

# Spatial Organization in Protein Kinase A Signaling Emerged at the Base of Animal Evolution

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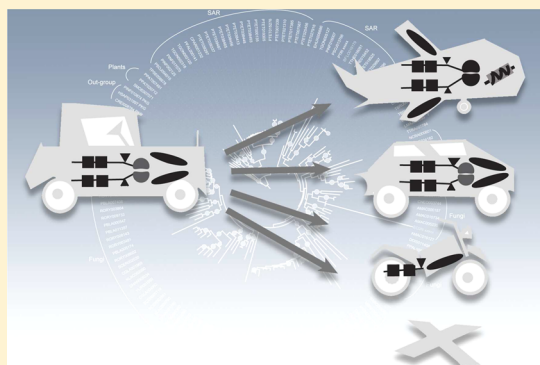
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## Supporting Information

**ABSTRACT:** In phosphorylation-directed signaling, spatial and temporal control is organized by complex interaction networks that diligently direct kinases toward distinct substrates to fine-tune specificity. How these protein networks originate and evolve into complex regulatory machineries are among the most fascinating research questions in biology. Here, spatiotemporal signaling is investigated by tracing the evolutionary dynamics of each functional domain of cAMP-dependent protein kinase (PKA) and its diverse set of A-kinase anchoring proteins (AKAPs). Homologues of the catalytic (PKA-C) and regulatory (PKA-R) domains of the  $(PKA-R)_2-(PKA-C)_2$  holoenzyme were found throughout evolution. Most variation was observed in the RIIa of PKA-R, crucial for dimerization and docking to AKAPs. The RIIa domain was not observed in all PKA-R homologues. In the fungi and distinct protist lineages, the RIIa domain emerges within PKA-R, but it displays large sequence variation. These organisms do not harbor homologues of AKAPs, suggesting that efficient docking to direct spatiotemporal PKA activity evolved in multicellular eukaryotes. To test this *in silico* hypothesis, we experimentally screened organisms with increasing complexity by cAMP-based chemical proteomics to reveal that the occurrence of PKA–AKAP interactions indeed coincided and expanded within vertebrates, suggesting a crucial role for AKAPs in the advent of metazoan multicellularity.

**KEYWORDS:** PKA, AKAP, RIIa, evolution, proteomics



## ■ BACKGROUND

In multicellular organisms, adequate transfer of information involves a sophisticated organization of signaling proteins in space and time.<sup>1,2</sup> One important way to achieve this goal is by forming multiprotein complexes uniting all aspects of a signaling node in close proximity.<sup>3</sup> A well-known example is represented by the various protein complexes involved in tyrosine phosphorylation (pY) signaling via the (receptor) tyrosine kinases (TyrK, writers), protein phosphatases (PTP, erasers), and Src Homology 2 (SH2) domains (readers).<sup>4</sup> Another example is the scaffold proteins KSR bringing together different combinations of MAP kinases to facilitate their activation at the specific cellular locations where these signaling units are sequestered.<sup>5</sup> A third, and one of the best-characterized, spatiotemporally controlled signaling mechanisms is that involved in cAMP-induced signaling in mammals. cAMP was identified over 50 years ago as the first a diffusible intracellular second messenger to play fundamental roles in

cellular responses to many hormones and neurotransmitters.<sup>6</sup> The principal intracellular cAMP target in animal cells is cAMP-dependent protein kinase (PKA), which exists as a symmetrical dimeric complex of two regulatory subunits (PKA-R) and two catalytic subunits (PKA-C).<sup>7</sup> When cAMP levels are at basal level, PKA is in an inhibited state as a heterotetrameric holoenzyme,  $(PKA-R)_2-(PKA-C)_2$ . When cAMP levels are elevated, two molecules of cAMP bind to each PKA-R, thereby releasing the active PKA-C.<sup>8</sup> Since PKA-C is a broad-spectrum serine/threonine kinase with many attributed physiological functions, specificity in space and time is required. This is primarily achieved through the interaction of the PKA-R dimer with the diverse family of A-kinase anchoring proteins (AKAPs).<sup>2</sup> By now, in mammals, well over 50 different AKAPs (including the AKAPs' splice variants and AKAPs

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lacking a confidently identified amphipathic helix) have been identified that each bring the PKA holoenzyme in close proximity to its substrates.<sup>9</sup> These AKAPs are often very large proteins with many different domains forming in the cell signaling hubs that, next to PKA, can harbor also adenylate cyclases (ACs; signal (cAMP) “generator”), phosphodiesterases (PDEs, signal (cAMP) “terminator”), phosphatases (“eraser”),<sup>2</sup> and other kinases to propagate or modulate the local cAMP signal.

Homologues of PKA-C and PKA-R are ubiquitously present throughout all branches of the tree of life;<sup>10</sup> however, little is known of the evolution of PKA's spatio- and temporal-controlled signaling observed in the mammalian system. To investigate this, here we zoom in on the evolutionary pattern of the PKA-AKAP system. Therefore, we traced the origin of each individual PKA/AKAP functional domain to elucidate crucial crossroads in the tree of life where the system evolved from its most simple form. To experimentally corroborate our bioinformatics analyses, we used cAMP-based chemical proteomics to reveal existing as well as putative cAMP-signaling nodes in 6 representative species, varying from unicell protist to human.

## METHODS

### Bioinformatics

**PKA-R and PKA-C Orthologues Definition and Phylogenetic Analyses.** PKA-R and PKA-C orthologues were defined using methodology described previously.<sup>11</sup> In short, we performed BLAST<sup>12</sup> searches for yeast PKA-R and PKA-C against a local database composed of genomes representative of all eukaryotic super groups. The conserved kinase domains of PKA-C and cNMP binding domains of PKA-R of the resulting hits were aligned using MAFFT<sup>13</sup> with option LINSI. Positions with too many gaps (>20%) were excluded from the alignment. Subsequently, an RAxML<sup>14</sup> tree with 100 bootstraps was generated (option PROTGAMMAWAG). From the resulting tree, a subcluster corresponding to the orthologous group of which PKA-C or PKA-R is a member was delineated. Potential RIIa domains in these homologues were searched for by constructing a HMMER3 profile<sup>15</sup> for the RIIa domain of vertebrate PKA-R homologues. Significant sequences from additional RIIa domain homologues were added to the profile in an iterative process until convergence. The resulting gene tree was visualized using iTOL (Interactive Tree of Life<sup>16</sup>). The putative RIIa domains of PKA-R were also manually checked in three domain databases: SMART,<sup>17</sup> Pfam,<sup>18</sup> and Superfamily.<sup>19</sup>

### AKAP Conservation and PKA Binding Motif Analysis.

In total, 24 well-known, documented AKAPs were collected (Figure S5). The homologues of these AKAPs were identified in 54 eukaryotic genomes by a BLAST<sup>12</sup> search with a threshold *e*-value of  $1 \times 10^{-3}$ . The resulting AKAP homologue hits were aligned using MAFFT<sup>13</sup> with option LINSI. The conservation of the specific PKA binding motif for each AKAP homologue was searched separately by a GL-search algorithm<sup>20</sup> (*e*-value 0.1) and subsequently manually checked.

For the discovery of potential new AKAPs, we performed a PKA binding motif scan with the MEME and FIMO tools from the MEME software suite (<http://meme.nbcr.net><sup>21</sup>). We first built the PKA binding amphipathic helix motif from the documented helices of the 24 known AKAPs mentioned above using the MEME program. The motif width was set to 18

residues and specified to one occurrence per sequence for the MEME search parameters. We then used the motif and PSPM (position specific probability matrix) generated by MEME to scan all other proteins with significant cAMP binding in chemical proteomics analyses (see below). All potential binding sites with FIMO *p*-value < 0.0001 were manually checked for validity based on information from the literature (e.g., ref 22).

Current bioinformatics tools to search and the predict novel AKAPs still face great challenges because of the large variation in AKAP sequences and the relatively short length of PKA binding motif.<sup>23,24</sup> To reduce the false positive identification rate, we have limited the motif search for AKAP candidates to proteins that either have homologue sequences to known AKAPs or that show significant cAMP binding in proteomics experiments.

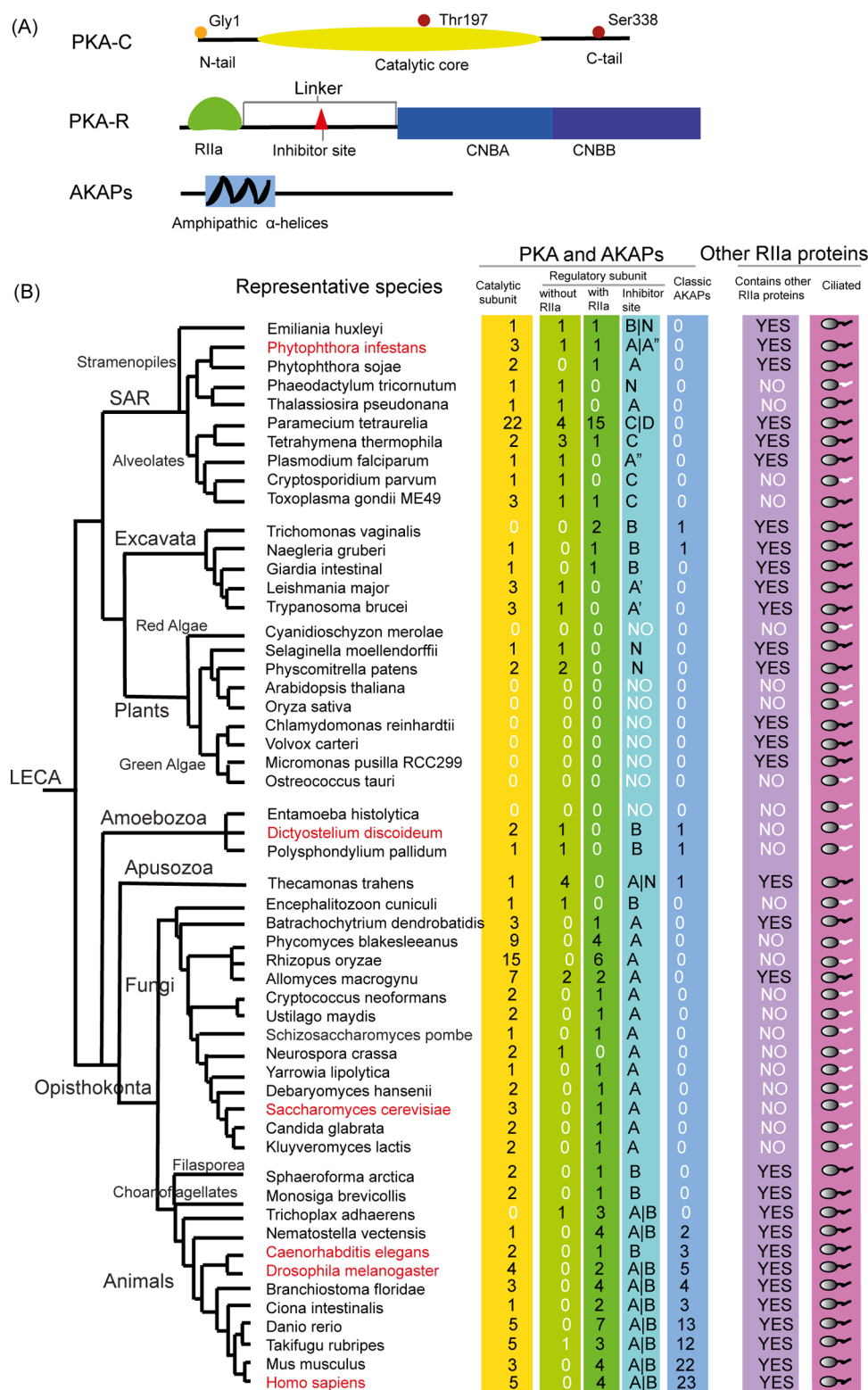
### Chemical Proteomics

**Materials.** The Rp-8-AHA-cAMPS coupled agarose beads were purchased from BiOLOG (Bremen, Germany). Protease inhibitor cocktail, complete mini, were from Roche (Basel, Switzerland). All other chemicals were purchased from commercial sources and were of analysis grade, unless otherwise stated.

**Sample Preparation.** For all species, identical buffer conditions were used with phosphate buffered saline, supplemented with 0.1% Tween-20 and the vendor recommended concentrations of protease inhibitors. Lysate preparation protocols for the different species were used according to published studies with minor modifications (*P. infestans*,<sup>25</sup> Human Jurkat T cell,<sup>26</sup> *Drosophila* S2 cell,<sup>26</sup> *C. elegans*,<sup>26</sup> *S. cerevisiae*,<sup>27</sup> and *D. discoideum*<sup>28</sup>). After lysis, centrifugation at 20 800g for 10 min was used to isolate the soluble fraction. Protein concentration was determined by the Bradford assay.

**Pulldown Assay.** Prior to the pulldown assay, each cell lysate (8–10 mg total protein) was aliquoted into two equal portions, and both were supplemented with 10 mM ADP/GDP and incubated for 30 min at 4 °C with rotary shaking to reduce nonspecific binding, mainly contributed by ADP and GDP binding proteins. One of the two aliquots was also supplemented with a competitive concentration of free cAMP (10 μM). A 10 μL dry volume 8-AHA-cAMPS agarose beads<sup>29</sup> was added per milligram of protein in the lysate aliquots and incubated for 2 h at 4 °C with rotary shaking. The beads were washed six times with ample lysis buffer containing ADP/GDP/10 μM cAMP (competed) and ADP/GDP (uncompeted). Subsequently, the bound proteins were eluted with SDS loading buffer, in ratio of 1:1 buffer/beads (v/v), and subjected to 4–12% SDS-PAGE electrophoresis (BioRad). After Coomassie and/or silver staining of the gel, each lane was cut into 6 gel slices of equal size. For each species, a cell lysate was also analyzed using the 6-band gel protocol. A standard in-gel digestion protocol with DTT (6.5 mM) reduction, iodoacetamide (54 mM) alkylation, trypsin digestion was used.

**LC-MS/MS Analysis.** The digested samples were analyzed on a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen) as described before.<sup>30</sup> Briefly, a Proxeon nanoLC system was equipped with a 20 mm Aqua C18 (Phenomenex, Torrance, CA) trapping column and a 500 mm ReproSil-Pur C18-AQ (Dr. Maisch-GmbH) analytical column (packed in-house, 50 μm i.d., 3 μm particle size). Solvent A consisted of 0.1 M acetic acid in deionized water, and solvent B consisted of 0.1 M acetic acid in 80% acetonitrile. Trapping was performed at 5 μL/min solvent A for 10 min. The flow rate over the



**Figure 1.** Representation of PKA and AKAP coding sequences in selected eukaryotic genomes. (A) Domain organization of PKA catalytic subunit (PKA-C), PKA regulatory subunit (PKA-R), and A-kinase anchoring proteins (AKAPs) in mammalian genomes. Crucial, conserved post-translational modifications in PKA-R include phosphorylations (red dots) and myristoylation (orange dot). (B) Distribution of PKA and AKAPs across eukaryotes and their specific domain architecture. A species tree according to Walker et al.<sup>78</sup> is drawn on the left. Six organisms, which were chosen for chemical proteomics assays, are highlighted in red. The number represents the total number of PKA-C (yellow column), PKA-R (green column), and AKAP (blue column) sequences found in each genome. Also indicated is the presence/absence of the RIIa domain in PKA-R as well as the type of autoinhibitory sequence (A = RRxS; A' = RRxT; A'' = [HK]RxS; B = RRx[AG]; C = [PNDFTQMG]RxS; D = [TN]Rx[AG]; N = noncanonical inhibitory sequence). The purple columns show the presence/absence of other, non-PKA, RIIa domain containing proteins and whether these species have flagella or cilia (black tails). The RIIa domains of PKA-R were manually checked in the SMART, Pfam, and Superfamily databases.



analytical column was set to 100 nL/min, and elution was achieved with a gradient of 7–30% solvent B within 90 min, with a total analysis time of 120 min. Nanospray was achieved using a distally coated fused silica emitter (360  $\mu\text{m}$  o.d., 20  $\mu\text{m}$  i.d., 10  $\mu\text{m}$  tip i.d.; constructed in-house) biased to 1.7 kV. The method was set up in data-dependent acquisition mode. Each sample was analyzed with a Top20 HCD method.

**Data Analysis.** MS/MS data were analyzed by MaxQuant<sup>31</sup> software (ver. 1.3.0.5) with the integrated Andromeda search against the UniProt database (ver. July 2013) for each species (*Homo sapiens*, 133 861 entries; *Drosophila melanogaster*, 39 828 entries; *Caenorhabditis elegans*, 25 985 entries; *Sacharomyces cerevisiae*, 6629 entries; *Dictyostelium discoideum*, 13 113 entries; *Phytophthora infestans*, 17 612 entries). The database searches were performed with the following parameters: a mass tolerance of  $\pm 6$  ppm for precursor masses and  $\pm 0.06$  Da for MS/MS fragment ions, allowing two missed cleavages, cysteine carbamidomethylation as fixed modification, and methionine oxidation as variable modification. The false discovery rate (FDR) at the peptide and protein levels was set to 1%. For stringency, only proteins identified with at least 2 unique peptides were considered for further, label-free quantitative analysis. This was performed using the raw intensities provided by the MaxQuant label-free algorithm. To determine specific cAMP binding proteins, the intensity of each protein in the uncompeted experiment (–cAMP) was divided by that of the competed experiment (+cAMP). Enrichment was determined on the basis of the intensity ratios between targeted experiment and total cell lysate. In the case of missing intensity in either experiment, an arbitrary ratio of 100-fold was assigned. To differentiate the cAMP binding specificity of enriched proteins, *p*-values were generated using significance B from the Perseus package (ver. 1.4.0.11) of the MaxQuant software.<sup>31</sup> For stringency, we considered proteins to be candidates only when they were quantified with a *p*-value  $\leq 0.005$  and a minimum summed intensity of  $1 \times 10^7$ . The contaminant proteins that are commonly identified in almost all AP–MS experiment (affinity purification coupled with mass spectrometry), including heat-shock proteins, tubulins, ribosomal proteins, and histones,<sup>32</sup> were discarded in our analysis.

## RESULTS

### Conserved Protein Domains Involved in PKA–AKAP Signaling

To investigate the evolutionary development of the most important players in PKA signaling we looked at PKA-C, PKA-R, and the AKAPs, zooming in on the conservation and evolution of functional domains that they harbor. Several specifically conserved domains on PKA-R, PKA-C, and AKAPs (Figure 1A) are responsible for different functionalities of the PKA holoenzyme. First, PKA-C contains the catalytic domain, composed of the ATP and substrate binding regions, as well as a domain used to interact with PKA-R. PKA-R contains a dimerization and docking domain at the N-terminus, which mediates dimerization and the interaction with AKAPs. Each AKAP contains a short 3–4 turn amphipathic helix that interacts with the RIIa domain.<sup>33</sup> Further downstream, PKA-R also contains a short pseudosubstrate autoinhibitory (AI) domain, which closely interacts with the catalytic core of PKA-C in the absence of cAMP. Two successive cAMP binding domains follow the AI domain.

For the evolutionary analysis, we first traced the origin of each of these crucial domains based on sequence homology and motif searching covering 54 organisms (Table S1A) that originated both before and after the emergence of metazoans from single-celled eukaryotic ancestors. Using conservative thresholds (see Methods), the number of proteins containing each of these domains was estimated (Figure 1B). Below, we discuss the evolution of these in more detail.

### PKA Catalytic Subunit, PKA-C

The history of protein kinase gene families is dominated by a large amount of gene duplications that predate the common ancestor of all eukaryotes. One such duplication occurred relatively shortly before the appearance of eukaryotes and gave rise to PKA-C. Our phylogenetic analysis of PKA-C revealed a large number of duplication events of different ages and several independent gene losses. For example, an ancient duplication took place in an early ancestor of animals and fungi, as suggested by the presence of two distinct groups of PKA-C genes in the phylogenetic tree (Figure S1): one group representing the evolution of the canonical PKA-C and the other representing the highly similar PRKX genes, as observed previously by Li et al.<sup>34</sup> Most species also underwent more recent independent or parallel duplications that resulted in multiple copies of possibly highly similar yet subtly specialized PKA-C genes.<sup>35</sup> These lineage-specific duplications, for instance, are the origin for the presence of 3 PKA-C genes in the human as well as the *Sacharomyces cerevisiae* genomes (PKA-C  $\alpha$ ,  $\beta$ , and  $\gamma$  and TPK1, 2, and 3, respectively). Our analysis confirmed that, except for flowering plants and green algae, all eukaryotic species contain a conserved PKA catalytic core (Figure 1B). Even species with small and reduced genomes, such as *Encephalitozoon cuniculi* and *Giardia intestinalis*, contain PKA-C, which is exceptional for a signaling system and underscores the importance of PKA-C signaling for many cellular processes. The lack of PKA-C evidence in most plants is consistent with previous studies that reported that cyclic nucleotide regulated kinases were absent in *Arabidopsis thaliana* and *Oryza sativa*.<sup>36</sup> Interestingly, we found PKA-C like genes in the early branching land plants *Selaginella moellendorffii* and *Physcomitrella patens*, suggesting that PKA existed in plant ancestors; however, later divergence may have caused the loss of the gene. We then focused on particular domains of PKA-C. The N- and C-terminal tails of PKA-C are both highly dynamic and play roles in substrate recognition and binding to PKA-R. Starting with the catalytic kinase domain, we observed this to be highly conserved, similar to that observed for other eukaryotic protein kinases.<sup>37</sup> Phosphorylation of Thr197 in the activation loop is a major regulatory site for human PKA-C.<sup>38</sup> This key threonine is extremely well-conserved, and the only variance observed is a serine, another phosphorylatable amino acid, in *Dictyostelium discoideum* and *Giardia intestinalis* (Figure S2) at this position. The sequence alignments of a large number of PKA-C genes across all eukaryotes show that key amino acids in the C-terminal region, important for substrate recognition, like the PxxP and FxxF motifs, are also highly conserved (Figure S2).<sup>39</sup> An exception is the FDDY-motif, an essential part of the ATP binding site,<sup>40</sup> which shows a high diversity across different eukaryotic kingdoms (Figure S2) and was also confirmed in a previous study performed on a limited set of species.<sup>41</sup> In conclusion, nearly all eukaryotic species contain one or even more highly conserved PKA-Cs. Next, we address whether these PKA-Cs can and do form a  $R_2C_2$

complex with PKA-R, as observed in mammals. We therefore first focused on the domain structure of PKA-R.

### PKA Regulatory Subunit, PKA-R

Our analysis reveals, in contrast to PKA-C, a highly dynamic and diverse evolution of PKA-R. Clustering based on phylogenetic analysis revealed the existence of five main groups of PKA-R types in the eukaryotic kingdom (Figure S3). Clearly visible within these groups is the separation of the animal subtypes PKA-RI and RII. The best-conserved domains on PKA-R are the cyclic nucleotide binding domains, which even date back to prokaryotes.<sup>42</sup> The cAMP binding domains have high sequence similarity, and all eukaryotic PKA-Rs in this study were observed with two consecutive cAMP binding domains to facilitate cooperative cAMP-binding<sup>43–45</sup> (Figure S4). One exception appears to be *E. cuniculi* that contains only one cAMP domain in its PKA-R, which may be explained by its highly reduced and compacted genome. The cAMP binding motif (FGELAL[ILVM]xxxPRAA), also called the phosphate binding cassette (PBC), is the hallmark of cAMP domains,<sup>10</sup> and we observed it to be very well-conserved in all PKA-Rs (Figure S4). Slightly upstream of the tandem cAMP domains is a pseudosubstrate autoinhibitory site (AI), which docks to the active site cleft of PKA-C in the absence of cAMP to block its activity. Previous analyses based on a limited set of eukaryotic PKA sequences revealed three types of AI: RRx[AG] $\Phi$  in animals, RRxS $\Phi$  for both animals and fungi, and a noncanonical TRxS $\Phi$  and xKxS $\Phi$  in paramecium and euplotes. On the basis of larger species samples, we now identified two other variants of this motif: [HK]RxS $\Phi$  and RRxT $\Phi$  in SAR and Amoebozoa ( $\Phi$  representing a hydrophobic residue) (Figure 1B). The co-occurrence of the cAMP binding domains with the AI domain slightly upstream in a single protein, the PKA-R prototype, is observed in almost all eukaryotic species that we investigated (Figure 1B). In prokaryotes, this combination is not observed,<sup>42</sup> indicating that cAMP-directed kinase action is a eukaryotic invention. Summarizing, it appears that the cAMP binding domain with the AI domain in front has been a successful evolutionary concept, as we observed it to be very stable throughout the tree of life. Next, we zoom in on the quaternary structure of the PKA holoenzyme.

### Holoenzyme Formation

The two subunit functionality of PKA as we know it in animals depends on the interaction of PKA-R with PKA-C. As mentioned, this occurs via the binding of the AI sequence to the catalytic core of PKA-C; however, recent crystal structures of PKA holoenzymes have revealed additional sites of interaction.<sup>46</sup> Therefore, we investigated these for sequence conservation as well. Starting with PKA-R, we first checked the B/C helix in the first cAMP binding (CNB-A) domain. This surface mediates the major conformational change of PKA-R upon binding to cAMP, and it docks onto the activation loop of PKA-C.<sup>46,47</sup> Interestingly, this motif (RxxxR[RK] $\Phi$  $\Phi$ xxx $\Phi$ -[RK][RK]RK, with  $\Phi$  representing a hydrophobic residue) is quite well-conserved in animals and fungi (Figure S4). However, the two basic residues in the middle of this motif are replaced by asparagine or histidine for most SAR and Excavata. A previous study showed that replacing these key residues in this motif can significantly change the interface interactions between PKA-R and PKA-C.<sup>43</sup> This suggests a different mode of interaction in these species, likely to fine-tune the cAMP concentrations required for activation toward the individual needs of the organism. At the other end of the

interaction, the N-terminal region and some post-translational modification sites of PKA-C play a key role in the *cis*-regulated activation of PKA.<sup>48</sup> In line with the sequence variability on PKA-R, these regions of PKA-C also showed much more diversity. For instance, the myristoylation site on the N-terminal glycine,<sup>49</sup> the phosphorylation site Ser338,<sup>50</sup> and the A helix<sup>51</sup> conserved in mammals are missing in PKA-C genes of other eukaryotic super groups, suggesting a stepwise evolution of PKA's molecular activation mechanism upon cAMP binding. The diversity of PKA holoenzymes is further illustrated by the observation that the crystal structures of the different mammalian tetrameric holoenzymes (RI $\alpha$ ,  $\beta$  and RII $\alpha$ ,  $\beta$ ) and their AKAP binding specificity are all rather distinct.<sup>7</sup> Whether this aspect may also contribute to appropriate localization of the holoenzyme in more distant species needs to be further investigated.

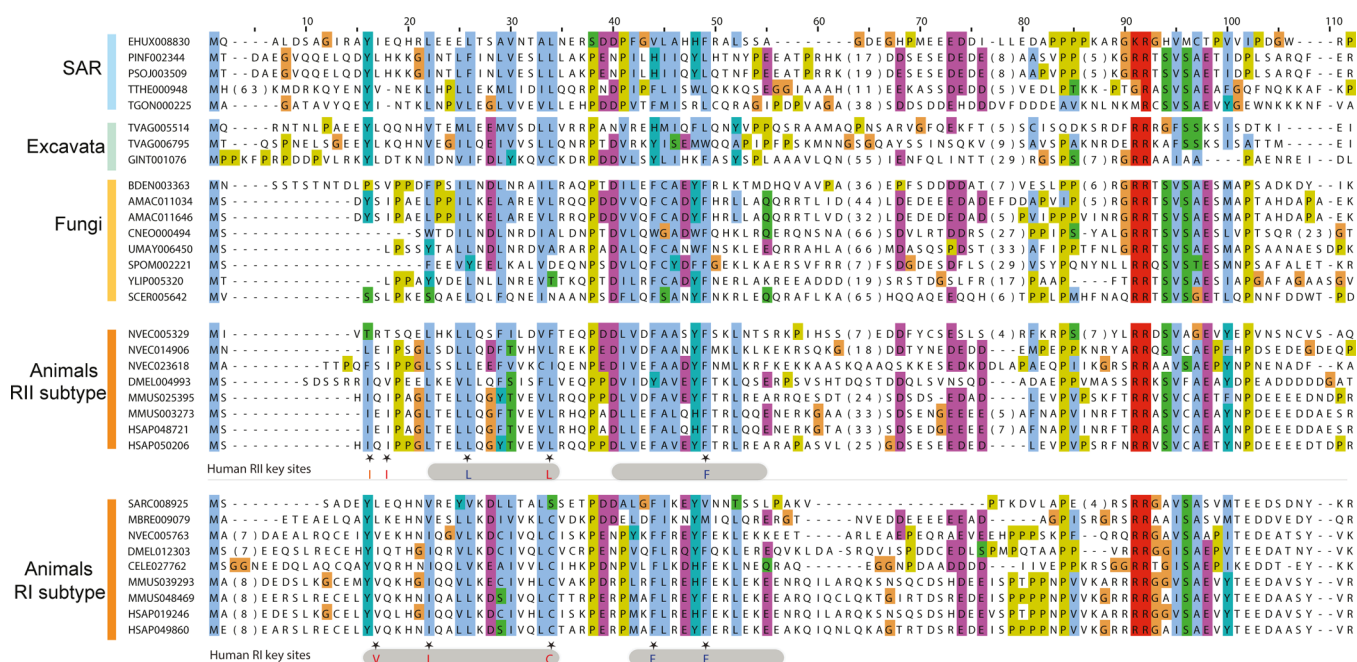
### The Origin of PKA's RII $\alpha$ Domain: the Key to PKA's Subcellular Localization in Mammalian Cells

The evolution of the RII $\alpha$  domain within PKA-R shows discordant behavior. In animals and fungi, it is closely connected to the AI/cAMP binding tandem (Figure 1B). In many SAR, excavate and amoebozoa lineages PKA-R lacks the RII $\alpha$  domain altogether, and several species in these lineages contain both PKA-R genes with and without the RII $\alpha$  domain. The absence of the RII $\alpha$  domain negates the formation of the PKA-R dimer and the canonical heterotetrameric holoenzyme (PKA-R)<sub>2</sub>-(PKA-C)<sub>2</sub>, as was indeed shown for *Dictyostelium discoideum*<sup>52</sup> and *Plasmodium falciparum*,<sup>53</sup> which have heterodimeric PKA holoenzymes. The scattered distribution of RII $\alpha$  domains in PKA-R proteins is indicative of independent loss on multiple occasions, multiple independent fusions of this domain, or horizontal gene transfer. The phylogeny of PKA-R does not indicate horizontal transfer, and the phylogeny of the RII $\alpha$  domain shows that RII $\alpha$  domains in PKA-R are monophyletic. This makes parallel domain fusion unlikely, especially because in organisms without a RII $\alpha$  domain in their PKA-R there are no separate RII $\alpha$  domains orthologous to the RII $\alpha$  domain in PKA-R.

Interestingly, the RII $\alpha$  domain itself is found in almost all eukaryotic lineages (Figure 1B), with a strong preference for ciliated eukaryotes; however, in these instances, the RII $\alpha$  domain is not connected to PKA-R. This indicates that this domain has duplicated before the last common ancestor and that one of the duplicates evolved separately from PKA, possibly to fulfill crucial functions in cilia function. Corroborating this finding, non-PKA RII $\alpha$  proteins can be found in mammalia today. Four proteins (SPI7, CABYR, ASP, and ROPN1) were recently discovered in flagella of mammalian sperm, and the former 3 proteins were also found in motile cilia of human bronchus and fallopian tubes.<sup>54</sup> These proteins also bind to AKAPs, indicating the retained functionality of this domain in other (signaling) systems.<sup>54</sup> Another non-PKA protein, the flagella radial spoke protein in green algae *Chlamydomonas reinhardtii* was also found to form a protein complex with amphipathic helices via its RII $\alpha$  domain.<sup>55</sup> Therefore, we suspect that the docking function of RII $\alpha$  domains may have an earlier origin, but its recombination into the PKA-R gene was crucial for spatiotemporal controlled PKA signaling.

The sequence alignments of all RII $\alpha$  domains revealed a large variation among mammals, fungi, and evolutionary more distant organisms such as SAR and Excavata (Figure 2), suggesting a





**Figure 2.** Sequence alignment of PKA-R N-termini, which contain the RIIa domain and the autoinhibitory site. Larger inserts have been removed and are represented by the number of residues removed given in parentheses. A solid gray bar at the bottom indicates residues involved in  $\alpha$ -helices in animal PKA-RI and RII. Therein, the key residues for mammalian PKA dimerization and AKAP binding are highlighted in blue and red in the gray bars below the alignment.

variable repertoire of docking sites. Fungi consistently contain the RIIa in their PKA-R, but sequence alignments show that key sites for AKAP docking and dimerization are different from the mammalian PKA-R sequences (Figure 2), possibly also influencing dimerization, such as in *Neurospora crassa*<sup>56</sup> and *Yarrowia lipolytica*.<sup>57</sup> One recent study in *S. cerevisiae* shows that the classic mammalian AKAPs cannot bind to yeast PKA-R.<sup>58</sup> Hence, although ancestrally the RIIa domain was likely able to bind amphipathic helices, it is unclear whether, besides animals, these other amphipathic helices are also able to this. One way to investigate this is to phylogenomically probe whether potential AKAPs can be detected in nonanimal species. From these *in silico* data on PKA alone, we cannot conclude the origin of localized AKAP signaling; therefore, we also traced the evolution of currently known AKAP proteins.

### A Kinase Anchoring Proteins, AKAPs

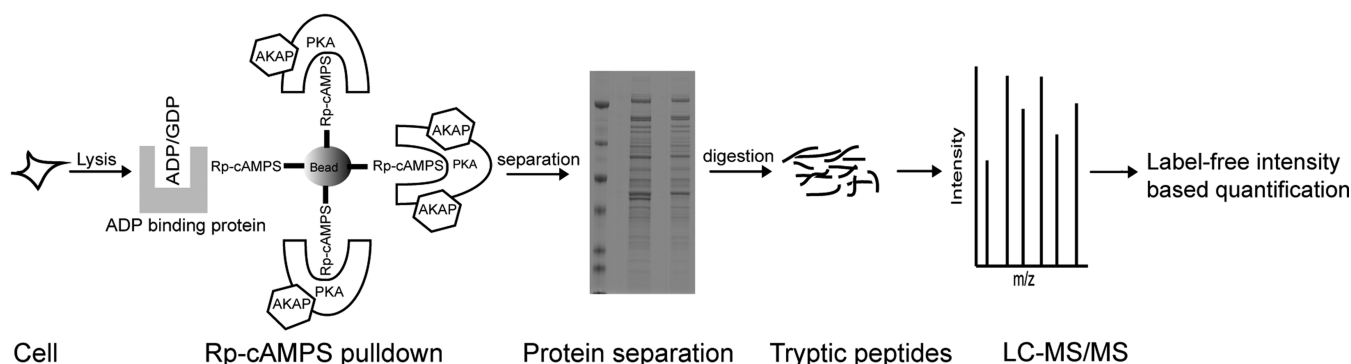
Using the same methodology, we traced back all known mammalian AKAP genes. Besides detecting homologous proteins, we paid particular attention to the presence and conservation of the amphipathic helix region on the AKAPs, as this is known to be the crucial site of PKA interaction. This analysis revealed that the large majority of helix-containing AKAPs are a relatively new invention, as close homologues were predominantly identified in animals, particularly vertebrates, whereas many fewer potential AKAPs can be identified in more distant species like *D. melanogaster* and *C. elegans* (Figure S5). Consistent with this observation, up to now, no classical AKAPs have been identified in fungi, neither in our *in silico* analyses nor in genome wide protein–protein interaction analyses in *S. cerevisiae*,<sup>59,60</sup> which contain the required PKA-R with an N-terminal RIIa domain. The lack of fungal AKAP genes is in line with the earlier mentioned sequence variation in the RIIa domains of PKA-R. This leaves the question of whether fungi and other more distant species may use a different repertoire of AKAPs or simply do not have any

AKAPs altogether. To answer this question, we set out to trace PKA binding proteins throughout the tree of life using well-established immobilized cAMP-based chemical proteomics assays.<sup>61</sup>

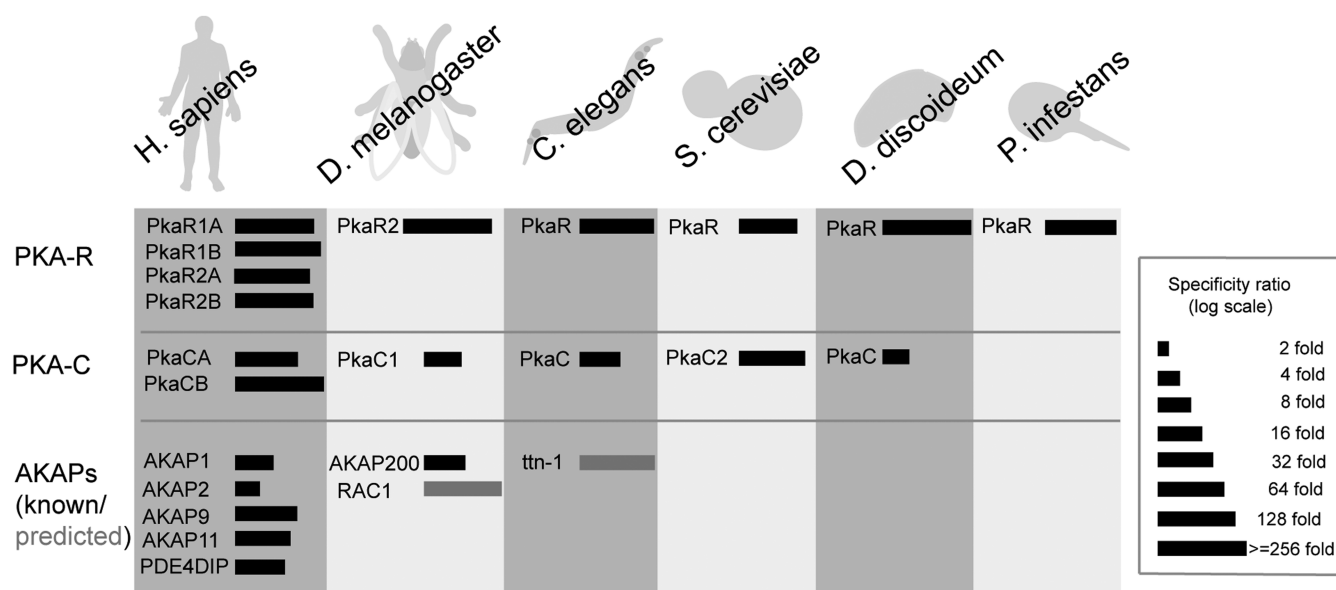
### Enrichment of cAMP-Binding Proteins Across the Tree of Life by Chemical Proteomics Experiments

In the chemical proteomics experiments, lysates are incubated with (agarose) beads in which Rp-cAMPS was covalently coupled via a flexible linker. The antagonist Rp-cAMPS was chosen because its binding to PKA-R does not disrupt the holoenzyme and thus enrich for both PKA-R and PKA-C.<sup>62</sup> Proteins that bind to the beads are either cAMP binders (e.g., PKA-R) or proteins that interact with these primary interactors (e.g., AKAPs). We performed the pulldown assay on lysates obtained from 6 different organisms, *Homo sapiens* (Jurkat T-cells), *Drosophila melanogaster* (S2 cells), *Caenorhabditis elegans* (whole organisms), *Sacharomyces cerevisiae*, *Dictyostelium discoideum*, and *Phytophthora infestans*, covering the four eukaryotic super groups of animals, fungi, amoebzoa, and SAR. Figure 3A showed the schematic workflow of the affinity pulldown. To distinguish cAMP-sensitive proteins from background and/or general nucleotide binders, we performed a parallel pulldown with 10  $\mu$ M cAMP as an in-lysate competitor. The cAMP binding specificities for individual proteins were calculated via intensity-based label-free quantitation and plotted versus the total intensity of each identified protein (Figure S6A–F). All identified proteins (1% FDR) and their cAMP ratio are listed in Table S2A–F. As shown in Figure S6A–F, more nonspecific binding proteins were observed in *D. melanogaster* and *D. discoideum* as compared to those in the other species. An explanation for this could be that there is a limited amount of PKA subunits present in those cell lysates, making it difficult to separate them from the background of nonspecific binders in identical pulldown experiments. The PKA subunit abundance in the total lysate was estimated

## (A) chemical proteomics workflow



## (B) cAMP binding specificity ratio (-/+cAMP)

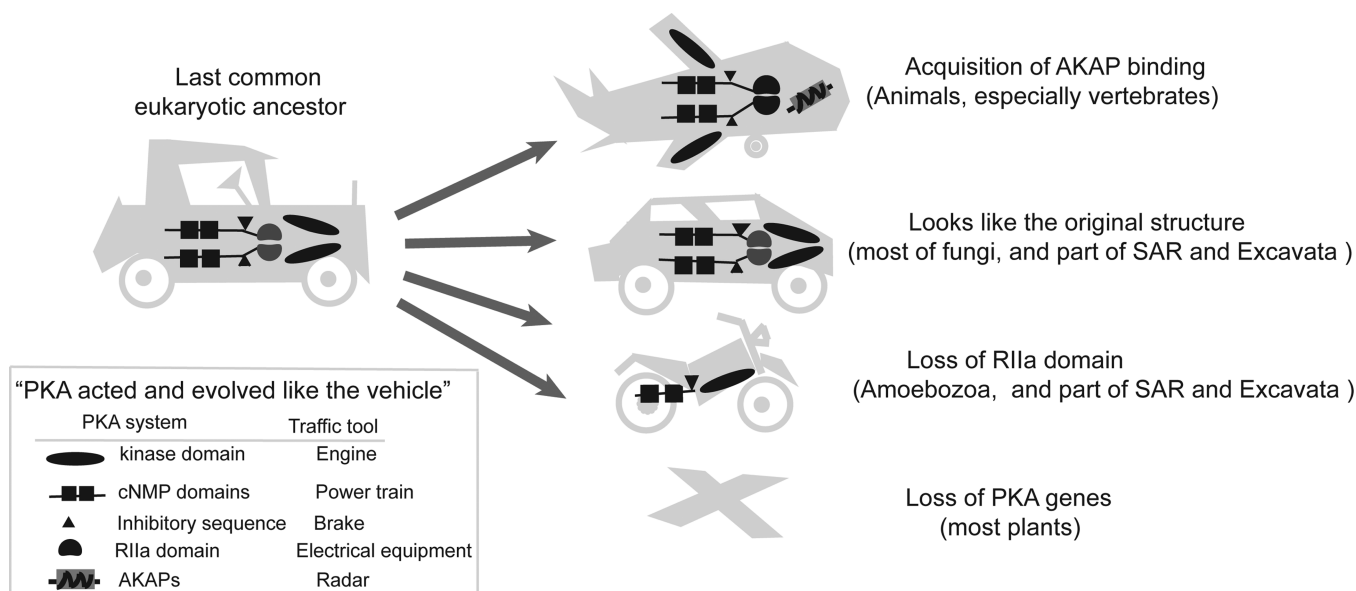


**Figure 3.** cAMP binding proteins identified by chemical proteomics experiments throughout the tree of life. (A) Schematic of the workflow for the enrichment of cAMP binding proteins and their interactors. The cells/tissue from six different organisms were lysed individually. Subsequently, chemical proteomics pulldowns were performed using Rp-8-AHA-cAMPS affinity agarose beads on two identical fractions: one supplemented with competitive amounts of cAMP (10  $\mu$ M) and one without. After incubation and washing, the bound fractions were separated by SDS-PAGE and followed by in-gel digestion and LC-MS/MS analysis. (B) Schematic representation of the enriched proteins and their interactors in three different categories based on their function in the cAMP pathway. The  $\pm$ cAMP enrichment ratios were calculated by protein intensity differences between the cAMP-competed and -uncompeted branches of the experiment. Predicted AKAPs are highlighted in dark gray.

(Table S3A–F) by calculating the abundance factor,  $F_{abb}^{63,64}$ . As shown in Figure S6G, the abundance of PKA subunits in *D. melanogaster* and *D. discoideum* was indeed lower as compared to that in other species.

In each species, we successfully identified one or more PKA-R species as cAMP-responsive proteins (Figures 3 and S6A–F), meaning that 10  $\mu$ M free cAMP was able to compete for binding to the beads; hence, in the experiment, these proteins displayed a differential intensity ratio ( $\pm$ cAMP). PKA-C was observed with similar ratios in *H. sapiens*, *D. melanogaster*, *C. elegans*, *S. cerevisiae*, and *D. discoideum*. In *P. infestans*, PKA-C was identified neither in the lysates nor in the cAMP pulldown experiment. This may be caused by a low abundance of PKA-C in this organism (Figure S6G). On the basis of the enrichment ratio, we identified several cAMP-responsive proteins in each of the studied species. After applying stringent criteria based on

both ratio and protein intensity, a subset was selected for further investigation. First, we performed BLAST analysis to check for homology with any of the currently known AKAPs or the presence of a cyclic nucleotide binding domain. Second, MEME and FIMO<sup>65</sup> software were used for searching for the AKAP amphipathic helix motif. In the human data set, we observed all 4 human PKA-R isoforms, two isoforms of PKA-C, 5 known AKAPs, and a known AKAP9 interactor, CDK5RAP2.<sup>66</sup> BLAST analyses did not reveal other candidates. All other proteins for which no evidence of cAMP or AKAP binding has been reported in the literature were screened for the presence of a putative amphipathic helix PKA binding domain. In the human data, no further candidates were observed. In the other species, there is a clear trend observed of a reduced number of AKAPs with increased simplicity. The multicellular *D. melanogaster* and *C. elegans* exhibit some known



**Figure 4.** Evolutionary history of PKA–AKAP signaling. The stepwise evolution of the PKA–AKAP system is visualized in a model in which we predict the last common eukaryotic ancestor (LECA) to possess the canonical PKA holoenzyme (antique car). This setup is still observed in almost all eukaryotic super groups containing PKA, although there are different versions of the system. The most simple lost the association of the RIIa domain with PKA-R, resulting in dimeric holoenzymes and likely also a loss of anchoring capabilities (motorcycle). This is followed by a version that contains all functional domains to perform anchoring; however, the emergence of AKAPs has not taken place in these organisms (modern car). The most evolved version is next, in which ample AKAPs are present and capable of associating through the RIIa domain of PKA (plane). A final version has lost the entire PKA gene set (plants). The prediction of origin and subsequent loss of PKA–AKAP signaling subunits is inferred from the phylogenetic tree of PKA-R and PKA-C as well as the presence/absence profiles of individual subunits and their key domains. Both bioinformatics and chemical proteomics showed that AKAP binding can be found only in animal genomes and therefore likely evolved later in conjunction with multicellularity, where tight regulation in space and time became more eminent.

or putative AKAPs, as shown in Figure S5, of which we identified three AKAPs (Figure 3). In *D. melanogaster*, a known AKAP, AKAP200,<sup>67</sup> and a putative AKAP, RAC1, with a conserved RIIa binding domain, were identified (Figure S7A,B). Consistent with our data, RAC1 has been reported as a potential human AKAP.<sup>68</sup> Similarly, the MEME search suggested Titin as a putative novel AKAP in *C. elegans* (Figure S7C). Interestingly, the amphipathic helix was not conserved in *Drosophila* and humans (Figure S7D). The cGMP-dependent kinase (PKG) in *C. elegans* and *D. melanogaster* (Figure S6B,C) was identified in cAMP pulldown experiments, which is consistent with previous report that cAMP and cGMP can cross-activate their respective kinases.<sup>69,70</sup> In contrast, none of the experiments in unicellular organisms yielded convincing AKAP candidates or more cyclic nucleotide domain containing proteins. In addition, we could not identify any cAMP-responsive proteins to be consistently present throughout the examined species, except for PKA-R and PKA-C, validating that AKAPs are a genuine multicellular invention. For fungi, this is somewhat surprising, as these do contain the canonical PKA-R with the RIIa domain at the N-terminus. It should be mentioned that these results are in strong agreement with the observations made in the large-scale protein–protein interaction networks by Gavin<sup>71</sup> and Breitkreutz,<sup>60</sup> who also have not observed major interaction networks for PKA-R (bcy1) or PKA-C (tpk1, 2, and 3) in yeast.

## DISCUSSION

### Stepwise Evolution of Localized PKA Signaling

Altogether, the bioinformatics and experimental data suggest a stepwise evolution of PKA/AKAP functional domains, which is

summarized in Figure 4. The distribution of seemingly intact proteins across the major groups of eukaryotes suggests that the last common ancestor of eukaryotes already possessed the canonical PKA-R/PKA-C kinase constellation. There is no intermediary species to tell us how the canonical PKA arose, but instead its origin is explained by the myriad duplications during eukaryogenesis, in line with what is observed for many other signature eukaryotic features. Nonetheless, this ancient system, which is absent in prokaryotes and flowering plants, has remained relatively constant in all other eukaryotes. The only exception is the N-terminal RIIa domain, and with it the ability to bind to AKAPs. The origin of the RIIa domain, its duplications, and its fusion with PKA-R also predate the last common ancestor of eukaryotes. As mentioned earlier, several RIIa domain containing proteins, apart from PKA-R, are still present in mammals. Surprisingly, the presence of the RIIa-containing PKA-R does not seem to be a determinant for the presence of AKAPs since proteomics and bioinformatics assays could not detect AKAPs in fungi or SAR but only in animals and their close unicellular relatives. Hence, the addition of AKAPs to the system appears to be an innovation of this group of organisms. From then on, it was an evolutionary success, as the number of AKAPs explodes linearly with the complexity/multicellularity of the animal (Figure S5). The dynamic evolution of AKAPs is also reflected in the fact that many vertebrate AKAPs do not have invertebrate homologues. Vice versa, the invertebrate *C. elegans* and *D. melanogaster* have documented AKAPs (e.g., AKAPce/AKA-1,<sup>72</sup> Neurobeachin<sup>73</sup>) that do not have vertebrate orthologues at all or have altered their AKAP properties. Also, the observation that Titin is a putative AKAP in *C. elegans* is interesting in this perspective, as the proposed anchoring domain seems to be lost in the



mammalian homologues (Figure S7D). This rapid emergence, and loss of AKAPs, started in the ancestors of animals and then continued throughout animal evolution to generate highly regulated subcellular localization profiles in mammalian systems.

How the unicellular species regulate cAMP/PKA specificity in space and time is currently unclear. We could not find consistent evidence for early AKAPs in our chemical proteomics enrichments. With the observed high sequence variation within, or even the absence of, the PKA-R-coupled RIIa domain, this suggests this type of organization to be absent in these species.

Compared to the evolution of PKA–AKAP complexes, other well-studied signaling complexes present quite different evolutionary histories. For example, the evolutionary model of phosphotyrosine (pTyr) signaling showed that the emergence of a complete toolkit of writers (TyrK), readers (SH2), and erasers (PTP) leads to rapid expansion and elaboration of each of these domain families.<sup>74,75</sup> Also, Wnt signaling shows a similar evolutionary pattern as that of pTyr signaling, and most members can be found in all clades of metazoans but not in fungi, plants, or unicellular eukaryotes.<sup>76</sup> Evolutionary studies of the signaling complex of GTP binding proteins suggest that the divergence of the Ras superfamily (Ras, Rho, Arf/Sar, Ran, and Rab) occurred before the emergence of eukaryotes other than those that started expanding from metazoans.<sup>77</sup> The evolutionary pathway of PKA–AKAP complexes is rather different. The core signaling machinery (cAMP-sensor (PKA-R) and -writer (PKA-C)) is strongly conserved throughout all eukaryotic species, whereas the AKAPs required for intracellular resolution expanded dramatically soon after ancestral animals harnessed the intrinsic (or ancestral) ability of the RIIa domain to bind amphipathic helices and a more versatile PKA-R emerged in animals. This explosive increase of AKAPs hardly affected the number of PKA isoforms and thus allowed the cAMP/PKA system to expand its functional/substrate repertoire without compromising specificity. We suspect this stepwise evolution model is not just restricted for PKA but is likely also to be applicable to other signaling proteins.

## CONCLUSIONS

Here, spatiotemporal regulation of PKA signaling is investigated by tracing the evolutionary dynamics of each functional domain of cAMP-dependent protein kinase (PKA) and its diverse set of A-kinase anchoring proteins (AKAPs) in combination with bioinformatics and chemical proteomics approaches. Homologues of the catalytic (PKA-C) and regulatory (PKA-R) domains of the (PKA-C)<sub>2</sub>–(PKA-R)<sub>2</sub> holoenzyme were found throughout eukaryotic evolution. Most variation was observed in the RIIa domain of PKA-R, crucial for dimerization and docking to AKAPs. The RIIa domain was not observed in all PKA-R homologues. In the PKA-R of fungi and distinct protist lineages, the RIIa domain emerges within PKA-R, but it displays large sequence variation. These organisms do not harbor homologues of AKAPs observed in animals, suggesting that efficient docking to direct spatiotemporal PKA activity evolved in multicellular eukaryotes. In order to verify this *in silico* hypothesis, we experimentally screened organisms with increasing complexity by cAMP-based chemical proteomics. The experimental approach revealed that the occurrence of PKA–AKAP interactions indeed coincided and largely expanded within vertebrates, suggesting a crucial role for AKAPs in the advent of metazoan multicellularity. The

take home message of this study is that although PKA is ubiquitously present throughout the tree of life its regulation in space and time has been remarkably different throughout evolution.

## ASSOCIATED CONTENT

### Supporting Information

Table S1 (XLSX): Data source of 54 genomes, and protein sequence of each predicted PKA-R, PKA-C, and AKAP in this study. Table S2 (XLSX): List of cAMP-enriched proteins identified in 6 organisms. The proteins are listed with their respective intensities, specificity ratios, and significance value. Primary binding proteins of cAMP, i.e., PKA-R and -C subunits, are highlighted with red, whereas secondary binding proteins are in green. Table S3 (XLSX): List of all identified proteins in cell lysate of 6 different organisms. The proteins are listed with respective intensities, number of PSM, and abundance factor. Primary binding proteins of cAMP, i.e., PKA-R and -C subunits, are highlighted with red. Figure S1 (TIFF): Phylogeny of PKA catalytic subunit. The phylogenetic tree was based on multiple alignments of all predicted PKA-C proteins in 54 species. Scale bar represents genetic distance reflected in branch length. Branches with bootstrap support values larger than 80% are labeled with black circles, representing high confidence for a given node of the observed tree. Figure S2 (TIFF): Protein sequence alignment of PKA catalytic subunits. In total, 281 PKA-C proteins identified from 54 species are aligned. The background coloring indicates conservation, with blue being the most conserved. The red and green lines around the important sites or motifs are used to indicate their high and low conservation separately in corresponding species. Positions with more than 20% gaps were excluded from the alignment. The full-length sequences of each PKA-C proteins obtained from each species are listed in Table S1B. Figure S3 (TIFF): Phylogeny of PKA regulatory subunit. The maximum likelihood (ML) tree was constructed with a multiple alignment approach, including the inhibitory domain and cAMP binding domains of all predicted PKA-R proteins in 54 species. The scale bar represents genetic distance reflected in branch length. Branches with bootstrap support values larger than 80% are labeled with black circles. Figure S4 (TIFF): Protein sequence alignment of PKA regulatory subunits. In total, 230 PKA-R proteins identified from 54 species are aligned. The background coloring indicates conservation, with blue being the most conserved. The red and green lines around the important sites or motifs are used to indicate the high and low conservation separately in corresponding species. Positions with more than 20% gap were excluded from the alignment. The full protein sequences are listed in Table S1C. Figure S5 (TIFF): Conservation of 24 known AKAPs. The distribution of AKAP homologues of 24 known AKAPs in 54 species is presented. The numbers shown in yellow indicate the presence of an AKAP homologous sequence, whereas the numbers in green indicate the PKA binding motif, which is conserved, while gray indicates the absence of proteins or motifs. The protein sequences of AKAPs and their PKA binding sites (also the corresponding reference for each AKAP) are tabulated in Table S1D. Figure S6 (TIFF): cAMP-binding proteins identified in different organisms by chemical proteomics experiments. (A–F) The SDS gels demonstrate the capture of primary binding proteins, PKA-R in the pulldown performed in the absence (–cAMP, left lanes) and presence (+cAMP, right lanes) of 10  $\mu$ M cAMP. The

dotted red lines indicate the area where PKA-R is expected based on the molecular weight. The cAMP binding specificity ratio ( $\pm$  cAMP) of each protein is plotted against the corresponding MS signal intensity. For stringency, only proteins with  $\geq 2$  unique peptides and a minimal intensity  $1 \times 10^7$  were considered to be putative candidates. All proteins with  $p$ -value  $\leq 0.005$  are labeled with their corresponding names. (G) The estimated protein abundance of PKA-R and -C subunits in the lysate is depicted. Figure S7 (TIFF): Predicted AKAPs and their putative amphipathic helix for binding to PKA. (A) Alignment of PKA RI $\alpha$  binding domain of three known AKAPs with the putative novel AKAP helix in the RAC1 protein of *D. melanogaster*. (B) Conservation of AKAP-PKA binding sequence of RAC1 protein among *C. elegans*, *H. sapiens*, *M. musculus*, and *D. melanogaster* (red dotted highlight). (C) Alignment of PKA RI binding domain of three known AKAPs with the putative novel AKAP helix in the Titin protein of *C. elegans*. (D) The conservation of AKAP-PKA binding sequence of Titin protein among *C. elegans* (red dotted highlight), *H. sapiens*, *M. musculus*, and *D. melanogaster*. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00370.

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

PKA, cAMP-dependent protein kinase; AKAP, A-kinase anchoring protein; PKA-C, PKA catalytic subunit; PKA-R, PKA regulatory subunit

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