Cell Chemical Biology

Homolog-Selective Degradation as a Strategy to Probe the Function of CDK6 in AML

Graphical Abstract



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In Brief

Brand et al. describe BSJ-03-123, a degrader with proteome-wide selectivity for CDK6. Specificity emerges from differential ternary complex formation with the E3 ligase CRL4^{CRBN}. BSJ-03-123 exploits a dependency of AML cells on CDK6, and rapid degradation allowed assaying the role of CDK6 in coordinating signaling and gene control in AML.

Highlights

- Development of a CDK6-selective small-molecule degrader
- Mechanistically understood selectivity via differential ternary complex formation
- Profiling of consequences of CDK6 degradation on signaling and gene regulation
- Precise exploitation of genetic dependencies through homolog-selective degradation



Cell Chemical Biology
Brief Communication

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SUMMARY

The design of selective small molecules is often stymied by similar ligand binding pockets. Here, we report BSJ-03-123, a phthalimide-based degrader that exploits protein-interface determinants to achieve proteome-wide selectivity for the degradation of cyclin-dependent kinase 6 (CDK6). Pharmacologic CDK6 degradation targets a selective dependency of acute myeloid leukemia cells, and transcriptomics and phosphoproteomics profiling of acute degradation of CDK6 enabled dynamic mapping of its immediate role in coordinating signaling and transcription.

INTRODUCTION

Cyclin-dependent kinases (CDKs) orchestrate fundamental cellular processes such as cell cycle and transcription. CDKs are serine/threonine kinases and their activity is dependent on association with cyclins, enabling temporal control over enzymatic function. CDK4 and CDK6 control G1-S transition by associating with D-type cyclins to phosphorylate the tumor suppressor retinoblastoma (Rb). This relieves Rb-mediated repression of E2F transcription factors (TFs) to trigger gene expression required for S-phase entry. While CDK4 and CDK6 were long perceived as redundant, recent studies have identified homolog-specific functions. In particular, CDK6 emerged as a regulatory hub connecting cell cycle with metabolism (Wang et al., 2017) and transcription (Tigan et al., 2016). In several contexts, the transcriptional role of CDK6 is independent of its kinase function, but relies on molecular scaffolding (Kollmann et al., 2013; Tigan et al., 2016).

ATP-competitive CDK4/6 inhibitors have shown significant clinical activity, leading to approval of three drugs for breast cancer (O'Leary et al., 2016). However, none of these inhibitors can discriminate between CDK4 and CDK6, as they share 94% amino acid sequence identity in their ATP binding pockets. In addition, current inhibitors cannot disrupt scaffolding functions (Kollmann et al., 2013). To address this, we turned to a ligand-

induced targeted protein degradation strategy based on heterobifunctional molecules that induce molecular proximity between a protein of interest (POI) and the CRL4^{CRBN} E3 ligase complex (Lu et al., 2015; Winter et al., 2015; Zengerle et al., 2015). Interestingly, studies of degraders derived from multi-targeted warheads revealed that only a subset of targets were efficiently degraded, indicating the possibility of engineering selectivity into poly-pharmacologic scaffolds (Bondeson et al., 2018; Gadd et al., 2017; Huang et al., 2018; Olson et al., 2018; Winter et al., 2015). Along those lines, it was demonstrated that selective degradation can be achieved by exploiting amino acid differences in the POI-E3 interface and thus differential ternary complex formation (Gadd et al., 2017; Nowak et al., 2018).

Here, we explored the feasibility of homolog-selective degradation of CDK6 versus CDK4 using ligands that bind the ATP sites of both enzymes. We describe the discovery and characterization of BSJ-03-123 (BSJ), a selective degrader of CDK6 that uniquely enables rapid pharmacological interrogation of CDK6dependent functions. Coupling acute CDK6 degradation with phosphoproteomic and transcriptomic profiling allowed us to establish a systems-level understanding of the sensitivity of acute myeloid leukemia (AML) cell lines to selective CDK6 ablation and to chart a network of CDK6-dependent signaling events.

RESULTS

Development of a Homolog-Selective CDK6 Degrader

Toward the development of CDK4/6 degraders, we chose palbociclib (palbo) as a starting point given its high selectivity and potency (Fry et al., 2004). Available crystal structures of palbo with CDK6 (PDB: 5L2I) highlighted the solvent-exposed piperazine moiety as an ideal site for linker conjugation (Chen et al., 2016). We installed a 3-polyethylene glycol linker conjugated to pomalidomide, resulting in the compound YKL-06-102 (YKL) (Figure 1A). Biochemical characterization indicated that phthalimide conjugation results in a comparable, albeit weakly reduced binding affinity to both CDK4 and CDK6 (Figure S1A; Table S1). Moreover, YKL bound to recombinant CRBN-DDB1 with comparable affinity as lenalidomide (Table S1). Unexpectedly, cellular treatment with YKL led to a selective destabilization of CDK6 (Figures S1B–S1D). To further survey degrader selectivity, we employed global proteomics following cellular treatment. While



Figure 1. Development of a Homolog-Selective Degrader of CDK6

(A) Structure of YKL-06-102.

(B) Quantification of 5,945 proteins following treatment with YKL-06-102 (500 nM, 5 hr). False discovery rate (FDR)-adjusted p values. Kinases inhibited by palbociclib *in vitro* are color-coded by magnitude of inhibition.

(C) Structure of BSJ-03-123 (BSJ).

(D) Immunoblot for CDK4, CDK6, and histone 3 after 4 hr BSJ treatment.

(E) As in (D), but time-resolved at 200 nM BSJ.

(F) Immunoblot for CDK6 and histone 3. 200 nM BSJ, 4 hr, in CRBN-deficient and wild-type MV4-11.

(G) Quantification of 5,995 proteins following BSJ treatment (100 nM, 2 hr). FDR-adjusted p values. Kinase labeling as in (B).

(H) Structure of BSJ-bump.

(I) As in (D), but for BSJ-bump.

See also Figure S1 and Tables S1 and S2.

recapitulating the selective degradation of CDK6, we detected significant destabilization of IKZF1 and IKZF3 ($p = 6.19 \times 10^{-6}$) (Figure 1B), suggesting warhead-independent CRBN substrate modulation (An et al., 2017; Gandhi et al., 2014; Kronke et al., 2014; Lu et al., 2014). To remove IKZF1/3 activity of YKL, we designed BSJ by employing a phenoxyacetamide linker without altering the initial linker length (Figure 1C). Measurements of *in vitro* binding to recombinant kinase domains (KINOMEScan) confirmed that phthalimide conjugation preserves the narrow selectivity profile (Figure S1G; Table S2). BSJ induced CRBN-dependent, potent, fast, and homolog-selective degradation of CDK6 (Figures 1D–1F and S1E). In proteome-wide selectivity studies, CDK6 was the only destabilized protein (Figures 1G and S1F).

Based on BSJ, we furthermore synthesized an N-methylated glutarimide analog (BSJ-bump) incapable of CRBN binding *in vitro* (Figure 1H; Table S1). Accordingly, BSJ-bump was un-

able to degrade CDK4 or CDK6, thus serving as excellent negative control with matched physiochemical properties (Figure 1I).

BSJ Triggers Homolog-Selective Degradation via Differential Ternary Complex Formation

Next, we sought to understand the mechanism of CDK6 selectivity. *In vitro* kinase assays confirmed comparable affinity to both kinases (Figure S1A; Table S1). Similar cellular thermal stabilization of CDK4/6 further suggested that selectivity does not emerge from differential cellular target engagement (Figure 2A). We thus hypothesized that selectivity might stem from differential ternary complex formation. To monitor tripartite assembly in real time in intact cells, we designed a luciferase complementation assay based on NanoBiT technology (Figure 2B). We expressed C-terminal CDK4/6 LgBit fusions along with an N-terminal SmBit-CRBN fusion in 293T^{CRBN-/-} cells. BSJ induced rapid, dose-dependent ternary complex formation with CDK6



Figure 2. BSJ Selectivity Is Explained by Differential Ternary Complex Formation

(A) Immunoblot for CDK4 and CDK6 after thermal shift assay in MV4-11 CRBN^{-/-} cells after cellular treatment with DMSO, palbociclib, or BSJ (both at 20 μM).
 (B) Schematic representation of the luciferase complementation assay.

(C) Measurement of CDK4:CRBN and CDK6:CRBN binding via NanoBiT assay. Averages of five replicates are plotted, shaded areas represent 95% confidence intervals.

(D) Measurement of CDK6:CRBN complex formation after 1 hr pre-treatment with 20 µM palbociclib or lenalidomide. Statistics as in (C).

and CRBN, but not with CDK4 and CRBN (Figure 2C). Treatment with BSJ-bump failed to induce CDK6:CRBN interactions (Figure 2C). Ternary complex formation was prevented by blocking binding sites on CDK6 or CRBN via pretreatment with palbo or lenalidomide (Figure 2D). We thus concluded that BSJ exploits structural differences between CDK4 and CDK6 to achieve homolog-selective degradation of CDK6 via differential ternary complex formation.

BSJ Can Exploit Homolog-Selective Dependency on CDK6

To identify a cellular system that is disproportionally dependent on CDK6 over CDK4, we turned to genome-scale CRISPR/Cas9 screens in 342 cancer cell lines (Meyers et al., 2017). We identified a pronounced enrichment of AML cell lines among the most CDK6-addicted models (Figure 3A). None of these cell lines showed a comparable dependency on CDK4 (Figure S2A). Intersection with gene expression data did not unveil a correlation between essentiality and mRNA transcript levels (Figures 3A and S2A) (Klijn et al., 2015). Accordingly, CRISPR/Cas9-mediated genetic depletion of CDK4 in the AML cell line MV4-11 revealed largely preserved growth kinetics and cell-cycle distribution, supporting a dispensable role of CDK4 in the proliferation of AML cell lines (Figures S2B–S2D).

Given their lack of homolog-selectivity, CDK4/6 inhibitors are incapable of exploiting such fine-tuned genetic dependencies. Concomitant inhibition of both homologs is thus expected to limit the achievable therapeutic window. Therefore, we wanted to test if BSJ could target the genetic dependency of AML cells on CDK6, while sparing CDK4-dependent cancer cells lines. As expected, dual CDK4/6 inhibition severely impaired proliferation of CDK4-dependent cell lines, whereas selective degradation of CDK6 did not trigger a comparable effect (Figures 3B and S2E). In contrast, BSJ caused a pronounced anti-proliferative effect in CDK6-dependent AML cell lines by inducing a G1 cell-cycle arrest without a measurable increase in apoptosis (Figures 3C-3E). Of note, this effect exceeded the anti-proliferative consequences of cellular treatment with BSJ-bump (Figures 3C and S2F). The observed benefit of CDK6 degradation over CDK6 inhibition could be due to the disruption of additional, kinase activity-independent molecular mechanisms (Kollmann et al., 2013), or due to pharmacologic advantages of degraders, such as catalytic target turnover. To address this, we compared BSJ with the clinically approved palbo in CDK4-deficient MV4-11 cells to enable a CDK6-centric readout. In this experimental setup, selective degradation of CDK6 was not superior to enzymatic inhibition (Figure 3F). While interpretation of this comparison is non-trivial given the physicochemical differences between the molecules, it suggests that the dependency of AML cells on CDK6 is mostly dependent on its kinase function.

Impairment of Selective Regulatory Networks after CDK6 Degradation

We next investigated drug impact on the known CDK4/6 Rb phosphorylation site S780. Both BSJ and palbo treatment reduced levels of p-S780 (Figure 4A). Notably, BSJ-bump treatment failed to affect phosphorylation of Rb, indicating that, at the assayed concentration, enzymatic CDK4/6 inhibition is insignificant (Figure 4A). Measurable impact of BSJ-bump was only observed upon dose escalation (Figure S2G). In line with this,



Figure 3. BSJ Exploits Homolog-Selective Dependencies

(A) Bottom: waterfall plot of 391 cell lines ranked by CDK6 dependency as determined in genome-wide CRISPR/Cas9 screens. CERES essentiality score is normalized for copy-number variation and scaled by setting the median of pan-essential genes to -1. Top: mRNA levels of CDK4, CDK6, and D-type cyclins. (B) Colony-formation assays (12 days, refreshing the treatment every 2 days).

(C) Growth curves of AML cell lines treated with 200 nM BSJ, BSJ-bump, or DMSO. Cells were counted and treatment refreshed every 2 days.

(D) Cell cycle after treatment with 200 nM BSJ or BSJ-bump for 24 hr.

(E) Percentage of apoptotic cells after 24 hr treatment with 200 nM palbociclib, BSJ, BSJ-bump, or DMSO (Caspase-Glo 3/7 assay). BET protein degrader dBET6 served as positive control.

(F) Growth curves of CDK4-deficient MV4-11 treated with 200 nM BSJ, palbociclib, or DMSO.

See also Figure S2. Data presented in (C–F): mean \pm SD, triplicate analysis.

the effect of BSJ treatment on Rb phosphorylation is rescued upon CRBN knockout (Figure S2H).

Given the plethora of functions assigned to CDK6, we globally extended our analysis to assay how acute and selective CDK6 disruption affects cellular signaling and gene activity in AML. Given the limited activity of BSJ-bump, we compared selective degradation of CDK6 via BSJ with catalytic CDK4/6 inhibition by palbo. We coupled acute drug treatment (2 hr, 200 nM each) to quantitative (Ser/Thr) phosphoproteomics to focus on direct drug effects. We quantified a total of 22,804 phosphopeptides in at least one technical replicate. In general, drug impact was modest, with 197 deregulated phosphopeptides after treatment with BSJ (44 up, 153 down), and 191 deregulated (57 up, 134 down) phosphopeptides following palbo treatment (Figures 4B and S3A). Changed sites were enriched for CDK motifs based on kinase-substrate enrichment analysis (Figures 4B and S3B), validating that short treatments biased for upstream events (Casado et al., 2013). Proteins with altered phosphosites were enriched for known regulators of cell-cycle progression (Figure S3C). Moreover, our data indicate a significant ($p = 4.49 \times$ 10^{-5}) overlap with previously reported CDK4/6 substrates based on *in vitro* assays (Anders et al., 2011), but also uncovered undescribed substrates such as the splicing factor SRRM2 and the RSF chromatin-remodeling component RSF1 (Figures S3D and S3E).

In parallel, we compared the transcriptional response elicited by palbo and BSJ. This allowed us to determine if CDK6 orchestrates transcription independent of its kinase domain. As expected, transcriptional impact of YKL significantly differed from BSJ/palbo owing to off-target effects on IKZF1 and IKZF3. In contrast, enzymatic CDK4/6 inhibition and CDK6 degradation yielded a very similar and highly correlated ($R^2 = 0.9375$) transcriptional response, arguing that the transcriptional role of CDK6 in AML is mostly limited to its kinase function (Figures S4A–S4D). To further explore the functional implications of immediate CDK6 degradation, we performed gene ontology-term analysis of transcriptional changes elicited by CDK6 degradation and CDK4/6 inhibition (Figures 4D and S4E). Again, transcriptional consequences were similar, indicating that both converge on known pathways such as cell-cycle regulation, but also

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Figure 4. An Integrated View of the Effects of Acute CDK6 Degradation on Cellular Signaling and Transcription

(A) Immunoblot for p-RB S780, RB, CDK4, CDK6, and histone 3 after treatment with 200 nM BSJ, palbociclib, or BSJ-bump.

(B) Global phosphoproteomics. Heatmap depicting fold changes in peptide phosphorylation after BSJ or palbociclib treatment (200 nM, 2 hr) compared with DMSO for 305 differentially phosphorylated peptides. Hits (log2 fold change [FC] > 0.5 or < -0.5, adjusted p value < 0.05) and peptides phosphorylated at canonical SP/TP CDK phosphorylation motifs are annotated.

(C) Heatmap of DMSO-normalized fold changes in gene expression after BSJ, YKL, or palbociclib treatment (200 nM, 6 hr) for 993 significantly deregulated genes (log2 FC > 1.5 or < -1.5, adjusted p value < 0.05).

(D) Functional network of BSJ treatment. Nodes represent gene ontology (GO) terms enriched among genes that are differentially expressed upon treatment, scaled by magnitude, and color coded by significance of enrichment. Edges represent parent-child relationships of GO terms.

(E) Molecular network of BSJ treatment. Hits identified via global phosphoproteomics were mapped on a protein-protein interaction network and expanded to include first-order neighbors limited to ENCODE transcriptional regulators. Node shape distinguishes transcriptional regulators (TR) (diamonds) from phosphoproteomics hits (round). Node color represents the number of quantified phosphopeptides. Diamonds are scaled proportional to percentage of dysregulated TR target genes upon treatment. Proteins phosphorylated at CDK consensus motif (SP/TP) are annotated by edge color. Edges represent physical interaction between proteins.

See also Figures S3 and S4.

influence processes such as DNA replication, the DNA damage response, or chromatin organization.

To derive a systems-level understanding of the function of CDK6, we condensed data from transcriptional and phosphoproteomic profiling into a network centered around proteins with altered phosphopeptides after immediate CDK6 degradation (Figure 4E). Proteins were differentiated based on presence of a consensus CDK phosphorylation motif (SP/TP) in the detected phosphopeptides. Next, we expanded this network by adding ENCODE transcriptional regulators with established protein-protein interactions to members from the experimentally derived network. Network expansion was limited to factors with available genome-wide binding data. Dynamic transcriptional changes after CDK6 depletion were calculated and altered target genes were assigned to the network-resident TFs, which were visually scaled based on the percentage of dynamically regulated target genes (Figure 4E). This integrated network allowed identification of known, consensus downstream signaling axes such as Rb-E2F1, but also nodes such as BCL11A and NCOR2, previously not linked to CDK6. Network topologies are largely overlapping between BSJ or palbo treatment (Figure S3F), again supporting the notion that, in AML, CDK6 coordinates signaling and gene control predominantly via its kinase function.

DISCUSSION

The development of highly selective small molecules is a longstanding challenge in ligand discovery given the structural similarities of substrate or cofactor binding sites. This is a particular concern with ATP-competitive kinase inhibitors where a lack of

selectivity can limit the achievable therapeutic window. This hinders pharmacologic exploitation of genetically defined dependencies for therapeutic indications. Here, we present BSJ, a homolog-selective CDK6 degrader. BSJ features proteome-wide selectivity for CDK6 via differential ternary complex formation. Selectivity of BSJ enables exploitation of genetic dependencies beyond a resolution achievable with dual CDK4/6 inhibitors. In particular, we show that BSJ is capable of exploiting a homolog-selective dependency of AML cells on CDK6, thus outlining the potential for selective CDK6 degraders for further translational investigation, conceivably at reduced overall toxicity. Degradation of CDK6 is fast and potent, allowing us to map global consequences of acute CDK6 disruption on downstream signaling networks and transcriptional programs. While we cannot rule out that, at saturating concentrations, BSJ also catalytically inhibits CDK4, no inhibition was measured at the assayed concentration. Comparative profiling of CDK6 degradation and CDK4/6 inhibition suggest that, in AML, CDK6 integrates signaling and gene activity predominantly via its kinase activity. Our analysis uncovered several signaling nodes and transcriptional hubs previously not linked to CDK6, such as BCL11A and NCOR2, and future research will be necessary to explore the functional relevance of these pathways in AML and beyond. CDK6 features a prominent role in other malignancies as well as hematopoietic and leukemic stem cells. Selective degradation of CDK6 will facilitate differentiating relevant molecular mechanisms and further untangle kinase-dependent and -independent functions. Future medicinal chemistry efforts will be necessary to expand on the presented concept and to develop a toolbox of selective degraders to investigate the role of protein kinases at unprecedented precision and kinetic resolution.

SIGNIFICANCE

Traditionally, drug discovery efforts focus on the development of small-molecule inhibitors that block accessible hydrophobic pockets such as substrate or cofactor binding sites. However, due to the often high sequence conservation of these pockets, the design of selective inhibitors remains a continuous challenge. Here, we report a degrader that exploits structural differences outside of the ligand binding pocket to induce differential E3 ligase recruitment and ensuing degradation of CDK6, but not CDK4. We employed this exquisitely selective probe to dissect CDK6-dependent signaling and gene expression in AML, and showed that it allows unprecedented exploitation of genetically defined dependencies. This work showcases a framework for engineering selectivity into small-molecule probes and paves the way for the development of tools to functionally understand proteins at high kinetic resolution.

STAR*METHODS

Detailed methods are provided in the online version of this paper and include the following:

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METHOD DETAILS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, two tables, and one data file and can be found with this article online at https://doi.org/10.1016/j.chembiol.2018.11.006.

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AUTHOR CONTRIBUTIONS

M.B. performed immunoblots, nluc-complementation assays, and proliferation assays. He also prepared samples for RNA sequencing and phosphoproteomics and expression proteomics, and performed the associated bioinformatic analysis. B.J. synthesized BSJ-03-123 and BSJ-bump. S.B. performed immunoblots and proliferation assays. K.A.D. performed, analyzed, and compared expression proteomics. Y.L. synthesized YKL-06-102. E.S.W. performed immunoblots and gave experimental advice. R.P.N. and J.C.Y. conducted recombinant CRBN binding assays. T.Z. and N.K. gave experimental and analytic advice. A.C.M. performed proteomics experiments. E.S.F. analyzed data and supervised aspects of the project. N.S.G. and G.E.W. wrote the manuscript, analyzed data, and have the overall project responsibility.

DECLARATION OF INTERESTS

E.S.F. is a member of the scientific advisory board of C4 Therapeutics and is a consultant to Novartis Pharmaceuticals. E.S.F. receives research funding from Novartis Pharmaceuticals and Astellas Pharma not related to this work. N.S.G. is equity holder and scientific advisor for Syros, Gatekeeper, Soltego, C4, Petra, and Aduro companies. N.S.G., B.J., T.Z., N.K., B.N., and E.S.F. are inventors on a patent covering CDK6 degraders owned by Dana-Farber.

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REFERENCES

Alanis-Lobato, G., Andrade-Navarro, M.A., and Schaefer, M.H. (2017). HIPPIE v2.0: enhancing meaningfulness and reliability of protein-protein interaction networks. Nucleic Acids Res. *45*, D408–D414.

An, J., Ponthier, C.M., Sack, R., Seebacher, J., Stadler, M.B., Donovan, K.A., and Fischer, E.S. (2017). pSILAC mass spectrometry reveals ZFP91 as IMiD-dependent substrate of the CRL4(CRBN) ubiquitin ligase. Nat. Commun. *8*, 15398.

Anders, L., Ke, N., Hydbring, P., Choi, Y.J., Widlund, H.R., Chick, J.M., Zhai, H., Vidal, M., Gygi, S.P., Braun, P., et al. (2011). A systematic screen for CDK4/6 substrates links FOXM1 phosphorylation to senescence suppression in cancer cells. Cancer Cell *20*, 620–634.

Bondeson, D.P., Smith, B.E., Burslem, G.M., Buhimschi, A.D., Hines, J., Jaime-Figueroa, S., Wang, J., Hamman, B.D., Ishchenko, A., and Crews, C.M. (2018). Lessons in PROTAC design from selective degradation with a promiscuous warhead. Cell Chem. Biol. *25*, 78–87.e5.

Casado, P., Rodriguez-Prados, J.C., Cosulich, S.C., Guichard, S., Vanhaesebroeck, B., Joel, S., and Cutillas, P.R. (2013). Kinase-substrate enrichment analysis provides insights into the heterogeneity of signaling pathway activation in leukemia cells. Sci. Signal. *6*, rs6.

Chen, P., Lee, N.V., Hu, W., Xu, M., Ferre, R.A., Lam, H., Bergqvist, S., Solowiej, J., Diehl, W., He, Y.A., et al. (2016). Spectrum and degree of CDK drug interactions predicts clinical performance. Mol. Cancer Ther. *15*, 2273–2281.

Fry, D.W., Harvey, P.J., Keller, P.R., Elliott, W.L., Meade, M., Trachet, E., Albassam, M., Zheng, X., Leopold, W.R., Pryer, N.K., et al. (2004). Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. Mol. Cancer Ther. 3, 1427–1438.

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

Gandhi, A.K., Kang, J., Havens, C.G., Conklin, T., Ning, Y., Wu, L., Ito, T., Ando, H., Waldman, M.F., Thakurta, A., et al. (2014). Immunomodulatory agents lenalidomide and pomalidomide co-stimulate T cells by inducing degradation of T cell repressors lkaros and Aiolos via modulation of the E3 ubiquitin ligase complex CRL4(CRBN.). Br. J. Haematol. *164*, 811–821.

Huang, H.T., Dobrovolsky, D., Paulk, J., Yang, G., Weisberg, E.L., Doctor, Z.M., Buckley, D.L., Cho, J.H., Ko, E., Jang, J., et al. (2018). A chemoproteomic approach to query the degradable kinome using a multi-kinase degrader. Cell Chem. Biol. *25*, 88–99.e6.

Klijn, C., Durinck, S., Stawiski, E.W., Haverty, P.M., Jiang, Z., Liu, H., Degenhardt, J., Mayba, O., Gnad, F., Liu, J., et al. (2015). A comprehensive transcriptional portrait of human cancer cell lines. Nat. Biotechnol. *33*, 306–312.

Kollmann, K., Heller, G., Schneckenleithner, C., Warsch, W., Scheicher, R., Ott, R.G., Schafer, M., Fajmann, S., Schlederer, M., Schiefer, A.I., et al. (2013). A kinase-independent function of CDK6 links the cell cycle to tumor angiogenesis. Cancer Cell *24*, 167–181.

Kronke, J., Udeshi, N.D., Narla, A., Grauman, P., Hurst, S.N., McConkey, M., Svinkina, T., Heckl, D., Comer, E., Li, X., et al. (2014). Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. Science *343*, 301–305.

Lu, G., Middleton, R.E., Sun, H., Naniong, M., Ott, C.J., Mitsiades, C.S., Wong, K.K., Bradner, J.E., and Kaelin, W.G., Jr. (2014). The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins. Science *343*, 305–309.

Lu, J., Qian, Y., Altieri, M., Dong, H., Wang, J., Raina, K., Hines, J., Winkler, J.D., Crew, A.P., Coleman, K., et al. (2015). Hijacking the E3 ubiquitin ligase cereblon to efficiently target BRD4. Chem. Biol. *22*, 755–763.

Meyers, R.M., Bryan, J.G., McFarland, J.M., Weir, B.A., Sizemore, A.E., Xu, H., Dharia, N.V., Montgomery, P.G., Cowley, G.S., Pantel, S., et al. (2017). Computational correction of copy number effect improves specificity of CRISPR-Cas9 essentiality screens in cancer cells. Nat. Genet. *49*, 1779–1784.

Nowak, R.P., DeAngelo, S.L., Buckley, D., He, Z., Donovan, K.A., An, J., Safaee, N., Jedrychowski, M.P., Ponthier, C.M., Ishoey, M., et al. (2018). Plasticity in binding confers selectivity in ligand-induced protein degradation. Nat. Chem. Biol. *14*, 706–714.

O'Leary, B., Finn, R.S., and Turner, N.C. (2016). Treating cancer with selective CDK4/6 inhibitors. Nat. Rev. Clin. Oncol. *13*, 417–430.

Olson, C.M., Jiang, B., Erb, M.A., Liang, Y., Doctor, Z.M., Zhang, Z., Zhang, T., Kwiatkowski, N., Boukhali, M., Green, J.L., et al. (2018). Pharmacological perturbation of CDK9 using selective CDK9 inhibition or degradation. Nat. Chem. Biol. *14*, 163–170.

Remillard, D., Buckley, D.L., Paulk, J., Brien, G.L., Sonnett, M., Seo, H.S., Dastjerdi, S., Wuhr, M., Dhe-Paganon, S., Armstrong, S.A., et al. (2017). Degradation of the BAF complex factor BRD9 by heterobifunctional ligands. Angew. Chem. Int. Ed. *56*, 5738–5743.

Tigan, A.S., Bellutti, F., Kollmann, K., Tebb, G., and Sexl, V. (2016). CDK6 - a review of the past and a glimpse into the future: from cell-cycle control to transcriptional regulation. Oncogene *35*, 3083–3091.

Wang, H., Nicolay, B.N., Chick, J.M., Gao, X., Geng, Y., Ren, H., Gao, H., Yang, G., Williams, J.A., Suski, J.M., et al. (2017). The metabolic function of cyclin D3-CDK6 kinase in cancer cell survival. Nature *546*, 426–430.

Winter, G.E., Buckley, D.L., Paulk, J., Roberts, J.M., Souza, A., Dhe-Paganon, S., and Bradner, J.E. (2015). Drug development. Phthalimide conjugation as a strategy for in vivo target protein degradation. Science *348*, 1376–1381.

Zengerle, M., Chan, K.H., and Ciulli, A. (2015). Selective small molecule induced degradation of the BET bromodomain protein BRD4. ACS Chem. Biol. *10*, 1770–1777.

STAR***METHODS**

KEY RESOURCES TABLE

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REAGENT OF RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CDK4	Cell Signaling	Cat#12790; RRID: AB_2631166
CDK6	Santa Cruz	Cat#sc-7961; RRID: AB_627242
Histone 3	Abcam	Cat#ab1791; RRID: AB_302613
β-actin	Sigma-Aldrich	Cat#A5441; RRID: AB_476744
p-Rb S780	Cell Signaling	Cat#8180; RRID: AB_10950972
Rb	Cell Signaling	Cat#9309; RRID: AB_823629
Peroxidase-conjugated AffiniPure Goat Anti- Mouse IgG	Jackson Immuno Research	Cat#115-035-003; RRID: AB_10015289
Peroxidase-conjugated AffiniPure Goat Anti- Rabbit IgG	Jackson Immuno Research	Cat#111-035-003; RRID: AB_2313567
Chemicals, Peptides, and Recombinant Proteins		
BSJ-03-123	This study	N/A
BSJ-bump	This study	N/A
YKL-06-102	This study	N/A
Palbociclib	Selleckchem	Cat#S1116
Critical Commercial Assays		
Caspase-Glo 3/7	Promeça	Cat#G8090
NanoBiT	Promeça	Cat#N2011
	i loniogu	
	Maximum at al. 0017	
Project Achilles: CHISPH-Casy Avana	Meyers et al., 2017	datasets/18/download
Gene expression data of 675 cell lines	Klijn et al., 2015	https://www.nature.com/articles/nbt.3080# supplementary-information
ENCODE transcription factor targets	ENCODE Consortium	http://amp.pharm.mssm.edu/Harmonizome/ dataset/ENCODE+Transcription+Factor+Targets
Human protein-protein interaction network	Alanis-Lobato et al., 2017	http://cbdm-01.zdv.uni-mainz.de/~mschaefer/ hippie/
Human reference genome NCBI build 38, GRCh38		http://hgdownload.cse.ucsc.edu/goldenPath/ hg38/bigZips/
RNA sequencing upon CDK6 degradation	This study	GEO: GSE116187
CDK4/6 targets	Anders et al., 2011	https://doi.org/10.1016/i.ccr.2011.10.001
Experimental Models: Cell Lines		
MV4-11	ATCC	BBID: CVCI 0064
THP-1	ATCC	BBID: CVCL_0006
MOL M13	DMSZ	BBID: CVCL_2119
	CCLE/Broad Instituto	
	ATCC	
NCI-H338	ATCC	RRID: CVCL_1559
	ATCO	
HEK2931	AICC	RRID: CVCL_0063
HS5781	AICC	RRID: CVCL_0332
Oligonucleotides		
sgCDK4 sense (5'- CACCGAGCCACTGGCTCAT ATCGAG-3')	Sigma-Aldrich	N/A
sgCDK4 antisense (5'- AAACCTCGATATGAGCC AGTGGCTC-3')	Sigma-Aldrich	N/A

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Continued			
REAGENT or RESOURCE	SOURCE	IDENTIFIER	
sgCRBN sense (5'- CACCGTAAACAGAC ATGGCCGGCGA-3')	Sigma-Aldrich	N/A	
sgCRBN antisense (5'- AAACTCGCCGGCCATG TCTGTTTAC-3')	Sigma-Aldrich	N/A	
Recombinant DNA			
Lenti_sgRNA_EFS_GFP	Addgene	https://www.addgene.org/65656	
pBit1.1-C [TK/LgBiT]	Promega	Cat#N2014	
pBit2.1-N [TK/SmBiT]	Promega	Cat#N2014	
Software and Algorithms			
Cytoscape	Institute for Systems Biology	http://cytoscape.org; RRID: SCR_003032	
GraphPad Prism		https://www.graphpad.com/scientific- software/prism/	
Proteome Discoverer	Thermo Fisher Scientific	https://www.thermofisher.com/order/catalog/ product/OPTON-30795	
KSEA App		https://casecpb.shinyapps.io/ksea	
STAR Aligner		https://github.com/alexdobin/STAR	
limma		https://bioconductor.org/packages/release/bioc/ html/limma.html	
GOrilla		http://cbl-gorilla.cs.technion.ac.il/	
networkx		https://networkx.github.io/	

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Georg Winter (gwinter@cemm.at).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

MV4-11 (RRID:CVCL_0064), THP-1 (RRID:CVCL_0006), MOLM13 (RRID:CVCL_2119), P31/FUJ (RRID:CVCL_1632), MOLT4 (RRID:CVCL_0013), NCI-H358 (RRID:CVCL_1559) and HT29 (RRID:CVCL_0320) cells were cultured in RPMI-1640 growth medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% Penicillin/Streptomycin solution (ThermoFisher Scientific). HEK293T (RRID:CVCL_0063) and Hs578T (RRID:CVCL_0332) cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% Penicillin/Streptomycin solution (ThermoFisher Scientific). HEK293T (RRID:CVCL_0063) and Hs578T (RRID:CVCL_0332) cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% Penicillin/Streptomycin solution (ThermoFisher Scientific). All cell lines were cultured at 37°C with 5% CO₂ and tested for mycoplasma contamination and cell identity.

Sex of human cell lines: MV4-11 (Male, 10 years old), THP-1 (Male, 1 year old), MOLM13 (Male, 20 years old), P31/FUJ (Male, 7 years old), MOLT4 (Male, 19 years old), NCI-H358 (Male, age unknown), HT29 (Female, 44 years old), HEK293T (Female, fetus), Hs578T (Female, 74 years old).

METHOD DETAILS

Knockout Generation

sgRNAs (CDK4: 5'-AGCCACTGGCTCATATCGAG-3', CRBN: 5'- TAAACAGACATGGCCGGCGA-3') were cloned into Lenti_ sgRNA_EFS_GFP (addgene #65656). Cells were transduced with lentiviral particles, selected by sorting for GFP positive cells and successful knockout verified by immunoblot and Sanger sequencing.

Proliferation Assays

For growth over time experiments of suspension cell lines, 300.000 cells per well were seeded in 24-well plates in triplicates. Cells were counted and treatments renewed every 2 days. For growth over time experiments of adherent cell lines, 100.000 cells per well were seeded in 12-well plates in triplicates. Cells were trypsinized and counted and treatments renewed every 3 days. For colony formation assays, 1000 cells per well were seeded in 6-well plates in duplicates. Every 2 days, culture medium was exchanged and treatments were renewed. After 12 days, cells were fixed in 1% PFA in PBS for 15 min at room temperature, washed 3 times with PBS and stained in Crystal Violet solution (0.1% in 10% EtOH) for 15 min at room temperature. The cells were again washed 3 times with PBS and left to dry overnight.

Cell Cycle and Apoptosis

For cell cycle measurement, 10^6 cells per well were seeded in 24-well plates in triplicates. After 24 h of drug treatment, cells were harvested by centrifugation at 500g for 5 min and washed in 1 ml cold PBS. Cells were spun down again, resuspended in 200 µl PBS and fixed by addition of 800 µl 70% EtOH and incubation for 20 min at -20°C. Cells were washed with 1 ml PBS and stained with 500 µl propidium iodide solution (50 µg/ml propidium iodide (Sigma Aldrich), 200 µg/ml RNase A (ThermoFisher Scientific), in PBS). Cellular DNA content was measured by flow cytometry using FACSCalibur (BD Biosciences) and analyzed using the FlowJo v10 software.

Analysis of apoptotic cells was performed using Caspase-Glo 3/7 (Promega) according to manufacturer's instructions. 20 000 cells per well were seeded in triplicate in a white 96-well plate in a total volume of 50 µl. After 24 h incubation with the treatment, 45 µl Caspase-Glo substrate were added per well. The plate was incubated at room temperature in the dark for 1 h and luminescence measured on a Victor X3 (Perkin Elmer) microplate reader. Luminescent signal was normalized to vehicle-treated cells.

In Vitro CRBN Binding Assay

Compounds in Atto565-Lenalidomide displacement assay were dispensed in a 384-well microplate (Corning, 4514) using D300e Digital Dispenser (HP) and normalized to 1% DMSO into 10 nM Atto565-Lenalidomide, 100 nM DDB1 Δ B-CRBN, 50 mM Tris pH 7.5, 200 mM NaCl, 0.1% Pluronic F-68 solution (Sigma). The change in fluorescence polarization was monitored using a PHERAstar FS microplate reader (BMG Labtech) for 30 cycles of 187s each. Data from three independent measurements (n = 3) was plotted and IC50 values estimated using variable slope equation in GraphPad Prism 7.

Immunoblotting

2x10⁶ cells were lysed in RIPA buffer (150 nM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0), protease inhibitors (Thermo Scientific), benzonase (Novagen)). Cell lysates containing 40 μg of protein were loaded on BoltTM 4-12% Bis-Tris Plus Gels (Thermo Scientific) and ran for SDS-PAGE before transfer onto 0.45 μM nitrocellulose membrane (GE Healthcare). Following antibodies were used for detection:

Name	Origin	Cat. No.
CDK4	Cell Signaling	#12790
CDK6	Santa Cruz	sc-7961
Histone 3	Abcam	ab1791
β-actin	Sigma-Aldrich	A5441
p-Rb S780	Cell Signaling	#8180
Rb	Cell Signaling	#9309
Peroxidase-conjugated AffiniPure Goat Anti- Mouse IgG	Jackson Immuno Research	115-035-003
Peroxidase-conjugated AffiniPure Goat Anti- Rabbit IgG	Jackson Immuno Research	111-035-003

Cellular Thermal Shift Assay

 $4x10^{6}$ MV4-11 CRBN-/- cells were treated for 3 h with 20 μ M palbociclib, BSJ-03-123 or vehicle. $1x10^{6}$ cells were spun down at 500 x g for 5 min, and the supernatant removed. Pellets were incubated at 46, 49, 52 or 55°C for 3 min followed by 3 min incubation at room temperature. 30 μ l of lysis buffer (20 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, protease inhibitors) were added and cells lysed by 3 rounds of snap freezing and thawing. Denatured proteins were removed by 20 min centrifugation at 14.000 x g at 4°C and supernatants analyzed by Western Blotting.

NanoBiT® Assay

The NanoBiT® assay (Promega) was performed following manufacturer's instructions. Full-length CDK4 and CDK6 were cloned into pBit1.1-C [TK/LgBiT] vector, full-length CRBN was cloned into pBit2.1-N [TK/SmBiT]. 400.000 CRBN-deficient HEK293T cells were seeded in a 6-well plate and transfected the day after with 750 μ g each of CDK4/6-LgBit and CRBN-SmBit using Lipofectamine (Thermo Fisher Scientific) according to manufacturer's instructions. After 24 h, cells were detached and 50.000 cells per well seeded in a 96-well plate and allowed to attach overnight. Before the assay, the medium was exchanged with 100 μ l of ambient temperature fresh medium supplemented with 25 mM HEPES and the plate incubated for 10 min at room temperature to equilibrate. 25 μ l Nano-Glo® Live Cell Reagent was added per well, incubated for 5 min and baseline luminescence measured on a Victor X3 (Perkin Elmer) with 2 s integration time. 10 μ l of 13.5X drug stock were added per well and emission of luminescent signal monitored by continuous measurement every 2 min. Signals were normalized to baseline and vehicle treated cells.

Expression Proteomics BSJ-03-123

Sample Preparation

15x10⁶ MOLT4 cells were treated with 100 nM BSJ-03-123 for 2 h, in triplicate. Cells were washed three times with PBS, the supernatant aspirated and pellets snap-frozen in liquid N₂. Each washed cell pellet was lysed separately in 40 µL of freshly prepared lysis buffer containing 50 mM HEPES (pH 8.0), 2% SDS, 0.1 M DTT, 1 mM PMSF, and protease inhibitor cocktail (Sigma-Aldrich). Samples rested at RT for 20 minutes before heating to 99°C for 5 min. After cooling down to RT, DNA was sheared by sonication using a Covaris S2 high performance ultrasonicator. Cell debris were removed by centrifugation at 20.000 × g for 15 min at 20°C. Supernatant was transferred to fresh eppendorf tubes and protein concentration determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). FASP was performed using a 30 kDa molecular weight cutoff filter (VIVACON 500). Fifty microliters of each cleared protein extract were mixed with 200 µL of freshly prepared 8 M urea in 100 mM Tris-HCI (pH 8.5) (UA-solution) in the filter unit and centrifuged at 14.000 × g for 15 min at 20°C to remove SDS. Any residual SDS was washed out by a second washing step with 200 µL of UA. The proteins were alkylated with 100 µL of 50 mM iodoacetamide in the dark for 30 min at RT. Afterward, three washing steps with 100 µL of UA solution were performed, followed by three washing steps with 100µL of 50 mM TEAB buffer (Sigma-Aldrich). Proteins were digested with trypsin overnight at 37°C. Peptides were recovered using 40 µL of 50 mM TEAB buffer followed by 50 μL of 0.5 M NaCl (Sigma-Aldrich). Peptides were desalted using C18 solid phase extraction spin columns (The Nest Group, Southborough, MA). After desalting, peptides were labeled with TMT 10plex™ reagents according to the manufacturer (Pierce, Rockford, IL). After quenching of the labeling reaction, labeled peptides were pooled, organic solvent removed in a vacuum concentrator at 45°C and reconstituted in 5% acetonitrile containing 20mM ammonia formate buffer, pH 10 for offline fractionation using high pH reversed phase liquid chromatography (2D-RP/RP-HPLC).

2D-RP/RP Liquid Chromatography Mass Spectrometry

Two-dimensional liquid chromatography was performed by reverse-phase chromatography at high and low pH. In the first dimension, peptides were separated on a Gemini-NX C18 (150 × 2 mm, 3 μm, 110 Å, Phenomenex, Torrance, USA) in 20 mM ammonia formate buffer, pH 10, and eluted over 45 min by a 5-70% acetonitrile gradient at 100 μL/min using an Agilent 1200 HPLC system (Agilent Biotechnologies, Palo Alto, CA, USA). Ninty-six time-based fractions were collected and consolidated into 40 fractions. After solvent removal in a vacuum concentrator, samples were reconstituted in 5% formic acid for LC-MS/MS analysis. Mass spectrometry was performed on an Orbitrap Fusion Lumos mass spectrometer (ThermoFisher Scientific, San Jose, CA) coupled to an Agilent 1200 HPLC nanoflow system (Agilent Biotechnologies, Palo Alto, CA) via nanoflex source interface. Tryptic peptides were loaded onto a trap column (Zorbax 300SB-C18 5 μm, 5 × 0.3 mm, Agilent Biotechnologies) at a flow rate of 45 μL/min using 0.1% TFA as loading buffer. After loading, the trap column was switched in-line with a 25 cm, 75 µm inner diameter analytical column (packed in-house with ReproSil-Pur 120 C18-AQ, 3 µm, Dr. Maisch, Ammerbuch-Entringen, Germany). Mobile-phase A consisted of 0.4% formic acid in water and mobile-phase B of 0.4% formic acid in a mix of 90% acetonitrile and 10% water. The flow rate was set to 230 nL/min and a 90 min gradient used (6 to 30% solvent B within 81 min, 30 to 65% solvent B within 8 min and, 65 to 100% solvent B within 1 min, 100% solvent B for 6 min before equilibrating at 6% solvent B for 18 min). Analysis was performed in a data-dependent acquisition mode using the multi-notch MS3-based TMT method (termed SPS-MS3 on the Lumos instrument). MS1 spectra were acquired within a mass range of 350 - 1550 m/z in the orbitrap at a resolution of 120,000 (at 200Th). Automatic gain control (AGC) was set to a target of 2×10^5 and a maximum injection time of 50 ms. Precursor ions for MS2/MS3 analysis were selected using a Top10 dependent scan approach. MS2 spectra were acquired in the linear quadrupole ion trap (IT) using a quadrupole isolation window of 0.8Da; collision induced dissociation (CID) for fragmentation; a normalized collision energy (NCE) of 35%; an AGC target of 4 × 10³; and a maximum injection time of 150 ms. For TMT quantitation, each MS2 scan followed a SPS-MS3 scan of the same precursor ion using the multiple frequency notches approach 3. The quadrupole isolation window for MS3 scans was set to 2 Da and the top 5 most intense MS2 fragment ions were isolated by SPS for fragmentation by higher energy collision-induced dissociation (HCD) using a NCE of 65%. Resulting fragment ions were analyzed in the Orbitrap at a resolution of 50,000 (at 200 Th). AGC settings were 5 × 10⁴ and a maximum injection time of 150 ms. Dynamic exclusion for selected ions was 60 s. A single lock mass at m/z 445.120024 was employed. Xcalibur version 4.0.0 and Tune 2.1 were used to operate the instrument.

Expression Proteomics YKL-06-102

MOLT4 cells were treated with DMSO or 500 nM of compound YKL-06-102 in biological triplicates for 5 hours and cells harvested by centrifugation. Lysis buffer (8 M Urea, 50 mM NaCl, 50 mM 4-(2hydroxyethyl)-1-piperazineethanesulfonic acid (EPPS) pH 8.5, Protease and Phosphatase inhibitors from Roche) was added to the cell pellets and homogenized by 20 passes through a 21 gauge (1.25 in. long) needle to achieve a cell lysate with a protein concentration between 1 – 4 mg mL-1. A micro-BCA assay (Pierce) was used to determine the final protein concentration in the cell lysate. 200 µg of protein for each sample were reduced and alkylated.

Proteins were precipitated using methanol/chloroform. In brief, four volumes of methanol were added to the cell lysate, followed by one volume of chloroform, and finally three volumes of water. The mixture was vortexed and centrifuged to separate the chloroform phase from the aqueous phase. The precipitated protein was washed with three volumes of methanol, centrifuged and the resulting washed precipitated protein was allowed to air dry. Precipitated protein was resuspended in 4 M Urea, 50 mM HEPES pH 7.4, followed by dilution to 1 M urea with the addition of 200 mM EPPS, pH 8. Proteins were first digested with LysC (1:50; enzyme:protein) for 12 hours at room temperature. The LysC digestion was diluted to 0.5 M Urea with 200 mM EPPS pH 8 followed by digestion with trypsin (1:50; enzyme:protein) for 6 hours at 37°C. Tandem mass tag (TMT) reagents (Thermo Fisher Scientific) were dissolved in anhydrous acetonitrile (ACN) according to manufacturer's instructions. Anhydrous ACN was added to each peptide sample to a final

Data were collected using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a Proxeon EASY-nLC 1200 LC pump (Thermo Fisher Scientific). Peptides were separated on a 75 μ M inner diameter microcapillary column packed with ~40 cm of Accucore C18 resin (1.6 μ M, 100 Å, Thermo Fisher Scientific). Peptides were separated using a 150 min gradient of 6–27% acetonitrile in 1.0% formic acid with a flow rate of 400 nL/min.

Each analysis used an MS3-based TMT method. The data were acquired using a mass range of m/z 340 – 1350, resolution 120,000, AGC target 1 x 106, maximum injection time 100 ms, dynamic exclusion of 120 seconds for the peptide measurements in the Orbitrap. Data dependent MS2 spectra were acquired in the ion trap with a normalized collision energy (NCE) set at 35%, AGC target set to 1.8 x 104 and a maximum injection time of 120 ms. MS3 scans were acquired in the Orbitrap with a HCD collision energy set to 55%, AGC target set to 1.5 x 105, maximum injection time of 150 ms, resolution at 50,000 and with a maximum synchronous precursor selection (SPS) precursors set to 10.

Phosphoproteomics

 $15x10^{6}$ MV4-11 cells were treated with 200 nM BSJ-03-123 or Palbociclib for 2 h, in triplicate. Cells were washed three times with PBS, the supernatant aspirated and pellets snap-frozen in liquid N₂.

Each washed cell pellet was lysed separately in 40 µL of freshly prepared lysis buffer containing 50 mM HEPES (pH 8.0), 2% SDS, 0.1 M DTT, 1 mM PMSF, phosSTOP and protease inhibitor cocktail (Sigma-Aldrich). Samples rested at RT for 20 minutes before heating to 99°C for 5 min. After cooling down to RT, DNA was sheared by sonication using a Covaris S2 high performance ultrasonicator. Cell debris was removed by centrifugation at 20.000 X g for 15 min at 20°C. Supernatant was transferred to fresh eppendorf tubes and protein concentration determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). FASP was performed using a 30 kDa molecular weight cutoff filter (VIVACON 500). In brief, 100 µg total protein per sample were reduced by adding DTT at a final concentration of 83.3 mM followed by incubation at 99°C for 5 min. After cooling to room temperature, samples were mixed with 200 µL of freshly prepared 8 M urea in 100 mM Tris-HCI (pH 8.5) (UA-solution) in the filter unit and centrifuged at 14.000 × g for 15 min at 20°C to remove SDS. Any residual SDS was washed out by a second washing step with 200 μL of UA. The proteins were alkylated with 100 µL of 50 mM iodoacetamide in the dark for 30 min at RT. Afterward, three washing steps with 100 µL of UA solution were performed, followed by three washing steps with 100µL of 50 mM TEAB buffer (Sigma-Aldrich). Proteins were digested with trypsin at a ratio of 1:35 overnight at 37°C. Peptides were recovered using 40 µL of 50 mM TEAB buffer followed by 50 µL of 0.5 M NaCl (Sigma-Aldrich). Peptides were desalted using C18 solid phase extraction spin columns (The Nest Group, Southborough, MA). After desalting, peptides were labeled with TMT 10plex™ reagents (label 128N was omitted) according to the manufacturer (Pierce, Rockford, IL). After quenching of the labeling reaction, labeled peptides were pooled, organic solvent removed in vacuum concentrator and labelled peptides loaded onto a SPE column. Peptides were eluted with 300µL 80% acetonitrile containing 0.1% trifluoroacetic to achieve a final peptide concentration of $\sim 1 \mu g/\mu l$. Eluate was then used for phosphopeptide enrichment via immobilized metal affinity chromatography (IMAC). Briefly, two times 100 μL of Ni-NTA superflow slurry (QIAGEN Inc., Valencia, USA) were washed with LCMS-grade water and Ni²⁺ stripped off the beads by incubation with 100 mM of EDTA, pH 8 solution for 1 hr at room temperature. Stripped NTA resin was recharged with Fe³⁺-ions by incubation with a fresh solution of Fe(III)Cl₃ and 2 x 110 µL of charged resin slurry used for the enrichment of a total of ~900 µg TMT-labelled peptide. The unbound fraction was transferred to a fresh glass vial and used for offline fractionation for the analysis of the whole chromatome proteome. After washing the slurry with 0.1% TFA, phosphopeptides were eluted with a freshly prepared ammonia solution containing 3mM EDTA, pH 8 and all used for offline fractionation for the analysis of the phophoproteome.

Offline Fractionation via RP-HPLC at High pH

Peptides were re-buffered in 20 mM ammonium formiate buffer shortly before separation by reversed phase liquid chromatography at pH 10. Phosphopeptides was separated into 20 fractions on a Dionex column (500 μ m × 50 mm PepSwift RP, monolithic, Dionex Corporation, Sunnyvale, CA, USA) using an Agilent 1200 series nanopump delivering solvent at 4 μ L/min. Peptides were separated by applying a gradient of 90% acconitrile containing 20 mM ammonium formiate, pH 10. After solvent removal in a vacuum concentrator, samples were reconstituted in 0.1% trifluroacetic acid (TFA) for LC-MS/MS analysis. Prepared samples were kept at -80°C until the analysis.

2D-RP/RP Liquid Chromatography Mass Spectrometry

Mass spectrometry was performed on an Orbitrap Fusion Lumos mass spectrometer (ThermoFisher Scientific, San Jose, CA) coupled to an Dionex Ultimate 3000RSLC nano system (ThermoFisher Scientific, San Jose, CA) via nanoflex source interface. Tryptic

peptides were loaded onto a trap column (Pepmap 100 5µm, 5 × 0.3 mm, ThermoFisher Scientific, San Jose, CA) at a flow rate of 10 µL/min using 2% ACN and 0.1% TFA as loading buffer. After loading, the trap column was switched in-line with a 30 cm, 75 µm inner diameter analytical column (packed in-house with ReproSil-Pur 120 C18-AQ, 3 µm, Dr. Maisch, Ammerbuch-Entringen, Germany). Mobile-phase A consisted of 0.4% formic acid in water and mobile-phase B of 0.4% formic acid in a mix of 90% acetonitrile and 10% water. The flow rate was set to 230 nL/min and a 90 min gradient used (6 to 30% solvent B within 81 min, 30 to 65% solvent B within 8 min and, 65 to 100% solvent B within 1 min, 100% solvent B for 6 min before equilibrating at 6% solvent B for 18 min). Analysis was performed in a data-dependent acquisition mode. Full MS scans were acquired with a mass range of 375 -1650 m/z in the orbitrap at a resolution of 120,000 (at 200Th). Automatic gain control (AGC) was set to a target of 2×10^5 and a maximum injection time of 50 ms. Precursor ions for MS² analysis were selected using a TopN dependant scan approach with a cycle time of 3 seconds. MS² spectra were acquired in the orbitrap (FT) using a quadrupole isolation window of 1 Da and higher energy collision induced dissociation (HCD) at a normalized collision energy (NCE) of 38%. AGC target was set to 5 × 10⁴ with a maximum injection time of 150 ms and MS² scans acquired at a resolution of 15,000 (at 200 Th). Dynamic exclusion for selected ions was 60 s. A single lock mass at m/z 445.120024 was employed. Xcalibur version 4.0.0 and Tune 2.1 were used to operate the instrument.

RNA Sequencing

3x10⁶ MV4-11 cells per well were seeded in triplicate in a 12-well plate and treated with 200 nM BSJ-03-123, YKL-06-102, Palbociclib or vehicle for 6 h. Cells were lysed using QIAshredder columns (QIAGEN) and RNA isolated using RNeasy kit (QIAGEN) according to manufacturer's instructions.

The amount of total RNA was quantified using the Qubit Fluorometric Quantitation system (Life Technologies) and the RNA integrity number (RIN) was determined using the Experion Auto-mated Electrophoresis System (Bio-Rad). RNA-seg libraries were prepared with the TruSeg Stranded mRNA LT sample preparation kit (Illumina) using both, Sciclone and Zephyr liquid handling robotics (PerkinElmer). Library concentrations were quantified with the Qubit Fluorometric Quantitation system (Life Technologies) and the size distribution was assessed using the Experion Automated Electrophoresis System (Bio-Rad). For sequencing, samples were diluted and pooled into NGS libraries in equimolar amounts. Expression profiling libraries were sequenced on Illumina HiSeq 3000/4000 instruments in 50-base-pair-single-end mode and base calls provided by the Illumina Real-Time Analysis (RTA) software were subsequently converted into BAM format (Illumina2bam) before de-multiplexing (BamIndexDecoder) into individual, sample-specific BAM files via Illumina2bam tools (1.17.3).

Chemical Synthesis of BSJ-03-123, BSJ-03-190/BSJ-bump, YKL-06-102

Synthesis of 6a and 6b

2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetic acid (6a). 6a was prepared according to the literature (Remillard et al., 2017). 3-Hydroxyphthalic anhydride (1) (1.64 g, 10 mmol) and 3-aminopiperidine-2,6-dione hydrochloride (2a) (1.65 g, 10 mmol) were dissolved in pyridine (40 mL, 0.25 M) and heated to 110°C. After 14h, the mixture was cooled to room temperature and concentrated under reduced pressure. The residue was purification by column chromatography on silica gel (0-10% MeOH/DCM) to give 2-(2,6-dioxopiperidin-3-yl)-4-hydroxyisoindoline-1,3-dione (3a) as a grey solid (2.41 g, 88%). LC-MS: m/z 275 [M+1]. To a solution of 3a (2.19 g, 8 mmol) in 8 mL of DMF was added K₂CO₃ (1.66 g, 12 mmol) and t-butyl bromoacetate (4) (1.18 mL, 8 mmol) respectively. The mixture was stirred at room temperature for 2h, then diluted with EtOAc and washed once with water then twice with brine. The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue was purification by column chromatography on silica gel (5-100%EtOAc/hexanes) to give tert-butyl 2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetate (5a) as a cream colored solid (2.70 g, 87%). LC-MS: m/z 389 [M+1]. 5a (2.06g, 5.3 mmol) was then dissolved in TFA (53 mL, 0.1M) at room temperature. After 4 hours, the solution was diluted with DCM and concentrated under reduced pressure to give 6a as a cream colored solid (1.5 g, 85%) was deemed sufficiently pure and carried onto the next step without further purification. LC-MS: m/z 333 [M+1]. ¹H NMR (500 MHz, DMSO-d6) δ 11.09 (s, 1H), 7.79 (dd, J = 8.4, 7.4 Hz, 1H), 7.48 (d, J = 7.4 Hz, 1H), 7.39 (d, J = 8.6 Hz, 1H), 5.10 (dd, J = 12.8, 5.4 Hz, 1H), 4.99 (s, 2H), 2.93-2.89 (m, 1H), 2.63-2.51 (m, 2H), 2.11-2.03 (m, 1H).

2-((2-(1-methyl-2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetic acid (6b). 6b was synthesized with similar procedures as 6a from 3-Hydroxyphthalic anhydride (1) (328 mg, 2 mmol), 3-amino-1-methylpiperidine-2,6-dione (2b) (357 mg, 2 mmol) and t-butyl bromoacetate (4) (0.295 mL, 2 mmol). 6b was obtained as an off-white solid (451 mg, 65% yield in 3 steps). LC-MS: m/z 347 [M+1]. ¹H NMR (500 MHz, DMSO-d₆) δ 13.24 (s, 1H), 7.80 (dd, J = 8.5, 7.3 Hz, 1H), 7.48 (d, J = 7.3 Hz, 1H), 7.40 (d, J = 8.5 Hz, 1H), 5.17 (dd, J = 13.0, 5.4 Hz, 1H), 4.99 (s, 2H), 3.02 (s, 3H), 2.99-2.91 (m, 1H), 2.80-2.73 (m, 1H), 2.59-2.52 (m, 1H), 2.09-2.02 (m, 1H). ¹³C NMR (125 MHz, DMSO) & 172.24, 170.14, 169.95, 167.17, 165.62, 155.61, 137.26, 133.70, 120.36, 116.76, 116.24, 65.46, 49.82, 31.56, 27.07, 21.65.

Synthesis of BSJ-03-123 and BSJ-03-190/BSJ-Bump

tert-butyl (2-(2-(2-(2-(4-(6-((6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl)ethoxy)ethoxy)ethoxy)ethyl)carbamate (9). To a suspension of Palbociclib (7) (100 mg, 0.22 mmol) in DMSO (5 mL) was added tert-butyl (2-(2-(2-(2-bromoethoxy)ethoxy)ethoxy)ethoxy)bamate (8) (156 mg, 0.44 mmol) and DIPEA (0.115 mL, 0.66 mmol). The mixture was heated to 80°C and kept stirring for 48h. The mixture was then cooled down to room temperature, extracted, dried, filtered and concentrated. The residue was purified by reverse phase HPLC (5-95% MeOH in H₂O) to give 9 (TFA salt) as a yellow solid (103mg, 65%). LC-MS: m/z 723 [M+1]. ¹H NMR (500 MHz, DMSO-d₆) δ 10.34 (s, 1H), 8.97 (s, 1H), 8.12 (d, J = 3.0 Hz, 1H), 7.90 (d, J = 9.1 Hz, 1H), 7.64-7.58 (m, 1H), 6.81-6.74 (m, 1H), 5.89-5.78 (m, 1H), 3.93-3.75 (m, 4H), 3.67-3.60 (m, 4H), 3.59-3.55 (m, 2H), 3.55-3.46 (m, 4H), 3.44-3.35 (m, 4H), 3.27 (br, 2H), 3.15-3.01 (m, 4H), 2.42 (s, 3H), 2.32 (s, 3H), 2.28-2.19 (m, 2H), 1.95-1.84 (m, 2H), 1.83-1.71 (m, 2H), 1.64-1.52 (m, 2H), 1.36 (s, 9H). ¹³C NMR (126 MHz, DMSO) δ 202.38, 160.69, 158.32, 158.08, 157.98, 155.59, 154.77, 145.07, 141.96, 141.83, 134.97, 129.58, 126.24, 115.21, 106.93, 77.61, 69.67, 69.60, 69.52, 69.47, 69.17, 64.24, 54.85, 52.96, 50.95, 45.48, 31.29, 28.21, 27.57, 25.14, 13.65.

N-(2-(2-(2-(2-(4-(6-((6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1yl)ethoxy)ethoxy)ethoxy)ethyl)-2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamide (10a). To a solution of the **9** (30.5 mg, 0.0422 mmol) in DCM (2 mL) was added TFA (1 mL) and the resulting solution was stirred at room temperature for 1h. The mixture was concentrated and the residue was then dissolved in DMF (1 mL) followed by adding **6a** (14mg, 0.0422 mmol), HATU (33 mg, 0.0844 mmol) and DIPEA (37 μL, 0.211 mmol). The resulting mixture was stirred for 1h at room temperature, then evaporated the solvent and purified by reverse phase HPLC (5-95% MeOH in H₂O) to give **10a** (TFA salt) as a yellow solid (34.4 mg, 87%). LC-MS: *m/z* 937 [M+1]. ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.2 (s, 1H, NH), 9.03 (s, 1H, AR-H), 8.09 (dt, *J* = 10.6, 3.1 Hz, 2H, AR-H), 7.87 (t, *J* = 8.5 Hz, 1H, AR-H), 7.79 (dd, *J* = 8.5, 7.3 Hz, 1H, AR-H), 7.47 (d, *J* = 7.2 Hz, 1H, AR-H), 7.40 (d, *J* = 8.5 Hz, 1H, AR-H), 5.89-5.80 (m, 1H, N-CH), 5.11 (dd, *J* = 12.8, 5.5 Hz, 1H, N-CH), 4.80 (s, 2H, CO-CH₂-O), 3.95-3.82 (m, 4H, 2xO-CH₂), 3.75-3.55 (m, 8H, 4xO-CH₂), 3.54-3.52 (m, 2H, N-CH₂), 3.51-3.45 (m, 2H, N-CH₂), 3.42-3.19 (m, 8H, 4xN-CH₂), 3.02-2.98 (br, 1H, H'_{N-C-CH2}), 2.65-2.52 (m, 2H, CO-CH₂), 2.44 (s, 3H, CO-CH₃), 2.35 (s, 3H, AR-CH₃), 2.28-2.12 (m, 2H, C-CH₂), 2.05 (ddt, *J* = 15.2, 7.8, 2.8 Hz, 1H, H'_{N-C-CH2}), 1.98-1.85 (m, 2H, C-CH₂), 1.82-1.74 (m, 2H, C-CH₂), 1.64-1.53 (m, 2H, C-CH₂), ¹³C NMR (126 MHz, DMSO) δ 202.43, 172.82, 169.90, 166.97, 166.72, 165.51, 160.70, 158.33, 158.23, 157.98, 154.92, 154.82, 144.95, 141.95, 141.83, 136.98, 133.03, 129.77, 126.77, 120.40, 116.78, 116.14, 115.35, 107.13, 69.71, 69.65, 69.63, 69.52, 68.83, 67.57, 64.19, 54.90, 53.00, 50.96, 48.85, 45.48, 38.40, 31.32, 30.98, 27.61, 25.19, 22.02, 13.70.

N-(2-(2-(2-(2-(4-(6-((6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl)ethoxy)ethoxy)ethoxy)ethyl)-2-((2-(1-methyl-2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamide (10b). **10b** was synthesized with similar procedures as **10a** from **9** (30.5 mg, 0.0422 mmol) and **6b** (14.6 mg, 0.0422 mmol). **10b** was obtained as a yellow solid (33.7 mg, 84%). LC-MS: m/z 951 [M+1]. ¹H NMR (500 MHz, DMSO- d_6) δ 9.75 (br, 1H, NH), 8.97 (s, 1H, AR-H), 8.10 (d, J = 3.1 Hz, 1H, AR-H), 8.03-7.93 (m, 1H, AR-H), 7.87 (d, J = 9.1 Hz, 1H, AR-H), 7.85-7.76 (m, 1H, AR-H), 7.64 (dd, J = 9.2, 3.2 Hz, 1H, AR-H), 7.50 (d, J = 7.3 Hz, 1H, AR-H), 7.40 (d, J = 8.5 Hz, 1H, H_{AR}), 5.87-5.76 (m, 1H, N-CH), 5.21-5.10 (m, 1H, N-CH), 4.79 (s, 2H, CO-CH₂-O), 3.91-3.82 (m, 2H, O-CH₂), 3.81-3.77 (m, 2H, O-CH₂), 3.65-3.59 (m, 2H, O-CH₂), 3.55 (m, 2H, O-CH₂), 3.54 (br, 4H, 2xO-CH₂), 3.47 (t, J = 5.8 Hz, 2H, N-CH₂), 3.43-3.36 (m, 2H, N-CH₂), 3.35-3.30 (m, 2H, N-CH₂), 3.30-3.18 (m, 2H, N-CH₂), 3.13-3.04 (m, 2H, N-CH₂), 3.02 (s, 3H, N-CH₃), 2.99-2.91 (m, 2H, N-CH₂), 2.80-2.74 (m, 2H, CO-CH₂), 2.74-2.70 (m, 1H, H'_{N-C-CH₂), 2.43 (s, 3H, CO-CH₃), 2.32 (s, 3H, Ar-CH₃), 2.29-2.16 (m, 2H, C-CH₂), 1.95-1.84 (m, 2H, C-CH₂), 1.83-1.72 (m, 2H, C-CH₂), 1.65-1.51 (m, 2H, C-CH₂). ¹³C NMR (126 MHz, DMSO) δ 202.37, 171.75, 169.62, 166.93, 166.67, 165.47, 160.67, 158.32, 158.20, 158.03, 157.95, 154.95, 154.79, 144.90, 141.92, 141.79, 136.98, 133.00, 129.75, 126.78, 120.40, 116.73, 116.12, 115.34, 107.12, 69.68, 69.61, 69.49, 68.81, 67.55, 64.17, 54.86, 52.97, 50.92, 49.38, 45.45, 38.37, 31.30, 31.09, 27.59, 26.61, 25.16, 21.21, 13.68.}

Synthesis of YKL-06-102

tert-butyl 2-(4-(6-((6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl) acetate (11). To a suspension of Palbociclib (7) (100 mg, 0.22 mmol) in DMSO (5 mL) were added *t*-butyl bromoacetate (4) (65 μ L, 0.44 mmol) and DIPEA (0.115 mL, 0.66 mmol). The mixture was heated to 80°C and kept stirring for 24h. The mixture was then cooled down to room temperature, extracted, dried, filtered and concentrated. The residue was purified by reverse phase HPLC (5-95% MeOH in H₂O) to give **11** (TFA salt) as a yellow solid (90 mg, 73%). LC-MS: *m/z* 562 [M+1]. ¹H NMR (500 MHz, DMSO-d6) δ 10.09 (s, 1H), 8.95 (s, 1H), 8.05 (d, J = 3.0 Hz, 1H), 7.85 (d, J = 9.0 Hz, 1H), 7.49-7.43 (m, 1H), 5.88-5.75 (m, 1H), 3.20-3.13 (m, 6H), 2.67 (t, J = 4.9 Hz, 4H), 2.42 (s, 3H), 2.31 (s, 3H), 2.27-2.18 (m, 2H), 1.89 (br, 2H), 1.82-1.73 (m, 2H), 1.65-1.52 (m, 2H), 1.43 (s, 9H). ¹³C NMR (126 MHz, DMSO) δ 202.91, 169.67, 161.22, 159.04, 158.74, 155.22, 144.77, 143.88, 142.55, 135.89, 125.21, 115.60, 107.02, 100.00, 80.72, 59.66, 53.36, 52.12, 48.78, 31.78, 28.29, 28.02, 25.58, 14.10.

DMSO) δ 202.22, 172.82, 170.07, 168.94, 167.27, 163.93, 160.54, 157.29, 157.19, 154.95, 146.39, 143.30, 141.74, 141.59, 136.25, 132.07, 130.81, 117.46, 116.43, 110.71, 109.23, 108.31, 72.16, 70.52, 69.81, 69.77, 69.60, 68.89, 68.71, 60.18, 55.90, 53.12, 50.89, 48.57, 44.97, 43.62, 41.71, 31.27, 30.99, 27.68, 25.27, 22.16, 13.79.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical parameters are reported in the figure legends of the paper.

Expression Proteomics BSJ-03-123

Acquired raw data files were processed using the Proteome Discoverer 2.2.0. platform, utilizing the Sequest HT database search engine and Percolator validation software node (V3.04) to remove false positives with a false discovery rate (FDR) of 1% on peptide and protein level under strict conditions. Searches were performed with full tryptic digestion against the human SwissProt database v2017.06 with up to two miscleavage sites. Oxidation (+15.9949 Da) of methionine was set as variable modification, whilst carbamidomethylation (+57.0214 Da) of cysteine residues and TMT 6-plex labelling of peptide N-termini and lysine residues were set as fixed modifications. Data was searched with mass tolerances of ± 10 ppm and 0.6 Da on the precursor and fragment ions (CID), respectively. Results were filtered to include peptide spectrum matches (PSMs) with Sequest HT cross-correlation factor (Xcorr) scores of ≥ 1 and high peptide confidence. PSMs with precursor isolation interference values of $\geq 50\%$ and average TMT-reporter ion signal-to-noise values (S/N) ≤ 10 were excluded from quantitation. Isotopic impurity correction and TMT channel-normalization based on total peptide amount were applied. For statistical analysis and p-value calculation, the integrated ANOVA hypothesis test was used. TMT ratios with p-values below 0.01 were considered as significant.

Expression Proteomics YKL-06-102

Proteome Discoverer 2.1 (Thermo Fisher Scientific) was used for .RAW file processing and controlling peptide and protein level false discovery rates, assembling proteins from peptides, and protein quantification from peptides. MS/MS spectra were searched against a Uniprot human database (September 2016) with both the forward and reverse sequences. Database search criteria are as follows: tryptic with two missed cleavages, a precursor mass tolerance of 20 ppm, fragment ion mass tolerance of 0.6 Da, static alkylation of cysteine (57.02146 Da), static TMT labelling of lysine residues and N-termini of peptides (229.16293 Da), and variable oxidation of methionine (15.99491 Da). TMT reporter ion intensities were measured using a 0.003 Da window around the theoretical m/z for each reporter ion in the MS3 scan. Peptide spectral matches with poor quality MS3 spectra were excluded from quantitation (summed signal-to-noise across 10 channels < 200 and precursor isolation specificity < 0.5), and resulting data was filtered to only include proteins that had a minimum of 3 unique peptides identified. Reporter ion intensities were normalised and scaled using in-house scripts in the R framework. Statistical analysis was carried out using the limma package within the R framework.

Phosphoproteomics

Acquired raw data files were processed using the Proteome Discoverer 2.2.0. platform, utilising the Sequest HT database search engine and Percolator validation software node (V3.04) to remove false positives with a false discovery rate (FDR) of 1% on peptide and protein level under strict conditions. Searches were performed with full tryptic digestion against the human SwissProt database v2017.12 with up to two miscleavage sites. Oxidation (+15.9949 Da) of methionine and phosphorylation (+79.9663 Da) of serine, threonine and tyrosine was set as variable modification, whilst carbamidomethylation (+57.0214 Da) of cysteine residues and TMT 10-plex labelling of peptide N-termini and lysine residues were set as fixed modifications. Data was searched with mass tolerances of \pm 10 ppm and 0.025 Da on the precursor and fragment ions (HCD), respectively. Results were filtered to include peptide spectrum matches (PSMs) with Sequest HT cross-correlation factor (Xcorr) scores of \geq 1 and high peptide confidence. The ptmRS algorithm was additionally used to validate phospopeptides with a set score cutoff of 90. PSMs with precursor isolation interference values of \geq 50% and average TMT-reporter ion signal-to-noise values (S/N) \leq 10 were excluded from quantitation. Isotopic impurity correction and TMT channel-normalization based on total peptide amount were applied. For statistical analysis and p-value calculation, the integrated ANOVA hypothesis test was used. TMT ratios with p-values below 0.01 were considered as significant.

Hits identified via global phosphoproteomics (log2 fold change > 0.5 or < -0.5, FDR adj. p-value < 0.05) were mapped on a proteinprotein interaction network using the networkx python package. The protein-protein interaction network was queried from the Human Integrated Protein-Protein Interaction rEference (HIPPIE) (Alanis-Lobato et al., 2017). Interactions without PubMed IDs were removed. The resulting subgraph was expanded to include first order neighbors limited to transcriptional regulators for which ENCODE genome localization data was available and visualized using Cytoscape. Kinase substrate prediction was performed with KSEA App (https://casecpb.shinyapps.io/ksea).

RNA Sequencing

NGS reads were trimmed based on quality and adapter sequence content with Trimmomatic in single-end (ILLUMINACLIP:TruSeq3-SE.fa:2:30:10:1:true, SLIDINGWINDOW:4:15, MINLEN:20) mode. The resulting reads were aligned with the "Spliced Transcripts Alignment to a Reference" (STAR) aligner to the hg38 reference genome assembly provided by the UCSC Genome Browser resembling the Genome Reference Consortium GRCh38 assembly. Ensembl transcript annotation from version e87 (December 2016) served as reference transcriptome. Reads were counted using htseq-count. Differential expression analysis was performed on

quantile-normalized read counts using the voom-limma R package. Following thresholds were applied for hit calling: fold change > 1.5 or < -1.5, FDR adj. p-value < 0.05. GO-term enrichment analysis was performed using GOrilla. For the network visualization of enriched GO-terms, parent-child relationships of terms were extracted from the go-basic.obo ontology and the network assembled using the python networkx package.

DATA AND SOFTWARE AVAILABILITY

The accession number for the RNA sequencing data reported in this paper is GEO: GSE116187.