Supporting Information

Validating STAT Protein-inhibitor Interactions using Biochemical and Cellular Thermal Shift Assays

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Supplementary Methods

Cloning and Plasmid DNA transfections. The mammalian expression vector pcDNA3-N-FLAG was a kind gift from SGC Oxford. Sub-cloning of the $STAT1^{132-683}$, $STAT3^{127-688}$, $STAT5A^{138-712}$ constructs into pcDNA3-N-FLAG plasmid was performed by the Protein Science Facility (PSF) at the Karolinska Institutet, Stockholm, using LIC-cloning methodology from a previously produced $STAT1^{132-683}$, $STAT3^{127-688}$, $STAT5A^{138-712}$ construct sub-cloned into the bacterial expression pNIC28-Bsa4 vector. Sequence was confirmed at Eurofins MWG with vector specific forward and reverse primers. Plasmid DNA transfection was performed using JetPEI (Polyplus Transfection) transfection reagents following the manufacturer's instructions.

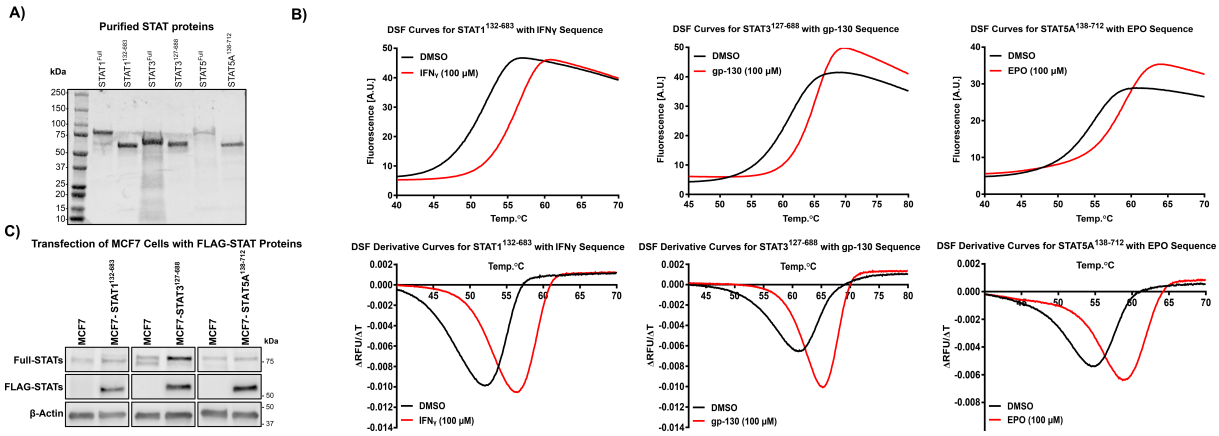
Cell lines and culturing conditions. MCF7 ($ER^+/PR^+/HER2^-$) breast adenocarcinoma cells and MB-231 ($ER^-/PR^-/HER2^-$) breast adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassass, VA, USA), A375 malignant melanoma cells were a generous gift from the Thomas Helleday Lab, Karolinska Institutet (Stockholm, Sweden). Cells were cultured in DMEM high glucose, GlutaMAX medium (Life Technologies/ThermoFisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were cultured at 37 °C with 5% CO2 in a humidified incubator.

Antibodies and reagents. The following antibodies were used in Western blots: rabbit anti-STAT1 (Cell Signaling, cat. no. 9172S), rabbit anti-STAT3 (Cell Signaling, cat. no. 4904), rabbit anti-STAT5 (Cell Signaling, cat. no. 94205S), mouse anti-β-actin (Abcam, cat. no. ab6276), mouse anti-FLAG (Sigma Aldrich, cat. no. F3165), mouse anti-SOD-1 (Santa Cruz, cat. no. sc-17767). The secondary antibodies for Western

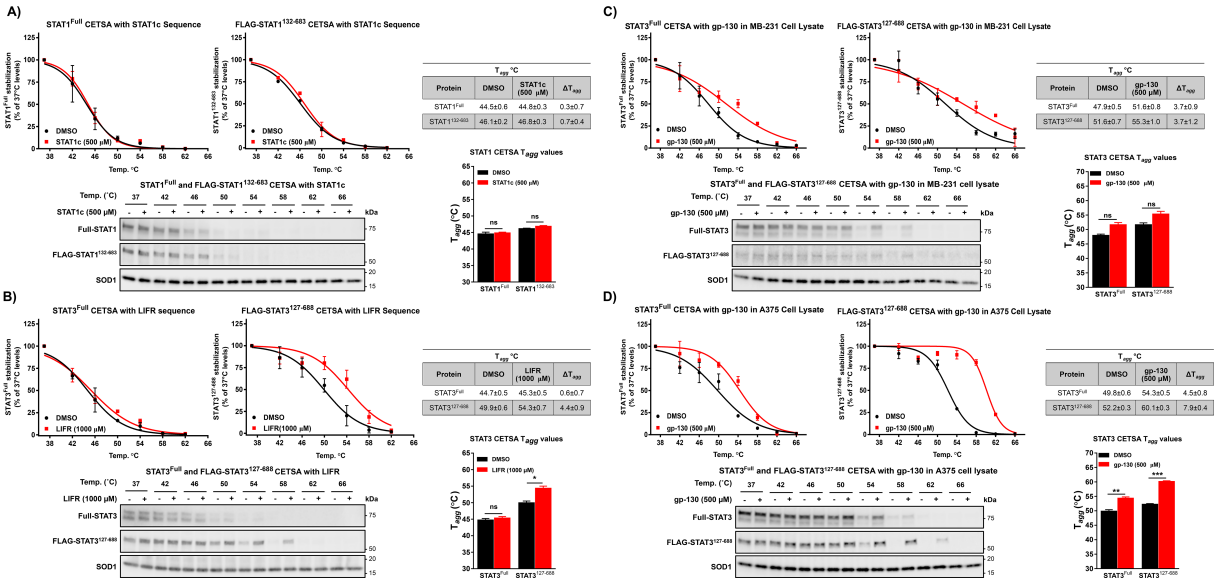
blot were: donkey anti-rabbit IgG IRDye 680RD (cat. no. 925-68073), donkey anti-rabbit IRDye 800CW (cat. no. 926-32213), donkey anti-mouse IRDye 800CW (cat. no. 926-32212) from Li-Cor Biosciences and goat anti-mouse IgG-HRP-conjugated (sc-2055), donkey anti-rabbit IgG-HRP-conjugated, Jackson (711-035-152). All STATs inhibitors were dissolved in DMSO to a stock concentration of 10 mM, and used at the final concentrations and incubation times indicated.

Western blot. Cells were lysed on ice for 10 min using the lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100) supplemented with protease inhibitor cocktail (Mini cOmplete, EDTA-free, Roche). Protein lysates were quantified using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific). Cell lysates were heated to 70 °C in NuPAGE LDS Sample Buffer (ThermoFisher Scientific) for 10 min. Proteins were separated by SDS-PAGE with 4–15% Mini- or Midi-PROTEAN® TGX precast gels (Bio-Rad) and transferred to nitrocellulose membrane using a Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked for 1 hour at room temperature in 1:1 Li-Cor Blocking Buffer/TBS + 0.05% Tween-20 (TBS-T)). Primary antibodies were incubated overnight at 4 °C at the following concentrations: anti-STAT1 (1:1000), anti-STAT3 (1:1000), anti-STAT5 (1:1000), β-actin (1:5000), SOD1 (1:1000). Secondary antibodies were diluted at 1:10,000 in 1:1 Li-Cor blocking buffer/TBS-T and incubated for 1 hour at room temperature. Bands were visualized with an Odyssey Fc Imager and analyzed with ImageJ or Image Studio™ Lite Software (Li-Cor Biosciences). All uncropped primary western blots from this manuscript and their duplicates are compiled in Supplementary Figure 7 and Supplementary Figure 8.

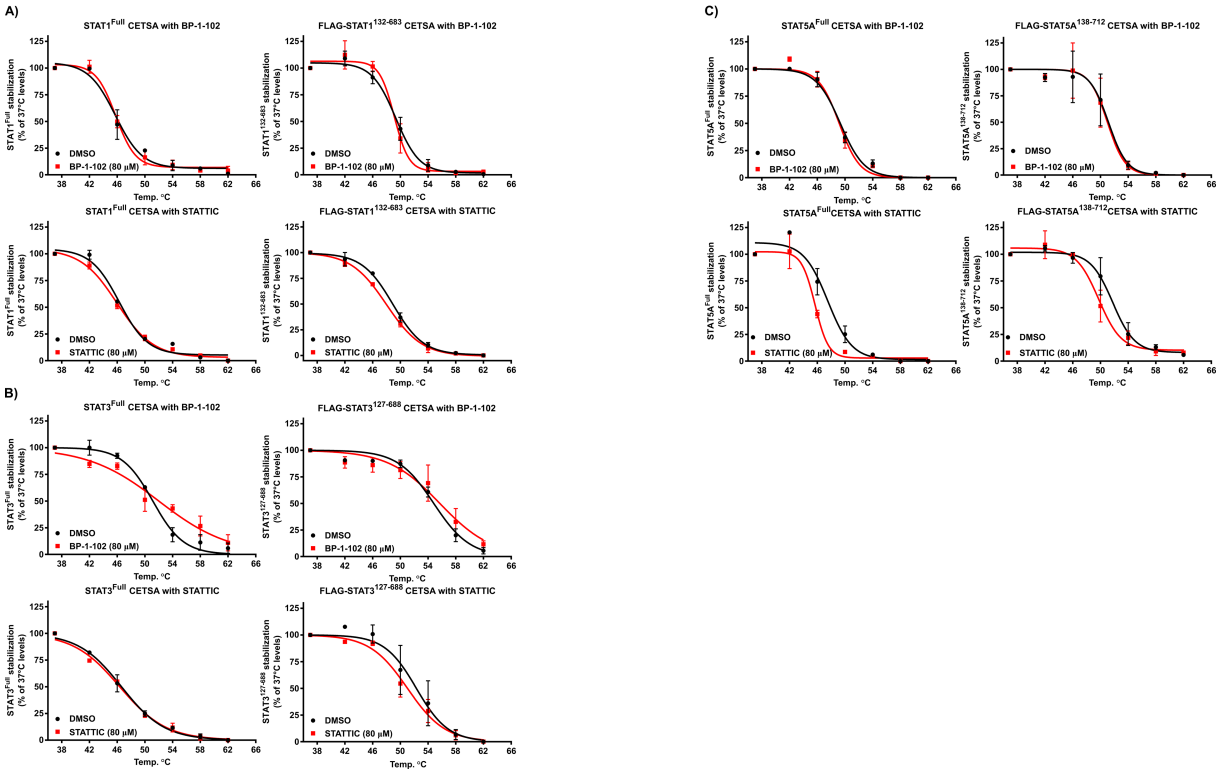
Supplementary Figures



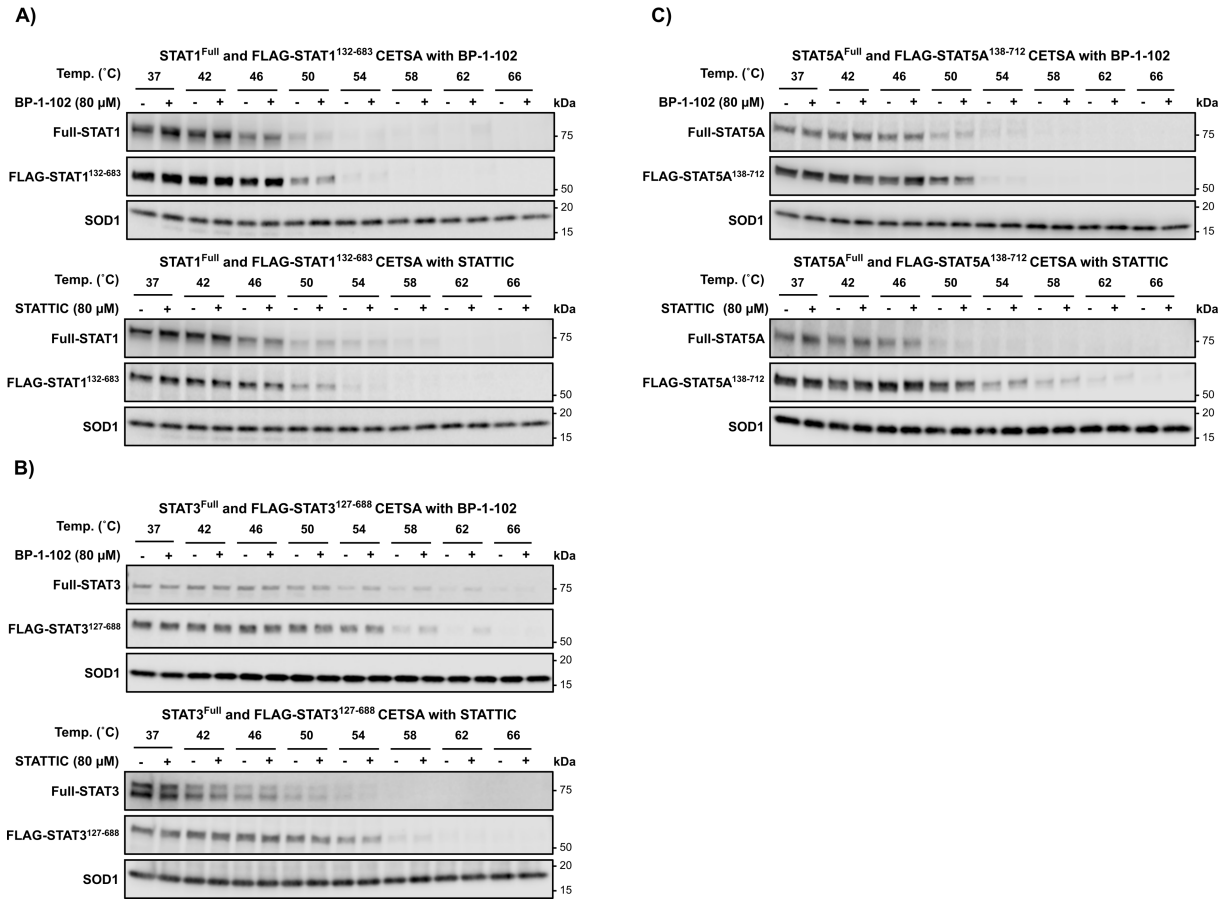
Supplementary Figure 1. Purified STAT proteins (A) DSF melting curves for truncated STATs by respective peptide inhibitor (B) expression of full and FLAG-STAT truncations of STAT1, 3 and 5 DNA plasmids in cancer cells (C). **A)** Purified STAT1^{Full}, STAT1$^{132-683}$, STAT3$^{127-688}$, STAT5A^{Full}, STAT5A$^{138-712}$ truncations and purchased STAT3^{Full} were analyzed by SDS-PAGE, followed by Blue Silver staining. **B)** *Top*, raw fluorescence data (RFU, relative fluorescence units); *bottom*, negative first derivative of fluorescence (-∂(RFU)/∂T) versus the temperature plot for visualization of the melting temperatures of truncated STAT proteins at 100 μM. **C)** Western blot showing expression of full-length STATs and truncated FLAG-STAT1$^{132-683}$, FLAG-STAT3$^{127-688}$ and FLAG-STAT5A$^{138-712}$ after 24 hours transfection in MCF7 transfected and non-transfected cells.



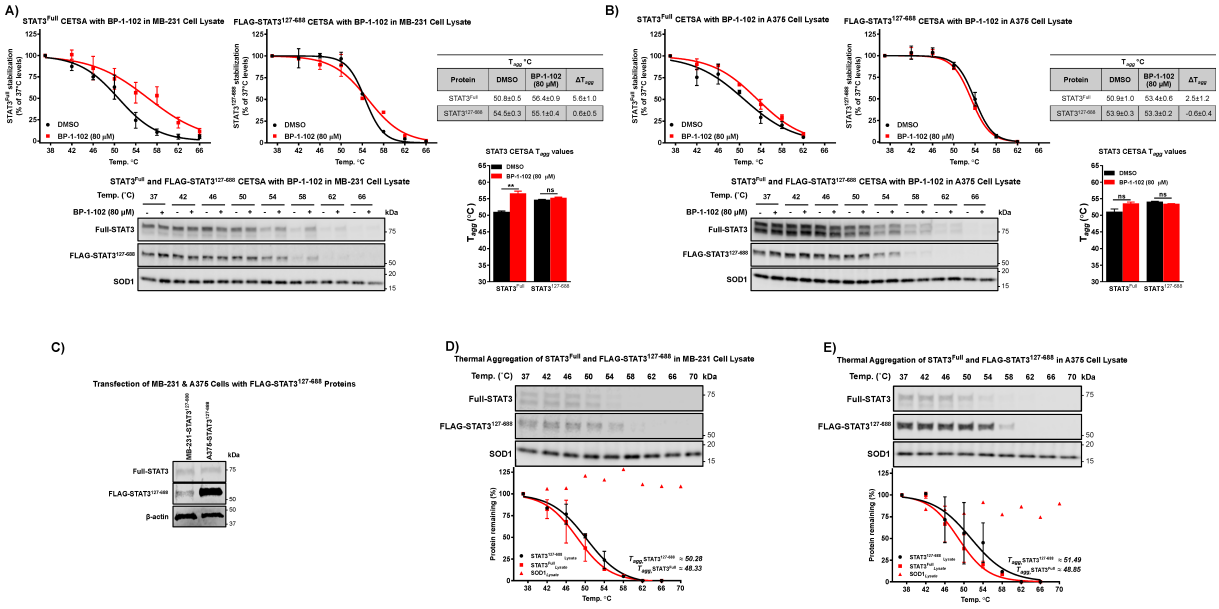
Supplementary Figure 2. **Assessment of peptide inhibitors STAT1c and LIFR for stabilization of STAT1$^{132\text{-}683}$ and STAT3$^{127\text{-}688}$ towards thermal denaturation in cell lysates by CETSA. A)** and **B)** Representative immunoblots and graphs (n = 2) to assess thermal stabilization of full-length and truncated STAT1 by STAT1c (A) and STAT3 by LIFR (B) at the specified concentrations in MCF7 cell lysates. STATs stabilization levels were relative to the 37 °C DMSO controls and normalized against SOD1 as a loading control. Curves to the *right,* thermal stabilization of FLAG-STAT truncations of STAT1 and 3 by STAT1c and LIFR when added to the cell lysates. While neither of peptide inhibitors were able to stabilize full-length STATs (curves to the *left*), LIFR significantly stabilized FLAG-STAT3$^{127\text{-}688}$ towards thermal denaturation. Apparent T_{agg} temperatures and ΔT_{agg} are shown in the table. **C)** and **D)** Immunoblots and graphs to assess thermal stabilization of full-length and truncated STAT3 by the gp-130 sequence in MB-231 (C) and A375 (D) cell lysates. ns not significant, *p < 0.05; Two-way ANOVA analysis.



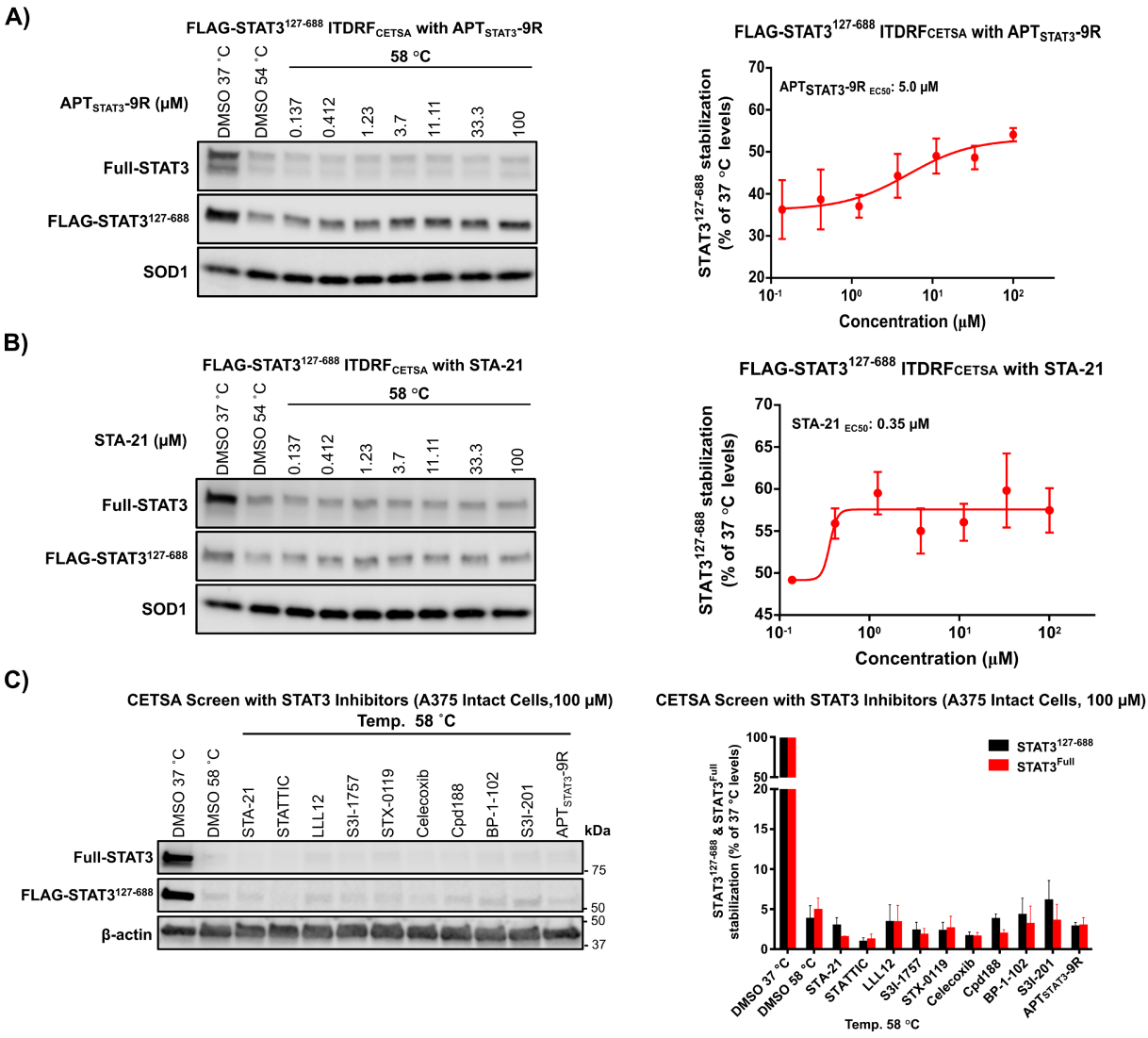
Supplementary Figure 3. CETSA melt curves for Figure 4. A–C) BP-1-102 and STATTIC were assessed for their ability to bind to the full-length (*left*) and FLAG-tagged truncated (*right*) STAT1 (A), 3 (B) and 5A (C) at 80 μM in MCF7 cell lysates. STATs stabilization levels were relative to the 37 °C controls. Points represent the means ± SEM from two independent experiments.



Supplementary Figure 4. Representative Immunoblots for Figure 4. A–C) Thermal stabilization of full-length and FLAG-tagged truncated STAT1 (A), 3 (B) and 5A (C) by BP-1-102 and STATTIC inhibitors by STAT CETSA at the specified concentrations in MCF7 cell lysates. STATs stabilization levels were relative to the 37 °C controls and normalized against SOD1 as a loading control.



Supplementary Figure 5. Evaluation of BP-1-102 by STAT CETSA in MB-231 and A375 Cell Lysates. A) and **B)** BP-1-102 was assessed for its ability to bind to the full-length (*left*) and truncated FLAG-STAT3$^{127\text{-}688}$ (*right*) at 80 μM in MB-231 (A) and A375 (B) cell lysates. STAT3 stabilization levels was relative to the 37 °C controls and normalized against SOD1 as a loading control. Points represent the means ± SEM from two independent experiments. Apparent T_{agg} temperatures and ΔT_{agg} are shown in the tables and plotted in the bar graphs. **C)** Western blot showing expression of full-length STAT3 and truncated FLAG-STAT3$^{127\text{-}688}$ after 24 hours transfection in MB-231 and A375 cell lines. **D)** and **E)** T_{agg} curves for endogenous STAT3^{Full} and transfected FLAG-STAT3$^{127\text{-}688}$ in MB-231 (D) and A375 (E) cell lysates. Comparable aggregation temperatures were observed between these two cell lines and MCF7 cells. Thermally stable SOD1 was used as a loading control.



Supplementary Figure 6. STAT3 inhibitor target engagement. MCF7 cell lysates were treated with **(A)** APT_{STAT3}-9R and **(B)** STA-21 serially diluted from 100 μM to 0.137 μM for $ITDRF_{CETSA}$. While both compounds could be confirmed as STAT stabilizers, $ITDRF_{CETSA}$ illustrated that APT_{STAT3}-9R was more potent stabilizer under the described conditions with an apparent EC50 of 5 μM. The dose response curve for STA-21 was not optimal as increasing concentrations of STA-21 gave a rather flat response. Data for both compounds comprise data points from 2 independent experiments. Representative cropped blots are shown. **C)** Compounds with known STAT3 inhibitory effects were screened for target engagement at 100 μM with intact

A375 cells by CETSA. None of the compounds stabilized FLAG-STAT3$^{127\text{-}688}$ in A375 cells relative to DMSO treated controls at 58 °C. Values are plotted relative to full-length STAT3 and FLAG-STAT3 at 37 °C and normalized to SOD1 (A and B) or β-actin (C). Data are means ± SEM from two independent experiments. Cropped representative blots are shown.

Supplementary Figure 7. All uncropped Western blots from other figures. Protein ladders are annotated as “M” for marker with molecular weight markers labeled, dotted lines indicate cropped area for figures and the primary/secondary antibodies used for each instance are labeled. Asterisks (*) denote irrelevant, misloaded samples or highlight non-specific bands recognized by the primary antibody.

Supplementary Figure 8. All uncropped Western blots from duplicate experiments of each figure. Protein ladders are annotated as “M” for marker with molecular weight markers labeled, dotted lines indicate cropped area for figures and the primary/secondary antibodies used for each instance are labeled. Asterisks (*) denote irrelevant samples or highlight non-specific bands recognized by the primary antibody.