# Supporting Information

## A Robust Strategy for Hit-to-Lead Discovery: NMR for SAR

Sacha T. Larda[1,‡], Yann Ayotte[1,2,‡], Maria M. Denk[1,2], Paul Coote[1,3,4], Gregory Heffron[1,3], David Bendahan[2], Fatma Shahout[2], Nicolas Girard[1], Mustapha Iddir[2], Patricia Bouchard[1], Francois Bilodeau[1], Simon Woo[1,2], Luc J. Farmer[1,2], Steven R. LaPlante[1,2,*]

[1]*NMX Research and Solutions Inc., Laval, Canada,* H7V 5B7

[2]*INRS – Centre Armand-Frappier Santé Biotechnologie, Laval, Canada,* H7V 1B7

[3]*Harvard Medical School, Boston, Massachusetts, United States,* 02115

[4]*Dana-Farber Cancer Institute, Boston, Massachusetts, United States,* 02215

*Corresponding author: steven.laplante@inrs.ca

‡ These authors contributed equally.

# Contents

# Supplementary figures

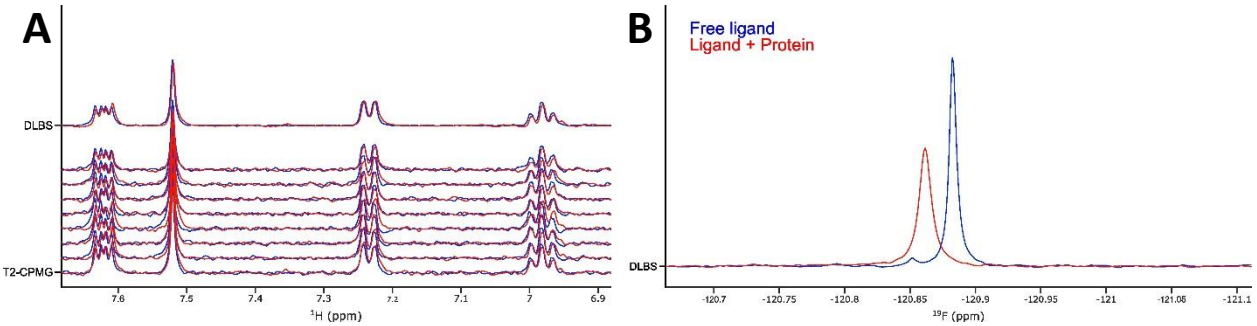


**Figure S1.** Comparison of Proton- vs Fluorine-Ligand Detected NMR for Series 1 hit FS-1255. (A) $^{1}$H Ligand-detected NMR spectra showing the differential line broadening (DLB) spectra followed by the stack of eight T2-CPMG spectra with different delay times (0, 25, 50, 100, 200, 300, 500, 800 msec). No binding is evident by $^{1}$H ligand-detected NMR. (B) $^{19}$F 1D Ligand-detected NMR spectra demonstrating clear binding for compound FS-1255, highlighting the advantage of $^{19}$F NMR for elucidation of binding of weak fragments.

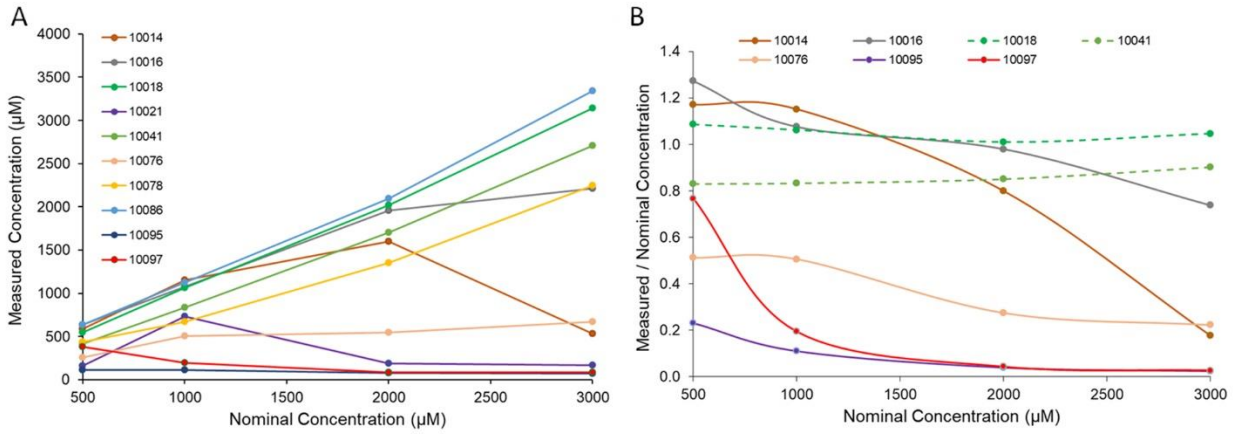


**Figure S2.** Compound behavior assay for monitoring differences between nominal and true solution concentrations of various ligands prior to dose-response assays (A). Compounds for which the ratio between measured and nominal concentration remains constant across the titration are typically well-behaved, whereas large changes in the ratio indicates potential solubility issues (B). In cases where the ratio slightly deviates from 1.0, but remains constant across a titration series (i.e. 10041, B), this is usually attributable to error in the nominal stock concentration (usually prepared by dissolving powder directly with solvent). Discrepancy between molecular weight and formula weight, along with poor measurement accuracy and precision are common sources of error in assumed nominal stock concentrations. Large initial (low concentration point) discrepancy between nominal and measured concentrations is often simply due to limited solubility of the compound. Dashed lines in (B) correspond to well-behaved compounds across the entire titration. Note the difference between nominal and measured solubility for 10095 and 10097, illustrating the importance of working within the measured solubility limits to obtain accurate binding and affinity data. Also note that differences can arise from the sample preparation procedure (e.g. diluting a concentrated DMSO compound solution directly into buffer and adding the remaining DMSO needed to

get the final desired DMSO content in the sample *versus* preparing a lower concentration sub-stock in DMSO and then transferring the required volume to get exactly the desired DMSO quantity). Such differences in preparation explain some slight differences with the maximal solubility limits reported in Figure 3.

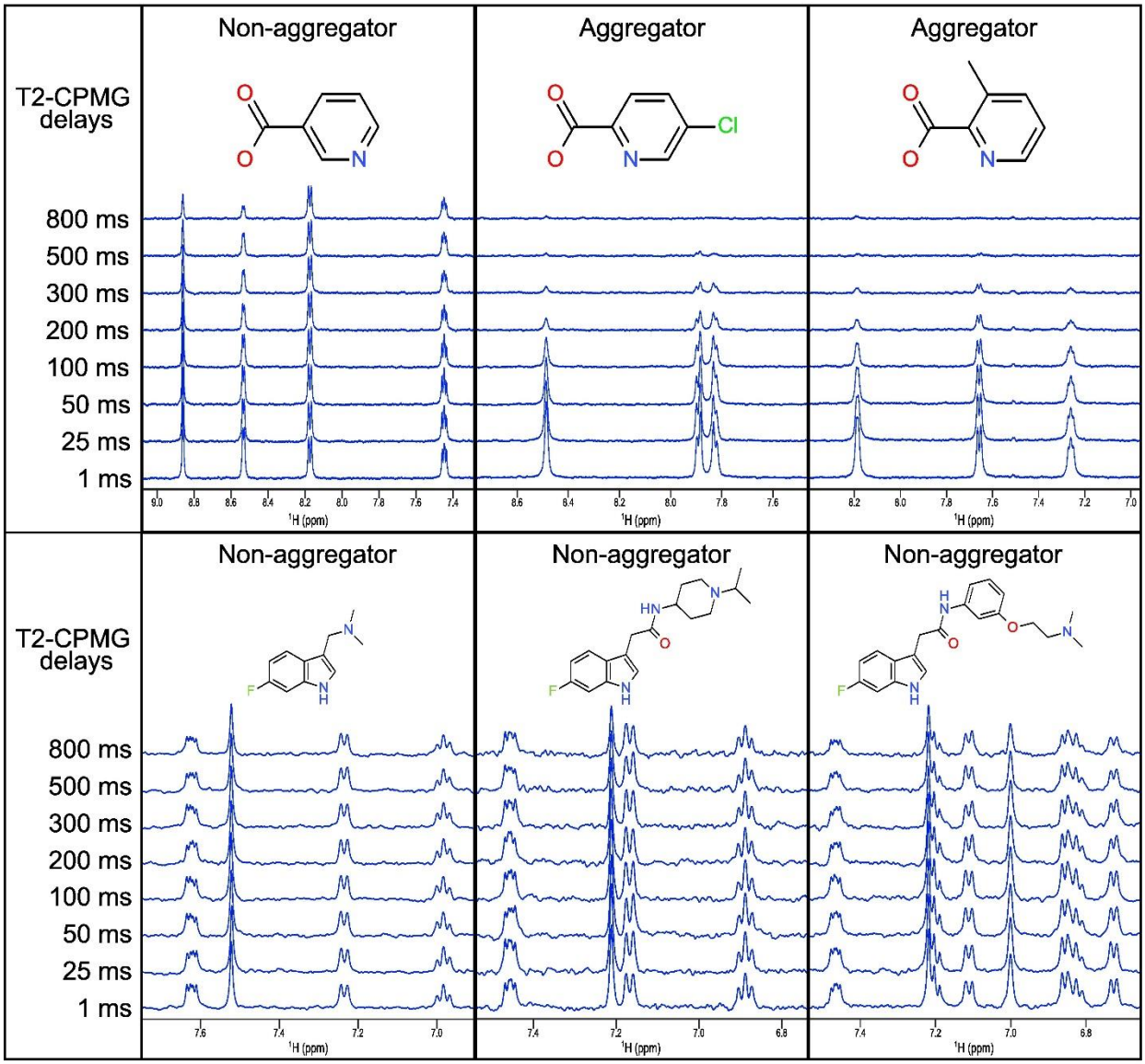


**Figure S3.** (Top three panels) Monitoring aggregation properties of related compounds via a high-throughput 1D NMR assay. Compounds were prepared to 300 µM in 50 mM sodium phosphate, 100 mM NaCl, pH 7.4 with 10% $D_2O$. Rapid signal decay is indicative of compound aggregation whereas long relaxation times are characteristic of non-aggregating, well-behaved compounds. (Bottom three panels) The initial fragment screening hit and related analogs are shown to be non-aggregating at the concentrations tested (240 µM in 25 mM sodium phosphate, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM TCEP-$d_{16}$, pH 7.4 with 10% $D_2O$) and were evaluated in binding assays under comparable conditions to mitigate false positives and false negatives.

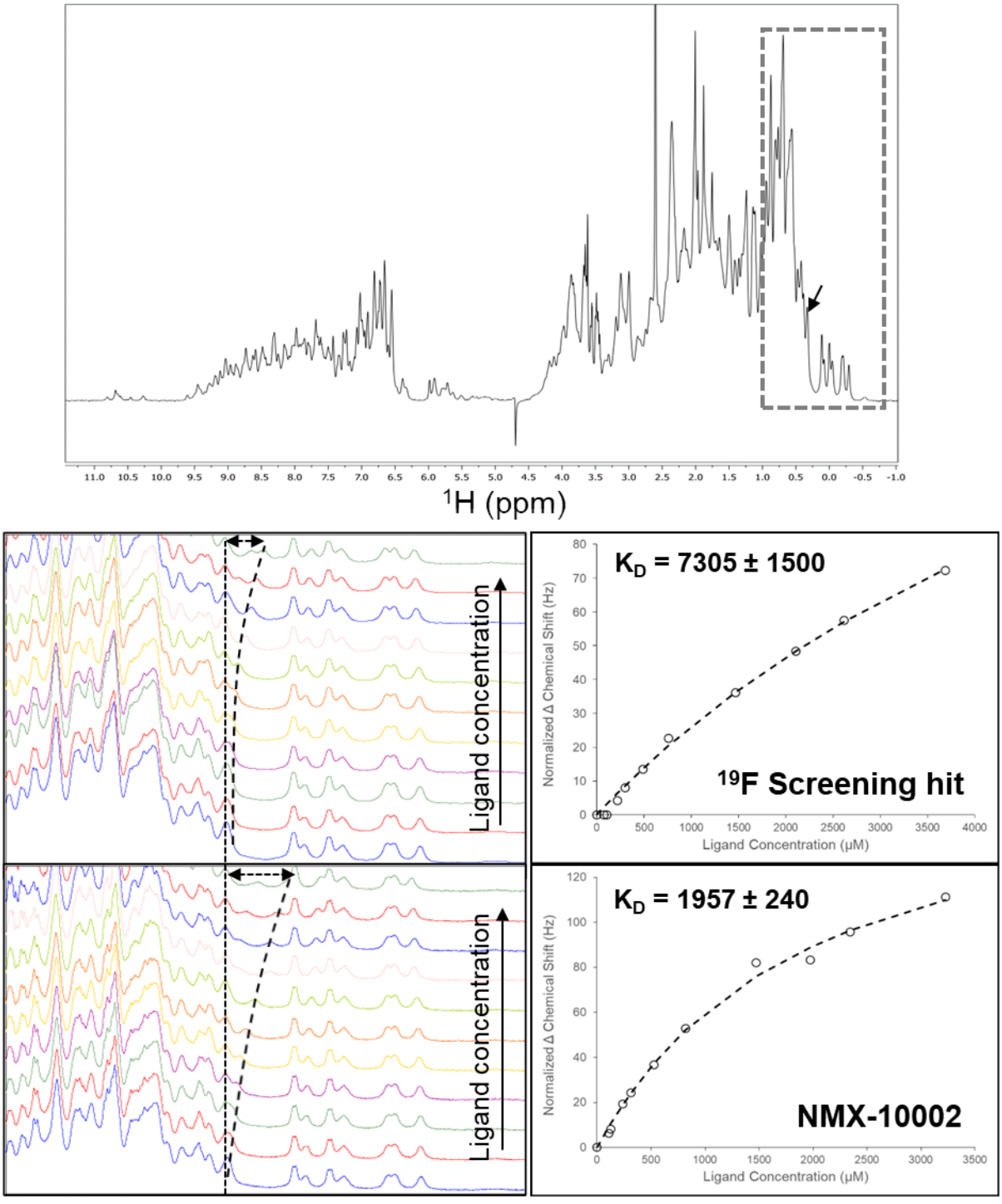


**Figure S4.** Apo HRas protein "fingerprint" spectrum (Top). Monitoring upfield protein methyl shifts with increasing ligand concentration provides direct insight into ligand binding (Bottom). Protein methyl shifts across a range of ligand concentrations provides $K_D$. While saturation could not be achieved with either the initial screening hit or NMX-10002, fitted $K_D$ estimates shown are consistent with orthogonal biophysical data, and most importantly, accurately reflect the relative affinity ranking of the compounds. Note that the ligand concentrations reported are measured rather than assumed nominal concentrations.

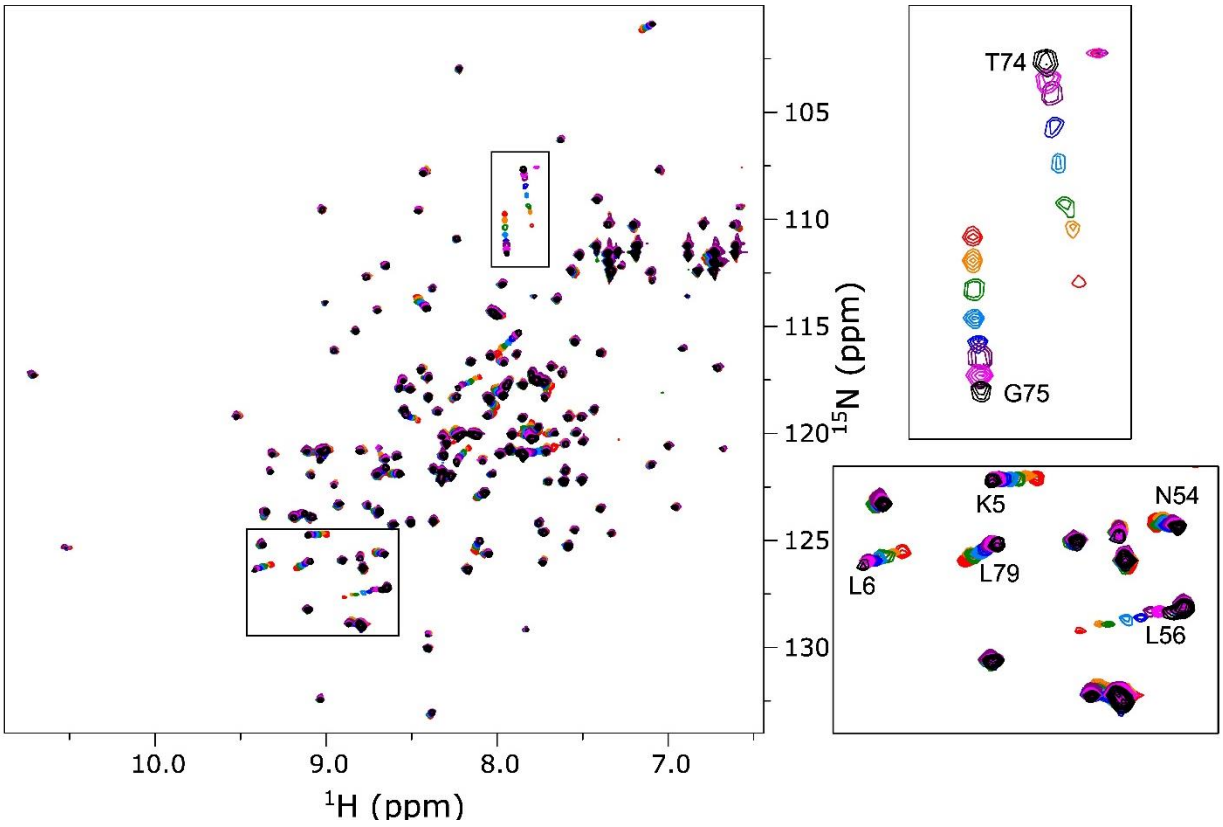


**Figure S5.** $^{1}$H,$^{15}$N HSQC titrations were used to estimate binding affinities for well-soluble, weaker affinity compounds. Titration of NMX-10086 is depicted here.

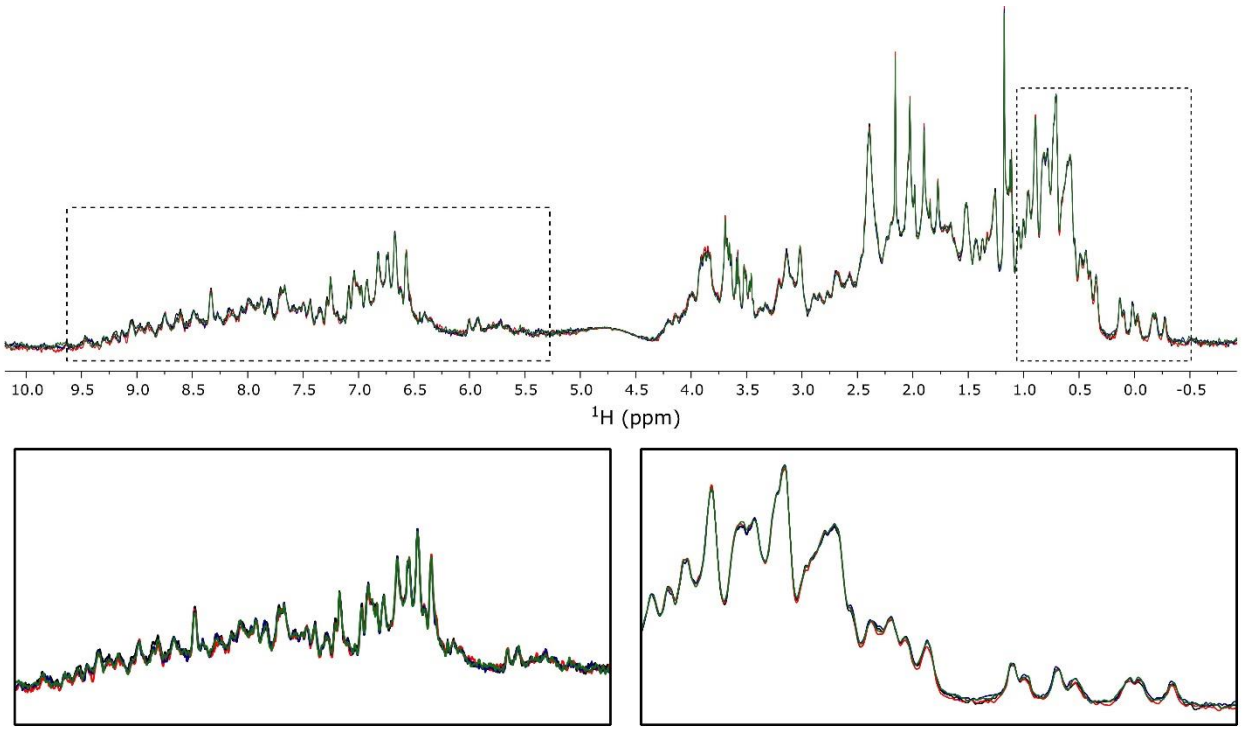


**Figure S6.** HRas stability by 1D protein-observed NMR over 96 hrs. Black, 0h; red, 48h; Blue, 72h; Green, 96h.

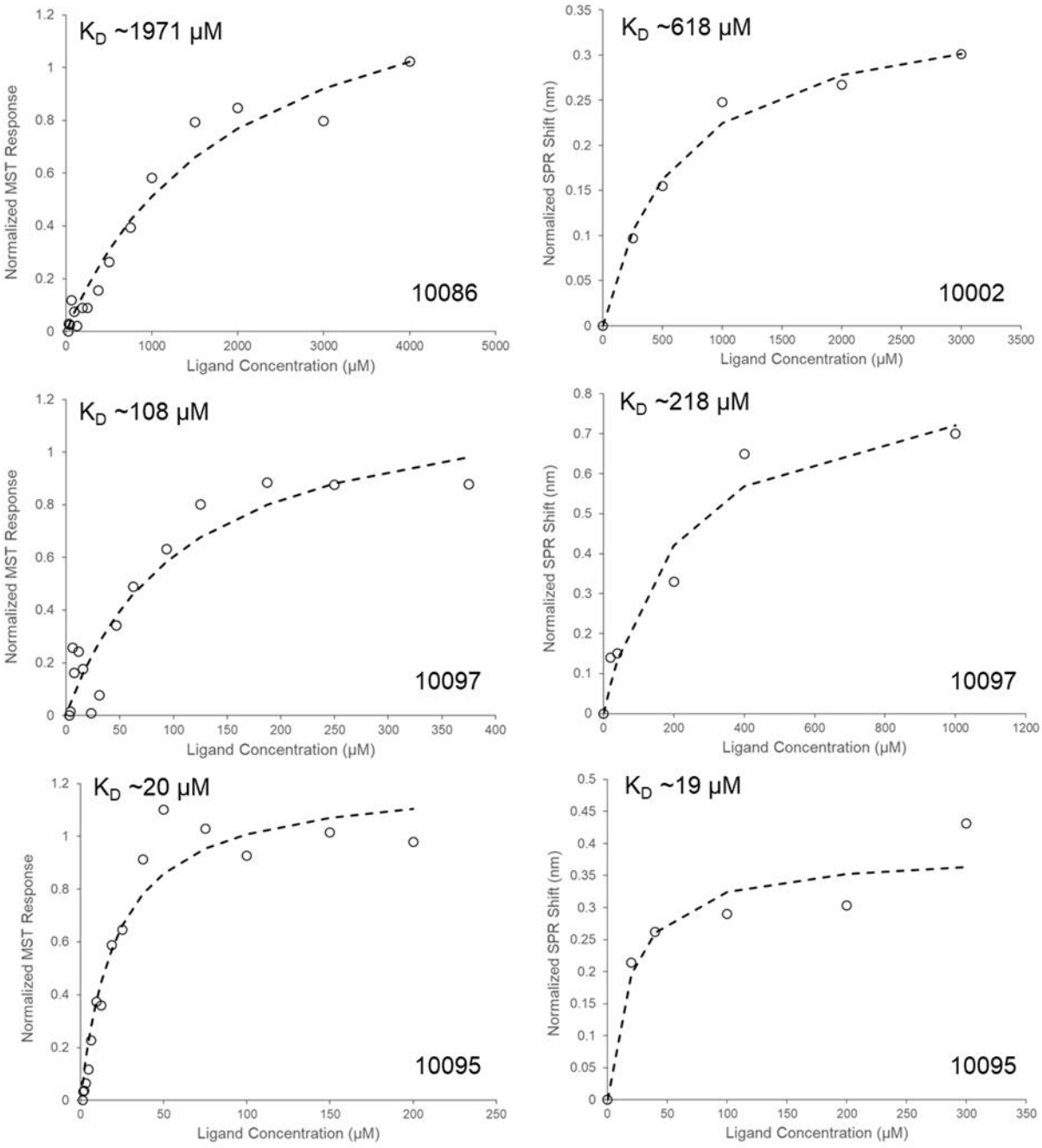


**Figure S7.** Orthogonal biophysical data for selected HRas binders. Left-hand column depicts MST data, whereas the right-hand column shows SPR data. Normalized data were fit in Excel using the solver function (GRG Non-linear method) and simulating the data with a comparable number of points to those measured in the titration.

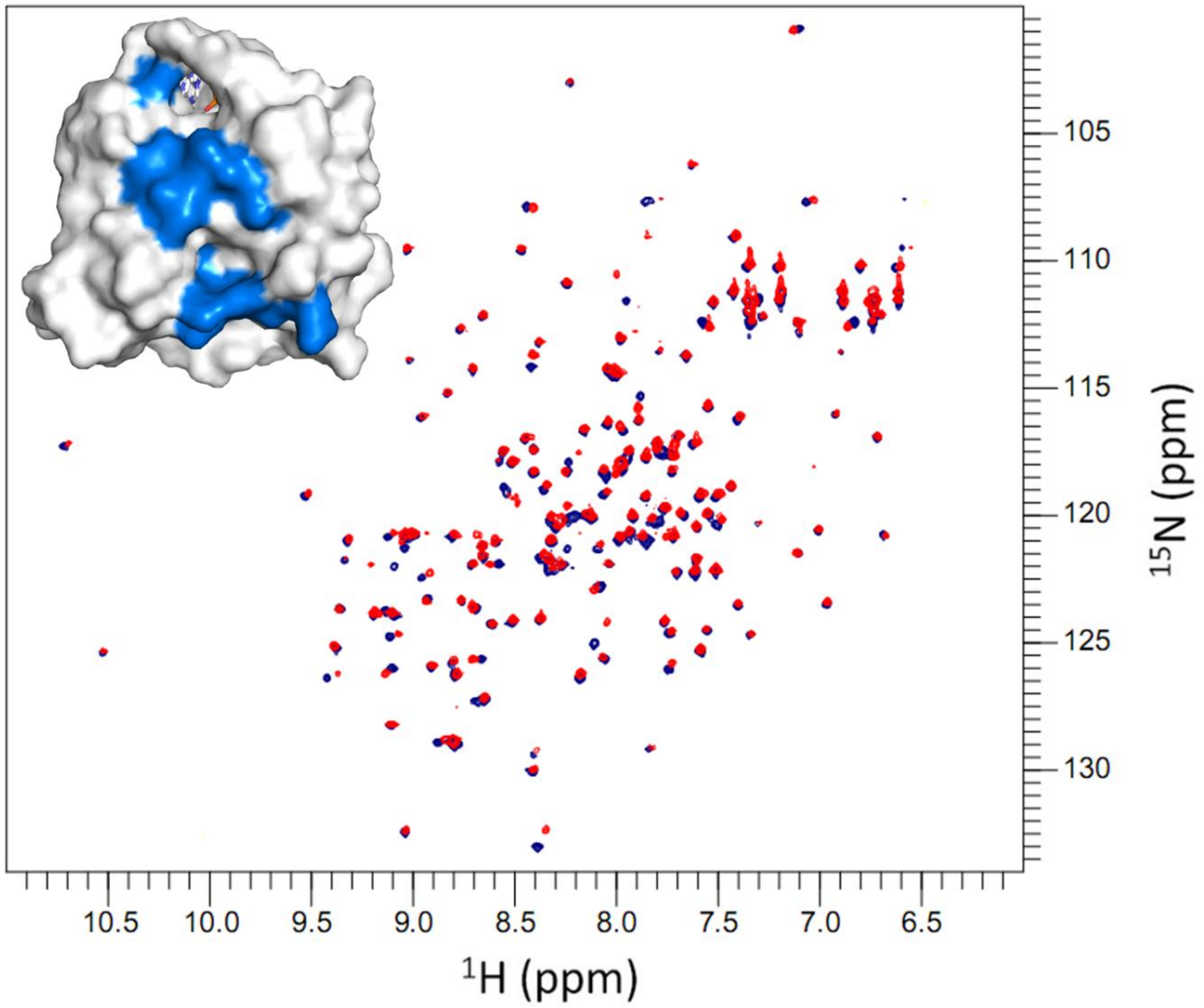


**Figure S8.** Binding site mapping based on backbone amide chemical shift perturbations between the free protein (blue) and protein in the presence of compound 10097 (red). Only weighted average chemical shift changes ≥ 0.02 ppm are mapped onto the structure of $HRas^{G12V}$ (3OIW). Binding of compound 10097 induces changes in the space between switch I and switch II.

# Supplementary methods

**MST**

MST experiments were performed with a Monolith NT.115 Pico (NanoTemper Technologies, Munich, Germany). Fluorescence labeling of GDP $HRas^{G12V}$ was achieved according to Nanotemper's protocol for use of the His Tag Labeling Kit RED tris NTA 2nd generation, or Kit RED-NHS 2nd Generation Labeling Kit (NanoTemper Technologies, Munich, Germany). Final protein concentrations were 20 nM fluorescently labeled GDP $HRas^{G12V}$. Compounds were prepared across a range of concentrations within the measured solubility ranges determined previously by NMR, and loaded into monolith capillaries. Data were acquired and analyzed with the NanoTemper MO.Control and MO. Affinity Analysis software. MST responses that passed the automated QC criteria were exported and normalized before fitting to a 1:1 binding model.

**SPR**

SPR was performed using a P4SPR (Affinité Instruments, Montreal, Canada) using His-tagged $HRas^{G12V}$. Ni-NTA-coated Au SPR chips were first rinsed with DI water. Protein was then immobilized by injecting ~300 µL of a 5 µM solution of protein in 1X PBS over the chip surface, followed by a 20 min incubation period. Wells were then washed and equilibrated with 1X PBS prior to dose-response experiments with small molecule analyte. Compounds were prepared in DMSO at various concentrations (5 points, dictated by expected affinity ranges given prior biophysical data). Compounds were then dried using a SpeedVac to obtain dry solid powders, which were resolubilized in 1X PBS. Solubilities in 1X PBS were previously known according to NMR data in nearly equivalent buffer conditions. Each ligand solution (~300 µL) was injected across the SPR chip surface and left to equilibrate for a minimum of 5 minutes before measuring changes in SPR wavelength, or RU. Subsequent concentrations of the same ligand were likewise tested in series starting from the lowest to highest concentration. Measured shifts in RU were then plotted and fit to a 1:1 binding model.