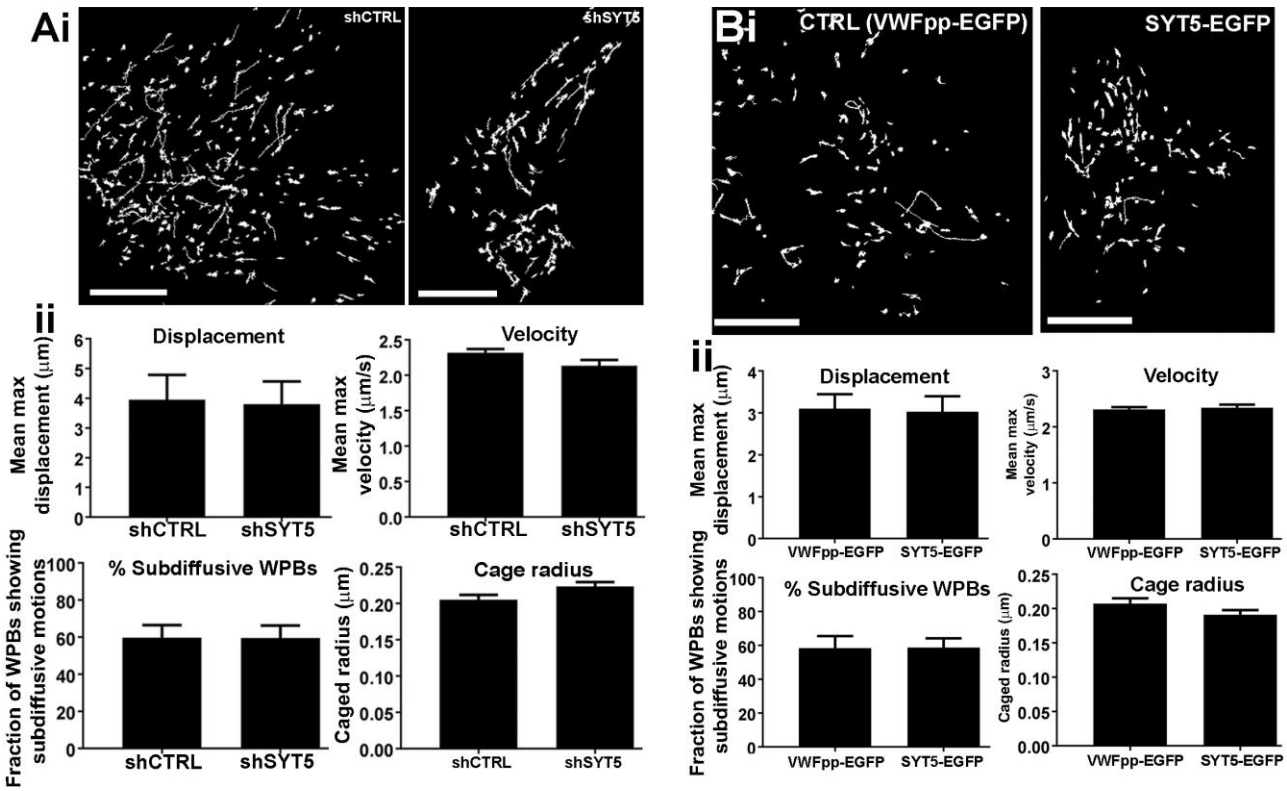

Figure S3. SYT5 depletion (A) or SYT5-EGFP overexpression (B) does not alter WPB trafficking close to the plasma membrane. (Ai). Representative X-Y trajectories of individual WPBs in single HUVEC expressing VWFpp-EGFP and following lentiviral transduction with shCTRL (**left**) or shSYT5 (**right**). Trajectories were determined here and elsewhere from TIRFM videos using the ASPT function of GMimPro software as described previously (Conte et al., 2016). Number of cells imaged and trajectories detected were: shCTRL, n=6 cells, 890 trajectories; shSYT5 n=6 cells, 740 trajectories. (**Aii).** Parameters determined from detected trajectories of long range (top panels) or short range (lower panels) WPB movements. Number of WPBs analysed for short range movements were: shCTRL, 135 trajectories; shSYT5, 149 trajectories. **(Bi).** Representative X-Y trajectories of individual WPBs in single HUVEC expressing VWFpp-EGFP (**i**) (control, n=7 cells, 693 trajectories) or SYT5-EGFP (**ii**) (n=7 cells, 735 trajectories) after lentiviral transduction. (**Aii).** Parameters determined from detected trajectories of long range (top panels) or short range (lower panels) WPB movements. Number of WPBs analysed for short range movements were: control, 76 trajectories; SYT5-EGFP, 103 trajectories.

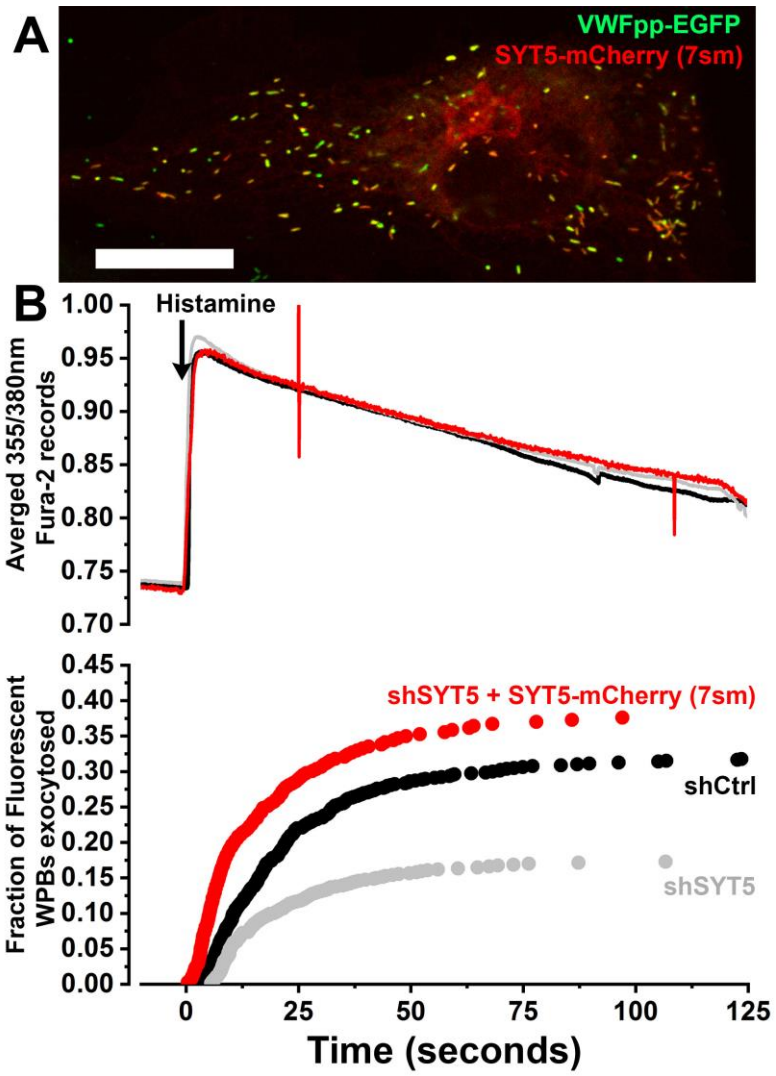

Figure S4. Overexpression of SYT5-mCherry (7sm) prevents shSYT5 mediated reduction in histamine-evoked WPB exocytosis in HUVEC. (A). Confocal fluorescence image of a HUVEC co-expressing VWFpp-EGFP (green) and SYT5-mCherry (7sm) (red) 24 hours post transfection. Scale bar is 10µm. (**B**) top panel shows averaged 355nm/380nm Fura-2 fluorescence ratios from shControl treated HUVEC (black, 10 cells), shSYT5 treated HUVEC (grey, 9 cells) and shSYT5 treated cells Nucleofected™ with SYT5-mCherry (7sm) (red, n=12 cells, 24 hrs post transfection). For clarity the ±95% confidence limits for the mean fluorescence ratios have been omitted. Histamine (100µM). Was added at the arrow. Lower panel in B shows cumulative plots of histamine-evoked WPB fusion times scaled by the mean fraction of WPBs that underwent exocytosis and colour coded as in (**A**). The mean (±SEM) maximal rates of WPB exocytosis in response to histamine were; shCtrl; 2.3 ± 0.3 WPBs/second, n=13 cells, shSYT5; 1.4 ± 0.2

WPBs/second, n=16 cells, and shSYT5 + SYT5-mCherry (7sm) 6.4 ± 1.1 WPBs/second, n=12 cells. The fraction of fluorescent WPBs that underwent exocytosis were; shCtrl; black trace 32.4 ± 1.4%, 136 fusion events, n=13 cells, shSYT5; grey trace,17.3 ± 2.9%, 129 fusion events, n=16 cells, and shSYT5 + SYT5-mCherry (7sm); red trace, 37.5 ± 3.1%, 158 fusion events, n=12 cells.

Supplementary Tables

Table S1. Antibody reagents.

Antigen	Manufacturer	Catalogue Number	Host Species	Optimum Dilution for ICC	Optimum Dilution for Western Blotting
VWF	DAKO	A0082	Rabbit	1:10000	N/A*
VWF	Serotec	AHP062	Sheep	1:10000	N/A
VWF	Serotec	MCA127	Mouse	1:100	N/A
Tubulin	Sigma-Aldrich	T9026	Mouse	N/A	1:5000
LAMP1	DSHB**	H4B4	Mouse	1:50	N/A
CD63	DSHB	H5C6	Mouse	1:200	N/A
EEA-1	BD Transduction Laboratories	610456/7	Mouse	1:100	N/A
TGN-46	Serotec	AHP500	Sheep	1:300	N/A
PDI	Stressgen	SPA-891	Mouse	1:500	N/A
tfR	Invitrogen	13-6800	Mouse	1:200	N/A
GFP	Molecular Probes	A-11122	Rabbit	1:300	Variable
GFP	Biogenesis	4745-1051	Sheep	1:250	Variable
GFP	Roche	11814460001	Mouse	1:500	N/A
PECAM	DSHB	PSB1	Mouse	1:10	N/A
SYT1	Synaptic Systems	105011	Mouse	1:200***	1:500
SYT5	Abcam®	ab116452	Rabbit	1:100	N/A
SYT 5	Abcam®	ab140432	Goat	N/D	1:300

Fluorophore- or horseradish peroxidase–coupled secondary Abs were from Jackson ImmunoResearch Europe (Newmarket, UK). Infrared dye secondary Ab were from LI-COR

Biosciences UK Ltd (Cambridge, UK). * N/A = Not Applicable. Western blotting or ICC was not performed for this antigen. ** DSHB = Developmental Studies Hybridoma Bank. *** Unless otherwise stated, the dilutions of the SYT antibodies for ICC were determined in HEK cells expressing fluorescent constructs of the SYT proteins.

Table S2. Primers sequence used for LIC.

SYT5	Forward Primer	Reverse Primer
	GCAGGGGCGCAACAGACCCCGGTATGTTCCCGGAGCCCCCAAC	CCACCAGGCCGGCCAGCACCCGGTCCGGGCGCAGGCAGCAGCCTCAC

Table S3. Synaptotagmin 5 shRNA used in this study

Synaptotagmin	shRNA clone MISSION® Library	shRNA target sequence
SYT5	TRCN0000000959	CCAGAGTTACATAGACAAGGT