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**Characterisation of two distinct binding modes  
between Syntaxin 4 and Munc18c.**

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**Synopsis**

Interaction of Sec1/Munc18 (SM) proteins with their cognate syntaxins represents an important regulatory mechanism of SNARE-mediated membrane fusion.

Understanding the conserved mechanism by which SM proteins function in this process has proved challenging, largely due to an apparent lack of conservation of binding mechanisms between different SM/syntaxin pairs. Here we have identified a hitherto uncharacterised mode of binding between syntaxin 4 and Munc18c that is independent of the binding mode previously shown to utilise the N-terminal peptide of syntaxin 4. Our data demonstrate that syntaxin 4/Munc18c interact via two distinct modes of binding, analogous to those employed by syntaxin 1a/Munc18a and syntaxin 16/Vps45. These data support the notion that all syntaxin/SM proteins bind using conserved mechanisms, and pave the way for the formulation of unifying hypotheses of SM protein function.

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## Introduction

SNARE proteins are central to the regulation of membrane trafficking in all eukaryotic systems, providing not only the energy to drive bilayer fusion, but also a degree of specificity to the process [1]. Although it has been established that formation of a SNARE complex, comprising of three coiled-coil SNARE motifs contributed by the t-SNARE (one from a syntaxin protein and two from light chain SNAREs) on the target membrane and another from the v-SNAREs on the donor vesicle, is sufficient to drive membrane fusion *in vitro*, it is clear that other factors are required for the regulation of this process in the cell. One family of proteins that are central to the regulation of SNARE complex formation are the Sec1p/Munc18 (SM) proteins, most of which bind to their cognate syntaxins with high affinity [2]. Understanding the mechanism(s) by which SM proteins function has proved problematic largely due to seemingly conflicting data concerning the way in which different SM family members interact with their cognate syntaxins [2, 3].

Structural studies have revealed that the neuronal syntaxin 1a (Sx1a) adopts two distinct conformations [4, 5]. In the closed conformation the autonomously-folded, N-terminal regulatory, Habc domain is folded back onto the juxtamembrane SNARE motif, rendering it unavailable for SNARE complex formation. In the open conformation, the Habc domain is moved away from the SNARE motif leaving it free to participate in the core complex. Munc18a was originally identified as a Sx1a-binding protein whose binding to Sx1a precludes SNARE complex formation [6, 7]. Consistent with this, crystallographic studies revealed that Munc18a is an arch shaped molecule that cradles monomeric Sx1a in its closed conformation with contacts being made between the inner arch of the SM protein and almost the entire length of the syntaxin's cytosolic domain [5]. These data support a model in which SM proteins bind their cognate syntaxins in a closed conformation and regulate SNARE complex assembly, perhaps facilitating a switch of syntaxins from their closed to their open conformation [4].

In striking contrast to the interaction between Munc18a and Sx1a captured by the crystal structure [5], the extreme N-terminal region, preceding the Habc domain, of other syntaxins is both necessary and sufficient to capture their cognate SM proteins [8-12]. For example, the N-terminal 44 residues of Sed5p are sufficient to bind the SM protein Sly1p [8, 13]. The crystal structure of this interaction reveals that the N-terminal peptide of the Sx inserts into a hydrophobic pocket on the outer face of the SM protein [8]. This 'pocket mode' of binding is consistent with the SM protein binding to either the closed or open conformations of the syntaxin.

The finding that different SM proteins bind their cognate syntaxins via strikingly different mechanisms has severely hampered formulation of a unifying hypothesis describing the mechanisms by which SM proteins regulate SNARE-mediated membrane fusion. We have recently demonstrated that the yeast SM protein Vps45p uses two distinct modes of binding to interact with its cognate SNARE proteins at different stages of the SNARE complex assembly/disassembly cycle [14, 15]. Vps45p dissociates from its monomeric syntaxin, Tlg2p, prior to *trans*-SNARE complex formation and then re-associates following membrane fusion and the conversion of *trans*-SNARE complexes to *cis*-SNARE complexes [14, 15]. We hypothesise that all syntaxin/SM pairs interact using both of these modes of binding at different stages of the SNARE assembly/disassembly cycle. In this model, the SM protein would prevent

futile reformation of *cis*-SNARE complexes following the action of the ATPase NSF and allow the SNARE proteins to recycle for further rounds of membrane fusion.

Consistent with the above, and in contrast to the long-held contention that Munc18a has binds only to the closed conformation of Sx1a [16], it has recently been demonstrated that Munc18a also binds to the syntaxins extreme N-terminus [17-19] and also to the assembled SNARE complex [20]. Importantly, deletion of the N-terminal 21 residues from a mutant version of Sx1a locked in the open conformation severely diminished binding of Munc18a indicating that, like Vps45p, Munc18a uses distinct modes of binding to interact with its cognate SNARE proteins [17-19].

In this study, we sought to characterise the binding between Munc18c and Syntaxin4 (Sx4), an interaction that mediates the insulin-stimulated delivery of GLUT4-vesicles to the plasma membrane of adipocytes and muscle cells (reviewed in [21]). Recent crystallographic studies have revealed that the binding of Munc18c to Sx4 is analogous to the interaction captured by the Sly1p/Sed5p crystal structure, with the N-terminus of Sx4 inserting into a hydrophobic pocket on the outer surface of the SM protein [11]. Here, we have used an *in vitro* binding approach to establish that Sx4 can interact with Munc18c, not only through this pocket-mode of binding as previously described, but also through a distinct, previously uncharacterised mode.

## Experimental

### Plasmids.

**N-terminally tagged Sx4 species.** Plasmid pGEX5-Sx4 encoding the cytosolic domain of Sx4 (residues 1 to 273) tagged at the N-terminus with GST was constructed using PCR to amplify the Sx4 coding sequence from a cDNA provided by Richard Scheller. The product of this reaction was sub-cloned into pGEX-5X1. The open mutant (L165A, E166A), the N $\Delta$  mutant (residues 37-273) and the N $\Delta$ -open mutant (residues 37-273, L165A, E166A) were then generated from this plasmid by PCR and/or site-directed mutagenesis (SDM).

### C-terminally tagged Sx4 species.

To create a plasmid allowing expression of C-terminally tagged GST fusion proteins, the coding sequence for GST was amplified from pGEX-5X1 with restriction sites *Xho*I and *Pac*I at either end. This was then used to replace the S-tag of pETDuet-1 (Novagen). Sx4 species were amplified from the plasmids described above and inserted in frame with the GST tag in pETDuet-1-GST. The L8K mutants were generated using SDM.

### Purification of GST-fusion proteins and Munc18c

GST fusion proteins were expressed in Rosetta 2(DE3) *E. coli*. Protein production was induced at 30°C overnight by the addition of 0.5 mM IPTG. *E. coli* were harvested by centrifugation at 4,000 g and lysed in GST purification buffer (100 mM Hepes, 500 mM NaCl, 5 mM MgCl<sub>2</sub>, 2 mM  $\beta$ -mercaptoethanol pH 7.4) containing complete protease inhibitors (Roche), by the addition of lysozyme and sonication. GST-tagged proteins were purified using Glutathione Sepharose (Amersham). Unbound protein was removed by washing with PBS. Bound protein was eluted from the beads with 50 mM Tris pH 8, 15 mM Glutathione. Munc18c was expressed as an N-terminal His<sub>6</sub>-fusion protein from the vector pQE30 in M15 cells co-transformed with a vector encoding GroEL. Protein production was induced at 22°C overnight by the addition of 0.2 mM IPTG. *E. coli* were harvested by centrifugation at 4,000 g and lysed in His<sub>6</sub>-purification buffer (25 mM Hepes pH7.4, 200 mM KCl, 10 % (w/v) glycerol, 2 mM  $\beta$ -mercaptoethanol, 15 mM Imidazole) containing EDTA-free complete protease inhibitors (Roche), by the addition of lysozyme and sonication. His<sub>6</sub>-tagged protein was purified using Ni-NTA resin (Qiagen). Unbound protein was removed from the resin by washing with His<sub>6</sub>-purification buffer. Bound protein was eluted from the resin with His<sub>6</sub>-elution buffer (25 mM Hepes pH7.4, 200 mM KCl, 10 % (w/v) glycerol, 2 mM  $\beta$ -mercaptoethanol, 400 mM Imidazole). Purified protein was dialysed against PBS and were analysed by SDS-PAGE and Coomassie staining.

### GST pull-downs

5  $\mu$ g of GST or GST-tagged protein, was incubated with 10  $\mu$ l glutathione Sepharose (50% slurry) in a volume of 100  $\mu$ l of binding buffer (150 mM Potassium acetate, 1 mM MgCl<sub>2</sub>, 0.05 % Tween 20, 20 mM Hepes pH 7.4) for 1 h in the cold room with end-over-end rotation. Unbound protein was removed by washing the beads three times with binding buffer and beads were collected by centrifugation at 500 g at 4°C for 2 min. 15  $\mu$ g Munc18c was added in a total volume of 500  $\mu$ l of binding buffer and incubated overnight at 4°C with continual mixing. Unbound protein was removed by washing three times with binding buffer containing 1 mg/ml fish skin gelatin, followed by three washes with binding buffer containing 5 % (w/v) glycerol then three washes with binding buffer alone. After the final wash the beads were

resuspended in 15  $\mu$ l SDS-PAGE sample buffer and heated to 95°C for 5 minutes prior to analysis by SDS-PAGE and Coomassie staining. Binding was quantified by densitometric scanning of non-saturating exposed films using TotalLab imaging software (Phoretix).

#### ***Circular dichroism.***

Circular dichroism (cd) measurements were obtained using a Jasco J-810 spectropolarimeter (Jasco, UK). For near uv cd analysis, data were collected from 320-250 nm in a 0.5 cm pathlength quartz cuvette using a protein concentration of 1mg/ml, a scan rate of 10 nm/min, 1 nm bandwidth and a response time of 2 sec. For far uv cd analysis, data were collected from 260-180 nm in a 0.02 cm pathlength quartz cuvette using protein concentration of 0.3 mg/ml, a scan rate of 50 nm/min, 1 nm bandwidth and a response time of 0.5 sec. Sx4 and Sx4-open were cleaved from the GST tag using thrombin prior to analysis.

#### ***Protease digestion experiments.***

Recombinant wild-type Sx4 or Sx4-open were incubated with chymotrypsin (1:100 w/w) in PBS and, at the time points indicated, samples were taken and immediately boiled in an equal volume of 2X SDS-PAGE sample buffer. Samples were then analysed by SDS-PAGE and Coomassie staining.

#### ***Surface plasmon resonance.***

Interactions between Munc18c and the GST-tagged Sx4 constructs were assessed at 25 degrees C using a Biacore 2000 instrument (Biacore AB). Syntaxin constructs were immobilised onto a CM5 sensorchip via GST antibodies covalently coupled using *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide chemistry. Following antibody immobilisation, residual binding sites on the sensorchip were blocked using 1M ethanolamine. All measurements were corrected using a double referencing correction procedure (subtraction of reference flow cell and buffer injections). Munc18c binding was assessed by injecting a series of increasing protein concentrations until a maximum binding concentration was achieved (40 $\mu$ l/min).

## ***Results and Discussion***

### ***Creation of an open mutant of Syntaxin4***

Mutation of residues L165 and E166 within the hinge region between the SNARE motif and the Habc domain of Sx1a both to alanines, results in a protein that is unable to adopt the closed conformation [4]. We mutated the analogous residues in the cytosolic domain of Sx4 (L173A, E174A) in the context of a GST-fusion protein. Analysis by circular dichroism demonstrated that these mutations do not cause any gross conformational changes in secondary structure (Fig. 1A; the small differences in the near UV spectra between the wild-type protein and the mutant protein likely reflect localised changes in the environments of aromatic amino acids in the Sx4<sub>L173A,E174A</sub> mutant). This is consistent with the introduced mutations affecting only the hinge region of Sx4, and not perturbing the structure of either the SNARE domain or the Habc domain, as has been reported for the Sx1a open mutant [4]. To further characterise the Sx4<sub>L173A,E174A</sub> mutant, we compared its sensitivity to chymotrypsin digestion with that of its wild-type counterpart. The Sx1a-open mutant is more sensitive to proteolysis [22], and Figure 1B shows that the Sx4<sub>L173A,E174A</sub> mutant was also substantially more sensitive to digestion, consistent with it predominantly adopting an open conformation. Through analogy with the Sx1a-open mutant, and the data presented in Figure 1, we will hereafter refer to this mutant as Sx4-open.

### ***Analysis of Munc18c binding to Syntaxin4***

To study the interaction(s) between Sx4 and Munc18c, we first examined the ability of the cytosolic domains of wild-type Sx4, Sx4-open, and a further mutant of Sx4 lacking the N-terminal 36 amino acids (Sx4-N $\Delta$ ) to bind Munc18c. Both wild-type Sx4 and Sx4-N $\Delta$  efficiently capture Munc18c in these experiments whereas Sx4-open exhibits a severely diminished capacity to do so (Fig. 2). The observation the Sx4-N $\Delta$  binds Munc18c was unexpected as this mutant lacks the N-terminal region of the syntaxin that has been shown, through crystallographic studies, to interact with Munc18c via the pocket mode of binding [11, 12]. Sx4-open contains this N-terminal region and therefore it was also surprising that this mutant displayed a drastically reduced ability to bind Munc18c compared to the full-length cytosolic domain (Fig. 2).

We considered the possibility that the bulky GST moiety at the N-terminus of the Sx4 constructs used in Figure 2 was interfering with the pocket-mode of binding, and generated the same collection of Sx4 constructs, tagged at the C-terminus. The ability of these to bind Munc18c was investigated as described for the N-terminally tagged constructs presented in Figure 2 (Fig. 3). Using the C-terminally tagged constructs, deletion of the N-terminal 36 amino acid residues of Sx4 drastically reduced the ability of Sx4 to capture Munc18c.

The crystal structure of the pocket-mode of binding between the N-terminal peptide of Sx4 and Munc18c identifies L8 of Sx4 as making a crucial contact with a hydrophobic pocket in domain 1 of Munc18c [11]. We therefore engineered the L8K mutation, previously shown to reduce Munc18c binding to Sx4 [12], into the C-terminally tagged cytosolic domain of Sx4. Figure 4 shows that the L8K mutation significantly reduced Munc18c binding to both wild-type Sx4 and Sx4-open indicating that these proteins both utilise the pocket-mode of binding to capture Munc18c. The data presented in Figures 2 - 4 suggest that the presence of a GST-tag



at the N-terminus of Sx4 prevents the pocket-mode of interaction with Munc18c, perhaps by steric hindrance.

Importantly, the observation that Sx4-open does not bind Munc18c when tagged at the N-terminus indicates that the GST-Sx4 and GST-Sx4-N $\Delta$  used in Figure 2 reveal an interaction with Munc18c that is independent of the pocket-mode of binding. This interaction is blocked by the N-terminal GST tag in the case of GST-Sx4 and by both the tag and the deletion of residues 1-36 in the case of GST-Sx4-N $\Delta$ ). The most obvious hypothesis is that this interaction is akin to that reported for Munc18a binding to the closed conformation of Sx1a [5]. Consistent with this, both the GST-Sx4-open and the GST-Sx4-open-N $\Delta$  mutants exhibit significantly reduced abilities to bind Munc18c (Fig. 2).

When the GST-tag is present at the C-terminus of the cytosolic domain of Sx4, the N-terminus is available to contribute to Munc18c binding. The Sx4-open mutant in this context binds Munc18c effectively because its N-terminus is unhindered and available to use the pocket-mode of binding to capture Munc18c (Fig. 3). Deletion of the amino terminal 36 residues (Fig. 3) or the introduction of L8K mutation (Fig. 4) from this mutant inhibits binding of Munc18c as the pocket-mode of binding is now abolished. Hence we propose that when tagged at the C-terminus, Sx4 is capable of binding via the pocket mode. Furthermore, the data presented in Figures 3 and 4 indicate that the C-terminally-tagged constructs bind predominantly through the pocket-mode of binding since its abolition drastically reduces the binding of Sx4-GST to Munc18c (Sx4-N $\Delta$ -GST and Sx4-L8K-GST). This suggests that, when tagged with GST at the C-terminus, the cytosolic domain of Sx4 either predominantly adopts an open conformation or is capable of making the closed to open transition effectively.

Figure 1 shows that introduction of two point mutations (L173A, E174A) opens the structure of GST-Sx4 and increases its sensitivity to protease digestion with chymotrypsin. To test the hypothesis that C-terminally-tagged Sx4 (Sx4-GST) exists in a predominantly open conformation, we compared its sensitivity to chymotrypsin to that of Sx4 tagged at the N-terminus (GST-Sx4) and the corresponding constitutively open-mutants (GST-Sx4open and Sx4open-GST). Figure 5 demonstrates that the C-terminally tagged Sx4-GST displays the same sensitivity to chymotrypsin as its corresponding open mutant (compare lanes 7 and 8). In contrast, N-terminally tagged GST-Sx4 is less sensitive to chymotrypsin than its corresponding open mutant (compare lanes 3 and 4). These data suggest that Sx4-GST is found predominantly in an open conformation in contrast to GST-Sx4.

Our finding that binding of Munc18c to C-terminally-tagged Sx4 occurs through the pocket-mode of binding utilising the N-terminal peptide of Sx4, and that the binding of Munc18c to N-terminally tagged Sx4 occurs through a second mode of binding independent of the pocket-mode of binding, perhaps akin to the binding of Munc18a to the closed conformation of Sx1a, afforded the opportunity to measure the affinities of these two interactions in isolation. Using Surface Plasmon Resonance we found that C-terminally tagged Sx4-GST has an apparent binding constant ( $K_d$ ) of  $\sim 28$ nM whereas the N-terminally tagged GST-Sx4 had a  $K_d$  of  $\sim 254$ nM. From these binding data, we propose that the interaction between the N-terminal peptide of Sx4 and Munc18c is a high-affinity interaction, as previously reported [12] whereas the second, previously uncharacterised interaction is approximately 10-fold weaker. It is

noteworthy that a recent study of *in vivo* Sx4/Munc18c interactions studied using Fluorescence Resonance Energy Transfer also concluded that regions of Sx4 outwith the N-terminus contribute to Munc18c binding [23]. However, the presence of a CFP-moiety at the N-terminus of Sx4 in this study may complicate analysis.

Formulation of a unifying hypothesis describing the action of SM proteins has been severely hampered by observations suggesting that different members of this family bind their cognate syntaxins via striking different mechanisms [2]. Recent studies however have shown that an increasing number of SM protein/syntaxin pairs use multiple modes of binding [15, 17-19]. Here we have shown that Sx4 and Munc18c also interact using multiple modes of binding with different affinities, allowing the field to move towards understanding the conserved mechanisms by which SM proteins control membrane fusion. A further important point arising from our study is that the positioning of affinity tags can significantly alter the binding profile of a syntaxin. This could explain why some interactions between SM proteins and syntaxins have been hitherto overlooked.

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### Figure legends

**Figure 1. Analysis of the Sx4<sub>L173A,E174A</sub> mutant.** (A) Near UV CD spectra (250 to 320 nm) were recorded for the cytosolic domains (generated by thrombin cleavage of GST-fusions) of wild-type Sx4 (dashed line) and Sx4<sub>L173A,E174A</sub> (solid line). Proteins were diluted to 1 mg/ml in 50 mM KH<sub>2</sub>PO<sub>4</sub>- K<sub>2</sub>HPO<sub>4</sub> pH 7.4. Data shown represent averaged spectra from 4 scans. Far UV CD spectra (insert) were recorded from 180 to 260 nm using proteins diluted to 0.3 mg/ml. Data shown represent averaged spectra from 8 scans. (B) Recombinant wild-type Sx4 or Sx4<sub>L173A,E174A</sub> (Sx4-LE/AA) were incubated with chymotrypsin (1:100 w/w). Samples were taken and immediately boiled in an equal volume of 2X SDS-PAGE sample buffer at the time points indicated prior to analysis by SDS-PAGE (12% gel) followed by Coomassie staining. Data from a representative experiment, repeated three times are shown.

**Figure 2. Munc18c binding to N-terminally tagged Syntaxin4.** (A) 5 µg GST, GST-Sx4, GST-Sx4-open, GST-Sx4-NΔ or GST-Sx4-open-NΔ were incubated with 10 µl of glutathione-Sepharose for 1 h and the beads extensively washed to remove unbound protein. 15 µg Munc18c was added and incubated overnight at 4°C with end-over-end rotation. Beads were then extensively washed and bound proteins eluted into SDS-PAGE sample buffer. Shown in the left panel are the purified proteins used in the experiment (input). Right panel shows proteins eluted from beads bound to the Sx4 species indicated (beads). Data shown are representative of four experiments of this type. Panel (B) shows quantification of these data, normalised to the amount of Munc18c bound to wild-type Sx4, expressed as mean ± SD.

**Figure 3. Munc18c binding to C-terminally tagged Syntaxin4.** (A) Binding of Munc18c to the same set of Sx4 species described in Figure 2 was examined, this time in the context of Sx4 tagged at the C-terminus with GST. Binding was performed exactly as outlined in the legend to Figure 2. Data from a typical experiment repeated three times are shown. Panel (B) shows quantification of these data, normalised to the amount of Munc18c bound to wild-type Sx4, expressed as mean ± SD.

**Figure 4. Munc18c binding to the Sx4-L8K mutant.** (A) Binding of Munc18c to Sx4, Sx4-L8K, Sx4-open, or Sx4-open-L8K (in the context of Sx4 tagged at the C-terminus with GST) was examined exactly as outlined in the legend to Figure 2. Data from a typical experiment repeated three times are shown. Panel (B) shows quantification of these data, normalised to the amount of Munc18c bound to wild-type Sx4, expressed as mean ± SD.

**Figure 5. Protease sensitivity of differentially tagged Sx4.** Recombinant wild-type Sx4 (WT) or Sx4-Open (Op) tagged at either the N-terminus (GST-Sx4; GST-Sx4-open) or the C-terminus (Sx4-GST; Sx4-open-GST) were incubated with or without chymotrypsin (ChT; 1:100 w/w) for 20 min. Samples were then boiled in an equal volume of 2X SDS-PAGE sample buffer prior to analysis by SDS-PAGE (12% gel) and Coomassie staining. Data from a representative experiment, repeated three times are shown.

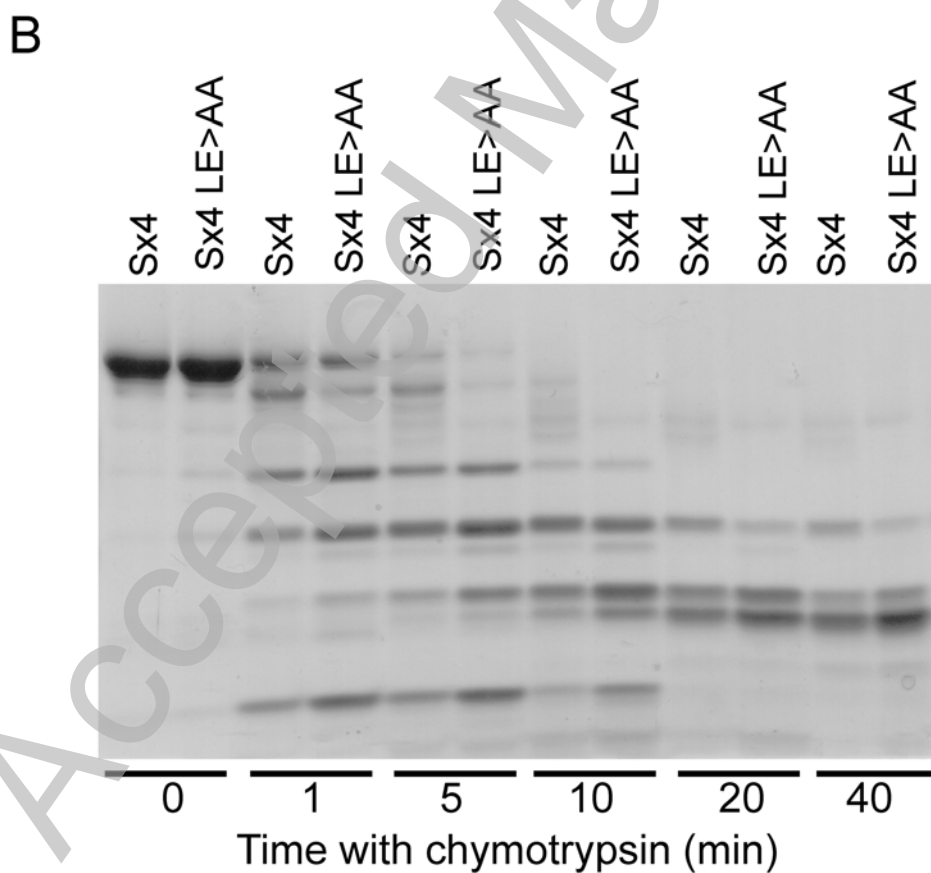
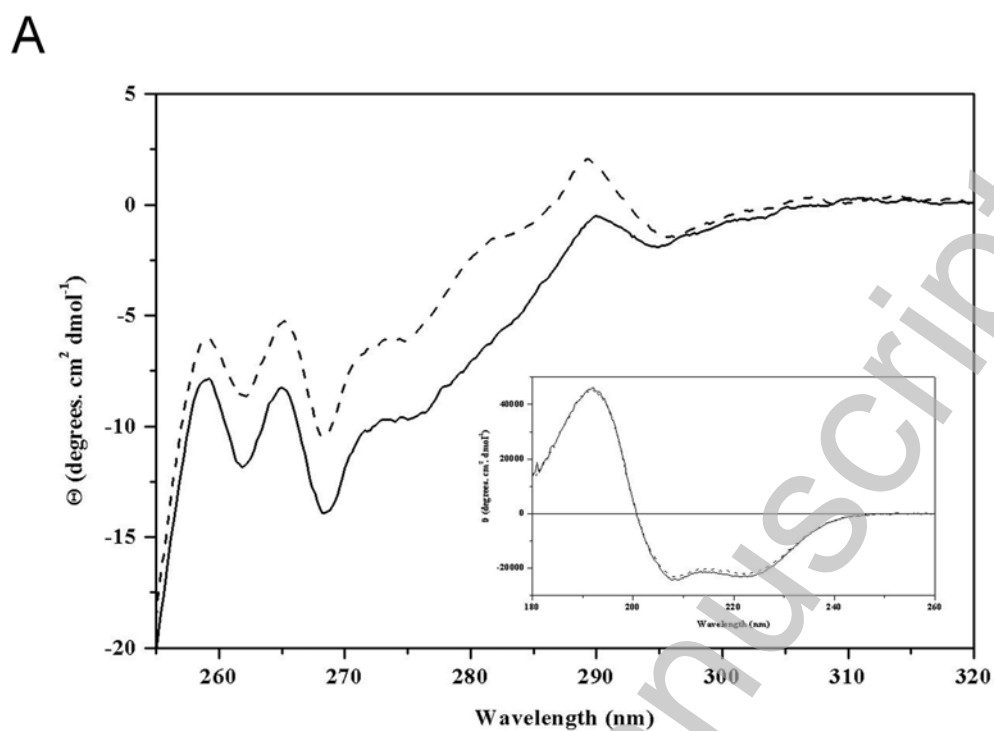
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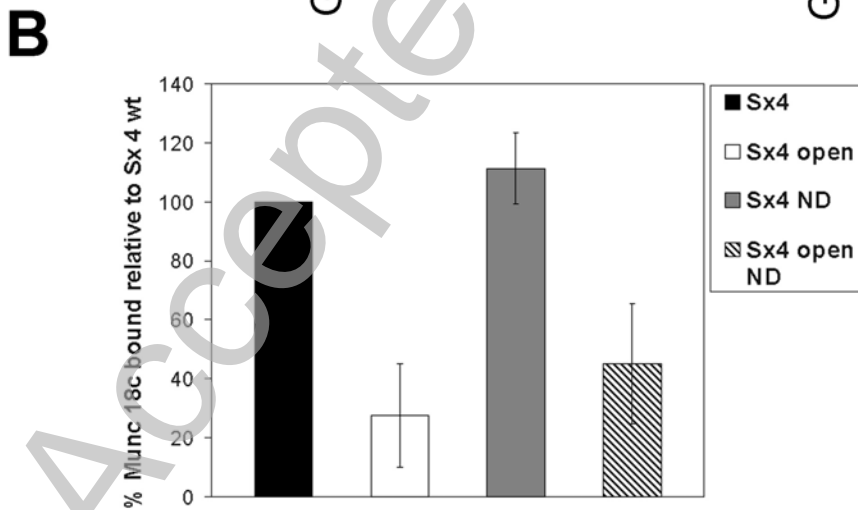
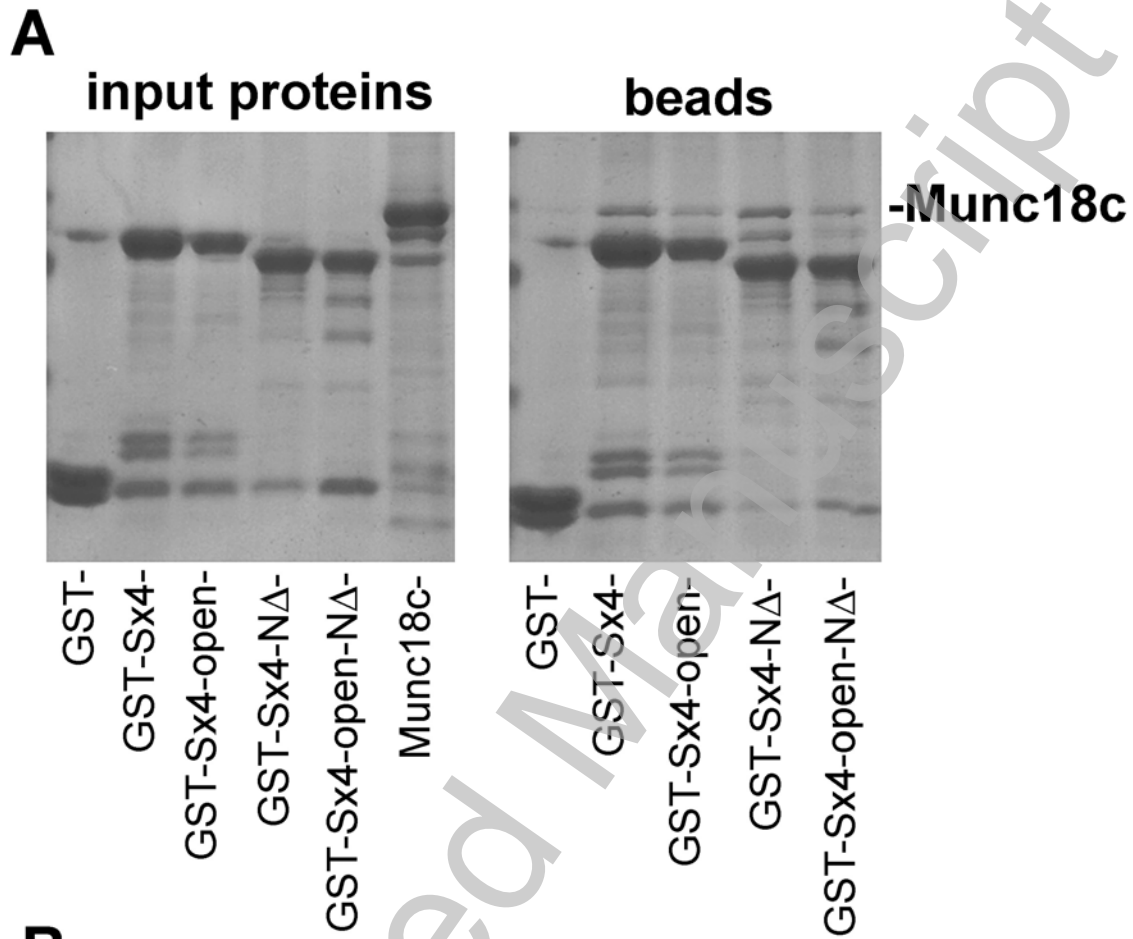
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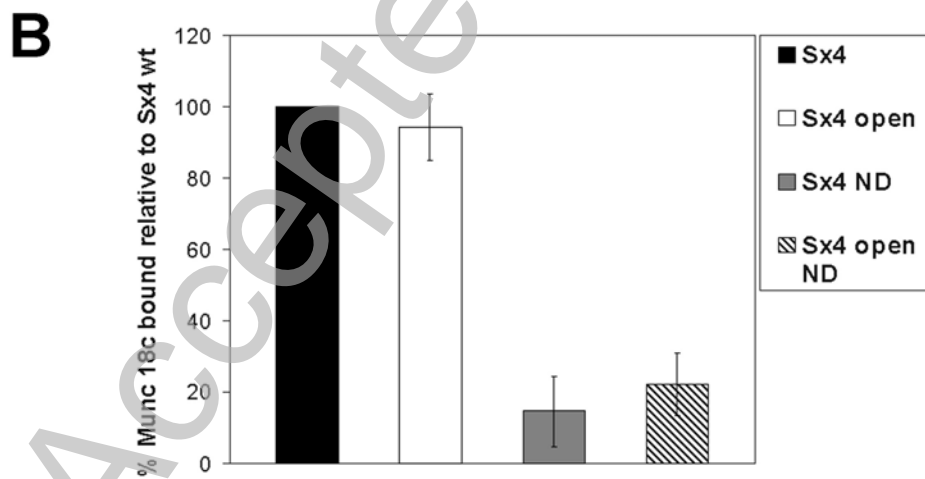
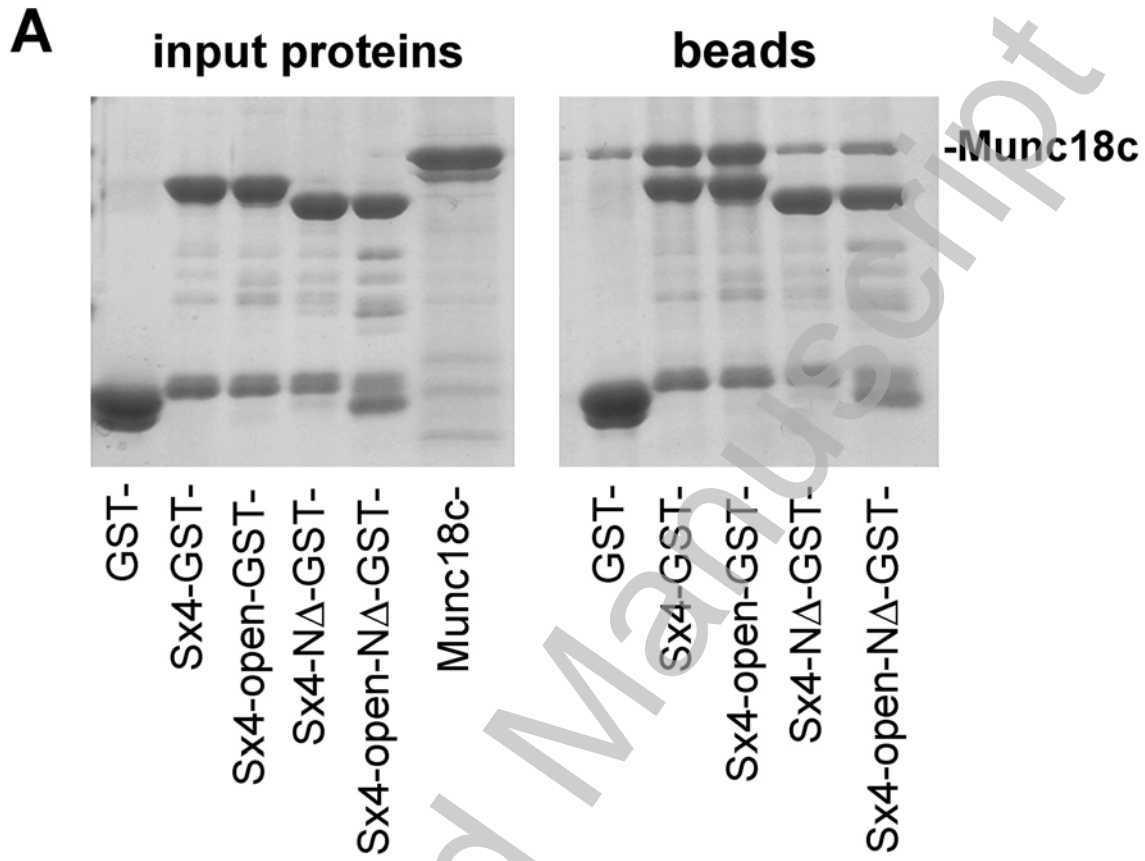
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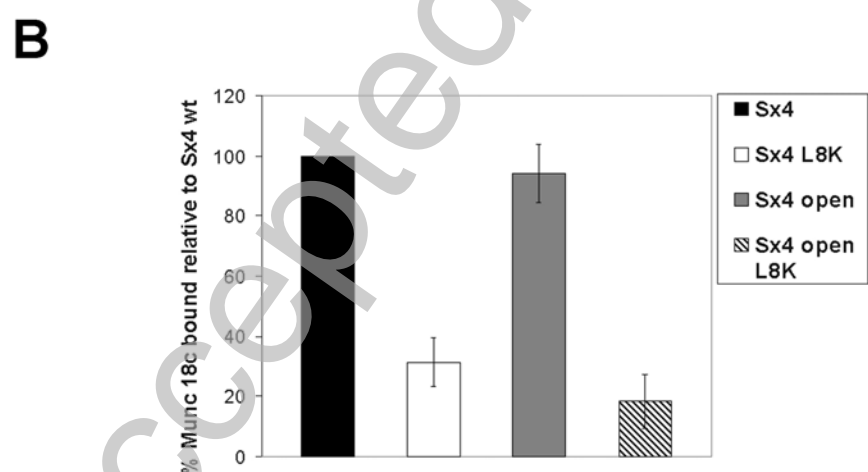
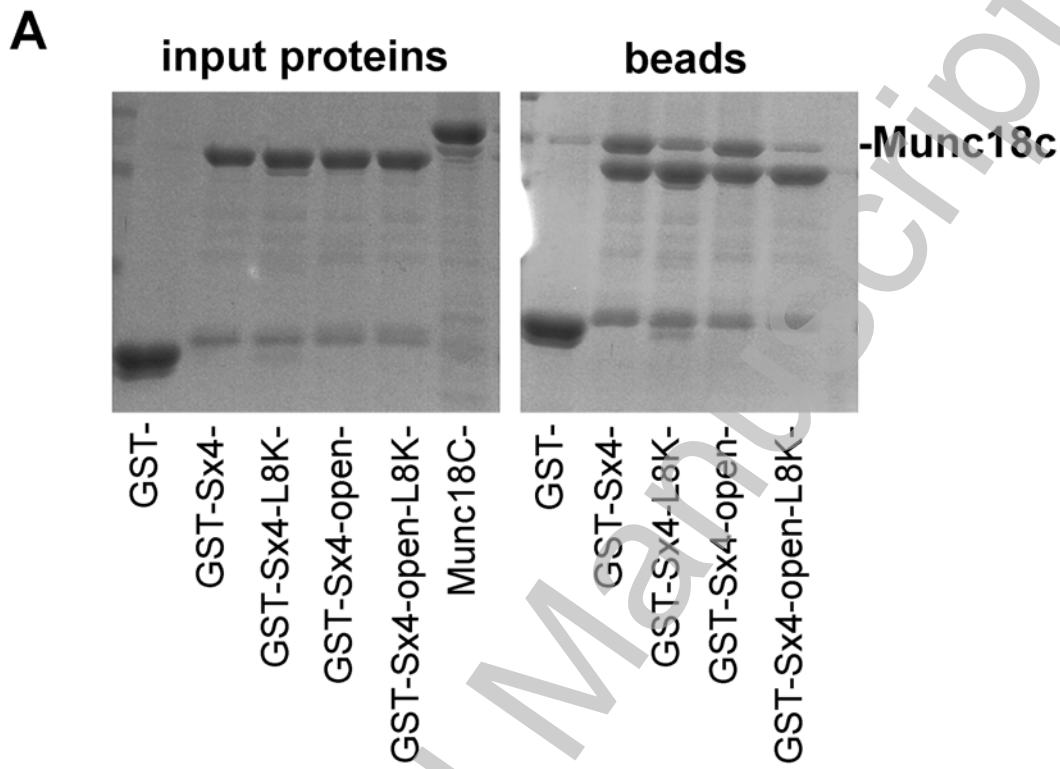
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