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Evidence for a conserved inhibitory binding mode between the membrane fusion assembly factors Munc18 and syntaxin in animals

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Running title: A common Munc18 and syntaxin binding mode

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ABSTRACT

The membrane fusion necessary for vesicle trafficking is driven by the assembly of heterologous SNARE proteins orchestrated by the binding of Sec1/Munc18 (SM) proteins to specific syntaxin SNARE proteins. However, the precise mode of interaction between SM proteins and SNAREs is debated, as contrasting binding modes have been found for different members of the SM protein family, including the three vertebrate Munc18 isoforms. While different binding modes could be necessary given their roles in different secretory processes in different tissues, the structural similarity of the three isoforms makes this divergence perplexing. Though the neuronal isoform Munc18a is well established to bind tightly to both the closed conformation and the *N*-peptide of Syntaxin 1a, thereby inhibiting SNARE complex formation, Munc18b and c, which have a more widespread distribution, are reported to mainly interact with the *N*-peptide of their partnering syntaxins and are thought to instead promote SNARE complex formation. We have re-investigated the interaction between Munc18c and Syntaxin 4 (Syx4). Using isothermal titration calorimetry, we found that Munc18c, like Munc18a, binds to both the closed conformation and the *N*-peptide of Syx4. Furtermore, using a novel kinetic approach, we found that Munc18c, like Munc18a, slows down SNARE complex formation through high-affinity binding to syntaxin. This strongly suggests that secretory Munc18s in general control the accessibility of the bound syntaxin, probably preparing it for SNARE complex assembly.

In eukaryotic cells, material exchange between the different compartments such as the endoplasmic reticulum (ER), the Golgi apparatus, lysosomes, endosomes, and the plasma membrane occurs via vesicle trafficking. The central molecular machine involved in the vesicle fusion process in each trafficking step is composed of a distinct set of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) and Sec1/Munc18 (SM) proteins. These distinct sets probably arose from duplication and diversification of a singular prototypic vesicle fusion machinery and adapted to the needs of specialized compartments during the rise of the eukaryotic cell (1-6). This suggests that a common mechanism of action of the fusion machine must exist; nevertheless, deciphering the precise mode of interaction between SNARE and SM proteins has proved rather challenging. Recently, a more detailed picture is beginning to emerge: When a transport vesicle reaches its target compartment, heterologous SNARE proteins assemble into tight membrane-bridging complexes that pull the two fusing membranes together. Their sequential assembly is orchestrated by SM proteins. These are thought to act primarily

through binding to one specific type of SNARE protein, the syntaxins (7-9). Recent findings indicate that several different SM protein types such as Munc18 (secretion)(10, 11), Sly1 (ER-Golgi)(12), and Vps45 (*trans*-Golgi network)(10, 13) are able to interact simultaneously with two distinct regions of syntaxin, the *N*-peptide (i.e. the very *N*-terminal region) and the remainder of the molecule in a closed conformation. In the closed conformation, the three-helix bundle formed by syntaxin's *N*-terminal Habc domain folds back onto its SNARE motif (14, 15), restricting its availability for SNARE complex formation. SM proteins probably shape the conformation of the bound syntaxin for its SNARE partners (10, 15-18). Subsequently, SM proteins are likely to facilitate the assembly of the SNARE complex by stabilizing a binding site for the vesicular R-SNARE (19). Possibly at the same time, the SM protein provides an additional R-SNARE interaction site that aligns two of the SNARE proteins for complex formation, as demonstrated for the vacuolar/lysosomal SM protein Vps33 (20).

As the focus has recently shifted towards the role of SM proteins during the final steps of SNARE complex assembly (e.g. (19-21)), less attention has been given to the fact that apparently contrasting syntaxin-binding modes have been found for the three Munc18 isoforms present in vertebrate genomes, namely Munc18a, Munc18b, and Munc18c (also called Munc18-1, -2, and -3) (22-24). All three vertebrate Munc18 isoforms are essential (25-27), but all three regulate the exocytosis of different types of secretory vesicles, often in different tissues, where they work together with their respective tissue-specific syntaxin (Syx) isoforms.

Munc18a is involved in neurotransmission and neuroendocrine release together with Syx1a or 1b (28-30). Mutations in Munc18a are linked to epilepsy and encephalopathy (31). By contrast, Munc18b and Munc18c show more widespread tissue distribution (32-35). Munc18b has mostly been studied in exocytosis, along with Syx3 and Syx11, in epithelial and hematopoietic cells (e.g. (27, 36, 37) (38-40)) and mutations are linked to hemophagocytic lymphohistiocytosis (41-43). Munc18c has mainly been studied in the exocytosis of the glucose transporter 4 (GLUT4) in adipocytes and skeletal muscle cells and in insulin exocytosis in the pancreas, which are both processes associated with Syx4 (44-46).

Munc18a is the best-studied SM protein physiologically and biochemically. The structure of Munc18a has been determined in a complex with the closed conformation (15) and the *N*-peptide (10) of Syx1a. Whether and how the two spatially separated binding sites communicate in the complex and how Munc18 prepares the bound syntaxin for SNARE complex assembly afterwards is unclear so far. The structures of the two other vertebrate isoforms, Munc18b (47) and Munc18c (48), have turned out to be highly similar to that of Munc18a. Notably, the overall structural conservation of different SM protein types is astonishing (7). However, in contrast to the Munc18a-Syx1a structure, the Munc18c structure was revealed in a complex with only the *N*-peptide of Syx4 (48). Interestingly, the Syx4 *N*-peptide binds with higher affinity to Munc18c than the Syx1 *N*-peptide to Munc18a (49). It has been brought forward that Munc18c mainly interacts with the *N*-peptide of Syx4 and the assembled SNARE complex and that it does not lock the bound syntaxin in a closed conformation (50), although this view is debated heavily (7, 46, 51-53). Liposome fusion experiments did not resolve this issue, as one study found that Munc18c, like Munc18a (54), promotes SNARE complex formation (55), whereas an earlier study had observed an inhibitory role for Munc18c during membrane fusion (56). As a way out, it has been suggested that Munc18a, which binds with high affinity to the closed syntaxin (10, 16), has undergone a specific evolution to satisfy the needs for fast neuronal secretion (57). But is this really the case? Invertebrates appear to sustain synaptic communication with only one Munc18. Did the three vertebrate Munc18s indeed diverge so much that the proteins changed their *modus operandi*?

We wanted to take a more detailed look at this conundrum. We have developed a fluorescence-based read-out for formation of SNARE complexes containing Syx4, as the role of Munc18c has been studied mainly through liposome fusion assays, which observe SNARE complex formation indirectly. With our new assay, we found that Munc18c, like Munc18a, interacts with the *N*-peptide and the closed conformation of syntaxin and slows down SNARE complex

formation, suggesting that all Munc18s share a common molecular mechanism, which is to prepare syntaxin for SNARE complex formation.

RESULTS

First, we extended our previous analysis on the phylogeny of Munc18s from all animals and their single-celled ancestors (11). In the current study, special attention was given to Munc18s from vertebrates in order to take a broader look at their conservation pattern and to resolve their phylogenetic relationships. The phylogenetic tree corroborates the notion that vertebrates generally possess three different secretory SM proteins: Munc18a, Munc18b, and Munc18c (11, 22-24), whereas many other animals (and most single-celled eukaryotes) often have only one Munc18 (Fig. 1). Overall, the sequences of all animal Munc18s are highly conserved, which is in agreement with the very high structural similarity observed (7, 47). The sequence conservation pattern reveals that both spatially separated binding regions for syntaxins, the *N*-peptide binding pocket, and the central cavity, which binds to the closed conformation, are maintained in all Munc18s (Fig. S1), suggesting that the binding mode that includes two binding surfaces is preserved in the three vertebrate isoforms. We therefore sought to re-investigate the binding mode of the most divergent vertebrate isoform, Munc18c, which is currently thought to bind predominantly to the *N*-peptide of its syntaxin partner.

Munc18c interacts with two regions of syntaxin - After establishing a procedure for purifying recombinant mouse (*Mus musculus*) Munc18c, we assessed its reactivity via isothermal titration calorimetry (ITC). As previous studies had shown that Munc18c binds to Syx4, we first mixed Munc18c with the entire cytoplasmic region of Syx4, Syx4 aa1-270. We determined a relatively high affinity of about 12 nM ($\Delta H \approx$ -14 kcal/mol, Fig. 2), which is about two orders of magnitude higher than that measured earlier via ITC for binding of the isolated *N*-peptide of Syx4 (Syx4 aa1-10) to Munc18c ($\approx$ 1.5 µM) (49). The stark difference in binding strength strongly suggests that not only the *N*-peptide region but also the remainder of Syx4 contributes to binding to Munc18c.

The sole Syx4 *N*-peptide binds to the outer surface of Domain 1 of Munc18c (48). This binding mode has been corroborated in several studies, but until now, it has not been fully understood how the remainder of Syx4 contributes to binding to Munc18c and whether its binding mode resembles that of the homologous pair Munc18a and Syx1a For the latter pair, it has been established that the *N*-peptide and the remainder of Syx1a bind to two different areas of Munc18a (10).

To gain deeper insights into the binding mode between Munc18c and Syx4, we tested other Syx 4 constructs for their ability to bind to Munc18c. When we used a Syx4 construct made up of the *N*-peptide and the Habc domain only (Syx4 aa1-191), we observed that it bound to Munc18c with lower affinity and with a smaller enthalpy change (Kd $\approx$ 546 nM, $\Delta H \approx$ -3.5 kcal/mol, Fig. 2) than the entire cytoplasmic domain of Syx4. As the affinity for Syx4 aa1-191 is only marginally higher than that of the isolated *N*-peptide, the finding reveals that the *C*-terminal SNARE motif of Syx4 contributes strongly to binding to Munc18c.

Interestingly, when we tested the isolated SNARE motif of Syx4, Syx4 aa192-270, we did not observe binding to Munc18c (Table 1, Fig. S2), ruling out that this portion of Syx4 can bind in the absence of the *N*-terminal portion of Syx4 to Munc18c. Remarkably, when we injected a premix of the SNARE motif (Syx4 aa192-270) and the Habc domain (Syx4 aa1-191) into Munc18c, an affinity comparable to the isolated Habc domain (Syx4 aa1-191) but with increased binding enthalpy (Table 1, Fig. S2) was observed, suggesting an increased binding surface. The increased binding enthalpy resembles that of the entire Syx4, suggesting that the two fragments of a split Syx4 bind together to Munc18. Probably, the two fragments of Syx4 interact when bound to Munc18c. Together, these results indicate that the *N*-peptide of Syx4 is not the sole binding site but the remainder of Syx4, possibly in a closed conformation, contributes to the overall binding strength.

Further we tested a construct lacking the *N*-peptide region of Syx4 (Syx4 aa25-270). We found that Munc18c was able bind to this construct, although with clearly reduced affinity (Kd $\approx$ 735 nM, $\Delta H \approx -$ 10 kcal/mol) than the entire cytoplasmic domain, Syx4 aa1-270 (Fig. 2).

We were able to verify this interaction via size exclusion chromatography, during which Munc18c co-eluted with Syx4 aa1-270 and also with Syx4 aa25-270 (Fig. S3). Note that an earlier study had failed to detect binding of Syx4 without N-peptide by ITC (58), whereas other studies had reported binding when using GST-binding assays (51, 59).

We obtained comparable ITC results when we used a Syx4 construct with two disruptive point mutations in the *N*-peptide region, R4A and L8A ($Syx4^{RL}$). These two conserved positions have been found to contribute strongly to the interaction with the *N*-peptide to the SM protein (48). Indeed, our ITC experiment corroborates that the mutations interfere strongly with the interaction between the Syx4 *N*-peptide and Munc18c. However, the point mutations did not prevent the interaction between the remaining part of Syx4 and Munc18c (Fig. S2).

Together, these results suggest that two regions of Syx4, the *N*-peptide and the remainder in a closed conformation, bind to Munc18c. This binding mode resembles that of Munc18a and Syx1a, but it is unclear whether Munc18c exerts a similar control to Munc18a over the ability of the bound syntaxin to engage in SNARE complex formation.

Munc18c inhibits SNARE complex assembly - In order to investigate the role of Munc18c during the SNARE complex formation of Syx4, we established a fluorescence-based kinetic approach to monitor SNARE complex formation, very similar to the one that was instrumental in observing the effect of Munc18a on neuronal SNARE complex formation (10). In adipocytes and muscle cells, the translocation of GLUT4 to the plasma membrane is thought to be mediated by the assembly of Syx4 with SNAP-23 and synaptobrevin 2 (Syb2). When a fluorescence-labeled cytosolic domain of Syb2 ($Syb1\text{-}96^{61OG}$) was mixed with Syx4 aa1–270 and SNAP-23, we observed an increase of fluorescence anisotropy, indicating successful ternary SNARE complex formation. However, the process took several hours to complete and is thus not suitable for a kinetic approach. We surmised that the slow reaction might be caused by the intrinsically low reactivity of recombinant SNAP-23, an effect we had noticed but not investigated further during an earlier study (60). Possibly, the lower reactivity is caused by the tendency of SNAP-23 to oligomerize (61). In the next set of experiments, we therefore replaced SNAP-23 with its close homolog, SNAP-25, which also interacts with Syx4 (16). SNAP-25 turned out to be much more reactive than SNAP-23 and was therefore used in all our further SNARE complex assembly experiments.

Interestingly, when Munc18c was added to the SNARE assembly reaction, we observed a clear inhibition of ternary SNARE complex formation (Fig. 3), although the inhibition exerted by Munc18c appeared to be somewhat less pronounced than the inhibition of neuronal SNARE complex formation by Munc18a reported earlier (10) and can probably attributed to a faster dissociation of the Munc18c/Syx4 complex compared to the Munc18a/Syx1a complex (Fig. S4). This finding corroborates the idea that Munc18c, very like Munc18a, binds to the closed conformation of its syntaxin partner and thereby inhibits its ability to engage in SNARE complex interactions.

Syx4 adopts a closed conformation - The cytoplasmic domain of neuronal Syx1a can adopt a closed conformation, in which the SNARE motif folds back onto the Habc domain (14, 15). In this conformation, the ability of Syx1a to engage in SNARE complex formation is inhibited. The binding of Munc18a to syntaxin stabilizes the closed conformation, whereas uncomplexed Syx1a can switch between a closed and an open conformation. We had shown earlier that removal of the Habc domain of Syx1a speeds up complex formation with SNAP-25 by a factor of about 7 (62). In order to assess whether Syx4 also switches between a closed and open conformation, we compared the assembly rate of the entire cytoplasmic region of Syx4 (Syx4 aa1–270) with that of the isolated SNARE motif (Syx4 aa191-270) using fluorescence-labeled SNAP-25 as the binding partner. Under pseudo-first-order conditions, the binding rate of the SNARE motif to SNAP-25 was about 18x faster than that of the entire cytoplasmic domain (Fig. S5). This suggests that the SNARE motif of Syx4 is autoinhibited by the Habc domain. Note that the degree of autoinhibition is somewhat stronger for Syx4 than for Syx1a, suggesting that the closed conformation of Syx4 is tighter than that of Syx1a. We observed a similar effect for other vertebrate syntaxins

involved in secretion, Syx2 and Syx3 (Fig. S5), suggesting that the open-closed conformational change is a conserved feature of all secretory syntaxins, as suggested earlier (63).

Comparison of Munc18 isoforms on their effect on SNARE assembly - As Munc18a does not interact with Syx4 (16), a direct comparison of the activities of Munc18a and Munc18c was not possible when the kinetic assays described above were used. However, according to earlier investigations, binding of the three vertebrate Munc18 isoforms is not restricted to one specific secretory syntaxin, as most Munc18s appear to be able to bind to at least another syntaxin isoform (16, 64, 65). In order to search for a common binding partner of both Munc18 isoforms, next we determined the binding specificities of Munc18a and Munc18c to different syntaxin isoforms via ITC (Fig. 4). Munc18a was found to bind tightly to Syx1a, as reported earlier (10, 16). We also found that Munc18a had a similarly high affinity for Syx2 (Kd ≈ 8 nM) but interacted with Syx3 with moderate affinity (Kd ≈ 62 nM). No interaction was detected between Syx4 and Munc18a (Fig. 4B), corroborating earlier results obtained via GST-binding experiments (16). We also found that Munc18c binds tightly not only to Syx4, as reported earlier (48), but also to Syx2 (Kd ≈ 8 nM). Munc18c also binds to Syx1a, although with a slightly lower affinity (Kd ≈ 20 nM) (Fig. 4A). Note that binding of the sole Syx1a *N*-peptide to Munc18c had been reported earlier (49), although with low affinity. We did not observe an interaction between Munc18c and Syx3, again corroborating earlier studies (64-66).

As both Munc18 isoforms can bind strongly to Syx2, we next established an assay to monitor the assembly of a SNARE complex containing Syx2 in order to find out whether the inhibitory binding mode is shared between all Munc18s. For this, we made use of the same fluorescence-based kinetic assay described above. When we mixed Syx2 with SNAP-25 and Syb1-96^{61OG}, a ternary SNARE complex, again visible by an increase in fluorescence anisotropy, formed readily (Fig. 5A). When Munc18a was added, SNARE complex formation was inhibited, revealing that the inhibitory activity of Munc18a is not restricted to its partner in synaptic secretion. Similar but somewhat more pronounced inhibitory activity was found for Munc18c, confirming that both Munc18 isoforms have a comparable effect: they slow down SNARE complex formation of Syx2.

In the next set of experiments, we compared the effect of both Munc18 isoforms on the assembly of a SNARE complex containing Syx1a. Again, we found that both Munc18 isoforms slowed SNARE complex formation, although Munc18c was clearly less effective than Munc18a (Fig. 5B). These data again support the notion that Munc18c acts in a very similar mode to Munc18a, although the inhibition of neuronal SNARE complex formation by Munc18c is less pronounced than by the inhibition of Munc18a.

Subtle differences between Munc18a and c - In a previous study, we showed that the inhibitory effect of Munc18a on SNARE complex formation can largely be bypassed when the Syx1a^{LE} mutant is used (10). The Syx1a^{LE} mutant carries two point mutations (L165A and E166A) in the linker helix between the Habc domain and the SNARE motif (14). Apparently, this mutation shifts the equilibrium between the open and closed state of Syx1a towards the open state (62). Nevertheless, Munc18a binds tightly to Syx1a^{LE}, probably in a state that resembles the closed conformation (10, 67) but does not stop Syx1a^{LE} from forming a SNARE complex (10). Interestingly, when we added Munc18c to the Syx1a^{LE} mutant, ternary SNARE complex formation was slowed down (Fig. 6A). We also tested the equivalent Syx4^{LE} mutant (L173A, E174A). Again, we observed that Munc18c slowed down the assembly rate of the Syx4^{LE} mutant (Fig. 6B). Apparently, the mutations in the linker helix of syntaxins does not bypass the tight controlling effect of Munc18c, hinting at a subtle structural and functional difference between Munc18a and Munc18c.

DISCUSSION

Compared with many other animals, vertebrates possess an extended repertoire of key secretory factors, including various SNARE (68) and SM proteins (11). Their increased number can be attributed to two rounds of genome duplication that occurred about 500 million years ago in the vertebrate lineage (69). Probably, this rich repertoire provided them with the ability to more finely regulate the secretory machinery in different tissues and appears to have facilitated the development of new specialized secretory cell types in vertebrates. Three different Munc18

proteins, Munc18a, Munc18b, and Munc18c, exist in vertebrates (22-24). They are thought to mediate exocytosis in different tissues by interacting with various different secretory syntaxins (68). Our study corroborates the idea that the different Munc18 and syntaxin isoforms are able to interact in a partially overlapping manner with a subset of different syntaxins. Rather exclusive interactions appear to be formed between Syx4 and Munc18c and between Syx3 and Munc18b, whereas Syx2 appears to be able to interact with all three Munc18s, for example.

Although the different vertebrate Munc18s arose from a common invertebrate ancestor that already formed a tight complex with a syntaxin, contrasting binding modes have been reported for the different vertebrate isoforms. The syntaxin *N*-peptide region was found to be a key determinant for binding to Munc18b (47) and to Munc18c (48, 49, 58). However, the isolated *N*-peptide of Syx1a binds only weakly to Munc18a (49), whereas the central cavity of Munc18a clasps around the closed conformation of Syx1a and contributes greatly to the overall affinity of the complex (10, 15, 67). In fact, it has been claimed that binding to the closed conformation reflects a specialized function of the neuronal Munc18-syntaxin pair (57) and several studies have attempted to functionally separate the assumed binding modes of Munc18s (e.g. (70-73)). The idea of the closed conformation binding mode of the neuronal Munc18a having a special function appears to be at odds with the high structural similarity of the three Munc18 isoforms. Likewise, functional investigation on the Munc18 homologs of invertebrates, the nematode *C. elegans*, Unc18 (74), and the fruitfly *Drosophila melanogaster*, Rop (75-77), have shown early on that these factors play a crucial role in synaptic transmission, a role comparable to neuronal Munc18a (25). Indeed, Unc18 and Rop are more similar to Munc18a than to the two other vertebrate Munc18s. For example, Unc18 shares ≈ 59% identity with mouse Munc18a, ≈ 54% with Munc18b, and ≈ 44% with Munc18c, suggesting that Munc18c has diverged greatly. This can be seen in the phylogenetic tree as well (Fig. 1). The higher similarity of Unc18 to Munc18a is also supported by the observation that the expression of mouse Munc18a but not Munc18c was able to compensate for severe locomotion disability in unc-18 mutants in *C. elegans* (78). Furthermore, Munc18b is able to partially rescue the lack of Munc18a in mouse chromaffin cells, suggesting that Munc18 vertebrate isoforms show some redundancy (79). This was corroborated in a recent study in which it was shown that expression of Munc18b supports the activity-dependent priming of synaptic vesicles in hippocampal neurons that lack Munc18a. Interestingly, Munc18c is unable rescue the lack of Munc18a (80), again exposing subtle differences among the three isoforms.

Interestingly, *D. melanogaster* Rop is not only expressed in neurons but is also found in several other tissues (81). Unc18, however, is mostly expressed in neurons, although it was also found to be expressed in the male gonad of the nematode (82). The more restricted expression pattern of Unc18 must be taken with a grain of salt, however, as *C. elegans* has a second Unc18-like gene (T07A9.10), which is more divergent, less explored, and - according to WormBase - is expressed in the body wall muscle cells, excretory cells, the hypodermis, germ lines, and intestines, and neurons. Thus Unc18 appears to be rather a neuronal isoform, whereas the Unc18-like might represent an ubiquitous form. Note that the Unc18-like gene is present in the genomes of several *Caenorhabditis* species and represents a gene duplication independent of the ones in vertebrates. The division of labor between different Unc18s in *C. elegans* is reminiscent of the situation in vertebrates, where the different Munc18 (and syntaxin) isoforms have distributed tasks. Although the lack of Munc18a specifically blocks neuronal secretion in mice and the animals dye after birth (25), knockouts of Munc18b and Munc18c are lethal and lead to developmental defects (26, 27). The expression profile of the sole Munc18 from the fruitfly, Rop, however, demonstrates that invertebrate Munc18s are probably not confined to a role in neuronal communication but control secretion in other cells as well. Not surprisingly, Rop mutations cause strong developmental defects, suggesting that Rop functions in constitutive and regulated secretion (76), possibly by interacting with different tethering complexes such as the exocyst complex (83, 84) or MUN domain-containing proteins such as (M)unc13s, BAP3/Baiap3, and CAPS/Unc31

(85, 86). It is possible that the three vertebrate Munc18s preferentially work together with certain tethering factors. For example, though Munc18a is controlled by Munc13 during neuronal secretion (87, 88), Munc18c is thought to interact with the exocyst complex during translocation of GLUT4 to the plasma membrane (89-91), suggesting that their activity can be orchestrated by different tethering factors.

In our study, we did not consider the complex physiological situation encountered by the different Munc18 isoforms in different cells and in different secretion events but focused on their core molecular activity, which is their interaction with syntaxin and their influence on SNARE complex formation. When we compared the molecular activity of the neuronal Munc18a with that of the more wide spread expressed isoform Munc18c, we found that, in general, Munc18c behaves similarly to Munc18a. Munc18c, like Munc18a, binds simultaneously to two spatially separated regions of syntaxin, the *N*-peptide and the remainder of syntaxin, probably in closed conformation. It is probable that not only the outer surface of Domain 1 of Munc18c, which contains the hydrophobic *N*-peptide binding pocket (48), participates in binding to syntaxin, but also the central cavity formed by the inner surfaces of Domain 1 and Domain 3a provides an interface for the closed conformation of syntaxin. Each binding region, the *N*-peptide (≈ 1.5 μM) and the remainder of Syx4 (≈ 735 nM), contribute a portion to the overall affinity of the Munc18c-Syx4 complex (≈ 12 nM). Similar observations were recently made for the interaction between Munc18b and Syx3 or Syx11. For the different syntaxins, the isolated *N*-peptides contributes only a portion (Syx3 *N*-peptide ≈ 11 μM; Syx11 *N*-peptide: ≈ 0.55 μM) to the overall high affinity of the Munc18b complexes with the entire cytoplasmic domain of the syntaxin (Syx3: ≈ 27 nM; Syx11: ≈ 30 nM)(47). Note that another study found that Munc18b binds to both regions of Syx3, the *N*-peptide and the closed conformation (36). This suggests a similar binding mode, involving two spatially separated binding regions for all vertebrate Munc18-Syx complexes.

They must have inherited this binding mode from an ancestral Munc18-Syx pair. Indeed, a very similar binding mode with two binding sites was discovered in the secretory Munc18-Syx complex in a choanoflagellate (11), a group of single-celled or colonial eukaryotes that are the closest living relatives of animals (92). Studies on *C. elegans* Unc18 (93) or *D. melanogaster* Rop (76, 77) also have indicated that two regions of syntaxin participate in binding, although it has never been demonstrated directly for these proteins that the two binding sites are occupied simultaneously. Moreover, comparable binding modes were also described for the SM proteins Vps45 (10, 13), which regulates *trans*-Golgi network trafficking, and Sly1, which acts in ER-Golgi trafficking (12). This suggests that a conformational switch between the closed and open conformations is at the heart of syntaxin's function. This conformational switch is tightly controlled by the SM protein. The syntaxin binding mode has been evolutionary conserved in several SM protein types, with the notable exception of vacuolar Vps33, which has no *N*-peptide binding pocket and does not seem to interact with a closed syntaxin (20). It is conceivable that the interaction mode of Vps33 represents a later stage of the reaction cascade of SM proteins and SNAREs, a stage that is less stable and more challenging to characterize for other SM protein types.

We suggested earlier that the binding of Munc18 to the closed conformation and binding to the *N*-peptide of syntaxin do not represent functionally distinct modes, but are rather two aspects of the same function, which is to prepare syntaxin for SNARE complex formation (10). Still, it is unclear how the two binding sites work together during this endeavor. In the closed conformation of syntaxins, the availability of syntaxin for its SNARE partners is restricted (15). This explains the biochemical observation why Munc18a prevents Syx1a from forming a SNARE complex (16, 17). We have now observed a similar effect of Munc18c: the bound syntaxin is inhibited from forming a SNARE complex. This inhibitory activity may have been less clear in earlier studies on Munc18c, because SNARE complex assembly was either not measured kinetically or was only indirectly measured by monitoring liposome fusion (55, 56). Given the high similarity among all animal Munc18s and the fact that we had observed a similar inhibitory activity for the choanoflagellate Munc18 (11), we thus conclude that all animal Munc18s have a similar molecular activity, which is to control the accessibility of the bound syntaxin. It remains

unclear, however, how Munc18 can open the bound syntaxin.

Admittedly, our analysis does not provide a snapshot of Syx4 with the *N*-peptide and the closed conformation bound to Munc18c, but our biochemical investigations strongly suggest that the remainder of Syx4 binds to the central cavity of Munc18c. Notably, in the structure of the Munc18c with the *N*-peptide of Syx4, the binding site for the closed conformation in the central cavity is obstructed by an extended conformation of the so-called "helical hairpin" at the tip of Domain 3a (48). A similar extended helical hairpin was found for Munc18b, which crystallized without bound syntaxin (47). Interestingly, Munc18a also adopts a conformation with an extended helical hairpin in the absence of a bound closed syntaxin in its cavity (49). The hairpin folds back, however, into the so-called furled conformation in the presence of the closed syntaxin (as seen in rat Munc18a-Syx1a (10) and choanoflagellate Munc18-Syx structures (11)). This suggests that this region undergoes a conformational change upon binding and unbinding of the closed conformation, and is key to our understanding of how SM proteins control the conformational switch of the bound syntaxin. In this regard, it is interesting that in the Munc18a/Syx1a structure the helical hairpin region of Munc18a is very close to the small linker helix of syntaxin 1a that is mutated in the LE variant (15). It is possible that the subtle differences observed between the effects of Munc18a and Munc18c on syntaxins with LE mutations might be due to small differences of their helical hairpin regions.

Currently, one can only speculate about how the conformation of the helical hairpin of different Munc18s impinges on the closed conformation of the bound syntaxin. Munc18a seems to be able to tightly control the transition from the bound closed Syx1a into a conformation that is compatible with SNARE complex formation. When we compared the level of control exerted by Munc18a and Munc18c using the same syntaxin, Syx1a or Syx2, we observed subtle differences but generally, Munc18c also slows SNARE complex assembly, suggesting that the ability of Munc18s to lock syntaxin is evolutionary conserved. Initially, this ability appears to be in conflict with the membrane fusion promoting activity of Munc18s *in vivo*, but establishing a complex with a syntaxin probably only reflects the first part of the mission of SM proteins (Fig. 7). Nevertheless, given the conflicting observations in studies using liposome assays (54-56), it cannot be excluded that the interaction of Munc18 with syntaxin is influenced by the presence of membranes. Indeed, it has been claimed very recently that the C-terminal region of Syx4 affects the activity of Munc18c (94). However, these experiments have not been carried out with membrane-inserted Syx4 but with a Syx4 carrying a 18 kDa T4 lysomzyme tag. Thus, before disregarding results from experiments with soluble SNARE portions altogether, the interaction of Munc18 with syntaxin need to be thoroughly studied in the presence of membranes. In addition, it should be noted that some experiment in the study by Rehman et al. (94) seem to have been performed with proteins of less activity compared to proteins used in our study (see Fig. S5). Munc18's second role is probably to make the bound syntaxin able to engage in SNARE complex assembly, for example by promoting the opening of syntaxin, as previously observed for Sly1 (12). On the basis of our kinetic analyses, we have speculated previously that syntaxin does not have to dissociate from Munc18 in order to form a SNARE complex; instead Munc18 prepares the bound syntaxin for SNARE complex formation by forming a transient Munc18-Syx-SNAP-25 complex. This was recently confirmed by isolating a stable tripartite complex with syntaxin and SNAP-25 that permits very fast binding of the vesicular synaptobrevin, thereby promoting fast membrane fusion (18, 19). It is likely that the flexible helical hairpin region plays an important role in catalyzing this transition, possibly controlled by tethering factors such as Munc13 (87, 88, 95).

EXPERIMENTAL PROCEDURES

Constructs and protein purification - All bacterial expression constructs were cloned into a *pET28a* vector that contains an *N*-terminal thrombin-cleavable His_6-tag. The constructs for SNARE proteins, cysteine-free SNAP-25b (aa 1‑206), the soluble portion of Syx1a (aa 1‑262), the SNARE domain of Syx1a (aa 180‑262) and the soluble portion of synaptobrevin 2 (Syb2, aa 1‑96), and full-length Munc18a (aa 1-594) were

derived from rat (*Rattus norvegicus*) and have been described before. Likewise, the single-cysteine SNARE protein variants used for labelling, Syb2 Cys61, as been described elsewhere (10). Expression constructs for full-length Munc18c (aa 1-592) and other syntaxin variants were derived from mouse (*Mus musculus*). The Munc18c construct was codon-optimized for bacterial expression. Syx4 constructs of different lengths were cloned: Syx4 (aa 1-270), Syx4 (aa 25-270), Syx4 (aa 192-270), and Syx4 (aa 1-191). In addition, two mutants of Syx4 (aa 1-270) were generated: L173A/E174A ($Syx4^{LE}$) and Syx4 R4A L8A ($Syx4^{RL}$). Furthermore, constructs for the cytoplasmic domain of Syx3 (aa1-263) and Syx2 (aa1-262) and their respective SNARE domains were made: Syx3 (aa183-261) and Syx2 (aa184-263). For fluorescence labeling, single cysteine variants at amino acid position 1 of Syx1a (aa 1–262) and of Syx4 (aa 1-270) were generated. All constructs were expressed in *E. coli*. Proteins were purified via Ni^{2+}-NTA affinity chromatography followed by ion exchange chromatography, essentially as described in (10). Munc18c was further purified via size exclusion chromatography on a Superdex-200 column. Note that Munc18s were used directly after purification and not stored at -80°C, as freezing and thawing reduces the activity of the protein.

Isothermal titration calorimetry - ITC was performed on a VP-ITC instrument (GE Healthcare) at 25°C. Samples were dialyzed against a degassed HEPES (60 mM HEPES, pH 7.0, 150 mM NaCl, 1 mM DTT) or PBS buffer (20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM DTT) as described in (10). Titrations were carried out via 10 µl injections. The measured heat released upon binding was integrated and analyzed with Microcal Origin 7.0 using a single-site binding model, yielding the equilibrium association constant (*Ka*), the enthalpy of binding (ΔH), and the stoichiometry (*N*).

Fluorescence spectroscopy - Fluorescence measurements were carried out in a Cary Eclipse spectrometer (Varian International AG, Zug) or a QuantaMaster 40 spectrometer in T-configuration (PTI, Birmingham, NJ 08011) equipped for polarization. Single-cysteine variants were labeled with Oregon Green 488 iodoacetamide (Invitrogen) according to the manufacturer's instructions. All experiments were performed at 25 °C in 1-cm quartz cuvettes in a HEPES buffer (60 mM HEPES, pH 7.0, 150 mM NaCl). Fluorescence anisotropy, which is used to indicate the local flexibility of the labeled residue, and which increases upon complex formation and decreases upon dissociation, was measured essentially as described in (10). The *G* factor was calculated according to $G = I_{HV}/I_{HH}$, where *I* is the fluorescence intensity, and the first subscript letter indicates the direction of the exciting light and the second subscript letter the direction of emitted light. The intensities of the vertically (V) and horizontally (H) polarized emission light after excitation by vertically polarized light were measured. The anisotropy *(r)* was determined according to $r = (I_{VV} - G\,I_{VH}) / (I_{VV} + 2\,G\,I_{VH})$.

Phylogenetic reconstruction - As described earlier, we collected Munc18 sequences from various animals (11), particularly from vertebrates. Alignments were made by using Muscle (96). We used the available 3D structures (10, 11, 15, 47-49, 67, 97, 98) to refine the alignments. For the phylogenetic reconstruction, we used a combination of IQ-TREE (99, 100) and Randomized Accelerated Maximum Likelihood (RAxML) (101). To be able to calculate the best trees, we first used IQ-TREE to estimate best model and model parameters. The JTT model (102) with gamma distribution for rate heterogeneity was found to be the most appropriate model. We executed IQ-TREE with 1000 rapid bootstrap replicates. For RAxML, we chose a random seed of 9 and 1000 bootstrap replicates. We then used RAxML to estimate site-wise log-likelihoods for all calculated trees and Consel (103) was used to estimate an Approximately Unbiased ranking. The highest-ranking tree was taken as a reference. Again making use of Consel, we corrected the support values of the different bootstrap replicates from RAxML by using the Approximately Unbiased test. IQ-TREE has a built-in correction and no further adjustment was necessary. The main edges in the tree are annotated in the following format: IQ-TREE support/RAxML support.

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Author contributions: DF conceived and coordinated the study and wrote the paper. CM designed, performed, and analyzed the experiments shown in Figures 2 to 6. PB designed, performed, and analyzed the experiments shown in Figure 4. CNK and THK designed, performed, and analyzed the experiments shown in Figure 1. All authors reviewed the results and approved the final version of the manuscript.

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Abbreviations: SM protein, Sec1/Munc18 protein; SNARE protein, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; Syx, syntaxin; GLUT4, glucose transporter 4; RAxML, Randomized Accelerated Maximum Likelihood

Tables

Table I. Thermodynamic parameters of the interaction of syntaxins and SM proteins measured via ITC. All isothermal calorimetric experiments were performed at 25 °C in a HEPES buffer.

Interaction of	K_d (nM)	ΔH° (kcal/mole)	*n*
Syx4 (1-270) - Munc18c	12.0 ± 1.7	- 14.02 ± 0.09	1.04
Syx4 (1-191) - Munc18c	546.5 ± 63.6	- 3.46 ± 0.09	0.99
Syx4 (25-270) - Munc18c	735.3 ± 78.7	- 10.06 ± 0.24	1.16
Syx4 (192-270) - Munc18c	-	-	-
Syx4 (1-191) + Syx4 (192-270)-Munc18c	787.4 ± 93.4	- 11.39± 0.69	0.81
$Syx4^{RL}$ - Munc18c	386.1 ± 32.9	- 7.04 ± 0.89	1.04
$Syx4^{LE}$ - Munc18c	16.1 ± 2.0	- 10.64 ± 0.84	0.92
Syx1a (1-262) - Munc18c	20.0 ± 3.9	- 7.5 ± 0.06	0.93
Syx2 (1-262) - Munc18c	7.6 ± 1.5	- 8.6 ± 0.06	0.91
Syx3 (1-263) - Munc18c	-	-	-
Syx1a (1-262) - Munc18a	1.5 ± 0.3	- 34.56 ± 0.19	1.03
Syx2 (1-262) - Munc18a	7.5 ± 1.0	- 31.10 ± 0.24	1.00
Syx3 (1-263) - Munc18a	62.1 ± 8.2	- 12.24 ± 0.19	1.02
Syx4 (1-270) - Munc18a	-	-	-

Figure Legends

Figure 1. Schematic outline of an evolutionary tree of the secretory SM protein Munc18 in animals. The tree shows that genome duplications in vertebrates resulted in three distinct Munc18 paralogs (Munc18a, Munc18b, and Munc18c) that are separated from other animal Munc18s. An additional duplication of Munc18a occurred in teleosts. Note that many other animals have only a single Munc18 gene. The tree was constructed from 551 sequences derived from 253 animal species (including 181 vertebrates) and three unicellular holozoans (Table S1). Statistical support values (IQ-TREE support and RAxML support) are given at selected inner edges. The original tree file is available as supplemental information in Nexus format (Fig. S6). The following crystal structures are shown at the corresponding positions in the tree: rat Munc18a-Syx1a complex (pdb code: 3C98)(10), human Munc18b (4CCA)(47), Munc18c-Syx4 N-peptide complex (3PUK)(49), squid Sec1 (1EPU)(97) and choanoflagellate Munc18-Syx1 complex (2XHE)(11). Note that only the structures of rat Munc18a and that of the choanoflagellate *Monosiga brevicollis* have been determined in complexes with the closed conformation of syntaxin and the *N*-peptide. The four-helix bundle of the closed syntaxin is accommodated in the cavity between Domains 1 and 3a.

Figure 2. The *N*-peptide and the remainder of Syx4 contribute to binding to Munc18c.
A) Schematic drawing of the domain structure of Syx4 and fragments used in this experiment. The short *N*-peptide (NP) motif, the three Habc helices, the SNARE motif, and the transmembrane region (TMR) are indicated.
B) Calorimetric titrations of different Syx4 variants into Munc18c. The integrated areas normalized to the amount of Syx4 (kcal/mol) versus the molar ratio of Syx4 to Munc18c are shown. The solid lines represent the best fit to the data for a single binding site model using nonlinear least squares fitting.
The affinity measured for binding of the soluble portion of Syx4 to Munc18c via ITC is about 12 nM. It also corroborates, as remarked earlier (58), that binding between Munc18c and Syx4 is somewhat weaker than between the neuronal pair Munc18a and Syx1a (Kd ≈ 1 nM, (10)). Of note, the affinity determined by us is higher than that previously reported via ITC (58) (Kd ≈ 95 nM), whereas an affinity of 32 nM had been determined by surface plasmon resonance (104). Note that the corresponding data are given in Table 1 and that all ITC experiments are shown in Fig. S2..

Figure 3. Munc18c inhibits SNARE complex formation of bound Syx4.
Ternary SNARE complex formation was followed by an increase in fluorescence anisotropy of 40 nM fluorescent Syb1-96^{61OG} upon mixing with 1 µM Syx4 (1-270) and 1.5 µM SNAP-25. In the presence of 1.5 µM Munc18c, ternary SNARE complex formation was slowed down.

Figure 4. Munc18a and Munc18c can interact with different syntaxins.
Calorimetric titration of different secretory syntaxins into Munc18a (A) and Munc18c (B). Note that the titration of Syx4 into Munc18c is also shown in Fig. 2. Munc18a can interact tightly with Syx1a and Syx2 but binds only with moderate affinity to Syx3. Munc18c can interact tightly with Syx1a, Syx2, and Syx4. Munc18a does not interact with Syx4, whereas Munc18c does not interact with Syx3.

Figure 5. Munc18a and Munc18c inhibit the SNARE complex formation of bound Syx2 and Syx1a.
Ternary SNARE complex formation was followed by an increase in the fluorescence anisotropy of 40 nM fluorescent Syb1-96^{61OG} upon mixing with SNAP25 and the soluble portion of Syx2 (1-262, *A*) or Syx1a (1-262, *B*). For the formation of the Syx1a SNARE complex, 0.5 µM Syx1a and 0.75 µM SNAP-25 were mixed. In the presence of 0.75 µM Munc18a or Munc18c, SNARE complex formation was slowed down. For the assembly of the Syx2 SNARE complex, 2 µM Syx2 and 3 µM SNAP-25 were mixed. In the presence of 3 µM Munc18a or Munc18c in the reaction mix, SNARE complex formation was slowed down.

Figure 6. Munc18c inhibits the SNARE complex formation of Syx^{LE} variants.
Ternary SNARE complex formation was followed by an increase in the fluorescence anisotropy of 40 nM fluorescent Syb1-96^{6IOG} upon mixing with SNAP25 and the LE mutants of Syx1 (Syx1a^{LE}) or Syx4 (Syx4^{LE}).
A) When 0.75 μM Munc18c was added, ternary SNARE formation of Syx1a^{LE} was slowed down, whereas addition of Munc18a had no visible effect, in agreement with our earlier report (10).
B) Munc18c slowed SNARE complex formation of the Syx4^{LE} mutant as well. Note that we did not add Munc18a to this reaction mix, because Munc18a does not bind to Syx4.

Figure. 7. Model of how Munc18 prepares syntaxin for SNARE complex formation.
A) Schematic showing that Munc18 binds first to the closed conformation and *N*-peptide region of syntaxin. Munc18 then makes the bound syntaxin able to engage with its partner SNAREs, SNAP-25 and synaptobrevin. This step is triggered by additional factors such as Munc13 (5) and possibly involves a conformational change of the helical hairpin region of Munc18. The crystal structure of Munc18 (cyan) bound to Syx1a (Habc domain, orange, SNARE domain, red) is shown at the top (10). The region shown below as a close-up image (B + C) is indicated by a dashed square. Close-up of the different conformations of the helical hairpin region of Munc18a in complex with the closed conformation of Syx1a ("furled", B) or bound to the *N*-peptide of Syx4 ("extended", C)(49).

Fig. 1 - Morey et al.

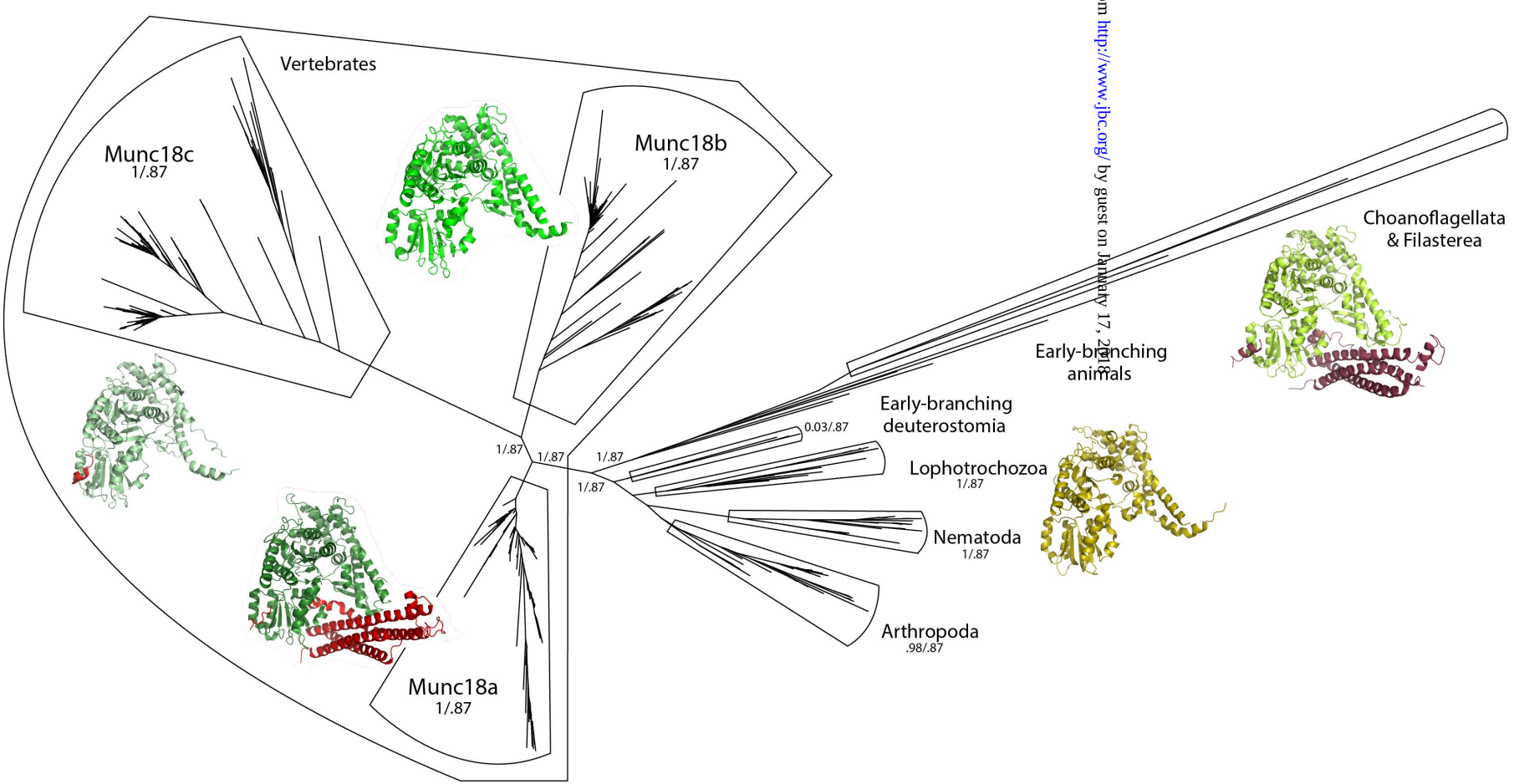
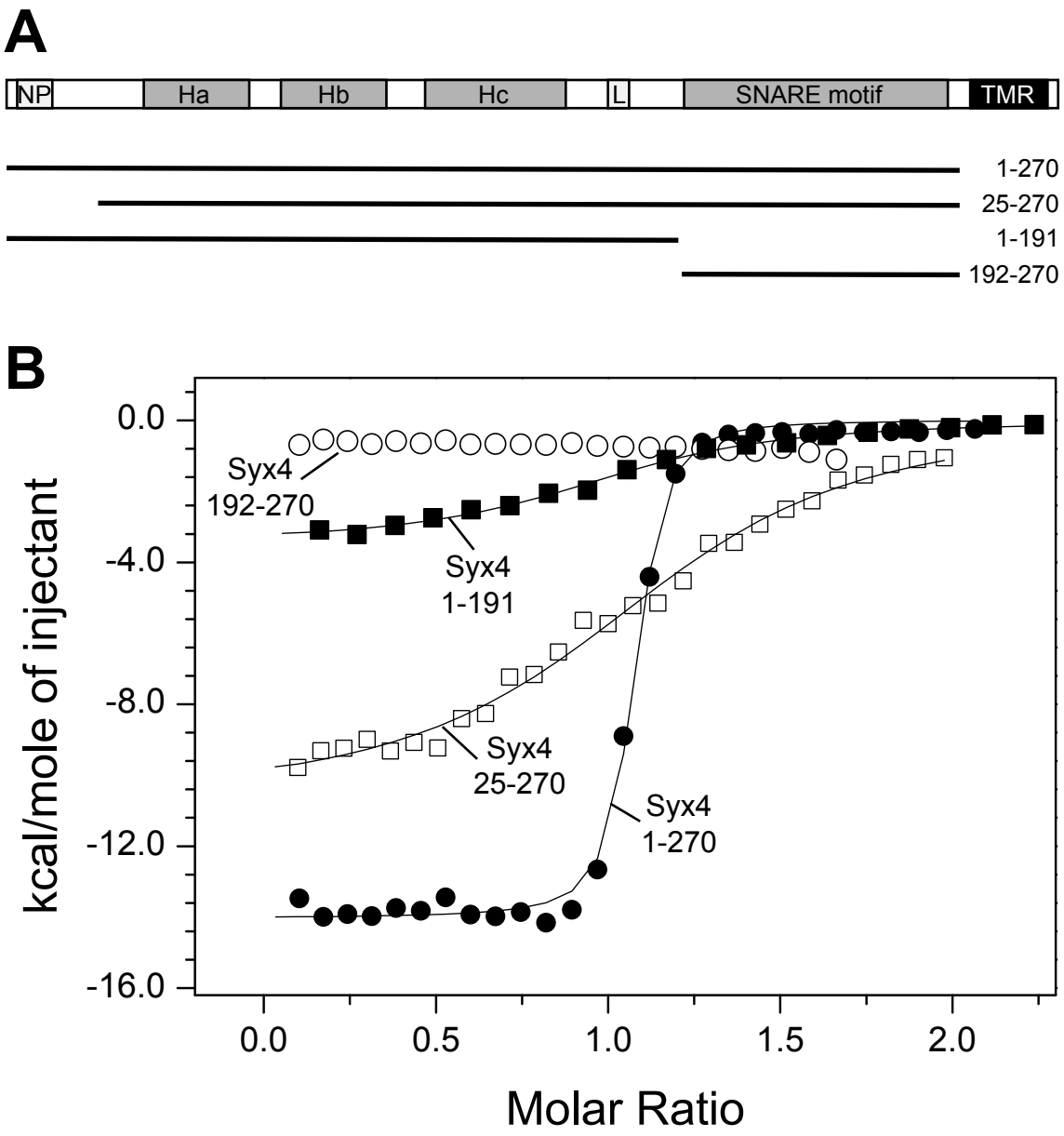


Fig. 2 - Morey et al.



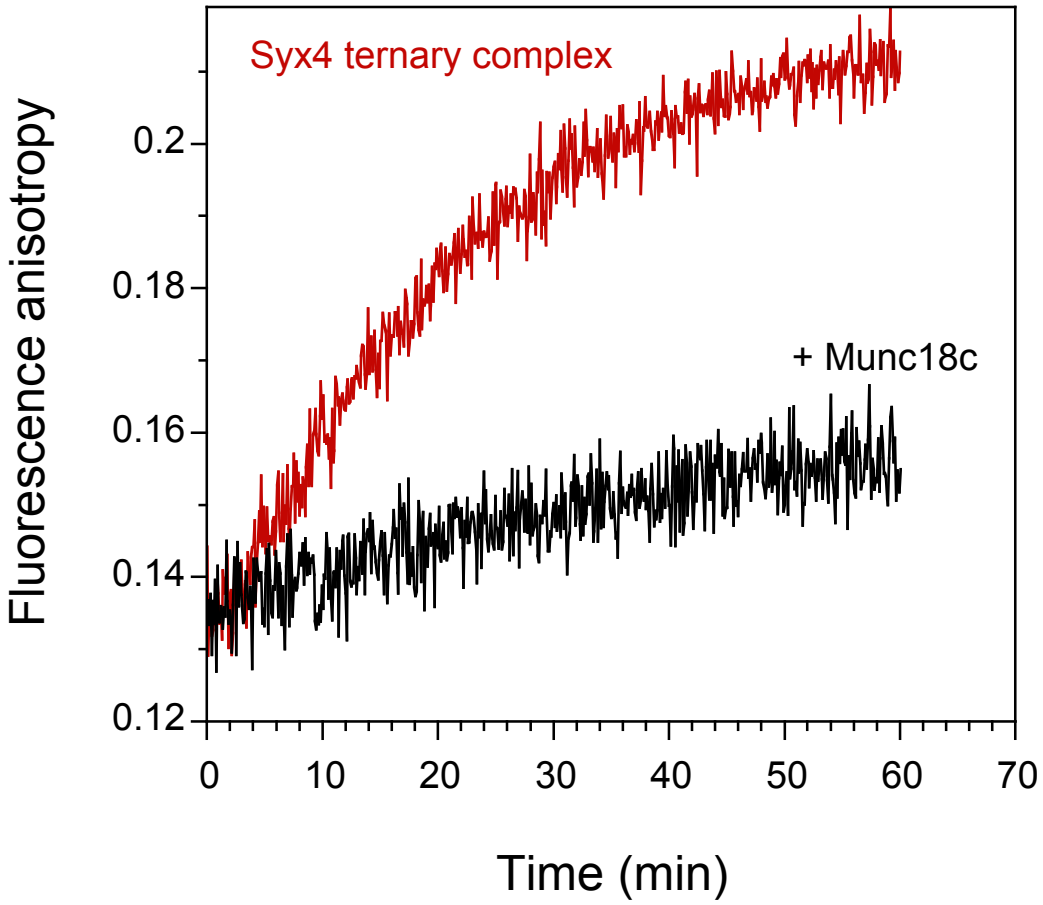
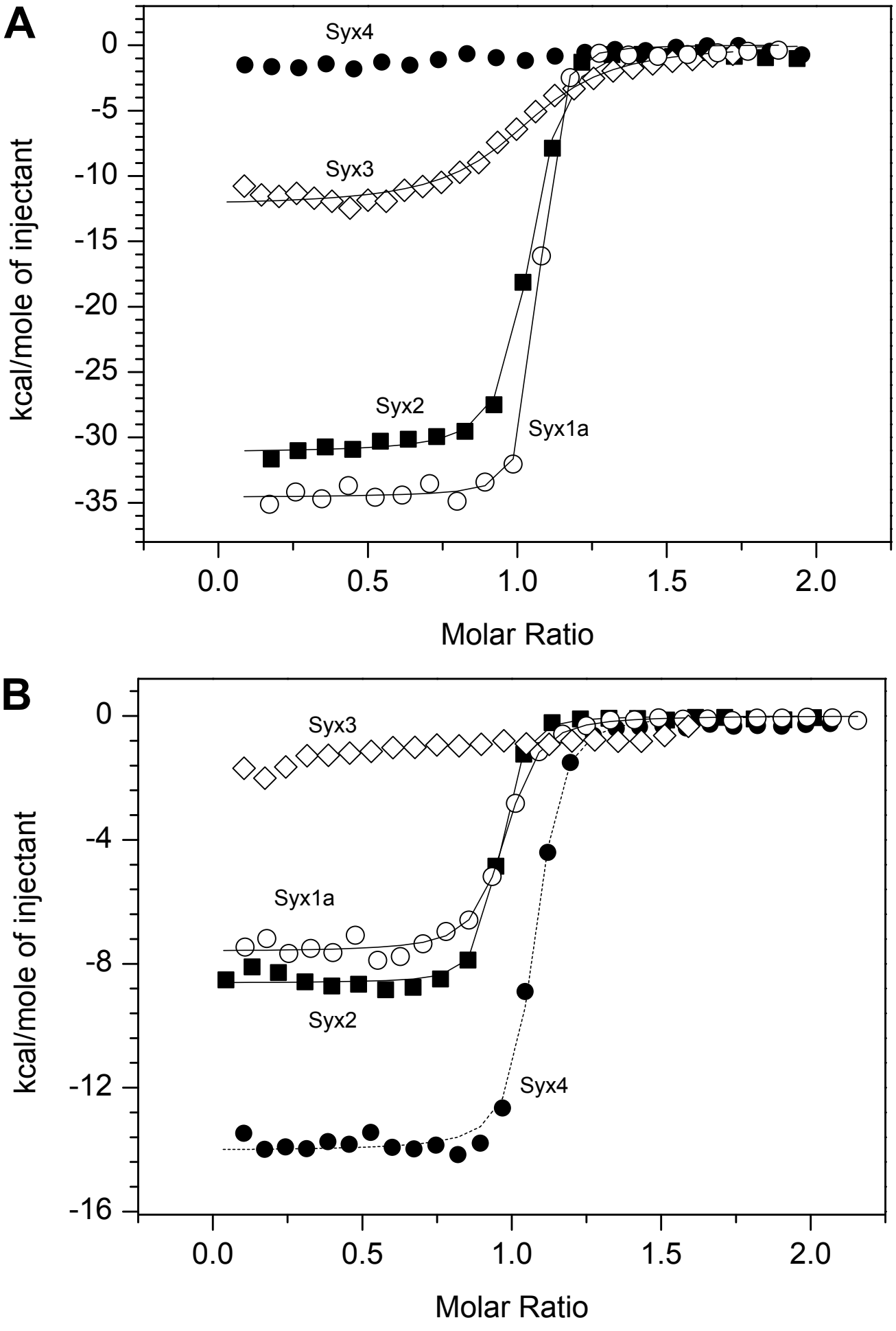
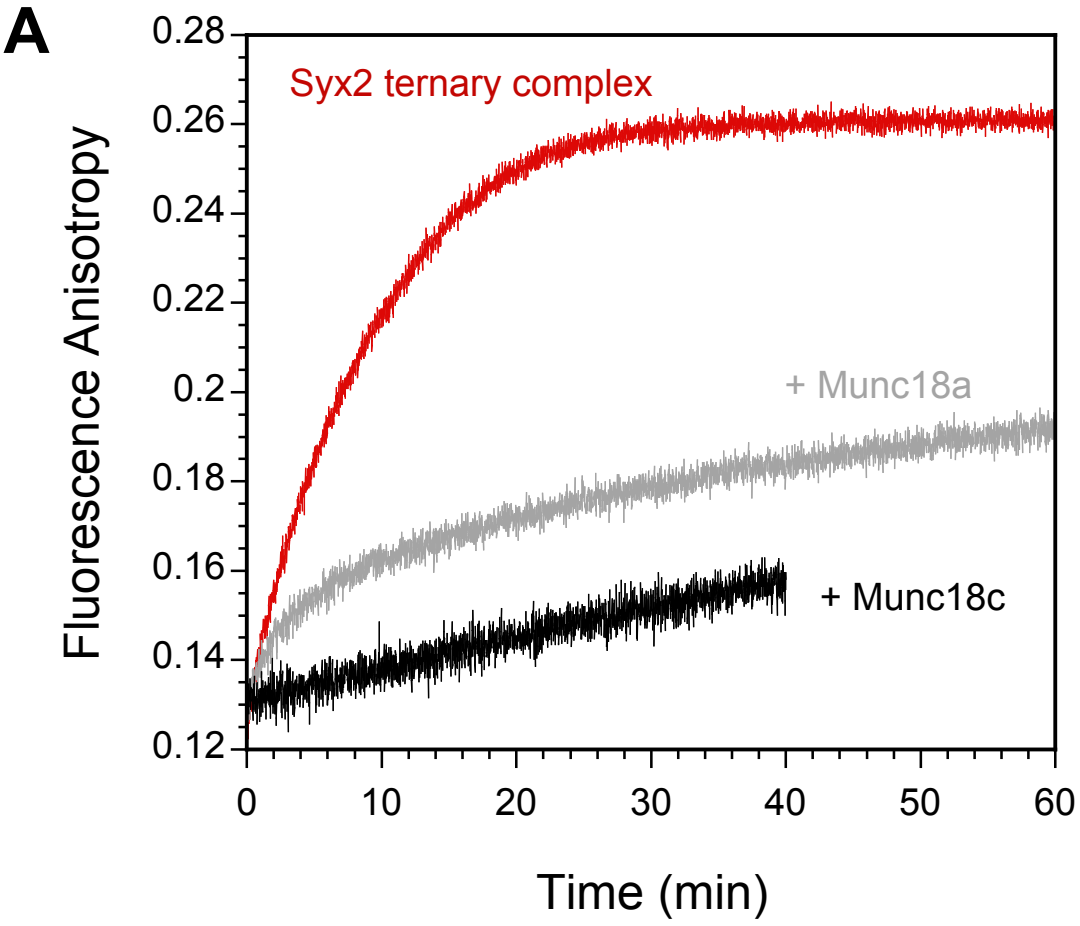
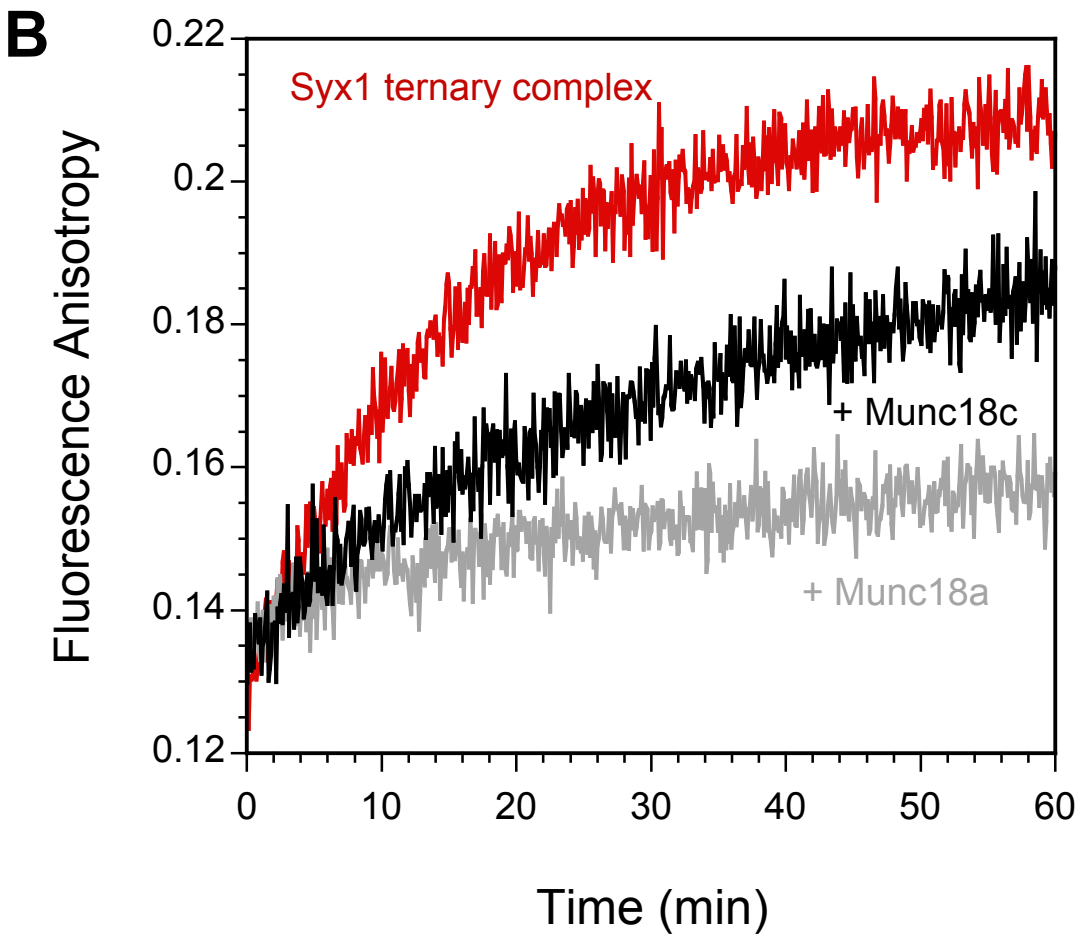


Fig. 3 - Morey et al.


A
Syx4
Syx3
Syx2
Syx1a
0
-5
-10
-15
-20
-25
-30
-35
kcal/mole of injectant
0.0
0.5
1.0
1.5
2.0
Molar Ratio
B
Syx3
Syx1a
Syx2
Syx4
0
-4
-8
-12
-16
kcal/mole of injectant
0.0
0.5
1.0
1.5
2.0
Molar Ratio


A
Syx2 ternary complex
+ Munc18a
+ Munc18c
Fluorescence Anisotropy
0.28
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0
10
20
30
40
50
60
Time (min)


B
Syx1 ternary complex
+ Munc18c
+ Munc18a
Fluorescence Anisotropy
0.22
0.2
0.18
0.16
0.14
0.12
0
10
20
30
40
50
60
Time (min)

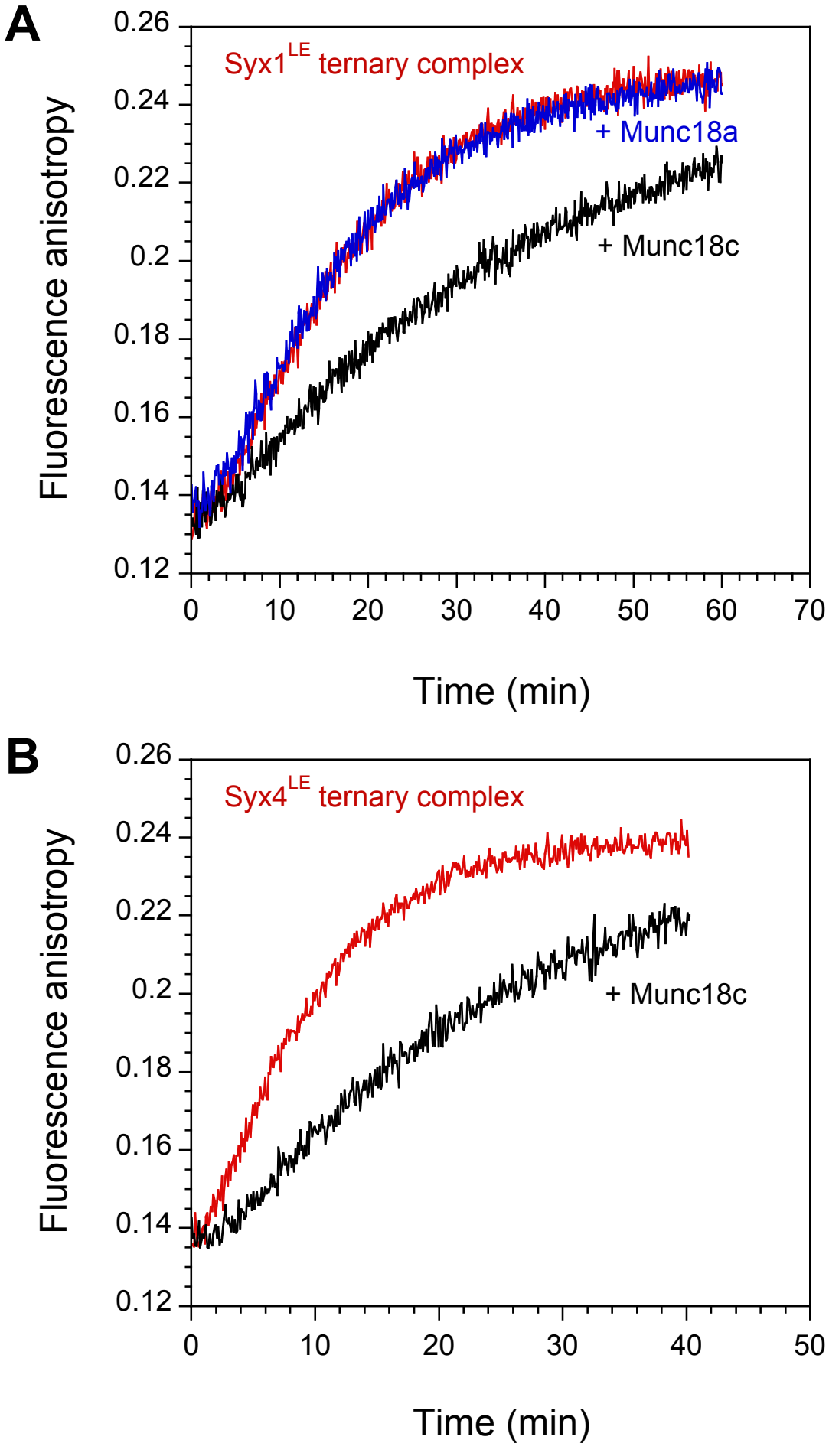
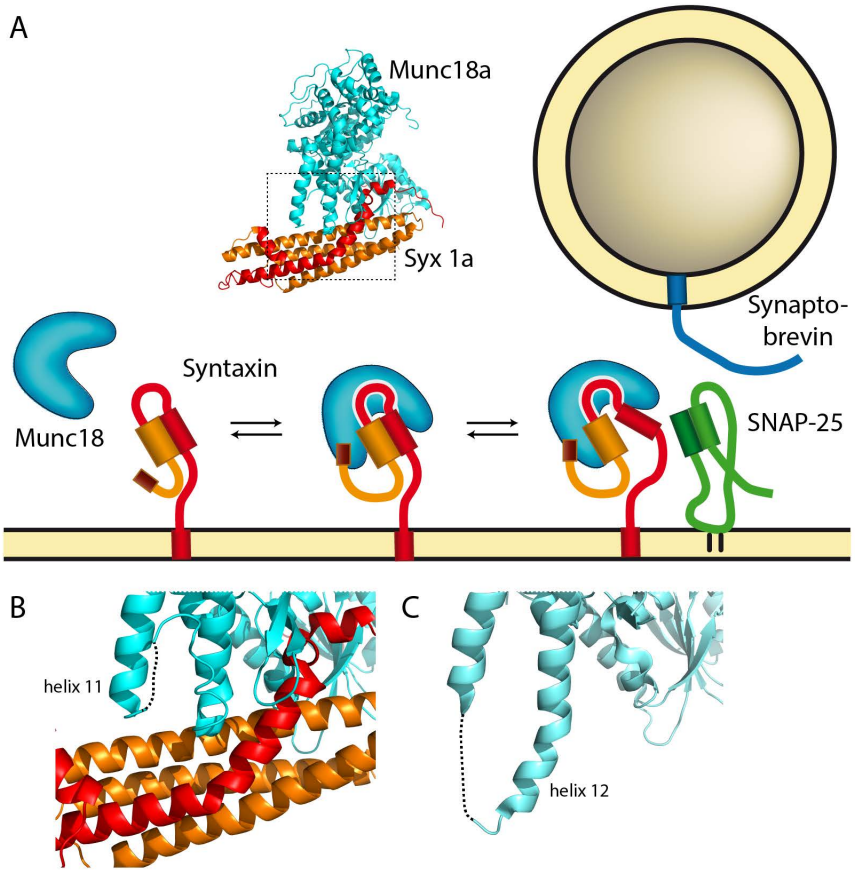

A
Syx1LE ternary complex
+ Munc18a
+ Munc18c
Fluorescence anisotropy
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0
10
20
30
40
50
60
70
Time (min)
B
Syx4LE ternary complex
+ Munc18c
Fluorescence anisotropy
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0
10
20
30
40
50
Time (min)

Fig. 7 - Morey et al.



Evidence for a conserved inhibitory binding mode between the membrane fusion assembly factors Munc18 and syntaxin in animals

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