1 Conservation of specificity in two low-specificity 2 proteins

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8 Abstract

9 S100 proteins bind linear peptide regions of target proteins and modulate their ac-
 10 tivity. The peptide binding interface, however, has remarkably low specificity and
 11 can interact with many target peptides. It is not clear if the interface discrimi-
 12 nates targets in a biological context, or whether biological specificity is achieved
 13 exclusively through external factors such as subcellular localization. To discriminate
 14 these possibilities, we used an evolutionary biochemical approach to trace the evolu-
 15 tion of paralogs S100A5 and S100A6. We first used isothermal titration calorimetry
 16 to study the binding of a collection of peptides with diverse sequence, hydrophobic-
 17 ity, and charge to human S100A5 and S100A6. These proteins bound distinct, but
 18 overlapping, sets of peptide targets. We then studied the peptide binding properties
 19 of S100A5 and S100A6 orthologs sampled from across five representative amniote
 20 species. We found that the pattern of binding specificity was conserved along all
 21 lineages, for the last 320 million years, despite the low specificity of each protein.
 22 We next used Ancestral Sequence Reconstruction to determine the binding speci-
 23 ficity of the last common ancestor of the paralogs. We found the ancestor bound
 24 the whole set of peptides bound by modern S100A5 and S100A6 proteins, suggesting
 25 that paralog specificity evolved by subfunctionalization. To rule out the possibility
 26 that specificity is conserved because it is difficult to modify, we identified a single
 27 historical mutation that, when reverted in human S100A5, gave it the ability to bind
 28 an S100A6-specific peptide. These results indicate that there are strong evolutionary

constraints on peptide binding specificity, and that, despite being able to bind a large number of targets, the specificity of S100 peptide interfaces is indeed important for the biology of these proteins.

Introduction

Many proteins have low specificity interfaces that can interact with a wide variety of targets (1–11). Such interfaces are difficult to dissect. Crucially, it is not obvious that their specificity is biologically meaningful: maybe such proteins are essentially indiscriminate, and biological specificity is encoded by external factors such as subcellular localization or expression pattern (3, 12, 13).

An evolutionary perspective allows us to probe whether specificity is, indeed, an important aspect of these interfaces (14). If there are functional and evolutionary constraints on binding partners, we would expect conservation of binding specificity similar to that observed for high-specificity protein families (15, 16). In contrast, if specificity is unimportant, we would expect it to fluctuate randomly over evolutionary time. Further, previous work on the evolution of specificity has revealed common patterns for the evolution of specificity (17–19), including partitioning of ancestral binding partners among descendant lineages (20–23) and transitions through more promiscuous intermediates (10, 24, 25). If low-specificity proteins exhibit similar patterns, it is strong evidence that the low specificity interface has conserved binding properties, and that the interface makes a meaningful contribution to biological specificity.

S100 proteins are an important group of low-specificity proteins (26, 27). Mem-

bers of the family act as metal sensors (28), pro-inflammatory signals (29–32), and antimicrobial peptides (33). Most S100s bind to linear peptide regions of target proteins via a short hydrophobic interface exposed on Ca^{2+} -binding (Fig 1A). S100s recognize extremely diverse protein targets (27, 34, 35). No simple sequence motif for discriminating binders from non-binders has yet been defined. The breadth of targets is much more extreme than other low-specificity proteins such as kinases and some hub proteins, which recognize well-defined, but degenerate, sequence motifs (1, 3, 6, 10, 11).

We set out to determine whether there was conserved specificity for two S100 paralogs, S100A5 and S100A6. These proteins arose by gene duplication in the amniote ancestor $\approx$ 320 million years ago (36, 37). S100A6 regulates the cell cycle and cellular motility in response to stress (38). It binds to many targets including p53 (39, 40), RAGE (31), Annexin A1 (35), and Siah-interacting protein (41). A crystal structure of human S100A6 bound to a fragment of Siah-interacting protein revealed that peptides bind via the canonical hydrophobic interface shared by most S100 proteins (41). The biology of S100A5 is less well understood. It binds both RAGE (31, 32) and a fragment of the protein NCX1 (42) at the canonical binding site. It is highly expressed in mammalian olfactory tissues (43–45), but its specific targets and their biological roles are not well understood.

Using a combination of *in vitro* biochemistry and molecular phylogenetics, we addressed three key questions regarding the evolution of specificity in S100A5 and S100A6. First: do the two human proteins exhibit specificity relative to one another? Second: is the set of binding partners recognized by each protein fixed over time,

74 or does the set of partners fluctuate? And, third: do we see similar patterns of
 75 specificity change after gene duplication for these low-specificity proteins compared
 76 to high-specificity proteins? Unsurprisingly, we find that S100A5 and S100A6 both
 77 bind to a wide variety of diverse peptides. Surprisingly, we find that the set of
 78 partners, despite being diverse, has been conserved over hundreds of millions of
 79 years. Further, we observe a pattern of subfunctionalization for these low-specificity
 80 proteins that is identical to that observed in high-specificity proteins. This suggests
 81 that these low-specificity interfaces are indeed constrained to maintain a specific—if
 82 large—set of binding targets.

83 Results

84 Human S100A5 and S100A6 interact with diverse peptides at the same 85 binding site

86 We first systematically compared the binding specificity of human S100A5 (hA5)
 87 relative to human S100A6 (hA6) for a collection of six peptides (Fig 1B). Peptide
 88 targets have been reported for both hA5 and hA6 (31, 32, 35, 39–42), but only two
 89 targets have been directly compared between paralogs. Using Isothermal Titration
 90 Calorimetry (ITC), Streicher and colleagues found that a peptide fragment of An-
 91 nexin 1 bound to hA6 but not hA5, and a peptide fragment of Annexin 2 bound to
 92 neither (35) (Fig 1B). To better quantify the relative specificity of these proteins, we
 93 used ITC to measure the binding of two additional peptides to recombinant hA5 and
 94 hA6. The first was a peptide from Siah-interacting protein (SIP) previously reported
 95 to bind to hA6 (41). We found that this peptide bound to hA6 with a K_D of 20 μM ,

but did not bind hA5 (Fig 1B, C). The second was a 12 amino acid fragment of the protein NCX1 that was reported to bind to hA5 (42). We found that this peptide bound with to hA5 with a K_D of 20 μM , but did not bind hA6 (Fig 1B, C).

To further characterize the specificity of the interface, we used phage display to identify two additional peptides that bound to each protein. We panned a commercial library of random 12-mer peptides fused to M13 phage with either hA5 or hA6. Phage enrichment was strictly dependent on Ca^{2+} (Fig S1). Three sequential rounds of binding and amplification with either hA5 or hA6 led to enrichment of the “A5cons” and “A6cons” peptides (Fig 2B, Fig S1). We then used ITC to measure binding of these peptides to hA5 and hA6. To ensure solubility, we added polar N- and C-terminal flanks before characterizing binding. A5cons bound to both hA5 and hA6 (Fig 1C). In contrast, A6cons, bound hA6 but not hA5 (Fig 1C). To verify that binding was driven by the central region, we re-measured binding in the presence and absence of different versions of the flanks (Table S1).

The peptides that bind to hA5 and hA6 are diverse in sequence, hydrophobicity, and charge (Fig 1B). One explanation for this diversity could be that the peptides bind at different interfaces on the protein. To test for this possibility, we used NMR to identify residues whose chemical environment changed on binding of peptide. We first verified the published assignments for hA5 using a 3D NOESY-TROSY experiment (46). We then collected $^1H - ^{15}N$ TROSY-HSQC NMR spectra of Ca^{2+} -bound protein in the presence of either the A5cons or A6cons peptide. By comparing the bound and unbound spectra, we could identify peaks whose location shifted dramatically or that broadened due to exchange. In addition to our own work,

we also included previously reported experiments probing the hA5/NCX1 peptide interaction in the analysis (42). For all three peptides, we observed a consistent pattern of perturbations in helices 3 and 4 and, to a lesser extent, helix 1 upon peptide binding (Fig 2A-C). These results suggest that all three peptides bind at the canonical interface. In addition to this spectroscopic evidence, binding of all of these peptides was strictly dependent on the presence of Ca^{2+} (Fig 2D-F)—consistent with binding at the interface exposed on Ca^{2+} binding (46).

The S100A5 and S100A6 clades exhibit conserved binding specificity

Although hA5 and hA6 exhibit distinct specificity relative to one another (Fig 1B). This could either result from functional constraints or, alternatively, simply be chance. These possibilities can be distinguished with an evolutionary perspective. If specificity at the interface is functionally important, we would expect conserved specificity between paralogs; if it is unimportant, we would expect it to fluctuate over evolutionary time. We therefore set out to study the evolution of the differences in peptide binding between the human proteins.

We first constructed a maximum-likelihood phylogeny of the clade containing S100A2, S100A3, S100A4, S100A5, and S100A6 (Fig 3A). We built the tree using the EX/EHO+ Γ_8 evolutionary model (47), which uses different evolutionary models for sites in different structural classes. As expected from previous phylogenetic and syntenic analyses (37, 48), S100A5 and S100A6 were paralogs that arose by gene duplication in the amniote ancestor, with S100A2, S100A3, and S100A4 forming a closely-related out group (Fig 3A). To set our expectation for conservation of

specificity, we then calculated the conservation of residues at the binding site across S100A5 and S100A6 homologs. Fig 3B and C show the relative conservation of residues on hA5 (Fig 3B) and hA6 (Fig 3C). Taken as a whole, the peptide binding region does not exhibit higher conservation than other regions in the protein. We therefore predicted substantial variability in the peptide binding specificity across S100A5 and S100A6 orthologs.

To test the prediction that specificity has fluctuated over time, we expressed and purified S100A5 and S100A6 orthologs from human, mouse (*Mus musculus*), tasmanian devil (*Sarcophilus harrisii*), American alligator (*Alligator mississippiensis*), and chicken (*Gallus gallus*). We then characterized the peptide binding specificity of these S100A5 and S100A6 orthologs against four peptides: A5cons, A6cons, SIP, and NCX1 (Fig 4A). We selected these peptides because there is direct evidence that these peptides bind at the canonical binding interface (Fig 2, as well as (41, 42)). Surprisingly, we found that the S100A5 and S100A6 clades exhibited broadly similar, ortholog-specific binding specificity (Fig 4A). All S100A5 orthologs bound NCX1, A5cons, and A6cons, but not SIP. In contrast, all S100A6 orthologs bound SIP and A6cons, but not A5cons. The only labile character is NCX1 binding to S100A6. The sauropsid and marsupial S100A6 orthologs bound NCX1, but not the eutherian mammal representatives. We also characterized binding of these peptides to human S100A4 as an outgroup. Binding for this protein was intermediate between the S100A5 and S100A6 clades: it bound A5cons and A6cons, but not SIP or NCX1. Thermodynamic parameters for these binding experiments are given in Table S2-S5. Representative ITC traces for each protein are shown in Fig S2.

164 The strong conservation of peptide binding suggested that other features—such
 165 as structural features—might be conserved between paralogs as well. To test for this,
 166 we characterized the secondary structure and response to Ca^{2+} for all proteins using
 167 far-UV circular dichroism (CD) spectroscopy. A Ca^{2+} -driven change in α -helical sec-
 168 ondary structure is a conserved feature of S100 proteins (26, 37). We asked whether
 169 this behavior was conserved across orthologs, which would indicate similar structural
 170 properties. As with peptide binding, we found that the CD spectrum and response to
 171 Ca^{2+} were diagnostic within each clade (Fig 4B-D, Fig S3). S100A5 orthologs exhib-
 172 ited deep minima at 208 and 222 nm, corresponding to a largely α -helical secondary
 173 structure (Fig 4B,D). This signal increased upon addition of saturating Ca^{2+} , con-
 174 sistent with the ordering of the C-terminus of the human protein reported by NMR
 175 (46). In contrast, all S100A6 orthologs exhibited a deeper minimum at 208 nm,
 176 likely corresponding to a mixture of α -helical and random coil secondary structure.
 177 The secondary structure of these proteins changed comparatively little on addition
 178 of Ca^{2+} (Fig 4C,D).

179 **Specificity evolved from an apparently promiscuous ancestor**

180 Surprisingly, despite the diversity of peptides that bind to each paralog, peptide
 181 binding specificity is conserved across across paralogs. We next asked whether
 182 these proteins exhibited comparable evolutionary patterns to those observed in high-
 183 specificity proteins, such as the partitioning of ancestral binding partners along du-
 184 plicate lineages (20–22). Using our phylogeny, we used ancestral sequence reconstruc-
 185 tion (ASR) to reconstruct the last common ancestors of S100A5 orthologs (ancA5)

186 and S100A6 orthologs (ancA6) (49). These proteins were well reconstructed, having
 187 mean posterior probabilities of 0.93 and 0.96, respectively. Their sequences are given
 188 in File S2. We expressed and purified both of these proteins. We found that they
 189 shared similar secondary structures and Ca^{2+} -binding responses with their descen-
 190 dants by far-UV CD (Fig 4C). We then measured binding to the suite of four peptides
 191 described above using ITC. These ancestors gave the pattern we would expect given
 192 the binding specificities of the derived proteins (Fig 4D). AncA5 is indistinguishable
 193 from a modern S100A5 ortholog, binding A5cons, A6cons, and NCX1, but not SIP
 194 (Fig 4D). AncA6 also behaves as expected, binding A6cons and SIP, but not A5cons.
 195 It does not bind NCX1, consistent with this character being labile in the S100A6
 196 lineage (Fig 4D).

197 We next characterized the last common ancestor S100A5 and S100A6 (ancA5/A6).
 198 This reconstruction had a mean posterior probability of 0.83 (File S2). AncA5/A6
 199 has a secondary structure content identical to ancA6 and the S100A6 descendants.
 200 It also responds to Ca^{2+} in a similar fashion (Fig 4C, Fig S2). Unlike any modern
 201 protein, however, ancA5/A6 binds to all four peptides (Fig 5). To verify that this
 202 result was not an artifact of the reconstruction, we also made an “AltAll” ancestor
 203 of ancA5/A6 in which we swapped all ambiguous sites in the maximum-likelihood
 204 ancestor with their next most likely alternative (50) (File S2, methods). This protein
 205 is quite different than ancA5/A6—differing at 21 of 93 sites—but the binding profile
 206 for the four peptides was identical to the maximum-likelihood ancestor. Thermody-
 207 namic parameters for these binding experiments are given in Table S2-S5.

208 **Binding specificity can be changed with a single mutation**

209 Our work revealed that S100A5 and S100A6, despite having low overall specificity,
 210 display the same basic evolutionary patterns as high-specificity proteins (20, 22, 23):
 211 they exhibit conserved partners across modern orthologs and display a pattern of
 212 subfunctionalization from a less specific ancestor. While suggestive, this does not
 213 establish that there are functional constraints on specificity. Another possibility is
 214 that switching specificity is intrinsically difficult, and that the pattern we observe re-
 215 flects this difficulty rather than selective pressure to maintain a particular specificity
 216 profile.

217 To distinguish these possibilities, we attempted to shift the binding specificity of
 218 hA5 by introducing mutations at the binding interface. We selected five historical
 219 substitutions that occurred along the branch between ancA5/A6 and ancA5: e2A,
 220 i44L, k54D, a78M, m83A (with the ancestral amino acid in lowercase and modern
 221 amino acid in uppercase). We chose these substitutions using three criteria: 1) the
 222 ancestral amino acid was conserved in S100A6 orthologs, 2) the derived amino acid
 223 was conserved in S100A5 orthologs, 3) and the mutations were located at the peptide
 224 binding interface. Fig 5A shows the positions of candidate substitutions mapped onto
 225 the structure of hA5 (46).

226 We reversed each of these sites individually to the ancestral state in hA5. We then
 227 measured binding of two clade-specific peptides, SIP and A5cons, to each mutant
 228 using ITC (Table S6). We found that reverting a single substitution (A83m) to its
 229 ancestral state in hA5 enabled it to bind the SIP peptide (Fig 5B). This reversion
 230 does not compromise binding to A5cons, thus recapitulating the ancestral specificity

(Table S3). Reversion to the ancestral methionine at residue 83 likely makes more favorable hydrophobic packing interactions with the SIP peptide than the extant alanine. This demonstrates that a single mutation at the peptide binding interface is capable of shifting specificity in S100A5. None of the remaining four ancestral reversions led to measurable changes in A5cons or SIP binding. Amino acids at these positions either do not interact with these peptides, or the ancestral and derived amino acids interact in roughly equivalent fashion.

Another way to view specificity is in terms of binding mechanism. If binding affinity is mostly due to the hydrophobic effect, we would predict it would be relatively easy to alter binding by small changes to packing interactions. To test for relative contributions of the hydrophobic effect versus polar contacts to binding affinity, we did a van't Hoff analysis for the binding of A5cons to hA5. We performed ITC at temperatures ranging from 10 °C to 25 °C and then globally fit van't Hoff models to the binding isotherms (Fig 5C-D). We first attempted fits using a fixed enthalpy of binding ($\Delta C_p^\circ = 0.0$), but the fits did not converge. When we allowed ΔC_p° to float, we found it was negative ($-0.40 \leq -0.36 \leq -0.32 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$), indicating that binding is driven by the hydrophobic effect (51). This observation is consistent with binding at the hydrophobic surface exposed by the Ca^{2+} -induced conformational change (46) and may help to explain why specificity can be readily altered via a single substitution in the interface.

251 Discussion

252 Our work highlights the paradoxical nature of peptide binding specificity for these
 253 low-specificity S100 proteins. The binding interface has low specificity, interacting
 254 with very diverse peptides with no obvious binding motif (Fig 1B). Further, the
 255 specificity is fragile, and can be altered with a single point mutation (Fig 5). One
 256 might therefore conclude that this binding specificity is only weakly constrained. In
 257 contrast, binding specificity has been conserved over 320 million years along both
 258 lineages, exhibiting a pattern of subfunctionalization similar to what has been ob-
 259 served previously for the evolution of high-specificity proteins (Fig 4). This strongly
 260 points to the binding specificity being important, despite being very broad.

261 Low specificity through a hydrophobic interface

262 The binding specificity of these proteins is likely driven almost entirely by shape
 263 complementarity and packing. The protein interface exposed on Ca^{2+} binding is
 264 hydrophobic and likely makes few protein-peptide polar contacts. This prediction is
 265 validated, at least for the hA5/A5cons interaction, by the negative ΔC_p° on binding,
 266 pointing to an important contribution from the hydrophobic effect on binding (Fig
 267 5C). The lack of polar contacts is the likely explanation for the low specificity of the
 268 interface. Peptides need only match hydrophobicity and packing, meaning that a
 269 large number of possible peptides bind with similar affinity.

270 The hydrophobic nature of the interface explains the low specificity, but makes
 271 the conservation of specificity over 320 million years quite surprising. There is likely
 272 no diagnostic set of polar contacts that can be conserved maintain specificity. It

273 should therefore be straightforward to change specificity with minimal perturbation.
 274 Indeed, we found that a single mutation, from a small to a large hydrophobic amino
 275 acid, is able to switch the specificity of the interface (Fig 5A). Yet, over evolutionary
 276 time, binding specificity—at least for this set of targets—has been maintained (Fig
 277 4). Amazingly, this is achieved without strict conservation of the binding site. The
 278 peptide binding region does not exhibit higher conservation than other residues in
 279 either S100A5 or S100A6 (Fig 3B-C).

280 Our work shows that protein binding specificity is likely an important feature
 281 of these proteins, but does not reveal the set of biological targets for S100A5 and
 282 S100A6. Identifying these targets will require further experiments. This could in-
 283 clude coupling S100A5 and S100A6 knockouts to proteomics or transcriptomics, pull
 284 downs followed by proteomics, and/or large-scale screens of peptide targets via a
 285 technique like phage display. We also anticipate that external factors—such as co-
 286 expression, large complex assembly, and subcellular localization—will add critical
 287 additional layers of specificity to the low-specificity binding interfaces of these pro-
 288 teins. Understanding the interplay between the biochemical specificity and these
 289 external factors will be important for dissecting the biology of these proteins.

290 **S100s may allow the evolution of new calcium regulation**

291 The existence of a conserved set of binding partners also has intriguing implications
 292 for the evolution of Ca^{2+} signaling pathways in vertebrates. This can be seen by
 293 contrasting S100 proteins with calmodulin, a protein that also exposes a protein
 294 interaction surface and regulates the activity of target proteins in response to Ca^{2+}

295 (2). It has been proposed that calmodulin provides a universal Ca^{2+} response across
 296 tissues, while S100 proteins allow for fine-tuned, tissue-specific responses (26, 27).
 297 Our results allow us to extend this idea along an evolutionary axis.

298 Our results suggest that S100 proteins may provide a minimally pleiotropic path-
 299 way for the evolution of new Ca^{2+} regulation. Calmodulin is broadly expressed across
 300 tissues. As a result, a mutation that causes a protein to interact with calmodulin will
 301 have the same effect in all tissues where that protein is expressed. This could lead
 302 to unfavorable pleiotropic effects that prevent fixation of the mutation. In contrast,
 303 S100 proteins have highly differentiated tissue expression. S100A5, for example, is
 304 expressed almost exclusively in olfactory tissues. This means that a protein that
 305 acquires an interaction with S100A5 will do so only in olfactory tissue, with minimal
 306 pleiotropic effects in other tissues. The pattern of subfunctionalization we observed
 307 is consistent with this idea (Fig 4D), as subfunctionalization is one way to escape
 308 adaptive conflict that arises due to pleiotropic effects of mutations (52, 53). This is
 309 only possible because S100A5 evolved a distinct binding profile relative to S100A6
 310 (and presumably other S100 proteins), meaning that acquisition of a new S100A5
 311 interaction does not imply an interaction with a large number of other S100 proteins,
 312 which would itself lead to extensive pleiotropy.

313 Additionally, our results suggest that S100 proteins would provide a much simpler
 314 path for the evolution of new Ca^{2+} regulation than calmodulin. The calmodulin se-
 315 quence has been conserved for over a billion years and is basically unchanged across
 316 fungi and animals. As a result, evolution of a new calmodulin-regulated target re-
 317 quires that the target change its sequence to bind to calmodulin. This would likely

mean that slowly evolving proteins would not be able to evolve Ca^{2+} regulation, as neither the calmodulin nor possible new target would be able to acquire the necessary mutations to form the new interaction. In contrast, S100 proteins are evolving rapidly. For example, human S100A5 and S100A6 only exhibit 53% sequence identity, despite sharing an ancestor ≈ 320 million years ago. This means that, particularly after gene duplication, S100 proteins can acquire new interactions through mutations to the S100 itself. This would allow them to capture slowly evolving target proteins, opening a different avenue for the evolution of Ca^{2+} regulation that would not be accessible by calmodulin alone.

Evolution of low-specificity proteins

Our results also shed light on the evolution of low specificity proteins in general. Many proteins besides S100 proteins exhibit low specificity including other signaling proteins (2, 12), hub proteins (3, 6, 9, 11), and many others (1, 4, 5, 8, 10). Further experiments will be required to determine the generality of our observations for low-specificity proteins, but our work suggests that low-specificity proteins can evolve with similar dynamics to the high-specificity proteins that have been studied in detail. Partners for low-specificity proteins can be strongly conserved and evolve by subfunctionalization, just like a high-specificity protein.

One important question is whether S100A5 and S100A6 did, indeed, gain specificity over time. The current study, like many others (17, 20, 54–58), revealed an ancestral protein that appears less specific than its descendants. Some have proposed this is a general evolutionary trend (17, 54, 58). Caution is warranted before

340 interpreting these data as evidence for this hypothesis. We selected a small set of
 341 peptides to study; therefore, other patterns may be consistent with our observations.
 342 For example, it could be that the proteins both acquired more peptides that we did
 343 not sample in this experiment (actual neofunctionalization), while becoming more
 344 specific for the chosen set of targets (apparent subfunctionalization). Particularly
 345 given the large number of targets for these proteins, distinguishing these possibil-
 346 ities will require an unbiased, high-throughout approach to measuring specificity.
 347 Advances in high-throughput protein characterization have made such experiments
 348 tractable (59–63). With the right method, we will be able to resolve whether the
 349 shifts in specificity we observed indeed reflect increased specificity over evolutionary
 350 time, or instead the small size of the binding set we investigated.

351 Whatever the precise evolutionary process, our results reveal that S100 pro-
 352 teins—despite binding diverse peptides at a low-specificity hydrophobic interface—have
 353 maintained the same binding profile for the last 320 million years. Low-specificity
 354 does not imply no specificity, nor a lack of evolutionary constraint.

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Materials and Methods

Molecular cloning, expression and purification of proteins

Synthetic genes encoding the S100 proteins and codon-optimized for expression in *E. coli* were ordered from Genscript. The accession numbers for the modern sequences are: *Homo sapiens* S100A5: P33763, S100A6: P06703; *Mus musculus* S100A5: P63084, S100A6: P14069; *Sarcophilus harrisi* S100A5: G3W581, S100A6: G3W4S8; *Alligator mississippiensis* S100A5: XP_006264408.1, S100A6: XP_006264409.1; *Gallus gallus* S100A6: Q98953. All accession numbers are for the uniprot database (64), with the exception of the *Alligator mississippiensis* accessions, which are for the NCBI database (65).

Genes were sub-cloned into a pET28/30 vector containing an N-terminal His tag with a TEV protease cleavage site (Millipore). Expression was carried out in Rosetta (DE3) pLysS *E. coli* cells. 1.5 L cultures were inoculated at a 1:100 ratio with saturated overnight culture. *E. coli* were grown to high log-phase ($OD_{600} \approx 0.8-1.0$) with 250rpm shaking at 37°C. Cultures were induced by addition of 1 mM IPTG along with 0.2% glucose overnight at 16°C. Cultures were centrifuged and the cell pellets were frozen at -20°C and stored for up to 2 months. Lysis of the cells was carried out via sonication in 25mM Tris, 100mM NaCl, 25mM imidazole, pH 7.4.

Purification of all S100s used in this study was carried out as follows. The initial purification step was performed using a 5 mL HiTrap Ni-affinity column (GE Health

384 Science) on an Äkta PrimePlus FPLC (GE Health Science). Proteins were eluted
385 using a 25mL gradient from 25-500mM imidazole in a background buffer of 25mM
386 Tris, 100mM NaCl, pH 7.4. Peak fractions were pooled and incubated overnight
387 at 4°C with $\approx 1:5$ TEV protease (produced in the lab). TEV protease removes the
388 N-terminal His-tag from the protein and leaves a small Ser-Asn sequence N-terminal
389 to the wildtype starting methionine. Next hydrophobic interaction chromatography
390 (HIC) was used to purify the S100s from remaining bacterial proteins and the added
391 TEV protease. Proteins were passed over a 5 mL HiTrap phenyl-sepharose column
392 (GE Health Science). Due to the Ca^{2+} -dependent exposure of a hydrophobic bind-
393 ing, the S100 proteins proteins adhere to the column only in the presence of Ca^{2+} .
394 Proteins were pre-saturated with 2mM Ca^{2+} before loading on the column and eluted
395 with a 30mL gradient from 0mM to 5mM EDTA in 25mM Tris, 100mM NaCl, pH
396 7.4. Peak fractions were pooled and dialyzed against 4 L of 25 mM Tris, 100 mM
397 NaCl, pH 7.4 buffer overnight at 4°C to remove excess EDTA. The proteins were
398 then passed once more over the 5 mL HiTrap Ni-affinity column (GE Health Science)
399 to removed any uncleaved His-tagged protein. The cleaved protein was collected in
400 the flow-through. Finally, protein purity was examined by SDS-PAGE. If any trace
401 contaminants appeared to be present we performed anion chromatography with a
402 5mL HiTrap DEAE column (GE). Proteins were eluted with a 50mL gradient from
403 0-500mM NaCl in 25mM Tris, pH 7.0–8.5 (dependent on protein isoelectric point)
404 buffer. Pure proteins were dialyzed overnight against 2L of 25mM TES (or Tris),
405 100mM NaCl, pH 7.4, containing 2 g Chelex-100 resin (BioRad) to remove divalent
406 metals. After final purification step, the purity of proteins products was assessed by

407 SDS PAGE and MALDI-TOF mass spectrometry to be > 95 . Final protein products
408 were flash frozen, dropwise, in liquid nitrogen to form frozen spherical pellets and
409 stored at -80°C . Protein yields were typically on the order of 25mg/1.5L of culture.

410 **Isothermal titration calorimetry**

411 ITC experiments were performed in 25 mM TES, 100mM NaCl, 2mM CaCl_2 , 1mM
412 TCEP, pH 7.4. Although most experiments were performed at 25°C , some were done
413 at cooler temperatures depending to ensure measurable binding heats and sufficient
414 curvature for fitting. Samples were equilibrated and degassed by centrifugation at
415 18,000 g at the experimental temperature for 30 minutes. Peptides (GenScript,
416 Inc.) were dissolved directly into the experimental buffer prior to each experiment.
417 All experiments were performed at on a MicroCal ITC-200 or a MicroCal VP-ITC
418 (Malvern). Gain settings were determined on a case-by-case basis to ensured qual-
419 ity data. A 750 rpm syringe stir speed was used for all ITC-200 experiments while
420 400rpm speed was used for experiments on the VP-ITC. Spacing between injections
421 ranged from 300s-900s depending on gain settings and relaxation time of the binding
422 process. These setting were optimized for each binding interaction that was mea-
423 sured. Titration data were fit to a single-site binding model using the Bayesian fitter
424 in pytc. For each protein/peptide combination, one clean ITC trace was used to fit
425 the binding model. Negative results were double-checked to ensure accuracy. Some
426 were done at lower temperatures (10°C or 15°C) to confirm lack of binding, because
427 peptide binding enthalpy should be dependent on temperature.

428 2D HSQC NMR experiments

429 We collected 2D $^1H - ^{15}N$ TROSY-HSQC NMR spectra for 2 *mM* hA5 in the
 430 presence of Ca^{2+} alone and with the addition of the 2 *mM* A5cons. We also collected
 431 the spectra of 0.5 *mM* hA5 with the addition of 0.5 *mM* A6cons peptide, which was
 432 done at lower concentration due to poorer solubility of A6cons in the aqueous buffer.
 433 We transferred published assignments to the Ca^{2+} -alone spectrum (BMRB: 16033,
 434 (46)), and then used 3D NOESY-TROSY spectra to verify the assignments. We
 435 were able to unambiguously assign 76 peaks of the 91 non-proline amino acids in
 436 the Ca^{2+} -bound form. We then added saturating A5cons or A6cons peptide to the
 437 sample and remeasured the TROSY-HSQC spectrum. We then noted which peaks
 438 had either shifted or entered intermediate exchange upon addition of the peptide. Of
 439 the 76 unambiguously assigned non-proline amino acids 26 shifted or disappeared in
 440 the A5cons-bound form, and 35 shifted or disappeared in the A6cons bound form.

441 All NMR experiments were performed at 25 °C on an 800 MHz (18.8T) Bruker
 442 spectrometer at Oregon State University. TROSY spectra were collected with 32
 443 transients, 1024 direct points with a signal width of 12820, and 256 indirect points
 444 with a signal width of 2837 Hz in ^{15}N . NOESY-TROSYs were run with 8 transients,
 445 non-uniform sampling with 15% of data points used, and a 150 ms mixing time. All
 446 spectra were processed using NMRPipe (66); data were visualized and assignments
 447 transferred using the CCPNMR analysis program (67).

448 **Far-UV CD spectroscopy**

449 Far-UV circular dichroism spectra (200–250nm) were collected on a J-815 CD spec-
 450 trometer (Jasco) with a 1 mm quartz cell (Starna Cells, Inc.). We prepared 20–40 μM
 451 samples in a Chelex (Bio-Rad) treated, 25mM TES (Sigma), 100mM NaCl (Thermo
 452 Scientific) buffer at pH 7.4. Samples were centrifuged at 18,000 x g at 25°C in
 453 a temperature-controlled centrifuge (Eppendorf) before experiments. Spectra were
 454 measured in the absence and presence of saturating Ca^{2+} . Reversibility of Ca^{2+} -
 455 induced structural changes was confirmed by subsequently adding a molar excess of
 456 EDTA to the Ca^{2+} -saturated samples and repeating the measurements. Five scans
 457 were collected for each condition and averaged to minimize noise. A buffer blank
 458 spectrum was subtracted with the built-in subtraction feature in the Jasco spectra
 459 analysis software. Raw ellipticity was later converted into mean molar ellipticity
 460 based on the concentration and residue length of each protein. These calculations
 461 were performed on the buffer-blanked data.

462 **Preparation of biotinylated proteins for phage display**

463 A small amount of the purified proteins were biotinylated in the following way us-
 464 ing the EZ-link BMCC-biotin system (ThermoFisher Scientific). This kit used a
 465 maleimide linker to attach biotin at a Cys residue on the protein. ≈ 1 mg BMCC-
 466 biotin was dissolved directly in 100% DMSO to a concentration of 8mM for labeling.
 467 Proteins were exchanged into 25mM phosphate, 100mM NaCl, pH 7.4 using a Nap-25
 468 desalting column (GE Health Science) and degassed for 30 minutes at 25°C using
 469 a vacuum pump (Malvern Instruments). While stirring at room temperature, 8mM

BMCC-biotin was added dropwise to a final 10X molar excess. Reaction tubes were sealed with PARAFILM (Bemis) and the maleimide-thiol reactions were allowed to proceed for 1 hour at room temperature with stirring. The reactions were then transferred to 4°C and incubated with stirring overnight to allow completion of the reaction. Excess BMCC-biotin was removed from the labeled proteins by exchanging again over a Nap-25 column (GE Health Science), and subsequently a series of 3 concentration-wash steps on a NanoSep 3K spin column (Pall corporation), into the Ca-TeBST loading buffer. Complete labeling was confirmed by MALDI-TOF mass spectrometry by observing the ≈ 540 Da shift in the protein peak. Final stocks of labeled proteins were prepared at 10 μM by dilution into the loading buffer.

Phage display panning

Phage display experiments were performed using the PhD-12 peptide phage display kit (NEB). All steps involving the pipetting of phage-containing samples was done using filter tips to prevent cross-contamination (Rainin). 100 μ L samples containing phage (2.5×10^{10} PFU) and biotin-protein 0.01 μM (or 0.01 μM biotin in the negative control) and 50 μM peptide competitor (in competitor samples) were prepared at room temperature in a background of Ca-TeBST loading buffer (25mM TES, 100mM NaCl, 2mM $CaCl_2$, 0.01% Tween-20, pH 7.4) to ensure saturation of the S100s with Ca^{2+} . Samples were incubated at room temperature for 1hr. Each sample was then applied to one well of a 96-well high-capacity streptavidin plate (previously blocked using PhD-12 kit blocking buffer and washed 6X with 150 μL loading buffer). Samples were incubated on the plate with gentle shaking for 20min. 1 μL of 10mM

biotin (NEB) was then added to each sample on the plate and incubated for an additional five minutes to compete away purely biotin-dependent interactions. Samples were then pulled from the plate carefully by pipetting and discarded. Each well was washed 5X with 200 μ L of loading buffer by applying the solution to the well and then immediately pulling off by pipetting. Finally, 100 μ L of EDTA-TeBST (25mM TES, 100mM NaCl, 5mM EDTA, 0.01% Tween-20, pH 7.4) elution buffer was applied to each well and the plate was incubated with gentle shaking for 1hr at room temperature to elute. Two replicates of the experiment were performed with each protein.

Eluates were pulled from the plate carefully by pipetting and stored at 4°C. Eluates were titrated to quantify enrichment as follows. Serial dilutions of the eluates from 1 : 10⁻¹ : 10⁻⁶ were prepared in LB medium. These were used to inoculate 200 μ L aliquots of mid-log-phase ER2738 *E. coli* (NEB) by adding 10 μ L to each. Each 200 μ L aliquot was then mixed with 3mL of pre-melted top agar, applied to a LB/agar/XGAL/IPTG (Rx Biosciences) plate, and allowed to cool. The plates were incubated overnight at 37°C to allow formation of plaques. The next morning, blue plaques were counted and used to calculate PFU/mL phage concentration. Enrichment was calculated as a ratio of experimental samples to the biotin-only negative control.

For subsequent rounds of panning the eluates were amplified as follows. 20mL 1:100 dilutions of an ER2738 overnight culture were prepared. Each 20mL culture was inoculated with one entire sample of remaining phage eluate. The cultures were incubated at 37°C with shaking for 4.5 hours to allow phage growth. Bacteria were

515 then removed by centrifugation and the top 80% of the culture was removed care-
 516 fully with a filtered serological pipette and transferred to a fresh tube containing
 517 1/6 volume of PEG/NaCl (20% w/v PEG-8000, 2.5M NaCl). Samples were incu-
 518 bated overnight at 4°C to precipitate phage. Precipitated phage were isolated by
 519 centrifugation and subsequently purified by an additional PEG/NaCl precipitation
 520 on ice for 1hr. Isolated phage were resuspended in 200 μ L each sterile loading buffer,
 521 titered to measure PFU/mL, and stored at 4°C for use in the next panning round.
 522 This process was repeated for 3 total rounds of panning. Plaques were pulled from
 523 final reound eluate titer plates and amplified in 1mL ER2738 culture for 4.5 hours.
 524 ssDNA was isolated from the phage cultures using the Qiagen M13 spin kit. 10
 525 plaques per replicate experiment were Sanger sequenced (GeneWiz, Inc.). These
 526 plaque sequences were used to construct the A5cons and A6cons consensus peptides.

527 **Phylogenetics and ancestral reconstruction**

528 We used targeted BLAST searches to build an database of 49 S100A2-S100A6 se-
 529 quences sampled from across the amniotes, as well as six telost fish S100A1 sequences
 530 as an outgroup. We attempted to achieve even taxonomic sampling across amniotes.
 531 Database accession numbers are in Table S7. We used MSAPROBS for the initial
 532 alignment (68), followed by manual refinement. Our final alignment is available as a
 533 supplemental stockholm file (File S1).

534 We constructed our phylogenetic tree using the EX/EHO+ Γ_8 model, which in-
 535 corporates information about secondary structure and solvent accessibility into the
 536 phylogenetic inference (47). We assigned the secondary structure and solvent ac-

537 cessibility of each site using 115 crystallographic and NMR structures of S100A2,
538 S100A3, S100A4, S100A5 and S100A6 paralogs: 1a03, 1a4p, 1b4c, 1bt6, 1cb1, 1cdn,
539 1cfp, 1clb, 1cnp, 1ig5, 1igv, 1irj, 1jwd, 1k2h, 1k8u, 1k9p, 1ksm, 1kso, 1m31, 1mq1,
540 1nsh, 1ozo, 1psb, 1psr, 1sym, 1uwo, 1yur, 1yus, 2bca, 2bcb, 2cnp, 2cxj, 2jpt, 2jtt,
541 2k8m, 2kax, 2ki4, 2ki6, 2kot, 2l0p, 2l50, 2l5x, 2le9, 2lhl, 2llt, 2llu, 2lnk, 2pru, 2rgi,
542 2wc8, 2wcb, 2wce, 2wcf, 3ko0, 3nsi, 3nsk, 3nsl, 3nso, 3nxa, 1b1g, 1e8a, 1gqm, 1j55,
543 1k96, 1k9k, 1mho, 1mr8, 1odb, 1qlk, 1xk4, 1xyd, 1yut, 1yuu, 1zfs, 2egd, 2h2k,
544 2h61, 2k7o, 2kay, 2l51, 2psr, 2q91, 2wnd, 2wor, 2wos, 2y5i, 3c1v, 3cga, 3cr2, 3cr4,
545 3cr5, 3czt, 3d0y, 3d10, 3gk1, 3gk2, 3gk4, 3hcm, 3icb, 3iqo, 3lk0, 3lk1, 3lle, 3m0w,
546 3psr, 3rlz, 4duq, 1mwn, 1qls, 2k2f, 2kbm, 3iqq, 3rm1, 3zwh, 4eto. We calculated
547 the secondary structure for each site using DSSP and the solvent accessibility us-
548 ing NACCESS (69, 70). To remove redundancy—whether from identical sequences
549 solved under slightly different conditions or from the multiple models in the NMR
550 models—we took the majority rule consensus secondary structure and the average
551 solvent accessibility for all structures with identical sequences before doing averages
552 across unique sequences. We then assigned the secondary structure for each column
553 using a majority-rule across unique sequences. We assigned the solvent accessibility
554 as the average across unique sequences at that site. Our structural annotation is
555 available in our alignment stockholm file (File S1).

556 We then constructed our tree using the EX/EHO+ Γ_8 model (47), enforcing cor-
557 rect species relationships within groups of orthologs (71). We compared the final
558 likelihood of this tree to trees generated using LG+ Γ_8 and JTT+ Γ_8 models (72, 73).
559 Although the EX/EHO model has seven more floating parameters than either LG or

JTT, the final tree had a log-likelihood 61 units higher than the next-best model. An AIC test strongly supports the more complex model ($p = 3 \times 10^{-30}$). One important output from an EX/EHO calculation is χ , a term that measures the fraction of sites that use the structural models relative to a linear combination of all of them (47). For our analysis, $\chi = 0.72$. We rooted the tree using the S100A1 sequences, which included S100s from several bony fishes.

To reconstruct ancestors using the EX/EHO+ Γ_8 model, we used PAML to reconstruct ancestors using each of the six possible EX/EHO matrices (49, 74), as well as their linear combination. We then mixed the resulting ancestral posterior probabilities using the secondary structure calls and apparent accessibility at each site, as well as χ (see Equation 3 in (47)). The code implementing this approach is posted on github: https://github.com/harmslab/exexo_phylo_mixer. We assigned gaps using parsimony. We generated the AltAll sequence as described in Eick et al (50). This incorporates uncertainty in the reconstruction by taking the next-best reconstruction at each all ambiguous sites. We took each site at which the posterior probability of the next-best reconstruction was greater than 0.20 and the introduced that alternate reconstruction at the site of interest. Our AltAll sequence differed from the maximum likelihood sequence at 21 positions (24% of sites). File S2 has the posterior probabilities of reconstructions at each site in the ancestor, as well as the final sequences characterized.

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Fig 1. Human S100A5 and S100A6 exhibit peptide binding specificity.

A) Published structures of S100 family members bound to both Ca^{2+} and peptide targets at the canonical hydrophobic interface (PDB: 3IQQ, 1QLS, 3RM1, 2KRF, 4ETO, 2KBM, 1MWN, 3ZWH). Structures are aligned to the Ca^{2+} -bound structure of human S100A5 (2KAY). Peptides are shown in red. Blue spheres are Ca^{2+} ions.

B) Binding specificity of hA5 and hA6. Boxes indicate whether the peptide binds to hA5 (purple) and/or hA6 (orange). If peptide does not bind by ITC ($K_D \gtrsim 100 \mu M$), the box is white. Peptide names are indicated on the left. Peptide sequences, aligned using MUSCLE (75), are shown on the right. Solubilizing flanks, which contribute minimally to binding (Table S1), are shown in lowercase letters. Annexin 1 (An1) and Annexin 2 (An2) binding measurements are from a published study (35).

C) ITC heats for the titration of NCX1 (blue) and SIP (red) peptides onto hA5 (top) and hA6 (bottom). Points are integrated heats extracted from each shot. Lines are 100 different fit solutions drawn from the fit posterior probability distributions. For the hA5/NCX1 and hA6/SIP curves, we used a single-site binding model. For hA5/SIP

798 and hA6/NCX1, we used a blank dilution model. Thermodynamic parameters for
799 these fits are in Table S2-S5.

800

801 **Fig 2. Diverse peptides bind at the human S100A5 peptide interface.**

802 Structures show NMR data mapped onto the structure of Ca^{2+} -bound hA5 (2KAY
803 (46)). To indicate the expected peptide binding location, we aligned a structure of
804 hA6 in complex with the SIP peptide (2JTT (41)) to the hA5 structure, and then
805 displayed the SIP peptide in red. Panels A-C show binding for NCX1, A5cons, and
806 A6cons respectively. In panel A, yellow residues are those noted as responsive to
807 NCX1 binding in (42). In panels B and C, yellow residues are those whose $^1H-^{15}N$
808 TROSY-HSQC peaks could not be identified in the peptide-bound spectrum because
809 the peaks either shifted or broadened. Panels D-E show ITC data for binding of the
810 peptides above in the presence of 2 mM Ca^{2+} (blue) or 2 mM EDTA (red). Points
811 are integrated heats extracted from each shot. Lines are 100 different fit solutions
812 drawn from the fit posterior probability distributions. For the Ca^{2+} curves, we used
813 a single-site binding model. For the EDTA curves, we used a blank dilution model.
814 Insets show raw ITC power traces for the Ca^{2+} binding curves. Thermodynamic
815 parameters for these fits are in Table S2-S5.

846

847 **Fig 5. Small changes are sufficient to alter binding specificity at the**
848 **interface.** A) Ca^{2+} -bound structure of human S100A5 (2KAY) (46) with ancestral
849 reversions marked in gray (no effect on SIP binding) and red (A83, which causes SIP
850 binding). Blue spheres are Ca^{2+} ions. B) ITC traces showing titration of SIP onto
851 hA5 A83m (red) versus wildtype hA5 (blue). Points are integrated heats extracted
852 from each shot. Lines are 100 different fit solutions drawn from the fit posterior
853 probability distributions. For the hA5/A83m curve, we used a single-site binding
854 model. For the hA5 curve, we used a blank dilution model, where the linear slope is
855 indicative of peptide dilution without binding. C) ITC traces for titrations of A5cons
856 onto hA5 for as a function of temperature: 10°C (purple), 15°C (green), 20°C (blue),
857 and 25°C (red). Points are integrated heats extracted from each shot. Lines are 100
858 different fit solutions drawn from the fit posterior probability distributions for a
859 global Van't Hoff model optimized on all four experiments simultaneously. D) Van't
860 Hoff plot showing temperature dependence of $\ln(K)$ determined from global fit in

861 panel C. Thick black line shows Maximum Likelihood curve, gray lines are 500 curves
862 drawn from the posterior distribution of the Bayesian fit.