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Binding of UNC-18 to the N-terminus of syntaxin is essential for neurotransmission in *Caenorhabditis elegans*.

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Running title: *C. elegans* syntaxin/UNC-18 interactions

Abbreviations: NEM, *N*-ethylmaleimide; NSF, NEM-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; SM, Sec1/Munc18-like; GST, glutathione *S*-transferase.

ABSTRACT

SNAP receptors (SNAREs) are widely accepted to drive all intracellular membrane fusion events. Sec1/Munc18 (SM) proteins bind to SNAREs and this interaction may underlie their ubiquitous requirement for efficient membrane fusion. SM proteins bind to SNAREs in at least 3 modes: to a closed conformation of syntaxin; to the syntaxin N-terminus; and to the assembled SNARE complex. Munc18-1 exhibits all three binding modes and recent *in vitro* reconstitution assays suggest that its interaction with the syntaxin N-terminus is essential for neuronal SNARE complex binding and efficient membrane fusion. To investigate the physiological relevance of these binding modes, we studied the UNC-18/UNC-64 SM/SNARE pair, which is essential for neuronal exocytosis in *Caenorhabditis elegans*. Mutations in the N-terminus of UNC-64 strongly inhibited binding to UNC-18, as did mutations targeting closed conformation binding. Complementary mutations in UNC-18 designed to selectively impair binding to either closed syntaxin or its N-terminus produced a similarly strong inhibition of UNC-64 binding. Therefore, high-affinity UNC18/UNC-64 interaction *in vitro* involves both binding modes. To determine the physiological relevance of each mode, *unc-18* null mutant worms were transformed with wild type or mutant *unc-18* constructs. The UNC-18(R39C) construct that is defective in closed syntaxin binding fully rescued the locomotion defects of the *unc-18* mutant. In contrast, the UNC-18(F113R) construct that is defective in binding to the N-terminus of UNC-64 provided no rescue. These data suggest that binding of UNC-18 to closed syntaxin is dispensable for membrane fusion, whereas interaction with the syntaxin N-terminus is essential for neuronal exocytosis *in vivo*.

Key words: membrane fusion/neurotransmission/SM protein/SNARE/UNC-64

INTRODUCTION

Intracellular membrane fusion is controlled by the same ubiquitous protein machinery from yeast to the human brain [1]. Key components of this machinery are the soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) [2]. First identified from brain as syntaxin 1, SNAP-25 and VAMP [2], this family of proteins is characterised by possession of one or more signature SNARE motifs, which mediate the interaction of the individual proteins to form a 4-helical heteromeric complex [3]. The zippering together of SNAREs localised to the vesicle (v-) and target (t-) membranes as complex formation proceeds is thought to pull the two membranes together and drive the fusion reaction [4]. Indeed, membrane fusion can be reconstituted *in vitro* between proteoliposomes containing appropriate SNAREs [5, 6].

However, SNAREs are not the only evolutionarily conserved proteins required for intracellular membrane fusion. A universal requirement for the Sec1/Munc18 (SM) protein family in vesicle fusion has been recognised for many years and is supported by genetic studies in a wide variety of organisms, including yeast (*SEC1*, *SLY1*, *VPS33*, *VPS45*), nematodes (*unc-18*), flies (*ROP*) and mice (*munc18-1*) [7, 8]. Their precise function in the membrane fusion process remains unclear, although the observation that SM proteins interact with cognate SNAREs involved in the corresponding membrane traffic steps - particularly syntaxin homologues - suggests that the conserved function of SM proteins in membrane fusion involves interactions with SNAREs.

This appealing idea has proved difficult to reconcile with the divergent binding modes of different SM/SNARE protein interactions, however [9]. For example, neuronal Munc18-1 was originally shown to bind with high affinity to a closed conformation of syntaxin 1a in isolation to form a complex that precludes syntaxin entering the SNARE complex (Mode 1) [10]. In contrast, mammalian Munc18c, yeast Sly1 and Vps45 bind to the extreme N-terminus of their cognate syntaxins, either in isolation or as part of a SNARE complex (Mode 2) [11-15]. A third binding mode is evident in yeast Sec1, which is claimed not to bind efficiently to its cognate syntaxin (Sso1/2) in isolation (but see [16]), and to only interact with the ternary SNARE complex (Mode 3) [17, 18]. Recently, however, evidence has emerged suggesting that individual SM proteins can employ multiple SNARE binding modes [19, 20]. For example, the binary interaction of Munc18-1 with syntaxin 1 and of Munc18c with syntaxin 4 appears to involve both Mode 1 and Mode 2 interactions [21-23]. Similarly, the Mode 3 interaction of Munc18-1 with the SNARE complex requires Mode 2 N-terminal binding to occur [24, 25].

The available structural information on SM-SNARE protein binding Modes 1 and 2 has enabled the design of mutations that disable these interactions. The Mode 1 interaction of Munc18-1 with the closed conformation of syntaxin can be inhibited by introduction of mutations that render syntaxin constitutively open [26]. However, yeast and *C. elegans* expressing such open mutants as the sole copy of their appropriate syntaxin are apparently normal under standard conditions (although a synthetic phenotype can be seen with yeast *sec9* and *C. elegans unc-13* or *unc-10* mutants) [27-29]. Similarly, mutations that disable the Mode 2 interaction between Sly1 and Sed5, and between Vps45 and Tlg2, show no defects in ER-Golgi or vacuolar trafficking in yeast [12, 14]. The physiological significance of these SNARE binding modes for SM function *in vivo* therefore remains uncertain.

Recent *in vitro* reconstitution experiments have revealed a role for Munc18-1 in accelerating SNARE-driven liposome fusion [24]. This stimulatory action is

eliminated by mutations that impair Mode 2 syntaxin N-peptide binding, which is in turn required for Mode 3 interaction with the SNARE complex. In contrast, mutations that interfere with Mode 1 closed syntaxin binding do not inhibit the stimulation of liposome fusion by Munc18-1 [24]. These *in vitro* data therefore suggest that the interaction of Munc18-1 with the N-terminus of syntaxin 1 is critical for neuronal exocytosis. We set out to test this *in vivo* using *C. elegans* as a model organism. This is a suitable model, as there is high sequence conservation between the worm UNC-18/UNC-64 and the mammalian Munc18-1/syntaxin 1 proteins (59% and 64% identical amino acids, respectively) and as all SM proteins examined to date have very similar crystal structures. Furthermore, Munc18-1 can substitute for UNC-18 *in vivo* [30], demonstrating functional homology between the worm and mammalian proteins. We report here that mutations in both UNC-18 and UNC-64 that interfere with either Mode 1 or Mode 2 binding strongly reduce the binary interaction of these proteins *in vitro*. Despite this, worms expressing an UNC-18 mutant defective in closed syntaxin binding are phenotypically normal, indicating that Mode 1 binding is dispensable for the essential function of UNC-18 in neurotransmission. In contrast, worms expressing an UNC-18 mutant defective in N-terminal syntaxin binding are severely uncoordinated, indicating that Mode 2 binding to syntaxin is essential for UNC-18 function *in vivo*

MATERIALS AND METHODS

Materials

Unless otherwise stated, all materials were obtained from Sigma.

Plasmid Construction and Mutagenesis

The *unc-18* rescuing construct, P_{unc-18}::*unc-18* (gift from Dr. H. Kitayama, Kyoto University) [30]), was mutated by site-directed mutagenesis to introduce *Nhe*I, *Bam*HI and *Xho*I, *Not*I sites at the 5′ and 3′ ends of the *unc-18* cDNA, respectively, to produce pAM-INJ-*unc-18*. The *Bam*HI-*Xho*I *unc-18* cDNA fragment was then cloned into the pENTR-1A Entry vector (Invitrogen) by standard techniques, producing pENTR1a-*unc-18*. The *unc-18* point mutants (R39C and F113R) were produced by site-directed mutagenesis of pENTR1a-*unc-18*. Mutant *Bam*HI-*Xho*I *unc-18* cDNA fragments were then transferred to pAM-INJ-*unc-18*. The N-terminal GST-fusion Destination vector, pG-GEX-6P-1, was created by insertion of a Gateway® Conversion Cassette (Invitrogen) at the (blunted) *Bam*HI site of pGEX-6P-1 (GE Healthcare). WT and point mutant *unc-18* cDNAs were transferred from pENTR1a-*unc-18* by LR reaction to create pG-GEX-*unc-18*. All site-directed mutagenesis reactions were performed using the GeneTailor™ method (Invitrogen), with Phusion™ DNA polymerase (Finnzymes).

Recombinant Protein Expression and Purification

Recombinant GST-UNC-18 proteins were expressed from pG-GEX-*unc-18* and purified using glutathione-Sepharose (GE Healthcare) as previously described [31].

Protein Interaction Studies

1.5 µg of GST-tagged proteins were bound to glutathione-coated beads (GE Healthcare) for 1 h at 4°C. Beads were washed with binding buffer (150 mM CH_3CO_2K, 20 mM HEPES, 1 mM $MgCl_2$, 1% TWEEN 20, pH 7.4) before incubation with *in vitro* translated ^{35}S-radiolabelled UNC-64 prepared from T7-promoter-containing PCR products, as previously described [32]. After washing, bound proteins were eluted by boiling in SDS-PAGE sample buffer. The samples were then subjected to SDS-PAGE and radiolabelled proteins visualised by autoradiography.

Circular Dichroism

GST-tagged fusion proteins were cleaved with PreScission Protease (GE Healthcare) to remove the GST tag. A Jasco-J810 spectropolarimeter was used for CD measurements in the far ultraviolet region, from 190 to 260 nm. Spectra were recorded at protein concentrations of approximately 0.25 mg/ml in a cuvette of 1 mm path length in a temperature-controlled cell holder at 25 °C. Averaged CD signals, corrected for the buffer, were converted to mean residue weighted molar elipticity as follows [33]:

$[\theta]_{MRW} = (100 \times \theta)/Cnl$

Where: C is the protein concentration in millimolar, θ is the measured elipticity in millidegrees, n is the number of residues and l is the pathlength in cm.

Protein 3D Structure Production

All 3D representations were produced in PyMOL (DeLano Scientific). UNC-18 and UNC-64 models were produced from their amino acid sequences using the SWISS-

MODEL web server [34], based on the previously published Munc18-1/Syntaxin-1a tertiary structure (PDB entry 3c98). The UNC-18/UNC-64 complex structure was produced by aligning UNC-18 and UNC-64 structures with PDB 3c98 in PyMOL. The UNC-64 N-peptide was too short to be modelled accurately so was produced by mutating the Syntaxin-1a N-peptide in PyMOL.

Nematode Culture and Strains

C. elegans were cultured on 60mm nematode growth media (NGM) agar plates at 20 °C with *Escherichia coli* OP50 as a food source, using standard methods [35]. The strain used in this study was *unc-18(e81)*.

Transformation of *C. elegans*

Germline transformation of *unc-18(e81)* worms with DNA was performed by microinjection [36]. *e81* worms were rescued with pAM-INJ-*unc-18*. Rescuing cDNA constructs (10 µg/µl) were co-injected with a reporter construct, pTG96 (*sur-5::GFP*) at 10 µg/µl, to identify transformed worms [37]. Total injected DNA concentration was made up to 130 µg/µl for all injections with pBluescript-SK(+) vector. At least 3 independent transformed lines were analysed for each mutant construct. Strains were confirmed by single worm PCR using specific primers and GoTaq® DNA polymerase (Promega).

Confirmation of transformation by RT-PCR

Animals were harvested from two 60mm NGM plates with chilled M9 buffer (42.3 mM Na_2HPO_4, 22 mM KH_2PO_4, 85.6 mM NaCl, 1 mM $MgSO_4$) and centrifuged at 5,000 *g*. The worm pellet was lysed in 400 µl TRI Reagent™ (Sigma) and RNA was purified according to the manufacturer's instructions. First strand cDNA was produced from 1 µg of total RNA template using AMV reverse transcriptase and random primers (Promega) following manufacturer's instructions. PCR was then performed with primers specific for the gene of interest, using GoTaq® DNA polymerase (Promega).

Protein Extraction and Western Blotting

Animals were harvested from twenty four 60 mm NGM plates in chilled M9 buffer and allowed to settle on ice for 10 min. After two washes in M9, the worm pellets were re-suspended in 800 µl worm lysis buffer (50 mM Tris, 150 mM NaCl, pH 7.6, 1.5 % (w/v) n-Octyl glucoside, protease-inhibitor cocktail (Sigma)) and lysed at 4,000 psi in a One-Shot cell disruptor (Constant Systems). The lysed worm suspension was then incubated at 4 °C with agitation before insoluble material was removed by centrifugation at 15,000 *g* for 30 min at 4 °C. Western blotting was performed using approximately 20 µg total protein of each strain. Membranes were blotted with anti-GFP (Clontech) or anti-UNC-18 ([38] kind gift of R. Hosono) antibodies, and proteins visualised using horseradish peroxidise (HRP)-conjugated anti-mouse IgG antibodies or HRP-conjugated anti-actin (clone AC-15).

Behavioural Assays

All assays were performed on young adult hermaphrodite animals from sparsely populated plates. Experiments were conducted in a temperature-controlled room at 20 °C. All data were expressed as mean ± SEM. Significance was tested by Mann-Whitney U Test.

Thrashing Assays

Locomotion was quantified by counting thrashes in solution over a 1 minute time period [39]. Young adult hermaphrodites were removed from NGM plates and placed in an untreated 96-well plate containing 100 µl freshly made Dent's solution (140 mM NaCl, 6 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, bovine serum albumin at 0.1 mg ml^{-1}, pH 7.4). A thrash was defined as one complete sinusoidal movement from maximum to minimum amplitude and back again. To control for any variation in environmental factors, the locomotion rates of individual transgenic worm lines being compared were measured alternately.

Dispersal Assays

OP50 was seeded around the edge of 60 mm NGM plates before approximately 50 worms were placed in the centre in a ~10 µl drop of Dent's solution. When the liquid had been absorbed, the plates were shaken briefly to reduce clumping. Plates were scored at intervals for the percentage of worms having reached the bacterial ring. This was repeated three times for each strain.

Pharyngeal pumping Assays

Worms were examined at 80x magnification under a dissecting microscope. The number of beats of the pharynx was counted over one minute. Ten worms per strain were used.

RESULTS

It has recently become clear that binding of mammalian Munc18-1 to syntaxin 1 involves both Mode 1 binding to the closed syntaxin conformation and Mode 2 binding to the syntaxin N-terminal peptide [21, 23]. To determine the relative contributions of these distinct interaction mechanisms to their *C. elegans* orthologues, we designed mutations in UNC-64 to block each mode of binding to UNC-18 (Fig 1A). For Mode 1 closed syntaxin binding, we made the previously described L166A/E167A 'open' mutant UNC-64 [29], itself based on the original L165A/E166A 'open' mutant syntaxin 1a [26]. We also created an I234A mutation in the SNARE motif, analogous to the I233A syntaxin 1a mutant that is severely defective in Munc18-1 binding, likely due to impairment of the Mode 1 interaction [10, 40, 41]. For Mode 2 N-terminal binding, we created a deletion mutant lacking the N-terminal 21 residues (Δ21N); and a point mutant, L9A, based on the L8A syntaxin 1a mutant that prevents Mode 2 binding and stimulation of liposome fusion by Munc18-1 [24] (Fig 1B). Both mutants are predicted to interfere with the binding of the UNC-64 N-peptide to the hydrophobic pocket in domain 1 of UNC-18 [11, 24] (Fig 1C). These mutations were introduced into untagged constructs encoding the cytoplasmic domain of UNC-64. Wild type and mutant ^{35}S-labelled UNC-64 proteins were then made by *in vitro* transcription/translation and their binding to GST-UNC-18 determined. Binding of wild type UNC-64 to GST-UNC-18 was readily observed, whereas negligible binding occurred with GST controls, confirming the specificity of the interaction (Fig 1D). All four UNC-64 mutations inhibited binding, although some specific binding remained with the open mutant (Fig 1D,E).

These data suggested that high-affinity binding of UNC-18 to UNC-64 involves both Mode 1 closed conformation binding and Mode 2 N-peptide binding. If so, reciprocal mutations in UNC-18 that interfere with Mode 1 or 2 binding to UNC-64 should also inhibit the UNC-64/UNC-18 interaction (Fig 1A). To target Mode 1 closed syntaxin binding, we made an R39C mutant, analogous to the well-characterised R39C Munc18-1 mutation [42], and itself based on the R50C *Drosophila Rop* mutant [43]. For Mode 2 N-terminal binding, we created a point mutation, F113R, at Site 1 of the putative hydrophobic interaction surface in Domain 1 (Fig 2A, B). This was chosen because substitution of the conserved hydrophobic residue at this position for charged amino acids inhibits Mode 2 binding of mammalian Munc18c, yeast Vps45 and Sly1 to their respective syntaxins [12, 14, 15]. These mutations were introduced into GST-UNC-18 and the effect on binding to ^{35}S-labelled UNC-64 proteins determined. Binding of wild type UNC-64 to GST-UNC-18 was greatly reduced by both the R39C and F113R mutations to near background levels (Fig 2C,D). To check that the novel F113R mutation does not result in gross conformational defects, we performed circular dichroism spectroscopy on the wild type and F113R recombinant proteins, after removal of their GST tags. This yielded essentially identical, superimposable spectra (Fig 2E), demonstrating that the F113R protein is properly folded, and thus ruling out this trivial explanation of the observed impaired binding. Thus, data from multiple mutations in both UNC-64 and UNC-18 strongly supports the idea that this high-affinity binary interaction involves both closed conformation and N-terminal syntaxin binding.

To determine the physiological relevance of each binding mode, we generated *C. elegans* expressing only wild type, R39C or F113R UNC-18. This was achieved by germline transformation of *e81 unc-18* null mutants with wild type or mutant *unc-18* plasmids along with *sur5::GFP* as a visible reporter [39]. UNC-18 expression from these constructs is controlled by the natural *unc-18* promoter [30], ensuring

physiologically relevant regulation of gene expression. To confirm that transformation had taken place, DNA was isolated from the various strains and PCR amplification performed using primers specific for the *unc-18* plasmid. This revealed bands of the predicted size for wild type-, F113R- and R39C-transformed worms, but not for the untransformed *e81* mutants (Fig 3A). Primers specific for the F113R mutation only produced a product in F113R-transformed worms, whereas endogenous *act-1* was amplified in all strains, confirming the specificity of the PCR. Analysis of mRNA expression by RT-PCR using primers specific for plasmid-borne *unc-18* confirmed that expression occurred only in transformed lines, whereas generic *unc-18* primers revealed amplification of the endogenous *unc-18* mRNA in all strains, as expected (Fig 3B). Finally, we assessed protein expression by western blotting using an UNC-18 antibody [38]. This identified a band of the predicted size (~65-kDa) in the wild type Bristol N2 strain that was absent in the untransformed *e81 unc-18* null mutant but reappeared in wild type-, F113R- and R39C-transformed *e81* mutants (Fig 3C). Anti-GFP antibody staining was observed only in transformed worms; whereas actin was present at similar levels in all strains, as expected (Fig 3C). From RT-PCR and western blotting, it appeared that F113R and R39C were expressed at lower levels than in wild type UNC-18 rescue lines. It is important to note, however, that the two mutants were expressed at the same level.

Having established *C. elegans* lines expressing only wild type, R39C or F113R UNC-18, we next determined their phenotypes. The severe inhibition of synaptic vesicle exocytosis in *unc-18* null mutants greatly impairs neuromuscular transmission, resulting in a classical uncoordinated (Unc) locomotion phenotype [35]. Microscopical observation clearly revealed that the severe Unc phenotype of the *e81* mutant was rescued by expression of wild type UNC-18, as expected (Supplementary movie; note fluorescent green staining from the SUR-5-GFP marker). Strikingly, R39C-expressing worms appeared indistinguishable from strains rescued with wild type UNC-18, whereas no rescue was evident with the F113R construct (Supplementary movie). To quantify these effects, thrashing assays in solution were performed, whereby the number of sinusoidal movements over a 1-minute period was determined. Untransformed *e81* mutants produced a very small number of severely uncoordinated thrashes and this was not improved by expression of F113R UNC-18; whereas expression of wild type or R39C UNC-18 was equally effective in restoring thrashing frequency to around 100/min (Fig 4A), similar to the Bristol N2 strain (Supplementary Fig 1). The data in Fig 4A were pooled from at least 3 independently-derived transformed lines for each muatnt, but analysis of each individual line produced essentially identical results, indicating that the effects are not due to strain variation (Supplementary Fig 1). To check that these differential quantitative effects on locomotion were not specific to thrashing in solution, we employed a dispersal assay, where the rate at which worms can detect and move to a food source on solid media is determined. Again, wild type- and R39C-transformed worms were indistinguishable, with complete migration into the food occurring within around 1 hour (Fig 4B); while untransformed and F113R-transformed worms exhibited similarly slow movement, with <20% of worms having encountered the food source even after 2 days. In contrast, there was no significant difference between any of the strains in frequency of pharyngeal pumping (Fig 4C), which is a predominantly myogenic process [44]. This therefore confirms that the severe phenotype of F113R UNC-18 worms is not due to general sickness or to post-synaptic muscle effects, but rather is due to a specific impairment of presynaptic neurotransmission.

DISCUSSION

Insight into SM protein function has been dramatically increased recently by the realisation that individual SM proteins can interact with their cognate SNAREs in at least 3 ways [19]. Indeed, the binary interaction of mammalian Munc18-1 with syntaxin 1 involves both Mode 1 binding to the closed syntaxin conformation and Mode 2 binding to the syntaxin N-terminal peptide [21, 23]. Using multiple mutations that affect UNC-18 interaction with the closed conformation (UNC-64 open, UNC-64 I234A; UNC-18 R39C) and the N-terminus (UNC-64 L9A, Δ21N; UNC-18 F113R) of UNC-64, we demonstrate here that the same is true for the orthologous *C. elegans* proteins. As mutation of either binding mode severely reduced UNC-64/UNC-18 binding, we conclude that this high-affinity *in vitro* interaction is the product of the two lower-affinity interaction modes. Interestingly, Mode 1 closed conformation binding has thus far only been demonstrated in SM/SNARE pairs involved in regulated exocytosis, i.e. Munc18-1/syntaxin 1 [10, 26, 45], Munc18c/syntaxin 4 [22], and UNC-18/UNC-64 (this study). In contrast, Mode 2 N-terminal binding is common to diverse SM/SNARE pairs, including those involved in constitutive ER-Golgi and prevacuolar membrane traffic in yeast [11, 14, 46]. This suggests that Mode 1 binding may have evolved as a regulatory mechanism, consistent with the modulation of this binding mode in higher organisms by post-translational modifications such as phosphorylation and *S*-nitrosylation [31, 47]. In contrast, Mode 2 binding likely serves a conserved role, possibly by facilitating the Mode 3 SNARE complex interaction that is thought to represent a fundamental function of SM proteins in membrane fusion [16, 24].

Despite the requirement for both binding modes for tight binary interaction *in vitro*, we demonstrate here that Mode 1 closed syntaxin binding is not essential for neuronal SM protein function in *C. elegans*. The ability of the R39C mutant to fully rescue the *unc-18* mutant phenotype is consistent with earlier work showing that *unc-64* mutants can be rescued by an open UNC-64 construct [29, 48]; and data from *in vitro* reconstitution assays, where R39C and wild type Munc18-1 are equally effective in promoting SNARE-mediated membrane fusion [24]. Although clearly not essential, the Mode 1 interaction plays some role in regulated exocytosis, as evidenced by the effects of R39C and other Mode-1-defective Munc18-1 mutants on dense-cored granule exocytosis [41, 42, 47] and the phenotype of the original R50C *Drosophila rop* mutant that R39C was based upon [43, 49]. Indeed, it was recently shown that knockin mice expressing open syntaxin 1b suffer seizures and exhibit alterations in synaptic vesicle releasable pool sizes [50]. Interestingly, these mice exhibit slightly increased synaptic vesicle docking, a phenotype shared by *C. elegans* expressing open UNC-64 [48]; and Mode 1 binding has recently been implicated in secretory granule docking via a Rab3-Munc18-1 interaction [51, 52]. The Mode 1 interaction may therefore serve a regulatory role in vesicle docking, but one that is too subtle to be detected by the behavioural assays used in this and previous studies [29, 48].

A major finding of our work is that only Mode 2 N-terminal syntaxin binding is essential for neuronal SM protein function *in vivo*. This conclusion is based on our observation that F113R UNC-18 provides no rescue of the Unc phenotype despite being expressed at similar levels to the R39C protein that confers full rescue (Fig 3). Although mutations have previously been introduced into the analogous Site 1 residues in yeast Sly1 (L137R) and Vps45 (L117R) that similarly block tight binding to their cognate syntaxins, these have no observable impact on membrane trafficking [12, 14]. Our findings therefore provide the first demonstration of an essential *in vivo* function for the Mode 2 SM-syntaxin interaction. While this manuscript was in

preparation, McEwan and Kaplan independently reported locomotion defects in UNC-64 L9A- and UNC-18 L116K-expressing worms, but not in open UNC-64- or UNC-18 R39C-expressing worms [53], thus confirming that only the Mode 2 interaction is important for normal synaptic transmission. In addition, they suggested that both interaction Modes were required for the putative chaperone function of UNC-18 in enabling transport of UNC-64 through the secretory pathway, as this was only inhibited in double mutants [53]. However, it is unclear if this represents a general mechanism, as syntaxin 1a trafficking in mammalian cells is unaffected by Mode 2 mutations, requiring only closed conformation binding [54]; and as syntaxin 1 still traffics to the plasma membrane in Munc18-1 knockout mice [55]. Since single mutations that inhibit UNC-18 interaction with the UNC-64 N-terminus exhibit locomotion defects despite fully rescuing this chaperone function, it seems safe to conclude that the late Mode 2-binding-dependent role of UNC-18 in exocytosis is phenotypically more prominent. This essential role of Mode 2 binding is also consistent with data from *in vitro* liposome fusion assays, where the analogous L8A syntaxin 1a mutation abolishes the stimulatory effect of Munc18-1 [24]; and from mammalian cell studies in which the inhibitory effects of syntaxin fragments require an intact N-terminus [56]. It is not clear why such dramatic effects of Mode 2 mutations on membrane fusion are seen with neuronal SNARE/SM isoforms from worms and mammals, but not with yeast homologues. Perhaps yet another SNARE binding mode exists in the yeast proteins that compensates for the loss of Mode 2 binding, as suggested for Vps45 [14].

In summary, our data clearly demonstrate an essential physiological role for the Mode 2 interaction of UNC-18 with UNC-64. However, the precise molecular function of this binding mode is unclear. It could be that the binary interaction of UNC-18 with the UNC-64 N-terminus serves a direct role, facilitating a novel function of this heterodimeric complex. However, it seems more likely that the Mode 2 interaction is an essential precursor that enables the distinct Mode 3 interaction with the SNARE complex, as previously suggested [24, 25]. Unfortunately, distinguishing between these possibilities is not possible at present, as there is no crystal structure of any Mode 3 SM/SNARE complex upon which to design specific Mode 3-disrupting mutations. Interestingly, SM mutants that are unaffected in Modes 1 and 2 binding yet are functionally defective, potentially due to impairment of Mode 3 binding, have recently been described [14, 39, 57]. Detailed analysis of the molecular defects in these mutants may enable the design of novel mutational tools required to resolve the outstanding mechanistic questions regarding SM-SNARE interactions and their contribution to synaptic membrane fusion *in vivo*.

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References

1 Ferro-Novick, S. and Jahn, R. (1994) Vesicle fusion from yeast to man. Nature **370**, 191-193

2 Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J. E. (1993) SNAP receptors implicated in vesicle targeting and fusion. Nature **362**, 318-324

3 Sutton, R. B., Fasshauer, D., Jahn, R. and Brunger, A. T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature **395**, 347-353

4 Jahn, R. and Scheller, R. H. (2006) SNAREs--engines for membrane fusion. Nat. Rev. Mol. Cell. Biol. **7**, 631-643

5 Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T. H. and Rothman, J. E. (1998) SNAREpins: minimal machinery for membrane fusion. Cell **92**, 759-772

6 McNew, J. A., Parlati, F., Fukuda, R., Johnston, R. J., Paz, K., Paumet, F., Sollner, T. H. and Rothman, J. E. (2000) Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. Nature **407**, 153-159

7 Halachmi, N. and Lev, Z. (1996) The Sec1 family: A novel family of proteins involved in synaptic transmission and general secretion. J. Neurochem. **66**, 889-897

8 Toonen, R. F. and Verhage, M. (2003) Vesicle trafficking: pleasure and pain from SM genes. Trends Cell Biol. **13**, 177-186

9 Gallwitz, D. and Jahn, R. (2003) The riddle of the Sec1/Munc-18 proteins - new twists added to their interactions with SNAREs. Trends Biochem. Sci. **28**, 113-116

10 Misura, K. M. S., Scheller, R. H. and Weis, W. I. (2000) Three-dimensional structure of the neuronal-Sec1-syntaxin1a complex. Nature **404**, 355-362

11 Bracher, A. and Weissenhorn, W. (2002) Structural basis for the Golgi membrane recruitment of Sly1p by Sed5p. EMBO J. **21**, 6114-6124

12 Peng, R. and Gallwitz, D. (2004) Multiple SNARE interactions of an SM protein: Sed5p/Sly1p binding is dispensable for transport. EMBO J. **23**, 3939-3949

13 Dulubova, I., Yamaguchi, T., Gao, Y., Min, S. W., Huryeva, I., Sudhof, T. C. and Rizo, J. (2002) How Tlg2p/syntaxin 16 'snares' Vps45. EMBO J. **21**, 3620-3631

14 Carpp, L. N., Ciufo, L. F., Shanks, S. G., Boyd, A. and Bryant, N. J. (2006) The Sec1p/Munc18 protein Vps45p binds its cognate SNARE proteins via two distinct modes. J. Cell Biol. **173**, 927-936

15 Hu, S. H., Latham, C. F., Gee, C. L., James, D. E. and Martin, J. L. (2007) Structure of the Munc18c/Syntaxin4 N-peptide complex defines universal features of the N-peptide binding mode of Sec1/Munc18 proteins. Proc. Natl. Acad. Sci. U S A. **104**, 8773-8778

16 Scott, B. L., Van Komen, J. S., Irshad, H., Liu, S., Wilson, K. A. and McNew, J. A. (2004) Sec1p directly stimulates SNARE-mediated membrane fusion in vitro. J. Cell Biol. **167**, 75-85

17 Carr, C. M., Grote, E., Munson, M., Hughson, F. M. and Novick, P. J. (1999) Sec1p binds to SNARE complexes and concentrates at sites of secretion. J. Cell Biol. **146**, 333-344

18 Togneri, J., Cheng, Y. S., Munson, M., Hughson, F. M. and Carr, C. M. (2006) Specific SNARE complex binding mode of the Sec1/Munc-18 protein, Sec1p. Proc. Natl. Acad. Sci. U S A. **103**, 17730-17735

19 Burgoyne, R. D. and Morgan, A. (2007) Membrane trafficking: three steps to fusion. Curr. Biol. **17**, R255-258
20 Toonen, R. F. and Verhage, M. (2007) Munc18-1 in secretion: lonely Munc joins SNARE team and takes control. Trends Neurosci. **30**, 564-572
21 Burkhardt, P., Hattendorf, D. A., Weis, W. I. and Fasshauer, D. (2008) Munc18a controls SNARE assembly through its interaction with the syntaxin N-peptide. EMBO J. **27**, 923-933
22 D'Andrea-Merrins, M., Chang, L., Lam, A. D., Ernst, S. A. and Stuenkel, E. L. (2007) Munc18c interaction with syntaxin 4 monomers and SNARE complex intermediates in GLUT4 vesicle trafficking. J. Biol. Chem. **282**, 16553-16566
23 Rickman, C., Medine, C. N., Bergmann, A. and Duncan, R. R. (2007) Functionally and spatially distinct modes of munc18-syntaxin 1 interaction. J. Biol. Chem. **282**, 12097-12103
24 Shen, J., Tareste, D. C., Paumet, F., Rothman, J. E. and Melia, T. J. (2007) Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. Cell **128**, 183-195
25 Dulubova, I., Khvotchev, M., Liu, S., Huryeva, I., Sudhof, T. C. and Rizo, J. (2007) Munc18-1 binds directly to the neuronal SNARE complex. Proc. Natl. Acad. Sci. U S A. **104**, 2697-2702
26 Dulubova, I., Sugita, S., Hill, S., Hosaka, M., Fernandez, I., Sudhof, T. C. and Rizo, J. (1999) A conformational switch in syntaxin during exocytosis: role of munc18. EMBO J. **18**, 4372-4382
27 Munson, M. and Hughson, F. M. (2002) Conformational regulation of SNARE assembly and disassembly in vivo. J. Biol. Chem. **277**, 9375-9381
28 Koushika, S. P., Richmond, J. E., Hadwiger, G., Weimer, R. M., Jorgensen, E. M. and Nonet, M. L. (2001) A post-docking role for active zone protein Rim. Nat. Neurosci. **4**, 997-1005
29 Richmond, J. E., Weimer, R. M. and Jorgensen, E. M. (2001) An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming. Nature **412**, 338-341
30 Gengyo-Ando, K., Kitayama, H., Mukaida, M. and Ikawa, Y. (1996) A murine neural specific homolog corrects cholinergic defects in *Caenorhabditis elegans unc-18* mutants. J. Neurosci. **16**, 6695-6702
31 Palmer, Z. J., Duncan, R., Johnson, J., Lian, L. Y., Mello, L. V., Booth, D., Barclay, J. W., Graham, M. E., Burgoyne, R. D., Prior, I. and Morgan, A. (2008) S-nitrosylation of syntaxin 1 at Cys145 is a regulatory switch controlling Munc18-1 binding. Biochem. J. **413**, 479-491
32 Craig, T. J., Ciufo, L. F. and Morgan, A. (2004) A protein-protein binding assay using coated microtitre plates: increased throughput, reproducibility and speed compared to bead-based assays. J. Biochem. Biophys. Methods **60**, 49-60
33 Luo, P. and Baldwin, R. L. (1997) Mechanism of helix induction by trifluoroethanol: a framework for extrapolating the helix-forming properties of peptides from trifluoroethanol/water mixtures back to water. Biochemistry **36**, 8413-8421
34 Schwede, T., Kopp, J., Guex, N. and Peitsch, M. C. (2003) SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res. **31**, 3381-3385
35 Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. Genetics **77**, 71-94

36 Mello, C. C., Kramer, J. M., Stinchcomb, D. and Ambros, V. (1991) Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J. **10**, 3959-3970
37 Yochem, J., Gu, T. and Han, M. (1998) A new marker for mosaic analysis in *Caenorhabditis elegans* indicates a fusion between hyp6 and hyp7, two major components of the hypodermis. Genetics **149**, 1323-1334
38 Gengyo-Ando, K., Kamiya, Y., Yamakawa, A., Kodaira, K., Nishiwaki, K., Miwa, J., Hori, I. and Hosono, R. (1993) The *C. elegans unc-18* gene encodes a protein expressed in motor neurons. Neuron **11**, 703-711
39 Graham, M. E., Edwards, M. R., Holden-Dye, L., Morgan, A., Burgoyne, R. D. and Barclay, J. W. (2008) UNC-18 Modulates Ethanol Sensitivity in *Caenorhabditis elegans*. Mol. Biol. Cell in press, DOI: 10.1091/mbc.E08-07-0689
40 Kee, Y., Lin, R. C., Shu-Chan, H. and Scheller, R. H. (1995) Distinct domains of syntaxin are required for synaptic vesicle fusion complex formation and dissociation. Neuron **14**, 991-998
41 Graham, M. E., Barclay, J. W. and Burgoyne, R. D. (2004) Syntaxin/Munc18 interactions in the late events during vesicle fusion and release in exocytosis. J. Biol. Chem. **279**, 32751-32760
42 Fisher, R. J., Pevsner, J. and Burgoyne, R. D. (2001) Control of fusion pore dynamics during exocytosis by munc18. Science **291**, 875-878
43 Wu, M. N., Littleton, J. T., Bhat, M. A., Prokop, A. and Bellen, H. J. (1998) ROP, the Drosophila sec1 homolog, interacts with syntaxin and regulates neurotransmitter release in a dosage-dependent manner. EMBO J. **17**, 127-139
44 Avery, L. and Horvitz, H. R. (1989) Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of *C. elegans*. Neuron **3**, 473-485
45 Yang, B., Steegmaier, M., Gonzalez, L. C. and Scheller, R. H. (2000) nSec1 binds a closed conformation of syntaxin1A. J. Cell Biol. **148**, 247-252
46 Dulubova, I., Yamaguchi, T., Arac, D., Li, H., Huryeva, I., Min, S. W., Rizo, J. and Sudhof, T. C. (2003) Convergence and divergence in the mechanism of SNARE binding by Sec1/Munc18-like proteins. Proc. Natl. Acad. Sci. U S A. **100**, 32-37
47 Barclay, J. W., Craig, T. J., Fisher, R. J., Ciufo, L. F., Evans, G. J. O., Morgan, A. and Burgoyne, R. D. (2003) Phosphorylation of Munc18 by Protein Kinase C Regulates the Kinetics of Exocytosis. J. Biol. Chem. **278**, 10538-10545
48 Hammarlund, M., Palfreyman, M. T., Watanabe, S., Olsen, S. and Jorgensen, E. M. (2007) Open syntaxin docks synaptic vesicles. PLoS Biol. **5**, e198
49 Harrison, S. D., Broadie, K., van de Goor, J. and Rubin, G. M. (1994) Mutations in the *Drosophila Rop* gene suggest a function in general secretion and synaptic transmission. Neuron **13**, 555-566
50 Gerber, S. H., Rah, J. C., Min, S. W., Liu, X., de Wit, H., Dulubova, I., Meyer, A. C., Rizo, J., Arancillo, M., Hammer, R. E., Verhage, M., Rosenmund, C. and Sudhof, T. C. (2008) Conformational switch of syntaxin-1 controls synaptic vesicle fusion. Science **321**, 1507-1510
51 Graham, M. E., Handley, M. T., Barclay, J. W., Ciufo, L. F., Barrow, S. L., Morgan, A. and Burgoyne, R. D. (2008) A gain-of-function mutant of Munc18-1 stimulates secretory granule recruitment and exocytosis and reveals a direct interaction of Munc18-1 with Rab3. Biochem. J. **409**, 407-416
52 van Weering, J. R., Toonen, R. F. and Verhage, M. (2007) The role of Rab3a in secretory vesicle docking requires association/dissociation of guanidine phosphates and Munc18-1. PLoS ONE **2**, e616

53 McEwen, J. M. and Kaplan, J. M. (2008) UNC-18 Promotes Both the Anterograde Trafficking and Synaptic Function of Syntaxin. Mol. Biol. Cell **19**, 3836-3846

54 Medine, C. N., Rickman, C., Chamberlain, L. H. and Duncan, R. R. (2007) Munc18-1 prevents the formation of ectopic SNARE complexes in living cells. J. Cell Sci. **120**, 4407-4415

55 Toonen, R. F., de Vries, K. J., Zalm, R., Sudhof, T. C. and Verhage, M. (2005) Munc18-1 stabilizes syntaxin 1, but is not essential for syntaxin 1 targeting and SNARE complex formation. J. Neurochem. **93**, 1393-1400

56 Khvotchev, M., Dulubova, I., Sun, J., Dai, H., Rizo, J. and Sudhof, T. C. (2007) Dual modes of Munc18-1/SNARE interactions are coupled by functionally critical binding to syntaxin-1 N terminus. J. Neurosci. **27**, 12147-12155

57 Boyd, A., Ciufo, L. F., Barclay, J. W., Graham, M. E., Haynes, L. P., Doherty, M. K., Riesen, M., Burgoyne, R. D. and Morgan, A. (2008) A random mutagenesis approach to isolate dominant-negative yeast *sec1* mutants reveals a functional role for domain 3a in yeast and mammalian Sec1/Munc18 proteins. Genetics **180**, 165-178

Figure Legends

Figure 1. UNC-18 binding is inhibited by UNC-64 mutations that impair either N-terminal or closed conformation binding modes

(A) Two views of a structural model of UNC-18 (grey) bound to UNC-64 (blue), based on the crystal structure of Munc18-1 bound to syntaxin 1a (pdb entry: 3c98). Mutations used in this study that target Mode 1 binding (*left*) and Mode 2 binding (*right*) are highlighted as pink spheres.

(B) Sequence alignment of syntaxins, highlighting the conserved N-terminal hydrophobic (shaded green) and charged (red) residues involved in Mode 2 SM protein binding. Underlined residues indicate previously characterised mutations that that inhibit this binding mode.

(C) *(left)* Close-up of the interaction of the wild type UNC-64 N-terminal peptide with Domain 1 of UNC-18, with Leu9 highlighted in green spheres. *(right)* Same view, but of the UNC-64 L9A mutant, with Ala9 in yellow spheres. Note the inability of the alanine residue to fill the hydrophobic pocket of UNC-18 (illustrated by light green surface fill)

(D), **(E)** GST or GST-UNC-18 was pre-bound to glutathione-Sepharose beads, washed and then incubated with ^{35}S-labelled wild type or mutant UNC-64 cytoplasmic domain. Bound and unbound UNC-64 was visualised by SDS-PAGE and subsequent autoradiography. Results from a representative experiment are shown in **(D)**; quantified data from 3 independent assays are shown as mean ± S.E.M in **(E).**

Figure 2. UNC-64 binding is inhibited by UNC-18 mutations that impair either N-terminal or closed conformation binding modes

(A) Sequence alignment of SM proteins, highlighting conserved hydrophobic (Sites 1-4, shaded green) and acidic (Site 5, red) residues involved in Mode 2 SM protein binding. Underlined residues indicate previously characterised mutations that inhibit this binding mode.

(B) *(left)* Close-up of the interaction of the UNC-64 interaction surface in Domain 1 of UNC-18, with Phe113 (Site 1) highlighted in green spheres. *(right)* Same view, but of the F113R mutant UNC-18, with Arg113 in red spheres. Note how the mutation is predicted to decrease the hydrophobic interaction surface of the UNC-18 binding pocket (illustrated by light green surface fill) and replaces this with the large basic Arg residue.

(C), **(D)** Wild type or mutant GST-UNC-18, or GST as control, was pre-bound to glutathione-Sepharose beads, washed and then incubated with ^{35}S-labelled wild type UNC-64 cytoplasmic domain. Bound and unbound UNC-64 was visualised by SDS-PAGE and subsequent autoradiography. Results from a representative experiment are shown in (C); quantified data from 3 independent assays are shown as mean ± S.E.M in **(D)**.

(E) The GST-tag was removed from wild type and F113R UNC-18 proteins using PreScission protease. These proteins were then analysed by CD spectroscopy.

Figure 3. Transformation of *C. elegans unc-18* null mutants with wild type or mutant *unc-18* constructs.
unc-18 e81 mutants were transformed with wild type, R39C or F113R plasmids along with a GFP reporter plasmid by germline microinjection.
(A) Total worm DNA extracts were prepared from the strains and used with specific PCR primers to verify the presence of *unc-18* plasmids in the appropriate transformed strains.
(B) Total RNA was purified from the strains and used to generate cDNA for subsequent PCR. Successful amplification of *unc-18* was achieved in all strains using generic *unc-18* primers (upper panel), but was restricted to transformed strains using plasmid-specific *unc-18* primers (middle panel).
(C) Total worm protein extracts were prepared from the strains and run on SDS-PAGE. Western blotting using antisera to UNC-18 revealed an ~65 kDa protein band in the control Bristol N2 strain that was absent in the *unc-18 e81* null mutant and was re-expressed in the transformed lines. Detection of GFP was specific for transformed worms only, whereas actin was present in all strains.

Figure 4. UNC-18 function *in vivo* requires Mode 2 interaction with the UNC-64 N-terminus, but not Mode 1 closed syntaxin binding.
(A) Thrashing assay. Locomotion rates of the untransformed *unc-18 e81* strain and isogenic transformed strains were quantified by counting thrashes per minute in solution. Both wild type (wt) and R39C UNC-18 constructs rescues restored coordinated locomotion in comparison to *e81* worms (*; $P<0.001$); however, F113R provided no phenotypic rescue. Data were analysed from 72 *e81*, 72 wt, 96 R39C and 108 F113R worms and are shown as mean ± SEM.
(B) Dispersal assay. Individual worms were deposited into the centre of an agar plate with OP50 *E. coli* lining the perimeter. The time taken for worms to enter the food was then recorded. Again, wild type (wt) and R39C UNC-18 constructs provided equal rescue of this phenotype, whereas F113R worms were indistinguishable from untransformed *e81* worms. Data were analysed from 150 worms for each strain and are shown as mean ± SEM.
(C) Pharyngeal pumping. The frequency of pharyngeal muscle contraction was counted and no significant difference was observed between the worms. Data were analysed from 10 worms for each strain and are shown as mean ± SEM.

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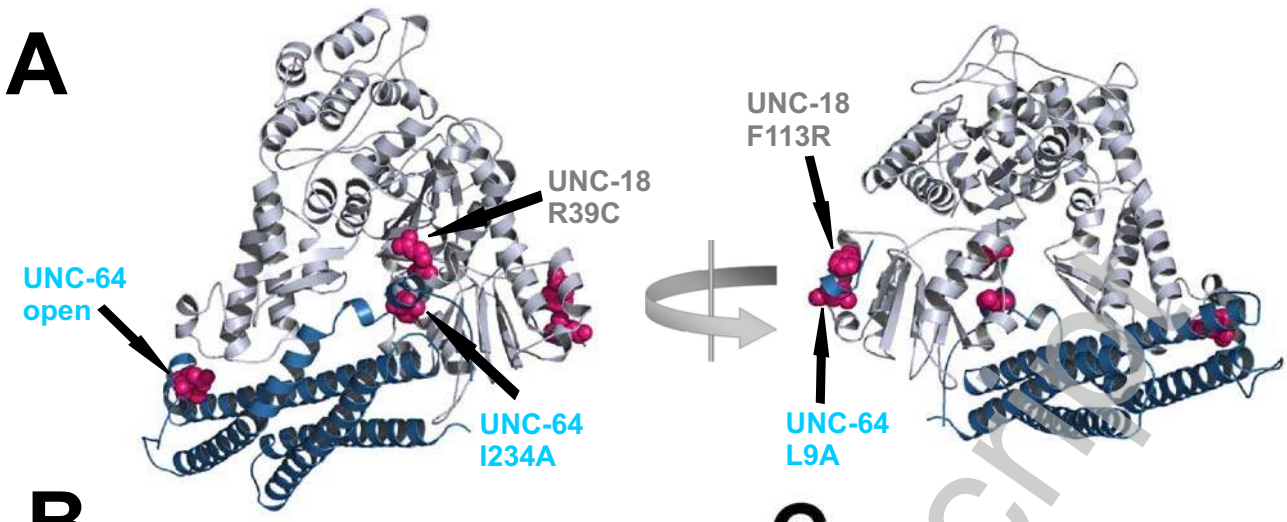

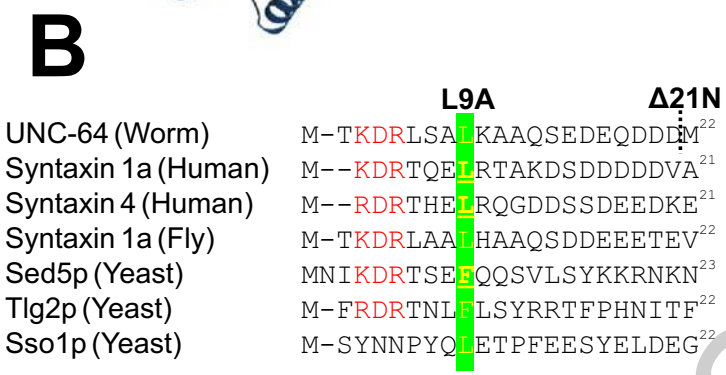

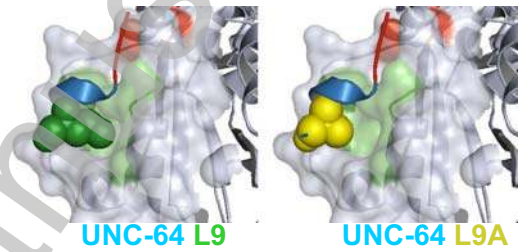

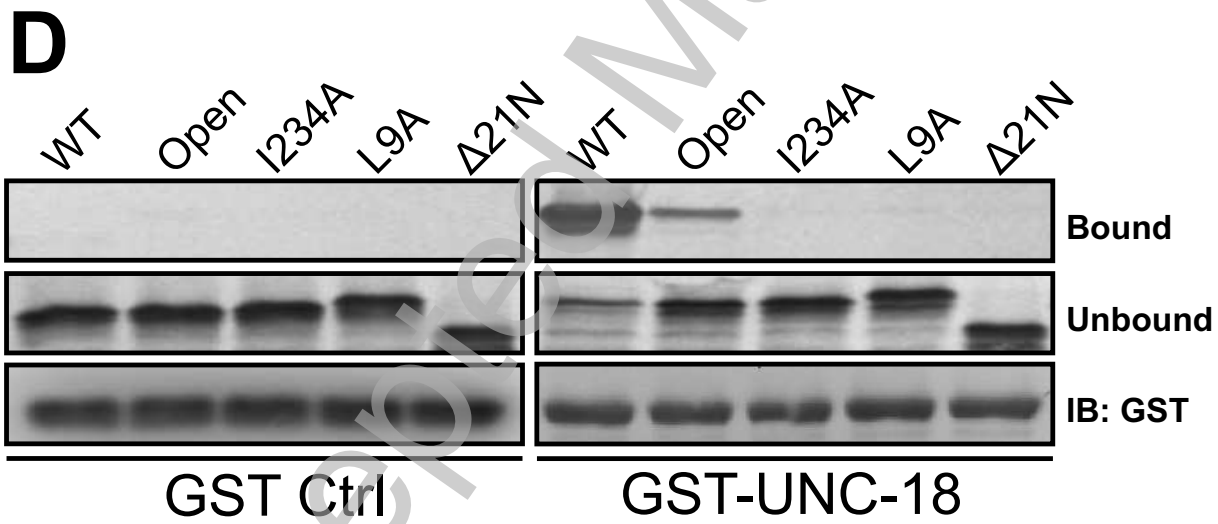

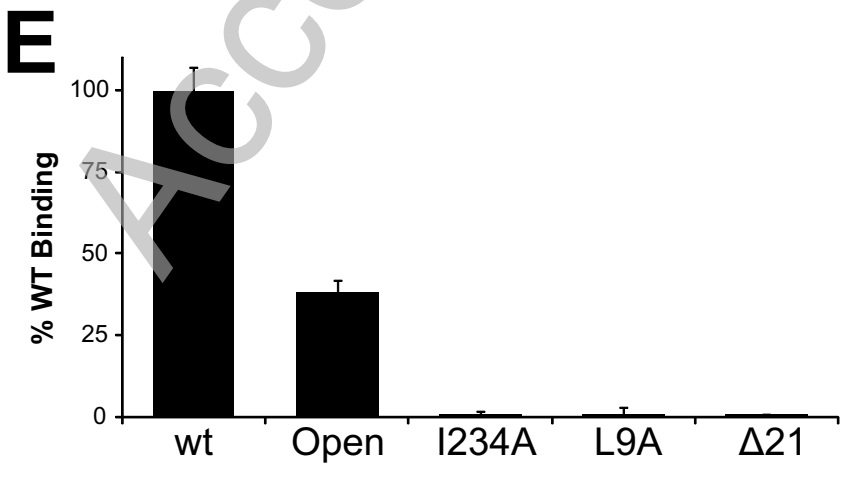

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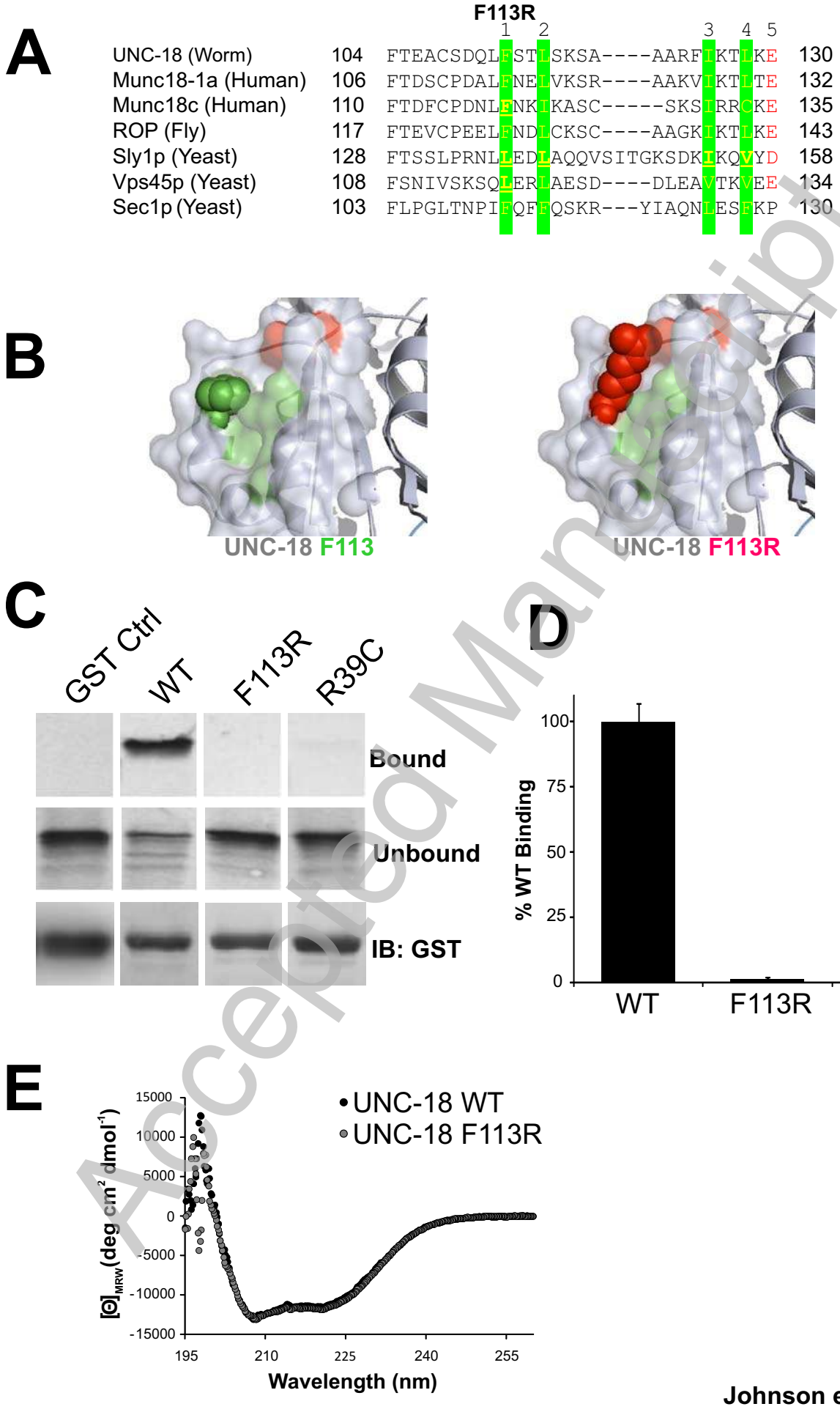

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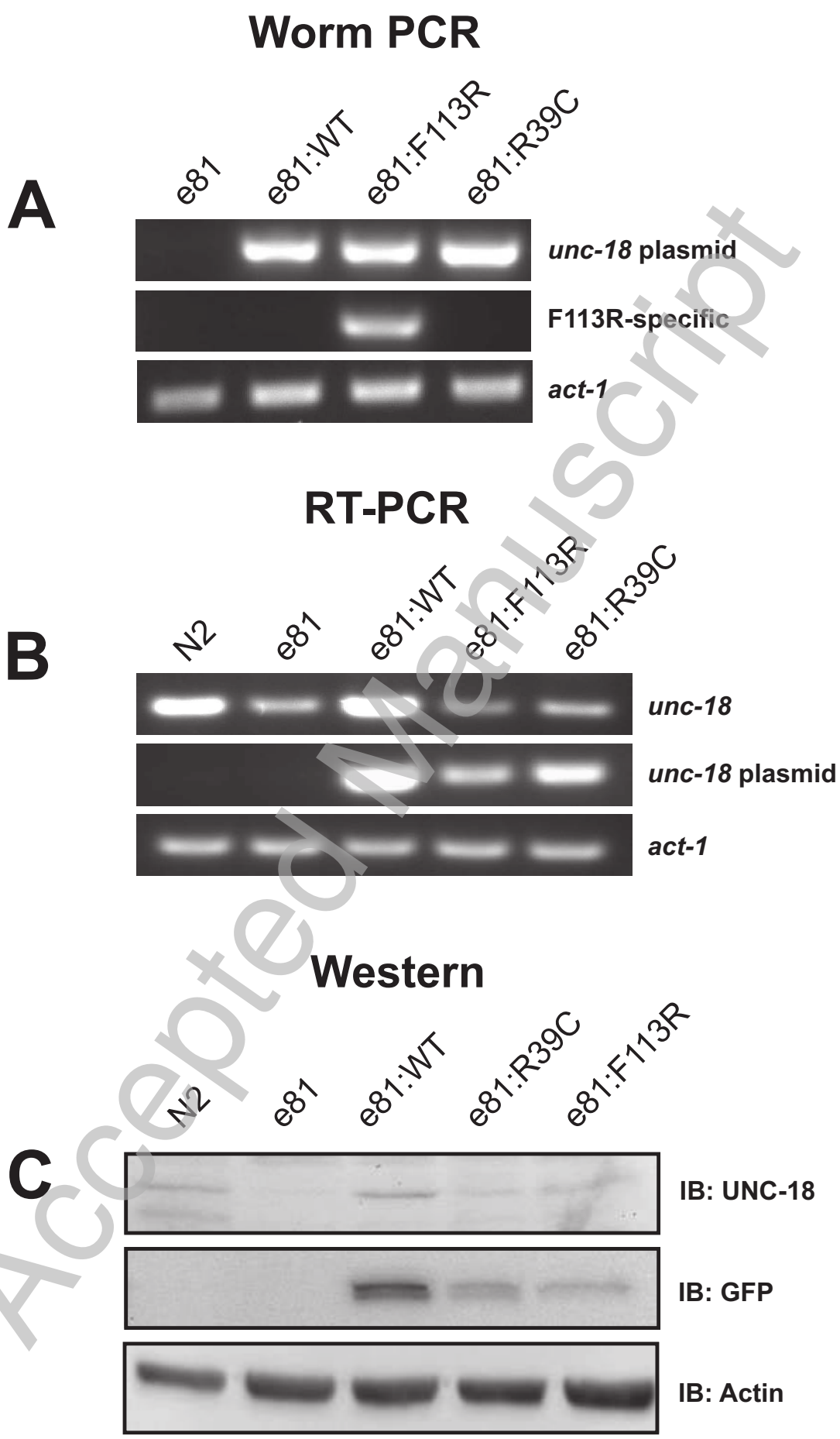

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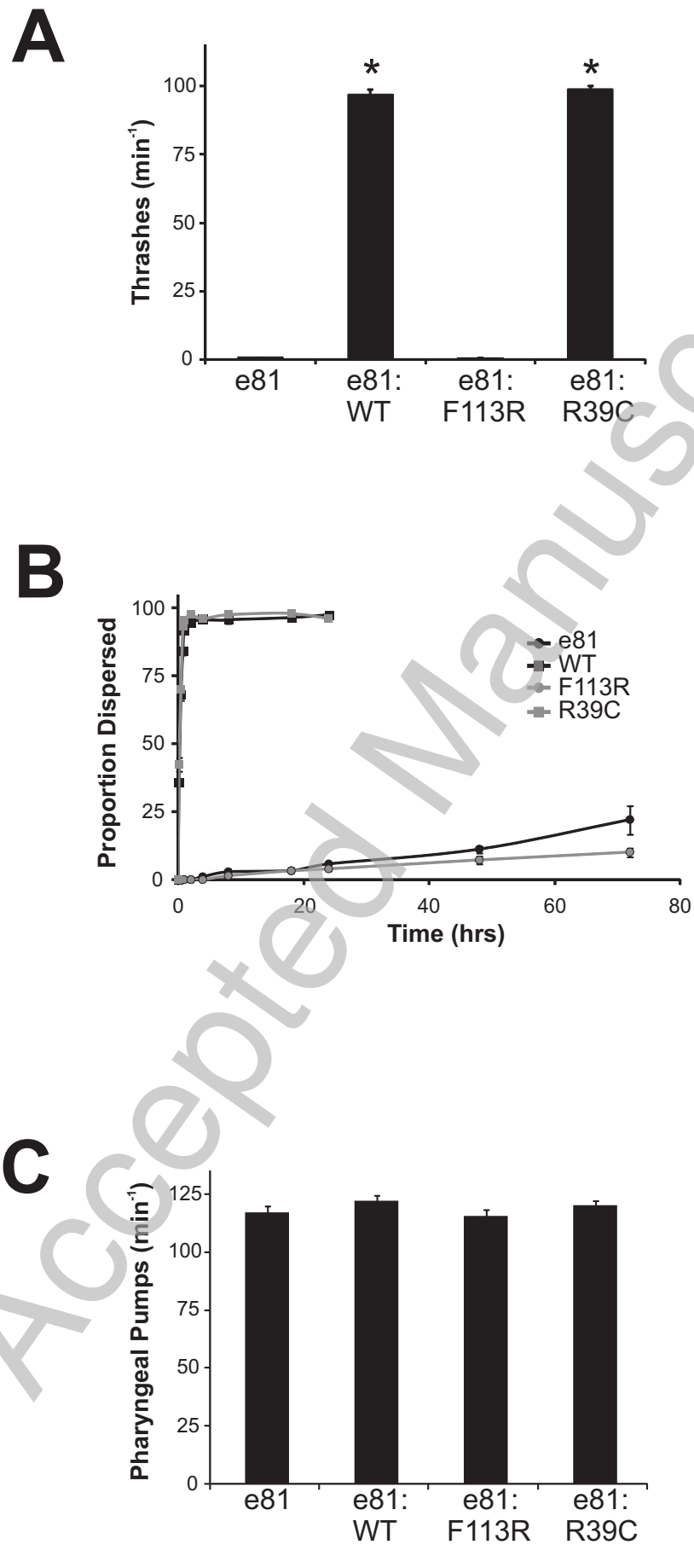

Johnson et al Fig 4