Rapid discovery of drug target engagement by isothermal shift assay

Authors: Kristofor J Webb[1]*, Kerri A Ball[1]*, Stephen J Coleman[1], Jeremy Jacobsen[1], Michael HB Stowell[1#], William M Old[1,2#]

[1] Department of MCD Biology, University of Colorado, Boulder, CO, USA.
[2] Linda Crnic Institute for Down Syndrome, University of Colorado School of Medicine, Anschutz Medical Campus, Aurora, CO, USA
* Equally contributing authors
[#] Corresponding authors: Address all correspondence to stowellm@colorado.edu or William.Old@colorado.edu

Determining protein targets directly bound by drug molecules remains challenging. Here we present the isothermal shift assay, iTSA, for rapid unbiased identification of drug targets. Compared with thermal proteome profiling, the prevailing method for target engagement, iTSA offers a simplified workflow, 4-fold higher throughput, and a multiplexed experimental design with higher replication. We demonstrate its application to determination of target engagement for several kinase inhibitors in lysates and living cells.

A key challenge in drug discovery is to identify the protein targets directly bound by small molecule drugs, i.e. target engagement. Many drugs in clinical use are known to promiscuously bind multiple targets in a manner thought to be necessary for therapeutic effect, or bind to off-targets that contribute to toxic side-effects[1–4]. Yet, many drugs approved for human use have not been evaluated for target binding on a proteome scale, as recently demonstrated in an analysis of 243 kinase inhibitors that revealed widespread off-target binding to non-kinase proteins[5].

Prevailing strategies to determine target engagement exploit the phenomenon of thermodynamic stabilization due to ligand binding[6]. A notable example is the thermal shift assay[7], which we have used to optimize membrane receptor stability for subsequent crystallographic studies[8]. Thermal shift assays have been adapted to complex lysates or cells (CETSA)[9], and, when coupled to mass spectrometry, allow for proteome-wide assessment of target engagement, a method called thermal proteome profiling (TPP) or MS-CETSA[10]. TPP involves estimation of the thermal melting temperature (T_M) shift between drug and vehicle samples for each protein quantified with a 10-temperature melting curve (ΔX in Fig. 1a). TPP requires at least two replicates per condition, totaling 40 samples, and consumes weeks of instrument time on hybrid Orbitrap mass spectrometry instrumentation[10]. This low throughput limits the number of drugs and conditions that can be interrogated, particularly for many laboratories without access to dedicated instrumentation.

To address these limitations, we developed the isothermal shift assay (iTSA) for proteome-wide assessment of target engagement, which requires 4-fold fewer samples and achieves comparable sensitivity to TPP experiments. This approach simplifies the experimental design and eliminates curve fitting for T_M. In developing our method, we reasoned that shifts in thermal stability could be detected by quantifying the difference in the soluble protein fraction at a single temperature, carefully selected to maximize detection sensitivity (ΔY in **Fig. 1a**). By employing neutron-encoded isobaric labeling, a direct comparison of drug and vehicle replicates can be made within a single multiplexed sample, accommodating up to 5 replicates per condition for increased statistical power.

We hypothesized that measurement close to the median proteome T_M would enable detection the maximum number of thermal shifts using a two-sample statistical test between vehicle and drug conditions. To estimate the median proteome T_M, we collected a 10-temperature melting curve analysis of vehicle (DMSO) treated K562 whole-cell lysates, which revealed a median T_M of ~50°C with a narrow interquartile range of 3.5°C (**Fig. 1b**). This proteome T_M distribution was largely comparable to those previously reported with TPP analysis of K562 lysates[10,11]. Next, we asked if the median vehicle T_M was the best temperature to perform iTSA. To answer this question, we further analyzed the published TPP staurosporine target data[10] as described in Methods (**Supplementary Fig. 1a)**. Our analysis of the TPP targets indicated that the maximal solubility difference between drug and vehicle was not highly correlated with vehicle T_M (**Supplementary Fig. 1b**), and instead was more highly correlated with a temperature near the drug T_M (**Supplementary Fig. 1b**). Based on this analysis, we selected a temperature that was offset from the proteome vehicle median: 52°C, as the target temperature to perform the iTSA proof of concept analysis. K562 lysates were treated with 20 μM staurosporine, a broad spectrum kinase inhibitor that allowed benchmarking against targets identified by TPP[10] (workflow illustrated in **Supplementary Fig. 2 and Methods)**. Using iTSA performed at 52°C, we identified 95 targets (0.1% FDR), compared with 60 targets identified previously[10] (**Fig. 1d & e, Supplementary Data File 1**).

The increase in iTSA target identifications over TPP was surprising given that single temperature profiling might be expected to miss proteins with large deviations from the median T_M. To evaluate the effect of the iTSA target temperature selection, we

performed iTSA at two additional temperatures: 48°C and 56°C. Both temperatures performed well (**Supplementary Fig. 3 a & b**), identifying 72 (48°C iTSA) and 66 (56°C iTSA) staurosporine targets with an FDR < 0.1%. As shown in **Fig. 1f**, when the three iTSA experiments are compared, iTSA performed near the median proteome T_M, 52°C, identified more targets overall and more unique targets. Combining the three temperatures together moderately increased the total number of observed targets from 95 to 130, but nevertheless confirmed many targets that were observed in multiple experiments (**Fig. 1f**). As would be expected for a kinase inhibitor, the staurosporine targets identified by the iTSA assay were enriched for kinases (**Fig. 1g and Supplementary Fig. 4)**.

Next, we performed an orthogonal validation of the iTSA, comparing the iTSA staurosporine targets to targets identified with a kinobead affinity profiling assay, in addition to the TPP comparison[10,12]. We observed a 55% overlap of iTSA targets with previously identified staurosporine targets (**Fig. 1h**). Evaluation of the 58 iTSA novel staurosporine targets revealed many kinases, kinase-binding proteins, and phosphoproteins known to be regulated by staurosporine-targeted kinases (**Supplementary Data File 1** for additional details). Some of these non-kinase staurosporine targets are known to stably interact with kinases, and would be expected to be indirectly stabilized by staurosporine binding to its cognate binding partner, as shown recently for MS-CETSA[13]. DCAF7, for example, is a stable interaction partner of the kinase DYRK1A[14], and both were found to be strongly stabilized by staurosporine (**Fig. 2a**). The non-catalytic regulatory subunits of PKA were destabilized by staurosporine, while the catalytic subunits of PKA were stabilized, as noted previously[10],

providing further confirmation of the ability of iTSA analysis to identify true binding targets.

Incidentally, the porphyrin binding proteins ferrochelatase (FECH) and heme-binding protein 1 (HEBP1), were not obvious targets of an ATP-competitive inhibitor. However, both were identified as targets in the iTSA analysis here, and in the TPP workflow. As illustrated in **Supplementary Fig. 5**, staurosporine is structurally similar to the native binding partners of these proteins. Moreover, the association of FECH with staurosporine, as well as with numerous other kinase inhibitors, has been further validated[5,10,15]. Together, this gives some validity to the six staurosporine targets that were uniquely identified using the iTSA method for which we could find no corroborating evidence to substantiate their statistical significance (RCN2, MIEF2, ALDH6A1, MRPL55, CCDC144C, and Q6ZSR9). Although we can't rule out that these are false positives, with our stringent FDR cutoff, we realistically expect no more than one false positive in our set of 130 putative targets. Thus, there is reason to focus on these six proteins for orthogonal validation as novel staurosporine targets.

As shown in **Fig. 1h**, the iTSA method failed to identify ~45% of the previously reported targets of staurosporine, with many iTSA false negative targets originating from the kinobead study, of which 64 were not observed in either the iTSA or TPP evaluations. To help explain these false-negatives, we explored the T_M range (**Fig. 1i**) and depth of coverage variables (**Fig. 1j**) in our data set. As shown is **Fig. 1i**, the iTSA T_M ranges of the significant targets (0.1% FDR) are shown for each temperature and are compared to the proteome, kinome, TPP, and kinobead ranges. The T_M ranges of all three iTSAs were surprisingly broad, however the minimum T_M of iTSA was higher

than the minimum of the TPP workflow and explains the few targets that were uniquely identified using that strategy. Neither the TPP nor the iTSA strategy demonstrated complete coverage of the kinase T_M range, however, all of the workflows demonstrated coverage that extended above and below the interquartile range of both the kinase family and the complete proteome. Moreover, the interquartile T_M range of the iTSA false negatives (kinobead targets but not iTSA targets) was well within the observed assay range of iTSA, suggesting that the iTSA T_M range alone could not account for missing these targets.

As shown in **Fig. 1j**, however, a much stronger correlation was observed with depth of coverage (MS/MS count), where the statistically significant iTSA targets had much higher MS/MS counts than the false negative targets. This results is indicative of high protein abundance biases of proteomics and may be overcome by increased fractionation, technical replication, or sequencing speed/sensitivity from the latest orbitrap instrumentation.

The assay T_M range and depth of coverage, however, do not explain the dramatic increase in the number of targets identified with iTSA in comparison to TPP. Additional analysis of our iTSA data suggest that the increased number of targets may be dependent on the increase in replication (2 replicates in TPP versus 5 replicates in iTSA). Using the 52°C staurosporine iTSA data, we performed sub-sampling (described in Methods) to illustrate the importance of replication to mitigate Type II errors. As shown in **Fig. 1k** (with results included in **Supplementary Data File 1**), sub-sampling our own data indicates that experimental designs requiring fewer replicates are more likely to dismiss real targets.

We next asked how iTSA performs with more selective kinase inhibitors. We used the 52°C in-lysate iTSA to survey the targets of harmine and H-89. Harmine is in an ATP-competitive inhibitor of DYRK family kinases, inhibiting DYRK1A most potently with an apparent Ki of 65 nM[16]. iTSA profiling of 20 μM harmine in K562 lysates revealed stabilization of DYRK1A and its known interacting protein, DCAF7 (**Fig. 2 a,d, Supplementary Data File 2**). Surprisingly, we found that the protein kinases CDK8, CDK9 and CSNK1G3 were also stabilized. Although harmine binding affinities have not been measured for these kinases to our knowledge, KinomeScan in-vitro screening of harmine has been reported[17], revealing that 10 μM harmine inhibited CDK8, CDK9 and CSNK1G3 by 79%, 43% and 34%, respectively, indicating that iTSA can detect lower affinity binding interactions with harmine. While harmine is well characterized, H-89 is an ATP-competitive inhibitor of protein kinase A (PKA) that is not well characterized. However, H-89 has been shown to have many PKA independent activities[18]. iTSA analysis with 100 nM H-89 identified the catalytic subunit of PKA (PRKACA) as the most significant target of H-89 (**Fig. 2b,e and Supplementary Data File 2**). Seven total high confidence targets were identified using an FDR < 0.1% with 107 potential targets identified using an FDR < 5% (and absolute fold change > 80th percentile) of which only a small percentage were kinases (**Fig. 2e**). Of the potential targets identified by iTSA, a majority are novel targets of H-89, but some have previously been validated, including the Sarcoplasmic/endoplasmic reticulum calcium ATPase (ATP2A2) that has strong evidence to support non-ATP-competitive interaction with H-89[19]. Rho-associated protein kinase (ROCK1) was also a previously identified H-89 target and an iTSA identified binding partner of H-89[18].

To validate iTSA for identification of intracellular target engagement, we performed 52°C iTSA in K562 cells treated with 1 μM Staurosporine (**Fig. 2c,f, Supplementary Data File 2**). This was a lower dose than that used in the in-lysate iTSA and represented a more experimentally relevant staurosporine concentration[20]. As expected with a lower dose and ATP concentration of 1-5 mM[21], fewer targets were observed (20); many of which were kinases (**Fig. 2f**). However, only eight of the in-cell iTSA targets overlapped with the in-lysate iTSA, and some targets that were stabilized in the in-lysate iTSA were destabilized in the in-cell iTSA (such as CDK5) suggesting that more complex interactions are observed when drug treatment is performed in biologically active cells. Thus, exploration of in-cell iTSA targets may offer relevant mechanism-of-action (MoA) information, but may also confound identification of primary drug-protein interactions.

Here, we have demonstrated that our iTSA workflow is a cost-effective method for high-confidence drug protein-target identifications. The single temperature method provided increased sensitivity and throughput relative to traditional approaches and sacrificed very little in terms of the proteomic T_M range. High throughput drug screening is a multi-billion-dollar market. Performed early in the drug development process, iTSA could rule out problematic compounds with undesirable off-target profiles before substantial development investments have been made. Furthermore, iTSA could be used to provide insight into the mechanism of action for compounds identified with phenotypic-screening. iTSA does not require chemical modification and immobilization necessary for molecular-affinity enrichment methods, requires fewer samples and

instrument time relative to TPP, and can be accommodated into higher-throughput workflows for identifying target engagement.

Methods

Methods are available in the Supplementary materials / online version of the paper.

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Author Contributions

MHBS, KJW, KAB, and WMO conceptualized the study. KJW, KAB, SJC & WMO designed the experiments. KJW, KAB, SJC, JJ & WMO designed data analysis strategies. KJW performed the experiments. KAB, JJ, SJC implemented analysis of the data. KAB, KJW, SJC, JJ, MHBS & WMO participated in the interpretation of the data and the writing of the manuscript. WMO and MHBS funding acquisition.

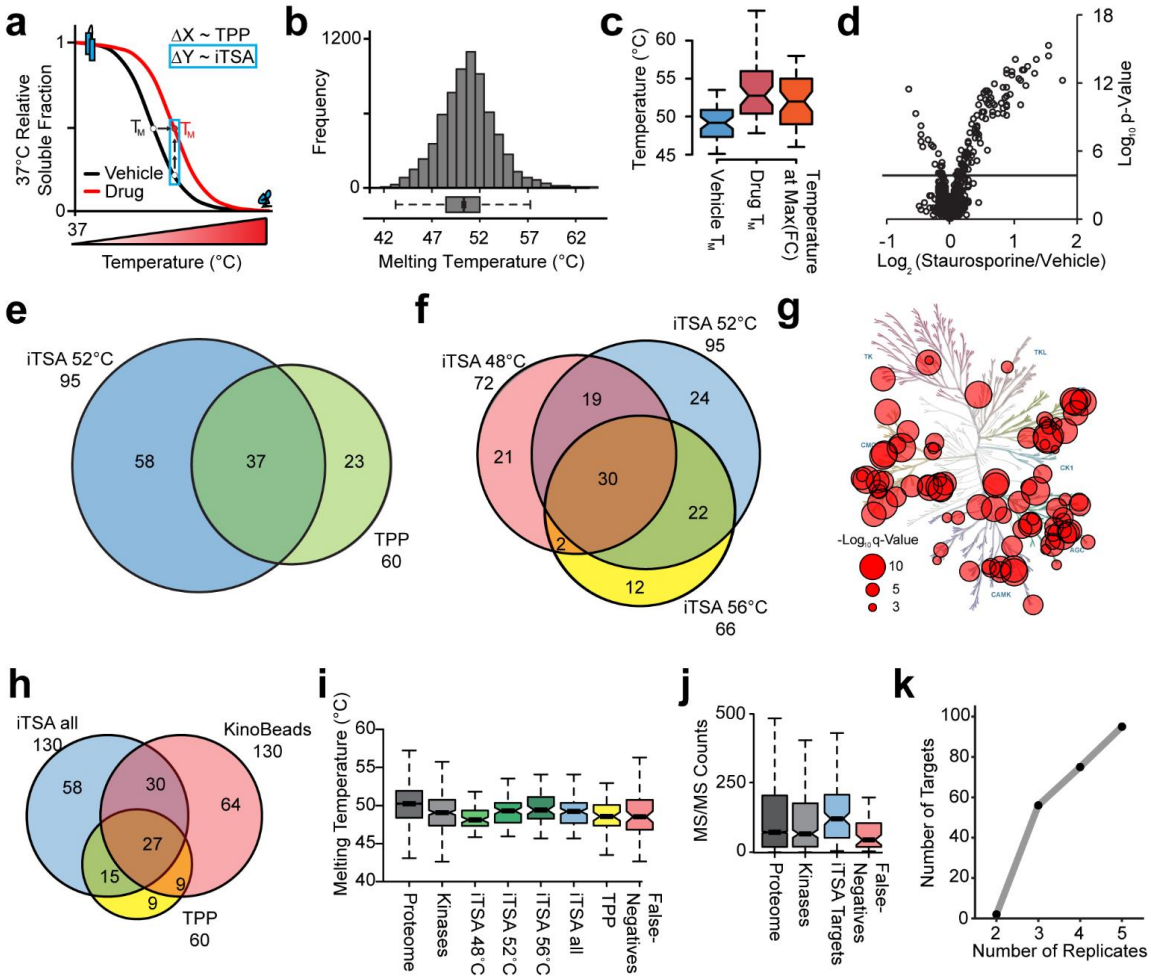

Figure 1 | iTSA Theory and Proof of Concept. **(a)** Representative Vehicle and Drug thermal gradient data is displayed and apparent T_M of each is indicated. **(b)** Thermal profile of K562 (vehicle) was performed and fitted as described in Methods. Distribution of K562 proteome T_M is shown with plot below indicating median (50.2), range, and quartiles (48.4 - 52.0) of the T_M distribution. **(c)** Savitski et al., 2014 staurosporine target data T_M box-plot distributions for vehicle, drug and the temperature at which the maximum difference between drug and staurosporine was observed when each temperature was analyzed by ΔY. **(d)** Volcano plot visualization of iTSA performed at 52°C using 20 μM Staurosporine. FDR = 0.1% is indicated with solid line. Venn diagram of **(e)** iTSA 52°C and Savitski et al., 2014 staurosporine targets and **(f)** targets from 48°C, 52°C, and 56°C iTSA experiments. **(g)** Kinome tree displaying all iTSA staurosporine kinase targets. Circle size is proportional to the -Log10 q-Value maximum from 48°C, 52°C, or 56°C. Illustration reproduced courtesy of Cell Signaling Technology, Inc. **(h)** Venn diagram of iTSA target (from all three iTSA experiments) overlap with previous staurosporine target assays. **(i)** Melting temperatures (T_M) range for each group is shown with a box-and-whisker plot. Proteome group contains all proteins with a calculated T_M in our K562 thermal gradient experiment. Kinases represent all proteins in the proteome that were identified by Uniprot as part of the kinase family. iTSA 48°C through iTSA 56°C groups include staurosporine drug targets (FDR 0.1%) identified in the respective experiments, TPP group is comprised of Savitski et al., 2014 identified drug targets. False-Negatives group encompasses the protein drug targets found in the Kinobead experiment (Werner et al., 2012) but not identified as targets in the iTSA experiment. **(j)** Box-and-whisker plot of MS/MS counts for the indicated protein groups shown with groups described in (i). **(k)** Number of changes identified with increasing replication, estimated by significance (0.1% FDR) in >80% of sub-sampled combinations from iTSA 52°C.

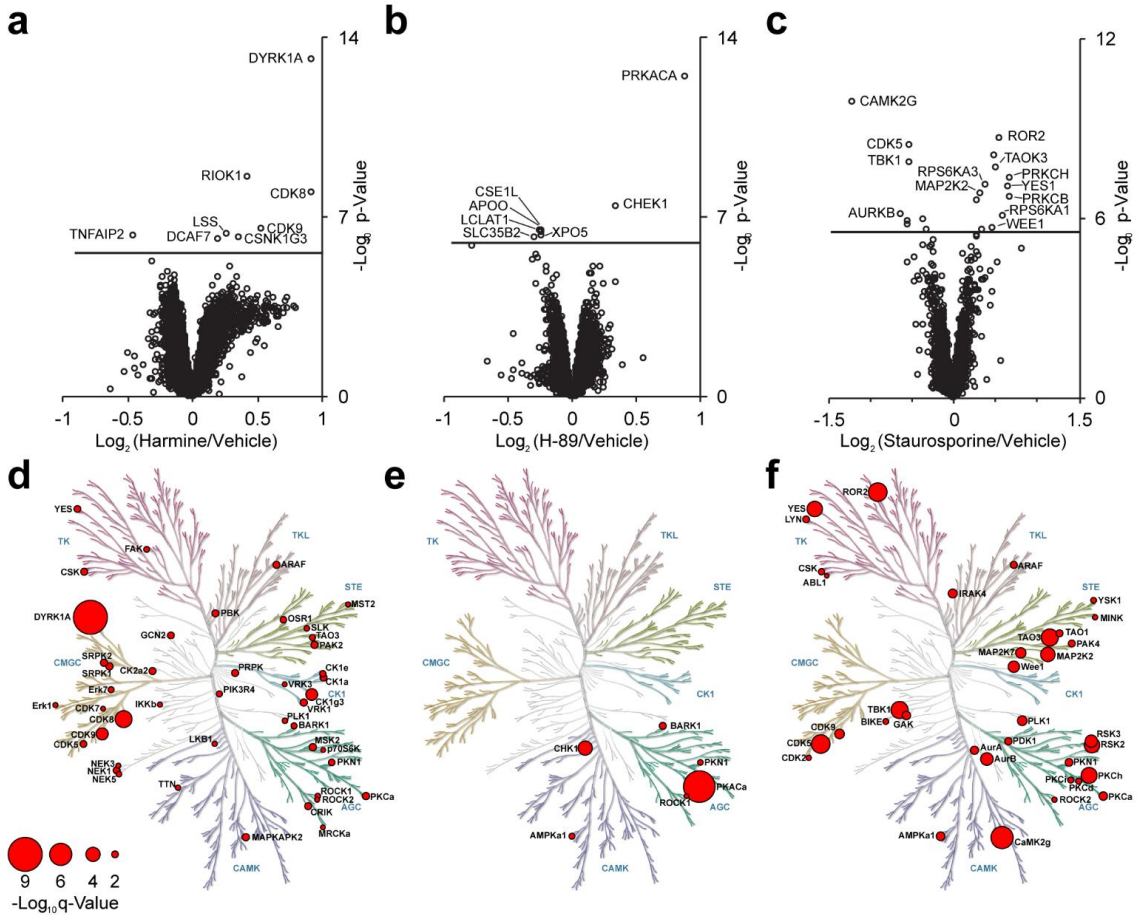

Figure 2 | Method Versatility. Volcano plot visualization of soluble protein levels using empirical Bayes statistical analysis. FDR = 0.1% is solid line. Kinome maps (illustration reproduced courtesy of Cell Signaling Technology, Inc.) display -log10 q-Value (size key displayed for all) of kinase targets (a, d) Using lysate, iTSA was performed at 52°C using 20 µM Harmine. (b, e) Using lysate, iTSA was performed at 52°C using 100 nM H-89. (c, f) Using living cells treated with 1 µM Staurosporine for 15 minutes, in-cell iTSA was performed at 52°C.

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Methods

Cell Culture

K562 cells were acquired from ATCC. Cells were cultured in RPMI GlutaMAX media containing 10% fetal bovine serum and 1% Anti-Anti antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA). Cells were expanded to 1.5 x 10^6 cells/mL. 50 million cells were washed two times with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM $NaHPO_4$, and 1.8 mM KH_2PO_4, pH 7.4) and snap frozen in liquid nitrogen for use in lysate iTSA experiments or were used immediately for in-cell iTSA experiments.

Preparation of lysate

Frozen cell pellets were suspended in 1 mL of Lysis Buffer (137 mM NaCl, 2.7 mM KCl, 10 mM $NaHPO_4$, and 1.8 mM KH_2PO_4, 0.4% Igepal CA-630, 1X cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma #11836170001), 1X Pierce™ Phosphatase Inhibitor Mini (Fisher # A32957), pH 7.4). The pellet was suspended by vortex then sonicated for 30 seconds, 3 times, with a 30-second rest in between in a 4°C Bioruptor water bath sonicator (Diagenode, Lorne, Australia). Lysate was centrifuged at 21,000 *g*, 4 °C, for 15 minutes and the supernatant collected. The final lysate protein concentration was then adjusted to 5 mg/mL as determined by BCA assay (Thermo Fisher Scientific, Waltham, MA). Samples were promptly used in thermal profiling experiments.

Thermal treatment of lysate

To reduce variability, minimal sample handling was a focus of the procedure. For thermal gradient profiling three gradient programs were created using a PTC-200 thermal cycler to cover the temperature points 37, 41.2, 44, 46.8, 50, 53.2, 56.1, 59.1

63.2, 66.9 °C (MJ Research, Reno, NV). Program 1 consisted of temperatures 37, 41.2, 56.1 °C, program 2: 44, 46.8, 50, 53.2 °C, and program 3: 59.1, 63.2, 66.9 °C. The heated lid was set to 95 °C. Three PCR plates were prepared with 40 µL per well of lysate and sealed (individually sealable and removable wells; 4titude Random Access, PN 4ti-0960/RA 96-well plate). The plates were spun at 500 *g* for two minutes at 4 °C, and then kept at 4 °C prior to use (less than 15 minutes). For each program, the thermal cycler program was set to run for a total of 20 minutes and started without the sample plate to preheat the block for a minimum of 2 minutes. During the preheating, one of the PCR plates was removed from 4 °C and placed at room temperature for 2 minutes. The plate was placed in the thermal cycler with the heated lid closed for 3 minutes. The heated plate was promptly removed and placed at 4 °C. This was repeated with the remaining plates using the additional programs. The plates were then spun at 500 *g* for two minutes to remove any condensation. The PCR tubes were removed from the PCR plate, carefully placed in 1.5 mL tubes, and spun at 21,000 *g* for 30 min at 4 °C to pellet the aggregate protein. Supernatant was carefully removed from each tube and placed in a clean, low-retention, 1.5 mL tubes. To reduce sample handling samples are not removed from this tube until isobaric labeling was completed. Illustration of the lysate to labeled mass spectrometry sample workflow can be found in Supplemental Material (**Supplementary Fig. 2**). 50 µL of Denaturation buffer (8 M Guanidine HCl, 100 mM HEPES pH 8.5, 10 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 40 mM 2-Chloroacetamide, all prepared fresh or stored for single use at -80 °C and thawed immediately prior to use) was added to each sample, and the samples were placed in a heat block at 90 °C for 10 minutes to denature, reduce, and alkylate the samples.

Samples were cooled to room temperature, 400 µL of cold acetone was added and the samples were placed at -20 °C overnight to precipitate protein. If a biphasic separation is observed, an addition of ddH_2O and repeated centrifugation will be necessary to achieve normal protein precipitation.

Single temperature thermal profiling in lysate

Workflow for iTSA is illustrated in **Supplementary Fig. 2**. Drug treated cell lysate was prepared by adding Staurosporine at final concentration of 20 µM or H-89 ((N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide) at a final concentration of 100 nM (Tocris Bioscience, Bristol, UK) or Harmine at a final concentration of 20 µM to prepared K562 lysate with a final DMSO concentration of 1%. A 1% DMSO vehicle control was prepared as well. Treated samples were incubated at room temperature for 10 minutes prior to the thermal shift. The PCR plate was treated as described above with the exception that the thermal cycler was held at a constant temperature with temperature indicated in the results section.

Single temperature drug profiling in cells

120 million K562 cells grown as described above were suspended in 240 mL of warm RMPI media and split in two. Drug treated cells were prepared by adding Staurosporine at final concentration of 1 µM, a DMSO vehicle control was prepared as well. The cells were placed at 37 °C for 15 minutes and then pelleted at 340 *g* for two minutes. Cells were washed twice in PBS then resuspended in a final volume of 3 mL. 100 µL/well, 5-replicates/condition of cell solution (~2 million cells) was utilized. The PCR plate was sealed and heated to 51 °C for 3 minutes as described above. The PCR plate was promptly flash frozen and stored at -80 °C. Samples were lysed by the addition of 100

µL of Lysis Buffer, vortexed, then sonicated for 30 seconds 3 times with a 30-second rest in between in a 4°C Bioruptor water bath sonicator. Lysate was centrifuged at 21,000 *g*, 4 °C, for 15 minutes and the supernatant collected. 200 µL of Denaturation buffer was added to each sample. The samples were placed in a heat block at 90 °C for 10 minutes to denature, reduce, and alkylate the samples. Samples were cooled to room temperature and precipitated in five-volumes of acetone as previously described for lysates.

Sample preparation and TMT labeling

Precipitated protein was collected by centrifugation at 21,000 *g*, -10 °C for 30 minutes. The acetone was carefully removed, and the pellets were washed two additional times by adding 150 µL 80% cold acetone and suspending with Bioruptor set at 4 °C for three 30 seconds sonication cycles on, 30 seconds off prior to centrifugation. (Note that if -10 °C centrifugation is not available, we recommend incubation of acetone washes at -20 °C for a minimum of 30 minutes prior to 4 °C centrifugation during the acetone wash steps). Acetone washed pellets were dried prior to suspension in 100 µL of TMT-label compatible Digestion Master Mix (10% 2,2,2-Trifluoroethanol, 50 mM HEPES pH 8.5, 2 µg Lys-C (Wako product number 129-02541) and 2 µg trypsin (Sigma product number T6567)). Suspension in Digestion Master Mix was aided by using the Bioruptor set at 4 °C for three 30 seconds sonication cycles on, 30 seconds off, repeating Bioruptor program as needed until a visibly homogenous suspension was formed. Samples were digested for 16 hours at 37 °C with 1,200 rpm agitation in a Thermal Mixer C (Eppendorf, Hamburg, Germany).

Samples were isobaric labeled (Thermo Fisher Scientific PN 90119) according to the manufacturer recommended procedure. Briefly, 10 µL solution of 20 µg/µL TMT label in anhydrous acetonitrile (ACN) was added to the sample. Samples were incubated in label at room temperature for 1 hour. Labeling was quenched by addition of 8 µL of 5% (w/v) hydroxylamine and incubation for 15 minutes at room temperature. At this point, the 10 samples were combined into a single tube and 10 µL of 10% trifluoroacetic acid (TFA) was added. Samples were dried by vacuum centrifuge to remove ACN added with TMT label and then cleaned up using 3 washes on an Oasis HLB column (Waters PN 186000383). Mobile phases used were (A) 0.1% TFA aqueous and (B) 70% acetonitrile (ACN). Briefly, HLB columns were activated 1X in 1 mL B, equilibrated 2X in 1 mL A, and the acidified sample added. Sample was then washed 3X with 1 mL A and then 200 µL of B was used to elute peptides. Samples were dried by vacuum centrifuge and stored dry at -20 °C until ready for reversed-phase fractionation.

RP/RP-MS/MS analysis

The TMT labeled tryptic peptides were fractionated on a high pH reversed-phase C18 column using an Agilent 1100 HPLC utilizing established methods[1]. Briefly, mobile phases used were (A) 10 mM ammonium formate, pH 10 water and (B) 10 mM ammonium formate, pH 10 in 80% (v/v) acetonitrile. Samples were loaded on a C18 column (Xbridge Peptide BEH C18, 130Å, 2.5 µm, 2.1 x 150 mm, Waters, Milford, MA) at a flow rate of 300 µL/minute equilibrated in 5% B. Peptides were eluted with a gradient from 5% B to 100% over 120 minutes. Fractions were collected for 1 minute

each, concatenating throughout the gradient for 24 mixed fractions of nearly equal complexity. Fractions were dried by vacuum centrifugation and stored at -80 °C.

All fractions for mass spectrometry were suspended in 20 µL of 5% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid and analyzed by direct injection on a Waters M-class Acquity column (BEH C18 column, 130 Å, 1.7 µm, 0.075 mm x 250 mm) at 0.3 µL/minute using a nLC1000 (Thermo Scientific). Mobile phases used were (A) 0.1% formic acid aqueous and (B) 0.1% formic acid acetonitrile (ACN). Peptides were gradient eluted from 3 to 20% B in 100 minutes, 20 to 32% B in 20 minutes, and 32 to 85% B in 1 minute. Mass spectrometry analysis was performed using an Orbitrap Fusion (Thermo Scientific). MS2 were collected at 50,000 FWHM resolution 2×10^5 automatic gain control (AGC), 120 ms max fill time, with 20 s dynamic exclusion +/- 10 ppm using the data dependent mode “Top Speed” for 3 s on the most intense ions.

Data Analysis

MS/MS Search Parameters

All Thermo MS/MS raw files were processed using MaxQuant version 1.6.3.3[2] with MS/MS spectra searched using the Andromeda search engine[3] against a Uniprot human database (downloaded August 30, 2017, 42,215 entries) and with the TMT lot-specific isotopic distributions added for corrected reporter ion intensities. The search was limited to peptides with a minimum length of six amino acid residues with a maximum of two missed cleavages with trypsin/P and LysC/P specificity. Variable modifications for methionine oxidation and fixed modifications for protein N-terminal acetylation and carbamidomethyl Cysteine were added, and search tolerances were set at 10 ppm for MS1 precursor ions and 20 ppm for MS/MS peaks. Protein and peptide

level false discovery rate thresholds were set at 1%. At least one unique or razor peptide was required for protein identification. Unique plus razor peptides were used for quantification.

iTSA Data Analysis

MaxQuant's "proteinGroups.txt" output file was utilized for bioinformatics analysis using R (version 3.5.2)[4]; R script is supplied in Supplemental Files. The data was analyzed with tools from the R package, `limma`[5]. Briefly, the data matrix was filtered to include only high confidence identifications, quantile normalized, and then analyzed with linear modeling, empirical Bayes statistics, and the Benjamini-Hochberg Procedure (BH) for false discovery correction[6–8]. Absolute fold change and *q*-value cutoffs were used to categorize significant protein groups for the "Sig_FDR.FC" and "rank" columns observed in the exported data files. Two-sample statistics with BH false discovery correction were performed using Perseus (version 1.6.1.3)[9] utilizing the quantile normalized $\log_2$ intensity data in the empirical Bayes script exported files. Kinome trees were built using the kinmap (http://kinhub.org/kinmap/) beta tool (**Figs. 1g & 2[d-f]**). Trees indicate kinase group target assignment and node diameter is proportional to significance of thermal shift.

To compare iTSA data analysis to TPP and kinobead data the protein IPI accession numbers were matched to UniprotKB identifiers and the data were then merged in R with significant columns and IPI accession numbers were retained are viewable in **Supplementary Data File 1**. TPP target proteins were considered significant (1) or not significant (0) if the protein fulfilled all 4 requirements as designated significant in the original publication[10]. Kinobead data was considered significant if a

pIC50 was reported and passed quality control filters as determined in the original publication[11].

Subsampling Data Analysis

We utilized the staurosporine iTSA 52°C data for subsampling data analysis. Our dataset contained 5 control sample and 5 drug-treated samples. Thus we can select sub-sets from both populations and calculate the significant changes (FDR < 0.1) we would have observed if our experimental design were comparing 2, 3, or 4 replicates. There were 5-choose-4 = 5 unique ways of sub-sampling each the control and drug treated samples, which means there were 5x5=25 4-replicate comparisons within our data. Likewise, for the 3-replicate or 2-replicate scenarios, there were 10x10=100 possible sub-sampled comparisons. We sub-sampled from all possible combinations within our data and followed the same analysis procedure as described above to identify which proteins were identified as significant in each sub-sample. A protein was tallied if it appeared significant in >80% of sub-sampled comparisons, corresponding to a statistical power of 0.8. There were few proteins that were not significant in the 4 or 5-replicate comparisons that appeared significant in >10% of the sub-samples in the 2 or 3-replicate comparisons. This indicates that Type I errors are well controlled.

Thermal Gradient Data Analysis

MaxQuant's output file was utilized for analysis and characterization of thermal profiles via Python. Thermal gradient data was median normalized prior to fitting with parametric sigmoidal function. This normalization is performed in order to remove or reduce any systematic effects introduced in the peptide handling or labeling. Briefly, the median solubility change is calculated across all proteins for each isobaric label. The set

of median solubilities is itself a thermal profile which can be fit to a curve. After the fit, a scale factor is calculated which can scale the median from a particular isobaric label to match the prediction from the best-fit curve. These scale factors should be very close to 1 (1 = no scaling); experiments with greater than 10% scale factor variance should not be evaluated. The calculated scale factor is then applied to all of the measurements quantified by that label.

The parametric model used to perform the normalization as well as to fit the normalized thermal profiles is the three-parameter log-logistic function:

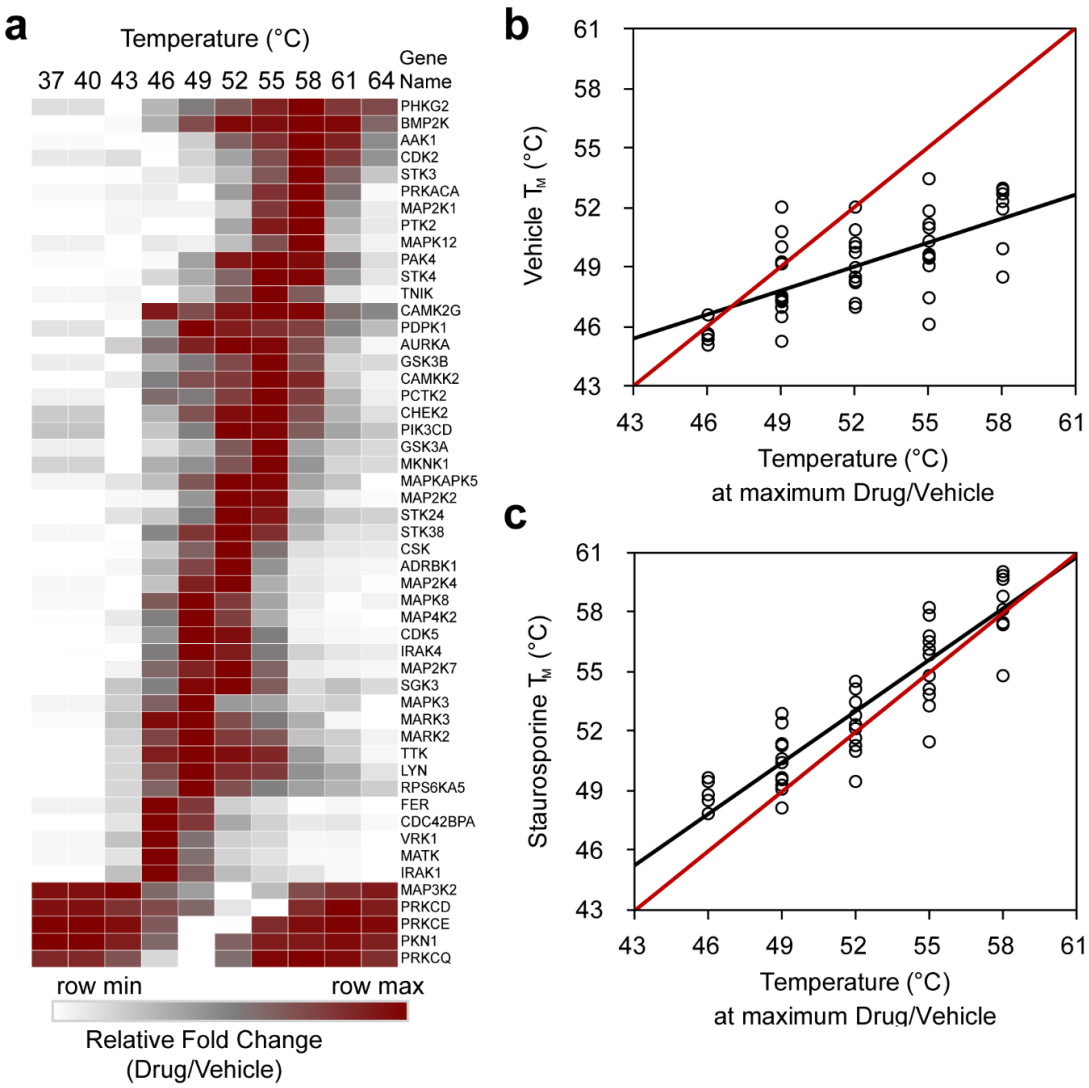
$$f(x) = c + \frac{1-c}{1+\exp(b(\log(x)-\log(T_M)))}$$

This function fits the soluble fraction relative to the lowest temperature measurement, and thus fixes the upper asymptote at 1.0. The parameter include the minimum relative solubility (the lower asymptote) is *c*, the melting temperature (T_M), and the slope-controlling parameter *b*. These fits were performed in Python, utilizing the SciPy[12] `optimization.curve_fit` package (version 1.0.1) and filtered by chi-square.

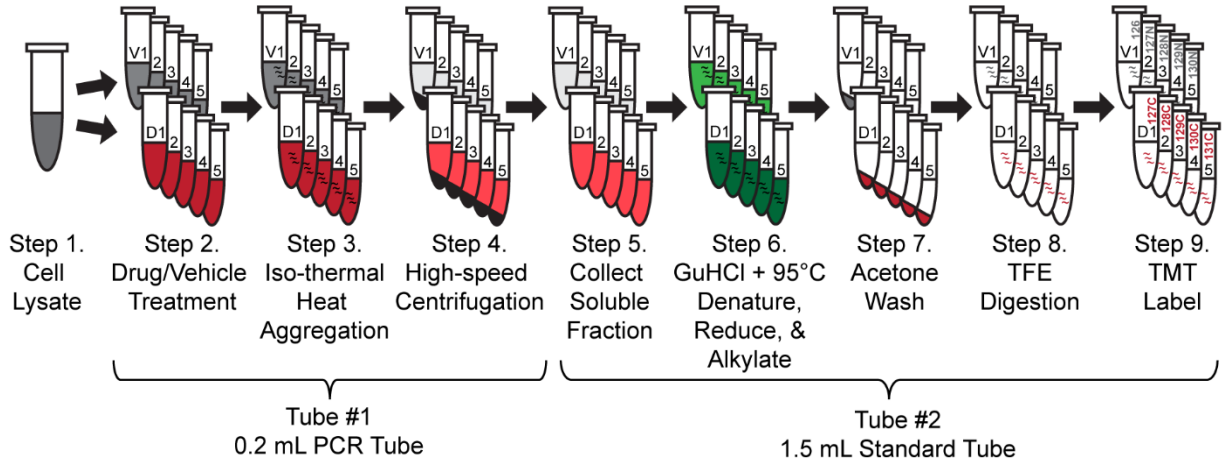
TPP Data Analysis

Supplemental Data containing staurosporine thermal profiling data was downloaded from Savitski et al., 2014[10] and the 60 targets that passed all four of their conditions for target identification were selected for additional analysis: Average vehicle T_M and average drug T_M were calculated for each target and the resulting T_M data was utilized for the box-plots in **Fig. 1C.** For each of the 10 temperatures utilized in the TPP thermal profiling the average relative soluble fraction from drug and vehicle was utilized

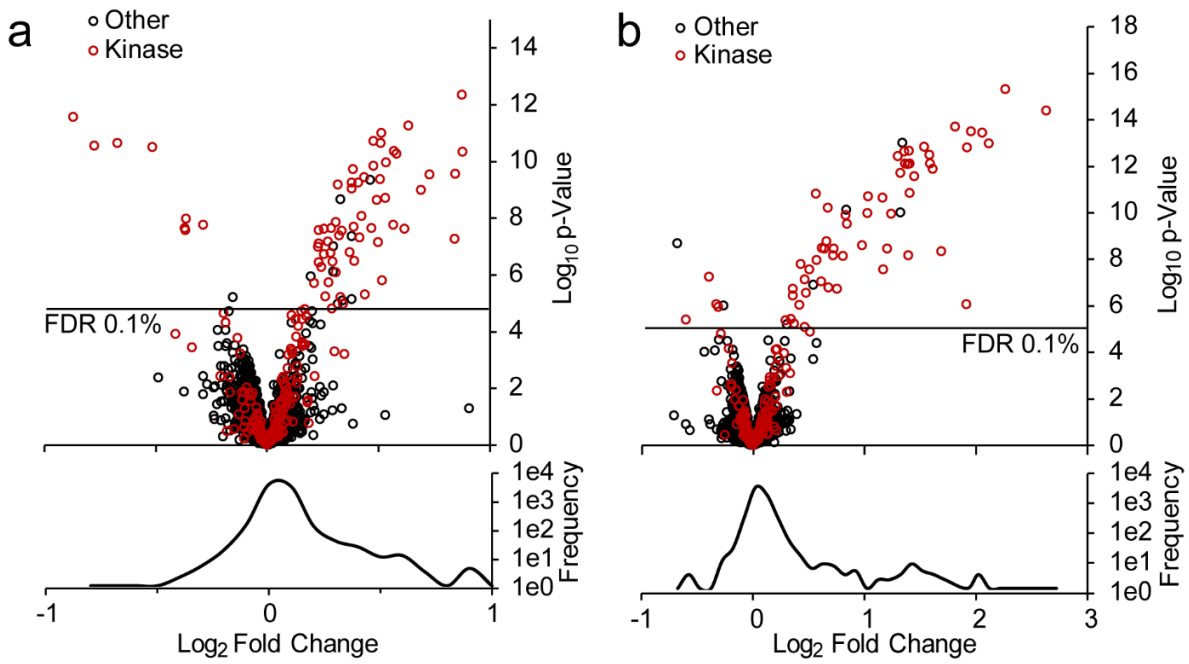
to calculate fold change (FC = average(Drug)/average(Vehicle)). This data is displayed in **Supplementary Fig. 1**. Then, the maximum absolute FC value for each target was determined and the temperature at which that maximum absolute FC occurred was recorded and the results were utilized for creation of the box-plot in **Fig. 1C** as well as the scatter-plots in **Supplementary Fig. 1 (b & c)**.



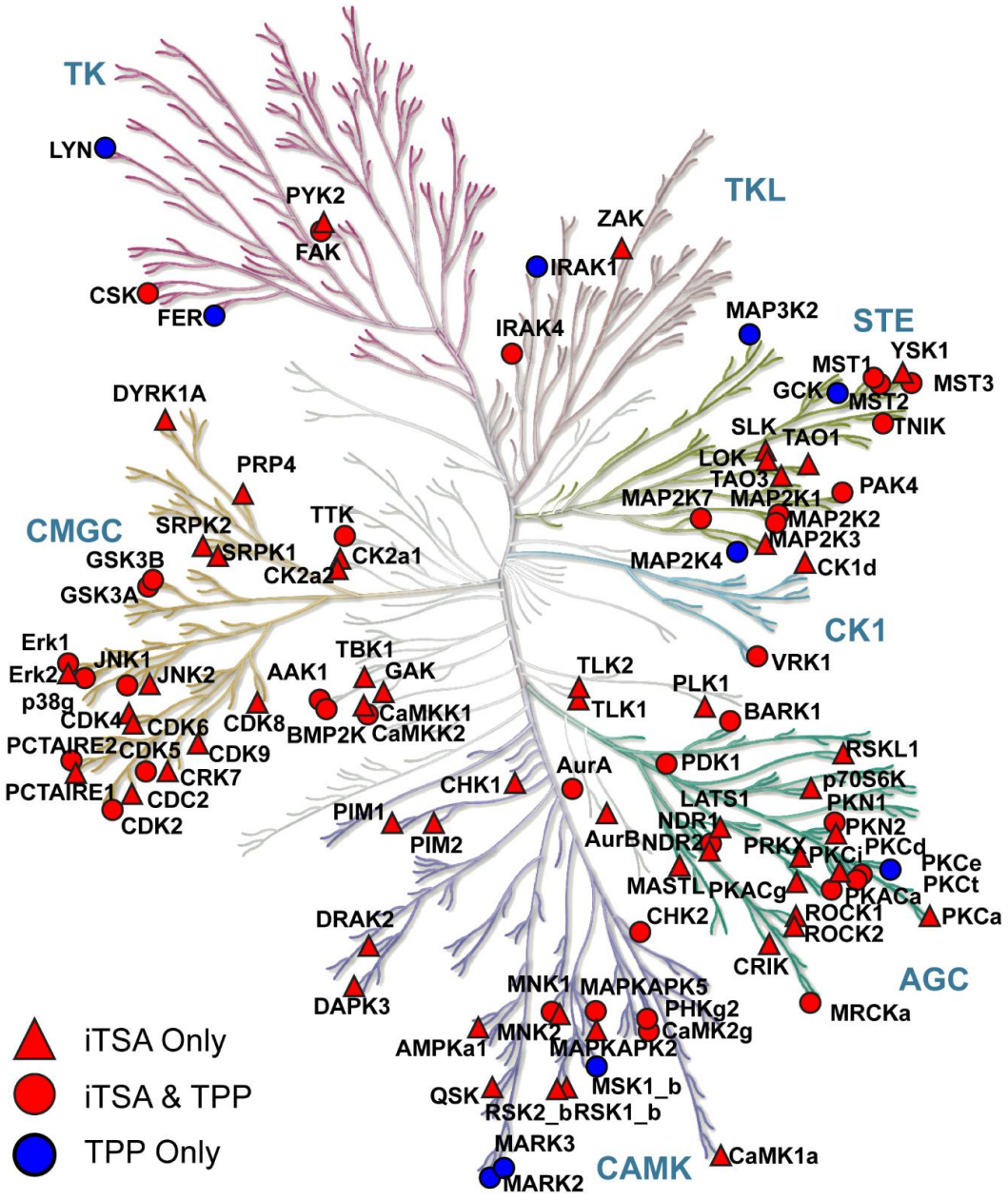
Supplementary Figure 1. Analysis of staurosporine thermal curves. (a) Staurosporine thermal curve data from staurosporine targets in [13] were downloaded and the relative fold change (Drug/Vehicle) calculated at each temperature. The temperature at the maximum fold was compared to the Vehicle T_M (b) and the T_M in the presence of staurosporine (c). Black line represents linear regression of the data points. Red line represents 1:1 correlation.



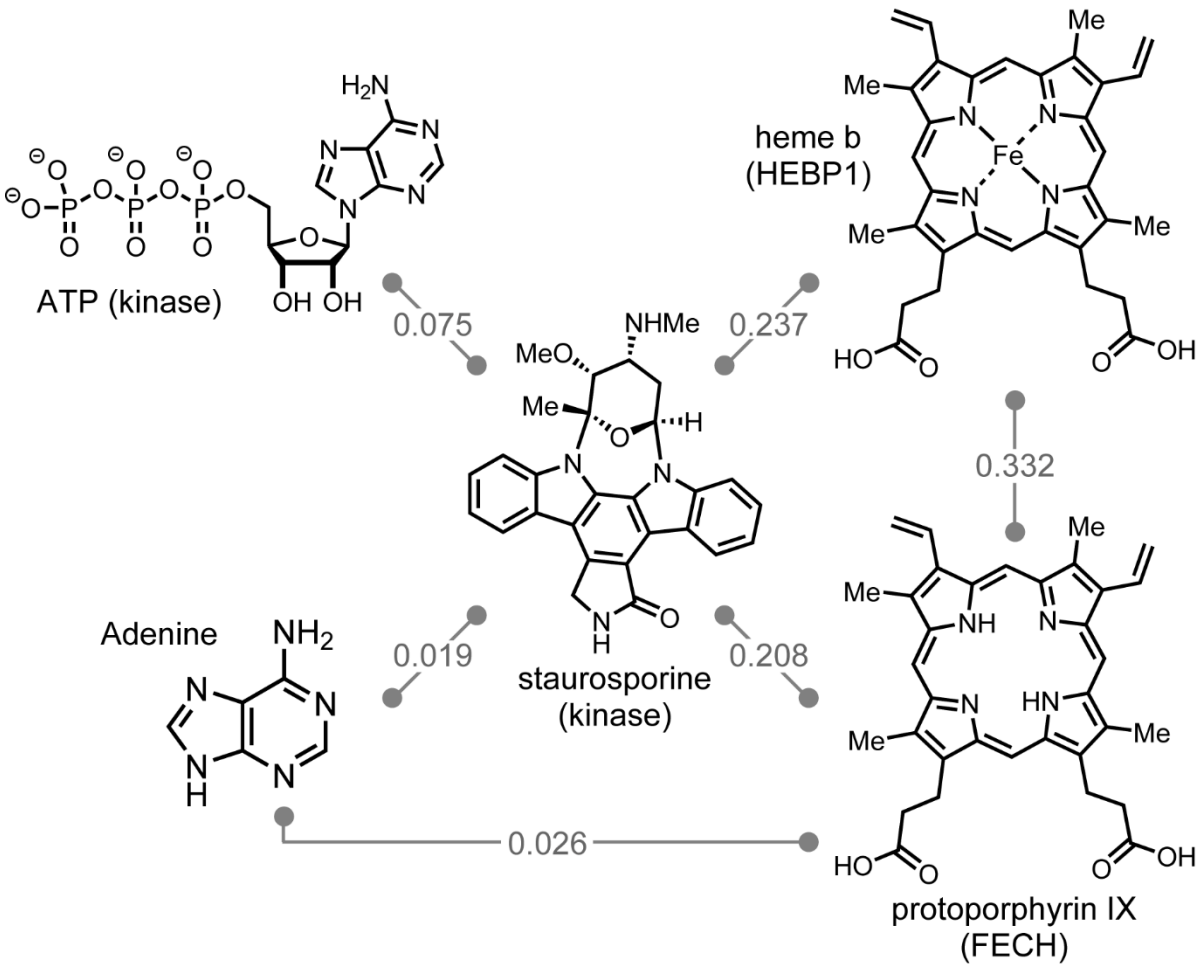
Supplementary Figure 2. Illustrated workflow for in lysate iTSA with emphasis placed on minimal sample handling to reduce variance between samples. Steps are described in the figure with details for each step in the Experimental Procedures section.



Supplementary Figure 3. iTSA was performed at 48°C **(A)** and 56°C **(B)** as described in Experimental Procedures using 20 μM Staurosporine. Volcano plot visualization of soluble protein levels using empirical Bayes statistical analysis. Proteins identified as kinases are red. FDR = 0.1% is indicated. Frequency (aka: density/distribution) of the volcano plot x-axis are shown as well. A positive fold change represents a more stable protein in drug condition and a negative fold change represents a less stable protein in drug condition.



Supplemental Figure 4. Kinome map of iTSA and TPP kinases. Blue circles represent kinases uniquely identified with TPP. Red represents kinases identified with iTSA where red triangles were uniquely identified in iTSA and red circles were targets identified by both thermal shift assays. Illustration reproduced courtesy of Cell Signaling Technology, Inc.



Supplemental Figure 5. Compound structures are shown with the known binding partners indicated and the Tanimoto coefficient similarity score displayed.

Supplemental Text File 1.

```
#####################################################################
## empirical Bayes Statistical Analysis - script by Kerri Ball and Stephen Coleman
#####################################################################
#User-defined variables are set below

setwd("directory/file")


#The output filename
prefix <- "date_sampleDescription"
drawplots <- TRUE


#Define the columnnames to be used for the experiment's multiplexing pattern
columnnames <-c("ID","Gene.names", "MS.MS.count",
            "V_log2.i._TMT_1", "D_log2.i._TMT_2",
            "V_log2.i._TMT_3", "D_log2.i._TMT_4",
            "V_log2.i._TMT_5", "D_log2.i._TMT_6",
            "V_log2.i._TMT_7", "D_log2.i._TMT_8",
            "V_log2.i._TMT_9", "D_log2.i._TMT_10",
            "Protein.IDs" )

#set up model.matrix according to label strategy/experimental design
design <- model.matrix(~ 0+factor(c(0,1,0,1,0,1,0,1,0,1))) #0=Vehicle; 1=Drug


#####################################################################
library(limma)
library(stringr)

##import data
proteinGroups <- read.table(file = "proteinGroups.txt",
                    header = TRUE,
                    sep = "\t",
                    quote = "\"",
                    dec = ".",
                    numerals = c("warn.loss"),
                    row.names = NULL,
                    na.strings = c("NA","NaN","Infinite"))

##Filter data rows
data <- proteinGroups
colnames(data)
data <- subset(data, Only.identified.by.site != "+")
data <- subset(data, Reverse != "+")
data <- subset(data, Potential.contaminant != "+")
data <- subset(data, Unique.peptides > 0)
data <- subset(data, MS.MS.count > 2)
data <- subset(data, Reporter.intensity.count.3 > 2)

##Filter data columns & define TMT data columns
# identifies column numbers that contain Label corrected intensity data (TMT lot-specific correction)
```

```
data.TMT <- grep("Reporter.intensity.corrected", colnames(data))
data.sort <- data[c("Protein.IDs", "Gene.names", "MS.MS.count")]
data.sort <- cbind(data.sort, data[,data.TMT])
data <- data.sort
# identify column numbers that contain intensity data post column filtering
data.TMT <- grep("Reporter.intensity.corrected", colnames(data))
data[,data.TMT] <- log(data[,data.TMT],base=2)  #log2 transform Reporter.intensity
View(data)

## Create Unique ID column & remove isoforms subjected to losing end of
#   MaxQuant's parsimonious distribution of peptides!
# use specific columns names to identify protein id columns
data.metanms <- grep("Protein.IDs", colnames(data))
colnames(data)[data.metanms] <-  "ID"   #change column names
data$Protein.IDs <- data$ID
data$last <- (regexpr(';', as.character(data$Protein.IDs))-1) # if no ";" then = -1
data$ID.i <- ifelse(data$last > 0, (substr(as.character(data$Protein.IDs),1,data$last)),
                (as.character(data$Protein.IDs)))
data$last <- (regexpr('-', as.character(data$ID.i))-1) # if no ";" then = -1
data$ID <- ifelse(data$last > 0, (substr(as.character(data$ID.i),1,data$last)), (as.character(data$ID.i)))
data$ID.i <- NULL
data$last <- NULL
data <- data[order(-data$MS.MS.count),] #sort by MS.MS. count (largest to smallest)
data$dup <- duplicated(data[,1]) #first occurrence is False, second and subsequent say True
data <- subset(data, data$dup != TRUE)
data$dup <- NULL

## Look at data prior to normalization:
# NOTE: if data doesn't look pretty good stop here - normalization should not be used to "fix" bad data
if(drawplots){
  pdf("BoxPlot_BeforeQuantileNormalization.pdf")
  boxplot(data[,data.TMT], main = "Before Normalization", names = TRUE,
          frame = FALSE, notch = TRUE,
          col =
c("gray80","gray75","gray70","gray65","gray60","gray55","gray50","gray45","gray40","gray35"))
  # Close the pdf file
  dev.off()
}
data.norm <- data

# identify column numbers that contain intensity data
data.TMT <- grep("Reporter.intensity.corrected", colnames(data.norm))
colnames(data.norm[,data.TMT])
data.norm[,data.TMT] <-  normalizeBetweenArrays(as.matrix(data.norm[,data.TMT]),method="quantile" )
if(drawplots){
  boxplot((data.norm[,data.TMT]), main = "After Quantile Normalization", names = TRUE,
          frame = FALSE, notch = TRUE,
          col =
c("gray80","gray75","gray70","gray65","gray60","gray55","gray50","gray45","gray40","gray35"))
  View(data.norm)
}

##Rename columns according to experimental design and set up model.matrix appropriately
data.norm <- setNames(data.norm, columnnames)
```

```
##Use limma for empirical bayes
colnames(design) <- c("V","D")   #0=Vehicle; 1=Drug
fit <- lmFit(data.norm[,data.TMT], design)
fit <- eBayes(fit)             ##Apply empirical Bayes smoothing to the standard errors.
contrast <- makeContrasts("D-V", levels=design)
fit2 <- contrasts.fit(fit, contrast)
fit2 <- eBayes(fit2, trend = TRUE)
ptopf <- topTableF(fit2, adjust="BH",genelist = data[,"ID"],number=Inf)
ptopf$rank <- 1:length(ptopf$F)
ptopf$numfp <- ptopf$rank * ptopf$adj.P.Val
metanms <- c("ID")
ftest.tab <- topTableF(fit2, adjust="BH",genelist = data[,metanms],number=Inf,p.value=1)
tab.t1 <- topTable(fit2, adjust="BH",genelist = data[,metanms], coef=1,p.value=1, number=Inf)
tab.t1[5:6] <- -log(tab.t1[5:6],base=10)

##preview data
tab1 <- tab.t1
if(drawplots){
  Xdata <- tab1$logFC
  Ydata <- tab1$P.Val
  plot(Xdata, Ydata,                    # plot the variables
       main = "",
       xlab="logFC",                    # x-axis label
       ylab="-Log10(P.Val)")         # y-axis label
}
  sum(tab.t1$adj.P.Val >= -log10(0.05))
  sum(tab.t1$adj.P.Val >= -log10(0.01))
  sum(tab.t1$adj.P.Val >= -log10(0.001))

  ###Change column names and merge EB results with normalized data
  tab.t1[ ,c(3,4,7)] <- list(NULL)
  tab.t1 <- setNames(tab.t1, c("ID","Log2FC","Log10P.Value","Log10adj.P.Val"))
  data.analysis <- merge(data.norm,        tab.t1, by = "ID" ,all = T, sort=F)
  data.analysis <- data.analysis[order(-data.analysis$Log10adj.P.Val),]
  if(drawplots){ View(data.analysis) }

  #Create FDR and FC columns
  data.analysis$FDR0.001Sig <- ifelse(data.analysis$Log10adj.P.Val>=3, 1, 0)
  data.analysis$FDR0.01Sig <- ifelse(data.analysis$Log10adj.P.Val>=2, 1, 0)
  data.analysis$FDR0.05Sig <- ifelse(data.analysis$Log10adj.P.Val>=1.30103, 1, 0)
  data.analysis$absFC <- abs(data.analysis$Log2FC)
  if(drawplots){ plot(data.analysis$absFC, data.analysis$Log10adj.P.Val) }
  quantile(data.analysis$absFC, probs = c(80,90)/100)
  percentile <- quantile(data.analysis$absFC, probs = c(80,90)/100)
  percentile80 <- data.frame(percentile[1])
  percentile90 <- data.frame(percentile[2])
  data.analysis$FC0.10Sig <- ifelse(data.analysis$absFC>= (percentile90[1,1]), 1, 0)
  data.analysis$FC0.20Sig <- ifelse(data.analysis$absFC>= (percentile80[1,1]), 1, 0)
  data.analysis$Sig_0.1FDR.FC <- ifelse(data.analysis$FDR0.001Sig >= 1,
(ifelse(data.analysis$FC0.10Sig >= 1, 1, 0)), 0)
  data.analysis$Sig_1FDR.FC <- ifelse(data.analysis$FDR0.01Sig >= 1, (ifelse(data.analysis$FC0.10Sig
>= 1, 1, 0)), 0)
  data.analysis$Sig_5FDR.FC <- ifelse(data.analysis$FDR0.05Sig >= 1, (ifelse(data.analysis$FC0.20Sig
>= 1, 1, 0)), 0)
  data.analysis$Sig_FDR.FC <- data.analysis$Sig_1FDR.FC + data.analysis$Sig_5FDR.FC +
data.analysis$Sig_0.1FDR.FC
```

```
  data.analysis$Direction <- ifelse(data.analysis$Log2FC >= 0, (1*data.analysis$Sig_FDR.FC), (-
1*data.analysis$Sig_FDR.FC))
  data.analysis <- data.analysis[order(-data.analysis$Sig_FDR.FC),]
  data.analysis$rank <- 1:length(data.analysis$Sig_FDR.FC)
  if(drawplots){ plot(data.analysis$rank, data.analysis$Log10P.Value) }

#Create gene.name column
data.analysis$last <- (regexpr(';', as.character(data.analysis$Gene.names))-1) # if no ";" then = -1
data.analysis$Gene.name <- ifelse(data.analysis$last > 0,
(substr(as.character(data.analysis$Gene.names),1,data.analysis$last)),
(as.character(data.analysis$Gene.names)))
#if no gene name is present then use ID
data.analysis$last <- nchar(data.analysis$Gene.name)
data.analysis$name <- data.analysis$Gene.name
data.analysis$Gene.name <- ifelse (data.analysis$last == 0, as.character(data.analysis$ID),
data.analysis$name)
data.analysis$last <- NULL
data.analysis$name <- NULL

#annotate data
##import annotation file:
## (create annotation file with https://www.uniprot.org/uniprot/ and edit in excel.
##  change "Entry" header to "ID" and I like yes/NA "annotation" columns)

annotation <- read.table(file = "UniprotID_ATPbinding_organism_isHuman_statis-isReviewed.txt",
                         header = TRUE,
                         sep = "\t",
                         quote = "\"",
                         dec = ".",
                         numerals = c("warn.loss"),
                         row.names = NULL,
                         na.strings = c("NA","NaN","Infinite"))
test2 <- merge(data.analysis,     annotation, by = "ID" ,all = T, sort=F)
test3 <- subset(test2, Sig_FDR.FC >= 0)
data.analysis <- test3

#Clean-up matrix for export: reorganize columns
V_TMT <- grep("V_log2.i._TMT_", colnames(data.analysis))
D_TMT <- grep("D_log2.i._TMT_", colnames(data.analysis))
colnames(data.analysis)  #"annotation" MUST MATCH COLNAMES! or error will occur
data.sort <- data.analysis[c("ID", "Gene.name", "rank", "Direction", "Sig_FDR.FC",
                           "ATP.binding", "Log2FC", "Log10P.Value", "Log10adj.P.Val",
                           "Protein.IDs", "Gene.names", "MS.MS.count")]
test2 <- cbind(data.sort, data.analysis[,V_TMT], data.analysis[,D_TMT])
colnames(test2)
data.analysis <- test2

##CSV files opened in excel will change Sept# and MAR# to dates;
## this addition tells excel that this is text and prevents change!
## (use find & replace in excel if desired)
data.analysis$Gene.name <- paste(data.analysis$Gene.name, ";", sep="")
data.analysis$Gene.names <- paste(data.analysis$Gene.names, ";", sep="")

write.csv(data.analysis,file=paste(prefix,"_empiricalBayesAnalysis.csv",sep=""),
        row.names = F)
```

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