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## Divalent cations influence the dimerization mode of murine S100A9 protein by modulating its disulfide bond pattern

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1 **Divalent cations influence the dimerization mode of murine S100A9 protein by**  
2 **modulating its disulfide bond pattern**

3  
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16

17 **Highlights**

18 The crystallographic structure of mS100A9 bound to calcium and zinc is reported

19 A novel Zn-binding site and a disulfide bridge rigidify mS100A9 C-terminus

20 In solution, mS100A9 exists both as non-covalent and disulfide-crosslinked homodimers

21 Divalent cations modulate the relative proportion of the different mS100A9 homodimers

22 **Abstract**

23 S100A9, with its congener S100A8, belongs to the S100 family of calcium-binding proteins found  
24 exclusively in vertebrates. These two proteins are major constituents of neutrophils. In response to  
25 a pathological condition, they can be released extracellularly and become alarmins that induce both  
26 pro- and anti-inflammatory signals, through specific cell surface receptors. They also act as  
27 antimicrobial agents, mainly as a S100A8/A9 heterocomplex, through metal sequestration. The  
28 mechanisms whereby divalent cations modulate the extracellular functions of S100A8 and S100A9  
29 are still unclear. Importantly, it has been proposed that these ions may affect both the ternary and  
30 quaternary structure of these proteins, thereby influencing their physiological properties. In the  
31 present study, we report the crystal structures of WT and C80A murine S100A9 (mS100A9),  
32 determined at 1.45 and 2.35 Å resolution, respectively, in the presence of calcium and zinc. These  
33 structures reveal a canonical homodimeric form for the protein. They also unravel an  
34 intramolecular disulfide bridge that stabilizes the C-terminal tail in a rigid conformation, thus  
35 shaping a second Zn-binding site per S100A9 protomer. In solution, mS100A9 apparently binds  
36 only two zinc ions per homodimer, with an affinity in the micromolar range, and aggregates in the  
37 presence of excess zinc. Using mass spectrometry, we demonstrate that mS100A9 can form both  
38 non-covalent and covalent homodimers with distinct disulfide bond patterns. Interestingly,  
39 calcium and zinc seem to affect differentially the relative proportion of these forms. We discuss  
40 how the metal-dependent interconversion between mS100A9 homodimers may explain the  
41 versatility of physiological functions attributed to the protein.

42

43 **Keywords**

44 S100 proteins; divalent cations; disulfide bridges; X-ray crystallography; mass spectrometry.

45 **Abbreviations**

46 alk: alkylation; ACN: acetonitrile; DTT: dithiothreitol; HPLC: high performance liquid  
47 chromatography; IAA: iodoacetamide; hS100A9: human S100A9; ICP-MS: Inductively-coupled  
48 plasma mass spectrometry; LC/ESI-TOF-MS: liquid chromatography/electrospray ionization  
49 time-of-flight mass spectrometry; MPD: 2-Methyl-2,4-pentanediol; MR: molecular replacement;  
50 mS100A9: murine S100A9; RAGE: receptor for advanced glycation end-products; r.m.s.d.: root  
51 mean square deviation; SEC: size exclusion chromatography; TFA: trifluoroacetic acid; TLR:  
52 Toll-like receptor; WT: wild-type.

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## 66 **1. Introduction**

67 S100 proteins are a group of small  $\text{Ca}^{2+}$ -binding proteins expressed exclusively in vertebrates  
68 (Marenholz et al., 2004; Donato et al., 2013; Gonzalez et al., 2020). Several members of this family  
69 also exist extracellularly where they act as alarmins and thereby play a crucial role in the  
70 modulation of inflammatory responses in various disease contexts, through their interactions with  
71 cell surface receptors such as the receptor for advanced glycation end-products (RAGE), Toll-like  
72 receptors (TLRs) or various cluster of differentiation molecules (Leclerc et al., 2011; Roh et al.,  
73 2018; Gonzalez et al., 2020).

74 S100A9, also known as calgranulin B or migration inhibitory factor-related protein 14 (MRP14), is  
75 one of the most well-studied members among this large family of proteins encoded by up to thirty  
76 genes (Kraemer et al., 2008; Gonzalez et al., 2020). *In vivo*, S100A9 is mostly present as a  
77 heterodimer with its congener S100A8 (aka calgranulin A or MRP8) (Longbottom et al., 1992). The  
78 extracellular form of this heterodimer is known as calprotectin. Homodimers of both proteins are  
79 also detected to some extent, but they seem to be much less stable than the heterodimeric form  
80 (Hunter et al., 1998; Kallberg et al., 2018; Wang et al., 2018b; Giudice et al., 2019). S100A8 and  
81 S100A9 are highly expressed in circulating neutrophils, as well as in monocytes (Lagasse et al.,  
82 1992; Striz et al., 2004). They can constitute up to 45% of the total cytosolic pool of proteins in these  
83 cells, being found either in the cytoplasm or associated with the plasma membrane (Striz et al., 2004;  
84 Wang et al., 2018b). Intracellularly, S100A8/A9 act as  $\text{Ca}^{2+}$ -sensors. In response to an increase in  
85 calcium levels, they regulate various processes including cytoskeleton rearrangement, cell migration,  
86 phagocytosis, exocytosis, and respiratory burst, the latter through the delivery of arachidonic acid to  
87 the membrane-bound NADPH oxidase (Goebeler et al., 1995; Kerkhoff et al., 2005; Wang et al.,  
88 2018b). In a pathological context, S100A8/A9 can be released in the extracellular space by

89 infiltrating leukocytes or following cell necrosis. Once these S100 proteins are outside the cells, their  
90 functions change dramatically: they mainly act as damage-associated molecular patterns (DAMPs),  
91 promoting leukocyte recruitment through chemotaxis and massive production of pro-inflammatory  
92 cytokines via their interactions with specific cell surface receptors. These processes participate in the  
93 maintenance of a high inflammatory state which aggravates the underlying pathology that led to  
94 S100 protein release (Wang et al., 2018b; Gonzalez et al., 2020). S100A8 and S100A9 have been  
95 shown to bind to both RAGE, TLR4, CD147, and neuropilin (Sakaguchi et al., 2016; Ma et al.,  
96 2017). Many other yet unraveled receptors may also serve as transducers of the pro-inflammatory  
97 signals elicited by the extracellular heterodimer and/or homodimers (Tomonobu et al., 2020).  
98 S100A8/A9 signaling has been linked to a plethora of inflammatory or inflammation-driven  
99 pathologies, including rheumatoid arthritis, myocardial infarction, Alzheimer's disease or cancers  
100 (Shabani et al., 2018; Wang et al., 2018a; Marinkovic et al., 2019; Wang et al., 2019). These two  
101 S100 proteins are therefore considered as valuable therapeutic targets (Bjork et al., 2009; Reeb et al.,  
102 2015; Pruenster et al., 2016). They are also utilized as biomarkers for the diagnostic of certain  
103 pathologies such as inflammatory bowel diseases, due to their increased plasma levels in an  
104 inflammatory context (Chaabouni et al., 2016).

105 S100 proteins fold into a four-helix globular domain that is sometimes followed by a C-terminal,  
106 unstructured extension of variable length (Donato et al., 2013). The minimal functional unit is a  
107 homo- or heterodimer, but higher order oligomers are also encountered (Leukert et al., 2006). As for  
108 other S100 proteins, S100A9 active conformation requires the presence of two  $\text{Ca}^{2+}$  ions, one in each  
109 EF-hand motif, for proper effector binding (Vogl et al., 2006). In addition to calcium, both human  
110 and murine S100A9 were shown to bind zinc (Raftery et al., 1996). Many S100 proteins possess two  
111 symmetrical  $\text{Zn}^{2+}$  binding motifs located at the interface between the two subunits of the S100

112 homodimer (Brodersen et al., 1999; Moroz et al., 2009a; Lin et al., 2016). These tetrahedral binding  
113 sites are formed by two histidine residues (or possibly an aspartic acid replacing the second His)  
114 surrounding the first EF-hand motif of one protomer, and two histidine residues (or equivalent  
115 positively charged residues) placed at the C-terminal end of the fourth helix in the second protomer.  
116 S100 proteins can also chelate other divalent cations such as manganese, iron, nickel or copper  
117 (Nakashige et al., 2015; Zackular et al., 2015; Gilston et al., 2016; Nakashige et al., 2017). Chelation  
118 of  $Mn^{2+}$ ,  $Ni^{2+}$  and  $Fe^{2+}$  by S100A8/A9 is part of a sequestration process known as nutritional  
119 immunity that aims at depriving invading pathogens from these essential nutrients (Damo et al.,  
120 2013; Nakashige et al., 2015; Nakashige et al., 2017). Thanks to the patch of histidine residues  
121 located in the long, flexible C-terminal tail of its S100A9 moiety, the S100A8/A9 heterodimer can  
122 generate an octahedral  $His_6$  binding motif for  $Mn^{2+}/Ni^{2+}$  coordination, thereby increasing the affinity  
123 of the protein for these metal ions (Damo et al., 2013; Nakashige et al., 2018). Therefore,  
124 S100A8/A9 is considered as a potent antimicrobial peptide (Corbin et al., 2008). Other S100 proteins  
125 have been shown to possess bactericidal properties (Glaser et al., 2005; Realegeno et al., 2016), but  
126 it is not known whether S100A9 also displays such ability to kill bacteria outside the S100A8/A9  
127 heterodimer (i.e. as a homodimer).

128 Metal ion chelation by S100 proteins may however not be restricted to an antimicrobial activity.  
129 Indeed, divalent cations other than calcium may act as modulators of the biological function of S100  
130 proteins, in particular zinc (Baudier et al., 1986; Nakatani et al., 2005; Moroz et al., 2011). Various  
131 processes may induce modifications in the structural organization of S100 proteins, including ion  
132 binding, which promotes protein oligomerization, as well as post-translational modifications and/or  
133 oxidation-dependent mechanisms that may lead for example to the formation of disulfide-  
134 crosslinked S100 oligomers having distinct functions than the non-covalent assemblies

135 (Winningham-Major et al., 1989; Haase-Kohn et al., 2011; Lim et al., 2011; Unno et al., 2011;  
136 Yatime, 2017). We and others have proposed that these effects may be the direct translation of a  
137 change in S100 proteins tertiary and/or quaternary structure (Moroz et al., 2009b; Yatime et al.,  
138 2016). Although these ideas are generally more and more well perceived by the scientific community  
139 working in the field, these forms have so far not been characterized in details. To date, only one  
140 structural study reports the finding of a novel homodimeric form of S100 proteins, observed for  
141 S100A6 in its complex with RAGE and stabilized by zinc and cysteine-dependent mechanisms  
142 (Yatime et al., 2016). In order to understand how these proteins can have many diverse and  
143 sometimes contradictory functions, differences between species should also be taken into account.  
144 For example, the C-terminal region of murine S100A9 (mS100A9) was shown to have  
145 antinociceptive functions and to inhibit macrophage functions through modulation of B1 cell  
146 function (Paccola et al., 2008; Pagano et al., 2014). Remarkably, these properties have so far not  
147 been ascribed to the human protein. Such distinct properties may arise from local differences in  
148 the 3D-architectures of human and murine proteins, possibly driven by divalent cations since  
149 S100A9 C-terminal region has been involved in metal chelation, at least as part of the S100A8/A9  
150 heterocomplex (Damo et al., 2013; Nakashige et al., 2015).

151 In order to gain deeper insight on how divalent cations may act as regulators of these processes at  
152 a structural level, we have undertaken the biophysical characterization of mS100A9 in the presence  
153 of various sets of cations. Using X-ray crystallography and mass spectrometry, we show that the  
154 mS100A9 homodimers exist, in solution, both as non-covalent and covalent, disulfide-crosslinked  
155 forms. The non-covalent homodimers contain an intramolecular disulfide bridge linking Cys91 to  
156 Cys111. The disulfide-crosslinked homodimers are characterized by distinct disulfide bond  
157 patterns depending on the metal present. We also demonstrate that the relative proportion of these



158 forms depends on the ions bound to the protein. Calcium appears to promote the non-covalent,  
159 canonical homodimer, whereas zinc enhances the formation of the SS-bridged homodimer(s).  
160 Furthermore, we observe that the C-terminal region of mS100A9 contains an additional Zn<sup>2+</sup>  
161 binding site, distinct from the one at the interface between the two monomers that is generally  
162 observed in the canonical S100 homodimers. Finally, we provide isothermal titration calorimetry  
163 (ITC) analyses of zinc binding to mS100A9 and dynamic light scattering (DLS) studies of the  
164 zinc-dependent aggregation of mS100A9. All these findings suggest that divalent cations may  
165 drastically affect the local conformation of mS100A9 C-terminal tail. In the present study, we  
166 discuss the possible consequences of these structural modulations in terms of physiological  
167 function.

## 168 **2. Material and Methods**

### 169 ***2.1. Expression and purification of mS100A9 WT and mutant proteins***

170 The gene coding for WT mS100A9, optimized for codon usage in *E. coli*, was synthesized by  
171 Genscript and further cloned into the *NcoI* – *HindIII* fragment of vector pETM11 (EMBL vector  
172 collection). The protein sequence of the mS100A9 sample obtained with this construct after  
173 purification is indicated in Supplementary Table S1. A theoretical molecular weight of 13177 Da  
174 is expected. Cysteine-to-alanine point mutants C80A, C91A and C111A, were obtained by PCR-  
175 based site-directed mutagenesis using High Fidelity Hot Start Phusion DNA Polymerase  
176 (Finnzymes) and anti-complementary oligonucleotides bearing the mutation to introduce  
177 (Supplementary Table S1). The resulting mS100A9:pETM11 constructs were used to transform  
178 *E. coli* BL21 (DE3) cells. Protein expression and purification was carried out as described  
179 previously (Yatime, 2019). Transformed cells were grown at 37°C for 4 hours and protein  
180 expression was induced overnight at 18°C by adding 1 mM IPTG in the culture medium. Cells

181 were harvested by centrifugation at 6000 g and disrupted by sonication. After clarification by  
182 centrifugation, the cell lysate was loaded onto a 5 ml HisTrap Ni-column (GE Healthcare)  
183 equilibrated with 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 30 mM imidazole, 1 mM PMSF.  
184 Following high salt wash with a buffer containing 1 M NaCl, to remove nonspecifically bound  
185 contaminants, the protein of interest was eluted with a buffer containing 500 mM imidazole. The  
186 N-terminal polyhistidine tag was then removed overnight by incubating the sample at 4°C with  
187 home-made recombinant Tobacco Etch Virus protease (rTEV), using a protein to rTEV massic  
188 ratio of 30 to 1. A second step of affinity chromatography on the His-Trap Ni-column was then  
189 performed to separate the cleaved mS100A9 protein, eluting in the flow-through, from the His<sub>6</sub>-  
190 tag and His-tagged rTEV that remained bound to the column. 10 mM EDTA/EGTA were then  
191 added to the sample to remove all traces of divalent cations and, after overnight incubation with  
192 the chelating agents, the protein was further purified by size exclusion chromatography (SEC) on  
193 a 24 ml Superdex 75 Increase column (GE Healthcare) equilibrated with Buffer 1 (20 mM Tris-  
194 HCl pH 7.5, 100 mM NaCl). Fractions corresponding to the dimeric form of mS100A9 were  
195 pooled and the purity of the sample was verified by SDS-PAGE (Supplementary Fig. S1A). The  
196 purified mS100A9 homodimer was flash frozen in liquid nitrogen and stored at -80°C until use.  
197 This purification protocol yielded in routine 10 mg of pure mS100A9 homodimer per liter of  
198 culture.

199 Before biophysical analyses, mS100A9 samples (WT and mutants) were freshly repurified on a  
200 Superdex 75 Increase SEC column equilibrated with Buffer 1, to ensure that a homogenous dimeric  
201 form of the protein was used (Supplementary Fig. S1B). SEC was performed at 18°C, at a flow  
202 rate of 0.4 ml/min. 2.5 mg of mS100A9 dimeric pool were injected per run (loading volume: 400  
203 µl of protein concentrated at 6.2 mg/ml). After pooling again the dimeric peak of mS100A9 and

204 concentrating, the different samples were prepared by incubating mS100A9 at a final protein  
205 concentration of 4.8 mg/ml (365  $\mu$ M) with either 1 mM CaCl<sub>2</sub> (mS100A9 + Ca), 1 mM ZnCl<sub>2</sub>  
206 (mS100A9 + Zn) or 1 mM CaCl<sub>2</sub> + 1 mM ZnCl<sub>2</sub> (mS100A9 + Ca/Zn), while the apo mS100A9  
207 sample did not contain any of these metals (as assessed by ICP-MS, Table 3). To prepare samples  
208 for MS analysis, these stock solutions were further diluted to a final mS100A9 concentration of  
209 50  $\mu$ M.

## 210 ***2.2. Crystallization, data collection and refinement***

211 Initial crystallization screening was performed by hand using the sitting-drop vapor diffusion  
212 technique at 18°C in 96-well Swissci MRC crystallization plates and commercial screens from  
213 Molecular Dimensions Ltd. mS100A9 (WT and C80A) crystallized at 10 mg/ml (760  $\mu$ M) in the  
214 presence of 5 mM CaCl<sub>2</sub> (6.6 eq) / 2 mM ZnCl<sub>2</sub> (2.6 eq) over a reservoir containing 0.2 M  
215 Ammonium sulfate, 4 mM spermine tetrahydrochloride, 50 mM Bis-Tris pH 7.0, 45% MPD.  
216 Crystals were directly flash frozen in liquid nitrogen and all datasets (native and anomalous) were  
217 collected at 100K on beamline ID23eh1 at ESRF (Grenoble, France) or beamline X06DA at SLS  
218 (Villingen, Switzerland). Datasets were processed with XDS (Kabsch, 2010) and the structure was  
219 determined by molecular replacement (MR) in PHASER (McCoy et al., 2007), using the structure  
220 of Ca<sup>2+</sup>-bound human S100A9 (PDB\_ID 1IRJ) (Itou et al., 2002) for the WT protein, and the newly  
221 determined structure of WT mS100A9 for the C80A mutant. Refinement of the models was carried  
222 out by alternating cycles of manual rebuilding in COOT (Emsley et al., 2004) and cycles of energy  
223 minimization in PHENIX.REFINE (Adams et al., 2010) including refinement of individual  
224 isotropic Atomic Displacement Parameters (ADP) using Translation–Libration–Screw (TLS)  
225 parameterization (Table 1 and Supplementary Table S2). The quality of the final models was

226 assessed with MOLPROBITY (Davis et al., 2007). All figures were made with the Pymol  
227 Molecular Graphics System (version 0.99rc6, DeLano Scientific LLC).

### 228 ***2.3. Inductively-coupled plasma mass spectrometry (ICP-MS)***

229 The metal content of our protein preparation of WT mS100A9 was analyzed by ICP-MS prior to  
230 cation binding studies. A 200  $\mu$ M stock solution of WT mS100A9 in Buffer 1 was used for  
231 measurements. Trace element concentrations were determined both in Buffer 1 alone and in the  
232 mS100A9 sample, with the Thermo Scientific iCAP TQ ICP-MS system (using the Kinetic Energy  
233 Discrimination mode and He as collision gas) on the AETE-ISO platform (OSU OREME,  
234 University of Montpellier, France). An internal solution, containing Be, Sc, Ge, Rh was added on-  
235 line to the samples to correct signal drifts. Certified water SLRS-6 (National Research Council of  
236 Canada, Metrology Research Centre) was used as a control of the correctness of the measurements.

### 237 ***2.4. Isothermal titration calorimetry (ITC)***

238 The affinity of mS100A9 for  $\text{ZnCl}_2$  in the presence or absence of  $\text{CaCl}_2$  was determined using a  
239 MicroCal iTC200 system (Malvern Panalytical, Malvern, UK). Experiments were performed at  
240 25°C in 20 mM Tris-HCl pH 7.5, 100 mM NaCl, +/- 1 mM  $\text{CaCl}_2$ . The mS100A9 protein solution  
241 (at concentrations ranging from 30  $\mu$ M to 120  $\mu$ M) was loaded in the calorimetric cell.  $\text{ZnCl}_2$  (at  
242 concentrations ranging between 0.6 and 1.2 mM) was titrated in the protein sample typically by  
243 performing 16 injections of 2.5  $\mu$ L or 20 injections of 2  $\mu$ L each. The dissociation constant ( $K_d$ ),  
244 enthalpy of binding ( $\Delta H$ ), and stoichiometry (N) were obtained after fitting the integrated and  
245 normalized data to a single-site binding model. The data were processed using Origin 7.0 (Malvern

246 Panalytical, Malvern, UK). All experiments were performed at least in duplicate to check for  
247 reproducibility of the data.

## 248 **2.5. Dynamic light scattering (DLS)**

249 DLS measurements were performed at 20°C using a Zetasizer Nano S from Malvern Instruments  
250 Ltd using a quartz cuvette from Hellma Analytics. We measured the hydrodynamic radius (Rh) of  
251 the protein mS100A9 at 120 µM, in the presence or absence of 1 mM CaCl<sub>2</sub>, at different mS100A9  
252 to ZnCl<sub>2</sub> molar ratios (1:0, 1:1, 1:2, 1:3 and 1:4).

## 253 **2.6. Liquid chromatography/electrospray-ionization mass spectrometry (LC/ESI-TOF-MS)**

254 The disulfide bond pattern of mS100A9 in the presence or absence of different divalent cations  
255 was investigated by Liquid Chromatography/Electrospray Ionization Mass Spectrometry (LC/ESI-  
256 TOF-MS) on a 6210 TOF mass spectrometer coupled to a HPLC system (1100 series, Agilent  
257 Technologies). The mass spectrometer was calibrated with tuning mix (ESI-L, Agilent  
258 Technologies). The following instrumental settings were used: gas temperature (nitrogen) 300 °C,  
259 drying gas (nitrogen) 7 L min<sup>-1</sup>, nebulizer gas (nitrogen) 10 psig, V<sub>cap</sub> 4 kV, fragmentor 250 V,  
260 skimmer 60 V, V<sub>pp</sub> (octopole RF) 250 V. The HPLC mobile phases were prepared with HPLC  
261 grade solvents. Mobile phase A composition was: H<sub>2</sub>O 95%, ACN 5%, TFA 0.03%. Mobile phase  
262 B composition was: ACN 95%, H<sub>2</sub>O 5%, TFA 0.03%.

263 For MS analysis of the intact protein, three different samples were prepared for each set of ions:  
264 1) *no treatment*: the protein sample at 10 µM concentration in the corresponding buffer, without  
265 any chemical treatment; 2) *alkylation (IAA)*: the protein sample at 10 µM concentration, after  
266 incubation with 55 mM IAA (30 min at room temperature, in the dark); 3) *reduction and alkylation*  
267 *(DTT+IAA)*: the protein sample at 10 µM concentration, first incubated with 10 mM DTT (45 min  
268 at 56°C, under gentle mixing) followed by treatment with 55 mM IAA (30 min at room

269 temperature, in the dark). For the mS100A9 protein without ions, a fourth sample was prepared:  
270 4) *unfolding, reduction and alkylation (urea+DTT+IAA)*: the protein sample at 10  $\mu$ M  
271 concentration, first incubated with 10 mM DTT in the presence of 8 M urea (45 min at 56°C, under  
272 gentle mixing) followed by treatment with 55 mM IAA (30 min at room temperature, in the dark).  
273 2  $\mu$ l of each protein sample (20 pmol) were injected for MS analysis and were first desalted on-  
274 line for 3 min with 100% of mobile phase A (flow rate of 50  $\mu$ l/ min), using a C8 reverse phase  
275 micro-column (Zorbax 300SB-C8, 5 $\mu$ m, 5  $\times$  0.3 mm, Agilent Technologies). The sample was then  
276 eluted with 70% of mobile phase B (flow rate of 50  $\mu$ l/ min) and MS spectra were acquired in the  
277 positive ion mode in the 300-3000  $m/z$  range. Data were processed with MassHunter software (v.  
278 B.02.00, Agilent Technologies) and GPMAW software (v. 7.00b2, Lighthouse Data, Denmark).  
279 The mass accuracy on the experimental mass values obtained for the mS100A9 samples from ESI  
280 mass spectra, after deconvolution, was of  $\pm$  0.06 Da (for the “monomeric” peak) and of  $\pm$  0.2 Da  
281 (for the “dimeric” peak). To verify whether monomeric and dimeric species have similar relative  
282 ionization efficiencies, we assessed the relative ionization efficiency of mS100A9 mutants C111A  
283 (that gives only monomeric species) and C91A (that gives only dimeric species). The results,  
284 displayed in Supplementary Figure S9, show that both species have relatively similar ionization  
285 efficiencies with an estimated average percent error of 5% max, which we consider acceptable.  
286 Based on these results, we then evaluated the relative proportions of monomeric and dimeric  
287 species in our LC/ESI-TOF MS experiments by measuring the peak area of each species in the  
288 deconvoluted ESI MS spectra and reporting their values as a percentage of the sum of all species  
289 observed (area % of deconvoluted peak) (see for ex. Figures 4A and 5A). The number of disulfide  
290 bonds versus free cysteines was determined for each set of ions by comparing the ESI MS spectra

291 recorded for the samples without any chemical treatment, with those after reaction with IAA alone  
292 or DTT + IAA.

### 293 ***2.7. Native mass spectrometry***

294 The samples were analyzed by native mass spectrometry (Boeri Erba et al., 2015; Boeri Erba et  
295 al., 2018; Boeri Erba et al., 2020). Protein ions were generated using a nanoflow electrospray  
296 (nano-ESI) source. Nanoflow platinum-coated borosilicate electrospray capillaries were bought  
297 from Thermo Electron SAS (Courtaboeuf, France). MS analyses were carried out on a quadrupole  
298 time-of-flight mass spectrometer (Q-TOF Ultima, Waters Corporation, Manchester, U.K.). The  
299 instrument was modified for the detection of high masses (Sobott et al., 2002; van den Heuvel et  
300 al., 2006). The following instrumental parameters were used: capillary voltage = 1.2-1.3 kV, cone  
301 potential = 40 V, RF lens-1 potential = 40 V, RF lens-2 potential = 1 V, aperture-1 potential = 0  
302 V, collision energy = 30-140 V, and microchannel plate (MCP) = 1900 V. All mass spectra were  
303 calibrated externally using a solution of cesium iodide (6 mg/mL in 50% isopropanol) and were  
304 processed with the Masslynx 4.0 software (Waters Corporation, Manchester, U.K.) and with  
305 Massign software package (Morgner et al., 2012).

## 306 **3. Results**

### 307 ***3.1. General overview of the Ca<sup>2+</sup>/Zn<sup>2+</sup>-bound mS100A9 structure***

308 To date, no structural data on mS100A9 are available. Quite a few structures of hS100A9 are  
309 deposited in the Protein Data Bank (PDB), either for the homodimeric form of the protein or for  
310 the S100A8/A9 heterotetramer (Itou et al., 2002; Korndorfer et al., 2007; Chang et al., 2016).  
311 However, none of these structures has been obtained in the presence of zinc. In fact, the sole  
312 information that may be derived on zinc chelation by S100A9 arise from the structures of  
313 manganese- and nickel-bound human S100A8/A9 (Damo et al., 2013; Gagnon et al., 2015;

314 Nakashige et al., 2017; Nakashige et al., 2018). No such data exist for the S100A9 homodimer,  
315 although this form is able to bind zinc as well (Raftery et al., 1999). Furthermore, differences in  
316 metal coordination between the human and murine proteins cannot be excluded. We therefore  
317 started investigating zinc binding to mS100A9 from a structural point of view. As for hS100A9,  
318 mS100A9 tends to form higher oligomers than just simple homodimers, as we indeed observed  
319 when we analyzed the concentrated protein by size exclusion chromatography (SEC)  
320 (Supplementary Figs. S1A and S1C-D). To avoid adding a level of complexity to the study, we  
321 focused our experiments on the dimeric pool of the protein, collected separately from the SEC  
322 column and rerun after long term storage at -80°C on the same column to evaluate its stability  
323 (Supplementary Fig. S1B). Despite extensive efforts, we could only obtain crystals of the protein  
324 in the presence of calcium and zinc. No crystals appeared if any of the two ions was absent from  
325 the protein buffer. The crystals belonged to space group C2 and diffracted to a maximum resolution  
326 of 1.45 Å (Table 1). The structure was determined by molecular replacement using calcium-bound  
327 hS100A9 (Itou et al., 2002) as the MR search model.

328 The final refined atomic model for Ca<sup>2+</sup>/Zn<sup>2+</sup>-bound mS100A9 is displayed in Figure 1A (Table 1  
329 for data statistics). Quality of the fit between this model and the experimental electron density map  
330 can be assessed in Figure 1B. As expected, mS100A9 arranges in a centro-symmetrical homodimer  
331 that corresponds to the canonical dimer reported for all other S100 proteins. Both subunits bind  
332 two calcium ions, in the two EF-hand motifs, and more surprisingly, two zinc ions, one at the  
333 interface between the two subunits and one within the C-terminal tail. The exact nature of these  
334 ions was confirmed by calculating anomalous difference Fourier maps from datasets collected at  
335 the Zn peak (wavelength of 1.27 Å), where both Zn and Ca display anomalous scattering of X-



336 rays, and just after the Zn peak (wavelength of 1.30 Å), where Zn anomalous signal is lost whereas  
337 calcium anomalous signal increases (Fig. 1C).

338 The overall architecture of mS100A9 closely resembles that of hS100A9. As depicted in Figure  
339 1D, the mS100A9 protomer (beige) superimposes quite well with the corresponding Ca<sup>2+</sup>-bound  
340 protomer of both WT (blue) and C3S (red) hS100A9 homodimer (Itou et al., 2002; Chang et al.,  
341 2016) as well as with the Ca<sup>2+</sup>/Mn<sup>2+</sup>-bound hS100A9 moiety (grey) of the hS100A8/A9  
342 heterocomplex (Damo et al., 2013). An overall r.m.s.d. on C $\alpha$  atoms of 1.05 and 1.42 Å can be  
343 measured between the murine protein and the WT or mutated hS100A9, respectively, for the core  
344 region encompassing helices H1 to H4 (Fig. 1D). Similarly, r.m.s.d. values on C $\alpha$  atoms comprised  
345 between 1.19 and 1.31 Å were obtained by comparing mS100A9 and the hS100A9 moiety from  
346 the different human S100A8/A9 structures. The major movements between these structures occur  
347 in the second half of helix H4 and in the C-terminal tail of the protein (Fig. 1D), a region that is  
348 not modeled in all available structures due to its high flexibility. In mS100A9, the long C-terminal  
349 tail adopts a rigidified architecture that will be discussed in more details below.

350 In contrast to the conserved conformation of the S100A9 protomer observed between human and  
351 murine proteins, the respective position of the two mS100A9 subunits within the homodimer  
352 differs quite substantially from what is observed for the Ca<sup>2+</sup>-bound hS100A9 homodimers (Fig.  
353 1E). Indeed, subunit B of mS100A9 is shifted away from subunit A by a rotation of approximately  
354 30° as compared to the equivalent subunits in both structures of the hS100A9 homodimer (Fig.  
355 1E). This differential positioning of the two S100A9 subunits may reflect intrinsic differences  
356 between the two S100A9 orthologues. Another possibility is that zinc binding triggers the  
357 reorientation of the two S100 subunits. To address this question, we then compared the relative  
358 orientation of the two S100 subunits in our Ca<sup>2+</sup>/Zn<sup>2+</sup>-bound mS100A9 homodimer with that of

359 other S100 homodimers bound to calcium alone or to calcium plus zinc. As depicted in Figure 1F  
360 and Supplementary Figure S2, our mS100A9 homodimer superimposes quite well with the  
361 hS100A7 (Brodersen et al., 1999), hS100A8 (Ishikawa et al., 2000; Lin et al., 2016), hS100A12  
362 (Moroz et al., 2001; Moroz et al., 2003), hS100A15 (Murray et al., 2012) and hS100B (Ostendorp  
363 et al., 2007; Ostendorp et al., 2011) homodimers, whether they are Ca<sup>2+</sup>-bound (hS100A8,  
364 hS100A12, hS100B), Ca<sup>2+</sup>/Zn<sup>2+</sup>-bound (hS100A7, hS100A8, hS100A15, hS100B) or even  
365 Ca<sup>2+</sup>/Cu<sup>2+</sup>-bound (hS100A12). In fact, no major repositioning of the two S100 subunits is observed  
366 in these different S100 homodimers upon zinc addition to the Ca<sup>2+</sup>-bound form. This suggests that  
367 the differential S100/S100 orientation we observe between Ca<sup>2+</sup>-bound hS100A9 and Ca<sup>2+</sup>/Zn<sup>2+</sup>-  
368 bound mS100A9 is not a direct consequence of zinc binding. The fact that the hS100A9 structures  
369 have been determined either by NMR (for the WT protein) or by X-ray crystallography but using  
370 a hS100A9 mutant form (for the C3S mutant) may also account for the observed differences. In  
371 any case, the quaternary organization of our Ca<sup>2+</sup>/Zn<sup>2+</sup>-bound mS100A9 homodimer is very similar  
372 to that of other Ca<sup>2+</sup>/Zn<sup>2+</sup>-bound s100 homodimers.

### 373 ***3.2. mS100A9 harbors two distinct types of zinc binding sites***

374 To our knowledge, no biophysical characterization of zinc binding to the S100A9 homodimer has  
375 been published so far, although S100A9 is known to bind zinc in the absence of its S100A8  
376 congener (Bjork et al., 2009). In particular, the stoichiometry of zinc binding to the S100A9  
377 homodimer and the exact nature of the cation binding sites remain unknown. Unexpectedly, our  
378 structure of Ca<sup>2+</sup>/Zn<sup>2+</sup>-bound mS100A9 revealed the presence of two Zn<sup>2+</sup> ions per S100A9  
379 protomer. The first binding site is positioned at the interface between the two mS100A9 subunits.  
380 The mS100A9 homodimer contains two equivalent sites of this type, arranged centro-  
381 symmetrically (Zn1 and Zn3 in Figure 1A) and displaying a tetrahedral geometry (Fig. 2A), with

382 average Zn-ligand bond distances between 1.9 and 2.0 Å (Table 2). The first mS100A9 subunit  
383 provides two of the coordinating residues, His21 at the end of helix H1, and Asp31 within the first  
384 Ca<sup>2+</sup> EF-hand. The two other Zn<sup>2+</sup>-chelating residues, His92 and His96, come from helix H4 in  
385 the second subunit. This site corresponds to the canonical His<sub>3</sub>Asp zinc binding motif which is  
386 encountered in the His-Zn class of S100 proteins, and its geometry is highly similar to that of the  
387 His-Zn sites present in hS100A7 (Brodersen et al., 1999; Supplementary Fig. S3A) and hS100A12  
388 (Moroz et al., 2009a; Supplementary Fig. S3B). The geometry of this site also corresponds well to  
389 that of the Zn-binding sites found in hS100B (Ostendorp et al., 2011; Supplementary Figure S3C)  
390 and hS100A8 (Lin et al., 2016; Supplementary Figure S3D), although in these two cases the  
391 coordinating motifs are respectively His<sub>3</sub>Glu and His<sub>4</sub>.

392 The second Zn-binding site observed in our structure involves the three histidine residues located  
393 in the C-terminal tail of mS100A9 (Fig. 2B, purple triangles). These three C-terminal His residues  
394 form a novel, tetrahedral Zn<sup>2+</sup>-binding motif (Fig. 2C), never encountered so far in other S100  
395 protein structures. The fourth coordinating residue, Glu65 at the end of helix H3, is provided by a  
396 symmetry-related mS100A9 molecule within the crystal. Average Zn-ligand bond distances for  
397 this second Zn-site range between 1.9 and 2.1 Å (Table 2). Due to the involvement of its three His  
398 residues in zinc chelation, the C-terminal tail folds back over the mS100A9 subunit core. This  
399 folding is further promoted by the presence of a disulfide bridge connecting Cys91, at the end of  
400 helix H4, and Cys111 at the end of the C-terminal tail. The C-terminal tail therefore adopts a  
401 rigidified architecture, forming a closed loop that protrudes at the extremity of helix H4 (Fig. 2D).  
402 This novel Zn-binding site is rendered possible in mS100A9, at least in the crystal structure,  
403 because the three His residues in its C-terminal portion are not placed contiguously but are instead  
404 spaced by a residue of different nature, giving rise to an HxHxH motif (Fig. 2B). hS100A9 likewise

405 contains a cluster of histidine residues in its C-terminal tail (Fig. 2B, orange circles). Since no  
406 structure of the hS100A9 homodimer bound to zinc is available, it is not possible to know if such  
407 a site can be formed within the human homodimer. However, the structures of the human  
408 S100A8/A9 heterocomplex bound to  $Mn^{2+}$  or  $Ni^{2+}$  (Damo et al., 2013; Nakashige et al., 2018)  
409 revealed that in hS100A9, these C-terminal His residues participate in the coordination of the  $Mn^{2+}$   
410 ion present at the interface between the two S100 subunits, thereby generating an octahedral  $His_6$   
411 coordination site with high affinity. In the context of the S100A8/A9 heterocomplex, this non-  
412 canonical  $His_6$  site was shown to bind both  $Zn^{2+}$ ,  $Mn^{2+}$ ,  $Ni^{2+}$  and  $Fe^{2+}$  (Damo et al., 2013;  
413 Nakashige et al., 2015; Nakashige et al., 2017). It remains unknown whether such an octahedral  
414 site would also be used for zinc chelation in the context of the hS100A9 homodimer or whether  
415 hS100A9 could also form a second Zn-binding site, similar to the one we observe for mS100A9.  
416 Obviously, since the three terminal histidines of hS100A9 are contiguous to each other (Fig. 2B),  
417 the geometrical constraint imposed on the protein backbone would only allow two of them to  
418 arrange simultaneously in a tetrahedral coordination motif, and the two remaining Zn-ligand would  
419 have to be provided by a distinct molecule/ion.

420 Since the novel Zn-site we observe in mS100A9 structure is promoted by crystal contacts, we  
421 investigated whether it was a crystallization artifact or whether it could also exist in solution. For  
422 this purpose, we first confirmed that our mS100A9 sample had been purified in the apo form by  
423 quantifying the residual content of divalent cations present in our protein preparation using  
424 inductively-coupled plasma mass spectrometry (ICP-MS). The measured metal/S100 ratios show  
425 that there is no significant metal-ion contamination in our sample (Table 3). We then performed  
426 ITC titrations of zinc binding to mS100A9, either in the absence or presence of calcium. As shown  
427 in Figures 3A and 3B, the thermograms were best fitted with a single-site model, indicating that

428 mS100A9 binds one  $\text{Zn}^{2+}$  ion per subunit with an average dissociation constant ( $K_d$ ) ranging  
429 between 16 and 25  $\mu\text{M}$  depending on the experiments. Thus, the mS100A9 homodimer possesses  
430 two equivalents Zn-binding sites with affinities in the medium  $\mu\text{M}$  range. Differences in  $K_d$  values  
431 between apo mS100A9 and  $\text{Ca}^{2+}$ -bound mS100A9 are within error range, suggesting that calcium  
432 has no effect on mS100A9 affinity for zinc. A few of the titrations curves exhibited a small, second  
433 phase that could indicate a biphasic binding mode but fitting with a two-sites model did not yield  
434 pertinent results in these cases (data not shown). Thus, even if a second Zn-binding site exists on  
435 the mS100A9 protomer in solution, we could not detect it with the experimental conditions  
436 employed here. Importantly, all titrations ended up at 2 molar equivalents of zinc as compared to  
437 mS100A9. When we increased further zinc concentration in the titrations, mS100A9 started  
438 precipitating. To analyze this metal-dependent aggregation of mS100A9 in more details, we  
439 performed DLS analysis of the sample, both in the presence and absence of calcium, using  
440 increasing concentrations of zinc. As depicted in Figures 3C and 3D, zinc-dependent aggregation  
441 of mS100A9 readily occurs as soon as the zinc to mS100A9 molar ratio equals or exceeds 3  
442 equivalents and this phenomenon is independent of calcium. This tendency to aggregate in the  
443 presence of excess zinc evidently complicates the analysis of the Zn-mS100A9 samples with native  
444 MS (see below). It may also impair the detection of low affinity Zn-binding sites by ITC.

### 445 ***3.3. In solution, mS100A9 exists as several homodimeric forms with distinct disulfide bond*** 446 ***patterns***

447 Our structure reveals that mS100A9 forms an intramolecular disulfide bridge between Cys91 and  
448 Cys111, at least in the presence of high calcium and zinc concentrations. By contrast, a disulfide  
449 bridge between Cys80 and Cys91 was previously reported for the protein isolated from native  
450 source or produced recombinantly in the absence of divalent cations (Raftery et al., 1999).

451 Presence of divalent cations may therefore influence SS link formation within mS100A9. In  
452 particular, zinc coordination at the C-terminal tail may preferentially stabilize the Cys91-Cys111  
453 SS bridge (or *vice versa*). To gain more insight into the disulfide bonding pattern of wild-type  
454 (WT) mS100A9, we analyzed the dimeric pool of mS100A9 under denaturing conditions using  
455 LC/ESI-TOF MS (Supplementary Fig. S4). This technique enables the characterization of both  
456 inter- and intramolecular disulfide crosslinks whereas all the non-covalent interactions are broken  
457 (Boeri Erba et al., 2018).

458 We first analyzed WT mS100A9 in the absence of divalent cations. When all three cysteines are  
459 reduced (i.e. are not engaged in an SS-bond), our mS100A9 protein has a theoretical mass of  
460 13177.1 Da as a monomer, and of 26354.2 Da as a homodimer. Under denaturing conditions and  
461 in the absence of DTT, WT mS100A9 generates an LC/ESI-TOF MS spectrum with one major  
462 peak (Fig. 4A; mass 13175.1 Da, 66% of the total protein signal) referred to as the “monomeric”  
463 peak and whose mass confirms the presence of one intramolecular disulfide bond per molecule.  
464 Since the mS100A9 sample was analyzed under denaturing conditions, the “monomeric” peak is  
465 composed of both native monomers and single subunits generated by the disassembly of non-  
466 covalent oligomers. The ESI MS spectrum also reveals a minor peak (Fig. 4A; mass 26349.5 Da,  
467 34% of the total protein signal) referred to as the “dimeric” peak. Upon reduction with DTT, the  
468 “dimeric” peak almost totally disappears (Fig. 4D), confirming that the mS100A9 form(s) giving  
469 rise to this peak contain intersubunit disulfide bonds.

470 To characterize the oligomeric state of these covalent and non-covalent forms of mS100A9, we  
471 analyzed the same samples using native MS. In both absence and presence of DTT, mS100A9 is  
472 mostly dimeric (Figs. 4B-C; mass:  $26351 \pm 2$  Da and  $26355 \pm 3$  Da, respectively), in agreement  
473 with our SEC experiments (Supplementary Fig. S1B). A small amount of monomer is also

474 observed in both conditions (Figs. 4B-C; mass:  $13175 \pm 2$  Da and  $13177 \pm 1$  Da, respectively).  
475 Due to the gentle experimental conditions employed, native MS is a technique that preserves non-  
476 covalent interactions (Boeri Erba et al., 2020). The monomeric state we observe can therefore not  
477 arise from the breaking of dimers during data acquisition. Interestingly, mS100A9 carries less  
478 charges in the presence of disulfide bridges (Figs. 4B-C). This reflects the higher degree of  
479 compactness of the disulfide crosslinked mS100A9 compared to the non-crosslinked one. This is  
480 also in agreement with our SEC experiments which show that in the presence of DTT, the peak  
481 corresponding to homodimeric mS100A9 elutes slightly earlier than under non-reducing  
482 conditions (Supplementary Fig. S1B), suggesting a less compact form in the presence of DTT.  
483 Addition of DTT does not yield a significantly higher proportion of monomeric mS100A9 (Fig.  
484 4C), suggesting that mS100A9 remains dimeric upon breakage of the intersubunit SS bridges.

485 To assess the number of free cysteines, we treated mS100A9 with iodoacetamide (IAA), in the  
486 absence or presence of DTT, and we analyzed these samples using LC/ESI-TOF MS (Figs. 4E-F).  
487 Reaction with IAA increases the mass of the “monomeric” peak to 13232.1 Da (+ 57 Da),  
488 indicating the covalent addition of one acetamide group per molecule (Figs. 4E, 4G). This suggests  
489 that only one cysteine per mS100A9 molecule is free to react with IAA. Treatment with DTT and  
490 IAA gives rise mainly to addition of 1 and 2 acetamide groups (Figs. 4F-G). Thus, DTT reduces  
491 two cysteines that were previously engaged in an intramolecular disulfide bridge. Species with 3  
492 acetamide groups were not detected after DTT and IAA treatment. There are several explanations:  
493 one of the three cysteines may be partly inaccessible to the solvent after cleavage of the SS bridge;  
494 alternatively, the reaction conditions may not be sufficient to reduce all cysteines. To address this  
495 question, we incubated mS100A9 with DTT in the presence of strong denaturant (8M urea) before  
496 performing alkylation with IAA using identical conditions as previously. In these conditions, we

497 obtained fully tri-alkylated protein (Fig. 4H, species at molecular mass 13348.3 Da). Thus, upon  
498 complete unfolding of the protein, all three cysteines reacted with IAA.

499 Reaction with IAA increases the mass of the “dimeric” peak of 114 Da, indicating a covalent  
500 addition of 2 acetamide groups per dimer (Figs. 4E, 4I). This means that only two cysteines (out  
501 of six available) are free to react. We may hypothesize two intersubunit SS bonds between two  
502 S100A9 protomers, forming a covalent dimer. Another possibility is that only one inter-subunit  
503 SS bond bridges the two mS100A9 protomers and one of the protomers also bears an  
504 intramolecular SS bridge.

505 Altogether MS data suggest that 1) WT mS100A9 forms both non-covalent and SS-crosslinked  
506 homodimers (Figs. 4A-4D); 2) the protein also exists as a monomer (Figs. 4B-C); 3) the non-  
507 covalent species all contain one intramolecular disulfide bridge per S100A9 subunit (Fig. 4G).

#### 508 ***3.4. The homodimerization mode of mS100A9 is differentially affected by calcium and zinc***

509 Next, we aimed to investigate the effect of divalent cations on the mS100A9 dimeric pool. Thus,  
510 we performed LC/ESI-TOF and native MS experiments on WT mS100A9 incubated in the  
511 presence of calcium alone, zinc alone, or both metals (Supplementary Fig. S4). As shown in Figure  
512 5, we observe the presence of both the “monomeric” and “dimeric” peaks in the LC/ESI-TOF  
513 spectra regardless of the metal present. However, when zinc is present, the equilibrium is  
514 significantly shifted towards the formation of SS-crosslinked homodimers (Figs. 5C, 5E).

515 In the absence of divalent cations or in the sole presence of calcium, the “monomeric” peak  
516 observed under denaturing conditions is predominant (Figs. 4A, 5A). In the native MS conditions,  
517 the non-covalent homodimer is in equilibrium with a monomeric form of the protein, whose  
518 abundance remains low (Fig. 4B, 5B). The use of IAA shows that calcium does not affect the  
519 disulfide bond pattern of the non-covalent and SS-crosslinked homodimers (Supplementary Fig.



520 S5). In the presence of calcium, the “monomeric peak” still contains an intramolecular SS bridge  
521 per subunit (Supplementary Figs. S5A-C), whereas the disulfide-crosslinked homodimer has two  
522 SS bonds and two free cysteines (Supplementary Fig. S5D). The addition of calcium seems only  
523 to loosen slightly the monomer packing in the homodimer as illustrated by the observed shift to  
524 higher charge states in the native MS spectrum (Fig. 5B).

525 Regardless of whether calcium is bound or not, the presence of zinc highly affects mS100A9  
526 behavior. The disulfide-crosslinked homodimer becomes predominant, representing 86 % and 80  
527 % of the total amount of protein in the presence of zinc alone or zinc and calcium, respectively  
528 (Figs. 5C, 5E). The observed molecular mass of this “dimeric” peak increases of +2 Da, suggesting  
529 that one of the two SS bridges is lost. Interestingly, native MS shows that the signals of mS100A9  
530 monomers are not observed anymore (Figs. 5D, 5F). Moreover, it was quite difficult to record  
531 native MS data when zinc was present since the sample tended to aggregate during injection into  
532 the mass spectrometer. Thus, as also suggested by our DLS experiments, zinc may promote the  
533 formation of high order insoluble oligomers and the recorded spectra may only reflect the soluble  
534 fraction of the sample.

535 In the sample with zinc alone, reaction with IAA generates species carrying one acetamide group  
536 for the “monomeric” peak (Supplementary Fig. S6A). Pre-treatment with DTT yields species  
537 carrying up to 2 acetamide groups (Supplementary Figs. S6B-C). The binding of 3 acetamide  
538 groups is not observed, indicating that a maximum of two cysteines per molecule is available. Zinc  
539 might hinder solvent accessibility of the third cysteine either because  $Zn^{2+}$ -binding sites may  
540 directly block the access to this residue or because zinc may promote mS100A9 oligomerization,  
541 thereby diminishing solvent exposure of the cysteines buried within the oligomers. Surprisingly,  
542 in the presence of both calcium and zinc, no alkylation of the non-covalent species occurs

543 (Supplementary Fig. S7A). Similarly, addition of DTT and IAA leads mainly to the formation of  
544 species with 1 acetamide group, and a smaller amount of species with 2 acetamide groups, as well  
545 as a certain amount of unreacted protein (Supplementary Figs. S7B-C).

546 Up to four acetamide groups are bound to the disulfide-crosslinked homodimer in the presence of  
547 zinc alone (Supplementary Fig. S6D), whereas only two acetamide groups are added in the sole  
548 presence of calcium (Supplementary Fig. S5D). This demonstrates loss of one SS bridge upon zinc  
549 addition. When calcium and zinc are present, the SS-linked homodimer is almost unreactive  
550 towards alkylation, the major form observed is the unreacted protein, whose mass corresponds to  
551 a dimer carrying one intersubunit SS bridge (Supplementary Fig. S7D). Small amounts of  
552 homodimers with 1 and 2 acetamide groups are also detected but clearly the cysteines are much  
553 less accessible to IAA when both cations are present. Taken together, these data suggest that zinc  
554 act as a modulator of the disulfide bonding pattern of mS100A9. It promotes the assembly of  
555 covalent, SS-crosslinked dimers and affects the accessibility of the other unliganded cysteines.

### 556 ***3.5. The formation of distinct SS bridges allows switching between the different homodimeric*** 557 ***forms of mS100A9***

558 In order to identify which of the three cysteines of mS100A9 are involved in the intramolecular  
559 and inter-subunit disulfide bridges, point mutations into alanine were introduced for each cysteine  
560 residue. Only the dimeric fraction of the samples was pooled from the SEC column  
561 (Supplementary Fig. S8). These dimeric pools were first analyzed by SDS-PAGE under non-  
562 reducing conditions for all four ionic conditions tested. As shown in Figure 6A, regardless of the  
563 metal present, almost no disulfide-crosslinked dimer is observed for the C111A mutant, whereas  
564 the C91A mutant forms almost exclusively the disulfide-crosslinked dimers. For both the WT  
565 protein and the C80A mutant, a mixture of monomers and SS-linked dimers is present. The relative

566 proportion of monomers and dimers is however difficult to evaluate and it is apparently not highly  
567 influenced by cations (Figure 6A).

568 In order to assess the mass of the species with high accuracy and sensitivity, the three Cys-to-Ala  
569 mutants were analyzed by LC/ESI-TOF-MS. C111A mutant is unable to form the disulfide-  
570 crosslinked homodimer, regardless of the cations present (Figure 6B), in agreement with our SDS-  
571 PAGE experiments. This indicates that Cys111 is responsible for the formation of inter-subunit  
572 SS bridge(s). In all four ionic conditions, the “monomeric” peak has an experimental molecular  
573 mass of 13145.2 Da. This mass indicates that the two non-mutated cysteines, Cys80 and Cys91,  
574 are in the reduced state, regardless of the presence or absence of metal ions. Consistently, treatment  
575 of the protein with IAA leads to the binding of two acetamide groups per subunit, when there is  
576 Ca only, Zn only or no metal (Supplementary Fig. S10A). When both zinc and calcium are present,  
577 one cysteine is less accessible to react with IAA. These data rule out the formation of an  
578 intramolecular bridge between Cys80 and Cys91, and suggest that Cys111 also participates in the  
579 intramolecular SS links. Alternatively, Cys111 mutation into alanine may affect the overall  
580 architecture of the S100A9 molecule in such a way that the SS bridge between Cys80 and Cys91  
581 cannot be formed. As Cys111 is located at the very end of mS100A9 C-terminal tail, in a highly  
582 flexible and unstructured region, it seems however unlikely that the C111A mutation may modify  
583 significantly the architecture of the protein core.

584 By contrast, the C91A mutant is exclusively present as disulfide-crosslinked dimers, in all ionic  
585 conditions screened (Fig. 6C). The measured molecular mass indicates a homodimer containing  
586 one inter-subunit SS bond and two reduced cysteines. Consistently, reaction with IAA leads mainly  
587 to the addition of 2 acetamide groups, except when both zinc and calcium are present. In that case,  
588 the protein is mostly present in its non-alkylated form (Supplementary Fig. S10B). These

589 observations suggest that Cys91 is not essential to the formation of the SS-linked homodimer and  
590 that Cys91 mutation frees two cysteines by removing one of the SS bonds present within the  
591 disulfide-crosslinked dimer.

592 In the case of the C80A mutant, different forms of mS100A9 are in equilibrium (Fig. 6D). In the  
593 absence of metals or in the presence of zinc, the disulfide-crosslinked homodimer represents the  
594 most abundant form of the protein whereas non-covalent species are slightly prevailing in the  
595 presence of calcium alone. The “monomeric” peak has a molecular mass consistent with one  
596 intramolecular SS bridge per mS100A9 subunit (Fig. 6D). Moreover, reaction with IAA does not  
597 result in alkylation of the protein, demonstrating that the two non-mutated cysteines, Cys 91 and  
598 Cys111, are not available to react due to their engagement in the intramolecular SS link  
599 (Supplementary Fig. S10C). The disulfide-crosslinked homodimer displays a mass indicating the  
600 presence of a single inter-subunit SS bridge and two reduced cysteines. These residues are  
601 available for alkylation by IAA, when there is calcium or zinc or no metals (Supplementary Fig.  
602 S10D). When both zinc and calcium are present, the unreacted protein represents the major species.  
603 This may be explained by oligomerization and/or local conformational changes hindering access  
604 to free cysteines.

605 In a further effort to unravel how the three cysteines interact with each other, in particular within  
606 the disulfide-crosslinked homodimer, we aimed to crystallize the three Cys-to-Ala mS100A9 point  
607 mutants in the various ionic conditions screened. We managed to obtain crystals for the mS100A9  
608 C80A mutant, in the presence of calcium and zinc, in the same crystallization conditions than for  
609 the WT protein. These crystals diffracted X-rays to 2.35 Å resolution (Table 1, Supplementary  
610 Table S2). The structure of the  $\text{Ca}^{2+}/\text{Zn}^{2+}$ -bound point mutant was determined by MR using the  
611 structure of the WT protein obtained in this study. The resulting structural model is reported in

612 Figure 7A and the quality of the fit with the electron density map is depicted in Supplementary  
613 Figure S11A. The overall structure of the mutant is highly similar to the structure of the WT  
614 protein, with an r.m.s.d. value on C $\alpha$  atoms of 0.2 Å between the two structures (Fig. 7A). As for  
615 the WT protein, the C80A mutant forms a non-covalent homodimer with two calcium and two zinc  
616 bound per protomer and one intramolecular SS bridge connecting Cys91 and Cys111 (Fig. 7A,  
617 Supplementary Fig. 11B). This second structure confirms that within the non-covalent species,  
618 Cys80 is not involved in the formation of intramolecular SS bonds.

619 In summary, our results indicate that Cys91 and Cys111 are able to form an intramolecular SS  
620 bridge in the absence or presence of calcium and/or zinc. Characterization of the SS inter-subunit  
621 bridges in the disulfide-crosslinked dimer is more complex. The dimer strongly relies on Cys111,  
622 whether metals are present or not. It contains two SS bridges, one of which is lost upon addition  
623 of zinc as well as when Cys80 or Cys91 are point-mutated. There may not be a single SS-linked  
624 homodimeric form of mS100A9 and the protein may display quite some flexibility in the way its  
625 subunits are connected through disulfide bridges.

#### 626 **4. Discussion**

627 In this study, we provided a detailed *in vitro* investigation of the homodimerization and disulfide  
628 bond pattern of murine S100A9 and we examined how divalent cations affect these properties. We  
629 described the first crystallographic structures of mS100A9 (WT and C80A mutant) obtained in the  
630 presence of calcium and zinc. They revealed a canonical architecture for the mS100A9  
631 homodimer, with both protomers arranged in a centro-symmetrical fashion, as observed for most  
632 other S100 proteins. The structures also suggested that mS100A9 is able to chelate two zinc ions  
633 per subunit, in contrast to what has been reported so far for other members of the family. Indeed,  
634 besides the canonical His<sub>3</sub>Asp Zn-motif, our structural data uncovered a second, His<sub>3</sub>Glu

635 tetrahedral  $Zn^{2+}$ -binding site formed by the HxHxH motif within mS100A9 C-terminal tail and a  
636 glutamate side chain coming from a symmetry-related molecule in the protein crystal. This second  
637 type of metal-binding site has not been observed in the structures of  $Mn^{2+}$ - or  $Ni^{2+}$ -bound human  
638 S100A8/A9 (Damo et al., 2013; Gagnon et al., 2015; Nakashige et al., 2017; Nakashige et al.,  
639 2018). Moreover, it has been proposed that S100A8/A9 also uses the octahedral His<sub>6</sub> site to chelate  
640 zinc (Nakashige et al., 2016).

641 The accurate mechanism and stoichiometry of zinc chelation by the S100A9 homodimer has not  
642 been characterized yet. It was reported that a truncated version of mS100A9 encompassing only  
643 the first 102 residues (i.e. lacking the three His residues from the C-terminal tail), binds  
644 substantially less zinc than the WT protein (Raftery et al., 1999). More recently, Harms and  
645 coworkers proposed a biphasic zinc binding mode for the hS100A9 homodimer (unpublished  
646 results). In their model, both the His20/Asp30/His91/His95 tetrad and the C-terminal histidines  
647 may contribute to zinc chelation, possibly through two distinct binding sites (Loes et al., 2019).  
648 These data are consistent with our structural observations of two independent Zn-sites per  
649 mS100A9 protomer but they still require confirmation. On the other hand, our ITC experiments  
650 (Figs. 3A-B) suggest that in solution, only one zinc ion is bound per mS100A9 subunit, with an  
651 affinity much lower than that reported for human calprotectin (Kehl-Fie et al., 2011; Brophy et al.,  
652 2012). If the affinity of the second Zn-site is even lower or if the binding is entropy driven, we  
653 may not detect it by ITC. In conclusion, we cannot evidence the existence of a second Zn-binding  
654 site per mS100A9 protomer in solution, but our structural data still highlight that such a site is  
655 geometrically possible. Whether this site is a crystallization artifact or whether it can be formed *in*  
656 *vivo* remains to be determined. This Zn-site may not exist in the context of the sole mS100A9  
657 homodimer, especially knowing that one of the coordinating residues would be missing. It could

658 for example be pertinent for mS100A9 interaction with specific binding partners, in particular  
659 those whose interaction has been described as zinc-dependent (Bjork et al., 2009).

660 The non-covalent mS100A9 homodimers we observe both in our crystal structure and in solution,  
661 through MS analysis, all contain an intramolecular bridge in each S100A9 subunit, between Cys91  
662 and Cys111. This finding correlates with older reports mentioning an intramolecular SS bridge in  
663 the protein (Raftery et al., 1998). It remains however uncertain whether all these non-covalent  
664 forms display a canonical S100A9/S100A9 arrangement, as reported in our crystallographic  
665 models. Remarkably, the Cys91-Cys111 bond leads the otherwise flexible C-terminal tail to adopt  
666 a rigidified architecture by folding back over the tip of helix H4. This mechanism seems to be zinc-  
667 independent since the disulfide bond pattern of the non-covalent homodimers is not affected by  
668 divalent cations. As a consequence, mS100A9 C-terminal tail adopts a conformation unique to the  
669 murine protein, regardless of the ions present. Behaviourial differences between murine and  
670 human S100 proteins have been described for several members of the family, including S100A8  
671 (Lackmann et al., 1993; Lim et al., 2009). This is also the case for S100A9: hS100A9 is generally  
672 considered as pro-inflammatory, especially in a cancer context (Gebhardt et al., 2006), whereas  
673 mS100A9 may rather display anti-inflammatory properties (Dale et al., 2006; Otsuka et al., 2009;  
674 Gomes et al., 2013; Wang et al., 2018b). These latter properties were attributed to mS100A9 C-  
675 terminal tail, which can inhibit hyperalgesia by modulating calcium channel signaling in sensory  
676 neurons (Dale et al., 2009). However, the mS100A9 C-terminal peptide used in these studies  
677 lacked both cysteines involved in the intramolecular SS-bridge. Consequently, it would not adopt  
678 the hairpin-like 3D-fold we observe in our structures. Modulation of the 3D-architecture of  
679 S100A9 C-terminal tail should therefore be taken into account when designing peptides with more  
680 potent antinociceptive effects. Furthermore, the unique architecture of mS100A9 C-terminal tail

681 should be kept in mind when comparing the physiological properties of hS100A9 and mS100A9.  
682 While the interconversion between pro- and anti-inflammatory functions for S100A9 is possibly  
683 influenced by both the pathological and the cell contexts, differential properties observed between  
684 human and murine S100A9 may also be inherent to their distinct 3D-architectures, especially in  
685 their C-terminal region known to be critical for effector binding.

686 Formation of disulfide-crosslinked S100A9 homodimers has already been described in the  
687 literature (Shibata et al., 2004). Our MS data provide novel insights into these disulfide-crosslinked  
688 species and how divalent cations may influence their formation. Indeed, we observed that the SS-  
689 linked homodimers bear one inter-subunit disulfide bridge, which strongly relies on Cys111, and  
690 a second SS bond, either inter- or intramolecular, which is lost when zinc is added or when Cys80  
691 or Cys91 are mutated. It is not straightforward to reconcile all these observations in a single model.  
692 The Cys111-Cys111 interaction is the sole inter-subunit SS bridge consistent with a canonical  
693 S100A9 homodimer (Fig. 7B). Other inter-subunit interactions would require a substantial  
694 repositioning of the two S100A9 protomers with respect to each other and/or conformational  
695 changes within each protomer. Other models for which the S100A9/S100A9 arrangement differs  
696 dramatically from the canonical configuration we observe in our crystal structures may also help  
697 describe these SS-linked homodimers. For example, some of these forms may adopt a two-fold  
698 symmetrical homodimeric arrangement, similar to the one we observed for S100A6 in complex  
699 with the full-length RAGE ectodomain (Yatime et al., 2016). Interestingly, we speculated at that  
700 time that the novel S100A6 conformation may be utilized by other S100 proteins, possibly  
701 stabilized by inter-subunit SS bond (Yatime et al., 2016; Yatime, 2017). The different SS-  
702 crosslinked mS100A9 dimers may also display other yet unraveled quaternary architectures. These  
703 models for the disulfide-crosslinked mS100A9 homodimer(s) are still speculative without



704 structural data. Nevertheless, our results clearly indicate that mS100A9 adopts multiple  
705 homodimeric arrangements that rely on distinct disulfide bond patterns and are modulated by the  
706 cations present. Local calcium/zinc concentrations may also influence the respective proportion of  
707 these forms, possibly through metal-induced crosslinking/oligomerization.

708 It becomes more and more evident that not only mS100A9, but also other S100 proteins exist *in*  
709 *vivo* as multiple forms, this plasticity helping understand how these proteins can achieve so many  
710 diverse and sometimes opposite functions. Disulfide-crosslinked homodimers have been reported,  
711 both *in vitro* and *in vivo*, for many other S100 proteins, including S100A2 (Yamaguchi et al.,  
712 2016), S100A4 (Haase-Kohn et al., 2011), S100A5 (Schafer et al., 2000), S100A6 (Wojda et al.,  
713 1994), S100A8 (Harrison et al., 1999), S100B (Winningham-Major et al., 1989), and the  
714 S100A8/A9 heterodimer (Stephan et al., 2018; Hoskin et al., 2019). None of these forms has been  
715 characterized from a structural point of view yet, due to the difficulty of isolating them *in vitro*.  
716 Nevertheless, evidence has accumulated suggesting that oxidation may contribute to the  
717 modulation of S100 function *in vivo*. In the case of the S100A8/A9 heterocomplex, both  
718 intramolecular and inter-subunit disulfide crosslinks have been identified (Stephan et al., 2018;  
719 Hoskin et al., 2019). The resulting SS-crosslinked complexes were shown *in vitro* to be more  
720 susceptible to protease degradation. This suggests a way to remove unnecessary S100A8/A9  
721 present in the extracellular space and thereby down-tune both the inflammatory and antimicrobial  
722 activities of the heterocomplex (Magon et al., 2015; Stephan et al., 2018; Hoskin et al., 2019).

723 An interplay between metal binding and oxidation-dependent regulation has also been reported for  
724 several S100 proteins. For example, disulfide crosslinking of S100A4 in the presence of copper  
725 was shown to enhance pro-inflammatory responses in the tumor microenvironment, through  
726 increased RAGE signaling (Haase-Kohn et al., 2011). Similarly, SS-linked S100B dimers promote

727 neuronal growth and proliferation of glial cells (Winningham-Major et al., 1989). S100B  
728 crosslinking is also induced by excess copper and may stimulate a toxic response, through nitric  
729 oxide production, in relevant neuropathological conditions (Matsui Lee et al., 2000). Cu<sup>2+</sup>-  
730 oxidized forms of S100A1, S100A2, S100A6, S100B and S100P were also described, leading to  
731 SS-crosslinked dimers at least for S100A2 (Yamaguchi et al., 2016). These forms were shown to  
732 modulate PP5 phosphatase activity, possibly impacting on apoptotic responses to oxidative stress.  
733 Copper-mediated oxidation of murine S100A8 also generated SS-crosslinked dimers that lost their  
734 chemotactic properties, thereby preventing leukocyte recruitment (Harrison et al., 1999).  
735 Conversely, SS bond formation may also regulate S100 metal binding properties. Formation of an  
736 intramolecular disulfide bridge within human S100A7 was shown to enhance metal sequestration  
737 by the canonical His3Asp Zn-binding site, through allosteric modulation, thereby increasing  
738 S100A7 antimicrobial activity (Cunden et al., 2017).

739 These examples demonstrate how subtle modulations of the 3D/4D-architecture of S100 proteins  
740 may dramatically affect their biological properties. Interestingly, reports also start emerging on  
741 possible crosslinking between molecules of RAGE, the cognate receptor for several members of  
742 the S100 family (Wei et al., 2012; Moysa et al., 2019). All these findings underline the importance  
743 of further studying S100 disulfide-crosslinked forms, in order to understand what is their function  
744 and which factors/mechanisms promote their formation *in vivo*. The in-depth characterization of  
745 these various forms and of their physiological functions will clearly be a prerequisite to propose  
746 efficient targeting strategies against S100 proteins in a context where their pro-inflammatory  
747 properties become deleterious, while preserving their beneficial functions such as their  
748 antimicrobial activity, these antagonistic effects being possibly orchestrated by distinct forms of  
749 the proteins.

750 **Accession codes**

751 The structure factors and atomic coordinates for the crystal structures of WT and C80A mS100A9  
752 in the presence of calcium and zinc have been deposited in the Protein Data Bank as entries 6ZDY  
753 and 6ZFE, respectively (<http://www.wwpdb.org/>).

754 **CRedit authorship contribution statement**

755 Luca Signor: Methodology, Investigation, Formal analysis, Visualization, Writing – original draft.  
756 Theo Paris: Investigation. Caroline Mas: Methodology, Investigation, Formal analysis,  
757 Visualization. Adrien Picard: Investigation. Georges Lutfalla: Funding acquisition. Elisabetta  
758 Boeri Erba: Methodology, Investigation, Formal analysis, Resources, Visualization, Writing –  
759 original draft. Laure Yatime: Conceptualization, Methodology, Investigation, Formal analysis,  
760 Supervision, Resources, Funding acquisition, Visualization, Writing – original draft.

761 **Declaration of Competing Interest**

762 The authors declare that they have no known competing financial interests or personal  
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## 778 **Appendix A. Supplementary data**

779 Supplementary data to this article can be found online.

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## 1204 **Figure legends**

1205 **Figure 1.** Structure of murine S100A9 in the presence of calcium and zinc. **(A)** General overview  
1206 of the structure of the  $\text{Ca}^{2+}/\text{Zn}^{2+}$ -bound murine S100A9 (mS100A9) homodimer at 1.45 Å  
1207 resolution. The two mS100A9 subunits are shown in beige and purple. Calcium and zinc ions are  
1208 displayed as green and yellow spheres, respectively. **(B)** Final electron density map contoured at  
1209  $1\sigma$  and final model superimposed to assess the quality of the fit. **(C)** Anomalous difference Fourier  
1210 maps calculated using phases and weight from the best refined atomic model obtained with the  
1211 native dataset and anomalous differences from the datasets collected at wavelengths of 1.27 Å  
1212 (blue mesh, contour at  $5\sigma$ ) and 1.30 Å (red mesh, contour at  $5\sigma$ ). The final model displayed as  
1213 cartoon is superimposed for comparison. **(D)** Superimposition of the mS100A9 protomer from the  
1214 X-ray structure of  $\text{Ca}^{2+}/\text{Zn}^{2+}$ -bound mS100A9 homodimer (beige, this study) with the hS100A9  
1215 moiety from the NMR structure of apo-hS100A9 homodimer (red, PDB\_ID 5I8N (Chang et al.,  
1216 2016)), from the X-ray structure of  $\text{Ca}^{2+}$ -bound hS100A9 homodimer (cyan, PDB\_ID 1IRJ (Itou  
1217 et al., 2002)) and from the X-ray structure of  $\text{Ca}^{2+}/\text{Mn}^{2+}$ -bound calprotectin heterocomplex (grey,  
1218 PDB\_ID 4GGF (Damo et al., 2013)). Superimposition was performed on C-alpha atoms  
1219 (mS100A9 residues Glu10 to Leu83). **(E)** Superimposition of the  $\text{Ca}^{2+}/\text{Zn}^{2+}$ -bound mS100A9  
1220 homodimer (beige, this study) with apo (red, PDB\_ID 5I8N (Chang et al., 2016)) and  $\text{Ca}^{2+}$ -bound

1221 hS100A9 homodimer (cyan, PDB\_ID 1IRJ (Itou et al., 2002)). All 3 homodimers are  
1222 superimposed on subunit A (superimposition on C-alpha atoms, mS100A9 residues Glu10 to  
1223 Leu83). **(F)** Superimposition of the Ca<sup>2+</sup>/Zn<sup>2+</sup>-bound mS100A9 homodimer (beige, this study)  
1224 with Ca<sup>2+</sup>-bound (grey, PDB\_ID 1MR8 (Chang et al., 2016)) and Ca<sup>2+</sup>/Zn<sup>2+</sup>-bound hS100A8  
1225 homodimer (purple, PDB\_ID 5HLO (Itou et al., 2002)). All 3 homodimers are superimposed on  
1226 subunit A (superimposition on C-alpha atoms, mS100A9 residues Glu10 to Leu83).

1227 **Figure 2.** The two Zn-binding modes of mS100A9: comparison with hS100A9 and influence on  
1228 the conformation of the C-terminal tail. **(A)** Zoom on the first type of Zn<sup>2+</sup>-binding site of  
1229 mS100A9, close to the first Ef-hand motif of subunit A. Anomalous difference Fourier maps  
1230 calculated using phases and weight from the best refined atomic model (without ions) obtained  
1231 with the native dataset and anomalous differences from datasets collected at different wavelengths  
1232 are shown as blue mesh ( $\lambda = 1.27 \text{ \AA}$ , contour at  $3.5 \sigma$ ) or red mesh ( $\lambda = 1.30 \text{ \AA}$ , contour at  $3.5 \sigma$ ).  
1233 Residues involved in zinc coordination are shown as sticks. **(B)** Sequence alignment of the C-  
1234 terminal region (Helix H4 + C-terminal tail) of murine and human S100A9. The alignment was  
1235 performed in Clustal Omega (Sievers et al., 2018; Madeira et al., 2019). The secondary structure  
1236 is displayed schematically above the alignment. The residue numbering indicated below the  
1237 alignment corresponds to mS100A9 sequence. Identical residues between the two sequences are  
1238 underlined in dark green whereas conserved mutations are highlighted in light green. The three  
1239 cysteines of mS100A9 are indicated with a red star. Histidine residues involved in zinc  
1240 coordination are marked with orange (Zn-binding site 1) or blue (Zn-binding site 2) triangles for  
1241 mS100A9 and with orange spheres (Zn-binding site 1) for hS100A9. **(C)** Same as panel **A** but with  
1242 the close-up view centered on the second type of Zn<sup>2+</sup>-binding site of mS100A9, within the C-  
1243 terminal tail of subunit A. **(D)** Overview of the C-terminal region of mS100A9 encompassing helix



1244 H4 and the C-terminal tail closing around the second Zn<sup>2+</sup> binding site. A disulfide bridge connects  
1245 Cys91 and Cys111 while Cys80 remains free.

1246 **Figure 3.** Biophysical investigation of zinc binding to the mS100A9 homodimer. **(A)** ITC titrations  
1247 and binding isotherms fitted with a one-site model for the binding of Zn<sup>2+</sup> to apo-mS100A9.  
1248 Experiments were performed at least in duplicates. The measured K<sub>d</sub> value and stoichiometry for  
1249 each particular experiment are indicated on the graph. **(B)** Same as panel **A** but with Ca<sup>2+</sup>-bound  
1250 mS100A9 homodimer. **(C)** DLS analysis of apo-mS100A9 in the presence of increasing  
1251 concentrations of zinc. Below 3 molar equivalents of zinc, mS100A9 remains homodimeric. At 3  
1252 or more equivalents, mS100A9 readily aggregates and forms large oligomers. **(D)** Same as panel  
1253 **C** but with Ca<sup>2+</sup>-bound mS100A9.

1254 **Figure 4.** Mass spectrometry analysis of WT mS100A9 in the absence of divalent cations. **(A)**  
1255 LC/ESI-TOF-MS deconvoluted spectrum of untreated WT mS100A9. The protein displays two  
1256 peaks, one at a mass of 13175.1 Da referred to as the “monomeric” peak (M), and one at 26349.5  
1257 Da referred to as the “dimeric” peak (D). **(B)** and **(C)** Native MS spectra of WT mS100A9 either  
1258 untreated (panel B) or after treatment with DTT (panel C) prior to MS analysis. Interestingly,  
1259 mS100A9 carries less charges in the presence of disulfide bridges. Specifically, the highest peaks  
1260 of disulfide-bond containing mS100A9 monomeric and dimeric forms are 7+ and 10+, respectively  
1261 (panel B). On the contrary, the highest peaks of the DTT-treated mS100A9 monomers and dimers  
1262 are 8+ and 11+, respectively (panel C). These differences in charge reflect the higher degree of  
1263 compactness of the disulfide cross-linked mS100A9 protein. **(D)**, **(E)** and **(F)** LC/ESI-TOF-MS  
1264 deconvoluted spectra of WT mS100A9 incubated with either DTT alone (DTT, panel D),  
1265 iodoacetamide alone (IAA, panel E) or DTT + IAA (panel F) prior to MS analysis. **(G)**  
1266 Superimposition of the LC/ESI-TOF-MS deconvoluted spectra obtained for WT mS100A9

1267 without any treatment (upper spectrum), incubation with IAA alone (middle spectrum) or with  
1268 DTT + IAA (lower spectrum). The spectra are centered on the region corresponding to the  
1269 “monomeric” peak. **(H)** Superimposition of the LC/ESI-TOF-MS deconvoluted spectra obtained  
1270 for WT mS100A9 without any treatment (upper spectrum) or incubation urea + DTT + IAA (lower  
1271 spectrum). The spectra are centered on the region corresponding to the “monomeric” peak. **(I)**  
1272 Superimposition of the LC/ESI-TOF-MS deconvoluted spectra obtained for WT mS100A9  
1273 without any treatment (upper spectrum) or after incubation with IAA alone (lower spectrum). The  
1274 spectra are centered on the region corresponding to the “dimeric” peak. The relative proportions  
1275 of the “monomeric” and “dimeric” peaks (peak area expressed in %) are indicated on the  
1276 corresponding graphs where appropriate.

1277 **Figure 5.** Mass spectrometry analysis of WT mS100A9 in the presence of divalent cations. **(A)**  
1278 and **(B)** LC/ESI-TOF-MS and native MS spectra of untreated WT mS100A9 in the presence of 1  
1279 mM CaCl<sub>2</sub>. **(C)** and **(D)** LC/ESI-TOF-MS and native MS spectra of untreated WT mS100A9 in  
1280 the presence of 1 mM ZnCl<sub>2</sub>. **(E)** and **(F)** LC/ESI-TOF-MS and native MS spectra of untreated  
1281 WT mS100A9 in the presence of 1 mM CaCl<sub>2</sub> + 1 mM ZnCl<sub>2</sub>. The relative proportions of the  
1282 “monomeric” and “dimeric” peaks (peak area expressed in %) are indicated on the LC/ESI-TOF-  
1283 MS spectra.

1284 **Figure 6.** Mass spectrometry analysis of the Cys-to-Ala mutants of mS100A9 in the absence or in  
1285 the presence of divalent cations. **(A)** SDS-PAGE analysis of WT and mutant mS100A9 in the four  
1286 different ionic conditions tested in the study. The sample deposited in each lane is indicated above  
1287 the gels. **(B)** Superimposition of the LC/ESI-TOF-MS deconvoluted spectra obtained for untreated  
1288 C111A mutant in the absence of ions (upper spectrum), in the presence of 1 mM CaCl<sub>2</sub> (upper  
1289 middle spectrum), in the presence of 1 mM ZnCl<sub>2</sub> (lower middle spectrum), or in the presence of

1290 1 mM CaCl<sub>2</sub> + 1 mM ZnCl<sub>2</sub> (lower spectrum). (C) Same as panel B but for mutant C91A. (D)  
1291 Same as panel B but for mutant C80A.

1292 **Figure 7.** Structure of Ca<sup>2+</sup>/Zn<sup>2+</sup>-bound mS100A9 C80A and possible models for the disulfide-  
1293 crosslinked mS100A9 homodimer. (A) General overview of the structure of the Ca<sup>2+</sup>/Zn<sup>2+</sup>-bound  
1294 murine S100A9 C80A mutant (mS100A9-C80A) homodimer at 2.35 Å resolution. The two  
1295 mS100A9-C80A subunits are shown in beige and purple. Calcium and zinc ions are displayed as  
1296 green and yellow spheres, respectively. The zinc coordinating residues are depicted as sticks. The  
1297 structure of the WT mS100A9 protein determined in this study has been superimposed for  
1298 comparison (dark blue cartoon). (B) Canonical homodimer conformation of mS100A9 as observed  
1299 in our crystal structure. In this configuration, only the two Cys111 from each monomer may reach  
1300 out to form an intramolecular SS bridge although they are separated by more than 50Å, due in part  
1301 to the side chains protruding from both helices H4. Inter-subunit interaction of Cys111 with Cys80  
1302 or Cys91 from the other subunit would require partial unfolding of the second S100A9 protomer  
1303 (interactions indicated with a question mark).

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	mS100A9 (WT) (native)	mS100A9 (WT) (anomalous)	mS100A9 (WT) (anomalous)	mS100A9 (C80A) (native)
<b>Data collection</b>				
Diffraction source	ID23eh1, ESRF	ID23eh1, ESRF	ID23eh1, ESRF	X06DA, SLS
Wavelength (Å)	1.27	1.27	1.30	1.28
Space group	C2	C2	C2	C2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	72.84, 40.62, 53.09	72.84, 40.62, 53.09	72.97, 40.65, 53.17	73.61, 38.80, 53.17
$\alpha$ , $\beta$ , $\gamma$ (°)	90, 125, 90	90, 125, 90	90, 124.99, 90	90, 124.75, 90
Mosaicity (°)	0.28	0.28	0.28	0.41
Resolution range (Å)	80 – 1.45 (1.51 – 1.45)	80 – 1.45 (1.51 – 1.45)	80 – 1.5 (1.57 – 1.5)	80 – 2.35 (2.44 – 2.35)
Total No. of reflections	141507 (13504)	143258 (13475)	130241 (14794)	34129 (3573)
No. of unique reflections	22267 (2406)	43051 (4657)	39043 (4809)	5224 (550)
Completeness (%)	97.6 (92.8)	96.9 (91.3)	96.9 (93.9)	98.5 (98.6)
Redundancy	6.35 (5.6)	3.3 (2.9)	3.3 (3.1)	6.5 (6.5)
<i>I</i> / $\sigma$ ( <i>I</i> )	14.57 (1.93)	14.18 (1.52)	16.31 (1.57)	8.73 (2.33)
<i>R</i> <sub>meas</sub>	5.9 (78.2)	4.4 (77.1)	3.8 (78.6)	13.2 (84.3)
Wilson B factor (Å <sup>2</sup> )	30.3	30.3	32.4	43.5
<b>Refinement</b>				
Resolution range (Å)	43 – 1.45			43 – 2.35
No. of unique reflections	22210			5198
Final <i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub> (%)	17.57 / 19.95			26.80 / 30.54
<i>No. of non-H atoms</i>				
Protein	868			867
Ions	9			20
Water	97			6
Total	974			893
<i>R.m.s. deviations</i>				
Bonds (Å)	0.004			0.002
Angles (°)	0.720			0.461
<i>Average B factors (Å<sup>2</sup>)</i>				
Protein	38.7			66.8
Ions	27.1			76.3
Water	47.1			59.3
<i>Ramachandran plot</i>				
Favored regions (%)	99.06			99.06
Allowed regions (%)	0.94			0.94
Outliers (%)	0			0

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1314 **Table 1.** Data collection and refinement statistics.

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<b>Zn-ligand bond distances</b>			
<b>Zn site</b>	<b>Ligand</b>	<b>Bond distance (Å)</b>	<b>Monodentate vs Bidentate ligand</b>
<b>Zn1/Zn3</b>	His21	1.92	nr
	Asp31	1.89 / 2.81	bidentate
	His92	1.99	nr
	His96	2.01	nr
<b>Zn2/Zn4</b>	Glu65	1.90	monodentate
	His103	2.08	nr
	His105	2.06	nr
	His107	2.04	nr

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<b>Zn-ligand bond angles</b>			
<b>Zn site</b>	<b>Ligand 1</b>	<b>Ligand 2</b>	<b>Angle (°)</b>
<b>Zn1/Zn3</b>	His21	Asp31	124.3
	His21	His92	107.4
	His21	His96	109.1
	Asp31	His92	114.2
	Asp31	His96	95.4
	His92	His96	103.8
<b>Zn2/Zn4</b>	Glu65	His103	111.9
	Glu65	His105	106.8
	Glu65	His107	118.5
	His103	His105	114.2
	His103	His107	104.6
	His105	His107	100.6

1327 **Table 2. Zn<sup>2+</sup>-ligand bond distances and angles measured for the two different types of Zn-**1328 **binding sites encountered in mS100A9 crystal structure. nr: not relevant.**

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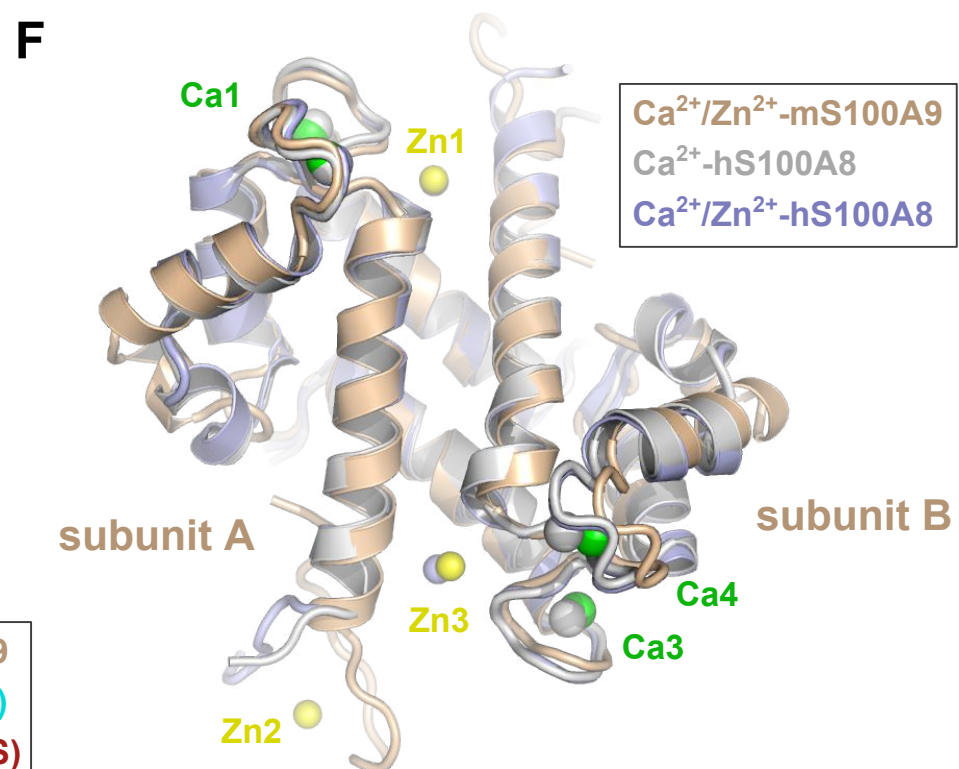
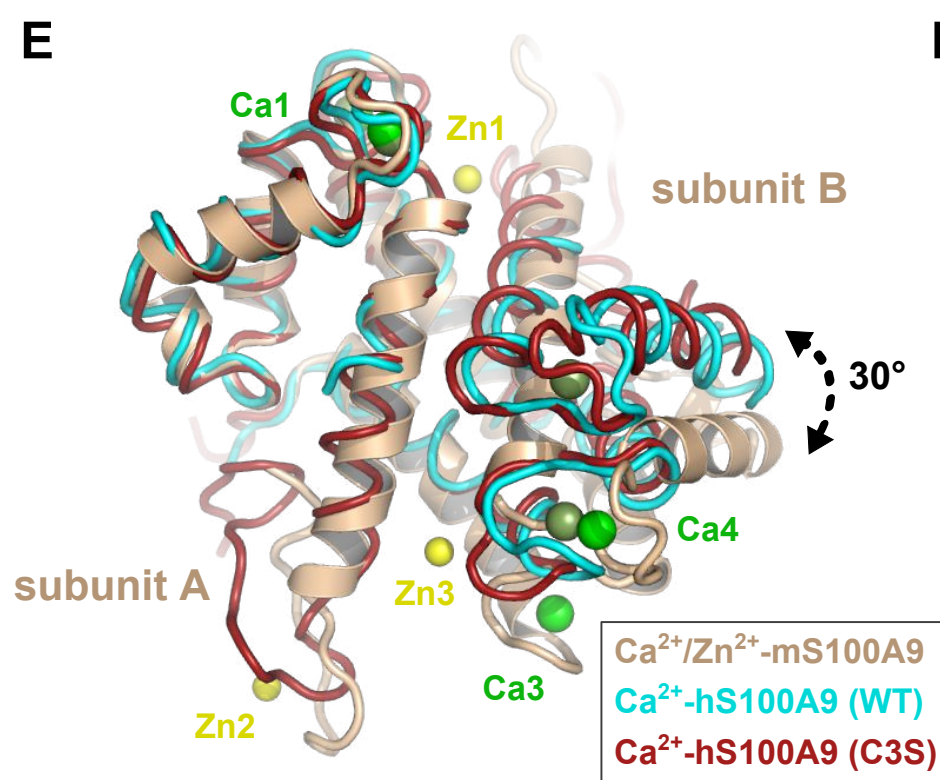
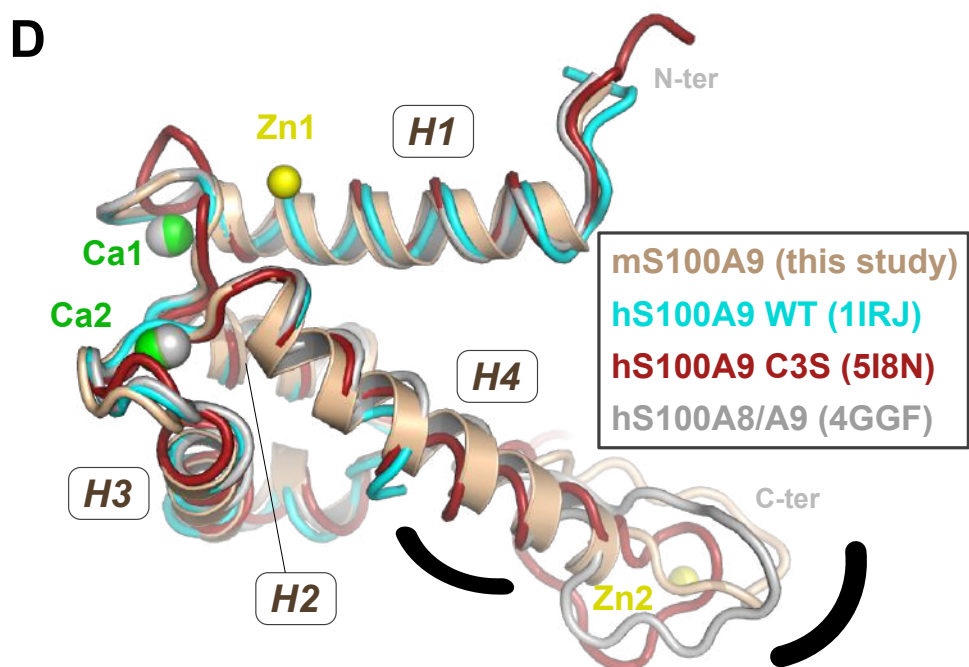
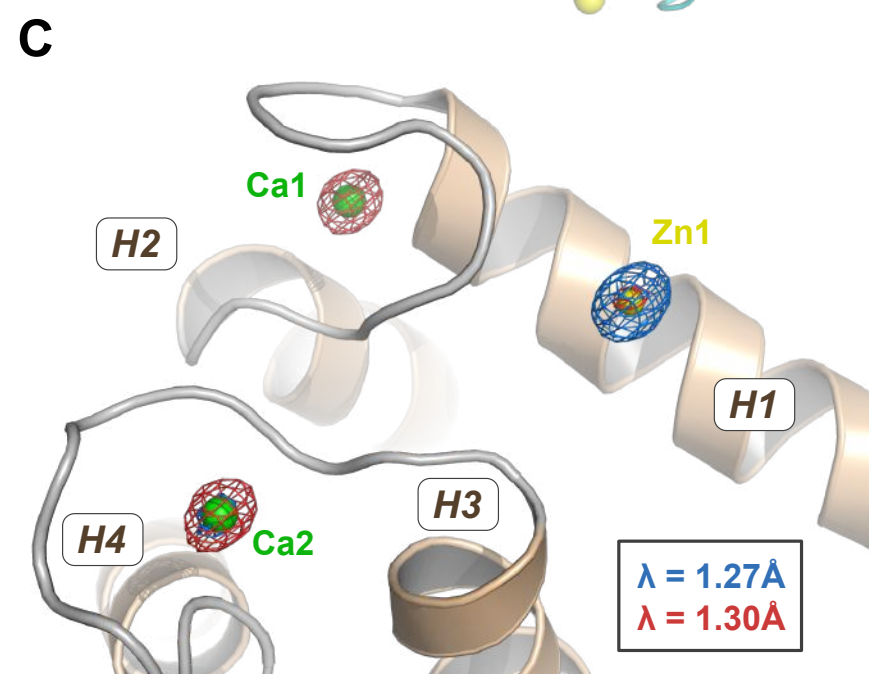
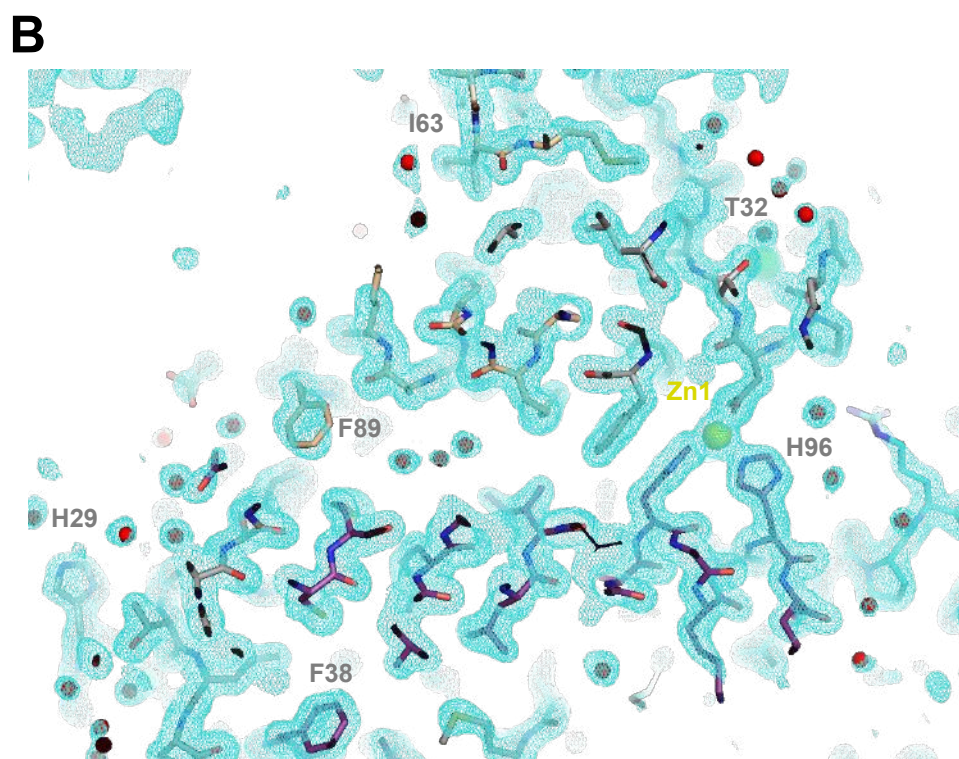
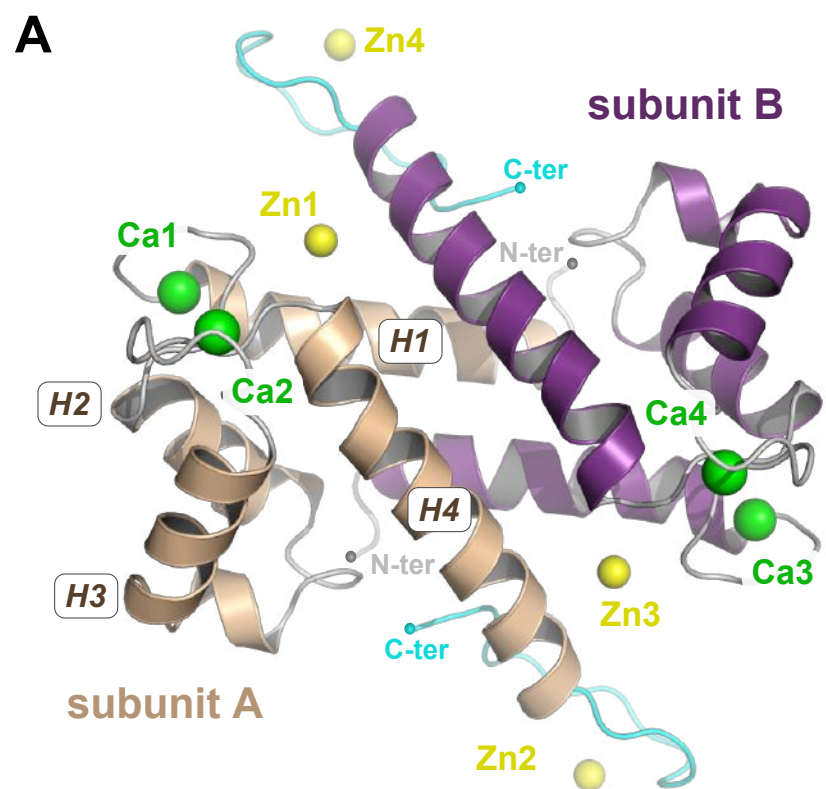
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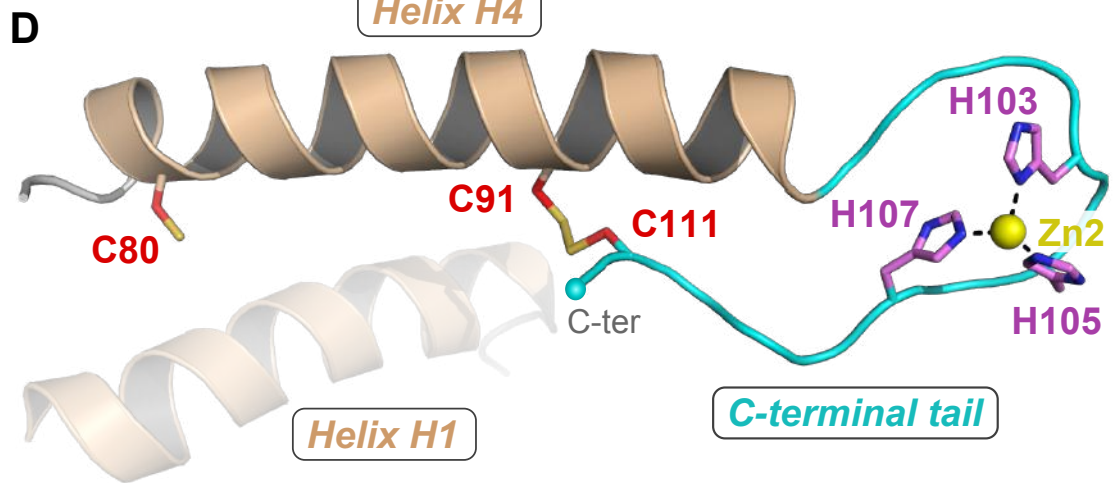
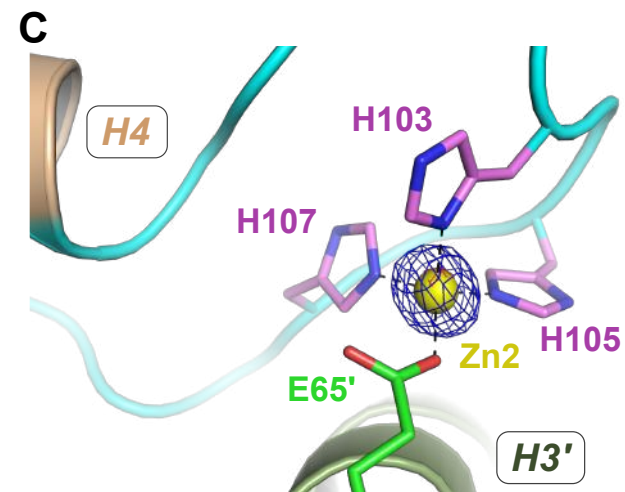
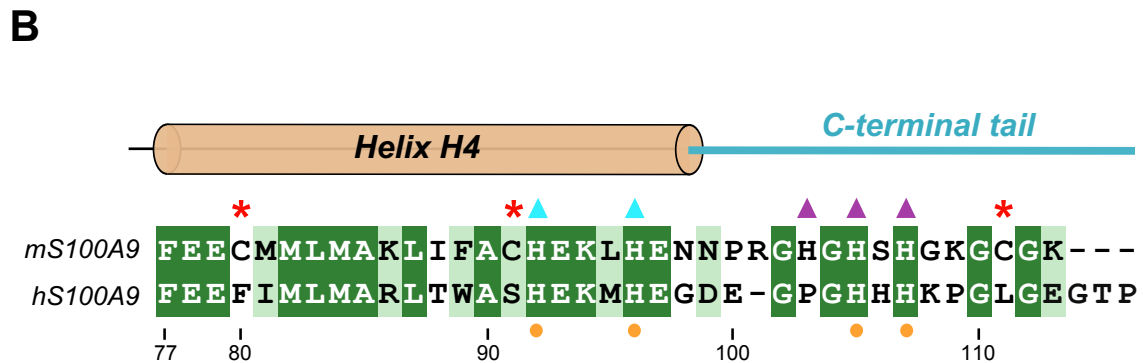
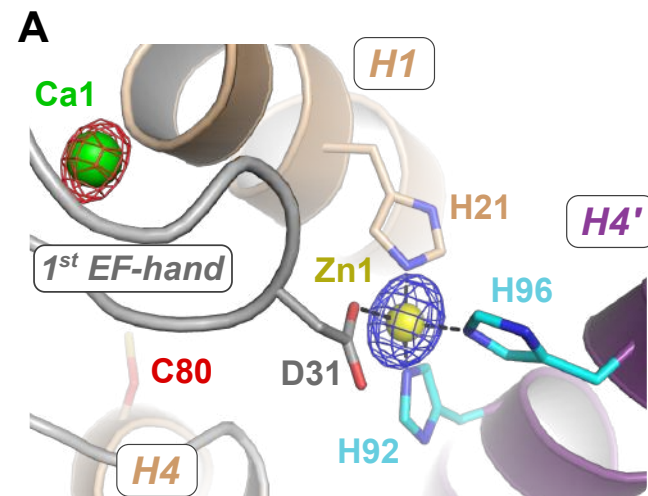
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Sample		Ca	Cr	Mn	Fe	Co	Ni	Cu	Zn
<b>Buffer 1</b>	ppb	151	0.061	Nd	Nd	0.122	4.760	0.554	1.787
	$\mu$ M	3.764	0.001	Nd	Nd	0.002	0.081	0.009	0.027
<b>mS100A9</b>	ppb	146	0.139	Nd	Nd	0.088	7.851	3.490	22.010
	$\mu$ M	3.644	0.003	Nd	Nd	0.001	0.133	0.055	0.336
	equiv./A9	0.018	1.34e-5	Nd	Nd	7.46e-6	7e-4	3e-4	0.002
<b>Control SLRS-6</b>									
<b>SLRS-6</b>	ppb	9161.5	0.260	2.218	85.513	0.059	0.612	25.76	1.960
<b>Certified values SLRS-6</b>	ppb	8770	0.252	2.12	84.5	0.053	0.617	24.0	1.76
	SD (ppb)	200	0.012	0.10	3.6	0.012	0.022	1.8	0.12

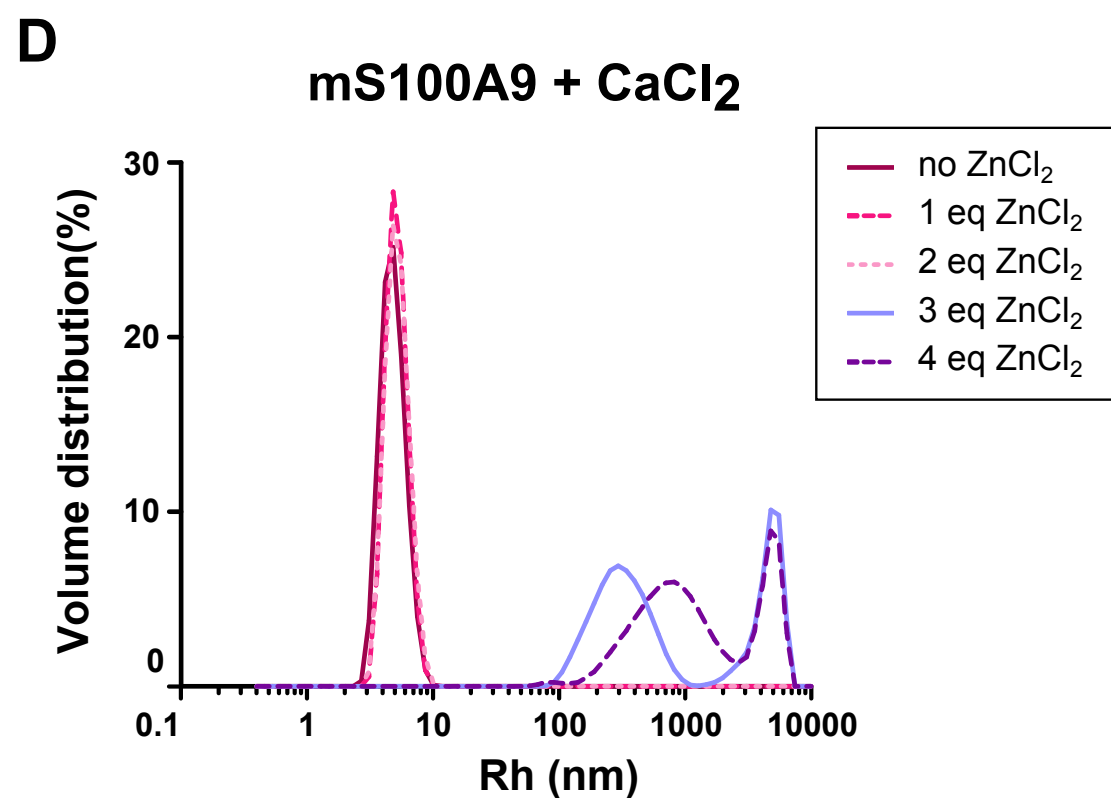
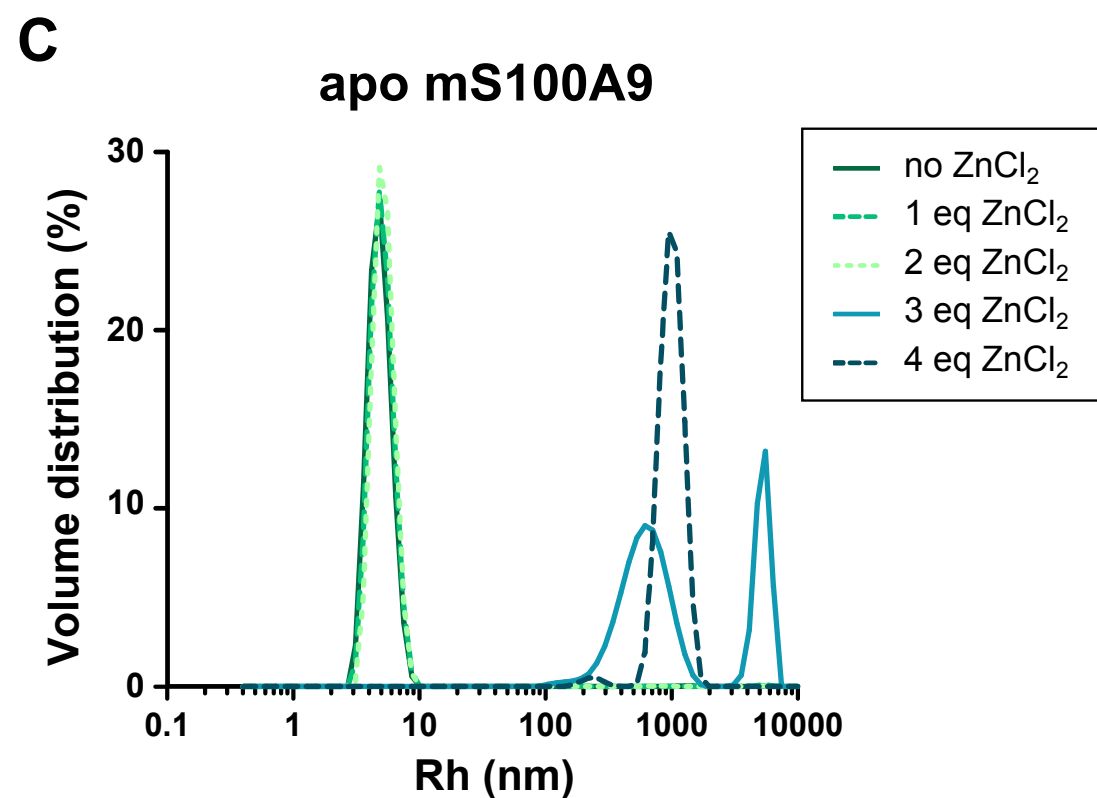
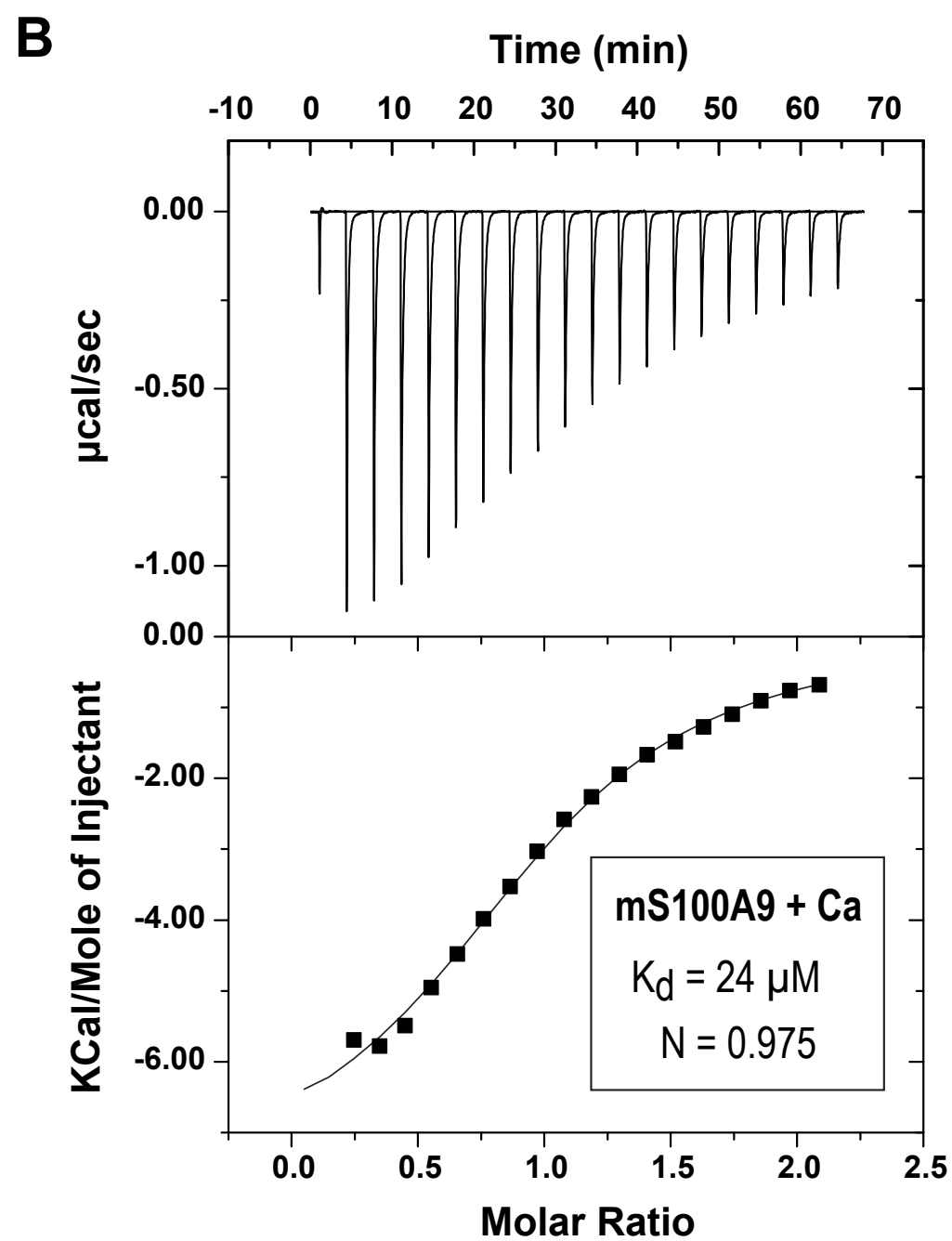
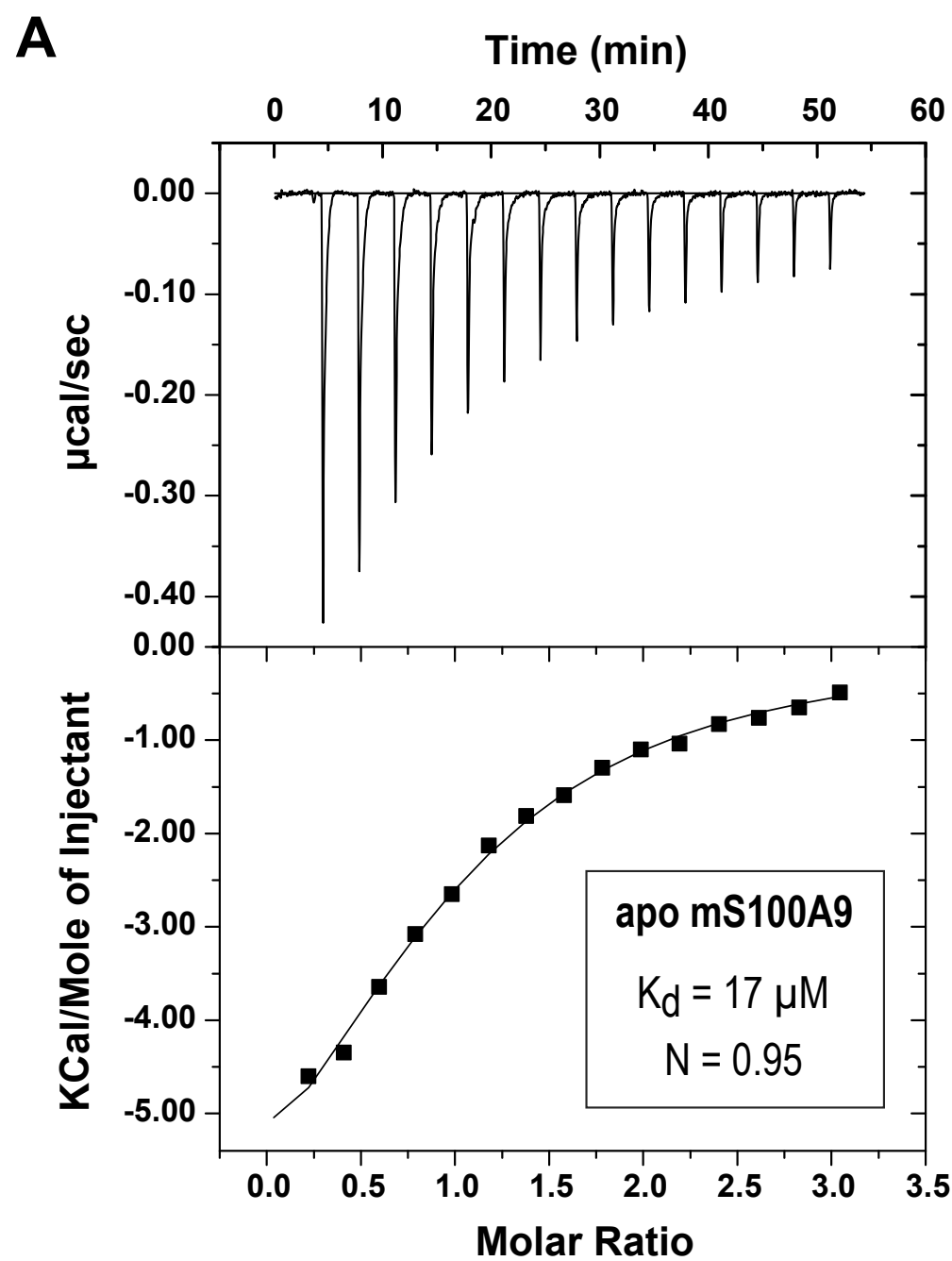
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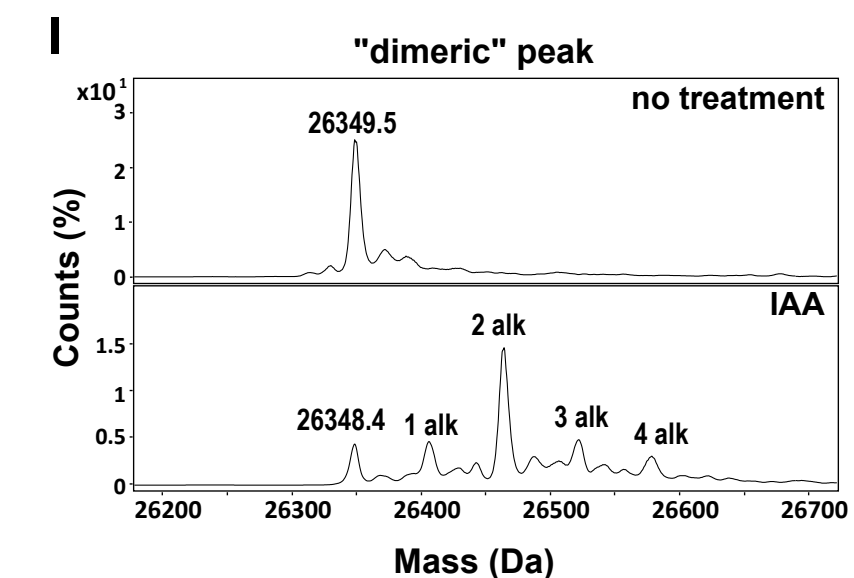
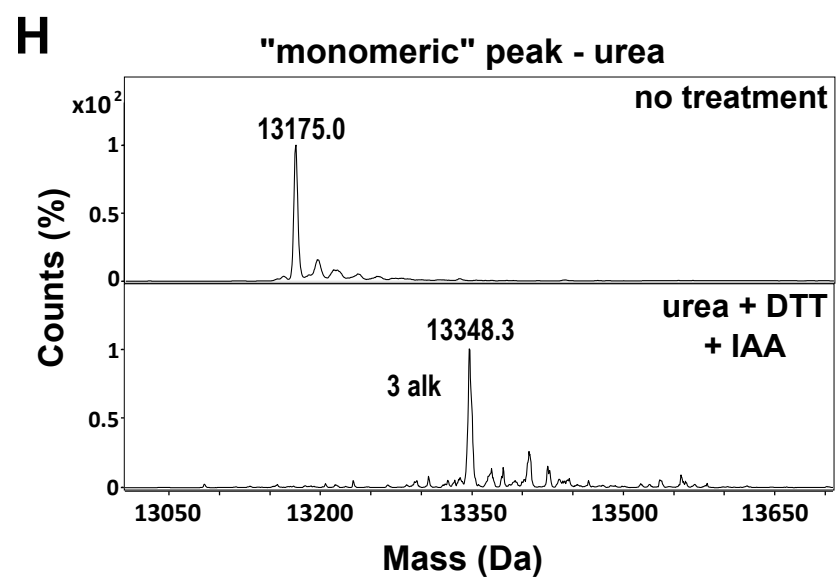
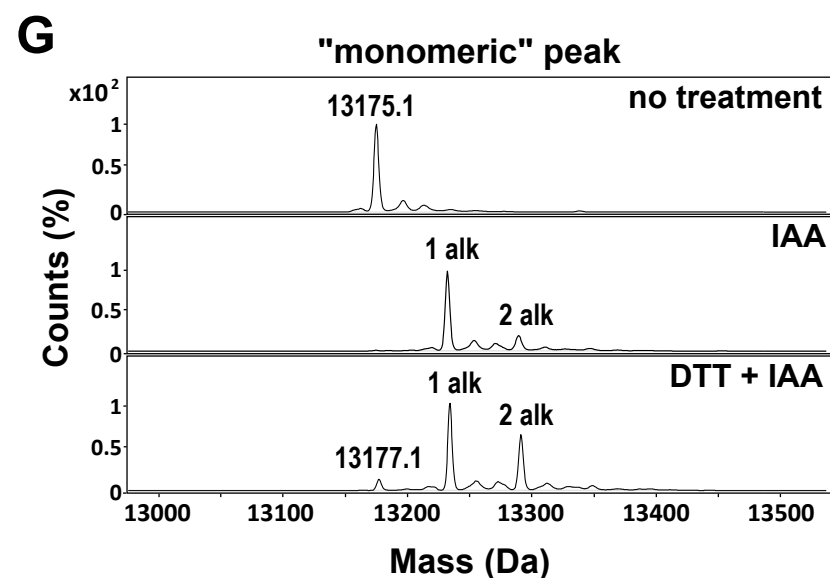
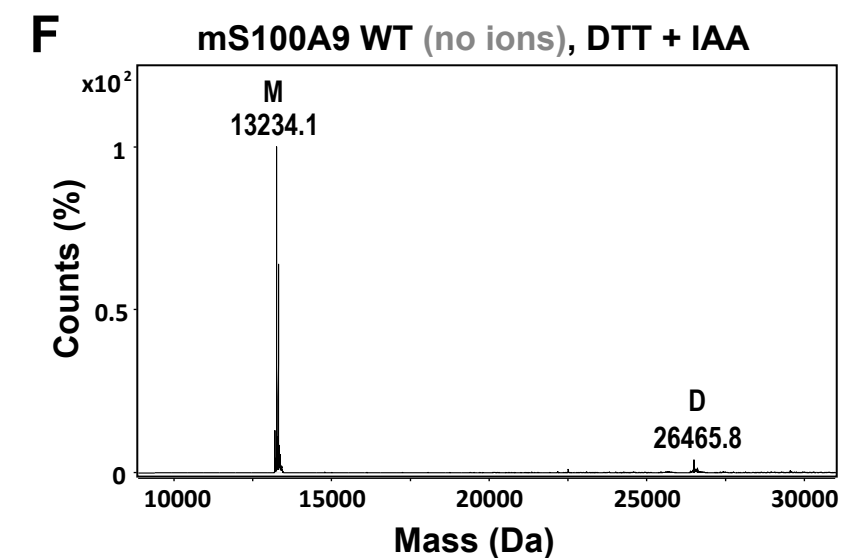
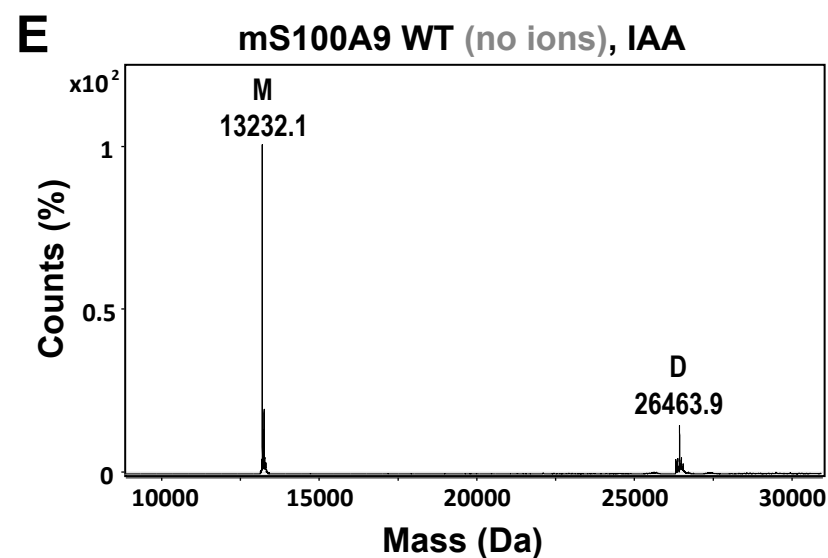
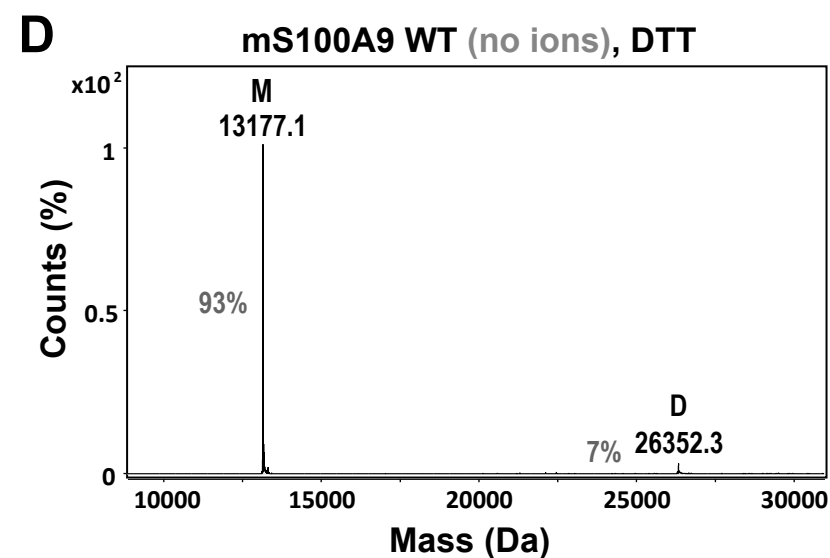
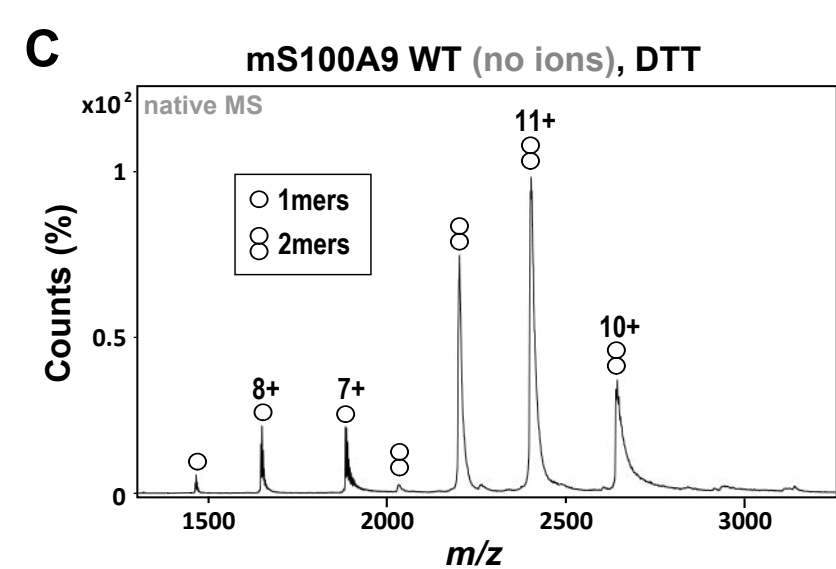
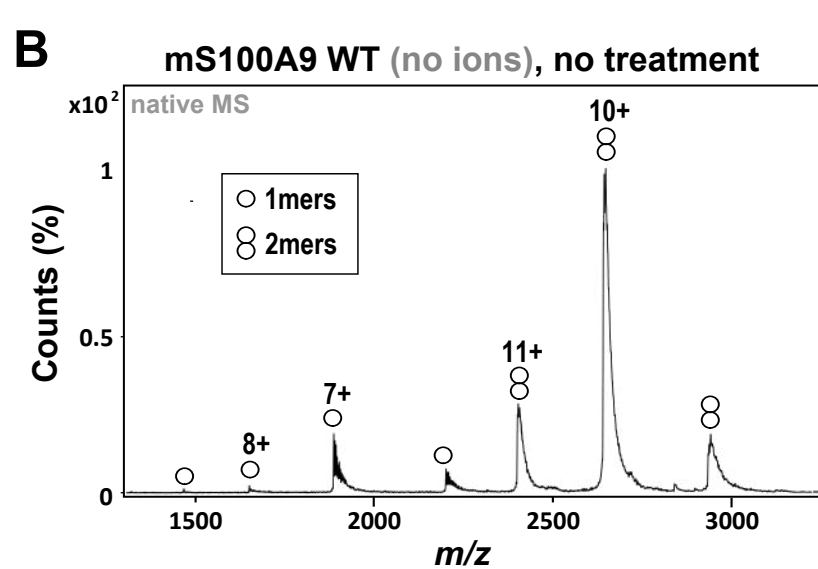
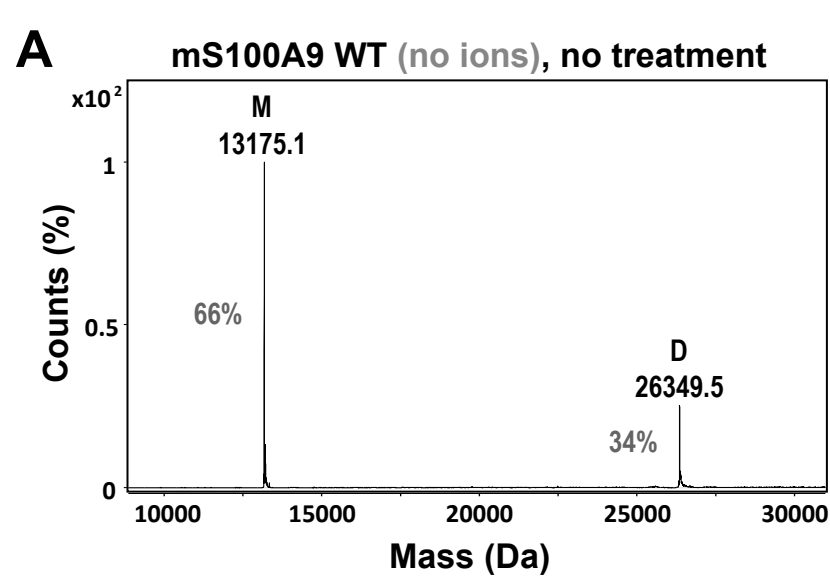
1336 **Table 3. Metal content of purified WT mS100A9 as analyzed by ICP-MS.** Each sample was  
1337 analyzed only once (averaging over 3 replicates). Metal content is given in parts per billion (ppb),  
1338 in concentration ( $\mu$ M), and as molar equivalents with respect to mS100A9. Nd indicates that the  
1339 concentration was below detection limit and could not be determined. Certified water SLRS-6 was  
1340 used as a control (both the values measured in our experiments, the certified values and the  
1341 standard deviation SD on certified values are indicated for SLRS-6).

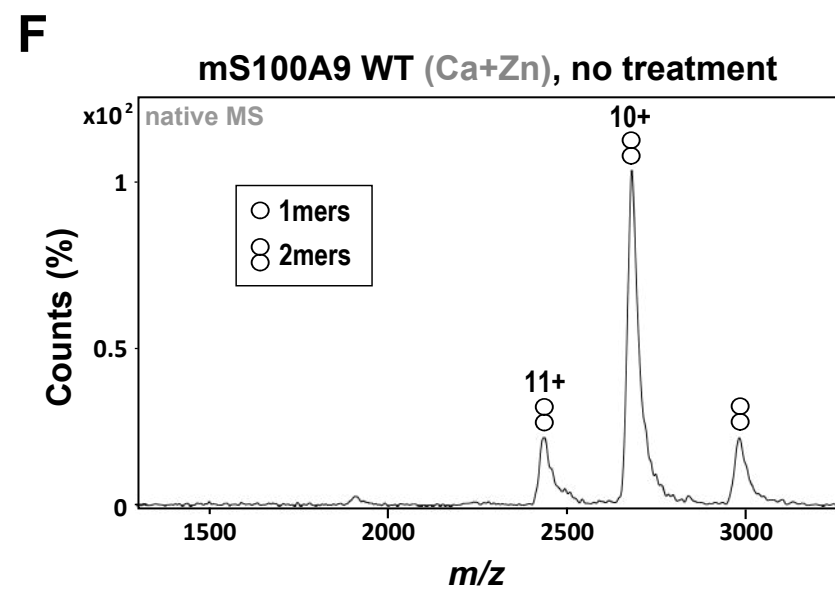
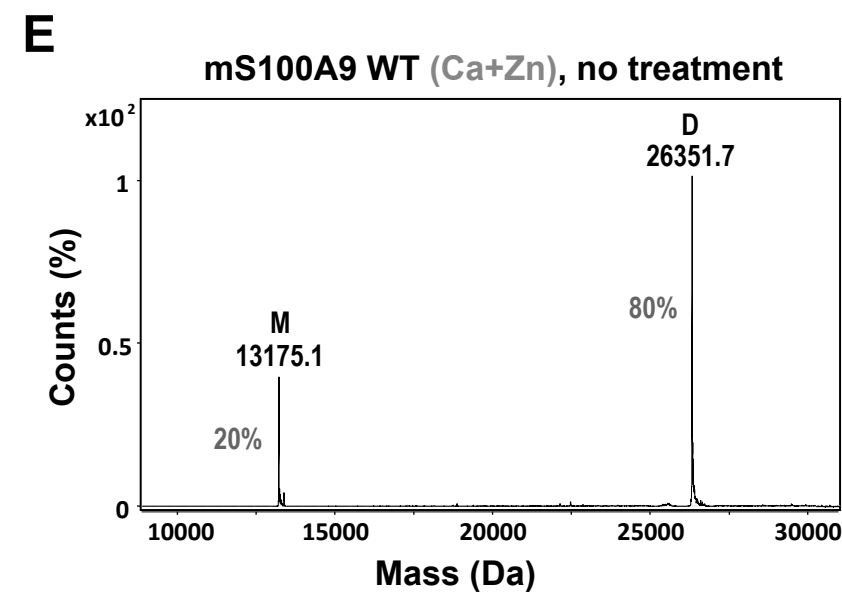
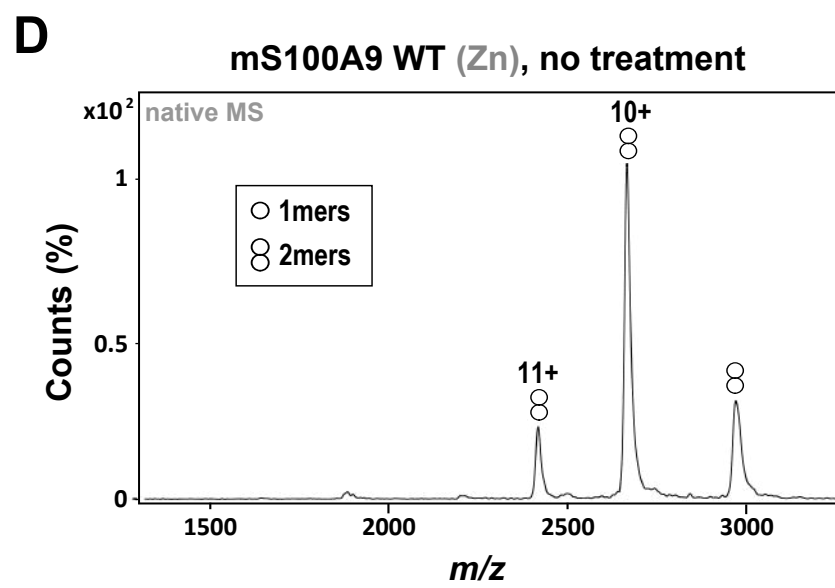
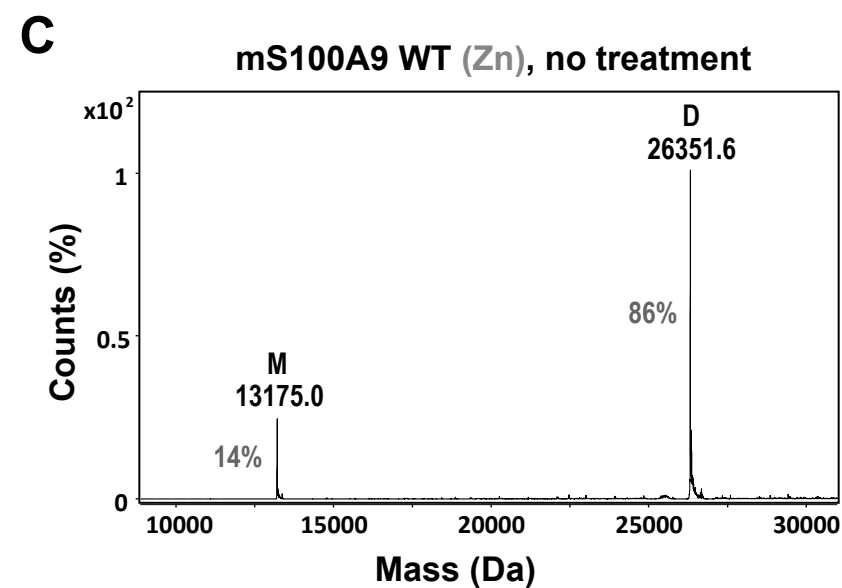
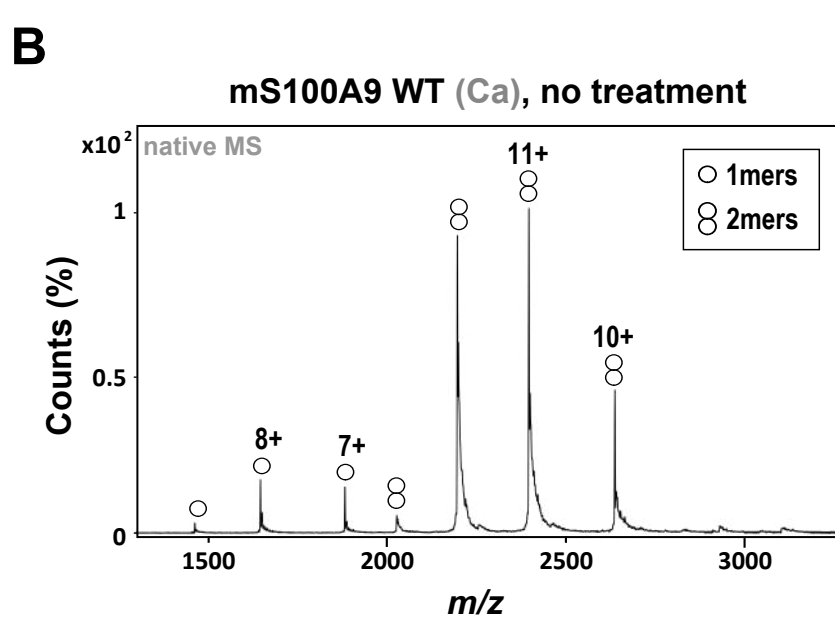
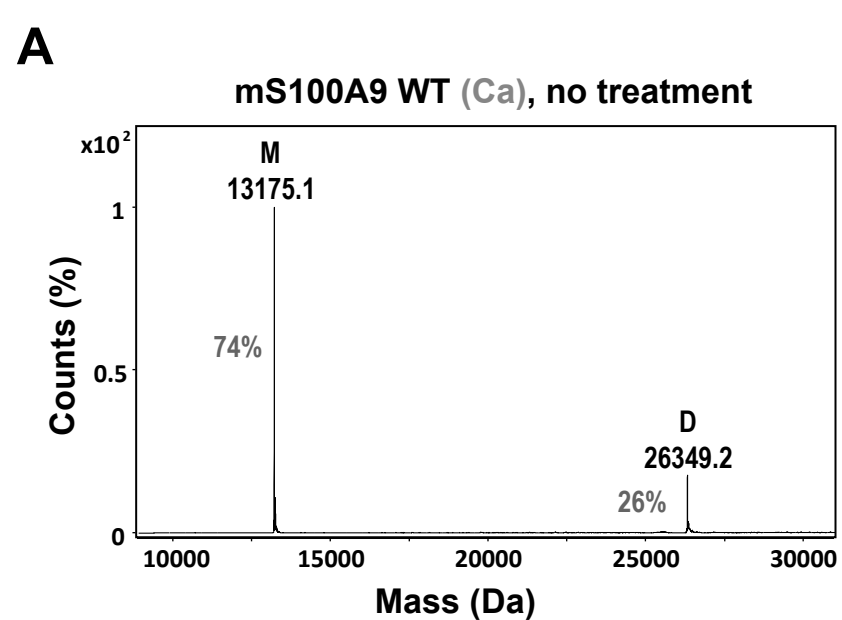


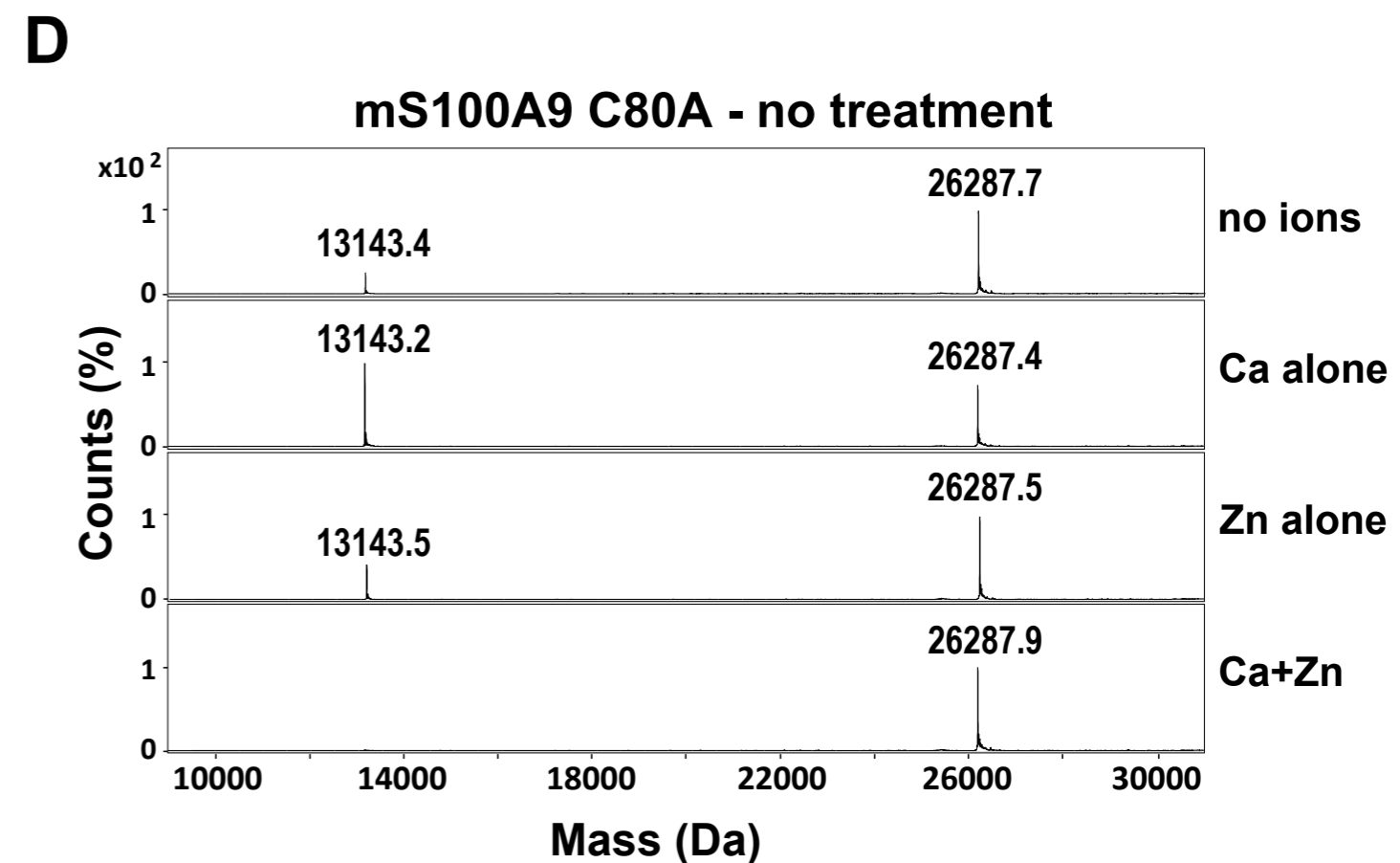
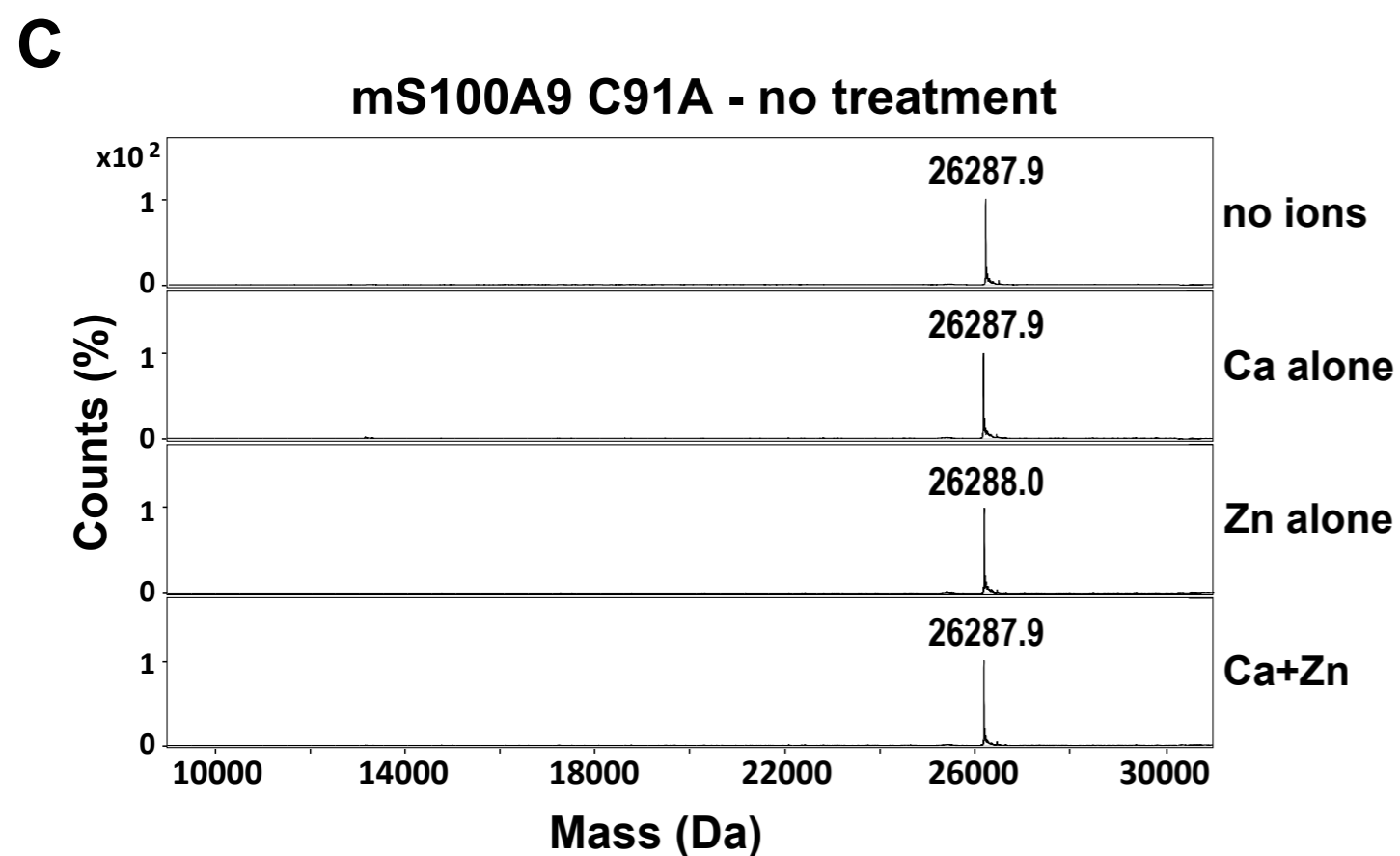
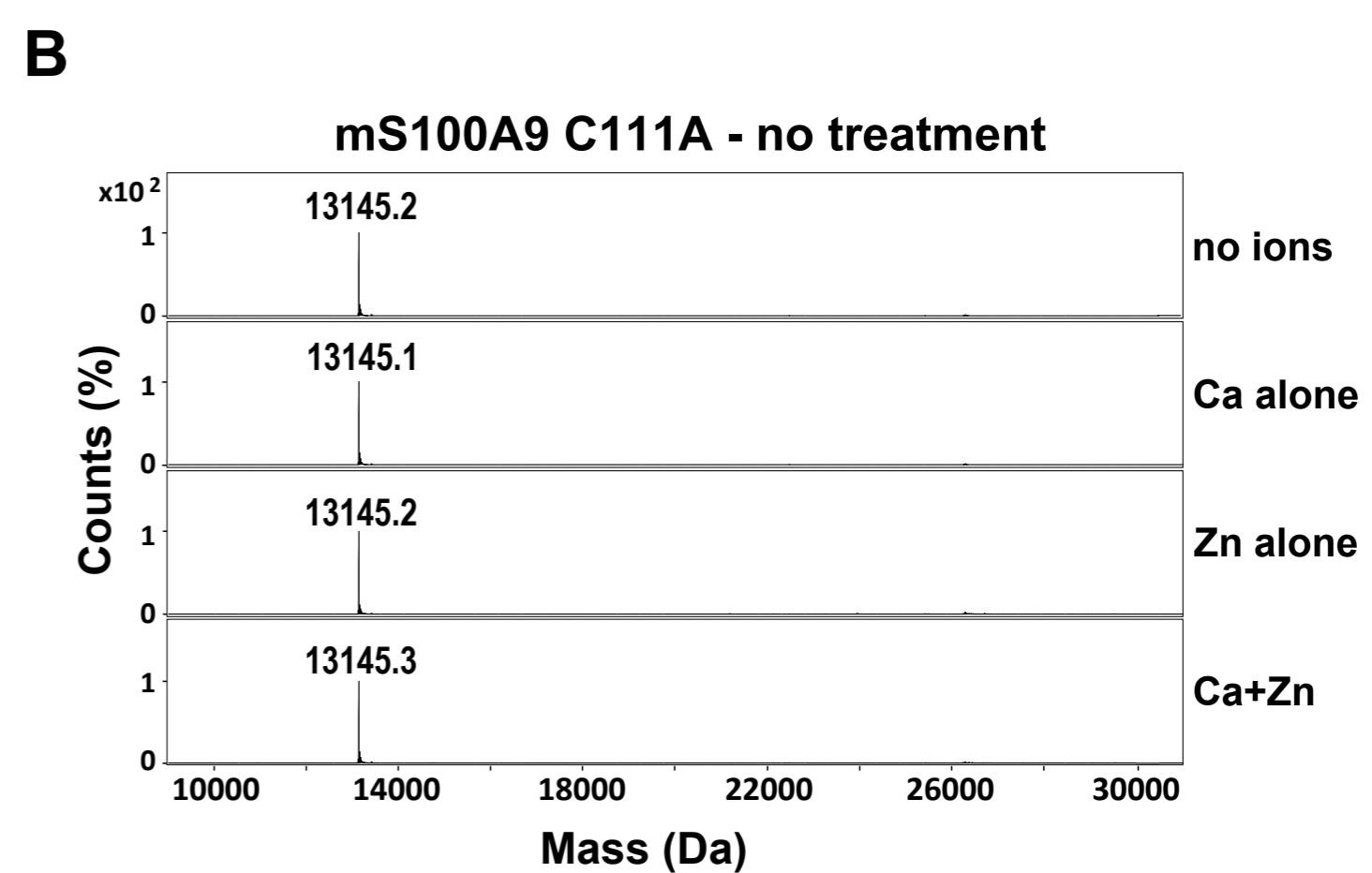
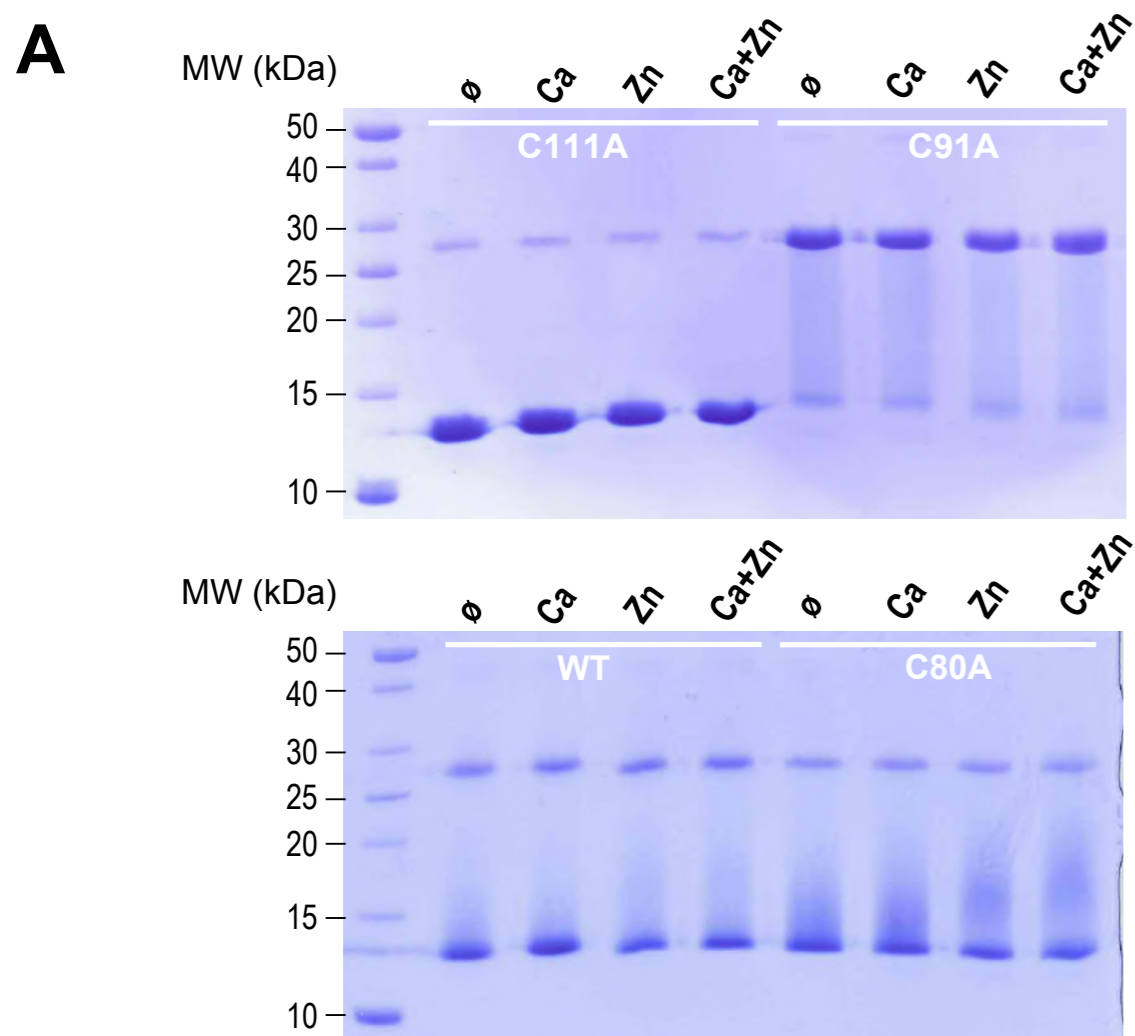


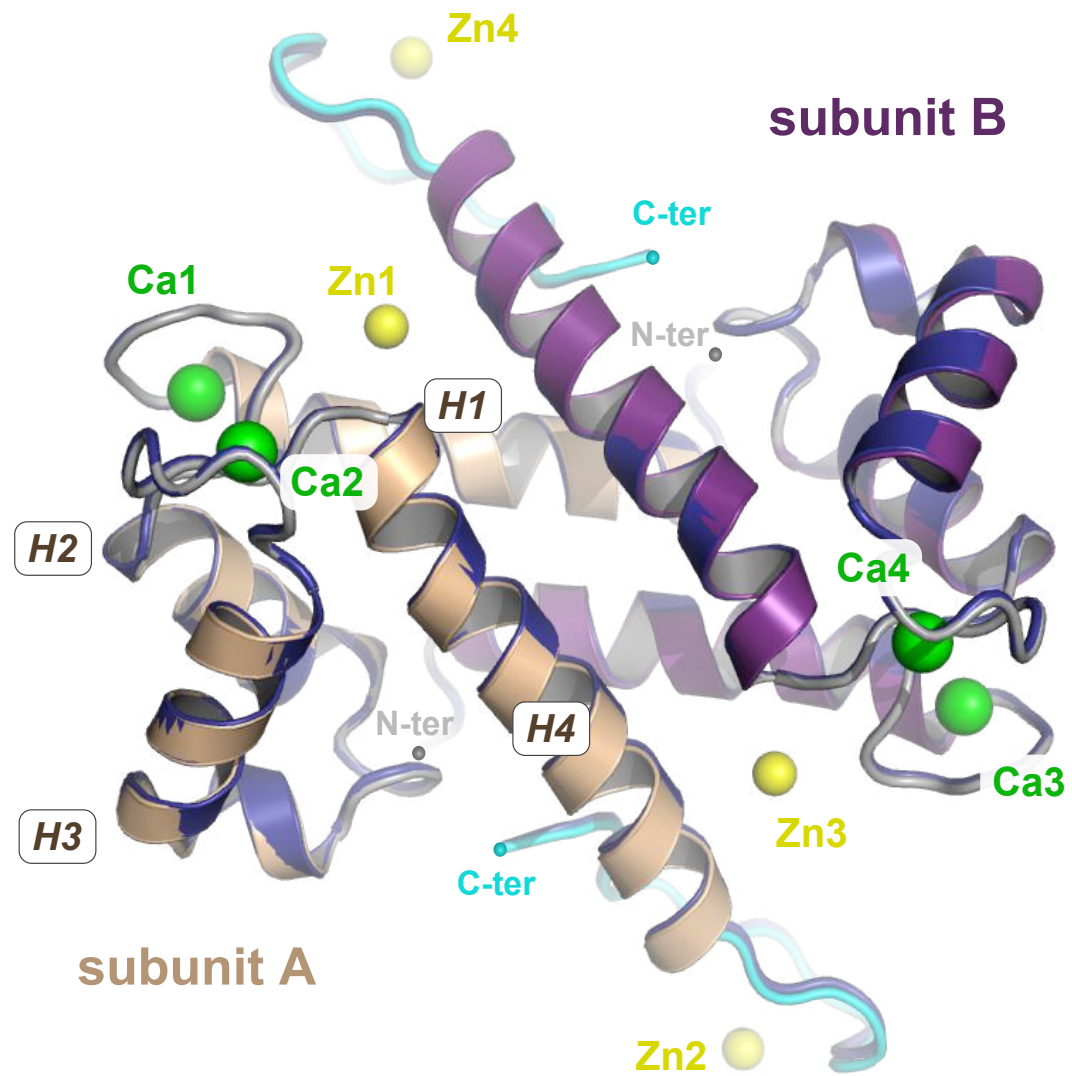










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