Supplemental Information

Table S1: Components of Existent protein footprinting methods. Related to **Figure 1**

Method	Label	Necessary Instrumentation	Resolution Level	Additional Considerations
HDX-MS	Deuterium	High-resolution MS + advanced LC for chromatography at ~0°	Peptide (Backbone amides)	Generally, buffers pH 6-8
FPOP*	Hydroxyl radical	High-resolution MS + standard LC + KrF laser or high-power UV light source	Residue (hydroxyl radical-reactive sidechains)	Choose buffer components that do not unintentionally quench hydroxyl radicals
Parallel Chemoselective Profiling	Residue dependent (e.g. iodoacetamide with cysteine)	High-resolution MS + standard LC	Residue (Installed/defined location)	Buffer conditions suitable for labeling reagent

*FPOP: Fast Photo-Chemical Oxidation of Proteins (Hambly and Gross, 2005)

Supplemental Figure 1

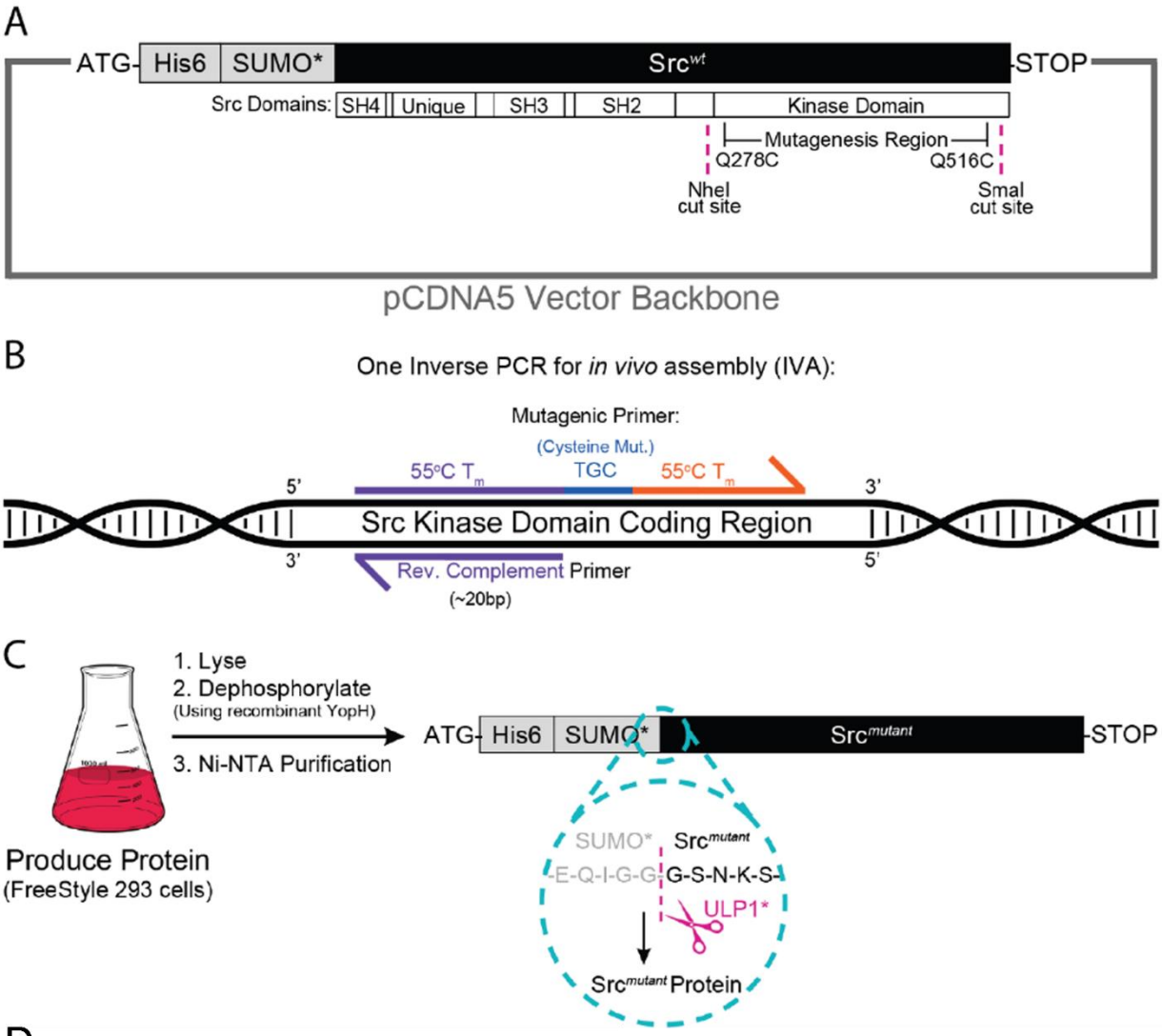

Figure S1 Key Features and Methods of Full-Length Src Mutant Protein Expression and Purification. Related to **Figure 3.**

(A) Mammalian expression plasmid with Src domain architecture and mutagenesis region noted. Silent NheI cut site was introduced to allow for the facile assembly of pooled mutant constructs with varying domain architecture (i.e. truncation mutants). (B) Primer design strategy for inverse PCR followed by transformation of linear DNA product into NEB5alpha bacteria for *in vivo* assembly. (C) Full-length Src mutants were expressed in a pooled format in suspension FreeStyle 293F cells. As the N- and C-termini of Src are important for its regulation, protein was expressed as a direct SUMO* fusion. Following standard Ni-NTA purification procedures, protein was cleaved yielding a pool of individual cysteine mutants with near native N-termini. (D) Western blot analysis of protein purification using phopsho- and non-

Supplemental Figure 2

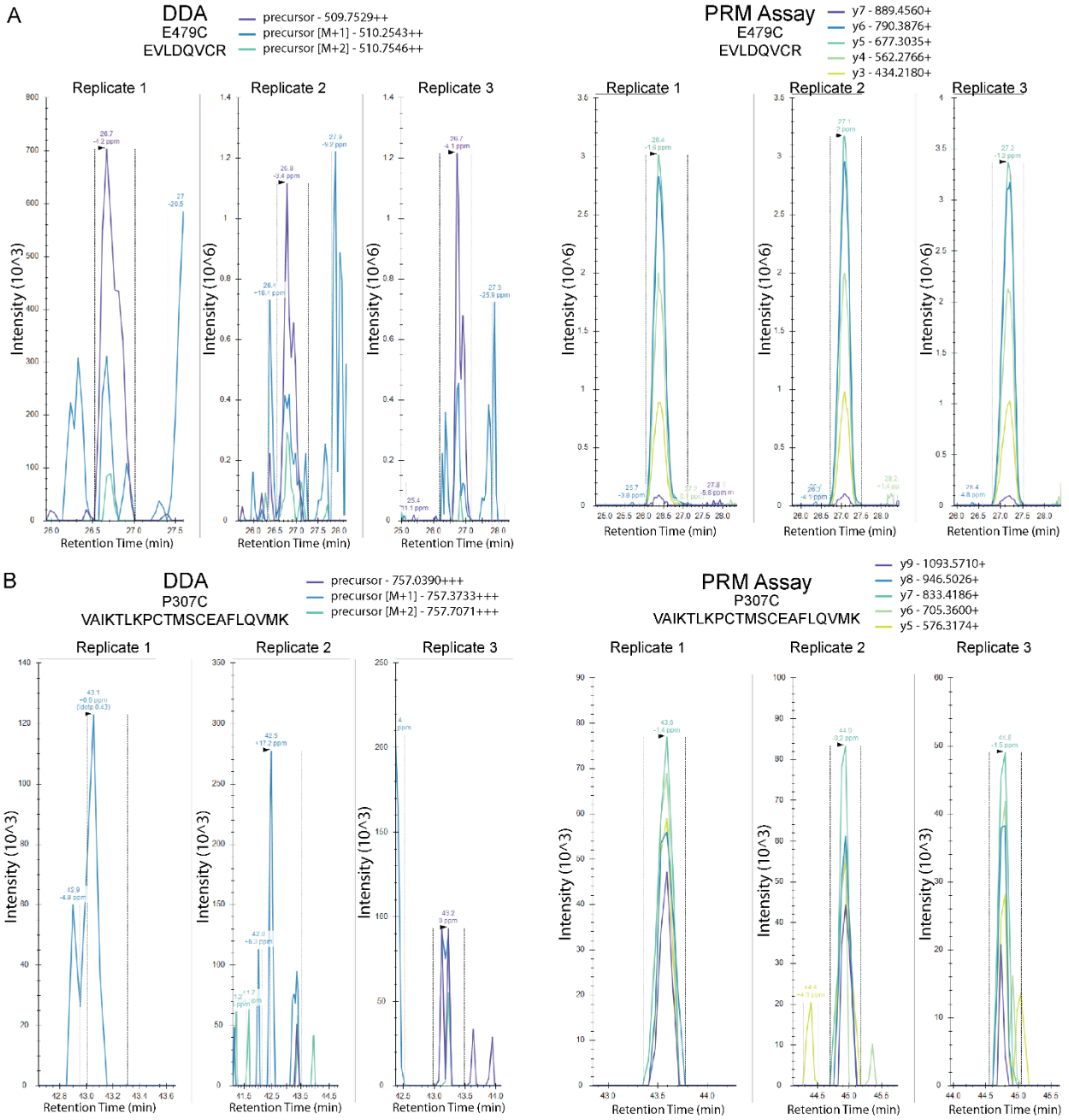

Figure S2 Comparison of extracted ion chromatograms from DDA and PRM experiments. Related to **Figure 3**

Ion chromatograms extracted using Skyline (A) Parent/precursor masses of EVLDQVCR peptide from E479C Src mutant from DDA experiment (left) compared to traces for fragment ions of the same peptide from the PRM assay (right). (B) Same analysis for the VAIKTLKPCTMSCEAFLWVMK peptides from P307C mutant of Src.

Supplemental Figure 3

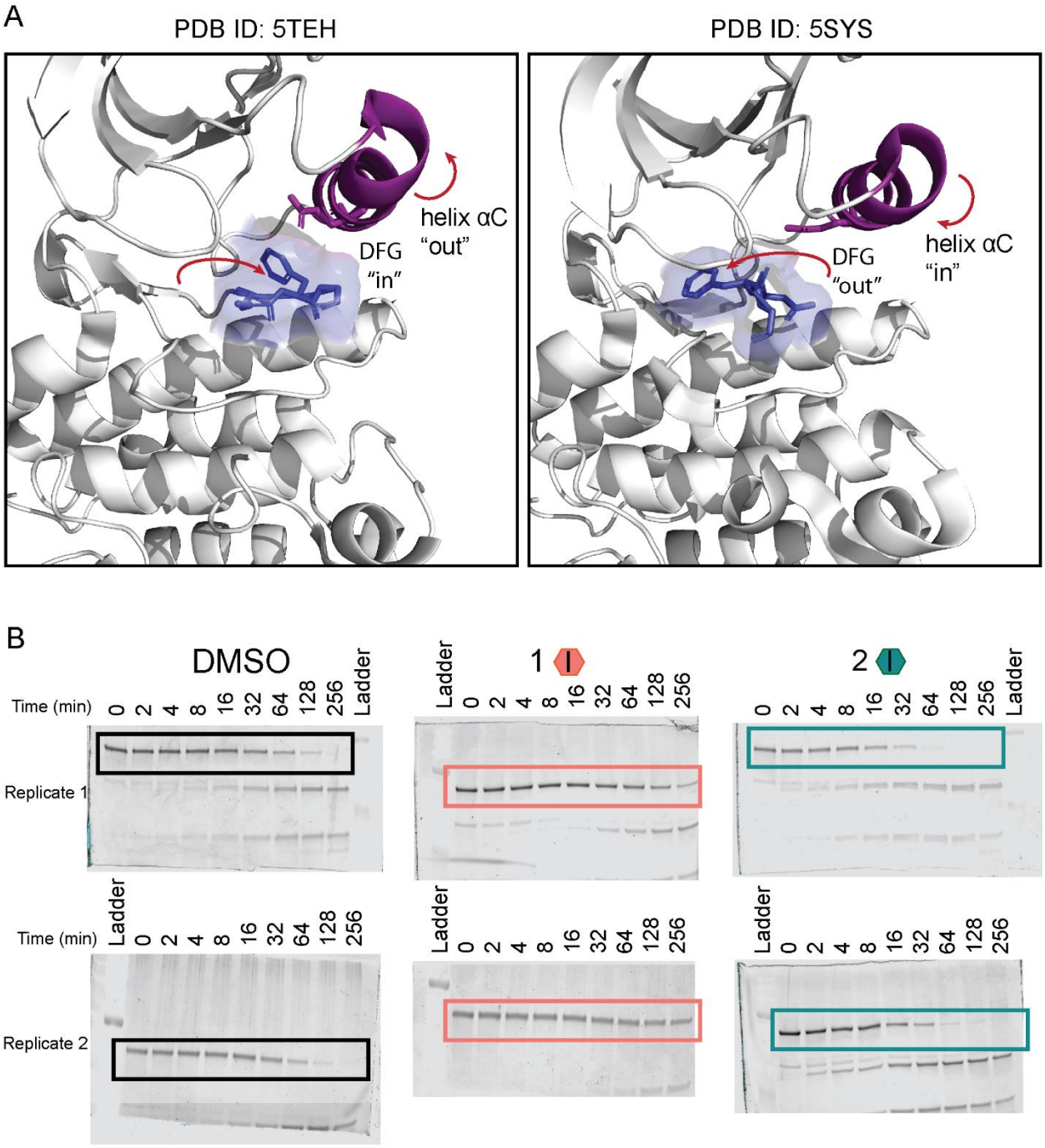

Figure S3 Active site conformations of Src stabilized by ATP-competitive conformation-selective inhibitors and raw thermolysin assay data. Related to **Figure 4.**

(**A**) Highlighted are the catalytically important helix αC and DFG-motif. The helix αC-out conformation is characterized by the rotation of the αC out of the active site and the DFG-motif in the active "in" conformation. Alternatively, the DFG-out conformation is characterized by the rotation of the helix αC into the active site, while the DFG-motif flips ~180º out of the active site. (**B**) Raw images of thermolysin assay SDS-PAGE gels. Boxes indicate regions quantified and represented in Figure 4A.

Supplemental Figure 4

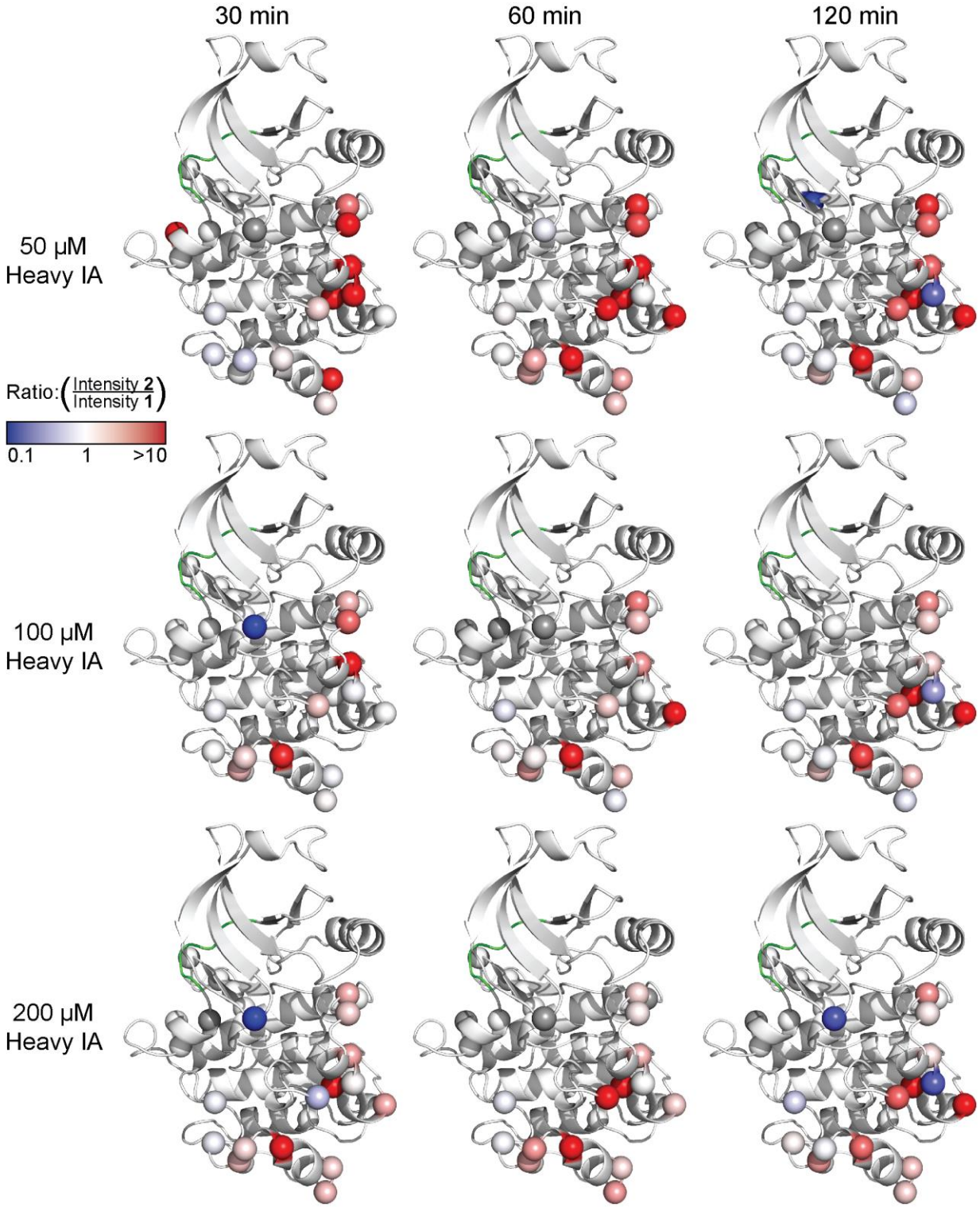

Figure S4 Parallel Chemoselective Profiling of conformation selective inhibitor bound Src. Related to **Figure 5.**

Complete dataset represented as ratios of heavy labeled peptides

Supplemental Figure 5

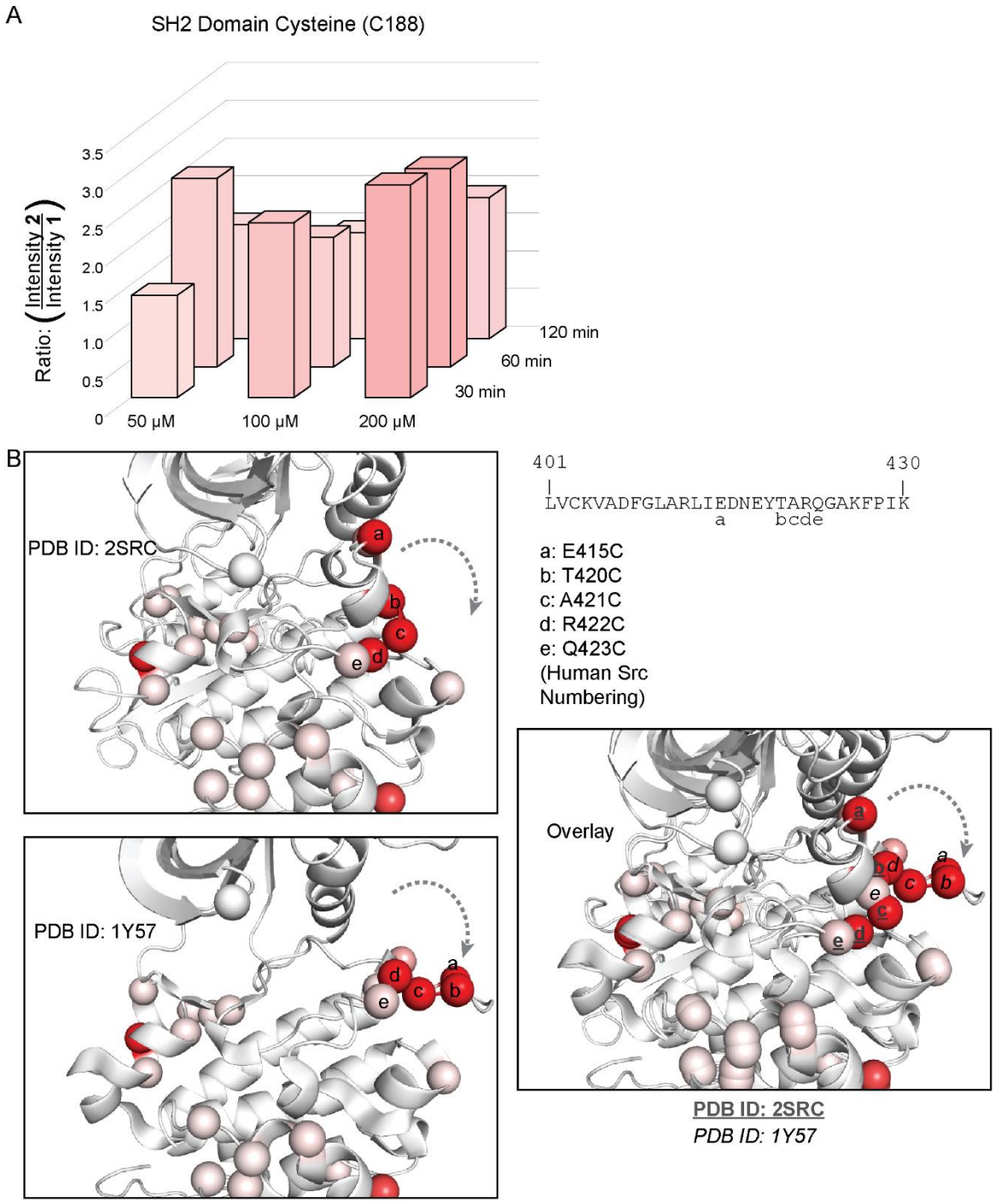

Figure S5 Movement of activation loop. Related to **Figure 5.**

(A) Quantification of WT wild-type cysteine residue C188 which residues on the SH2 domain. (B) 50uM 60min data mapped to crystal structures PDB ID: 2SRC and 1Y57. Indicated residues move out into solution in the open global conformation (PDB ID: 1Y57) relative to the closed global conformation (PDB ID: 2SRC).

Supplemental Figure 6

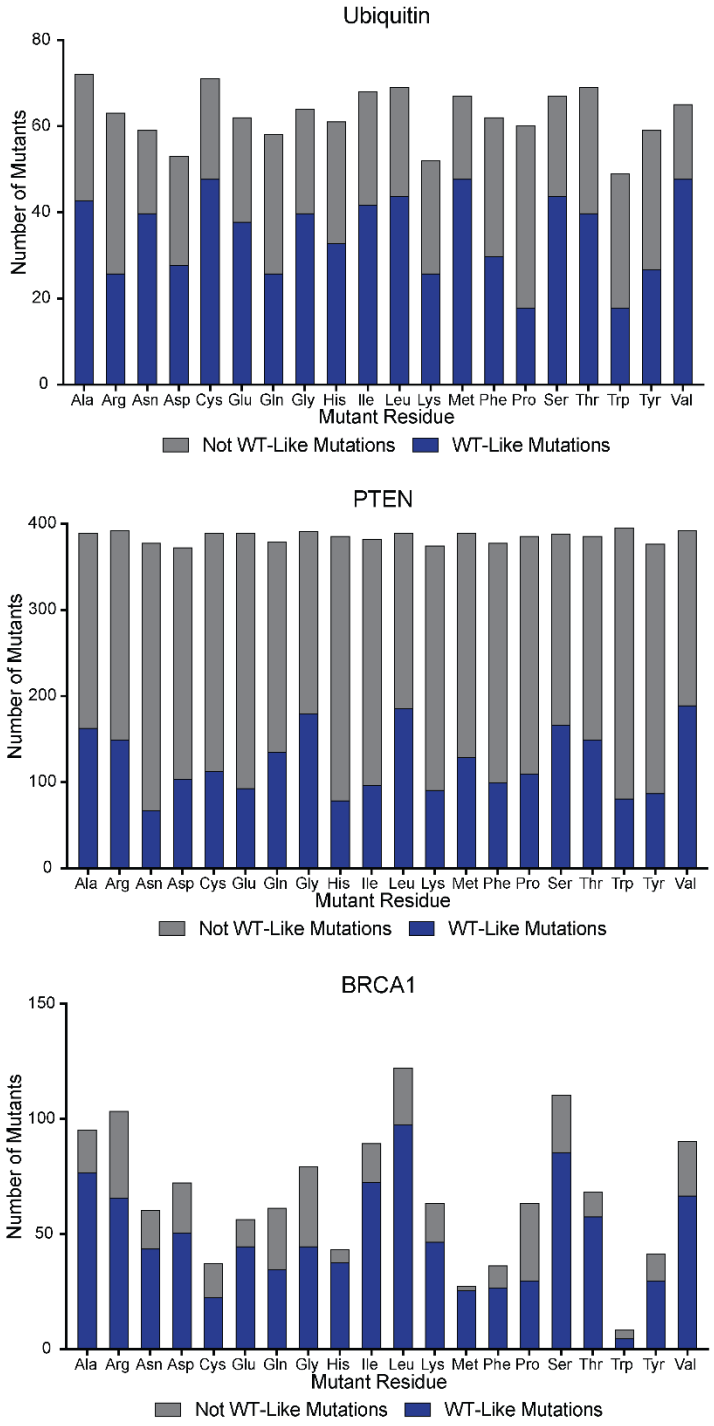

Figure S6 Distribution of all mutations contained in the Deep Mutational Scans analyzed. Related to **Figure 6.**

All mutational effect scores were extracted from the original datasets and binned as WT-like or not WT-like using the definition provided in the original reports (relative to synonymous distribution).