

1 **Bimodal evolution of Src and Abl kinase substrate specificity revealed using**
2 **mammalian cell extract as substrate pool**

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26 **Abstract**

27 The specificity of phosphorylation by protein kinases is essential to the integrity of
28 biological signal transduction. While peptide sequence specificity for individual kinases
29 has been examined previously, here we explore the evolutionary progression that has led
30 to the modern substrate specificity of two non-receptor tyrosine kinases, Abl and Src. To
31 efficiently determine the substrate specificity of modern and reconstructed ancestral

32 kinases, we developed a method using mammalian cell lysate as the substrate pool,
33 thereby representing the naturally occurring substrate proteins. We find that the oldest
34 tyrosine kinase ancestor was a promiscuous enzyme that evolved through a more specific
35 last common ancestor into a specific human Abl. In contrast, the parallel pathway to
36 human Src involved a loss of substrate specificity, leading to general promiscuity. These
37 results add a new facet to our understanding of the evolution of signaling pathways, with
38 both subfunctionalization and neofunctionalization along the evolutionary trajectories.

39

40 **Introduction**

41 The human genome contains 32 non-receptor tyrosine kinases (NRTKs) that are
42 tightly involved in a multitude of cellular processes including differentiation, apoptosis,
43 and proliferation¹⁻³. The interaction between each NTRK and its substrate comprises a
44 fundamental cellular signal that consequently required the evolution of specificity between
45 signaling pathways. To prevent unwanted signaling 'crosstalk', NRTKs have evolved two
46 main strategies to ensure substrate insulation^{4,5}: kinase localization⁶⁻¹² and active-site
47 peptide specificity¹³⁻¹⁸. The localization process is achieved through binding interactions
48 on the NTRKs' SH2/SH3 domains, which complex with either phosphotyrosines or poly-
49 prolines, respectively¹⁹⁻²². The differences in the active site of NRTK kinase domains
50 results in specificity where only a subset of substrates can bind and thus get
51 phosphorylated.

52 Unlike the serine/threonine family of kinases, NRTKs possess relatively
53 promiscuous active-site peptide specificities with a broad range of potential substrates⁵.
54 In high-throughput substrate screens, catalytic domains of NRTK members
55 phosphorylated hundreds of distinct peptide sequences, highlighting the promiscuity of
56 these kinases. Nevertheless, comparisons within the family show unique sequence
57 preferences and a consequent range of substrate selectivity. For two of the most well-
58 studied members, Abl and Src, narrow and broad selectivities are reported,
59 respectively^{13,17}. Because substrates bind at the active site in an elongated fashion, the
60 primary peptide sequence largely dictates the description of selectivity⁵. Abl has a clear
61 preference for hydrophobic residues flanking the tyrosine of interest (I/L/V₋₁, A₊₁, and
62 P₊₃)^{14,23}. In contrast, little sequence selectivity is observed for Src other than the relatively

63 weaker preferences for a bulky aliphatic residue (I/V/L-1) at the residue preceding the
64 phosphoacceptor, a phenylalanine three residues away from the phosphoacceptor (F_{+3}),
65 and a negatively charged residue on the N-terminal side of the phosphoacceptor (D/E-4, -
66 3, -2)^{17,24,25}.

67 As Src and Abl are sister clades within the NRTK phylogeny, their distinct
68 sequence preferences beget the question: how did peptide selectivity arise throughout
69 evolution? Herein we answer this question using ancestral sequence reconstruction
70 (ASR) for the catalytic domains. ASR uses modern sequences and an evolutionary model
71 to infer the sequences of internal nodes in a phylogenetic tree (Figure 1A, Figure 1 — figure
72 supplement 1)²⁶⁻³¹. Resurrection of ancestral kinases bridging Src and Abl allows the
73 evolution of peptide selectivity to be directly traced. The differences in protein sequence
74 between modern kinases and ancestors are spread through the kinase domain and the
75 oldest ancestor only has ~65% similarity with Src (Figure 1B).

76 Src and Abl display lower sequence selectivity than other kinases such as Aurora
77 A and B-RAF serine/threonine kinases^{5,24,32}, and, consequently, obtaining an accurate
78 description of the primary sequence determinants for each tyrosine kinase is a greater
79 statistical challenge³³. Comparison of ancestral and modern kinases requires a
80 comprehensive library of substrates since ancestral kinases likely refined their sequence
81 preferences over time. To ensure biological relevance, the peptide library should ideally
82 be composed of naturally occurring proteins. To construct such a library, we took
83 advantage of the diversity of sequences present in mammalian whole cell lysate (HEK293
84 cell line)^{34,35}. After endogenous kinases are covalently inhibited, the proteome of
85 mammalian cells presents a convenient substrate library containing thousands of
86 potential protein substrates. Here we use this comprehensive library to examine the
87 evolution of substrate selectivity in Abl/Src tyrosine kinases. We find that kinase substrate
88 preferences evolved in a complex manner involving two different modes: a promiscuous
89 progenitor specialized into the modern specific Abl, whereas evolution of Src involved
90 relaxing selectivity via a specific ancestral intermediate. We find that kinase substrate
91 preferences evolved in a complex manner involving two different modes: a promiscuous
92 progenitor specialized into the modern specific Abl (subfunctionalization), whereas
93 evolution of Src involved relaxing selectivity via a specific ancestral intermediate

94 (neofunctionalization). Therefore, our results shed light into a critical open question in
95 signaling, how new protein kinases with novel substrate specificities have evolved.

96

97 **Results**

98 **Whole-cell lysate phosphoproteomics-based approach**

99 A large and diverse protein library is necessary to readily screen the primary
100 sequence determinants of ancestral and modern NRTKs. An easily accessible, cheap,
101 and biologically relevant pool of substrates was created by inactivating endogenous
102 kinases in HEK293 lysate using the covalent, nonspecific inhibitor 5'-[p-
103 (fluorosulfonyl)benzoyl]adenosine (FSBA) (Figure 1C). After dialyzing out unreacted
104 inhibitor, purified kinases were added to the treated lysate and phosphorylation was
105 initiated with the addition of Mg²⁺ and ATP. To assess the required reaction time, total
106 phosphorylation was monitored by western dot blot experiments. Constant
107 phosphorylation levels were found to occur between two to four hours (Figure 1D,E). After
108 protein digestion with trypsin, peptide fragments were enriched for phosphorylation using
109 immobilized metal affinity chromatography (IMAC) and then analyzed by liquid
110 chromatography-mass spectrometry (LC/MS/MS, Figure 1C). Peptides were associated
111 with their full protein sequence based on the known HEK293 proteome, and results were
112 focused on a 15-amino acid sequence window centered on the phosphorylated tyrosine.

113 To determine a kinase's sequence specificity, each amino acid frequency must be
114 compared between the phosphorylated dataset and the background HEK293 proteome³³.
115 A background dataset was generated by proteomic analysis of lysate that was treated
116 with kinase inhibitor, but where no kinase was added and no phosphorylation enrichment
117 was performed³⁶. From this analysis, the amino acid frequencies of all surrounding
118 tyrosine residues (not only phosphorylated tyrosines) were calculated (Figure 1 — figure
119 supplements 2 and 3).

120 To determine the extent of endogenously phosphorylated peptides present, we
121 analyzed a lysate sample that was enriched for phosphorylated peptides but lacked
122 exogenous kinase. A total of 26 phosphorylated tyrosines were found in this control and
123 these peptides were excluded from the ancestral/modern NRTK list of substrates in which
124 kinase was added (Figure 1 — figure supplement 4).

125 In the data set where Src was added to the cell lysate, 8208 unique phosphorylated
126 sequences were identified (Figure 2A). These peptides include the characteristic
127 preferences for large aliphatic residues directly preceding the phosphotyrosine (V₋₁),
128 negatively charged residues in multiple positions N-terminal to the phosphotyrosine (D/E-
129 _{3, -2}), and a glycine following the phosphorylation site (G₊₁). An inclination for other
130 aliphatic residues preceding the phosphotyrosine (I/P/T₋₁) is also seen (Figure 2B).
131 Notably, a preference for proline at the -1 position identified in our data was not observed
132 previously. For Abl, specificity for large aliphatic residues preceding the phosphotyrosine
133 is found (I/V₋₁), as well as the canonical proline at the +3 position (Figure 2B). Additionally,
134 Abl exhibits a high preference for proline at the -2 position, which had not been identified
135 previously.

136 We can compare the results obtained here with the substrate specificity that is
137 observed when only natural substrates are considered. PhosphoSitePlus is a database
138 which annotates all known phosphorylation sites *in vivo* and *in vitro* that a given kinase
139 phosphorylates²⁴. Overall, our results confirm the previously found descriptions for both
140 Src and Abl's substrate specificities based on substrates in the PhosphoSitePlus
141 database, but additionally identify a few new preferences (Figure 2B, C). Our HEK293
142 lysate has a much larger number of phosphorylated substrates than the PhosphoSitePlus
143 database, which allows us to ascertain the residues dictating phosphorylation specificity
144 with greater accuracy and statistical significance than is possible with the
145 PhosphoSitePlus data (Figure 2B,C). Some of the differences may be due to the larger
146 number of substrates in our whole cell lysate. However, many of the observed
147 discrepancies likely result from differences in experimental design. PhosphoSitePlus is
148 based on *in vivo* substrates for the full-length kinase, whereas we are interested in the
149 intrinsic specificity of the kinase domain. In our experiments, we do not have the full-
150 length kinases and, therefore, we only find substrates that are selected by the kinase
151 domain itself. In contrast, phosphorylation within the cellular framework, as reported by
152 PhosphoSitePlus, is strongly determined by regulation and co-localization events, and
153 intrinsic kinase domain specificity plays a relatively smaller role. Indeed, Shah *et al.*
154 studied the specificity of NRTK kinase domains with a high-throughput, cell-surface based

155 experiment and found similar discrepancies between PhosphoSitePlus based logos and
156 their experimentally determined sequence determinants¹⁷.

157 **Evolution of specificity between Src and Abl**

158 Having established now the accuracy and statistics of our methodology on the
159 modern kinases, we next chose to determine the sequence specificity of three resurrected
160 ancestral kinases (Figure 1A). Anc-AS and Anc-S1 were previously resurrected for
161 investigating the mechanism of Abl selectivity for Gleevec²⁹, while the newly resurrected
162 Anc-AST (Figure 1 — figure supplement 1) is the oldest common ancestor of the Abl/Src
163 branch and the Tec family. Using our whole cell lysate phosphoproteomics-based
164 method, we identified a total of 12,056 unique sequences phosphorylated by the ancestral
165 and modern proteins (Figure 3A, Figure 3 – figure supplement 2). The common ancestor
166 of Src and Abl, Anc-AS, phosphorylated the least number of substrates (2495), which was
167 comparable to Abl (3073). The relative dearth of substrates for Anc-AS hinted that this
168 ancestor might be more specific than the promiscuous Src, which phosphorylated a total
169 of 8208 substrates. In contrast, the ancestors preceding (Anc-AST) and following (Anc-
170 S1) the common ancestor of Src and Abl each phosphorylated a significantly greater
171 number of substrates (8189 and 7242, respectively), indicating these ancestors were
172 likely more promiscuous.

173 General kinase specificity was then quantified by calculating the substrate
174 sequence entropy for each position in the 15-residue window (Figure 3B)³⁷. Lower
175 entropy, with fewer potential amino acid possibilities, indicates higher specificity. As
176 expected from the sequence logos (Figure 2B), Abl possesses the lowest entropy at
177 residues close to the phosphorylated tyrosine (-3, -2, -1, +1, and +3), with the most
178 specificity occurring at the signature proline position (+3). In contrast, the promiscuity of
179 Src manifests itself as increased entropy across almost all positions and a more limited
180 region of high specificity (-2, -1, and +1). The ancestral proteins give entropy plots that
181 agree with what was suggested by the observed substrate counts: The common ancestor
182 (Anc-AS) possessed entropy akin to Abl, albeit with a higher entropy at +3 and lower
183 entropy at positions -2 and +5. The two additional ancestors, Anc-AST and Anc-S1, both
184 exhibited 'hybrid' specificity with higher entropy than Abl, but less than Src. Notably, only
185 Src lacks specificity at the +3 position.

186 To analyze each enzyme's positional specificity in more detail, specificity heat
187 maps were created to illustrate the relative specificity for each amino acid at every position
188 in the 15-residue window (shown as a 20x15 matrix of positional normalized amino acid
189 log probabilities, Figure 3C)^{17,38}. Qualitatively, Abl's specificity is apparent from the high-
190 intensity signals for both preferred residues (red, P₊₃ and I₋₁) and unfavorable residues
191 (blue, S₋₁, P₊₁, and D/E₊₃), while Src has more white space and overall less intense signal.
192 We note that under substrate saturating conditions the enzyme would phosphorylate even
193 the less favorable substrates, which could result in an apparent low specificity. To ensure
194 that the observed promiscuity is not due to substrate saturation, experiments with Src
195 were repeated with a much shorter incubation period of the cell extract and the kinase
196 (10 minutes versus 4 hours). In this control, less phosphorylation was observed (Figure
197 1 C,D); however, the same primary sequence determinants were found (Figure 3 — figure
198 supplements 1 and 2), validating our findings.

199 Tracing individual amino acid preferences at specific positions provides a clear
200 picture of how specificity evolved for Abl (Figure 4A). Focusing on residues which are
201 preferred in Abl, but disfavored in Src (P₊₃, A/V₊₂, A₊₁, and P₋₂), we see that moderate
202 preference is already observed in ancestral kinases. The evolutionary path from the
203 oldest ancestor (Anc-AST) to Abl involves further increasing specificity either at the Anc-
204 AS node (P₋₂ and A/V₋₁) or in the final transition to Abl (A₊₁ and P₊₃). In contrast, the
205 pathway from the more specific Anc-AS to Src involves a corresponding loss of specificity
206 for each of these residues, with Anc-S1 possessing intermediary preferences (Figure 4A).

207 The evolution of the few positions favored by Src followed a different trend. The
208 preference for P₋₁ appears late, only in Src. Such recent evolution is in agreement with
209 the lack of preference for proline at position -1 of its close homolog Lck¹⁷. Other Src
210 sequence preferences were already present in the oldest ancestor, then lost in Anc-AS
211 and regained in Anc-S1 and Src (Figure 4B).

212 The increased promiscuity of Src is revealed in overall lower log probability values
213 than that of Abl's specific residues. Despite these differences in substrate specificity, there
214 are multiple positions where all modern and ancestral kinases prefer the same residues,
215 most of which are well-known features of NRTKs (e.g., I₋₁, D/E₋₃, and S₊₂) (Figure 4C). As
216 these characteristics are observed in all ancestral and modern proteins in our study and

217 are common among most NRTKs, they likely represent the oldest features of substrate
218 specificity for the NRTK family.

219 **Validation of evolutionary trends of primary sequence determinants via enzyme** 220 **kinetics**

221 Having determined the primary sequence determinants for the ancestral and
222 modern kinases, *in vitro* peptide enzyme turnover experiments were performed to relate
223 these bulk specificity experiments to quantitative enzymatic parameters. Compressing
224 thousands of substrates into residue-by-residue descriptions is compelling (i.e.,
225 preference for P₊₃), but how these preferences relate to enzymatic properties remains
226 unclear. We therefore measured the Michaelis-Menten kinetics of four distinct peptide
227 substrates with each of the five ancestral and modern kinases.

228 Previous microarray specificity experiments had determined optimized substrates
229 for Src and Abl, known as Srctide and Abltide, respectively^{13,18,25}. While both substrates
230 are ideal for their respective modern kinases, each also has residues favored by the
231 opposite kinase, which allow it to be phosphorylated to a certain degree by each kinase.
232 Therefore, we designed modified versions of Srctide and Abltide, called Srctide2 and
233 Abltide2, to test our evolutionary trends (Figure 5A,B). Srctide2 was intended to be
234 favored by both Src and Anc-S1, with changes made to Srctide to include residues that
235 occurred more frequently in substrates for these two kinases (D₋₂, A₊₂, and I₊₃). Abltide2
236 was designed to be preferred by both Abl and Anc-AS by mutating the alanine at position
237 -2 into a proline. P₋₂ is favored by all the kinases, except for Src (Figure 5A, B).

238 As substrates in signaling cascades are generally present at low concentrations *in*
239 *vivo*, the k_{cat}/K_M likely represents a more fundamentally important parameter than k_{cat} for
240 substrate specificity. As can be seen from the measured Michaelis-Menten curves (Figure
241 5C), the measured differences in the kinetics corroborate the evolutionary trends found
242 before but suggest additional features for substrate specificities. Starting with a
243 promiscuous Anc-AST, Anc-AS becomes more selective, particularly for substrates with
244 P₋₂, Abltide2 (which was identified as a highly preferred residue for Anc-AS and Abl from
245 our phosphoproteomics data, Figure 5B). Moving to Abl, the specificity for Abltide and
246 Abltide2 further increases as seen with high increases in k_{cat}/K_M . This is primarily due to
247 the strong preference for P₊₃ (Figure 5B), which are both present in Abltide and Abltide2.

248 At higher concentrations of these two well-optimized peptides we observe partial
249 inhibition, due to the negative cooperativity of ATP and peptide substrate found for Abl³⁹.

250 Following the evolutionary branch towards Src, Anc-S1 becomes more
251 promiscuous, mainly due to its ability to catalyze Src-preferred substrates in addition to
252 the Abltide substrates. Furthermore, the strong preference of I₊₃ observed in the
253 proteomics data (Figure 5B) can be directly recapitulated by the preference for Srctide2
254 (Figure 5C). The strong preference of Srctide and Srctide2 over the Abltide substrates
255 only appears in Src, primarily due to a complete loss of preference for P₊₃ leading to poor
256 activity for the Abltide substrates, combined with a subtle preference for F₊₃.

257 **Discussion**

258 There are multiple methods described in the literature for assessing substrate
259 specificity of protein kinases, including non-receptor tyrosine kinases and
260 serine/threonine kinases. Several studies look only at known natural substrates, such as
261 in PhosphoSitePlus, to determine which residues occur more frequently in known
262 phosphorylation sites^{24,40}. Other studies, including the one presented here, use the whole
263 cell lysate as a pool of substrates to test the specificity of kinases^{34,35}. The HAKA-MS
264 method reported by Muller *et al.* used a similar whole cell lysate, yet only found a P₊₃
265 preference from 104 Abl substrates (Figure 2 – figure supplement 2)³⁵. In contrast, our
266 study found ~30-fold more substrates and several residues that are preferred or
267 disfavored at multiple positions. The large difference in detected phosphorylated
268 substrates could be due to different methods to enrich for phosphorylated peptides: Muller
269 *et al.* use multiple phosphotyrosine binding antibodies, whereas we use IMAC. We also
270 tried using phosphotyrosine binding antibodies for enrichment, but we found peptide bias
271 and artifacts using this method. Lastly, one of the most popular methods uses peptide
272 libraries to determine the preference of kinases^{13,14,16-18,38}.

273 Our experimental method, which exploits the substrate-rich, kinase-inactive whole
274 cell lysate and has improved statistical significance, discovered new determinants of Abl
275 and Src specificity in addition to those previously reported¹⁷: Src shows significant
276 favorability for proline or threonine at the -1 position and a serine at the -2 location, while
277 Abl shows a preference for proline at the -2 position and a serine at the -2 position. These
278 new features were subsequently verified by *in vitro* enzyme kinetic experiments. The most

279 important advantage of the increased sensitivity of our assay has been the ability to
280 explore how Src and Abl kinases evolved their sequence preferences.

281 How these kinases have differing specificities can be partially rationalized based on
282 the details of kinase sequence and structure. Y569 in Abl has previously been determined
283 to be required for its preference for proline in the +3 position of substrates⁴¹. Leucine at
284 the homologous position (L475) in Src was previously shown to disfavor proline⁴¹. A437
285 has been proposed to be responsible for Src's preference for phenylalanine at position
286 +3, along with L475¹⁷. All ancestors contain an isoleucine at position 475 and show
287 intermediate specificity towards P₊₃. Moreover, Anc-S1 differs from Src in both positions,
288 I475 and L437 (Figure 5 – figure supplement 1), identical to another Src family kinase
289 member, Lck (I412 and I450 in Lck). Interestingly, when comparing the Anc-S1 substrate
290 preference to that of Lck, as investigated by Shah *et al.*¹⁷, we see a high similarity in the
291 +3 position preferences. Both Anc-S1 and Lck show a strong preference for L₊₃ and P₊₃,
292 suggesting that Anc-S1 is more Lck-like, and that the substitution to the less bulky A437
293 in Src causes its preference for F₊₃ (Figure 5 – figure supplement 1). These structural
294 differences between Src and the ancestors explain why the Src tide is less effective at
295 being phosphorylated by other kinases. Anc-S1 was unable to effectively phosphorylate
296 Src tide potentially due to F₊₃, wherein its substitution to the less bulky isoleucine in
297 Src tide2 is favored by Anc-S1. We elect to steer clear from additional structural
298 explanations for other detected specificities, as these are just coarse models of
299 kinase/substrate complexes, and more collective, long-range effects often underlie such
300 specificity changes. For example, in an appealing study of the evolution of CMGC
301 kinases, Howard *et al.* identified a key residue for imparting specificity at the +1 position²⁸.
302 Tests of their hypothesis via mutations in the corresponding modern kinases resulted in
303 partial changes in specificities. Since the authors were unable to achieve a full swap in
304 specificity, they concluded that there must be additional residues in play that are not
305 readily apparent by looking at the differences in active-site residues.

306 The different trajectories we find in the evolution of Src and Abl substrate specificity
307 add a new facet to our understanding of the evolution of signaling pathways. It has been
308 postulated that much of biological diversity, including metazoan complexity, has been
309 driven by the evolution of new regulatory networks and signaling pathways, such as those

310 controlled by the post-translational modification of kinase phosphorylation^{42,43}. A key
311 evolutionary challenge in creating a new signaling pathway is ensuring kinase specificity
312 to minimize crosstalk with other pathways, many of which are vital to cellular fitness. One
313 critical open question is how new protein kinases with novel substrate specificities have
314 evolved.

315 Gene duplication has been the major force driving the evolutionary diversity of
316 signaling pathways and kinase specificity⁴²⁻⁴⁴. There are two main ways that gene
317 duplication can evolve enzymes with new and different functions: (1)
318 “subfunctionalization”, the specialization of previously existing functions, and (2)
319 “neofunctionalization”, the creation of a novel function through the accumulation of
320 beneficial, gain-of-function mutations^{45,46}. The evolution of sequence specificity does not
321 cleanly fall in either of these categories, because specificity is not an “all-or-nothing” gain
322 or loss of a function. Nevertheless, for kinases, subfunctionalization most closely aligns
323 with evolution from a non-specific ancestor (which can bind and use many different
324 substrates) to descendants with differing, specialized specificities (which can bind and
325 use only a subset of the ancestral substrates), whereas neofunctionalization involves
326 evolution from a specific ancestor to a descendant with a broad, promiscuous specificity
327 or with a different specificity. Though neofunctionalization is the older of the two
328 hypotheses^{47,48}, it is now widely viewed as relatively improbable and hence less frequent
329 in evolution than subfunctionalization mechanisms⁴⁹⁻⁵³. For instance, in the evolution of
330 specificity in CMGC protein kinases, the ancestral kinase was a promiscuous bispecific
331 enzyme in respect to the +1 position, unlike the modern kinases which are specific for a
332 single amino acid²⁸. Currently, the evolutionary mechanisms by which gene duplications
333 evolve new functions are controversial, and there are relatively few examples of classic
334 neofunctionalization⁵⁴⁻⁵⁶.

335 Intriguingly, we see both mechanisms of gene duplication in the evolution of Abl
336 and Src. With Abl, subfunctionalization converted a promiscuous, non-specific ancestor
337 (ANC-AST) into specific descendants (ANC-AS and modern Abl); with Src,
338 neofunctionalization transformed a surprisingly specific ancestor (ANC-AS, the last
339 common ancestor of the Abl and Src kinase families) into progressively more

340 promiscuous descendants (ANC-S1 followed by modern Src). In fact, the particular
341 lineage leading from ANC-AST to modern Src appears to involve both mechanisms.

342 In the toxin-antitoxin signaling systems of bacteria, Aakre *et al.*⁵⁷ found that the
343 evolution of some enzymes passes through promiscuous intermediates before
344 developing strong substrate specificity. Our results suggest that eukaryotic protein
345 kinases similarly evolve through waves of promiscuous and specific effectors. Periods of
346 increased promiscuity may allow kinases to access new substrates while maintaining their
347 current function, which could explain the redundancy of kinase networks²⁴. To insulate
348 individual pathways from others, sometimes this promiscuity may be selected out. In other
349 cases, the overlap may provide a fitness advantage resulting in modern protein kinases
350 with overlapping substrates.

351 The present work has addressed one component of kinase substrate specificity –
352 the intrinsic specificity of the kinase domain – but it is important to emphasize the rich
353 literature about the crucial role of the additional regulatory domains found within most
354 NRTKs for kinase specificity. Proteins containing SH2 or SH3 domain binding sites are
355 more likely to be phosphorylation targets for NRTKs, due to the selective activation of the
356 kinases^{7,20,58-60}. SH2 and SH3 domains have their own unique specificity⁶¹⁻⁶⁵, and bring
357 the kinases to their target substrates. Studies using kinase domains in isolation have not
358 identified all the known substrates that are found in databases like PhosphoSitePlus. For
359 example, Shah *et al.* limited their library to only known phosphorylation sites, yet they
360 could not see a preference for all known kinase substrates for a given kinase¹⁷. We fully
361 agree with their discussion¹⁷ that intrinsic kinase domain specificity^{13,14,25} acts in concert
362 with the selective activation and localization provided by the SH2 and SH3 domains in a
363 cellular context^{4,7,8,12,20,21,64,66-70} to provide the full specificity of the NRTKs.

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369 **Materials and Methods**

370 **Kinase Specificity in Whole Cell Lysate Experiment**

371 HEK293 cells were grown in CytoOne 150X20mm TC dishes with DMEM (High
372 Glucose, No Glutamine; Fisher Sci) containing HyClone bovine growth serum (Fisher
373 Sci), glutamine (Fisher Sci), fungizone (Fisher Sci), and penicillin-streptomycin (Fisher
374 Sci). At ~90% confluency, cells were washed with 5mL of PBS before being harvested.
375 Cells were centrifuged at 4500g for 6 minutes to pellet. PBS was then decanted from the
376 pellet. Pelleted cells were washed by repeating the previous step. The pellet was then
377 resuspended in ~3 mL of assay buffer (20 mM Tris, Fisher; 500 mM NaCl, Fisher; 1 mM
378 MgCl₂, Fisher; 1mM TCEP, Fisher; pH 8) per 10 plates harvested. Cells were lysed by
379 sonication followed by centrifugation at 30,000g. The supernatant was pipetted from the
380 pellet and 20 mM 5'-(4-Fluorosulfonylbenzoyl)adenosine hydrochloride (FSBA; Sigma
381 Aldrich) was added to the supernatant. The lysate was incubated with FSBA at 25 °C for
382 2 hours. Lysate was dialyzed in 2 L of assay buffer for 5 hours at room temperature
383 followed by a second dialysis at 4 °C overnight in 2 L of assay buffer. The protein
384 concentration in the lysate was then calculated with BCA assay Kit (~9 mg/mL; Pierce).

385 Wilson et al.²⁹ previously published the construction of an alignment and
386 phylogenetic model using BALi-Phy⁷¹, which was used to resurrect ancestral protein
387 sequences with PAML⁷². The robustness of these ancestral kinases have been previously
388 tested by investigating activities of alternate sequences of the same nodes²⁹. Ancestral
389 and modern kinases were expressed and purified as previously reported²⁹. The reaction
390 was setup with 10 μM kinase, 20 mM MgCl₂, 10 mM ATP, Phosphatase Inhibitor Cocktail
391 #2 (Cal Biotech), and ~1.3 mL of lysate (~12 mg of protein). Reaction went for up to 4
392 hours at 25 °C. This was repeated three times for each kinase along with a background
393 sample where no kinase was added. For the western dot blot time course of Src, 5 μL of
394 sample was quenched with 15 μL of 8 M urea (Fisher Sci) to make the 4-fold dilutions.
395 Other dilutions were made accordingly for 2-fold and 8-fold dilution samples. 1μL samples
396 were loaded directly onto a nitrocellulose membrane (GenScript). The protocol was
397 followed for the iBind Automated Western Systems (ThermoFisher) with Phosphotyrosine
398 antibody (P-Tyr-1000 MultiMab™ Rabbit mAb mix; Cell Signaling Technology) as the
399 primary antibody (1:2000 dilution) and ScanLater anti-rabbit antibody (Molecular Devices)
400 as the secondary antibody (1:5000 dilution). The western dot blot was then imaged on a

401 SpectraMax i3x Multi-Mode Microplate Reader with a ScanLater Western Blot cartridge
402 (Molecular Devices).

403 **Mass Spectrometry of Phosphorylated Kinase-Inactive Lysate**

404 For analysis of phosphorylated peptides, ~1 mL of lysate (~10 mg of protein) which
405 has been reacted with the kinase previously was quenched with a 1:1 ratio of 8 M urea
406 (Fisher). Followed by digestion with MS grade trypsin protease (Pierce) as instructed by
407 the manufacturer. The reaction was then quenched with 10% trifluoroacetic acid (TFA,
408 Sigma) bringing the final concentration to 0.5% TFA. Samples were desalted under
409 vacuum using Sep Pak tC18 cartridges (Waters). Each cartridge was activated with 1 mL
410 80% acetonitrile (ACN)/0.1% TFA, then equilibrated with 3 × 1 mL of 0.1% TFA. Following
411 sample loading, cartridges were washed with 4 × 1 mL of 0.1% TFA, and samples were
412 eluted with 4 × 0.5 mL 50% ACN/0.25% formic acid (FA). 20 µg of each sample was kept
413 for protein abundance measurements, and the remainder was used for phosphopeptide
414 enrichment. Samples were dried by vacuum centrifugation. The digested peptides were
415 enriched for phosphopeptides using ion metal affinity column (IMAC). FeCl₃-NTA beads
416 were prepared from Ni-NTA super flow slurry beads (QIAGEN) by first stripping the beads
417 by incubating with 100 mM EDTA in a vacuum manifold three times. The beads were then
418 washed with water before incubating with 15 mM FeCl₃ (Sigma) for one minute three
419 times. Excess FeCl₃ was washed with water before rinsing the beads with 0.5% formic
420 acid (FA). A slurry was prepared by adding water to the beads. 60 µL of slurry was added
421 into a C18 NEST column that had been equilibrated with 150 µL of 80% ACN, 0.1% TFA.
422 100 µL of 50% ACN was added to the lyophilized lysate pellet to dissolve it. 100 µL of
423 100% ACN and 3 µL of 10% TFA was added after lysate was dissolved. 1 mg of peptide
424 is added to Fe-NTA beads in the desalting tip and then incubated for 1-2 minutes followed
425 by mixing and incubation for another 1-2 minutes. After incubating liquid is drained. Then
426 200 µL of 80% ACN and 0.1% TFA was used to wash the beads 3 times. 200 µL of 0.5%
427 FA is used twice to wash the beads. the beads are incubated for 2-3 minutes with 200 µL
428 of 500 mM phosphate buffer pH7 before eluting peptides to C18 column. This is repeated
429 one more time to fully elute the peptides from the beads. The beads are incubated for 15
430 seconds with 200 µL 0.5% FA before the C18 column is used in a centrifuge to elute the

431 phosphorylated peptides with 75 μ L of 50% ACN and 0.1% TFA twice before the mass
432 spectrometry run.

433 The LC/MS/MS was performed on all the samples prepared with phosphopeptide
434 enrichment and a background sample with no enrichment. We used a 90-minute
435 separation by nano reversed-phase HPLC gradient over a 75- μ m ID X 25-cm precolumn
436 packed with Reprosil C18 1.9- μ m (Waters). The sample was run on a Q-Exactive Plus
437 mass spectrometer (ThermoFisher) and the top 20 ions were selected for MS2
438 sequencing. Resulting data was then searched with MaxQuant⁷³ to against the human
439 proteome to identify phosphorylated peptides. The mass spectrometry proteomics data
440 have been deposited to the ProteomeXchange Consortium via the PRIDE partner
441 repository⁷⁴ with the dataset identifier PXD020299.

442 **Specificity Calculations**

443 The results from MaxQuant⁷³ were analyzed with an in-house script written in
444 python. For each kinase, a set of substrate sequences was generated from the
445 phosphorylated peptides found in at least one of the three trials. To generate the set of
446 substrates, first the substrate peptides would be extended or shortened to 7 residues on
447 each side of the phosphorylation site. If the sequence was too close to the beginning or
448 end of a protein it would be rejected immediately. Next, if the sequence is already in the
449 set of substrate sequences or was found in the control experiment, where no kinase was
450 added, it would be rejected. Lastly, the localization probability must be greater than or
451 equal to 70% and the MS intensity must be greater than 0. A background dataset was
452 generated by applying the same rules to tyrosine containing peptides, from samples that
453 were not enriched for phosphorylation. The background sequence logo was generated
454 using WebLogo⁷⁵.

455 Heatmaps were calculated by taking the log frequency of an amino acid occurring
456 at a specific position minus the log of the frequency in the background. Each position and
457 amino acid pair were tested using the logs odd ratio estimate used in pLogos and any
458 significant value was marked with a black square in the heatmap.¹⁰ This allows for
459 intensities of significant residues to be compared between data sets accurately. Residues
460 below a 1.3-fold effect size were masked even if significant to focus only on residues

461 which have the largest effect on specificity. Using the amino acid frequencies for each
462 position the sequence entropy was calculated using the SciPy stats module⁷⁶.

463 **Activity Assay**

464 Initial rates were measured by a continuous colorimetric assay⁷⁷. The reactions
465 contained 20-100 nM of purified kinase along with 20 mM MgCl₂ (Fisher), 525 μM β-
466 Nicotinamide adenine dinucleotide (Sigma), 4 mM phosphoenolpyruvate (Sigma), 2.5 μL
467 of PK/LDH (PK 600 U/mL – 1000 U/mL, LDH 900 U/mL-1400 U/mL; Sigma Aldrich), 0.3
468 mg/mL Bovine Serum Albumin (Fisher), and substrate peptide (GenScript). Reactions
469 were initiated with 5 mM ATP, by pipetting the solution up and down, and then the
470 absorbance was read at 340 nm for the course of the reaction (20 minutes) at 25 °C.

471 **Homology Model of Bound Peptide**

472 An initial homology model was created from a crystal structure of Abl bound to an
473 ATP-peptide conjugate (PDB: 2G2I). Using PyRosetta⁷⁸, the initial structure was mutated
474 to the sequence for either Src or Anc-S1. The bound peptide was then mutated to the
475 sequence of Src tide. The backbone of the protein and peptide was set to be constrained
476 before running the Fast Relax protocol using the ref2015 score function.

477

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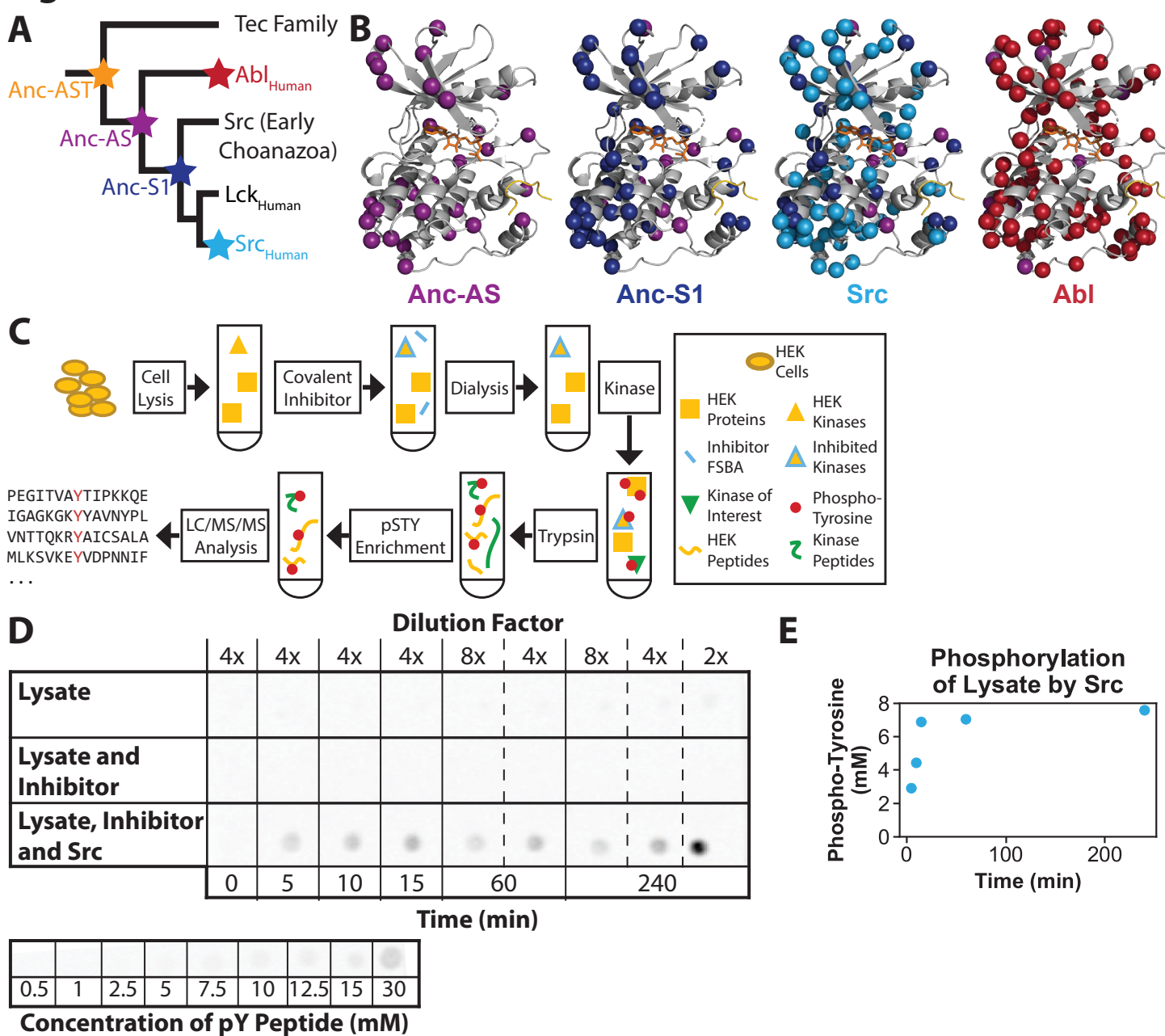
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683

Figure 1



Analysis of the evolution of non-receptor tyrosine kinase specificity with HEK lysate based approach. (A) Phylogenetic tree of non-receptor tyrosine kinase domains constructed with Bali-Phy (Suchard *et al.* 2006). The reconstructed nodes and modern kinases used herein are marked with stars. For complete tree, sequences, testing of alternate sequences, and statistics see (Wilson *et al.* 2015). (B) Crystal structure of Abl (PDB ID: 2G2I; Levinson *et al.* 2006) with peptide substrate (yellow) and ADP (orange) bound. The additive differences in primary sequence between Anc-AST and Anc-AS (purple; 87.3% identity), Anc-S1 (dark blue; 85.8% identity), Src (blue; 65.2% identity), and Abl (red; 67.0% identity) are shown. (C) Flow chart displaying how cell lysate was prepared and used for kinase specificity assays. See methods for a detailed description. (D) Western dot blots using anti-pY1000 antibody to detect phosphorylated tyrosine in proteins. (Top) Cell lysate incubated without Src does not show any phosphorylation, whereas lysate treated with the kinase does. Dilutions of the 60-minute and 240-minute time points illustrate that measurements are in the linear range of the dot-blot. (Bottom) Control of phosphorylated peptide blotted at a range of concentrations, diluted 4-fold. (E) Phosphorylation of the cell lysate over time by Src kinase.

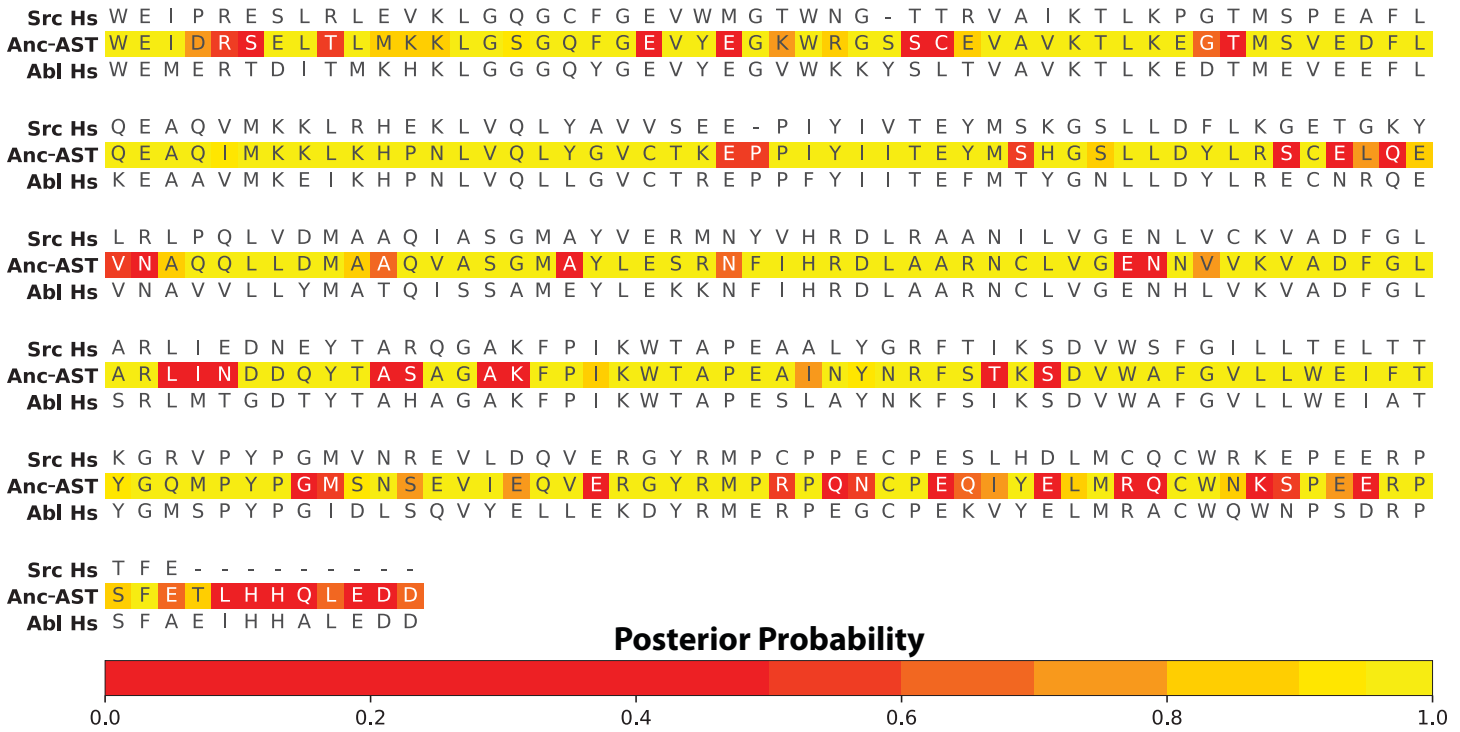


Figure 1 — figure supplement 1: Posterior probabilities for Anc-AST reconstruction aligned with Src and Abl from *Homo sapiens*. Wilson *et al.* (2015) performed ancestral sequence reconstruction using PAML (Yang *et al.* 2007) and reported all other ancestral statistics and alignments. Here we report the Anc-AST sequence which has an average posterior probability of 0.86 across all positions. Anc-AST is aligned to the Src and Abl kinase domains and each position displays the given residue's posterior probability from PAML.

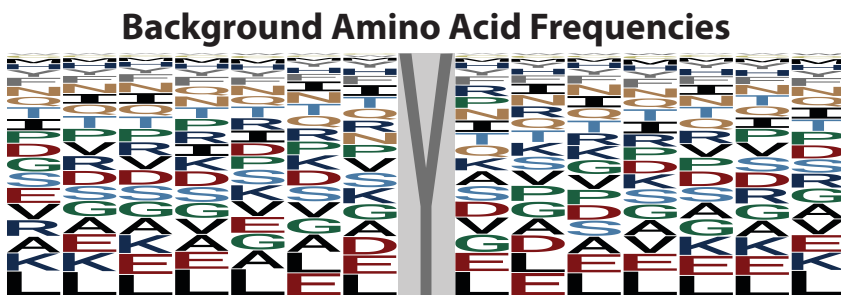


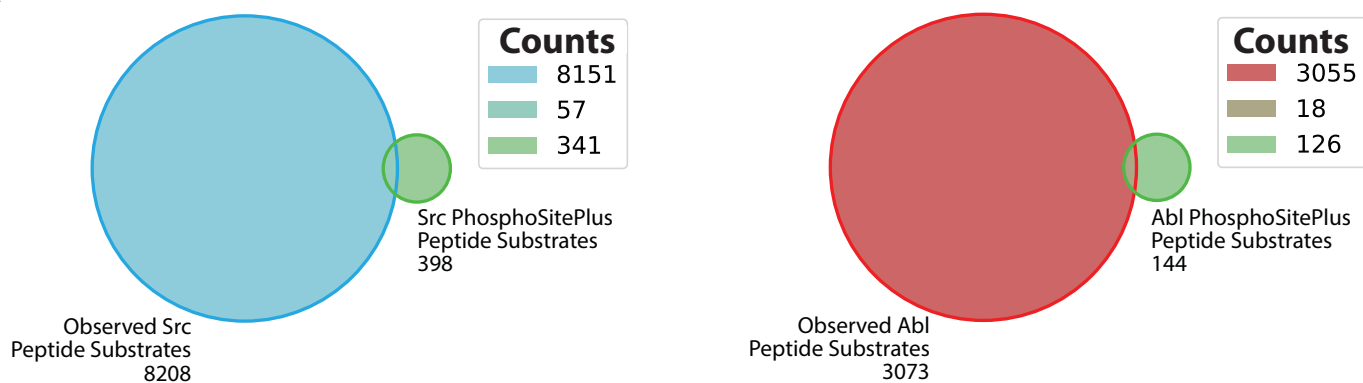
Figure 1 — figure supplement 2: Frequencies of each amino acid from the unenriched background sample. Sequence logo generated by WebLogo (Crooks *et al.* 2004) displaying frequency of amino acids in peptides containing a phosphotyrosine from an unenriched sample, that was also not treated with any target kinases.

Figure 1 — figure supplement 3: BackgroundUnenrichedProteomics.csv MaxQuant (Cox *et al.* 2008) results from a sample that was not enriched for phosphotyrosine. This data set was used to create the background amino acid frequencies.

Figure 1 — figure supplement 4: ControlPhosphoproteomicsData.csv MaxQuant (Cox *et al.* 2008) results from phosphoproteomics experiment where no kinase was added.

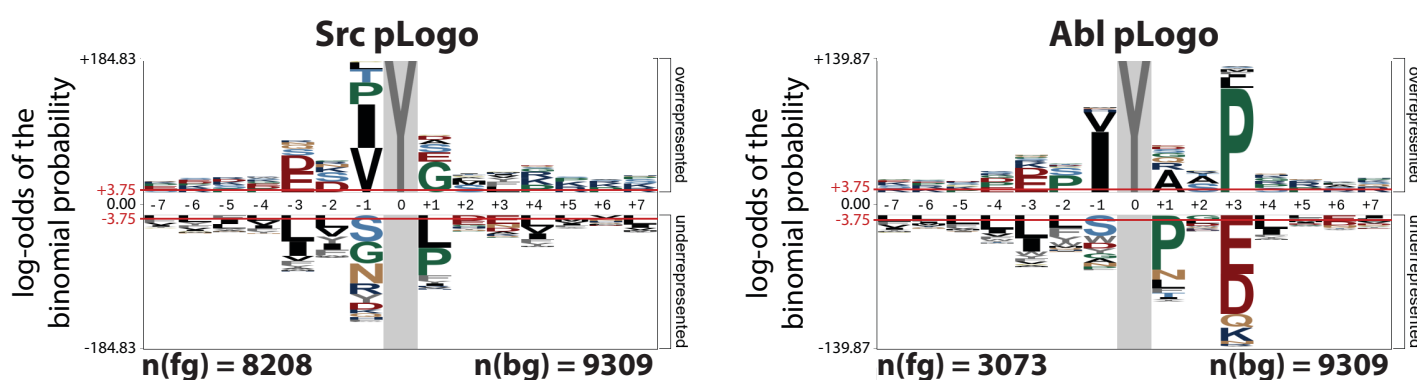
Figure 2

A



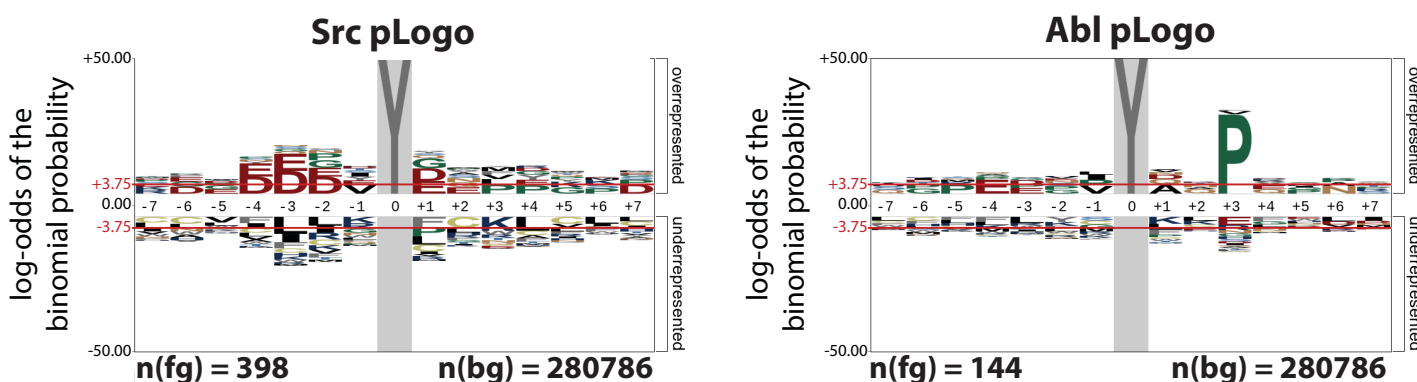
B

Observed Substrates



C

PhosphoSitePlus Substrates



HEK cell lysate library approach finds large number target substrates and recapitulates known specificities of Src and Abl with improved statistics. (A) Venn diagrams of sequences that were observed in our Src and Abl kinase specificity experiments compared to the substrates listed in PhosphoSitePlus (Hornbeck *et al.* 2015) for the respective kinase. **(B)** pLogo's for the prevalence of each amino acid in Src and Abl's individual set of substrates (8208 and 3073 sequences, respectively) relative to the background experiment (9309 sequences). **(C)** In comparison, pLogo's generated from the list of human substrates on the PhosphoSitePlus database (Hornbeck *et al.* 2015). Statistical significance level is shown as red line in B and C.

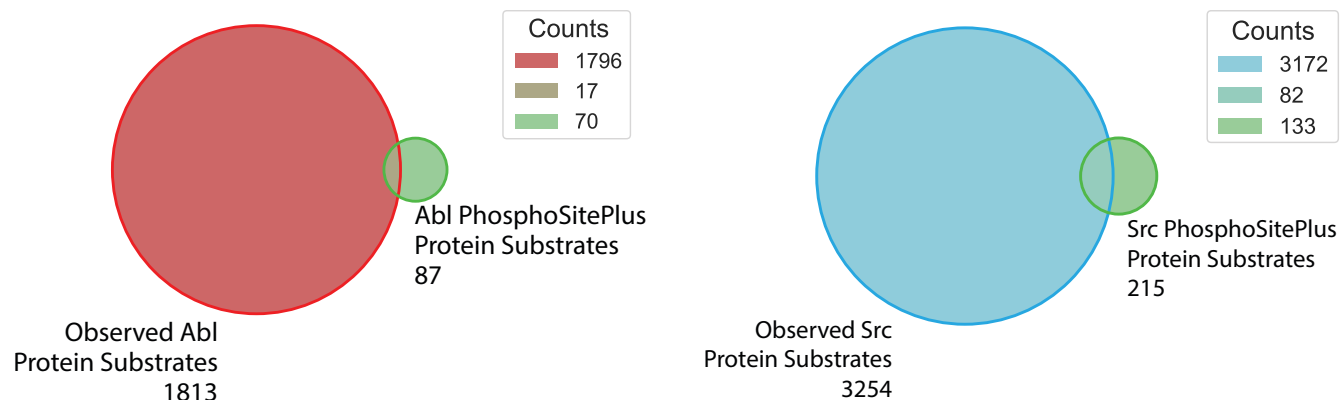


Figure 2 — figure supplement 1: Natural protein substrates found in phosphoproteomics experiments. Venn diagrams of proteins that were observed in our kinase specificity experiments compared to the proteins listed in PhosphoSitePlus (Hornbeck *et al.* 2015) for the respective kinase.

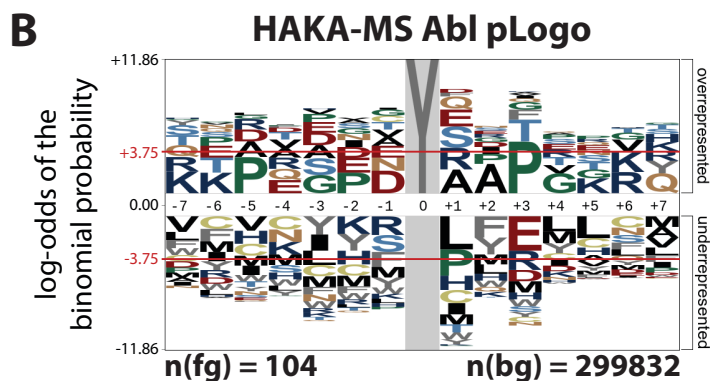
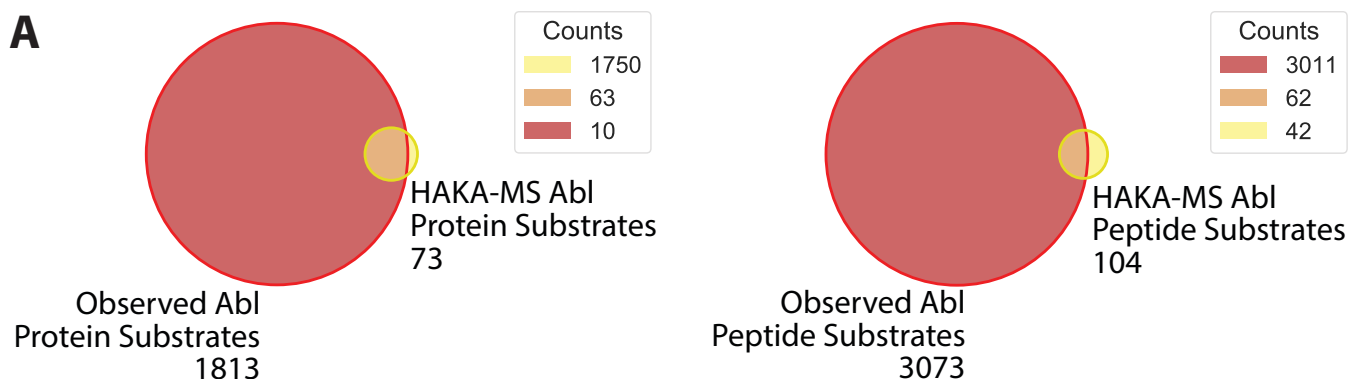
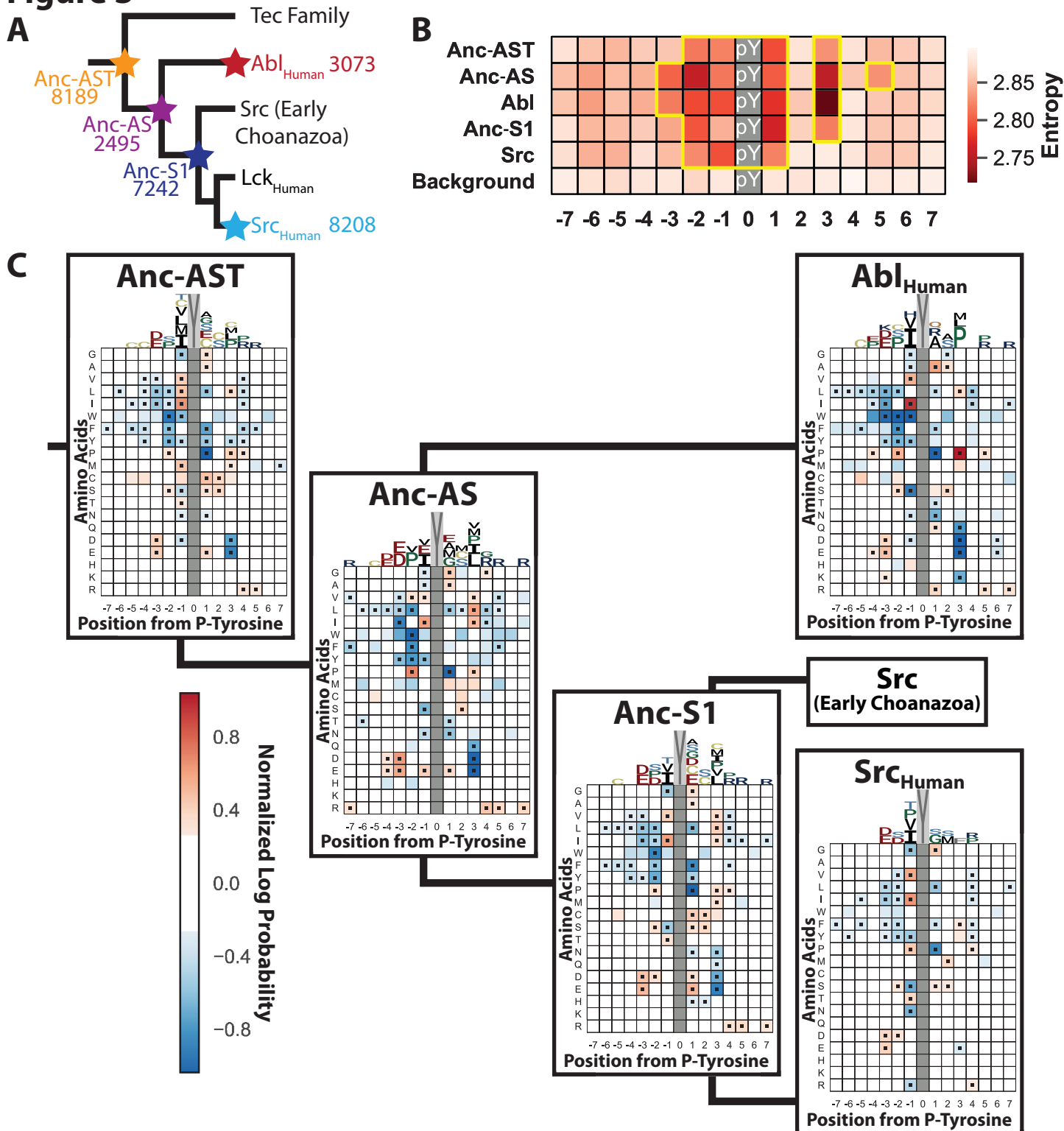


Figure 2 — figure supplement 2: Comparison of our results with published data from the HAKA-MS experimental procedure (Muller *et al.* 2016). (A) Comparison of how many substrates (proteins and peptide fragments) were found in either our whole cell lysate assay or the HAKA-MS method. (B) pLogo showing the preference of Abl based on the peptides from the HAKA-MS method. The background used in their study was all tyrosines in the human proteome. Only P₊₃ was found to be statistically significant.

Figure 3



Anc-AST and Anc-S1 are promiscuous, but are bridged by a relatively specific Anc-AS. (A) Total number of phosphopeptides found for each of the five kinases are plotted onto the gene tree. **(B)** Positional entropy description for each kinase, where a lower entropy indicates higher specificity. Highlighted in yellow are values below the ~30th percentile for sequence entropy, illustrating the positions with the highest specificity. **(C)** 20x15 positional/amino acid heatmap displaying the normalized log probability of each amino acid at a given position for modern and ancestral kinases. Significant residues at each position are marked with black squares ($p < 0.05$). The sequence logo above the heatmap displays positions with positive values only, and the height of the character is equal to the normalized log probability.

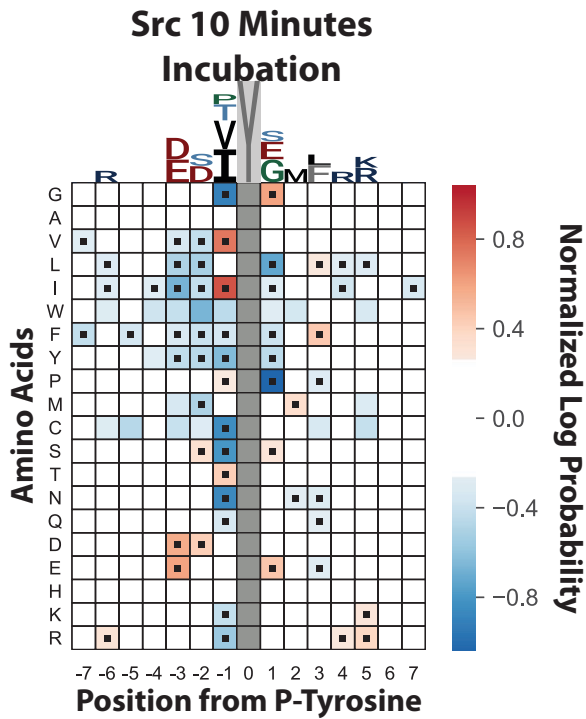
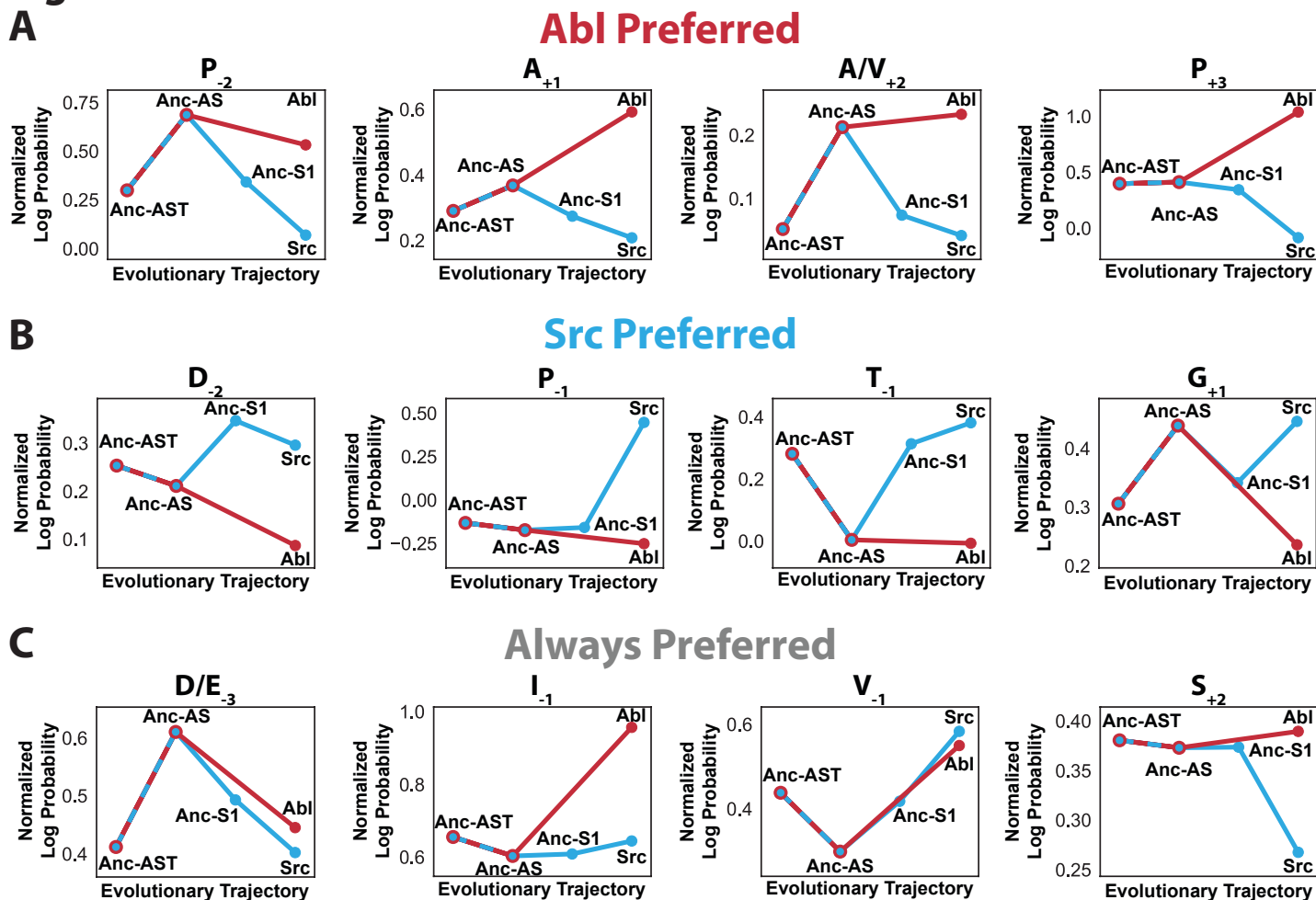


Figure 3 — figure supplement 1: Substrate specificity is not affected by saturation phosphorylation. Heatmap displaying enrichment of amino acids at each position in a set of Src substrates after only 10 minutes of incubation. This short time point retains all major features of the heatmap from Src's 4-hour time point in Figure 2C.

Figure 3 — figure supplement 2: PhosphoproteomicsData.csv MaxQuant results from phosphoproteomic experiments where kinase was added for the 4-hour incubation.

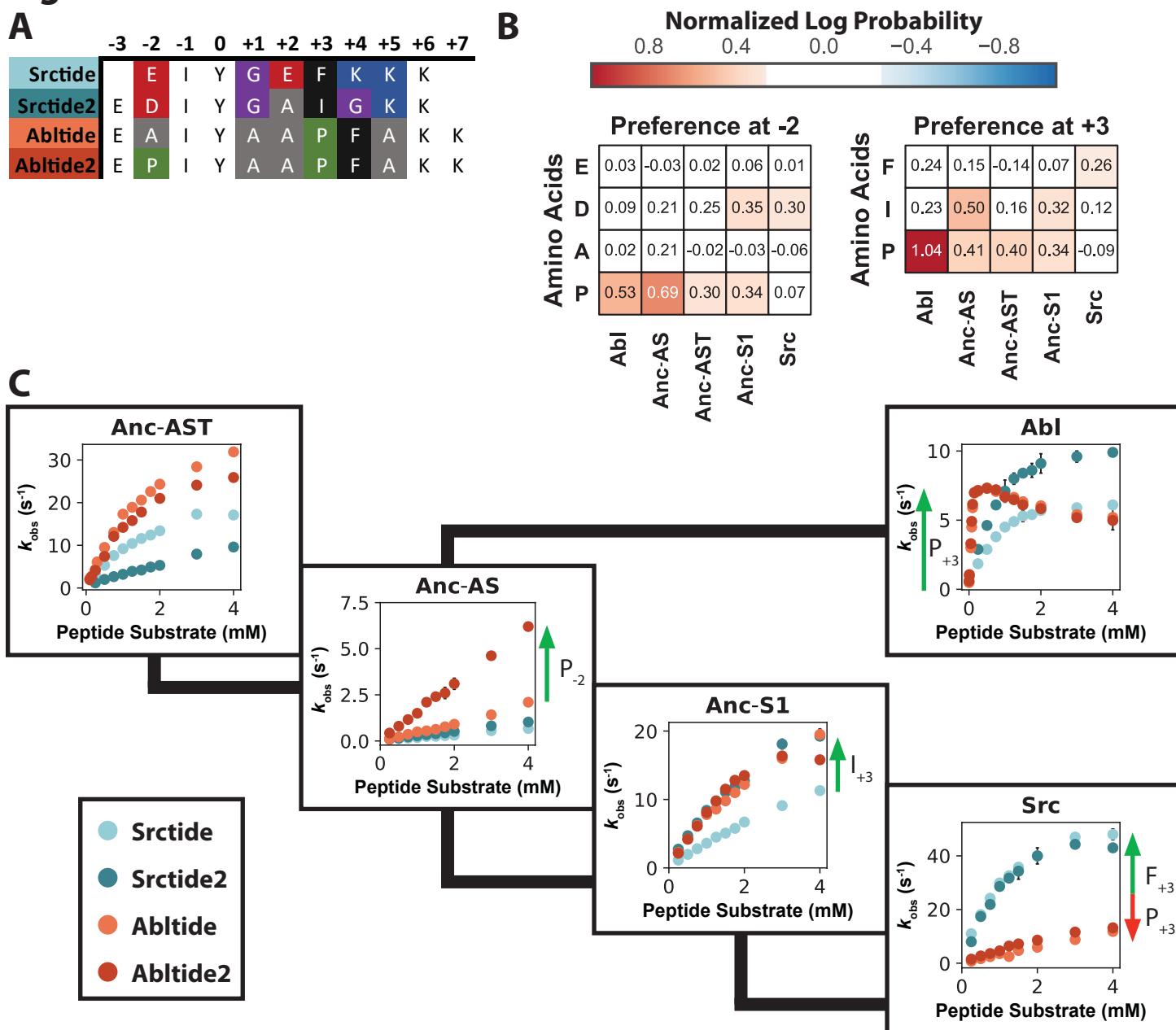
Figure 3 — figure supplement 3: PhosphoproteomicsSrcShortTimePoint.csv MaxQuant results from the phosphoproteomic experiment where Src was added for only 10 minutes.

Figure 4



Evolutionary trajectories of sequence specificity show both subfunctionalization and neofunctionalization. The normalized log probability of an amino acid occurring in a pool of substrates demonstrates the evolutionary progression for **(A)** Abl specific residues, **(B)** Src specific residues, and **(C)** residue determinants common to all all kinases in this study.

Figure 5



Specificity of kinases correlate with individual peptide turnover parameters. (A) Sequence alignment of the substrate peptides used with differences between peptides highlighted. Coloring of the peptides are used in the Figure 5C. **(B)** Normalized log probability of amino acid preferences for -2 and +3 positions for the five ancestral and modern kinases determined from the cell lysate experiments, see also Fig. 3C. **(C)** Michaelis-Menten curves of phosphorylation for the four peptide substrates and the five different kinases measured with a coupled assay for detecting ADP production (see methods). While it is not possible to saturate and measure accurate K_M values for many of the substrates, it is possible to observe how the rates are affected under k_{cat}/K_M conditions. Each kinase was assayed at 20-200 nM, and error bars represent the standard deviation from three measurements. Green arrows indicate the key primary sequence determinants in the different substrates responsible for the large changes in observed rates.

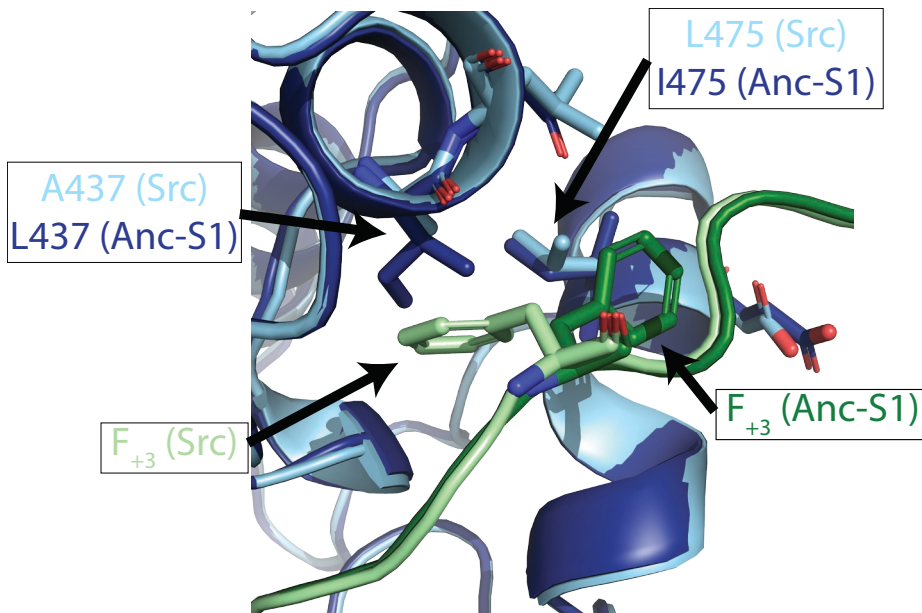


Figure 5 — figure supplement 1: Homology modelling of protein/substrate complexes suggests amino acid differences between modern and ancestral proteins responsible for the differing substrate specificity in the +3 position. A crystal structure of Abl containing a peptide bound to the active site (PDB: 2G2I; Levinson *et al.* 2006) was used to build homology models of Src and Anc-S1 bound to Src-tide. The bound peptide in PDB 2G2I already contained F₊₃, but the rest of the Src-tide sequence was modelled in. A Fast Relax protocol was ran in Rosetta with full constraints on the backbone. The zoom-in indicates how the L475I and A437L substitutions in Anc-S1 could prohibit to bind F₊₃ in the same pocket as Src.