

Supplementary Material for

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

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This PDF file includes:

Materials and Methods

Figs. S1 to S12

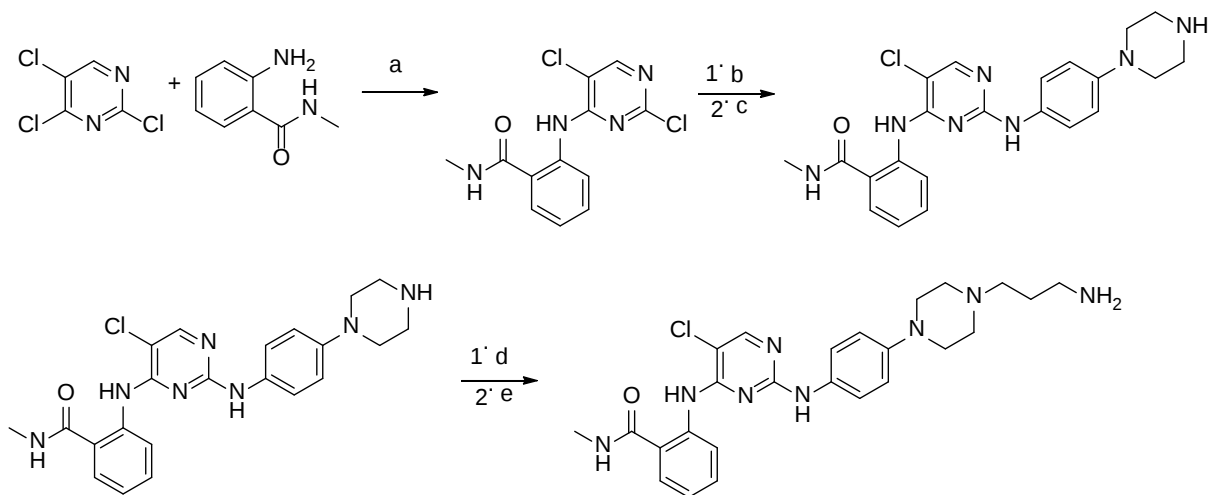
Other Supplementary Material for this manuscript includes the following:
(available at www.sciencemag.org/content/346/6205/1255784/suppl/DC1)

Tables S1 to S13 as separate Excel files

Supplementary material and methods: synthesis of GSK3182571

General Chemistry Procedures

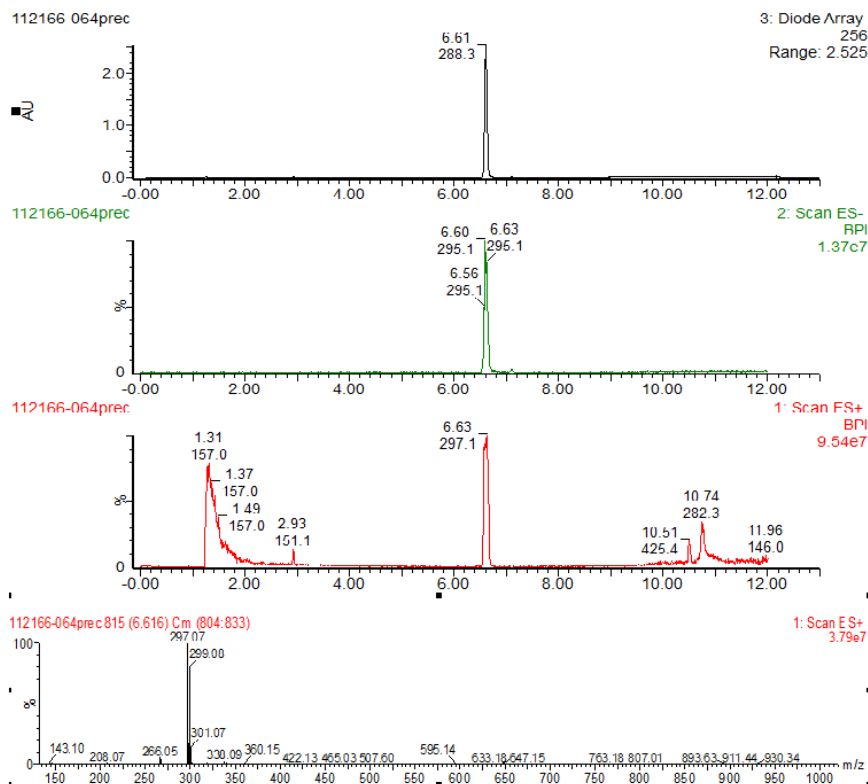
Commercially available starting materials, reagents and dry solvents were used as supplied. Flash column chromatography was performed using a Biotage Isoleara four purification system using Biotage Flash silica cartridges. ^1H NMR spectra were recorded on a Bruker Avance 400. Samples were prepared as solutions in a deuterated solvent and referenced to the appropriate internal nondeuterated solvent peak. LC/MS analysis was performed using a Waters X-Bridge C18-column, 5 μm particle size, 4,6 x 150 mm (diameter x length) at a flow rate of 1,75 mL/min with a linear gradient (water to acetonitrile, 0.2% formic acid as modifier) from initially 99:1 to 1:99 over 9.10min, then hold for 1.80 min. Mass signals were determined using a Waters 3100 Mass Detector.



(a) DIPEA, secbutanol, room temperature, 18 h (b) *tert*-butyl 4-(4-aminophenyl)piperazine-1-carboxylate, HCl, 120°C, 18 h (c) DCM, TFA, room temperature, 1 h (d) *tert*-butyl (3-oxopropyl)carbamate, NaCNBH_3 , Methanol, room temperature, 4h (e) HCl, dioxane, room temperature, 3h

2-((2,5-dichloropyrimidin-4-yl)amino)-N-methylbenzamide

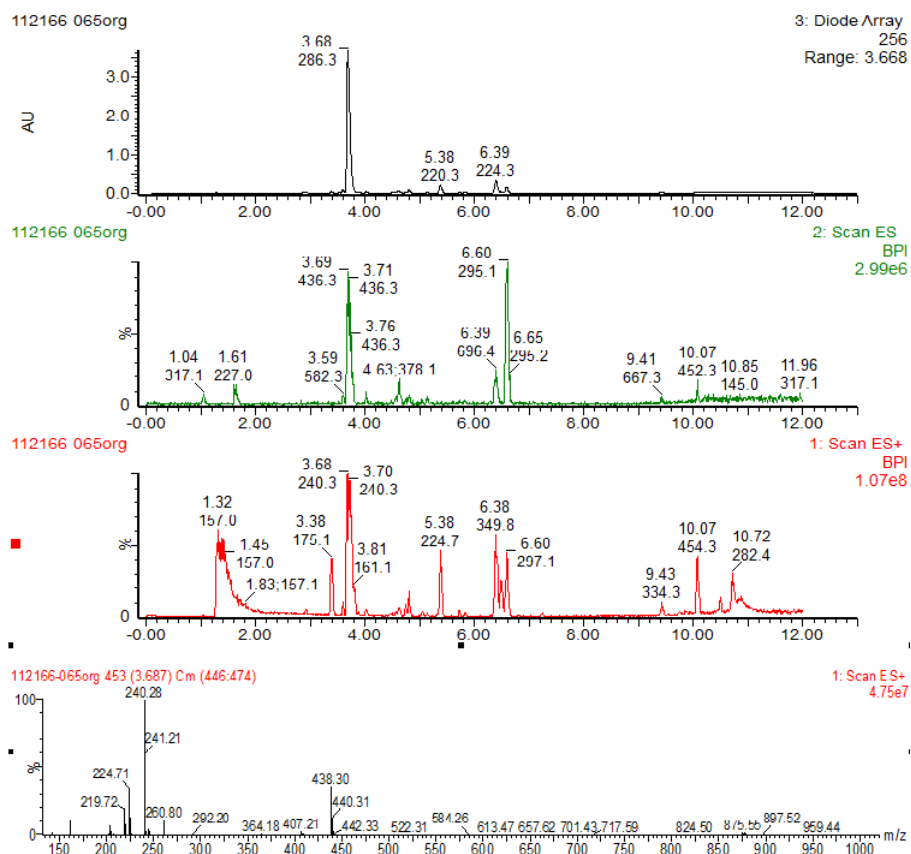
2,4,5-Trichloro pyrimidine (2.50 g, 13.70 mmol) was dissolved in secbutanol (25ml) and to this was added 2-amino-N-methyl benzamide (2.05g, 13.70 mmol) and diisopropylethylamine (2.9 ml, 16.44 mmol). The reaction mixture was stirred at room temperature for 18 h. The precipitate was collected by filtration and washed with diethyl ether. This afforded 2-((2,5-dichloropyrimidin-4-yl)amino)-N-methylbenzamide (1.776 g, 44%). LC/MS (ESI, m/z) t_R = 6.61 min, 297, 298, 299, 300, 301 ($[M+H]^+$, 2Cl isotopic pattern).



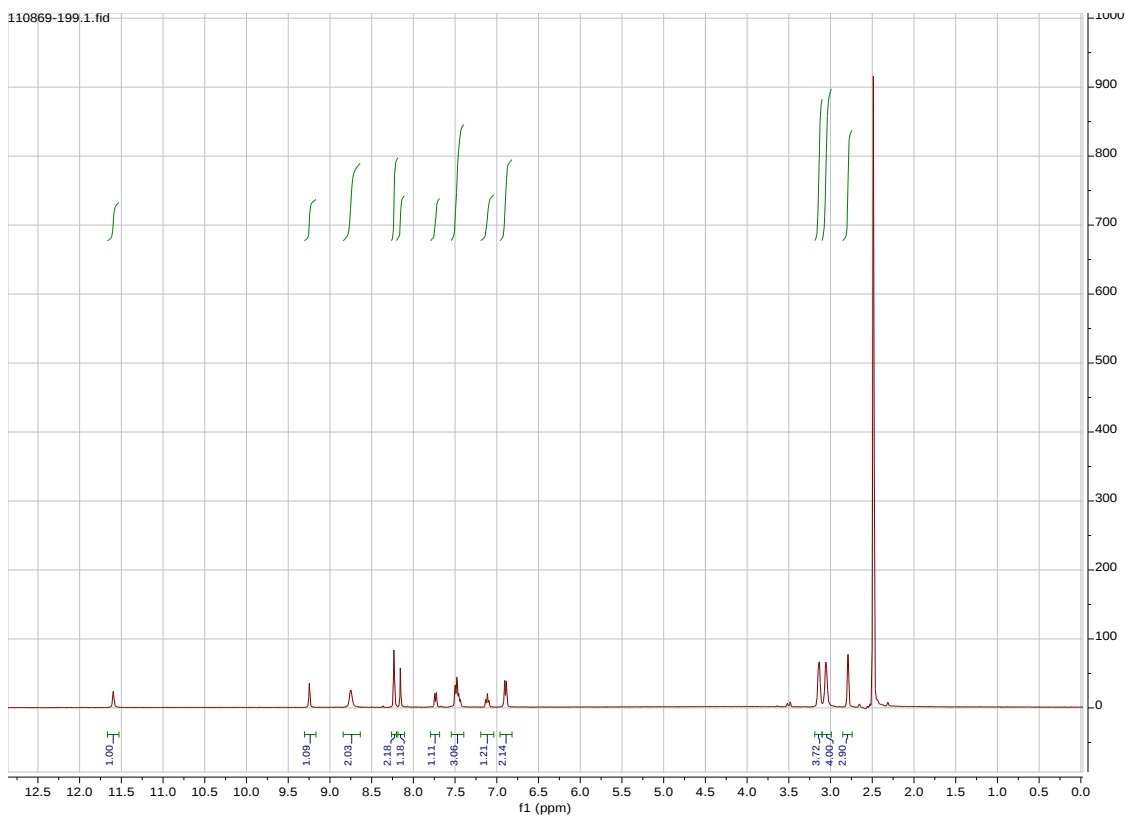
LC/MS analysis of 2-((2,5-dichloropyrimidin-4-yl)amino)-N-methylbenzamide

2-((5-chloro-2-((4-piperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)-N-methylbenzamide

2-((2,5-dichloropyrimidin-4-yl)amino)-N-methylbenzamide (1.776 g, 5.98 mmol) was dissolved in *sec*butanol (20 ml). To this was added *tert*-butyl 4-(4-aminophenyl)piperazine-1-carboxylate (1.656 g, 5.98 mmol) and 4M HCl in dioxane (10 drops). The reaction mixture was heated to 120°C for 18 h. The solvent was removed *in vacuo* and the residue was dissolved in DCM (10 ml) and TFA (10 ml). The reaction mixture was stirred at room temperature for 1 h and then the solvent was removed *in vacuo*. The residue was partitioned between ethyl acetate (50 ml) and saturated aqueous NaHCO₃ (50 ml). The aqueous phase was extracted with further ethyl acetate (2 x 50 ml) and the combined organic phases were dried over Na₂SO₄, filtered and evaporated. This afforded 2-((5-chloro-2-((4-piperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)-N-methylbenzamide (1.621 g, 62%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.60 (s, 1H), 9.24 (s, 1H), 8.84 – 8.64 (m, 2H), 8.15 (s, 1H), 7.73 (d, *J* = 7.6 Hz, 1H), 7.55 – 7.40 (m, 3H), 7.19 – 7.04 (m, 1H), 6.90 (d, *J* = 8.9 Hz, 2H), 3.16-3.12 (m, 4H), 3.07-3.03 (m, 4H), 2.79 (s, 3H). LC/MS (ESI, *m/z*) *t*_R = 3.68 min, 438, 440, ([M+H]⁺, Cl isotopic pattern).



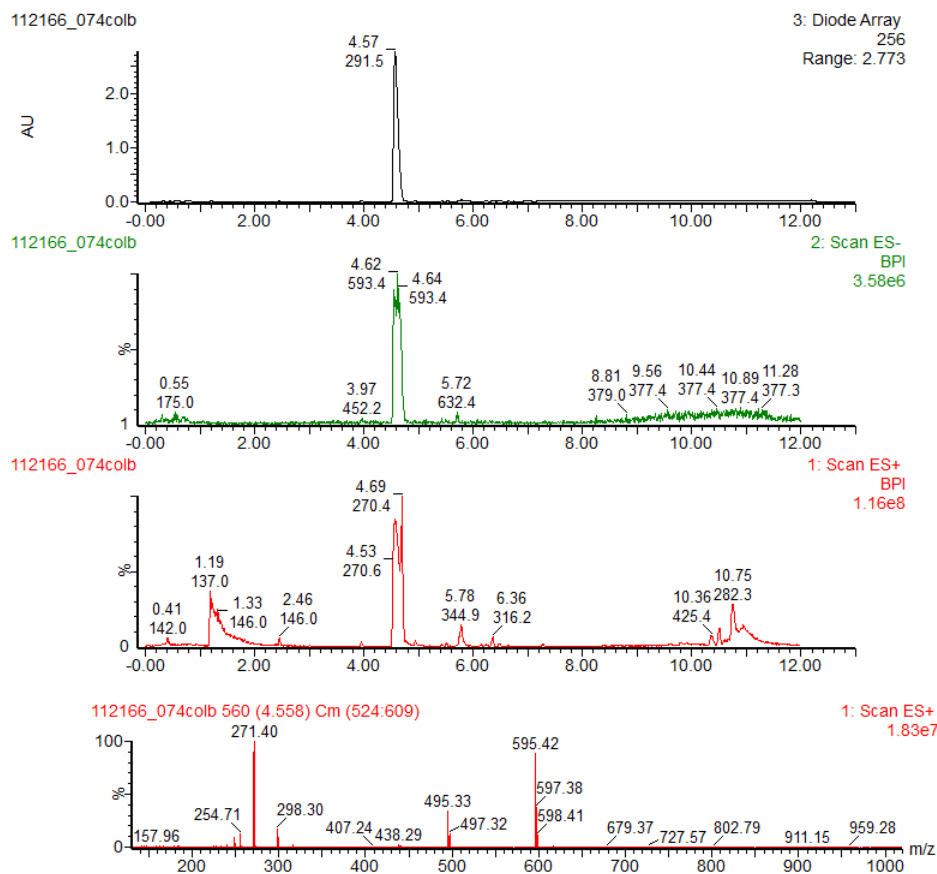
LC/MS analysis of 2-((5-chloro-2-((4-piperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)-N-methylbenzamide



^1H -NMR spectrum of 2-((5-chloro-2-((4-piperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)-*N*-methylbenzamide

Tert-butyl (3-(4-(4-((5-chloro-4-((2-(methylcarbamoyl)phenyl)amino)pyrimidin-2-yl)amino)phenyl)piperazin-1-yl)propyl)carbamate

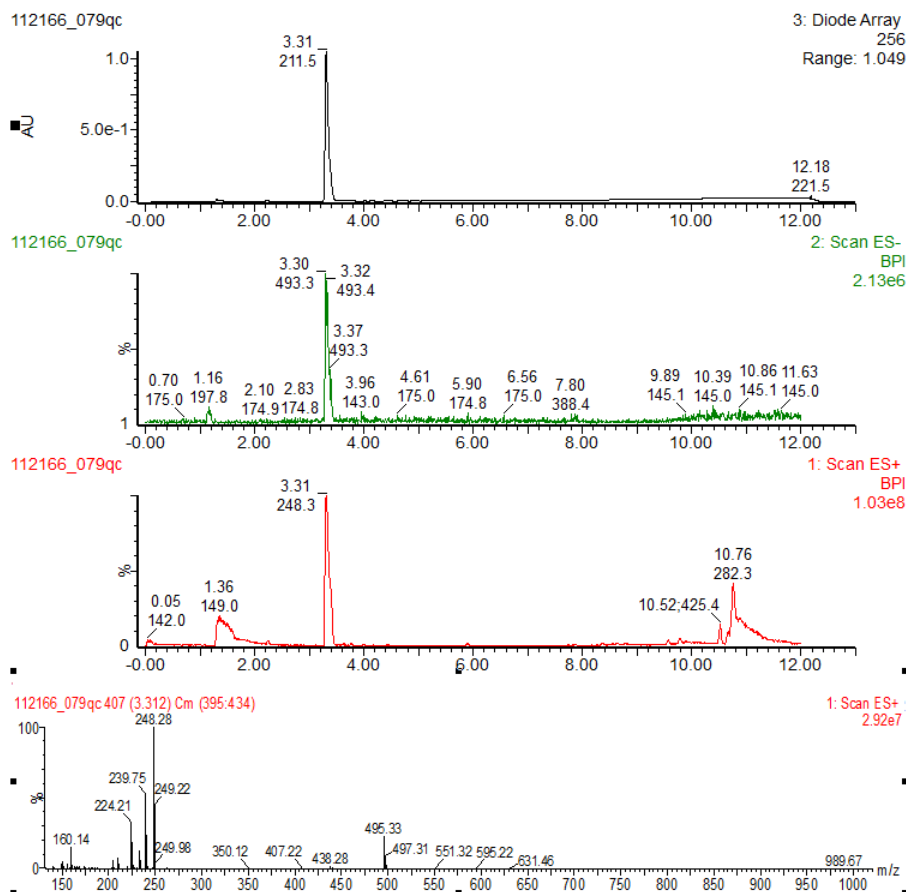
2-((5-chloro-2-((4-piperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)-*N*-methylbenzamide (1.621g, 3.71 mmol) was dissolved in methanol (20 ml). To this was added *tert*-butyl (3-oxopropyl)carbamate (0.705 g, 4.08 mmol) and acetic acid (0.1 ml, 1.85 mmol). The reaction mixture was stirred at room temperature for 15 min. before adding sodium cyanoborohydride (0.350 g, 5.56 mmol). The reaction mixture was stirred at room temperature for a further 4 h. This was then poured into ethyl acetate (100 ml) and washed with saturated aqueous NaHCO₃ (100 ml). The organic phase was then washed with water (2 x 100 ml), dried over Na₂SO₄, filtered and evaporated. The residue was purified by column chromatography using an automated biotage apparatus with a gradient of ethyl acetate in cyclohexane of 0-100%. This afforded *tert*-butyl (3-(4-(4-((5-chloro-4-((2-(methylcarbamoyl)phenyl)amino)pyrimidin-2-yl)amino)phenyl)piperazin-1-yl)propyl)carbamate (0.88 g, 40%). LC/MS (ESI, m/z) *t*_R = 4.57 min, 595, 597, ([M+H]⁺, Cl isotopic pattern).



LC/MS analysis of *Tert*-butyl (3-(4-(4-((5-chloro-4-((2-(methylcarbamoyl)phenyl)amino)pyrimidin-2-yl)amino)phenyl)piperazin-1-yl)propyl)carbamate

2-((2((4-(4-(3-aminopropyl)piperazin-1-yl)phenyl)amino)-5-chloropyrimidin-4-yl)amino)-N-methylbenzamide (GSK3182571)

Tert-butyl (3-(4-(4-((5-chloro-4-((2-(methylcarbamoyl)phenyl)amino)pyrimidin-2-yl)amino)phenyl)piperazin-1-yl)propyl)carbamate (0.88 g, 1.48 mmol) was dissolved in dioxane (7 ml) and to this 4 M HCl in dioxane (7 ml) was added. The reaction mixture was stirred at room temperature for 3 h. The solvent was removed *in vacuo* to afford 2-((2((4-(4-(3-aminopropyl)piperazin-1-yl)phenyl)amino)-5-chloropyrimidin-4-yl)amino)-*N*-methylbenzamide.HCl. (0.72 g, 98%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.02 (s, 1H), 11.17 (s, 1H), 9.82 (s, 1H), 8.86 (s, 1H), 8.62 (s, 1H), 8.26 (s, 1H), 8.09 (s, 2H), 7.79 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.43-7.47 (m Hz, 3H), 7.16-7.18 (m, 1H), 7.00 (d, *J* = 9.2, 2H), 3.77 (d, *J* = 11.0 Hz, 2H), 3.55 (s, 3H), 3.29 – 3.03 (m, 6H), 2.95-2.90 (m, 2H), 2.79 (d, *J* = 4.4 Hz, 2H), 2.11-2.04 (m, 2H). LC/MS (ESI, *m/z*) *t*_R = 3.31 min, 495, 497, ([M+H]⁺, Cl isotopic pattern).



LC/MS analysis of 2-((2((4-(4-(3-aminopropyl)piperazin-1-yl)phenyl)amino)-5-chloropyrimidin-4-yl)amino)-*N*-methylbenzamide (GSK3182571)



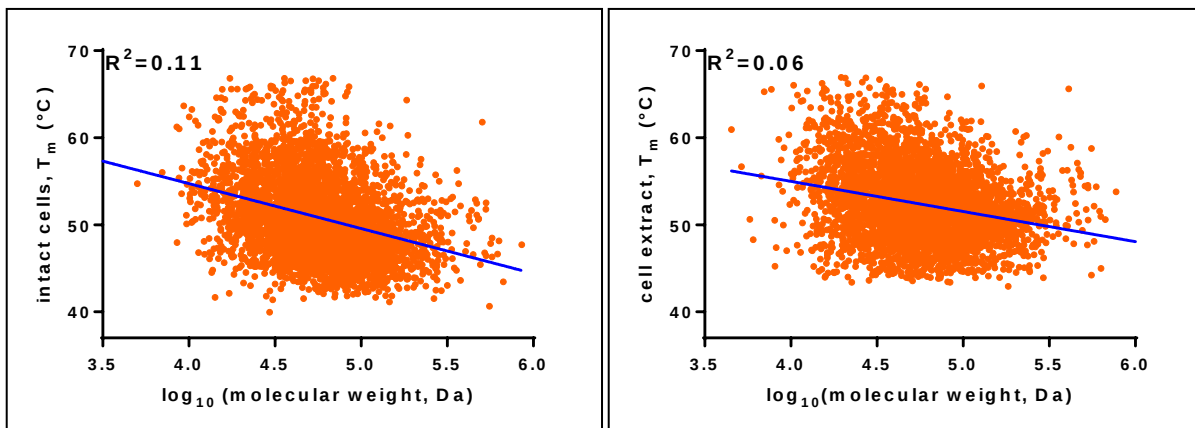


Fig. S1. T_m values show a weak but significant anti-correlation with protein molecular weight in both intact cells and cell extract.

Correlation of T_m values and protein molecular weight for intact cells data (left), and cell extract data (right). A weak but significant (p-value <<0.01 in both cases, correlation test) anti-correlation trend is observed.

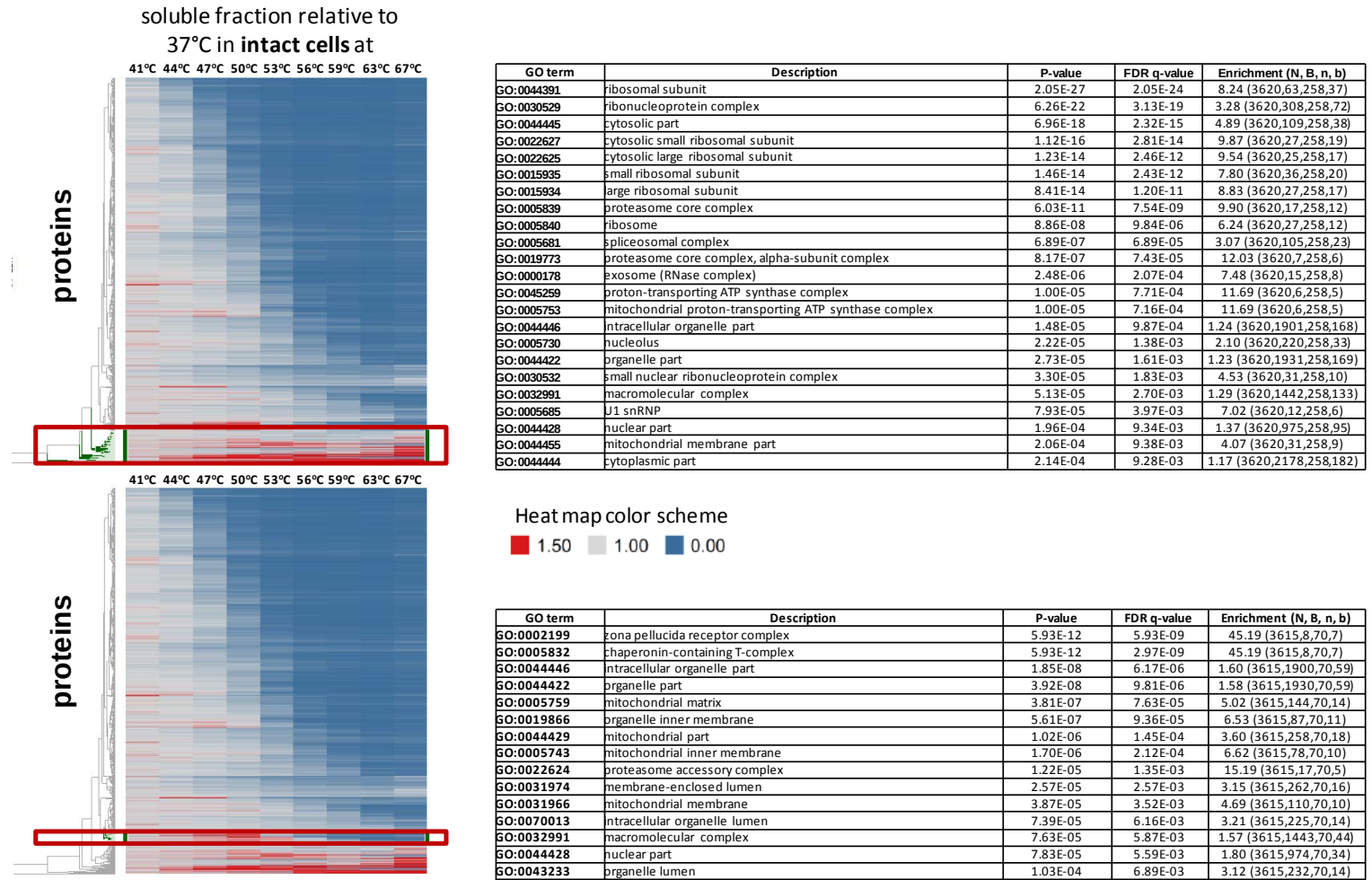


Fig. S2. Proteins exhibiting a transient concentration increase with temperature are annotated to be part of organelles or large protein assemblies.

The web tool Gorilla (cbl-gorilla.cs.technion.ac.il/) was used to identify enriched GO terms in the two clusters in the intact cells melting proteome heat map where proteins had an upward going trend during the whole or the first half of the heating phase, using all the proteins in the intact cells heat map as background. GO terms (cellular component) corresponding to large protein assemblies were strongly enriched in the cluster with the strongest upward going protein trend (upper panel). Different organelle terms including mitochondria were enriched in the second cluster where the upward going trend was broken at around 50°C (lower panel).

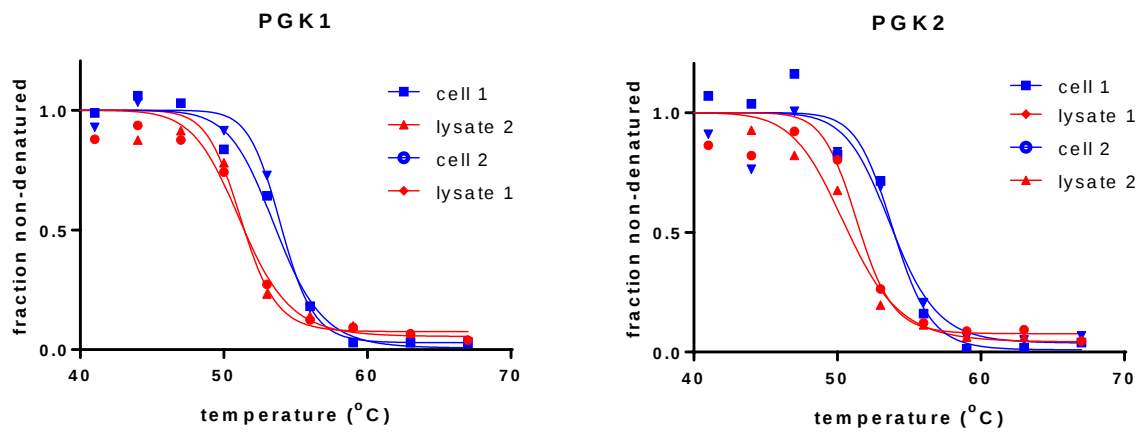


Fig. S3. Phosphoglycerate kinase 1 (PGK1) and 2 (PGK2) show an apparent stabilization in intact cells compared to cell extract

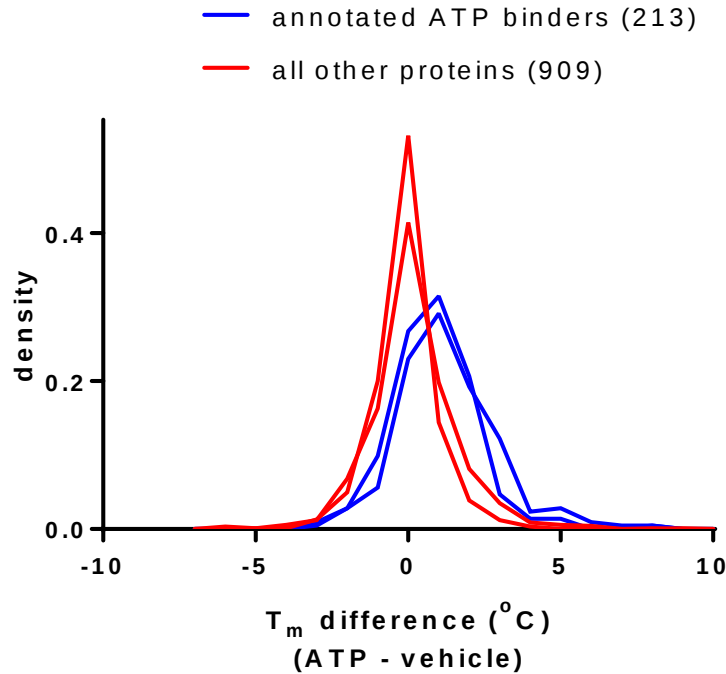


Fig. S4. Proteins annotated as ATP binders are stabilized in cell extract with MgATP added at 2 mM as compared to vehicle.

The plots show density distributions of differences in protein T_m in cell extract with added MgATP compared to vehicle. A set of 213 proteins annotated as ATP binders, reproducibly identified with proper melting curves (see Methods section) in two replicate experiments, shows a significant shift in the mean of its T_m difference distribution in both experiments (mean 0.88, 1.28, SEM 0.093, 0.112, p-value for the mean being greater than zero is <0.01 in both cases, t-test) towards increased apparent stability in cell extract with MgATP compared to vehicle. Also the means of the distributions of the T_m shifts of ATP binding proteins is significantly different compared to the means of the distribution of the T_m shifts of the 909 non-ATP binding proteins reproducibly identified with proper melting curves (see Methods section) in the two replicates (ATP binders: mean 0.88, 1.28, SEM 0.093, 0.112, non ATP binders: mean -0.097, 0.19 SEM 0.036, 0.047, p-value for significance in difference between the means of the distributions of ATP binders and non ATP binders is <0.01 in both cases, t-test).

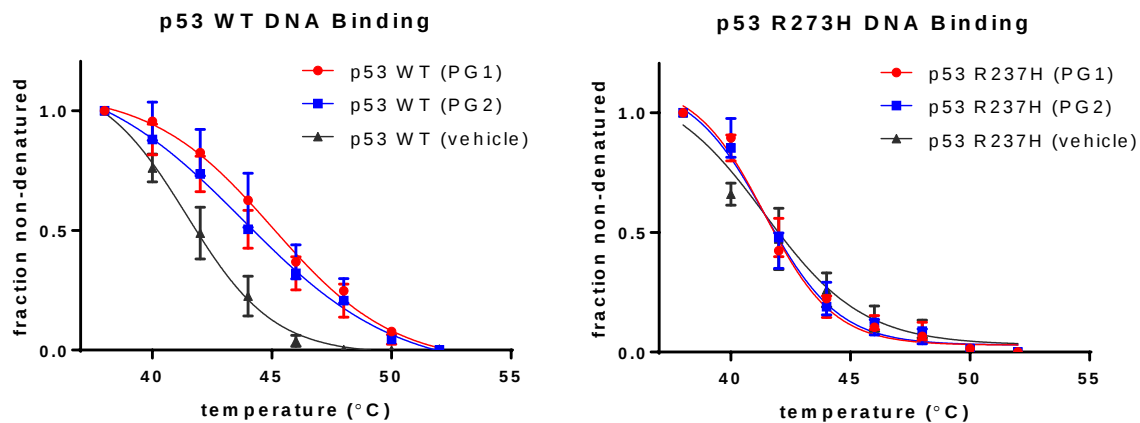


Fig. S5. p53 with two cognate duplex-DNA oligonucleotides (PG1; red, and PG2; blue) added to A549 cell extract containing wild-type p53 and HT-29 cells containing the p53 R273H mutant.

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. N=3; error bars indicate SEM.

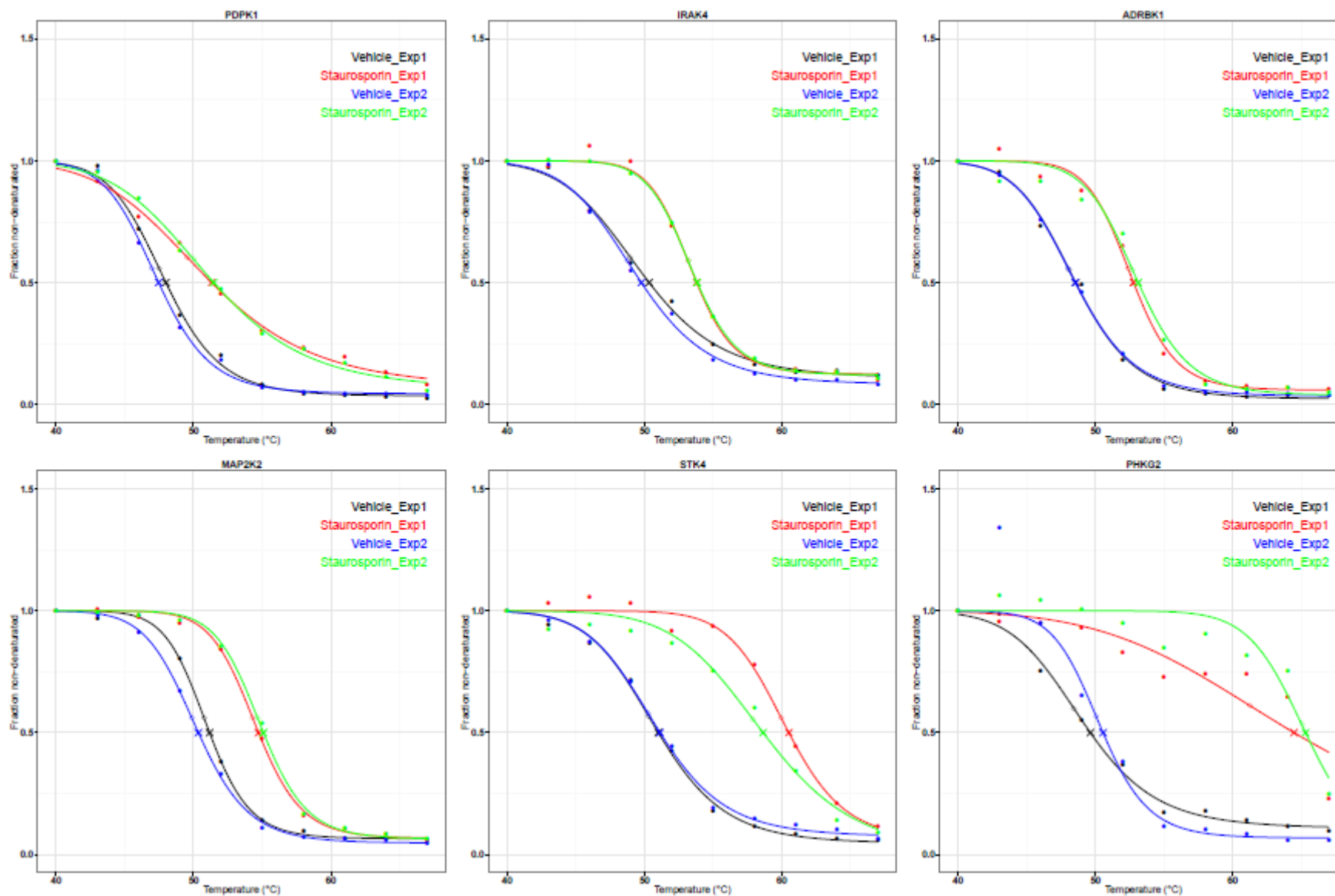


Fig. S6a. Examples of melting curves for proteins affected by staurosporine.

Panel of melting curves for all 60 proteins, that yielded a significant T_m shift upon treatment with 20 μ M staurosporine in thermal profiling experiments in cell extract.

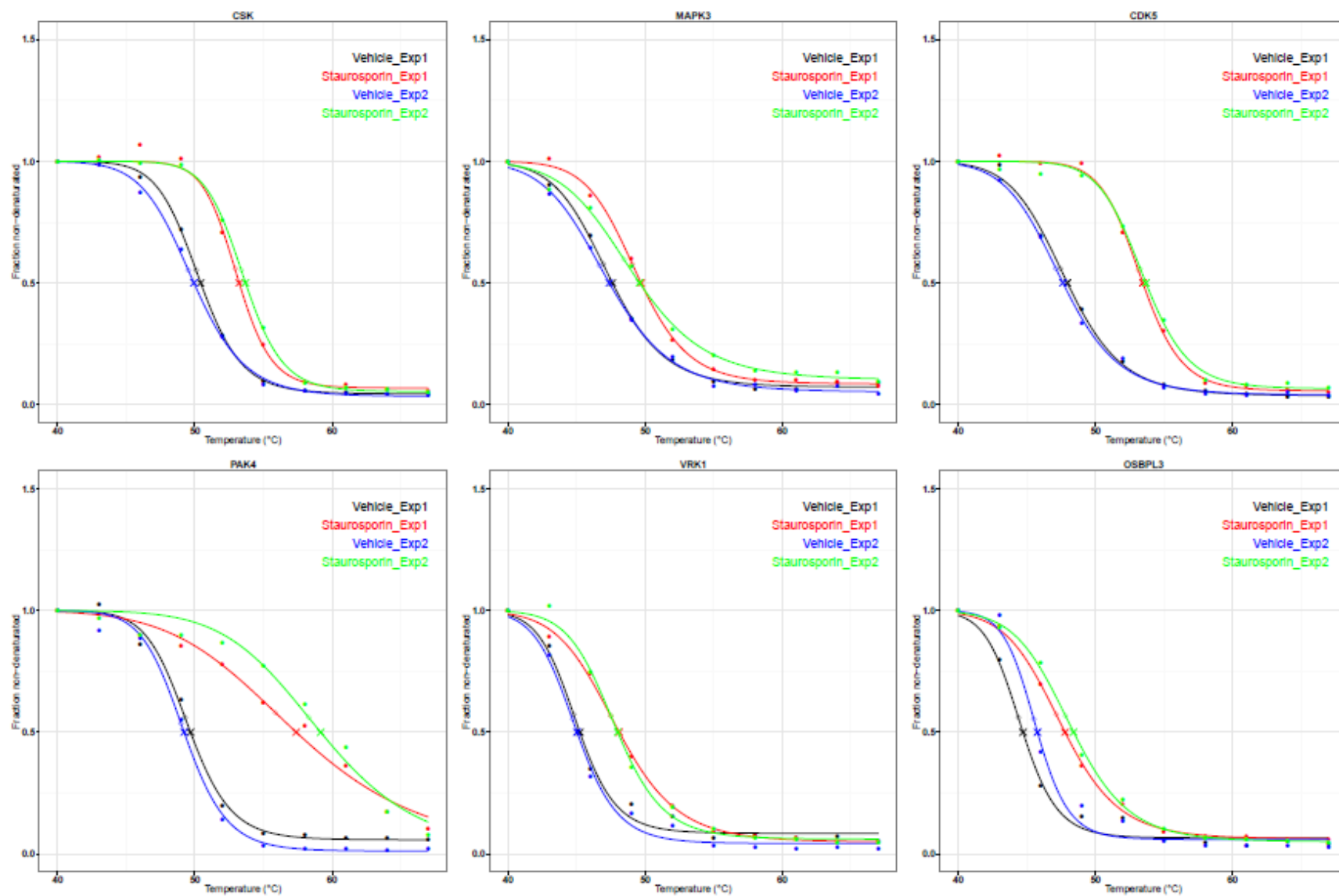
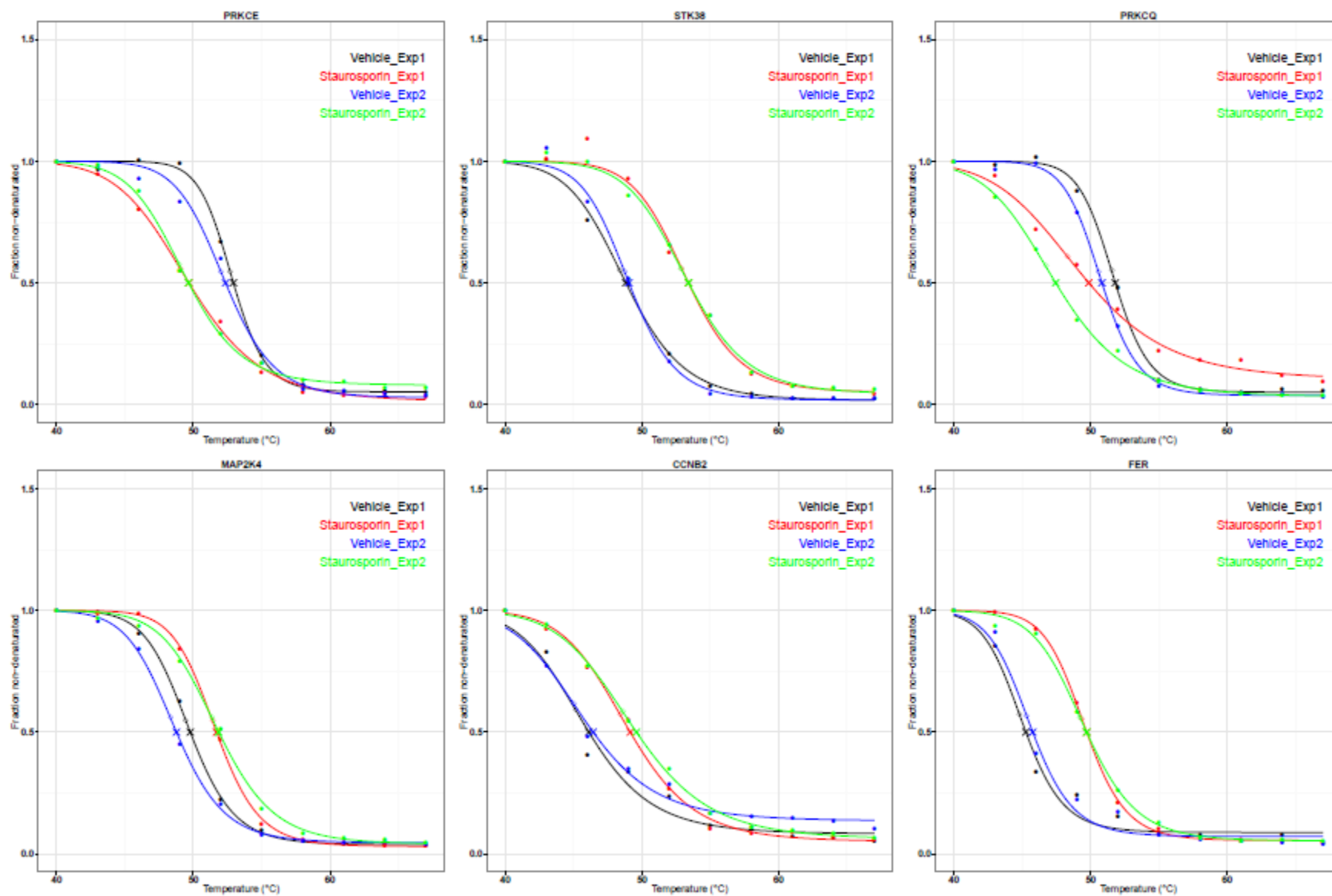
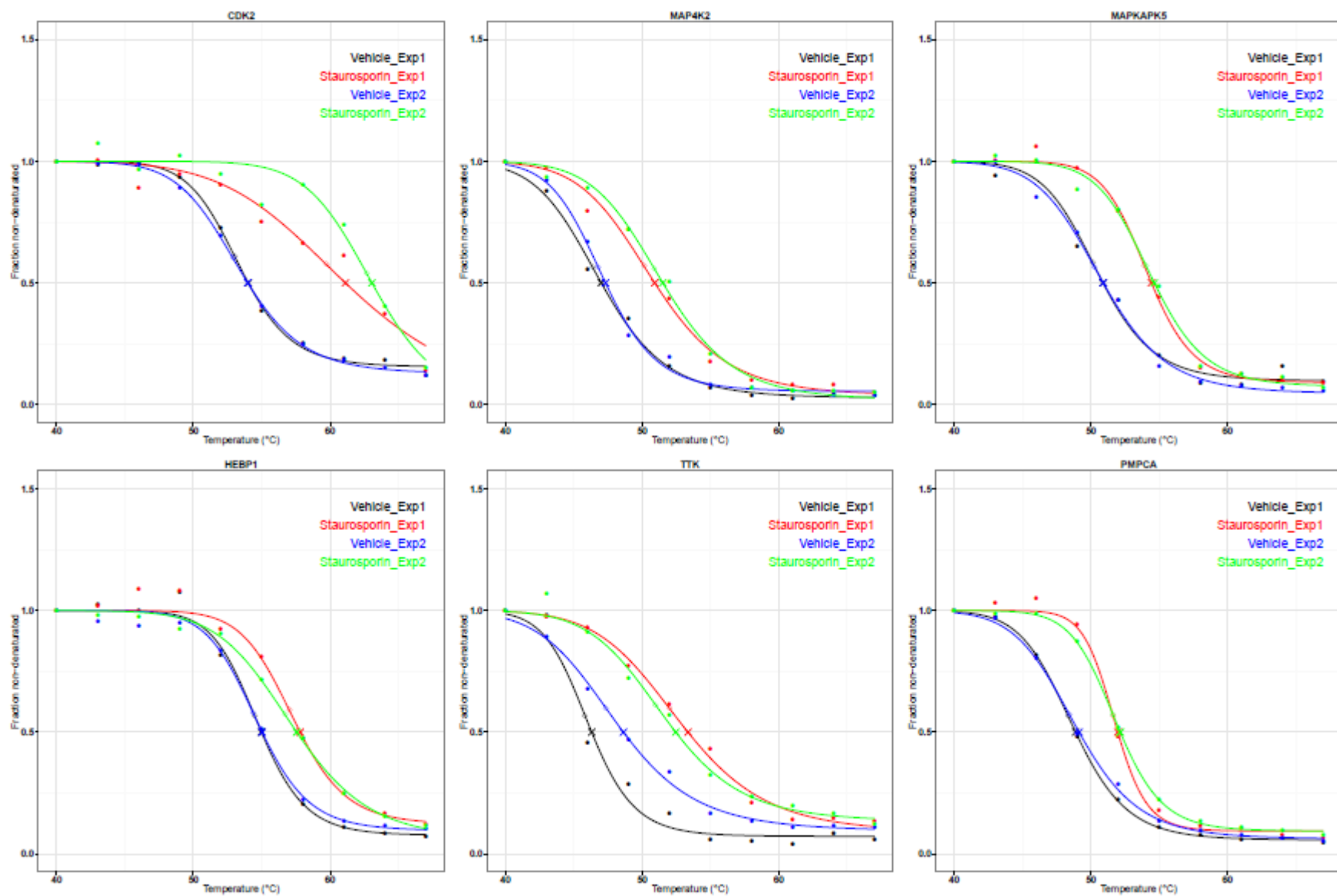


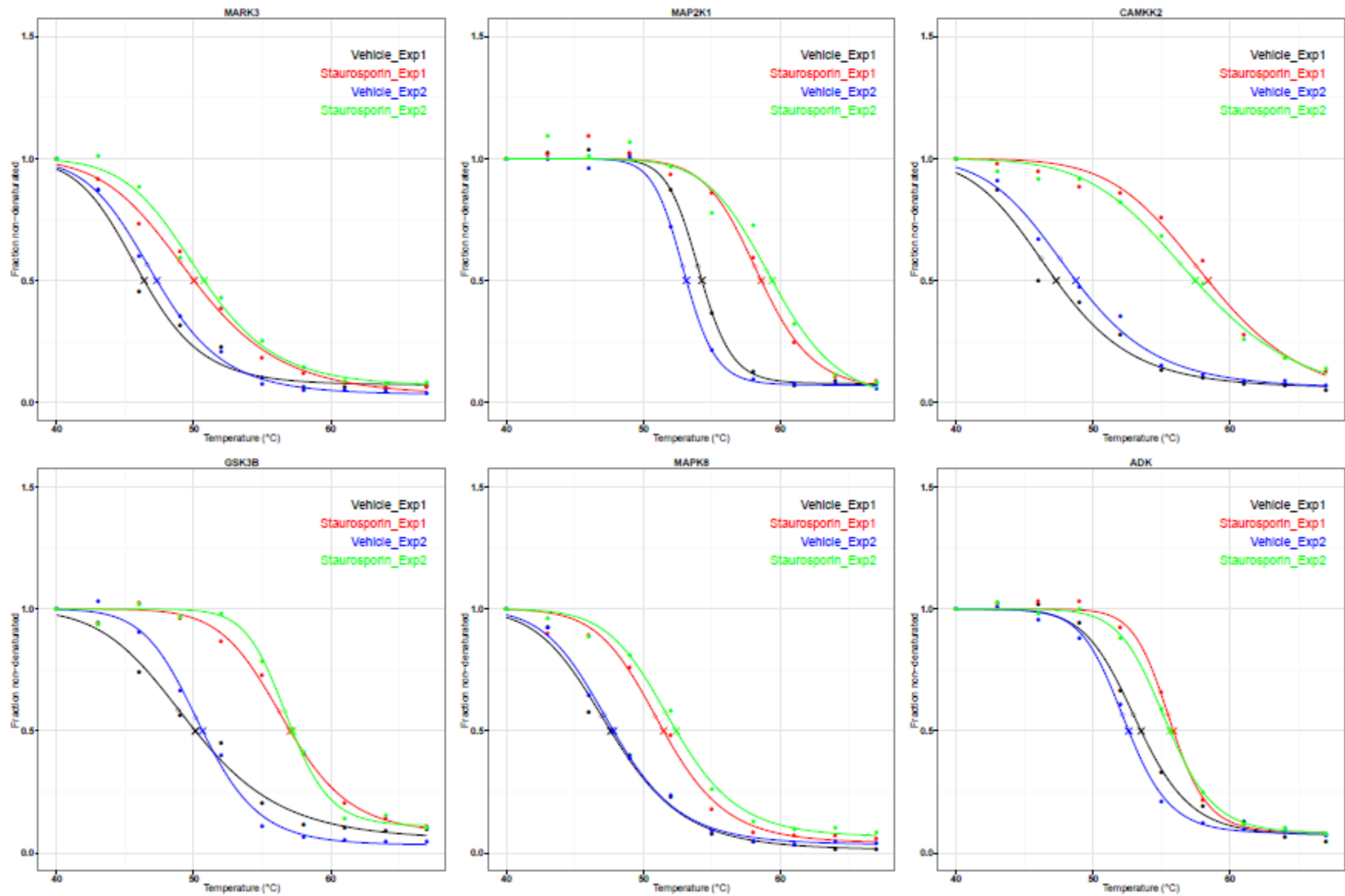
Fig. S6b. Examples of melting curves for proteins affected by staurosporine. Fig.



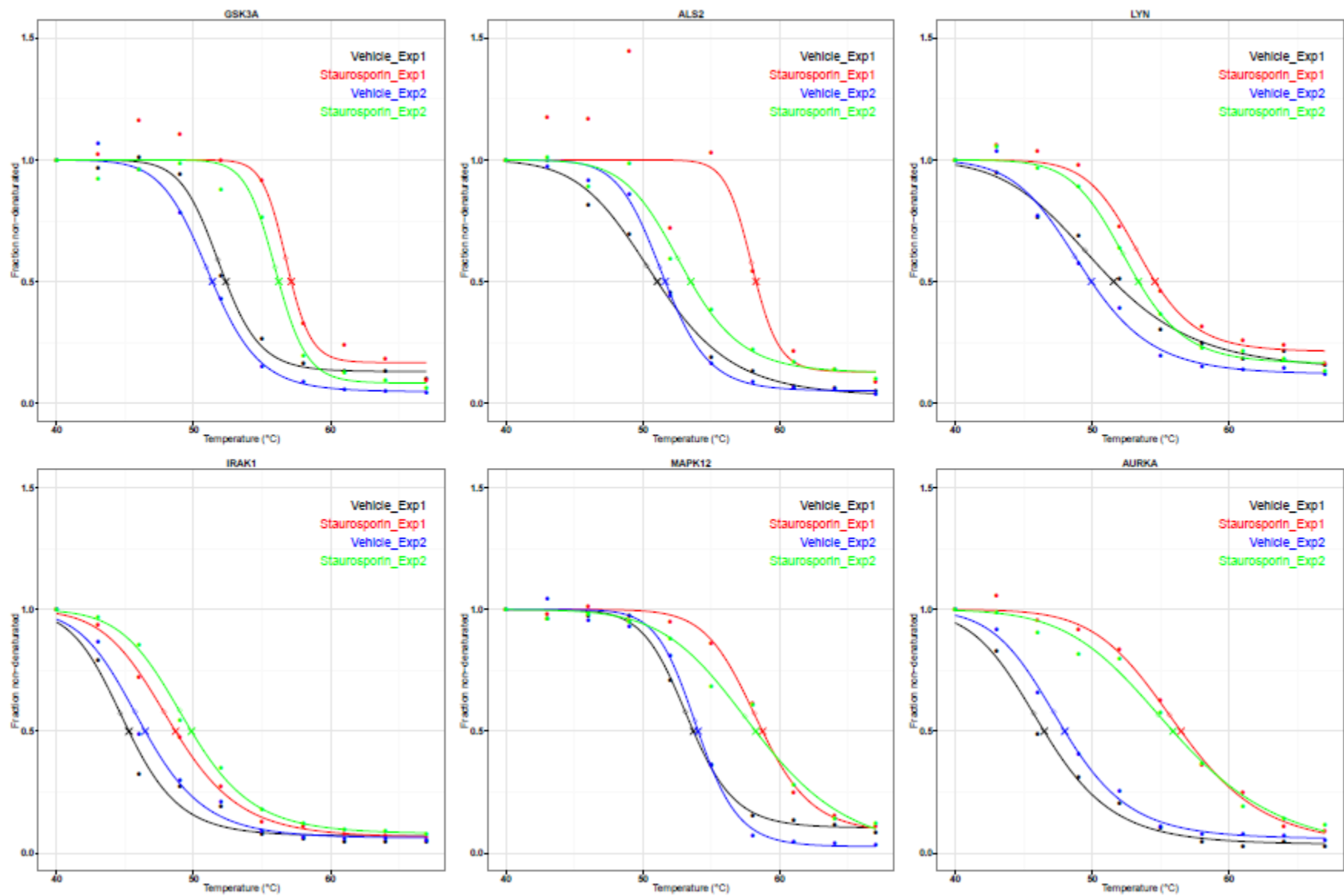
S6c. Examples of melting curves for proteins affected by staurosporine



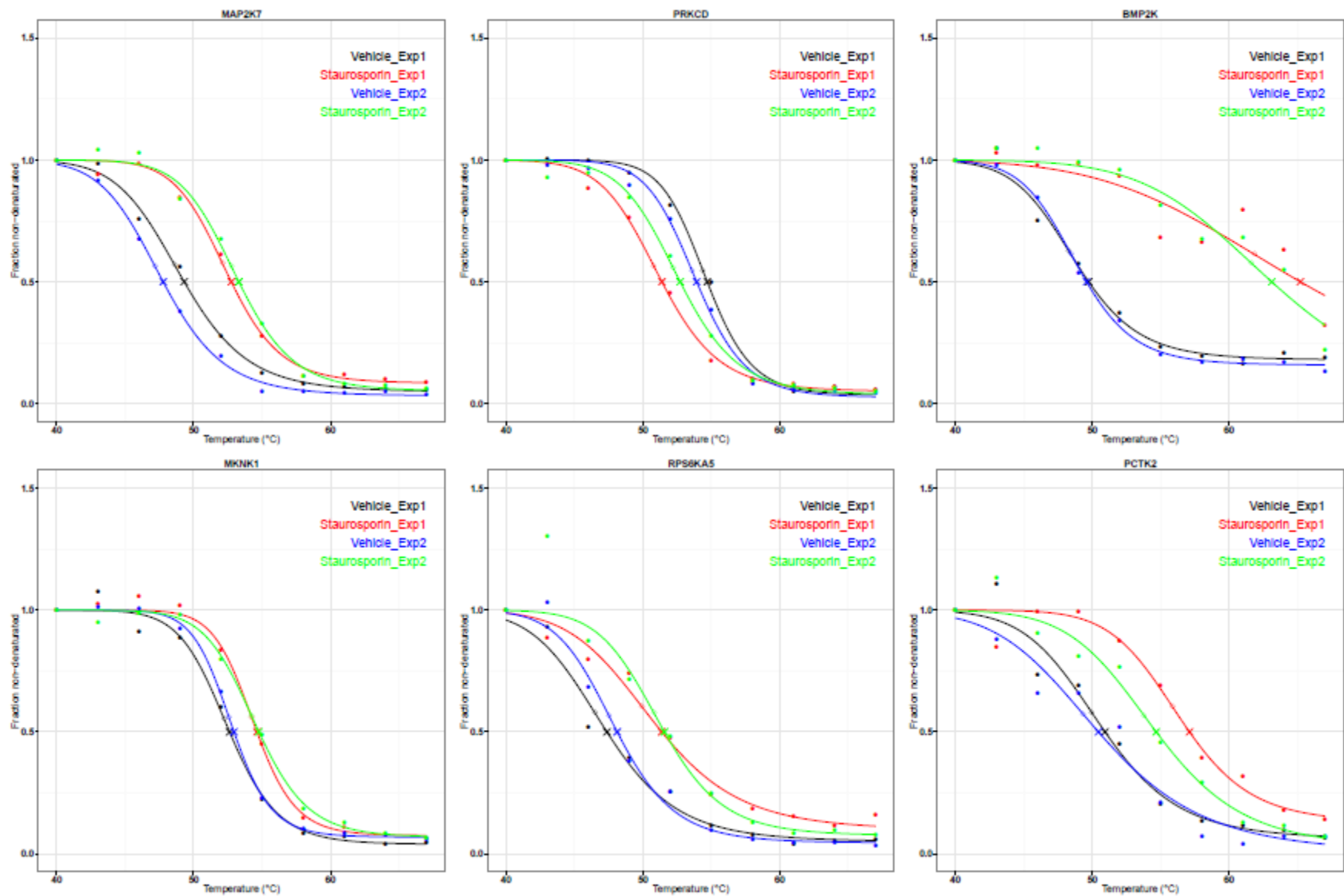
S6d. Examples of melting curves for proteins affected by staurosporine



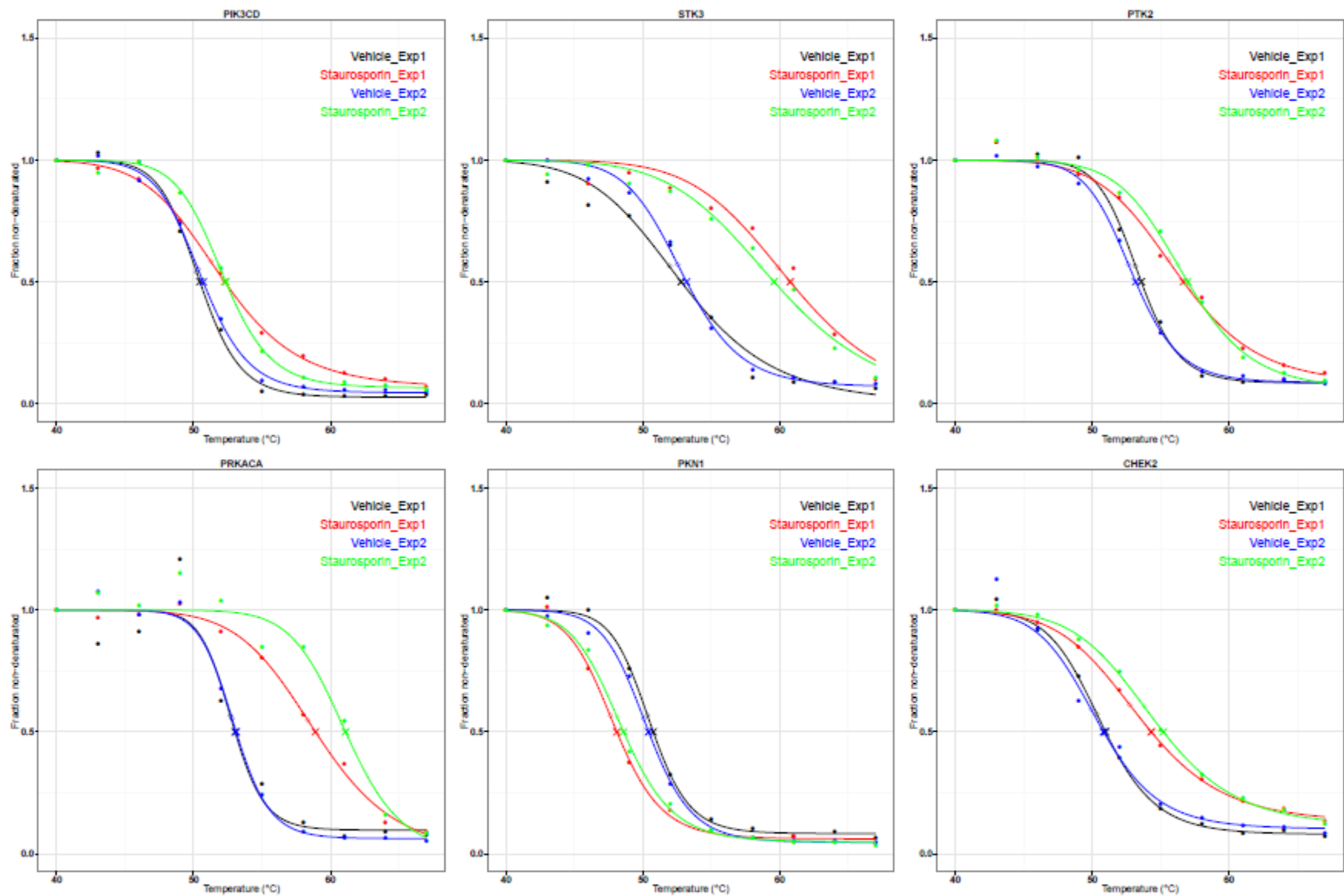
S6e. Examples of melting curves for proteins affected by staurosporine



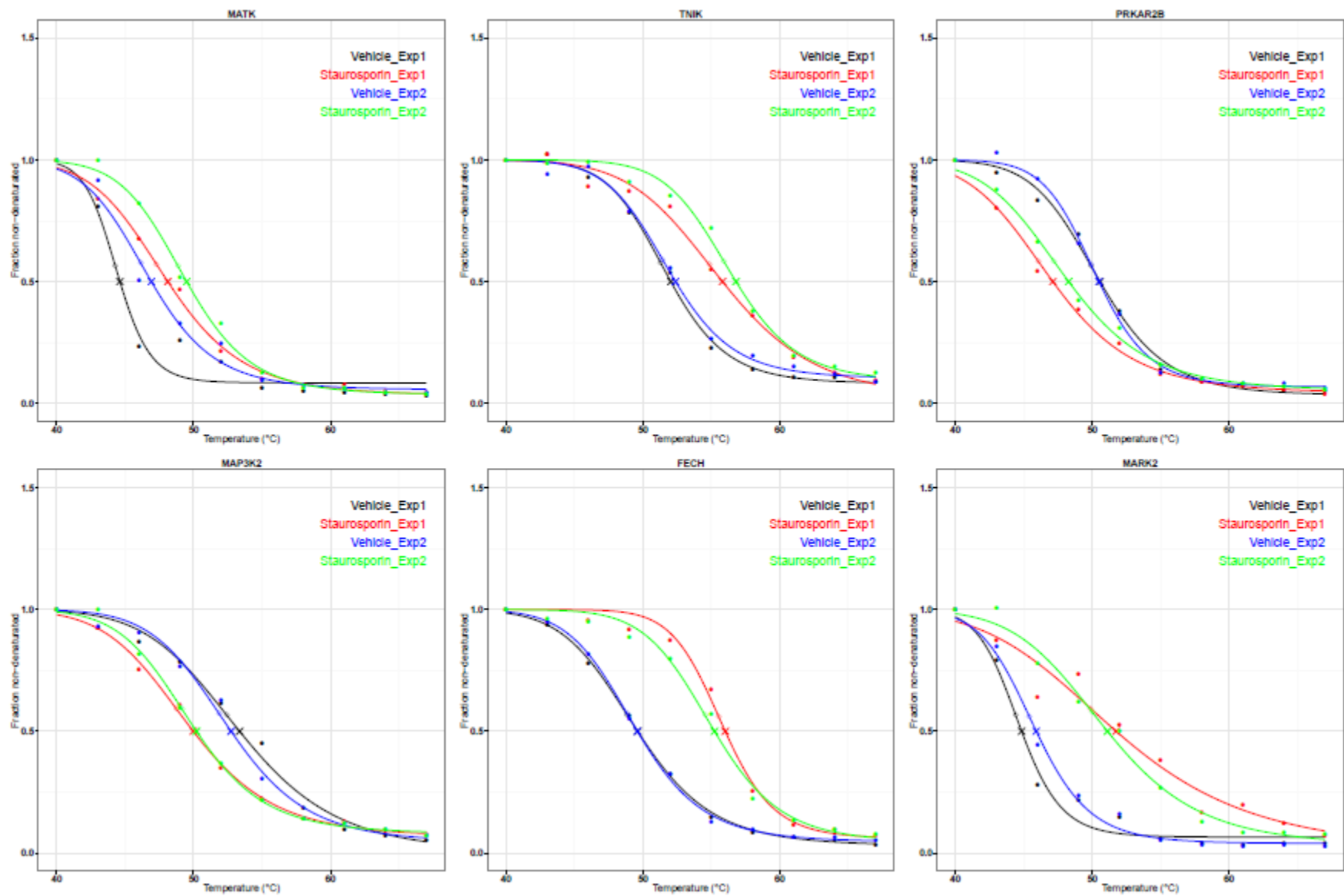
S6f. Examples of melting curves for proteins affected by staurosporine



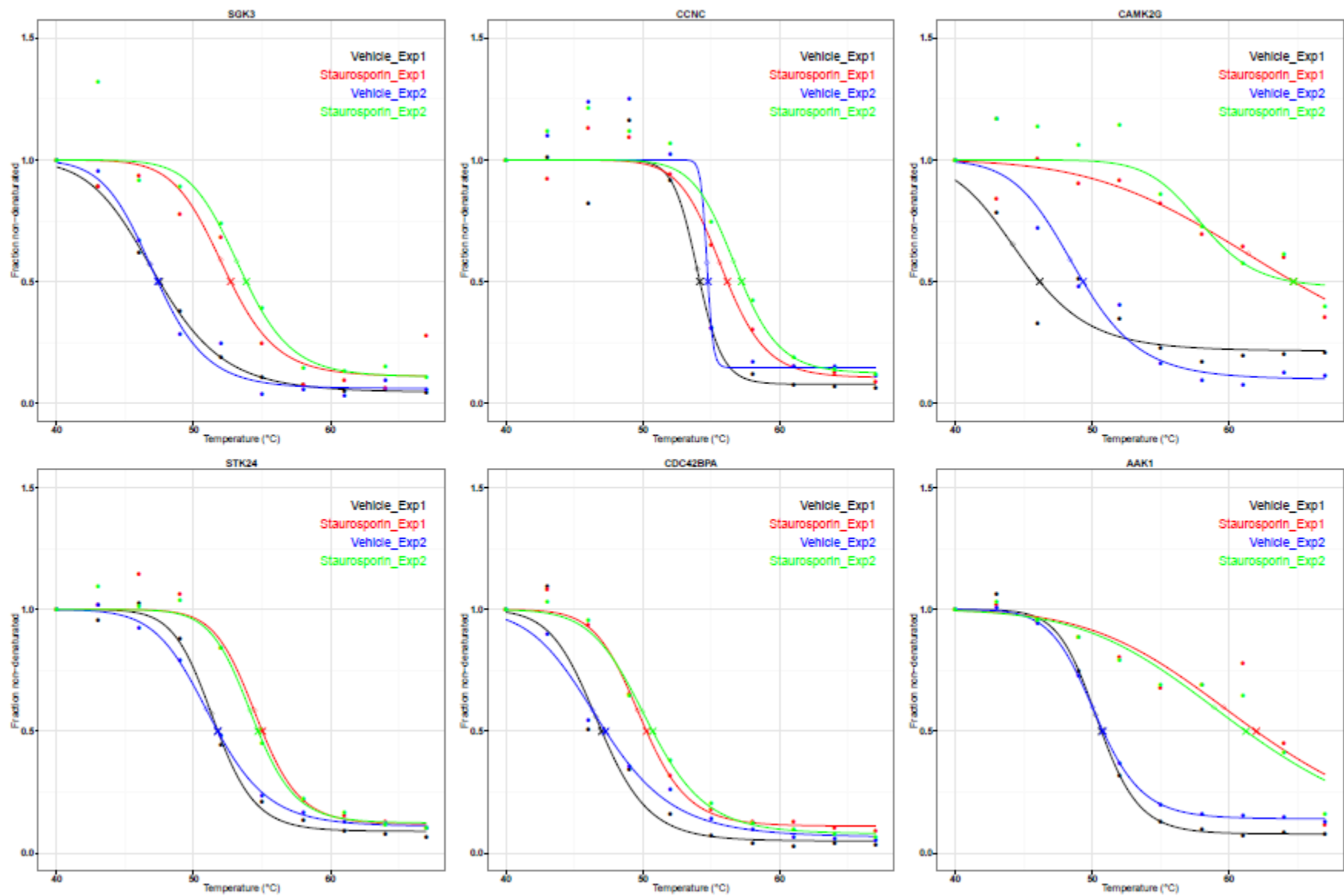
S6g. Examples of melting curves for proteins affected by staurosporine



S6h. Examples of melting curves for proteins affected by staurosporine



S6i. Examples of melting curves for proteins affected by staurosporine



S6j. Examples of melting curves for proteins affected by staurosporine

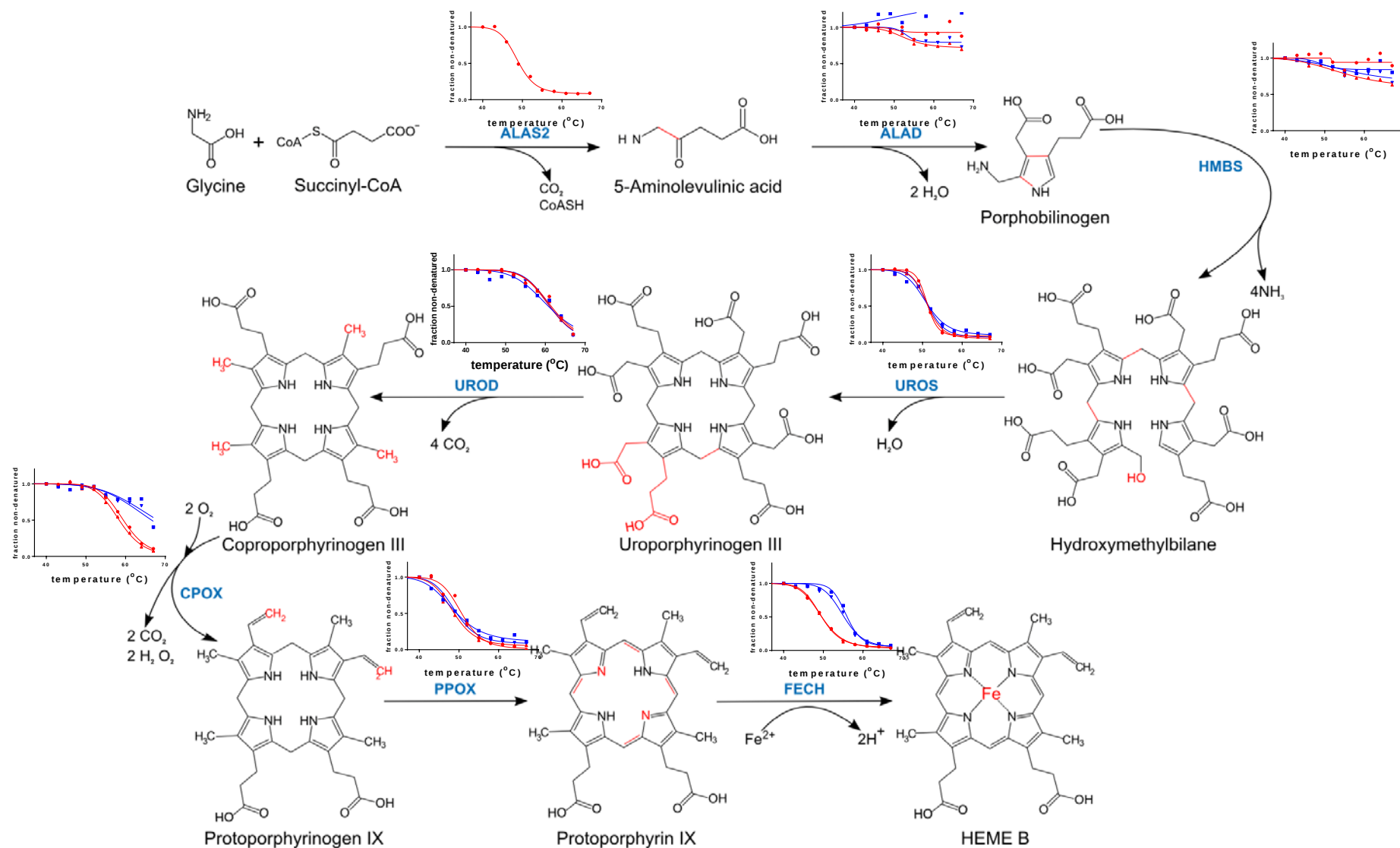


Fig. S7. Melting curves for the enzymes in the heme biosynthesis pathway in the absence (red) and presence (blue) of staurosporine in K562 cell extract.

The graphs show data from independent replicates. Staurosporine stabilized coproporphyrinogen-III oxidase and ferrochelatase. Ala synthase (ALAS2) was only detected in one of the four datasets.

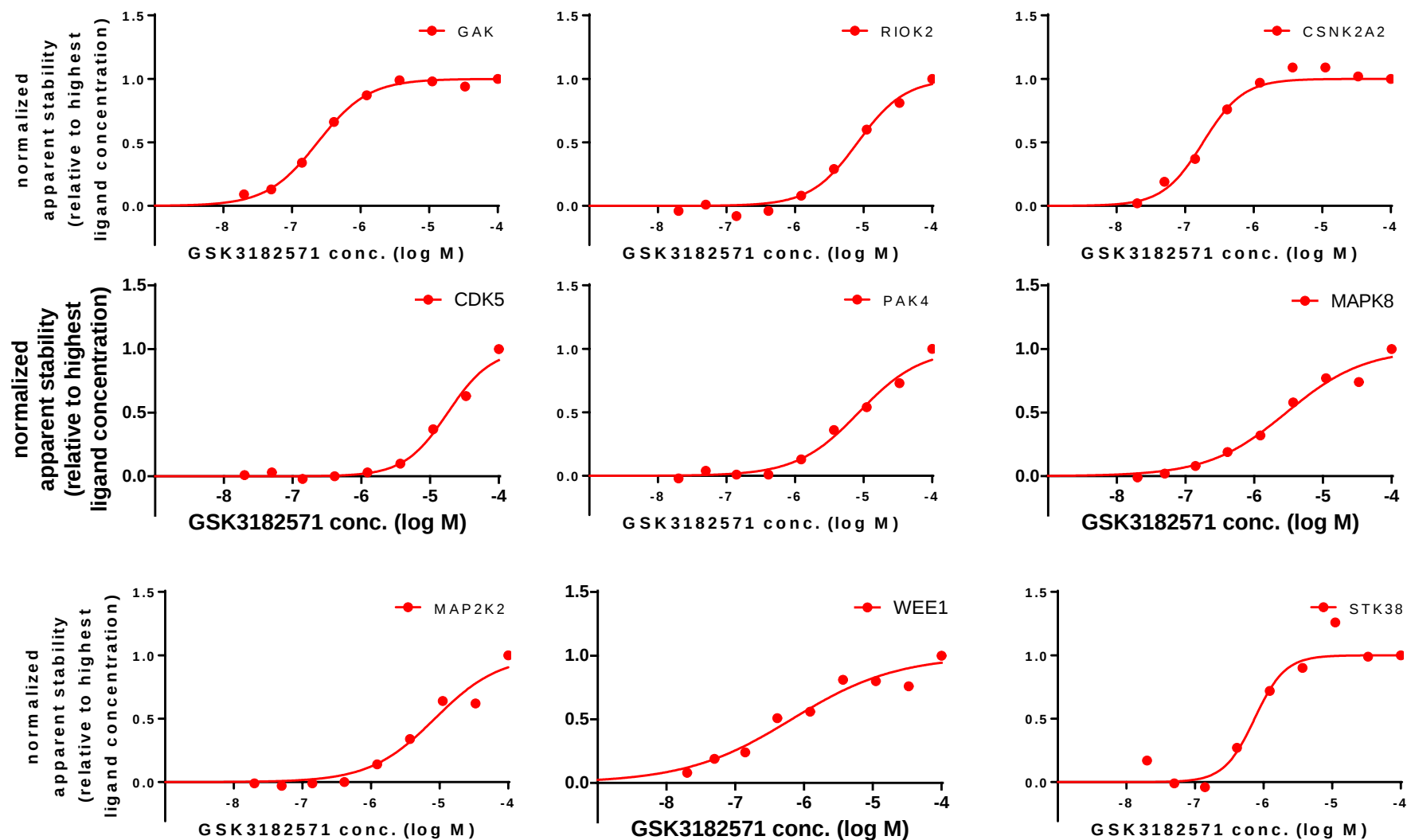


Fig. S8a. Isothermal Dose Response (ITDR) profiles for GSK3182571 at 53°C.

The panels show ITDR curves for all 27 proteins stabilized and the 4 proteins destabilized by GSK3182571 at 53°C.

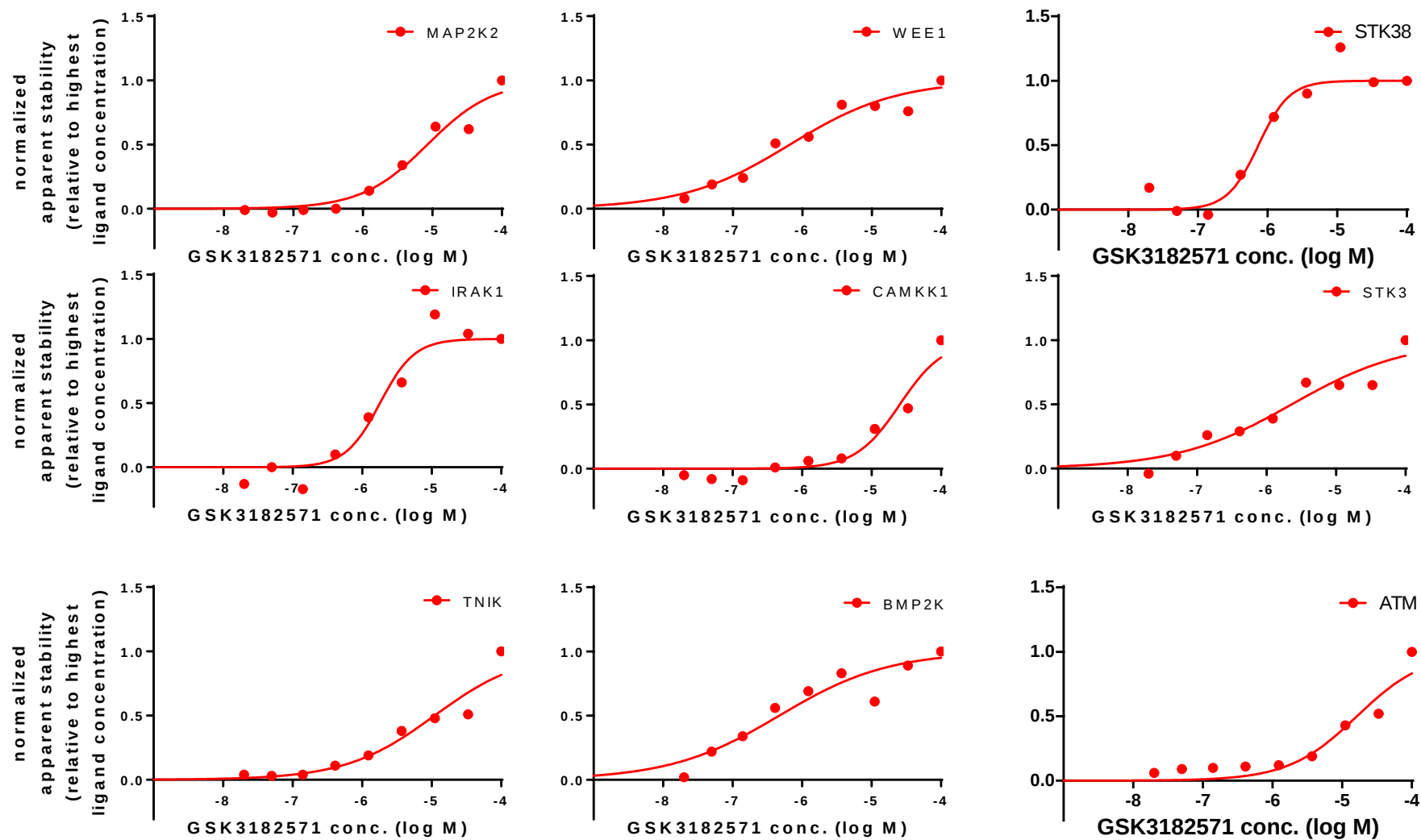


Fig. S8b. Isothermal Dose Response (ITDR) profiles for GSK3182571 at 53°C.

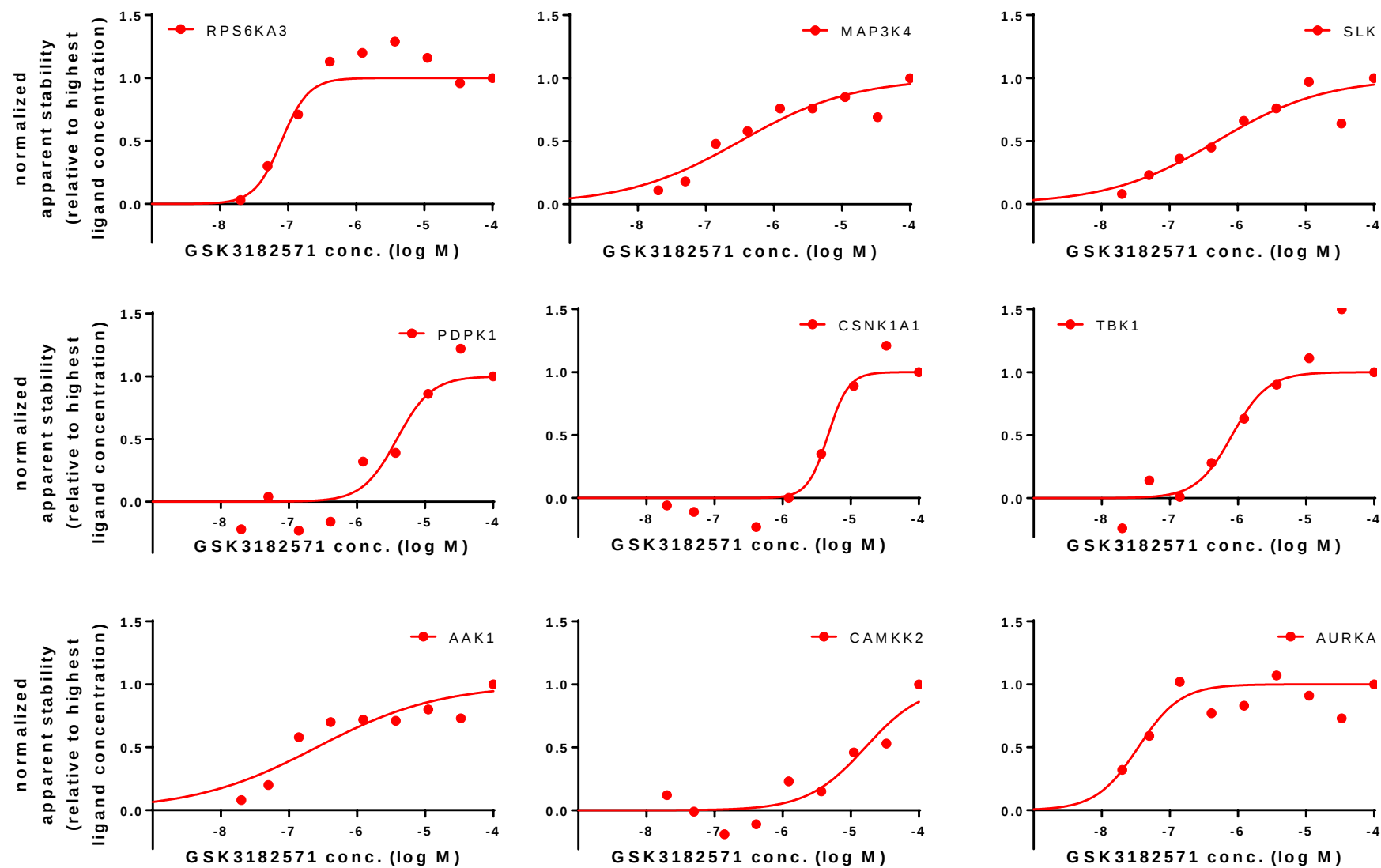


Fig. S8c. Isothermal Dose Response (ITDR) profiles for GSK3182571 at 53°C.

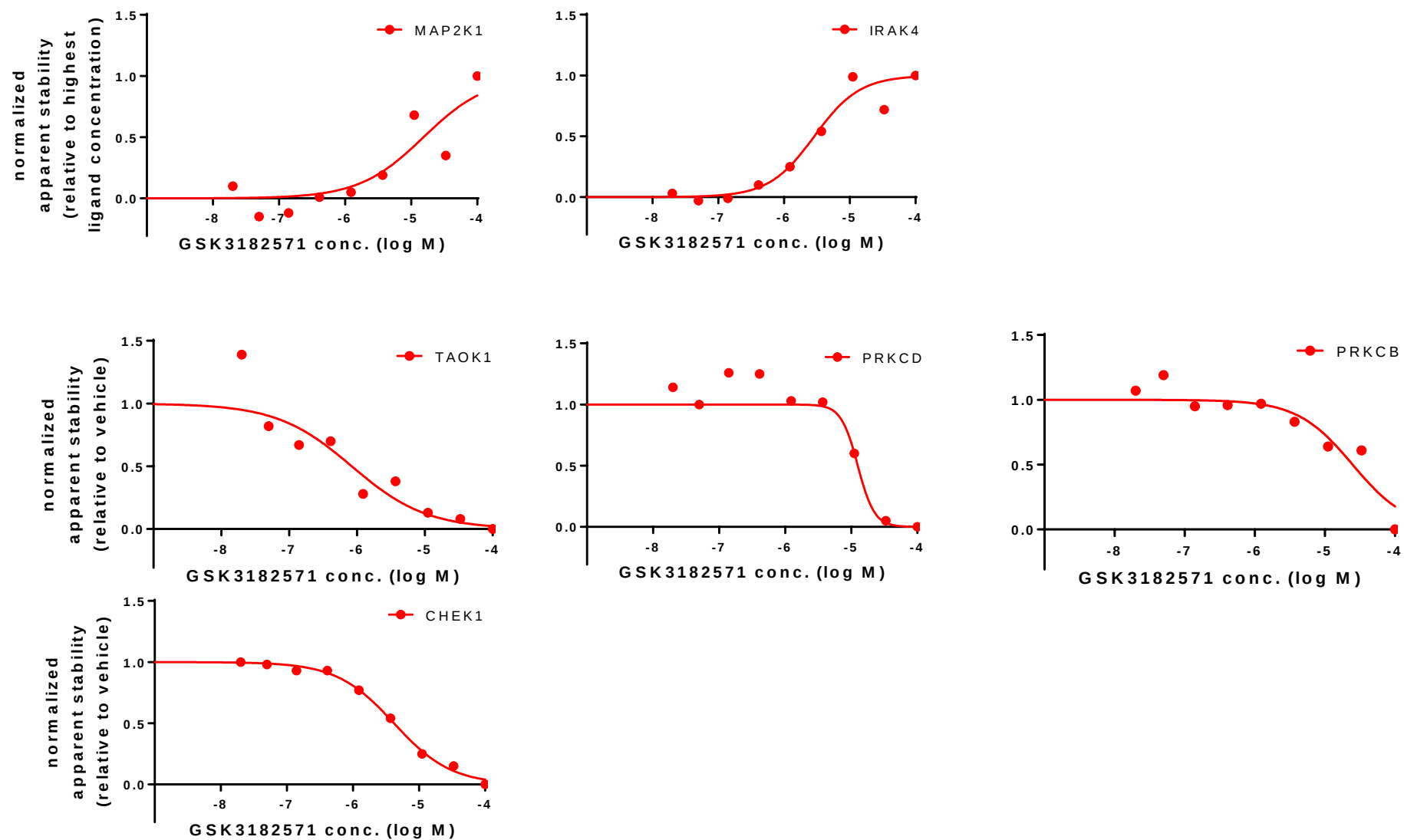


Fig. S8d. Isothermal Dose Response (ITDR) profiles for GSK3182571 at 53°C.

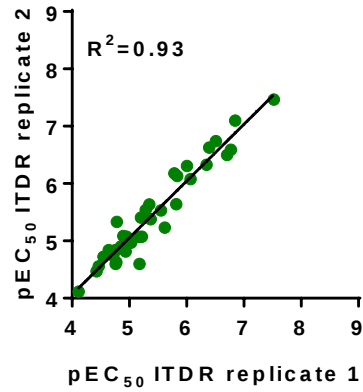


Figure S8e. pEC₅₀ values derived from curve fits fulfilling the specified requirements (see methods section) reveal excellent correlation between biological replicates of isothermal dose-response profiles of GSK3182571

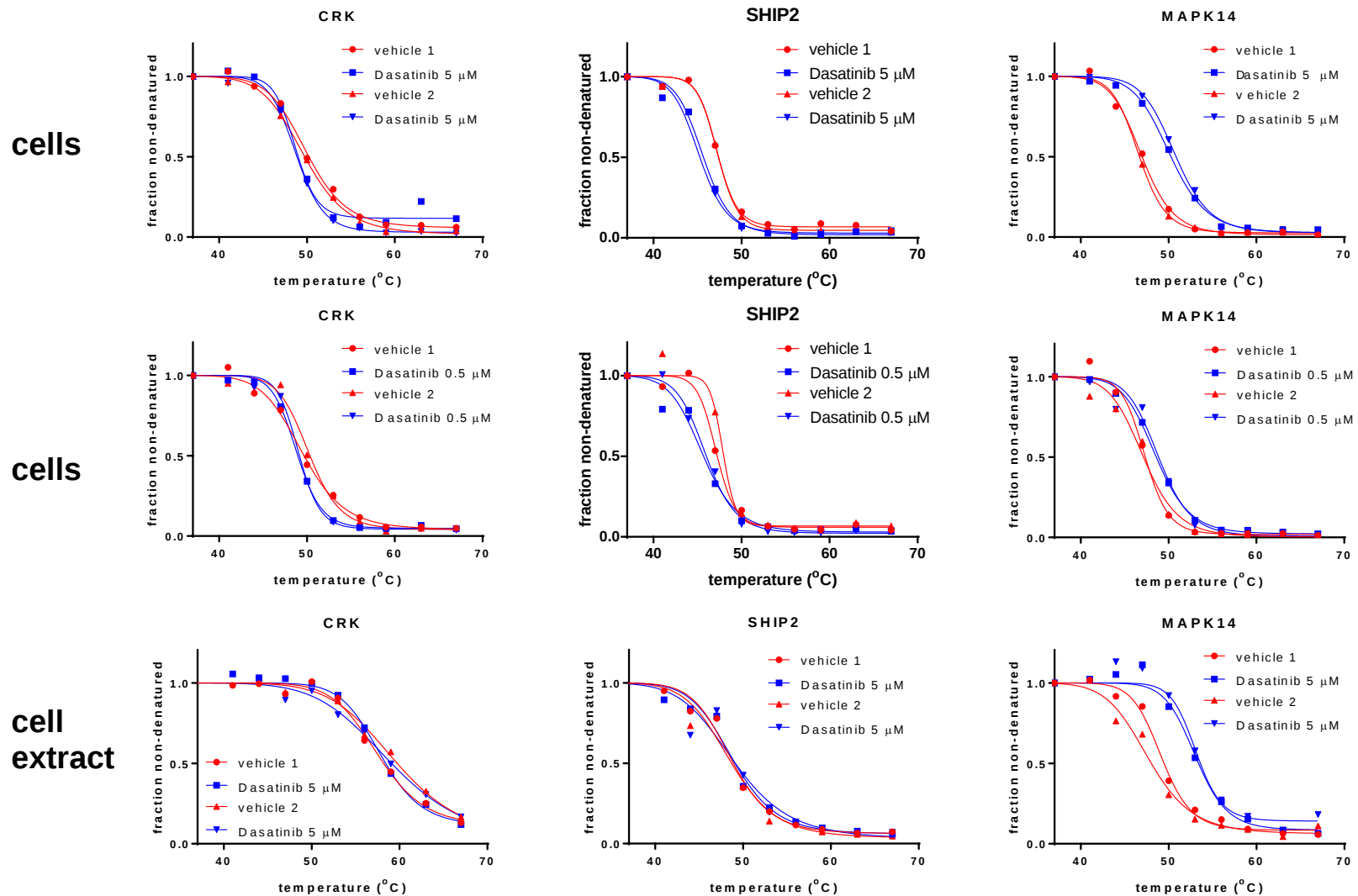


Fig. S9. Dasatinib induces reproducible T_m shifts or changes in the slope of the melting curve also for the indirect targets CRK and SHIP2 and the direct target MAPK14.

Treatment of cells with dasatinib at either 0.5 μM or 5 μM results in a change in the melting curve slope or T_m shift for the two indirect BCR-ABL pathway targets CRK and SHIP2 (INPPL1) whereas no shift can be observed for the two proteins when the experiment is performed in cell extract. However, the direct target MAPK14/p38 α is shifted in both the cell extract and intact cells experiments. The T_m shift in intact cells is more pronounced for the 5 μM dasatinib experiment compared to 0.5 μM .

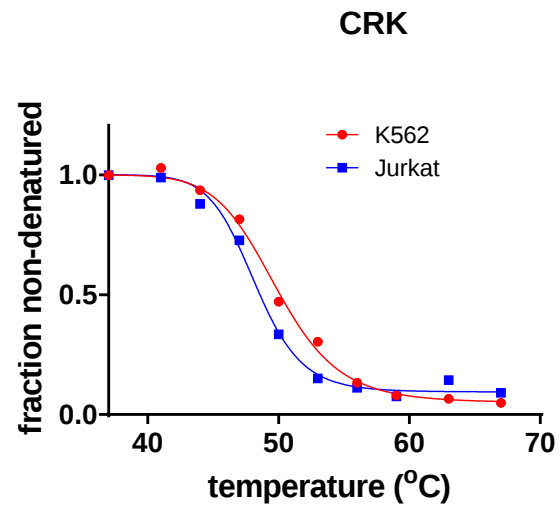
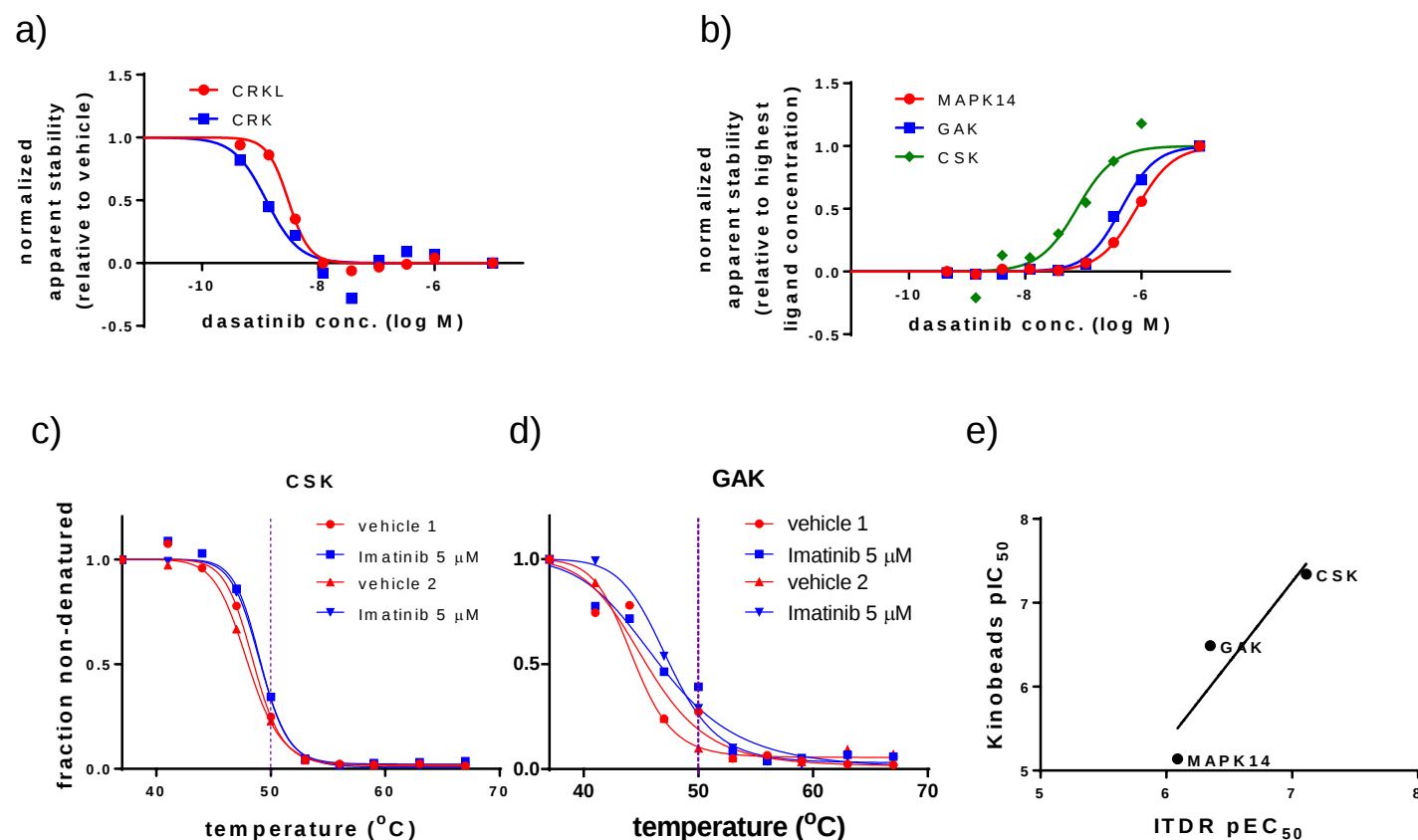


Fig. S10. Comparison of melting curves for CRK between Jurkat and K562 cells



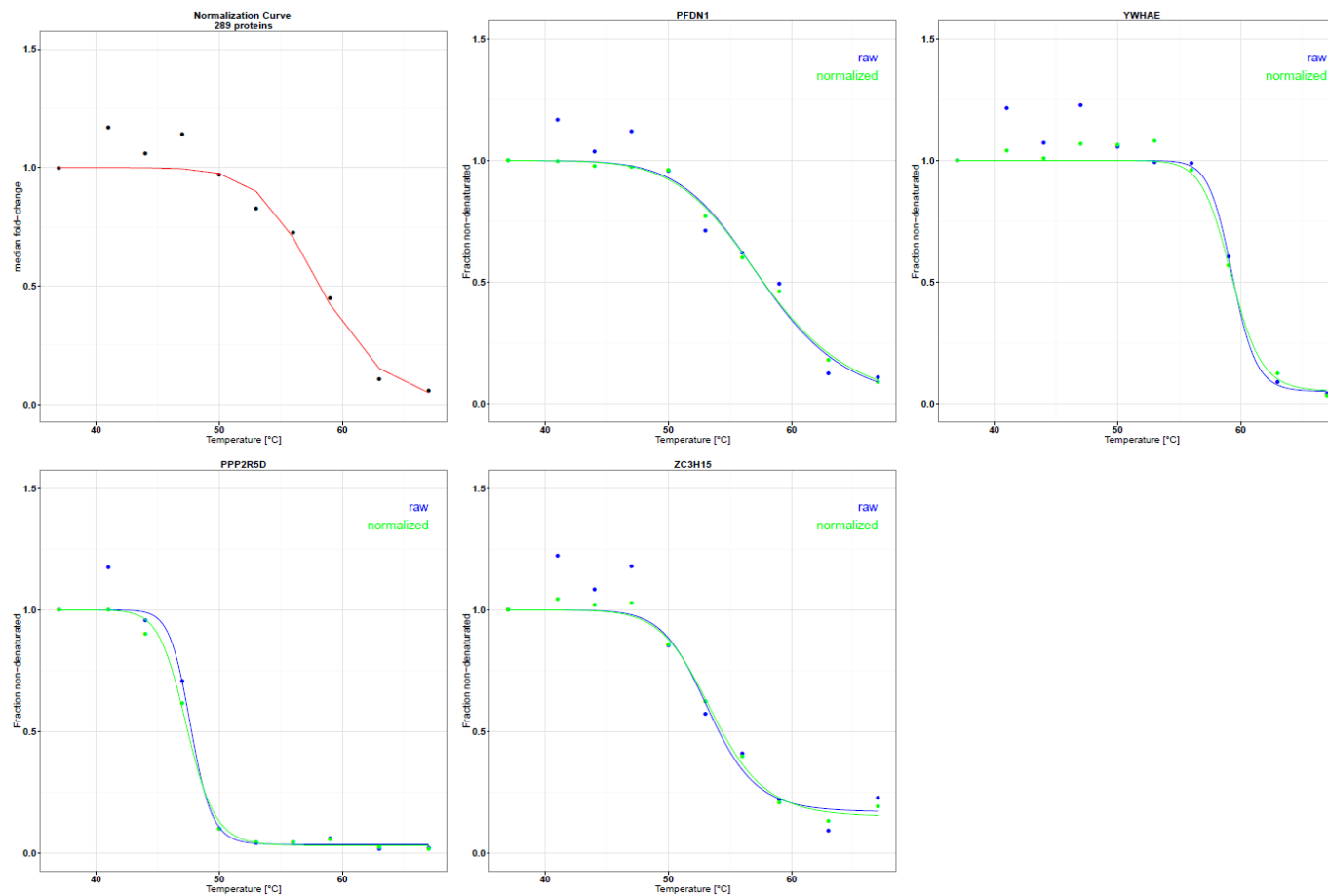


Fig. S12. Example of the normalization procedure for raw thermal profiling data

a) Normalization curve used to calculate normalization coefficients. b-e) Effect of normalization on a subset of proteins, the net effect in all cases is a better agreement between the individual data points and the fitted curve.