Chemical proteomic profiling reveals protein interactors of the alarmones diadenosine triphosphate and tetraphosphate

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The nucleotides diadenosine triphosphate (Ap3A) and diadenosine tetraphosphate (Ap4A) are formed in prokaryotic and eukaryotic cells. Since their concentrations increase significantly upon cellular stress, they are considered to be alarmones triggering stress adaptive processes. However, their cellular roles remain elusive. To elucidate the proteome-wide interactome of Ap3A and Ap4A and thereby gain insights into their cellular roles, we herein report the development of photoaffinity-labeling probes and their employment in chemical proteomics. We demonstrate that the identified ApₙA interactors are involved in many fundamental cellular processes including carboxylic acid and nucleotide metabolism, gene expression, various regulatory processes and cellular response mechanisms and only around half of them are known nucleotide interactors. Our results highlight common functions of these ApₙAs across the domains of life, but also identify those that are different for Ap₃A or Ap₄A. This study provides a rich source for further functional studies of these nucleotides and depicts useful tools for characterization of their regulatory mechanisms in cells.
inucleoside polyphosphates (Ap₄Ns) are a family of nucleotides which were discovered in bacteria and eukaryotic cells already in the 1960s, but their cellular roles and functions remain mostly elusive. The most widely studied family members are diadenosine polyphosphates (Ap₄As), like diadenosine triphosphate Ap₃A and diadenosine tetraphosphate Ap₄A. As their cellular concentrations increase in response to extracellular cues such as pH, temperature and oxidative stress from a nanomolar to a lower micromolar range, Ap₄As have been considered to be alarmones that signal cellular stress. However, the underlying mechanisms and pathways remain to be elucidated.

It is assumed that the main source of intracellular Ap₄As is a side reaction of aminocyl-tRNA synthetases (aaRSs), since several studies have shown that across species aaRSs can produce Ap₄As. Recently, it was reported that activation of the post-translational modifiers ubiquitin and ubiquitin-like proteins (i.e., SUMO, NEDD8) is accompanied by the formation of Ap₃A and Ap₄A linking one of the most prevalent eukaryotic protein modification systems to Ap₃A formation.

In higher eukaryotes, two proteins have been identified that are responsible for the degradation of Ap₃A and Ap₄A. In humans, Ap₃A is cleaved into AMP and ADP by the tumor suppressor protein fragile histidine triad (FHIT), whereas Nudix (nucleoside diphosphate linked to X) type motif 2 (NUDT2) hydrolyzes Ap₄A to AMP and ATP. The malfunction of these enzymes results in severe consequences for the affected cells or organisms. Dysfunction or absence of FHIT are frequently observed in various types of cancer. NUDT2 has been proposed as a prognostic marker for breast and lung carcinoma, due to its enhanced oxidative stress from a nanomolar to a lower micromolar range, in response to extracellular cues such as pH, temperature and oxidative stress.

In this work, we report the development and chemoproteomic evaluation of Ap₃A- and Ap₄A-based photoaffinity-labeling probes (PALPs) (Fig. 1) to elucidate the interactome of Ap₃A and Ap₄A and eventually gain insights into their intracellular roles. These PALPs are equipped with diazirine (DA) as photoreactive group, enabling the formation of a covalent bond between the probe and an interacting protein upon UV irradiation, and desthiobiotin (DTB) as affinity tag. By applying these PALPs in photoaffinity enrichment assays followed by LC-MS/MS (Fig. 1a), we identified 61 significantly enriched proteins for Ap₃A and 26 for Ap₄A from human embryonic kidney (HEK293T) cell lysates. Interestingly, while nine proteins were identified with both Ap₃A baits, 52 proteins were exclusively found for Ap₃A and 17 for Ap₄A, respectively. Gene ontology (GO) analysis reveals the involvement of these potential Ap₃A and Ap₄A interactors in different fundamental cellular processes, including carboxylic acid and nucleotide metabolism, gene expression, and various regulatory processes. In addition, we used our PALPs to probe for interactors in E. coli cells and identified overall 20 significantly enriched proteins. These proteins are also mainly associated with metabolism highlighting a common role of these Ap₄As across the domains of life. Moreover, only about half of the identified proteins are known nucleotide binders which indicates roles of Ap₃A and Ap₄A in cellular pathways that are distinguishable from those of other nucleotides. Taken together, this study demonstrates the power of our functionalized non-hydrolysable Ap₃A and Ap₄A derivatives used as PAL probes (PALP).

Unlabeled, non-hydrolysable Ap₃A and Ap₄A derivatives and a control substance (con-1) lacking the Ap₄A scaffold served as controls in PAL experiments.

Fig. 1 Overview of photoaffinity-labeling (PAL) approach for identifying Ap₄A interactors. a Schematic workflow of PAL experiments. Probes were incubated with cell lysates and irradiated with UV light to initiate photo-crosslinking. Labeled proteins were affinity-purified using desthiobiotin (DTB) as affinity tag. Eluted fractions were digested and analyzed by LC-MS/MS and label-free quantification (LFQ). b Functionalized, non-hydrolysable Ap₃A and Ap₄A derivatives used as PAL probes (PALP). c Unlabeled, non-hydrolysable Ap₃A and Ap₄A derivatives and a control substance (con-1) lacking the Ap₄A scaffold served as controls in PAL experiments.
chemical proteomics approach based on the synthesized PALPs and uncovers proteome-wide interaction maps for Ap₃A and Ap₄A. This provides a rich source for further functional studies directed towards a deeper understanding of the physiological and pathophysiological roles of these nucleotides.

Results
Design and synthesis of PALPs. To unravel the interactome of Ap₃A and Ap₄A we designed and employed photoaffinity-labeling probes that contain surrogates of the phosphoanhydride bond in order to confer hydrolytic stability, as we observed that natural Ap₃As are hydrolyzed to different extent in cell lysates (Supplementary Fig. 1). Previous studies showed that the stability of Ap₃As in cell lysates is increased by exchanging the P₂O₅ oxygen for sulfur and replacing the bridging oxygen with a methylene group. Therefore, we synthesized the Ap₃A-analog PALP-1 and Ap₄A-analog PALP-2 (Fig. 1b) that contain thio- phosphate at the α-phosphate positions and methylene bisphosphonate linkages.

Due to the proficiency of several enzymes to use N6-modified ATP and N6-modified Ap₃A as substrates, the N6 positions of the adenine moieties were chosen for the attachment of the photocrosslinking diazirine (DA) and the affinity tag desthiobiotin (DTB) in our probes. The used DA represents a small functional group that undergoes carbene formation under UV light that rapidly inserts into nearby C–H, N–H, and O–H bonds, rendering DAs well-established tools for photoaffinity labeling. DTB as affinity tag allows enrichment of tagged proteins by exploiting the affinity of DTB to (strept)avidin, and its specific elution with biotin for subsequent analysis by high-resolution mass spectrometry (MS).

The synthesis of both probes was straightforward and is described in detail in Supplementary Figs. 2 and 3. To further increase the specificity of our approach, two non-photoreactive derivatives comp-1 and comp-2 (Fig. 1c) were synthesized following described routes and employed in competition experiments. Similarly, to exclude proteins from the interactomes that bind to the diazirine or desthiobiotin groups of our Ap₃A and Ap₄A probes, molecule con-1 was synthesized (Fig. 1c) and applied as an additional control.

Ap₃A and Ap₄A are present in HEK293T cell lysates. As HEK293 cells are commonly used in interactome analyses and already have been employed to study the correlation between intracellular Ap₃A concentrations and apoptosis, we chose the HEK293T cell line for our experiments. To obtain further insights into the responsiveness of HEK293T cells towards external stress, we determined the cellular levels of Ap₃A and Ap₄A and their changes upon exposure to different stress conditions such as mitomycin C (MMC)–induced DNA damage and oxidative stress by treatment with H2O2. To do so, we developed a method to quantify Ap₃A and Ap₄A levels in cell lysates via high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) employing synthesized 13C-labeled compounds and 13C-10-Ap₃A and 13C-10-Ap₄A as internal standards (Fig. 2a). By this, we determined the level of Ap₃A to be 0.7 ± 0.1 pmol per 10⁶ cells and of Ap₄A level at 0.9 ± 0.4 pmol per 10⁶ cells under unstressed conditions, while their levels significantly increased upon treatment of cells with MMC or H₂O₂ (Fig. 2b). These results confirm that Ap₃A and Ap₄A are present in HEK293T cells and their concentration increases upon exposure to stressors.

Proteomic profiling of the Ap₃A and Ap₄A protein interactome. Next, we tested the synthesized probes for PAL of proteins with HEK293T cell lysates. To this end, the Ap₃A derivatives PALP-1 and PALP-2 were first incubated with HEK293T cell lysates. The samples were then irradiated with UV light (365 nm) to initiate the photo-crosslinking reaction and the labeled proteins were affinity-enriched via streptavidin-coated Sepharose™ beads. After washing the beads thoroughly, the bound proteins were eluted with biotin. The eluted proteins were resolved by SDS-PAGE and subsequently analyzed by western blotting using ExtrAvidin®-Peroxidase.

For LC-MS/MS analysis of bound proteins, triplicates of PAL experiments with PALP-1 and PALP-2 were performed using HEK293T cell lysates. We conducted several control experiments including (i) experiments without UV irradiation, (ii) PAL experiments using con-1 as control substance, and (iii) experiments without addition of any PALPs to the cell lysates. After data processing (see below), only proteins that were enriched compared to these negative controls were included in our lists (Supplementary Data 1). In order to further limit the number of false positive hits, we conducted competition experiments using PALP-1 and PALP-2 in the presence of the respective unlabeled Ap₃A analogs comp-1 and comp-2. For the final lists, only proteins that were enriched with PALP-1 and PALP-2 in the absence of comp-1 and comp-2, but not in their presence, were considered (Fig. 3c).

After affinity enrichment, proteins in the eluted fractions were resolved by SDS-PAGE and digested for LC-MS/MS analysis. Three replicates of this process were analyzed and measured as technical duplicates by LC-MS/MS to result in a total of six measurements per applied condition. PA-labeled proteins were identified by MaxQuant and quantified by LFQ. The data was further analyzed by Perseus resulting in 732 identified proteins for PALP-1 and 802 identified proteins for PALP-2. For these proteins, missing value imputation (downshift 1.8, width 0.3) in the total matrix mode and multiple-sample tests (FDR ≤ 0.01, S₀ = 0.2) were performed. Under these conditions, 56 proteins were identified as significant by ANOVA statistics for the PALP-1 experiment, 53 of which were significantly enriched after pairwise comparison to Tukey’s honest significant difference (THSD, FDR ≤ 0.05) and subsequent filtering against the three negative control experiments. These proteins were further filtered for significance against proteins enriched by the competitor control, resulting in 17 potential interaction partners for Ap₃A (Fig. 3c). Usage of the Ap₄A analog PALP-2 revealed 86 significantly enriched proteins by ANOVA of which 81 showed significant enrichment in PALP-2 towards the three negative control experiments by the post hoc THSD test (FDR ≤ 0.05). Of these proteins 26 showed significant enrichment against the competitor control (Fig. 3c).

Due to the low number of proteins identified with PALP-1, we decided to develop an additional PALP for Ap₄A. Since C2-modifications in adenosines have been shown to be accepted by several nucleotide processing enzymes, we synthesized (Supplementary Fig. 5) and applied PALP-3 (Fig. 3) as described above. With PALP-3, 538 proteins were identified, of which 341 were enriched against the negative controls, applying the same workflow as for PALP-1 and PALP-2. Comparison with the competitor experiments led to 49 significantly enriched proteins for PALP-3.

Proteins that were significantly enriched against all four controls after the ANOVA analysis (S₀ = 0.2, FDR ≤ 0.01) followed by a Tukey’s post hoc THSD test were then visualized in heatmaps (Fig. 3; for heatmaps including proteins enriched by the control experiments see Supplementary Figs. 6 and 7). The
The majority of the identified proteins appear to be involved in metabolic processes ranging from those of carboxylic acids to nucleotides and carbohydrates (Fig. 3e and Supplementary Data 2). Exemplarily, proteins contributing to the metabolism of carboxylic acids were enriched in all our samples (41% for PALP-1 and PALP-3 and 38% for PALP-2). Another process that seems to be connected to both Ap3A and Ap4A is RNA processing. Taken together, 11 proteins (14%) were identified with the three PALPs that are associated with RNA processing, of which 7 (9%) are involved in mRNA splicing. In addition, several molecular functions were found to be enriched in our data sets (Supplementary Data 2). Interestingly, out of the 78 overall identified proteins, only 36 (46%) are known nucleotide binders whereas 25 (32%) are ATP binders. This indicates that Ap3A and Ap4A are not just ATP surrogates but have roles in cellular pathways that are distinguishable from those of other nucleotides.

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thus, UbcH7–Ub thioester complex formation was not observed for ATP, while it was detected when Ap4A was employed.

**LDHA and PGK1 bind to a fluorescent labeled Ap4A analog.**

As mentioned before, the majority of the proteins enriched with the used PALPs are involved in metabolic processes. Among the most prominent hits were LDHA (found for all PALPs) and PGK1 (found with PALP-3), which showed strong enrichment in our PAL experiments compared to the controls (Fig. 6a). Additionally, recent reports document that besides their functions as (post-)glycolytic enzymes, both proteins are reported to have non-metabolic functions including involvement in tumorigenesis and stress response\(^{(38,39)}\). To investigate the interactions between these proteins and Ap\(_e\)As, we developed a method to measure
binding affinities via fluorescence polarization (FP). Based on the principle that the tumbling rate of a small fluorescently labeled molecule decreases upon binding to a larger interaction partner, which is mirrored in an increase in FP, FP assays present a well-established method to quantify such interactions.\textsuperscript{40,41} First, we synthesized a fluorescein-labeled Ap3A analog (F-Ap3A) that is similar to PALP-3, bears a 5-carboxyfluorescein dye linked to the C2 position of one adenine moiety (Fig. 6b).

F-Ap3A was then used to measure binding affinities with LDHA and PGK1. We incubated increasing concentrations of these proteins with F-Ap3A and plotted the obtained FP values against the protein concentration (Fig. 6c). This revealed that LDHA and PGK1 bind to F-Ap3A with $K_D$ values of $35.27 \pm 0.69 \mu M$ and $37.39 \pm 1.62 \mu M$, respectively (for further details, see Supplementary Table 1).

Proteomic profiling of the Ap3A and Ap4A protein interactome in \textit{E. coli}. Next, we investigated the Ap3A interactome of the \textit{E. coli} K12 strain by applying the PAL approach using PALP-2 and PALP-3 (Fig. 7a). The data obtained by LC-MS/MS was analyzed as described above resulting in the identification of a total of 46 proteins significantly enriched against the three negative control experiments, of which 20 were significantly enriched against the respective competitor controls. Significance was determined as described before by...
ANOVA statistics (FDR $\leq 0.01$, $S_0 = 0.2$) and subsequent analysis of the hits by THSD test (FDR $\leq 0.05$). The proteins identified and their Z-score normalized median LFQ values measured in the different conditions were displayed in a combined heatmap (Fig. 7b, Supplementary Data 3 and Supplementary Fig. 10). Interestingly, the distribution of the hits retrieved with the Ap3A-based and Ap4A-based probes with E. coli K12 lysates was similar to the one obtained with eukaryotic HEK293T cells. GO analysis showed significant enrichment for various metabolic pathways, including carboxylic acid metabolism (more than 50% in both cases), nucleotide metabolism (more than 40% in both cases), and carbohydrate catabolism (more than 20% in both cases) (Fig. 7d and Supplementary Data 4). Also, out of the 20 identified hits, 11 (55%) are known nucleotide binders and 8 (40%) are known ATP binders. Noteworthy, 40% of the hits are overlapping for PALP-2 and PALP-3 probes (Fig. 7c), which is significantly higher than for the proteins identified from HEK293T cell lysates, where only 12% were overlapping.

We finally investigated the human orthologues of the bacterial hits using the PANTHER (Protein ANalysis THrough Evolutionary Relationship) bioinformatics resource. Two of the most prominent hits, enriched in PALP experiments using HEK293T and E. coli lysates, were the phosphoglycerate kinase, encoded by the pgk gene.

Fig. 5 Investigation of the interaction of ApnAs with UBA1. a Extract of ANOVA-heatmap (Z-scores) for UBA1 under different PAL conditions. b ATP-dependent Ub activation and transthioesterification reactions catalyzed by UBA1. c Detection of Ub transfer from UBA1 to UbcH7 in the presence of Ap3A, Ap4A or ATP, analyzed by non-reducing SDS-PAGE (15%) followed by Coomassie blue staining. d The nucleotides were incubated with SAP before performing the transthioesterification reaction to remove possible contaminants of ATP in the Ap3A and Ap4A samples. The experiments were performed twice with matching results. Source data are provided as a Source Data file.

Fig. 6 Investigation of the binding affinity of LDHA and PGK1 to the fluorescent labeled Ap3A analog F-Ap3A via fluorescence polarization (FP). a Extract of ANOVA-heatmap (Z-scores) for LDHA and PGK1 under different PAL conditions. b Chemical structure of F-Ap3A. c FP-based quantification of binding between LDHA (orange) or PGK1 (green) to F-Ap3A. Increasing concentrations of protein were incubated with F-Ap3A (25 nM) for 15 min on ice and 30 min at 30 °C before measurement. FP values were determined and plotted against the protein concentrations. Data presented are mean ± SD, n = 3 technical replicates. Source data are provided as a Source Data file.
in bacteria and by PGK1 in humans, and the malate dehydrogenase, encoded by mdh in bacteria and MDH1 and MDH2 in humans. Interestingly, phosphoglycerate kinase and malate dehydrogenase are both involved in carbohydrate metabolism, as well as LDHA and GALK1 that we identified in HEK293T cell lysates, highlighting potential common roles of Ap nAs in bacteria and eukarya.

Furthermore, the bacterial orthologue of the aaRS QARS (glnS) was found, showing further similarities in our results.

Discussion
Even though Ap3A and Ap4A were discovered in the 1960s, their cellular roles and functions remain enigmatic. Here, we report proteome-wide interaction maps of Ap3A and Ap4A by employing probes for PAL in combination with MS-based proteomics. In comparison to previous affinity enrichment studies that relied on non-covalent interactions between Ap nAs and potential binders, our study is based on covalently connecting Ap3A-based and Ap4A-based probes to their target proteins allowing for both trapping transient interactions and thorough washing steps in order to minimize false positive hits. Given the relatively small size of the chemical modifications in the PALPs in comparison to the dimensions of an average protein also renders covalent "trapping" of indirect interaction partners of Ap nA unlikely, even though it cannot fully be excluded.

The earlier studies exclusively investigated Ap3A and mostly E. coli cells. Albeit less rigorous investigations and analysis were conducted (i.e., fewer repetitions and less rigorous statistical analysis), only 6–13 proteins were identified. Furthermore, there is little overlap in identified proteins between these studies and compared to our studies.

Overall, we identified 78 proteins from HEK293T cell lysates that potentially interact with Ap3A and Ap4A after rigorous Fig. 7 Proteomic analysis of PAL experiments using E. coli cell lysates. a Western blot analysis with ExtrAvidin®-Peroxidase showing enrichment of labeled proteins under optimized conditions using PALP-2 and PALP-3 as indicated after irradiation with UV light (365 nm) for 5 min. b Heatmap representation (Z-scores) of proteins that were quantified in the respective experiments in at least four out of six measurements and that passed a one-way ANOVA-based multiple-sample test for statistically significant enrichment, with a permutation-based false discovery rate (FDR) below or equal to 0.01 and SD value set to 0.2, followed up by two-sided post hoc Tukey's HSD test (FDR ≤ 0.05) to determine proteins enriched against all performed control experiments. c Venn diagram illustrating the overlap of identified proteins with PALP-3 (green) and PALP-2 (blue) and the global E. coli proteome (gray) according to GO analysis using a one-sided hypergeometric test performed with the Cytoscape BiNGO resource (Supplementary Data 4). Global frequency represents the number of genes annotated to a GO term in the entire E. coli proteome, while sample frequency represents the number of genes annotated to that GO term in the protein list obtained by PAL. Source data are provided as a Source Data file.
statistical analysis. Only about half of these are known nucleotide binding proteins, indicating a broader scope of the roles of ApnAs and ApnA in cellular processes beyond nucleotide-processing and speaking against the hypothesis that ApnAs merely act as ATP-surrogates. In fact, our chemical proteomics study uncovered interactions that are specific for either ApnA or ApnA and interactions that are shared by both ApnAs, again pointing towards broader and specific roles of the respective nucleotides.

The majority of the proteins found to interact with ApnA and ApnA probes appear to be involved in metabolic processes ranging from those of carboxylic acid to nucleotides and carbohydrates (Fig. 3e and Supplementary Data 2). Besides these common interactions, ApnA appears to play a role in cellular response to e.g., chemical stimuli and changing oxygen levels, which may correlate to the observed changes in ApnA levels in HEK293T cells lysates upon stress induced by mitomycin C and H2O2. For ApnA we identified a variety of proteins involved in gene expression, translation and protein targeting, as well as UBA1, an essential enzyme of the Ub system, whose interaction with ApnA could demonstrate in a case study (Fig. 5). Taken together, our results support the hypothesis that ApnA and ApnA are alaramones that signal various stress conditions.

Our PAL studies also discovered the involvement of ApnA and ApnA in RNA-associated processes. Proteins involved in tRNA aminoclylation and mRNA splicing were overrepresented among the proteins found with either probe. We also identified several proteins that are connected to regulation of translation. This is particularly interesting since recent studies report on the involvement of dinucleoside polyphosphates in RNA cap formation in bacteria. Luciano et al. showed that NpAs can be used as precursors for RNA cap structures that serve as primers for the E. coli RNA polymerase. Furthermore, Hudeček et al. reported that both ApnA and ApnA can be incorporated into bacterial RNA caps. Likewise, our PAL study identified the decapping scavenger enzyme DcpS as ApnA-binder. Using additional follow-up assays, we demonstrated that ApnA and ApnA can act as alternative substrates for the latter (Fig. 4), and thereby slow down the cleavage of cap analogs. All these results point at a potential role of these ApnAs in the regulation of RNA stability, metabolism, and/or transport.

Investigations of E. coli K12 cells revealed a total of 20 target proteins for ApnA and/or ApnA. Again, only about half of the identified proteins were known nucleotide binders, comparable to the results obtained for HEK293T cells. Moreover, similar to the results obtained with HEK293T cells, proteins involved in various metabolic pathways, including carboxylic acid metabolism, nucleotide metabolism, and carbohydrate catabolism were found to be enriched using E. coli extracts indicating potentially conserved functions of these ApnAs in bacterial and eukaryotic cells. Two metabolic enzymes identified from E. coli lysates were the phosphoglycerate kinase and the malate dehydrogenase, proteins whose human orthologues we also found in HEK293T. Interestingly, both enzymes are involved in carbohydrate metabolism, which is the case for several proteins we identified in E. coli and HEK293T, such as pgk, mdh, PGK1, MDH2, LDHA, and GALK1. Recent reports showed that various metabolic enzymes including PGK1, LDHA, and MDH2 have non-metabolic functions in processes including tumorigenesis and stress response, making them interesting candidates for follow-up experiments.

To further investigate the interaction between ApnAs and these proteins, we measured binding affinities between PGK1 and LDHA and a fluorescein-labeled ApnA probe and obtained Kd values in the 10−5 M range.

Overall, our approach based on PAL experiments with cell extracts combined with proteomics provides proteome-wide interaction maps for ApnA and ApnA in eukaryotic and bacterial cells. Still the proteins we identified as potential interaction partners of ApnA and ApnA likely represent only a subset of their full interaction networks. One piece of evidence supporting this assumption is that the use of the differently modified ApnA-based probes in PALP-1 and PALP-3 resulted in overlapping but also distinct hits, indicating a substrate bias that is inherent in the use of such chemical probes. This might also be the reason why several known interactors such as NUDT2 and FHIT were not identified. However, the applied control experiments and the rigorous statistics that we used for evaluation make it likely that significantly enriched proteins are also true positive interactors. Nonetheless, already at the present state, these maps represent a rich source that will hopefully spur and guide further investigations aimed at the elucidation of the hitherto enigmatic roles and functions of these nucleotides. Moreover, the ApnA-PAL approach presented here opens up an avenue to discover the function and roles of these ApnAs e.g., by applying these PALPs and eventually new PALP scaffolds to different cell types exposed to external cues.

**Methods**

**Chemical synthesis and characterization of compounds**

Chemical synthesis and characterization of compounds are described in the Supplementary Methods.

**Cell culture**

**Growth conditions for human cells.** HEK293T cells (ATCC) were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and 100 units/mL penicillin/streptomycin at 37 °C and 5% CO2. Cells were passaged every 2–3 days. For PAL experiments only passages up to 20 were used.

**Growth conditions for E. coli.** K12 BW25113. Bacterial cells were grown in LB medium at 37 °C and 200 rpm overnight. For the main culture, LB medium was inoculated to a calculated OD600 of 0.01 with the overnight culture and grown for 5 h at 37 °C to reach the exponential growth phase. Cells were harvested by centrifugation (3220 x g) for 5 min.

**Quantification of intracellular ApnA and ApnA in HEK293T cells under stress conditions**

**No external stress.** At 90% confluence, the cell dish was directly put on ice. The medium was removed and the cells were washed twice with 2 mL of ice-cold isotonic NaCl solution. One milliliter of −80 °C lysis solution (90/9/1 H2O/MeOH/DCM, (v/v/v)), as well as the internal standard solution (20 μL of 5 μM 13C6-ApnA and 13C6-ApnA in water) was added to the cells and distributed evenly. After 15 min on ice, the lysed cells were scraped down and collected. The plate and the scraper were washed with 1 mL of lysis solution and the suspensions were combined. After centrifugation (21,882 x g, 4 °C, 30 min) the supernatant was stored on ice and the pellet was ultrasonicated in 500 μL lysis solution (5 min) and centrifuged again (21,882 x g, 4 °C, 30 min). The combined supernatants were evaporated in a SpeedVac (Thermo Fisher Scientific) and the residue was stored at −20 °C until further purification.

**DNA damage via mitomycin C (MMC).** At 90% confluence of the cells, MMC in 1 x PBS was added to the dish to result in a final concentration of 100 nM MMC in 10 mL medium. The cells were incubated for 18 h at 37 °C. After the treatment, the cells were treated in the same way as the unstressed samples.

**Oxidative damage via hydrogen peroxide.** At 90% confluence, the medium was removed, the cells were washed twice with 1 x PBS and 5 mL of a 500 μM H2O2 solution in 1 x PBS was added. The cells were incubated for 20 min at 37 °C. The resulting cell suspension was transferred to a 15 mL falcon, the plate was washed with 4 mL 1 x PBS and the suspensions were combined and centrifuged (500 x g, 4 °C, 3 min). The cell pellet was resuspended in 1 mL of −80 °C lysis solution and after 20 min of incubation on ice, the suspension was centrifuged (3220 x g, 4 °C, 5 min). The supernatant was transferred in an Eppendorf tube and the pellet was resuspended and ultrasonicated in 1 mL lysis solution. After centrifugation (3220 x g, 4 °C, 5 min) the supernatants were combined and evaporated in a SpeedVac (Thermo Fisher Scientific) and the residue was stored at −20 °C until further purification.

**Purification via tC18 columns.** The SepPak® tC18 column (Waters) was washed with 1 mL MeCN and equilibrated with 2 mL 5% MeCN in water. The cell extract was dissolved in 500 μL 5% MeCN in water and applied to the column. The eluted solution was collected, the column was washed with 1 mL 5% MeCN in water and the...
the combined extracts were freeze-dried. The resulting residue was dissolved in 50 μL water and directly measured via LC-HR-ESI-MS.

Quantification of intracellular Ap3A and Ap4A. The purified cell extract was analyzed and quantified via LC-HR-ESI-MS (Bruker MicroTOFII). A Hyperscar Porous Graphitic Carbon LC column (5 μm, 100 × 2.1 mm) was used in combination with a Hypercarb drop-in guard pre-column (5 μm, 10 × 2.1 mm). As mobile phase, 10 mM NH4HCO3 + 0.1% diethylamine (pH 10) and MeCN (B) was used (gradient shown in Supplementary Table 2). For the calibration curve, different volumes of known concentrations of ApA and ApA were injected and linearly fitted. In every measured calibration sample, the same amount of internal standard (13Cl10-Ap3A and 13Cl10-Ap4A) was present. The calibration curve was renewed for every experiment. To measure technical duplicates, every sample was injected twice (20 μL each). For the calculation of the concentrations, the EICs of ApA and ApA (m/z = 755.0747 ± 0.02 and 835.040 ± 0.02) were integrated and quantified. For the internal standard, the EICs of 13Cl10-Ap3A and 13Cl10-Ap4A (m/z = 765.1082 ± 0.02 and 847.0400 ± 0.02) were integrated and quantified. The resulting calculated concentrations of the technical duplicates were averaged. Exclusively measurements with a S/N bigger than S/N = 7 were considered for the calculation of concentrations. In case of insufficient spectrum quality or S/N ratio of one of the duplicates only the duplicate of sufficient quality was considered for the calculation. The quantification of the samples and the standardization of the concentrations with the aid of the internal standards was done via Bruker Compass DataAnalysis (version 4.1) and Bruker Compass QuantAnalysis (version 2.1). Statistical tests were done using a one-way ANOVA in combination with the Dunnett test in GraphPad Prism (version 6.01).

Cell lysate stability assay. The stability of ApA and ApA in HEK293T cell lysate was measured by RP-HPLC. To this end, 200 μM nucleotides were incubated with or without 2 mg/mL HEK293T cell lysates in 100 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM MgCl2, and 1 mM DTT at 37 °C for 1 h in a volume of 45 μL. 15 μL aliquots were taken at 0 and 60 min followed by the removal of proteins and salts by U-C4 ZipTips (Merck Millipore). The resulting mixture was diluted with 100 μL water. 110 μL sample was injected and analyzed by analytical RP-HPLC with a 2500/4 mm NUCLEOSIL C18 HTC column. The run was performed with 50 mM aqueous triethylammonium acetate buffer (TEA, pH 7.0) and MeCN as eluents (gradient shown in Supplementary Table 3).

PAL experiments
Preparation of HEK293T cell lysate. Cells were grown to 90% confluence. Before lysis, cells were centrifuged at 500×g at 4 °C for 10 min. Cell pellets were washed with cold 1× PBS (3 x 2 mL), resuspended in cold lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1% NP40, 0.5% glycerol, 0.1 M Pefabloc, 1 μg/mL aprotinin/leupeptin) and sonicated on ice at an amplitude of 10% for 10 s, three times. Lysates were centrifuged at 4 °C and 19,064×g for 30 min. The protein content of the supernatant was determined by BCA assay (Kit, Thermo Fisher Scientific). The supernatant was stored on ice until further use.

Preparation of E. coli K12 cell lysate. Pellets were stored on ice and washed with cold 1× PBS (10 mL). After repeating steps of centrifugation and washing in total of three times, the pellet was resuspended in cold lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1% NP40, 0.5% glycerol, 0.1 M Pefabloc, 1 µg/mL aprotinin/leupeptin). The cell mixture was sonicated on ice at an amplitude of 20% for 30 s, five times. The mixture was centrifuged at 4 °C and 19,064×g for 30 min. The protein concentration in the supernatant was determined by BCA assay (Kit, Thermo Fisher Scientific). The supernatant was stored on ice until further use.

Photoaffinity labeling. In a total volume of 100 μL the photoaffinity-labeling probe (PALP-1, PALP-2, or PALP-3, 20 μM) was incubated with lysate (3.0 mg/mL) in the presence of 13Cl10-Ap3A and 13Cl10-Ap4A (500 nM). The mixture was centrifuged at 4 °C and 19,064×g for 30 min. The protein concentration in the supernatant was determined by BCA assay (Kit, Thermo Fisher Scientific). The supernatant was stored on ice until further use.

Control experiments. The control samples were treated as described above. In case of the UV control, the sample was handled in a black Eppendorf tube to exclude UV irradiation. In the beads assay, water was added as a negative control to exclude cross-reactivity to PALPs. The control substance (con-1) was applied in the same concentration as the PALPs (20 μM). For the competition experiment a 100-fold excess of the competitor (comp-1 or comp-2, respectively, 2 mM) derivative was incubated with cell lysate for 1 h on ice before adding the corresponding PALPs.

In-gel digestion. For in-gel digestion each gel lane was cut into small pieces and destained repetitively in acetonitrile/water (3:2, v/v, 50 μL) for 30 min and NH4HCO3 (25 mM, 50 μL) for 15 min. Then, the gel pieces were incubated with NH4HCO3 (20 mM, 50 μL) for 15 min. After washing, proteins were reduced with 50 mM NH4HCO3, 20 mM DTT for 60 min at 56 °C, followed by alkylation with iodoacetamide (50 mM in NH4HCO3, 20 mM, 100 μL) at room temperature for 60 min under protection from UV light. After washing in NH4HCO3 (20 mM, 100 μL) for 15 min and in NH4HCO3/acetonitrile (20 mM, 1/1, v/v, 100 μL) for 10 min and dehydration in pure acetonitrile (100 μL) for 10 min, proteins were digested overnight at 37 °C with trypsin (1/50, w/w) (Promega V5111). Peptides were extracted twice from the gel with acetonitrile/TFA in water (0.1%, 1/1) for 60 min. After desalting using ZipTips (Merck Millipore) the peptides were subjected to mass spectrometric analysis with nano-LC-MS/MS (Proteomics facility, University of Konstanz).

Mass spectrometry. The digests were analysed on a QXactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) operated with Tune (version 2.0.9) and interfaced with an Easy-nLC 1200 nanoflow liquid chromatography system (Thermo Fisher Scientific, Bremen, Germany). The peptide digests were reconstituted in 0.1% formic acid and loaded onto the analytical column (50 μm × 15 cm). Peptides were resolved at a flow rate of 300 nL/min using a linear gradient of 6–40% solvent B (0.1% formic acid in 80% acetonitrile) over 45 or 60 min. Data-dependent acquisition with full scans in a 350–1200 m/z range was carried out at a mass resolution of 120,000. The 20 most intense precursor ions were selected for fragmentation. Peptides with charge states 2–7 were selected and dynamic exclusion was set for 30 s. MS2 scans were triggered at a mass resolution of 1500. Precursor ions were fragmented using higher-energy collision dissociation (HCD) set to 28%. Each of the three independent biological replicates was measured as technical duplicates.

Data analysis and quantification. Raw files from LC-MS/MS measurements were analyzed using MaxQuant (version 1.6.2.6) with the andromeda search engine with default settings and match between runs and label-free quantification (LFQ) (minimum ratio count 2) enabled.26-27. For protein identification, the human and the E. coli reference proteome downloaded from the UniProt database (https://www.uniprot.org/uniprot#) (download date: 02.04.2019 and 01.10.2019) and an integrated database of common contaminants were used. Oxidation, N-acetylation, and carboxamidomethylation were selected as modifications. Identified proteins were filtered for reverse hits, common contaminants and hits only identified by site at a false discovery rate (FDR) of equal or below 0.01. Further data processing was performed using Perseus software (version 1.6.14.0).29 LFQ intensities were log2 transformed. The proteins were filtered to be detected in at least 60% of the replicate experiments (n = 6) and missing values were imputed from a normal distribution (width = 0.3 and shift = 1.8) in the total matrix mode, based on the assumption that these proteins were below the detection limit. Enriched proteins were identified by a one-way ANOVA-based multiple sample test with S0 adjusted to 0.2, the number of randomizations set to 250 and the permutation-based FDR accepted equal or below 0.01 with technical replicates grouped for randomization. A two-sided Tukey’s post hoc test with an accepted FDR value equal or below 0.05 was performed on all ANOVA significant hits. The Tukey’s Honest Significant Difference (THSD) performs a pairwise comparison of all conditions and calculates the mean difference between each condition pair. If the difference is greater than or equal to the corresponding THSD, which depends on the number of treatments, the degrees of freedom, the mean squared error and the number of data points in each group, the difference is considered significant. For further analysis only proteins enriched in a PAL experiment against all four corresponding controls were considered. Z-scoring of the median LFQ intensities was performed without logarithmic transformation and used for unsupervised clustering analysis. In significant hits, the distance was set to Euclidian, the linkage to average, and the maximal numbers of clusters to 300.

GO term and abundance analysis. The obtained protein hits were analyzed using ClueGO (version 3.8.2) with the BINGO api (version 3.0.3) to compare the abundance of GO terms in the identified proteins to their frequency in the whole proteome. A one-sided hypergeometric test was performed with a significance level of 0.05 and Benjamini Hochberg False Discovery Rate (FDR) (looks at the frequency with which that GO term appears, the p-value consequently falls as the frequency increases)29. Global frequency is the number of genes annotated to a GO term in the entire background set, while sample frequency is the number of genes annotated to that GO term in the input list. (cf. http://geneontology.org/docs/go-enrichment-analysis/). Human and E. coli gene association (gaf) and ontology files (obo) were downloaded from geneontology.org (http://geneontology.org)
Expression and purification of human DcpS. The protein expression plasmid pET28b containing the sequence for His-tagged DcpS was kindly provided by A. Rentmeister (Institute of Biochemistry, WWU Münster). After transformation into BL21 (DE3) E. coli cells, the cells were grown in LB medium containing 100 mg/mL kanamycin at 37 °C overnight. The pre-culture was diluted with medium containing 100 mg/mL kanamycin to an OD600 value of 0.1. Cells were grown at 37 °C until they reached an OD600 of approximately 1.1. Protein expression was induced by addition of 1 mM IPTG at 25 °C for 4 h. Then, cells were pelleted, resuspended in lysis buffer (50 mM Tris·HCl (pH 8.0), 350 mM NaCl, 20% sucrose, 20 mM imidazole, 1 mM β-mercaptoethanol) and lysed by sonication at an amplitude of 20% for 5 s, five times. The lysate was clarified by high speed centrifugation and purified by immobilized metal affinity chromatography (IMAC) using a HisTrap™-FM column (1 mL, GE Healthcare) on an AKTA system. The protein was eluted in 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl, with linear increasing concentrations of imidazole (30–500 mM). Fractions containing pure PGK1 protein were identified by 12.5% SDS-PAGE followed by Coomassie blue staining and pooled. The elution buffer was replaced by PGK1 storage buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM DTT, 10% glycerol) by dialysis overnight. The protein concentration was determined by BCA assay (Kit, Thermo Fisher Scientific). The protein was stored at -80 °C until use.

Determination of binding affinities by fluorescence polarization. Prior to the experiments the protein storage buffers were replaced by the respective fluorescence polarization assay buffers for LDHA (20 mM HEPES (pH 7.5), 200 mM NaCl, 0.01% Triton X-100) and PGK1 (20 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM MgCl2, 0.01% Triton X-100). The protein concentrations were determined by BCA assay (Kit, Thermo Fisher Scientific). The protein expression series using the same buffers were prepared. C2 (5-S-FAM)-AP-A (F-AP-A) was added to the protein samples, yielding reaction mixtures containing 25 nM F-AP-A and varying protein concentrations (as noted) in an overall volume of 110 µL. For each reaction mixture 30 µL were transferred to black 384-well non-binding microplates no. 781900 (Greiner Bio-One) in three separate wells. Samples were centrifuged (200g, 2 min, 4 °C) and incubated on ice for 15 min followed by 30 min incubation at 30 °C. FP measurements were subsequently recorded on a Tecan infinite 500 plate reader. Emission was measured at 535 nm with two filters transmissive for polarized light, either parallel or perpendicularly to the plane of excitation. The FP values were directly provided as milli- polarization (mP). To correct for instrument intrinsic differences of the sensitivity towards detecting parallelly and perpendicularly emitted polarized light, the G-factor was determined and used for subsequent measurements. For this calibration, the FP value of a 1 nM 5-FAM solution in 0.01 M NaOH was measured and set to 20 mP. The mean and the standard deviation were calculated for each sample. The binding curves were corrected by subtracting the background FP value. Data was analyzed with GraphPad Prism (version 6.01) and curve fittings were calculated by using the equation for one site specific binding (Kd) with the maximum specific binding (Bmax) and the equilibrium constant (Kp). The obtained binding parameters are shown in Supplementary Table 1.

Data availability. The data that support this study are available from the corresponding authors upon reasonable request. The mass spectrometry proteomics data generated in this study have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository under accession code PXD020740. Figures with associated raw data: Figs. 2 A–D and Supplementary Figs. 1, 2-7 and 10. Source data are provided with this paper. For protein identification, the human and the E. coli reference proteome downloaded from the Uniprot database [https://www.uniprot.org/protbasea/ (download date: 02.04.2019 and 01.10.2019)] and an integrated database of common contaminants were used. For GO term analysis Human and E. coli gene association (gaf) and ontology files (obo) were downloaded from geneontology.org (http://geneontology.org) (27.01.2020). Source data are provided with this paper.

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Author contributions
A.M., L.K., C.J.A. designed and L.K., C.J.A., H.K.S., F.M.S., Y.Y., and J.W. performed the experiments and L.K., C.J.A., M.L.N., and F.S. analyzed the data. A.M., L.K., and C.J.A. wrote the manuscript. K.S. and M.S. provided UBA1 and UbcH7 and assisted with these experiments and edited the manuscript.

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