Expanded View Figures

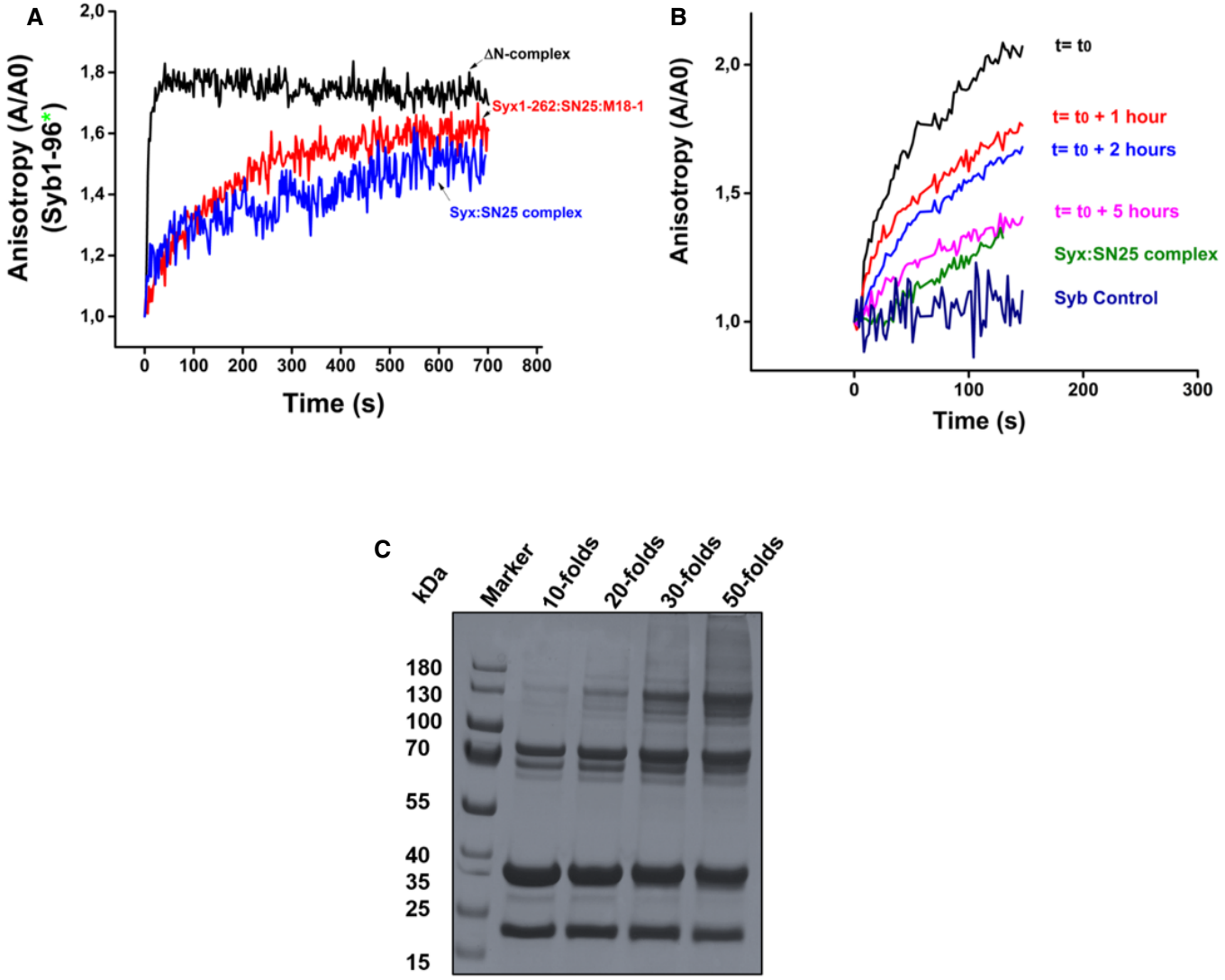


Figure EV1. A syntaxin1:SNAP25:Munc18-1 complex containing the transmembrane domain of syntaxin1 serves as a labile, yet efficient acceptor for synaptobrevin binding.

A Binding of synaptobrevin to the syntaxin1 (1-262):SNAP25:Munc18-1 complex (red curve) was slower as compared to the ΔN complex (black curve) and resembled binding to the binary syntaxin1a:SNAP25 (2:1) complex (blue curve). This was in contrast to the complex containing the transmembrane domain of syntaxin1. Precise time-point measurements using the syntaxin1 (1-262):SNAP25:Munc18-1 complex were, however, not performed.

B Binding of fluorescently labeled synaptobrevin (1-96) to the syntaxin1 (1-288): SNAP25:Munc18-1 complex was measured over increasing time intervals using fluorescence anisotropy. The complex showed a decrease in the rate of synaptobrevin binding with increasing periods of time.

C The syntaxin1 (1-288):SNAP25:Munc18-1 complex could be cross-linked by the chemical cross-linker BS3. A titration with increasing amounts of the cross-linker showed that a 50-fold excess of the cross-linker was optimum for cross-linking the syntaxin1:SNAP25:Munc18-1 complex. The "folds" indicate the molar excess of BS3. The cross-linked band appeared around a molecular weight of 130 kDa.

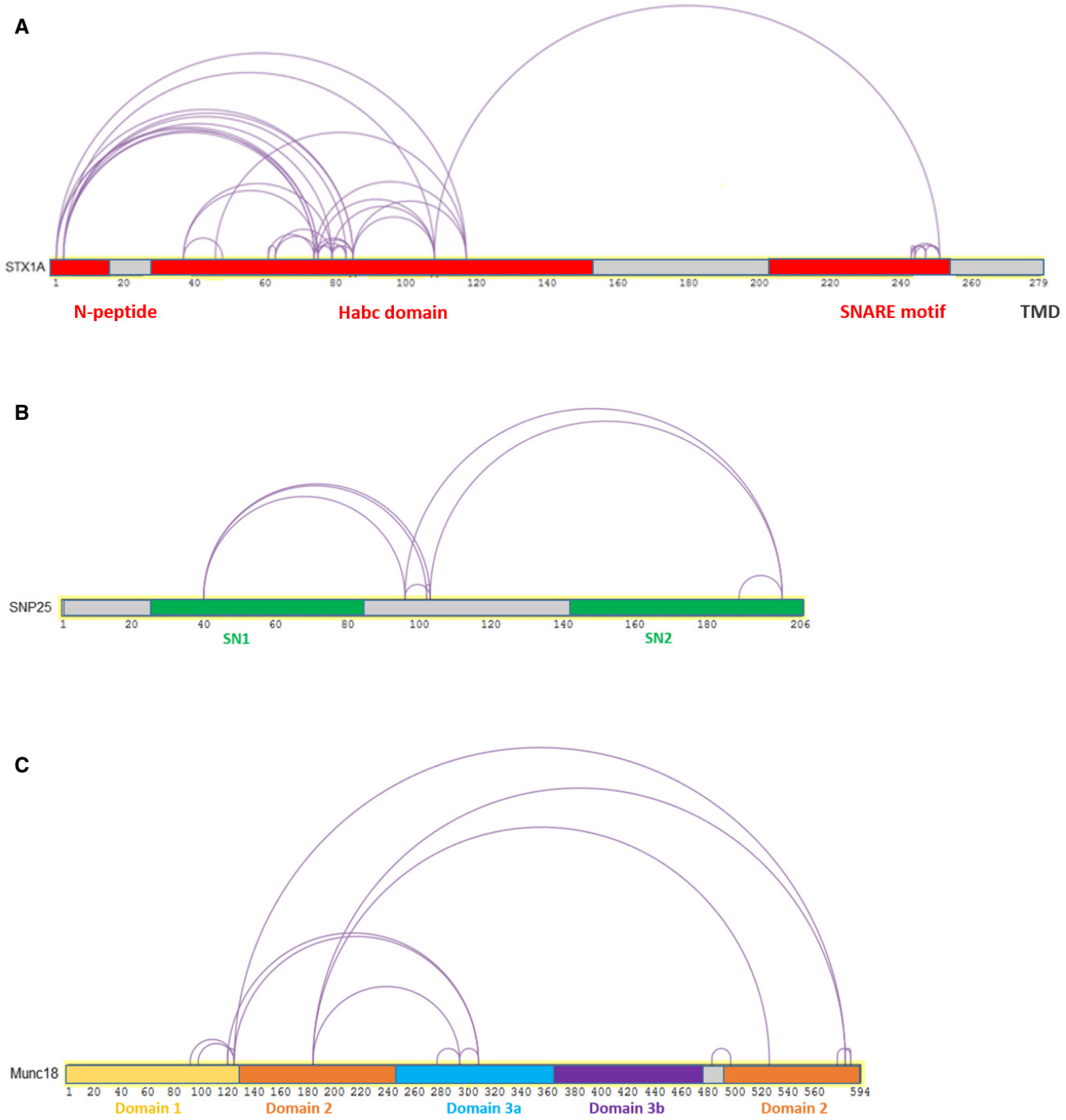


Figure EV2. Intra-cross-links between the monomeric constituents of the syntaxin1:SNAP25:Munc18-1 complex.

A-C Representative intra-cross-links between (A) syntaxin1, (B) SNAP25, and (C) Munc18-1, respectively, in the syntaxin1:SNAP25:Munc18-1 complex.

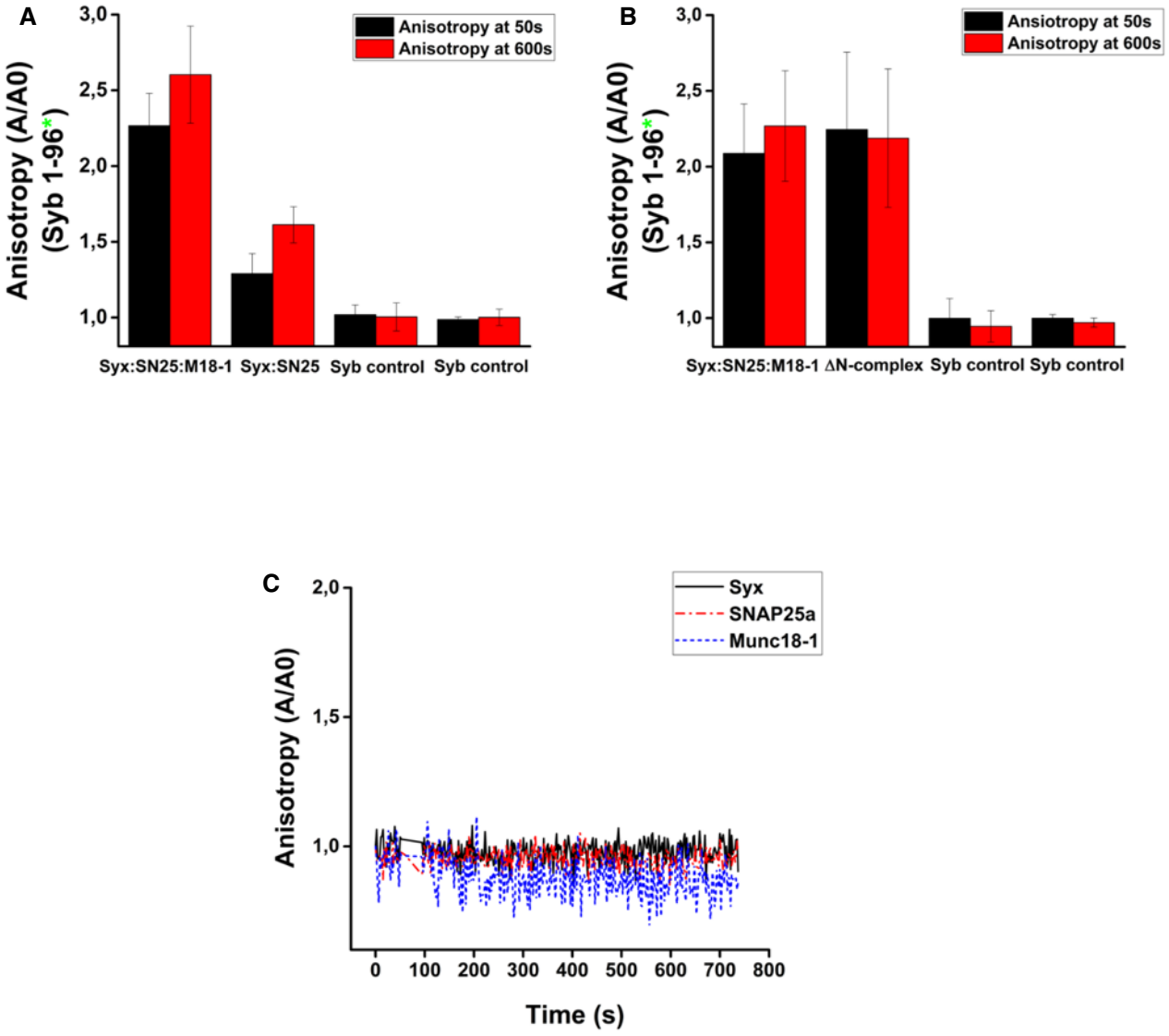


Figure EV3. Synaptobrevin binds efficiently to the syntaxin1:SNAP25:Munc18-1 complex, but not to monomeric syntaxin1, SNAP25a or Munc18-1.

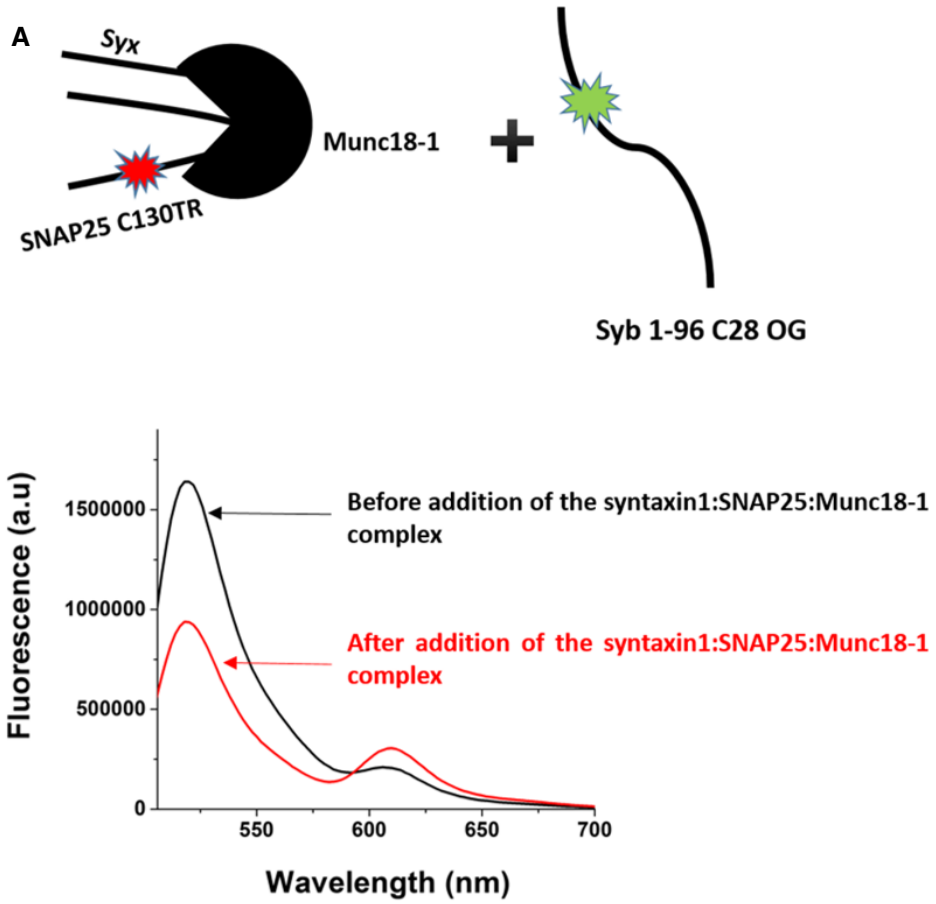
A, B (A) Quantification of synaptobrevin binding to the syntaxin1:SNAP25 complex and the syntaxin1:SNAP25:Munc18-1 complex as measured by fluorescence anisotropy, and (B) quantification of synaptobrevin binding to the ΔN complex and the syntaxin1: SNAP25:Munc18-1 complex. Error bars in both (A) and (B) indicate the range of values (n = 3).

C Synaptobrevin does not bind to the monomeric constituents of the syntaxin1:SNAP25:Munc18-1 complex. No increase in anisotropy was observed upon the addition of unlabeled syntaxin1, SNAP25, or Munc18-1 to fluorescently labeled synaptobrevin. Note that a twofold excess of the monomers with respect to synaptobrevin was used to perform this reaction.

Figure EV4. The association of synaptobrevin with the syntaxin1:SNAP25:Munc18-1 complex does not displace Munc18-1.

A Fluorescence spectra showing FRET upon addition of the syntaxin1:SNAP25-TR: Munc18-1 complex to fluorescently labeled synaptobrevin (Syb-OG). The decrease in the donor emission was accompanied by an increase in the acceptor emission (TR indicates Texas Red, and OG indicates Oregon Green).

B The incubation of the syntaxin1:SNAP25:Munc18-1 complex with synaptobrevin, followed by chemical cross-linking resulted in an increase in the molecular size/hydrodynamic radius of the sample. The separation of the cross-linked samples was performed on an analytical Superose 6 column (GE Healthcare). The cross-linked sample after synaptobrevin incubation (red curve) showed a lower retention volume as compared to the one without synaptobrevin incubation (black curve), indicating continued association of Munc18-1 after synaptobrevin binding.



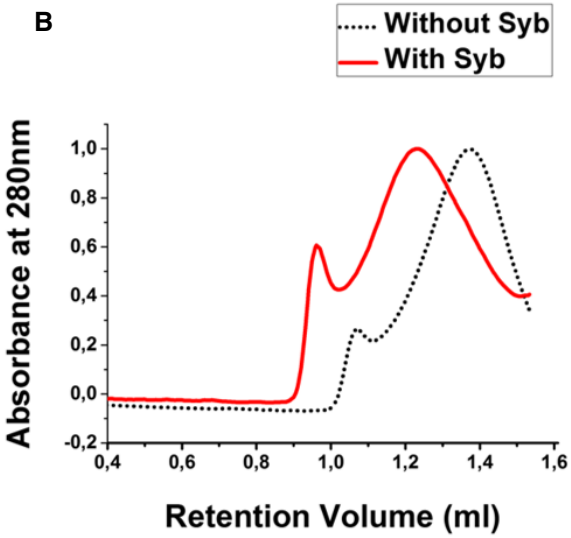


Figure EV4.

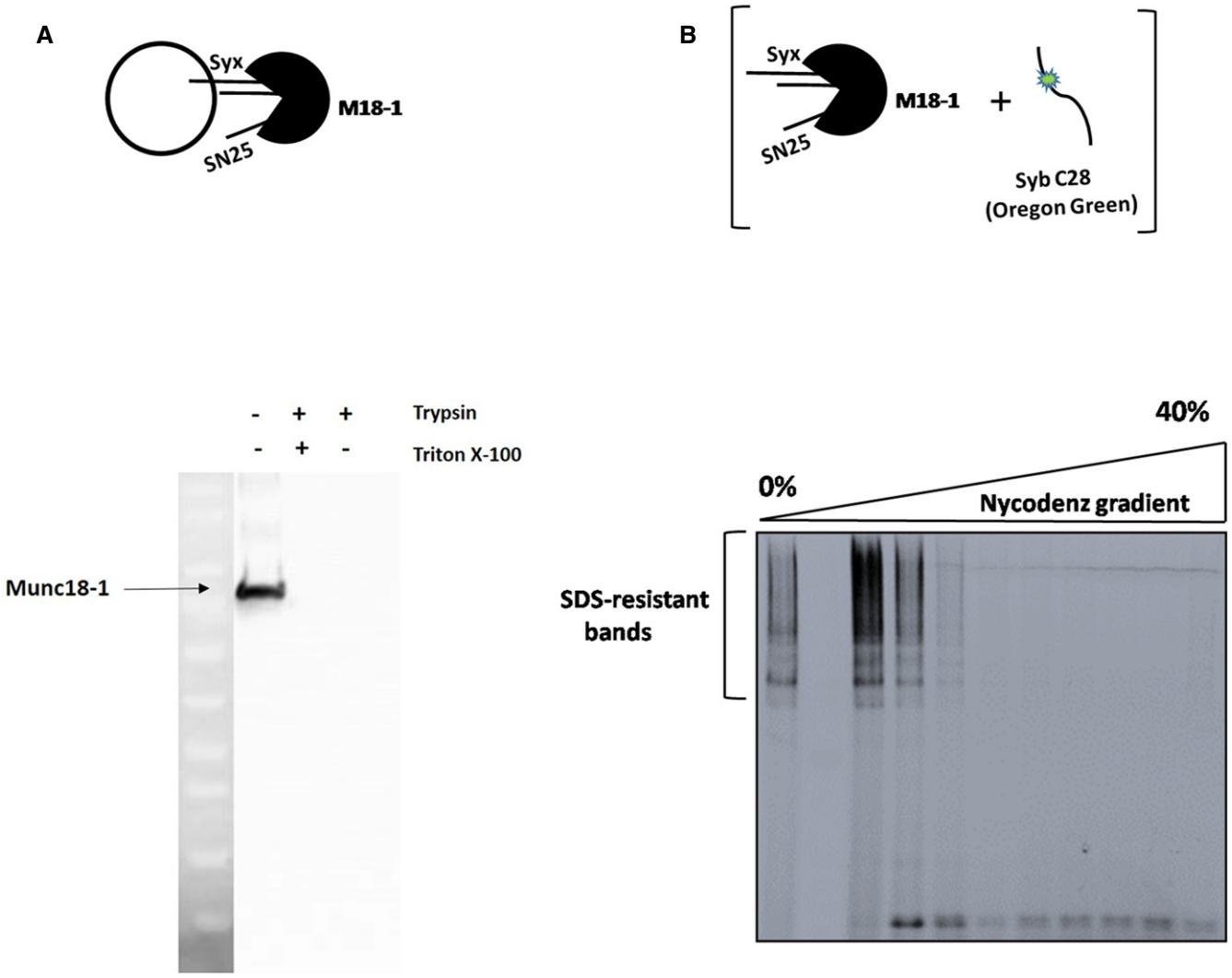


Figure EV5. Assessment of orientation of the syntaxin1:SNAP25:Munc18-1 complex on liposomes, and SNARE-complex assembly upon synaptobrevin binding to this complex.

A The schematic on the top represents a liposome containing the syntaxin1:SNAP25:Munc18-1 complex. Trypsin-digestion assay (see Materials and Methods) followed by Western blotting against Munc18-1 indicated that the syntaxin1:SNAP25:Munc18-1 complex is inserted into liposomes with a nearly 100% "right-side out" orientation.

B SDS-resistant SNARE complexes are formed after synaptobrevin binding to the syntaxin1:SNAP25:Munc18-1 complex. Fluorescently-labeled synaptobrevin was added to the syntaxin1:SNAP25:Munc18-1 complex (as depicted in the cartoon above), and the mixture was reconstituted into liposomes. SDS–PAGE analysis of the samples after co-flotation (without prior boiling of the samples) revealed the presence of multiple high-molecular-weight, SDS-resistant bands, marked by the fluorescence of synaptobrevin. The second lane from the left indicates the marker lane and hence does not show any fluorescence.

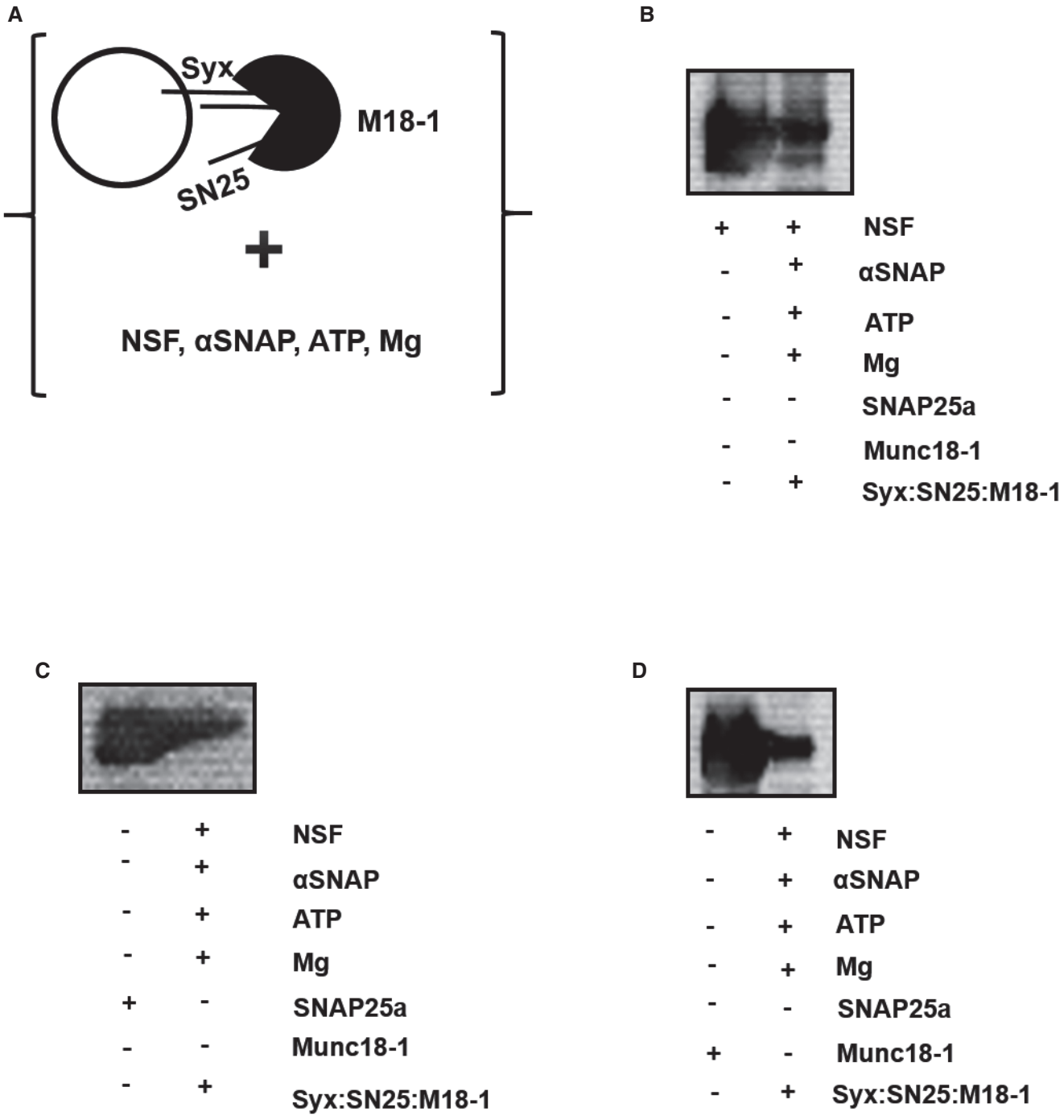


Figure EV6. Resistance of the syntaxin1:SNAP25:Munc18-1 complex to disassembly by NSF-αSNAP.

A Freshly prepared syntaxin1:SNAP25:Munc18-1 complex was reconstituted into liposomes and was thereafter incubated with NSF, αSNAP, ATP, and magnesium. The mixture was then analyzed by co-flotation analysis and subsequent immunoblotting (see Materials and Methods).

B–D The top liposomal fractions were immunoblotted using (B) α-NSF, (C) α-SNAP25a, and (D) α-Munc18-1 antibodies. The presence of all these three proteins in the liposomal fractions clearly demonstrated the resistance of the syntaxin1:SNAP25:Munc18-1 complex to disassembly by NSF-αSNAP. The left lane in each case represents the monomeric proteins as a control, and the right lanes indicate the liposomal fractions obtained after the co-flotation assay.