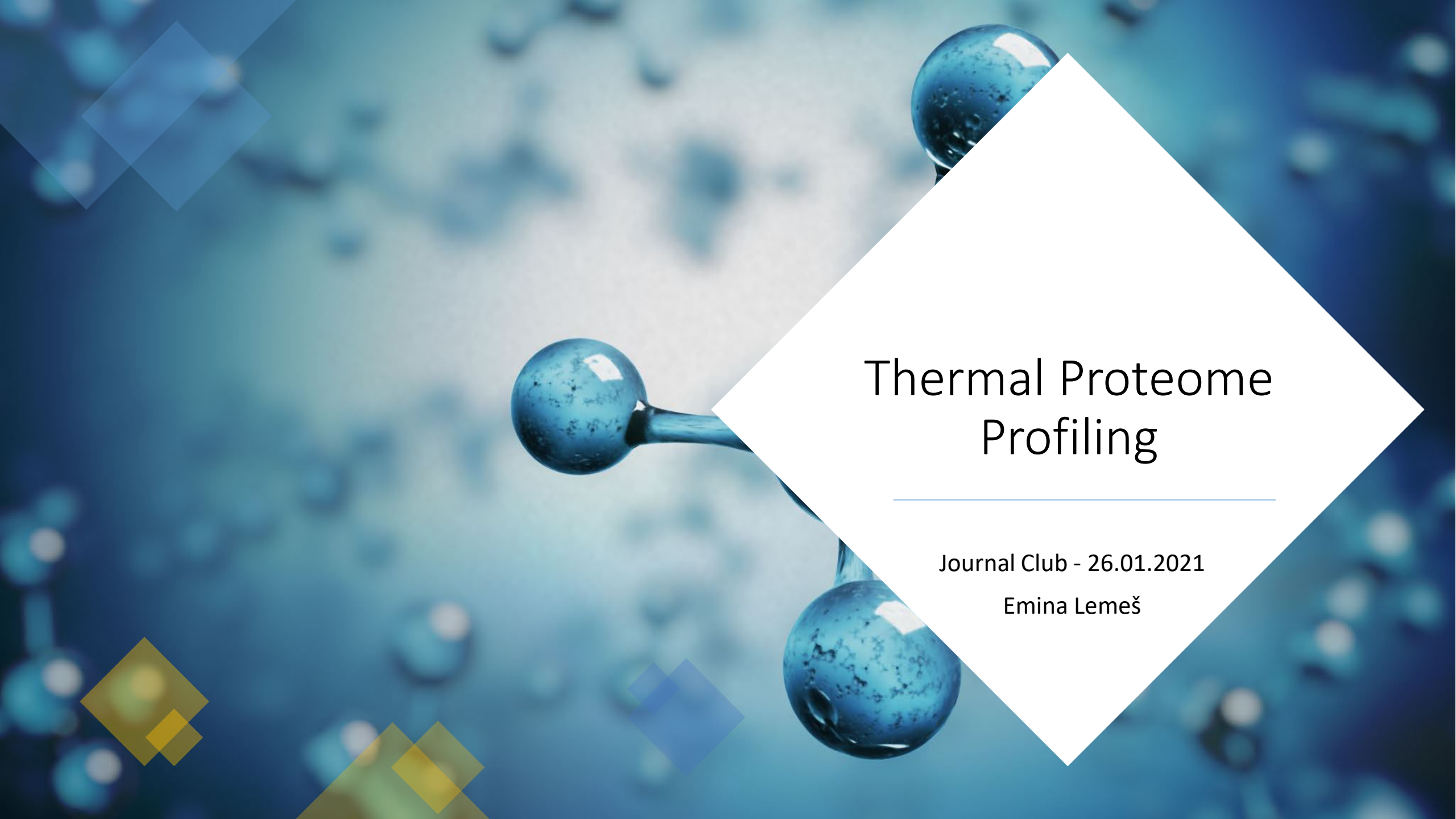


Thermal Proteome Profiling

Journal Club - 26.01.2021

Emina Lemeš

The Holy Grail:
Identifying Effective and Safe Therapies

- Small molecule drugs
  - Main challenge: identification of molecular targets underlying drug therapeutic effects
  - and/or adverse side effects

Global Investigation of Protein-ligand Interactions

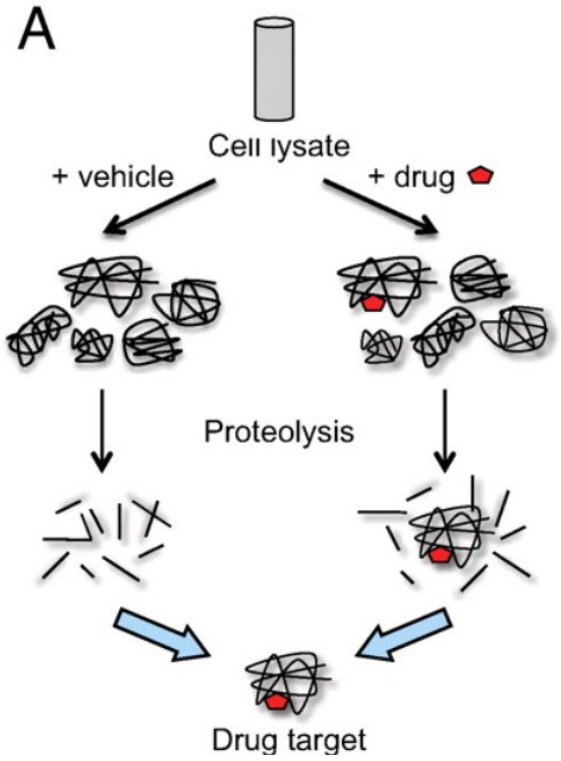
- Affinity-based target identification techniques
- Drug affinity responsive target stability (DARTS)
- Limited proteolysis mass spectrometry
- Thermal proteome profiling (TPP)t

Global Investigation of Protein-ligand Interactions

- Affinity-based target identification techniques
  - Limited by necessity to modify each drug individually (without losing bioactivity)
- Drug affinity responsive target stability (DARTS)
- Limited proteolysis mass spectrometry
- Thermal proteome profiling (TPP)

Global Investigation of Protein-ligand Interactions

- Affinity-based target identification techniques
- Drug affinity responsive target stability (DARTS)
  - Drugs binding to target = stabilization
  - Reducing protease sensitivity by masking recognition sites
- Limited proteolysis mass spectrometry
- Thermal proteome profiling (TPP)



(Lomenick et al 2009, PNAS)

Mass Spectrometry

Global Investigation of Protein-ligand Interactions

- Affinity-based target identification techniques
- Drug affinity responsive target stability (DARTS)
- Limited proteolysis mass spectrometry
- Thermal proteome profiling (TPP)

Thermal Proteome Profiling (TPP) - Paper 1

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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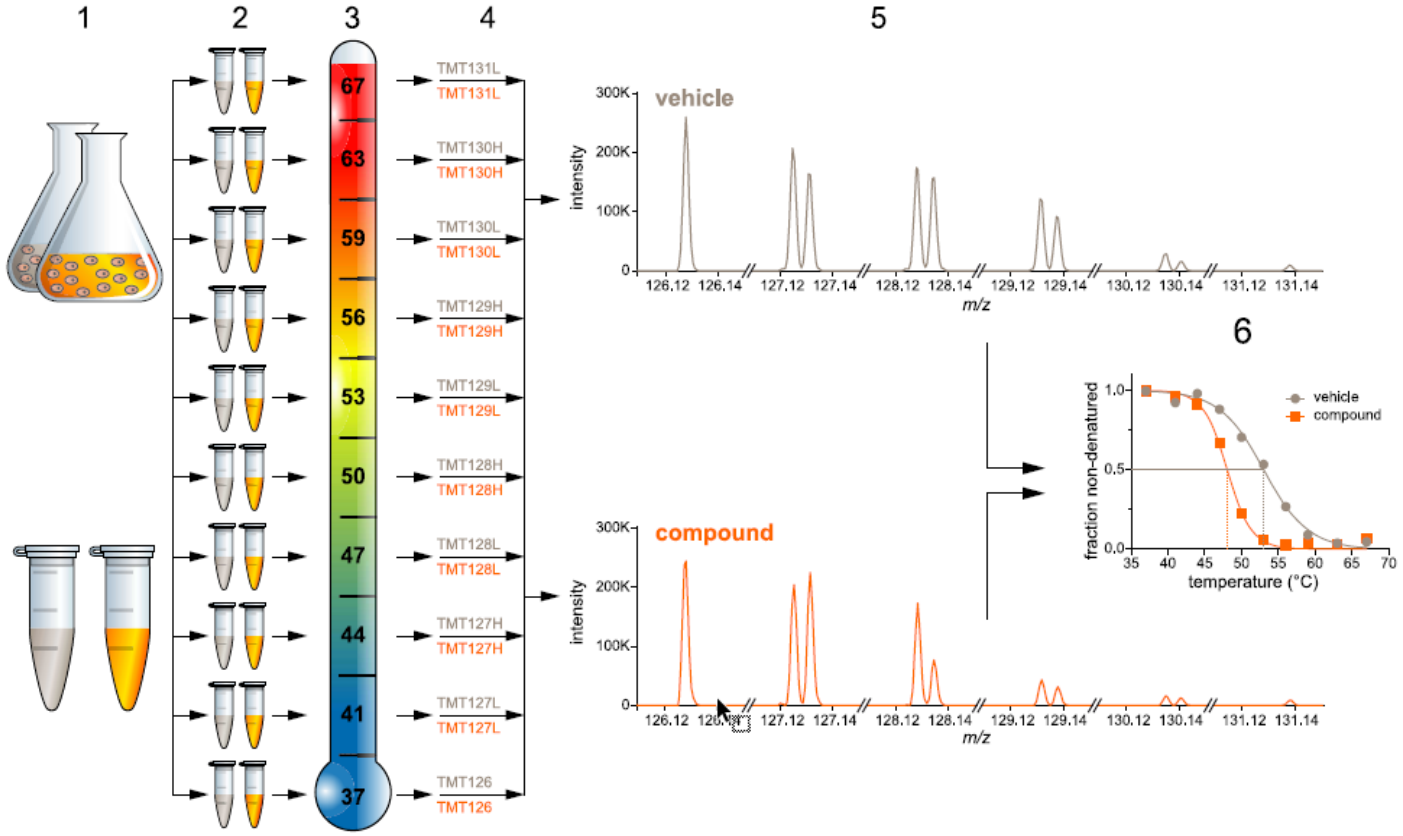
Thermal Proteome Profiling – Workflow

Cells cultured under differential conditions → Each condition -> cells divided into 10 aliquots → Briefliy heated to a different temperature → Extraction: PBS; Digestion: trypsin; Labeling: TMT10 isotope tag → Analyzed by LC-MS/MS → Reporter ion intensities used to fit a melting curve for each protein



Around intrinsic melting temperature proteins denature and aggregate

Gradual disappearance from PBS-extracted samples with rising temperaure

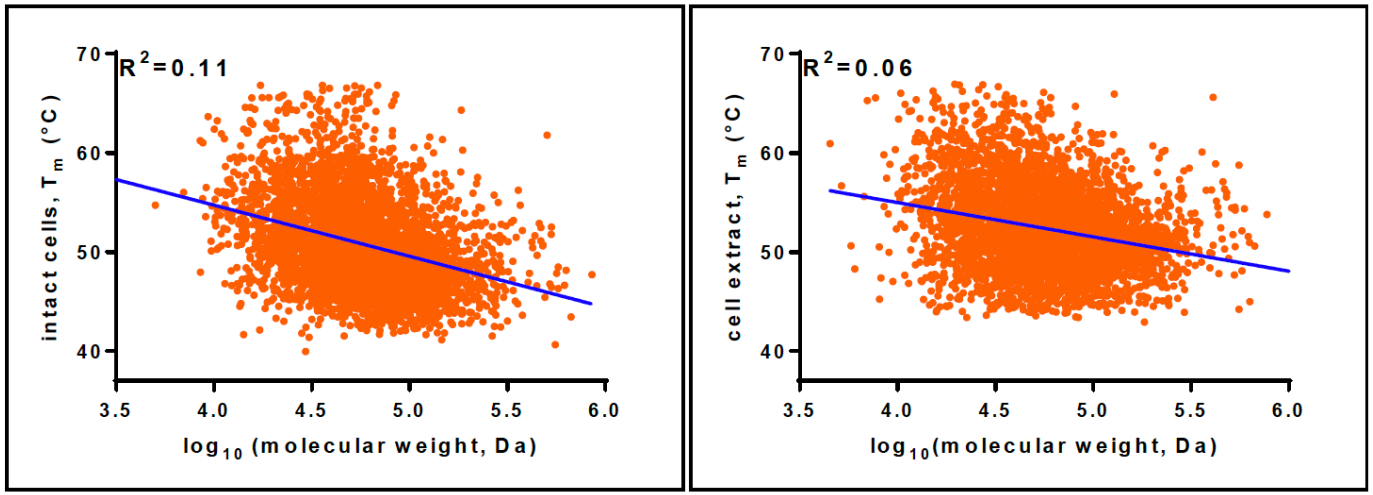
! Only for soluble proteome fraction

Differences Between Cells and Extract

- K562 cells (human, suspension, chronic myeloid leukemia)
- Quantitative thermal stability data for 5299 proteins across 10 different temperatures

⇨ 1st description of a melting proteome «meltome»

- 2 exp setting: heating of intact cells or cell extracts
  - In both: weak but significant anticorr of thermal stability with molecular weight -> smaller proteins tend to be more stable



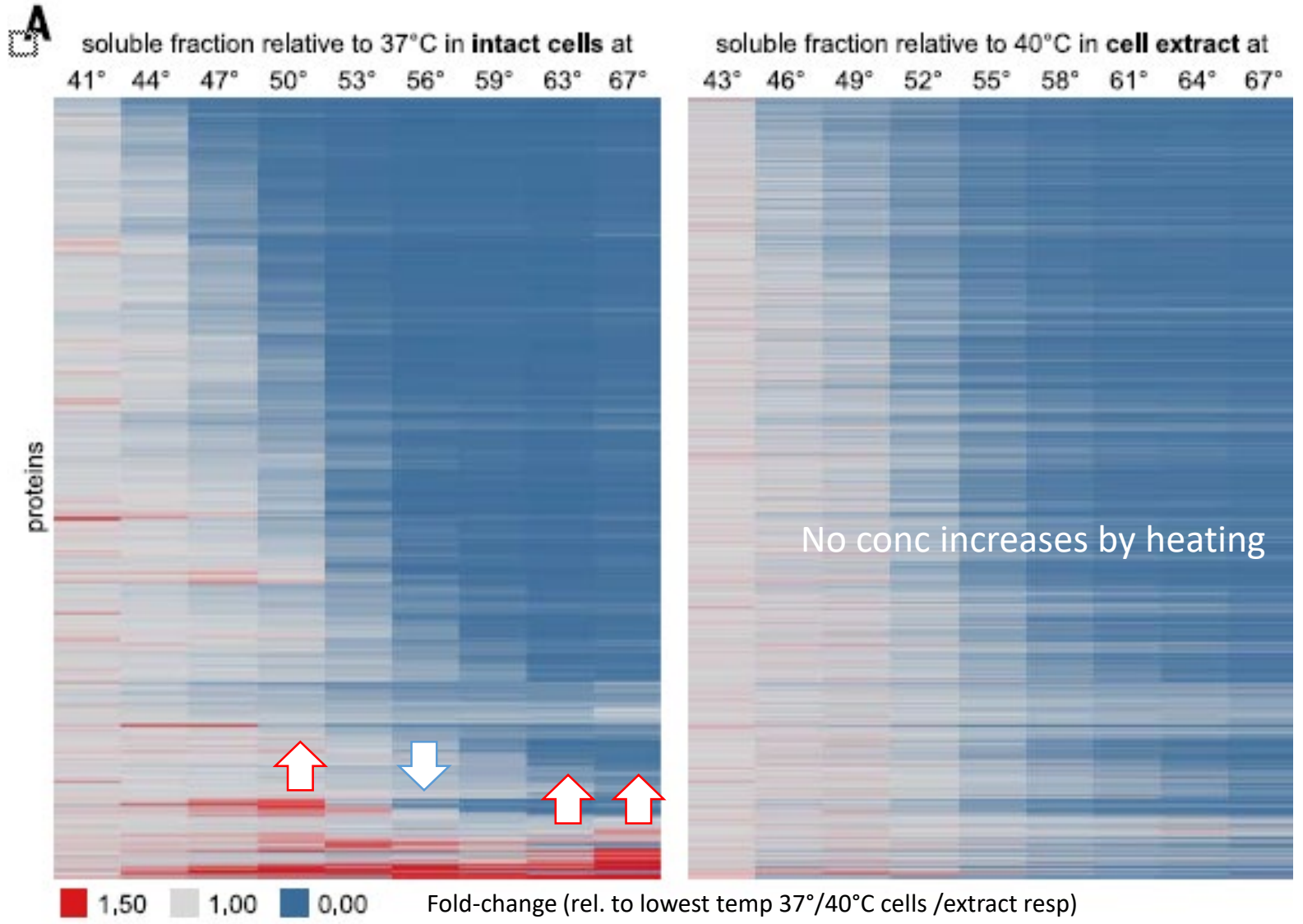
Differences Between Cells and Extract

- 3204 proteins robustly quantified in both cells and cell extracts
- Hierarchical cluster analysis of the temperature-dependent relative protein concentrations

Observed:

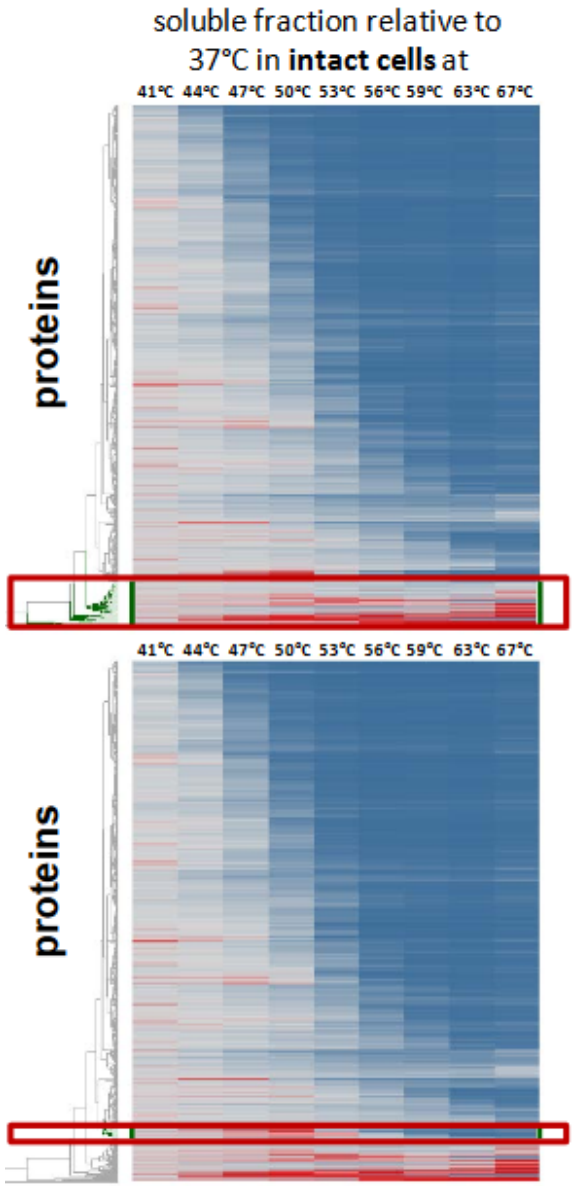
- Group 1) Increased stability at 50°C and pronounced decrease at 56°C
- Group 2) Increase at 63°C
  - Due to initial solubilization followed by aggregation at higher temp

⇨ GO analysis



Gene Ontology Analyis in Intact Cells:

- Proteins in these Clusters are released from:
  - or large protein assemblies (ribosomes)
  - disintegrating organelles (eg mitochondria)



GO term	Description	P-value	FDR q-value	Enrichment (N, B, n, b)
GO:0044391	ribosomal subunit	2.05E-27	2.05E-24	8.24 (3620,63,258,37)
GO:0030529	ribonucleoprotein complex	6.26E-22	3.13E-19	3.28 (3620,308,258,72)
GO:0044445	cytosolic part	6.96E-18	2.32E-15	4.89 (3620,109,258,38)
GO:0022627	cytosolic small ribosomal subunit	1.12E-16	2.81E-14	9.87 (3620,27,258,19)
GO:0022625	cytosolic large ribosomal subunit	1.23E-14	2.46E-12	9.54 (3620,25,258,17)
GO:0015935	small ribosomal subunit	1.46E-14	2.43E-12	7.80 (3620,36,258,20)
GO:0015934	large ribosomal subunit	8.41E-14	1.20E-11	8.83 (3620,27,258,17)
GO:0005839	proteasome core complex	6.03E-11	7.54E-09	9.90 (3620,17,258,12)
GO:0005840	ribosome	8.86E-08	9.84E-06	6.24 (3620,27,258,12)
GO:0005681	spliceosomal complex	6.89E-07	6.89E-05	3.07 (3620,105,258,23)
GO:0019773	proteasome core complex, alpha-subunit complex	8.17E-07	7.43E-05	12.03 (3620,7,258,6)
GO:0000178	exosome (RNase complex)	2.48E-06	2.07E-04	7.48 (3620,15,258,8)
GO:0045259	proton-transporting ATP synthase complex	1.00E-05	7.71E-04	11.69 (3620,6,258,5)
GO:0005753	mitochondrial proton-transporting ATP synthase complex	1.00E-05	7.16E-04	11.69 (3620,6,258,5)
GO:0044446	intracellular organelle part	1.48E-05	9.87E-04	1.24 (3620,1901,258,168)
GO:0005730	nucleolus	2.22E-05	1.38E-03	2.10 (3620,220,258,33)
GO:0044422	organelle part	2.73E-05	1.61E-03	1.23 (3620,1931,258,169)
GO:0030532	small nuclear ribonucleoprotein complex	3.30E-05	1.83E-03	4.53 (3620,31,258,10)
GO:0032991	macromolecular complex	5.13E-05	2.70E-03	1.29 (3620,1442,258,133)
GO:0005685	U1 snRNP	7.93E-05	3.97E-03	7.02 (3620,12,258,6)
GO:0044428	nuclear part	1.96E-04	9.34E-03	1.37 (3620,975,258,95)
GO:0044455	mitochondrial membrane part	2.06E-04	9.38E-03	4.07 (3620,31,258,9)
GO:0044444	cytoplasmic part	2.14E-04	9.28E-03	1.17 (3620,2178,258,182)

Heat map color scheme

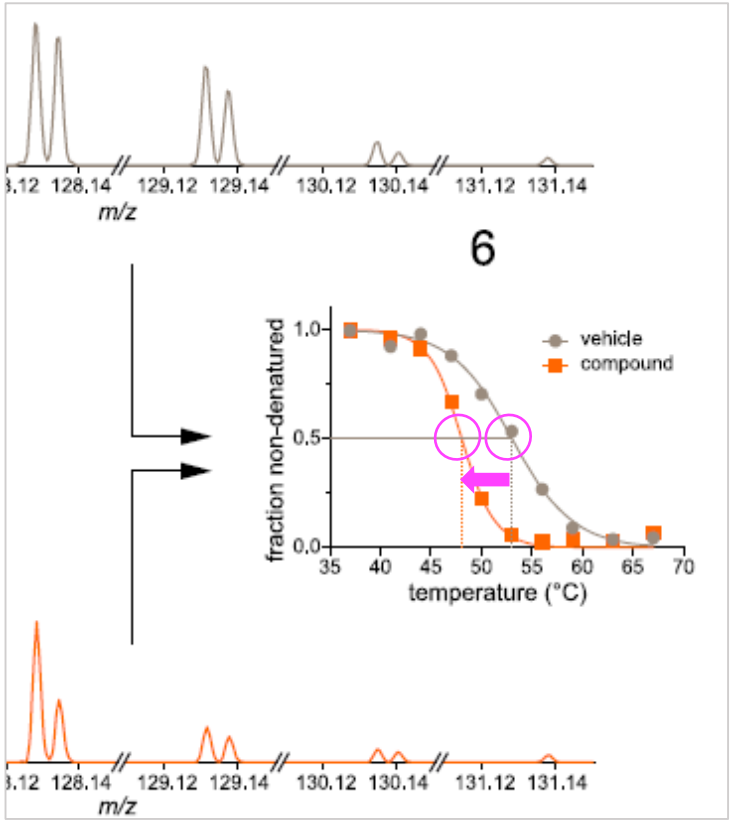
1.50 1.00 0.00

GO term	Description	P-value	FDR q-value	Enrichment (N, B, n, b)
GO:0002199	zona pellucida receptor complex	5.93E-12	5.93E-09	45.19 (3615,8,70,7)
GO:0005832	chaperonin-containing T-complex	5.93E-12	2.97E-09	45.19 (3615,8,70,7)
GO:0044446	intracellular organelle part	1.85E-08	6.17E-06	1.60 (3615,1900,70,59)
GO:0044422	organelle part	3.92E-08	9.81E-06	1.58 (3615,1930,70,59)
GO:0005759	mitochondrial matrix	3.81E-07	7.63E-05	5.02 (3615,144,70,14)
GO:0019866	organelle inner membrane	5.61E-07	9.36E-05	6.53 (3615,87,70,11)
GO:0044429	mitochondrial part	1.02E-06	1.45E-04	3.60 (3615,258,70,18)
GO:0005743	mitochondrial inner membrane	1.70E-06	2.12E-04	6.62 (3615,78,70,10)
GO:0022624	proteasome accessory complex	1.22E-05	1.35E-03	15.19 (3615,17,70,5)
GO:0031974	membrane-enclosed lumen	2.57E-05	2.57E-03	3.15 (3615,262,70,16)
GO:0031966	mitochondrial membrane	3.87E-05	3.52E-03	4.69 (3615,110,70,10)
GO:0070013	intracellular organelle lumen	7.39E-05	6.16E-03	3.21 (3615,225,70,14)
GO:0032991	macromolecular complex	7.63E-05	5.87E-03	1.57 (3615,1443,70,44)
GO:0044428	nuclear part	7.83E-05	5.59E-03	1.80 (3615,974,70,34)
GO:0043233	organelle lumen	1.03E-04	6.89E-03	3.12 (3615,232,70,14)

Melting Point Determination For Each Protein

Passing quality control criteria

Greater thermal stability
(higher T_m values)



Greater Thermal Stability in Cell Extract Compared to Intact Cells

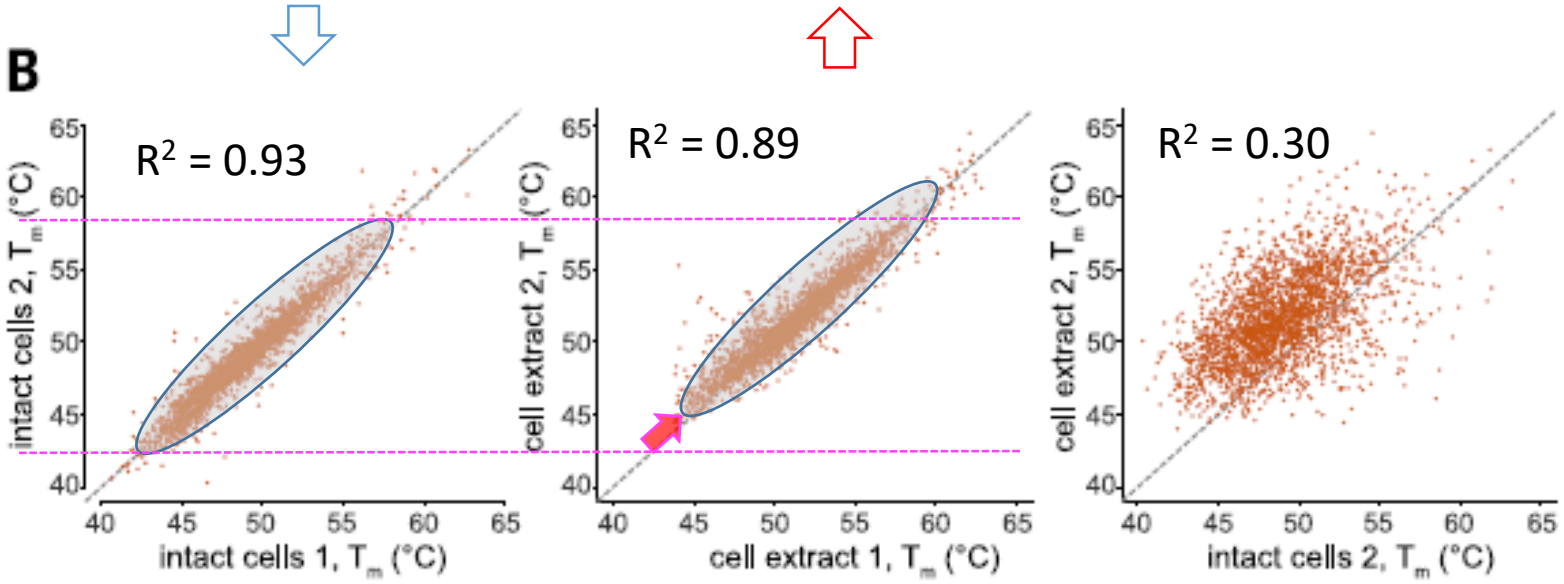
Average of 2.7°C higher T_m values in cell extract compared with intact cells.

Disruption of the cellular context heterogeneously affects protein stability
- due to lower protein concentration?

Cellular context should have a stabilizing effect because -> molecular crowding.
But results are contrary.

Prev studies showed: phosphoglycerate kinase more stable in intact cell.

Hypothesis: Phosphoglycerate kinase stabilization could also be explained by binding of endogenous co-substrate ATP



Then extraction might cause dissociation and show lower T_m

Greater Thermal Stability in Cell Extract Compared to Intact Cells

Cellular context should have a stabilizing effect because -> molecular crowding. But results are contrary.

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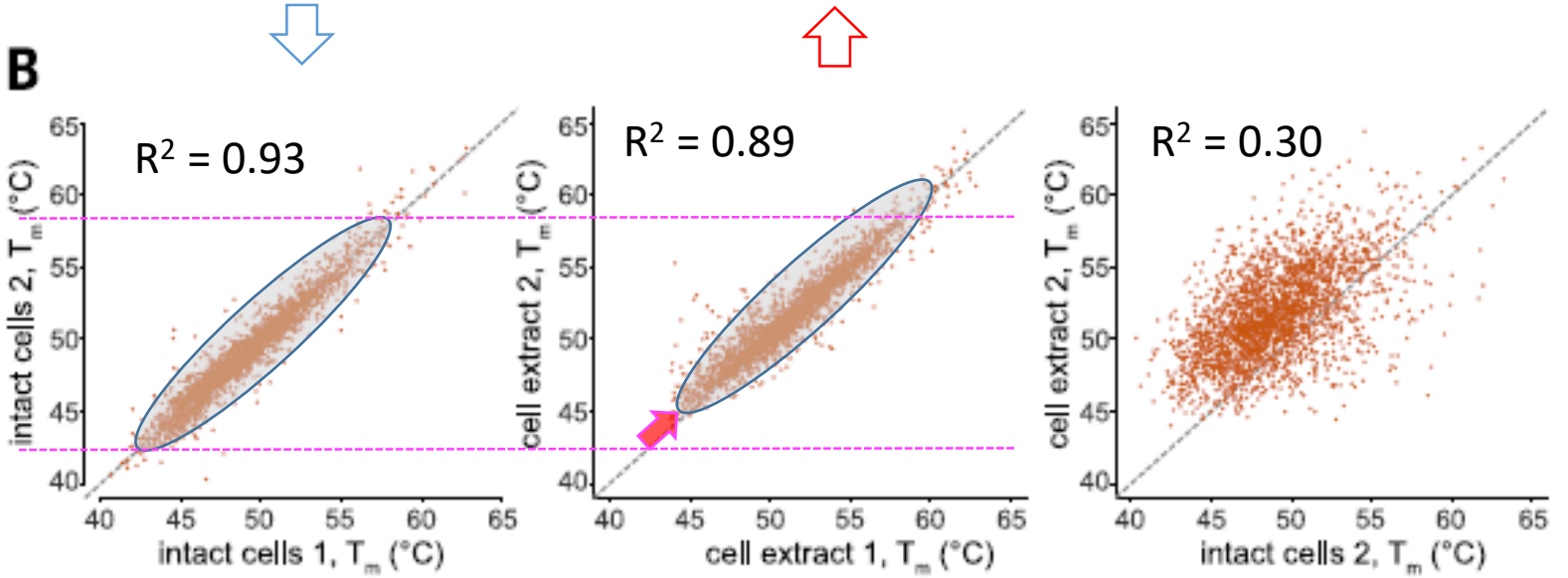
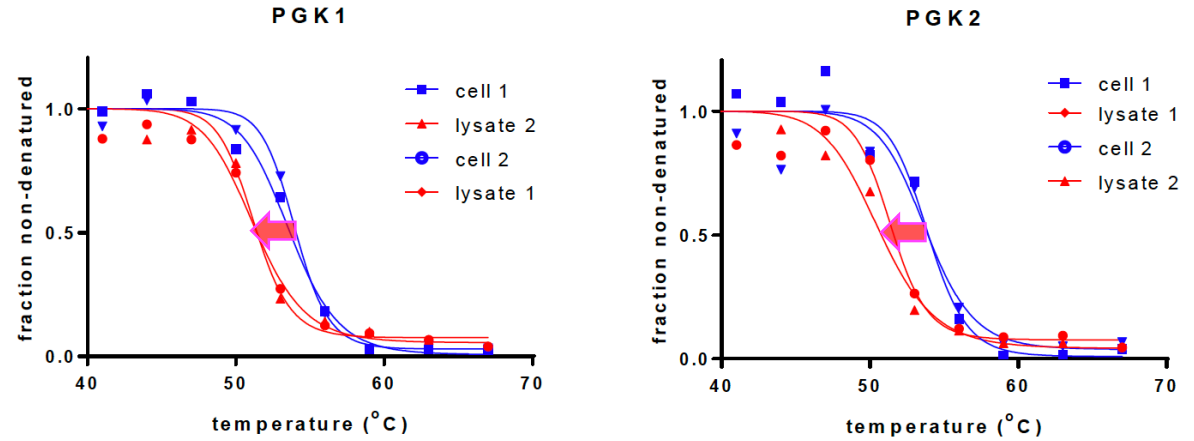
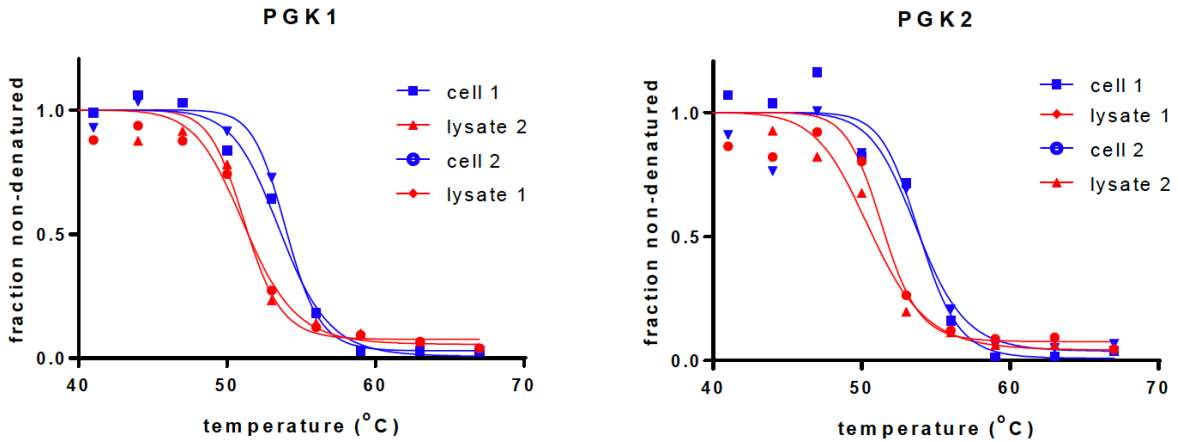


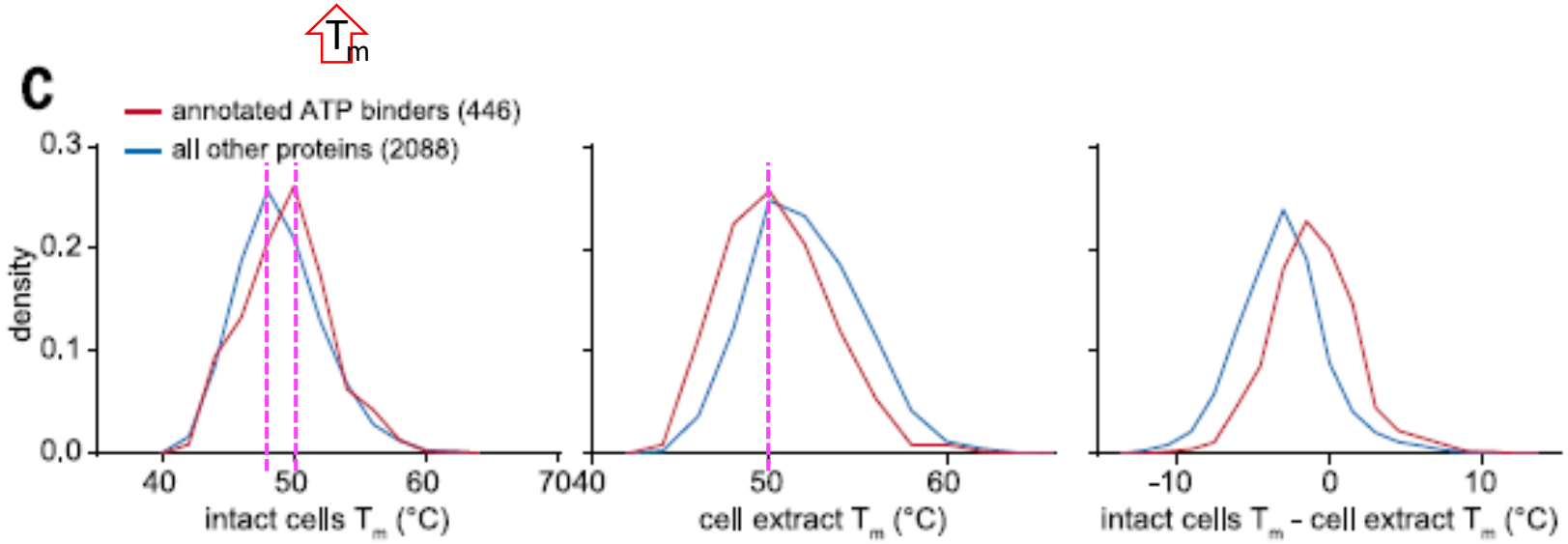


Or Does Cellular Context Have an Impact on Thermal Stability? – ATP-Binders

Analysis of T_m values of 440 annotated ATP binders ->

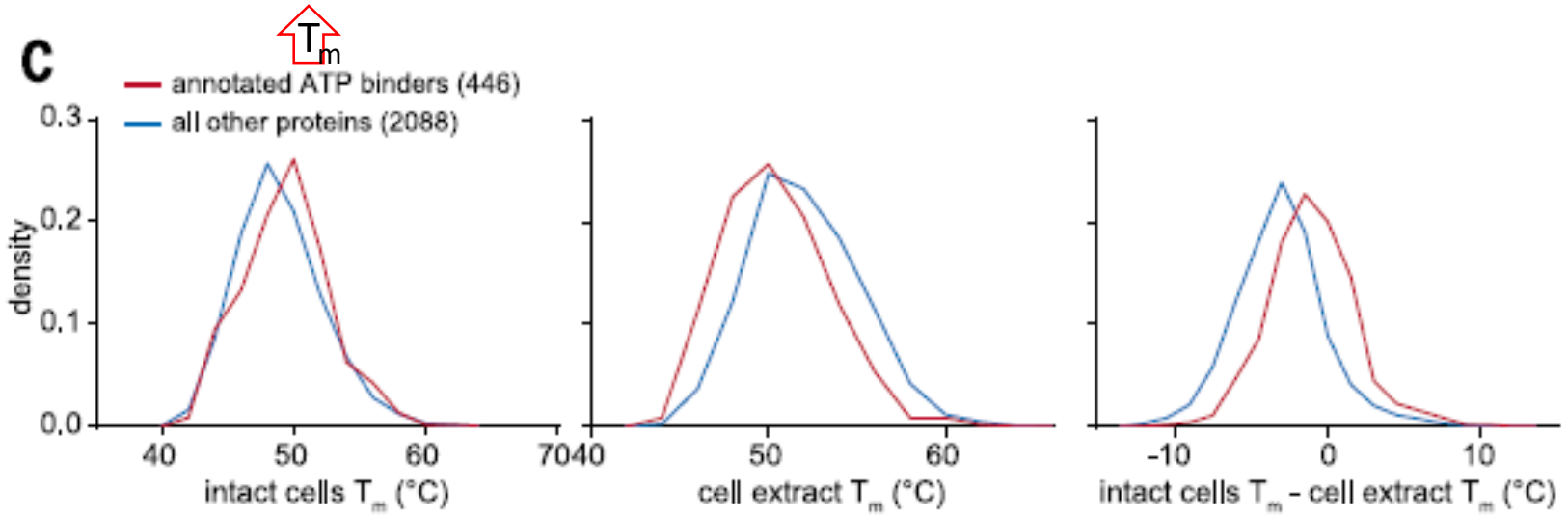


higher T_m (increased stability) in intact cells when compared with all other proteins



ATP-Binders Are Stabilized in Cell Extract

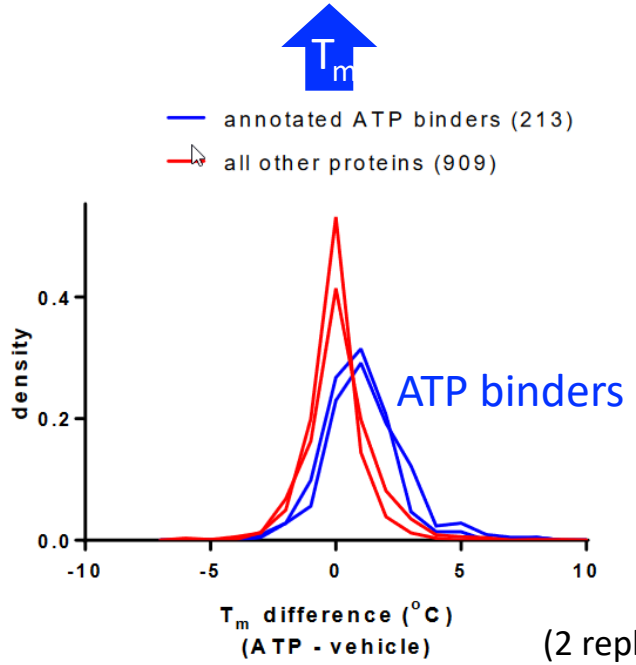
With the addition of MgATP



Analysis of Tm values of 440 annotated ATP binders -> higher T_m in intact cells compared to cell extract

K562 cell extract supplemented with MgATP (2mM) -> only ATP binders show trend towards higher T_m

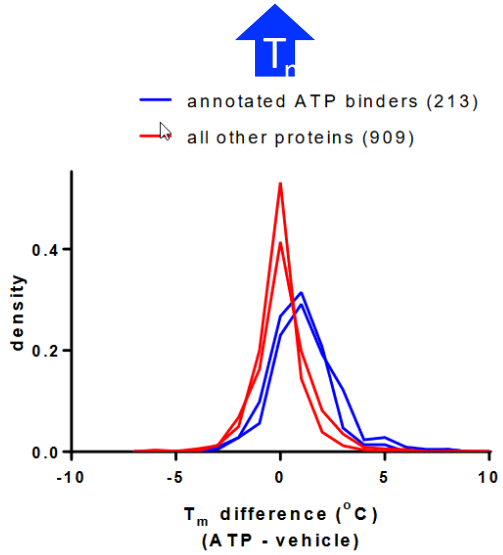
Another Experiment with DNA – binders (p53 and cognate DNA) ->



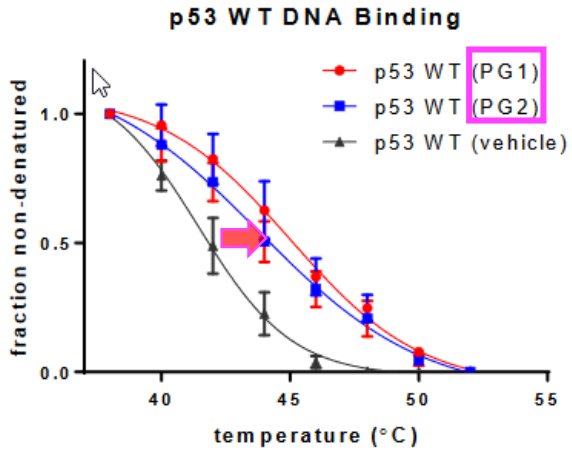
(2 replicate experiments)

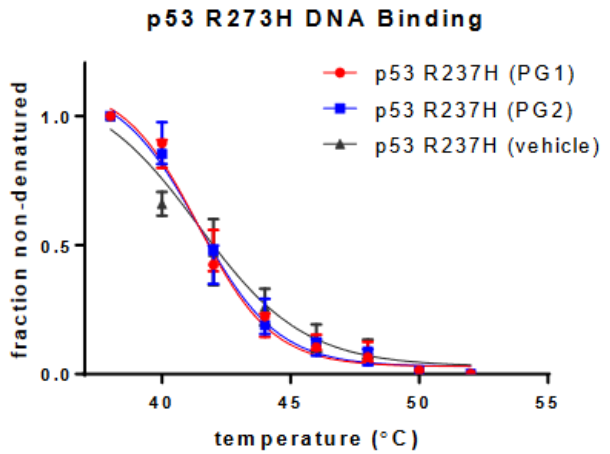
DNA-Binders Are Stabilized Upon Ligand Binding

When supplemented with MgATP -> only ATP binders show trend towards higher T_m



Another Experiment with DNA – binders (p53 and cognate DNA) -> same results

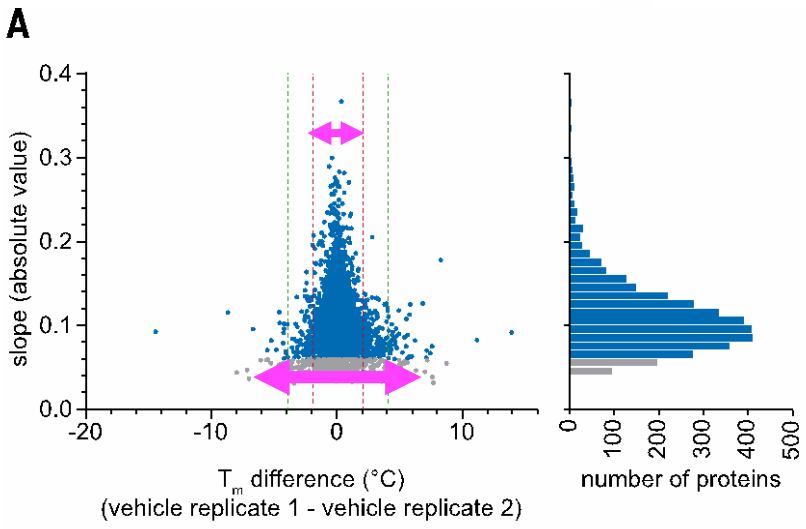




(Binding deficient mutant)

Proof of Principle – Kinase Inhibitors

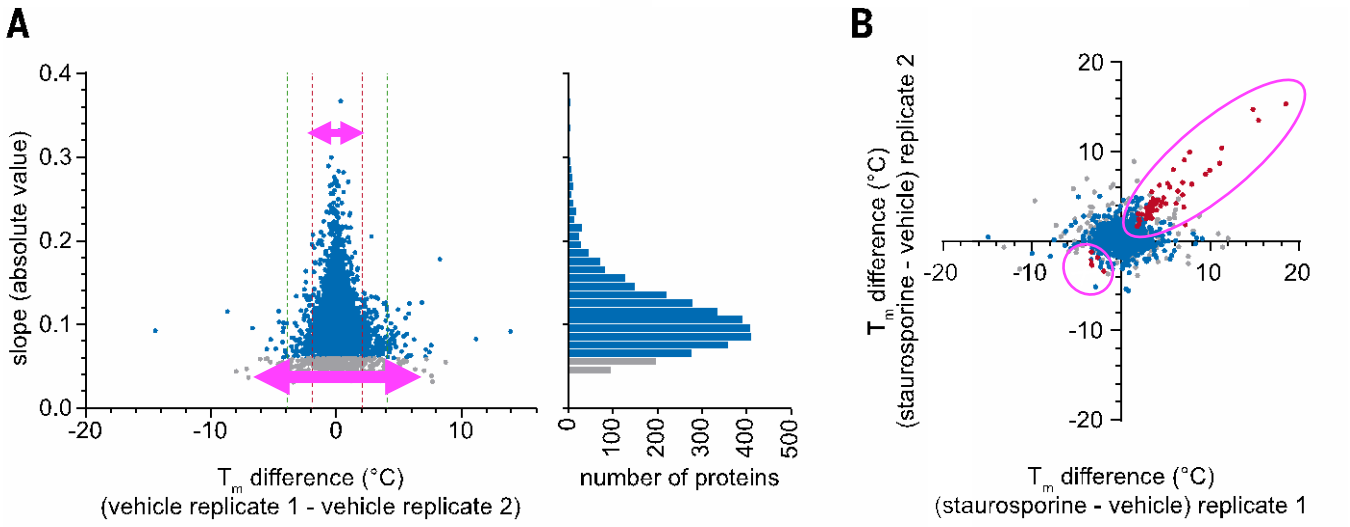
- Test: promiscuous kinase inhibitors with a known spectrum of targets: staurosporine and GSK318257
- K562 cell extract treated with staurosporine or vehicle



- Shallow slope -> less reproducibility
  - Because deviations in MS analysis will have bigger effects on T_m
- 92% of detected proteins yielded sufficiently steep slopes

Proof of Principle – Kinase Inhibitors

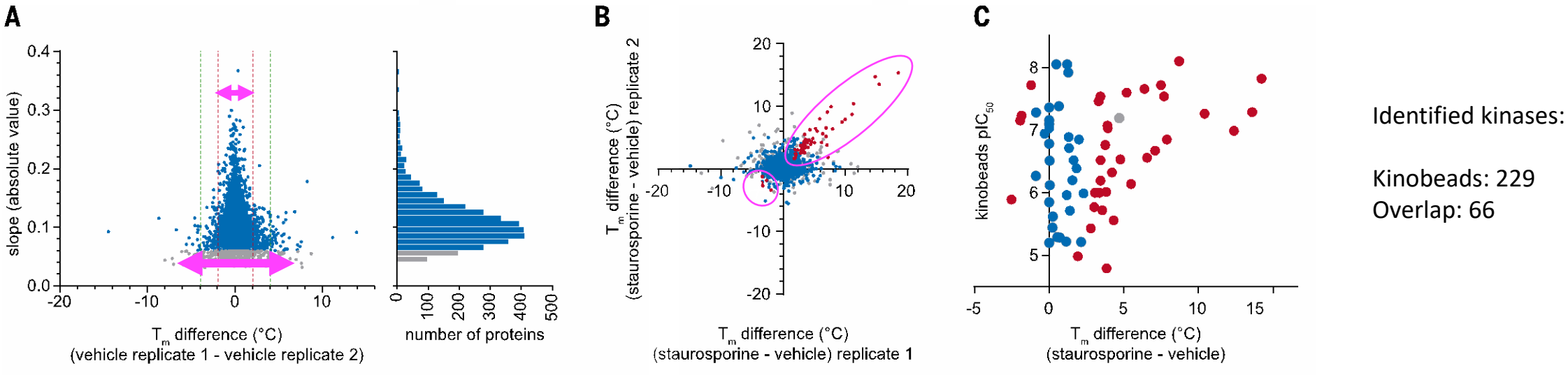
- Test: promiscuous kinase inhibitors with a known spectrum of targets: staurosporine and GSK318257
- K562 cell extract treated with staurosporine or vehicle



- Most affected proteins show positive shift
- Some: destabilization (protein kinase C family)

Proof of Principle – Kinase Inhibitors

- Test: promiscuous kinase inhibitors with a known spectrum of targets: staurosporine and GSK318257
- K562 cell extract treated with staurosporine or vehicle
- Comparison to previous study using chemoproteomics “kinobeads” profiling

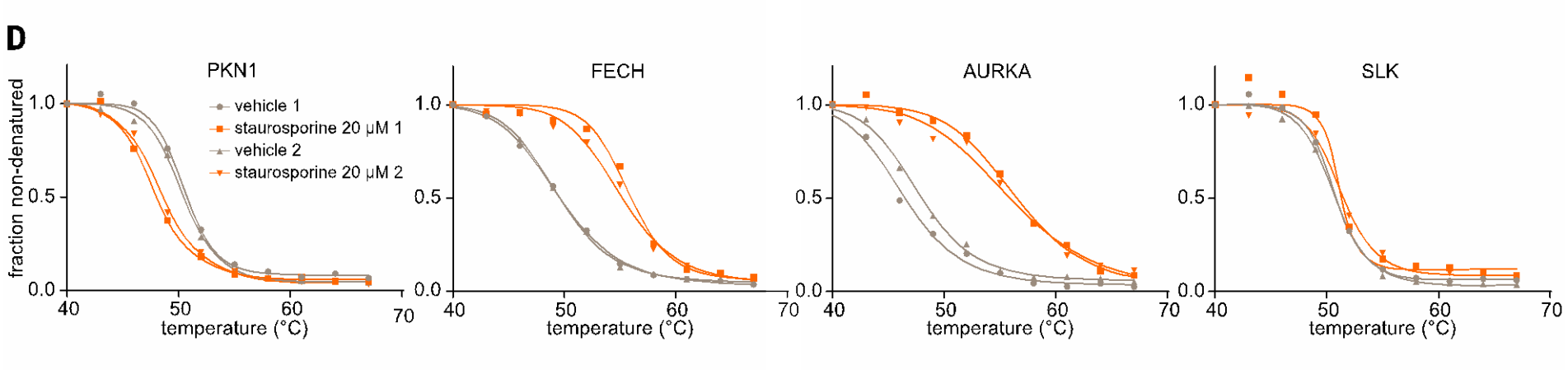


Identified kinases:

Kinobeads: 229
Overlap: 66

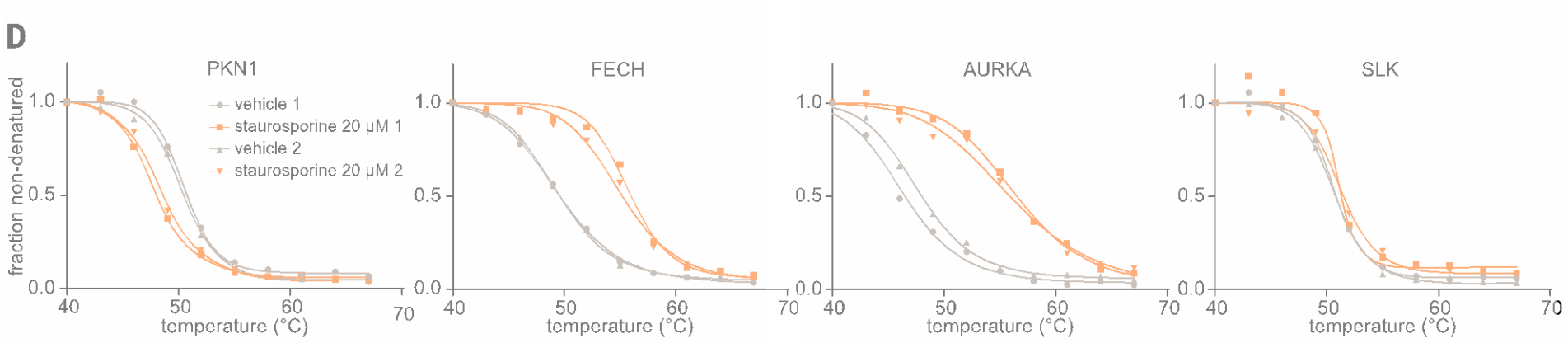
Proof of Principle – Kinase Inhibitor Promiscuity

- Thermal shifts identified for proteins **other** than kinases
  - coproporphyrinogen- III oxidase and ferrochelatase (FECH) (2/8 enzymes in **heme biosynthesis pathway**)

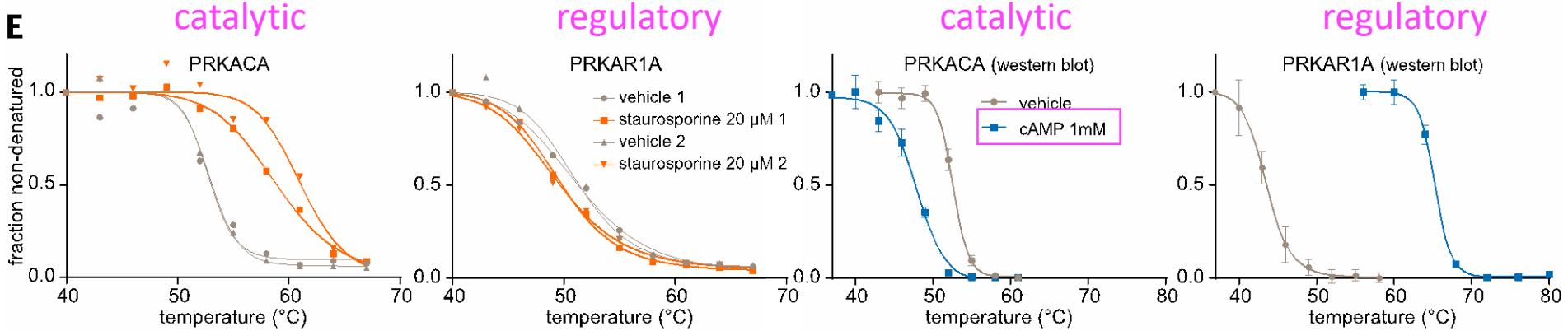


Proof of Principle – Kinase Inhibitor Promiscuity

- Thermal shifts identified for proteins other than kinases
  - coproporphyrinogen- III oxidase and ferrochelatase (FECH) (2/8 enzymes in heme biosynthesis pathway)
- Regulatory components of other kinase complexes -> eg protein kinase A (PKA) complex
  - Inhibition by staurosporine appeared to stabilize the catalytic subunit but destabilize the regulatory subunit.



Subunit: catalytic regulatory catalytic regulatory



cAMP causes dissociation of reg from cat subunit

Suggests: Catalytic subunit released from regulatory

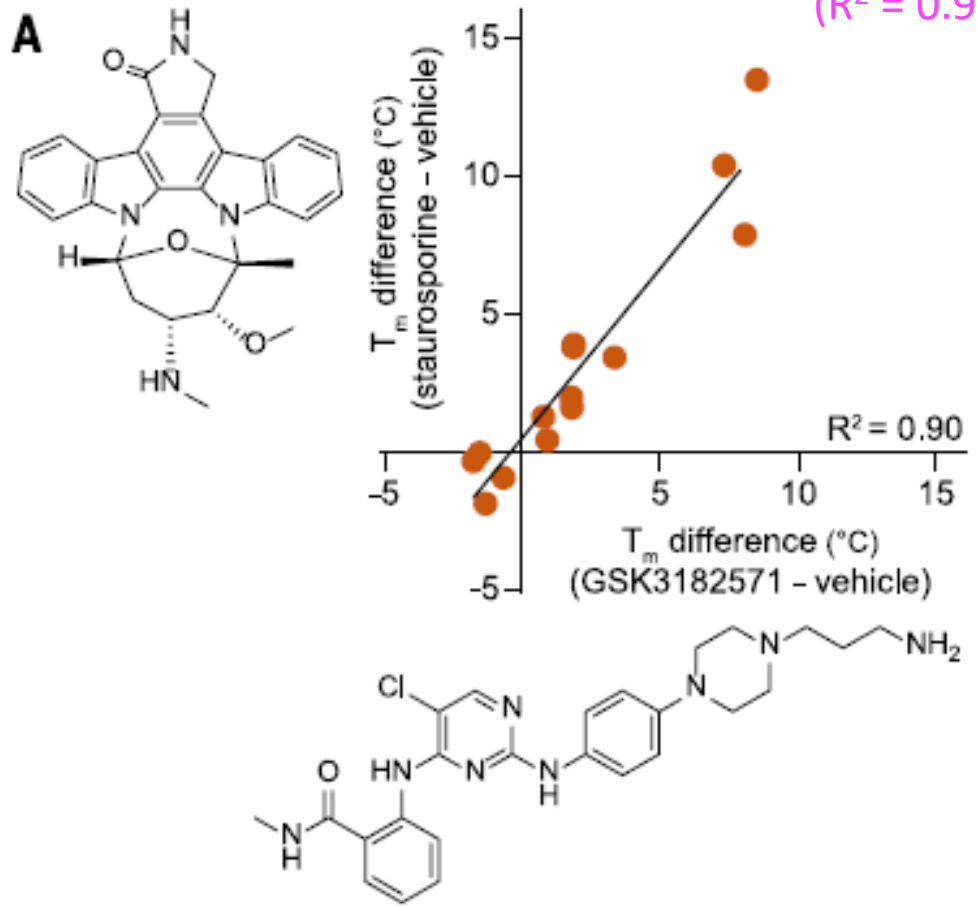
Correlation Between Ligand Affinity and Thermal Shift

Comparing Staurosporine with GSK3182571: structurally divergent promiscuous kinase inhibitor

Identified 13 common targets

For these targets: both compounds showed similar T_m shifts

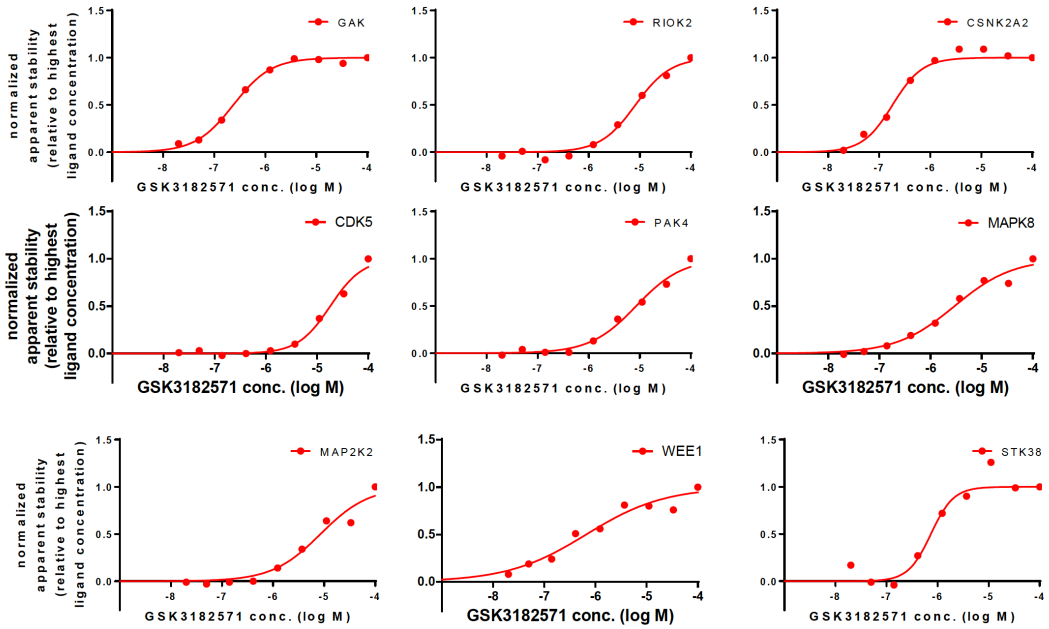
-> suggesting that for a given protein, the T_m shift at saturating ligand concentrations is dependent on the intrinsic affinity of the ligand.

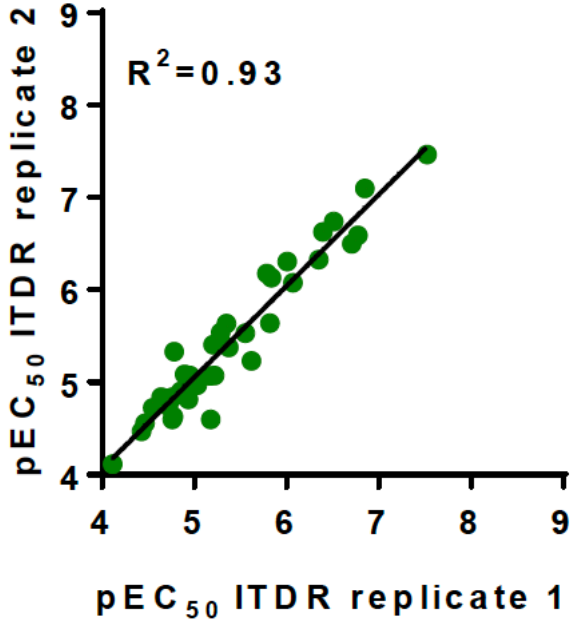


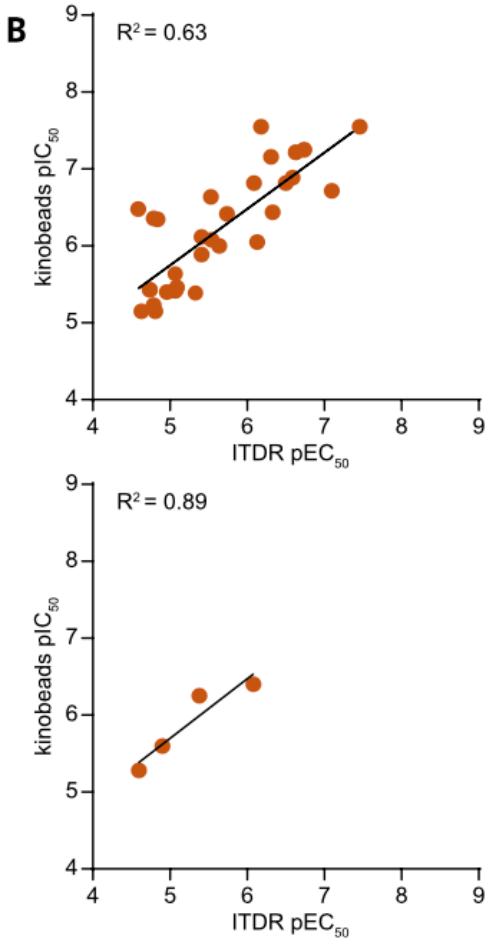
affinity below 1 mM and a less than 10-fold difference in affinity.

Isothermal Dose-Response (ITDR)

- ITDR is generated at a defined temperature , over a range of compound concentrations
- Affinity data for GSK3182571
- Good reproducibility, comparison with kinobeads -> good aggreement

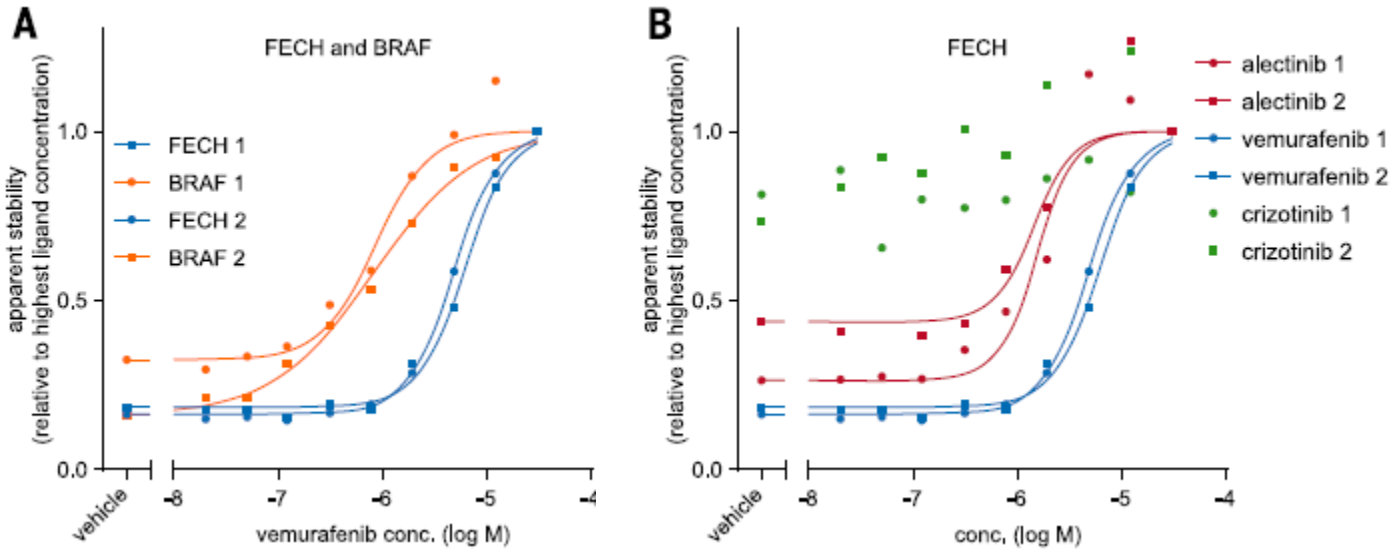






Testing Known Drugs For Affinity to Targets

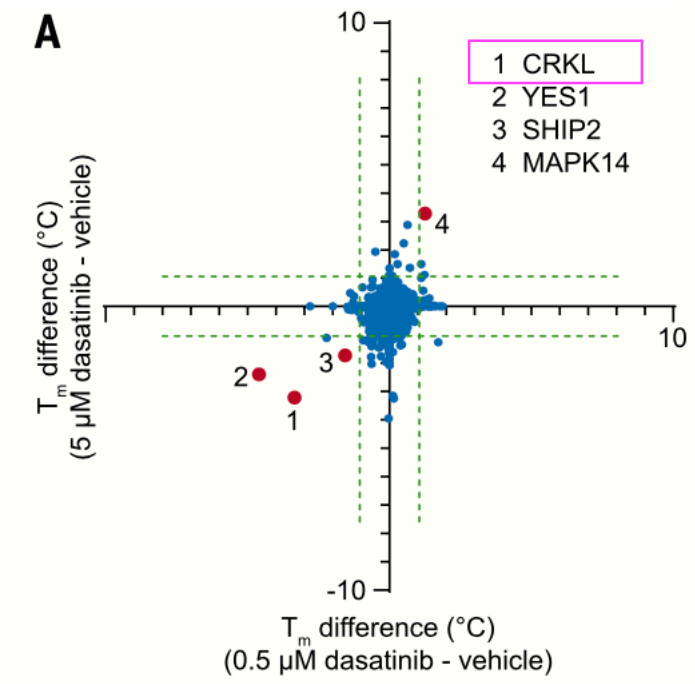
- Deficiency in FECH and results in high tissue levels of protoporphyrins
- **Vemurafenib** (melanoma drug) often causes photosensitivity and increased levels of protoporphyrins ->
  - Profiling identifies known target BRAF and also FECH
- **Alectinib** -> anaplastic lymphoma kinase (ALK) inhibitor (non-small lung cancer) -> also causes photosensitivity
  - FECH **affected most potently by alectinib, then vemurafenib and not by crizotinib**

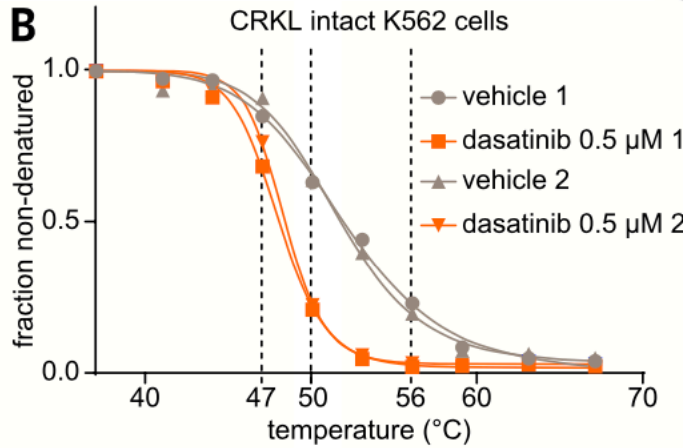


Demonstrates: TPP can serve as a standalone technique for obtaining quantitative affinity data for target-ligand interactions in a cell-based setting.

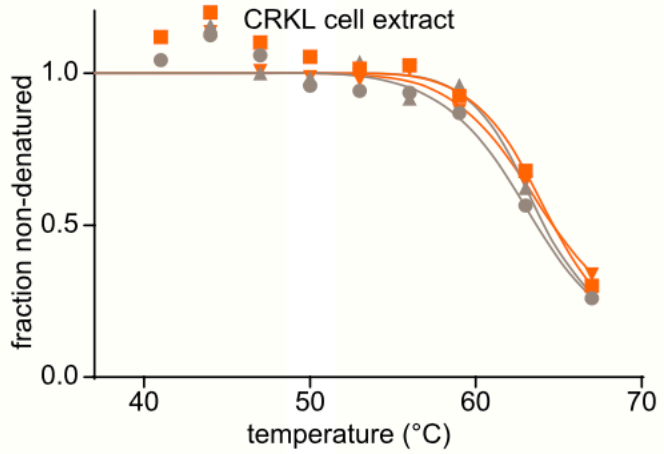
Identifying Induced Tm Shifts in Downstream Proteins

- Hypothesis:
  - Cell extract -> no downsream effects can be detected
  - Intact cells -> active signalling takes place -> downstream signalling could be detected





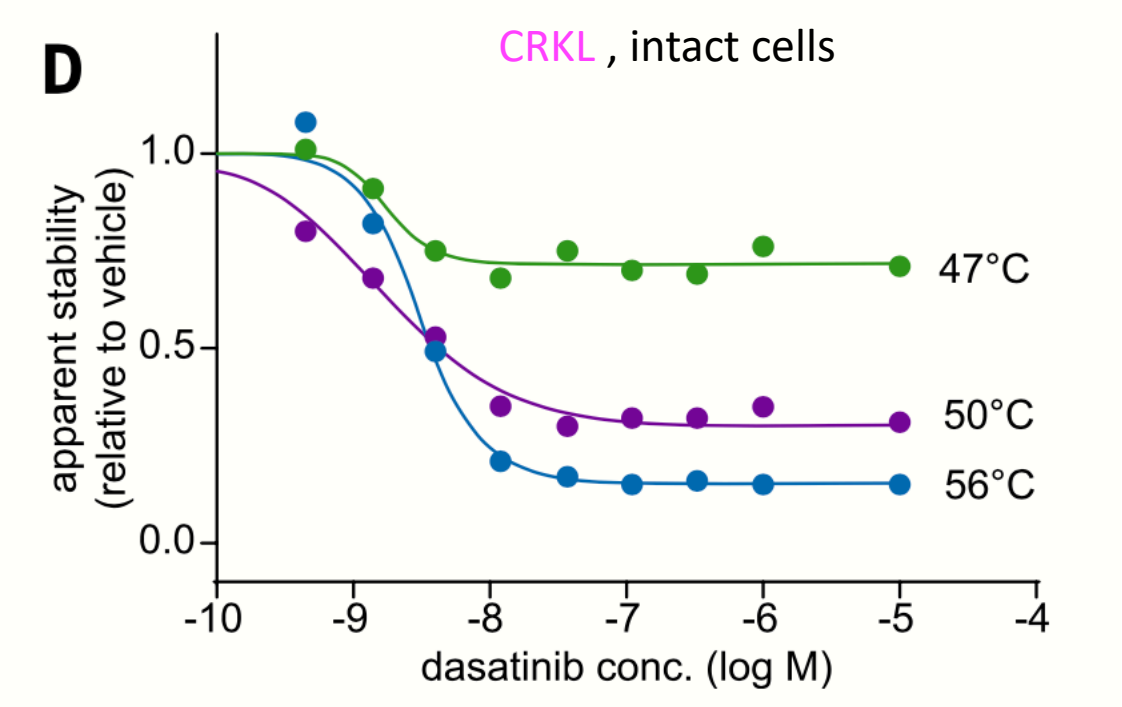
Destabilized in intact cells



No impact in cell extract

Drug Concentration Dependance of Effektors

- ITDR profiles at 3 temperatures
- Half maximal reponse of CRKL marker was between 1.5 and 3.2 nM (agrees with known potency for inhibiting cell growth)



Destabilization of Effector with increase in desatinib concentration

Positives and Drawbacks – TPP

Positive

- Identification of off-targets
- Identification of direct targets and their effectors
- Dose dependency of targets and their effectors
- Possible identification of post-translational modifications, fusion proteins, splice variants (typically undersamples in MS-based proteomics)
- bound ligands, cofactors, metabolites, drugs
- Can provide general view of proteomic state or proteotype
- Avoiding the design of affinity-tagged chemical probes
- Hypothesis Free target engagement studies
- Within constraints: mechanism of action studies

Drawback

- False negative: Some ligands don't provide a t_m shift, therefore identification of all targets is not guaranteed (dasatinib –BCR, Savitski et al)
- Thermal profiling will miss proteins owing to insufficient abundance and/or solubility or the absence of a significant ligand effect.
- Application not developed in adherent cell systems yet
- **No detection of membrane proteins**

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Paper 2

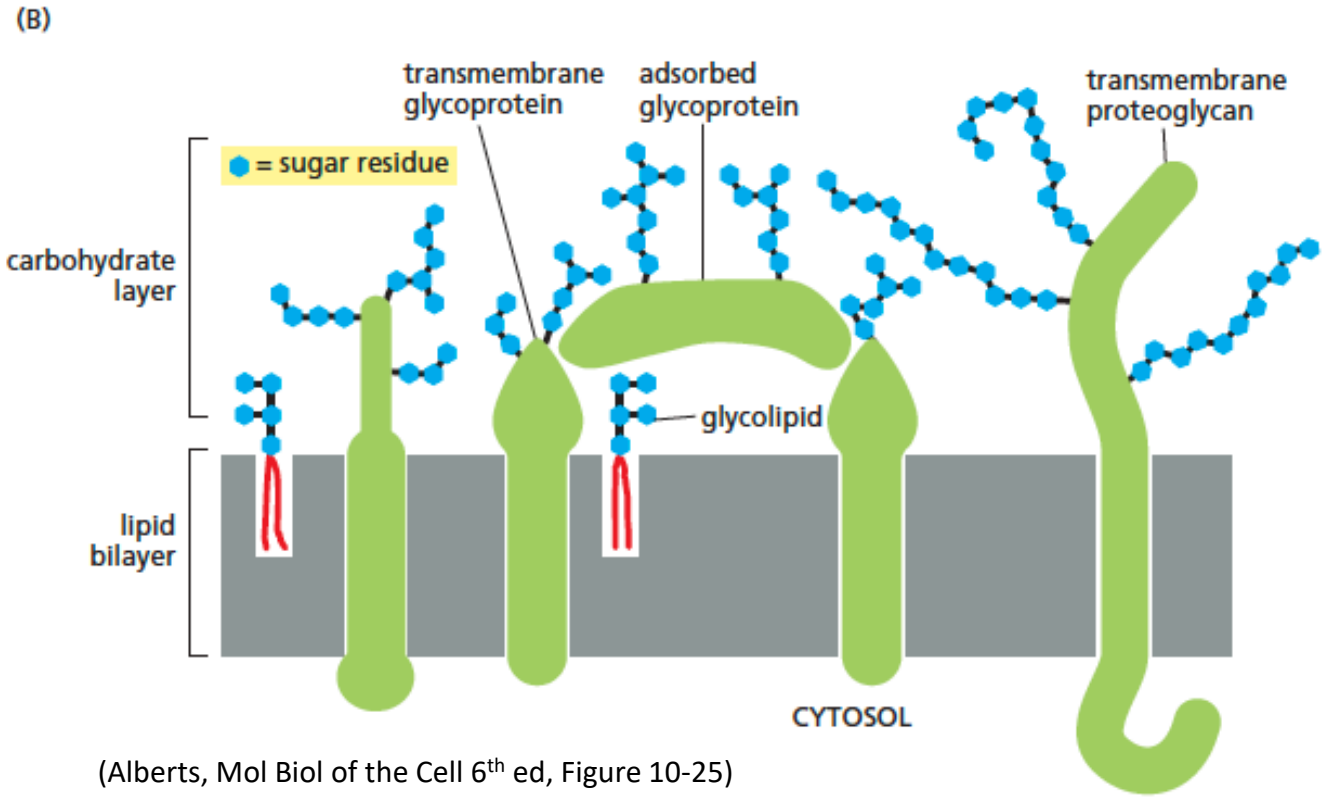
ARTICLES
https://doi.org/10.1038/s41592-020-01022-1

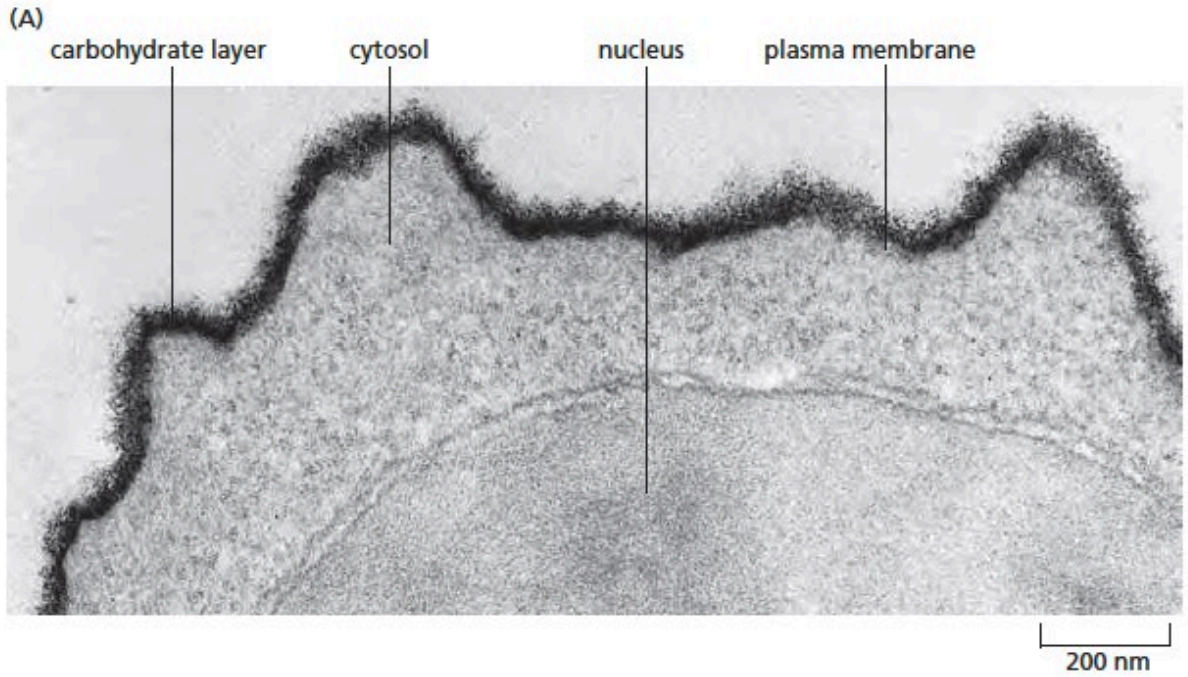
nature methods

Cell surface thermal proteome profiling tracks perturbations and drug targets on the plasma membrane

Mathias Kalxdorf[1], Ina Günthner[1], Isabelle Becher[2], Nils Kurzawa[2,3], Sascha Knecht[1], Mikhail M. Savitski[2], H. Christian Eberl[1]✉ and Marcus Bantscheff[1]✉

Most Membrane Proteins are Glycosylated

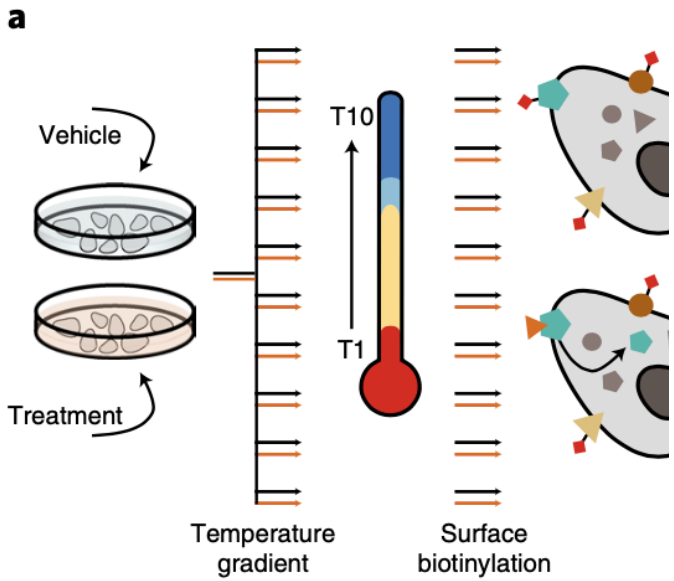


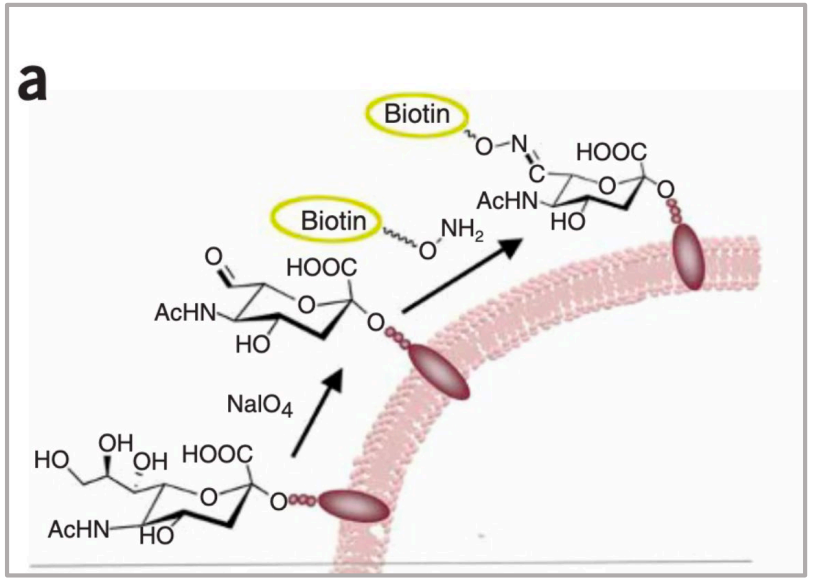


(Alberts, Mol Biol of the Cell 6th ed, Figure 10-25)

Cell Surface Thermal Protein Profiling (CS-TPP) Through Enrichment of Glycosylated Proteins

Live cells incubated with/ without Compund → Oxidized using sodium metaperiodate → Biotinilated using oxime ligation reaction

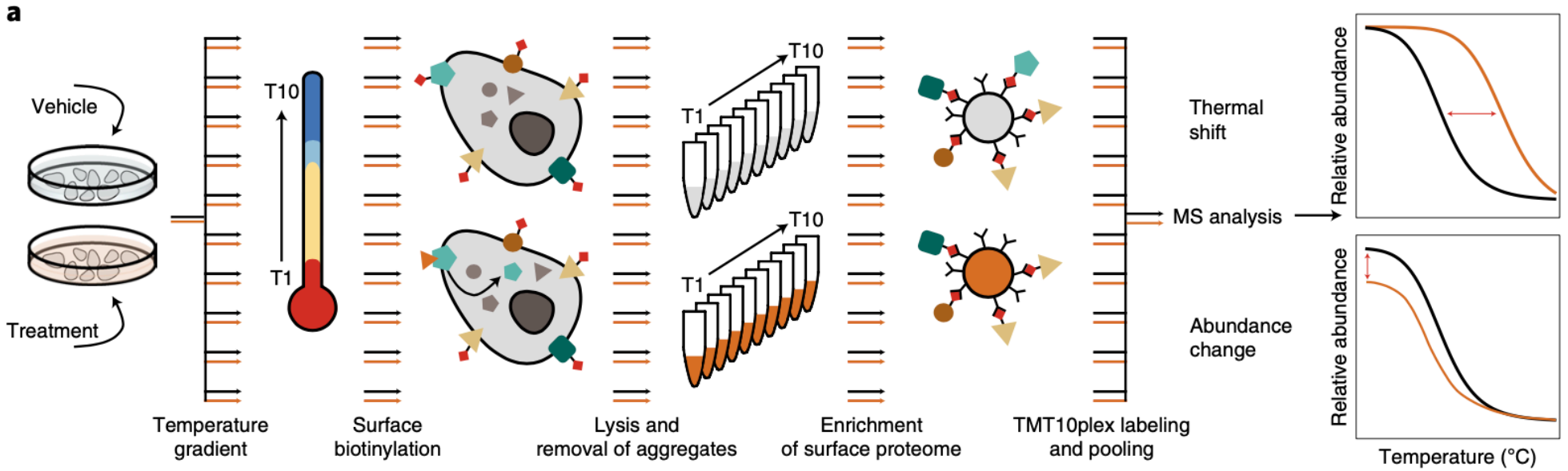




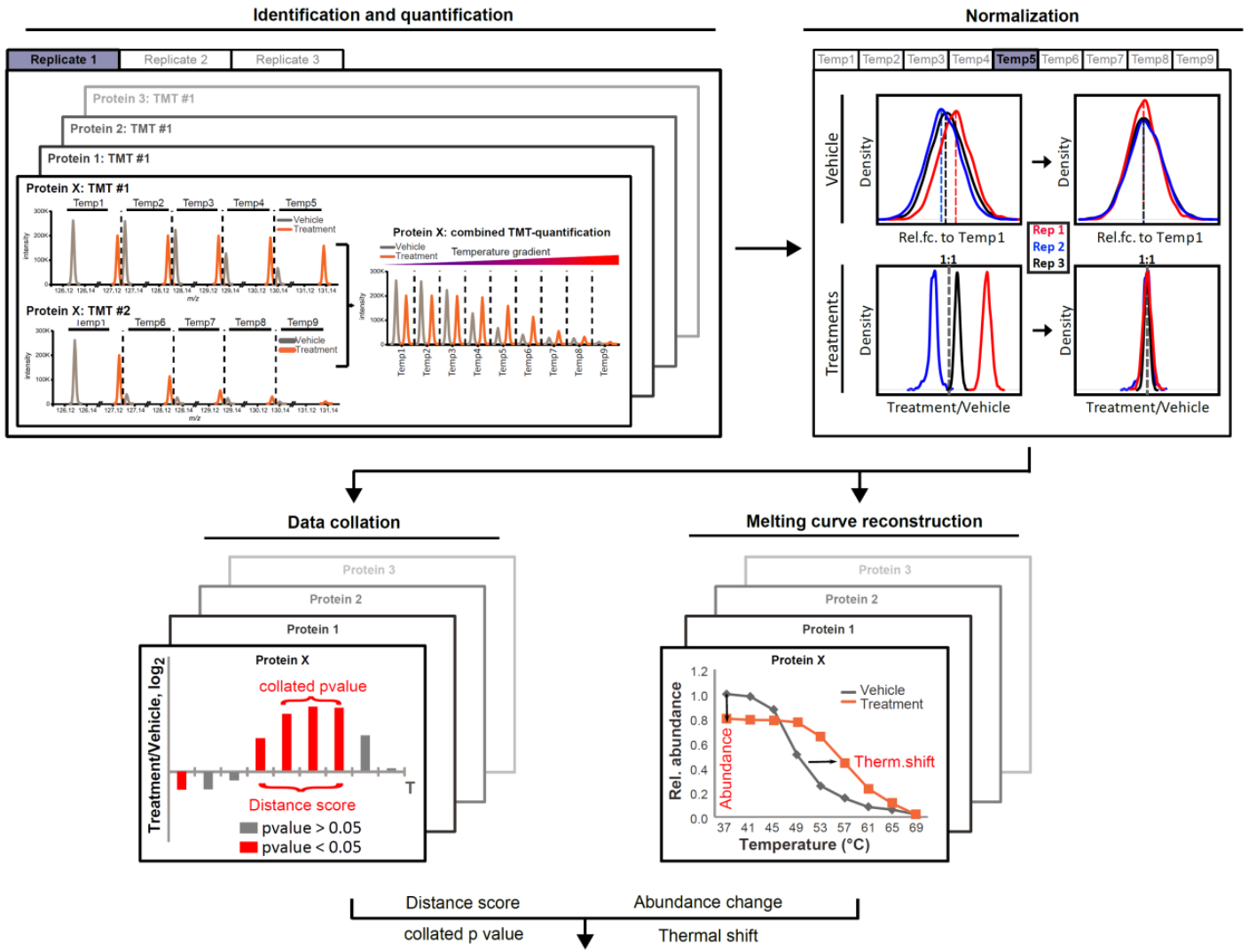
Zeng, Y., Ramya, T. N. C., Dirksen, A., Dawson, P. E. & Paulson, J. C. High-efficiency labeling of sialylated glycoproteins on living cells. *Nat. Methods* **6**, 207–209 (2009).

Cell Surface Thermal Protein Profiling (CS-TPP) Through Enrichment of Glycosylated Proteins

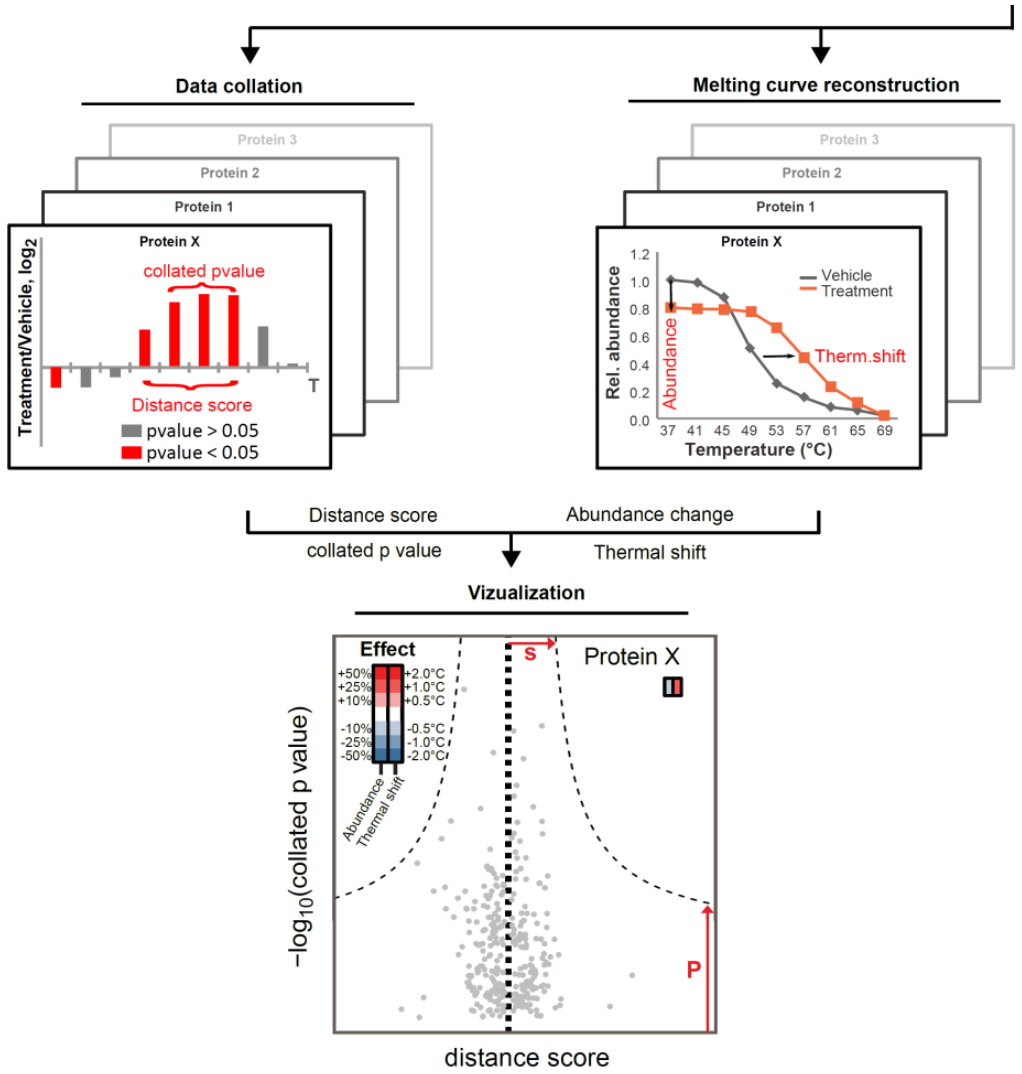
Live cells incubated with/ without Compund → Oxidized using sodium metaperiodate → Biotinilated using oxime ligation reaction → Filtration: removal of aggregated proteins → Enrichment of biotinilated proteins on neutravidin beads → Trypsinolysis and multiplexed quantitative mass spectrometry



CS-TPP Analysis Workflow



CS-TPP Analysis Workflow - How To Read The Graphs

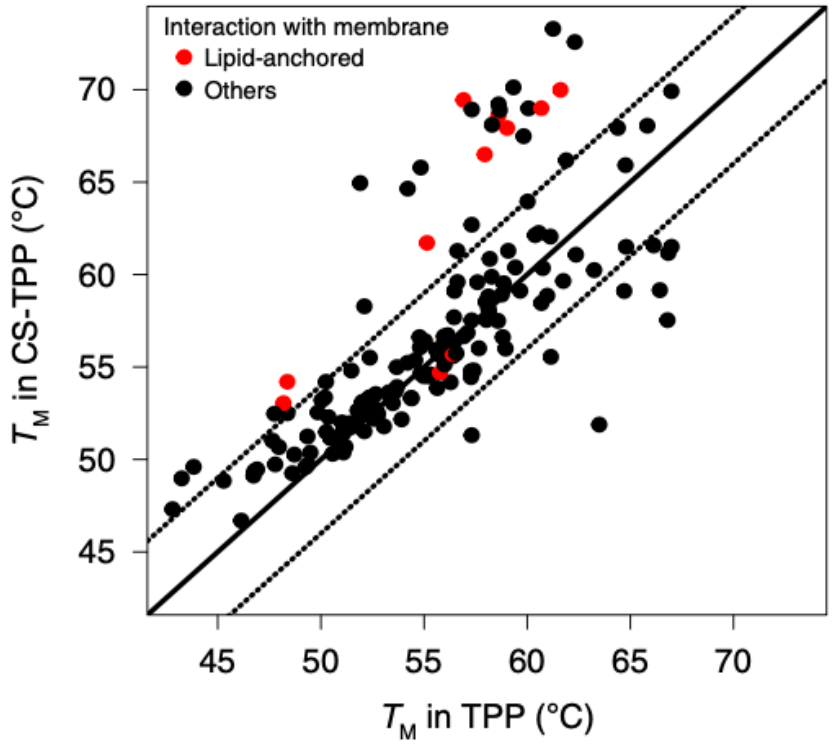


Collated p-value: significance of the abundance changes
(p-Value at most significant window (Browns method, sliding window over 3 temperatures))
Distance score: significant abundance changes
(tells the distance from each individual point to the mean (z-scored))
Cut-offs: defined by median std dev of ratios between replicates and by max p-value of 0.05
2 Boxes: left: significant abundance changes, right: significant thermal shifts

Cell Surface Protein Enrichment Influence on Melting Temperature

melting points, T_M : temperatures at which 50% of the respective protein is aggregated

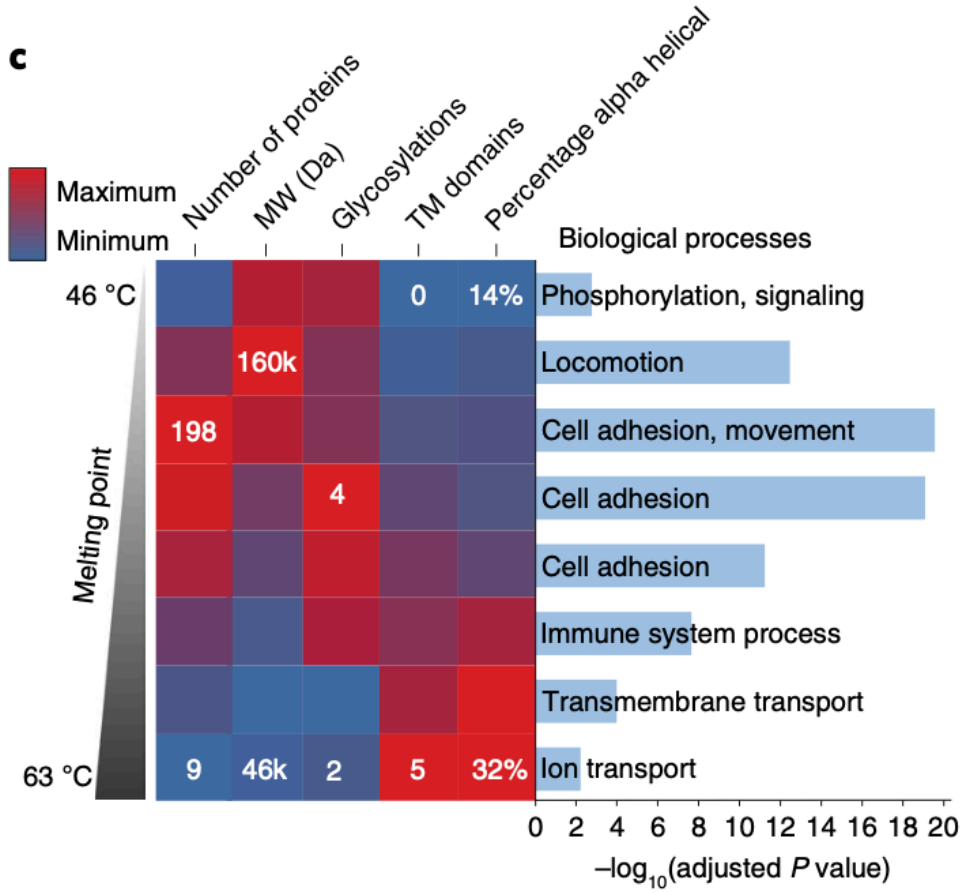
b



- median T_M -> 0.6 °C higher in the cell surface focused TPP
- Lipid-anchored +8.3°C
- Suggest intracellular subpopulations, major difference in conformation

Cell Surface Protein Thermal Stability

- Across 4 suspension cell lines
- With regard to protein properties

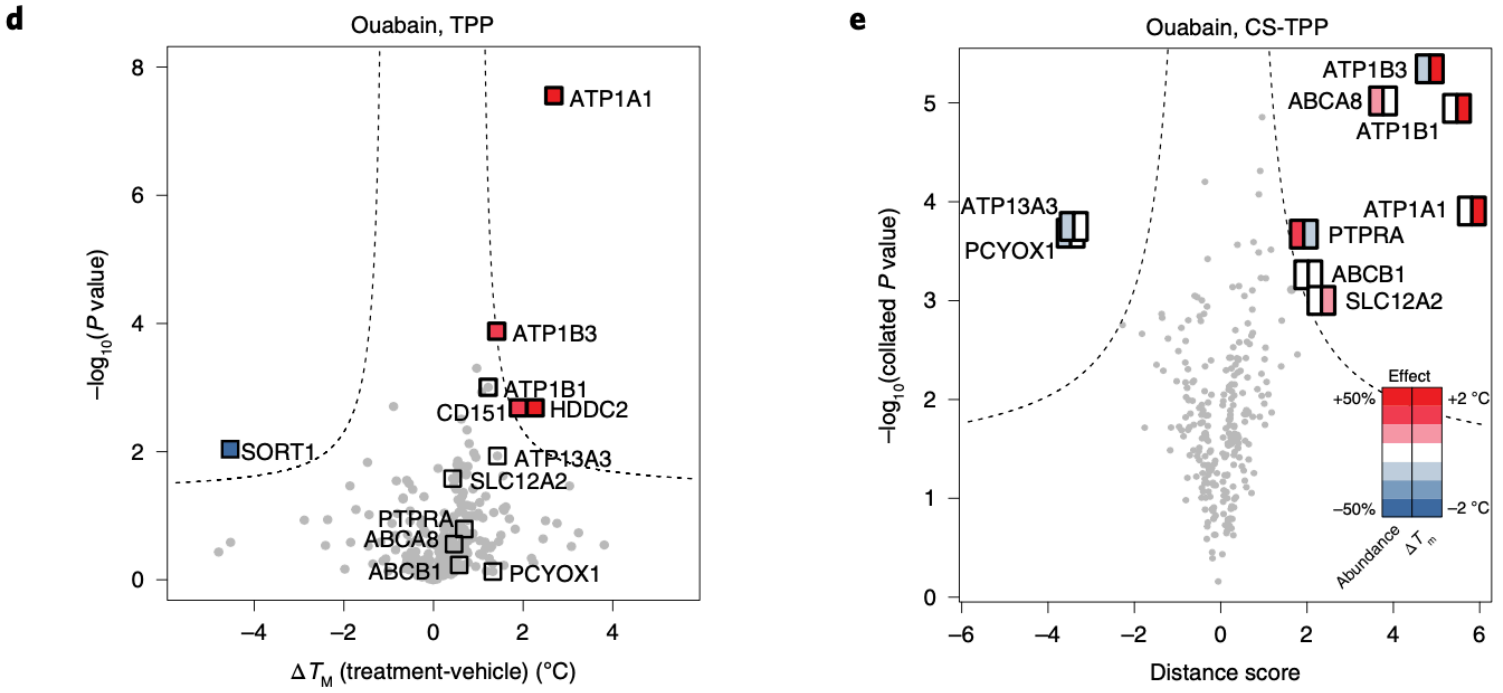


Most significantly enriched gene ontology term of biological processes

- Negative correlation with molecular weight
  - Smaller proteins -> more stable
- Positive correlation with number of transmembrane domains and alpha-helical content
- Less stable Proteins involved in:
  - Signalling
  - Locomotion and adhesion
- More stable:
  - Transporters, solute carriers

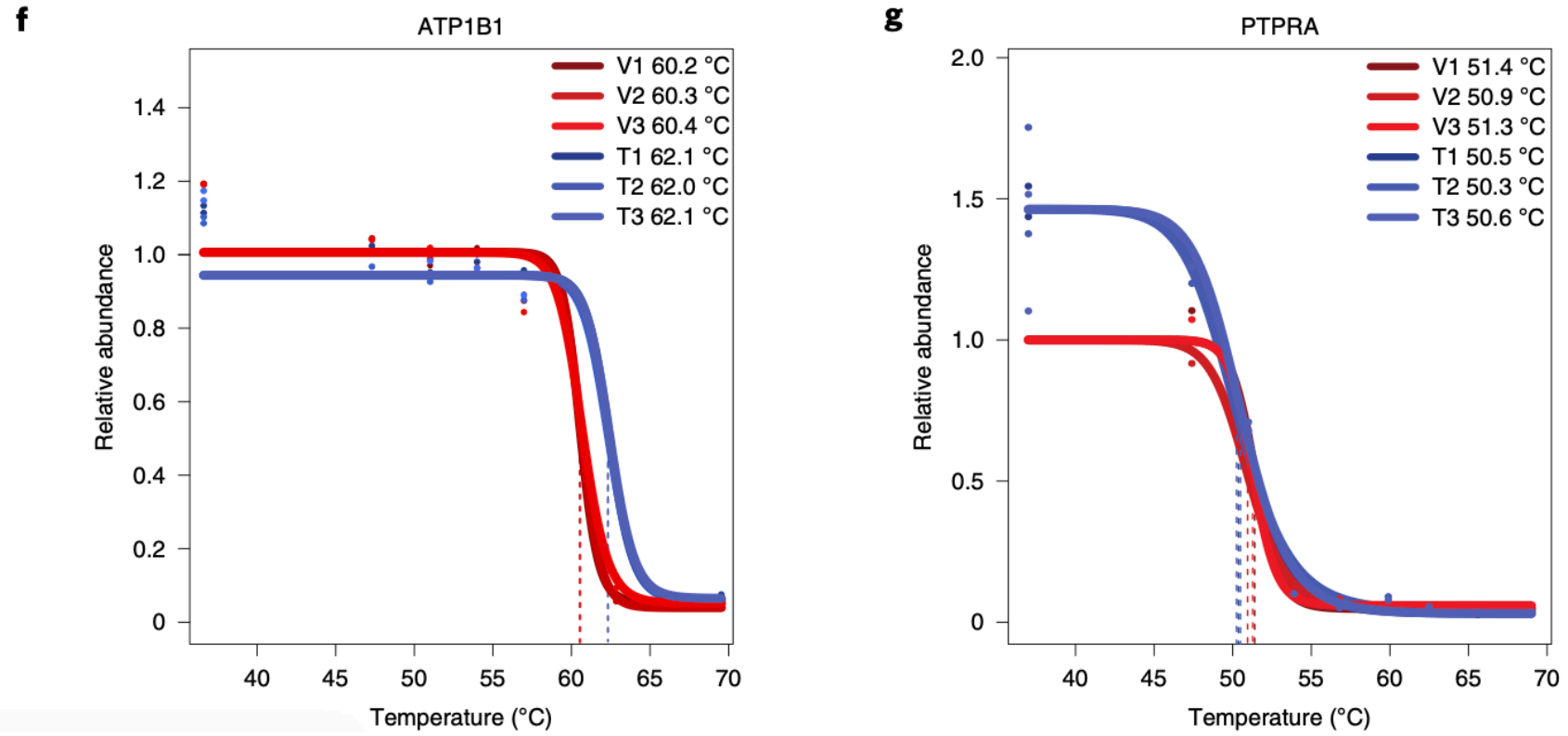
Comparisoon TPP With CS-TPP – Using Oubain

K562 cells treated with 1 µM ouabain (*n* = 3 independent experiments)
Oubain: Cardiac glycoside



- Main ouabain targets, Na^+/K^+-ATPase subunits
  - ATP1A1, ATP1B1, ATP1B3
- In TPP: only alpha subunit identified
- In CS-TPP: all identified

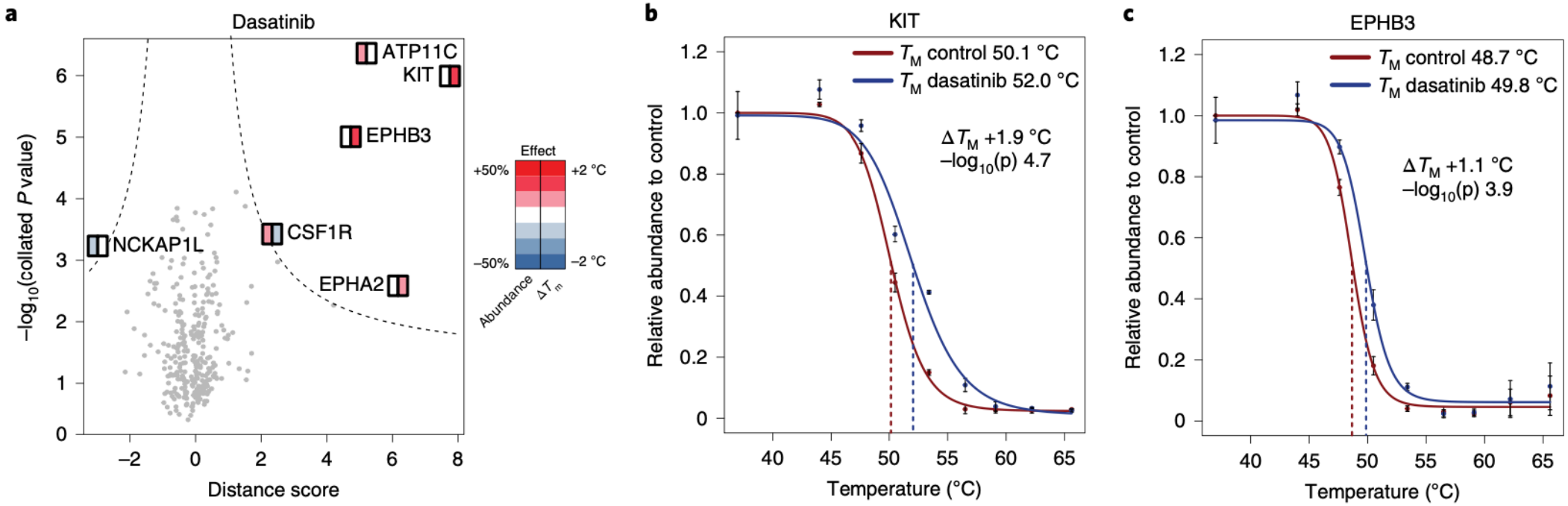
Thermal Stability Changes Can Be Distinguished from Abundance Changes



Identification of Direct and Additional Targets

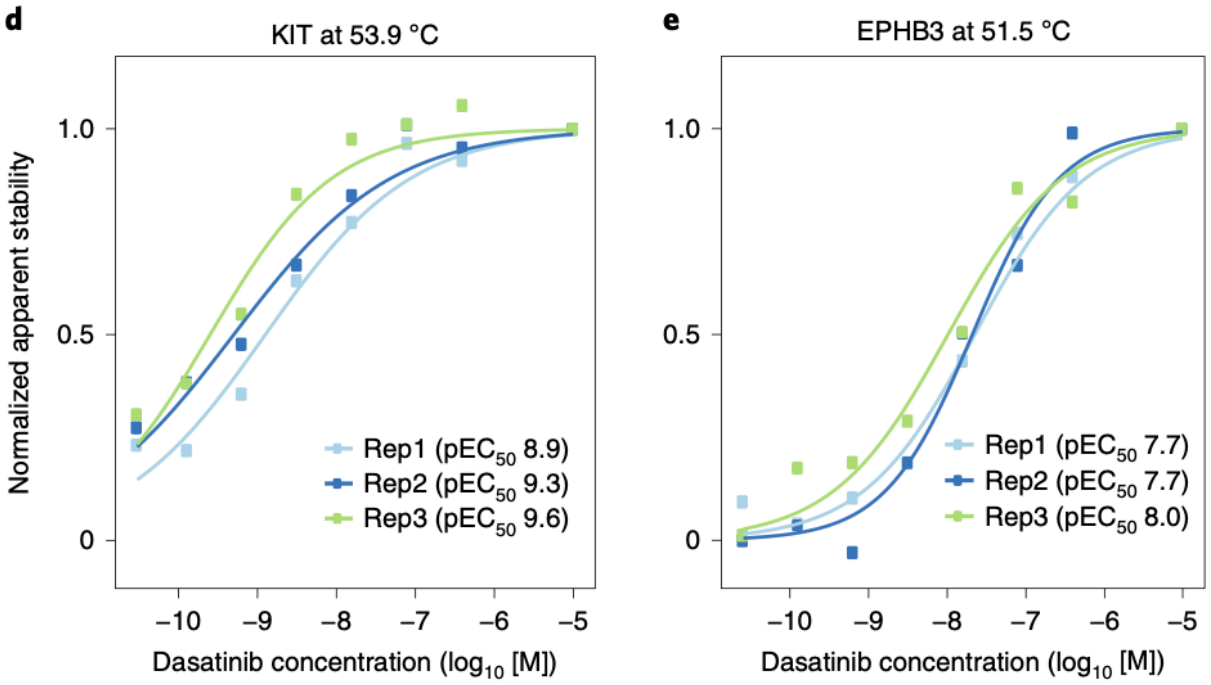
THP-1 cells treated with 1 µM dasatinib (*n* = 3 independent experiments)
Dasatinib: ABL inhibitor, drug against chronic myelogenous leukemia (CML)

Melting curves for significantly stabilized dasatinib targets KIT and EPHB3



Conventional TPP was not able to detect effects on direct targets c-KIT and DDR1

Isothermal Dasatinib Titration



Halfbinding concentrations similar as in previous TPP experiments (Savitski et al)

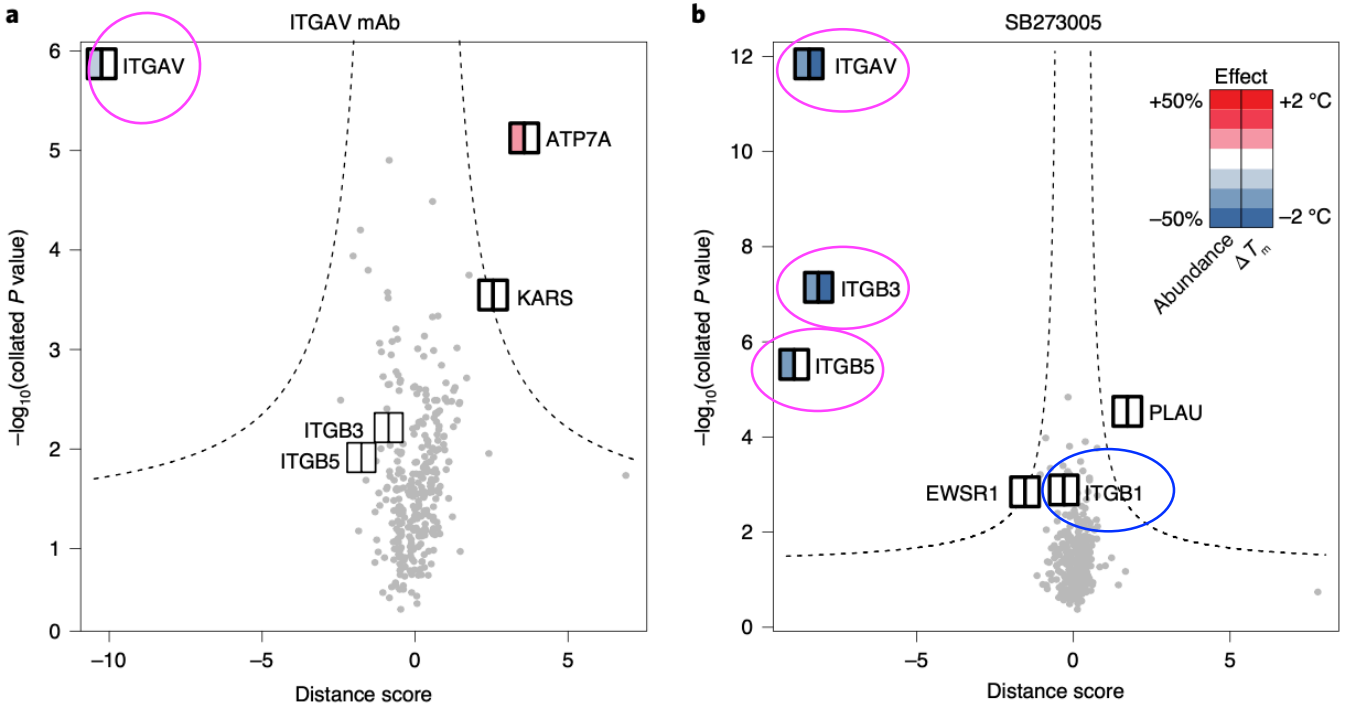
Testing of Multiple Systems

- TGF-beta signalling
- Copper Transport
- Cell surface remodeling upon T-cell receptor stimulation
- Identification of chaperone dependencies.
- **Differentiation of mechanisms for receptor modulation**

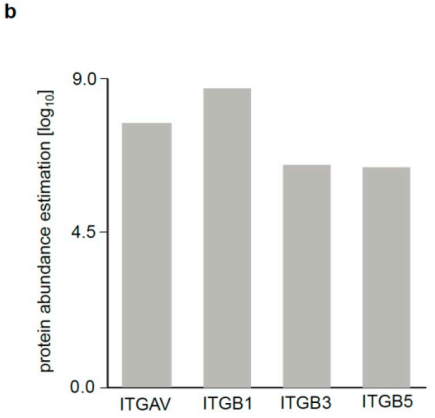
Internalization of Integrins Upon Target Binding

ITGAV: Integrin subunit alpha V
Integrins: heterodimeric integral membrane proteins, function in cell surface adhesion and signalling
Drug targets for treatment of multiple diseases, including Crohn's disease and ulcerative colitis

K562 cells -> presented with an ITGAV-directed monoclonal antibody -> selective internalization of ITGAV
-> SB273005: small molecule inhibitor -> ITGAV internalization + subunits



In contrast: subunit β1 was not affected. Its substantially higher abundance suggests that a large proportion of it is not associated with the αV subunit



GPCR – Inhibitor Interferes With Endogenous Ligand

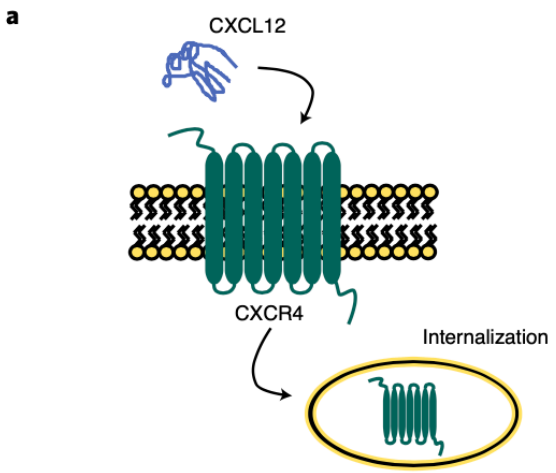
Jurkat cells
VLDL: Very low density lipoprotein
CXCL12: chemokine that binds to CXCR4
CXCR4: GPCR
IT1t: Small molecule inhibitor of CXCR4

Specific internalization of the corresponding cell surface receptors

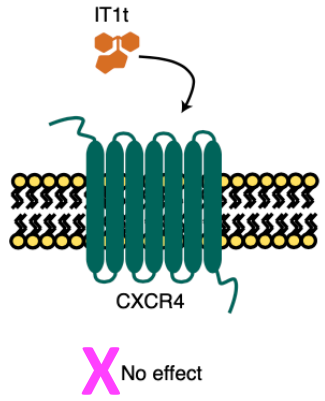
But CXCR4 was not affected by the inhibitors IT1t and WZ811

However, endogenous ligand-induced internalization of the target can be blocked in the presence of the small molecule inhibitor

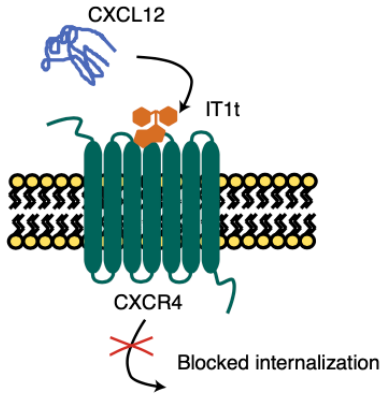
20 nM endogenous ligand CXCL12

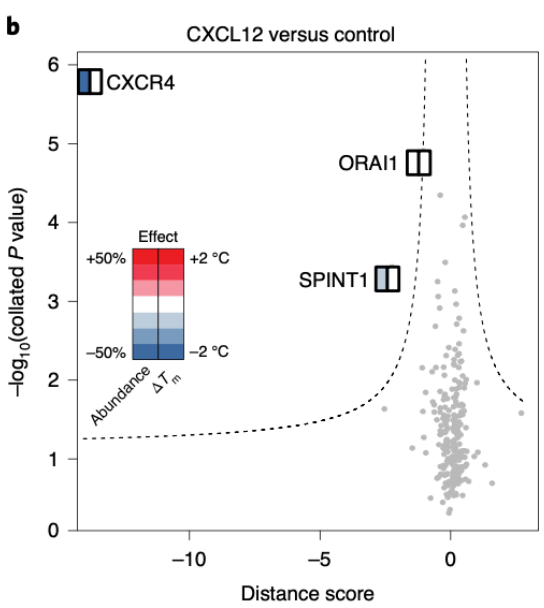


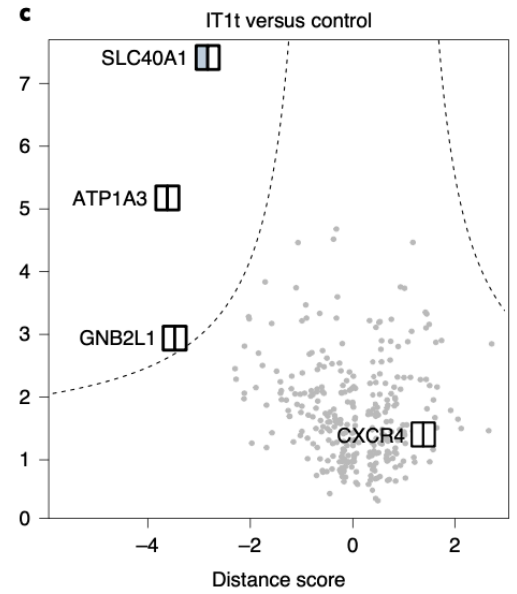
10 µM IT1t

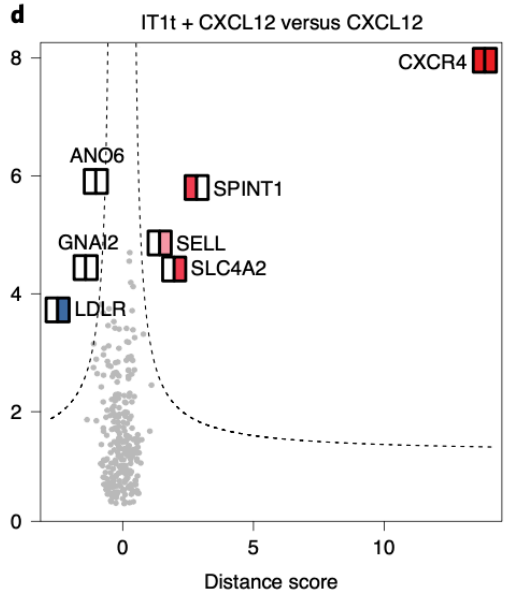


Preincubation 10 µM IT1t followed by treatment with 20 nM CXCL12 compared to cellular treatment with 20 nM CXCL12









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