

## Phosphorylation-Inducing Chimeric Small Molecules

Sachini U. Siriwardena,<sup>⊥</sup> Dhanushka N. P. Munkanatta Godage,<sup>⊥</sup> Veronika M. Shoba,<sup>⊥</sup> Sophia Lai,<sup>⊥</sup> Mengchao Shi, Peng Wu, Santosh K. Chaudhary, Stuart L. Schreiber, and Amit Choudhary\*Cite This: *J. Am. Chem. Soc.* 2020, 142, 14052–14057

Read Online

ACCESS |



Metrics &amp; More



Article Recommendations



Supporting Information

**ABSTRACT:** Small molecules have been classically developed to inhibit enzyme activity; however, new classes of small molecules that endow new functions to enzymes via proximity-mediated effect are emerging. Phosphorylation (native or neo) of any given protein-of-interest can alter its structure and function, and we hypothesized that such modifications can be accomplished by small molecules that bring a kinase in proximity to the protein-of-interest. Herein, we describe phosphorylation-inducing chimeric small molecules (PHICS), which enable two example kinases—AMPK and PKC—to phosphorylate target proteins that are not otherwise substrates for these kinases. PHICS are formed by linking small-molecule binders of the kinase and the target protein, and exhibit several features of a bifunctional molecule, including the hook-effect, turnover, isoform specificity, dose and temporal control of phosphorylation, and activity dependent on proximity (i.e., linker length). Using PHICS, we were able to induce native and neo-phosphorylations of BRD4 by AMPK or PKC. Furthermore, PHICS induced a signaling-relevant phosphorylation of the target protein Bruton's tyrosine kinase in cells. We envision that PHICS-mediated native or neo-phosphorylations will find utility in basic research and medicine.

The appendage of a phosphoryl group to many proteins profoundly influences their structures and functions.<sup>1</sup> Unsurprisingly, small molecules that *block* protein phosphorylation via kinase inhibition have had a transformative impact in basic science and medicine.<sup>2</sup> We hypothesize that small molecules that *induce* phosphorylation of any given protein-of-interest on-demand will also be useful in myriad scenarios. For example, such molecules can be used to trigger cell-signaling events or neo-phosphorylations that are not observed in native cellular environment.<sup>3</sup> Neo-phosphorylation can also alter protein structure and function,<sup>4</sup> evoke an immune response,<sup>5,6</sup> or affect the protein's interaction with other biomolecules, particularly with RNA/DNA that have negatively charged phosphodiester backbones.<sup>7,8</sup>

To design such phosphorylation-inducing molecules, we drew inspiration from chemical inducers of dimerization<sup>9,10</sup> and ubiquitination-inducing small molecules (e.g., PROTACs).<sup>11</sup> The latter increase the effective molarity of the ubiquitin ligase around the target protein, triggering ubiquitination even when the target protein is otherwise not a substrate of the given ligase. Herein, we describe a new class of bifunctional molecules that we term as phosphorylation-inducing chimeric small molecules (PHICS) formed by linking a kinase activator with a small-molecule binder of the target protein. Using PHICS, we demonstrate rewiring of substrate specificity of two kinases, AMP-activated protein kinase (AMPK) and protein kinase C (PKC), to induce phosphorylation (native and neo) of bromodomain-containing protein 4 (BRD4), which is not known to be a substrate of AMPK or PKC. Furthermore, an AMPK-based PHICS was able to induce a signaling-relevant phosphorylation (S180) on Bruton's tyrosine kinase (BTK) in a non-native cellular environment. While kinase specificity has been rewired using

adaptor proteins,<sup>12,13</sup> to the best of our knowledge, these studies provide first examples of induction of phosphorylation by rational rewiring of kinase specificity using small molecules.

To generate PHICS for AMPK and PKC, we designed small-molecule kinase binders with functional groups for linker attachment. For AMPK, we modified its allosteric activator,<sup>14</sup> PF-06409577, by replacing the cyclobutyl ring with the more synthetically amenable aminoethyl handle (Figure S1), while for PKC 9-(4-aminomethylbenzyloxy)-substituted benzolactam activator was used.<sup>15</sup> These chemical modifications did not perturb the ability of the binders to activate AMPK or PKC as assessed by the ADP-Glo assay,<sup>16</sup> which measures the amount of ADP produced from the kinase reaction (Figure S2). Next, we generated PHICS by conjugating these binders to (S)-JQ1, a BRD4 binder, with linkers of varying length (Figure S3 and S4). The AMPK PHICS were synthesized using a modular, click-chemistry-based convergent synthesis<sup>17</sup> (Figure S3), while the PKC PHICS were constructed using two consecutive amidation steps (Figure S4).

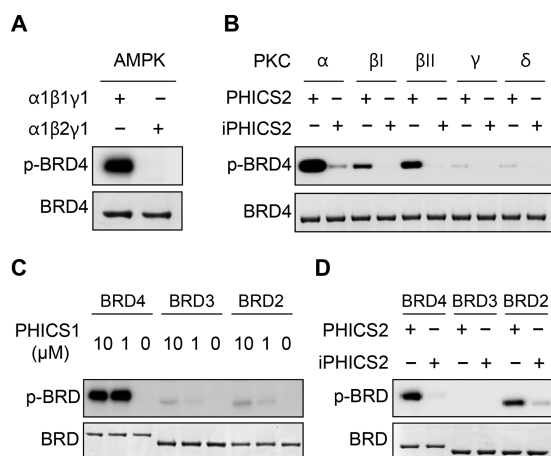
We assessed both ternary complex formation and levels of BRD4 phosphorylation (Figure S5 and S6) induced by these PHICS to identify PHICS1 and PHICS2 as the most optimal for AMPK and PKC, respectively (Figure 1A). We used (R)-JQ1, the inactive enantiomer of the BRD4 binder,<sup>18</sup> to synthesize iPHICS1 and iPHICS2 to serve as negative controls. Using the ADP-Glo assay, we confirmed that

Received: May 20, 2020

Published: August 4, 2020







**Figure 2.** PHICS exhibits isoform and paralog specificity. (A) Effect of AMPK isoforms on PHICS1-mediated BRD4 phosphorylation. (B) Effect of PKC isoforms on PHICS2-mediated BRD4 phosphorylation. (C,D) Phosphorylation of BRD4 paralogs by AMPK (C) or PKC (D).

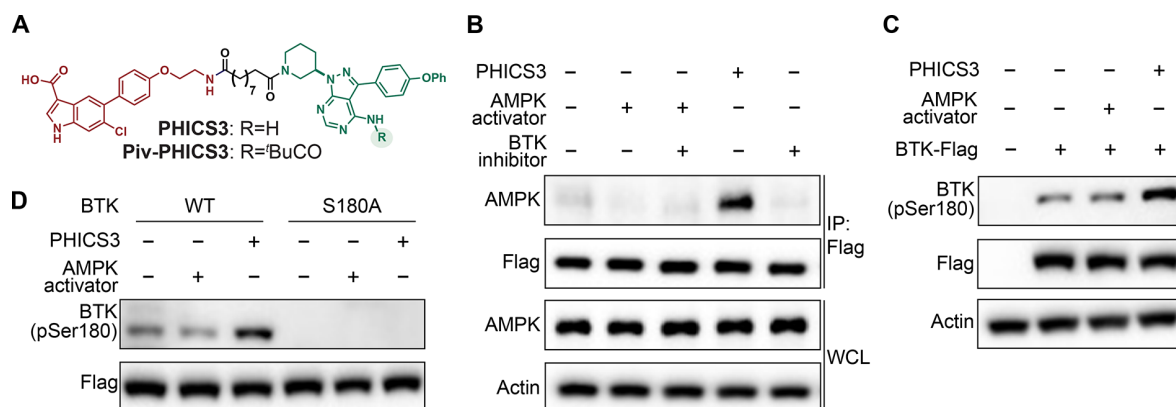
phosphorylation with PKC $\beta I$  and II, and only minor phosphorylation with the PKC $\gamma$  and  $\delta$  isoforms (Figure 2B).<sup>32</sup> Paralog specificity was also observed for the target proteins BRD (2/3/4), with BRD4 showing the highest level of phosphorylation (Figure 2C, 2D and S13).

We were unable to observe PHICS-mediated BRD4 phosphorylation in cells perhaps owing to different localization of the kinases and BRD4—while the former are mostly cytosolic, the latter primarily resides in the nucleus. We chose Bruton's Tyrosine Kinase (BTK), a protein widely expressed in B cells<sup>33,34</sup> for several reasons. First, BTK is a cytoplasmic protein and thus available for interactions with cytoplasmic AMPK. Second, while BTK can interact with PKC, it is not known to interact with AMPK.<sup>35</sup> Third, high-quality chemical probes of BTK and their co-crystal structures are available, allowing rational design of PHICS and inactive controls by engineering BTK or PHICS.<sup>36</sup> Fourth, BTK possesses a phosphorylation site (S180) that not only lies in substrate-like motif of AMPK but plays an important role in negative regulation of BTK.<sup>35</sup> Finally, BTK is undetectable in

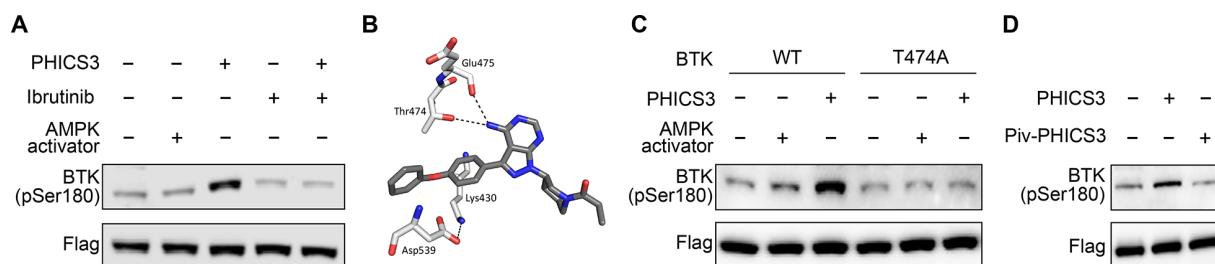
HEK293T cells allowing us to assess the ability of PHICS to induce BTK phosphorylation in a non-native cellular environment.

We linked AMPK binder with a noncovalent analogue of Ibrutinib via various linkers<sup>37</sup> (Figure S14) and found eight carbon alkyl linker (PHICS3, Figure 3A) to be optimum for the *in vitro* phosphorylation (Figure S15), which was monitored using phospho-BTK (Ser180) antibody. To demonstrate the ternary complex formation inside the cells, we transfected HEK293T with BTK-Flag and performed co-immunoprecipitation of AMPK with BTK in the presence of PHICS3 (Figure 3B). Furthermore, we were able to detect PHICS-mediated BTK phosphorylation at Ser180 (Figure 3C and S16A) and we validated this site of phosphorylation using S180A variant, where the phosphorylation was not detected (Figure 3D). We note that potent AMPK activator, PF-06409577, alone did not induce the same levels of BTK phosphorylation as PHICS3, even at 4-fold higher concentration (Figure S16B). Finally, we were able to dosably control PHICS-induced S180 phosphorylation with fast kinetics (Figure S16C and D).

To confirm that the observed BTK phosphorylation in the non-native environment was mediated by PHICS, we leveraged several chemical genetics approaches. First, the pretreatment of cells with covalent BTK binder, Ibrutinib, demolished the ability of PHICS3 to induce BTK phosphorylation (Figure 4A and S17A). Second, we mutated the residues purported to be involved in the binding of PHICS to BTK (Thr474, Lys430, and Asp539). Notably, Thr474 forms hydrogen bond with the 4-amino group of Ibrutinib (Figure 4B)<sup>38</sup> and we observed drastically lowered BTK phosphorylation for T474A variant (Figure 4C). Alterations in the ATP binding pocket by D539N and K430R mutations also markedly reduced the PHICS3 induced phosphorylation (Figure S17B and C). Finally, we designed an inactive analogue of PHICS3, by placing a bulky pivaloyl (Piv) group on the 4-aminopyrazolo[3,4-d]pyrimidine that should sterically prevent the binding to BTK. Indeed, the pivaloyl-bearing PHICS (Piv-PHICS3, Figure 3A) was unable to induce a significant BTK phosphorylation (Figure 4D). Taken together, these studies confirm that the Ser180 phosphorylation of BTK arises owing to proximity-effects induced by PHICS.



**Figure 3.** PHICS induces proximity and phosphorylation *in cellulo*. (A) Structures of PHICS3 for phosphorylation of BTK via AMPK and the inactive analogue, Piv-PHICS3. (B) Detection of ternary complex formation in HEK293T cells by co-immunoprecipitation of AMPK and BTK-Flag in the presence of PHICS3. WCL: Whole Cell Lysate (C,D) Western blot analysis of BTK phosphorylation by PHICS3 in HEK293T cells expressing wild-type BTK-Flag (C) or S180A variant (D). See Figures S3 and S14 for structures of AMPK activator and BTK inhibitor.



**Figure 4.** Cellular validation of PHICS. (A) Competition experiment with covalent BTK inhibitor, Ibrutinib. (B) Key interactions of Ibrutinib with BTK (PDB ID: 5P9I). (C) Western blot analysis of PHICS3-induced phosphorylation of wild-type BTK-Flag and T474A variant. (D) Western blot analysis of BTK phosphorylation with PHICS3 and its inactive analogue, Piv-PHICS3.

Herein, we successfully rewired both AMPK and PKC kinases using chimeric small molecules to induce novel phosphorylation events, including neo-phosphorylations of BRD4 and a signaling-relevant phosphorylation of BTK. PHICS exhibited the hallmarks of a typical bifunctional molecule, including the hook effect, turnover, dependence on proximity (linker length), isoform specificity, and dose- and temporal-control of phosphorylation. The turnover likely arises from the reversible binding of the PHICS to the kinase and target protein, which is also observed for PROTACs. Kinase specificity has been previously rewired using adaptor proteins,<sup>12,13</sup> but we focused on small molecules since they are cell-permeable and non-immunogenic. Furthermore, they afford facile dose and temporal controls, exhibit fast kinetics and turnover, and possess modular design allowing rapid assembly. While these studies have focused on serine/threonine-kinases, we are investigating the PHICS-mediated rewiring of tyrosine kinases. Our future studies will deploy PHICS to induce signaling-relevant phosphorylation as well as neo-phosphorylation of oncogenic proteins that could evoke an immune response against a tumor or the PHICS-mediated deposition of a negative charge on the DNA-binding domains of transcription factors (which are often deemed chemically undruggable)<sup>39</sup> that could adversely impact their ability to bind to DNA. Overall, PHICS expands the toolkit of chimeric small molecules that can be used to induce various post-translational modifications.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.0c05537>.

Supplementary Figures S1–S17; Materials and Methods; Characterization of compounds: <sup>1</sup>H and <sup>13</sup>C spectra (Figures S18–S70); Supplementary References (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

**Amit Choudhary** – Chemical Biology and Therapeutics Science, Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, United States; Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, United States; Divisions of Renal Medicine and Engineering, Brigham and Women's Hospital, Boston, Massachusetts 02115, United States; [orcid.org/0000-0002-8437-0150](https://orcid.org/0000-0002-8437-0150); Email: [achoudhary@bwh.harvard.edu](mailto:achoudhary@bwh.harvard.edu)

## Authors

**Sachini U. Siriwardena** – Chemical Biology and Therapeutics Science, Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, United States; Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, United States

**Dhanushka N. P. Munkanatta Godage** – Chemical Biology and Therapeutics Science, Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, United States; Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, United States

**Veronika M. Shoba** – Chemical Biology and Therapeutics Science, Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, United States; Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, United States; [orcid.org/0000-0003-0402-7414](https://orcid.org/0000-0003-0402-7414)

**Sophia Lai** – Chemical Biology and Therapeutics Science, Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, United States; Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138, United States

**Mengchao Shi** – Chemical Biology and Therapeutics Science, Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, United States; Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, United States; Divisions of Renal Medicine and Engineering, Brigham and Women's Hospital, Boston, Massachusetts 02115, United States

**Peng Wu** – Chemical Biology and Therapeutics Science, Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, United States; Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, United States; Divisions of Renal Medicine and Engineering, Brigham and Women's Hospital, Boston, Massachusetts 02115, United States; [orcid.org/0000-0002-0186-1086](https://orcid.org/0000-0002-0186-1086)

**Santosh K. Chaudhary** – Chemical Biology and Therapeutics Science, Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, United States; Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, United States

**Stuart L. Schreiber** – Chemical Biology and Therapeutics Science, Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, United States; Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138, United States; [orcid.org/0000-0003-1922-7558](https://orcid.org/0000-0003-1922-7558)

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/jacs.0c05537>

## Author Contributions

<sup>†</sup>S.U.S., D.N.P.M.G., V.M.S., and S.L. contributed equally to this work and are listed arbitrarily.

## Notes

The authors declare the following competing financial interest(s): Broad Institute has filed a patent application pertaining to the work described herein. S.L.S. serves on the Board of Directors of the Genomics Institute of the Novartis Research Foundation (GNF); is a shareholder and serves on the Board of Directors of Jnana Therapeutics; is a shareholder of Forma Therapeutics; is a shareholder and advises Decibel Therapeutics and Eikonizo Therapeutics; serves on the Scientific Advisory Boards of Eisai Co., Ltd., Ono Pharma Foundation, Exo Therapeutics, and F-Prime Capital Partners; and is a Novartis Faculty Scholar.

## ■ ACKNOWLEDGMENTS

V.M.S. and S.L. were supported by the Damon Runyon Postdoctoral Fellowship Award and the NSF GRFP (DGE-1745303), respectively. This work was supported by the Merkin Institute of Transformative Technologies in Healthcare and DARPA (N66001-17-2-4055). We thank Prof. C. M. Chiang (UT Southwestern Medical Center) for providing the BRD4 phospho-Ser484/488 antibody and Dr. P. K. Tiwari (Broad Institute) for assistance with synthesis. This work is dedicated to Prof. Laura L. Kiessling on the occasion of her 60th birthday.

## ■ REFERENCES

- (1) Tarrant, M. K.; Cole, P. A. The chemical biology of protein phosphorylation. *Annu. Rev. Biochem.* **2009**, *78*, 797–825.
- (2) Ferguson, F. M.; Gray, N. S. Kinase inhibitors: the road ahead. *Nat. Rev. Drug Discovery* **2018**, *17*, 353.
- (3) McCormick, J. W.; Pincus, D.; Resnekov, O.; Reynolds, K. A. Strategies for Engineering and Rewiring Kinase Regulation. *Trends Biochem. Sci.* **2020**, *45* (3), 259–271.
- (4) Ku, N. O.; Azhar, S.; Omary, M. B. Keratin 8 phosphorylation by p38 kinase regulates cellular keratin filament reorganization: modulation by a keratin 1-like disease causing mutation. *J. Biol. Chem.* **2002**, *277* (13), 10775–82.
- (5) McLaughlin, R. J.; Spindler, M. P.; van Lummel, M.; Roep, B. O. Where, How, and When: Positioning Posttranslational Modification Within Type 1 Diabetes Pathogenesis. *Curr. Diabetes Rep.* **2016**, *16* (7), 63–63.
- (6) Neugebauer, K. M.; Merrill, J. T.; Wener, M. H.; Lahita, R. G.; Roth, M. B. SR proteins are autoantigens in patients with systemic lupus erythematosus. Importance of phosphopeptides. *Arthritis Rheum.* **2000**, *43* (8), 1768–78.
- (7) Thapar, R. Structural basis for regulation of RNA-binding proteins by phosphorylation. *ACS Chem. Biol.* **2015**, *10* (3), 652–66.
- (8) Garcia-Garcia, T.; Poncet, S.; Derouiche, A.; Shi, L.; Mijakovic, I.; Noirot-Gros, M. F. Role of Protein Phosphorylation in the Regulation of Cell Cycle and DNA-Related Processes in Bacteria. *Front. Microbiol.* **2016**, *7*, 184.
- (9) Gerry, C. J.; Schreiber, S. L. Unifying principles of bifunctional, proximity-inducing small molecules. *Nat. Chem. Biol.* **2020**, *16* (4), 369–378.
- (10) Stanton, B. Z.; Chory, E. J.; Crabtree, G. R. Chemically induced proximity in biology and medicine. *Science* **2018**, *359* (6380), eaao5902.
- (11) Lai, A. C.; Crews, C. M. Induced protein degradation: an emerging drug discovery paradigm. *Nat. Rev. Drug Discovery* **2017**, *16* (2), 101–114.
- (12) Hobert, E. M.; Schepartz, A. Rewiring kinase specificity with a synthetic adaptor protein. *J. Am. Chem. Soc.* **2012**, *134* (9), 3976–8.
- (13) Bhattacharyya, R. P.; Remenyi, A.; Yeh, B. J.; Lim, W. A. Domains, motifs, and scaffolds: the role of modular interactions in the evolution and wiring of cell signaling circuits. *Annu. Rev. Biochem.* **2006**, *75*, 655–680.
- (14) Cameron, K. O.; Kung, D. W.; Kalgutkar, A. S.; Kurumbail, R. G.; Miller, R.; Salatto, C. T.; Ward, J.; Withka, J. M.; Bhattacharya, S. K.; Boehm, M.; Borzilleri, K. A.; Brown, J. A.; Calabrese, M.; Caspers, N. L.; Cokorinos, E.; Conn, E. L.; Dowling, M. S.; Edmonds, D. J.; Eng, H.; Fernando, D. P.; Frisbie, R.; Hepworth, D.; Landro, J.; Mao, Y.; Rajamohan, F.; Reyes, A. R.; Rose, C. R.; Ryder, T.; Shavnya, A.; Smith, A. C.; Tu, M.; Wolford, A. C.; Xiao, J. Discovery and Preclinical Characterization of 6-Chloro-5-[4-(1-hydroxycyclobutyl)-phenyl]-1H-indole-3-carboxylic Acid (PF-06409577), a Direct Activator of Adenosine Monophosphate-activated Protein Kinase (AMPK), for the Potential Treatment of Diabetic Nephropathy. *J. Med. Chem.* **2016**, *59* (17), 8068–81.
- (15) Mach, U. R.; Lewin, N. E.; Blumberg, P. M.; Kozikowski, A. P. Synthesis and pharmacological evaluation of 8- and 9-substituted benzolactam-V8 derivatives as potent ligands for protein kinase C, a therapeutic target for Alzheimer's disease. *ChemMedChem* **2006**, *1* (3), 307–314.
- (16) Zegzouti, H.; Zdanovskaia, M.; Hsiao, K.; Goueli, S. A. ADP-Glo: A Bioluminescent and homogeneous ADP monitoring assay for kinases. *Assay Drug Dev. Technol.* **2009**, *7* (6), 560–72.
- (17) Wurz, R. P.; Dellamaggiore, K.; Dou, H.; Javier, N.; Lo, M. C.; McCarter, J. D.; Mohl, D.; Sastri, C.; Lipford, J. R.; Cee, V. J. A “Click Chemistry Platform” for the Rapid Synthesis of Bispecific Molecules for Inducing Protein Degradation. *J. Med. Chem.* **2018**, *61* (2), 453–461.
- (18) Filippakopoulos, P.; Qi, J.; Picaud, S.; Shen, Y.; Smith, W. B.; Fedorov, O.; Morse, E. M.; Keates, T.; Hickman, T. T.; Felletar, I.; Philpott, M.; Munro, S.; McKeown, M. R.; Wang, Y.; Christie, A. L.; West, N.; Cameron, M. J.; Schwartz, B.; Heightman, T. D.; La Thangue, N.; French, C. A.; Wiest, O.; Kung, A. L.; Knapp, S.; Bradner, J. E. Selective inhibition of BET bromodomains. *Nature* **2010**, *468* (7327), 1067–73.
- (19) Bielefeld-Sevigny, M. AlphaLISA immunoassay platform- the “no-wash” high-throughput alternative to ELISA. *Assay Drug Dev. Technol.* **2009**, *7* (1), 90–2.
- (20) Beaudet, L.; Rodriguez-Suarez, R.; Venne, M.-H.; Caron, M.; Bédard, J.; Brechler, V.; Parent, S.; Bielefeld-Sevigny, M. Alpha LISA immunoassays: the no-wash alternative to ELISAs for research and drug discovery. *Nat. Methods* **2008**, *5*, A10.
- (21) Douglass, E. F., Jr.; Miller, C. J.; Sparer, G.; Shapiro, H.; Spiegel, D. A. A comprehensive mathematical model for three-body binding equilibria. *J. Am. Chem. Soc.* **2013**, *135* (16), 6092–9.
- (22) Rodbard, D.; Feldman, Y.; Jaffe, M. L.; Miles, L. E. Kinetics of two-site immunoradiometric (‘sandwich’) assays-II. Studies on the nature of the ‘high-dose hook effect’. *Immunochemistry* **1978**, *15* (2), 77–82.
- (23) Johanns, M.; Lai, Y. C.; Hsu, M. F.; Jacobs, R.; Vertommen, D.; Van Sande, J.; Dumont, J. E.; Woods, A.; Carling, D.; Hue, L.; Viollet, B.; Foretz, M.; Rider, M. H. AMPK antagonizes hepatic glucagon-stimulated cyclic AMP signalling via phosphorylation-induced activation of cyclic nucleotide phosphodiesterase 4B. *Nat. Commun.* **2016**, *7*, 10856.
- (24) Wang, Z.; Ma, J.; Miyoshi, C.; Li, Y.; Sato, M.; Ogawa, Y.; Lou, T.; Ma, C.; Gao, X.; Lee, C.; Fujiyama, T.; Yang, X.; Zhou, S.; Hotta-Hirashima, N.; Klewe-Nebenius, D.; Ikkyu, A.; Kakizaki, M.; Kanno, S.; Cao, L.; Takahashi, S.; Peng, J.; Yu, Y.; Funato, H.; Yanagisawa, M.; Liu, Q. Quantitative phosphoproteomic analysis of the molecular substrates of sleep need. *Nature* **2018**, *558* (7710), 435–439.
- (25) Wu, S. Y.; Lee, A. Y.; Lai, H. T.; Zhang, H.; Chiang, C. M. Phospho switch triggers Brd4 chromatin binding and activator recruitment for gene-specific targeting. *Mol. Cell* **2013**, *49* (5), 843–57.
- (26) Hornbeck, P. V.; Zhang, B.; Murray, B.; Kornhauser, J. M.; Latham, V.; Skrzypek, E. PhosphoSitePlus, 2014: mutations, PTMs

and recalibrations. *Nucleic Acids Res.* **2015**, *43* (Database issue), D512–D520.

(27) Davies, S. P.; Carling, D.; Hardie, D. G. Tissue distribution of the AMP-activated protein kinase, and lack of activation by cyclic-AMP-dependent protein kinase, studied using a specific and sensitive peptide assay. *Eur. J. Biochem.* **1989**, *186* (1–2), 123–8.

(28) Hardie, D. G.; Schaffer, B. E.; Brunet, A. AMPK: An Energy-Sensing Pathway with Multiple Inputs and Outputs. *Trends Cell Biol.* **2016**, *26* (3), 190–201.

(29) Weekes, J.; Ball, K. L.; Caudwell, F. B.; Hardie, D. G. Specificity determinants for the AMP-activated protein kinase and its plant homologue analysed using synthetic peptides. *FEBS Lett.* **1993**, *334* (3), 335–9.

(30) Schaffer, B. E.; Levin, R. S.; Hertz, N. T.; Maures, T. J.; Schoof, M. L.; Hollstein, P. E.; Benayoun, B. A.; Banko, M. R.; Shaw, R. J.; Shokat, K. M.; Brunet, A. Identification of AMPK Phosphorylation Sites Reveals a Network of Proteins Involved in Cell Invasion and Facilitates Large-Scale Substrate Prediction. *Cell Metab.* **2015**, *22* (5), 907–21.

(31) Gadd, M. S.; Testa, A.; Lucas, X.; Chan, K. H.; Chen, W.; Lamont, D. J.; Zengerle, M.; Ciulli, A. Structural basis of PROTAC cooperative recognition for selective protein degradation. *Nat. Chem. Biol.* **2017**, *13* (5), 514–521.

(32) Kozikowski, A. P.; Wang, S.; Ma, D.; Yao, J.; Ahmad, S.; Glazer, R. I.; Bogi, K.; Acs, P.; Modarres, S.; Lewin, N. E.; Blumberg, P. M. Modeling, Chemistry, and Biology of the Benzolactam Analogs of Indolactam V (ILV). 2. Identification of the Binding Site of the Benzolactams in the CRD2 Activator-Binding Domain of PKC $\delta$  and Discovery of an ILV Analog of Improved Isoenzyme Selectivity. *J. Med. Chem.* **1997**, *40* (9), 1316–1326.

(33) Pal Singh, S.; Dammeijer, F.; Hendriks, R. W. Role of Bruton's tyrosine kinase in B cells and malignancies. *Mol. Cancer* **2018**, *17* (1), 57.

(34) Burger, J. A.; Wiestner, A. Targeting B cell receptor signalling in cancer: preclinical and clinical advances. *Nat. Rev. Cancer* **2018**, *18* (3), 148–167.

(35) Kang, S. W.; Wahl, M. I.; Chu, J.; Kitaura, J.; Kawakami, Y.; Kato, R. M.; Tabuchi, R.; Tarakhovsky, A.; Kawakami, T.; Turck, C. W.; Witte, O. N.; Rawlings, D. J. PKC $\beta$  modulates antigen receptor signaling via regulation of Btk membrane localization. *EMBO journal* **2001**, *20* (20), 5692–702.

(36) Liang, C.; Tian, D.; Ren, X.; Ding, S.; Jia, M.; Xin, M.; Thareja, S. The development of Bruton's tyrosine kinase (BTK) inhibitors from 2012 to 2017: A mini-review. *Eur. J. Med. Chem.* **2018**, *151*, 315–326.

(37) Johnson, A. R.; Kohli, P. B.; Katewa, A.; Gogol, E.; Belmont, L. D.; Choy, R.; Penuel, E.; Burton, L.; Eigenbrot, C.; Yu, C.; Ortwine, D. F.; Bowman, K.; Franke, Y.; Tam, C.; Estevez, A.; Mortara, K.; Wu, J.; Li, H.; Lin, M.; Bergeron, P.; Crawford, J. J.; Young, W. B. Battling Btk Mutants With Noncovalent Inhibitors That Overcome Cys481 and Thr474 Mutations. *ACS Chem. Biol.* **2016**, *11* (10), 2897–2907.

(38) Bender, A. T.; Gardberg, A.; Pereira, A.; Johnson, T.; Wu, Y.; Grenningloh, R.; Head, J.; Morandi, F.; Haselmayer, P.; Liu-Bujalski, L. Ability of Bruton's Tyrosine Kinase Inhibitors to Sequester Y551 and Prevent Phosphorylation Determines Potency for Inhibition of Fc Receptor but not B-Cell Receptor Signaling. *Mol. Pharmacol.* **2017**, *91* (3), 208–219.

(39) Koehler, A. N. A complex task? Direct modulation of transcription factors with small molecules. *Curr. Opin. Chem. Biol.* **2010**, *14* (3), 331–340.