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K-BILDS: A kinase substrate discovery tool

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Abstract

Kinases catalyze protein phosphorylation to regulate cell signaling events. However, identifying kinase substrates is challenging due to the often low abundance and dynamic nature of protein phosphorylation. Development of novel techniques to identify kinase substrates is necessary. Here, we report K-BILDS (Kinase-catalyzed Biotinylation with Inactivated Lysates for Discovery of Substrates) as a tool to identify direct substrates of a kinase. As a proof of concept, K-BILDS was applied to PKA (cAMP-dependent protein kinase A) using HeLa cell lysates. Subsequent enrichment and MS/MS analysis identified 279 candidate PKA substrates, including 56 previously known PKA substrates. Out of the candidate substrates, NASP (Nuclear Autoantigenic Sperm Protein), BAG3 (BCL2 Associated Athanogene 3), and YWHAQ (14-3-3 Protein Tau) and were validated as novel PKA substrates. K-BILDS provides a valuable tool to identify direct substrates of any protein kinase.

Graphical Abstract

Kinase substrate identification represents a significant challenge in cell signaling research. Kinase-catalyzed biotinylation with inactivated lysates for discovery of substrates (K-BILDS) offers a simple, yet powerful, method for detecting kinase substrate pairs.

Keywords

Kinase; Substrate discovery; ATP-biotin; FSBA; Mass Spectrometry

Introduction

Phosphorylation catalyzed by kinases can change the activity and localization of protein substrates to regulate cellular functions, including signaling and metabolism.^[1–2] Deregulation of kinase activity can result in abnormal protein phosphorylation events, which are linked to various disease states, such as cancer and immunosuppression. To understand the underlying mechanism behind phosphorylation-related biological events and diseases,

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identifying the protein substrate of kinases is necessary. With more than 500 protein kinases known,^[3] decoding kinase-substrate pairs is challenging, especially due to the low abundance, low stoichiometry, and transient nature of protein phosphorylation.^[4] Therefore, it is possible that the complete list of kinase substrates is yet to be defined.

Several techniques are available to identify kinase substrates from cellular lysates.^[5] Analogue-sensitive kinase alleles (ASKAs) developed by Shokat and coworkers utilize engineered kinases that accept a bulky ATP (5'-adenosine triphosphate) analog to label and identify substrates in cell lysates.^[6] ASKAs are available for roughly 30 kinases. For kinases where ASKAs are not available, phosphoprotein detection after immunoprecipitation using phospho-specific antibodies is another method used to identify kinase substrates.^[7] While phospho-specific antibodies recognizing phosphotyrosine have been used widely for substrate detection, phosphoserine and phosphothreonine antibodies have had limited success due to their poor binding efficiency. A new method that is broadly applicable to all kinases and substrates would provide an appealing alternative to characterization of kinase-substrate relationships.

In previous work, our lab and others discovered that a variety of γ -phosphate modified ATP analogs act as kinase cosubstrates.^[8–13] Specifically, ATP modified with a biotin tag at γ -phosphate acts as a kinase cosubstrate to transfer a phosphoramidyl biotin group onto protein substrates (Figure 1a).^{[14],[15]} Kinase-catalyzed biotinylation has been used to visualize proteins,^[16] but has not yet been exploited in kinase-substrate identification. Here we report a new method for kinase substrate identification entitled, “Kinase-catalyzed Biotinylation with Inactivated Lysates for Discovery of Substrates” or K-BILDS.

In the K-BILDS method, endogenous cellular kinases in lysates are first inactivated by incubating with the covalent inhibitor, FSBA (5'-(4-fluorosulfonylbenzoyl) adenosine hydrochloride).^[17–19] FSBA reacts with a catalytic lysine in the kinase active site to irreversibly inhibit activity (Figure 1b).^[18] After removal of excess FSBA, exogenous active kinase and ATP-biotin are added to biotinylate kinase substrates in the lysates (Figure 1c). Biotinylated proteins are then enriched using streptavidin resin, separated by SDS-PAGE (sodium dodecyl sulfate- polyacrylamide gel electrophoresis), and analyzed by LC-MS/MS (liquid chromatography-tandem mass spectrometry, Figure 1d). The biotinylated proteins identified are candidate kinase substrates, which must be validated in secondary studies.

The advantages of K-BILDS include the generality to all kinases and substrates,^[15] the strong biotin-streptavidin interaction for purification, and the use of highly sensitive LC-MS/MS for substrate detection.

To establish K-BILDS as a substrate identification tool, we focused on a well-studied kinase, PKA (cAMP dependent protein kinase A/PRKACA). PKA is a serine/threonine kinase that regulates multiple cellular events, such as signaling, transcription, metabolism, transport and homeostasis.^[20] Importantly, PKA has many known kinase substrates, which will test the utility of K-BILDS. Application of K-BILDS to PKA resulted in identification of 56 known and more than 200 candidate substrates. *In vitro* validation studies confirmed that the K-

BILDS hits, BAG3, NASP and YWHAQ, are PKA substrates. These results document the diversity of PKA-mediated signaling and the sensitivity of the K-BILDS strategy.

Results and Discussion

Kinase-catalyzed biotinylation with FSBA-treated lysates

To initially develop K-BILDS, we confirmed that endogenous cellular kinase activity was inhibited by FSBA treatment. Lysates were treated with FSBA and then incubated with ATP-biotin to observe possible residual endogenous kinase activity. As a positive control, untreated (DMSO) HeLa lysates were also incubated with ATP-biotin. As expected, robust kinase-catalyzed biotinylation was observed with untreated HeLa lysates (Figure 2, lane 2, SA-Cy5). In contrast, only a low level of biotinylation was observed with FSBA-treated lysates (Figure 2, lane 3, SA-Cy5), consistent with kinase inactivation by FSBA. After FSBA inactivation, lysates were subjected to kinase-catalyzed biotinylation using ATP-biotin and exogenous active PKA. A small increase in biotin signal was observed in the presence of PKA (Figure 2, lane 4, SA-Cy5, see brackets), compared to inactivated lysates incubated with ATP-biotin alone (Figure 2, lane 3, SA-Cy5). When comparing biotinylation with PKA versus all cellular kinases (Figure 2, lane 4 vs. 2, SA-Cy5), the reduced signal observed with PKA suggested selective biotinylation of only PKA substrates, and not global labeling of high abundance proteins. These results with inactivated lysates and PKA motivated us to apply K-BILDS on a larger scale for substrate identification using LC-MS/MS analysis.

K-BILDS with PKA

To identify PKA substrates in a full K-BILDS experiment, FSBA-treated cell lysates were incubated with ATP-biotin in the presence or absence of PKA. Biotinylated proteins were then enriched using avidin resin, separated by SDS-PAGE (Figure S2), in-gel digested with trypsin, and analyzed by LC-MS/MS. Proteins enriched in the PKA reaction were compared to the control reaction to generate a list of candidate substrate hits. In two independent trials, 279 proteins were reproducibly enriched in the PKA reactions (Table S1, Figures S3–S6), making them candidate PKA substrates.

To compare previously known substrates of PKA to K-BILDS candidate hits, the phosphosite-plus database and published journals were searched. Among the K-BILDS hits, 56 proteins are previously documented PKA substrates, including twenty-five from Phosphosite Plus that have been validated both *in vitro* and *in vivo* (Table S2). The isolation of known PKA substrates validated K-BILDS for kinase substrates identification.^[21–27] In addition, K-BILDS hits were further analyzed by Scansite to detect PKA consensus motifs (R/K,R/K,X,S/T).^[28] Remarkably, 91% of K-BILDS hits contained possible PKA consensus sites. The presence of PKA consensus sites among the K-BILDS hits strongly implicates the candidate proteins as PKA substrates.

According to statistics on kinase complexes, roughly 95% of known PKA substrates either directly associate with PKA or indirectly interact with PKA through three or fewer other intermediate proteins (three degrees of separation).^[29–30] Thus, to further analyze the 279

K-BILDS hits, a PKA interactome analysis was conducted. The 279 K-BILDS hits were compared to protein-protein interaction data in the GeneMANIA database to identify direct binding partners, as well as proteins that indirectly interact with PKA through direct interactors with only three degrees of separation.^[31] Remarkably, 78% of K-BILDS hits interact with PKA (PRKACA) directly or within three degrees of separation (Figure 3a). Furthermore, 14 direct interacting hits (Figure 3a, inner circle, blue) and 37 indirect interacting hits (Figure 3a, outer circle, blue) are previously documented PKA substrates. Consequently, 51 out of 56 known PKA substrates among the K-BILDS hits are present in the PKA interactome (91%), as predicted.^[29–30] Therefore, the PKA-centered interactome established that the majority of K-BILDS hits are direct or indirect interacting proteins and, as a result, likely PKA substrates.

To gain functional insight into K-BILDS candidate PKA substrates, the 279 hit proteins were analyzed by gene ontology (GO). GO analysis categorized K-BILDS hits into twelve biological processes, including metabolism, cellular enzymatic functions, and localization (Figure S8). The fact that PKA controls diverse cellular events, including all twelve identified biological processes, provided further evidence that candidate K-BILDS hits contain potential PKA substrates.

Next, an abundance analysis of K-BILDS hits was performed to assess how protein quantities in a cell can affect the ability of K-BILDS to identify substrates. We hypothesized that the phosphatase-insensitive biotin tag will allow K-BILDS to enrich substrates irrespective of cellular abundance.^[32] The known abundances of K-BILDS hits in HeLa cells were obtained from Pax-db database (pax-db.org).^[33] The observed abundance range for K-BILDS hits was 0.7 to 9,000 ppm (Figure S9), which is comparable to the distribution of all cellular proteins in HeLa cells (0.01 to 10,000 ppm), confirming that K-BILDS identified proteins irrespective of abundance. Interestingly, some of the K-BILDS hits were low abundance proteins, such as transcription factors and signaling molecules (Figure S8).

Validation of new PKA substrates

Based on the global physical interactome (Figures 3a and S7) and biological functions of proteins (Figure S8), three candidate hits were selected for biochemical validation. First, we selected NASP (Nuclear Autoantigenic Sperm Protein) for validation because it is a direct interacting protein of PKA (Figure 3a and S4),^{[34],[35]} and is a possible PKA substrate based on curated MS/MS data in phosphosite plus and phosphonetworks.^[34] However, NASP has not been validated as a direct PKA substrate. For the validation study, an *in vitro* kinase assay was performed by incubating NASP with ATP and PKA. Phosphorylated protein products were analyzed by SDS-PAGE and Pro-Q phosphoprotein stain. NASP was phosphorylated by PKA (Figure 4a, lane 2, Pro-Q) compared to the negative control without PKA (Figure 4a, lane 1, Pro-Q). The fact that NASP physically interacts and coexpresses with PKA in HeLa cells is consistent with NASP being a substrate of PKA.^{[36],[37]}

As a second K-BILDS hit for validation, we studied BAG3, which is an indirect interactor of PKA (Figure 3a and S5). BAG3 is a Bcl2 (B-cell lymphoma 2) family protein that regulates several biological processes, including apoptosis.^[38] BAG3 functions as an anti-apoptotic protein through other Bcl2 family proteins, such as Bcl2. Phosphorylation of Bcl2 by PKA

causes Bcl2 inactivation and induction of apoptosis.^[39] However, no phosphorylation events have directly linked BAG3 to its anti-apoptotic function. Therefore, validating BAG3 as a substrate of PKA would provide the first evidence of phosphorylation-mediated regulation of BAG3 and an expanded role of PKA in Bcl2-mediated apoptosis. *In vitro* kinase assays were performed by incubating BAG3 with ATP and PKA. Pro-Q analysis of the phosphorylated products confirmed that BAG3 is phosphorylated by PKA (Figure 4b, lane 4, Pro-Q). Given the precedence of PKA in regulating apoptosis through Bcl2, we hypothesize that phosphorylation of BAG3 by PKA plays a role in regulating its anti-apoptotic function.^[40]

YWHAQ is another indirect interacting protein of PKA (Figure 3a and S6) that was selected for validation. YWHAQ is a member of the 14-3-3 family, which are adaptor proteins involved in cell signaling. Interestingly, all seven 14-3-3 isoforms (YWHA proteins) were identified by K-BILDS as potential PKA substrates (Figure 3b). Three isoforms (YWHAQ, YWHAH, and YWHAZ) are previously documented PKA substrates,^[21–22] which suggests that YWHAQ is also a substrate. Importantly, previous reports claimed that all 14-3-3 (YWHA) proteins act as adaptors and regulate the function of PKA.^[41] *In vitro* kinase assays confirmed that YWHAQ is phosphorylated after incubation with PKA (Figure 4c, lane 6, Pro-Q), in contrast to a reaction without PKA (Figure 4c, lane 5, Pro-Q), confirming that YWHAQ is a substrate of PKA. Based on the K-BILDS results, we hypothesize that phosphorylation of all seven YWHA (14-3-3) isoforms may be involved in signaling mediated by PKA.

In addition to identifying novel substrates, we discovered several complexes in HeLa cells using K-BILDS. Substrate-containing complexes were discovered by analyzing the PKA K-BILDS interactome using MCODE in the Cytoscape program. In addition to the 14-3-3 family members (Figure 3b), proteins belonging to the proteasome core (Figure 3c) and ribosome (Figure 3d) were identified by K-BILDS as candidate PKA substrates. Previous literature reported that PKA-dependent phosphorylation regulates proteasome and ribosome function,^[42–43] consistent with these proteasomal and ribosomal proteins being PKA substrates. The interactome analysis showed that K-BILDS not only identified kinase substrates, but also uncovered complexes containing substrates, which will assist in defining the full activity of kinases in a cell.

Conclusions

Here we established K-BILDS for kinase substrate identification using PKA as a well-studied model system. Fifty-six previously known PKA substrates and more than 200 candidate substrates were identified, demonstrating the success of the method. *In vitro* validation confirmed that BAG3, NASP and YWHAQ are PKA substrates, suggesting that K-BILDS efficiently identified new PKA substrates. While K-BILDS represents a useful kinase substrate discovery tool, we must emphasize that validation studies are critical to confirm that putative substrates are modified by PKA. Moreover, thorough *in cellulo* or *in vivo* studies are needed to explore the physiological relevance of PKA phosphorylation on the function of identified substrates. Given the general ability for kinases to accept ATP-

biotin as a cosubstrate,^[15] K-BILDS represents an enabling tool to identify substrates of any protein kinase.

Experimental Section

Synthesis of ATP-biotin

The synthesis of ATP-biotin has been published elsewhere.^[44]

Kinase-inactivation of HeLa lysates using FSBA

HeLa cells (20×10^6) obtained from National Cell Culture Center (Biovest) were lysed in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, and 1X protease inhibitor cocktail (GenDepot); 1 mL) at 4°C for 15 minutes. Cell debris was removed by centrifugation at 4°C for 20 minutes. The protein concentration of the lysates was measured by Bradford assay (Biorad). HeLa cell lysates were aliquoted and stored at -80 °C until use. For kinase inactivation, HeLa lysates (200 µg or 1 mg) were diluted to 2 mg/mL with lysis buffer and then treated with FSBA (10 mM in a final concentration of 20% DMSO) at 31°C for 2 hours with 800 rpm shaking. As a control, untreated lysates were prepared by adding DMSO to 20% concentration. The total volumes were 100 µL for a 200 µg scale reaction or 500 µL for a 1 mg scale reaction. After kinase inactivation, samples were centrifuged for 2 min at 1 rcf to remove any precipitate. Excess FSBA was further removed by diluting the resulting supernatant in PKA/DTT buffer (500 µL for a 200 µg scale reaction or 2 mL for a 1 mg scale reaction; 50 mM Tris-HCl, 10 mM MgCl₂, 0.1 mM EDTA, and 2 mM DTT) and filtering through a 3 KDa centriprep spin column (Milipore) twice at 14,200 rcf for 45 min. The resulting FSBA-treated HeLa lysates were used immediately or stored at -80°C until further use.

Kinase-catalyzed biotinylation with FSBA-treated lysates with PKA

FSBA-treated HeLa lysates (~200 µg) were incubated with ATP-biotin (2 mM) and PKA (1250 U, catalytic subunit of cAMP-dependent Protein Kinase, NEB) at 31°C for 2 hours. Control reactions were performed by incubating FSBA-treated or untreated lysates with ATP-biotin (2 mM) only. The total volume of each reaction was 30 µL. After kinase reaction, samples were boiled at 95°C for 1 min in Laemmli sample buffer (10 µL; 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 2.5 % beta-mercaptoethanol, and 0.01% bromophenol blue) and separated by 12% SDS-PAGE. Total proteins were assessed by Sypro Ruby staining (Invitrogen), whereas biotinylation was assessed using SA-Cy5 staining (Invitrogen), which were performed according to the manufacturer's instructions. Data are shown in Figures 2 and S1.

K-BILDS with PKA

FSBA-treated lysates (~1 mg) were incubated with ATP-biotin (2 mM) and PKA (2500 U) at 31°C for 2 hours. A negative control was performed by incubating FSBA-treated lysates (~1 mg) with ATP-biotin (2 mM) only. The total volume of each reaction was 60 µL. After kinase reaction, samples were diluted with phosphate binding buffer (300 µL; 0.1 M phosphate pH 7.2, 0.15 M NaCl) and excess ATP-biotin was removed by filtering through 3 KDa centriprep spin columns (Milipore) at 14,200 rcf for 45 min twice. After filtration,

samples were diluted with phosphate binding buffer to a total volume of 300 μ L and then incubated with streptavidin resin (200 μ L of packed resin, Genscript) for 1 hour at room temperature with rotation. The streptavidin resin was washed twice with phosphate binding buffer (400 μ L) before use. After incubation, the resin was washed ten times with phosphate binding buffer (400 μ L) and then five times with water (400 μ L). The bound proteins were eluted by boiling the resin in 2% SDS in water (200 μ L) for 7 minutes. The eluate was concentrated using a speedvac (Thermo scientific). The concentrated eluate was boiled in Laemmli sample buffer (final volume 40 μ L) at 95°C for 1 min and separated by 12% SDS-PAGE. Total proteins were assessed by Sypro Ruby, whereas biotinylation was assessed using SA-Cy5 staining, according to the manufacturers protocols. A representative gel image is shown in Figure S2.

In-gel trypsin digestion and LC-MS/MS analysis

In-gel trypsin digestion was performed on the Sypro Ruby stained gel (Figure S2) by excising both PKA reaction (Figure S2, lane 4) and negative control (Figure S2, lane 3) lanes from two independent trials, following the previously published protocol.^[45] Digested peptides were initially separated using EASY Nlc-1000 UHPLC system (Thermo) under acidic conditions (0.1% formic acid). Peptides were then analyzed on Q-Exactive mass spectrometer (Thermo). MS1 profiling was performed in a 375–1600 m/z range at a resolution of 70,000. MS2 fragmentation was carried out on the top fifteen ions using a 1.6 m/z window and normalized collision energy of 29 using higher energy collision induced dissociation (HCD) with a dynamic exclusion of 15s. Peak lists were generated using Proteome Discoverer (Thermo, version 1.4). MS2 spectra were scored using Mascot (Matrix Science; version 2.4.0) and Sequest HT against human protein sequences within the UniProt database and scrambled decoy database. Then data were imported into Scaffold software. Proteins were considered a positive identification if they achieved a 1% FDR (false discovery rate).

To identify PKA candidate substrates, spectral count ratios were calculated by dividing the number of peptide spectra for each protein in the PKA reaction by the number of spectra of that protein in the negative (no PKA) control. As a first screen, the spectral count ratio was calculated independently for each trial and only proteins that displayed a spectral count ratio of ≥ 1.0 in both trials were selected. Then, as a second more rigorous screen, the spectral counts of each protein in both trials were summed and a new ratio was calculated by dividing the summed number of spectral counts for each protein in the PKA reactions by the summed number of spectral counts of that protein in the negative (no PKA) controls (Table S1). The final 279 K-BILDS hit proteins demonstrated summed spectral count ratios of ≥ 1.2 (Table S1). The 1.2-fold summed spectra count ratio cut-off was chosen because known PKA substrates were observed at enrichment levels as low as 1.2-fold (Table S1, see GFPT1 protein).

Interactome analysis

The previously identified physical protein-protein interactions among K-BILDS hits were mapped with Cytoscape 3.3.0, using the GeneMANIA application (Figure 3a and S7). The PKA-centered interactome in Figure 3a was generated using only proteins that interact with

PKA within three degrees of protein separation. The full interactome map of all 279 hit proteins is provided (Figure S7). Protein complexes were identified using the MCODE application in Cytoscape (Figure 3b–d).

***In vitro* Kinase Assays**

In vitro kinase reactions were carried out by incubating BAG3 (1 µg, Prospectbio) or YWHAQ (1 µg, Prospectbio) proteins with ATP (2 mM, MP Biomedicals) and PKA (2500 U) in PKA buffer (50mM Tris-HCL, 10 mM MgCl₂, pH 7.5) at 31°C for 3 hours. Negative controls were performed by omitting PKA from the reactions. A positive control was carried out by incubating MBP (2 µg, Invitrogen) with ATP (2 mM) and PKA (2500 U) in PKA buffer at 31°C for 3 hours. In the case of NASP, the material purchased from Origene was phosphorylated. Dephosphorylated NASP was initially generated by treating NASP (1 µg, Origene) with CIP (10 U, Calf Intestinal Phosphatase, NEB) in CIP buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 100 µg/mL BSA) at 31°C for 3 hours, followed by addition of sodium orthovanadate (40 mM, Sigma) to inhibit CIP. The kinase reaction was then carried out by incubating the dephosphorylated NASP generated in the previous reaction with ATP (2 mM), sodium orthovanadate (40 mM), and PKA (2500 U) in PKA buffer at 31°C for 3 hours. Total volume for each reaction was 20 µL. After kinase reaction, protein products were analyzed by SDS-PAGE (12%). The gel was stained with Pro-Q (Invitrogen) to visualize phosphoproteins, whereas Sypro Ruby (Invitrogen) was used to confirm equal protein loading. Data is shown in Figures 4, S10, S11, and S12

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Lawrence DS. Accounts of chemical research. 2003; 36:353–354. [PubMed: 12809521]
2. Hunter T. Cell. 1995; 80:225–236. [PubMed: 7834742]
3. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. Science. 2002; 298:1912–1934. [PubMed: 12471243]
4. Jin LL, Sydorsky Y, Tong J, Taylor P, Moran MF, St-Germain JR. Current protocols in chemical biology. 2011; 9:2752–2761.
5. Manning BD, Cantley LC. Sci STKE. 2002; 2002:pe49. [PubMed: 12475999]
6. Shah K, Liu Y, Deirmengian C, Shokat KM. Proc Natl Acad Sci USA. 1997; 94:3565–3570. [PubMed: 9108016]
7. Mann M, Ong SE, Gronborg M, Steen H, Jensen ON, Pandey A. Trends in biotechnology. 2002; 20:261–268. [PubMed: 12007495]

8. Suwal S, Pflum MH. *Angew Chem Int Ed Engl*. 2010; 49:1627–1630. [PubMed: 20108289]
9. Suwal S, Senevirathne C, Garre S, Pflum MK. *Bioconjugate chemistry*. 2012; 23:2386–2391. [PubMed: 23116557]
10. Garre S, Senevirathne C, Pflum MK. *Bioorg Med Chem*. 2014; 22:1620–1625. [PubMed: 24529309]
11. Song, H., Kerman, K., Kraatz, HB. *Chemical communications*. 2008. p. 502-504.
12. Wang N, She Z, Lin YC, Martic S, Mann DJ, Kraatz HB. *Chemistry*. 2015; 21:4988–4999. [PubMed: 25676877]
13. Lee SE, Elphick LM, Anderson AA, Bonnac L, Child ES, Mann DJ, Gouverneur V. *Bioorganic & medicinal chemistry letters*. 2009; 19:3804–3807. [PubMed: 19410453]
14. Green KD, Pflum MH. *Journal of the American Chemical Society*. 2007; 129:10–11. [PubMed: 17199263]
15. Senevirathne C, Embogama DM, Anthony TA, Fouda AE, Pflum MK. *Bioorg Med Chem*. 2016; 24:12–19. [PubMed: 26672511]
16. Senevirathne C, Green KD, Pflum MK. *Current protocols in chemical biology*. 2012; 4:83–100. [PubMed: 25177527]
17. Xue L, Wang P, Cao P, Zhu J, Tao WA. *Molecular & Cellular Proteomics*. 2014; 13:3199–3210. [PubMed: 25022875]
18. Knight JD, Tian R, Lee RE, Wang F, Beauvais A, Zou H, Megeney LA, Gingras AC, Pawson T, Figeys D, Kothary R. *Skeletal Muscle*. 2012; 2:1–12. [PubMed: 22221535]
19. Menzel N, Chari A, Fischer U, Linder M, Raabe T. *Anal Biochem*. 2007; 368:178–184. [PubMed: 17597574]
20. Shabb JB. *Chemical Reviews*. 2001; 101:2381–2411. [PubMed: 11749379]
21. Hornbeck PV, Zhang B, Murray B, Kornhauser JM, Latham V, Skrzypek E. *Nucleic Acids Res*. 2015; 43:D512–520. [PubMed: 25514926]
22. Giansanti P, Stokes MP, Silva JC, Scholten A, Heck AJ. *Mol Cell Proteomics*. 2013; 12:3350–3359. [PubMed: 23882029]
23. Hu J, Rho HS, Newman RH, Hwang W, Neiswinger J, Zhu H, Zhang J, Qian J. *Biochim Biophys Acta*. 2014; 1844:224–231. [PubMed: 23524292]
24. Amano M, Hamaguchi T, Shohag MH, Kozawa K, Kato K, Zhang X, Yura Y, Matsuura Y, Kataoka C, Nishioka T, Kaibuchi K. *J Cell Biol*. 2015; 209:895–912. [PubMed: 26101221]
25. Cai R, Kwon P, Yan-Neale Y, Sambucetti L, Fischer D, Cohen D. *Biochem Biophys Res Commun*. 2001; 283:445–453. [PubMed: 11327722]
26. Ghosh-Dastidar P, Fox CF. *J Biol Chem*. 1984; 259:3864–3869. [PubMed: 6323445]
27. Matthies HJG, Miller RJ, Palfrey HC. *JBC*. 1993; 268:11176–11187.
28. Obenauer JC, Cantley LC, Yaffe MB. *Nucleic Acids Res*. 2003; 31
29. Lee TY, Bo-Kai Hsu J, Chang WC, Huang HD. *Nucleic Acids Res*. 2011; 39:D777–787. [PubMed: 21037261]
30. Colinge J, Cesar-Razquin A, Huber K, Breitwieser FP, Majek P, Superti-Furga G. *J Proteomics*. 2014; 107:113–127. [PubMed: 24704859]
31. Warde-Farley D, Donaldson SL, Comes O, Zuberi K, Badrawi R, Chao P, Franz M, Grouios C, Kazi F, Lopes CT, Maitland A, Mostafavi S, Montojo J, Shao Q, Wright G, Bader GD, Morris Q. *Nucleic Acids Res*. 2010; W214–W220. [PubMed: 20576703]
32. Senevirathne C, Pflum MH. *ChemBioChem*. 2013; 14:381–387. [PubMed: 23335220]
33. Wang M, Weiss M, Simonovic M, Haertinger G, Schrimpf SP, Hengartner MO, MCV. *Mol Cell Proteomics*. 2012; 11:492–500. [PubMed: 22535208]
34. Hu J, Rho HS, Newman RH, Zhang J, Zhu H, Qian J. *Bioinformatics*. 2013; 30:141–142. [PubMed: 24227675]
35. Newman RH, Hu J, Rho HS, Xie Z, Woodard C, Neiswinger J, Cooper C, Shirley M, Clark HM, Hu S, Hwang W, Jeong JS, Wu G, Lin J, Gao X, Ni Q, Goel R, Xia S, Ji H, Dalby KN, Birnbaum MJ, Cole PA, Knapp S, Ryazanov AG, Zack DJ, Blackshaw S, Pawson T, Gingras AC, Desiderio S, Pandey A, Turk BE, Zhang J, Zhu H, Qian J. *Mol Syst Biol*. 2013; 9:655. [PubMed: 23549483]

36. Wang Q, Diskin S, Rappaport E, Attiyeh E, Mosse Y, Shue D, Seiser E, Jagannathan J, Shusterman S, Bansal M, Khazi D, Winter C, Okawa E, Grant G, Cnaan A, Zhao H, Cheung N, Gerald W, London W, Matthay K, Brodeur GM, Maris JM. *Cancer Research*. 2006; 66:6050–6062. [PubMed: 16778177]
37. Kristensen AR, Gsponer J, Foster LJ. *Nature methods*. 2012; 9:907–909. [PubMed: 22863883]
38. Rosati A, Graziano V, Laurenzi VD, Pascale M, Turco M. *Cell Death and Disease*. 2011;2.
39. Srivastava RK, Srivastava AR, Korsmeyer SJ, Nesterova M, Cho-chung YS, Longo DL. *Molecular & Cellular biology*. 1998; 18:3509–3517. [PubMed: 9584191]
40. Li X. *Biochim Biophys Acta*. 2011; 1812:1219–1224. [PubMed: 21392577]
41. Kent CB, Shimada T, Ferraro GB, Ritter B, Yam PT, McPherson PS, Charron F, Kennedy TE, Fournier AE. *J Neurosci*. 2010; 30:14059–14067. [PubMed: 20962227]
42. Myeku N, Clelland CL, Emrani S, Kukushkin NV, Yu WH, Goldberg AL, Duff KE. *Nat Med*. 2016; 22:46–53. [PubMed: 26692334]
43. Ashe MP, De Long SK, Sachs AB. *Mol Biol Cell*. 2000; 11:833–848. [PubMed: 10712503]
44. Senevirathne C, Pflum MK. *ChemBioChem*. 2013; 14:381–387. [PubMed: 23335220]
45. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. *Nature Protocols*. 2006; 1:2856–2860. [PubMed: 17406544]

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Figure 2.

Kinase-catalyzed biotinylation of FSBA-treated lysates with PKA. HeLa cell lysates were untreated (DMSO, lanes 1 and 2) or treated with FSBA (lanes 3 and 4). Subsequently, samples were filtered to remove FSBA, and then ATP-biotin (lane 2, 3, and 4) and PKA (lane 4) were added. After kinase reaction, protein products were separated by SDS-PAGE and stained with SA-Cy5 to visualize biotinylation (top) and Sypro Ruby for total protein analysis (bottom). PKA is labeled with an arrow, whereas possible biotinylated PKA substrate bands are indicated with brackets. Replicate trials are shown in Figure S1.

Figure 3.

Interactome analysis of candidate PKA substrates from the K-BILDS study. (a) The 279 hits enriched in K-BILDS PKA reactions but not in the negative controls (Table S1) were analyzed using GeneMANIA in Cytoscape to identify physical protein-protein interactions between PKA and K-BILDS substrate hits. From the 279 hits, 78% (214 proteins) are represented in the interactome. The inner circle represents direct interactors of PKA (PRKACA), while the outer circles represent proteins that indirectly interact with PKA through three or fewer intermediate proteins. The lines between proteins document the interactions, with the thickness and length of lines arbitrarily drawn for clarity. The full interactome, including the 59 proteins that either interact indirectly with PKA beyond three degrees of separation or have no documented interaction with PKA, is shown in Figure S7. (b) All seven 14-3-3 proteins were identified by K-BILDS as candidate PKA substrate. (c) Candidate PKA substrate of the proteasome core identified by K-BILDS. (d) Candidate PKA substrate in the ribosome complex identified by K-BILDS. PKA (PRKACA), known PKA substrates, and the three substrates validated here are indicated in red, blue, and yellow, respectively, while all remaining candidate substrates shown in green.

Figure 4.

Validation of select K-BILDS hits as PKA substrates using *in vitro* kinase assays. NASP (a), BAG3 (b), or YWHAQ (c) were incubated with ATP either without (lanes 1, 3, and 5) or with PKA (lanes 2, 4, and 6) before SDS-PAGE separation and protein visualization with ProQ phosphoprotein stain (top) or Sypro Ruby total protein stain (bottom). Full gel images and repetitive trials are shown in Figures S10–S12.