# The structure of SALM5 suggests a dimeric assembly for the presynaptic RPTP ligand recognition

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**Running title:** Structure of SALM synaptic adhesion protein

**Keywords:** crystal structure, synapse. adhesion, LRR, leucine rich reapeat, SALM

**ABSTRACT**

Synaptic adhesion molecules play a crucial role in the regulation of synapse development and maintenance. Recently several families of leucine rich repeat domain containing neuronal adhesion molecules have been characterized, including netrin G-ligands, LRRTMs, and the SALM family proteins. Most of these are expressed at the excitatory glutamatergic synapses, and dysfunctions of these genes have been shown to be genetically linked with cognitive disorders, such as autism spectrum disorders and schizophrenia. The SALM family proteins SALM3 and SALM5, similar to SLITRKs, have been shown to bind to the presynaptic receptor protein tyrosine phosphatase (RPTP) family ligands. Here we present the 3 Å crystal structure of SALM5 LRR-Ig domain construct, and biophysical studies that verify the crystallographic results and show that both SALM3 and SALM5 extracellular domains form similar dimeric structures, and bind to RPTP lg-domains with micromolar affinity. SALM3 shows a clear preference for the RPTP-ligands with the meB splice insert. The LRR domains form the dimer interface, and based on previous results it is known the LRR domain is required also for the ligand binding. Our structural studies and sequence conservation analysis suggests a ligand binding site and mechanism for RPTP binding via the dimeric LRR domain region.

## 1. Introduction

Synaptic adhesion molecules play important role in the regulation of synapse development and maintenance, including the formation of early synapses and their differentiation into mature synapses (Missler *et al.*, 2012; Südhof, 2008). These molecules are present on either presynaptic or postsynaptic side of the synaptic cleft on the neuronal cell membrane and contribute to the regulation of synapse development through trans-synaptic adhesion, either via homophilic interactions, as in the case of NCAMs (Kallapur and Akeson, 1992) or heterophilic interactions, such in the case of the e.g. the neurexin-neuroligin interactions (Sudhof, 2017). A large number of leucine rich repeat (LRR)-containing synaptic adhesion molecules such as the LRRTMs (Lauren *et al.*, 2003), netrin-G ligands (NGLs) (Woo *et al.*, 2009), synaptic adhesion-like molecules (SALMs) (Nam *et al.*, 2011) and SLITRKs (de Wit and Ghosh, 2014) have been identified to be involved in various aspects of synapse development.

The SALM family of proteins consists of five members (SALM1-5) (Nam *et al.*, 2011). They share a similar domain structure, containing seven LRRs, an immunoglobulin (Ig) domain, a fibronectin III (FN) repeat at the extracellular region, a single transmembrane region, and a cytoplasmic tail. In particular, a subset of SALMs (SALM1-3) possess a C-terminal type I-PDZ binding motif that binds to PDZ domains of postsynaptic density protein PSD-95. In addition, all five SALMs are mainly expressed in brain.

SALMs have been implicated in the regulation of synapse development and function. SALM1 has been reported to heterophilically interact with other SALM proteins, and to interact with GluN1 subunit of NMDA receptors (NMDARs), it furhter promotes

dendritic clustering of NMDARs in cultured neurons (Wang *et al.*, 2006). SALM2 associates with both NMDARs and AMPA receptors (AMPARs), and promotes the development of excitatory synapses (Ko *et al.*, 2006). SALM3 and SALM5 (but not the other SALMs) specifically induce both excitatory and inhibitory presynaptic differentiation in contacting axons via trans-synaptic interactions with type IIa presynaptic leukocyte antigen related (LAR)-family receptor protein tyrosine phosphatases (RPTPs) (Choi *et al.*, 2016; Li *et al.*, 2015).

The LAR-RPTPs consist of three members in vertebrates: LAR, RPTPσ, and RPTPδ, and each member contains three Ig and four-eight FN type III repeats and have multiple splice variants with short inserts atmini exons A, B and C (meA, meB and meC) in the extracellular region, with meA and meB in the Ig2 domain and between the Ig3 and Ig3 domains, respectively, and possess two tandem intracellular protein tyrosine phosphatase domains (see e.g. Takahashi and Craig, 2013, Um and Ko, 2013). LAR-RPTPs form multiple trans-synaptic adhesion complexes with various postsynaptic binding partners, including NGL-3 (Woo *et al.*, 2009), SALM3 and SALM5 (Choi *et al.*, 2016; Li *et al.*, 2015) neurotrophic receptor tropomyosin-related kinase C (TrkC) (Takahashi *et al.*, 2011), interleukin-1-receptor accessory protein-like1 (IL1RAPL1) (Yamagata *et al.*, 2015), interleukin-1 receptor accessory protein (IL1RAcP) and SLITRKs (1–6) (Takahashi *et al.*, 2012; Um and Ko, 2013; Yim *et al.*, 2013), and as such appear to act as presynaptic hubs for synaptic adhesion and signalling.

SALM3 and SALM5 interact with the Ig-domains of all three members of LAR-RPTP family to promote the excitatory and inhibitory synapse development (Choi *et al.*, 2016; Li *et al.*, 2015). Intriguingly, SALM3 binds to a specific LAR-RPTP splice variants that

contain an insert at the MeB site (Li *et al.*, 2015), whereas SALM5 appears to bind to LAR-RPTPs independent of alternative splicing events at the MeB site (Choi *et al.,* 2016). A recent study showed that SALM4 suppresses excitatory synapse development by cis-inhibiting trans-synaptic SALM3/LAR-RPTP synaptic adhesion (Lie *et al.,* 2016). Further, SALM1 and SALM5 have been implicated in severe progressive autism and familial schizophrenia (Choi *et al.,* 2016; Morimura *et al.,* 2017).

The lack of a high-resolution structure of the synaptic adhesion molecules SALM proteins has limited our understanding of how SALMs act in synapse development. Investigating the detailed molecular mechanisms underlying the SALM/LAR-RPTP synaptic adhesion pathways will be crucial for the further analysis of the biological function of these proteins. In the current study, we report the crystal structure of SALM5 full extracellular region containing the LRRs and Ig domains, together with biophysical characterization of the protein family and binding to presynaptic ligand RPTPσ and its meB splice variant, based on which we suggest a molecular model for the oligomerization and function of the SALM proteins in synaptic adhesion.

## 2.Methods

### 2.1 Cloning, expression and purification SALM and RPTP protein constructs

The mouse SALM gene constructs (SALM1 LRR-Ig$_{20-378}$, SALM1 LRR-Ig-Fn$_{20-518}$,SALM3 LRR-Ig$_{17-367}$, SALM3 LRR-Ig-Fn$_{17-510}$, and SALM5 LRR-Ig$_{18-376}$ and SALM5 LRR-Ig-Fn$_{18-505}$ and mouseRPTPσ constructs (PTPσ Ig1-3$_{33\ -327}$, andRPTPσ Ig1-3meB$_{33-331}$) were cloned into Drosophila pRMHA3 expression vector (Bunch *et al.,* 1988). The cDNAs for SALM3 and SALM5 were obtained from ImaGenes GmbH, SALM1 and RPTPσ cDNAs were a kind gift from Dr. Juha Kuja-Panula. The expression constructs included a CD33 signal

sequence at the N-terminus of the insert and a C-terminal Fc tag with a preceding the Prescission protease cleavage site. The oligo pairs used for cloning are listed in the Table S1.

The SALM andRPTPσ protein constructs were expressed from stably transfected *Drosophila* S2 cells. Expression was verified by transient transfection using western blot method with goat polyclonal horse radish peroxidase (HRP) conjugated antibody (Abcam ab98567) against human IgG-Fc. All constructs except SALM5 LRR-Ig-$Fn_{18-505}$ were found to be expressed, and were further produced from stable cell lines. For expression from stable cell line of S2 cells, $1.25 \times 10^6$ cells per well were plated on a six-well plate at room temperature. After 24 hours, the cells were transfected with 4μg of DNA containing 1:20 part of selection plasmid pCoHygro. The DNA was diluted into 400μL of the medium; 8uL of TransIT insect reagent (Mirus Bio LLC) was mixed with the DNA, and the mixture was incubated for 20 min and added to the cells. After 3 days, the selection was started; the cells and medium were centrifuged and the cells were resuspended into medium with 0.3 mg/mL hygromycin and replated into the same wells. The selection was conducted every 6 days for 3 weeks in same plate. After 3 weeks, the cells were amplified by splitting them first in low split ratios. The cells were amplified every 6 days until the cell viability was above 95%.

For large scale purification from stable cell lines, the S2 cells were divided 1:10 into HyQ-SFX (ThermoFisher) medium supplemented with 0.15 mg/mL hygromycin, grown in shaker at 25°C for 1 day, and induced with 0.7 mM CuSO4, and expression was conducted for further 6 days, after which the medium was harvested and cells were pelleted by centrifugation at 7000 rpm for 20 min at 4°C. The protein was purified using

the C-terminal Fc fusion tag with protein-A sepharose (Invitrogen). Samples were eluted with 0.1 M glycine (pH 3.0) directly to neutralizing buffer, 60 mM Tris (pH 7.4) and 300 mM NaCl. The tagged proteins were incubated with Prescission protease for 16 h at 4°C to remove the Fc tag. Prescission protease was produced as a GST fusion in Escherichia coli BL21 (DE3) using plasmid construct developed in pGEX-6P-1 vector (Addgene), and affinity purified with glutathione sepharose (Macherey-Nagel). Cleaved Fc-fusion proteins were passed through a protein-A column, and flow-through containing the cleaved SALM or RPTPσ was collected and gel filtered with Superdex 200 10/300 (GE Healthcare) in 60 mM Tris (pH 7.5) and 300 mM NaCl and concentrated, flash frozen in liquid nitrogen and stored at -80 C for further use.

**2.3. Binding affinity measurements and biophysical characterization**

Surface plasmon resonance measurements on the interaction of SALM3 and SALM5 with RPTPσ variants were carried out using the Biacore T100 system (GE Healthcare) at 25 $^{o}$C. Fc-tagged proteins of SALM3 LRR-Ig-FN (25 ug/ml) and SALM5 LRR-Ig (25 ug/ml), were immobilized on the CM5 chip (GE Healthcare) with amide coupling chemistry according to manufacturer's instructions, and using the running buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl and 0.005% Tween-20. Two ligands, the RPTPσ Ig1-3 and RPTPσ Ig1-3meB constructs, were tested for the binding affinity against SALM-Fc proteins. The concentrations of wild typeRPTPσ andRPTPσ-meB varied from 0.02 μM to 50 μM.

The thermal stability of the SALM1 LRR-Ig , SALM3 LRR-Ig and SALM5 LRR-Ig were measured by differential scanning fluorimetry using Mx3005P RT-PCR machine (Agilent Technologies). Samples for the fluorescence measurement were prepared in 60 mM

Tris buffer pH 7.4 and 300 mM NaCl, at 1mg/ml concentration of protein mixed with 1:200 diluted SYPRO-Orange.

The size exclusion chromatography-coupled multiangle static laser light scattering (SEC-MALLS) was used for the characterization of the oligomerization, and monodispersity of the SALMs and RPTP variants. The measurements were done at 0.5 mL/min over an S-200 Superdex 10/300 column (GE Healtcare) in 20 mM Tris (pH 7.4) and 150 mM NaCl with a HPLC system (Shimadzu) and a MiniDAWN TREOS light scattering detector, and Optilab rEX refractive index detector (Wyatt Technology Corp.). Data were then analyzed with ASTRA 6 software (Wyatt Technology Corp.).

**2.4. Crystallization and structure determination and refinement**

SALM5 LRR-Ig construct was concentrated to 8.5 mg/ml and exchanged to 20 mM Tris pH 7.4 and 100 mM NaCl for crystallization. Initial crystals appeared from 0.1 M sodium citrate pH 5.5, 20% w/v PEG 1500 at +20 °C, and were further optimized to 0.1 M sodium citrate pH 5.0, 20% w/v PEG 1500, 0.01 M $CuCl_2$ at +4 °C. Crystals were harvested with addition of 10% ethylene glycol or glycerol and flash frozen for data collection. The crystal diffracted to 3.0 Å at best and crystallized in space group $F4_132$, with one monomer in the asymmetric unit (Table 1).

The SALM5 crystal structure was solved using the BALBES automated molecular replacement pipeline with CCP4 (https://www.ccp4.ac.uk/ccp4online/) (Long *et al.,* 2008). Molecular replacement was able to find an initial solution for the LRR-domain of SALM5, using the LRR domain of AMIGO-1 (PDB:2XOT) (Kajander *et al.,* 2011) as a template, but not the Ig-domain. After molecular replacement and initial refinement

with REFMAC (Murshudov *et al.,* 2011), the R-factors for the initial LRR domain model were $R_{work}/R_{free}$ = 43.2%/45.9%. Subsequently several runs of the autobuild function in phenix software suite (Terwilliger *et al.*, 2008) and "morphing" (Terwilliger *et al.,* 2013) were used to initially build the model from scratch using the molecular replacement solution to correct the model and trace partially the Ig-domain during the refinement. Finally the Ig-domain was modeled using Swiss-modeller (Bordoli *et al.,* 2008), and the best minimal model was aligned with the partial crystal structure model built in the 2Fo-Fc electron density tracable for the Ig-domain and re-refined with the autobuild "rebuild-in-place" and "morphing" options in phenix. All of this was done with several iterative cycles of model building with Coot (Emsley and Cowtan, 2004) with most of the surface side chains and the loop between Ile328 to Leu335 removed during building due to lack of density. This way a model for the Ig-domain was completed and final rounds of refinement were done with BUSTER (Bricogne *et al.,* 2017). Final R-factors were $R_{work}/R_{free}$ = 25.2%/28.2% and model had geometry restrained to standard expected quality (Table 1).

### 2.4. Small-angle X-ray scattering data collection and analysis

Small-angle X-ray scattering (SAXS) data on the proteins were measured for SALM5 in batch mode at Diamond Lightsource on the B21 beamline, and for SALM3 at the ESRF beam line BM29 (see Table 3). SALM5 sample was measured in 30 mM Tris-Cl pH 7.5, 150 mM NaCl, 3% glycerol at 1, 3 and 5 mg/ml in 50 ul volumne. The SALM3 samples were measured by SEC-SAXS. Sample compartment and exposure cell were cooled to 4°C (Table 3). An SD200 10/300 GL (GE Healthcare) column was used at 0,5 ml/min in 20 mM Tris HCl pH7.5, 100 mM NaCl, 0,02% $NaN_3$. For the SALM3 LRR-Ig construct 45 uL of sample at 8,8 mg/ml, was used for SEC-SAXS analysis Data processing was

performed automatically using the EDNA online data analysis pipeline using tools from the ATSAS 2.5.1 (Incardona *et al.,* 2009) generating radially integrated, calibrated, and normalised one-dimensional profiles for each frame. DATASW (Shkumatov and Strelkov, 2015) was used for calculation of the invariants (I(0), Rg, and molecular weights (Mw).

For SALM3 constructs, an elution profile was generated with the I(0)/Rg variation plotted versus recorded frame number. The averaged data, corresponding to frames where Rg is stable and shows linearity, were further processed using ATSAS package (Petoukhov *et al.*, 2012).

For the SALM3 data was collected at 1 frame per second from the elution. Rg and I(0) as a function of the frames collected showed a stable Rg in the top of the desired peak so frames 1100 to 1200 were selected for averaging for SALM3 LRR-Ig and frames 1540-1600 for the SALM3 LRR-Ig-FN construct. The 100 first frame of each run were averaged and used as buffer baseline for substraction. PRIMUS software from the ATSAS software suite version 2.8.0 was used for primary data processing (Franke *et al.,* 2017). Parameters for each sample are given in Table 3. Rigid body fit of models derived based on SALM5 LRR-Ig construct crystal structure were calculated using SASREF with the online version of ATSAS (https://www.embl-hamburg.de/biosaxs/atsas-online/) for both SALM3 and SALM5 LRR-Ig domain constructs. Glycans were added to the models with the GlyProt server (www.glycosciences.de/modeling/glyprot/php/main.php).

## 3. Results and Discussion

### 3.1. Overall Structure of SALM5 reavels a dimeric assembly

We have solved the structure of SALM5 at 3 Å resolution. The protein crystallized in the cubic space group $F4_132$ (see Table 1). The structure reveals a typical extracellular LRR-

domain with the LRRNT and LRRCT capping subdomains with two stabilizing disulphides each, and seven LRR repeat between these. Based on sequence data it has been unclear whether there was six or seven LRR repeats, the structure confirms the presence of seven LRR repeats. Overall, the extracellular LRR domains tend to be highly variable in size, e.g. the neuronal LRRTMs have 10 LRR-repeats (Lauren *et al.,* 2003), while others have less, e.g. Slit LRR domains have 4-6 repeats (Rothberg *et al.,* 1990), while Toll-like receptors have 19-25 repeats (Botos *et al.,* 2011) and several proteins have consecutive separate LRR-domains in tandem, such as SLITRKs and Slits (Rothberg *et al.,* 1990; Beaubien and Cloutier, 2009). While all eukaryotic extracellular LRR domains share the disulphide-linked capping domains at the N- and C-termin of the LRR-domains. Also, combinations of LRR and Ig-domains and LRR and fibronection (FN) domains, such as in the SALM-family proteins, are common among the neuronal cell surface LRR-protens, which typically reside on the postsynaptic membrane.

In SALM5, the N-terminal LRRNT capping subdomain has the typical structure of extracellular chordate N-terminal capping motifs (Bella *et al.,* 2008), apart from the fact that the β -hairpin capping the LRR domain β-sheet structure is longer than the rest of the LRR β-sheet. The C-terminal LRRCT-capping subdomain (Gln216-Cys284) is quite divergent from the typical LRRCT structure: it starts with an unsual large insertion disordered in the crystal structure (Gln217-Phe233) before the β-strand connecting to the LRR domain concave surface rest of the LRRCT forms a partly helical capping bundle with the two disulphide brigdes stabilizing it. The capping LRRCT appears to be more loosely packed than usual with somewhat irregular secondary structure with only one regular α-helix. The looser packing possible follows from the large insertion in the LRRCT. The LRR domain ends in the last Cys of the second LRRCT disuphide (Cys285-

Cys246), as is typical. The other disulphide in the LRRCT is formed by the Cys244 and Cys263.

The mouse SALM5 LRR domain has one N-glycosylation site at Asn73 for which extra electron density can be seen, but the sugar moiety could not be modeled, and two in the Ig-domain at residues Asn330 and Asn339, which are disordered in the crystal structure, overall based on their location it would appear that the glycans do not have functional significance.

The SALM5 Ig-domain (Glu285-His373) follows immediately after the LRRCT-capping subdomain. The Ig-domain appears fairly disordered overall and apparently somewhat mobile in the crystal and has poor electron density with higher B-factors compared to the LRR domain (Table 1), nevertheless it was possible to trace its position, based on intial partial model (see methods) up to the last reisdue of the fold, His373. Some parts of the Ig-domain are weak in the density and loop between two β-sheets of the β-sandwich fold at Ile328 and Leu335 is disordered.

The refined crystal structure forms a dimer with C2 symmetry with a subunit interface formed by the LRR-domains edge-to-edge, the top surfaces of the LRR β-sheets packed against each other (Fig. 1), with a surface area of 1046 $Å^2$, which is clearly the largest intermolecular interface found in the crystal, as measured with the PISA-server (Krissinel and Henrick, 2007). Interestingly though, the Ig-domains form trimeric assemblies in the crystal, which might be able to further contribute to possible clustering of the molecules on the membrane. In the full length protein the Ig-domain is followed by a linker region of ca. 40 residues from Ile374 to Thr413 and fibronectin

type-III domain on the extracellular side before the the transmembrane helix and cytosolic tail. The dimeric LRR-Ig structure has dimensions of ca. 80 Å x 65 Å x 55 Å.

The LRR domains in the dimer forms a shallow bowl-shaped surface towards the synaptic cleft formed (Fig 1.) by the two LRR domain concave surfaces, while the Ig-domains pointing "downwards" at almost 90 degree angle towards the cell-membrane from the putative LRR-dimer concave ligand binding platform, which shows high degree of conservation between the known RPTP binding SALM3 and SALM5 proteins (Fig. 1). In total we find 31 highly conserved residues on the concave surface on the LRR-domain beta-sheet from LRRNT to the seven LRR repeat (Table 2). In practice, the whole surface appears highly conserved (Fig 1.), while the overall sequence identity between the SALM3 and SALM5 proteins is ca. 50-60% and the convex side of the LRRs (or the Ig-domain) do not show significant continuous areas of conservation (Fig. 1).

The dimer interface is formed by hydrophobic interactions at the N- and C-termini on the LRR domain (Fig. 2) involving residues Phe62 and Leu 43 on one side and Leu268, Ala2264 and Thr262 on the other side, partly packing against the peptide backbone turns of the opposing domain, and by hydrogen bonding interactions in the middle of the interface by side chains of Arg110 and Asn158 from opposing monomers, Arg 110 also hydrogen bonds to the backbone carbonyl of Asn157. Also Gln134 is possibly involved in the hydrogen-bonding network (Fig. 2) but its position could not be accurately modeled in the 3 Å resolution 2Fo-Fc electron density map.

### 3.2. Comparison to other LRR proteins

Based on a DALI-search (Holm *et al.,* 2008) we found several LRR domain proteins with high Z-scores, the highest Z-scores were found for the LRR domain for the Hagfish variable lymphocyte receptor (VLR) (Z-score 27.6) domain with seven LRRs (PDB:2O6Q) and the platelet glycoprotein Ib alpha (GP1ba; PDB:1M0Z) (Z-score 25.9), also with seven LRRs. The GP1ba LRR domain aligns with an r.m.s.d of 1.462 Å with the SALM5 LRR domain (Fig 4). Interestingly, both SALM5 and GP1ba have a large insertion loop in the LRRCT subdomain which in the GP1ba wraps around the von WIllebrand factor ligand, structuring into a β–hairpin structure on ligand binding (Huizinga *et al.*, 2002); similarly the β-sheet of LRRNT is extended in both, and in GPIba bent towards the binding site on the concave surface of its LRR domain inducing larger curvature, and wrapping around the ligand. It can be hypothesized that the long insertion loop in SALM5 also might have a functional role in recognition of the ligand, the insertion is present in the whole SALM family. Also the SLTRK 1st LRR domain, with six LRRs, aligns well (r.m.s.d. 1.462 Å) with our SALM5 structure – this is notable as both recognize the same RPTP-ligands (see below). Previously we have characterized the structures of the neuronal AMIGO family proteins (Kajander *et al.,* 2011) which have six LRR followed by and Ig-domain, but with a different type of dimer arrangement, where the LRR concave surfaces form the dimer interface. The SALM LRR domain dimer appears unique in the known cell surface LRR-protein proteins by forming a continuous surface by the two LRR domains (Fig. 1).

### 3.3. Biophysical characterization of SALM family proteins

Solution measurements by SEC-MALLS and SAXS analysis confirm the dimeric nature of SALM-family proteins in solution, as dimers were observed for all $SALM1_{LRR\text{-}Ig\text{-}FN}$, $SALM3_{LRR\text{-}Ig}$, $SALM3_{LRR\text{-}Ig\text{-}FN}$ and $SALM5_{LRR\text{-}Ig}$ by SEC-MALLS (Fig. 3), with observed

molecular weights (Mw) of 80.7 and 83.2 kDa for SALM3 and $SALM5_{LRR-Ig}$ constructs, and 121 kDa for the FN-domain containing constructs. These match well with expected theoretical dimer Mws based on the calculated monomer MWs of 39.8 and 39.1 kDa for the short constructs, as well as the longer ectodomain constructs, which have theoretical MWs of 52 and 54 kDa for a monomer. All theoretical values are without glycans, which contribute ca. 3-4 kDa per monomer, due to the paucimannose-type glycans present in insect cell-produced proteins (Shi and Jarvis, 2007).

Similarly, SAXS measurements support the observation of SALM-proteins being obligate dimers (Fig. 5, Table 2). The SAXS data can be fitted well to present a model for SALM3, which forms the same type of dimer as SALM5. The LRR domains would form the dimer interface and an extended "top"-surface of the dimer assembly, as in the SALM5 crystal structure (also the SALM5 SAXS data fit the predicted dimer from the crystal structure), rigid body modeling of $SALM3_{LRR-Ig}$ and $SALM5_{LRR-Ig}$ glycosylated LRR and Ig-domains give a very good fit to the SAXS data (Fig. 5) with $\chi 2$-values of 1.2 and 2.4, respectively. Therefore, taking together the SEC-MALLS and SAXS data, we suggest this dimeric organization is conserved in the SALM-family. The conserved structural arrangement as well as the sequence conservation (Fig. 1) suggests a conserved mechanism of action between at least the SALM3 and SALM5 proteins for ligand binding, as they both bind the same presynaptic RPTPσ-ligands.

A differential scanning fluorimetry assay revealed that all three SALM proteins unfold with single transition event in the fluorescence curve, suggesting probably a cooperative unfolding event for the dimer (Suppl. Fig 1.). SALM3 appears to slightly less

stable than SALM1 and SALM5, with a $T_m$-value of 45.5 °C while the other have $T_m$-values of 52 and 54 °C, respectively (Suppl. Fig 1.)..

### 3.4. Ligand binding by SALM3 and SALM5 and model for binding site for RPTP-ligands

We determined the binding affinities of SALM3 and SALM5 extracellular domains about RPTPσ and RPTPσ-meB splice variant by surface plasmon resonance. SALM3 showed a clear preference for RPTPσ-meB splice variant, while for SALM5 the data indicated similar affinities for both (Fig 6.). Overall, SALM3 had slightly higher affinity and was more specific towards the RPTPσ–meB ligand, with 10-fold lower affinity observed by SPR for the RPTPσ lacking the mini-exon B between the Ig2- and Ig3-domains of the RPTPσ ligand. For SALM5 similar affinities were observed with or without the meB-insert of RPTPσ. Both proteins had similar low micromolar range affinities for the meB-containing splice variant ligand (Table 3). Previously, SALM3 have been reported to have a preference for the meB splice variant (Li *et al.,* 2015).

Based on the earlier reported structures of SLITRK in complex with RPTP Ig-domains (Um *et al.,* 2014; Yamagata *et al.*, 2015) we decided to compare the two systems. Given the clear sequence conservation pattern among SALM3 and SALM5 proteins when displayed on SALM5 surface (Fig. 1), it seems obvious that SALMs will also recognize their ligand via the unique concave dimeric LRR domain surface, possibly suggesting a new type of ligand recognition for the LRR-adhesion receptors. Overall, LRR domains typically do recognize their targets utilizing the concave surface (Helft *et al.,* 2011). In order to compare the two systems, we aligned the SALM5 LRR domain and the SLITRK LRR domains from N-terminus onwards and observed how the RPTP ligand would fit on

the SALM dimer "top" concave surface (Fig. 7). Obviously no direct fit is expected, but based on the alignment, it can be suggested that the RPTP ligand would not fit very well in a 2:2 complex onto the SALM5 dimer, unless the binding occurs in a very different manner; which would involve the non-conserved regions on the LRR domains, away from the dimer interface and the "top"-surface (Fig. 7). We then performed assays to verify the stoichiometry, and based on the SEC-MALLS analysis (Fig 3.) of SALM3-PTPσ Ig1-Ig3 complex peaks corresponding to 2:1 complex and dissociated RPTPσ ligand were observed (Fig. 3). We therefore suggest that the RPTPσ-ligand is recognized with 2:1 stoichiometry. It still possible that there is a second binding event with lower affinity that we are unable to observe, that would result in some form of 2:2 complex. But it would appear that 2:1 complex is the major quaternary state for the complex, excluding possible clustering effects by other factors.

## 4. Conclusions

We show that SALM5 appears to function as a dimeric synaptic organizer molecule - the crystal structure revealing a bowl-shaped top surface formed by the dimerized LRR-domains. We find that also SALM3, as well as SALM, and therefore most likely the whole protein family form dimeric structures, presumably via their LRR-domains. Our SAXS analysis confirms that SALM3 LRR-Ig fragment has a very similar structure to the SALM5 crystal structure. Both SALM3 and SALM5 in our studies are able to bind the RPTP ligands with meB splice site with similar affinity while SALM3 appears more selective. We suggest a 2:1 stoichiometry for RPTP-ligand binding, and based on sequence conservation and structural organization locate the ligand binding site at the extended concave LRR "double β-sheet" surface of the dimeric SALM ectodomain.

**Funding**

This work was supported by grants from the Academy of Finland (256049 and 251700) and Jane and Aatos Erkko Foundation (to TK).

**Acknowledgements**

We thank the staff at Diamond Lightsource and ESRF beamlines for help with data collection on beam lines BM29 (ESRF), Diamond B21, and ESRF ID30B. Crystals were initially screened at the Biocenter Finland crystallization core facility at Institute of Biotechnology, Helsinki, Finland. We also thank wish to thank Dr. Juha Kuja-Panula for the gift of RPTPs and SALM1 cDNAs, Dr. Katja Rosti for work in the initial phases of the project and Seija Mäki for help with crystallization. The coordinates and structure factors have been deposited in the protein data bank with accession code 6F2O.

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## Figure Legends

**Figure 1. The SALM5 crystal structure and the dimer assembly of the LRR-Ig fragment.** A) The monomeric SALM5 LRR-Ig construct structure. The β-sheets are displayed in blue (N-terminal concave face β-stands of LRR repeats 1-7 labeled β1-7), helices red, and disulphide brigdes drawn as sticks and missing parts for the LRR domain shown as dashed lines. LRRNT and LRRCT capping domains are labeled for their position the N- and C-termin are marked with "N" and "C" and the residue number. B) Dimeric assembly observed in the crystal structure as a side view with transparent surface. C) The conserved residues plotted on the surface indicate the top-concave LRR dimer surface (right) as the most conserved area. Red and light red indicate the most conserved residues as calculated by CONSURF (Ashkenazy, Abadi et al. 2016) for SALM3 and SALM5. Convex side view (left) shows litlle conservation in the LRR domain or the Ig-domain. D) A 2Fo-Fc electron density contoured at 1 σ for a representative part of the LRR domain. Molecular images made with PyMoL.

**Figure 2. SALM5 dimer interface formed by the LRR-domains.** Residues involved in dimer interface contacts are labelled, for further details see text. Monomers are shown in light grey and blue, N-T = N-terminus. Ig-domain is hidden behind the plane of the paper.

**Figure 3. Characterization of SALM proteins by SEC-MALLS.** A) SALM5 LRR-Ig domain construct B) The RPTPR Ig1-3-mEB construct. C) SALM3 LRR-Ig:RPTPmeB complex (solid line) and SALM3 LRR-Ig construct alone (dashed line). D) An SDS-PAGE analysis of the first ("A") and second ("B") peaks of the SALM3:RPTPmeB complex, verifying the presense of both proteins in the complex peak ("A").

**Figure 4. Superimposition of SALM5 with closely related structure of platelet Glycoprotein Ib alpha (GPIba).** The alignment of the LRR domains (Upper figure, GPIba in yellow and SALM5 in grey). Below, the crystal structure of the SALM5 LRR-Ig fragment with the disordered loop$_{215\text{-}235}$ modelled (in dark grey). The arrow indicates the loop insertion at the start of the LRRCT capping subdomain in SALM5. N-T, N-terminus. Structures were aligned and molecular images made with PyMoL.

**Figure 5. Solution structures of SALM5 and SALM3 support a conserved dimer interface and assembly.** A) SALM3 LRR-Ig domain rigid body domain fit to SEC-SAXS data (gray, observed data; solid orange line, fit) B) SALM5 LRR-Ig model fit to SAXS data (gray, observed data; solid blue line, fit). Protein models fitted to SAXS data are shown as cartoon models and the predicted N-glycans as sticks.

**Figure 6. Binding of SALM3 and SALM5 to the RPTPσ ligand as measured by surface plasmon resonance.** Binding of SALMs to RPTPσ/RPTPσ-meB. SALM3 LRR-Ig-Fn and SALM5 LRR-Ig were tested for binding to RPTPσ and RPTPσ-meB by surface plasmon resonance (SPR). A) SPR sensorgrams (left) and equilibrium binding curve plotted (right) for binding of SALM5 LRR-Ig to RPTPσ. B) SPR sensorgrams (left) and equilibrium binding curve plotted (right) for binding of SALM5 LRR-Ig to RPTPσ meB. C) SPR sensorgrams (left) and equilibrium binding curve plotted (right) for binding of SALM3 LRR-Ig-Fn toRPTPσ. D) SPR sensorgrams (left) and equilibrium binding curve plotted (right) for binding of SALM3 LRR-Ig-Fn to RPTPσ meB.

**Figure 7. A model for the interaction complex of SALM5 with RPTP ligand and comparison with SLITRK complex.** A) The monomeric SLITRK:RPTP complex (with the SLTRK and SALM5 LRR domains aligned, see text). (SLITRK in grey, SALM5 in, blue), RPTP, red. B) Potential interaction model for SALM5 dimer and RPTP ligand. Arrow indicates changes in the orientation of the Ig3-domain of RPTPσ (in red, with transparent surface) required to avoid steric clashes in the hypothetical SALM5:RPTPσ complex. Approximate position of the ligand is consistent with surface conservation in SALM5 and SALM3 proteins (See Fig. 1). C) Schematic interaction model at the synapse for SALM5 interaction with presynaptic monomeric RPTP. rectangles = FN-domains, spheres = Ig-domains, ellipses the SALM LRR repeats. Further clustering of the complex could occur via other interactions by either partner.

## Tables

**Table 1.** Crystallographic data collection and refinement

| | |
|---|---|
| Beam line | ID30B ESRF |
| λ (Å) | 0.9677 |
| Space group | $F4_132$ |
| *Unit cell* | |
| a, b, c (Å) | 254.0, 254.0, 254.0 |
| *α, β, γ* (°) | 90, 90 , 90 |
| Resolution (Å) | 50-3.0 |
| Observations | 159812 |
| Unique | 14488 |
| Rmerge (%) | 13.6 (222.6) |
| CC(1/2) | 98.30 (40.90) |
| I/(I)σ | 10.59 (1.10) |
| *Refinement* | |
| Resolution (Å) | 30-3.0 |
| Rwork / Rfree (%) | 25.20 / 28.20 |
| No. Reflections / Rfree-set reflections | 14438 / 721 |
| Average B-factor ($Å^2$)<br>LRR-domain ($Å^2$)<br>Ig-domain ($Å^2$) | 142.1<br>126 .1<br>200 .2 |
| No. of atoms (protein) | 2457 |
| r.m.s.d bond lengths (Å) | 0.01 |
| r.m.s.d bond angles (°) | 1.36 |
| Ramachandran plot (favored/outliers) (%) | 91.60 / 1.24 |

**Table 2.** Most conserved residues on the concave SALM5 LRR surface.

| LRR region | Conserved residues§ |
|---|---|
| LRRNT | Asn32 *, Ala34 *, Leu36 |
| LRR1 | Val54, Glu55, Arg57, Asp60 |
| LRR2 | Val78, Asp79, Thr81, Ser83, Arg84 |
| LRR3 | Arg102, Ala103*, His105 |
| LRR4 | His126*, His127, Ile129, Asn131 |
| LRR5 | Glu150, Glu151*, Asp153, Ser155 |
| LRR6 | His174, Thr175*, Ser177*, Asp179 |
| LRR7 | Arg199, Asp201, Thr203, Ser204 |

§Residues with Consurf-server scores: 9* and 10.

**Table 3.** SAXS data collection and structural parameters

| | **Salm3 (LRR-Ig)** | **Salm5 (LRR-Ig)** |
|---|---|---|
| Beamline | BM29 (ESRF) | B21 (DLS) |
| Wavelength (Å) | 0.9919 | 1.005 |
| $q$ range (Å$^{-1}$) * | 0.004-0.5 | 0.004-0.4 |
| Temperature (K) | 277.85 | 288 |
| *I(0)* (from *P(r)*) | 17.6 ± 0.1 | 0.0067 ± 0.0001 |
| $R_g$ (Å) (from *P(r)*) | 37.5 ± 0.1 | 36.5 ± 0.1 |
| *I(0)* (from Guinier) | 18.3 ± 0.1 | 0.0067 ± 0.0001 |
| $R_g$ (Å) (from Guinier) | 37.2 ± 0.1 | 36.4 ± 0.1 |
| $D_{max}$ (Å) | 121.3 ± 10 | 135.0 ± 10 |
| Porod volume $V_p$ (Å$^3$) | 182.59 | 155.00 |
| Excluded volume $V_{ex}$ (Å$^3$) (*DAMMIN*/P1) | 191,600 ± 1,600 | 171,800 ± 1,500 |
| Mw From Porod volume ($V_p$/1.7) (Da) | 101,400 | 91,100 |
| Mw From excluded volume ($V_{ex}$/1.7) (Da) | 112,700 ± 950 | 101,061 ± 870 |
| From Sequence + Glycans (Da)§ | 39,603 + 4,000 | 41,018 + 3,000 |

**Abbreviations:** *I(0)*, extrapolated scattering intensity at zero angle; $R_g$, radius of gyration calculated using either Guinier approximation (from Guinier plot) or the indirect Fourier transform package GNOM [from *p(r)*]; $M_w$, molecular mass; $D_{max}$, maximal particle dimension; $V_p$, Porod volume; $V_{ex}$, particle excluded volume.

***Momentum transfer $|q| = 4\pi\sin(\theta)/\lambda$.**

**§Monomer Mw and the approximate Mw of insect cell N-glycans**

**Table 4.** The binding affinities ($K_d$-values) of SALM3 LRR-Ig-FN and SALM5 LRR-Ig for RPTPRσ and RPTPRσ-meB.

| | PTPσ | PTPσ meB |
|---|---|---|
| SALM3 LRR-Ig-FN | 23.34 ± 2.042 uM | 2.242 ± 0.20 uM |
| SALM5 LRR-Ig | 10.6 ± 1.78 uM | 5.42 ± 0.66 uM |

**Figure 1.**

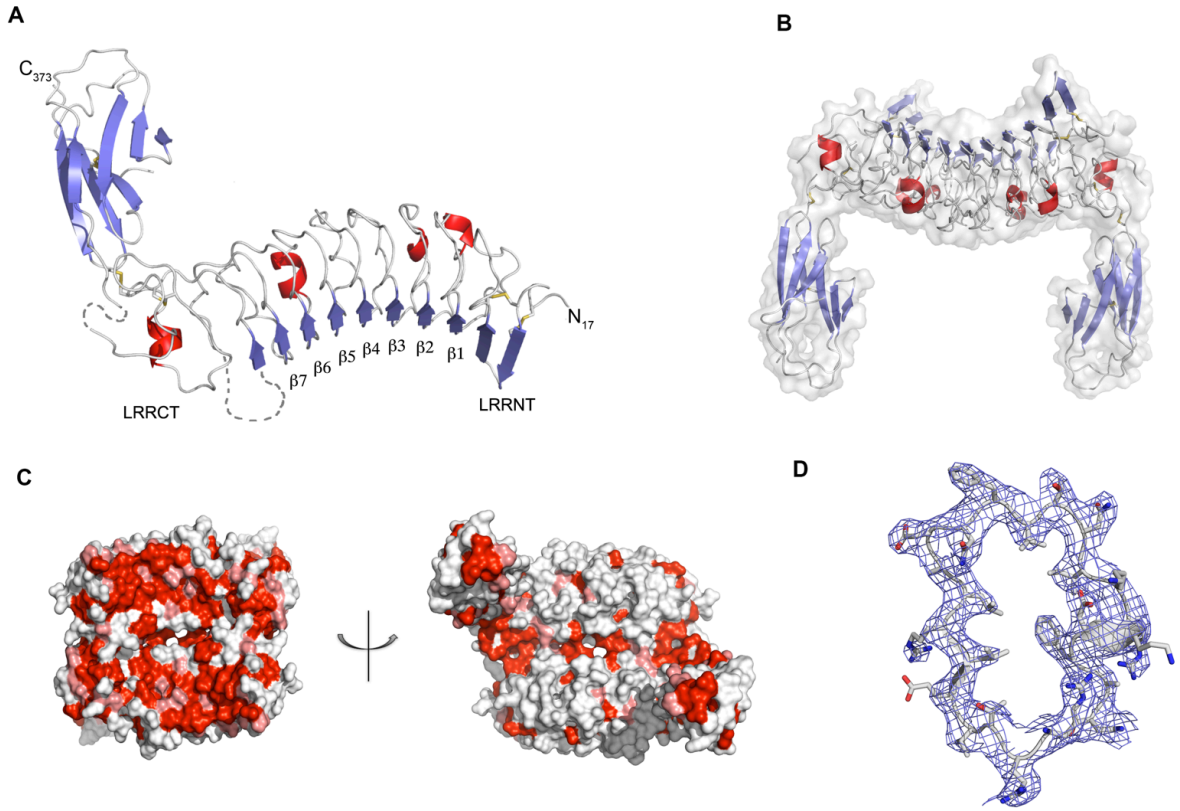


**Figure 2.**

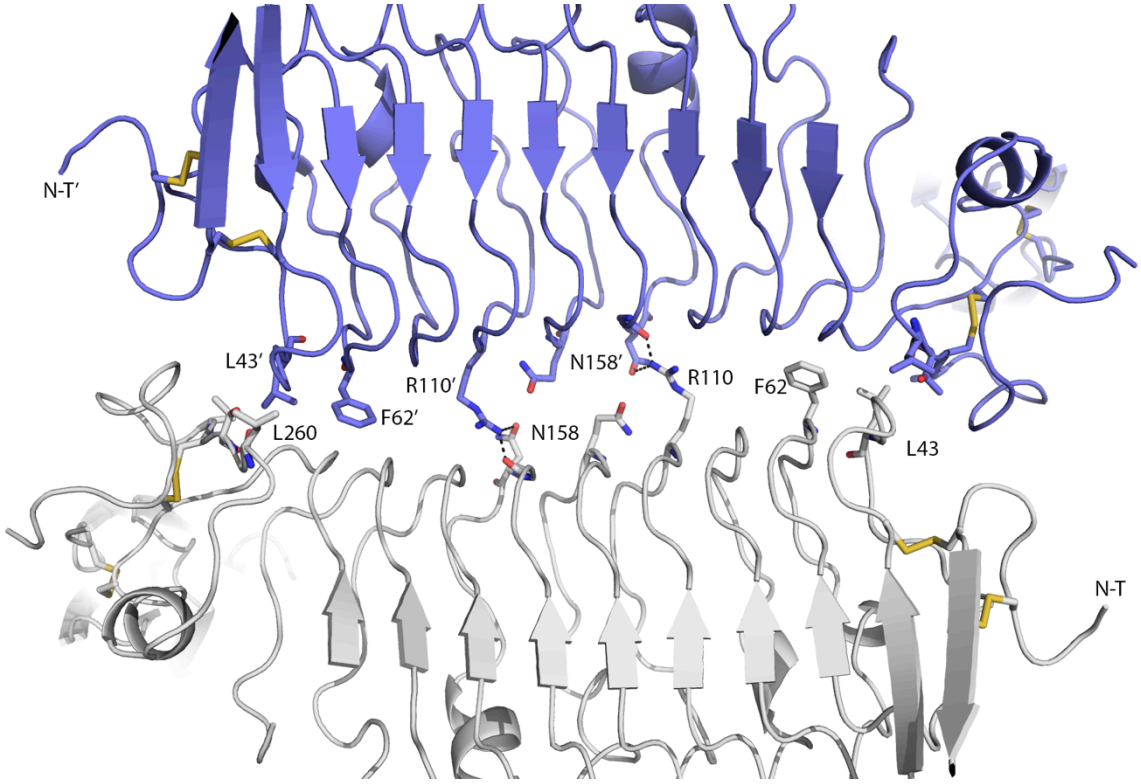


**Figure 3.**

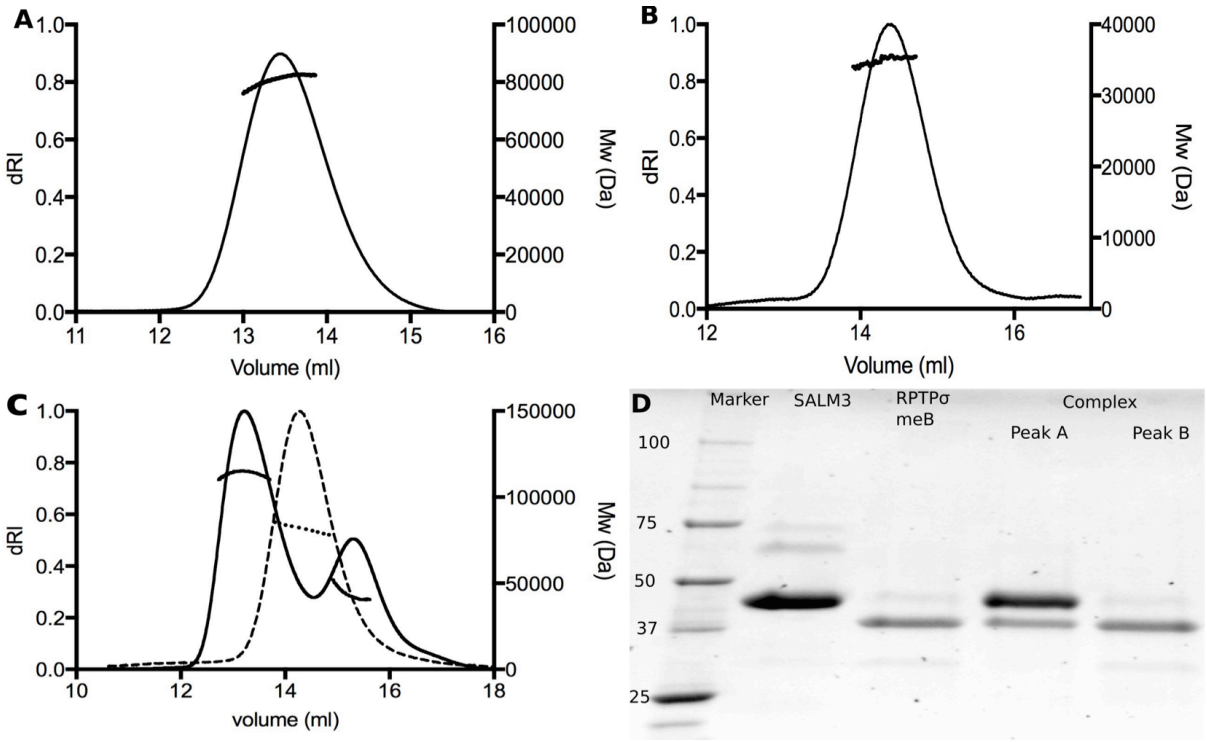

A
dRI
Mw (Da)
Volume (ml)
B
dRI
Mw (Da)
Volume (ml)
C
dRI
Mw (Da)
volume (ml)
D
Marker
SALM3
RPTPσ meB
Complex
Peak A
Peak B
100
75
50
37
25

**Figure 4.**

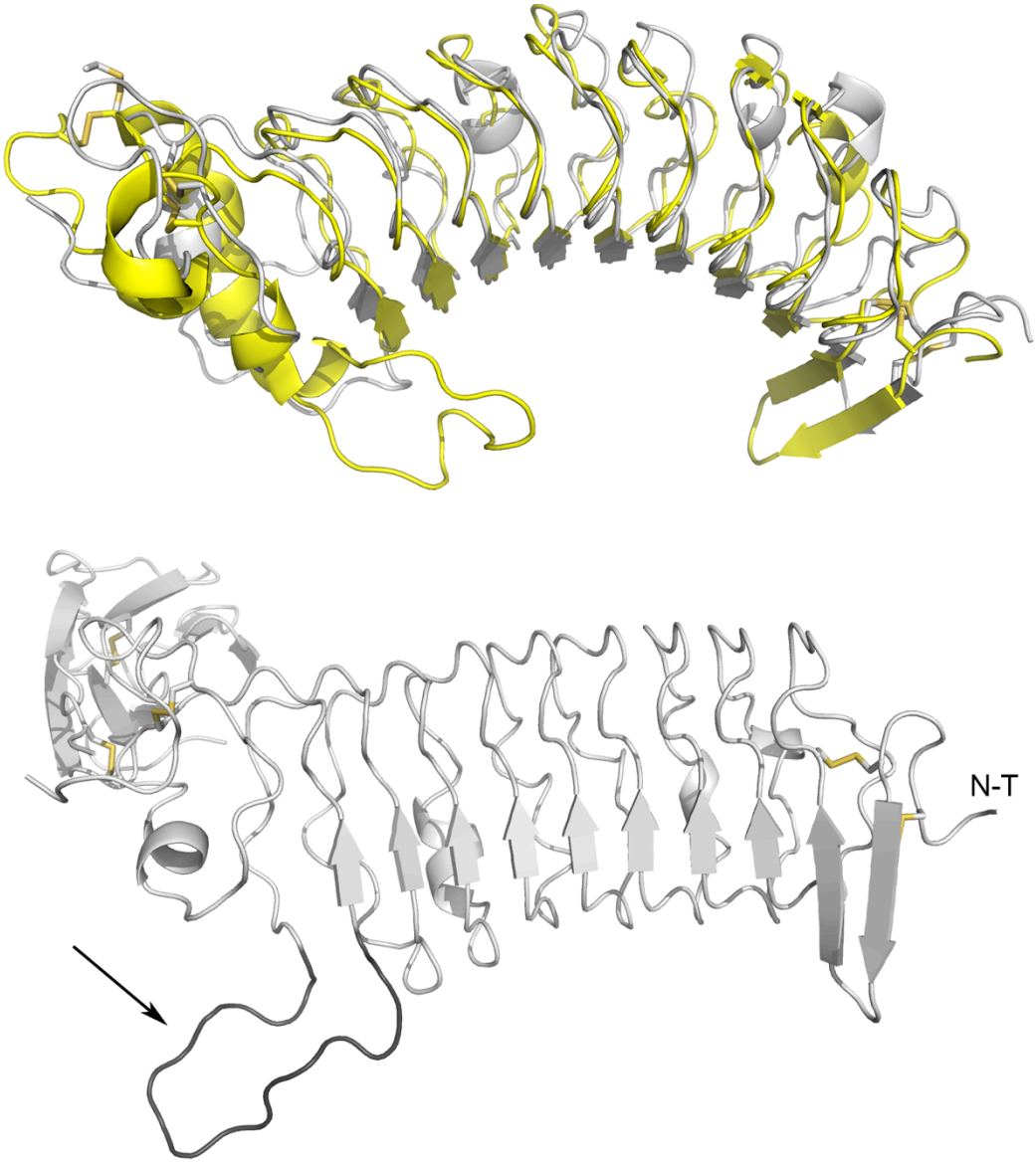
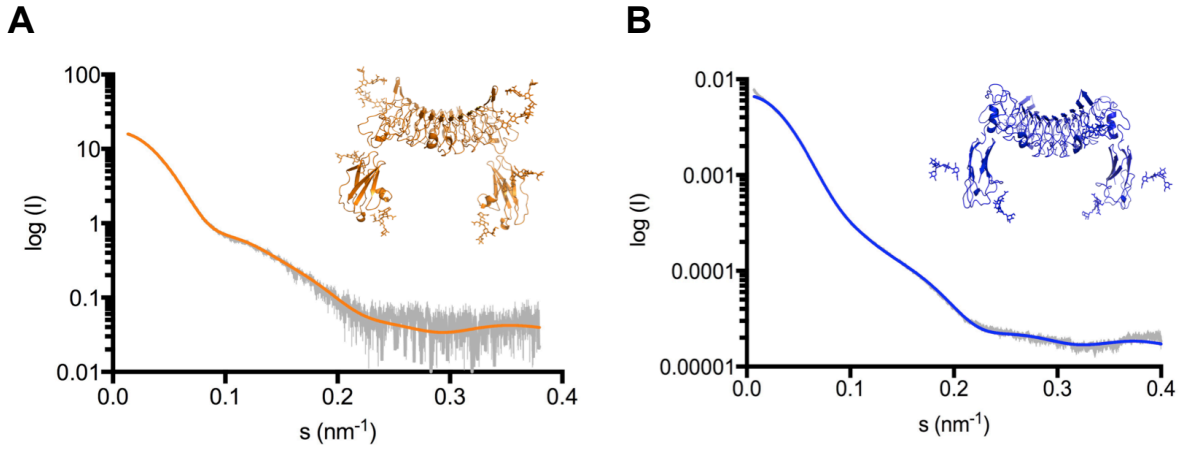


**Figure 5.**


A
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s (nm-1)
B
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0.001
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0.00001
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0.1
0.2
0.3
0.4
s (nm-1)

**Figure 6.**

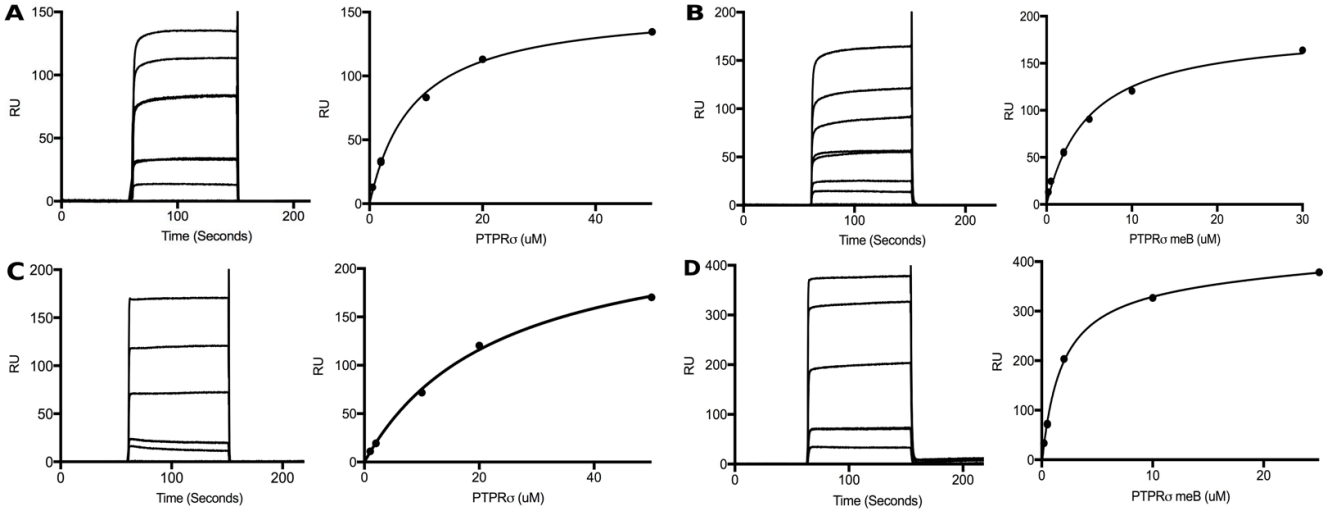
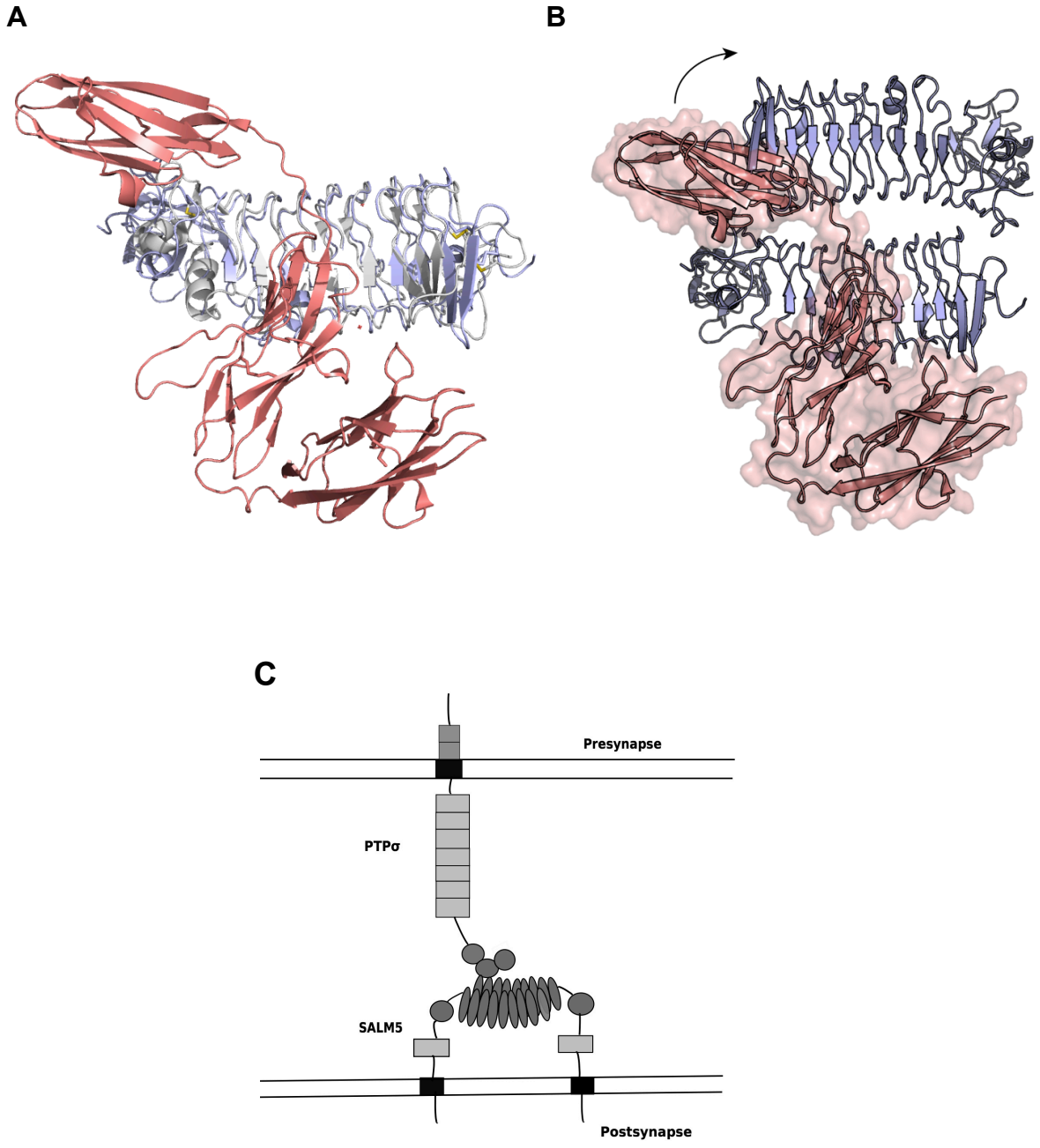


**Figure 7.**



## Supplementary Material

**Table S1.** Oligonucleotide pairs for used cloning the gene constructs.

| **Proteins Expression Constructs** | **Oligonucleotides for cloning** |
|---|---|
| **SALM1 LRR-Ig- Fn** $_{20-518}$<br>**SALM1 LRR-Ig-Fn** $_{20-378}$ | Forward: TATATAGAATTCTGCCCCAAGTACTGCGTC<br>Reverse: TATATGGTACCCTGCACAATGGAGACCTC<br>Reverse: TATATGGTACCGTCGGCCTTGGTGAAGAAC |
| **SALM3 LRR-Ig** $_{17-367}$<br>**SALM3 LRR-Ig-Fn** $_{17-502}$ | Forward: TTTTGAATTCTGCCCGCTACCCTGTGTGTG<br>Reverse:TTTTGGTACCCTGTAGGGCATGGCACAGGG<br>Reverse:TTTTGGTACCCAAGGCCCAGTACCCGGAGCTC |
| **SALM5 LRR-Ig** $_{18-376}$<br>**SALM5 LRR-Ig-Fn** $_{18-505}$ | Forward: ATATATGGATCCCCTCCGCTCATCACTC<br>Reverse ATATGGATCCTTTAATTATGTGAAGATCCACGG<br>Reverse: ATATGGATCCGCAACCCACGACTCTTGTG |
| **PTPσ Ig1-3** $_{33-327}$<br>**PTPσ Ig1-3 meB** $_{33-331}$ | Forward: TTTTGAATTCGAAGAACCACCCAGGTTTATC<br>Reverse: TTTTGGTACCTTTGGGGAGAGATTTTACAG |

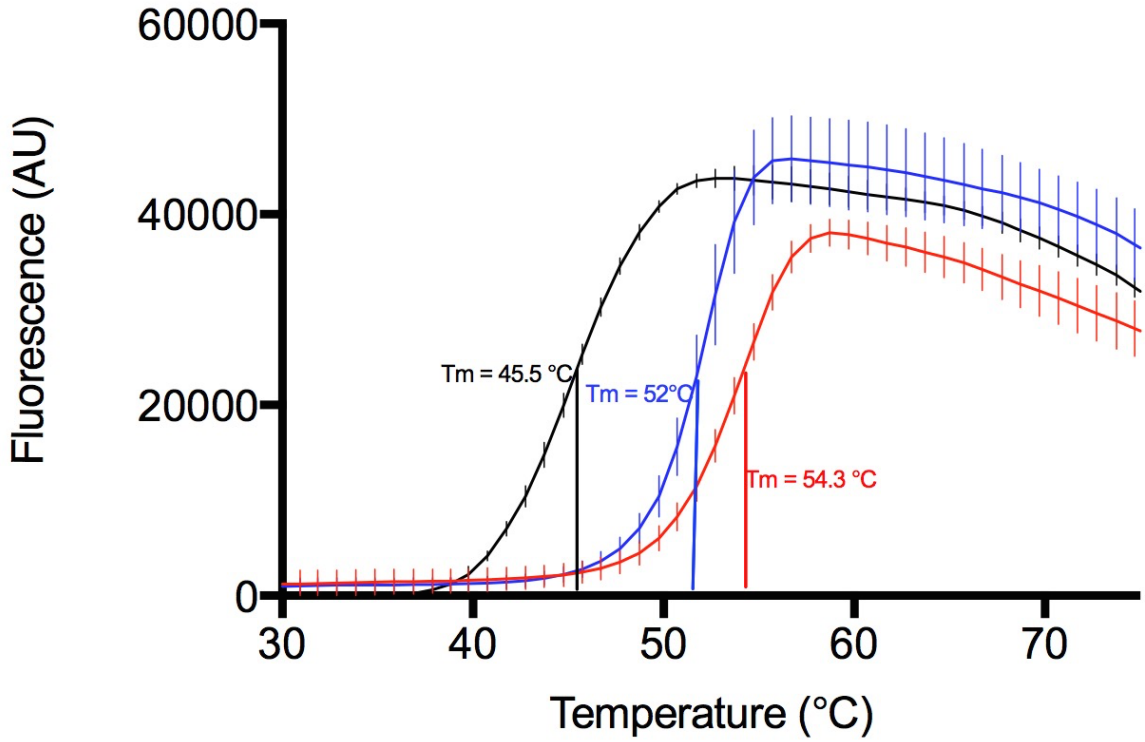



**Figure S1. Thermal stability and unfolding of SALMs proteins.** Unfolding curves from differential scanning fluorimetry for SALM1 (red line), SALM3 (black line) and SALM5 (blue line).