Technical Journal Club

UniversitätsSpital Zürich

Heating up the target deconvolution strategies: thermal proteome profiling for identification of drug targets

Assunta Senatore

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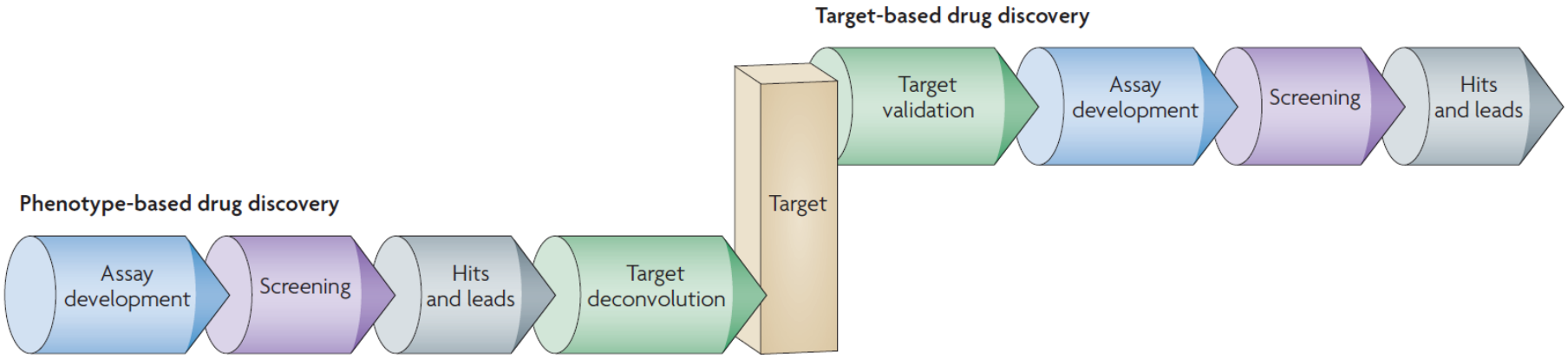
Phenotype-based versus target-based drug discovery



Highly challenging

High costs

Long time for development

Target deconvolution strategies

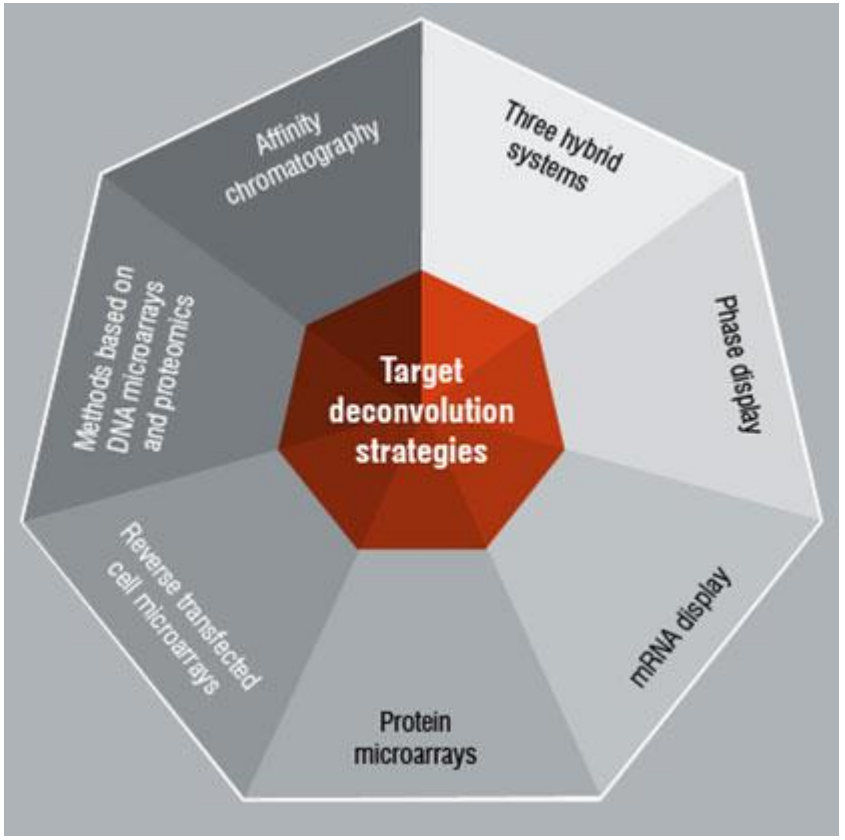
Target deconvolution:

The retrospective identification of the molecular targets that underlie the observed phenotypic responses

knowledge of the molecular targets:

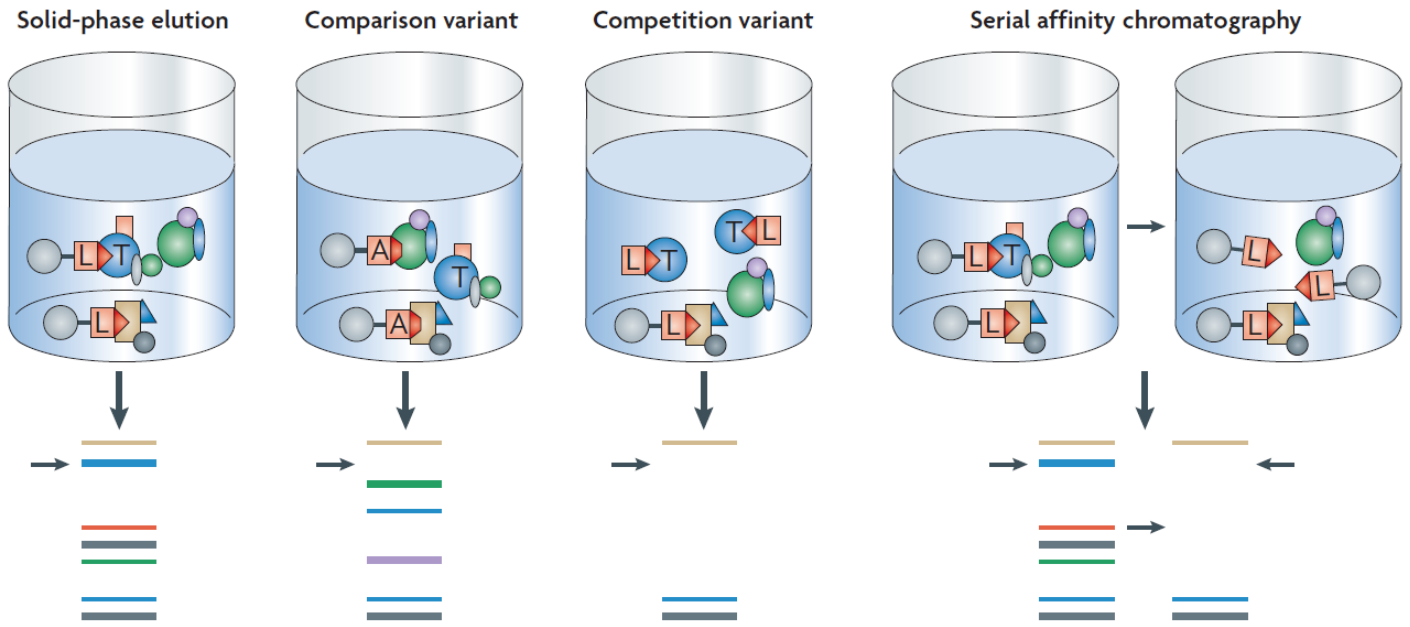
- will help to understand the (patho) mechanism(s) of the disease.
- will also aid rational drug design and allow efficient Structure-Activity Relationship (SAR) studies in a chemical optimisation programme, thereby developing target-specific assays.

In addition, the aspects related to target-specific toxicity and side effects can be addressed, reducing later-stage "attrition" early on.”



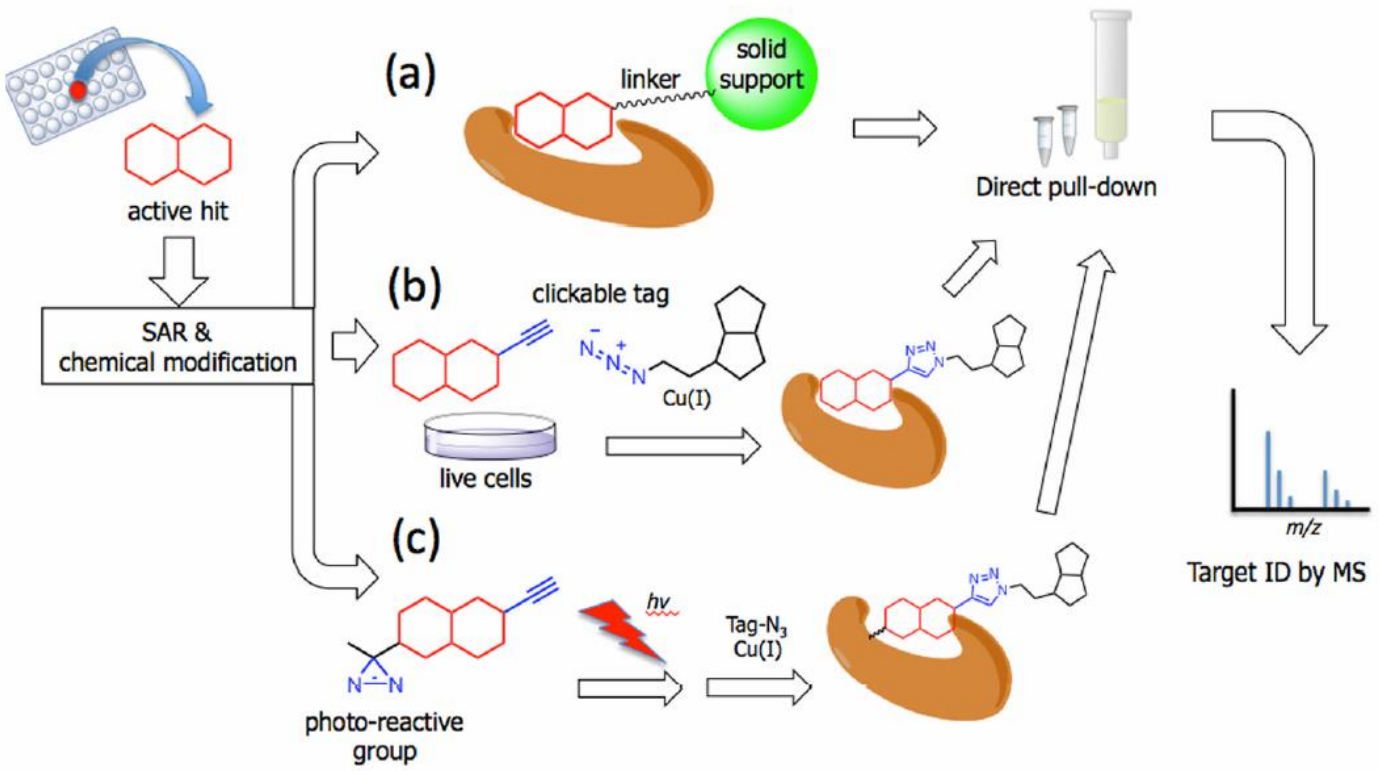
Affinity-chromatography-based methods for target deconvolution

The small molecule ligand (L) is tethered to a matrix and incubated with a protein extract that includes the target protein (T).



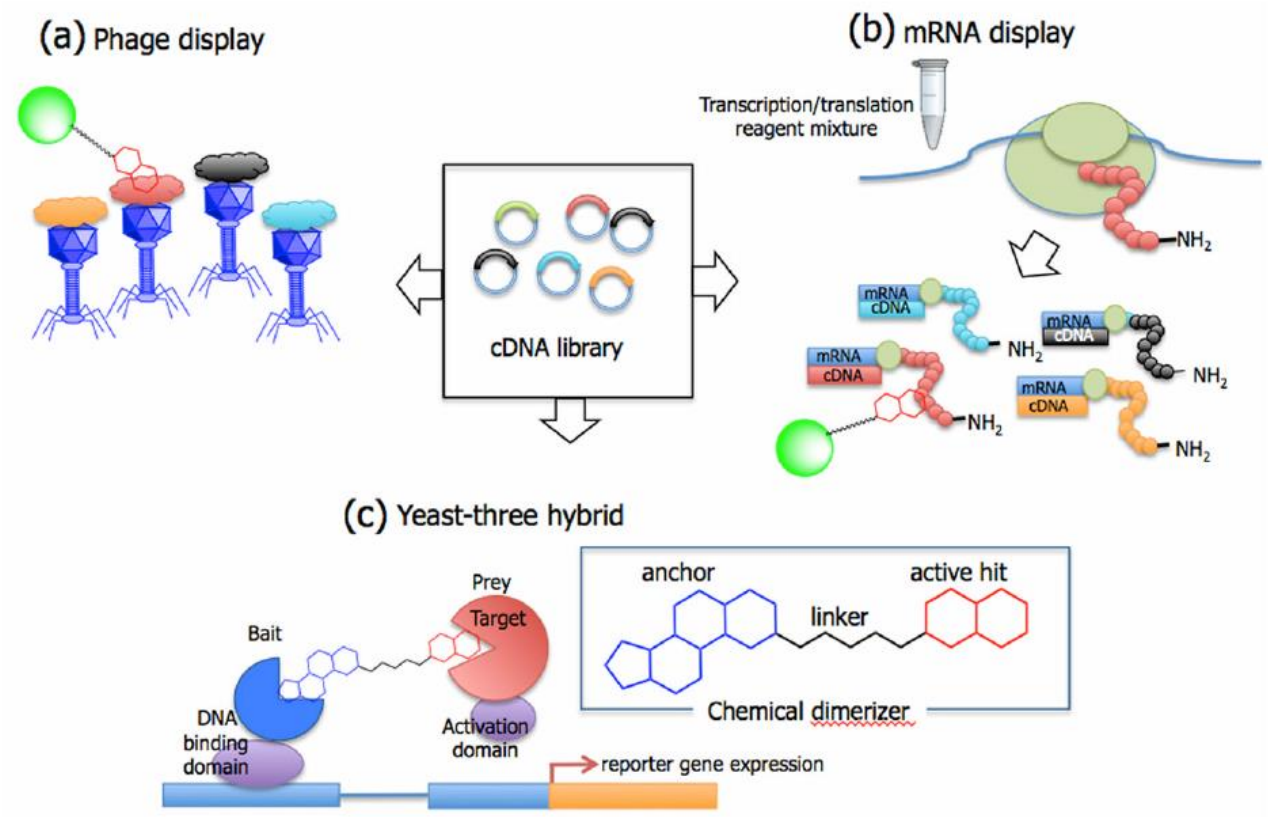
- Solid-phase elution: ligand-bound proteins are eluted using buffers that disrupt intermolecular interactions.
- The protein pattern that is obtained with an inactive ligand analogue (A) is also determined (the ‘comparison variant’), and the two outcomes are compared.
- Competition variant’, protein elution is accomplished by an excess of free ligand.
- Serial affinity chromatography, the matrix is incubated with protein extract which is then incubated with fresh matrix. Most of the proteins that bind specifically are captured by the first matrix, whereas the amounts of nonspecific binding proteins are similar for both matrices.

Labeling strategies

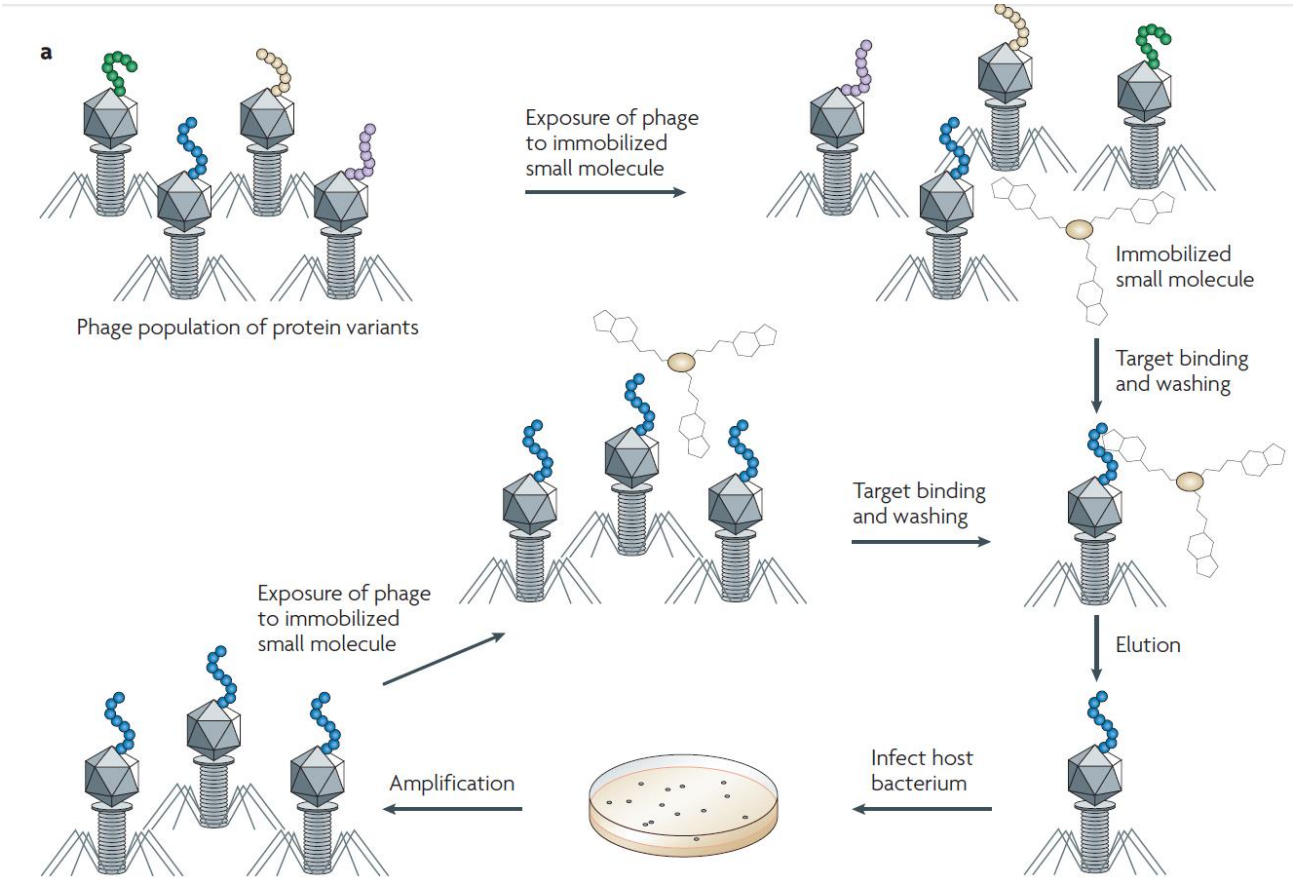


Expression cloning techniques

Proteins can be expressed using cloning vectors containing cDNA library, and these proteins exposed to small molecules for affinity selection

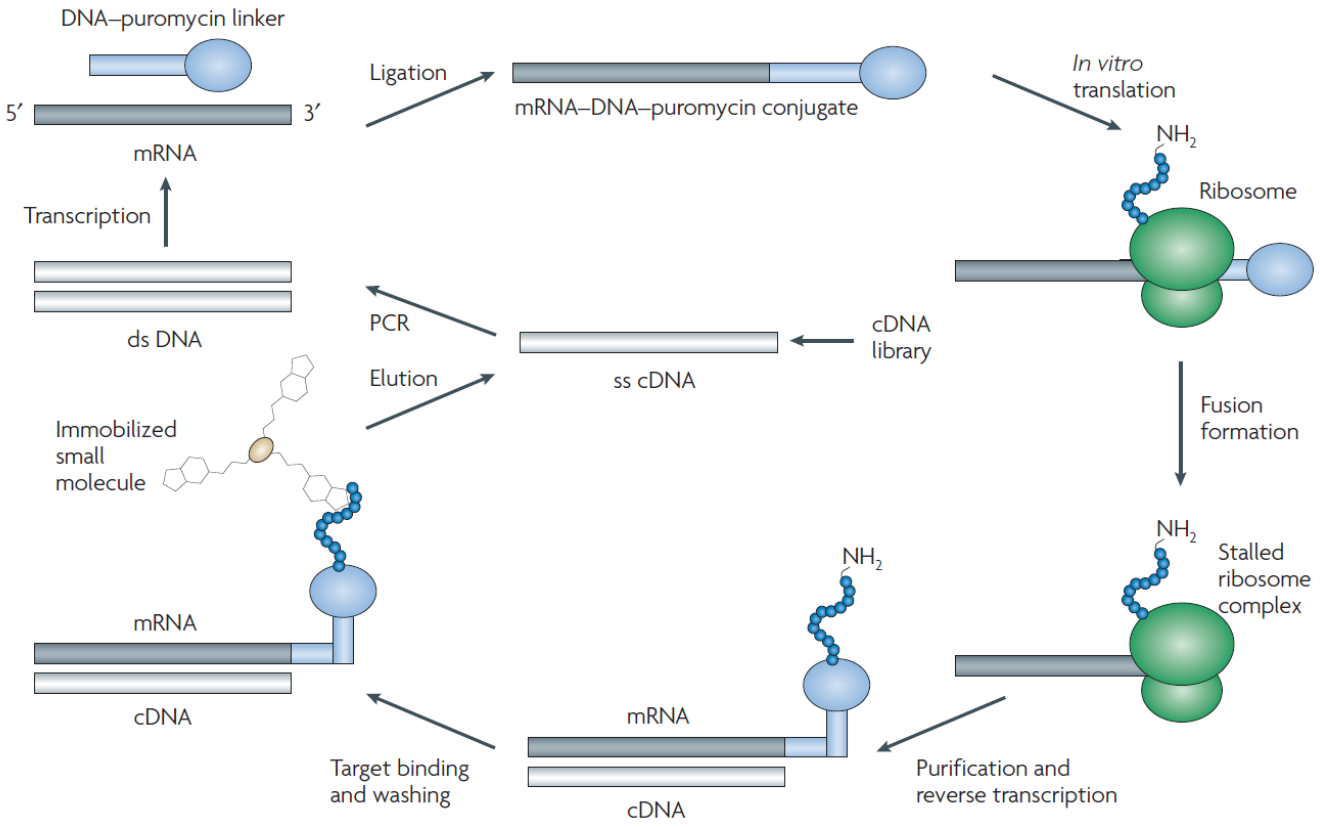


Phage Display technology for target deconvolution



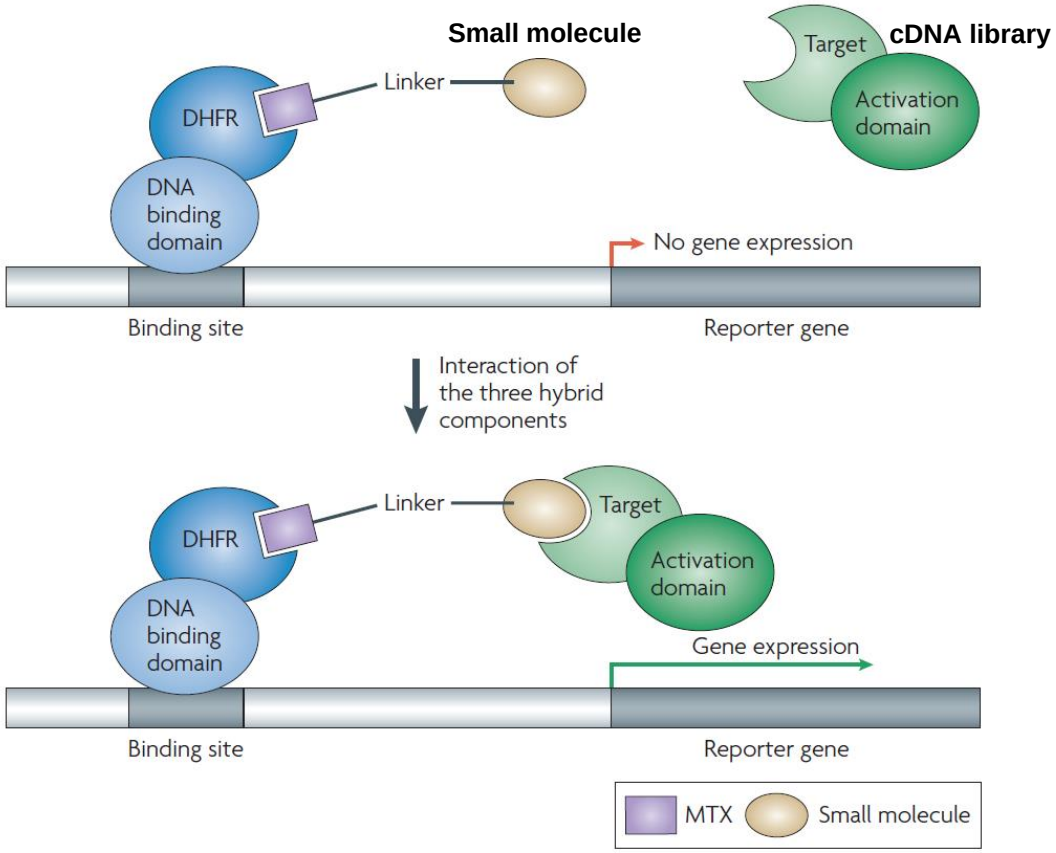
A **phage population displaying potential target proteins** on their surface is exposed to an immobilized small molecule. After affinity selection, the eluted phage population is amplified and subjected to further rounds of affinity enrichment. At the end of the procedure, the monoclonal phage population can be analysed for target identification.

mRNA display technology for target deconvolution



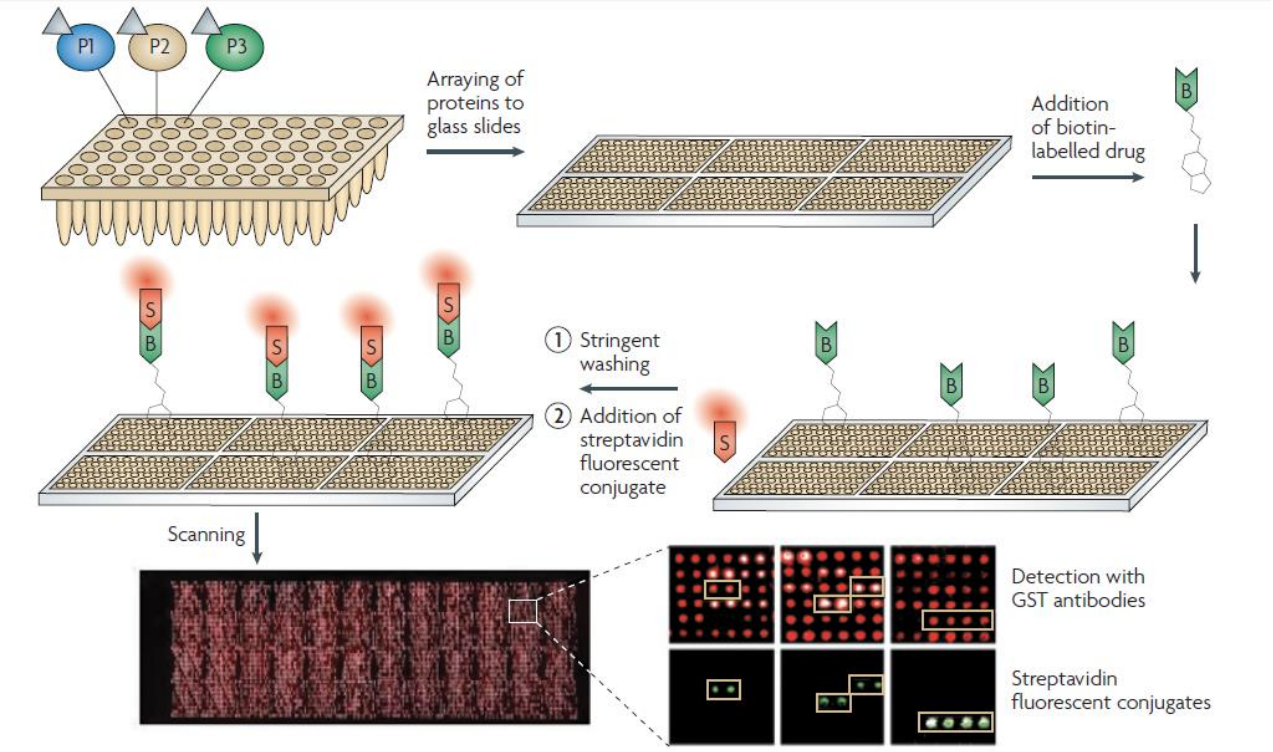
An **mRNA-displayed protein library** is created in vitro and exposed to an immobilized small molecule. After affinity selection, cDNA target molecules are amplified by PCR and used in the next selection round to generate a new library that is enriched for drug-binding proteins. Several rounds of reiteration lead to the identification of target molecules. ds, double stranded; ss, single stranded

Three-hybrid systems for target deconvolution



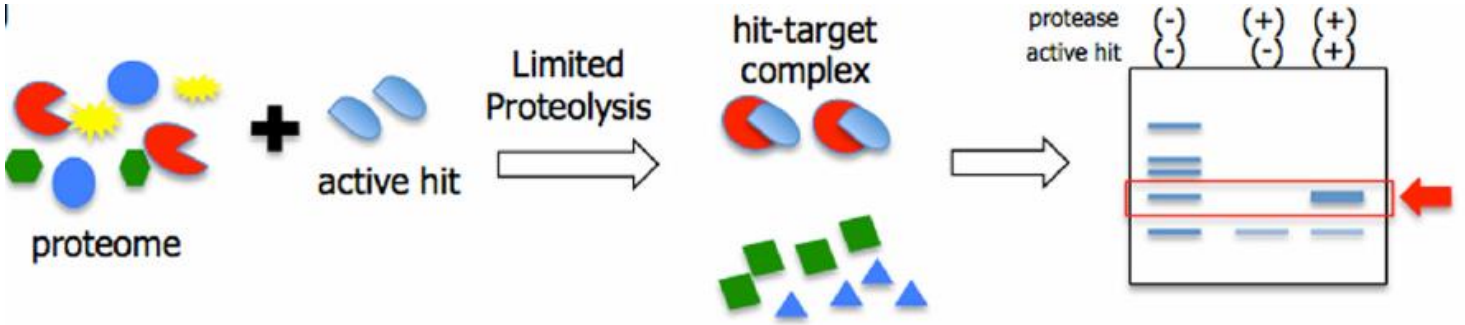
The three-hybrid system comprises one component that consists of a DNA-binding domain fused to a ligand binding domain (DHFR), one component that consists of a ligand molecule (MTX) linked to a **small molecule**, and one component that consists of a transcriptional activation domain fused to a protein from a cDNA library (which might be a **target protein**).
The binding of the small molecule to its target protein results in the interaction of the three hybrid components, which form a trimeric complex. This complex then activates the expression of a reporter gene, providing a measure of the interaction.

Microarray technologies for target deconvolution



Protein microarrays.
Proteins are immobilized on a glass slide through a glutathione S-transferase (GST) tag and exposed to a labelled small molecule (in this example, a biotinylated form of the small molecule). Bound target proteins are detected by adding a fluorescently labelled streptavidin (S) conjugate, and then identified by their positions on the array. As a 'loading' control, the array is probed with GST-specific antibodies.

Label-free techniques for target deconvolution



Limited proteolysis techniques such as DARTS (drug-assisted responsive target stability) and pulse proteolysis utilize stability of protein-ligand complex under proteolytic condition. Ligand bound proteins are more resistant to proteolysis in the presence of denaturant (pulse-proteolysis) or without denaturant (DARTS), and non-binding proteins are hydrolyzed to small peptides and amino acids. All proteolysis resistant proteins can be analyzed by SDS-PAGE and identified by mass spectrometry

"The efficacy of therapeutics is dependent on a drug binding to its cognate target. Optimization of target engagement by drugs in cells is often challenging, because drug binding cannot be monitored inside cells."

Monitoring Drug Target Engagement in Cells and Tissues Using the Cellular Thermal Shift Assay

Daniel Martinez Molina,[1]* Rozbeh Jafari,[1]* Marina Ignatushchenko,[1]* Takahiro Seki,[2]
E. Andreas Larsson,[3] Chen Dan,[3] Lekshmy Sreekumar,[3] Yihai Cao,[2,4] Pär Nordlund[1,3]†

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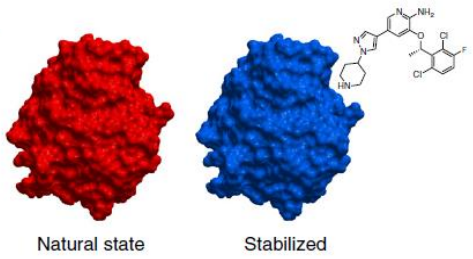
Cellular thermal shift assay (CETSA)

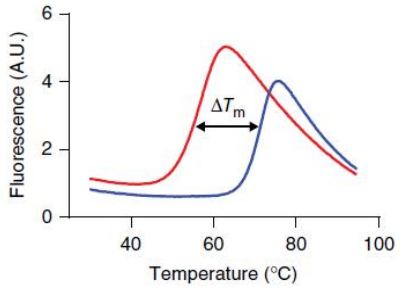
Based on the biophysical principle of ligand-induced thermal stabilization of target proteins, as described by Koshland (1958) and Linderstrom-Lang and Schellman (1959).

Few crucial components:

- a heating step in which target proteins denature and precipitate unless stabilized through ligand binding and,
- a step in which proteins that remain stable during heating are distinguished from proteins that denature and precipitate

A **thermal shift assay** quantifies the change in thermal denaturation temperature of an <u>isolated</u> protein under varying conditions (pH, salts, additives, drugs, drug leads, oxidation/reduction, or mutations).



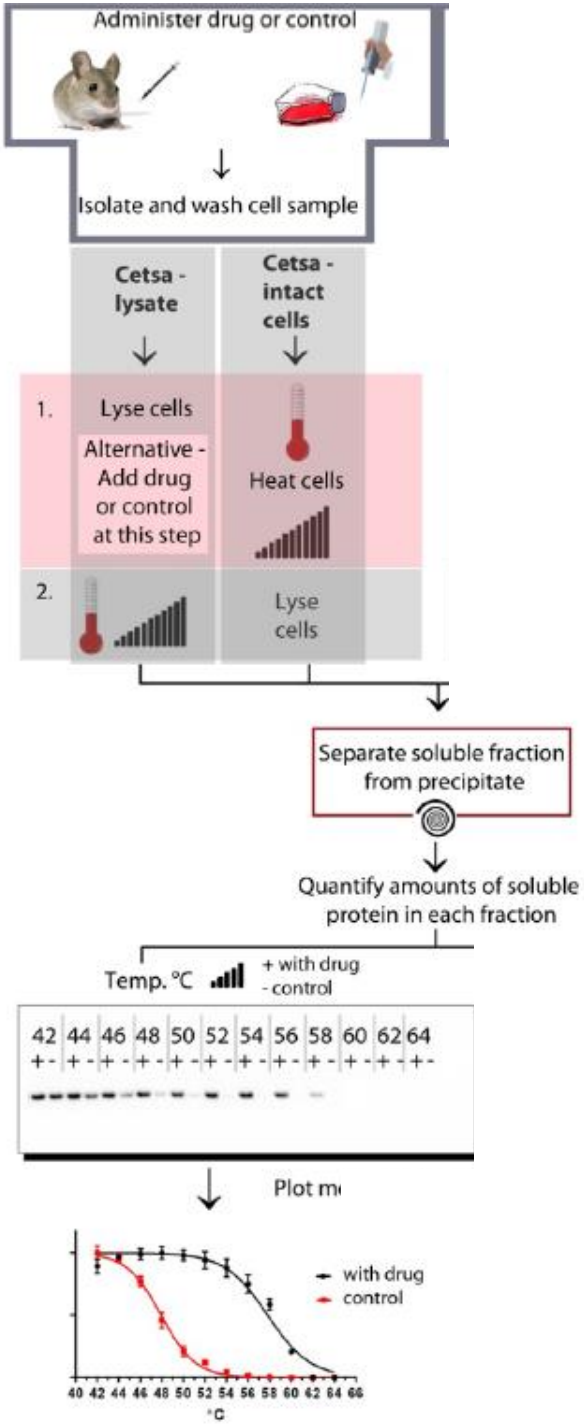


Thermal shifts assays (for example fluorescence or light scattering based techniques) are widely used for characterization of ligand binding in structural biology and drug screening in a broad range of affinities.
<u>These methods have been applied **only to purified proteins**</u>.

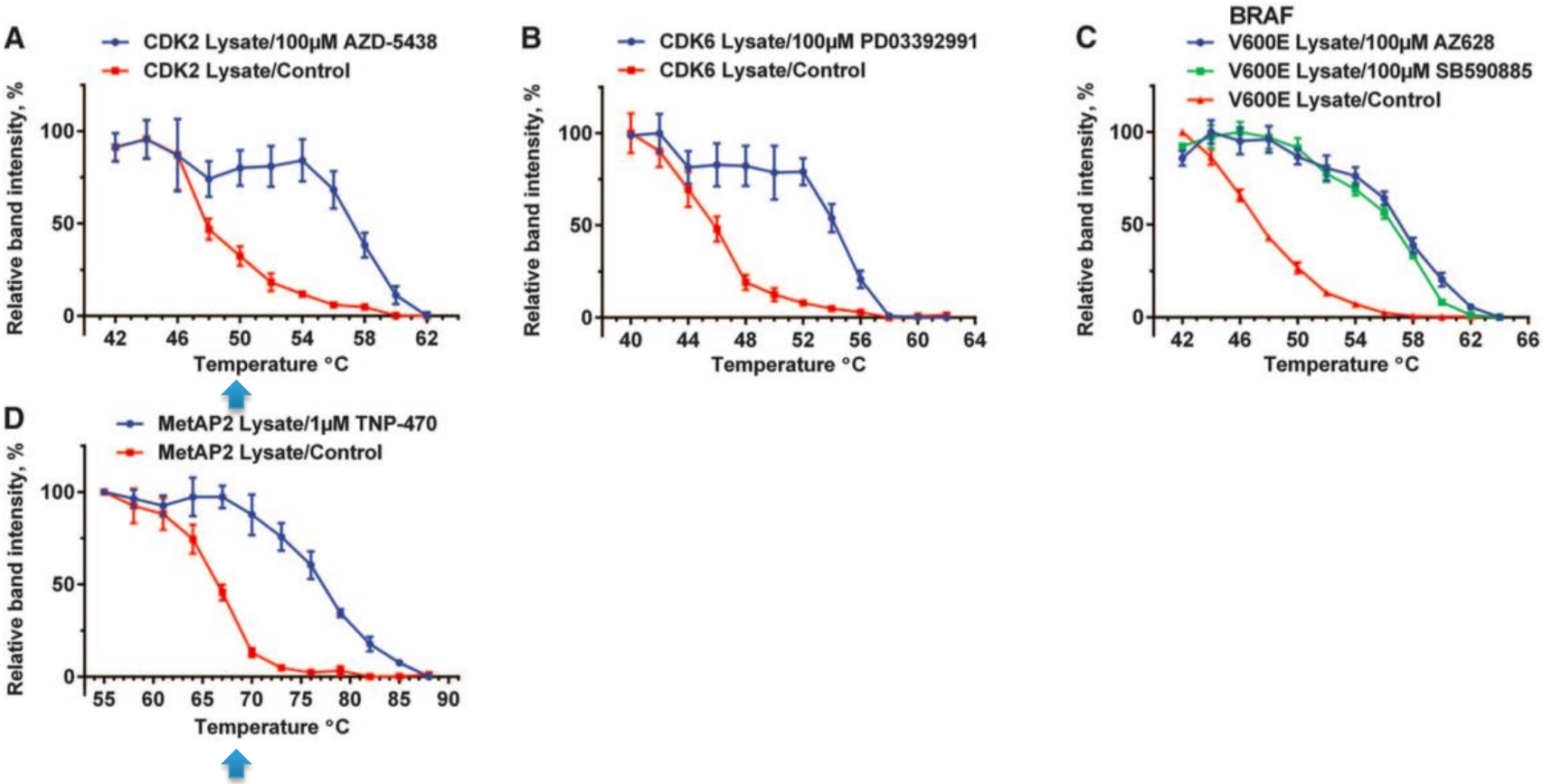
Melting temperature:ΔGfolding(Tm) = 0,
the temperature at which half of the population is unfolded and the other half is folded.

CETSA mealting curves

- treatment of cells with a compound of interest: critical incubation time
- heating to denature and precipitate proteins
- cell lysis
- the separation of cell debris and aggregates from the soluble protein fraction, by centrifugation or filtration of the samples to remove denatured and precipitated material
- Whereas unbound proteins denature and precipitate at elevated temperatures, ligand-bound proteins remain in solution.

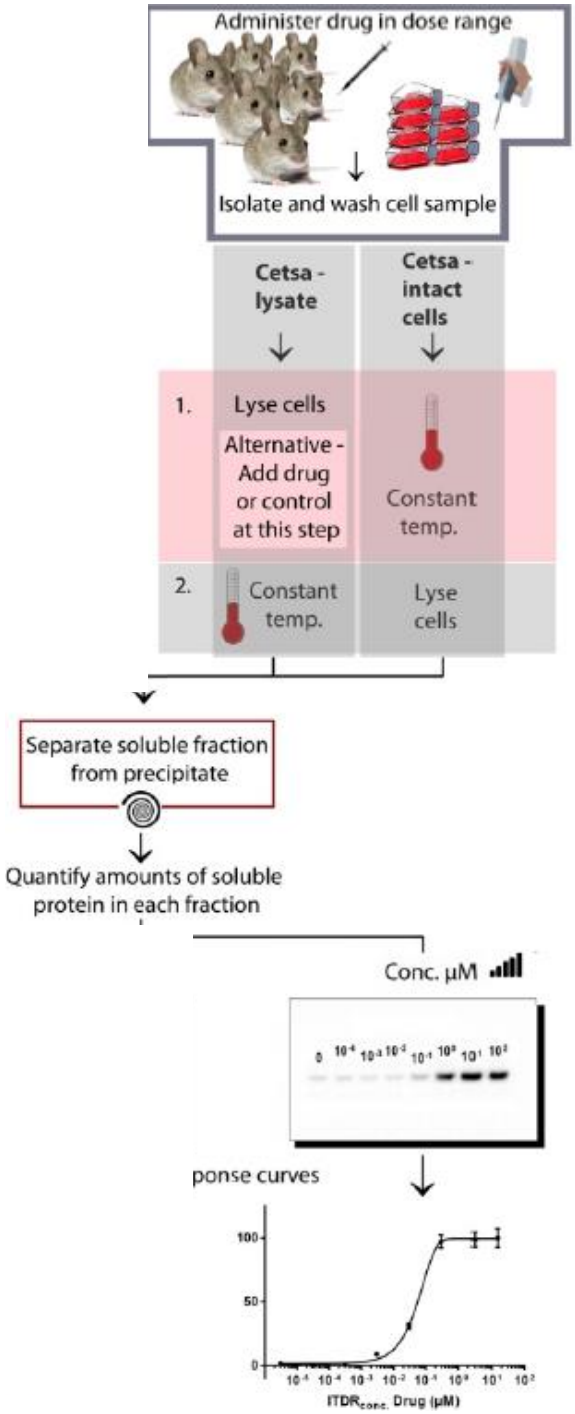


CETSA melt curves in cell lysate

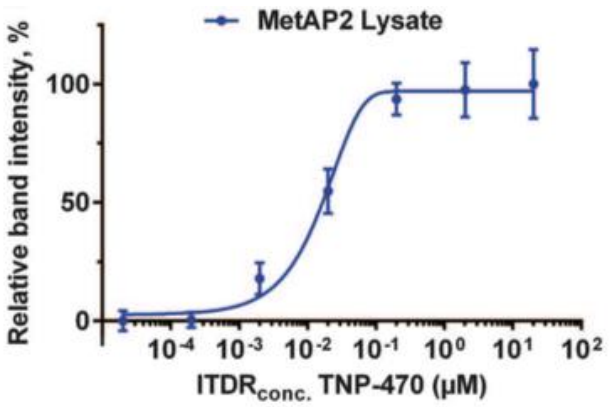


$ITDRF_{CETSA}$

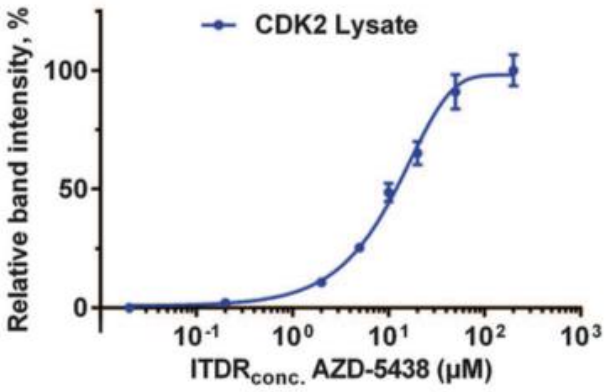
- Isothermal dose-response procedure to investigate drug concentration effects.
- Lysate aliquots were exposed to different concentrations of drug while time of heating and **temperature were kept constant.**
- The temperature at which the unbound proteins denature and precipitate needs to be known



$ITDRF_{CETSA}$ in cell lysate



72°C



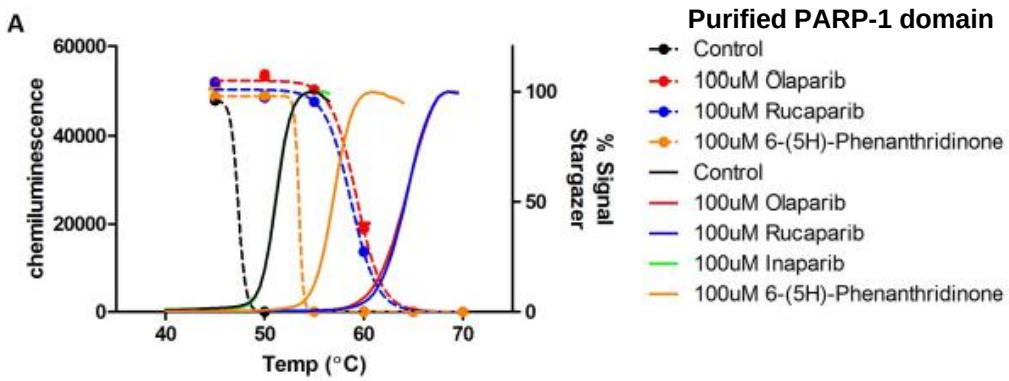
52°C

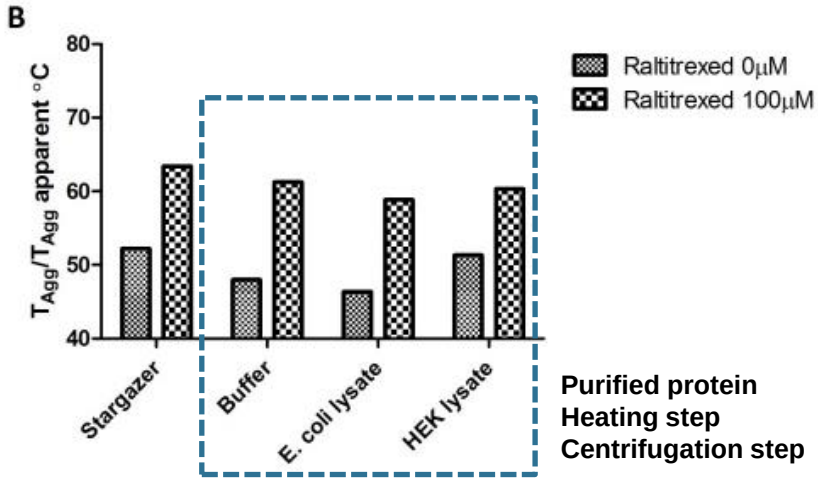
The response here is typically reached at higher drug concentrations, unlike traditional dose-response experiments in which half-saturation points are related to affinities.

This procedure yields a **characteristic fingerprint of the target engagement along the drug concentration** axis. This isothermal dose-response fingerprint was used to estimate relative differences in drug concentration required to establish a similar extent of target engagement.

Correlation of CETSA with TSA by DSLS on purified proteins

The drop in signal in the centrifugation experiments follows the increase of Stargazer signal (which monitors the light scattering of the aggregates formed upon precipitation).

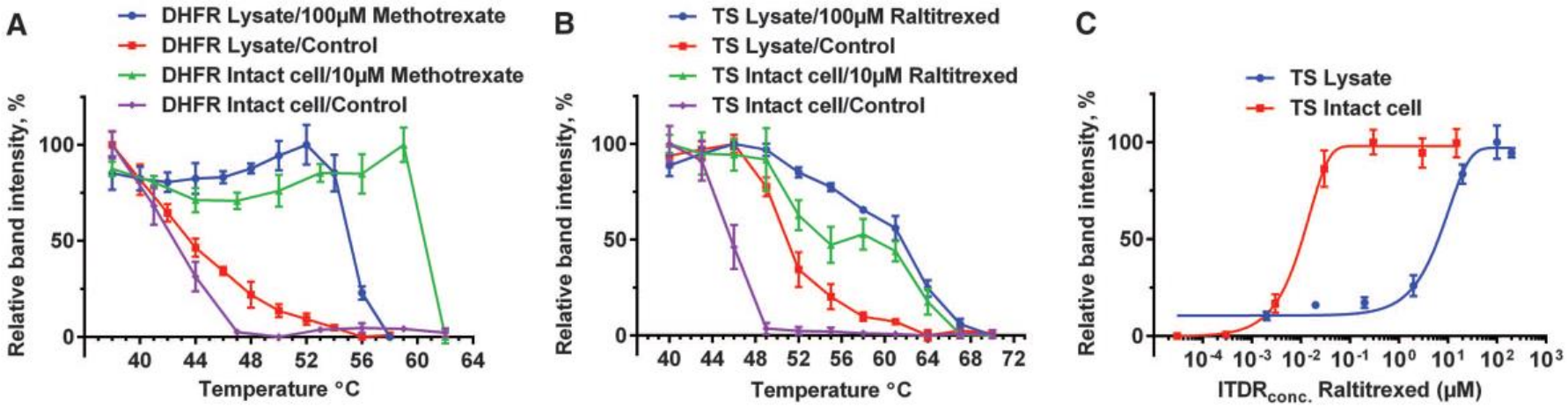




Static light scattering (DSLS) allows monitoring temperature-dependent protein aggregation. Since proteins aggregate upon denaturation the detected species size will go up.

Stargazer-2 measures SLS while heating samples. This is label-free and independent of specific residues in the protein or buffer composition. The only requirement is that the protein actually aggregates/fibrillates after denaturation and that the protein of interest has been purified.

Monitoring of antifolate drug transport and activation in intact cells (1)



52°C

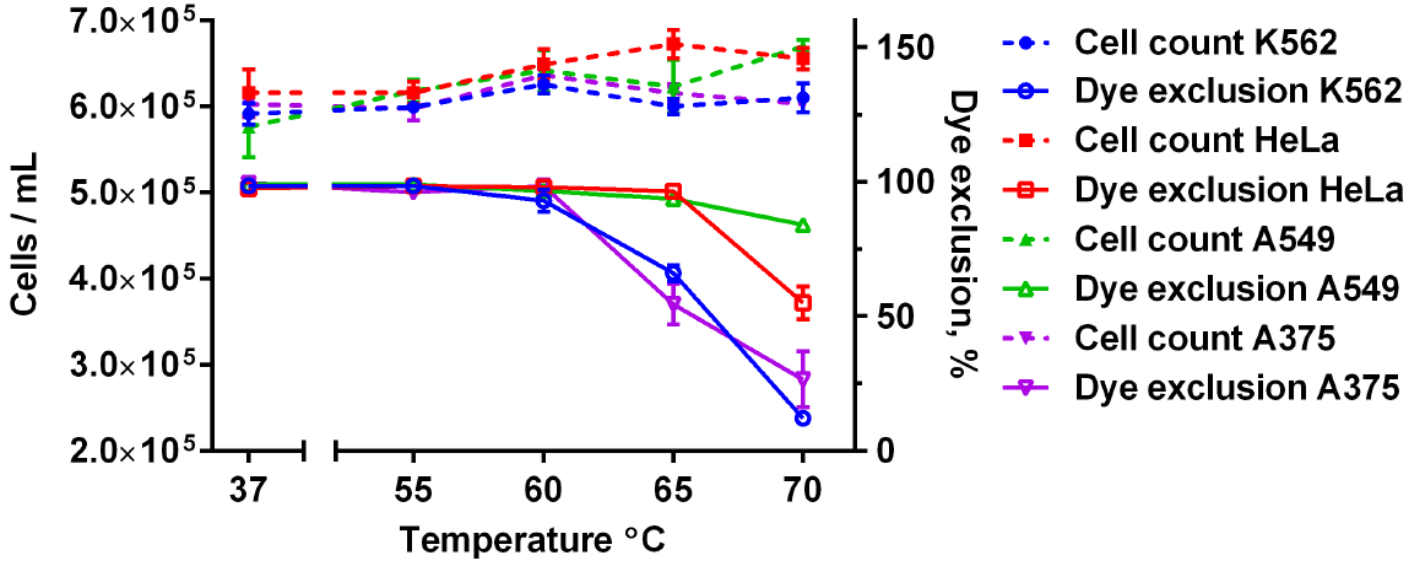
3 h of exposure

CETSA curves in intact cells versus lysate for DHFR (A) and TS (B) with methotrexate and raltitrexed respectively.

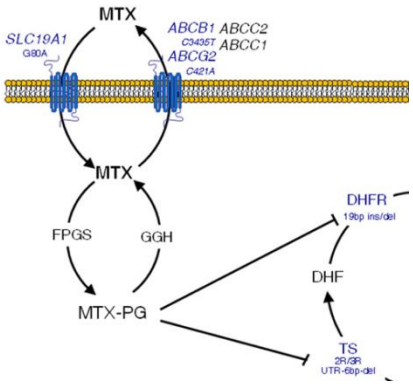
(C) ITDRFCETSA at 52°C in intact cells versus lysate for TS using raltitrexed to assess the relative binding in cells

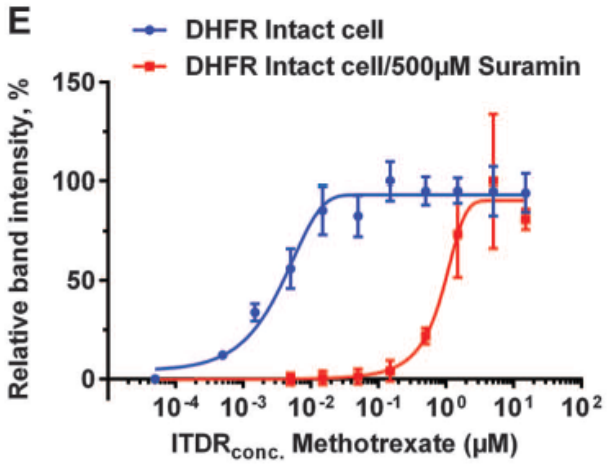
Cell count and cell membrane integrity

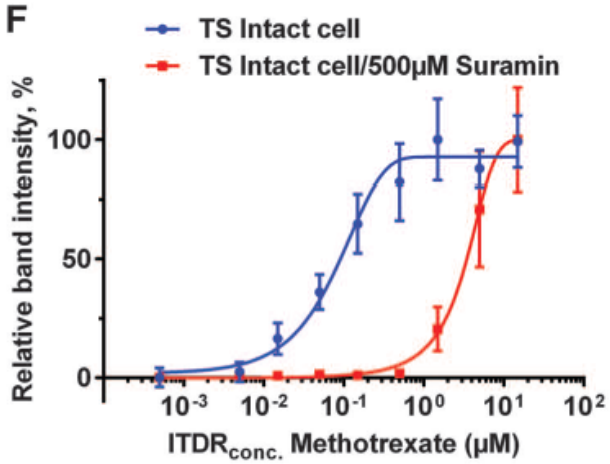
Cell count and dye exclusion by four different cell lines after heating **for 3 minutes** to different temperatures**.**



Monitoring of antifolate drug transport and activation In intact cells (2)

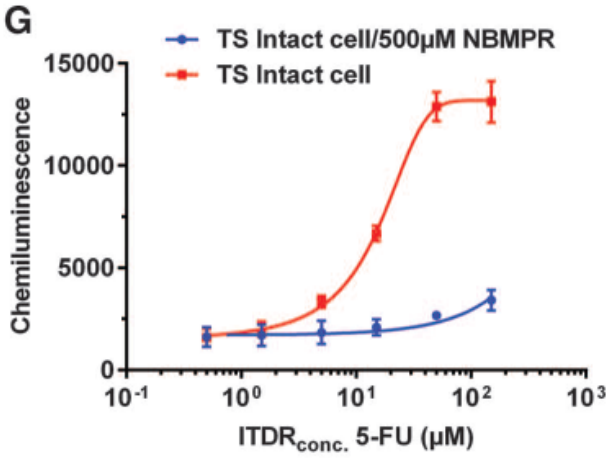






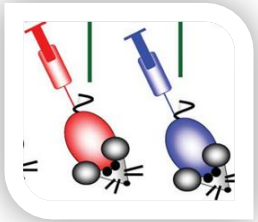
(E) And (F) $ITDRF_{CETSA}$ at 52°C for DHFR and TS in intact cells after inhibition of methotrexate activation by blocking polyglutamate synthetase with suramin.

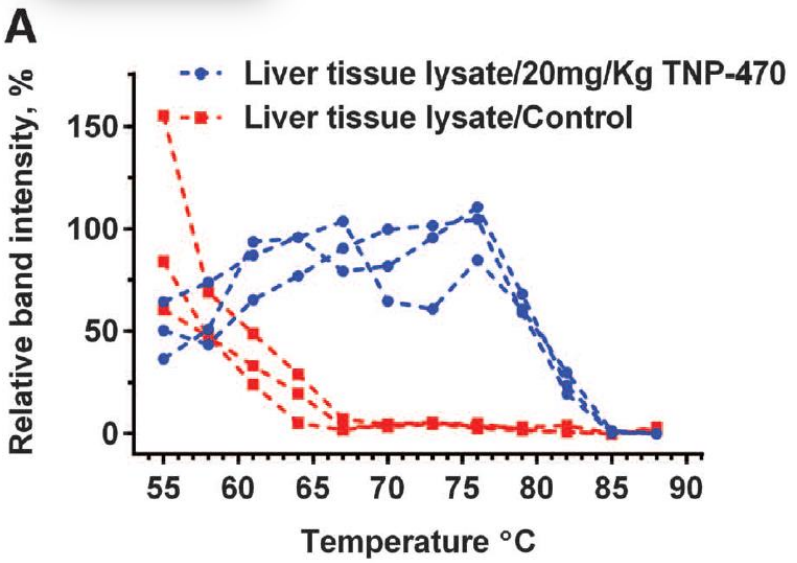
(G) $ITDRF_{CETSA}$ at 52°C showing the effect of nucleoside transporter inhibitor NBMPR on import of 5-FU and target engagement for TS in intact cells.

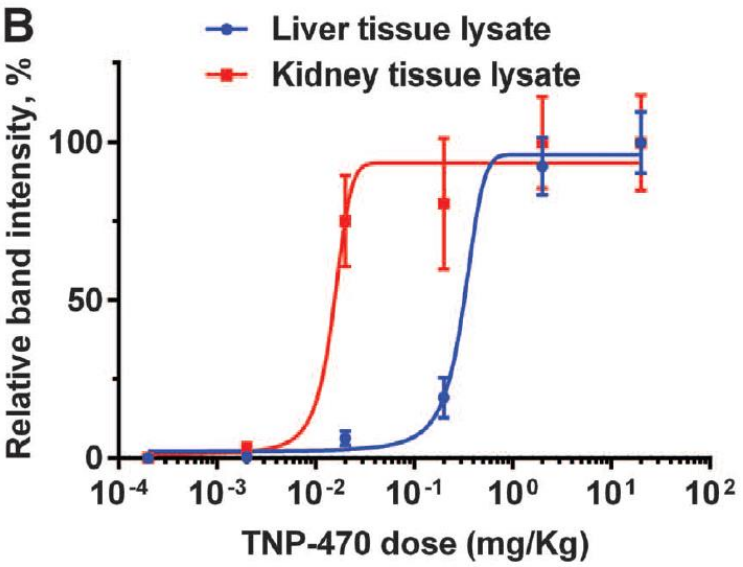


CETSA can monitor processes involved in drug resistance

Monitoring of methionine aminopeptidase-2 (MetAP-2) inhibitor TNP-470 target engagement in tissue samples from mice.



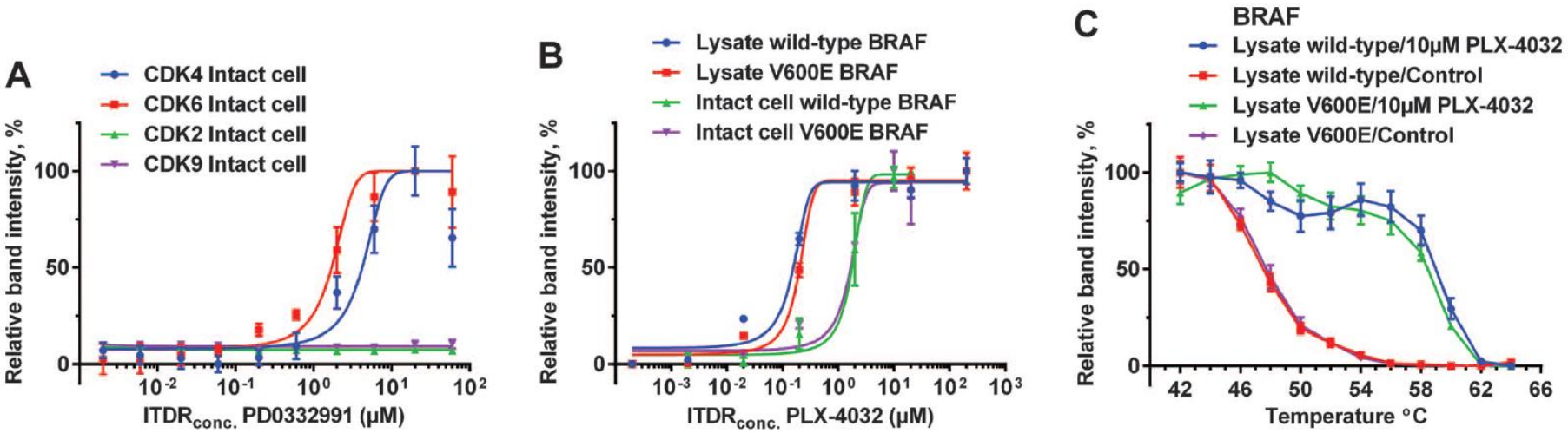




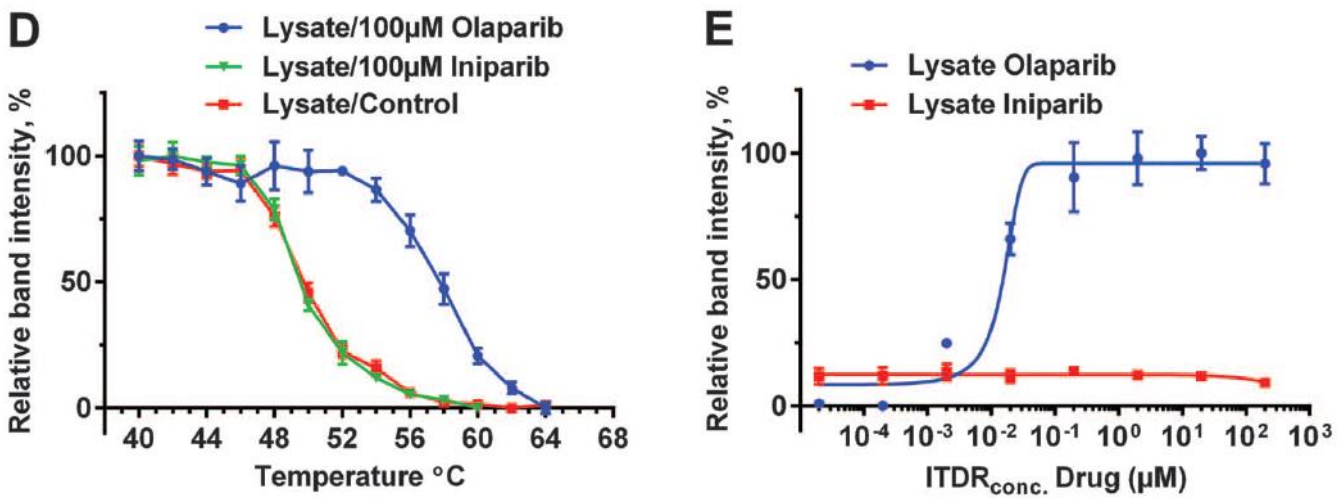
(A) CETSA curves of MetAP2 in mouse liver lysates from untreated mice and mice treated with TNP-470 at 20 mg per kilogram of body weight.

(B) $ITDRF_{CETSA}$ at 72°C of MetAP2 in liver and kidney at six different TNP-470 dosage levels.

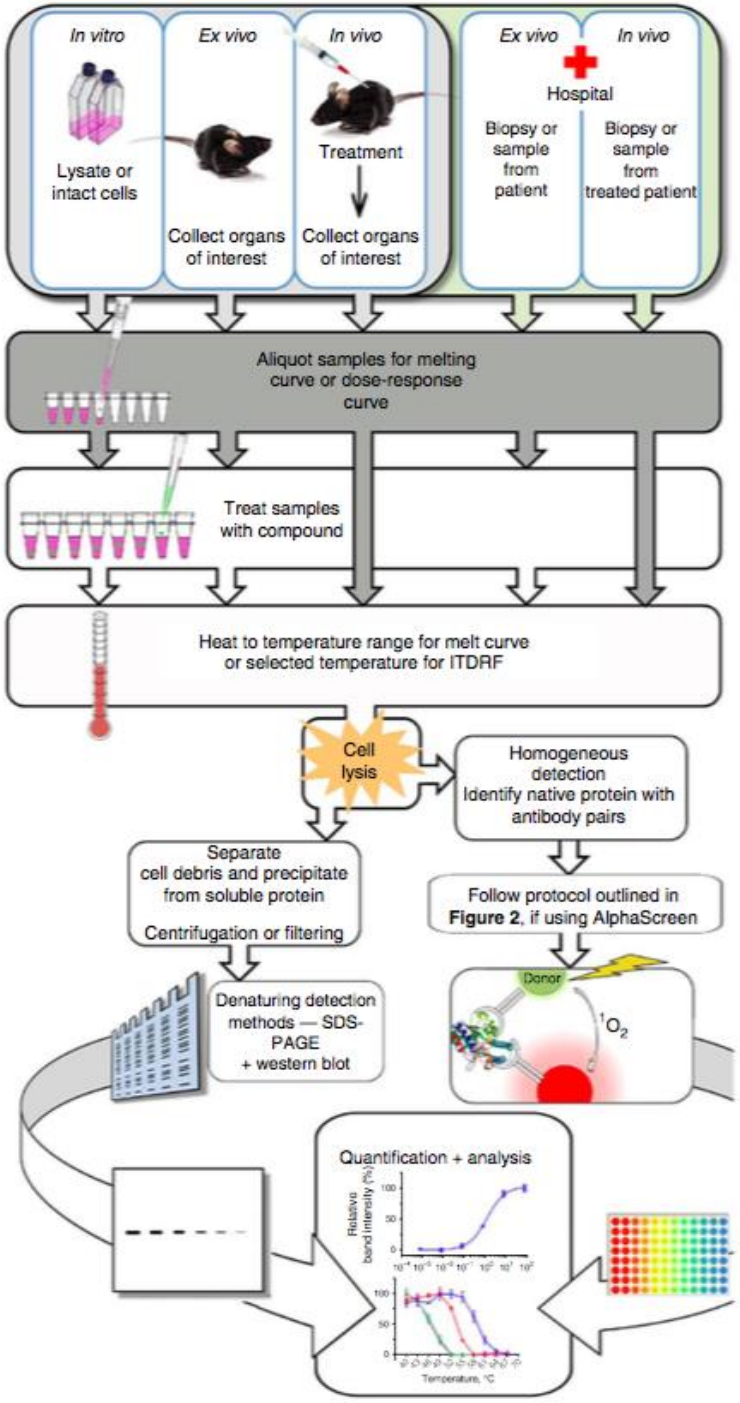
Monitoring of drug target specificity



CETSA for validation of clinical drug target



The case of the PARP-1 inhibitor iniparib



Two procedures for detecting the stabilized protein in the soluble fraction of the samples.

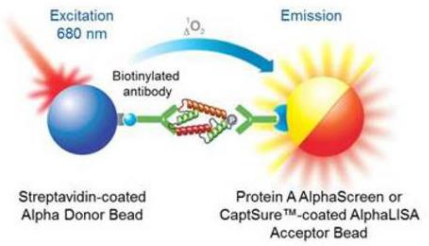
1) Sample workup and detection using quantitative western blotting;

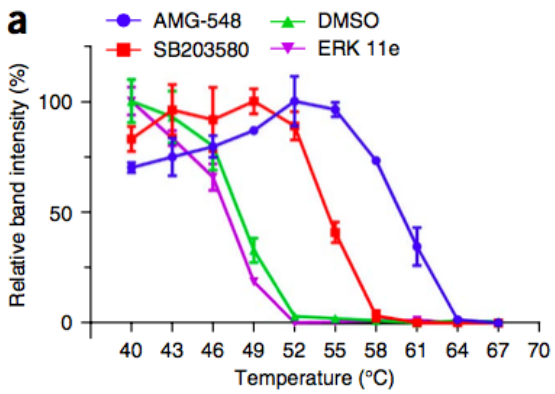
1) Direct detection in solution approach relying on the **induced proximity of two target-directed antibodies** upon binding to soluble protein (increased throughput)

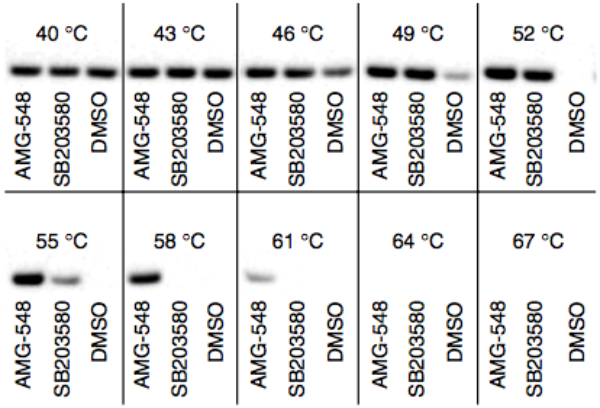
Both approaches can be completed in a day.

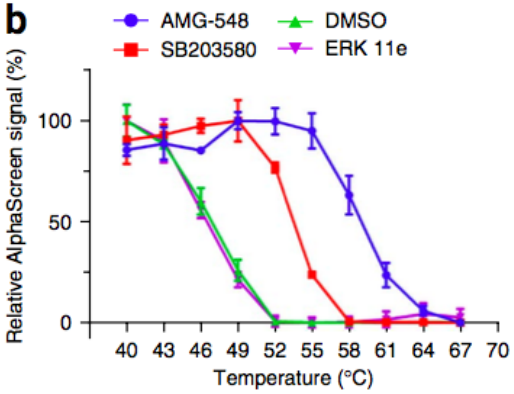
The latter can be achieved either through separation of the soluble material, e.g., by centrifugation or filtration of the samples to remove denatured and precipitated material, or by using a detection method capable of distinguishing between these entities (e.g., one based on antibodies recognizing only the folded protein).

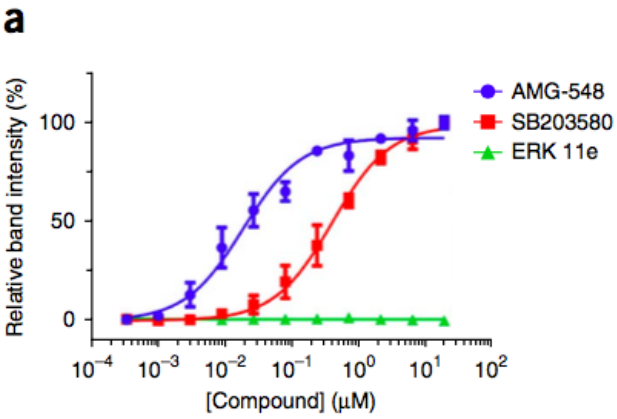
CETSA: WB and direct in solution detection

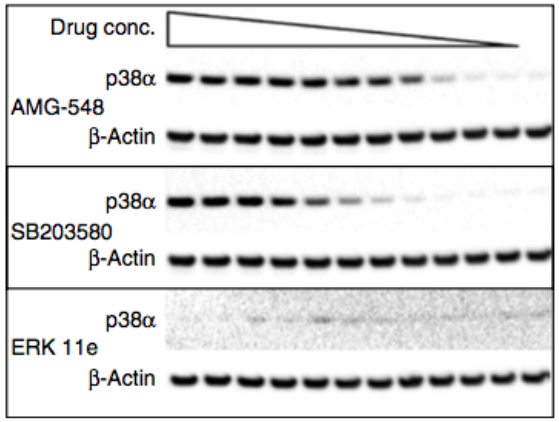


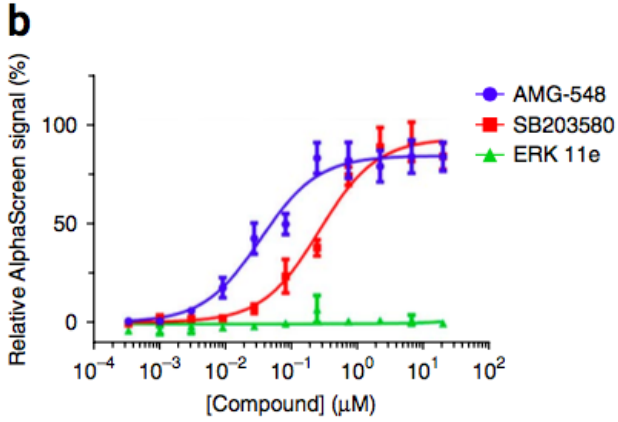




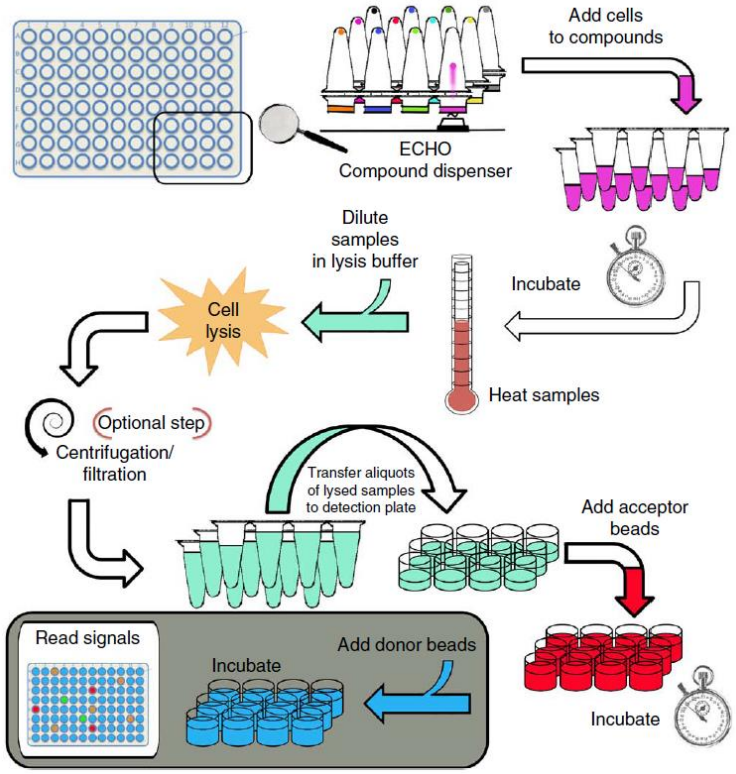


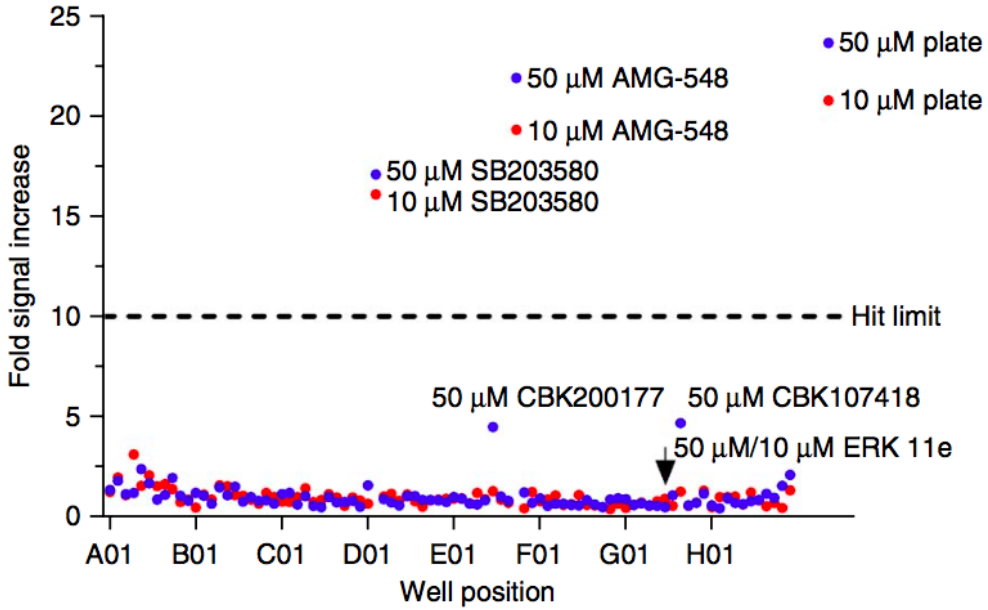






CETSA: the screen format assay procedure





CETSA advantages and limitations

- ✓ Ligand-induced stabilization is investigated at target level, in complex systems
- ✓ Preservation of target subcellular localization, post translational modification, interaction with other cellular partners
- ✓ Direct measure
- ✓ Easy, use of common laboratory techniques and standard equipment
- ✓ Useful for targets that are difficult to express and purify in a biologically active and relevant form.
- ✓ Few false-positive

- ❖ An intrinsic limitation of this assay is that not all proteins aggregate upon unfolding but might instead populate highly soluble, relatively compact molten-globule (like) conformations.
- ❖ In addition, it is possible that proteins will co-precipitate with their less stable protein interaction partners and therefore show lower apparent stability than their actual thermodynamic stability.
- ❖ False negative
- ❖ It lacks the potential for detecting unexpected targets

RESEARCH ARTICLE

PROTEOMICS

Tracking cancer drugs in living cells by thermal profiling of the proteome

Mikhail M. Savitski,[1*†] Friedrich B. M. Reinhard,[1†] Holger Franken,[1] Thilo Werner,[1] Maria Fälth Savitski,[1] Dirk Eberhard,[1] Daniel Martinez Molina,[2] Rozbeh Jafari,[2] Rebecca Bakszt Dovega,[2] Susan Klaeger,[3,4] Bernhard Kuster,[3,4] Pär Nordlund,[2,5] Marcus Bantscheff,[1*] Gerard Drewes[1*]

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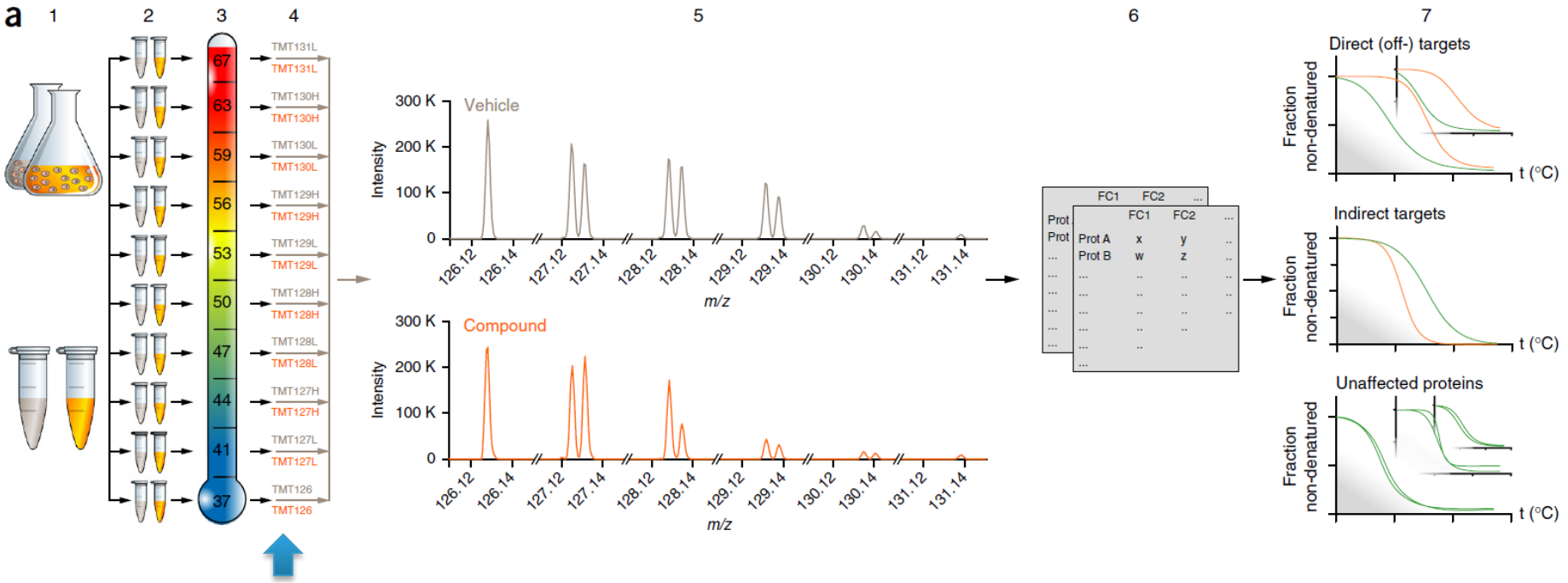
"They monitored the unfolding or "melting" of over 7000 human proteins and measured how small-molecule binding changes individual melting profiles. As a proof of principle, over 50 targets were identified for an inhibitor known to bind a broad spectrum of kinases.
Two cancer drugs, vemurafib and Alectinib, are known to have a side effect of photosensitivity. The thermal profiling approach identified drug-protein interactions responsible for these side effects."

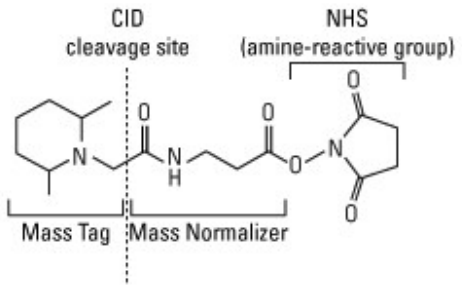
Thermal proteome profile (TPP) by combining CETSA with quantitative mass spectrometry



- Monitor the thermal stability of proteins at 10 different temperatures by labeling the peptides with **neutron-encoded isobaric mass tagging reagents (TMT10) in conjunction with high- resolution MS**.

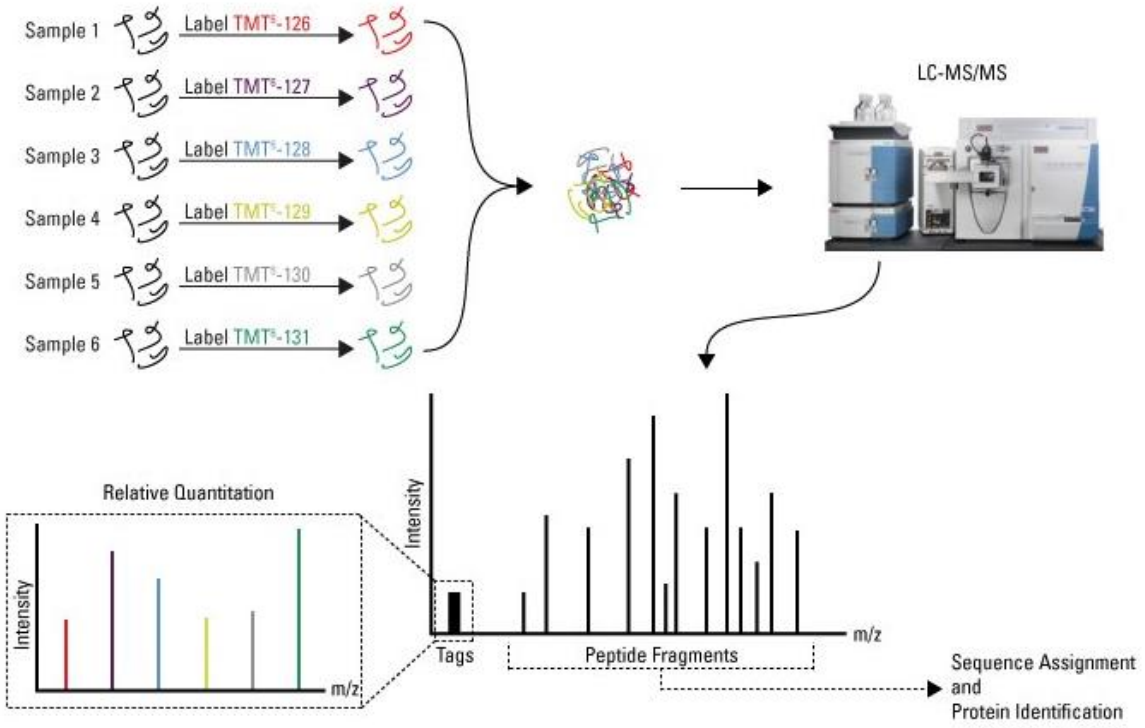
- The reporter ion intensities in MS/MS spectra are used to fit a curve and calculate melting curves for a large proportion of expressed soluble proteins in a **single (LC-MS)/MS experiment.**

TMT labeling



- mass reporter region (M),
- cleavable linker region (F),
- mass normalization region (N)
- a protein reactive group (R).

The chemical structures of all the tags are identical but each contains isotopes substituted at various positions.



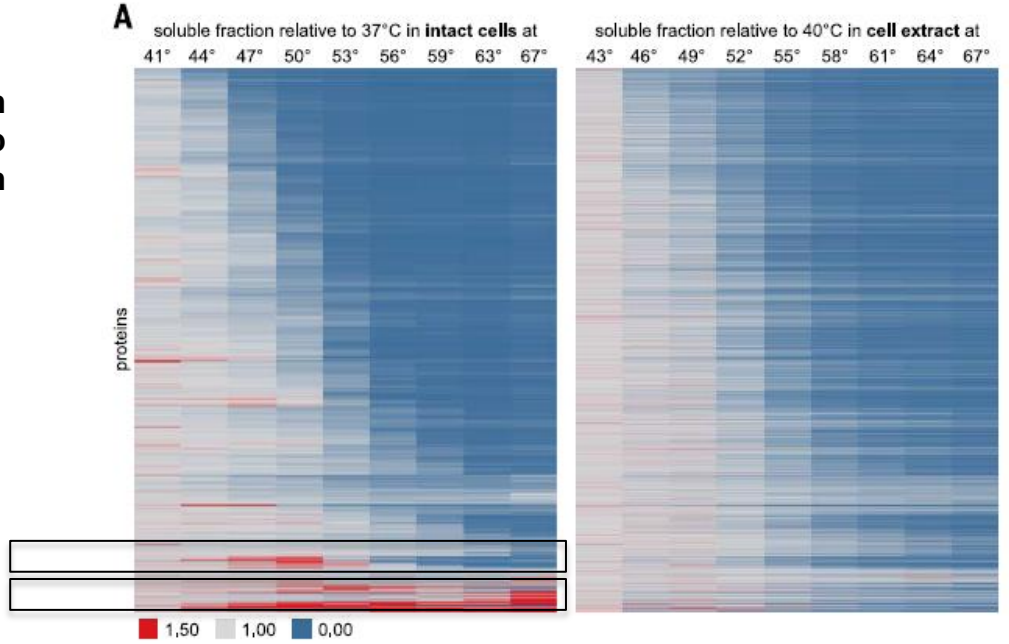
The combined M-F-N-R regions of the tags have the same total molecular weights and structure so that during chromatographic separation and in single MS mode, molecules labeled with different tags are indistinguishable.

The tags are then cleaved from the peptides by collision-induced dissociation (CID) during MS/MS

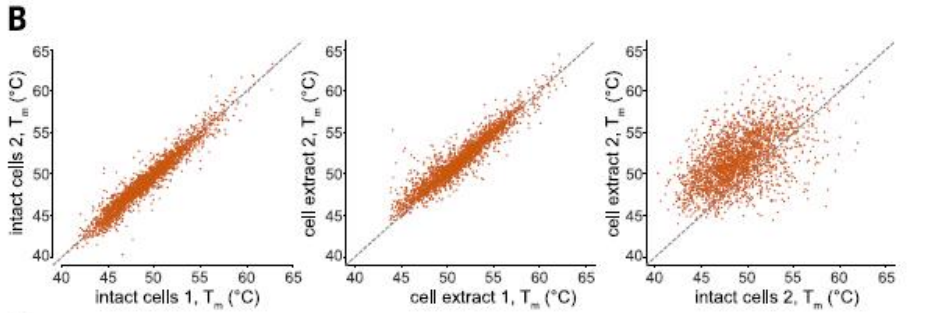
After CID, the peptide fragment ions are analyzed for sequence assignment and the isobaric tags are quantitated, resulting in concurrent peptide identification and relative quantitation.

Thermal proteome profile (TPP) of K562 cells

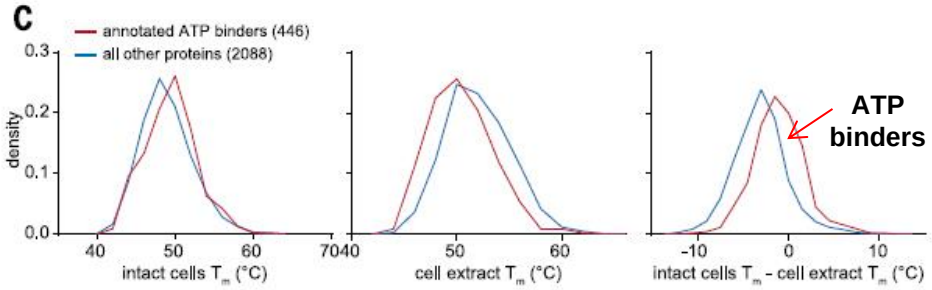
Proteins exhibiting a transient concentration increase with temperature are annotated to be part of organelles or large protein assemblies.



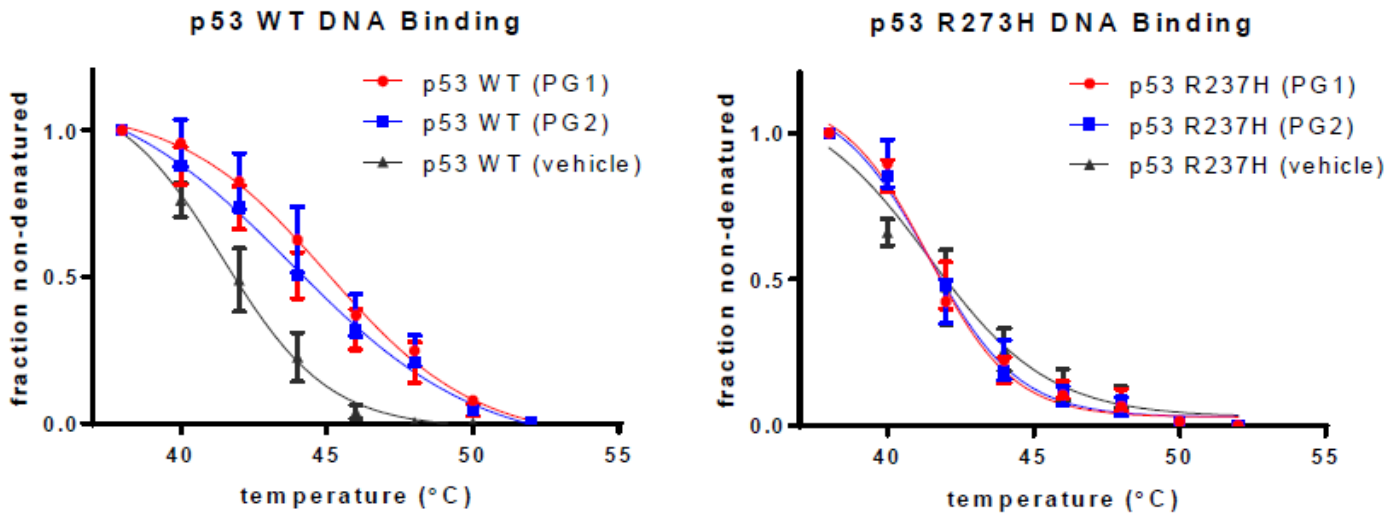
Reproducibility of thermal proteome profiles and direct comparison of proteomes from intact cells and cell-extract experiments. Most proteins showed greater thermal stability (higher Tm values) in cell extract as compared with intact cells.



A set of 440 ATP-binding proteins show a trend toward increased stability in intact cells as compared with cell extract.



Potential of thermal proteome profiling for the large-scale analysis of proteome-ligand interactions, including endogenous ligands such as cofactors or metabolites

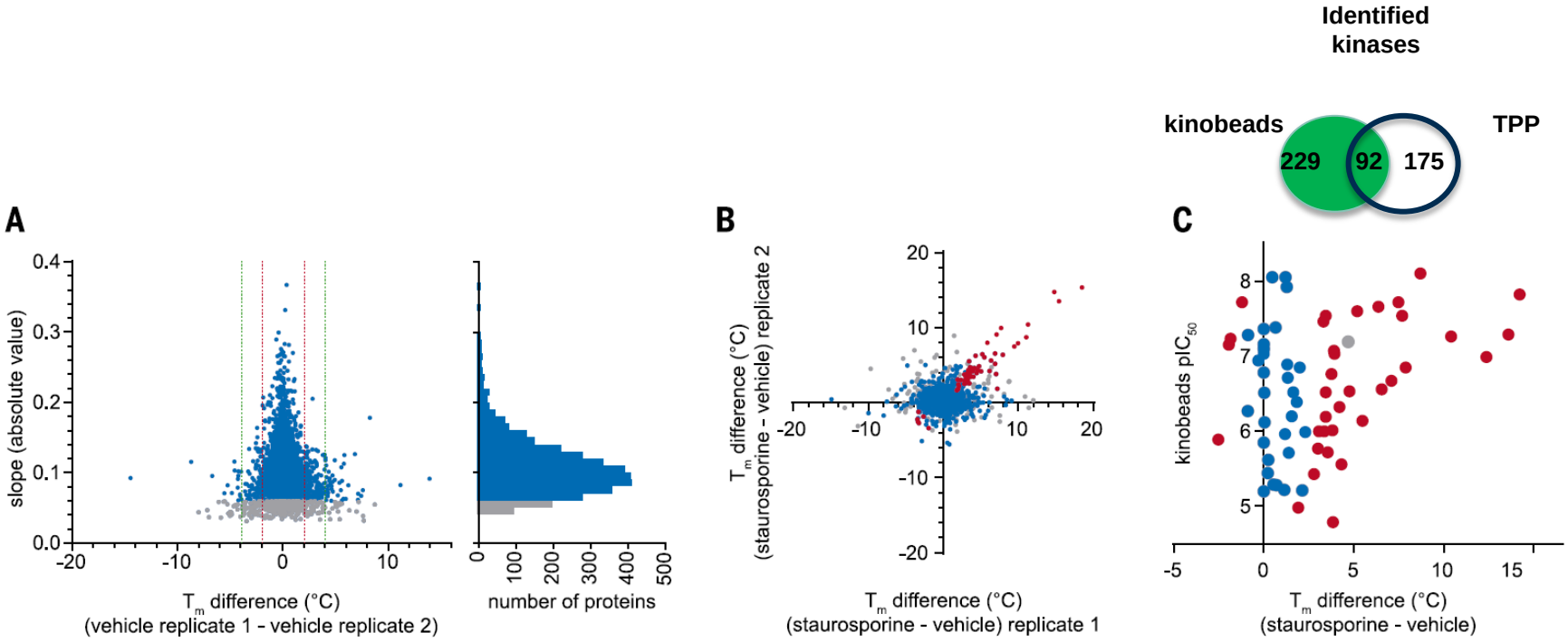


p53 with two cognate duplex-DNA oligonucleotides (PG1; red, and PG2; blue) added to A549 cell extract containing wild-type p53 and HT-29 cells containing the p53 R273H mutant.

p53 is a global transcription regulator for stress and when wild-type p53 was exposed to its cognate effector DNAs it was stabilized, while the p53 R273H mutant, known to not bind these effector sequences, was not stabilized.

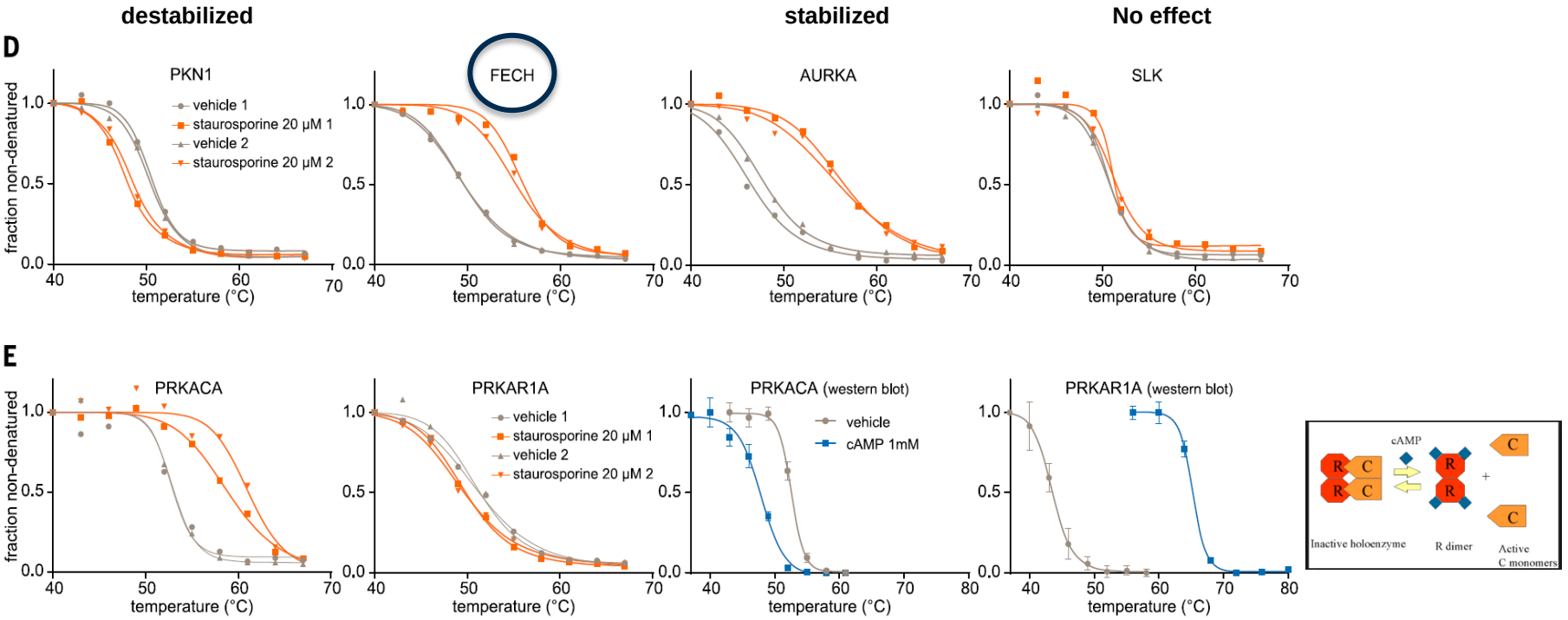
Monitoring the drug effects on thermal proteome profile (TPP)

Study of two structurally divergent promiscuous kinase inhibitors with a known spectrum of targets:
staurosporine and GSK3182571 to investigate the reliability of thermal proteome profiling



Staurosporine treatment of cell extract yields reproducible thermal shifts, allowing robust target identification.
Cell extract was treated with vehicle or staurosporine. Both experiments were performed as two independent replicates. A flat slope of the melting curve relates to lower melting point reproducibility. Proteins with an absolute slope below 0.06 are plotted in gray. The histogram shows the distribution of proteins versus slope values. Of all proteins, 92% yielded curves with sufficiently large slopes.

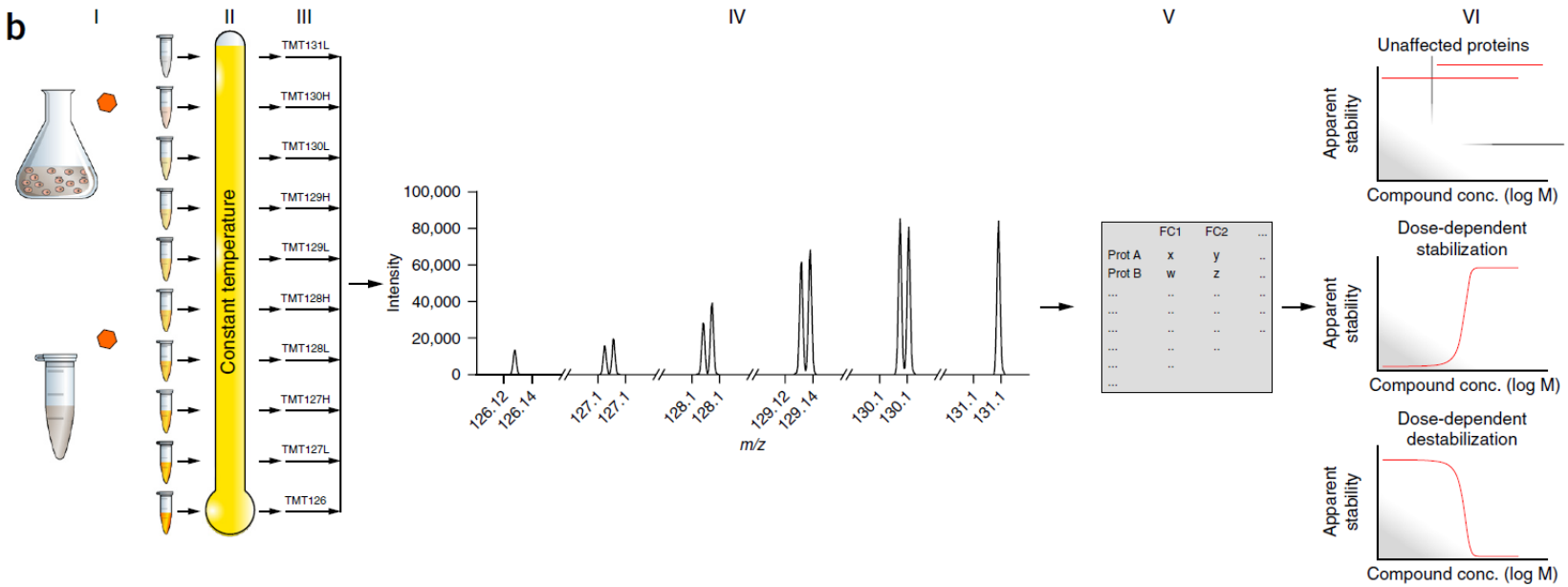
Differential profiling of drug effects on the thermal proteome profile



(D) **Examples of melting curves** for PKN1, FECH, AURKA, and SLK with and without staurosporine treatment.
(E) The catalytic subunit of PKA is stabilized by staurosporine, whereas the regulatory subunit is destabilized. Addition of cAMP followed by Western blot detection revealed destabilization of the catalytic subunit and stabilization of the regulatory subunit.

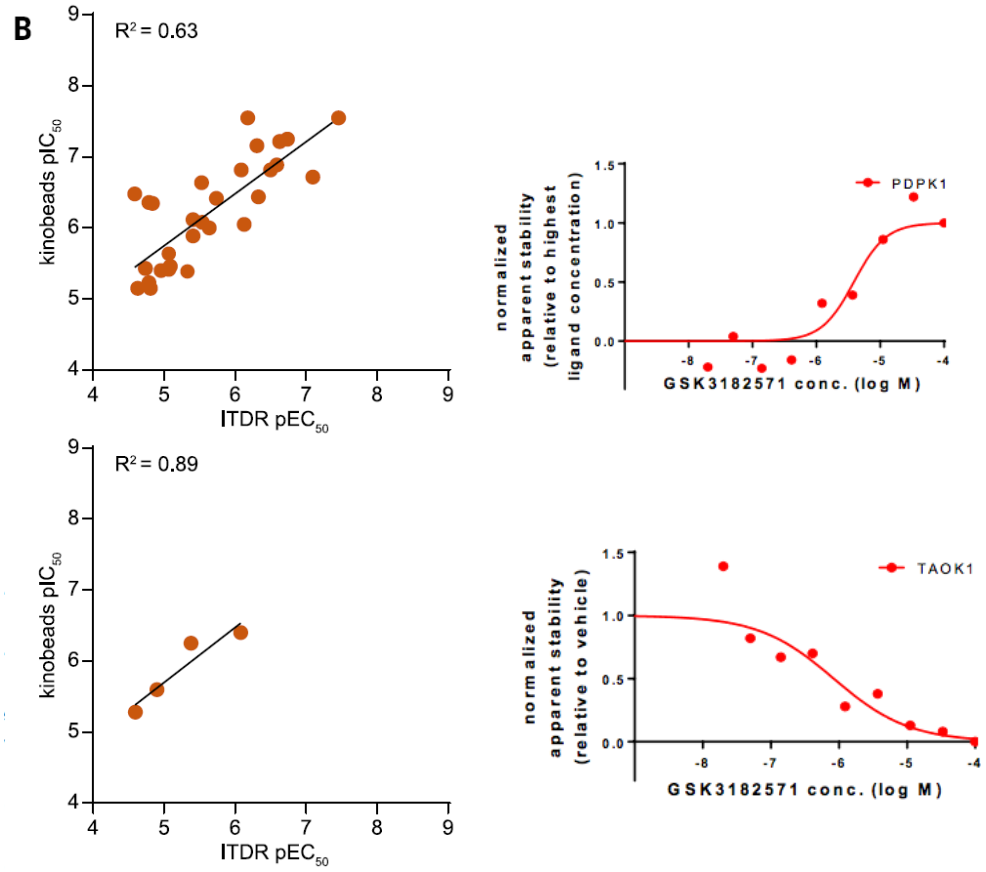
Thermal proteome profiling: drug concentration series

To infer the concentration of the ligand at which 50% of the total stabilizing effect has been observed (EC50), in order to rank the potency of the different protein targets



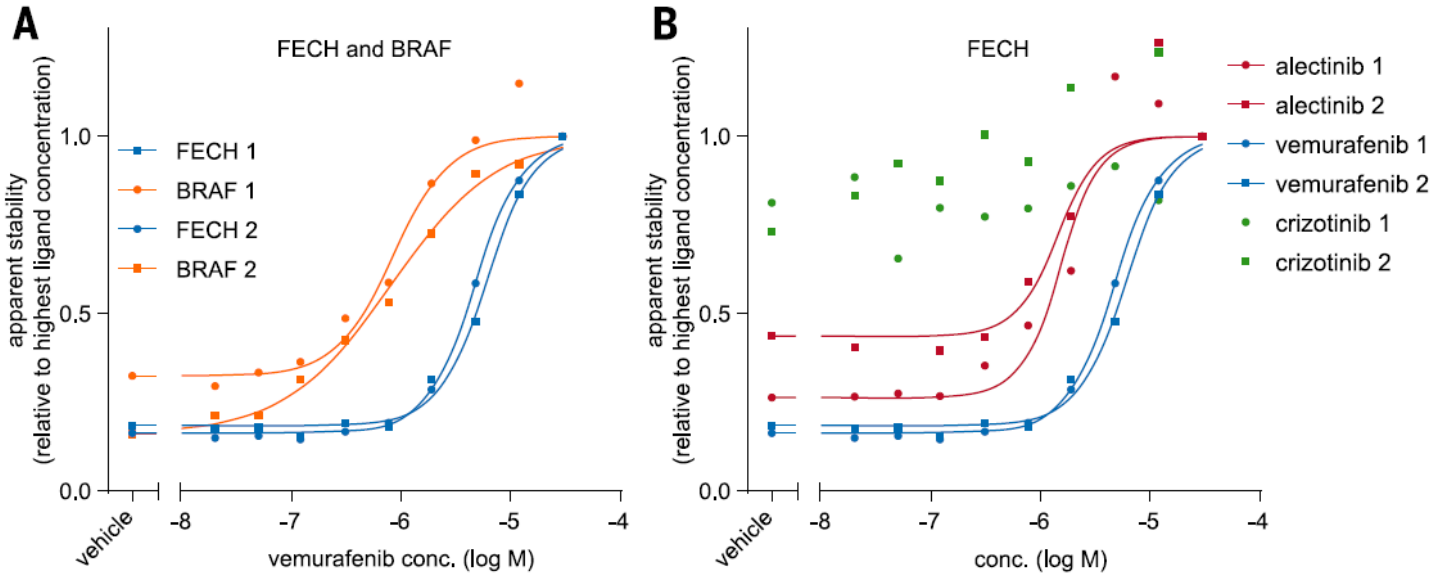
The selection of the temperature is the most crucial parameter. TPP-CCR experiments are typically conducted slightly above the melting temperature of the protein(s) of interest such that the protein will just have largely disappeared in the absence of the stabilizing compound, but it is easily detectable if the compound is added.

Assessment of ligand affinity requires ITDR measurements



Assessment of ligand affinity requires ITDR measurements.
Good agreement between GSK3182571 pEC50 values determined by means of ITDR, with pIC50 values determined with kinobeads. (Top) Proteins stabilized by GSK3182571 treatment. (Bottom) Proteins destabilized by GSK3182571 treatment.

Identification of off-targets of drug compounds



Vemurafenib is a B-Raf enzyme inhibitor
Side effects: photosensitivity and increased levels of protoporphyrins.

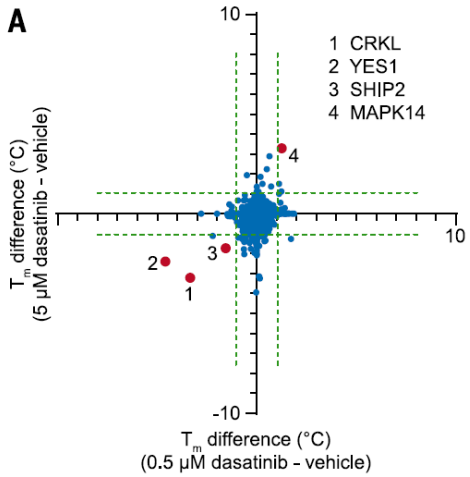
Alectinib is a ALK kinase inhibitor
Side effects: photosensitivity and increased levels of protoporphyrins.

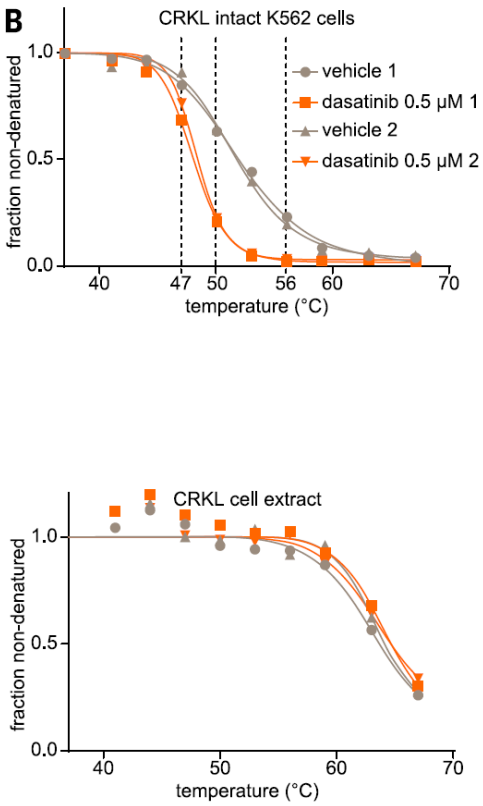
The clinical kinase drugs vemurafenib and alectinib induce Tm shifts in the heme biosynthesis enzyme FECH.

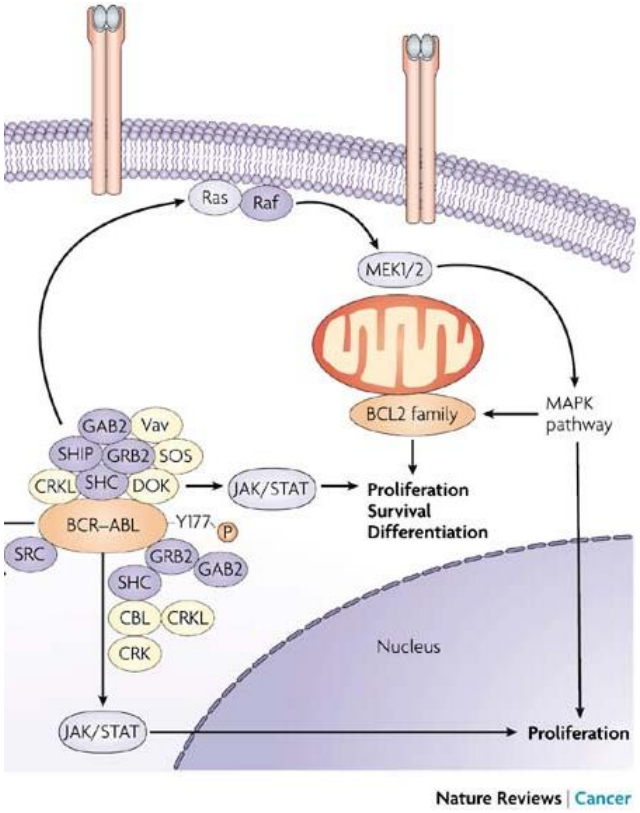
(A) ITDR profiling was performed at 55°C with vemurafenib-treated K562 cells and showed concentration-dependent thermal stabilization of FECH and BRAF.
(B) ITDR performed at 55°C with K562 cells treated with vemurafenib, alectinib, or crizotinib over a range of concentrations. Alectinib displays a more potent effect on FECH as compared with that by vemurafenib, whereas crizotinib, a drug not known to cause photosensitivity, has no effect.

Identification of downstream effector proteins of drug compounds

comparison of Tm shifts in cell extract, where ligand binding but no downstream effects occur, with Tm shifts in intact cells, where active signaling takes place, might reveal effector proteins downstream of the target

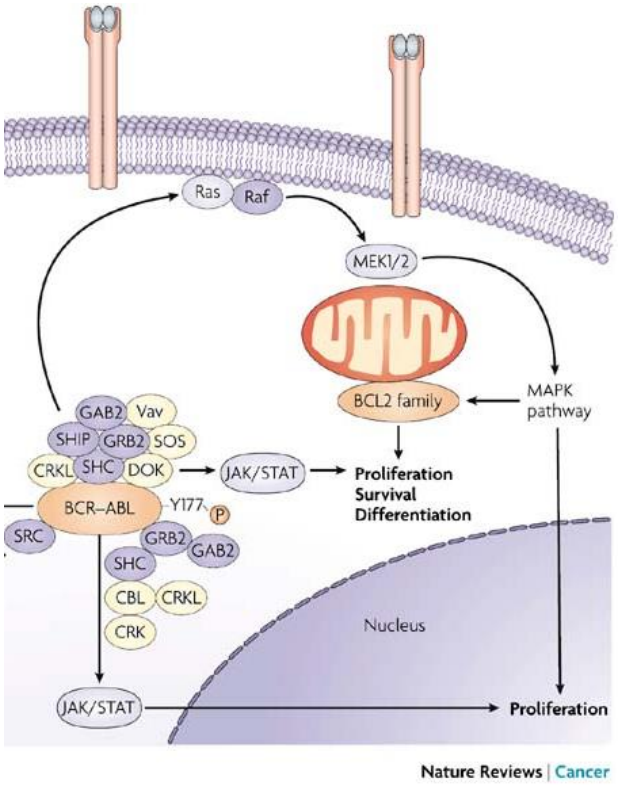


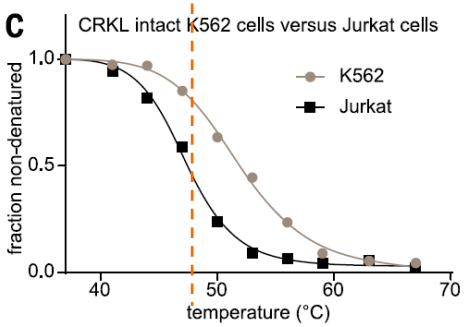


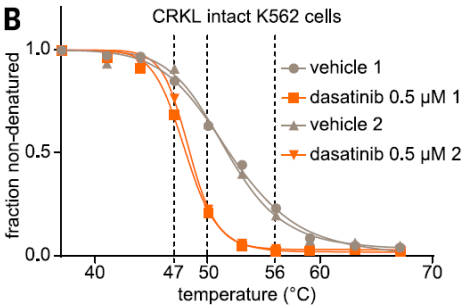


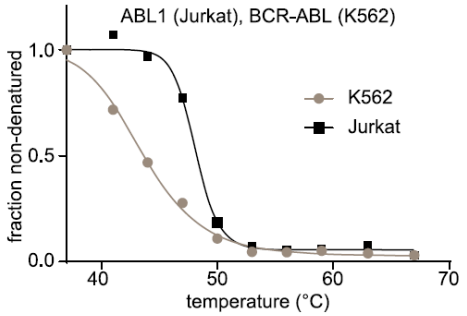
Treatment of K562 cells with **dasatinib** induces Tm shifts for downstream effector proteins in the BCR-ABL pathway.
(A) Cell-wide assessment of Tm shifts induced by dasatinib (0.5 and 5 mM) in K562 cells. Four proteins showing significant melting point differences both in the 0.5 and 5 mM dasatinib data sets are marked in red.
(B) Effect of dasatinib on the melting curves for the effector protein CRKL, determined in two biological replicates in intact cells (top) and cell extracts (bottom).

Identification of downstream effector proteins of drug compounds









(C) The melting curve for CRKL in Jurkat cells closely matches the curve obtained in dasatinib-treated K562 cells (top). The ABL1 protein in Jurkat cells is much more thermostable compared with the BCR-ABL fusion protein in K562 cells.

- The **thermal proteome profile** of a human cell can provide a general view of the proteomic state, or proteotype and identification of:

  - bound ligands such as other proteins, cofactors, metabolites, or drugs
  - posttranslational modifications
  - fusion proteins, splice variants

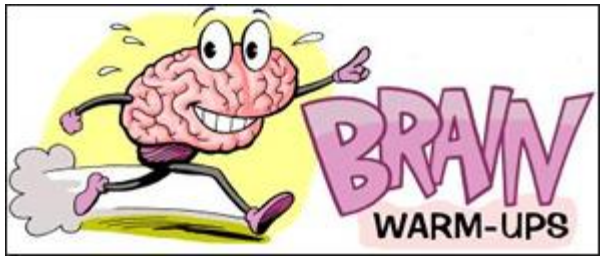
Which are typically undersampled and therefore not comprehensively detected in MS-based proteomics.

- **Low amount of cell material** required thus applicable to primary cells and tissues

- beneficial **to reduce late-stage failure of compounds in clinical development** either because of a lack of target engagement and therefore efficacy, or because of adverse effects caused by drug interaction with unexpected targets that mediate toxic effects

- The future scope and applicability of thermal proteome profiling will substantially benefit from continued advances in the sensitivity and **accuracy of MS-based proteomics**.

- Future developments should include the development of protocols for **membrane proteins** and the application to tissues from animal studies or clinical biopsies.



Thank you for your attention!