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A gain of function mutant of Munc18-1 stimulates secretory granule recruitment and exocytosis and reveals a direct interaction of Munc18-1 with Rab3.

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Synopsis

Munc18-1 plays a crucial role in regulated exocytosis in neurons and neuroendocrine cells through effects on vesicle docking and membrane fusion. The molecular basis for Munc18 function is still unclear as are the links with Rabs and SNARE (soluble NSF-attachment protein receptors) proteins that are also required. Munc18-1 can bind to the SNAREs through at least three modes of interaction, including binding to the closed conformation of syntaxin 1. Using a gain of function mutation in Munc18-1 (E466K), based on a mutation in the related yeast Sly1p protein, we have identified a direct interaction of Munc18-1 with Rab3A which is increased by the mutation. Expression of Munc18-1 with the E466K mutation increases exocytosis in adrenal chromaffin and PC12 cells and was found to increase the density of secretory granules at the periphery of PC12 cells suggesting a stimulatory effect on granule recruitment through docking or tethering. Both the increase in exocytosis and changes in granule distribution appears to require Munc18-1 E466K binding to the closed form of syntaxin 1 suggesting a role for this interaction in bridging Rab- and SNARE-mediated events in exocytosis.

Short title: Munc18-1 and Rabs in vesicle docking

Key words: SNARE proteins, secretory granules, exocytosis, Sec8

Abbreviations: GH, growth hormone; GST, glutathione-S-transferase (GST); SM, Sec1/Munc18-like; SNARE, soluble NSF-attachment protein receptor; VAMP, vesicle-associated membrane protein

Introduction

Intracellular membrane fusion throughout the exocytic and endocytic pathways is mediated by members of the conserved SNARE (soluble NSF-attachment protein receptors) protein family [1] and is driven by the assembly of a SNARE complex between vesicle (v-) and target (t-) SNAREs [2, 3]. Additional conserved protein families are involved in SNARE-dependent vesicle fusion including the Rab [4] and Sec1/Munc18-like (SM) proteins. The Rab GTPases are involved in all membrane traffic steps and, along with their specific effectors, play key roles in vesicle tethering and docking prior to fusion [5, 6]. The functional link between Rabs and SNARE proteins is unclear but is mediated in part by Rab effectors [7, 8]. There is an essential requirement for an SM protein in every vesicular traffic step in cells [9-12] which has been suggested to be related to their ability to interact with their appropriate syntaxin SNARE as first observed for Munc18-1 [13, 14]. A link between Rabs and SM proteins has been inferred from genetic evidence [15] but direct interactions have been not observed. Recent work has shown that increased vesicle docking due to Rab3 over-expression requires Munc18-1 but the mechanism underlying this is unknown [16]. Indeed, the exact functions of SM proteins and how they fit into the steps between Rab and SNARE functioning are still to be resolved.

The most specialised form of vesicle fusion is in regulated exocytosis in neurons and neuroendocrine cells [17] which is tightly regulated both temporally and spatially and in the case of neuronal exocytosis can occur with sub-millisecond kinetics. In neuronal and neuroendocrine exocytosis the key SNARE proteins are syntaxin 1 and SNAP-25 on the plasma membrane and vesicle-associated membrane protein (VAMP) 1 or 2 on the vesicle membrane [2, 18] with an involvement of Munc18-1 [11] and the Rab proteins Rab3 [19] or Rab3 and Rab27 working in concert [6, 20]. Munc18-1 is an essential protein for neurotransmission [11, 12, 21] and in neuroendocrine cells it has been suggested to be required for dense-core vesicle docking [22] or to have a role in regulating the fusion event itself [23]. A late role in fusion is supported

by observations revealing a stimulatory effect of Munc18-1 and Sec1 in an *in vitro* fusion assay [24, 25]. It is not yet understood at a molecular level how Munc18-1 stimulates vesicle docking and fusion.

SM proteins have been observed to interact with their syntaxin using one or more of three modes of binding [26]. Mode 1, seen only with Munc18-1, involves binding of Munc18-1 to syntaxin 1 in a closed conformation that precludes its assembly into a SNARE complex [27-29]. Mode 2 binding, originally described for the SM proteins, Sly1p and Vps45 [30-34] and subsequently for Munc18c [35] involves binding of the SM proteins to the extreme N-terminus of the relevant syntaxin. Mode 3 binding, first described for the yeast Sec1 [36], involves preferential binding to the assembled SNARE complex [24, 37]. It is unclear whether all SM proteins undergo all three modes of interaction and whether all three modes are physiologically relevant. It has recently been observed, however, that Vps45 may participate in two modes of binding [34]. In addition, Munc18-1 may undergo all three modes of interaction [25, 38, 39] and Modes 2 and 3 are required for the ability of Munc18-1 to stimulate the kinetics of membrane fusion *in vitro* [25]. The Mode 1 binding has only been observed with Munc18-1 and its role in exocytosis has been debated. It has been suggested that this mode of interaction might be required to chaperone syntaxin during its traffic to the cell surface [38]. A requirement for this interaction for syntaxin 1 expression on the plasma membrane has, however, been ruled out as syntaxin 1 can reach the plasma membrane in Munc18-1 null organisms [21, 40] or if it is mutated to prevent Mode 1 binding [41].

We have previously examined the effect of various mutations in Munc18-1 on Mode 1 binding to syntaxin 1 and also on dense-core granule exocytosis following expression of mutated proteins in adrenal chromaffin cells [23, 42]. These mutations were based on those that have been discovered to produce an altered phenotype in yeast, *Drosophila* or *C. elegans*. One of these (E466K)

was modelled on a mutation found in yeast Sly1p that suppressed the need for the Rab protein Ypt1 in ER to Golgi traffic [15, 43]. The E466K mutation in Munc18-1 is sited well away from known or postulated interaction sites for syntaxin 1 [32] and does not affect Mode1 binding to syntaxin 1 in vitro [42]. Interestingly, E466K acted as a gain-of-function mutation so that expression of mutated Munc18-1 resulted in an increase in the number of exocytotic events after stimulation. We have set out to characterise the molecular basis of this gain of function and this has revealed a direct interaction of Munc18-1 with Rab3A. In addition, expression of Munc18-1 with the gain of function mutation stimulated secretory granule recruitment to the cell periphery possible through docking or tethering in a manner requiring interaction of Munc18-1 with closed syntaxin1. These findings raise the possibility that Munc18-1 act as a direct link between Rabs and SNARE function.

Materials and Methods

Plasmid construction and recombinant protein production. Munc18 was cloned into pcDNA3.1 vector for expression in mammalian cells and into the pGEX vector for glutathione-S-transferase (GST) fusion protein expression [42]. His₆-Rab3A was made as described [44]. The cytoplasmic domain of syntaxin encoding residues 4-266 was expressed and purified as a His₆-tagged protein [45]. GST-complexin II was prepared as previously described [46].

Binding of proteins to immobilised GST-fusion proteins. Extracts of bovine brain were prepared as described previously [47]. Glutathione-Sepharose beads (GH Healthcare Biosciences, UK) were washed 3 times with binding buffer (150 mM potassium acetate, 1 mM MgCl₂, 20 mM Hepes, pH 7.4, 0.05% Tween 20). Beads were incubated with 140 mg of E. coli protein extract per ml of a 50% slurry of beads in binding buffer for 1 h, then washed 3 times. All incubations were carried out at 4°C with rotation. GST-recombinant proteins were added to the beads to a final concentration of 2 µM in a total volume of

200 μ l and incubated for 30 min. 200 μ l of bovine brain extract was added to the beads and incubated for 2 h. Beads were washed using CytoSignal spin filters (CytoSignal, Irvine, CA) initially with 2 washes of buffer containing 1 mg/ml gelatin then with 3 washes of buffer containing 5% glycerol. Proteins were eluted from the beads by the addition of 100 μ l of SDS-dissociation buffer for 15 min. Samples were separated on a 12.5% SDS-PAGE gel and transferred to nitrocellulose paper for immunoblotting with mouse monoclonal anti-Rab3A, SNAP-25, Munc18 (BD Biosciences, Oxford, UK), Hsc70 and syntaxin 1 (Sigma, Poole, UK), or rabbit anti-VAMP2 (a gift from Prof M Takahashi, University of Tokyo, Japan).

For analysis of the binding of His₆-tagged syntaxin 1A (4-266) to immobilised GST-Munc18-1 proteins, Gluathione-Sepharose beads were washed 3 times with binding buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 1mM DTT, 2mM DTT, 2 mM MgCl₂, 0.5% Triton-X-100) and incubated with E. coli extract. GST recombinant proteins were incubated with beads at a final concentration of 1 μ M and in a final volume of 100 μ l for 30 min. His₆-syntaxin 1A (4-266) at a final concentration of 1 μ M was added to the GST-protein bound beads for 2 h. Beads were washed using CytoSignal filters and samples eluted, run on SDS-gels and transferred for immunoblotting as described above using syntaxin monoclonal antibody at 1:100,000 dilution. For analysis if binding of His₆-tagged Rab3A this was incubated with immobilised GST Munc18-1 proteins at a final concentration of 1 μ M and in a final volume of 100 μ l for 30 min. The guanyl nucleotide dependency of binding was examined by performing binding studies in the presence of either 50 μ M GDP β S or GTP γ S with 1 mM ATP.

Amperometric Recording and Analysis

Electrochemical recording conditions were as previously described [45].

Briefly, cells were incubated in bath buffer (139 mM potassium glutamate, 0.2 mM EGTA, 20 mM PIPES, 2 mM ATP and 2 mM MgCl₂; pH 6.5) and a 5 μ m

diameter carbon fiber electrode was positioned in direct contact with a targeted adrenal chromaffin cell. Exocytosis from cells was stimulated with pressure ejection of a permeabilization/stimulation buffer (139 mM potassium glutamate, 20 mM PIPES, 5 mM EGTA, 2 mM ATP, 2 mM MgCl₂, 20 μM digitonin and 10 μM free calcium; pH 6.5) from a glass pipette oriented on the opposite side of the cell. Amperometric responses were monitored with a VA-10 amplifier (NPI Electronic, Tamm, Germany) and saved to computer by Axoscope 8 (Axon Instruments). Individual experiments were conducted in parallel on control (untransfected) and transfected cells from the same cell batch. Transfection was identified by expression of EGFP as previous studies have established a 95% rate of co-expression. Amperometric data were exported from Axoscope and subsequently analyzed using Origin (Microcal Software, Northampton, MA). Spikes were selected for analysis, provided that the spike amplitude was greater than 40 pA, to remove any confounding effects of including those fusion events not occurring directly underneath the carbon fibre end. All of the data are shown as mean ± S.E. Significance was tested with nonparametric Mann-Whitney U-tests.

Growth Hormone Release Assay. The effect of Munc18-1 proteins on exocytosis was analysed using the release of expressed human growth hormone (GH) from PC12 cells. Transient transfections of GH (pXGH5) and pcDNA3 vector alone or containing wild-type Munc18, Munc18-E466K or Munc18-E466K/R39C were made using LipofectAmine 2000 with using 1 μg of pXGH5 and 1 μg test plasmid per well of a poly-D-lysine coated 24-well plate (BD Biocoat, VWR, UK), containing 0.35 × 10⁶ cells. Seventy-two hours later cells were washed in Krebs buffer (145 mM NaCl, 5mM KCl, 1.2 mM NaH₂PO₄, 10 mM glucose, 20 mM Hepes, pH 7.4, 1.3 mM MgCl₂ and 3 mM CaCl₂) and challenged for 15 minutes with either Krebs alone or Krebs containing 300 μM ATP. Cells were solubilised with PBS containing 0.5% Triton-X-100 and both secreted and intracellular GH content was assayed using a human GH ELISA (Roche Diagnostics, Lewes, UK).

Immunostaining. PC12 cells were seeded onto 24-well plates at $\sim 4 \times 10^5$ cells per well on Biocoat poly-D-lysine-coated glass cover-slips (BD Biosciences, Bedford, MA, USA) and cells allowed to adhere overnight. Prior to transfection, the growth medium was replaced with 400 μ l/well Optimum 1 media (Invitrogen, Paisley, UK). Transfection mixes consisting of 3 μ l of Lipofectamine 2000 reagent (Invitrogen) per μ g plasmid in 100 μ l Optimum 1 media were prepared and incubated at room temperature for 20 min before being added dropwise to the cells. Five hours following their addition, transfection mixes were removed and replaced with normal growth medium. The transfected cells were washed in PBS and then fixed in 0.5ml 4% (v/v) paraformaldehyde for 30 min at room temperature. Coverslips were then washed twice in PBS, and exposed to anti-Munc18 (Calbiochem, CA, USA) at 1:400 dilution in PBS containing 0.3% BSA and 0.1% Triton-X-100 (PBT) for 1h. and then washed three times with PBT. The coverslips were exposed to appropriate Alexa Fluor 594-conjugated secondary antibodies at a 1:100 dilution in PBT for 1h. After a final washing step, coverslips were air dried prior to mounting on glass slides using Mowiol.

Confocal microscopy and quantification. Confocal microscopy following immunostaining was carried out on a Leica TCS-SP microscope (Leica Microsystems, Heidelberg, Germany) using a 63x oil immersion objective with a 1.4 numerical aperture and pinhole set to airy1. EGFP-containing constructs were excited using a 488nm laser and light collected between 500-550nm. Alexa Fluor 488 was excited using a 488nm laser and light collected between 500-550nm. Alexa Fluor 594 was excited using a 594nm laser and light collected between 625-675nm. Regions of interest were drawn around the outside of each cell, immediately beneath the cell periphery, and around the nucleus. Peripheral fluorescence was calculated by subtraction of cytoplasmic and nuclear fluorescence from the outer region of interest. Cytosolic fluorescence was calculated by subtraction of nuclear fluorescence. Ratios of

peripheral to cytosolic fluorescence were calculated on the basis of the mean fluorescence per pixel in the respective regions rather than total fluorescence to avoid problems arising from variations in the size of the selected regions of interest. To determine overall expression levels of overexpressed Munc18-1 and its mutants or EGFP-Rab3A, the mean pixel intensity over the whole cell was determined using Image J.

Results

The E466K mutation increases the interaction of Munc18-1 with Rab3A

Expression of Munc18-1 bearing the E466K mutation (Figure 1a) in adrenal chromaffin cells increases the number of exocytotic events [42]. The E466K mutation does not, however, affect the expression of Munc18-1 in transfected cells or its binding to the known interacting proteins syntaxin1, granuphilin, Mint1 or Mint2 [42]. In order to investigate the molecular basis of the E466K gain of function mutation seen in chromaffin cells we set out to search for new or increased protein interactions with Munc18-1 E466K. The R39C mutation was also introduced on the E466K background as this has been shown to inhibit the binding of Munc18-1 to the closed form of syntaxin1 [23, 42] and would allow us to probe the importance of this mode of interaction with syntaxin. In the crystal structure of the Munc18-1/syntaxin 1A complex [28], the R39 is present in a cleft within which the closed form of syntaxin1A resides and makes a direct contact with syntaxin 1A (Figure 1a). As expected from the structure, the R39C mutation substantially reduces direct binding to GST-syntaxin 1A constructs in assays in which the binding is predominantly binding to the closed syntaxin 1A [23, 42]. As seen for the R39C mutation alone the Munc18-1 protein with the double mutation bound markedly less His6-syntaxin 1A (4-266) in a direct binding assay with a 4.6 fold reduction in binding compared to wild-type Munc18-1 (Fig 1b).

GST-fusion proteins for wild-type, E466K and R39C/E466K Munc18-1 were initially used in pull-down assays in which proteins bound from a brain extract were detected using western blotting to search for new or increased protein interactions with E466K or the double mutant. In initial screens, no binding or differences for the mutants compared to wild-type Munc18-1 were seen for amisyn, Csp, Doc2, NSF, β -adaptin, sec6, sec8, SNAP-25, synapsin, synaptotagmin I or syntrophophysin. Increased binding by E466K was observed, however, for Rab3A (Figure 1c). In such experiments, equal amounts of the Munc18-1 proteins were bound to the glutathione-Sepharose beads (Figure 1c). An increased level of binding of Rab3A from brain extract compared to wild type Munc18-1 was also found with the double mutant R39C/E466K protein (Figure 1c,d) indicating that this was not dependent on Munc18-1 binding to closed syntaxin 1A. In independent pull-down experiments, increased binding of Rab3A to E466K was always observed with low but detectable binding to wild-type Munc18-1 over GST controls also seen in 4 of 6 experiments. From quantification of western blotting using recombinant Rab3A standards it was estimated that the Rab3A concentration in the brain extracts was around 80nM and 5-8% of the input Rab3A became bound to immobilised R39C/E466K Munc18-1 in these experiments. Many other proteins tested from brain extracts did not bind to wild-type or mutant Munc18-1 but another novel interaction was identified. Specific binding of Sec8 was observed but this was not consistently different for wild-type vs mutant Munc18-1 (Figure 1c). Specificity of the Rab3A interaction with Munc18-1 was suggested by the finding that in contrast to Rab3A, Rab5 was only detected at background levels in all incubations (Figure 1d). As an additional control, the binding of the chaperone protein Hsc70 was tested as this often binds to recombinant proteins. Only low levels of Hsc70 were detected bound to wild type Munc18-1 and the level was not increased by the double mutation (Figure 1d), indicating that these mutations did not result in misfolding of the protein.

In order to assess whether Munc18-1, Rab3A and syntaxin 1 associate in the same complex, GST-syntaxin 1A(4-266) was incubated with brain extract in pull down experiments to see if both native Munc18-1 and Rab3A could be found associated with the syntaxin construct (Fig 2a). Munc18-1 was efficiently pulled-down by GST-syntaxin 1A(4-266). In contrast, although Rab3A was readily detected in the brain extract no Rab3A was detectably bound to GST-syntaxin 1A(4-266). To check if this was the case for native syntaxin in SNARE complexes we also used GST-complexin II in pull-downs as this binds efficiently only to the assembled SNARE complex [48]. As a control for this experiment we used the R59H mutation in complexin II [46]. This residue is involved in direct interaction with VAMP in the SNARE complex [49] and the mutation abolishes binding of SNARE proteins in the pull down assay (Figure 2b). Munc18-1 was found specifically associated along with the SNARE complex in the GST-complexin II pull down whereas other control proteins were not detected. In contrast, no Rab3A was detected bound to GST- complexin II (Figure 2b) even though it was readily detected in the input brain extract. These data suggest that Rab3A can not bind to Munc18-1 when it is bound to closed syntaxin (GST-syntaxin1A(4-266)) or the SNARE complex.

The binding of Rab3A to Munc18-1 could have been direct or due to indirect interactions with complexes containing other proteins in the brain extract. To assess this possibility, pull-down assays were performed using recombinant Rab3A. For comparison pull-downs were also carried out using the known Rab3A effector Noc2 which binds Rab3A in vitro [44]. In this direct binding assay both wild-type and R39C/E466K Munc18-1 appeared to bind Rab3A equally well while little binding was detected to control GST (Figure 3a). The difference compared to the assay with brain extract may be due to Rab3A being present at a higher concentration (1 μ M) than in brain extracts. The level of Munc18-1 binding to Rab3A was similar to that seen with GST-Noc2 and, from quantification of Western blots of bound Rab3A compared to the input

Rab3A, a stoichiometry of around 0.7 moles of Rab3 per mole of Munc18-1 was found. In contrast to the situation with Noc2, where binding to Rab3A was 5.5 fold higher in the presence of GTP γ S compared to GDP β S, binding of wild-type or R39C/E466K Munc18-1 to Rab3A was not dependent on the guanine nucleotide-loaded status of Rab3A (Figure 3b).

The stimulation of exocytosis by E466K is dependent on binding to closed syntaxin.

Expression of Munc18-1 bearing the E466K mutation in adrenal chromaffin cells increased the rate of exocytotic events measured using carbon-fibre amperometry on single transfected cells [42]. Unlike studies based on very high over-expression of wild-type Munc18-1 in immature chromaffin cells using viral constructs [22], we and others have not observed such a stimulatory effect of the wild-type protein in studies on chromaffin [23] or PC12 cells [50, 51]. We used our well characterised amperometric assay [52] in order to test whether the specific stimulation of exocytosis by E466K required high-affinity binding to closed syntaxin 1, we examined the effect of the additional R39C mutation. Expression of Munc18-1 with only the single R39C mutation was previously found to have no effect on the number of exocytotic events measured by amperometry [23]. Example traces are shown immediately after stimulation and the onset of amperometric spikes which were largely undetectable in unstimulated cells (Figure 4a). Due to variability between individual cells and recording electrodes, data were derived from at 17-37 cells per condition and control recordings were taken in parallel from non-transfected cells in the same dishes with the same carbon-fibre electrodes [52]. The spike frequency was expressed as a percentage of the appropriate controls for each Munc18-1 protein. We have previously established that transfection with empty plasmid and EGFP does not itself affect exocytosis [53]. Expression of Munc18-1 E466K increased the frequency of amperometric spikes in chromaffin cells measured over a 210 second period after stimulation (Figure 4c). In order to test whether the specific stimulation of exocytosis by

E466K required the ability of Munc18-1 to bind to closed syntaxin 1, we examined the effect of the additional R39C mutation. Introduction of this second mutation on the E466K background abolished the stimulatory effect of E466K on exocytosis (Figure 4a-c).

An alternative assay was used with PC12 cells based on release of newly synthesised growth hormone (GH) expressed from a co-transfected plasmid. Cells were stimulated with ATP and release of GH above basal levels was determined. In these experiments over-expression of wild type Munc18-1 did not affect evoked GH release but GH release was significantly increased by 20% following expression of E466K (Figure 4d). Again this stimulation apparently required syntaxin binding as the double mutant was without effect despite it being expressed in the PC12 cells. The difference in the effect of E466K versus R39C/E466K was not due to differences in expression levels of the proteins (see below).

Expression of the Munc18-1 E466K mutant increases secretory granule recruitment to the cell periphery.

Rab3A is associated with secretory granules but not other organelles in PC12 cells and we have shown that Rab3A cycles rapidly between the granule and cytosol under resting conditions [54]. In order to examine whether interaction between the Munc18-1 E466K mutant and Rab3A would affect Rab3A cycling, PC12 cells were transfected to express both the Munc18-1 mutant and EGFP-Rab3A and the cycling of EGFP-Rab3A was assessed by analysis of fluorescence recovery after photobleaching as previously described [54]. Following the photobleaching of a small region of the cell, recovery of EGFP-Rab3A fluorescence on granules did not differ in cells with or without expression of Munc18-1 E466K (Figure 5a). In these experiments EGFP-Rab3A labelled granules appeared to be distributed throughout the control cells consistent with only a proportion (~30%) of the granules being docked in the

particular PC12 cells that we used. We noticed, however, that in these experiments more labelled granules appeared to be present at the cell periphery in cells expressing Munc18-1 E466K (Figure 5b). Previous work has implicated both Munc18-1 [22] and Rab3A [6, 55] in granule tethering at the plasma membrane or docking in neuroendocrine cells and so we investigated whether the E466K gain-of-function mutation could have its effect through increasing granule recruitment. Cells were transfected to express a Munc18-1 protein and EGFP-Rab3A and were fixed and immunostained with anti-Munc18 antiserum. Cells over-expressing Munc18-1 following transfection can be detected by immunostaining due to higher fluorescence intensity compared to non-transfected cells [42]. This experiment was based on using a carefully titrated concentration of anti-Munc18 antiserum that does not detect the endogenous Munc18-1 in the cells but only that due to over-expression (Figure 5b). In the initial analysis of EGFP-Rab3A distribution, cells were selected as high Munc18-1 expressing cells based on immunostaining and compared to cells separately transfected with empty vector. Under standardised imaging conditions on the same day, regions of interest were drawn around the cell periphery and the nucleus (Figure 5c) and mean pixel intensity at the cell periphery and that in the rest of the cell (minus the nucleus) was determined and expressed as a ratio. We determined mean pixel intensity rather than total fluorescence in the defined areas to rule out error due to the size of the compartments within the regions of interest. Over-expression of wild-type Munc18-1, which did not increase exocytosis, did not affect the fluorescence ratio compared to low expressing cells. In contrast, in Munc18-1 E466K expressing cells, there was a significant increase in peripheral fluorescence intensity for the granule associated EGFP-Rab3A compared to controls (Figure 5d). In contrast, the R39C/E466K double mutant was without effect on granule distribution. The lack of effect of R39C/E466K was not due to a lower expression level as the levels of wild-type, E466K and R39C/E466K Munc18-1 were very similar and also expression of EGFP-Rab3A was similar in cells expressing each of the Munc18-1 constructs (Figure 5de. Interestingly,

therefore, the increase in peripheral granules following expression of E466K appeared to require the ability of Munc18-1 to interact with closed syntaxin 1.

In a subsequent alternative analysis, of an independent experiment, low Munc18 expressing cells, with fluorescence levels comparable to untransfected cells, were selected to act as controls compared to high Munc18-1 expressing cells on the same coverslips to rule out variations between coverslips. Very similar findings were obtained in this analysis with E466K but not wild-type or R39C/E466K Munc18-1 increasing the proportion of labelled granules at the cell periphery in high expressing cells (Figure 6a,b). Unlike Rab3A, Rab27A does not cycle on and of secretory granules [54]. In an additional experiment using EGFP-Rab27A instead of EGFP-Rab3A to label the secretory granules, similar findings were obtained (Figure 6c,d) indicating the reproducibility of the effect on granule distribution.

In neuroendocrine cells, the cortical actin network is able to act as a barrier to prevent granules reaching the plasma membrane [56]. It has been suggested that, in addition to direct effects on granule tethering and docking, another mechanism by which Munc18-1 could increase the number of tethered granules is by disrupting cortical actin [57]. This was based on the observation of gross changes in the cortical actin in chromaffin cells in which Munc18-1 levels had been modified. We examined the effect of expression of Munc18-1 E466K on cortical actin visualised using staining with phalloidin. No difference was seen in the overall organisation of the peripheral actin, or in the intensity of actin staining in the cell cortex, ruling out this indirect mechanism for the redistribution of granules to the cell periphery (Figure 7).

Discussion

One regulated step in vesicle exocytosis is the recruitment of vesicles to the plasma membrane. This has been characterised as due to vesicle tethering or

docking although the two processes have been difficult to define. The data presented here addresses two key questions regarding Munc18-1 function. First, what is the basis for Munc18-1 being able to stimulate vesicle docking alongside Rab3? Second, is the Mode 1 interaction of Munc18-1 with closed syntaxin 1 functionally relevant? It has been shown in a number of systems that Rab proteins acting through their effectors can mediate vesicle tethering or docking [5]. The additional requirement for Munc18-1 for vesicle docking and tethering suggests a possible link between Munc18-1 and Rabs. Significantly, recent work has established that increased secretory granule recruitment due to Rab3 over-expression requires Munc18-1 [16]. The molecular basis for this was not explored, however. One possibility is that this could involve interaction of Rab effectors with Munc18-1 such as that described between granuphilin and Munc18-1 [58]. We now provide an alternative explanation by showing that Munc18-1 can interact directly with Rab3A. While we detected novel interactions of Munc18-1 with Rab3A and Sec8 only the binding of Rab3A was increased by the E466K gain of function mutant. Expression of Munc18-1 E466K increased exocytosis in adrenal chromaffin and PC12 cells. In squid Sec1 [59] and rat Munc18-1 [28], E466 is in domain III of the protein and is present in an exposed residue on a surface loop suggesting that this residue could potentially influence protein interactions. A peptide corresponding to the loop region inhibits neurotransmitter release in the squid giant synapse [60] and Munc18c-dependent translocation of glut4 vesicles to the plasma membrane in adipocytes [61] supporting the functional significance of the loop and suggesting that the E466K mutation could influence crucial protein interactions involving this region. We set out, therefore, to search for any protein interactions that are affected by the E466K mutation and found a specific increase in Rab3A binding from brain extracts to Munc18-1 E466K. This interaction was due to direct binding of Rab3A to Munc18-1. Expression of the E466K gain of function mutant was also found to increase the proportion of secretory granules at the cell periphery following expression in PC12 cells consistent with an increase in granule tethering or docking. The method used

was confocal microscopy to quantify peripheral fluorescence intensity in cells expressing EGFP-tagged Rab3A or Rab27A which associate preferentially with newly synthesised granules [54]. The same approach has also been used by others and shown to detect changes in granule recruitment (interpreted as increase docking), following manipulation of granuphilin [8], rabphilin [7], Rab3A or Rab27A [6] with expression with granules being detected by expression of tagged granule proteins. This approach is valid as the newly synthesised granules which are labelled are those that form the most readily releasable pool [62]. We have observed the same phenomenon of increased granule redistribution to the cell periphery following granuphilin over-expression using our assay in PC12 cells (unpublished observations) as previously described for insulin secreting cells [8].

Knock-out studies have shown that Munc18-1 is normally required for vesicle tethering and docking [21, 22]. Wild-type Munc18-1 can bind Rab3A as well as the E466K mutant in direct binding assays. Since Munc18-1 is required for Rab-stimulated granule recruitment [16] this suggests that one of the physiological functions of Munc18-1 may be to link Rab-dependent tethering with SNARE dependent docking and fusion. The E466K mutation increased Rab3A binding in brain extracts compared to wild type Munc18-1 to which the binding was low, but using higher Rab3A concentrations in a direct binding assay, it was possible to show that wild type Munc18-1 also binds Rab3A directly. One possibility is that the E466K mutation removes a mechanism that normally limits the Munc18-1/Rab3 interaction within cells or extracts or alternatively that it increases the overall affinity of the interaction with Rab3A and this leads to the gain of function. The Sly1-20 mutation on which E466K was modelled was discovered as a suppressor of a loss of function mutation in the yeast Rab protein Ypt1 suggesting that it by-passed the requirement for Ypt1 [43]. It may seem counterintuitive for the E466K mutation to enhance granule recruitment and exocytosis via an increased interaction with Rab3A.

These two situations can be reconciled, however, as more detailed analysis of the Sly1-20 mutation suggested that it allowed another closely related Rab protein, Ypt6p, to substitute for Ypt1p [63] consistent with altered Rab binding properties due to the mutation. It is possible that the loop in domain III of Munc18-1 and other SM proteins could form a Rab-binding site.

Munc18-1, Rab3A and syntaxin 1 were not all found together in the same complex but the functional effects of the E466K mutation required the ability of Munc18-1 to interact with syntaxin based on the effect of the R39C mutation. We have used the R39C mutation to probe the importance of the Mode 1 Munc18-1 interaction with the closed form of syntaxin 1. This mutation disrupts binding to syntaxin 1 constructs *in vitro* [23, 42] but has no effect on the ability of Munc18-1 to stimulate fusion in a manner which is dependent on Mode 2 and 3 binding [25]. We have observed no differences between E466K and R39C/E466K in their binding properties apart from the reduced binding of closed syntaxin 1 to the double mutant. For, example, both proteins bind Rab3A and Sec8. In addition, low levels of binding of the Hsc70 chaperone protein from brain extract to the double mutant showed that it was unlikely to be misfolded compared to wild type Munc18-1. Finally, we confirmed that both mutants were expressed to the same extent as wild type protein after transfection. It is notable that the effects of the E466K mutant on exocytosis and vesicle distribution were both lost when the R39C mutation was introduced suggesting a dependence on the Mode 1 interaction with closed syntaxin. Despite the finding that the E466K mutant required syntaxin binding for its functional effect, it appeared that Munc18-1, Rab3A and syntaxin 1A may not stably assemble into the same complex. One speculative possibility is the existence of a sequential series of interactions in which Munc18-1 initially interacts with Rab3A and is then handed on during the process of tethering as the tethered state is stabilised through the closed syntaxin interaction. Munc18-1 would thus form a transient bridge between plasma membrane syntaxin and vesicle Rab3 to allow tethering before the alternative modes of interaction of

Munc18-1 with syntaxin and the SNARE complex come into play in later stages leading to membrane fusion.

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Stage 2(a) POST-PRINT

Figure legends

Figure 1. Analysis of Munc18-1 and Munc18-1 mutant binding to Rab3A and syntaxin 1A. (a) Structure of the Munc18-1/syntaxin 1A complex showing the position of the two Munc18-1 residues (R39 and E466) mutated in this study. Syntaxin 1A is shown in green. (b) GST, GST-Munc18-1 or GST-Munc18-1 R39C/E466K were incubated with His-tagged syntaxin 1A (4-266) and bound syntaxin detected by western blotting with anti-syntaxin 1 antiserum. (c) Bovine brain extract was incubated with equal concentrations of immobilised GST, GST-Munc18-1 or GST-Munc18-1 with the indicated mutations and bound proteins were analysed by western blotting with anti-Rab3A or anti-Sec8. One fifth of the bound material was run in each lane and a sample of the input brain extract is shown that represents 5% on the total input in the incubations. (d) An alternative pull-down experiment in which a brain extracts were incubated with GST, GST-Munc18-1 or GST-Munc18-1 R39C/E466K and the bound proteins analysed by western blotting with anti-Rab3A, anti-Rab5 or anti-Hsc70. In this experiment a low level of binding of Rab3A to wild-type Munc18-1 was detected.

Figure 2. Rab3A does not associate with Munc18-1 bound to syntaxin or the native SNARE complex. (a) Brain extract was incubated with GST or GST-syntaxin 1A(4-266) and the bound proteins analysed by western blotting with anti-Munc18-1 and anti-Rab3A. (b) Native SNARE complexes pulled-down from brain extract using GST-complexin II. Bovine brain extract was incubated with GST, GST-complexin II (CPXII) or GST-complexin II (R59H) and bound proteins were analysed by western blotting with antisera as shown. All the incubations were run at the same time and carried out in duplicate. Unlike other proteins (SCAMP, dynamin, Csp, Hsc70), Munc18-1 was specifically bound to complexin. The need for SNARE complex association for Munc18-1 binding was shown by its abolition by the R59H mutation in complexin II that inhibits

SNARE complex binding to complexin. Despite the presence of Munc18-1, no binding of Rab3A was detected in the pull downs.

Figure 3. Direct binding of Rab3A to Munc18-1. (a) GST, GST-Munc18-1 proteins or GST-Noc2 were incubated with Rab3A and bound Rab3A was detected by western blotting. The final concentration of Rab3A in the assay was 1 μ M and samples of the input at various concentrations are shown. (b) GST, GST-Munc18-1 proteins or GST-Noc2 were incubated with Rab3A in the presence of GDP β S or GTP γ S and bound Rab3A was detected by western blotting.

Figure 4. The increase of evoked exocytosis by Munc18-1 E466K in adrenal chromaffin and PC12 cells requires Mode 1 binding to closed syntaxin 1. (a) Typical amperometric responses from an untransfected adrenal chromaffin cell (control) and a cell transfected with the Munc18-1 E466K mutant after addition of digitonin and Ca²⁺ to elicit exocytosis. (b) Typical amperometric responses from an untransfected adrenal chromaffin cell (control) and a cell transfected with the Munc18-1 R39C/E466K double mutant. (c) Analysis of the frequency of exocytotic fusion events (amperometric spikes) in cells expressing the indicated Munc18-1 mutants over a 210 second period following stimulation expressed as a percentage of values from the corresponding control cells in the same experiment. The data are shown as means \pm SEM and are derived from 33 E466K expression cells and 37 control cells and from 17 R39C/E446K expressing cells and 17 corresponding control cells. (d) PC12 cells were co-transfected with plasmids encoding growth hormone and control empty vector (pcDNA3) or plasmids encoding the indicated Munc18-1 proteins. Cells were treated with or without 300 μ M ATP for 15 min. GH release was calculated as a percentage of total cellular GH and is shown as evoked release after subtraction of basal release values in the absence of stimulation.

Figure 5. Expression of Munc18-1 E466K does not affect the dynamics of Rab3A cycling but redistributes secretory granules to the cell periphery. (a) PC12 cells were transfected to express EGFP-Rab3A alone (control) or with Munc18-1 E466K. During live cell imaging, selected regions in individual cells were bleached with high intensity laser (arrow), and the fluorescence recovery of these areas was followed over time. The data are shown as mean \pm SEM for 10 control cells and 7 cells expressing Munc18-1 E466K. The data were corrected for general photo-bleaching for each cell at each time point and normalised by setting the initial fluorescence value for each cell to 100. No difference was seen between the two groups of cells. (b) PC12 cells were transfected to express EGFP-Rab3A along with wild-type Munc18-1 or Munc18-1 mutants and immunostained with anti-Munc18-1. Cells were co-transfected with 0.9 μ g of Munc18-1 plasmid or empty vector (pcDNA3) and 0.1 μ g of EGFP-Rab3A plasmid. Highly expressing transfected cells were identified due to higher level of immunostaining with anti-Munc18-1 and expression of EGFP-Rab3A. Nearby untransfected cells with endogenous levels of Munc18-1 expression are indicated by asterisks and are not detectably stained above background at the concentration of antiserum used. (c) For quantification of EGFP-Rab3A distribution, regions of interest were drawn around the cell periphery and nucleus as shown in the example which is a cell expressing EGFP-Rab3A and co-transfected with empty vector (pcDNA3) from the same experiment as those cells shown expressing Munc18-1 proteins. The scale bar represents 10 μ m. (d) Quantification of the ratio of mean pixel intensity at the cell periphery compared to the cytosol for cells from each condition (n=10). (e) Quantification of the overall expression levels of Munc18-1 and its mutants and EGFP-Rab3A. The mean pixel intensity over the whole cell for anti-Munc18-1 staining and for EGFP-Rab3A fluorescence was determined in cells transfected to express both proteins (n=15).

Figure 6. Localisation of EGFP-Rab3A and EGFP-Rab27A in control and cells expressing high levels of wild-type Munc18-1 or Munc18-1 with the indicated

mutations. (a) images of EGFP-Rab3A in transfected cells. In each case cells were co-transfected with 0.8 μ g of Munc18-1 plasmid and 0.2 μ g of EGFP-Rab3A plasmid. (b) Quantification of the ratio of mean pixel intensity at the cell periphery compared to the cytosol for EGFP-Rab3A. Cells were selected as showing high or background levels of Munc18-1 expression and data for high expressing cells is shown next to low expressing cells from the same coverslips. The data are derived from 10 cells for each condition. (c) Localisation of EGFP-Rab27A in control and cells expressing high levels of wild-type Munc18-1 or Munc18-1 with the indicated mutations. In each case cells were co-transfected with 0.8 μ g of Munc18-1 plasmid and 0.2 μ g of EGFP-Rab27A plasmid. (d) Quantification of the ratio of mean pixel intensity at the cell periphery compared to the cytosol for EGFP-Rab27A. The scale bars represent 10 μ m.

Figure 7. Expression of Munc18-1 E466K does not affect the organisation of the cortical actin cytoskeleton. (a) Cells were co-transfected with plasmid encoding Munc18-1 E466K. After fixation cells were incubated with anti-Munc18-1 and actin filaments were stained with 25 nM TRITC-conjugated Phalloidin (Molecular Probes, Paisley, UK) which was visualised with a 543nm laser and light collected between 500-535nm. (b) Quantification of the ratio of mean pixel intensity at the cell periphery compared to the cytosol. Cells from the same coverslips were selected as showing high or background levels of Munc18-1. The data are derived from 10 cells for each condition. The scale bar represents 10 μ m.

Figure 1

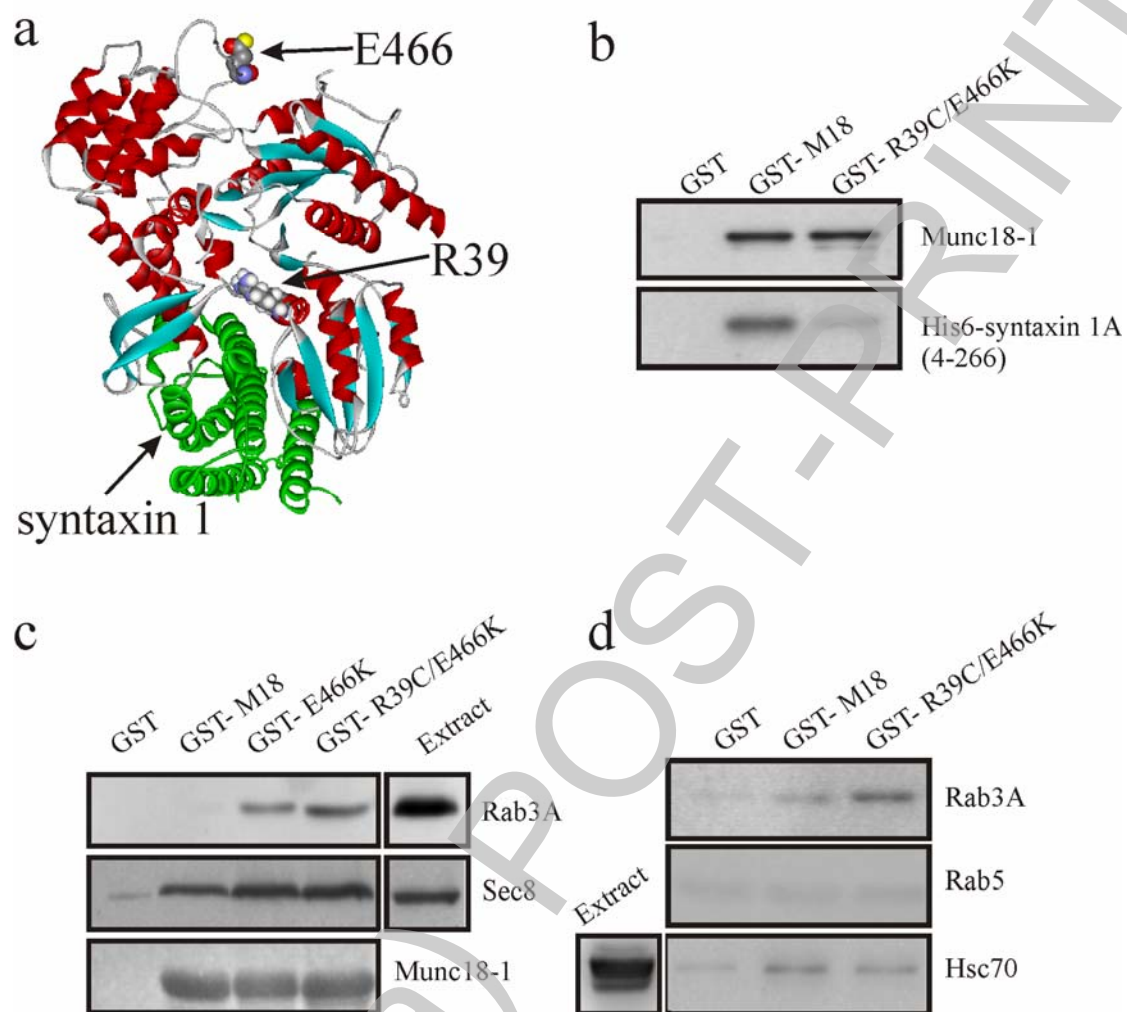


Figure 2

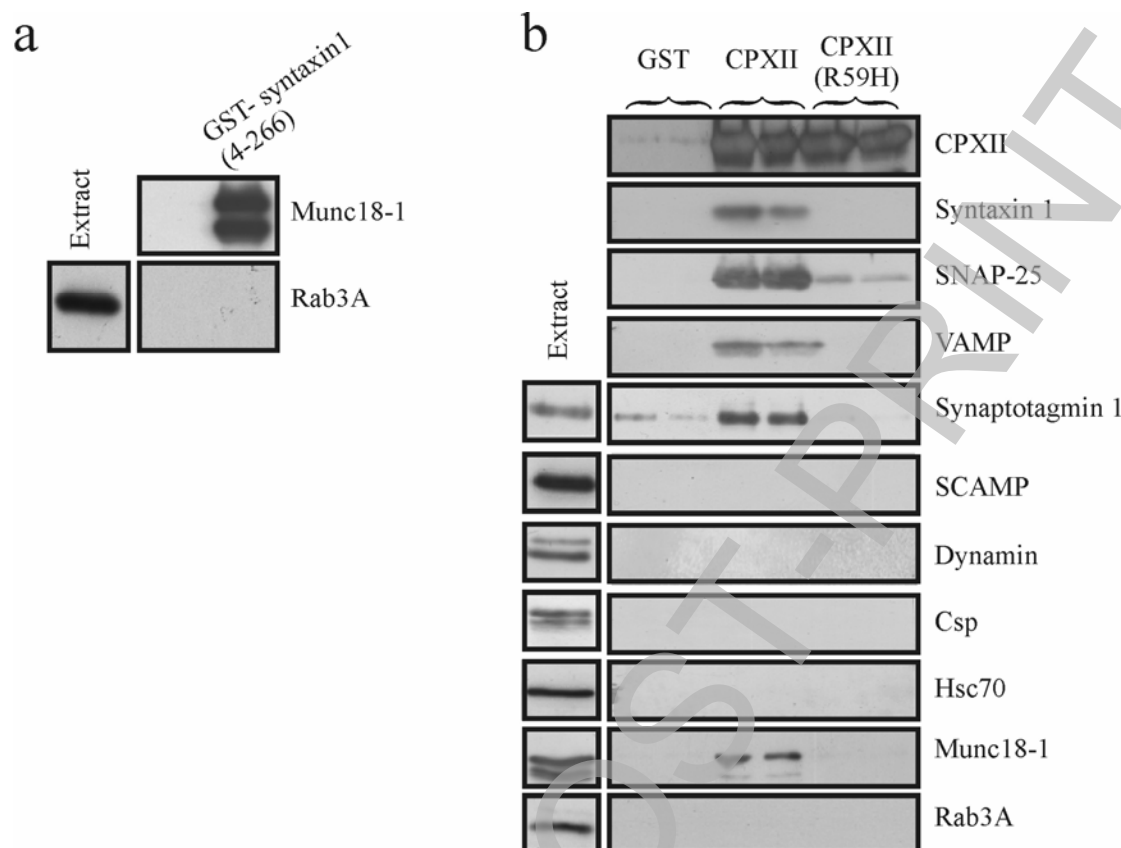
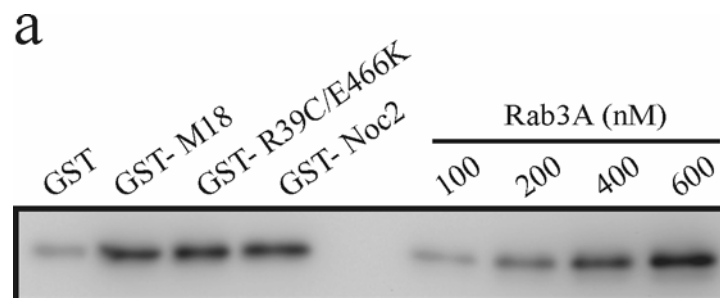


Figure 3

a



b

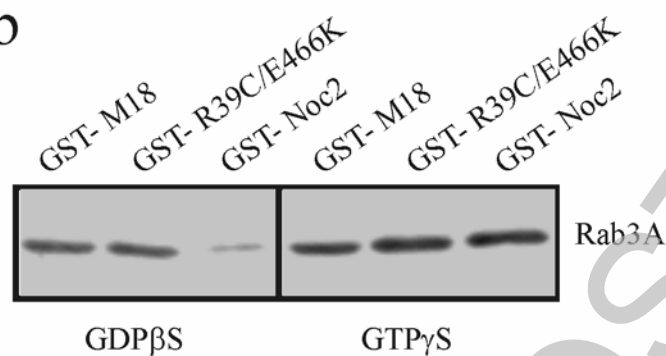


Figure 4

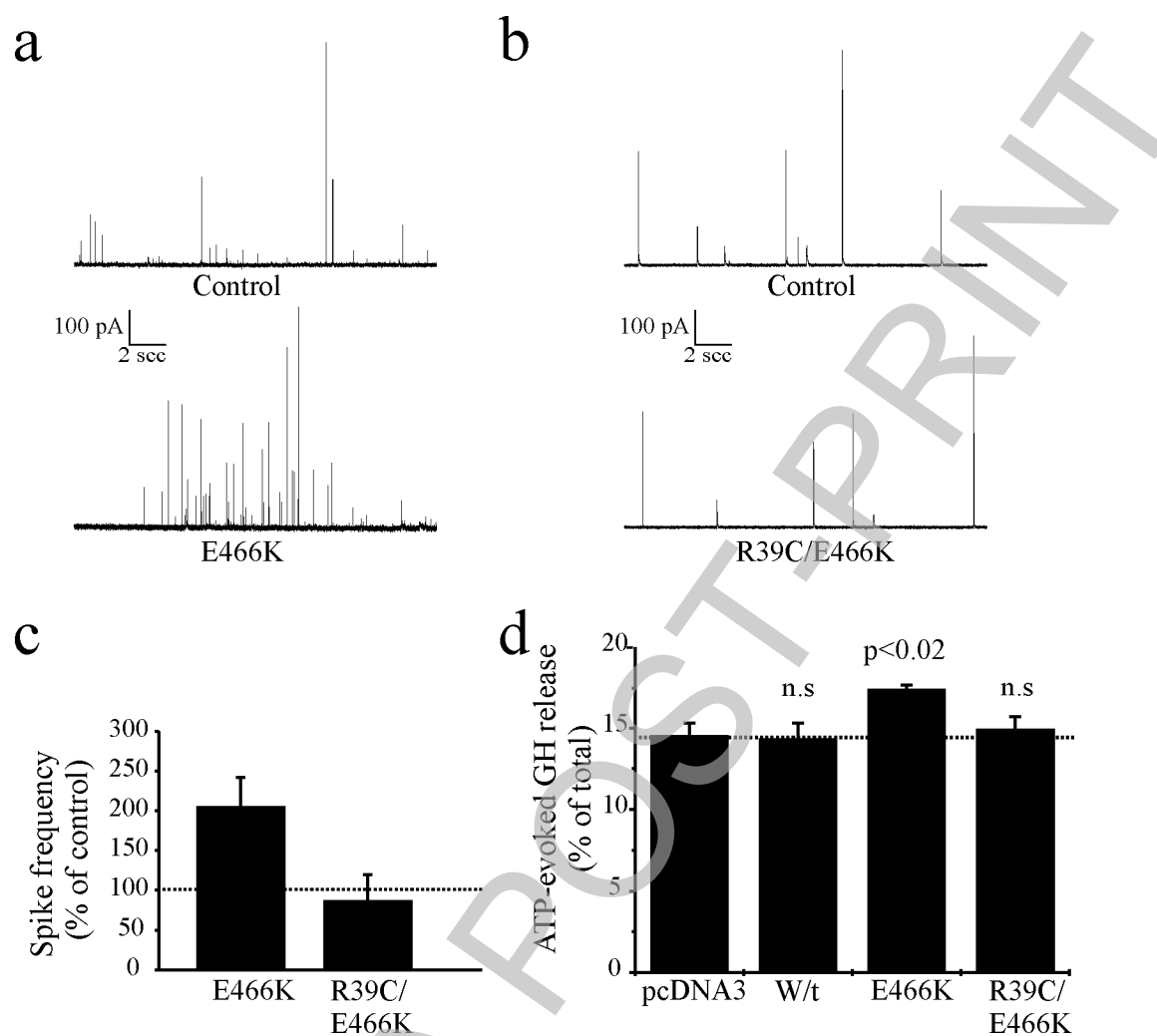
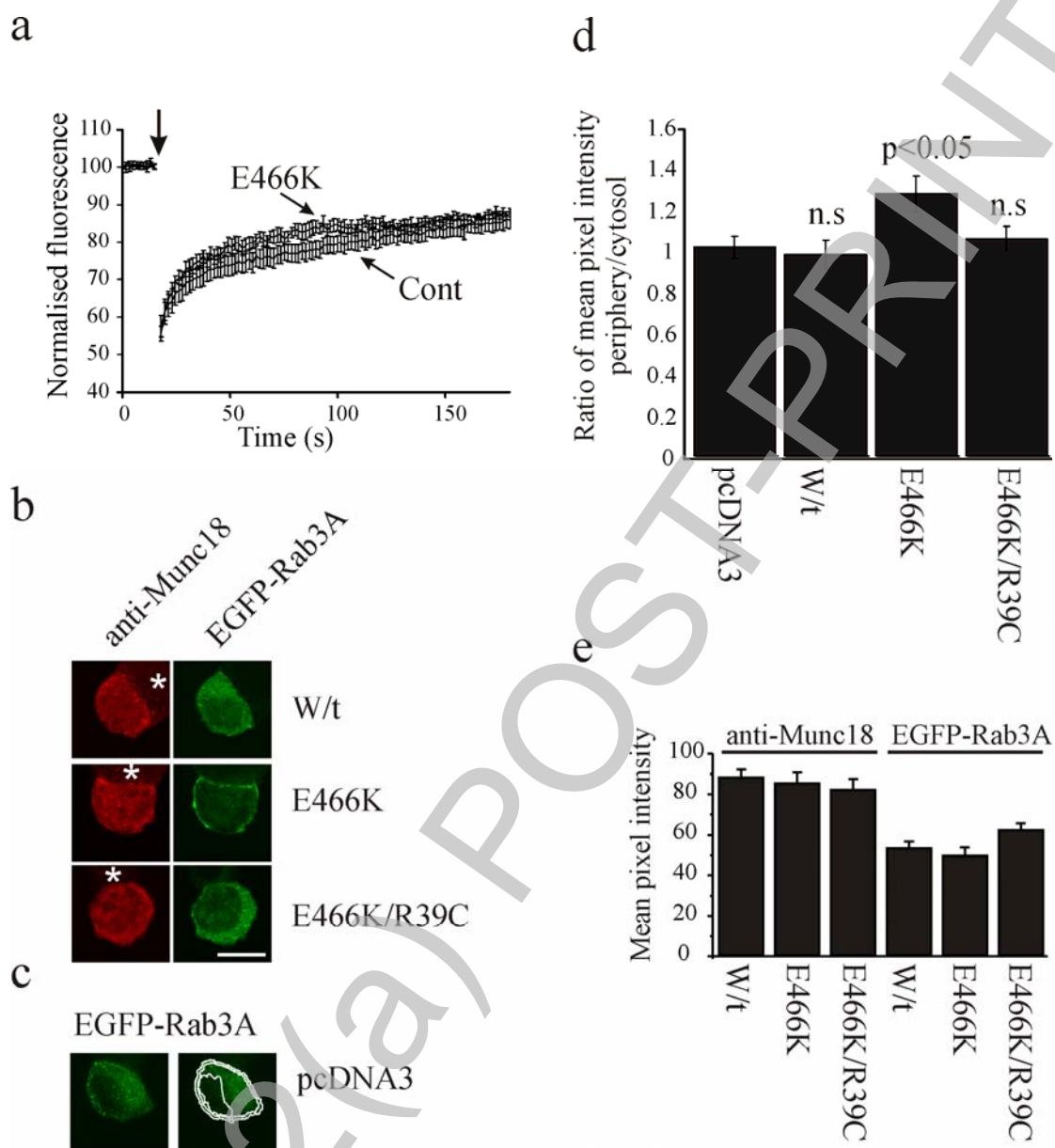


Figure 5



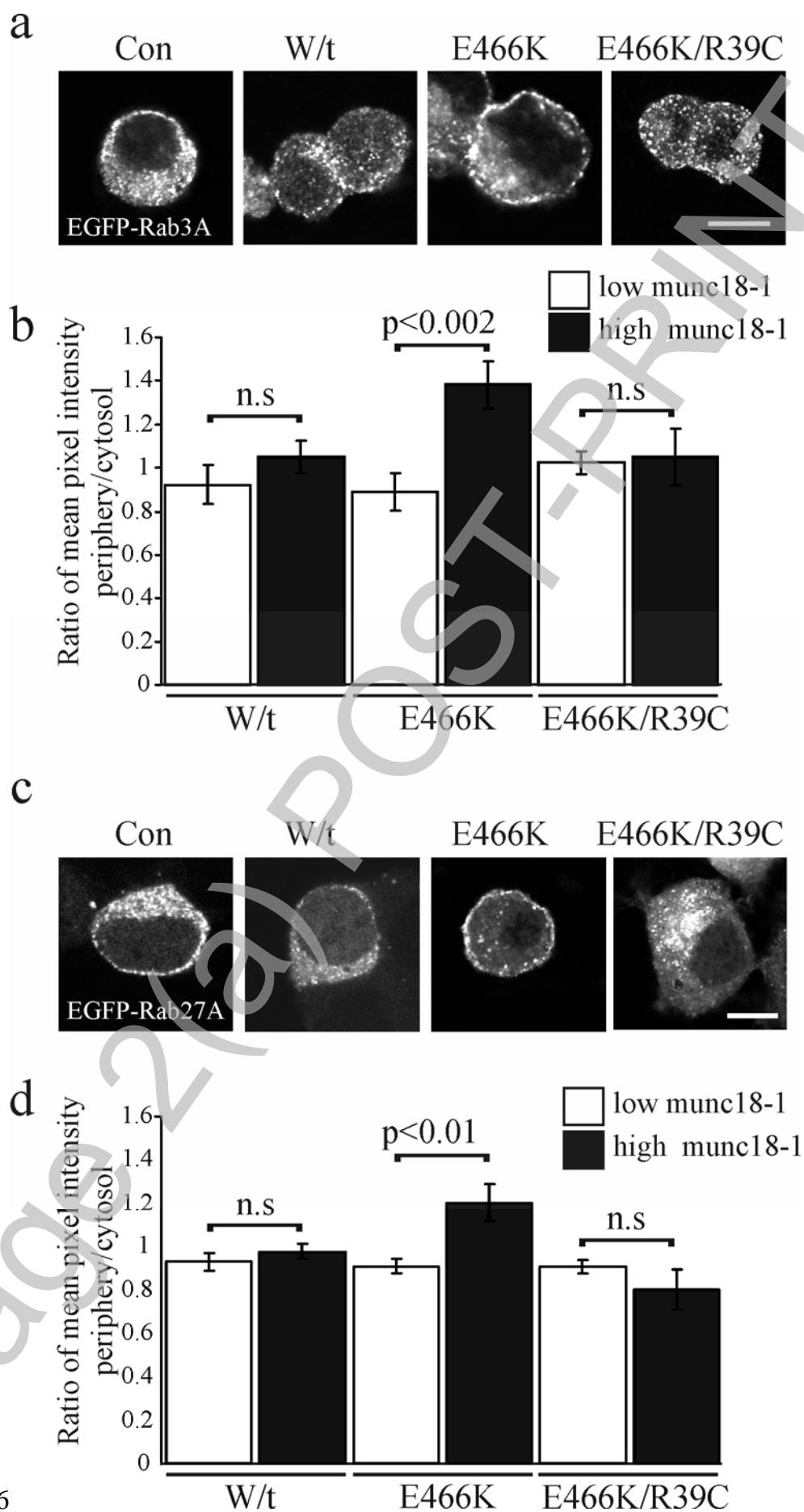


Figure 6

Figure 7

