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(Article begins on next page)

1 **Structural characterization of the**
2 **third scavenger receptor cysteine-rich domain**
3 **of murine Neurotrypsin**

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25 **Abstract**

26 Neurotrypsin (NT) is a multi-domain serine-protease of the nervous system with only one
27 known substrate: the large proteoglycan Agrin. NT has seen to be involved in the
28 maintenance/turnover of neuromuscular junctions and in processes of synaptic plasticity in
29 the central nervous system. Roles which have been tied to its enzymatic activity, localized in
30 the C-terminal serine-protease (SP) domain. However the purpose of NT's remaining 3-4
31 scavenger receptor cysteine-rich (SRCR) domains is still unclear. We have determined the
32 crystal structure of the third SRCR domain of murine NT (mmNT-SRCR3), immediately
33 preceding the SP domain and performed a comparative structural analysis using homologous
34 SRCR structures. Our data and the elevated degree of structural conservation with
35 homologous domains highlight possible functional roles for NT SRCRs. Computational and
36 experimental analyses suggest the identification of a putative binding region for Ca²⁺ ions,
37 known to regulate NT enzymatic activity. Furthermore, sequence and structure comparisons
38 allow to single out regions of interest that, in future studies, might be implicated in Agrin
39 recognition/binding or in interactions with as of yet undiscovered NT partners.

40

41 **Keywords**

42 Scavenger receptor cysteine-rich domain; SRCR; Protease; Neurotrypsin; Neuromuscular
43 Junctions.

44

45

46 **Introduction**

47 Neurotrypsin (NT), also known as PRSS12 and motopsin, is a multi-domain extracellular
48 serine-protease of the nervous system first described in the late 90's (1; 2). Spanning 761
49 amino acids, the murine variant encompasses an N-terminal proline rich segment, a kringle
50 domain (Kr), three scavenger receptor cysteine rich (SRCR) domains and a C-terminal
51 serine-protease domain (SP) (1; 2). In comparison to the human homolog (875 a.a.), sharing
52 an overall sequence identity of 87%, the murine ortholog is shorter in virtue of an additional
53 N-terminal SRCR domain (3) (Figure 1 A, Supplementary Fig. 1).

54 Produced predominantly by neurons of the central and peripheral nervous systems, NT is
55 stocked in synaptic vesicles and released in an activity-dependant manner (4; 5). Upon
56 secretion, NT cleaves the large proteoglycan Agrin, its only known substrate, generating two
57 C-terminal fragments. This event is likely responsible for the roles played by NT in
58 neuromuscular junction maintenance/turnover and in neuronal plasticity (4-7). Such is the
59 importance of this cleavage event that de-regulation and/or inactivation result, respectively,
60 in Sarcopenia (muscle wasting disease of the elderly) (7) and non-syndromic mental
61 retardation (4).

62 While the significance of the catalytic domain has been touched upon, the role and relevance
63 of the accessory domains remain unclear. Nonetheless it would be reasonable to attribute
64 them with a role in protein-protein interaction and/or substrate recognition. Indeed, literature
65 reports two potential NT interactors, in addition to the substrate Agrin, identified through
66 yeast two-hybrid screening experiments: the product of seizure related gene 6 (Sez-6) (8) and
67 the Integral Membrane Protein 2A (Itm2a) (8). These have been seen to influence,
68 respectively, neuronal development and function (9; 10), and the differentiation of different
69 tissue/cell types (11-13), among which skeletal muscle (14). NT's interaction with Sez-6 has

70 been tied to one of the accessory domains: the Kr domain (8). Conversely, the domain(s)
71 responsible for interacting with Itm2a has yet to be identified.

72 The scavenger receptor cysteine-rich (SRCR) domain is a small (90-110 residues)
73 distinguishing element of a broad superfamily of proteins whose members span different
74 functional roles (15-17). It displays a highly conserved structure with five anti-parallel beta
75 strands cradling an alpha helix and a distinct disulfide bridge architecture (18; 20-23). A
76 feature which has been used to identify two types of SRCR domains, A and B, bearing
77 respectively 6 and 8 cysteines. While the number of cysteines differs, the relative pairing is
78 surprisingly consistent and tends to form as follows: C1-C4, C2-C7, C3-C8 and C5-C6. The
79 first pair distinguishes types A and B as it is present exclusively in the latter (16). Members
80 of the SRCR superfamily can share little functional similarity playing roles in immune
81 response (18), cell differentiation (24), apoptosis (25) and tumor suppression alike. While it
82 has been difficult to assign a univocal function to SRCR domains, a commonality can be
83 drawn across several SRCR superfamily members, broadly attributing the SRCR domain with
84 a role in protein-protein/protein-substrate interaction (26). It is therefore likely NT SRCR
85 domains may mediate interactions with the surrounding environment. They could be
86 responsible for the reported glycosaminoglycan and heparin binding capabilities (27),
87 contribute to its specificity, mediate its localized lingering at the synapse (19), contribute to
88 its specificity, mediate its localized lingering at the synapse (20) and/or be involved in
89 binding to, as of yet, unidentified partners. Of NT's accessory domains only the structure of
90 the Kr domain has been successfully determined and assigned a possible role (21; 8). The
91 lack of structural information pertaining to the remaining domains limits our functional
92 understanding of NT. As such new structural data on the SP or SRCR domains might provide
93 key insights on the mechanisms underlying NT's biological role.

94 Here we report the recombinant production, purification, crystallization and crystal structure
95 determination of the third SRCR domain of murine NT (mmNT-SRCR3), and analysis of its
96 molecular architecture based on comparisons with homologous SRCR domains. Our
97 structural data constitute a solid additional step towards the understanding of NT's molecular
98 interactions and biological functions.

99

100 **Results**

101 *Purification of mmNT-SRCR3*

102 Owing to the high cysteine content of extracellular SRCR domains, we produced the mmNT-
103 SRCR3 in soluble form using a *E. coli* strain particularly adapted to facilitate disulfide bond
104 formation. Purification of mmNT-SRCR3 was performed using a two-step immobilized Ni-
105 affinity chromatography (Ni-IMAC) followed by size-exclusion chromatography (SEC)
106 (Supplementary Fig. 2). The purified material was used for crystallization experiments and
107 protein characterization studies in solution.

108

109 *Crystal structure of mmNT-SRCR3*

110 A single mmNT-SRCR3 crystal allowed complete X-ray data collection and structure
111 determination (Figure 1 B); data processing and structure refinement statistics are reported in
112 table 1. This process highlighted the presence of two mmNT-SRCR3 monomers in the
113 asymmetric unit assembling into what appeared to be a crystallographic dimer (Figure 1 C).
114 Each monomer presents a very compact fold with a central alpha helix ($\alpha 1$) nested in five
115 beta strands ($\beta 1$ - $\beta 5$) forming a twisted anti-parallel cradle (Figure 1 B). The B-factor
116 distribution across the structure highlights the high stability of the central core and
117 progressive flexibility of the peripheral loops interconnecting the secondary structure
118 elements (Figure 1 C). Of particular interest is the Val466–Asn474 segment, whose flexibility

119 is such that the electron density map of that region is very poor despite the 1.7-Å resolution
120 (Supplementary Fig. 3).

121 Each mmNT-SRRCR3 model spans 107 residues and lacks the final C-terminal “KKASS”
122 sequence, owing to the high mobility of this five-residue tail. Superposition of the two
123 monomers found in the asymmetric unit and analysis of the root mean square deviation
124 (r.m.s.d) shows that the only visible significant difference is located in the aforementioned
125 Val466–Asn474 loop, further supporting its flexible nature (Figure 1 D). Calculations of
126 surface electrostatic potentials using CCP4mg (22) displayed a disc-like shape with mixed
127 charge distribution along its sides and two opposite faces, one almost fully hydrophobic and
128 the other displaying a patch of negative charges (Figure 1 E).

129

130 *SEC-SAXS analysis*

131 In order to assess the oligomeric state of the purified protein sample in solution, and thus
132 ascertain the nature of the mmNT-SRRCR3 dimer observed in the crystal packing (Figure 1 C),
133 we performed a SEC-small angle x-ray scattering (SEC-SAXS) experiment. The initial
134 chromatogram displayed a well-defined peak with good scattering intensities and a very
135 homogeneous mass distribution (Figure 2 A). mmNT-SRRCR3 presents as a monodisperse
136 species with an MW of 12 kDa calculated from averaged peak intensities (Table 2). This is in
137 line with the 12.5 kDa calculated for a monomer of this construct and incompatible with
138 higher order oligomeric species. *CRY SOL* (23) was used to compare the averaged scattering
139 curve of the SEC-SAXS peak to a theoretically calculated scattering curve for our
140 crystallographic structure. The single mmNT-SRRCR3 monomer displayed a very good fit (χ^2
141 = 1.4) with the in-solution data (Figure 2 B), ruling the dimer as induced by the
142 crystallization process.

143

144 *Comparison with other SRCR domains*

145 To better understand the functional role of NT's SRCR domains, we used the *DALI* server
146 (24) to compare this structure with other entries in the Protein Data Bank (PDB). This search
147 returned several high scoring matches which were manually filtered to remove redundancies
148 resulting in a list of only 8 hits (Table 3). While limited in sequence identity, structural
149 superpositions with mmNT-SRCR3 (Figure 3 A) evidenced a high degree of structural
150 conservation, strongest within the central core and with greater variability in the peripheral
151 loops. Comparison with an expanded pool of homologs using *ConSurf* (25) highlighted a
152 similar trend for sequence conservation. When mapped to the structure of mmNT-SRCR3, it
153 was possible to see how the areas of greater sequence identity, for the most part,
154 corresponded to highly conserved secondary structure elements forming the domain core
155 (Figure 3 B). Conversely the more flexible external loops displayed a significantly greater
156 compositional variability. Of particular note is the β 4 strand that, while being one of five core
157 β strands displays little to no amino acid conservation (Figure 3 C).

158 *DALI* matches with > 30% identity evidenced several perfectly conserved residues which
159 were mirrored in ConSurf alignments. Among these is a six-cysteine network (residues 411,
160 424, 455, 465, 475, and 485) responsible for the archetypical SRCR domain disulfide bridges
161 (Figure 3 B). This canonical pairing is expressed relative to type B SRCR domains and while
162 type A domains lack the C1-C4 pair, the relative numbering of the other pairs remains
163 conventionally and structurally unaltered. Therefore, in mmNT-SRCR3 we found that
164 Cys411-Cys475 corresponds to the C2-C7 pair, while Cys424-Cys485 and Cys455-Cys465
165 correspond to the C3-C8 and C5-C6 pairs, respectively. Residues Gly391, Gly397, Glu400,
166 Ala420, Val422, Leu427, Gly457, Glu459 and Val483 are also fully conserved (Figure 3 C).
167 Of these, Ala420, Val422 and Leu427 can be mapped to the central helix while Gly397,
168 Glu400 and Val483 can be found on strands β 2 and β 5, respectively (Figure 3 B).

169 Conversely, the remaining Gly391, Gly457 and Glu459 locate on the more flexible, and
170 generally less conserved, external loops (Figure 3 B).

171 Comparison of mmNT-SRCR3 with other mmNT SRCR domains evidenced a certain extent
172 of conservation (44-58% sequence identity) comparable to that of the highest *DALI* matches
173 (Supplementary Fig. 1 B). Extending this analysis to include human NT evidenced how its
174 SRCR domains 2-4 correspond, respectively, to mmNT SRCR domains 1-3, while the first
175 human SRCR domain is not conserved across the two homologs (Supplementary Fig. 1B).

176

177 *Evidence for Ca²⁺ binding*

178 Given the significant contributions of Ca²⁺ to NT activity (5), and the reported Ca²⁺-based
179 modulation of SRCR-mediated interactions (18; 26), we investigated whether mmNT-SRCR3
180 might contribute in similar fashion. Therefore, an Isothermal Titration Calorimetry (ITC)
181 experiment was conducted in presence of CaCl₂. This evidenced a Ca²⁺ binding to mmNT-
182 SRCR3 (Figure 4 A), with $K_d = 10.1$ mM, $\Delta H = -7.7$ Kcal/mol, $\Delta S = -16.8$ cal/mol/deg.

183 A comparison of mmNT-SRCR3 with the structure of the monomeric MARCO SRCR
184 domain (18) highlighted a cluster of highly conserved residues coordinating a bivalent metal-
185 ion in MARCO (Figure 4 B). These correspond to amino acids Asp412-Asp413, His473,
186 Asn474 and Glu479, that significantly contribute mmNT-SRCR3's negatively charged patch.
187 Interestingly the same residues were seen to be perfectly conserved in the DMBT1 SRCR-1
188 domain, known as well to display Ca²⁺ binding (27) (Figure 4 C).

189

190 **Discussion**

191 The scavenger receptor cysteine-rich domain groups numerous proteins with different
192 functions in a large superfamily (15). However, the understanding of the specific function of
193 SRCR domains is, to date, limited to a general consensus of protein-protein/protein-ligand

194 interaction. The solution of the structure of the third SRCR domain of murine Neurotrypsin
195 (mmNT-SRCR3) and its analysis highlighted several interesting features that might provide a
196 functional insight both in regards to NT and SRCR domains in general. Most striking of all
197 was the degree of similarity with the available homologous structures despite a generally
198 poor sequence conservation (Table 3).

199 At the core of mmNT-SRCR3 is an extremely stable secondary structure element
200 organization which seems to be perfectly conserved across available structures. Such
201 variability could be traced to the more flexible external loops, most notably the Val466-
202 Asn474 loop. This region not only displayed the highest mobility across homologs but also
203 within the mmNT-SRCR3 monomers observed in the crystallographic dimer. Mapping of
204 sequence conservation to the structure of mmNT-SRCR3 showed that the least conserved
205 residues were located on this and other peripheral flexible loops, while the core secondary
206 structure elements bore the majority of highly conserved amino acids. Of particular note are:
207 a six-cysteine network responsible for the typical disulfide bond architecture of SRCR
208 domains, and several perfectly conserved residues (Gly397, Glu400, Ala420, Val422,
209 Leu427, and Val483) mapped to the highly stable core. These, owing to their localization and
210 conservation, are likely to provide essential contributions to the fold of SRCR domains.
211 Conversely, three additional highly conserved residues (Gly391, Gly457 and Glu459),
212 mapped to the more flexible loop regions, and the β 4 secondary structure element, which
213 displays structural but not compositional conservation, might contribute to SRCR domain
214 function.

215 Among the more conserved regions two stretches, spanning Gly397–Val401 and Trp407–
216 Asp412 respectively, should be mentioned owing to their documented evolutionary
217 conservation (28). The first likely contributes functionally, as similar consensus sequences
218 were seen to mediate protein-target interaction in other SRCR-SF members including

219 MARCO (29), DMBT1 (35), CD163 (30), as well as structurally, given its mapping to the
220 highly conserved β 1 secondary structure element. Conversely, the second has yet to be
221 assigned with a functional role but it is thought that the conservation of this stretch is
222 structurally related.

223 Owing to structural conservation it is possible to suppose a protein-target role for mmNT-
224 SRCR3. Additional observations drawn from that structure and other SRCR-SF members
225 might hint at its more specific function in relation to NT. Notably, analysis of surface charge
226 distribution of mmNT-SRCR3 evidenced a negatively charged patch corresponding to a
227 cluster of residues (Asp412, Asp413, His473, Asn474 and Glu479), conserved in the metal-
228 ion binding MARCO SRCR domain (18), that might be involved in Ca^{2+} ion binding known
229 to be important for NT enzymatic activity (31). A plausible hypothesis, given that ITC
230 experiments showed Ca^{2+} binding for mmNT-SRCR3 and that other SRCR-SF members
231 (MARCO, DMBT1 and CD163) also display a Ca^{2+} dependant modulation mapped to their
232 SRCR domains (32; 30; 18). Further analogies can be speculated regarding the nature of
233 possible binding partners. As several SRCR-SF members are known to bind to bacteria (32;
234 27), lipopolysaccharides (LPS) directly (29; 33) or glycoproteins (34) via their SRCR domains.
235 Thus it is possible that NT SRCR domains might mediate similar interactions between NT
236 and its heavily glycosylated substrate Agrin (19).

237 Finally, cross-comparison of human and murine NT evidenced how the first SRCR domain in
238 the human NT sequence represents the principal source of divergence between the two
239 homologs. Surprisingly, also the residues putatively coordinating Ca^{2+} in mmNT-SRCR3 are
240 well conserved across all NT SRCR domains, except for human NT-SRCR1 (Supplementary
241 Fig. 4). An observation which hints at a possible lack of Ca^{2+} binding for that SRCR domain
242 and that, in conjunction with its absence in murine NT, opens to speculation regarding the
243 function and relevance of the human NT SRCR1.

244 In conclusion, it seems plausible that the broad protein-target interaction attributed to SRCR
245 domains might be mediated by the highly conserved nature of their core structure. While the
246 more structurally and compositionally variable regions could be responsible for more
247 nuanced and specific functional aspects such as ligand recognition. Finally in regards to NT,
248 our characterization of mmNT-SRCR3 allowed us to identify a Ca^{2+} binding site and infer, by
249 comparison, a possible role for this domain in Agrin recognition.

250

251 **Materials and methods**

252 *Molecular cloning, recombinant expression and purification*

253 The cDNA encoding for the third SRCR domain of murine Neurotrypsin (UniProt id O08762,
254 residues 383-494) was obtained from Source Bioscience (I.M.A.G.E. clone ID 3665834),
255 amplified with a Phusion DNA polymerase (Thermo Fisher Scientific) using oligos
256 AAAGATCTGGTTTTCCCATCAGACTAGTGGATG (forward) and
257 AAGCGGCCGCACTTGATGCTTTCTTCTCTAAATAG (reverse), and inserted into the
258 pCIOX recombinant expression vector (Addgene), yielding the final construct bearing an N-
259 terminal 8-His-SUMO tag. This plasmid was transformed into chemically competent
260 *Escherichia coli* SHuffle T7 cells (New England Biolabs), which were used for protein
261 production.

262 A small culture of transformed cells was grown over night shaking at 30 °C in LB +
263 kanamycin (50 µg/ml) and used to inoculate, in a 1:100 ratio, a larger volume (1 - 6 L) of
264 autoinducing ZYP-5052 media (35) for large-scale production. Inoculated media was
265 incubated shaking at 30 °C for 5 h, after which the temperature was lowered to 20 °C to
266 induce recombinant protein expression, and further incubated for 20 h.

267 Cells were harvested by centrifugation (5000 x g, 15 min, 4 °C) with a swinging bucket
268 centrifuge (Beckman Coulter). The supernatant was discarded and the cells were resuspended
269 in buffer A (25 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
270 (HEPES)/NaOH, 0.5 M NaCl, pH 8) in a 1:5 (w/v) wet cell pellet-to-buffer ratio. This
271 suspension was placed on ice for 30 minutes and then sonicated (80% amplitude, 8 cycles
272 with 40 s on/20 s off pulses) to lyse the cells. Cell debris was removed by centrifugation
273 (10'000 x g, 50 min, 4 °C) and the supernatant was filtered with a 0.45 µm syringe filter
274 (Sartorius). The clarified lysate was loaded on a 5 mL HisTrap (GE Healthcare) column, pre-
275 conditioned with buffer A, at a rate of 1 mL/min. Unbound material was washed from the

276 column with buffer A and non-specifically bound contaminants were removed with buffer A
277 supplemented with 25 mM imidazole. mmNT-SRCR3 was eluted by further increasing the
278 imidazole concentration to 250 mM. The eluted fractions were pooled, supplemented with
279 SUMO protease (1.2 mg/mL stock, 1:300 v/v) and dialyzed overnight at 4 °C against buffer
280 A. Following dialysis the sample was subject to centrifugation (5000 x g, 4 °C, 15 min) and
281 the supernatant was loaded onto a 1 mL HisTrap column (GE Healthcare). Successful
282 removal of the His-SUMO tag was evaluated through sample recovery in the flow through
283 fractions, which were further purified by gel filtration on a Superdex 75 10/300 GL column
284 (GE Healthcare) equilibrated with 25 mM HEPES/NaOH, 100 mM NaCl, pH 8. At each step
285 of protein purification, samples were collected and analysed using SDS-PAGE. The final
286 yield was \approx 2 mg of pure protein per gram of bacterial cells.

287

288 *Crystallization, X-ray Data collection and processing*

289 For crystallization screening the protein was concentrated to 20 mg/mL with a 5 kDa MWCO
290 Vivaspin12 concentrator (Sartorius). Crystallization was performed using the sitting-drop
291 vapor diffusion method at both 4 and 20 °C. Drops were set up in a volumetric 1:1 protein-to-
292 precipitant solution ratio using an Oryx8 crystallization robot (Douglas Instruments) with
293 MRC 96-well PS plates (SwissSci). Initial screening performed with commercial screens
294 yielded thin microcrystals, not suitable for diffraction testing, in numerous conditions.
295 Optimization of initial crystallisation hits was performed by hand using the sitting drop
296 method. The best crystals were obtained at 4 °C, over the span of a month, by mixing
297 mmNT-SRCR3 concentrated at 19 mg/mL with a solution composed of 16% PEG3350, 0.1
298 M Tri-Sodium citrate, pH 7.5. Prior to flash-cooling in liquid nitrogen, crystals were
299 harvested with nylon cryoloops (Hampton Research) and briefly soaked into a cryo-
300 protectant solution (19% PEG3350, 0.1 M Tri-Sodium citrate, 20% Glycerol). X-ray

301 diffraction data were collected at 100 K at the ID30A-3 beamline of the ESRF synchrotron.
302 Data were indexed and integrated using *XDS* (36) and scaled using *Aimless* (37). Data
303 collection statistics are summarized in Table 1.

304

305 *Structure determination and refinement*

306 The structure of mmNT-SRCR3 was solved at 1.7 Å by molecular replacement using the
307 structure of the Mac-2 binding protein (M2BP) SRCR domain (pdb: 1BY2) (38) as search
308 model. This model (50% sequence identity to mmNT-SRCR3) was selected based on
309 conservation of sequence identity as evaluated using *NCBI BLAST* (39). Prior to molecular
310 replacement, the sequences of mmNT-SRCR3 and M2BP SRCR were aligned using *EBI*
311 *MUSCLE* (40) and non-conserved residues were adjusted using *CHAINSAW* (41). The
312 resulting model was used in molecular replacement with *PHASER* (42). Two copies of the
313 search model were found constituting the asymmetric unit, with a V_m of 3.11 Å³ and 60%
314 solvent content. The structure was refined with successive steps of manual building in *COOT*
315 (43) and automated refinement with *REFMAC5* (44). Model validation was performed with
316 *MolProbity* (45). Refinement statistics for the final model are reported in Table 1 as
317 deposited to the PDB under accession code 6H8M. Electrostatic surface calculations and
318 representations were generated with *CCP4mg* (22). Other structural images were generated
319 with *UCSF Chimera* (46).

320

321 *SEC-SAXS analysis*

322 Size exclusion chromatography coupled to small-angle X-ray scattering (SEC-SAXS)
323 analysis was performed at the BM29 beamline of the ESRF synchrotron in Grenoble (France)
324 using a protocol adapted from (47). The protein was concentrated to 15 mg/mL and a 15 µL
325 sample was run on a Superdex 75 Increase 3.2/300 column (GE Healthcare) equilibrated in

326 25 mM HEPES/NaOH, 0.1 M NaCl, pH 8 and mounted on a Nexera High Pressure
327 Liquid/Chromatography (HPLC; Shimadzu) system. SAXS data were collected from the
328 sample capillary mounted on line with the HPLC system, using a Pilatus 1 M detector
329 (Dectris) positioned at distance of 2.87 m allowing a global q range of 0.03–4.5 nm with 12.5
330 keV energy. Analysis of scattering intensities was performed using *CHROMIXS* (48) and the
331 *ATSAS* suite (49). Comparison of in-solution scattering to crystallographic data was carried
332 out with CRY SOL (23). Details of SEC-SAXS data collection and analysis are summarized
333 in Table 2 as deposited in SASBDB under accession code SASDES5.

334

335 *Isothermal Titration Calorimetry*

336 Titrations were carried out at 25°C in 100 mM NaCl 25 mM HEPES/NaOH pH8 using the
337 high feedback mode of a MicroCal ITC200 instrument (Malvern Instruments, Worcestershire,
338 UK). The concentration of mmNT-SRRCR3 in the cell was 1.2 mM, whereas the CaCl₂
339 solution in the syringe was at 120 mM. The first injection was kept to the minimum volume
340 of 0.1 uL to allow complete equilibration of the cell. The following 19 titrations of CaCl₂
341 were performed using a 2 uL injection volume with a 120 sec. time interval while
342 maintaining stirring at 750 rpm. The heat of dilution of 120 mM CaCl₂ was determined by
343 performing a second titration in which the cell was filled only with buffer while all other
344 experimental parameters remained unaltered. The net contribution of binding of CaCl₂ to
345 mmNT-SRRCR3 was calculated by subtracting the heat of dilution of CaCl₂. Data were
346 analyzed using the “One Set of Sites” curve fitting model (MicroCal ITC200 Origin).

347

348 **Supplementary Material**

349 Supplementary figures and tables are available and linked through the text.

350

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359 University of Pavia.

360

361 **Conflict of interest statement**

362 The authors declare no competing financial interests.

363

364 **Author Contributions**

365 FF conceived and supervised the project. AC cloned, expressed, purified, crystallized and
366 solved the structure of mmNT-SRCR3. AC and FF analyzed crystallographic and SEC-SAXS
367 data. GC performed ITC measurements and data processing. AC prepared the figures. AC
368 and FF wrote the paper. All authors read and approved the final manuscript.

369

370 **Table 1. X-ray data collection and refinement statistics**

Data Collection	
Diffraction source	ESRF ID30A-3
Wavelength (Å)	0.983
Space group	P 2 ₁ 2 ₁ 2 ₁
Cell parameters <i>a</i> , <i>b</i> , <i>c</i> (Å)	<i>a</i> = 57.8 Å, <i>b</i> = 62.9 Å, <i>c</i> = 84.5 Å, $\alpha = \beta = \gamma = 90^\circ$
Resolution range (Å) ^{1,2}	42.54 - 1.70 (1.73 - 1.70)
Unique reflections	34576 (1785)
Completeness (%)	100.0 (100.0)
Multiplicity	6.9 (6.9)
Mean (<i>I</i> /σ(<i>I</i>))	15.5 (0.5)
CC(1/2)	0.999 (0.434)
<i>R</i> _{sym} ³	0.049 (3.37)
Refinement	
<i>R</i> _{work} / <i>R</i> _{free}	0.19 / 0.21
No. of non-H atoms	1747
Protein	1662
Ligands	0
Waters	85
r.m.s. deviations	
Bonds (Å)	0.016
Angles (°)	1.699
Average <i>B</i> factors (Å ²)	
Protein	65.3
Water	54.0
Ramachandran favoured (%)	97.6
Ramachandran allowed (%)	2.4

371

372 ¹ Values for the outer shell are given in parentheses.373 ² Resolution limits were determined by applying a cut-off based on the mean intensity correlation coefficient of
374 half-datasets (CC1/2) approximately of 0.5 (50).375 ³ $R_{sym} = \Sigma |I - \langle I \rangle| / \Sigma I$, where *I* is the observed intensity for a reflection and $\langle I \rangle$ is the average intensity
376 obtained from multiple observations of symmetry-related reflections.

377

378 **Table 2. Summary of SEC-SAXS data collection and analysis**

<i>Data Collection</i>	
Light source and beamline	ESRF BM29
Beam energy (keV)	12.5
Sample-detector distance (m)	2.867
Exposure time (s)	1
Sample cell thickness (mm)	1
Temperature (°)	20
Final q range (nm^{-1})	0.01 - 4
<i>Data Analysis</i>	
Points used for Guinier analysis	27-173
Guinier qR_g limits	0.24
Guinier R_g (nm)	1.54 ± 0.03
$I(0)$ (nm^{-1})	13.48 ± 0.02
Dmax (nm)	5.8
MW estimation (V_c based) (kDa)	12

379

380

381 **Table 3** List of homologous SRCR structures identified by *DALI*.

<i>Protein</i>	<i>PDB</i>	<i>Z-Score</i>	<i>r.m.s.d.</i>	<i>% id.</i>
Lysyl oxidase homolog 2	5ZE3	18.2	2.1	49
CD6	5A2E	18.0	1.5	49
Mac-2 binding protein (M2BP)	1BY2	18.0	1.8	50
Scavenger receptor cysteine-rich type 1 protein m	5JFB	16.6	1.8	38
Macrophage receptor (MARCO)	2OYA	13.7	1.7	52
Human complement factor I	2XRC	11.2	2.2	31
T-cell surface glycoprotein CD5	2JA4	7.5	2.4	27
Serine protease Hepsin	3T2N	7.2	3.0	16

382

383 **Figure Legends:**

384 *Figure 1* - Three-dimensional structure of the third SRCR domain of murine NT. (A) domain
385 organization of mouse and human NT, highlighting the localization of mmNT-SRCR3 within
386 the multidomain enzyme architecture. Colouring of the domains highlights sequence
387 conservation between homologous enzymes, as described in Supplementary Fig. 1. (B)
388 Cartoon representation of mmNT-SRCR3 displaying its main secondary structure elements,
389 α -helix ($\alpha 1$) in blue and β -strands ($\beta 1 - \beta 5$) in cyan. (C) Cartoon “putty” representation of
390 the two mmNT-SRCR3 monomers found in the crystallographic asymmetric unit, coloured
391 blue-red by B-factors ($34.2 - 162.5 \text{ \AA}^2$). (D) Superposition of the two mmNT-SRCR3
392 molecules found in the asymmetric unit. chain A is shown in red, chain B is shown in cyan.
393 (E) Surface representation with colouring by electrostatic potential ($-2.0 - +2.0 \text{ V}$), negative
394 charges in red, positive charges in blue and neutral areas in white. Monomer is presented
395 orientations highlighting main features: uncharged bottom, mixed charges on sides and
396 negatively charged patch on top.

397
398 *Figure 2* – mmNT-SRCR3 SEC-SAXS analysis. (A) SEC-SAXS chromatogram showing
399 mmNT-SRCR3 scattering intensities and profile of mass distribution across the peak. (B)
400 Averaged experimental peak scattering curve, after buffer subtraction, plotted superimposed
401 to a theoretical, *CRY SOL*-derived, scattering curve for an mmNT-SRCR3 monomer ($\chi^2 =$
402 1.4).

403
404 *Figure 3* - Structural comparison of mmNT-SRCR3 with other SRCR domains. (A)
405 Superposition with non-redundant *DALI* matches (Table 3) coloured red-yellow by r.m.s.d.
406 ($0.39 - 6.32 \text{ \AA}$). (B) Cartoon representation of mmNT-SRCR3 in two orientations, coloured
407 cyan-brown ($0 - 100\%$) by residue conservation after *ConSurf* analysis. The left panel maps

408 highly conserved residues from SRCR structure sequence alignments, while the right panel
409 maps the principal secondary structure elements and the archetypical SRCR disulfide bridge
410 organization. The C1-C4 pair is absent in SRCR type A domains, residues Cys411-Cys475
411 correspond to the C2-C7 pair, Cys424-Cys485 to the C3-C8 pair and Cys455-Cys465 to the
412 C5-C6 pair. (C) Sequence alignment of SRCR structures identified by *DALI* analysis of
413 mmNT-SRCR3. Identical residues boxed in red, residues with 70% conservation boxed in
414 yellow. mmNT-SRCR3 secondary structure elements displayed above alignment and
415 consensus sequence with 70% conservation under alignment. Image created using *ESPRIT3*
416 (51).

417

418 *Figure 4* – Evidence for mmNT-SRCR3 Ca²⁺ binding. (A) Isothermal calorimetric titration of
419 CaCl₂ (120 mM) with purified mmNT-SRCR3. Heat variation generated by each injection of
420 titrant at each time interval (top panel) and the integration of each peak and the amount of
421 heat produced (lower panel). (B) Structural inference of mmNT-SRCR3 binding of metal
422 ions. Top panel, residues contributing to the formation of the negatively charged patch on
423 mmNT-SRCR3 likely bind metal ions. Bottom panel, superposition of mmNT-SRCR3 (blue)
424 with monomeric MARCO (cyan, PDB: 2OY3) coordinating Mg²⁺ (green sphere) via those
425 same amino acids. Side chains of residues Asp412, Asp413 and Glu479 display almost
426 identical conformations, while Asn474 and His473 show higher flexibility. (C) Sequence
427 alignment of mmNT-SRCR3 against MARCO and DMBT1 SRCR domains known to bind
428 Ca²⁺. Blue arrows and boxes indicate residues evidenced in (B) which are perfectly conserved
429 across all three proteins. Perfectly conserved residues boxed in red, residues with 70%
430 conservation are boxed in yellow. Evaluation of sequence conservation is shown under
431 alignment; symbols indicate: ! = Ile or Val, \$ = Leu or Met, % = Phe or Tyr, # = Asn, Asp,
432 Glu or Gln. Residue numbering refers to mmNT-SRCR3.

433

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