


Evolution of protein kinase substrate recognition at the active site

Journal Article**Author(s):**

Bradley, David; Beltrao, Pedro 

Publication date:

2019-06-24

Permanent link:

<https://doi.org/10.3929/ethz-b-000531932>

Rights / license:

[Creative Commons Attribution 4.0 International](#)

Originally published in:

PLoS Biology 17(6), <https://doi.org/10.1371/journal.pbio.3000341>

RESEARCH ARTICLE

Evolution of protein kinase substrate recognition at the active site

David Bradley ^{*}, Pedro Beltrao ^{*}

European Molecular Biology Laboratory, European Bioinformatics Institute (EMBL-EBI), Wellcome Genome Campus, Cambridge, United Kingdom

* davidbr@ebi.ac.uk (DB); pbeltrao@ebi.ac.uk (PB)



Abstract

Protein kinases catalyse the phosphorylation of target proteins, controlling most cellular processes. The specificity of serine/threonine kinases is partly determined by interactions with a few residues near the phospho-acceptor residue, forming the so-called kinase-substrate motif. Kinases have been extensively duplicated throughout evolution, but little is known about when in time new target motifs have arisen. Here, we show that sequence variation occurring early in the evolution of kinases is dominated by changes in specificity-determining residues. We then analysed kinase specificity models, based on known target sites, observing that specificity has remained mostly unchanged for recent kinase duplications. Finally, analysis of phosphorylation data from a taxonomically broad set of 48 eukaryotic species indicates that most phosphorylation motifs are broadly distributed in eukaryotes but are not present in prokaryotes. Overall, our results suggest that the set of eukaryotes kinase motifs present today was acquired around the time of the eukaryotic last common ancestor and that early expansions of the protein kinase fold rapidly explored the space of possible target motifs.

OPEN ACCESS

Citation: Bradley D, Beltrao P (2019) Evolution of protein kinase substrate recognition at the active site. *PLoS Biol* 17(6): e3000341. <https://doi.org/10.1371/journal.pbio.3000341>

Academic Editor: Benjamin E. Turk, Yale University, UNITED STATES

Received: January 23, 2019

Accepted: June 12, 2019

Published: June 24, 2019

Copyright: © 2019 Bradley, Beltrao. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: The kinase phylogeny presented in [Fig 1A](#) is fully accessible via the Interactive Tree of Life (iTOL) resource. This tree includes full classifications (group, family, and subfamily) for each kinase and the mapping of switch events to the phylogeny at the family and subfamily levels (as shown in [S2 Fig](#)). The tree can be accessed from the following link: <https://itol.embl.de/shared/db534>. The kinase sequence alignment and phylogeny file (unannotated) are available on GitHub (<https://github.com/DBradley27/Kinase-specificity-evolution>). The ancestral sequence reconstructions and kinase divergence scores (at family and subfamily levels)

Introduction

Protein kinases are essential for signal transduction and have been found in every eukaryotic species so far examined. They are required for almost all cellular processes [1], and mutations in protein kinases are often associated with diseases such as cancer and diabetes [2–4]. Kinases are often described in terms of their ‘specificity’, which refers to the set of substrates that the kinase is able to phosphorylate *in vivo*. Multiple factors define the specificity of the kinase [5]. The kinase and substrate must be coexpressed and colocalised, for example, and their interaction may be mediated by adaptor or scaffold proteins [6,7]. Docking sites on the substrate may also be employed to recruit the kinase and the substrate directly [8,9]. Fundamentally, selectivity is often defined by the structural interface between the kinase active site and the residues flanking the target serine, threonine, or tyrosine—the so-called peptide specificity of the kinase.

The kinase peptide specificity is usually described in terms of a short linear motif [10,11]. The substrate motif of PKA (protein kinase A), for example, is R-R-x-S/T, meaning that an

are also available on GitHub, as are the eukaryotic and prokaryotic phosphorylation data used for the analysis presented in Fig 4. Finally, the R code used for the analyses in this manuscript is available in the form of R markdown reports, which are also available on GitHub (<https://github.com/DBradley27/Kinase-specificity-evolution>).

Funding: The authors received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: AC, ancestral conservation; AGC, (PKA, PKG, PKC); AUR, Aurora Kinase; BADASP, Burst After Duplication with Ancestral Sequence Predictions; CAMK, Calcium- and Calmodulin-regulated kinases; CAMK2, Calcium/calmodulin-dependent protein kinase type 2; CDC7, Cell Division Cycle 7-related protein kinase; CDK, Cyclin-Dependent Kinase; CK1, Casein Kinase 1; CK2, Casein Kinase 2; CLK, CDK-Like Kinase; CMGC, cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK) and CDK-like kinases (CLKs); Dbf4, Dumbbell former 4 protein; ELK, ePK-like kinase; GRK, G-protein-coupled Receptor Kinases; HPRD, Human Protein Reference Database; MS, Mass spectrometry; MSA, multiple sequence alignment; mya, million years ago; PDB, Protein Data Bank; PDZ, Post-synaptic density protein 95 (PSD-95), Drosophila disc large tumor suppressor (Dlg1), Zona occludens 1 (ZO-1); Pfam, Protein families; PHK, Phosphorylase Kinase; PIC, phylogenetic independent contrast; PKA, Protein Kinase A; PKC, Protein Kinase C; PKG, Protein Kinase G; PKIA, cAMP-dependent protein kinase inhibitor alpha; PLK, Polo-like kinase; PRKCG, Protein Kinase C gamma; PWM, position weight matrix; RC, recent conservation; RGC, Receptor Guanylate Cyclase; RSK, Ribosomal S6 Kinases; RSKp70, Ribosomal S6 Kinases p70; RSKp90, Ribosomal S6 Kinases p90; SDP, specificity-determining positions; SDR, specificity-determining residue; SGK, Serum and Glucocorticoid-regulated Kinase; SH2, Src Homology 2; SH3, Src Homology 3; SRPK, SR-rich protein kinase; S/T, serine/threonine; STE, sterile mutant; TK, tyrosine kinase; TKL, Tyrosine Kinase-Like.

arginine is preferred 2 and 3 positions N terminal to the target serine/threonine in the PKA active site. Conceptually, different substrate motifs can be thought of as different channels of communication within the cell, allowing for kinases that are simultaneously active to regulate a specific set of substrates. Mitotic kinases with overlapping localisations, for example, tend to have mutually exclusive substrate motifs, presumably to prevent the aberrant phosphorylation of nontargets during cell-cycle progression [12].

It is possible that the range of selectivities at the active site is restricted by the structure of the kinase domain itself. In turn, the capacity of the kinase fold to create novel specificity preferences through mutations may determine the maximum effective number of kinases possible for a genome, because it has been suggested for transcription factors [13]. In this analogy to a communication channel, the full set of possible substrate motifs can be thought of as the full bandwidth or ‘communication potential’ of the kinase fold. Understanding how this communication potential was explored over evolutionary time can reveal insights into the evolution of cell pathways and cell signalling. However, although the proliferation of the kinase domain itself has been well documented, much less is known about the evolution of new kinase specificities at the active site [14]. One study found that the frequency of tyrosine kinases (TKs) in the proteome correlates negatively with the frequency of tyrosine residues in the proteome, implying some extent of coevolution between kinases and substrates [15]. Another study found that the evolution of a new specificity in the CMGC (cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK) and CDK-like kinases (CLKs)) group proceeded through an intermediate of broad specificity (P + 1/R + 1) before later specialisation into distinct target preferences (P + 1 and R + 1) [16].

Currently, the scarcity of kinase-substrate interaction data outside of a few model organisms (human, mouse, and budding yeast) is limiting for further research. However, other sources of data can yield insights more indirectly. An evolutionary analysis of the kinase domain can be informative provided that the specificity-determining positions (SDPs) are known [17]. This applies to phosphoproteome data also, provided that motifs can be extracted and linked to the known specificities of kinase families or subfamilies. Here, we collect kinase sequence data, kinase specificity data, and phosphorylation data from several species to perform an evolutionary analysis of kinase specificity. Collectively, the results suggest that most specificities arose early in the evolution of protein kinases, followed by a long period of relative stasis.

Results

Global kinase phylogeny for 9 different species

The eukaryotic protein kinase superfamily by convention is divided hierarchically at the level of ‘groups’, ‘families’, and ‘subfamilies’ [18,19]. The most up-to-date classification is based primarily upon sequence similarity between kinase domains but also takes into account the kinase sequence outside of the kinase domain, the known function(s) of the kinase, and previous manual classifications of known orthologs [19,20].

The 8 canonical kinase groups (AGC [PKA, PKG, PKC], CAMK [Calcium- and Calmodulin-regulated kinases], CK1 [Casein Kinase 1], CMGC [cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK) and CDK-like kinases (CLKs)], RGC [Receptor Guanylate Cyclases], STE [sterile mutant], TKL [Tyrosine Kinase-Like], and TK) evolved the earliest and, with the exception of TKs and the RGC group, are thought to have arisen in an early eukaryotic ancestor [21]. Kinase families, and then subfamilies, generally emerged later during evolution and reflect more distinct features of the kinase’s function (specificity, regulation, localisation, etc.) [18]. In order to study the

evolution of kinase specificity, we first performed a systematic phylogenetic analysis to predict kinase functionally divergent residues for every kinase family and subfamily where possible. To this end, a global kinase domain phylogeny was constructed for the 9 manually curated opisthokont kinomes (*Homo sapiens*, *Mus musculus*, *Strongylocentrotus purpuratus*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Amphimedon queenslandica*, *Monosiga brevicollis*, *Saccharomyces cerevisiae*, and *Coprinopsis cinerea*) present in the kinase database KinBase (<http://kinase.com/web/current/kinbase/>).

The resulting phylogeny contains 2,094 kinase sequences spanning 8 different groups (Fig 1A). Although based just on the kinase domain sequence, the grouping of kinase sequences accords well with the group/family/subfamily classifications provided by KinBase. For example, out of 102 families tested, 70 show exact correspondence to the KinBase classifications in that all family members group together to the exclusion of kinase sequences from any other family (i.e., the family is monophyletic). This was found to be the case also for 69 out of 105 subfamily kinases tested. For the remaining families and subfamilies tested (32 and 36, respectively), we consider for further analysis (described below) only the largest clade containing the family/subfamily sequences of interest. These contain relatively few 'spurious' sequences on average from other families and subfamilies (median clade purity 91.5% and 66.7%, respectively) and tend to cover a large proportion of all kinases annotated by KinBase to a particular family or subfamily (median family coverage: 95.1%, median subfamily coverage: 100%).

Residues implicated in the differentiation of duplicated kinases

For 99 kinase families and 83 kinase subfamilies, we identified residues that are conserved within a clade but differ from the sister clade in the phylogeny. These residues are implicated as functionally divergent residues and are expected to underlie functional differences between kinase sister clades. This was achieved by calculating divergence scores (s) for each alignment position and each family and subfamily using an adaptation of the phylogenetic BADASP (Burst After Duplication with Ancestral Sequence Predictions) method [22]. Among other methods [23–26], we selected BADASP given that it enables ancestral family sequences to be compared directly (Fig 1B; Materials and methods), allowing determinant positions arising at a specific point in time to be captured using user-defined protein family definitions [22].

Functionally divergent residues were first predicted across all kinase families. A detailed analysis of the results suggests multiple ways in which novel kinase functions have evolved at the family level via changes to functionally relevant residues. In the SRPK (SR-rich protein kinase) family (CMGC), for example, 2 substitutions to negatively charged amino acids (D and/or E) map to parts of the kinase structure (α F- α G region) that have been shown previously to bind to a positively charged docking peptide (Fig 1C; [27]). In the CDC7 (Cell Division Cycle 7-related protein kinase) family (CMGC) also, 2 of the identified functionally divergent residues bind to the CDC7 activator protein named Dbf4 (Dumbbell former 4 protein) (Fig 1D; [28]) and are therefore important for kinase regulation. In other examples, the functionally divergent residues identified can help to account for the specificity of the kinase. Two substitutions in the CAMK2 family (CAMK), for example, bind to a preferred D/E residue at the substrate +2 position (PDB [Protein Data Bank]: 5H9B). A glycine substitution in the activation loop has also been shown to be important for kinase function [29] and may explain why CAMK2 kinases do not require activation loop phosphorylation for activity ([30], Fig 1E). Finally, many substitutions for the acidophilic PLK (Polo-like kinase) family map to SDRs (specificity-determining residues) and are convergent with those identified for the unrelated GRK (G-protein-coupled Receptor Kinase) family (AGC), which contains some acidophilic kinases ([17,31], Fig 1F). These examples illustrate how the predicted functionally divergent

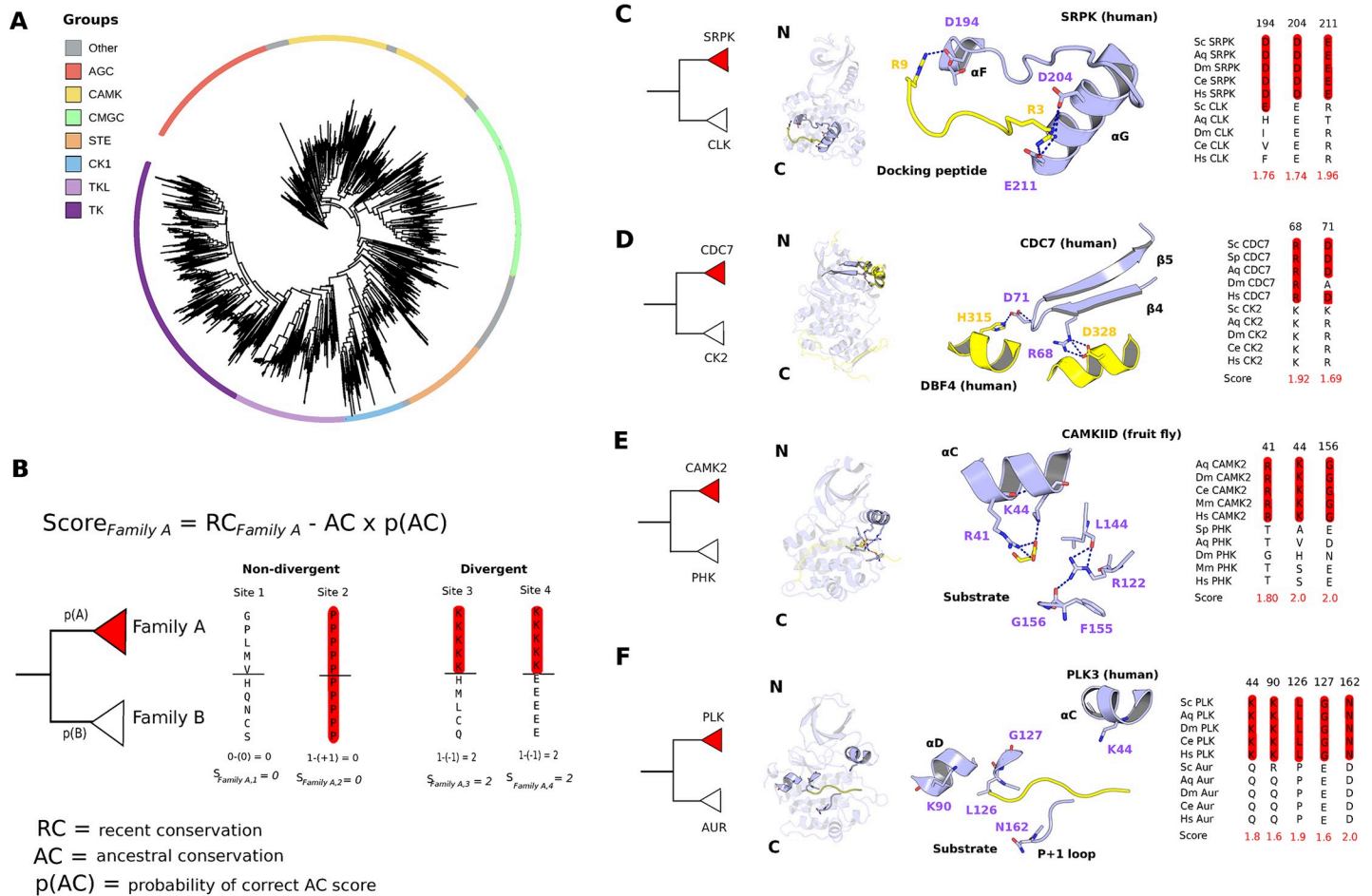


Fig 1. Family and subfamily divergent residues. (A) Kinase domain phylogeny for 2,094 different kinase domains, spanning 9 species. Each sequence has been coloured according to its group membership. (B) Explanation of the score used to identify divergent residues. RC: conservation of the residue within the family of interest. AC: extent to which the ancestral residues are conserved between sister clades in the phylogeny. p(AC): a measure of confidence in the ancestral sequence prediction. A full explanation is given in the Materials and methods section. (C–F) Examples of residues predicted to be functionally divergent in the SRPK (PDB: 1WBP), CDC7 (PDB: 4F9A), CAMK2 (PDB: 5H9B), and PLK (PDB: 4B6L with peptide binding modelled) families. In these 4 examples, kinase residues have been numbered according to their position in the protein kinase domain (Pfam: PF00069). The peptides and/or proteins that physically interact with the kinase have been coloured in yellow. AC, ancestral conservation; AGC, (PKA, PKG, PKC); AUR, Aurora Kinase; CAMK, Calcium- and Calmodulin-regulated kinases; CAMK2, Calcium/calmodulin-dependent protein kinase type 2; CDC7, Cell Division Cycle 7-related protein kinase; CK1, Casein Kinase 1; CK 2, Casein Kinase 2; CLK, CDK-Like Kinase; CMGC, (cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK) and CDK-like kinases (CLKs)); PDB, Protein Data Bank; PHK, Phosphorylase Kinase; Pfam, Protein families; PLK, Polo-like kinase RC, recent conservation; SRPK, SR-rich protein kinase; STE, sterile mutant; TK, tyrosine kinase; TKL, Tyrosine Kinase-Like.

<https://doi.org/10.1371/journal.pbio.3000341.g001>

sites between families or subfamilies of kinases can map to functionally relevant residues. We next studied whether this would be a general feature of these residues across many families and subfamilies.

Functionally important residues are often divergent across kinase families and subfamilies

Across all kinase families, we aggregated the total number of predicted functionally divergent residues or ‘switches’ at each position in the kinase domain. This allows us to predict positions that often determine the functional differences between kinase families. These were mapped across the kinase catalytic domain sequence and fold (Fig 2, left). The distribution of residues

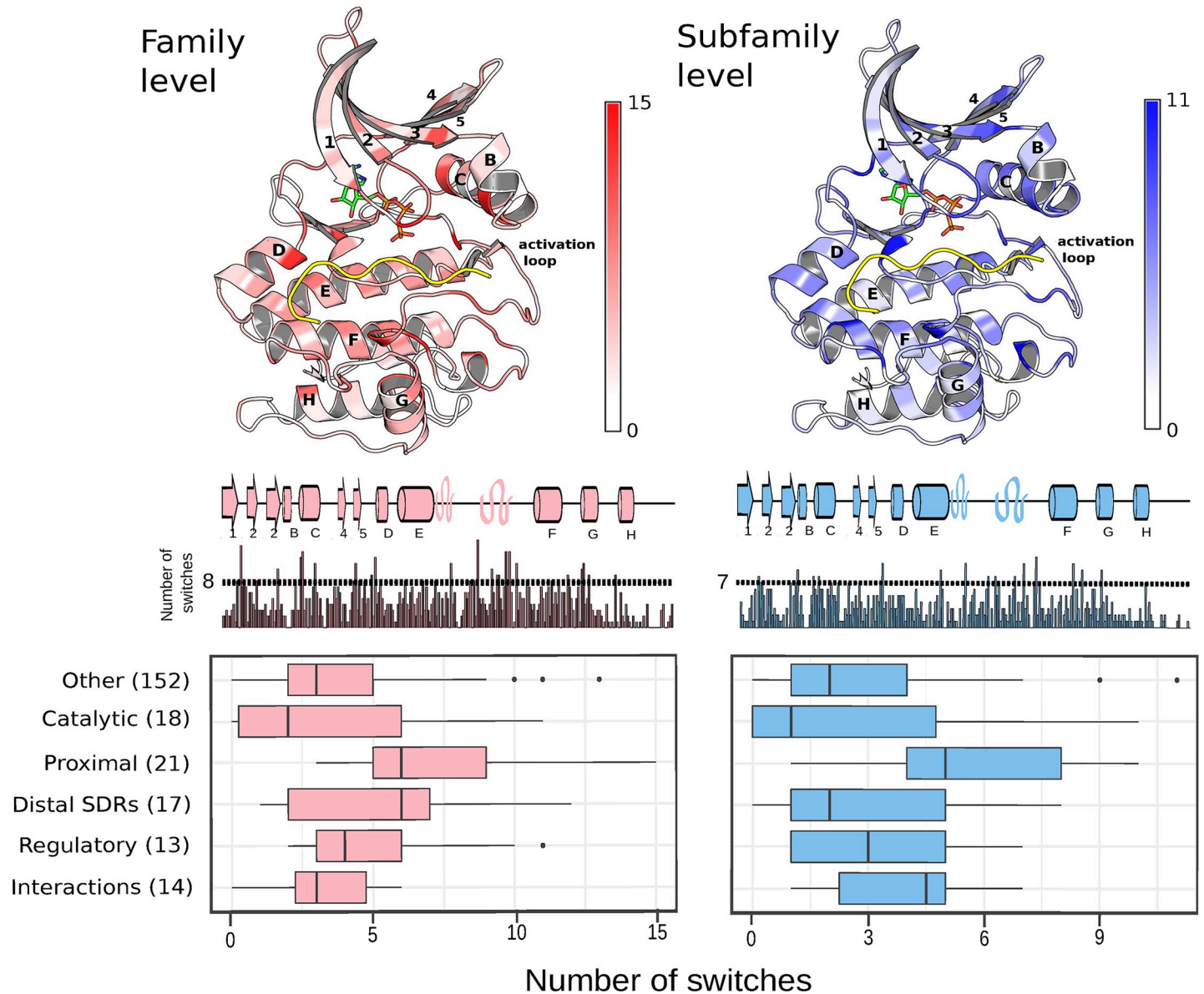


Fig 2. Aggregated analysis of sequence divergence across kinase families (left) and subfamilies (right). For each kinase domain position, the total number of ‘switches’ (score > 95th percentile of scores) was counted across all families or subfamilies considered (see [Materials and methods](#)). (Top) Results mapped to kinase structures (mouse protein kinase A: PDB 1ATP). The kinases are represented in complex with an ATP molecule (green, orange, blue, red) and a substrate-mimicking inhibitor (PKIA, yellow). Darker shades of red or blue represent a higher number of switches (see colour bar, right). (Middle) Total number of switches mapped to the kinase primary sequence, with secondary structure elements represented above the bar plot. A domain position is considered to be ‘frequently switching’ if the number of switches lies above a 90th percentile threshold for the kinase domain. The threshold is 8 for families and 7 for subfamilies. (Bottom) The values for each domain position have been grouped according to the functional category (‘catalytic’, ‘regulatory’, ‘proximal’, etc.) and the distribution plotted separately at the family and subfamily level. The numbers in brackets represent the number of residues in each category. PDB, Protein Data Bank; PKIA, cAMP-dependent protein kinase inhibitor alpha; SDR, specificity-determining residue.

<https://doi.org/10.1371/journal.pbio.3000341.g002>

that are often implicated in kinase family functional differences is not uniform and strongly enriched within or close to the kinase activation segment, the α C helix, the β 5- α D region, and the α F- α G regions (Fig 2, left). For further analysis, we divided kinase residues into functional categories: ‘catalytic’ (catalytic residues and the catalytic spine), ‘proximal’ (within 4 Angstroms of the peptide substrate but excluding ‘catalytic’ positions), ‘distal SDRs’ (distal SDRs

implicated in [17]), ‘regulatory’ (regulatory spine residues and those within and surrounding the activation loop), ‘interaction’ residues (those most frequently in contact with other protein domains), and ‘other’ (residues not belonging to any of the previous categories). All residues belonging to these categories are defined in [S1 Table](#), using mappings to a PKA reference (PDB: 1ATP) and the Pfam (Protein families) protein kinase domain (Pfam: PF00069). We defined as frequently switching residues those above the 90th percentile of residues with most changes. The majority (14/21) of frequently switching residues can be assigned to a functional category (catalysis, specificity, regulation, etc.), which is more than would be expected by chance ($p = 0.0048$; Fisher’s exact test, one-sided). This suggests that this approach can successfully identify residues that are of relevance for the functional divergence of kinases. Of these residues, 8 have been implicated in determining differences in kinase specificity: domain position 84 [32–34], 86 [35], 144 [16,36,37], 157 [37,38], 158 [35,37], 162 [35], 164 [39,40], and 205 [35]. The number of substitutions for ‘proximal’ residues is generally higher than that for residues without an assigned function (Mann-Whitney, one-tailed, $p = 3.2 \times 10^{-6}$). These residues lie at the kinase-substrate interface and are likely to perturb kinase specificity when mutated. We find similar results when using SDR annotations from a global analysis of kinase specificity [41] (Mann-Whitney, one-tailed, $p = 9.0 \times 10^{-3}$) and when using a literature-curated set of SDRs provided by the same study [41] (Mann-Whitney, one-tailed, $p = 3.6 \times 10^{-5}$).

A similar analysis was performed for kinase subfamily comparisons ([Fig 2](#), right). Similar to kinase family evolution, a large fraction (73%, 11 out of 15) of residues frequently implicated in the functional differences between kinase subfamilies were also annotated to a functional category ($p = 0.0043$; Fisher’s exact test, one-sided). All 15 frequently switching residues have been mapped to the protein kinase fold in [S1 Fig](#), alongside the 21 frequently switching residues at the family level. We also observed a higher than expected number of switches for ‘proximal’ residues at the subfamily level when compared with residues not annotated with a function (Mann-Whitney, one-tailed, $p = 2.2 \times 10^{-5}$). This was found to be the case also when using the SDR annotations in [41] (Mann-Whitney, one-tailed, $p = 2.2 \times 10^{-4}$) and a set of literature-curated SDRs from the same study (Mann-Whitney, one-tailed, $p = 1.7 \times 10^{-3}$).

All switch events within the ‘proximal’ category at the family and subfamily levels have been mapped to the kinase phylogeny in [S2 Fig](#). Taken together, these results suggest that substrate-determining residues often undergo substitutions as new kinase families and subfamilies emerge. We note, however, that the probability that any given residue in the ‘proximal’ category will diverge between sister families is relatively low; the most frequently substituted residue was found to have switched in only 17.6% of families analysed, for example ([S3 Fig](#)). The relationship between kinase sequence divergence and kinase specificity divergence is discussed further below.

Evolution of experimentally determined kinase target preferences

The above results suggest that residues important for kinase specificity differ often across kinase families and subfamilies. We then studied the extent to which these changes in kinase residues impact upon their target specificity. To study this, we derived specificity models for 101 S/T kinases from human and mouse, using experimentally determined target sites listed in the literature-curated databases HPRD (Human Protein Reference Database), Phospho.ELM, and PhosphoSitePlus [42–44]. This approach tends to produce PWMs (position weight matrices) that are similar to those generated using a peptide-screening approach [35].

We then tested the extent to which kinase specificities differ within and between groups, families, and subfamilies for kinases of known specificity. In line with expectation, the differences in specificity are larger across groups than across families and also larger across families

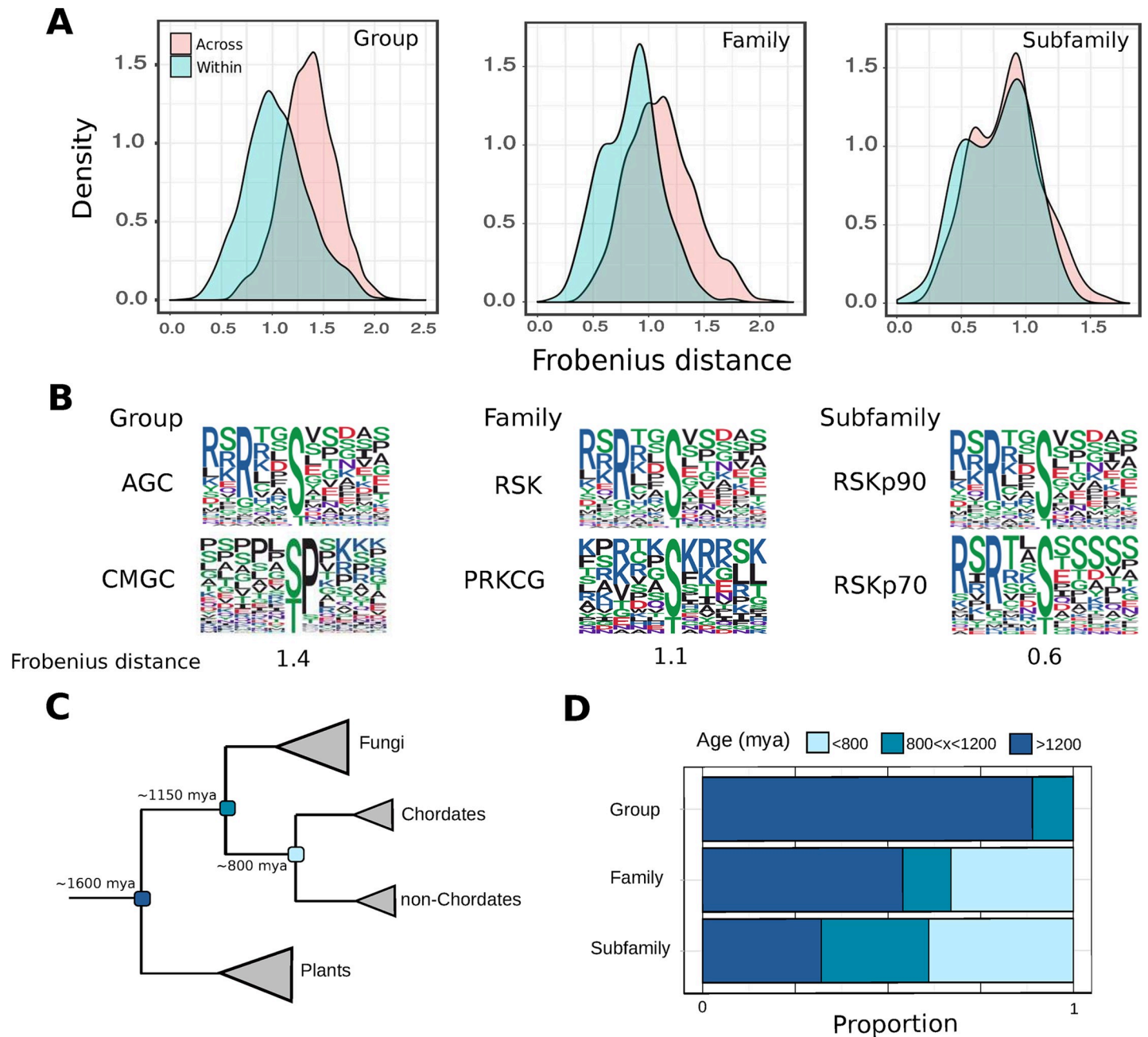


Fig 3. Evolution of kinase specificity at the group, family, and subfamily level. (A) Differences in S/T kinase specificity models at the group, family, and subfamily levels. The Frobenius distance was calculated for all possible pairwise comparisons within and between groups, families, and subfamilies. (B) Representative kinase pairs belonging to different groups (left), families (centre), and subfamilies (right). Frobenius distances for each of the 3 pairs are given beneath the logos. (C) A simplified tree of life with 3 important divergence times (plant-opisthokont, fungi-metazoa, chordate-nonchordate) marked. (D) Phylogenetic estimation of kinase ages at the group, family, and subfamily level for S/T kinases. AGC, (PKA, PKG, PKC); CMGC, cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK) and CDK-like kinases (CLKs); mya, million years ago; PRKCG, Protein Kinase C gamma; RSK, Ribosomal S6 Kinases; RSKp70, Ribosomal S6 Kinases p70; RSKp90, Ribosomal S6 Kinases p90; S/T, serine/threonine.

<https://doi.org/10.1371/journal.pbio.3000341.g003>

than across subfamilies (Fig 3A). For subfamilies, the differences are not statistically different from the distances measured within subfamilies ($p = 0.19$, Kolmogorov-Smirnov test, two-sided). These results suggest that kinase specificity often diverges at the level of the group, less

so at the family level, and rarely when new subfamilies emerge. We show in Fig 3B some examples of typical differences in kinase specificity for the 3 classifications. Although the differences in specificity across families is statistically different from expectation ($p \ll 0.01$, Kolmogorov-Smirnov test, two-sided), the 'typical' differences observed are smaller than at the group level. This is illustrated by the RSK (Ribosomal S6 Kinases) and PKC (Protein Kinase C) families (Fig 3B, centre), which both have a preference for arginine at the -3 position, but PKC additionally has a preference for R at position $+2$ whereas RSK has a modest preference for the same residue at position -5 .

We next considered the relationship between the divergence in kinase sequence and divergence in kinase specificity. Even for kinase sequences of unknown function ('other' category, $n = 152$), the sequence identity between kinase pairs was found to correlate with the distances between their PWMs ($r = -0.39$, $p \ll 0.01$). The correlation between the two was even greater for residues of the 'proximal' category ($n = 21$, $r = -0.48$, $p \ll 0.01$) and for a 'high-confidence' set of residues ($n = 10$, $r = -0.48$, $p \ll 0.01$; S5 Fig) close to the substrate that covary strongly with specificity [17]. We then considered the extent of sequence change required before divergence in specificity could be observed, which we define here using a PWM Frobenius distance of 1.06 (see Materials and methods). Generally, for the 'other', 'proximal', and 'high-confidence SDR' categories, new specificities emerge as sequence identities fall below approximately 55%, approximately 70%, and approximately 80%, respectively (S5 Fig), corresponding to 68, 6, and 2 required switches, respectively. These data suggest that specificity changes at the family and subfamily level should be relatively rare, because only 9 kinase families and 4 kinase subfamilies were found with at least 6 'proximal' switch residues. However, we note that the impact of residue mutation will likely depend upon the nature of the substitution ('conservative' or 'nonconservative'), the substrate position affected (e.g., $+1$ and -3 tend to be much more important for specificity than -1), and the existence of compensatory mutations in the kinase domain that might buffer the effect of SDR mutations, because other studies have demonstrated experimentally that a single SDR mutation can be sufficient to radically alter specificity [36,38,40].

To put the previous results into the context of evolutionary time scales, we sought to date the emergence of many kinase groups, families, and subfamilies. To this end, the presence or absence of every S/T kinase group, family, and subfamily was predicted for several species across the tree of life (Fig 3C). The phylogenetic origin of kinase groups, families, and/or subfamilies was then predicted using ancestral state reconstructions, which allowed their emergence to be dated based on the known divergence times between species [45]. Overall, we estimated that most kinase groups arose in a universal eukaryotic ancestor, in line with a previous study [21]. For kinase families, around 55% are estimated to have arisen in a universal ancestor, and up to 65% have arisen before the split between chordates and nonchordates (approximately 800 million years ago [mya]). Around 60% of subfamilies were similarly estimated to have arisen before the split between chordates and nonchordates (Fig 3D). Together with the analysis of kinase specificity differences, this result suggests that relatively few kinase specificities are likely to have arisen in the past 800 million years of kinase evolution.

Kinase motif enrichment across 48 eukaryotic species

The analysis of kinase specificity differences described above can only be performed for kinases with many experimentally determined targets. For most kinases, this information is not available [17,46]. As an alternative way to study the evolution of kinase specificity, we analysed MS (mass spectrometry)-derived phosphorylation sites from a broad range of species. The phosphoproteome of any given species represents an ensemble of kinase activities. Many

of these kinases will have preferred target site sequence motifs that are required for optimal substrate phosphorylation. The signature of several different kinases may therefore be encoded in each phosphoproteome.

For this study, we were interested in determining the extent to which different kinase-substrate motifs have been exploited during the evolution of the eukaryotes. To this end, phosphoproteome data were collected from 48 eukaryotic species, including species from the alveolates (4), amoebozoa (1), excavates (3), fungi (19), heterokonts (1), metazoa (12), and plants (8). We first measured the enrichment of 3 well-established substrate signatures (R-x-x-S/T, S/T-P, and S/T-x-x-D/E) and found them to be strongly enriched in nearly all of the 48 species (Fig 4, top). This suggests that these 3 common preferences are likely to have been present very early on during the evolution of the eukaryotes. To extend this to other kinase preferences, target site S/T sequence motifs were extracted from each species phosphoproteome using the motif-x tool [47]. Motifs without consistent enrichment across related species were filtered from any further analysis (see [Materials and methods](#)). In total, 29 motifs were (Fig 4) identified, which account for approximately 54% of all phosphosites analysed (S6A Fig).

Among the 29 motifs identified, 11 have been characterised previously in the literature and have been assigned to at least 1 kinase family or subfamily. This includes well-known motifs such as the CDK (Cyclin-Dependent Kinase) family motif (S/T-P-X-K) and the CK2 family motif (S/T-D/E-x-D/E). Eight of the motifs feature either a proline at position +1 or an arginine at position -3. Other motifs were identified in addition to those that are well characterised (Fig 4, motifs in black). Here, multiple constraints were imposed to ensure that the selected motifs were likely to represent bona fide kinase target motifs. For example, motifs with simple S/T additions to a classical motif were filtered from the analysis, because they could result from phosphosite misassignment within phosphopeptides from the mass spectrometry analysis or potential clustering of phosphorylation sites in the substrate primary sequences [48,49]. Overall, 18 uncharacterised motifs were selected using the protocol described in the Materials and methods section. Some of these motifs feature 'new' substrate specificity determinants such as asparagine (N) and glycine (G).

In Fig 4, enrichment p -values for each motif were calculated for each species relative to a background set of shuffled phosphorylation site sequences, with the S/T retained at the centre. This analysis suggests that the majority of motifs (Fig 4) are pervasive across the eukaryotic tree of life. This finding is even more evident when phosphorylation data are pooled from each of the major taxonomic clades (animals, fungi, plants, etc.) and the enrichment p -values are recalculated (S6B Fig and S6C Fig). Most of the motifs analysed are distributed across clades that diverged early during the evolution of the eukaryotes. For example, 18 out of 29 motifs (62%) are highly enriched ($p < 1 \times 10^{-6}$) in animals, fungi, and plants, indicating that they are likely to be of ancient origin.

The distribution of motif enrichments between related species is nonrandom, as supported by tests for the phylogenetic signal of phosphorylation motifs (S2 Table). We tested whether kinase motif enrichments correlate with the frequency of their expected effector kinases in the kinome, including, for example, the frequency of CDKs with the frequency of S/T-P-x-K motifs. However, our analysis suggests that kinase family or subfamily frequencies are not generally correlated with motif enrichment values when the phylogenetic interdependence of data points is taken into account [50] (S7 Fig). In spite of this, there is local evidence of kinase-substrate coevolution for some clades and motifs. In the plants, for example, the lack of enrichment of the basophilic R-R-x-S/T motifs can likely be explained by the depletion of its cognate effector kinase (PKA/PKG (Protein Kinase G)) (S8 Fig), as has been suggested previously [51,52]. For the CDK family also, the pattern of S/T-P-x-K evolution and CDK evolution is similar across many species (S9 Fig). However, many other patterns cannot be similarly

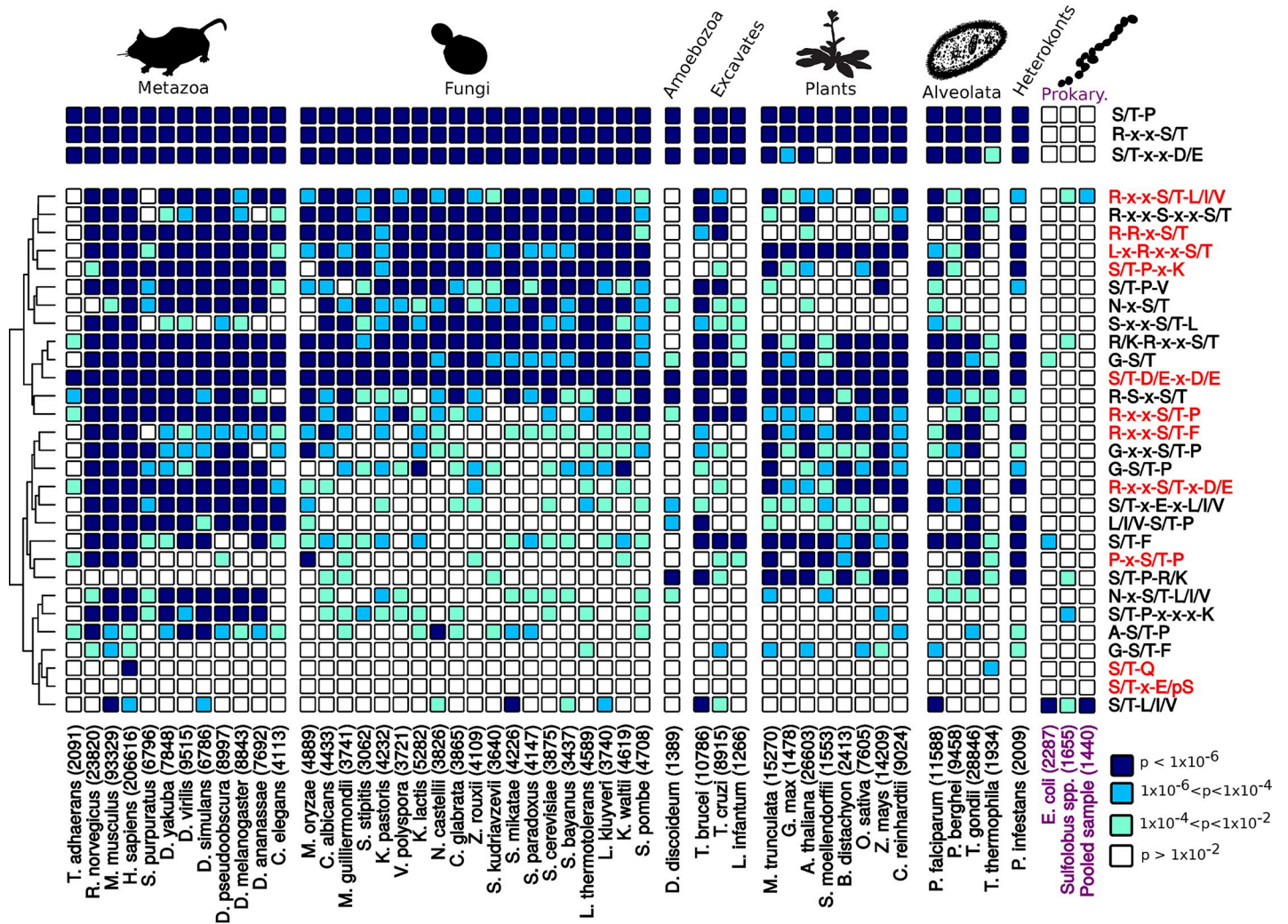


Fig 4. Enrichment of S/T phosphorylation motifs across several species. Binomial *p*-values were calculated for each motif and each species considered. The heat map cells are coloured according to the extent of enrichment for that particular motif and species (see legend, bottom right). The numbers in the column labels correspond to the sample size of unique S/T phosphorylation sites (15-mer). Prokaryotic phosphosite samples are coloured in purple. (Top) Enrichment of 3 common phosphorylation signatures (S/T-P, R-x-x-S/T, and S/T-x-x-D/E). (Bottom) Enrichment of 29 motifs discovered using the motif-x tool. Motifs in which the effector kinase has already been described in the literature are coloured in red. S/T, serine/threonine.

<https://doi.org/10.1371/journal.pbio.3000341.g004>

accounted for, which suggests that there are multiple factors that can affect the fold enrichment values calculated.

Kinase motif enrichment in prokaryotes

Some kinases encoded in the genome of prokaryotes are homologous to eukaryotic protein kinases and are currently referred to as ePK-like kinases (ELKs) [53,54]. Current genomic data now suggest that these eukaryotic-like kinases are as prevalent as histidine kinases in the prokaryotes [55]. Until recently however, the S/T phosphoproteomes of archaean and bacterial species had remained poorly characterised [56]. We repeated the motif analysis on the species *Escherichia coli* (bacteria) and *Sulfolobus* spp. (archaea), which are currently the only 2 organisms with more than 1,000 determined S/T phosphosites [56–58]. This analysis suggests that a large majority of the eukaryotic phosphorylation motifs discussed previously are not

significantly enriched in these 2 species (Fig 4). For *E. coli*, there is a moderate enrichment for some single-site motifs, which could have evolved convergently with those motifs found in eukaryotes. For *Sulfolobus*, we observe only weak enrichment for 5 eukaryotic motifs (S/T-L/I/V, S/T-P-x-x-x-K, S/T-P-R/K, R/K-R-x-x-S/T, and R-x-x-S/T-L/I/V). We note also that the S/T-P and R-x-x-S/T motifs that are highly prevalent in the eukaryotes show no evidence of enrichment across the prokaryotic species tested (Fig 4). Although it could be argued that the low phosphosite sample sizes (2,287 and 1,655 for *E. coli* and *Sulfolobus*, respectively) precludes the reliable detection of weaker motifs, we note that both of these signatures (S/T-P and R-x-x-S/T) were found to be strongly enriched in eukaryotic species with small sample sizes (*Dictyostelium discoideum*: 1,389, *Leishmania infantum*: 1,266). A similar lack of enrichment was found for a pooled sample of prokaryotic phosphorylation sites ($n = 1,440$) from several species [57] (Fig 4).

Prokaryotic motifs were then identified de novo using the motif-x tool for *E. coli* and the *Sulfolobus* genus. None of the motifs identified overlap with the motifs recovered previously for eukaryotic species (S3 Table and S4 Table). For *Sulfolobus*, in particular, we also found that 5 out of the 7 motifs identified contain a positively charged residue (R/K), implying the existence of basophilic *Sulfolobus* kinases.

Discussion

Here, we have explored the evolution of protein kinase specificity at the active site, using a combination of kinase sequence data, phosphorylation data, and kinase specificity models. Using the sequence of protein kinases across several species, we have shown that the evolution of new kinase families is dominated by sequence changes that are likely to impact upon kinase function, including kinase peptide specificity. This is in line with our observation that kinases belonging to different groups and families typically show significant differences in their specificity. In contrast, kinases belonging to sister subfamilies do not show significant differences in their specificity. A phylogenetic analysis revealed that most kinase groups and families (89% and 54%, respectively) are of ancient origin among the eukaryotes, whereas subfamilies generally emerged later during evolution (only 32% are of ancient origin). Finally, phosphorylation motifs determined for 48 eukaryotic species were found to be broadly distributed across divergent species and likely emerged soon before or after the last eukaryotic common ancestor. Taking these different observations together, we suggest that the majority of the kinase active site specificities present today in eukaryotic species have emerged early on during the evolution of eukaryotes.

The analysis here of divergent residues across kinase families follows a similar analysis employing a BLAST-based approach [59]. This study is focused on the kinase catalytic domain, and we did not take into account the evolution of kinase domain composition or sequence changes outside the catalytic domain, which may have a significant impact on catalytic function [60]. Kinase docking interfaces also are not amenable to the aggregated analysis attempted here because their location in the kinase domain tends to differ significantly between families [8]. In general, the approach used here assumes that a given kinase domain position will adopt a given function (catalytic, regulatory, proximal, etc.) across all kinase families. However, examples are known already of modes of regulation or specificity that are particular to a given family or subfamily [61,62]. The important residues may be functionally misannotated in such cases, which would underestimate the extent of divergence in regulatory or substrate-specific functions. Such kinase-specific examples of residue function may account for many of the switching events currently placed in the 'other' category.

From the analysis of kinase specificity models, it is apparent that new specificities are often generated following the emergence of a new kinase group or family, but not following the emergence of a new kinase subfamily. This is not a surprising result given that kinase groups and families tend to be older than kinase subfamilies (Fig 3D). We note, however, that the sample size of PWMs for this analysis was relatively low ($n = 101$) and that many of the kinase targets gathered from in vitro studies tend to lack strong in vivo support [44]. The approach used for PWM construction relies on literature-curated targets sites and therefore tends to be biased towards medically relevant and/or well-studied kinases [63,64]. However, more systematic approaches have recently been developed [40,65,66], which may in the future enable the characterisation of many kinases in parallel, in a similar vein to a 2010 specificity-based study of 61 *S. cerevisiae* kinases [35].

The observation that subfamilies from the same family tend to have the same specificity is, however, in conflict with the finding that SDR substitutions are also present throughout the evolution of subfamilies (Fig 2, subfamilies). There are 2 known cases in the literature (PLK and GRK) in which moderate differences in specificity are observed between sister subfamilies [31,67–69]. We suggest that differences in peptide specificity can exist between subfamilies, but on average and based on the current small sample size of specificity models, these tend to be modest at the subfamily level.

Finally, the analysis of phosphorylation motifs across 48 different eukaryotic species suggests that most arose either in an early eukaryotic ancestor or shortly before the emergence of the last eukaryotic common ancestor. The phosphorylation data set spans the tree of life but is unsurprisingly biased towards animal, fungal, and plant species. Ongoing projects for increased representation of the protist superkingdoms could help to address this problem in the future [70]. From this analysis, we also conclude that most eukaryotic phosphomotifs post-date the divergence of eukaryotes and prokaryotes. The acquisition of phosphoproteome data from several more prokaryotic species will be required, however, to strengthen this conclusion. In general, the increase in statistical power enabled by larger data sets will enable the reliable identification of weakly enriched motifs ('false negatives'), many of which are likely missing from this analysis. However, this would not eliminate the possibility that the phosphomotifs observed may reflect the specificities of phospho-binding domains such as the WW domain, Polo-box domain, and 14-3-3 domains [71,72]. A more definitive analysis would require the acquisition of direct kinase-substrate relationship data across several species.

Collectively, the results suggest that the evolution of new kinase specificities was characterised by a 'burst' around the time of the last eukaryotic common ancestor, followed by a period of relative stasis. Most gene duplicates will be quickly silenced [73], and diversification of function is often considered a primary means for the 'survival' of newly duplicated genes in the genome. The capacity of the kinase fold to generate diverse target preferences at the active site interface through mutations may have been an important factor underlying the success of this fold. Our analysis suggests that, over the past 800 million years, there have been relatively few novel motifs emerging in eukaryotic kinases. It is interesting to speculate why this is the case. It is possible that no new distinct mode of interaction can be accommodated at the active site or that such novel motifs are not easily reached via mutations of existing kinases. As mentioned above, kinase specificity is determined via multiple mechanisms, including docking interactions, expression differences, localisation differences, activation modes, etc. Duplicated kinases can, therefore, be made nonredundant by diversifying the way by which they regulate their substrates to avoid misregulation in multiple different ways [12]. Additional research will be needed to study how the different kinase specificity mechanisms have evolved in kinases.

Protein kinases are just one of many peptide-binding domain types that can recognize diverse sets of peptide motifs. Other such domains include, for example, the PDZ (Post-

synaptic density protein 95 (PSD-95), *Drosophila* disc large tumor suppressor (Dlg1), Zona occludens 1 (ZO-1), SH2 (Src Homology 2), SH3 (Src Homology 3), and WW, among many other families. It remains to be seen whether the findings described here relating to the evolution of different target motifs will apply to other such important peptide-binding domains.

Materials and methods

Kinase phylogenetic analysis

Kinase domain sequences were collected for all 9 opisthokont species in KinBase with an annotated kinome (*H. sapiens*, *M. musculus*, *S. purpuratus*, *D. melanogaster*, *C. elegans*, *A. queenslandica*, *M. brevicollis*, *S. cerevisiae*, and *C. cinerea*) [19]. Kinases of the 'atypical' group were excluded from the analysis. The kinase domain sequences were then aligned using MAFFT [74], were filtered to remove pseudokinases (kinases without expected residues at domain positions 30, 48, 123, 128, and 141), and then were realigned using MAFFT L-INS-i [74]. Manual corrections were then made to the multiple sequence alignment (MSA), and the trimAl tool was employed to remove positions with 20% or more of 'gap' characters among the sequences [75]. Finally, a further filter was applied to remove truncated sequences with fewer than 190 kinase domain positions.

The resulting MSA (2,094 sequences) was used to generate a maximum-likelihood kinase domain phylogeny with the RaxML tool [76]. Amino acid substitutions were modelled using the LG matrix, and a gamma model was employed to account for the heterogeneity of rates between sites. A neighbour-joining phylogeny generated with the R ape package was used as the starting tree [77].

Ancestral sequence reconstructions were performed with the CodeML program (part of the PAML package) using an LG substitution matrix [78]. No molecular clock was assumed (clock = 0), and a gamma model was employed again to account for rate heterogeneity between sites. The alpha parameter of the gamma distribution was estimated (fix_alpha = 0) with a starting value of 0.5 (alpha = 0.5), and 4 categories of the gamma distribution were specified (ncatG = 4). The physicochemical properties of the amino acids were not taken into account when performing the ancestral sequence reconstructions (aaDist = 0).

For the analysis of kinase evolution, each family and subfamily was assessed iteratively, and a divergence score (s) was assigned to each position of the MSA. The divergence scores are calculated by comparing the family/subfamily of interest (clade A) with the closest sister clade (clade B) in the phylogeny. The score calculated is adapted from the BADX score of a previous publication by Edwards and colleagues [22], specifically:

$$S = RC_A - AC_x \cdot p(AC).$$

Recent conservation (RC) represents the sequence conservation for the clade of interest (clade A) and is calculated here on the basis of substitution matrix similarity in the R package bio3d [79]. AC_x represents the conservation of ancestral nodes for the clade of interest (clade A) and the ancestral node for the nearest sister clade (clade B); this is given as a 1 if the predicted residues are identical to each other and a -1 otherwise. Finally, the score is weighted by the value $p(AC)$, which represents the probability that the AC value was correctly assigned. For matching residues ($AC = 1$), this is the posterior probability of the predicted residue for clade B; for differing residues ($AC = -1$), this is the summed posterior probability of all residues in clade B besides from the predicted residue for clade A. Therefore, scores for suspected divergence would be down-weighted if there is ambiguity concerning the nature (matching or mismatching) of the clade B ancestor.

Where the sequences of interest were divided into 2 or more clades in the phylogeny, only the largest clade was considered for further analysis. In some cases, also, the clade of interest contained spurious sequences from the wrong family or subfamily. Spurious sequences were tolerated only if they comprised less than 15% of the clade sequences; otherwise, the largest 'pure' subclade (with the sequences of interest only) was selected for further analysis. For the calculation of divergence scores, the nearest sister clade to the clade of interest was selected. However, scores were only calculated if both clades contained 5 or more sequences and belonged to the correct category (e.g., 2 subfamilies that are being compared must belong to the same family). All searching and/or manipulation of the phylogeny was performed using a custom script in R with the aid of the ape package.

Aggregated analysis across kinase families and subfamilies

For the global analysis represented in Fig 2, the total number of switches for each alignment position was calculated at the family and subfamily levels. For this aggregated analysis, 'duplicate comparisons' (i.e., in which the same 2 ancestral nodes were compared) were filtered out to ensure that the same switch event was only counted once. A substitution is considered a switch if it is above the 95th percentile for all subfamily ($s_{\text{subfamily}(95)} = 1.904$) or family ($s_{\text{family}(95)} = 1.793$) scores. For the one-sided Fisher test described in the Results section, a site is considered to be 'frequently switching' if the number of switches is above the 90th percentile of switch frequencies for the 246 alignment positions. This was calculated separately at the family (90th percentile = 8) and subfamily (90th percentile = 7) level.

In Fig 2, the aggregated number of switches have been grouped according to the functional categories 'catalytic', 'proximal', 'distal', 'regulatory', 'interactions', and 'other'. The 'catalytic' residues refer to those that are needed for catalysis or form the catalytic spine [80]. The 'proximal' category refers to noncatalytic residues within 4 Angstroms of the substrate peptide of PKA (PDB: 1ATP), excluding substrate positions N terminal to the -6 position. The 'distal SDRs' are the predicted SDRs in Bradley and colleagues [17] more than 4 Angstroms from the substrate (in PDB:1ATP). The 'regulatory' category refers to regulatory spine residues and those within and surrounding the activation loop [81]. The 'interaction' category refers to residues often found to be in contact with other protein domains (at least 10) in cocrystal structures, as determined using the 3DID database of protein-protein interactions [82]. Finally, 'other' represents any residue not belonging to any of the categories described above. The residues belonging to each category are defined in S1 Table.

When using the SDR annotations from the Creixell and colleagues [41] study, SDRs with a score >0.8 and within 5 Angstroms of the substrate peptide were used for this definition. When using the literature-curated SDRs from the same study, the 'proximal' and 'distal' categories were merged because only 5 of the literature-curated SDRs were distal from the substrate.

Analysis of kinases with known specificity

For the analysis of kinase specificity, 101 high-confidence specificity models of human and mouse S/T kinases were collected as described in Bradley and colleagues (2018). These models are derived from the literature-curated kinase targets given in the databases HPRD, PhosphoELM, and PhosphoSitePlus [42–44]. Each kinase was annotated at the group, family, and subfamily level (as required) using the manual annotations given in the kinase.com website [19]. The analysis of specificity divergence was performed separately at each of the 3 levels. For each level, all pairwise distances within a grouping are computed, and then all possible pairwise distances are calculated between groupings. Importantly, the higher-level categorisation is

retained for all pairwise comparisons. For example, at the family level, all between-family distance comparisons would occur for kinases belonging to the same 'group'. For each pairwise comparison, the Frobenius distance between specificity models was calculated using the 'norm (type = 'F')' function in R after subtracting one PWM from the other. The Frobenius distance represents the sum of squared differences between matrix values, followed by square rooting [83].

For the comparison of kinase sequence divergence with kinase specificity divergence (S5 Fig), a Frobenius distance threshold of 1.06 was used to separate 'specificity-conserved' kinase-kinase pairs from 'specificity-diverged' kinase-kinase pairs. This threshold was derived by taking the maximum distance between PWMs generated for the same kinase, which were constructed by subsampling known targets ($n = 25$) from kinases with 50 or more annotated targets.

Dating the emergence of kinase groups, families, and subfamilies

First, all known kinase groups, families, and subfamilies present in animal and fungal species were retrieved from the kinase database KinBase [19]. The list was then filtered to remove atypical kinases and tyrosine protein kinases. The presence or absence of each kinase group, family, and subfamily across several species of the eukaryotic tree of life was then predicted using the KinAnnotate tool [84]. For this purpose, we used all species from a recently published tree of life for which a publicly available genome/proteome sequence was available [85]. The tree of life was then pruned in R using the ape package to retain these species only (55 in total). The origin of each kinase group, family, and subfamily was then predicted using maximum-likelihood-based ancestral state reconstruction with the ace function of the ape package [77]. The reported divergence times between species in the literature was then used to estimate ages for each group, family, and subfamily [45].

Where multiple origins were predicted for a kinase group, family, and subfamily, we traced the kinase emergence to the most recent common ancestral node between the predicted nodes of origin. This approach assumes no horizontal gene transfer between species or convergent evolution of kinase groups, families, and subfamilies.

Kinase motif enrichment across eukaryotic species

The phosphorylation site data were collected from a range of sources. They are as follows: *Trypanosoma brucei* [86,87], *T. cruzi* [88,89], *L. infantum* [90], *Trichoplax adhaerens* [91], *H. sapiens*/*M. musculus*/*Rattus norvegicus* [46], *S. purpuratus* [92], *Drosophila* spp. [93], *C. elegans* [94], *Magnaporthe oryzae* [95], 18 fungal species [96], *D. discoideum* [97], *Medicago truncatula* [98,99], *Glycine max* [99,100], *Arabidopsis thaliana* [99,101], *Selaginella moellendorffii* [102], *Brachypodium distachyon* [103], *Oryza sativa* [99,104], *Zea mays* [99,105], *Chlamydomonas reinhardtii* [106], *Plasmodium falciparum*/*P. berghei*/*Toxoplasma gondii* [107,108], *Tetrahymena thermophila* [109], and *Phytophthora infestans* [51].

For each species, redundant phosphosite 15-mers (centred on S or T) were filtered from the analysis. Phosphorylation motifs (S/T) for each of the 48 species were obtained by running r-motif-x using its default parameters (p -value of 1×10^{-6} and a minimum of 20 motif occurrences). This tool takes as its input a 'foreground' set of known target sites and a 'background' set of sites known not to be target sites [110]. For the background set, we randomly shuffled the flanking sequences of known phosphorylated target sites (central S/T retained). The amino acid composition of the foreground and background sets were therefore identical. This approach is expected to generate fewer spurious motif predictions than simply sampling S/T

sites randomly from the proteome [111]. To generate the background set, each known target site was randomly shuffled 10 times.

For further analysis, we selected only those motifs appearing in at least a third of species within one or more superphyla (i.e., fungi, metazoa, and plants). For the excavates (3 species represented here), the motif had to be present in at least 2 of the examined species. Motifs exclusive to the amoebozoa or heterokonts were not considered because both superphyla are represented here by only a single species. Other constraints were imposed to filter out potentially spurious motifs. Serine or threonine additions to a classical motif were not considered, because they may result from phosphosite misassignment within phosphopeptides or the clustering of phosphorylation sites in the substrate primary sequence [48,49]. We also considered R/K, D/E, and L/I/V/M to be synonymous when identifying new motifs. Finally, D/E additions to the classic casein kinase 2 motif 'S/T-D/E-x-D/E' were not considered because weak D/E preferences outside the +1 and +3 positions have already been described for this kinase [38]. Motifs detected here that do not match the list of motifs given in Amanchy and colleagues [112] or Miller and colleagues [113] are declared to be 'new' motifs with an unknown upstream regulator.

The enrichment of kinase motifs was calculated relative to the background set of randomised peptides. The significance of motif enrichments in each species was determined by calculating binomial p -values. Here, the null probability of the motif is taken to be equal to the total frequency of motif matches (e.g., P-x-S/T-P) to the background set, divided by the total number of background matches for the superset motif (e.g., S/T-P). The calculation of equivalent frequencies for the foreground set enables an analytical p -value to be calculated using the binomial distribution. The calculated p -value therefore gives an indication, for each motif, of the extent of enrichment of the motif against the background set relative to that of the most frequent superset motif (e.g., the enrichment of P-X-S/T-P relative to S/T-P).

Number of motif matches as a percentage of the phosphoproteome

Each of the motifs previously identified using motif-x was screened against the known target sites of each species, and the total number of target sites matching at least one motif was counted and then divided by the total number of known target sites in each species (S6A Fig). For this analysis, we do not consider motifs with only one constrained flanking position (e.g., G-S/T), because matches to the foreground set are likely to arise just by chance. These patterns may represent incomplete sequence motifs. Exceptions are made for the classic S/T-P and R-x-x-S/T signatures, which by themselves can be sufficient for kinase targeting [5,114].

Kinase motif enrichment for prokaryotic phosphorylation sites

The prokaryotic phosphorylation data were collected from multiple sources. Phosphorylation data for *E. coli* derives from [56–58]. Phosphorylation data for *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* comes from the dbPSP database [57]. The pooled species in Fig 4 represents 180 unique phosphorylation sites from 8 prokaryotic species—*Halobacterium salinarum*, *Bacillus subtilis*, *Mycobacterium tuberculosis*, *Streptomyces coelicolor*, *E. coli*, *Synechococcus* sp., *S. solfataricus*, *S. acidocaldarius*—all of which derive from the dbPSP database also [57].

Enrichment values and binomial p -values were calculated using the same methods described in the previous section. The motif-x tool was executed using its default parameters, as described above.

Coevolution between the kinome and phosphoproteome

A starting phylogeny for the 48 eukaryotic species was assembled using the NCBI taxonomy tool [115]. Unresolved branches (polytomies) for particular clades were then resolved

manually after referring to previous phylogenetic studies in the literature [116–120]. Kinome annotations for each species were generated automatically using the KinAnnotate tool [84], which employs BLAST- and HMM-based searches to identify and classify eukaryotic protein kinases.

The relationship between kinase motifs and their cognate kinases (e.g., S/T-P-x-K and CDKs) was modelled with phylogenetic independent contrasts (PICs) in R using the ape package [50,77]. This method generates phylogenetic contrasts between variables on a tree to account for the nonindependence of data points [50]. In S7 Fig, contrasts were generated for motif enrichment values on the y-axis and for relative kinase frequencies (number of kinases of interest divided by the total number of kinases detected in the proteome) on the x-axis.

Tests for the phylogenetic signal of different motifs were conducted in R using the Phylo-signal package [121]. The phylogenetic plots in S8 Fig and S9 Fig were also generated using Phylo-signal.

Supporting information

S1 Fig. Frequently switching residues in kinases. Residues coloured in red and blue mark ‘frequently switching’ residues (number of switches above the 90th percentile of switch frequencies across the kinase domain) at the family (A) and subfamily level (B), respectively. The kinases (mouse protein kinase A: PDB 1ATP) are represented in complex with an ATP molecule (green, orange, blue, red) and a substrate-mimicking inhibitor (PKIA, yellow). Kinase residues have been numbered according to their position in the protein kinase domain (Pfam: PF00069). PDB, Protein Data Bank; Pfam, Protein families; PKIA, cAMP-dependent protein kinase inhibitor alpha.

(TIF)

S2 Fig. Global kinase domain phylogeny with 2,094 different sequences represented. Red and blue circles represent families and subfamilies (respectively) where divergent residues are found in kinase residues close to the substrate (i.e., the ‘proximal’ category). The size of the circle is proportional to the number of switches found in the ‘proximal’ category.

(TIF)

S3 Fig. Relative number of switches for each kinase domain position at the family and sub-family level. Here, the number of switches has been divided (normalised) by the total number of families ($n = 85$) and subfamilies ($n = 64$) considered when aggregating the number of switches. As in Fig 2, the values for each domain position have been grouped according to the functional category (‘catalytic’, ‘regulatory’, ‘proximal’, etc.) of the residues.

(TIF)

S4 Fig. Frobenius distances between PWMs generated for the same kinase. On the left (‘same’), PWMs of the same kinase were generated by subsampling all known kinase target sites derived from literature-curated databases. The left-hand box plot represents the distribution of matrix distances between PWMs generated using this same method. The right-hand box plot (‘different’) represents matrix distances between PWMs generated using 2 different methods: phosphosite-based and peptide-screening-based [35]. Only 13 kinases characterised in [35] have sufficient known substrates for PWM construction (therefore, $n = 13$). As expected, PWMs generated using different approaches are more different on average than those generated using the same method. However, in most cases, the inter-PWM distances are comparable to those found for the ‘same’ category. PWM, position weight matrix.

(TIF)

S5 Fig. Relation between kinase sequence and specificity differences. (Top) Plot between the kinase sequence identity (x-axis) and Frobenius distance (y-axis) for all possible kinase-kinase pairs among the 101 S/T kinases for which specificity models have been constructed. This has been plotted for residues of the ‘other’ category (left), ‘proximal’ category (centre), and 10 high-confidence SDRs (right) identified in [17]. (Bottom) For the same residue descriptions, plot of kinase sequence identity (x-axis) against the specificity divergence (y-axis). ‘Specificity divergence’ represents the proportion of Frobenius distances above the 1.06 threshold used to separate ‘specificity-conserved’ kinase-kinase pairs from ‘specificity-diverged’ kinase-kinase pairs (see [Materials and methods](#)). SDR, specificity determining residue; S/T, serine/threonine.

(TIF)

S6 Fig. Motif presence across species. (A) Proportion of phosphorylation sites in each species that match a phosphorylation motif (see [Materials and methods](#)). (B) A simplified version of the eukaryotic tree of life presented in the [85] study. The numbers in brackets correspond to the number of different species represented by phosphorylation data in this study. (C) Calculation of binomial *p*-values (as in [Fig 4](#)) for each motif in each major clade (metazoa, fungi, plants, etc.) after phosphorylation sites within a clade were pooled across species. The figure legend (bottom right) is the same as in [Fig 4](#).

(TIF)

S7 Fig. Phylogenetic independence tests. PICs between 5 different kinase clades (AKT/SGK, CAMK2, CDK, CK2, PKA/PKG) and their corresponding substrate motifs (R-x-x-S/T-F, R-x-x-S/T-x-D/E, S/T-P-x-K, S/T-D/E-x-D/E, and R-R-x-S/T, respectively). This approach accounts for the phylogenetic nonindependence between data points when comparing 2 continuous variables [50]. CAMK2, Calcium/calmodulin-dependent protein kinase type 2; CDK, Cyclin-Dependent Kinase; CK2, Casein Kinase 2; PIC, phylogenetic independence contrast; PKA, Protein Kinase A; PKG, Protein Kinase G; SGK, Serum and Glucocorticoid-regulated Kinase.

(TIF)

S8 Fig. Mapping of PKA/PKG family relative kinase frequencies and substrate motif enrichments to a phylogeny of 48 eukaryotic species. Relative kinase frequencies across the 48 species were calculated for the PKA/PKG family, and motif enrichments were calculated for their cognate substrate motif (R-R-x-S/T). The red box highlights species where the absence of PKA and PKG kinases in the proteome corresponds to a lack of R-R-x-S/T motif enrichment. PKA, Protein Kinase A; PKG, Protein Kinase G.

(TIF)

S9 Fig. Mapping of CDK family relative kinase frequencies and substrate motif enrichments to a phylogeny of 48 eukaryotic species. Relative kinase frequencies across the 48 species were calculated for the CDK family, and motif enrichments were calculated for its cognate substrate motif (S/T-P-x-K). CDK, Cyclin-Dependent Kinase.

(TIF)

S1 Table. A table mapping the kinase MSA used for all sequence analysis (column 1) to the sequence of human protein kinase A (column 2), to the PDB numbering (PDB: 1ATP) of protein kinase A (column 3), and to the kinase domain positions (Pfam: PF00069). MSA, multiple sequence alignment; PDB, Protein Data Bank; Pfam Protein families.

(XLSX)

S2 Table. Five tests for the phylogenetic signal (Cmean, I, K, K.star, and Lambda) of 9 different eukaryotic motifs. Numbers in the table represent *p*-values for each one of the tests. Low *p*-values (e.g., $p < 0.01$) suggest that the motif in question is nonrandomly distributed with respect to the species phylogeny of 48 eukaryotic species (as presented in [S3 Fig](#) and [S4 Fig](#)). All tests were performed using the Phylosignal package in R [[121](#)].
(XLSX)

S3 Table. Motifs identified from phosphorylation sites in *E. coli* ($n = 2,287$) using the motif-x tool. In both instances, motif-x was executed using its default parameters ($p < 1 \times 10^{-6}$ and at least 20 occurrences). The motif-x scores for each of the motifs are displayed in the second column.
(XLSX)

S4 Table. Motifs identified from phosphorylation sites in *Sulfolobus* spp. ($n = 1,655$) using the motif-x tool. In both instances, motif-x was executed using its default parameters ($p < 1 \times 10^{-6}$ and at least 20 occurrences). The motif-x scores for each of the motifs is displayed in the second column.
(XLSX)

Author Contributions

Conceptualization: David Bradley, Pedro Beltrao.

Formal analysis: David Bradley.

Investigation: David Bradley.

Project administration: Pedro Beltrao.

Supervision: Pedro Beltrao.

Writing – original draft: David Bradley, Pedro Beltrao.

Writing – review & editing: Pedro Beltrao.

References

1. Endicott JA, Noble MEM, Johnson LN. The structural basis for control of eukaryotic protein kinases. *Annu Rev Biochem.* 2012; 81: 587–613. <https://doi.org/10.1146/annurev-biochem-052410-090317> PMID: [22482904](#)
2. Stenberg KA, Riikonen PT, Vihinen M. KinMutBase, a database of human disease-causing protein kinase mutations. *Nucleic Acids Res.* 2000; 28: 369–371. <https://doi.org/10.1093/nar/28.1.369> PMID: [10592276](#)
3. Lahiry P, Torkamani A, Schork NJ, Hegele RA. Kinase mutations in human disease: interpreting genotype-phenotype relationships. *Nat Rev Genet.* 2010; 11: 60–74. <https://doi.org/10.1038/nrg2707> PMID: [20019687](#)
4. Torkamani A, Kannan N, Taylor SS, Schork NJ. Congenital disease SNPs target lineage specific structural elements in protein kinases. *Proc Natl Acad Sci U S A.* 2008; 105: 9011–9016. <https://doi.org/10.1073/pnas.0802403105> PMID: [18579784](#)
5. Ubersax JA, Ferrell JE Jr. Mechanisms of specificity in protein phosphorylation. *Nat Rev Mol Cell Biol.* 2007; 8: 530–541. <https://doi.org/10.1038/nrm2203> PMID: [17585314](#)
6. Pawson T, Scott JD. Signaling through scaffold, anchoring, and adaptor proteins. *Science.* 1997; 278: 2075–2080. <https://doi.org/10.1126/science.278.5346.2075> PMID: [9405336](#)
7. Faux MC, Scott JD. Molecular glue: kinase anchoring and scaffold proteins. *Cell.* 1996; 85: 9–12. PMID: [8620541](#)
8. Biondi RM, Nebreda AR. Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions. *Biochem J.* 2003; 372: 1–13. <https://doi.org/10.1042/BJ20021641> PMID: [12600273](#)

9. Goldsmith EJ, Akella R, Min X, Zhou T, Humphreys JM. Substrate and docking interactions in serine/threonine protein kinases. *Chem Rev*. 2007; 107: 5065–5081. <https://doi.org/10.1021/cr068221w> PMID: 17949044
10. Pearson RB, Kemp BE. Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. *Methods Enzymol*. 1991; 200: 62–81. PMID: 1956339
11. Pinna LA, Ruzzene M. How do protein kinases recognize their substrates? *Biochim Biophys Acta*. 1996; 1314: 191–225. [https://doi.org/10.1016/s0167-4889\(96\)00083-3](https://doi.org/10.1016/s0167-4889(96)00083-3) PMID: 8982275
12. Alexander J, Lim D, Joughin BA, Hegemann B, Hutchins JRA, Ehrenberger T, et al. Spatial exclusivity combined with positive and negative selection of phosphorylation motifs is the basis for context-dependent mitotic signaling. *Sci Signal*. 2011; 4: ra42. <https://doi.org/10.1126/scisignal.2001796> PMID: 21712545
13. Itzkovitz S, Tlusty T, Alon U. Coding limits on the number of transcription factors. *BMC Genomics*. 2006; 7: 239. <https://doi.org/10.1186/1471-2164-7-239> PMID: 16984633
14. Ochoa D, Bradley D, Beltrao P. Evolution, dynamics and dysregulation of kinase signalling. *Curr Opin Struct Biol*. 2018; 48: 133–140. <https://doi.org/10.1016/j.sbi.2017.12.008> PMID: 29316484
15. Tan CSH, Pasculescu A, Lim WA, Pawson T, Bader GD, Linding R. Positive selection of tyrosine loss in metazoan evolution. *Science*. 2009; 325: 1686–1688. <https://doi.org/10.1126/science.1174301> PMID: 19589966
16. Howard CJ, Hanson-Smith V, Kennedy KJ, Miller CJ, Lou HJ, Johnson AD, et al. Ancestral resurrection reveals evolutionary mechanisms of kinase plasticity. *Elife*. 2014; 3. <https://doi.org/10.7554/eLife.04126> PMID: 25310241
17. Bradley D, Vieitez C, Rajeev V, Cutillas PR, Beltrao P. Global analysis of specificity determinants in eukaryotic protein kinases [Internet]. *bioRxiv*. 2018. p. 195115. <https://doi.org/10.1101/195115>
18. Hanks SK, Hunter T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J*. 1995; 9: 576–596. PMID: 7768349
19. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science*. 2002; 298: 1912–1934. <https://doi.org/10.1126/science.1075762> PMID: 12471243
20. Manning G, Plowman GD, Hunter T, Sudarsanam S. Evolution of protein kinase signaling from yeast to man. *Trends Biochem Sci*. 2002; 27: 514–520. PMID: 12368087
21. Miranda-Saavedra D, Barton GJ. Classification and functional annotation of eukaryotic protein kinases. *Proteins*. 2007; 68: 893–914. <https://doi.org/10.1002/prot.21444> PMID: 17557329
22. Edwards RJ, Shields DC. BADASP: predicting functional specificity in protein families using ancestral sequences. *Bioinformatics*. 2005; 21: 4190–4191. <https://doi.org/10.1093/bioinformatics/bti678> PMID: 16159912
23. Studer RA, Dessailly BH, Orengo CA. Residue mutations and their impact on protein structure and function: detecting beneficial and pathogenic changes. *Biochem J*. 2013; 449: 581–594. <https://doi.org/10.1042/BJ20121221> PMID: 23301657
24. Chagoyen M, García-Martín JA, Pazos F. Practical analysis of specificity-determining residues in protein families. *Brief Bioinform*. 2016; 17: 255–261. <https://doi.org/10.1093/bib/bbv045> PMID: 26141829
25. de Juan D, Pazos F, Valencia A. Emerging methods in protein co-evolution. *Nat Rev Genet*. 2013; 14: 249–261. <https://doi.org/10.1038/nrg3414> PMID: 23458856
26. Chakraborty A, Chakrabarti S. A survey on prediction of specificity-determining sites in proteins. *Brief Bioinform*. 2015; 16: 71–88. <https://doi.org/10.1093/bib/bbt092> PMID: 24413183
27. Ngo JCK, Chakrabarti S, Ding J-H, Velazquez-Dones A, Nolen B, Aubol BE, et al. Interplay between SRPK and Clk/Sty kinases in phosphorylation of the splicing factor ASF/SF2 is regulated by a docking motif in ASF/SF2. *Mol Cell*. 2005; 20: 77–89. <https://doi.org/10.1016/j.molcel.2005.08.025> PMID: 16209947
28. Hughes S, Elustondo F, Di Fonzo A, Leroux FG, Wong AC, Snijders AP, et al. Crystal structure of human CDC7 kinase in complex with its activator DBF4. *Nat Struct Mol Biol*. 2012; 19: 1101–1107. <https://doi.org/10.1038/nsmb.2404> PMID: 23064647
29. LeBoeuf B, Gruninger TR, Garcia LR. Food deprivation attenuates seizures through CaMKII and EAG K+ channels. *PLoS Genet*. 2007; 3: 1622–1632. <https://doi.org/10.1371/journal.pgen.0030156> PMID: 17941711
30. Bhattacharyya M, Stratton MM, Going CC, McSpadden ED, Huang Y, Susa AC, et al. Molecular mechanism of activation-triggered subunit exchange in Ca(2+)/calmodulin-dependent protein kinase II. *Elife*. 2016; 5. <https://doi.org/10.7554/eLife.13405> PMID: 26949248

31. Onorato JJ, Palczewski K, Regan JW, Caron MG, Lefkowitz RJ, Benovic JL. Role of acidic amino acids in peptide substrates of the beta-adrenergic receptor kinase and rhodopsin kinase. *Biochemistry*. 1991; 30: 5118–5125. <https://doi.org/10.1021/bi00235a002> PMID: 1645191
32. Gibbs CS, Zoller MJ. Rational scanning mutagenesis of a protein kinase identifies functional regions involved in catalysis and substrate interactions. *J Biol Chem*. 1991; 266: 8923–8931. PMID: 2026604
33. Huang C-YF, Yuan C-J, Blumenthal DK, Graves DJ. Identification of the Substrate and Pseudosubstrate Binding Sites of Phosphorylase Kinase γ -Subunit. *J Biol Chem*. 1995; 270: 7183–7188. <https://doi.org/10.1074/jbc.270.13.7183> PMID: 7706257
34. Batkin M, Shaltiel S. The negative charge of Glu-127 in protein kinase A and its biorecognition [Internet]. *FEBS Letters*. 1999. pp. 395–399. [https://doi.org/10.1016/s0014-5793\(99\)00500-1](https://doi.org/10.1016/s0014-5793(99)00500-1)
35. Mok J, Kim PM, Lam HYK, Piccirillo S, Zhou X, Jeschke GR, et al. Deciphering protein kinase specificity through large-scale analysis of yeast phosphorylation site motifs. *Sci Signal*. 2010; 3: ra12. <https://doi.org/10.1126/scisignal.2000482> PMID: 20159853
36. Chen C, Ha BH, Thévenin AF, Lou HJ, Zhang R, Yip KY, et al. Identification of a major determinant for serine-threonine kinase phosphoacceptor specificity. *Mol Cell*. 2014; 53: 140–147. <https://doi.org/10.1016/j.molcel.2013.11.013> PMID: 24374310
37. Kannan N, Neuwald AF. Evolutionary constraints associated with functional specificity of the CMGC protein kinases MAPK, CDK, GSK, SRPK, DYRK, and CK2 α . *Protein Sci*. Cold Spring Harbor Laboratory Press; 2004; 13: 2059–2077.
38. Sarno S, Vaglio P, Marin O, Issinger OG, Ruffato K, Pinna LA. Mutational analysis of residues implicated in the interaction between protein kinase CK2 and peptide substrates. *Biochemistry*. 1997; 36: 11717–11724. <https://doi.org/10.1021/bi9705772> PMID: 9305961
39. Zhu G, Fujii K, Belkina N, Liu Y, James M, Herrero J, et al. Exceptional disfavor for proline at the P+ 1 position among AGC and CAMK kinases establishes reciprocal specificity between them and the proline-directed kinases. *J Biol Chem*. *ASBMB*; 2005; 280: 10743–10748. <https://doi.org/10.1074/jbc.M413159200> PMID: 15647260
40. Lubner JM, Dodge-Kafka KL, Carlson CR, Church GM, Chou MF, Schwartz D. Cushing's syndrome mutant PKAL205R exhibits altered substrate specificity. *FEBS Lett*. 2017; 591: 459–467. <https://doi.org/10.1002/1873-3468.12562> PMID: 28100013
41. Creixell P, Palmeri A, Miller CJ, Lou HJ, Santini CC, Nielsen M, et al. Unmasking determinants of specificity in the human kinome. *Cell*. 2015; 163: 187–201. <https://doi.org/10.1016/j.cell.2015.08.057> PMID: 26388442
42. Prasad TSK, Kandasamy K, Pandey A. Human Protein Reference Database and Human Proteinpedia as discovery tools for systems biology. *Methods Mol Biol*. 2009; 577: 67–79. https://doi.org/10.1007/978-1-60761-232-2_6 PMID: 19718509
43. Dinkel H, Chica C, Via A, Gould CM, Jensen LJ, Gibson TJ, et al. Phospho.ELM: a database of phosphorylation sites—update 2011. *Nucleic Acids Res*. Oxford University Press; 2011; 39: D261–D267. <https://doi.org/10.1093/nar/gkq1104> PMID: 21062810
44. Hornbeck PV, Kornhauser JM, Latham V, Murray B, Nandhikonda V, Nord A, et al. 15 years of PhosphoSitePlus: integrating post-translationally modified sites, disease variants and isoforms. *Nucleic Acids Res*. 2019; 47: D433–D441. <https://doi.org/10.1093/nar/gky1159> PMID: 30445427
45. Kumar S, Stecher G, Suleski M, Hedges SB. TimeTree: A Resource for Timelines, Timetrees, and Divergence Times. *Mol Biol Evol*. 2017; 34: 1812–1819. <https://doi.org/10.1093/molbev/msx116> PMID: 28387841
46. Hornbeck PV, Zhang B, Murray B, Kornhauser JM, Latham V, Skrzypek E. PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Res*. 2015; 43: D512–20. <https://doi.org/10.1093/nar/gku1267> PMID: 25514926
47. Schwartz D, Gygi SP. An iterative statistical approach to the identification of protein phosphorylation motifs from large-scale data sets. *Nat Biotechnol*. 2005; 23: 1391–1398. <https://doi.org/10.1038/nbt1146> PMID: 16273072
48. Moses AM, Hériché J-K, Durbin R. Clustering of phosphorylation site recognition motifs can be exploited to predict the targets of cyclin-dependent kinase. *Genome Biol*. 2007; 8: R23. <https://doi.org/10.1186/gb-2007-8-2-r23> PMID: 17316440
49. Schweiger R, Linial M. Cooperativity within proximal phosphorylation sites is revealed from large-scale proteomics data. *Biol Direct*. 2010; 5: 6. <https://doi.org/10.1186/1745-6150-5-6> PMID: 20100358
50. Felsenstein J. Phylogenies and the Comparative Method. *Am Nat*. 1985; 125: 1–15.
51. Resjö S, Ali A, Meijer HJG, Seidl MF, Snel B, Sandin M, et al. Quantitative label-free phosphoproteomics of six different life stages of the late blight pathogen *Phytophthora infestans* reveals abundant

- phosphorylation of members of the CRN effector family. *J Proteome Res.* 2014; 13: 1848–1859. <https://doi.org/10.1021/pr4009095> PMID: 24588563
52. Frades I, Resjö S, Andreasson E. Comparison of phosphorylation patterns across eukaryotes by discriminative N-gram analysis. *BMC Bioinformatics.* 2015; 16: 239. <https://doi.org/10.1186/s12859-015-0657-2> PMID: 26224486
 53. Oruganty K, Talevich EE, Neuwald AF, Kannan N. Identification and classification of small molecule kinases: insights into substrate recognition and specificity. *BMC Evol Biol.* 2016; 16: 7. <https://doi.org/10.1186/s12862-015-0576-x> PMID: 26738562
 54. Pereira SFF, Goss L, Dworkin J. Eukaryote-like serine/threonine kinases and phosphatases in bacteria. *Microbiol Mol Biol Rev.* 2011; 75: 192–212. <https://doi.org/10.1128/MMBR.00042-10> PMID: 21372323
 55. Kannan N, Taylor SS, Zhai Y, Venter JC, Manning G. Structural and functional diversity of the microbial kinome. *PLoS Biol.* 2007; 5: e17. <https://doi.org/10.1371/journal.pbio.0050017> PMID: 17355172
 56. Lin M-H, Sugiyama N, Ishihama Y. Systematic profiling of the bacterial phosphoproteome reveals bacterium-specific features of phosphorylation. *Sci Signal.* 2015; 8: rs10. <https://doi.org/10.1126/scisignal.aaa3117> PMID: 26373674
 57. Pan Z, Wang B, Zhang Y, Wang Y, Ullah S, Jian R, et al. dbPSP: a curated database for protein phosphorylation sites in prokaryotes. *Database.* 2015; 2015: bav031. <https://doi.org/10.1093/database/bav031> PMID: 25841437
 58. Potel CM, Lin M-H, Heck AJR, Lemeer S. Widespread bacterial protein histidine phosphorylation revealed by mass spectrometry-based proteomics. *Nat Methods.* 2018; 15: 187–190. <https://doi.org/10.1038/nmeth.4580> PMID: 29377012
 59. Kalaivani R, Reema R, Srinivasan N. Recognition of sites of functional specialisation in all known eukaryotic protein kinase families. *PLoS Comput Biol.* 2018; 14: e1005975. <https://doi.org/10.1371/journal.pcbi.1005975> PMID: 29438395
 60. Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol.* 2010; 11: 9–22. <https://doi.org/10.1038/nrm2822> PMID: 20027184
 61. Sang D, Pinglay S, Vatansever S, Lou HJ, Turk BE. Ancestral resurrection reveals mechanisms of kinase regulatory evolution. *bioRxiv.* biorxiv.org; 2018; Available from: <https://www.biorxiv.org/content/early/2018/05/25/331637.abstract> [cited 2018 May 25].
 62. Simon B, Huat A-S, Temmerman K, Vahokoski J, Mertens HDT, Komadina D, et al. Death-Associated Protein Kinase Activity Is Regulated by Coupled Calcium/Calmodulin Binding to Two Distinct Sites. *Structure.* 2016; 24: 851–861. <https://doi.org/10.1016/j.str.2016.03.020> PMID: 27133022
 63. Invergo BM, Beltrao P. Reconstructing phosphorylation signalling networks from quantitative phosphoproteomic data. *Essays Biochem.* 2018; 62: 525–534. <https://doi.org/10.1042/EBC20180019> PMID: 30072490
 64. Needham EJ, Parker BL, Burykin T, James DE, Humphrey SJ. Illuminating the dark phosphoproteome. *Sci Signal.* 2019; 12. <https://doi.org/10.1126/scisignal.aau8645> PMID: 30670635
 65. Barber KW, Miller CJ, Jun JW, Lou HJ, Turk BE, Rinehart J. Kinase Substrate Profiling Using a Proteome-wide Serine-Oriented Human Peptide Library. *Biochemistry.* 2018; 57: 4717–4725. <https://doi.org/10.1021/acs.biochem.8b00410> PMID: 29920078
 66. Imamura H, Sugiyama N, Wakabayashi M, Ishihama Y. Large-scale identification of phosphorylation sites for profiling protein kinase selectivity. *J Proteome Res.* 2014; 13: 3410–3419. <https://doi.org/10.1021/pr500319y> PMID: 24869485
 67. Franchin C, Cesaro L, Pinna LA, Arrigoni G, Salvi M. Identification of the PLK2-dependent phosphopeptidome by quantitative proteomics [corrected]. *PLoS ONE.* 2014; 9: e111018. <https://doi.org/10.1371/journal.pone.0111018> PMID: 25338102
 68. Loudon RP, Benovic JL. Expression, purification, and characterization of the G protein-coupled receptor kinase GRK6. *J Biol Chem.* 1994; 269: 22691–22697. PMID: 8077221
 69. Kunapuli P, Onorato JJ, Hosey MM, Benovic JL. Expression, purification, and characterization of the G protein-coupled receptor kinase GRK5. *J Biol Chem.* 1994; 269: 1099–1105. PMID: 8288567
 70. Waller RF, Cleves PA, Rubio-Brotons M, Woods A, Bender SJ, Edgcomb V, et al. Strength in numbers: Collaborative science for new experimental model systems. *PLoS Biol.* 2018; 16: e2006333. <https://doi.org/10.1371/journal.pbio.2006333> PMID: 29965960
 71. Yaffe MB, Smerdon SJ. PhosphoSerine/threonine binding domains: you can't pSERious? *Structure.* 2001; 9: R33–8. PMID: 11286893
 72. Reinhardt HC, Yaffe MB. Phospho-Ser/Thr-binding domains: navigating the cell cycle and DNA damage response. *Nat Rev Mol Cell Biol.* 2013; 14: 563–580. <https://doi.org/10.1038/nrm3640> PMID: 23969844

73. Lynch M, Conery JS. The evolutionary fate and consequences of duplicate genes. *Science*. 2000; 290: 1151–1155. <https://doi.org/10.1126/science.290.5494.1151> PMID: 11073452
74. Katoh K, Kuma K-I, Toh H, Miyata T. MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Res*. 2005; 33: 511–518. <https://doi.org/10.1093/nar/gki198> PMID: 15661851
75. Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics*. 2009; 25: 1972–1973. <https://doi.org/10.1093/bioinformatics/btp348> PMID: 19505945
76. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*. 2014; 30: 1312–1313. <https://doi.org/10.1093/bioinformatics/btu033> PMID: 24451623
77. Paradis E, Schliep K. ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics*. 2018; <https://doi.org/10.1093/bioinformatics/bty633> PMID: 30016406
78. Yang Z. PAML 4: phylogenetic analysis by maximum likelihood. *Mol Biol Evol*. 2007; 24: 1586–1591. <https://doi.org/10.1093/molbev/msm088> PMID: 17483113
79. Grant BJ, Rodrigues APC, ElSawy KM, McCammon JA, Caves LSD. Bio3d: an R package for the comparative analysis of protein structures. *Bioinformatics*. 2006; 22: 2695–2696. <https://doi.org/10.1093/bioinformatics/btl461> PMID: 16940322
80. Kornev AP, Taylor SS, Ten Eyck LF. A helix scaffold for the assembly of active protein kinases. *Proc Natl Acad Sci U S A*. 2008; 105: 14377–14382. <https://doi.org/10.1073/pnas.0807988105> PMID: 18787129
81. Taylor SS, Kornev AP. Protein kinases: evolution of dynamic regulatory proteins. *Trends Biochem Sci*. 2011; 36: 65–77. <https://doi.org/10.1016/j.tibs.2010.09.006> PMID: 20971646
82. Mosca R, Céol A, Stein A, Olivella R, Aloy P. 3did: a catalog of domain-based interactions of known three-dimensional structure. *Nucleic Acids Res*. 2014; 42: D374–9. <https://doi.org/10.1093/nar/gkt887> PMID: 24081580
83. Ellis JJ, Kobe B. Predicting protein kinase specificity: Predikin update and performance in the DREAM4 challenge. *PLoS ONE*. 2011; 6: e21169. <https://doi.org/10.1371/journal.pone.0021169> PMID: 21829434
84. Goldberg JM, Griggs AD, Smith JL, Haas BJ, Wortman JR, Zeng Q. Kinannotate, a computer program to identify and classify members of the eukaryotic protein kinase superfamily. *Bioinformatics*. 2013; 29: 2387–2394. <https://doi.org/10.1093/bioinformatics/btt419> PMID: 23904509
85. Burki F, Kaplan M, Tikhonenkov DV, Zlatogursky V, Minh BQ, Radaykina LV, et al. Untangling the early diversification of eukaryotes: a phylogenomic study of the evolutionary origins of Centrohelida, Haptophyta and Cryptista. *Proc Biol Sci*. 2016; 283. <https://doi.org/10.1098/rspb.2015.2802> PMID: 26817772
86. Nett IRE, Martin DMA, Miranda-Saavedra D, Lamont D, Barber JD, Mehlert A, et al. The phosphoproteome of bloodstream form *Trypanosoma brucei*, causative agent of African sleeping sickness. *Mol Cell Proteomics*. 2009; 8: 1527–1538. <https://doi.org/10.1074/mcp.M800556-MCP200> PMID: 19346560
87. Urbaniak MD, Martin DMA, Ferguson MAJ. Global quantitative SILAC phosphoproteomics reveals differential phosphorylation is widespread between the procyclic and bloodstream form lifecycle stages of *Trypanosoma brucei*. *J Proteome Res*. 2013; 12: 2233–2244. <https://doi.org/10.1021/pr400086y> PMID: 23485197
88. Amorim JC, Batista M, da Cunha ES, Lucena ACR, Lima CV de P, Sousa K, et al. Quantitative proteome and phosphoproteome analyses highlight the adherent population during *Trypanosoma cruzi* metacyclogenesis. *Sci Rep*. 2017; 7: 9899. <https://doi.org/10.1038/s41598-017-10292-3> PMID: 28852088
89. Marchini FK, de Godoy LMF, Rampazzo RCP, Pavoni DP, Probst CM, Gnad F, et al. Profiling the *Trypanosoma cruzi* phosphoproteome. *PLoS ONE*. 2011; 6: e25381. <https://doi.org/10.1371/journal.pone.0025381> PMID: 21966514
90. Tsigankov P, Gherardini PF, Helmer-Citterich M, Späth GF, Zilberstein D. Phosphoproteomic analysis of differentiating *Leishmania* parasites reveals a unique stage-specific phosphorylation motif. *J Proteome Res*. 2013; 12: 3405–3412. <https://doi.org/10.1021/pr4002492> PMID: 23688256
91. Ringrose JH, van den Toorn HWP, Eitel M, Post H, Neerinx P, Schierwater B, et al. Deep proteome profiling of *Trichoplax adhaerens* reveals remarkable features at the origin of metazoan multicellularity. *Nat Commun*. 2013; 4: 1408. <https://doi.org/10.1038/ncomms2424> PMID: 23360999

92. Guo H, Garcia-Vedrenne AE, Isserlin R, Lugowski A, Morada A, Sun A, et al. Phosphoproteomic network analysis in the sea urchin *Strongylocentrotus purpuratus* reveals new candidates in egg activation. *Proteomics*. 2015; 15: 4080–4095. <https://doi.org/10.1002/prot.201500159> PMID: 26227301
93. Hu Y, Sopko R, Chung V, Studer RA, Landry SD, Liu D. iProteinDB: an integrative database of Drosophila post-translational modifications. *bioRxiv*. [bioRxiv.org; 2018](https://www.biorxiv.org/content/early/2018/08/07/386268.abstract); Available from: <https://www.biorxiv.org/content/early/2018/08/07/386268.abstract>. [cited 2018 August 7].
94. Rhoads TW, Prasad A, Kwiecien NW, Merrill AE, Zawack K, Westphall MS, et al. NeuCode Labeling in Nematodes: Proteomic and Phosphoproteomic Impact of Ascarioside Treatment in *Caenorhabditis elegans*. *Mol Cell Proteomics*. 2015; 14: 2922–2935. <https://doi.org/10.1074/mcp.M115.049684> PMID: 26392051
95. Franck WL, Gokce E, Randall SM, Oh Y, Eyre A, Muddiman DC, et al. Phosphoproteome Analysis Links Protein Phosphorylation to Cellular Remodeling and Metabolic Adaptation during *Magnaporthe oryzae* Appressorium Development. *J Proteome Res*. 2015; 14: 2408–2424. <https://doi.org/10.1021/pr501064q> PMID: 25926025
96. Studer RA, Rodriguez-Mias RA, Haas KM, Hsu JI, Viéitez C, Solé C, et al. Evolution of protein phosphorylation across 18 fungal species. *Science*. 2016; 354: 229–232. <https://doi.org/10.1126/science.aaf2144> PMID: 27738172
97. Charest PG, Shen Z, Lakoduk A, Sasaki AT, Briggs SP, Firtel RA. A Ras signaling complex controls the RasC-TORC2 pathway and directed cell migration. *Dev Cell*. 2010; 18: 737–749. <https://doi.org/10.1016/j.devcel.2010.03.017> PMID: 20493808
98. Rose CM, Venkateshwaran M, Volkening JD, Grimsrud PA, Maeda J, Bailey DJ, et al. Rapid phosphoproteomic and transcriptomic changes in the rhizobia-legume symbiosis. *Mol Cell Proteomics*. 2012; 11: 724–744. <https://doi.org/10.1074/mcp.M112.019208> PMID: 22683509
99. Yao Q, Ge H, Wu S, Zhang N, Chen W, Xu C, et al. P³DB 3.0: From plant phosphorylation sites to protein networks. *Nucleic Acids Res*. 2014; 42: D1206–13. <https://doi.org/10.1093/nar/gkt1135> PMID: 24243849
100. Nguyen THN, Brechenmacher L, Aldrich JT, Clauss TR, Gritsenko MA, Hixson KK, et al. Quantitative phosphoproteomic analysis of soybean root hairs inoculated with *Bradyrhizobium japonicum*. *Mol Cell Proteomics*. 2012; 11: 1140–1155. <https://doi.org/10.1074/mcp.M112.018028> PMID: 22843990
101. Lin L-L, Hsu C-L, Hu C-W, Ko S-Y, Hsieh H-L, Huang H-C, et al. Integrating Phosphoproteomics and Bioinformatics to Study Brassinosteroid-Regulated Phosphorylation Dynamics in *Arabidopsis*. *BMC Genomics*. 2015; 16: 533. <https://doi.org/10.1186/s12864-015-1753-4> PMID: 26187819
102. Chen X, Chan WL, Zhu F-Y, Lo C. Phosphoproteomic analysis of the non-seed vascular plant model *Selaginella moellendorffii*. *Proteome Sci*. 2014; 12: 16. <https://doi.org/10.1186/1477-5956-12-16> PMID: 24628833
103. Lv D-W, Subburaj S, Cao M, Yan X, Li X, Appels R, et al. Proteome and phosphoproteome characterization reveals new response and defense mechanisms of *Brachypodium distachyon* leaves under salt stress. *Mol Cell Proteomics*. 2014; 13: 632–652. <https://doi.org/10.1074/mcp.M113.030171> PMID: 24335353
104. Hou Y, Qiu J, Tong X, Wei X, Nallamilli BR, Wu W, et al. A comprehensive quantitative phosphoproteome analysis of rice in response to bacterial blight. *BMC Plant Biol*. 2015; 15: 163. <https://doi.org/10.1186/s12870-015-0541-2> PMID: 26112675
105. Marcon C, Malik WA, Walley JW, Shen Z, Paschold A, Smith LG, et al. A high-resolution tissue-specific proteome and phosphoproteome atlas of maize primary roots reveals functional gradients along the root axes. *Plant Physiol*. 2015; 168: 233–246. <https://doi.org/10.1104/pp.15.00138> PMID: 25780097
106. Wang H, Gau B, Slade WO, Juergens M, Li P, Hicks LM. The global phosphoproteome of *Chlamydomonas reinhardtii* reveals complex organellar phosphorylation in the flagella and thylakoid membrane. *Mol Cell Proteomics*. 2014; 13: 2337–2353. <https://doi.org/10.1074/mcp.M114.038281> PMID: 24917610
107. Invergo BM, Brochet M, Yu L, Choudhary J, Beltrao P, Billker O. Sub-minute Phosphoregulation of Cell Cycle Systems during Plasmodium Gamete Formation. *Cell Rep*. 2017; 21: 2017–2029. <https://doi.org/10.1016/j.celrep.2017.10.071> PMID: 29141230
108. Trecek M, Sanders JL, Elias JE, Boothroyd JC. The phosphoproteomes of *Plasmodium falciparum* and *Toxoplasma gondii* reveal unusual adaptations within and beyond the parasites' boundaries. *Cell Host Microbe*. 2011; 10: 410–419. <https://doi.org/10.1016/j.chom.2011.09.004> PMID: 22018241
109. Tian M, Chen X, Xiong Q, Xiong J, Xiao C, Ge F, et al. Phosphoproteomic analysis of protein phosphorylation networks in *Tetrahymena thermophila*, a model single-celled organism. *Mol Cell Proteomics*. 2014; 13: 503–519. <https://doi.org/10.1074/mcp.M112.026575> PMID: 24200585

110. Wagih O, Sugiyama N, Ishihama Y, Beltrao P. Uncovering Phosphorylation-Based Specificities through Functional Interaction Networks. *Mol Cell Proteomics*. 2016; 15: 236–245. <https://doi.org/10.1074/mcp.M115.052357> PMID: 26572964
111. Cheng A, Grant CE, Noble WS, Bailey TL. MoMo: Discovery of statistically significant post-translational modification motifs [Internet]. *bioRxiv*. 2018. p. 410050. <https://doi.org/10.1101/410050>
112. Amanchy R, Periaswamy B, Mathivanan S, Reddy R, Tattikota SG, Pandey A. A curated compendium of phosphorylation motifs. *Nat Biotechnol*. 2007; 25: 285–286. <https://doi.org/10.1038/nbt0307-285> PMID: 17344875
113. Miller CJ, Turk BE. Homing in: Mechanisms of Substrate Targeting by Protein Kinases. *Trends Biochem Sci*. 2018; 43: 380–394. <https://doi.org/10.1016/j.tibs.2018.02.009> PMID: 29544874
114. Errico A, Deshmukh K, Tanaka Y, Pozniakovskiy A, Hunt T. Identification of substrates for cyclin dependent kinases. *Adv Enzyme Regul*. 2010; 50: 375–399. <https://doi.org/10.1016/j.advenzreg.2009.12.001> PMID: 20045433
115. NCBI Resource Coordinators. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res*. 2018; 46: D8–D13. <https://doi.org/10.1093/nar/gkx1095> PMID: 29140470
116. Cavalier-Smith T, Chao EE, Snell EA, Berney C, Fiore-Donno AM, Lewis R. Multigene eukaryote phylogeny reveals the likely protozoan ancestors of opisthokonts (animals, fungi, choanozoans) and Amoebozoa. *Mol Phylogenet Evol*. 2014; 81: 71–85. <https://doi.org/10.1016/j.ympev.2014.08.012> PMID: 25152275
117. Drosophila 12 Genomes Consortium, Clark AG, Eisen MB, Smith DR, Bergman CM, Oliver B, et al. Evolution of genes and genomes on the Drosophila phylogeny. *Nature*. 2007; 450: 203–218. <https://doi.org/10.1038/nature06341> PMID: 17994087
118. Mathews S, Tsai RC, Kellogg EA. Phylogenetic structure in the grass family (Poaceae): evidence from the nuclear gene phytochrome B. *Am J Bot*. 2000; 87: 96–107. PMID: 10636833
119. Shen X-X, Zhou X, Kominek J, Kurtzman CP, Hittinger CT, Rokas A. Reconstructing the Backbone of the Saccharomycotina Yeast Phylogeny Using Genome-Scale Data. *G3*. 2016; 6: 3927–3939. <https://doi.org/10.1534/g3.116.034744> PMID: 27672114
120. Telford MJ, Budd GE, Philippe H. Phylogenomic Insights into Animal Evolution. *Curr Biol*. 2015; 25: R876–87. <https://doi.org/10.1016/j.cub.2015.07.060> PMID: 26439351
121. Keck F, Rimet F, Bouchez A, Franc A. phylosignal: an R package to measure, test, and explore the phylogenetic signal. *Ecol Evol*. 2016; 6: 2774–2780. <https://doi.org/10.1002/ece3.2051> PMID: 27066252