

Large-scale characterization of drug mechanism of action using proteome-wide thermal shift assays

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Abstract

In response to an ever-increasing demand of new small molecules therapeutics, numerous chemical and genetic tools have been developed to interrogate compound mechanism of action. Owing to its ability to approximate compound-dependent changes in thermal stability, the proteome-wide thermal shift assay has emerged as a powerful tool in this arsenal. The most recent iterations have drastically improved the overall efficiency of these assays, providing an opportunity to screen compounds at a previously unprecedented rate. Taking advantage of this advance, we quantified more than one million thermal stability measurements in response to multiple classes of therapeutic and tool compounds (96 compounds in living cells and 70 compounds in lysates). When interrogating the dataset as a whole, approximately 80% of compounds (with quantifiable targets) caused a significant change in the thermal stability of an annotated target. There was also a wealth of evidence portending off-target engagement despite the extensive use of the compounds in the laboratory and/or clinic. Finally, the combined application of cell- and lysate-based assays, aided in the classification of primary (direct ligand binding) and secondary (indirect) changes in thermal stability. Overall, this study highlights the value of these assays in the drug development process by affording an unbiased and reliable assessment of compound mechanism of action.

eLife Assessment

The study provides a **valuable** showcase of a workflow to perform large-scale characterization of drug mechanisms of action using proteomics in which on-target and off-targets of 166 compounds using proteome solubility analysis in living cells and cell lysates were determined. The evidence supporting the claims of the authors is **solid**, however, the inclusion of more replicate experiments and more statistical rigor would have strengthened the study. This will be of broad interest to medicinal chemists, toxicologists, computational biologists and biochemists

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Introduction

A critical aspect of any drug development effort is the ability to define compound mechanism of action. Most small molecule therapeutics function by engaging a protein (target) through a direct physical interaction, thereby inhibiting or activating a specific target in order to modulate enzymatic activity, cell signaling pathways, or gene expression. Because of its unique ability to probe cell function on a proteome-wide scale, mass spectrometry (MS) has emerged as a powerful tool for interrogating compound mechanism of action by aiding in the validation of known targets and the identification of new ones (Moellering and Cravatt, 2012 [↗](#); Daub, 2015 [↗](#); Schürmann et al., 2016 [↗](#); Frantzi et al., 2019 [↗](#); Robers et al., 2020 [↗](#); Kuljanin et al., 2021 [↗](#); Meissner et al., 2022 [↗](#)). In addition to traditional proteomic approaches that profile protein expression and post-translational modifications (PTMs), the mass spectrometer has also become indispensable for unbiased assessments of compound target engagement and, by extension, mechanism of action. This has led to the development of numerous MS-based methods to screen the proteome for evidence of ligand binding (Lomenick et al., 2009 [↗](#); Molina et al., 2013 [↗](#); Jafari et al., 2014 [↗](#); Savitski et al., 2014 [↗](#); Liu et al., 2017 [↗](#); Gaetani et al., 2019 [↗](#); Ball et al., 2020 [↗](#); Van Vranken et al., 2021 [↗](#); Beusch et al., 2022 [↗](#)).

All proteins have a characteristic melting temperature, and this physical property can be influenced by numerous factors (ligand-binding, protein-protein interactions, and PTMs) that make a given protein more or less resistant to thermal denaturation (Koshland, 1958 [↗](#)). Classically, the thermal shift assay was used to study a single protein of interest, however, by coupling the traditional thermal shift assay with modern quantitative proteomics and sample multiplexing, it is possible to assess changes in protein thermal stability on a proteome-wide scale (Molina et al., 2013 [↗](#); Jafari et al., 2014 [↗](#); Savitski et al., 2014 [↗](#)). As such, MS-coupled thermal shift assays have emerged as a powerful tool for studying compound target engagement and mechanism of action in lysates, cells, and even tissues (Molina et al., 2013 [↗](#); Savitski et al., 2014 [↗](#); Jafari et al., 2014 [↗](#); Franken et al., 2015 [↗](#); Huber et al., 2015 [↗](#); Reinhard et al., 2015 [↗](#); Becher et al., 2016 [↗](#); Mateus et al., 2017 [↗](#); Sridharan et al., 2019a [↗](#); Mateus et al., 2020 [↗](#); Perrin et al., 2020 [↗](#)).

The cellular thermal shift assay (CETSA) and thermal proteome profiling (TPP) use tandem mass tag (TMT)-based quantitative proteomics to quantify complete melting curves and assign melting temperatures (T_M) to thousands of proteins, simultaneously, making it possible to screen the proteome for ligand-induced changes in protein thermal stability (Molina et al., 2013 [↗](#); Savitski et al., 2014 [↗](#); Jafari et al., 2014 [↗](#); Perrin et al., 2020 [↗](#); Zinn et al., 2021 [↗](#)). More recently, the proteome integral solubility alteration (PISA) assay was proposed to improve the efficiency of the proteome-wide thermal shift assay (Gaetani et al., 2019 [↗](#)). Rather than building complete melting curves, PISA uses TMT-based quantitative proteomics to estimate, or integrate, the area under a protein melting curve (**Figure 1 – figure supplement 1A** [↗](#)). Instead of TMT labelling the soluble fraction from each individual temperature—as in CETSA and TPP—PISA pools the soluble fractions of multiple samples heated across a thermal gradient such that a single TMT reporter represents an entire integrated melting curve. So, while CETSA and TPP measure a change in melting temperature (ΔT_M), PISA measures a change in solubility (ΔS_M). Critically, there is a strong correlation between ΔT_M and ΔS_M , which makes PISA a reliable, if still imperfect, surrogate for measuring direct changes in protein thermal stability (Gaetani et al., 2019 [↗](#); Li et al., 2020 [↗](#)). Thus, in the context of PISA, a change in protein thermal stability (or a thermal shift) can be defined as a fold change in the abundance of soluble protein in a compound-treated sample vs. a vehicle-treated control after thermal denaturation and high-speed centrifugation. Therefore, an increase in melting temperature, which one could determine using CETSA or TPP, will lead to an increase in the area under the curve and an increase in the soluble protein abundance relative to controls (positive $\log_2$ fold change). Conversely, a decrease in melting temperature will result in a

decrease in the area under the curve and a decrease in the soluble protein abundance relative to controls (negative $\log_2$ fold change). In the end, the ability to compress entire melting curves into a single TMT channel enables the synchronized interrogation of multiple compounds at multiple concentrations with multiple replicates in one experiment (Gaetani et al., 2019 [DOI](#)).

Owing to improved scalability, PISA results in an 8-fold theoretical improvement in throughput compared to TPP and CETSA. Therefore, PISA makes it feasible to readily approach large-scale chemical perturbation experiments, such as chemical library screening. With this in mind, we sought to establish an efficient platform for screening chemical libraries for drug-induced changes in protein thermal stability and test the ability of this workflow to provide meaningful compound-specific data when performed at scale. We selected 96 compounds with known mechanisms of action for PISA screening in live K562 cells and further assayed 70 of the compounds in native lysates. Using these data, we identified evidence of on-target binding, as well as putative examples of off-target engagement. In addition to direct drug binding, we were able to identify secondary changes in protein thermal stability that did not result from direct engagement and, instead, likely stemmed from other factors such as PTMs or changes in macromolecular interactions. Overall, this study serves as a roadmap for the scalable implementation of protein thermal stability quantitation to interrogate compound mechanism of action.

Results

Establishing a workflow for large-scale chemical perturbation screening

To enable large-scale chemical perturbation screening, we first sought to establish a robust workflow for assessing protein thermal stability changes in living cells. We chose K562 cells, which grow in suspension, because they have been frequently used in similar studies and can easily be transferred from a culture flask to PCR tubes for thermal melting (Savitski et al., 2014 [DOI](#); Jarzab et al., 2020 [DOI](#)). In a PISA experiment, a change in melting temperature or a thermal shift is approximated as a significant deviation in soluble protein abundance following thermal melting and high-speed centrifugation. Throughout this manuscript, we will interpret these observed alterations in solubility as changes in protein thermal stability. Most commonly this is manifested as a $\log_2$ fold change comparing the soluble protein abundance of a compound treated sample to a vehicle-treated control (**Figure 1 – figure supplement 1A** [DOI](#)). The ranges of temperatures used in a PISA experiment can impact the magnitude of the fold-change measurements, with narrower temperature ranges often resulting in larger fold changes (Li et al., 2020 [DOI](#)). After testing a number of ranges experimentally, we ultimately settled on a temperature range of 48°C–58°C, which encompasses the back half of most protein melting curves in K562 cells (**Figure 1 – figure supplement 1B** [DOI](#); Jarzab et al., 2020 [DOI](#)). Importantly, similar temperature ranges have been utilized in other studies that employ PISA for target deconvolution (Sabatier et al., 2022 [DOI](#)).

To highlight the advantage of this window, K562 cells were treated with each of the clinically-available CDK4/6 inhibitors—ribociclib, abemaciclib, and palbociclib—or DMSO (10 μ M, 30 minutes; Figure 1 – source data 1). Notably, all three compounds and a DMSO control were able to be assayed with four biological replicates in a single TMTPro 16plex experiment, requiring just 24 hours of instrument time. The cells were either heated across a range of 37°C–62°C (to approximate a full melting curve) or 48°C–58°C (thermal window) and any changes in protein thermal stability (based on a deviation in soluble protein abundance after thermal denaturation) were assessed using PISA. In cells heated across the full melting curve, CDK4 and CDK6 experienced minimal $\log_2$ fold changes, whereas utilizing the narrower range yielded a statistically-significant change in CDK4 and CDK6 abundance following thermal denaturation and centrifugation, which, in a PISA experiment, is consistent with a ligand-induced thermal shift or, more specifically, a thermal stabilization (**Figure 1 – figure supplement 1C** [DOI](#)). In addition to CDK4/6, several other kinases

experienced a significant change in solubility (thermal stability) in response to treatment with each compound (**Figure 1 – figure supplement 1D-G** [↗](#)). In fact, each of the three CDK4/6 inhibitors appear to engage a unique set of kinases, which might contribute to their disparate clinical and molecular phenotypes (**Figure 1 – figure supplement 1G** [↗](#); [Hafner et al., 2019](#) [↗](#)). Finally, we observed a consistent inhibitor-induced negative $\log_2$ fold change in soluble protein abundance for RB1, a well-established phosphorylation target of CDK4/6, which is consistent with a thermal destabilization (**Figure 1 – figure supplement 1C-F** [↗](#); [Narasimha et al., 2014](#) [↗](#)). The simplest explanation of this change is that inhibition of CDK4/6 prevents phosphorylation of RB1 thereby inducing a change in its thermal stability.

A large-scale chemical perturbation screen in K562 cells

We next sought to execute a large-scale chemical screen in live K562 cells. Specifically, we hoped to test the ability of PISA to provide meaningful insights into compound mechanism of action when performed at scale in the context of a chemical library screen. To that end, we curated a custom chemical library comprised of 96 commonly used cancer drugs and tool compounds. Each compound selected for the library had at least one well-annotated target and a known mechanism of action (Table 1). Of these 96 compounds, 70 targeted protein kinases, while the remaining 26 compounds targeted other classes of proteins including histone deacetylases (HDACs), lysine demethylases, poly (ADP-ribose) polymerases (PARPs), and others (**Figure 1A** [↗](#)). The library also contained multiple compounds targeting certain proteins—eight HDAC inhibitors, four aurora kinase inhibitors, six BRAF inhibitors, etc. To initiate the screen, K562 cells were treated with 10 μ M of each compound for 30 minutes in biological duplicate, which is consistent with previous thermal proteome profiling work (**Figure 1A** [↗](#); [Savitski et al., 2018](#) [↗](#); [Herneisen and Lourido, 2021](#) [↗](#); [Johnson et al., 2023](#) [↗](#)). The live cells were heated across a thermal gradient (48°C–58°C) and any changes in protein thermal stability were approximated using PISA (**Figure 1 – figure supplement 2** [↗](#)). This resulted in a total of 256 samples, which were arranged into 16 TMTpro 16-plex experiments and required a minimum of 384 hours of instrument time (**Figure 1 – figure supplement 3A** [↗](#)).

In total, we quantified approximately 6,800 proteins per treatment for a total of 871,120 drug-protein thermal stability measurements (**Figure 1B** [↗](#), **Figure 1 – figure supplement 3B** [↗](#), and **Figure 1 – source data 2**). Compared to the full dataset, annotated targets of the library compounds had significantly different solubility (thermal stability) compared to non-targets (Wilcoxon rank sum test, $p=3.56 \times 10^{-17}$). To define high-confidence thermal stability changes, we developed an empirically derived framework (see Methods; **Figure 1 – figure supplement 3C** [↗](#)). Briefly, we determined the $\log_2$ fold change of each compound treatment in reference to the vehicle-treated control and then quantified the trimmed standard deviation of all solubility measurements for a given protein across all treatments. In order to be considered a hit, the $\log_2$ fold change needed to be 3.5 standard deviations from the mean and have an absolute value greater than or equal to 0.2 ($|\log_2 \text{Cmpd/DMSO}| > 0.2$, $|\text{nSD}| > 3.5$; **Figure 1 – figure supplement 3C-G** [↗](#)). The fold change filtering removed background proteins while the per protein standard deviation filtering removed proteins that were observed to engage in large numbers of non-specific interactions, such as the kinase GAK (**Figure 1** [↗](#) – **source data 2**). While, we believe this strategy is capable of identifying meaningful compound-dependent changes, there are examples of compound-target pairs that surpass the standard deviation cutoff, but not the $\log_2$ fold change cutoff and vice versa. This suggests that this filtering strategy might be overly conservative (**Figure 1 – figure supplement 3F-H** [↗](#)). Nonetheless, from the 871,120 total protein solubility measurements, filtering the data resulted in 3,156 putative hits ($|\log_2 \text{Cmpd/DMSO}| > 0.2$, $|\text{nSD}| > 3.5$; **Figure 1B** [↗](#)), each of which point to a compound-dependent change in protein thermal stability.

Upon assembling compound-centric PISA solubility (thermal stability) profiles, known compound targets could be easily resolved. For example, in cells treated with OTS167, the primary target, MELK, had one of the largest negative $\log_2$ fold changes ($\log_2 \text{Cmpd/DMSO}$: -0.840; nSD : -5.688), which is consistent with a thermal destabilization (**Figure 1C** [↗](#)). In addition to its intended target,

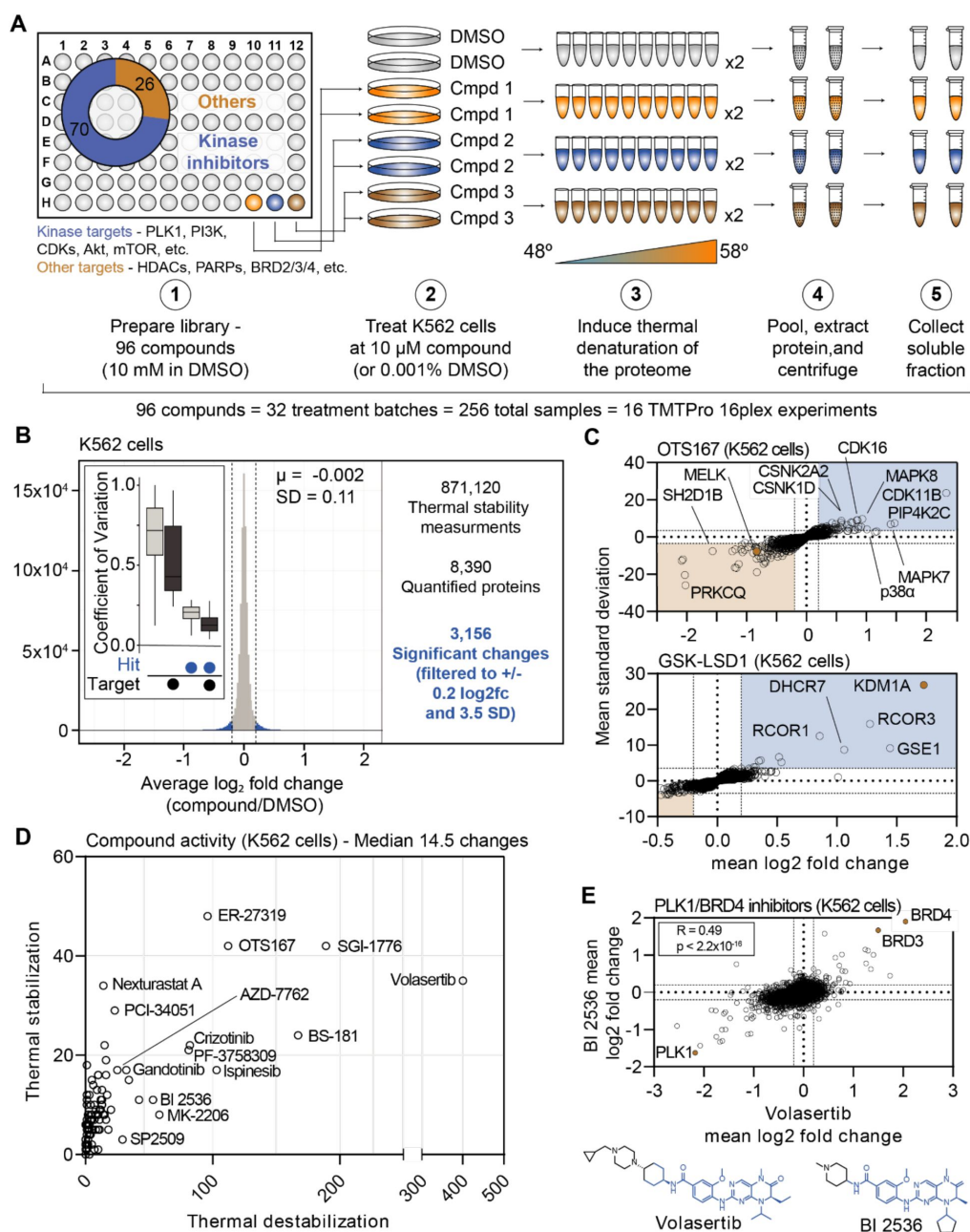


Figure 1.

Establishing a robust and efficient workflow for assessing changes in protein thermal stability in living cells.

A. Schematic of the cell-based PISA workflow utilized in the large-scale chemical perturbation screen. B. Histogram plotting the $\log_2$ fold change values for all solubility (thermal stability) measurements (main panel). Box plot depicting the %CV of curated targets vs. all other proteins (inset). C. Plots highlighting the significant changes in protein solubility following treatment with OTS167 (top) or GSK-LSD1 (bottom). The mean $\log_2$ fold change of duplicate measurements is plotted on the x-axis and the mean nSD is plotted on the y-axis. Blue boxes contain proteins that exhibit an increase in solubility (increase in melting temperature) and orange boxes contain proteins that exhibit a decrease in solubility (decrease in melting temperature). Orange points represent the known target of each compound. D. The total number of significant solubility changes quantified upon treatment with each compound. The number of stabilizing events (increase in solubility) is plotted on the y-axis and destabilizing events (decrease in solubility) are plotted on the x-axis. E. Plot comparing the $\log_2$ fold changes measurements for each protein in BI 2536 (y-axis)-and volasertib (x-axis)-treated K562 cells.

OTS167 also induced consistent protein solubility changes in other kinases (WEE1, PRKD3, CDK11B, AURKA, and others). These findings are consistent with previous reports demonstrating that the mechanism of action for OTS167 is in part driven by off-target protein engagement (Giuliano et al., 2018 [\[1\]](#)). Similarly, treatment with GSK-LSD1 caused an increase in the solubility of its primary target—the lysine demethylase, KDM1A, which likely results from a compound-dependent thermal stabilization ($\log_{2Cmpd/DMSO}$: 1.726; nSD: 26.825; **Figure 1C** [\[2\]](#)). In addition to the primary target, GSK-LSD1 also impacted several proteins known to form a complex with KDM1A, including GSE1 and RCOR1/3 (**Figure 1C** [\[2\]](#)). Finally, we observed disparities in how different molecules targeting the same protein impacted the target. The library contained two compounds known to target AKT1—MK-2206, an allosteric inhibitor and CCT128930, an ATP-competitive inhibitor (Amiran et al., 2023 [\[3\]](#); Hirai et al., 2010 [\[4\]](#); Yap et al., 2011 [\[5\]](#)). Interestingly, engagement of the allosteric ligand, MK-2206, induced a large change in AKT1 solubility, while the ATP-competitive inhibitor appeared to have no effect (**Figure 1 – figure supplement 4A,B** [\[6\]](#)).

Beyond the known targets, these data define a set of high-confidence ligand-induced thermal stability changes for each of the 96 compounds assayed in this screen (**Figure 1D** [\[7\]](#)). OTS167 caused a significant change in the solubility (thermal stability) of 154 proteins, including other protein kinases, which likely stem from off-target engagement (**Figure 1C,D** [\[2\]](#)). GSK-LSD1 treatment, on the other hand, resulted in only ten filtered changes (**Figure 1C,D** [\[2\]](#)). Although OTS167 was among the most active compounds in the screen (as defined by total filtered changes), treatment with the PLK1 inhibitor volasertib resulted in the most changes (435, **Figure 1D** [\[7\]](#)). While there were several other compounds that caused >100 changes in (ER-27319, BS-181, SGI-1776, etc.), the median number of filtered changes per compound was 14.5 (**Figure 1D** [\[7\]](#)).

Our screening library contained two dual PLK1/BRD4 inhibitors—volasertib and BI 2536—which have similar chemical structures (**Figure 1E** [\[8\]](#)). Appropriately, the PISA solubility profiles that resulted from treatment with each compound correlated well ($r_{\text{spearman}} = 0.49$, $p < 1.0 \times 10^{-15}$), highlighted by consistent changes in the solubility (thermal stability) of PLK1, BRD4, and the proteome in general (**Figure 1E** [\[8\]](#)). In addition to these dual PLK1/BRD4 inhibitors, the library also contained two compounds—JQ-1 and CPI-203—which target BRD4, but not PLK1. While the PISA profiles of the dual PLK1/BRD4 inhibitors were correlated, this was not the case for volasertib and JQ-1. Indeed, there was clear evidence that JQ-1 could engage BRD4 but not PLK1 however, there was little overall correlation, suggesting that the observed volasertib-dependent changes are largely PLK1-dependent (**Figure 1 – figure supplement 4C** [\[6\]](#)).

Finally, we wanted to determine if the set of high-confidence solubility (thermal stability) alterations could provide insights related to compound mechanism of action. PF-3758309 is designated as a PAK4 inhibitor. Including PAK4, treatment with this inhibitor caused a total of 102 proteins to undergo a significant change in solubility (**Figure 1D** [\[7\]](#)). Expectedly, this list included a number of protein kinases including MELK, TBK1, WEE1, and others (**Figure 1 – figure supplement 5A** [\[9\]](#)). Unexpectedly, there was also a significant enrichment of spliceosome subunits among these proteins (**Figure 1 – figure supplement 5A** [\[9\]](#)). These changes could stem from upstream inhibition of a kinase that regulates splicing and that the mechanism of action of this compound might, at least, in part, works through an alteration of this process. This result is consistent with a recent study that identified PF-3758309 as a potent modulator of pre-mRNA splicing (Shi et al., 2020 [\[10\]](#)). Overall, these data provide further evidence that PF-3758309 might be capable of impacting the assembly and/or activity of the spliceosome.

A protein-centric view of compound-dependent changes in thermal stability

Having established criteria for identifying significant compound-dependent changes in protein thermal stability, we focused on the known targets of the library compounds in order to identify key factors affecting the interpretation of our screening data. In total, we quantified at least one

known target for 79 of the 96 compounds assayed in the primary screen (84%) and found that 56 (71%) of these compounds induced a change in the solubility (thermal stability) of an annotated target (**Figure 2A**). While compound treatment more commonly resulted in an increase in the solubility (thermal stabilization) of known targets, we also observed a number of examples in which inhibitor binding stimulated a decrease in solubility (thermal destabilization). A consistent increase in solubility was observed for proteins including BRD4, HDAC1, and AURKA. Conversely, compound engagement by PLK1 resulted in a consistent decrease in solubility (**Figure 2A**). Therefore, both positive and negative $\log_2$ fold changes represent evidence of target engagement and specific proteins are consistently impacted by inhibitor binding. This result is consistent with previous studies and serves to corroborate the quality of our screening data (Sabatier et al., 2022).

Next, we characterized the magnitude of solubility (thermal stability) changes that we measured for known targets. Having treated cells with each compound at 10 μM (a concentration commonly used in similar studies; Savitski et al., 2014; Gaetani et al., 2019; Mitchell et al., 2023), we assumed that each target was fully saturated with a given compound. Therefore, the measurements likely reflect the maximal ligand-induced solubility (thermal stability) changes for each target. We observed a large range of solubility measurements for known compound-target pairs, from a four-fold reduction in protein solubility after thermal denaturation to a four-fold increase in protein solubility upon compound engagement (**Figure 2A**). We also observed many proteins with small (15%) but consistent solubility changes following compound treatment. p38 α -targeting compounds ralimetinib and doramapimod caused a large apparent increase in p38 α solubility (thermal stability; $\log_2\text{Cmpd/DMSO}$ = 1.52 and 1.94, respectively), while compounds targeting PI3K (pictilisib, duvesilib, and IC-87114), on the other hand, caused smaller solubility (thermal stability) changes ($\log_2\text{Cmpd/DMSO}$ = 0.258, 0.319, and 0.213, respectively; **Figure 2B,C** and **Figure 2 – figure supplement 1A,B**). In all cases, each of these measurements surpassed the nSD cutoff of 3.5 for each particular target. Taken at face value, one might conclude that the greater $\log_2$ fold change in solubility that doramapimod exerts on p38 α is more meaningful than the much smaller change that pictilisib exerts on PIK3CB, however, this is not necessarily true as both compounds can engage their targets at nanomolar concentrations. Instead, our data appears to be consistent with the previous observation that the maximum ligand-induced change in thermal stability is target-specific (Savitski et al., 2014; Becher et al., 2016). Therefore, a small $\log_2$ fold change for one protein (PIK3CB, $\log_2\text{Cmpd/DMSO} \geq 0.2$), can be just as meaningful as a large $\log_2$ fold change for another (p38 α , $\log_2\text{Cmpd/DMSO} \geq 2$).

Using the cell-based screening data to identify off-target compound engagement

Having defined a set of high-confidence solubility (thermal stability) changes for each compound, we next looked for potential examples of off-target engagement. In addition to volasertib and BI 2536 (PLK1/BRD inhibitors), several other compounds from the screen also impacted the solubility of PLK1 to a similar extent (**Figure 2D**). These included two promiscuous kinase inhibitors—OTS167 and PF-3758309. In addition to these compounds, two other ATP-competitive kinase inhibitors also impacted the solubility of PLK1—NVP-TAE-226, a FAK inhibitor and palbociclib, one of the CDK4/6 inhibitors used to establish our workflow. Because these two compounds induced a change in solubility to a similar magnitude as BI 2536 and volasertib, we hypothesized that these molecules might bind the active site and inhibit PLK1.

To further explore the impact of NVP-TAE-226 and palbociclib on PLK1 thermal stability, we treated K562 cells with increasing concentrations of BI 2536, NVP-TAE-226, and palbociclib, ranging from 100 nM to 10 μM and performed cell-based PISA profiling to approximate the changes in thermal stability that occurred at each inhibitor concentration (**Figure 2** – source data 1 and source data 2). As supported by previous reports, this would provide a relative assessment of the ability of each molecule to engage (and inhibit) PLK1 (Savitski et al., 2014;

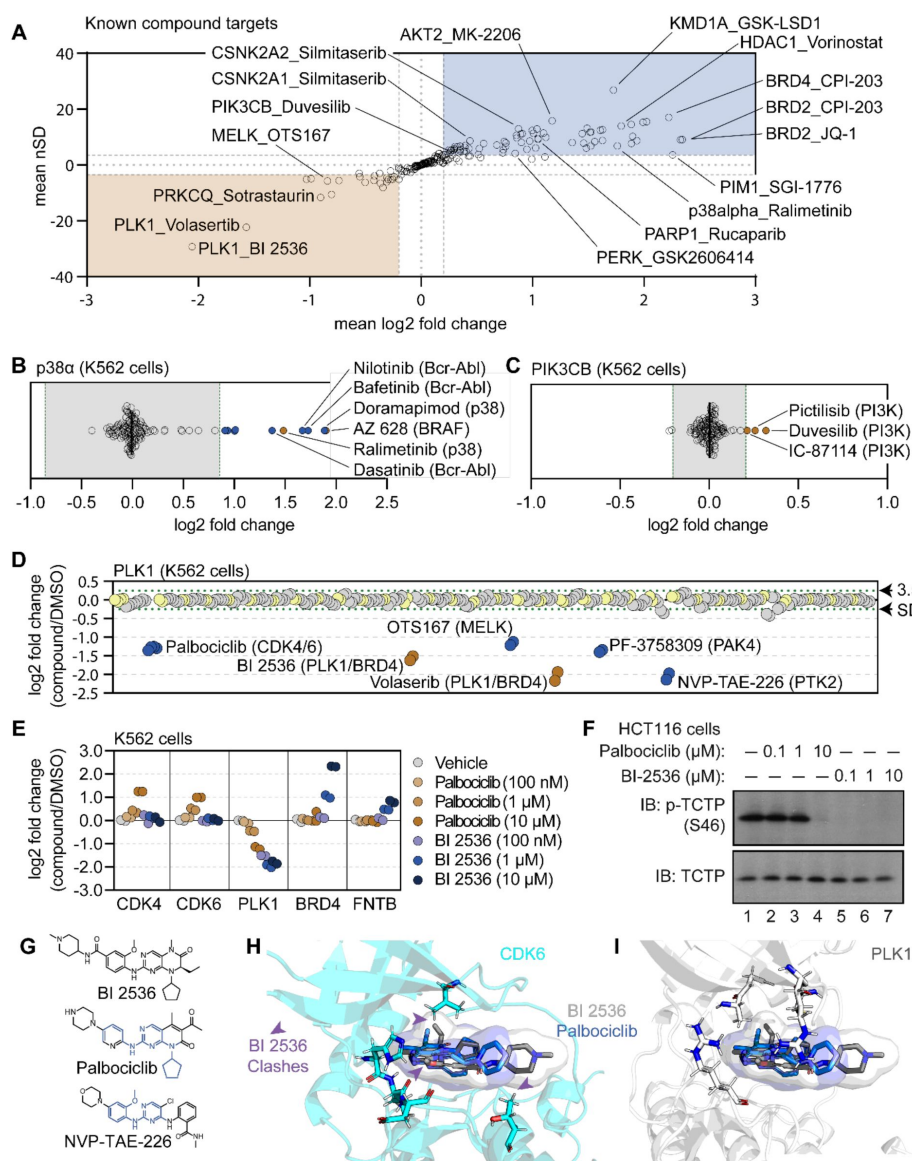


Figure 2.

Using cell-based PISA data to assess compound target engagement.

A. A plot of the mean log₂ fold change (x-axis) and mean nSD (y-axis) of each known compound-target pair that were quantified in the screen. Blue boxes contain proteins that exhibit an increase in solubility (increase in melting temperature) and orange boxes contain proteins that exhibit a decrease in solubility (decrease in melting temperature). B and C. Plots depicting the mean log₂ fold change of p38α (B) and PIK3CB (C) in response to each of the compound and DMSO treatments. Orange points indicate compounds known to target each protein. Blue points indicate other compounds that induce a significant change in thermal stability for each protein. Green dashed lines mark a SD of 3.5 for each protein. D. A protein-centric view of PLK1 solubility (thermal stability) in response to all treatments. Log₂ fold change is plotted on the y-axis. The points represent each of the 256 treatments that were performed. Orange points indicate compounds known to target PLK1. Blue points indicate other compounds that result in a significant decrease in solubility of PLK1. Green dashed lines mark a SD of 3.5 cutoff for PLK1. E and F. K562 cells were treated with the indicated concentrations of palbociclib or BI 2536 for 15 minutes. Changes in protein thermal stability are represented as a log₂ fold change in soluble protein abundance for each treatment in reference to a DMSO-treated control. (E). PLK1 activity was determined using a western blot for p-TCTP (S46) and total TCTP (F). G. Chemical structures of BI 2536, palbociclib and NVP-TAE-226. Common structural features with BI 2536 are highlighted in blue for Palbociclib and NVP-TAE-226. H. Co-crystal structure of CDK6 bound to palbociclib (blue) with BI 2536 (gray) modeled into the active site. I. Co-crystal structure of PLK1 bound to BI 2536 (gray) with palbociclib (blue) modeled into the active site.

Becher et al., 2016 [↗](#); Mateus et al., 2017 [↗](#); Sabatier et al., 2022 [↗](#)). Appropriately, BI 2536 caused a dose-dependent decrease in PLK1 solubility down to the lowest dose tested (100 nM; **Figure 2E** [↗](#) and **Figure 2 – figure supplement 1C** [↗](#)). Treatment with palbociclib and NVP-TAE-226 also caused a dose-dependent decrease in PLK1 solubility, however, each of these compounds appeared to impact PLK1 to a lesser extent than BI 2536. In fact, 100 nM BI 2536 treatment impacted PLK1 to the same extent as 10 μ M treatments with palbociclib and NVP-TAE-226 (**Figure 2E** [↗](#) and **Figure 2 – figure supplement 1C** [↗](#)). Next, we synchronized HCT116 cells in G2/M phase to test the ability of each compound to directly inhibit PLK1 in a cell-based assay. During this phase of the cell cycle, PLK1 is active and known to phosphorylate TCTP (at serine 46), a protein involved in microtubule stabilization (Ulise Cucchi et al., 2010 [↗](#)). Following synchronization, these cells were treated with increasing concentrations of each compound and phosphorylation of TCTP at S46 was assayed to read out PLK1 activity. BI 2536 completely abolished PLK1-dependent phosphorylation of TCTP at all tested doses. Palbociclib and NVP-TAE-226 also inhibited TCTP phosphorylation however, these compounds were much less potent than BI 2536 and were only able to inhibit PLK1 at doses between 1 μ M and 10 μ M (**Figure 2F** [↗](#) and **Figure 2 – figure supplement 1D** [↗](#)). Importantly, these results closely mirror the PISA data, providing further evidence that proteomic thermal stability measurements accurately recapitulate target engagement. Therefore, palbociclib and NVP-TAE-226 appear to be weak inhibitors of PLK1, which is consistent with previous studies that suggested these compounds as off-target inhibitors of this kinase (Hafner et al., 2019 [↗](#)).

The PISA data suggests that palbociclib was able to engage both CDK4/6 and PLK1, while BI 2536 was only able to engage PLK1 (**Figure 2E** [↗](#)). This is despite the fact that both compounds have a similar chemical structure (**Figure 2G** [↗](#)). In order to further interrogate this disparity, we modeled palbociclib and BI 2536 into co-crystal structures of PLK1 (PDB: 2rku) and CDK6 (PDB: 5l2i) with their ‘specific’ inhibitors (**Figure 2H,I** [↗](#); Chen et al., 2016 [↗](#); Kothe et al., 2007 [↗](#)). When we overlaid BI 2536 in the active site of CDK6, we observed three points of steric clashing with the protein density of the CDK6 active site. These findings were consistent with the lack of CDK4/6 solubility changes in cells that were treated with BI 2536. From overlaid projections of palbociclib and BI 2536 in PLK1, we did not observe steric interference in binding suggesting that both of these inhibitors could readily bind to the active site of PLK1. These data are again consistent with our observed decrease in solubility of PLK1 in cells that were treated with either BI 2536 or palbociclib.

PISA screening in crude cell extracts

Thus far we have focused exclusively on living cells, but PISA can also be used to screen for changes in cell lysates or crude extracts (Molina et al., 2013 [↗](#); Savitski et al., 2014 [↗](#); Franken et al., 2015 [↗](#); Becher et al., 2016 [↗](#); Sridharan et al., 2019b [↗](#); Liang et al., 2022 [↗](#)). Having already assembled one of the largest drug-based thermal denaturation studies in cells, we wanted to further profile a subset of these compounds in native extracts. We selected 70 compounds from the cell-based screen for further interrogation in native cell lysates. For this screen, we prepared crude extracts by dounce homogenizing K562 cells in PBS (Sridharan et al., 2019b [↗](#)). The crude extracts were treated with each of the 70 compounds in biological duplicate at 10 μ M for 30 minutes and any changes in thermal stability were approximated using PISA. In total, the 70 compounds and DMSO controls generated a total of 160 samples, which were arrayed across 10 TMT 16-plex experiments and quantified using approximately 240 hours of instrument time (**Figure 3A** [↗](#) and **Figure 3 – figure supplement 1A** [↗](#)).

We quantified an average of 7,840 proteins per treatment (**Figure 3B** [↗](#) and **Figure 3 – figure supplement 1B** [↗](#)), which resulted in a total of 627,176 solubility (thermal stability) measurements from all K562 lysate-based experiments (**Figure 3B** [↗](#) and Figure 1 – source data 2). After applying the same filters used in the cell-based screen, our dataset consisted of 2,176 protein solubility (thermal stability) changes (**Figure 3B** [↗](#) and Figure 3 – figure supplement 1C). Similar to the cell-based studies, the known targets of library compounds frequently experienced a significant change in thermal stability (**Figure 3 – figure supplement 1C-G** [↗](#)). For example,

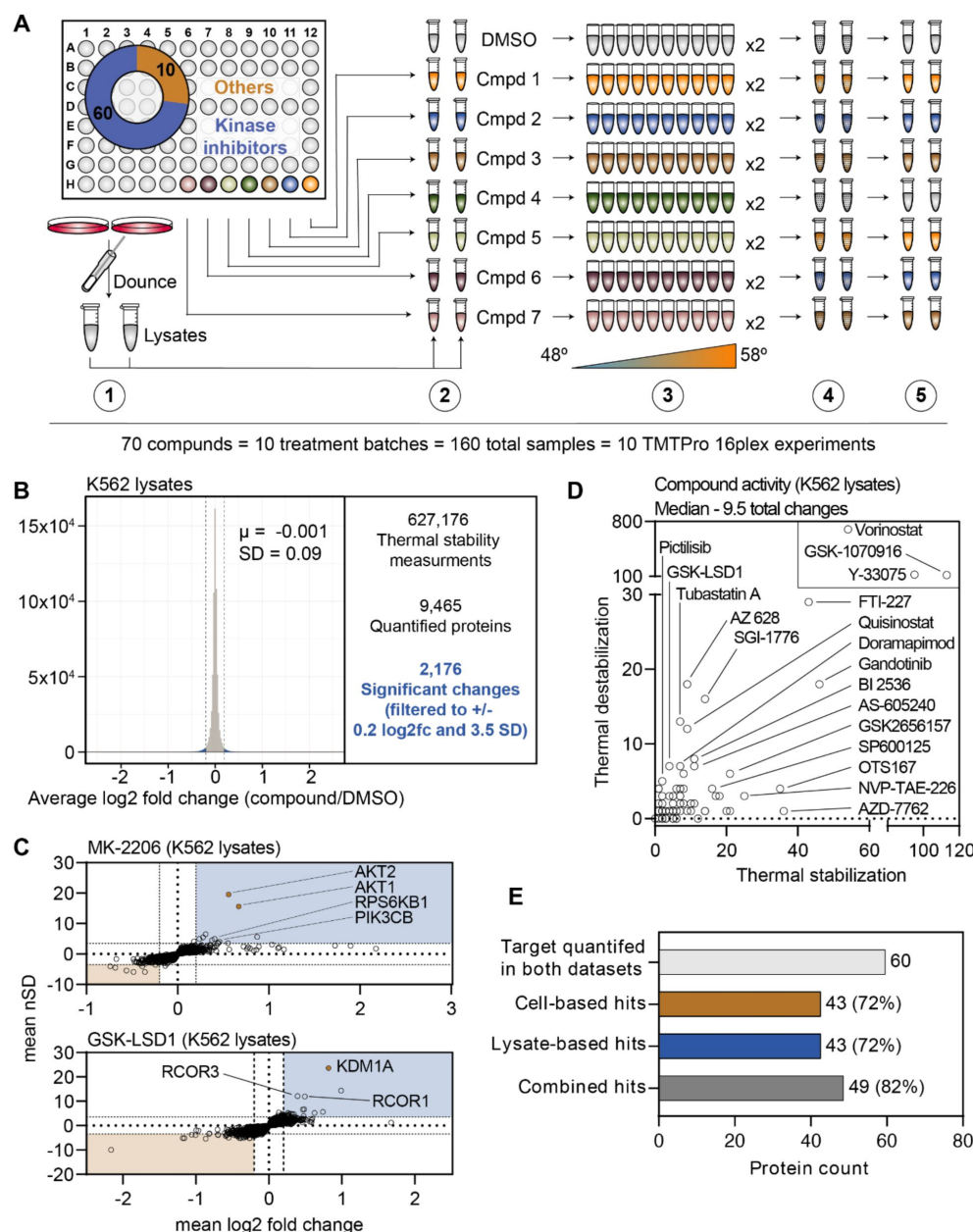


Figure 3.

A chemical perturbation screen of 70 compounds in K562 native extracts.

A. Schematic of the lysate-based PISA workflow utilized in the large-scale chemical perturbation screen. B. Histogram plotting the log₂ fold change values for all solubility measurements in lysate-based screen. C. Plots highlighting the significant changes in protein solubility (thermal stability) following treatment with MK-2206 (top) or GSK-LSD1 (bottom). The mean log₂ fold change of duplicate measurements is plotted on the x-axis and the mean nSD is plotted on the y-axis. Blue boxes contain proteins that exhibit an increase in solubility (increase in melting temperature) and orange boxes contain proteins that exhibit a decrease in solubility (decrease in melting temperature). Orange points represent the known target of each compound. D. Plot depicting the total solubility changes quantified upon treatment with each compound. The number of stabilizing events (increase in solubility) is plotted on the x-axis and destabilizing events (decrease in solubility) are plotted on the y-axis. E. Plot depicting the number of compounds for which a target was quantified in both cell- and lysate-based experiments (light gray), the number of compounds that caused a significant change in a known target in cell-based experiments (orange), the number of compounds that caused a significant change in a known target in lysate-based experiments (blue), and the number of compounds that caused a significant change in a known target in cell-or lysate-based experiments.

treatment of K562 lysates with MK-2206 and GSK-LSD1 increased the solubility (thermally stabilized) AKT1/2 and KDM1A, respectively (**Figure 3C**). In addition to AKT1/2, MK-2206 also increased the solubility of additional kinases, including subunits of RPS6K and PI3K, which highlights the ability of this approach to find putative examples of off-target engagement (**Figure 3C**). Finally, we observed a GSK-LSD1-dependent changes in the solubility of several KDM1 binding partners, which echoes the similar observation made in cell-based experiments (**Figure 3C**).

Since changes in thermal stability in lysate-based experiments stem almost exclusively from direct ligand binding events, we expected to observe fewer overall solubility (thermal stability) changes in this setting compared to cell-based experiments (Savitski et al., 2014; Franken et al., 2015; Huber et al., 2015; Becher et al., 2016). As expected, the lysate-based screen (2,176) yielded fewer significant changes than the cell-based screen (3,156). It is also important to note that three compounds (vorinostat, Y-33075, and GSK-1070916) contribute 1,200 (55%) of all the significant changes in the lysate-based screen. It is highly unlikely that these three molecules actively engage so many proteins and, therefore, the 2,176 hits in the lysate-based screen were likely affected in part by consistent, but artefactual effects of lysate-based analyses that we do not fully understand (Van Vranken et al., 2021). Overall, the median number of protein solubility changes in lysates was 66% of those observed in cells, with 9.5 proteins in lysate-based experiments compared to 14.5 in cell-based experiments (**Figure 3D**).

In order to directly compare the ability of each approach to identify an on-target hit, we focused on the 60 compounds for which a known target was quantified in both cell- and lysate-based experiments. Of these 60 compounds, both approaches identified a change in a known target of 43 compounds (~72%; **Figure 3E**). Importantly, these were not the same 43 compounds (**Figure 3 – figure supplement 2A**). For example, an alisertib-dependent change in AURKA solubility occurred in cells, but not lysates, while an AZ 628-dependent change in BRAF solubility was observed in lysates, but not cells (**Figure 3E** – figure supplement 3A,B). Consideration of each dataset in isolation results in an on-target hit rate of ~72%. Combining the datasets, on the other hand, results in an on-target hit rate of ~82% (49/60; **Figure 3E** and **Figure 3 – figure supplement 2A**). Therefore, combining the two approaches increases the chances of finding the target of a compound of interest.

Cell- and lysate-based PISA are complimentary approaches to determine compound engagement

Having re-screened dozens of compounds in lysate-based experiments, we wanted to assess the complementarity of these approaches. We focused on the known compound targets quantified in both cell- and lysate-based experiments and compared the solubility (thermal stability) changes measured using each approach (**Figure 4A**). Encouragingly, we observed compounds that impacted their known targets in both cell- and lysate-based approaches. These include GSK-1070916 and MK-2206, which increased the solubility of AURKA and AKT1, respectively, in both cell- and lysate-based experiments (**Figure 4A,B**). Conversely, we observed compounds that failed to impact the solubility of their target proteins in either the cell-based or lysate-based experiments (**Figure 4A** and **Figure 4 – figure supplement 1A**). These include compounds such as IRAK4-Inhibitor-1 and entospletinib which are known to target IRAK4 and SYK kinase, respectively. We note that the magnitudes of the solubility changes varied between the cell- and lysate-based approaches. Yet, compounds that increased the solubility of their annotated protein targets in cells also tended to increase the solubility their targets in lysates with one notable exception. Sotrastaurin induces an increase in the solubility (thermal stabilization) of its target, PRKCA, in lysates ($\log_2 \text{Cmpd/DMSO} = 0.924$), but a decrease in the solubility (thermal destabilization) in cells ($\log_2 \text{Cmpd/DMSO} = -0.502$; **Figure 4A,B**). These data suggest that the decrease in solubility observed in cell-based experiments might stem from a complex biophysical rearrangement.

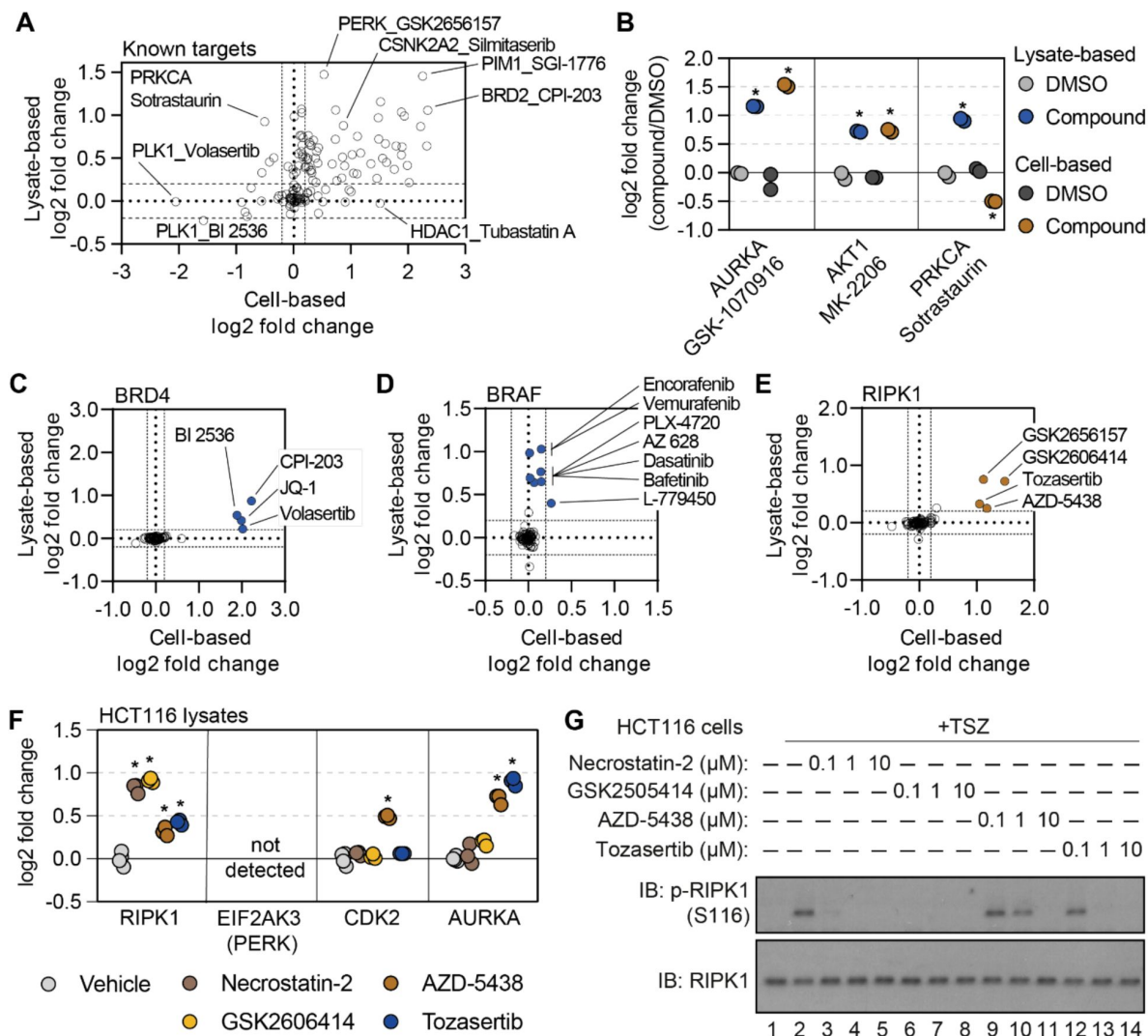


Figure 4.

Cell- and lysate-based PISA are complimentary approaches for assessing mechanism of action.

A. A plot depicting the mean $\log_2$ fold change for all compound-target pairs quantified in cell-(x-axis) and lysate-based (y-axis) PISA. Dashed lines indicate a $\log_2$ fold change of ± 0.2 . B. The $\log_2$ fold change of a selected number of compound-target pair in cell- and lysate-based PISA. C-E. Plots depicting the $\log_2$ fold change values for BRD4 (C), BRAF (D), and RIPK1 (E) in response to treatment with all compounds assayed using cell- and lysate-based PISA. Dashed lines indicate a $\log_2$ fold change of ± 0.2 . F. HCT116 cell lysates (N=3) were treated with each compound or DMSO for 15 minutes and assayed using PISA. The plot indicates the $\log_2$ fold change of several proteins in response to treatment with each compound. G. HCT116 cells were treated with the indicated concentration of each compound for 1 hour following the initiation of necroptosis using TSZ treatment. RIPK1 autophosphorylation was assayed by western blot using antibodies targeting p-RIPK1 (S116) or total RIPK1.

While many compounds significantly altered their known targets using both approaches, there were some compounds that preferentially altered the solubility (thermal stability) of known targets in one experimental setting but not the other (**Figure 4A**). For example, there were four compounds known to target BRD4 (JQ-1, CPI-203, BI 2536, and volasertib) that were assayed in both cell- and lysate-based experiments. Treatment of cells with any of these four inhibitors resulted in a $\log_{2} \text{Cmpd/DMSO} \sim 2$, while the lysate-based assays resulted in smaller changes (**Figure 4C** and **Figure 4 – figure supplement 1A**). Conversely, multiple inhibitors caused a significant change in BRAF solubility in lysates (AZ 628, PLX-4720, L-779450) but did not impact BRAF in cell-based assays (**Figure 4D** and **Figure 4 – figure supplement 1A**). Thus, certain protein targets were more prone to solubility (thermal stability) changes in one experimental setting compared to the other (Huber et al., 2015).

Combining cell- and lysate-based data to discover off-target engagement

Having demonstrated the complementarity of the cell- and lysate-based approaches, we sought to explore the corroborative value of integrating these data. We reasoned that any unexpected ligand-induced changes in solubility (thermal stability) that were shared between the two approaches would provide strong evidence of compound engagement. Despite an absence in the library of compounds designated as RIPK1 kinase inhibitors, there were four compounds that significantly increased the solubility of RIPK1 in cells and lysates (**Figure 4E** and **Figure 4 – figure supplement 1B**). Although three of the compounds—GSK-2656167, GSK-2606414, and tozasertib—had previously been shown to be off-target inhibitors of RIPK1, AZD-5438, a cyclin-dependent kinase (CDK) inhibitor, had never been attributed such activity (Byth et al., 2009; Martens et al., 2018; Rojas-Rivera et al., 2017). Nonetheless, this compound was capable of increasing RIPK1 solubility to a similar extent as the other three known RIPK1 off-target inhibitors (**Figure 4E** and **Figure 4 – figure supplement 1B**).

In order to gain a sense of the potency of AZD-5438 (and the other molecules), we assayed these compounds alongside necrostatin-2, a bona fide RIPK1 inhibitor, in HCT116 lysates (**Figure 4 – source data 1**). Treatment with necrostatin-2 or GSK-2606414 resulted in a similar solubility change for RIPK1, that was far greater than both tozasertib and AZD-5438 (**Figure 4F** and **Figure 4 – figure supplement 1C-F**). Treatment of HCT116 cells with TNF α , a Smad mimetic, and a pan-caspase inhibitor (zVAD) will lead to an initiation of necroptosis and, importantly, autophosphorylation of S116 by RIPK1 (Laurien et al., 2020). While each of our putative RIPK1 inhibitors was capable of inhibiting this autophosphorylation, AZD-5438 and tozasertib were less potent than necrostatin-2 and GSK-2606414 (**Figure 4G**). Therefore, the thermal stability measurements made in our lysate-based PISA experiment correlated with compound potency. This demonstrates that the combination of cell- and lysate-based screening data can be employed to identify instances of off-target engagement, even for weak inhibitors.

In addition to compound potency, the combined cell and lysate data provided valuable information regarding compound specificity. While it is difficult to separate necrostatin-2 and GSK-2606414 based on potency, necrostatin-2 had a greater apparent specificity with respect to RIPK1 engagement. Indeed, necrostatin-2 only impacted the solubility of RIPK1 in the lysate-based experiments (**Figure 4 – figure supplement 1C**). GSK-2606414, on the other hand, engaged other kinases including CSK, LIMK1, and TBK1 (**Figure 4 – figure supplement 1D**). Finally, AZD-5438 and tozasertib were less potent and less specific for RIPK1 than necrostatin-2 (**Figure 4 – figure supplement 1E,F**).

Disparities in cell-and lysate-based data pinpoint secondary changes in protein thermal stability

In comparison to lysate-based approaches, cell-based experiments have added potential to identify secondary changes in protein thermal stability that occur independent of direct ligand binding (Savitski et al., 2014 [↗](#); Almqvist et al., 2016 [↗](#); Becher et al., 2016 [↗](#); Sridharan et al., 2019b [↗](#); Liang et al., 2022 [↗](#); Becher et al., 2018 [↗](#); Dai et al., 2018 [↗](#)). These changes could occur as a result of changes in interactions with other proteins, ligands, nucleic acids, or the effects of PTMs.

Importantly, all of these factors can help to define compound mechanism of action. This type of change was previously highlighted in the context of the CDK4/6 inhibitors. Indeed, treatment of cells with CDK4/6 inhibitors induced a decrease in the solubility (thermal destabilization) of RB1, which we attributed to a change in phosphorylation due to upstream target inhibition (**Figure 1 – Figure supplement 1C** [↗](#)). Other CDK inhibitors (AZD-5438 and flavopiridol) also had a similar effect on RB1. In lysates, on the other hand, when cellular signaling networks are disrupted, RB1 was unaffected by these compounds (**Figure 5A** [↗](#)). This concept is also apparent when comparing the PF-3758309-dependent PISA profiles in each experimental setting (**Figure 5 – figure supplement 1A** [↗](#)). Indeed, there is a striking difference in the number of significant changes in cells (102) vs. lysates (21). This disparity is driven by spliceosome subunits, which are significantly impacted in cell-based experiments but unaffected in lysates. This supports the interpretation that the apparent thermal destabilization of the spliceosome in cells is the result of secondary changes rather than direct ligand binding.

Exploiting the disparities between cell-and lysate-based data can be a powerful tool in determining compound mechanism of action in the absence of reliable evidence of target engagement. These studies were performed in K562 cells/lysates, a chronic myelogenous leukemia cell line that expresses the BCR-ABL fusion. Consistent with previous reports, treatment of K562 cells with BCR-ABL-targeted compounds did not induce any apparent change in the solubility (thermal stability) of their primary target (Savitski et al., 2014 [↗](#)). We were, however, able to detect a change in solubility (thermal stability) for well-known BCR-ABL substrates—most notably CRKL and CRK (**Figure 5B** [↗](#) and **Figure 5 – figure supplement 2A-D** [↗](#)). Importantly, the change in CRKL solubility only occurred in cell-based experiments and not in lysates (**Figure 5 – figure supplement 2A,B** [↗](#); Savitski et al., 2014 [↗](#)). These data are consistent with a distal effector of thermal stability. A number of other compounds that largely target tyrosine kinases also caused a significant decrease in the solubility (thermal destabilization) of CRKL in K562 cells but not lysates (**Figure 5B** [↗](#)). We treated K562 cells with a small panel of these inhibitors and found that compounds that induce a decrease in the solubility of CRKL also inhibit phosphorylation at Y207 (**Figure 5C** [↗](#)). Therefore, the change in CRKL solubility correlates with a change in phosphorylation and is likely dependent on the inhibition of a primary compound target—such as BCR-ABL—that then inhibits CRKL phosphorylation. These data highlight that protein thermal stability measurements can reveal critical insights into primary and secondary effectors of compound mechanism of action, including kinase-substrate relationships (Franken et al., 2015 [↗](#)).

Using secondary changes to identify putative off-target effects

Previously, we demonstrated that the combined cell and lysate data could identify off-target inhibition through direct target engagement. Next, we wanted to determine if secondary effects could be used to determine putative off-target engagement in our screening data. In addition to tyrosine kinase inhibitors, we also observed a strong decrease in the solubility (thermal destabilization) of CRKL in response to treatment with the CHEK1 inhibitor AZD-7762 (**Figure 5B,C** [↗](#) and **Figure 5 – figure supplement 2A** [↗](#)). Importantly, our screen contained two additional inhibitors known to engage CHEK1 (CCT241533 and BML-227), neither of which had any impact on CRKL solubility (**Figure 5D** [↗](#)). Therefore, the AZD-7762-dependent decrease in CRKL solubility is unlikely to be related to on-target inhibition of CHEK1 and, instead, likely stems from off-target engagement of other kinases. Consistent with our hypothesis, the lysate-based screening

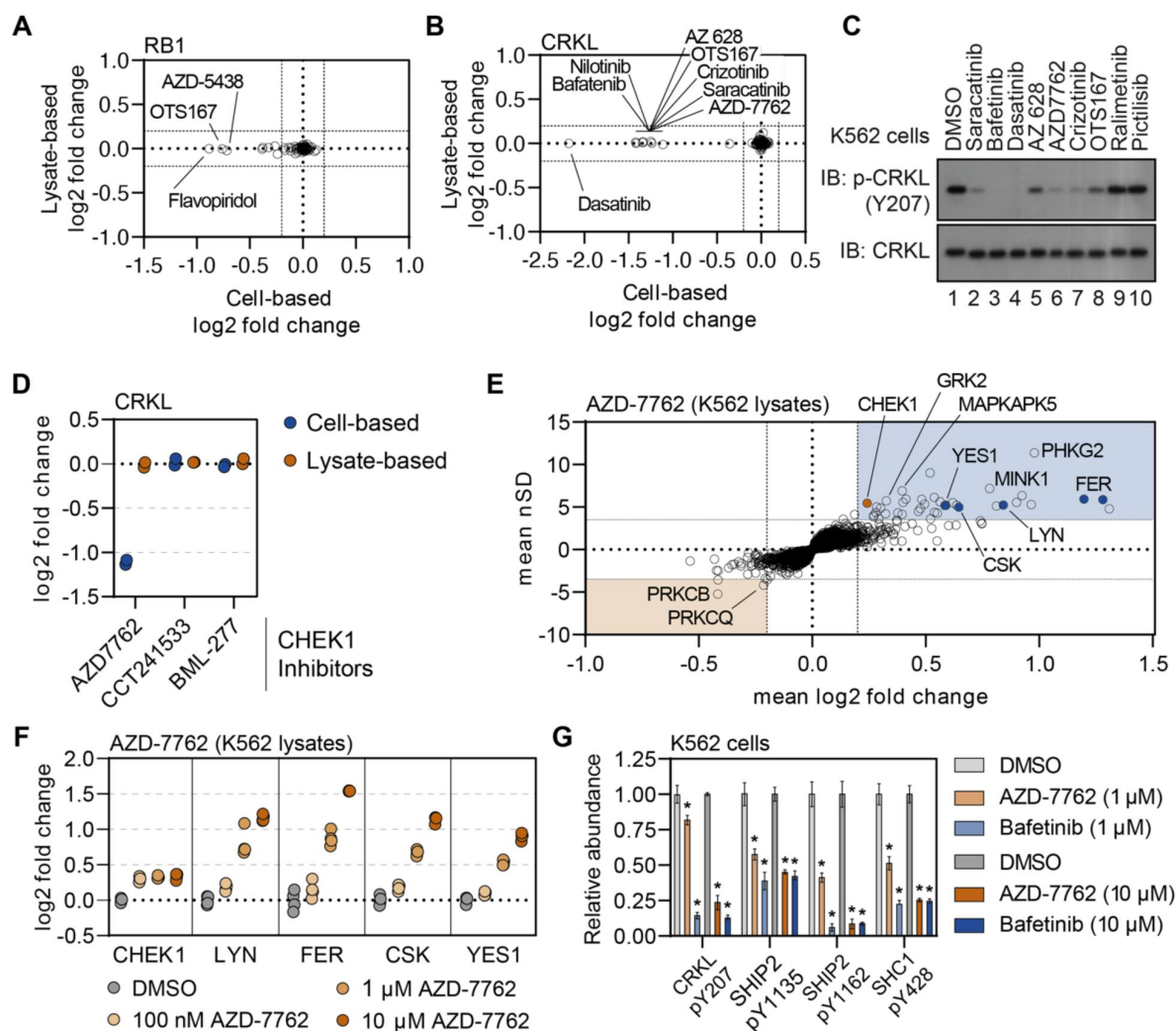


Figure 5.

Disparities in cell- and lysate-based PISA can point toward secondary changes in protein thermal stability.

A and B. Plots depicting the mean $\log_2$ fold change values for RB1 (A) and CRKL (B) in response to treatment with all compounds assayed using cell (x)- and lysate (y)-based PISA. Dashed lines indicate a $\log_2$ fold change of ± 0.2 . C. K562 cells were treated with each indicated compound at 10 μM for 15 minutes. Western blots were used to assess levels of p-CRKL (Y207) and total CRKL. D. A plot of the $\log_2$ fold change of CRKL in response to each indicated compound. E. Plots highlighting the significant changes in protein solubility (thermal stability) following treatment with AZD-7762. The mean $\log_2$ fold change of duplicate measurements is plotted on the x-axis and the mean nSD is plotted on the y-axis. Blue boxes contain proteins that exhibit an increase in solubility (increase in melting temperature) and orange boxes contain proteins that exhibit a decrease in solubility (decrease in melting temperature). Orange points represent the known target of AZD-7762 and the blue points represent tyrosine kinases that experience a significant change in soluble protein abundance (thermal stability). F. K562 lysates were treated with the indicated concentrations of AZD-7762 for 15 minutes and assayed using PISA. Changes in protein thermal stability are represented as a $\log_2$ fold change in soluble protein abundance for each treatment in reference to a DMSO-treated control. G. K562 cells were treated with the indicated concentration of bafetinib or AZD-7762 for 15 minutes and assayed using phosphoproteomic profiling. Plots depict the relative abundance of several phosphorylation sites following treatment with the indicated compounds. * indicate significant changes, which were determined using a permutation-based FDR (FDR = 0.05, S0 = 0.1).

data revealed that AZD-7762 induced a change in the solubility of many tyrosine kinases including FER, LYN, CSK, and YES1 (**Figure 5E,F**). These putative interactors were not limited to tyrosine kinases but also included dozens of serine/threonine kinases.

In order to further corroborate the ability of AZD-7762 to engage and inhibit tyrosine kinases, we treated K562 lysates with an increasing concentration of this compound, ranging from 100 nM to 10 μ M, and performed lysate-based PISA (**Figure 5 – figure supplement 3A-C** and **Figure 5** – source data 1). Consistent with the screening data, we observed a dose-dependent increase in the solubility of FER, LYN, CSK, YES1, and other kinases (**Figure 5F** and **Figure 5 – figure supplement 3A-C**). The annotated target of AZD-7762, CHEK1, on the other hand, experienced a similar change in solubility in response to each dose of the compound, which implies that it is fully saturated even at the lowest dose (**Figure 5F**).

Previously, we showed that treatment of K562 cells with AZD-7762 prevented the phosphorylation of CRKL Y207 (**Figure 5C**). In order to further implicate the ability of AZD-7762 to inhibit tyrosine kinases *in vivo*, we treated K562 cells with 1 μ M or 10 μ M of AZD-772 or bafetinib (a known tyrosine kinase inhibitor employed in the primary screen) and performed phosphoproteomic profiling to quantify compound-dependent changes in phosphopeptide abundance (**Figure 5 – figure supplement 4A-D** and **Figure 5** – source data 2 and 3). In addition to CRKL Y207, both bafetinib and AZD-7762 also caused a significant decrease in the abundance of other pY residues including SHIP2 Y1135 and Y1162 and SHC1 Y428 (**Figure 5G**). In addition to these specific pY sites, we also noticed that AZD-7762 caused considerably more total changes than bafetinib, which is consistent with AZD-7762 PISA profile and suggests that this compound is a highly promiscuous kinase inhibitor (**Figure 5 – figure supplement 4A-F**).

Library-scale assays connect consistent thermal stability changes

Binary comparisons of compounds with shared targets such as volasertib and BI 2536 (**Figure 1E**) revealed consistent thermal stability responses for known targets PLK1 and bromodomain proteins. Next, we set out to determine if small but consistent effects on the proteome due to drug treatment could reveal new information concerning compound engagement of proteins, protein classes, or protein complexes. To address this, we implemented a network-based approach using the correlation of solubility changes for each protein. To this end, we mapped the all-by-all correlation of proteins in the cell-based dataset. We filtered the 70,392,100 binary comparisons to include only the correlations in the top ~5% of absolute magnitude ($r_{\text{spearman}} > 0.35$). Because 70 of the 96 compounds used were kinase inhibitors, we further focused on correlated solubility changes within the 396 human kinases we quantified in our dataset (**Figure 6A**).

We first sought to determine if the network-based approach could reveal consistent thermal stability responses for kinases with conserved sequences or general kinase families (Eid et al., 2017). Strikingly, for the highly conserved 90 kDa ribosomal S6 kinases, we observed highly correlated solubility profiles for the three members, RPS6KA1, RPS6KA2, and RPS6KA3 (**Figure 6B**). We reasoned that this may be a function of the sequence conservation between these proteins or direct binding interactions (Huttlin et al., 2021, 2017). To this end we compared the cell and lysate data. In cells, RPS6KA1, RPS6KA2, and RPS6KA3 generally experienced a decrease in solubility following compound treatment. However, in lysates, these proteins generally experienced an increase in solubility, even by the same compound that decreased the solubility of proteins in cells, such as NVP-TAE-226. These data were in line with what we observed for sotrastaurin engagement of PRKCA (**Figure 4B**). Interestingly, within our thermal stability network, we observed that PRKCA solubility changes were significantly correlated with PRKCB and PRKCQ (**Figure 6 – figure supplement 1A,B**).

Both RPS6K and PRKC subnetworks reveal correlation between kinases of the same kinome group. RPS6K proteins belong to the CAMK kinases, and PRKC proteins belong to the AGC kinases. To explore this within group correlation, we annotated the network of quantified human protein

kinases based on their established groupings (AGC, Atypical, CAMK, CK1, CMGC, STE, TK, TKL, Other) along with a set of non-protein kinases (**Figure 6A**). Within the kinase network, we observed correlated solubility changes within and between families of kinases. For example, we observed correlated profiles between isoforms of p38 α /MAPK14 (a CMGC kinase) and the MAPKAPK2/3 (CAMK kinases, **Figure 6C,D**). MAPKAPK2 and MAPKAPK3 both experienced an increase in solubility with treatment of the p38 kinase inhibitor doramapimod. Indeed, these proteins are known to both act as substrates of p38 and to form complexes in divergent human cancer cells (**Figure 6E**). We further observed interconnected correlation networks within larger kinase groups. The AGC kinases, including AKT1/2 and ROCK1/2 were particularly well correlated (**Figure 6F**). AKT1 and AKT2 was driven in part by their solubility change due to AKT inhibitor MK-2206 (**Figure 6G**).

We reasoned that because protein kinases and non-protein kinases share similar structures and cofactor binding pockets, that non-protein kinases may represent an important class of off-target effectors for our library compounds. Within the correlated thermal stability profile network, we observed strong correlation due to compound-based solubility changes within the non-protein kinase group. PIP4K2A, PIP4K2B, and PIP4K2C solubilities were highly correlated in both cells and lysates (**Figure 6 – figure supplement 1C-E**). For example, the PLK1 inhibitor BI 2536 caused an increase in the solubility of all three PIP4K proteins in both cells and lysates. It is worth noting that the magnitude of the solubility changes for these PIP4K proteins was higher in cells than in lysates consistent with loss of membranes and cellular metabolite concentrations (Sridharan et al., 2019b). Yet the thermal stability responses in both assays were highly consistent for all three proteins using either approach. We explored the observation of small but consistent changes further and observed significant correlation of the SRPK1, SRPK2, and RIOK2 kinases (**Figure 6 – figure supplement 1F,G**). In our final datasets, we did not call any of these proteins as hits due to either the large numbers of solubility effects (SRPK1, SRPK2) or the small magnitude of changes (RIOK2). However, with correlations of 0.95 and 0.77 for SRPK1-SPRK2 and SPRK1-RIOK2, it is clear that these proteins respond consistently to chemical perturbation suggesting that our initial filters for full PISA datasets may mask some real and important protein-compound engagements.

Finally, we observed a strong correlation between the CMGC kinases CDK16, GSK3A, and GSK3B solubilities (**Figure 6A**). CDK inhibitors have been reported as active for GSK3 kinases owing to phylogenetic and structural conservation (**Figure 6H**; Fischer, 2003; Meijer et al., 2003). In our cell-based approach the inhibitor AZD-5438 has dual efficacy for GSK3A and CDK16 and we observed that the solubility responses for CDK16, GSK3A, and GSK3B were correlated (**Figure 6H**). This effect was driven in part by annotated inhibitors of CDKs (AZD-5438, flavopiridol) or GSKs (AZD-5438, LY2090314). Yet, known inhibitors of several kinases increased the solubility of both GSKs and CDK16, including MELK (OTSSP167), JAK (LY2784544), FAK (defactinib), and PAK4 (PF-3738309), as well as the PARP inhibitor rucaparib (**Figure 6I**). Interestingly, compounds such as VE821 (ATR), A205804 (ICAM-1), CC-401 (JNK), and L-779450 (Raf) exhibited GSK3 specific increases in solubility in cells. The consistent and differential responses of CDK16 and the GSKs offers clues into compound specificity or the utilization of combined effects in cells.

Discussion

The determination of protein-compound engagement and mechanism of action is an essential aspect of the drug development process. Herein we present approachable methods for the implementation and interpretation of proteome-wide thermal shifts assays on a 96-well plate scale. Taking advantage of the 8-fold increase in efficiency afforded by PISA, we catalogued compound-dependent changes in protein thermal stability in response to 96 compounds in living K562 cells and 70 compounds in native extracts. This study resulted in a total of 871,120 differential solubility (thermal stability) measurements in living cells and an additional 627,176 measurements in lysates. Exploiting this wealth of data, we were able to find clear evidence of on-

target engagement for the majority of compounds tested in both cell-and lysate-based experiments. Furthermore, there were numerous potential examples of off-target engagement. In addition to direct target engagement, there were many secondary changes in protein thermal stability, as evidenced by changes in solubility, that occurred independent of direct binding. Finally, using an all-by-all analysis of compounds and proteins, we demonstrated how structurally related proteins and protein complexes engage consistently with our library of compounds. Our study serves as an unbiased assessment of the current technology and serves as a comprehensive guide for ongoing work to enable library-scale study of protein-compound and protein-ligand interactions.

One of the most valuable features of the proteome-wide thermal shift assay, including PISA, is the ability to screen compounds in both living cells and native lysates (Molina et al., 2013 [DOI](#); Savitski et al., 2014 [DOI](#); Franken et al., 2015 [DOI](#); Huber et al., 2015 [DOI](#); Becher et al., 2016 [DOI](#), 2018 [DOI](#); Dai et al., 2018 [DOI](#); Gaetani et al., 2019 [DOI](#); Sridharan et al., 2019b [DOI](#); Sabatier et al., 2021 [DOI](#), 2022 [DOI](#)). In this study, we further demonstrate that these two approaches are not redundant, but, rather, provide complimentary data that can be employed to help establish compound mechanism of action. First and foremost, each approach was similarly proficient in identifying compound-dependent changes of known targets (**Figures 1** [DOI](#) and **3** [DOI](#)). In both cell-and lysate-based PISA experiments, about 70% of all compounds (whose target was quantified) induced a significant change in the solubility of its well-established target(s). Furthermore, when the two datasets were combined, that figure increased to over 80%. This is because certain targets, like BRAF, for example were more prone to undergo a change in solubility in one experimental setting rather than the other (**Figure 4D** [DOI](#)).

In general, a compound-dependent thermal shift that occurs in a lysate-based experiment is almost certain to stem from direct target engagement (Savitski et al., 2014 [DOI](#); Becher et al., 2016 [DOI](#)). This is because cell signaling pathways and cellular structures are disrupted and diluted. Cell-based studies, on the other hand, have the added potential to identify the targets of pro-drugs that must be metabolized in the cell to become active and secondary changes that occur independent of direct engagement (Savitski et al., 2014 [DOI](#); Franken et al., 2015 [DOI](#); Almqvist et al., 2016 [DOI](#); Becher et al., 2016 [DOI](#); Liang et al., 2022 [DOI](#)). Consistent with this dichotomy, we found that, on average, compounds caused a greater number of thermal shifts (as evidenced by changes in solubility) in cells than lysates. This is further supported by the PF-3758309-dependent thermal destabilization of spliceosome subunits. Finally, we documented a number of examples of phosphorylation-dependent thermal shifts. For example, compounds targeting CDKs tended to impact the thermal stability of RB1. Likewise, compounds targeting tyrosine kinases induced a thermal destabilization of downstream phosphorylation targets like CRKL and CRK. In these examples, an inhibitor engages and inhibits a direct target, which, in turn, prevents the phosphorylation of a downstream target thereby inducing a change in thermal stability (Savitski et al., 2014 [DOI](#)).

The idea of area under the curve analysis (PISA) remains a relatively recent modification of the traditional proteome-wide thermal shift assay (Gaetani et al., 2019 [DOI](#)). Using this dataset, we were able to benchmark many aspects of this advance and highlight many important considerations when designing and interpreting these experiments. First, we highlight the importance of selecting an appropriate thermal window (Li et al., 2020 [DOI](#)). In this study we selected a temperature range of 48°C-58°C, however, it is possible that deploying multiple thermal windows could be a viable strategy for focused interrogation of a small number of high-value compounds. In utilizing thermal windows, the goal is to maximize the potential magnitudes of the final fold change measurements. Still, we observed a large range in the magnitudes of the log₂ fold changes that were quantified for known compound targets. This is despite the fact, that we utilized a high treatment dose (10 μM) to maximize the magnitude of the fold change of the primary target. Ultimately, the data seemed to be consistent with previous studies that indicate the maximal change in thermal stability in protein specific (Savitski et al., 2014 [DOI](#); Becher et al., 2016 [DOI](#); Sabatier et al., 2022 [DOI](#)). Therefore, a log₂ fold change of 2 for one compound-protein pair could be

just as meaningful as a $\log_2$ fold change of 0.2 for another. In either case though we find that it is essential to optimize the thermal denaturation to ensure maximally useful effect sizes when comparing compounds.

While the primary screen was carried out at fixed dose, the increased throughput of PISA allowed for certain compounds to be assayed at multiple doses in a single experiment. In these instances, there was a clear dose-dependent change in thermal stability of primary targets, off-targets, and secondary targets. This not only helped corroborate observations from the primary screen, but also seemed to provide a qualitative assessment of relative compound potency in agreement with previous studies (Savitski et al., 2014 [DOI](#); Becher et al., 2016 [DOI](#); Mateus et al., 2017 [DOI](#)). Specifically, the compounds that most strongly impacted the thermal stability of targets, also acted as the most potent inhibitors. In order to be a candidate for this type of study, a target must have a large maximal thermal shift (magnitude of $\log_2$ fold change in solubility) because there must be a large enough dynamic range to clearly resolve different doses. From this study, PIK3CB (maximal $\log_2$ fold change ~ 0.2) would make a bad candidate, while p38 α (maximal $\log_2$ fold change ~ 2.0) would be a good candidate. Importantly, however, it should be possible to tune the thermal window in order to specifically increase the maximal $\log_2$ fold change of PI3KCB for dose-dependency studies.

Each of the compounds used in this study have a well-defined target and known mechanism of action. Furthermore, many of the compounds have been used extensively as tool compounds in basic research and as therapeutics in the treatment of multiple cancers. Despite the frequency with which many of these compounds have been used, the data generated in this study suggests that the current mechanistic understanding of many (if not all) of these compounds remains incomplete. For example, we found that the clinically-deployed CDK4/6 inhibitor palbociclib has a dramatic impact on PLK1 thermal stability in live cells, is capable of inhibiting PLK1 activity in cell-based assays, and can be modelled into the PLK1 active site. Importantly, this activity could contribute to the mechanism of action of palbociclib and further supports the conclusions of a previous study that implicated palbociclib as a potential inhibitor of PLK1 (Hafner et al., 2019 [DOI](#)). Similarly, PISA helped reveal that the CHEK1 inhibitor AZD-7762 is, in fact, a highly promiscuous kinase inhibitor that, amongst many other kinases, appears to engage a number of tyrosine kinases and inhibit downstream signaling. While this compound has not been approved for clinical use, it has been used frequently as a tool compound to study the biological impact of CHEK1 inhibition. Additionally, the PLK1 inhibitor BI 2536 engages non-protein kinases such as PIP4K2A/B/C. As inhibition of PIP4K2s has been shown in hematological cells, this potentially suggests the mechanism of dose-limiting hematologic events upon patient treatment with BI 2536 (Frost et al., 2012 [DOI](#); Lima et al., 2022 [DOI](#)). Overall, these examples, and the dataset as a whole, suggest that proteome-wide thermal shift assays and PISA, specifically, are valuable tools to help resolve the full range of compound activities. Despite this utility, however, it is important to note that these assays, alone, are unlikely to paint a full picture of mechanism of action. Instead, they represent a valuable tool that can be coupled with other MS-based and genetic approaches.

In the end, this study highlights the immense power of thermal stability assays in screening large collections of molecules to help define target engagement and mechanism of action on a proteome-wide scale. While this type of study is ideal for finding the targets of compounds that have been identified in high-throughput screening, they can provide valuable information in almost any context. This includes compounds that seemingly already have a well-defined target. Despite finding many ways to utilize this data, we expect that this dataset contains many interesting observations that can not only be used to better understand specific drugs, but also has the potential to reveal new biology. Moving forward, we hope that this data will serve as a valuable resource for those interested in drug development and the research community at large.

Acknowledgements

We would like to acknowledge the Gygi and Schweppe labs for advice and technical assistance concerning methods development, experimental implementation, and data interpretation. We would also like to acknowledge our funding sources: R35GM150919-01 (DKS), R01GM067945-20 (SPG), Andy Hill CARE Distinguished Researcher Award (DKS), Cancer Consortium New Investigator Award (DKS), the Pew Charitable Trusts (DKS). JGV is the Mark Foundation for Cancer Research Fellow of the Damon Runyon Cancer Research Institute (DRG-2359-19).

Methods

Cell culture

K562 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. For cell-based PISA experiments, cells were grown to approximately 1×10^6 cells/mL and immediately used for the assay. For lysate-based experiments, cells were grown to approximately 1×10^6 cells/mL, washed with phosphate-buffered saline, and flash frozen in liquid nitrogen. Cell pellets were stored at -80°C until ready for use.

HCT116 cell were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1X penicillin-streptomycin. Cells were grown until $\sim 80\%$ confluency, harvested by scraping, washed with phosphate-buffered saline, and flash frozen in liquid nitrogen. Cell pellets were stored at -80°C until ready for use.

SDS-PAGE and western blotting

K562 and HCT116 cell lysates were combined with Laemmli buffer and resolved on Novex WedgeWell 4-20% Tris-Glycine gels (Invitrogen). Gels were transferred to an Immuno-Blot PVDF membrane (Bio-Rad). Membranes were immunoblotted with antibodies against CRKL (Cell Signaling Technologies (CST), 38710), p-CRKL Y207 (CST, 3490), RIPK1 (CST, 3493), p-RIPK1 S116 (CST, 65746), TCTP (CST, 5128), and p-TCTP S46 (CST, 5251). Following primary antibody, membranes were incubated in goat anti-rabbit IgG-HRP secondary antibody (Santa Cruz Biotechnology, sc-2004).

Cell cycle synchronization

HCT116 cells were grown to approximately 50% confluency. The media was removed and replaced with media containing 100 ng/mL nocodazole (Cayman, 13857). The cells were allowed to incubate for 20 hours. After 20 hours, each compound was added at the desired concentration. The cells were allowed to further incubate for 1 hour. After 1 hour the cells were washed three times with PBS and lysed in RIPA buffer. 20 μg of each lysate was separated by SDS-PAGE and analyzed with immunoblot.

Initiation of necroptosis

HCT116 cells were grown to approximately 50% confluency. The media was removed and replaced with media containing 20 μM Z-VAD(Ome)-FMK (Cayman, 14463), 100 nM Birinapant (Cayman, 19699), and 10 ng/mL soluble recombinant human TNF- α (Cayman, 32020) (Laurien et al., 2020 [DOI](#)). Cells were incubated for 6 hours in the presence of various inhibitors or vehicle (DMSO). In the end, cells were washed three times with PBS and lysed in RIPA buffer. 20 μg of each lysate was separated by SDS-PAGE and analyzed with immunoblot.

Cell-based PISA

K562 cells were grown to a concentration of 1×10^6 cells/mL. The cells were pelleted and resuspended on a 1:1 mixture of conditioned media and fresh media for a final concentration of 6×10^6 cells/mL. Each compound was added to fresh media at a 3X concentration (30 μ M). In order to initiate the experiment, 500 μ L of cell suspension was mixed with 1 mL of 3X treatment media and plated in a 24 well untreated tissue culture plate to achieve a final cell concentration of 2×10^6 cells/mL and a compound concentration of 10 μ M. The cells were allowed to incubate for 30 minutes. After incubation, an equal volume of each culture was transferred to 10 PCR tubes. The PCR tubes were heated across a thermal gradient ranging from 48°C-58°C (or 37°C-62°C) for 3 minutes to induce thermal denaturation. The samples were allowed to cool to room temperature and then an equal volume from each PCR tube was pooled. The cells in each pooled sample were washed once with PBS and then an equal volume of extraction buffer (1X PBS pH 7.4, 0.5% NP-40, protease inhibitors) was added to each pellet. Samples were incubated for 10 minutes at 4°C on a roller. Extracted samples were spun at 21,000xg for 90 minutes to separate insoluble aggregates from soluble protein. An equal volume from each soluble fraction was collected and prepared for LC-MS/MS analysis.

Crude extract PISA (Lysate-based PISA)

Frozen K562 or HCT116 pellets were thawed on ice and resuspended in lysis buffer (1X PBS pH 7.4, 1 mM $MgCl_2$, protease inhibitor). The proteomes were extracted using a dounce homogenizer (20 strokes). The extracts were spun at 300xg for 3 minutes to remove any unbroken cells. The resulting crude extract was diluted to 2 mg/mL in lysis buffer. Each compound was added to lysis buffer at a 2X concentration (usually 20 μ M). In order to initiate the experiment, an equal volume of crude extract and treatment buffer were combined, to achieve a final protein concentration of 1 mg/mL and compound concentration of 10 μ M, and incubated for 30 minutes. After incubation, an equal volume of each sample was transferred to 10 PCR tubes. The PCR tubes were heated across a thermal gradient ranging from 48°C-58°C for 3 minutes to induce thermal denaturation. An equal volume from each PCR tube was pooled. An equal volume of extraction buffer (1X PBS pH 7.4, 1% NP-40, protease inhibitors) was added to each pooled sample to achieve a final NP-40 concentration of 0.5%. Samples were incubated for 10 minutes at 4°C on a roller. Extracted samples were spun at 21,000xg for 90 minutes to separate insoluble aggregates from soluble protein. An equal volume from each soluble fraction was collected and prepared for LC-MS/MS analysis.

LC-MS sample preparation

Samples (20 μ g protein) were diluted in prep buffer (400 mM EPPS pH 8.5, 1% SDS, 10 mM tris(2-carboxyethyl)phosphine hydrochloride) and incubated at room temperature for 10 minutes. Iodoacetimide was added to a final concentration of 10 mM to each sample and incubated for 25 minutes in the dark. Finally, DTT was added to each sample to a final concentration of 10 mM. A buffer exchange was carried out using a modified SP3 protocol (Hughes et al., 2019, 2014). Briefly, ~250 μ g of Cytiva SpeedBead Magnetic Carboxylate Modified Particles (65152105050250 and 4515210505250), mixed at a 1:1 ratio, were added to each sample. 100% ethanol was added to each sample to achieve a final ethanol concentration of at least 50%. Samples were incubated with gentle shaking for 15 mins. Samples were washed three times with 80% ethanol. Protein was eluted from SP3 beads using 200 mM EPPS pH 8.5 containing trypsin (ThermoFisher Scientific, 90305R20) and Lys-C (Wako, 129-02541). Samples were digested overnight at 37°C with vigorous shaking. Acetonitrile was added to each sample to achieve a final concentration of ~33%. Each sample was labelled, in the presence of SP3 beads, with ~60 μ g of TMTPro 16plex reagents (ThermoFisher Scientific). Following confirmation of satisfactory labelling (>97%), excess TMT was quenched by addition of hydroxylamine to a final concentration of 0.3%. The full volume from each sample was pooled and acetonitrile was removed by vacuum centrifugation for 1 hour. The

pooled sample was acidified using formic acid and peptides were de-salted using a Sep-Pak 50mg tC18 cartridge (Waters). Peptides were eluted in 70% acetonitrile, 1% formic acid and dried by vacuum centrifugation. The peptides were resuspended in 10mM ammonium bicarbonate pH 8, 5% acetonitrile and fractionated by basic pH reverse phase HPLC. In total 24 fractions were collected. The fractions were dried in a vacuum centrifuge, resuspended in 5% acetonitrile, 1% formic acid and desalted by stage-tip. Finally, peptides were eluted in, 70% acetonitrile, 1% formic acid, dried, resuspended in 5% acetonitrile, 5% formic acid, and analyzed by LC-MS/MS.

For analysis of phosphopeptides, samples (100 ug protein) were prepared and digested as described above. Following digestion, acetonitrile was added to each sample to achieve a final concentration of ~33%. Each sample was labelled, in the presence of SP3 beads, with ~300 µg of TMTPro 16plex reagents (ThermoFisher Scientific). Following confirmation of satisfactory labelling (>97%), excess TMT was quenched by addition of hydroxylamine to a final concentration of 0.3%. The full volume from each sample was pooled and acetonitrile was removed by vacuum centrifugation for 1 hour. The pooled sample was acidified using trifluoroacetic acid (TFA) and peptides were de-salted using a Sep-Pak 200 mg tC18 cartridge (Waters). Peptides were eluted in 70% acetonitrile, 1% formic acid and dried by vacuum centrifugation. Phosphopeptides were enriched using a High Select Phosphopeptide Enrichment Kit (ThermoFisher Scientific, A32992). Following elution, phosphopeptides were acidified with formic acid and dried by vacuum centrifugation. The flow through from the phosphopeptide enrichment columns, which was retained for total proteome analysis, was fractionated as described previously. The dried phosphopeptides were solubilized in 5% acetonitrile, 0.1% TFA, desalted by stage-tip, and analyzed by LC-MS/MS.

Offline basic reversed phase fractionation

TMT labeled peptides were solubilized in 5% acetonitrile/10 mM ammonium bicarbonate, pH 8.0 and ~300 µg of TMT labeled peptides were separated by an Agilent 300 Extend C18 column (3.5 µm particles, 4.6 mm ID and 250 mm in length). An Agilent 1260 binary pump coupled with a photodiode array (PDA) detector (ThermoFisher Scientific) was used to separate the peptides. A 45-minute linear gradient from 10% to 40% acetonitrile in 10 mM ammonium bicarbonate pH 8.0 (flow rate of 0.25 mL/min) separated the peptide mixtures into a total of 96 fractions (36 seconds).

A total of 96 Fractions were consolidated into 24 samples in a checkerboard fashion and vacuum dried to completion.

Mass spectrometry data acquisition

Total proteome data were collected on Orbitrap Eclipse mass spectrometer (ThermoFisher Scientific) coupled to a Proxeon EASY-nLC 1000 (or 1200) LC pump (ThermoFisher Scientific). Peptides were separated using a 90-120 minute gradient at 500-550 nL/min on a 30 cm column (i.d. 100 µm, Accucore, 2.6 µm, 150 Å) packed inhouse. High-field asymmetric-waveform ion mobility spectroscopy (FAIMS) was enabled during data acquisition with compensation voltages (CVs) set as -40 V, -60 V, and -80 V (Schweppe et al., 2019). MS1 data were collected using the Orbitrap (Resolution – 60,000; Scan range – 400-1600 Th; Automatic gain control (AGC) -4×10⁵; Normalized AGC target – 100%; maximum ion injection time – 50 ms). Determined charge states between 2 and 6 were required for sequencing, and a 90 s dynamic exclusion window was used. Data dependent mode was set as cycle time (1 s). MS2 scans were collected in the orbitrap after high-energy collision dissociation (HCD) fragmentation (Resolution – 50,000; AGC target – 1×10⁵; Normalized AGC target – 200%; Normalized collision energy – 36; Isolation window – 0.5 Th; Maximum ion injection time – 86 ms).

Phosphorylation data were collected on Orbitrap Eclipse mass spectrometer (ThermoFisher Scientific) coupled to a Proxeon EASY-nLC 1000 (or 1200) LC pump (ThermoFisher Scientific). Peptides were separated using a 90-120 minute gradient at 500-550 nL/min on a 30 cm column (i.d.

100 μm , Accucore, 2.6 μm , 150 $\text{\AA}$) packed inhouse. High-field asymmetric-waveform ion mobility spectroscopy (FAIMS) was enabled during data acquisition with compensation voltages (CVs) set as -40 V, -60 V, and -80 V for the first shot and -45 V and -75 V for the second shot. MS1 data were collected using the Orbitrap (resolution – 120,000; maximum injection time – 50 ms; AGC target – 4×10^5). Determined charge states between 2 and 5 were required for sequencing, and a 120 second dynamic exclusion window was used. Data dependent mode was set as cycle time (1 second). MS2 scans were performed in the Orbitrap after HCD fragmentation (resolution – 50,000; isolation window – 0.5 Da; collision energy – 36%; maximum injection time – 250 ms; AGC – 1.5×10^5 ; Normalized AGC target – 300%).

Mass spectrometry data analysis

Raw files were first converted to mzXML, and monoisotopic peaks were assigned using Monocle (Rad et al., 2021 [DOI](#)). Database searching included all human entries from Uniprot (downloaded on February 25th, 2020). The database was concatenated with one composed of all protein sequences in the reversed order (Elias and Gygi, 2007 [DOI](#)). Sequences of common contaminant proteins (e.g., trypsin, keratins, etc.) were appended as well. Searches were performed with Comet (Eng et al., 2013 [DOI](#)) using a 50 ppm precursor ion tolerance and 0.02 Da product ion tolerance. TMT on lysine residues and peptide N termini (+304.207 Da) and carbamidomethylation of cysteine residues (+57.0215 Da) were set as static modifications, while oxidation of methionine residues (+15.9949 Da) was set as a variable modification. For phosphorylation searches, a variable modification of 79.9663 was set for serine, threonine, and tyrosine residues. Phosphorylation site localization was determined using AScorePro (Gassaway et al., 2022 [DOI](#)). Peptide-spectrum matches (PSMs) were adjusted to a 1% false discovery rate (FDR; Elias and Gygi, 2007 [DOI](#)). PSM filtering was performed using linear discriminant analysis (LDA) as described previously (Huttlin et al., 2010 [DOI](#)) while considering the following parameters: XCorr, ΔCn , missed cleavages, peptide length, charge state, and precursor mass accuracy. Each run was filtered separately. Protein-level FDR was subsequently estimated at a data set level. For each protein across all samples, the posterior probabilities reported by the LDA model for each peptide were multiplied to give a protein-level probability estimate. Using the Picked FDR method (Savitski et al., 2015 [DOI](#)) proteins were filtered to the target 1% FDR level. TMT reporter ion intensities were measured using a 0.003 Da window around the theoretical m/z of each reporter ion. Proteins were quantified by summing reporter ion counts across all matching PSMs. Reporter ion intensities were adjusted to correct for the isotopic impurities of the different TMT reagents according to manufacturer specifications. Peptides were filtered to exclude those with a summed signal-to-noise (SN) < 160 across all TMT channels. To control for different total protein loading within a TMT experiment, the summed protein quantities of each channel were adjusted to be equal within the experiment. For phosphorylation experiments, the normalization factors that were applied to the associated proteome we also applied the phosphoproteome.

Protein engagement hit calling

Compound engagement was determined based on relative thermal stability to DMSO controls. Owing to the fact that the duplicate analyses used in the initial screen would result in common statistical tests (Welch's t-test) being underpowered, we used a combination of fold changes compared to DMSO and individual protein variance to call hits. First, relative thermal stability to the DMSO controls was determined. Second, for each protein across all cells or lysate assays, the number of standard deviations away from the mean thermal stability measurement (z-score) for a given protein was quantified. Cutoffs for fold change and z-score were determined to limit the number of hits derived from DMSO-treated samples. We maintained the same cutoffs across both datasets. We considered proteins to engage a compound if both replicates of the compound treatments resulted when the thermal stability fold change compared to DMSO greater than an absolute value of $\log_2 0.2$ and an absolute z-score greater than 3.5. This resulted in a false hit rate ($n_{\text{DMSO-Hits}}$) of 1% across all lysate-based assays and 3% for all cell-based assays.

Statistical analyses

Follow-up PISA data were analyzed using Perseus (Tyanova et al., 2016 [DOI](#)). Significant changes were determined using a permutation-based FDR with the following settings – FDR – 0.05, S0 – 0.1, and number of randomizations – 250. Individual fold change values were calculated in reference to the mean of the vehicle-treated samples.

Correlation analyses were run in R version 4.3.1 using rank-based Spearman's rho (r_{spearman}) to minimize the effects of thermal stability-based outliers. The list of binary comparisons was filtered to include the top 5% of highly correlated and anticorrelated protein pairs. Significant pairwise correlations for individual comparisons were determined using the psych package.

Structural modeling

Protein structures and associated ligands were modeled and aligned using the ICMBrowser v3.9 (Molsoft) and displayed either using the either Pymol v2.5.1 (**Figure 2H** [DOI](#)–**2I** [DOI](#)) or ICMBrowser (**Figure 6H** [DOI](#)). PDB files were downloaded from RSCB PDB for the following identifiers: 2rku, 5l2i, 5g6v.

Data availability

The mass spectrometry data have been deposited to the ProteomeXchange Consortium with the data set identifier PDX048009 (initial submission) and PDX053138 (revised submission). Information regarding TMT channel assignments and plex layout can be found in Table 2.

Table 1. Library compounds. A list of all compounds utilized in the cell-and lysate-based PISA screens.

Table 2. Sample multiplexing layout. This table contains the TMTPro channel assignments for all samples generated in the cell-and lysate-based screens.

Table 3. Cell-based PISA full dataset. This table contains all the data from the cell-based screen in an easy to graph format.

Table 4. Lysate-based PISA full dataset. This table contains all the data from the lysate-based screen in an easy to graph format.

Figure 1 – source data 1. This table contains cell-based PISA data for K562 cells treated with ribociclib, palbociclib, and abemaciclib.

Figure 1 – source data 2. This table contains the PISA data for cell-and lysate-based screens.

Figure 2 – source data 1. This table contains cell-based PISA data for K562 cells treated with increasing concentrations of BI 2536 and palbociclib.

Figure 2 – source data 2. This table contains cell-based PISA data for K562 cells treated with increasing concentrations of BI 2536 and NVP-TAE-226.

Figure 2 – source data 3. This document contains the unedited western scans for the panels displayed in **Figure 2F** [DOI](#) and **Figure 2G** [DOI](#) – figure supplement 1D.

Figure 4 – source data 1. This table contains lysate-based PISA data for HCT116 lysates treated with necrostatin-2, GSK2606414, AZD-5438, and tozasertib.

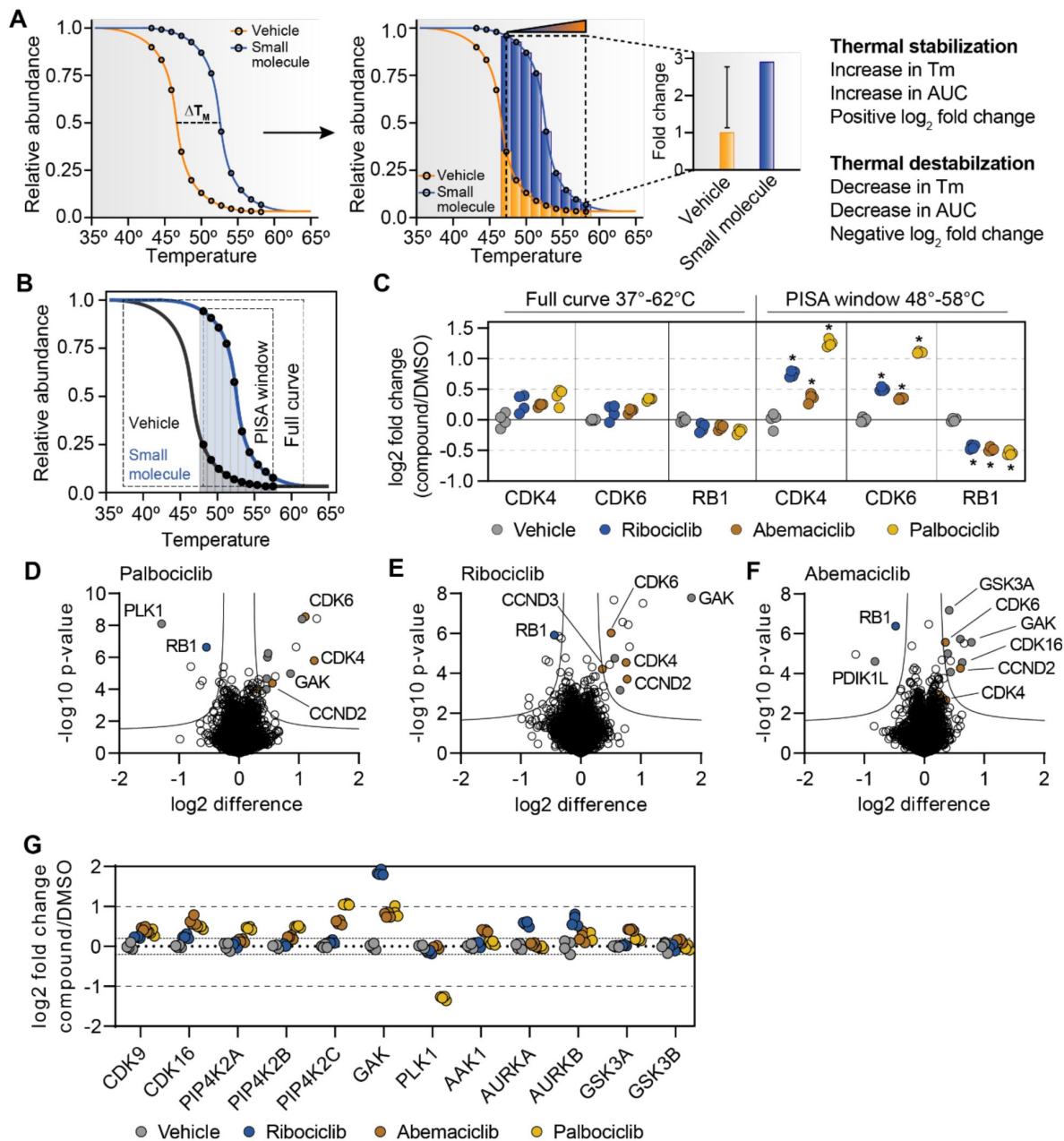


Figure 1 – figure supplement 1.

Establishing a robust and efficient workflow for assessing changes in protein thermal stability in living cells.

A. Schematic depicting how thermal shifts are measured in a PISA experiment. An increase in melting temperature (thermal stabilization) will result in an increase in the AUC and a positive $\log_2$ fold change in soluble protein abundance. A decrease in melting temperature (thermal destabilization) will result in a decrease in the AUC and a negative $\log_2$ fold change in soluble protein abundance. B. PISA schematic depicting a thermal window vs. a full melting curve. C–G. K562 cells (N=4) were treated with 10 μ M palbociclib (D), ribociclib (E), or abemaciclib (F) for 15 minutes and assayed using PISA. (C) The $\log_2$ fold changes for specific proteins are plotted for each treatment using a full melting curve (left) or thermal window (48°C–58°C; right). (D–F). Data is presented as a volcano plot to highlight significant changes in abundance utilizing the thermal window (48°C–58°C). Significant changes were determined using a permutation-based FDR (FDR = 0.05, S0 = 0.1). (G). $\log_2$ fold change values for selected proteins following treatment with each compound in D–F.

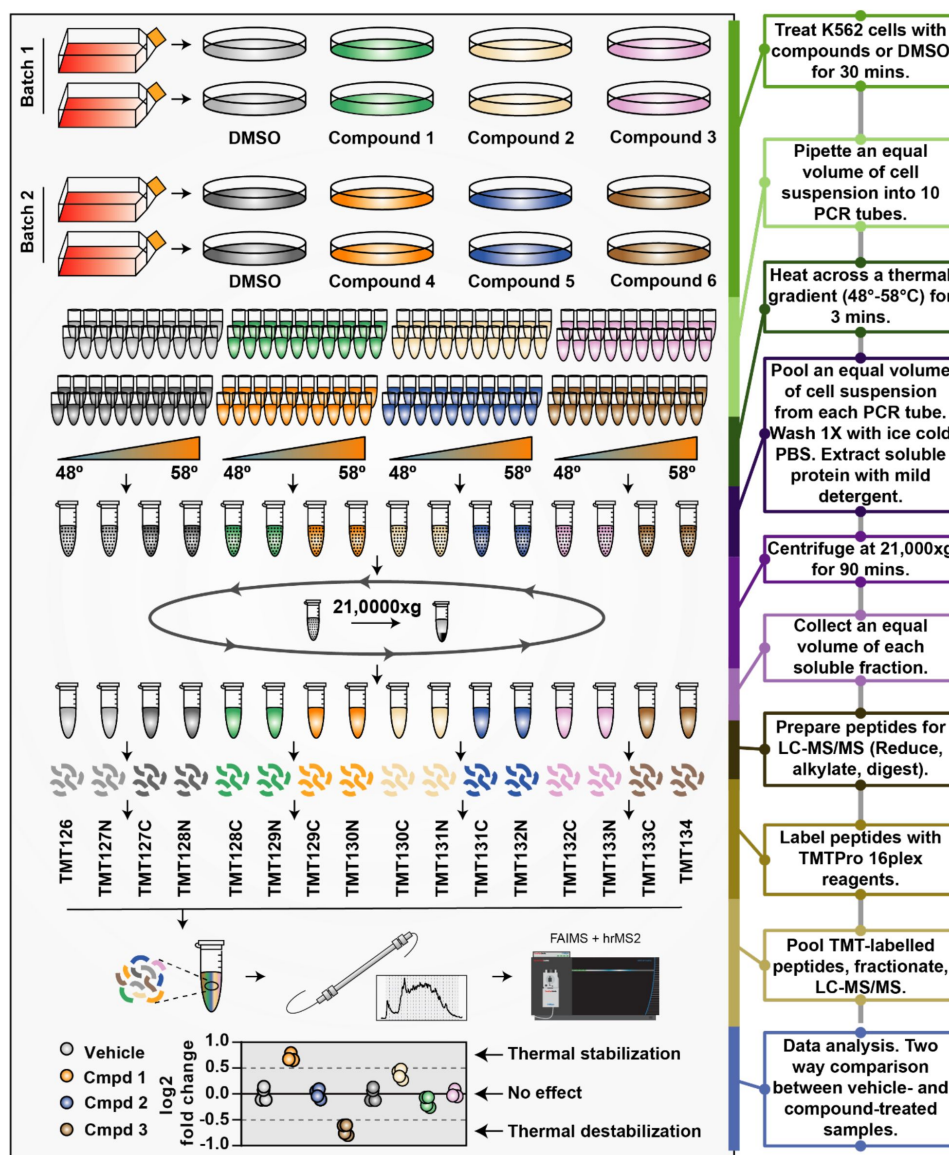


Figure 1 – figure supplement 2.

Establishing a robust and efficient workflow for assessing changes in protein thermal stability in living cells.

K562 cells were treated with each of the 96 compounds at 10 μ M for 30 minutes. Three compounds and a DMSO control were assayed in duplicate (batch). All 96 compounds and 64 DMSO controls were assayed using thirty-two treatment batches. Following treatment, an equal number of cells were transferred to 10 PCR tubes. The cells were placed in a thermal cycler and heated across a thermal gradient from 48°C-58°C for 3 minutes. The cells were allowed to cool to room temperature for 5 minutes. An equal volume from each PCR tube was pooled and spun at 300 x g for 3 minutes to pellet cells. Cells were washed one with PBS and lysed in a buffer containing 0.5% NP-40, which will dissolve membranes without disrupting heat-induced protein aggregates. The lysates were centrifuged for 90 minutes at 21,000 x g to separate soluble protein from aggregates. An equal volume of each soluble fraction (~20 μ g) was prepared for LC-MS/MS analysis. Two treatment batches were combined for each TMTpro 16-plex. Each soluble fraction was reduced and alkylated. Each sample was precipitated onto SP3 carboxylate-coated beads to facilitate a buffer exchange. Proteomes were eluted off the SP3 beads into digestion buffer and digested with a combination of Lys-C and trypsin. Peptides from each sample were labeled with a unique TMTpro reagent. TMT-labelled peptides were pooled into a single sample, which was desalted using a sep-pak. Dried peptides were resuspended in HPLC buffer A and fractionated by basic reverse-phase HPLC. Twelve to twenty-four fractions were stage-tipped and analyzed on an Orbitrap Eclipse with a FAIMS device enabled (Thermo Fisher). Changes in thermal stability were determined by comparing soluble protein abundance in a compound-treated samples to vehicle-treated controls.

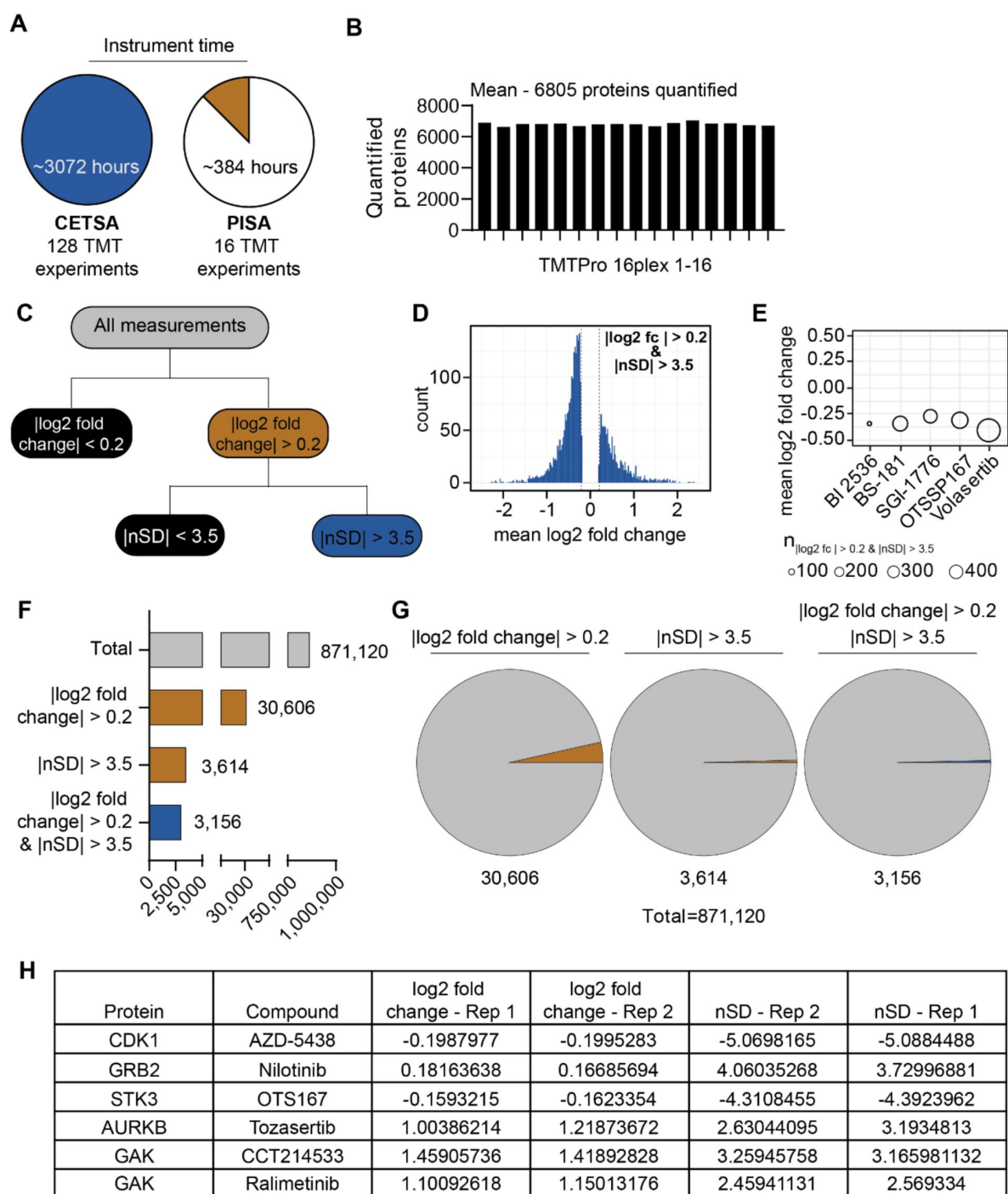


Figure 1 – figure supplement 3.

Establishing a robust and efficient workflow for assessing changes in protein thermal stability in living cells.

A. Minimal instrument time (assuming 12 fractions per plex) required to assay 96 compounds and 32 DMSO controls in duplicate using CETSA (left) or PISA (right). B. Total proteins quantified per TMTpro 16-plex. C. Schematic depiction of the filtering scheme used to define significant changes. D. Histogram depicting the $\log_2$ fold change measurements that were greater than 3.5 standard deviations from the mean. E,F. Total number of proteins passing each filter. G. Pie charts depicting the fraction of all measurements that pass each filter. H. Table highlighting proteins that pass the $\log_2$ fold change filter, but not the nSD filter and vice versa.

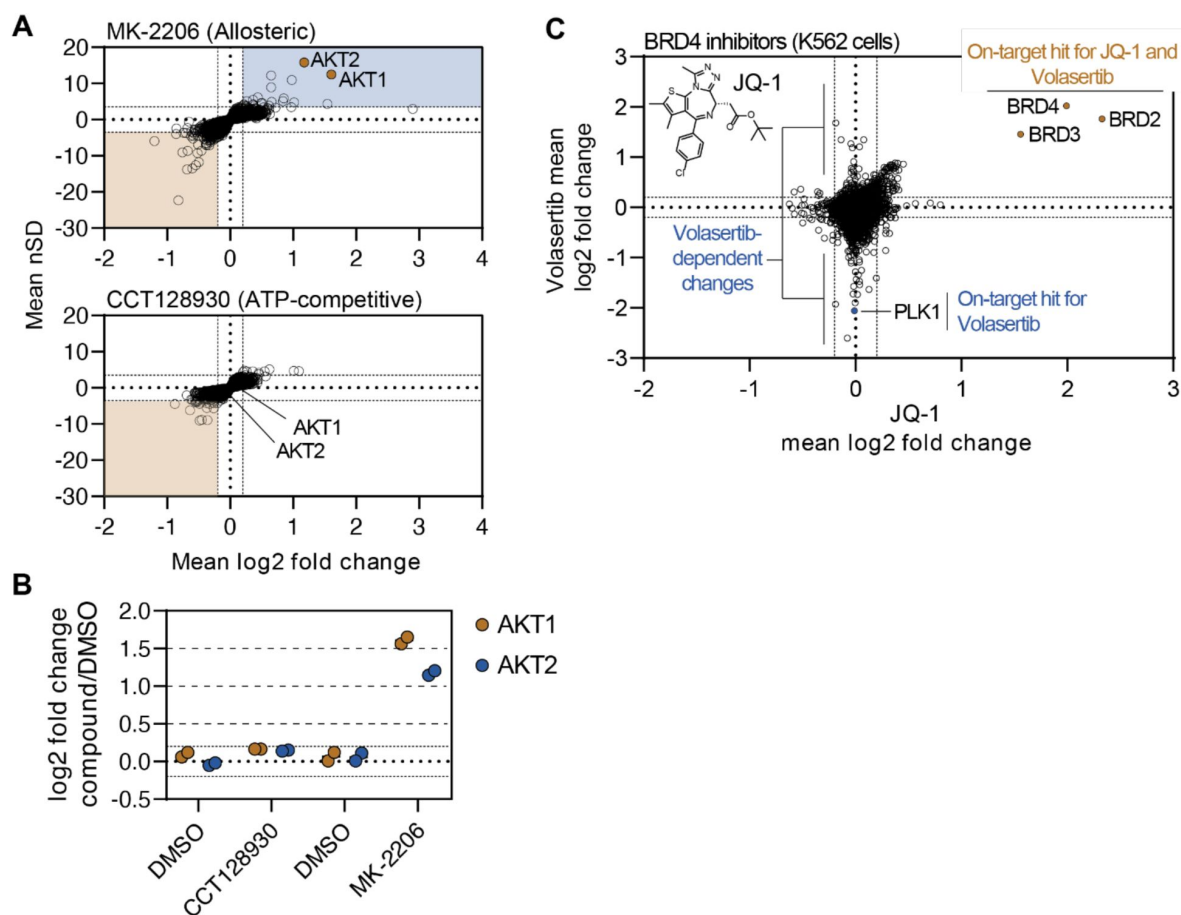


Figure 1 – figure supplement 4.

Establishing a robust and efficient workflow for assessing changes in protein thermal stability in living cells.

A-B. K562 cells (N=2) were treated with MK-2206 (top) or CCT128930 (bottom) at 10 μ M for 30 minutes and assayed using PISA. The mean log₂ fold change of duplicate measurements is plotted on the x-axis and the mean nSD is plotted on the y-axis. Blue boxes contain proteins that exhibit an increase in solubility (increase in melting temperature) and orange boxes contain proteins that exhibit a decrease in solubility (decrease in melting temperature). Orange points represent the known target of each compound. B. The plot displays the log₂ fold change values for selected proteins following treatment with each compound in A. C. Plot comparing the log₂ fold changes measurements for each protein in JQ-1 (x-axis)-and volasertib (y-axis)-treated K562 cells. Orange points represent the known targets of the compounds.

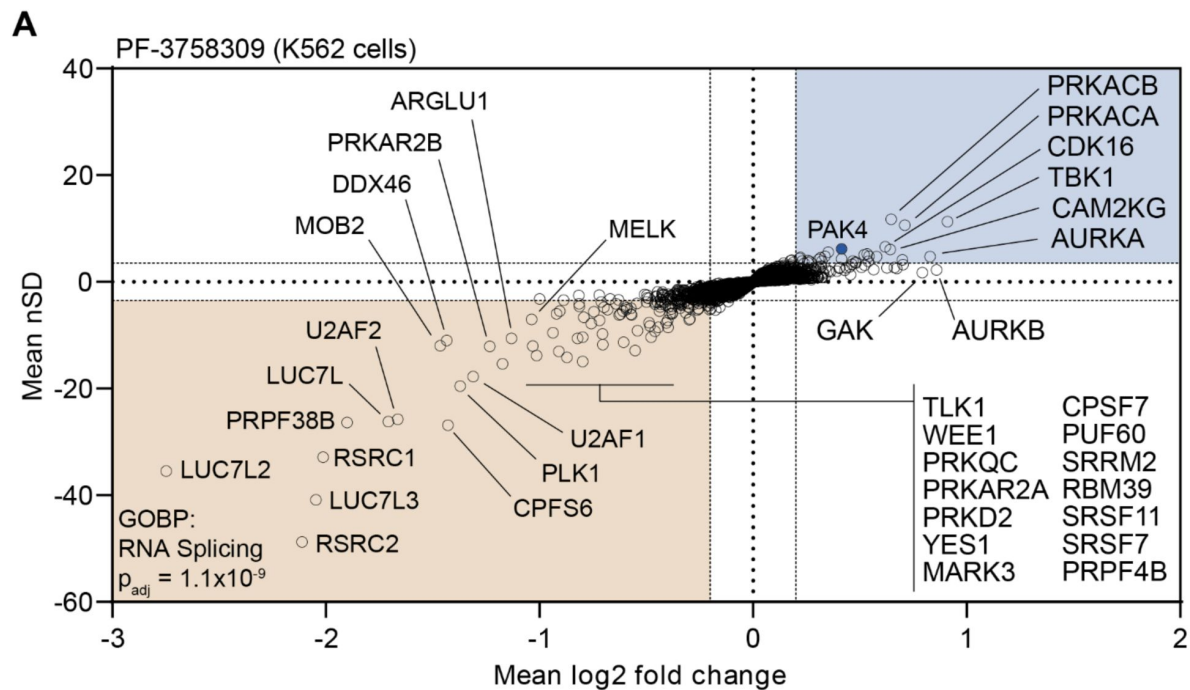


Figure 1 – figure supplement 5.

Establishing a robust and efficient workflow for assessing changes in protein thermal stability in living cells.

A. Plot highlighting the significant changes in protein thermal stability following treatment with PF-3758309. The mean log₂ fold change of duplicate measurements is plotted on the x-axis and the mean nSD is plotted on the y-axis. Blue boxes contain proteins that exhibit an increase in solubility (increase in melting temperature) and orange boxes contain proteins that exhibit a decrease in solubility (decrease in melting temperature). The blue point represents the known target.

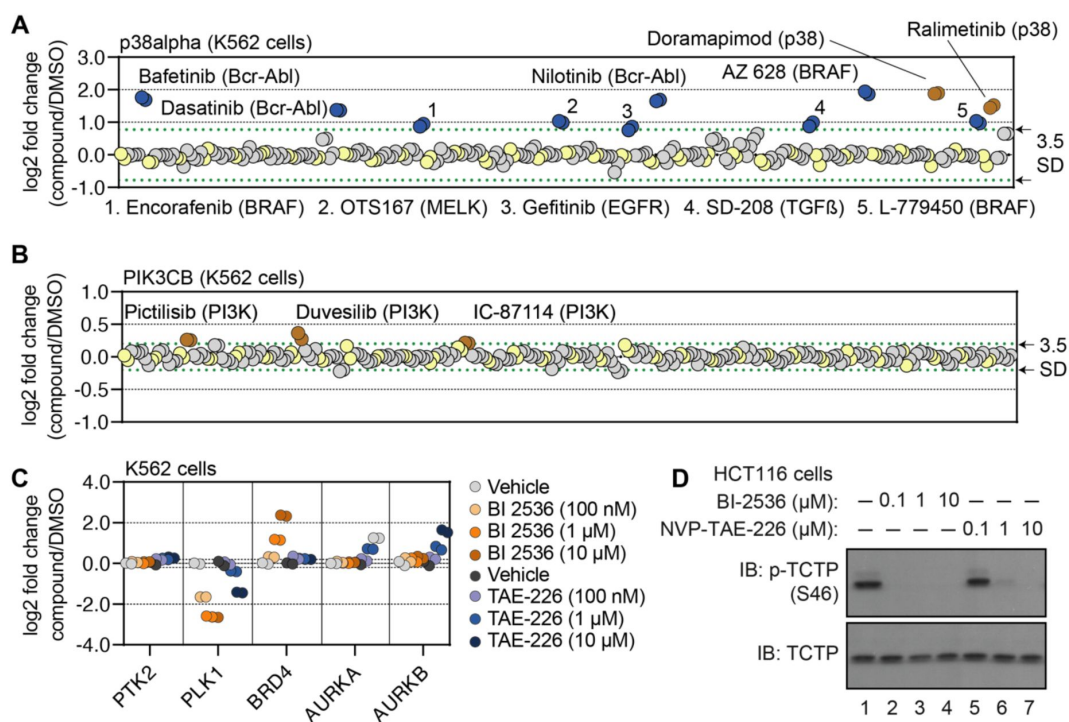


Figure 2 – figure supplement 1.

Using cell-based PISA data to assess compound target engagement.

A-B. A protein-centric view of p38α (A) and PIK3CB (B) solubility (thermal stability) in response to all treatments. Log₂ fold change is plotted on the y-axis. The points represent each of the 256 treatments that were performed. Orange points indicate compounds known to target each respective protein. Blue points indicate other compounds that result in a significant thermal stabilization. Green dashed lines mark a SD of 3.5 cutoff for each treatment. C-D. K562 cells were treated with the indicated concentrations of NVP-TAE-226 or BI 2536 for 15 minutes. Changes in protein thermal stability are represented as a log₂ fold change in soluble protein abundance for each treatment in reference to a DMSO-treated control (C). PLK1 activity was determined using a western blot for p-TCTP (S46) and total TCTP (D).

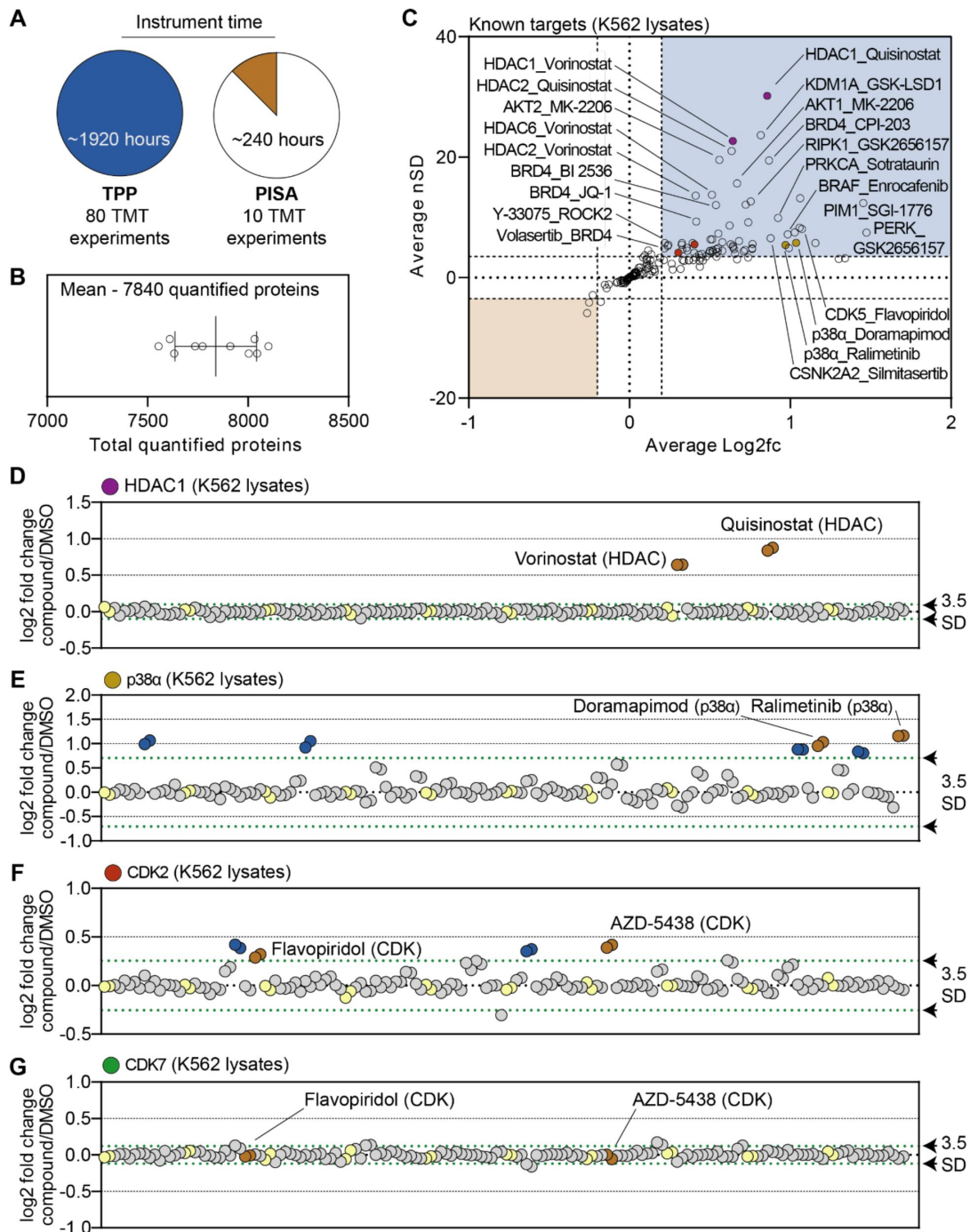


Figure 3 – figure supplement 1.

A chemical perturbation screen of 70 compounds in K562 native extracts.

A. Minimal instrument time required to assay 70 compounds and 10 DMSO controls in duplicate using TPP (left) or PISA (right). B. Total number of proteins quantified in each plex in the lysate-based screen. C. A plot of the mean $\log_2$ fold change (x-axis) and mean nSD (y-axis) of each known compound-target pair that were quantified in the lysate-based screen. Blue boxes contain proteins that exhibit an increase in solubility (increase in melting temperature) and orange boxes contain proteins that exhibit a decrease in solubility (decrease in melting temperature). D-G. Protein-centric view of HDAC1 (D), p38 α (E), CDK2 (F), and CDK7 (G) solubility (thermal stability) in response to all treatments. Orange points indicate compounds known to target each respective protein. Blue points indicate other compounds that result in a significant thermal destabilization of each respective protein. Green dashed lines mark a SD of 3.5 cutoff for each compound.

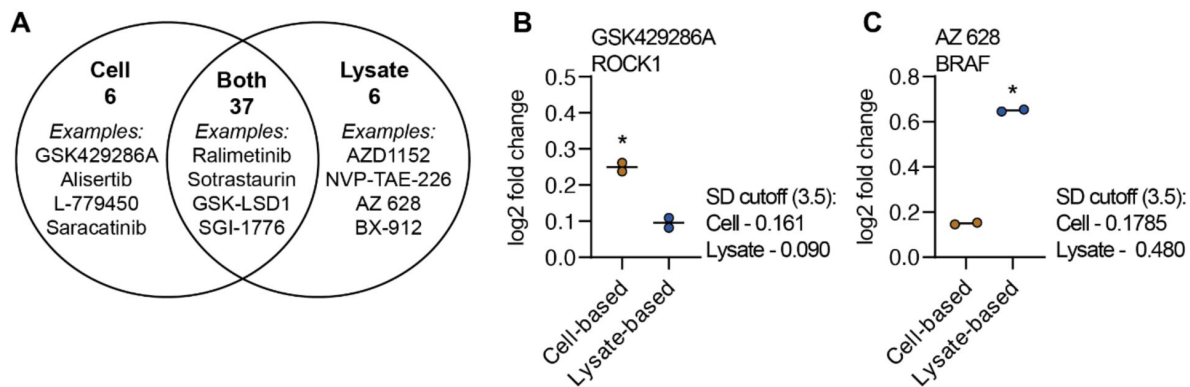


Figure 3 – figure supplement 2.

A chemical perturbation screen of 70 compounds in K562 native extracts.

A. A venn diagram depicting the total number of compounds that cause a change in a known target in cell-and lysate-based PISA. B,C. Log2 fold change measurements for the indicated drug-target pair (GSK429286A_ROCK1 [A] and AZ 628_BRAF [B]) in cell-and lysate-based PISA.

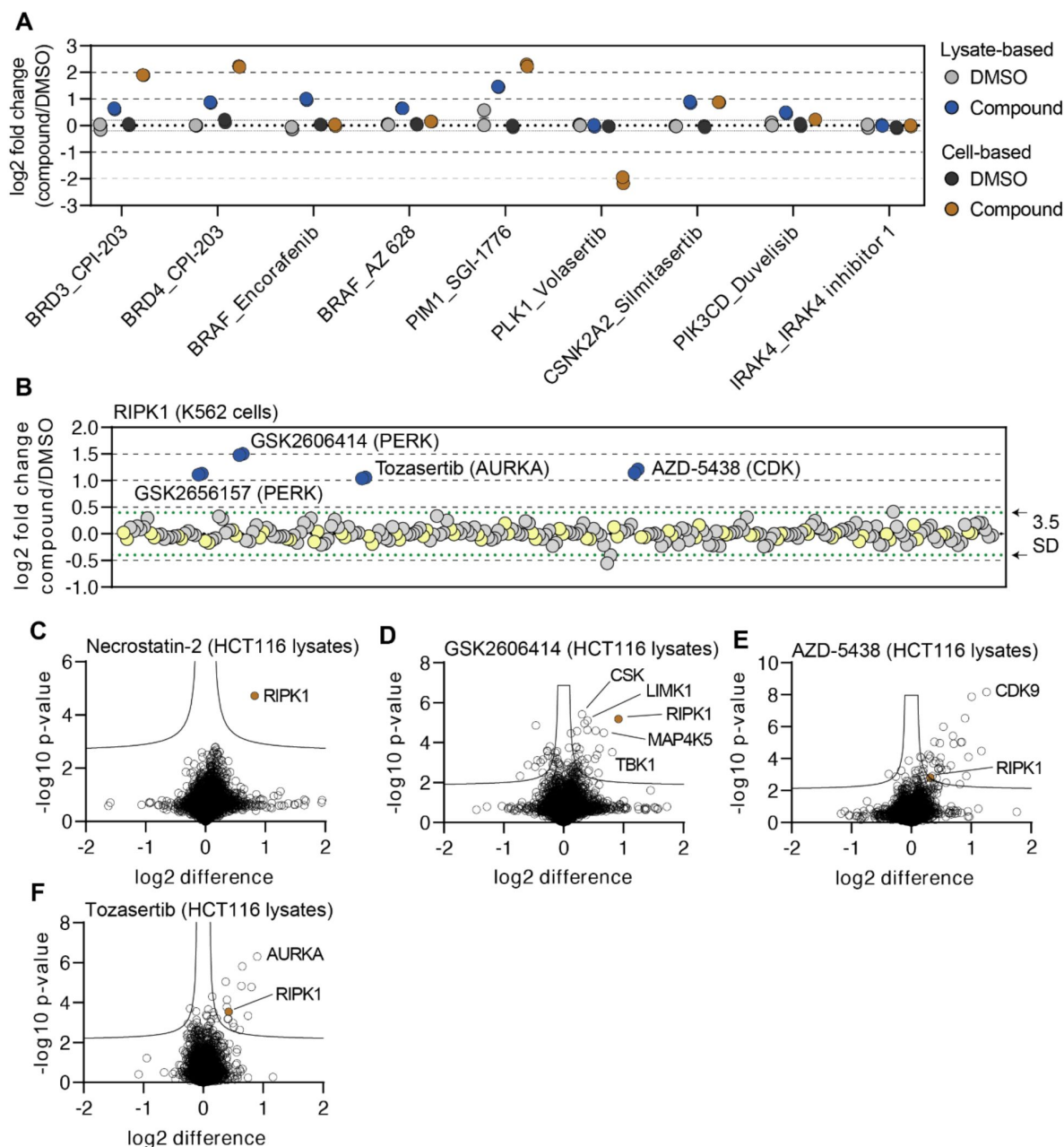


Figure 4 – figure supplement 1.

Cell-and lysate-based PISA are complimentary approaches for assessing mechanism of action.

A. Changes in protein solubility (thermal stability) for the indicated proteins in cell-and lysate-based PISA are represented as a log₂ fold change in protein abundance for each treatment in reference to a DMSO-treated control. Dashed lines indicate a log₂ fold change of +/-0.2. B. A protein-centric view of RIPK1 solubility (thermal stability) in response to all treatments. Log₂ fold change is plotted on the y-axis. The points represent each of the 256 treatments that were performed. Blue points highlight compounds that result in a significant increase in solubility (thermal stabilization). Green dashed lines mark a SD of 3.5 cutoff for RIPK1. C-F. HCT116 lysates were treated with necrostatin-2 (C), GSK2606414 (D), AZD-5438 (E), or tozasertib (F) (N=3) or DMSO (N=4) for 15 minutes and any changes in thermal stability were determined using PISA. Data is presented as a volcano plot to highlight significant changes in abundance. Significant changes were determined using a permutation-based FDR (FDR = 0.05, S0 = 0.1).

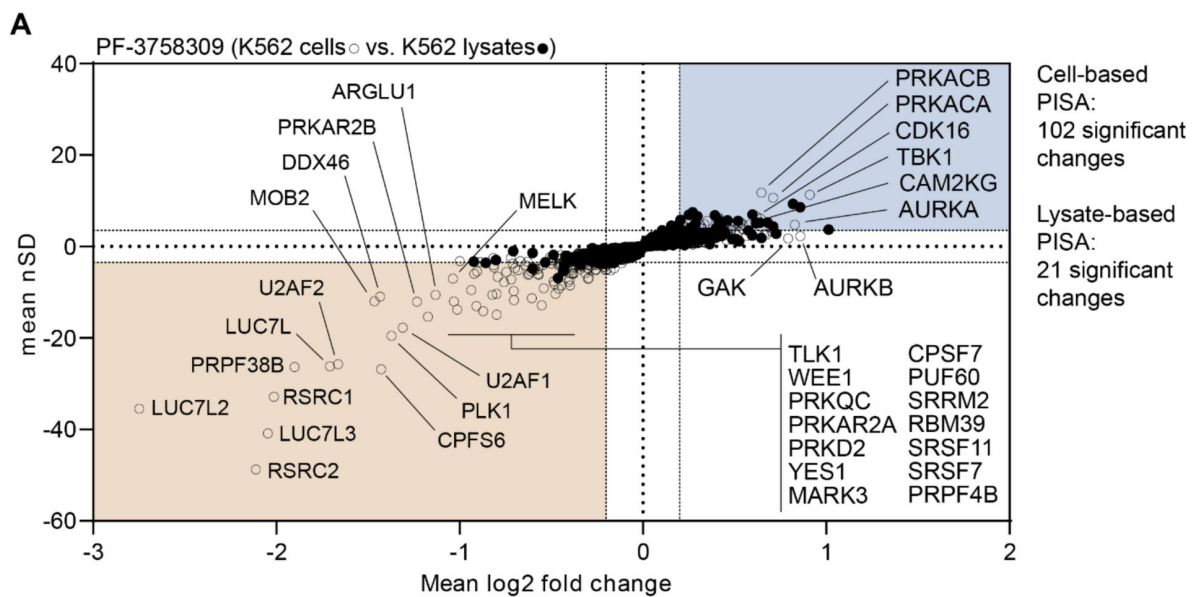


Figure 5 – figure supplement 1.

Disparities in cell-and lysate-based PISA can point toward secondary changes in protein thermal stability.

A. Plot highlighting the significant changes in protein solubility (thermal stability) following treatment with PF-3758309 in cells (no fill) and lysates (black points). The mean log₂ fold change of duplicate measurements is plotted on the x-axis and the mean nSD is plotted on the y-axis. Blue boxes contain proteins that exhibit an increase in solubility (increase in melting temperature) and orange boxes contain proteins that exhibit a decrease in solubility (decrease in melting temperature).

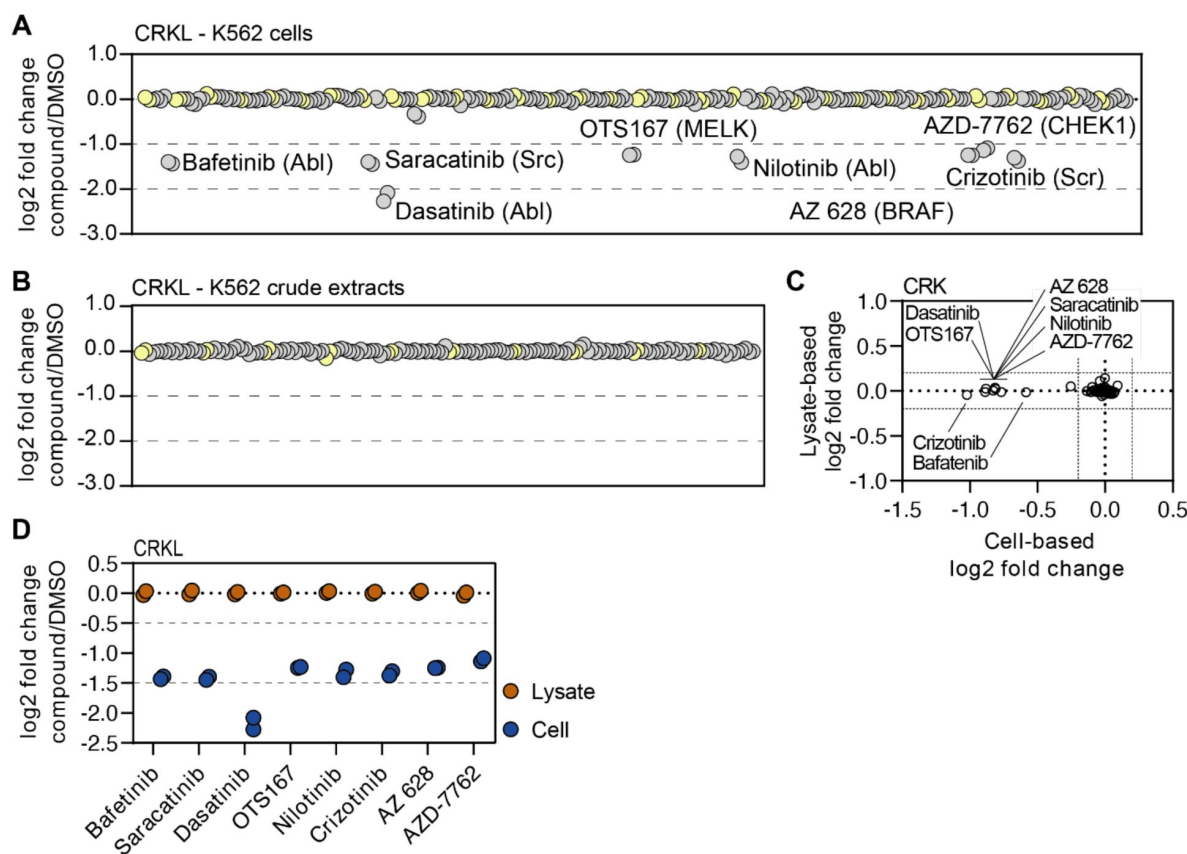


Figure 5 – figure supplement 2.

Disparities in cell-and lysate-based PISA can point toward secondary changes in protein thermal stability.

A and B. A protein-centric view of CRKL solubility (thermal stability) in K562 cells (A) and lysates (B) in response to all treatments. Log₂ fold change is plotted on the y-axis. The points represent each treatment that was performed in each screen. Green dashed lines mark a SD of 3.5 cutoff for each treatment. C. Plots depicting the log₂ fold change values for CRK in response to treatment with all compounds assayed using cell-and lysate-based PISA. D. Changes in protein solubility (thermal stability) for the indicated proteins in cell-and lysate-based PISA are represented as a log₂ fold change in soluble protein abundance for each treatment in reference to a DMSO-treated control.

Figure 5 – figure supplement 3.

Disparities in cell-and lysate-based PISA can point toward secondary changes in protein thermal stability.

A-C. K562 lysates were treated with the indicated concentrations of AZD-7762 for 15 minutes and assayed using PISA. Changes in protein thermal stability are represented as a $\log_2$ fold change in protein soluble abundance for each treatment in reference to a DMSO-treated control. Significant changes were determined using a permutation-based FDR (FDR = 0.05, S_0 = 0.1).

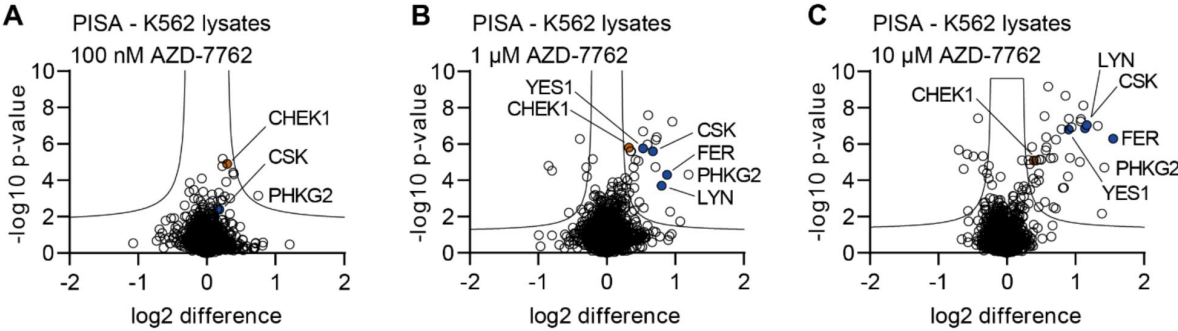
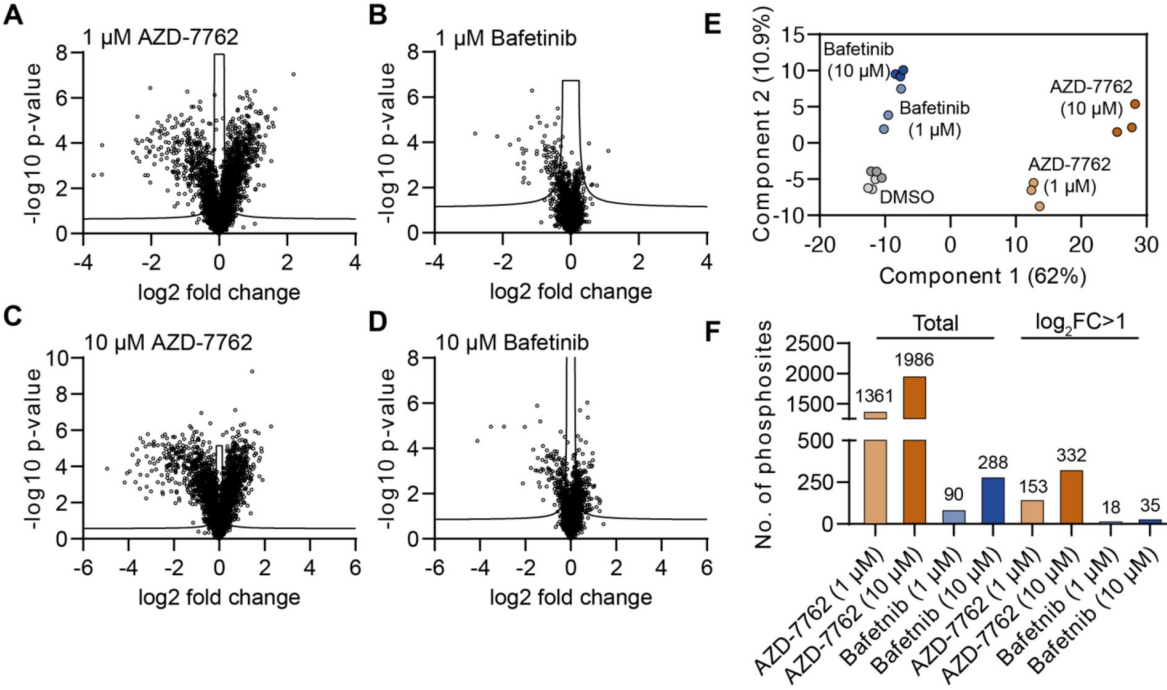


Figure 5 – figure supplement 4.

Disparities in cell-and lysate-based PISA can point toward secondary changes in protein thermal stability.

A-F. K562 cells were treated with the indicated concentration of bafetinib or AZD-7762 for 15 minutes and assayed using phosphoproteomic profiling. A-D. Data is displayed as a volcano plot. Significant changes were determined using a permutation-based FDR (FDR = 0.05, S_0 = 0.1). E. PCA plot of phosphoproteomic data. F. Total number of significant changes that result from each treatment (left) or significant changes with a $\log_2$ fold change > 1 or < -1 (right).



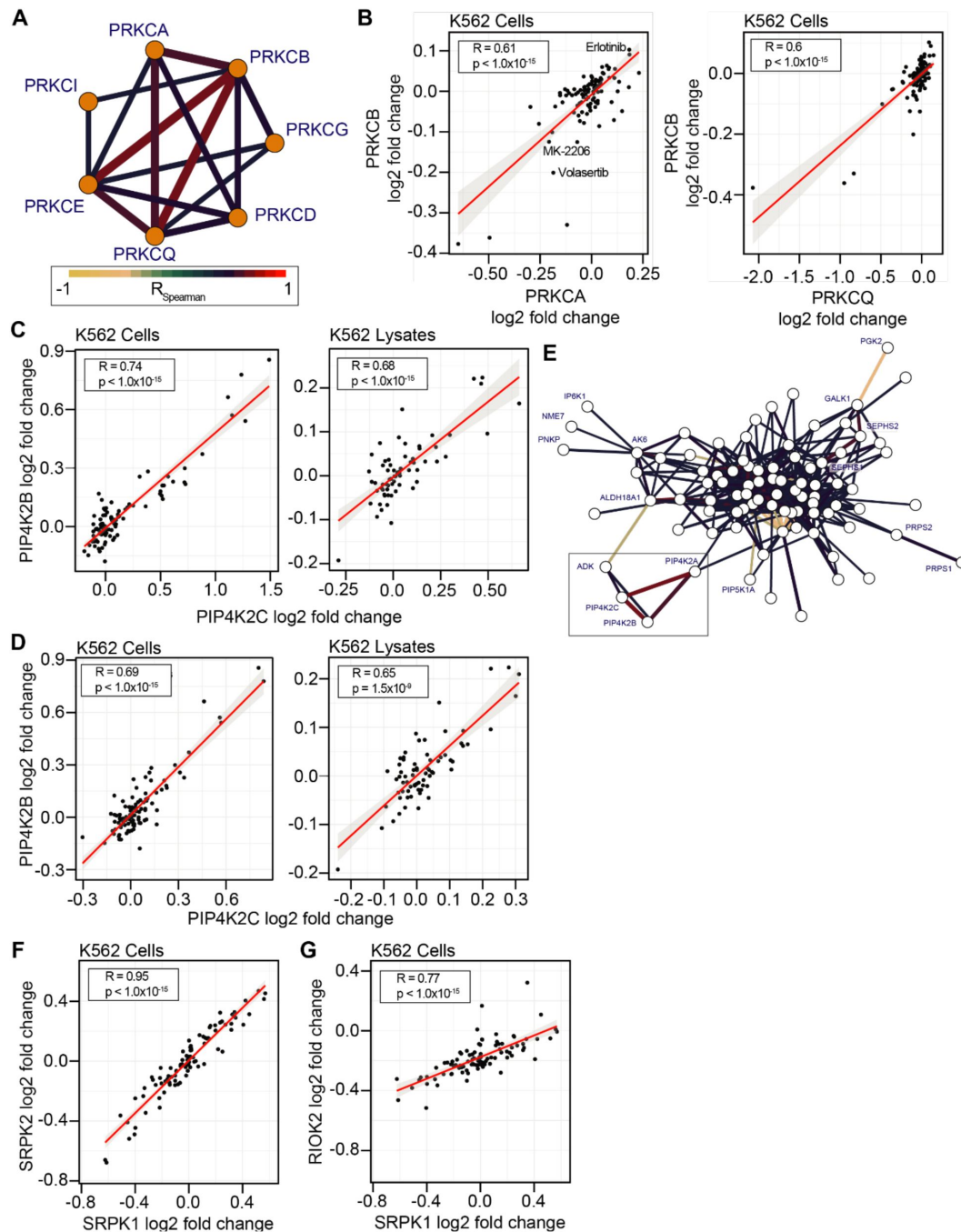


Figure 6 – figure supplement 1.

Concordance of protein engagement for protein complex members and structurally similar kinases.

A. Subgraph from Figure 7A highlighting the correlation between PRKC kinases. Edges are colored based on R_{spearman} . B. Correlation of PRKCB and either PRKCA or PRKCQ. These correlations are driven by consistent but relatively small changes in thermal stability. C. Non-protein kinases of the correlation network graph. Library compounds did not specifically target any non-protein kinases though these compounds elicited consistent solubility (thermal stability) changes in these kinases. Comparison of compound engagement for PIP4K2 non-protein kinases (D) PIP4K2A and PIP4K2B as well as (E) PIP4K2B and PIP4K2C. Compound engagement generates statistically significant correlations in both cells and lysates, with a larger solubility (thermal stability) effect size in cell-based assays. F-G. Significant correlation with small solubility (thermal stability) fold changes for the non-protein kinases SRPK1, SRPK2, and RIOK2.

Figure 4 – source data 2. This document contains the unedited western scans for the panel displayed in **Figure 4G** [↗](#).

Figure 5 – source data 1. This document contains the unedited western scans for the panels displayed in **Figure 5C** [↗](#).

Figure 5 – source data 2. This table contains lysate-based PISA data for K562 lysates treated with 100 nM, 1 μ M, or 10 μ M AZD-7762.

Figure 5 – source data 3. This table contains phosphoproteomic data for K562 cells treated with 1 μ M AZD-7762, 10 μ M AZD-7762, 1 μ M bafetinib, or 10 μ M bafetinib.

Figure 5 – source data 4. This table contains proteomic data for K562 cells treated with 1 μ M AZD-7762, 10 μ M AZD-7762, 1 μ M bafetinib, or 10 μ M bafetinib.

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Reviewer #1 (Public review):

This paper describes proteome solubility analysis (PISA) of 96 compounds in living cells and 70 compounds in cell lysates. A wealth of information related to on- and off-target engagement is uncovered. This work fits well the eLife profile, will be of interest to a large community of proteomics researchers, and thus is likely to be reasonably highly cited.

<https://doi.org/10.7554/eLife.95595.2.sa2>

Reviewer #3 (Public review):

Summary:

This work aims to demonstrate how recent advances in thermal stability assays can be utilised to screen chemical libraries and determine compound mechanism of action. Focusing on 96 compounds with known mechanisms of action, they use the PISA assay to measure changes in protein stability upon treatment with a high dose (10uM) in live K562 cells and whole cell lysates from K562 or HCT116. They intend this work to showcase a robust workflow which can serve as a roadmap for future studies.

Strengths:

The major strength of this study is the combination of live and whole cell lysates experiments. This allows the authors to compare the results from these two approaches to identify novel ligand-induced changes in thermal stability with greater confidence. More usefully, this also enables the authors to separate primary and secondary effects of the compounds within the live cell assay.

The study also benefits from the number of compounds tested within the same framework, which allows the authors to make direct comparisons between compounds.

These two strengths are combined when they compare between CHEK1 inhibitors and suggest that AZD-7762 likely induces secondary destabilisation of CRKL through off-target engagement with tyrosine kinases.

Weaknesses:

One of the stated benefits of PISA compared to the TPP in the original publication (Gaetani et al 2019) was that the reduced number of samples required allows more replicate experiments to be performed. Despite this, the authors of this study performed only duplicate experiments. They acknowledge this precludes use of frequentist statistical tests to identify significant changes in protein stability. Instead, they apply an 'empirically derived framework' in which they apply two thresholds to the fold change vs DMSO: absolute z-score (calculated from all compounds for a protein) > 3.5 and absolute log2 fold-change > 0.2 . They state that the fold-change threshold was necessary to exclude non-specific interactors. While the thresholds appear relatively stringent, this approach will likely reduce the robustness of their findings in comparison to an experimental design incorporating more replicates. Firstly, the magnitude of the effect size should not be taken as a proxy for the importance of the effect. They acknowledge this and demonstrate it using their own data for PIK3CB and p38 α inhibitors (Figure 2B-C). They have thus likely missed many small, but biological relevant changes in thermal stability due to the fold-change threshold. Secondly, this approach relies upon the fold-changes between DMSO and compound for each protein being comparable, despite them being drawn from samples spread across 16 TMT multiplexes. Each multiplex necessitates a separate MS run and the quantification of a distinct set of peptides, from which the protein-level abundances are estimated. Thus, it is unlikely the fold-changes for unaffected proteins are drawn from the same distribution, which is an unstated assumption of their thresholding approach. The authors could alleviate the second concern by demonstrating that there is very little or no batch effect across the TMT multiplexes. However, the first concern would remain. The limitations of their approach could have been avoided with more replicates and use of an appropriate statistical test. It would be helpful if the authors could clarify if any of the missed targets passed the z-score threshold but fell below the fold-change threshold.

The authors use a single, high, concentration of 10 μ M for all compounds. Given that many of the compounds may have low nM IC₅₀s, this concentration could be orders of magnitude above the one at which they inhibit their target. This makes it difficult to assess the relevance of the off-target effects identified to clinical applications of the compounds or biological experiments. The authors acknowledge this and use ranges of concentrations for follow-up studies (e.g. Figure 2E-F). Nonetheless, this weakness is present for the vast bulk of the data presented.

Aims achieved, impact and utility:

The authors have achieved their main aim of presenting a workflow which serves to demonstrate the potential value of this approach. However, by using a single high dose of each compound and failing to adequately replicate their experiments and instead applying heuristic thresholds, they have limited the impact of their findings. Their results will be a useful resource for researchers wishing to explore potential off-target interactions and/or mechanisms of action for these 96 compounds but are expected to be superseded by more robust datasets in the near future. The most valuable aspect of the study is the demonstration that combining live cell and whole cell lysate PISA assays across multiple related compounds can help to elucidate the mechanisms of action.

<https://doi.org/10.7554/eLife.95595.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

This is an interesting and potentially important paper, which however has some deficiencies.

Strengths:

A significant amount of potentially useful data.

Weaknesses:

One issue is a confusion of thermal stability with solubility. While thermal stability of a protein is a thermodynamic parameter that can be described by the Gibbs-Helmholtz equation, which relates the free energy difference between the folded and unfolded states as a function of temperature, as well as the entropy of unfolding. What is actually measured in PISA is a change in protein solubility, which is an empirical parameter affected by a great many variables, including the presence and concentration of other ambient proteins and other molecules. One might possibly argue that in TPP, where one measures the melting temperature change ΔT_m , thermal stability plays a decisive or at least an important role, but no such assertion can be made in PISA analysis that measures the solubility shift.

We completely agree with the insightful comment from the reviewer and we are very grateful that the point was raised. Our goal was to make this manuscript easily accessible to the entire scientific community, not just experts in the field. In an attempt to simplify the language, we likely also simplified the underlying physical principles that these assays exploit. In defense of our initial manuscript, we did state that PISA measures “a fold change in the abundance of soluble protein in a compound-treated sample vs. a vehicle-treated control after thermal denaturation and high-speed centrifugation.” Despite this attempt to accurately communicate the reviewer’s point, we seem to have not been sufficiently clear. Therefore, we tried to further elaborate on this point and made it clear that we are measuring differences in solubility and interpreting these differences as changes in thermal stability.

In the revised version of the manuscript, we elaborated significantly on our original explanation. The following excerpt appears in the introduction (p. 3):

“So, while CETSA and TPP measure a change in melting temperature (ΔT_m), PISA measures a change in solubility (ΔSM). Critically, there is a strong correlation between ΔT_m and ΔSM , which makes PISA a reliable, if still imperfect, surrogate for measuring direct changes in protein thermal stability (Gaetani et al., 2019; Li et al., 2020). Thus, in the context of PISA, a change in protein thermal stability (or a thermal shift) can be defined as a fold change in the abundance of soluble protein in a compound-treated sample vs. a vehicle-treated control after thermal denaturation and high-speed centrifugation. Therefore, an increase in melting temperature, which one could determine using CETSA or TPP, will lead to an increase in the area under the curve and an increase in the soluble protein abundance relative to controls (positive log2 fold change). Conversely, a decrease in melting temperature will result in a decrease in the area under the curve and a decrease in the soluble protein abundance relative to controls (negative log2 fold change).”

And the following excerpt appears in the results section (p. 4):

“In a PISA experiment, a change in melting temperature or a thermal shift is approximated as a

significant deviation in soluble protein abundance following thermal melting and high-speed centrifugation. Throughout this manuscript, we will interpret these observed alterations in solubility as changes in protein thermal stability. Most commonly this is manifested as a log2 fold change comparing the soluble protein abundance of a compound treated sample to a vehicle-treated control (Figure 1 – figure supplement 1A).”

We have now drawn a clear distinction between what we were actually measuring (changes in solubility) and how we were interpreting these changes (as thermal shifts). We trust that the Reviewer will agree with this point, as they rightly claim that many of the observations presented in our work, which measures thermal stability, indirectly, are consistent with previous studies that measured thermal stability, directly. Again, we thank the reviewer for raising the point and feel that these changes have significantly improved the manuscript.

Another important issue is that the authors claim to have discovered for the first time a number of effects well described in prior literature, sometimes a decade ago. For instance, they marvel at the differences between the solubility changes observed in lysate versus intact cells, while this difference has been investigated in a number of prior studies. No reference to these studies is given during the relevant discussion.

We thank the reviewer for raising this point. Our aim with this paper was to test the proficiency of this assay in high-throughput screening-type applications. We considered these observations as validation of our workflow, but admit that our choice of wording was not always appropriate and that we should have included more references to previous work. It was certainly never our intention to take credit for these discoveries. Therefore, we were more than happy to include more references in the revised version. We think that this makes the paper considerably better and will help readers better understand the context of our study.

The validity of statistical analysis raises concern. In fact, no calculation of statistical power is provided.

As only two replicates were used in most cases, the statistical power must have been pretty limited. Also, there seems to be an absence of the multiple-hypothesis correction.

We agree with the reviewer that a classical comparison using a t-test would be underpowered comparing all log2 normalized fold changes. We know from the data and our validation experiments that stability changes that generate log2 fold changes of 0.2 are indicative of compound engagement. When we use 0.2 to calculate power for a standard two-sample t-test with duplicates, we estimated this to have a power of 19.1%. Importantly, increasing this to n=3 resulted in a power estimate of only 39.9%, which would canonically still be considered to be underpowered. Thus, it is important to note that we instead use the distribution of all measurements for a single protein across all compound treatments to calculate standard deviations (nSD) as presented in this work. Thus, rather than a 2-by-2 comparison, we are comparing two duplicate compound treatments to 94 other compound treatments and 18 DMSO vehicle controls. Moreover, we are using this larger sample set to estimate the sampling distribution. Estimating this with a standard z-test would result in a p-value estimate $\lll 0.0001$ using the population standard deviation. Additionally, rather than estimate an FDR using say a BenjaminiHochberg correction, we estimated an empirical FDR for target calls based on applying the same cutoffs to our DMSO controls and measuring the proportion of hits called in control samples at each set of thresholds. Finally, we note that

several other PISA-based methods have used fold-change thresholds similar to, or less than, those employed in this work (PMID: 35506705, 36377428, 34878405, 38293219).

Also, the authors forgot that whatever results PISA produces, even at high statistical significance, represent just a prediction that needs to be validated by orthogonal means. In the absolute majority of cases such validation is missing.

We appreciate this point and we can assure the reviewer that this point was not lost on us. To this point, we state throughout the paper that the primary purpose of this paper was to execute a chemical screen. Furthermore, we do not claim to present a definitive list of protein targets for each compound. Instead, our intention is to provide a framework for performing PISA studies at scale. In total, we quantified thousands of changes and feel that it would be unreasonable to validate the majority of these cases. Instead, as has been done for CETSA (PMID: 34265272), PISA (PMID: 31545609), and TPP (PMID: 25278616) experiments before, we chose to highlight a few examples and provide a reasonable amount of validation for these specific observations. In Figure 2, we show that two screening compounds—palbociclib and NVP-TAE-226—have a similar impact on PLK1 solubility as the two known PLK1 inhibitors. We then assay each of these compounds, alongside BI 2536, and show that the same compounds that impact the solubility of PLK1, also inhibit its activity in cell-based assays. Finally, we model the structure of palbociclib (which is highly similar to BI 2536) in the PLK1 active site. In Figure 4, we show that AZD-5438 causes a change in solubility of RIPK1 in cell- and lysate-based assays to a similar extent as other compounds known to engage RIPK1. We then test these compounds in cell-based assays and show that they are capable of inhibiting RIPK1 activity *in vivo*. Finally, in Figure 5, we show that treatment with tyrosine kinase inhibitors and AZD-7762 result in a decrease in the solubility of CRKL. We showed that these compounds, specifically, prevented the phosphorylation of CRKL at Y207. Next, we show that AZD-7762, impacts the thermal stability of tyrosine kinases in lysate-based PISA. Finally, we performed phosphoproteomic profiling of cells treated with bafetinib and AZD-7762 and find that the abundance of many pY sites is decreased after treatment with each compound. It is also worth stating that an important goal of this study was to determine the proficiency of these methods in identifying the targets of each compound. We do not feel that comprehensive validation of the “absolute majority of cases” would significantly improve this manuscript.

Finally, to be a community-useful resource the paper needs to provide the dataset with a user interface so that the users can data-mine on their own.

We agree and are working to develop an extensible resource for this. Owing to the size and complexities there, that work will need to be included in a follow-up manuscript. For now, we feel that the supplemental table we provide can be easily navigated the full dataset. Indeed, this has been the main resource that we have been emailed about since the preprint was first made public. We are glad that the Reviewer considers this dataset to be a highly valuable resource for the scientific community.

Reviewer #2 (Public Review):

Summary:

Using K562 (Leukemia) cells as an experimental model, Van Vracken et. al. use Thermal Proteome Profiling (TPP) to investigate changes in protein stability after exposing either live cells or crude cell lysates to a library of anti-cancer drugs. This was a large-scale and highly ambitious study, involving thousands of hours of mass spectrometry instrument time. The authors used an innovative combination of TPP together with Proteome Integral Solubility Alternation (PISA) assays to reduce the amount of instrument time needed, without compromising on the amount of data obtained.

The paper is very well written, the relevance of this work is immediately apparent, and the results are well-explained and easy to follow even for a non-expert. The figures are well-presented. The methods appear to be explained in sufficient detail to allow others to reproduce the work.

We thank the reviewer. One of our major goals was to make these assays and the resulting data approachable, especially for non-experts. We are glad that this turned out to be the case.

Strengths:

Using CDK4/6 inhibitors, the authors observe strong changes in protein stability upon exposure to the drug. This is expected and shows their methodology is robust. Further, it adds confidence when the authors report changes in protein stability for drugs whose targets are not well-known. Many of the drugs used in this study - even those whose protein targets are already known - display numerous offtarget effects. Although many of these are not rigorously followed up in this current study, the authors rightly highlight this point as a focus for future work.

Weaknesses:

While the off-target effects of several drugs could've been more rigorously investigated, it is clear the authors have already put a tremendous amount of time and effort into this study. The authors have made their entire dataset available to the scientific community - this will be a valuable resource to others working in the fields of cancer biology/drug discovery.

We agree with the reviewer that there are more leads here that could be followed and we look forward to both exploring these in future work and seeing what the community does with these data.

Reviewer #3 (Public Review):

Summary:

This work aims to demonstrate how recent advances in thermal stability assays can be utilised to screen chemical libraries and determine the compound mechanism of action. Focusing on 96 compounds with known mechanisms of action, they use the PISA assay to measure changes in protein stability upon treatment with a high dose (10uM) in live K562 cells and whole cell lysates from K562 or HCT116. They intend this work to showcase a robust workflow that can serve as a roadmap for future studies.

Strengths:

The major strength of this study is the combination of live and whole cell lysates experiments. This allows the authors to compare the results from these two approaches to identify novel ligand-induced changes in thermal stability with greater confidence. More usefully, this also enables the authors to separate the primary and secondary effects of the compounds within the live cell assay.

The study also benefits from the number of compounds tested within the same framework, which allows the authors to make direct comparisons between compounds.

These two strengths are combined when they compare CHEK1 inhibitors and suggest that AZD-7762 likely induces secondary destabilisation of CRKL through off-target engagement with tyrosine kinases.

Weaknesses:

One of the stated benefits of PISA compared to the TPP in the original publication (Gaetani et al 2019) was that the reduced number of samples required allows more replicate experiments to be performed. Despite this, the authors of this study performed only duplicate experiments. They acknowledge this precludes the use of frequentist statistical tests to identify significant changes in protein stability. Instead, they apply an 'empirically derived framework' in which they apply two thresholds to the fold change vs DMSO: absolute z-score (calculated from all compounds for a protein) > 3.5 and absolute log2 fold-change > 0.2. They state that the fold-change threshold was necessary to exclude nonspecific interactors. While the thresholds appear relatively stringent, this approach will likely reduce the robustness of their findings in comparison to an experimental design incorporating more replicates. Firstly, the magnitude of the effect size should not be taken as a proxy for the importance of the effect.

They acknowledge this and demonstrate it using their data for PIK3CB and p38a inhibitors (Figures 2BC). They have thus likely missed many small, but biologically relevant changes in thermal stability due to the fold-change threshold. Secondly, this approach relies upon the fold-changes between DMSO and compound for each protein being comparable, despite them being drawn from samples spread across 16 TMT multiplexes. Each multiplex necessitates a separate MS run and the quantification of a distinct set of peptides, from which the protein-level abundances are estimated. Thus, it is unlikely the fold changes for unaffected proteins are drawn from the same distribution, which is an unstated assumption of their thresholding approach. The authors could alleviate the second concern by demonstrating that there is very little or no batch effect across the TMT multiplexes. However, the first concern would remain. The limitations of their approach could have been avoided with more replicates and the use of an appropriate statistical test. It would be helpful if the authors could clarify if any of the missed targets passed the z-score threshold but fell below the fold-change threshold.

The authors use a single, high, concentration of 10uM for all compounds. Given that many of the compounds likely have low nM IC50s, this concentration will often be multiple orders of magnitude above the one at which they inhibit their target. This makes it difficult to assess the relevance of the offtarget effects identified to clinical applications of the compounds or biological experiments. The authors acknowledge this and use ranges of concentrations for follow-up studies (e.g. Figure 2E-F). Nonetheless, this weakness is present for the vast bulk of the data presented.

We agree that there is potential to drive off-target effects at such high-concentrations. However, we note that the concentration we employ is in the same range as previous PISA/CETSA/TPP studies. For example, 10 μ M treatments were used in the initial descriptions of TPP (Savitski et al., 2014) and PISA (Gaetani et al., 2019). We also note that temperature may affect off-rates and binding interactions (PMID: 32946682) potentiating the need to use compound concentrations to overcome these effects.

Additionally, these compounds likely accumulate in human plasma/tissues at concentrations that far exceed the compound IC50 values. For example, in patients treated with a standard clinical dose of ribociclib, the concentration of the compound in the plasma fluctuates between 1 μ M and 10 μ M. (Bao, X., Wu, J., Sanai, N., & Li, J. (2019). Determination of total and unbound ribociclib in human plasma and brain tumor tissues using liquid chromatography coupled with tandem mass spectrometry. *Journal of pharmaceutical and biomedical analysis*, 166, 197–204. <https://doi.org/10.1016/j.jpba.2019.01.017>)

The authors claim that combining cell-based and lysate-based assays increases coverage (Figure 3F) is not supported by their data. The '% targets' presented in Figure 3F have a

different denominator for each bar. As it stands, all 49 targets quantified in both assays which have a significant change in thermal stability may be significant in the cell-based assay. If so, the apparent increase in % targets when combining reflects only the subsetting of the data. To alleviate this lack of clarity, the authors could update Figure 3F so that all three bars present the % targets figure for just the 60 compounds present in both assays.

We spent much time debating the best way to present this data, so we are grateful for the feedback. Consistent with the Reviewer's suggestion, we have included a figure that only considers the 60 compounds for which a target was quantified in both cell-based and lysate-based PISA (now Figure 3E). In addition, we included a pie chart that further illustrates our point (now Figure 3 – figure supplement 2A). Of the 60 compounds, there were 37 compounds that had a known target pass as a hit using both approaches, 6 compounds that had a known target pass as a hit in only cell-based experiments, and 6 compounds that had a known target pass as a hit in only lysate-based experiments.

Within the Venn diagram, we also included a few examples of compounds that fit into each category. Furthermore, we highlighted two examples of compound-target pairs that pass as a hit with one approach, but not the other (Figure 3 – figure supplement 2B,C). We would also like to refer the reviewer to Figure 4D, which indicates that BRAF inhibitors cause a significant change in BRAF thermal stability in lysates but not cells.

Aims achieved, impact and utility:

The authors have achieved their main aim of presenting a workflow that serves to demonstrate the potential value of this approach. However, by using a single high dose of each compound and failing to adequately replicate their experiments and instead applying heuristic thresholds, they have limited the impact of their findings. Their results will be a useful resource for researchers wishing to explore potential off-target interactions and/or mechanisms of action for these 96 compounds, but are expected to be superseded by more robust datasets in the near future. The most valuable aspect of the study is the demonstration that combining live cell and whole cell lysate PISA assays across multiple related compounds can help to elucidate the mechanisms of action.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

More specifically:

P 1 | 20, we quantified 1.498 million thermal stability measurements.

It's a staggering assertion, and it takes some reading to realize that the authors mean the total number of proteins identified and quantified in all experiments. But far from all of these proteins were quantified with enough precision to provide meaningful solubility shifts.

We can assure the reviewer that we were not trying to deceive the readers. We stated '1.498 million thermal stability measurements.' We did not say 1.498 million compound-specific thermal stability shifts.' We assume that most readers will appreciate that the overall quality of the measurements will be variable across the dataset, e.g., in any work that describes quantitation of thousands of proteins in a proteomics dataset. In accordance with the Reviewer's suggestion, we have weakened this statement. The revised version of the manuscript now reads as follows (p. 1):

"Taking advantage of this advance, we quantified more than one million thermal stability measurements in response to multiple classes of therapeutic and tool compounds (96

compounds in living cells and 70 compounds in lysates).”

P 7 | 28. We observed a large range of thermal stability measurements for known compound-target pairs, from a four-fold reduction in protein stability to a four-fold increase in protein stability upon compound engagement (Figure 2A).

PISA-derived solubility shift cannot be interpreted simply as a "four-fold reduction/increase in protein stability".

We thank the Reviewer for highlighting this specific passage and agree that it was worded poorly. As such, we have modified the manuscript to the following (p. 8):

“We observed a large range of thermal stability measurements for known compound-target pairs, from a four-fold reduction in protein solubility after thermal denaturation to a four-fold increase in protein solubility upon compound engagement (Figure 2A).”

P 8, | 6. Instead, we posit that maximum ligand-induced change in thermal stability is target-specific.

Yes, that's right, but this has been shown in a number of prior studies.

We agree with the reviewer and accept that we made a mistake in how we worded this sentence, which we regret upon reflection. As such, we have modified this sentence to the following:

“Instead, our data appears to be consistent with the previous observation that the maximum ligand-induced change in thermal stability is target-specific (Savitski et al., 2014; Becher et al., 2016).”

P 11 | 7. Combining the two approaches allows for greater coverage of the cellular proteome and provides a better chance of observing the protein target for a compound of interest. In fact, the main difference is that in-cell PISA provides targets in cases when the compound is a pro-drug that needs to be metabolically processed before engaging the intended target. This has been shown in a number of prior studies, but not mentioned in this manuscript.

While our study was not focused on the issue of pro-drugs, this is an important point and we would be happy to re-iterate it in our manuscript. We thank the Reviewer for the suggestion and have modified the manuscript to reflect this point (p. 19):

“Cell-based studies, on the other hand, have the added potential to identify the targets of pro-drugs that must be metabolized in the cell to become active and secondary changes that occur independent of direct engagement (Savitski et al., 2014; Franken et al., 2015; Almqvist et al., 2016; Becher et al., 2016; Liang et al., 2022).”

While we are happy to make this change, we also would like to point out that the reviewer’s assertions that, “the main difference is that in-cell PISA provides targets in cases when the compound is a prodrug that needs to be metabolically processed before engaging the intended target” also may not fully capture the nuances of protein engagement effectors in the cellular context. Thus, we believe it is important to highlight the ability of cell-based assays to identify secondary changes in thermal stability.

P 11 | 28. These data suggest that the thermal destabilization observed in cell-based experiments might stem from a complex biophysical rearrangement. That's right because it is not about thermal stability, but about protein solubility which is much affected by the environment.

We agree that the readout of solubility is an important caveat for nearly every experiment in the family of assays associated with ‘thermal proteome profiling’. Inherently complex biophysical arrangements could affect the inherent stability and solubility of a protein or complex. Thus, we would be happy to make the following change consistent with the reviewer’s suggestion (p. 12):

“These data suggest that the decrease in solubility observed in cell-based experiments might stem from a complex biophysical rearrangement.”

P 12 | 7 A). Thus, certain protein targets are more prone to thermal stability changes in one experimental setting compared to the other. Same thing - it's about solubility, not stability.

We thank the Reviewer for the recommendation and have modified the revised manuscript as follows (p. 13):

“Thus, certain protein targets were more prone to solubility (thermal stability) changes in one experimental setting compared to the other (Huber et al., 2015).”

P13 | 15. While the data suggests that cell- and lysate-based PISA are equally valuable in screening the proteome for evidence of target engagement... No, they are not equally valuable - cell-based PISA can provide targets of prodrugs, which lysate PISA cannot.

We have removed this sentence to avoid any confusion. We will not place any value judgments on the two approaches.

P 18 | 10. In general, a compound-dependent thermal shift that occurs in a lysate-based experiment is almost certain to stem from direct target engagement. That's true and has been known for a decade. Reference needed.

We recognize this oversight and would be happy to include references. The revised manuscript reads as follows:

“In general, a compound-dependent thermal shift that occurs in a lysate-based experiment is almost certain to stem from direct target engagement (Savitski et al., 2014; Becher et al., 2016). This is because cell signaling pathways and cellular structures are disrupted and diluted. Cell-based studies, on the other hand, have the added potential to identify the targets of pro-drugs that must be metabolized in the cell to become active and secondary changes that occur independent of direct engagement (Savitski et al., 2014; Franken et al., 2015; Almqvist et al., 2016; Becher et al., 2016; Liang et al., 2022).”

P 18 | 29. the data seemed to indicate that the maximal PISA fold change is protein-specific. Therefore, a log2 fold change of 2 for one compound-protein pair could be just as meaningful as a log2 fold change of 0.2 for another. This is also not new information.

We again appreciate the Reviewer for highlighting this oversight. The revised manuscript reads as follows:

“Ultimately, the data seemed to be consistent with previous studies that indicate the maximal change in thermal stability in protein specific (Savitski et al., 2014; Becher et al., 2016; Sabatier et al., 2022). Therefore, a log2 fold change of 2 for one compound-protein pair could be just as meaningful as a log2 fold change of 0.2 for another.”

P 19 | 5. Specifically, the compounds that most strongly impacted the thermal stability of targets, also acted as the most potent inhibitors. I wish this was true, but this is not

always so. For instance, in Nat Meth 2019, 16, 894-901 it was postulated that large ΔT_m correspond to biologically most important sites ("hot spots") - the idea that was later challenged and largely discredited in subsequent studies.

Indeed, we agree with the Reviewer that there may be no essential connection between these. Rather, we are simply drawing conclusions from observations within the presented dataset.

Saying nothing about the work presented in the paper that the reviewer notes above, the referenced definition is also more nuanced "...we hypothesized that 'hotspot' modification sites identified in this screen (namely, those significantly shifted relative to the unmodified, bulk and even other phosphomodiforms of the same protein) may represent sites with disproportionate effects on protein structure and function under specific cellular conditions." Indeed, in the response to that work, Potel et al. (<https://doi.org/10.1038/s41592-021-01177-5>) "agree with the premise of the Huang et al. study that phosphorylation sites that have a significant effect on protein thermal stability are more likely to be functionally relevant, for example, by modulating protein conformation, localization and protein interactions."

Anecdotally, we also speculate that if we observe proteome engagement for two compounds (let's say two ATP-competitive kinase inhibitors) that bind in the same pocket (let's say the ATP binding site) and one causes a greater change in solubility, then it is reasonable to assume that it is a stronger evidence and we see evidence supporting this claim in Figure 2, Figure 3, Figure 4, and Figure 5.

It is also important to point out that previous work has also made similar points. This is highlighted in a review article by Mateus et al. (10.1186/s12953-017-0122-4). The authors state, "To obtain affinity estimates with TPP, a compound concentration range TPP (TPP-CCR) can be performed. In TPPCCR, cells are incubated with a range of concentrations of compound and heated to a single temperature." In support of this claim, the authors reference two papers—Savitski et al., 2014 and Becher et al., 2016. We have updated this section in the revised manuscript (p. 20):

"While the primary screen was carried out at fixed dose, the increased throughput of PISA allowed for certain compounds to be assayed at multiple doses in a single experiment. In these instances, there was a clear dose-dependent change in thermal stability of primary targets, off-targets, and secondary targets. This not only helped corroborate observations from the primary screen, but also seemed to provide a qualitative assessment of relative compound potency in agreement with previous studies (Savitski et al., 2014; Becher et al., 2016; Mateus et al., 2017). Specifically, the compounds that most strongly impacted the thermal stability of targets, also acted as the most potent inhibitors. In order to be a candidate for this type of study, a target must have a large maximal thermal shift (magnitude of log2 fold change) because there must be a large enough dynamic range to clearly resolve different doses."

Also, the compound efficacy is strongly dependent upon the residence time of the drug, which may or may not correlate with the PISA shift. Also important is the concentration at which target engagement occurs (Anal Chem 2022, 94, 15772-15780).

In our study, the time and concentration of treatment and was fixed for all compounds at 30 minutes and 10 μ M, respectively. Therefore, we do not believe these parameters will affect our conclusions.

P 19 | 19. For example, we found that the clinically-deployed CDK4/6 inhibitor palbociclib is capable of directly engaging and inhibiting PLK1. This is a PISA-based prediction that needs to be validated by orthogonal means.

As we demonstrate in this work, the PISA assays serve as powerful screening methods, thus we agree that validation is important for these types of studies. To this end, we show the following:

- Proteomics: Palbociclib causes a decrease in solubility following thermal melting in cells.
- Chemical Informatic: Palbociclib is structurally similar to BI 2536.
- Protein informatics: Modeling of palbociclib in empirical structures of the PLK1 active site generates negligible steric clashes.
- Biochemical: Palbociclib inhibits PLK1 activity in cells.

We have changed this text to the following to clarify these points:

“For example, we found that the clinically-deployed CDK4/6 inhibitor palbociclib has a dramatic impact on PLK1 thermal stability in live cells, is capable of inhibiting PLK1 activity in cell-based assays, and can be modelled into the PLK1 active site.”

Reviewer #2 (Recommendations For The Authors):

I am wondering why the authors chose to use K562 (leukaemia) cells in this work as opposed to a different cancer cell line (HeLa? Panc1?). It would be helpful if the authors could present some rationale for this decision.

This is a great question. Two reasons really. First, they are commonly used in various fields of research, especially previous studies using proteome-wide thermal shift assays (PMID: 25278616, 32060372) and large scale chemical perturbations screens (PMID: 31806696). Second, they are a suspension line that makes executing the experiments easier because they do not need to be detached from a plate prior to thermal melting. We think this is a valuable point to make in the manuscript, such that non-experts understand this concept. We tried to communicate this succinctly in the revised manuscript, but would be happy to elaborate further if the Reviewer would like us to.

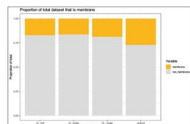
“To enable large-scale chemical perturbation screening, we first sought to establish a robust workflow for assessing protein thermal stability changes in living cells. We chose K562 cells, which grow in suspension, because they have been frequently used in similar studies and can easily be transferred from a culture flask to PCR tubes for thermal melting (Savitski et al., 2014; Jarzab et al., 2020).”

I note that integral membrane proteins are over-represented among targets for anti-cancer therapeutics. To what extent is the membrane proteome (plasma membrane in particular) identified in this work? After examining the methods, I would expect at least some integral membrane proteins to be identified. Do the authors observe any differences in the behaviour of water-soluble proteins versus integral membrane proteins in their assays? It would be helpful if the authors could comment on this in a potential revision.

We agree this is an important point when considering the usage of PISA and thermal stability assays in general for specific classes of therapeutics. To address this, we explored what effect the analysis of thermal stability/solubility had on the proportion of membrane proteins in our data (Author response image 1). Annotations were extracted from Uniprot based on each protein being assigned to the “plasma membrane” (07/2024). We quantified 1,448 (16.5% of total proteins) and 1,558 (17.3% of total proteins) membrane proteins in our cell and lysate PISA datasets, respectively. We also compared the proportion of annotated proteins in these datasets to a recent TMTpro dataset (Lin et al.; PMID: 38853901) and found that the PISA

datasets recovered a slightly lower proportion of membrane proteins (~17% in PISA versus 18.9% in total proteome analysis). Yet, we note that we expect more membrane proteins in urea/SDS based lysis methods compared to 0.5% NP-40 extractions.

Author response image 1.



We were not able to find an appropriate place to insert this data into the manuscript, so we have left it here in the response. If the Reviewer feels strongly that this data should be included in the manuscript, we would be happy to include these data.

A final note: I commend the authors for making their full dataset publicly available upon submission to this journal. This data promises to be a very useful resource for those working in the field.

We thank the Reviewer for this and note that we are excited for this data to be of use to the community.

Reviewer #3 (Recommendations For The Authors):

There is no dataset PDX048009 in ProteomeXchange Consortium. I assume this is because it's under an embargo which needs to be released.

We can confirm that data was uploaded to ProteomeXchange.

MS data added to the manuscript during revisions was submitted to ProteomeXchange with the identifier – PDX053138.

Page 9 line 5 refers to 59 compounds quantified in both cell-based and lysate-based, but Figure 3E shows 60 compounds quantified in both. I believe these numbers should match.

We thank the Reviewer for catching this. In response to critiques from this Reviewer in the Public Review, we re-worked this section considerably. Please see the above critique/response for more details.

Page 10, lines 26-28: It would help the reader if some of the potential 'artefactual effects of lysatebased analyses' were described briefly.

We thank the Reviewer for raising this point. The truth is, that we are not exactly sure what is happening here, but we know that, at least, for vorinostat, this excess of changes in lysate-based PISA is consistent across experiments. We also do not see pervasive issues within the plexes containing these compounds. Therefore, we do not think this is due to a mistake or other experimental error. We hypothesize that the effect might result from a change in pH or other similar property that occurs upon addition of the molecule, though we note that we have previously seen that vorinostat can induce large numbers of solubility changes in a related solvent shift assays (doi: 10.7554/eLife.70784). We have modified the text to indicate that we do not fully understand the reason for the observation (p. 11):

“It is highly unlikely that these three molecules actively engage so many proteins and, therefore, the 2,176 hits in the lysate-based screen were likely affected in part by consistent, but artefactual effects of lysate-based analyses that we do not fully understand (Van Vranken et al., 2021).”

Page 24, lines 29-30 appear to contain a typo. I believe the '>' should be '<' or the 'exclude' should be 'retain'.

The Reviewer is completely correct. We appreciate the attention to detail. This mistake has been corrected in the revised manuscript.

Page 25, lines 5-7: The methods need to explain how the trimmed standard deviation is calculated.

We apologize for this oversight. To calculate the trimmed standard deviation, we used proteins that were measured in at least 30 conditions. For these, we then removed the top 5% of absolute log2 foldchanges (compared to DMSO controls) and calculated the standard deviation of the resulting set of log2 fold-changes. This is similar in concept to the utilization of “trimmed means” in proteomics data (<https://doi.org/10.15252/msb.20145625>), which helps to overcome issues due to extreme outliers in datasets. We have added the following statement to the methods to clarify this point (p. 27):

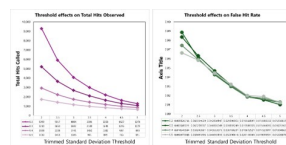
“Second, for each protein across all cells or lysate assays, the number of standard deviations away from the mean thermal stability measurement (z-score) for a given protein was quantified based on a trimmed standard deviation. Briefly, the trimmed standard deviation was calculated for proteins that were measured in at least 30 conditions. For these, we removed the top 5% of absolute log2 foldchanges (compared to DMSO controls) and calculated the standard deviation of the resulting set of log2 fold-changes.”

Page 25, lines 9-11 needs editing for clarity.

We tested empirical hit rates for estimation of mean and trimmed standard deviation (trimmedSD) thresholds to apply, to maximize sensitivity and minimizing the ‘False Hit Rate’, or the number of proteins in the DMSO control samples called as hits divided by the total number of proteins called as hits with a given threshold applied.

Author response image 2.

Hit calling threshold setting based on maximizing the total hits called and minimizing the False Hit Rate in cells (number of DMSO hits divided by the total number of hits).



Author response image 3.

Hit calling threshold setting based on maximizing the total hits called and minimizing the False Hit Rate in lysates (number of DMSO hits divided by the total number of hits).

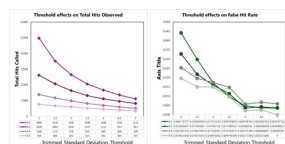


Figure 1 supplementary 2a legend states: '32 DMSO controls'. Should that be 64?

We thank the Reviewer for catching our mistake. This has been corrected in the revised manuscript.

I suggest removing Figure 1 supplementary 3c which is superfluous as only the number it presents is already stated in the text (page 5, line 9).

We thank the Reviewer for the suggestion and agree that this panel is superfluous. It has been removed from the revised manuscript.

New data and tables added during revisions:

- (1) Table 3 – All log₂ fold change values for the cell-based screen. Using this table, proteincentric solubility profiles can be plotted (as in Figures 2D and others).
- (2) Table 4 – All log₂ fold change values for the lysate-based screen. Using this table, proteincentric solubility profiles can be plotted (as in Figures 2D and others).
- (3) Figure 1 – Figure supplement 3H – Table highlighting proteins that pass log₂ fold change cutoffs, but not nSD cutoffs and vice versa.
- (4) Figure 2 – Panels H and I were updated with a new color scheme.
- (5) Figure 3 – Updated main figure and supplement at the request of Reviewer 3.
 - Figure 3E – Compares on-target hits for the cell- and lysate-based screens for all compounds for which a target was quantified in both screens.
 - Figure 3 – Figure supplement 2 – Highlights on-target hits in both screens, exclusively in cells, and exclusively in lysates.
- (6) Figure 5 – PISA data for K562 lysates treated with AZD-7762 at multiple concentrations.
 - Figure 5F
 - Figure 5 – Figure supplement 3A-C
 - Figure 5 – Source data 2
- (7) Figure 5 – Phosphoproteomic profiling of K562 cells treated with AZD7762 or Bafetinib.
 - Figure 5G
 - Figure 5 – Figure supplement 4A-F
 - Figure 5 – Source data 3 (phosphoproteome)
 - Figure 5 – Source data 4 (associated proteome data)

<https://doi.org/10.7554/eLife.95595.2.sa0>