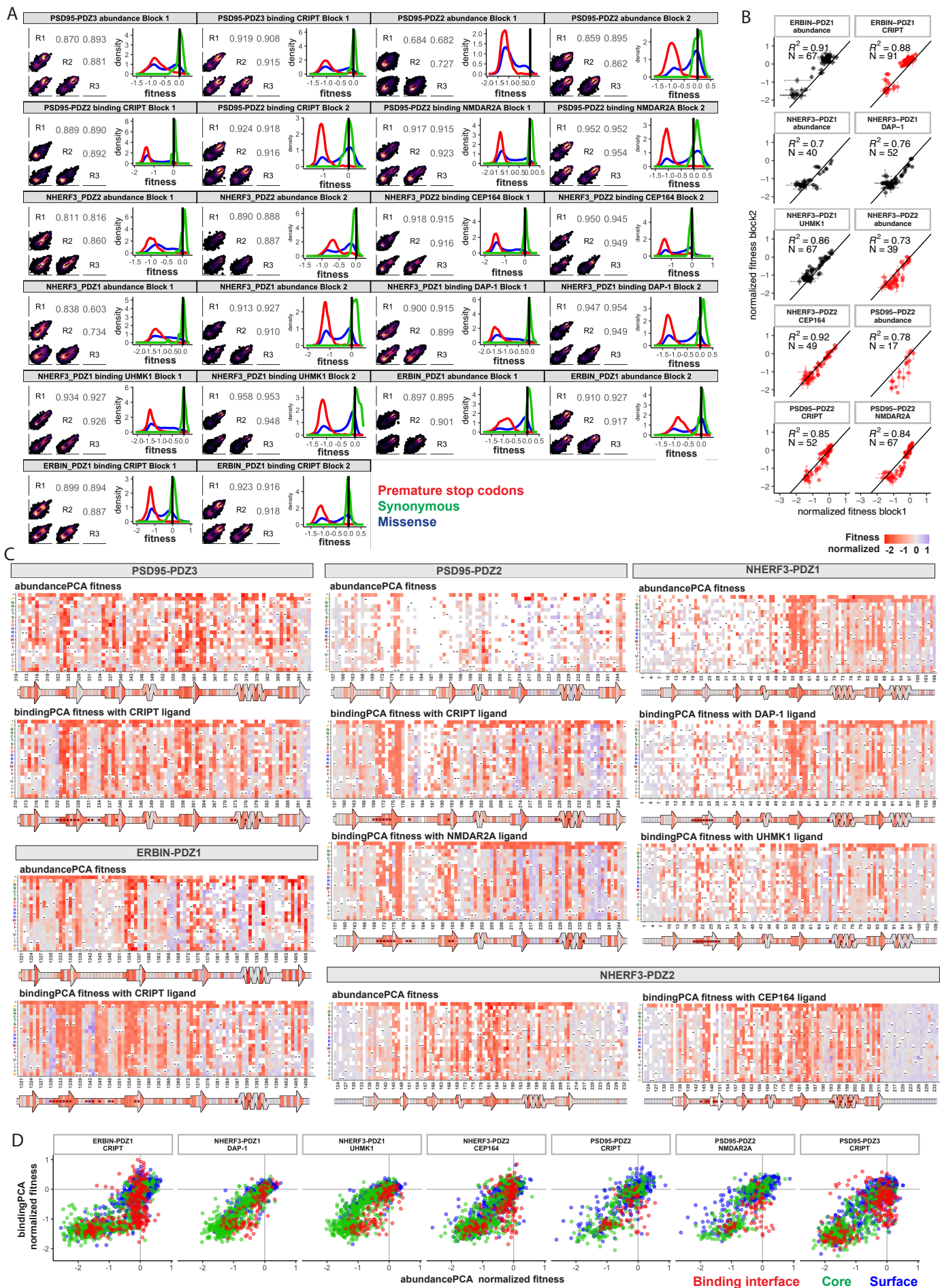


Supplementary Information

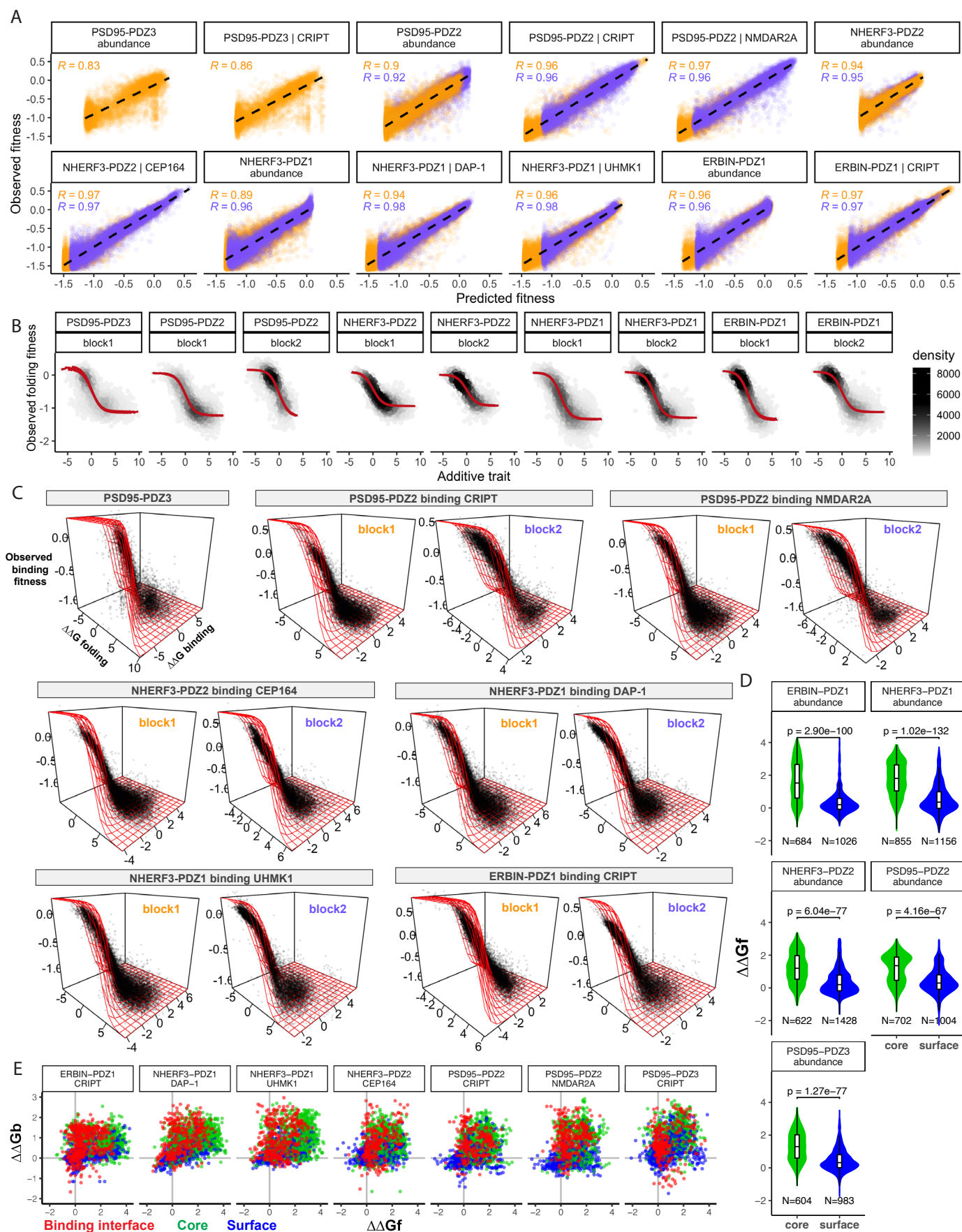
Seven complete comparative maps of allosteric mutations in a protein family

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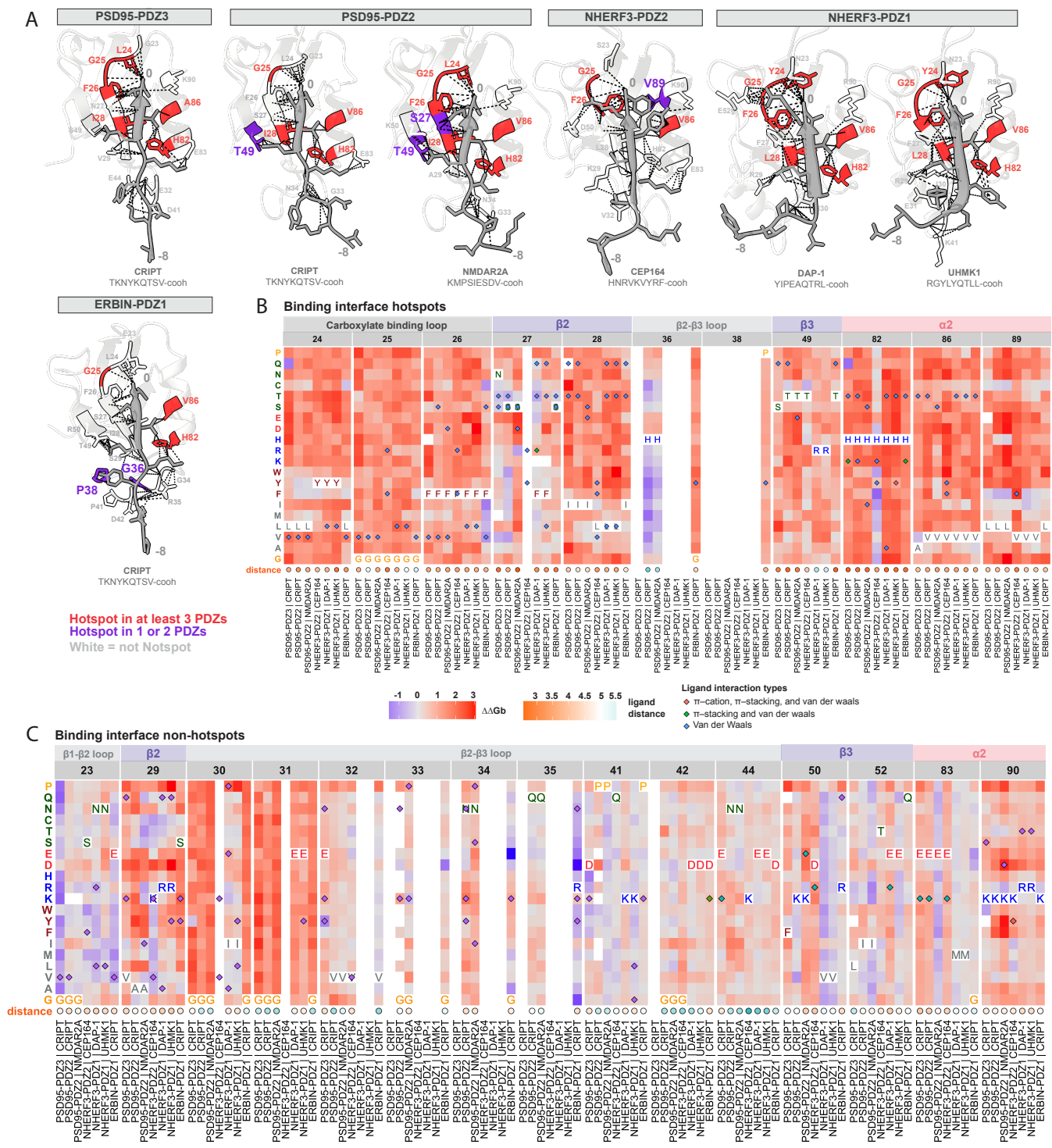
Supplementary Figure 1: Energetic landscapes of multiple PDZ domains homologs

a. Triplicate fitness Pearson correlations (biological replicates) and fitness density distributions for all libraries, including AbundancePCA (aPCA), BindingPCA (bPCA), and two blocks for each domain and interaction. **b.** Coefficient of determination (R^2) for Pearson correlations of normalized fitness measurements in overlapping regions between blocks (i.e. repeated variant measurements in independent experiments). Red points represent libraries lacking single amino acid substitutions in the wild-type background; for visualization, data from the most wild-type-like background ($\Delta\Delta G_b$ of single-mutant background closest to 0) are shown. Error bars indicate 95% confidence intervals ($n=3$ biological replicates). **c.** Fitness heat maps for single mutants for aPCA and bPCA results. As in panel b, for blocks lacking single variants on the wild-type background, data is shown for the most wild-type-like background. Dashes indicate wild-type residues. Residue numbering follows UniProt protein sequence annotation **d.** Scatter plots comparing aPCA and bPCA fitness measurements for single amino acid substitutions from the same PDZ domain.



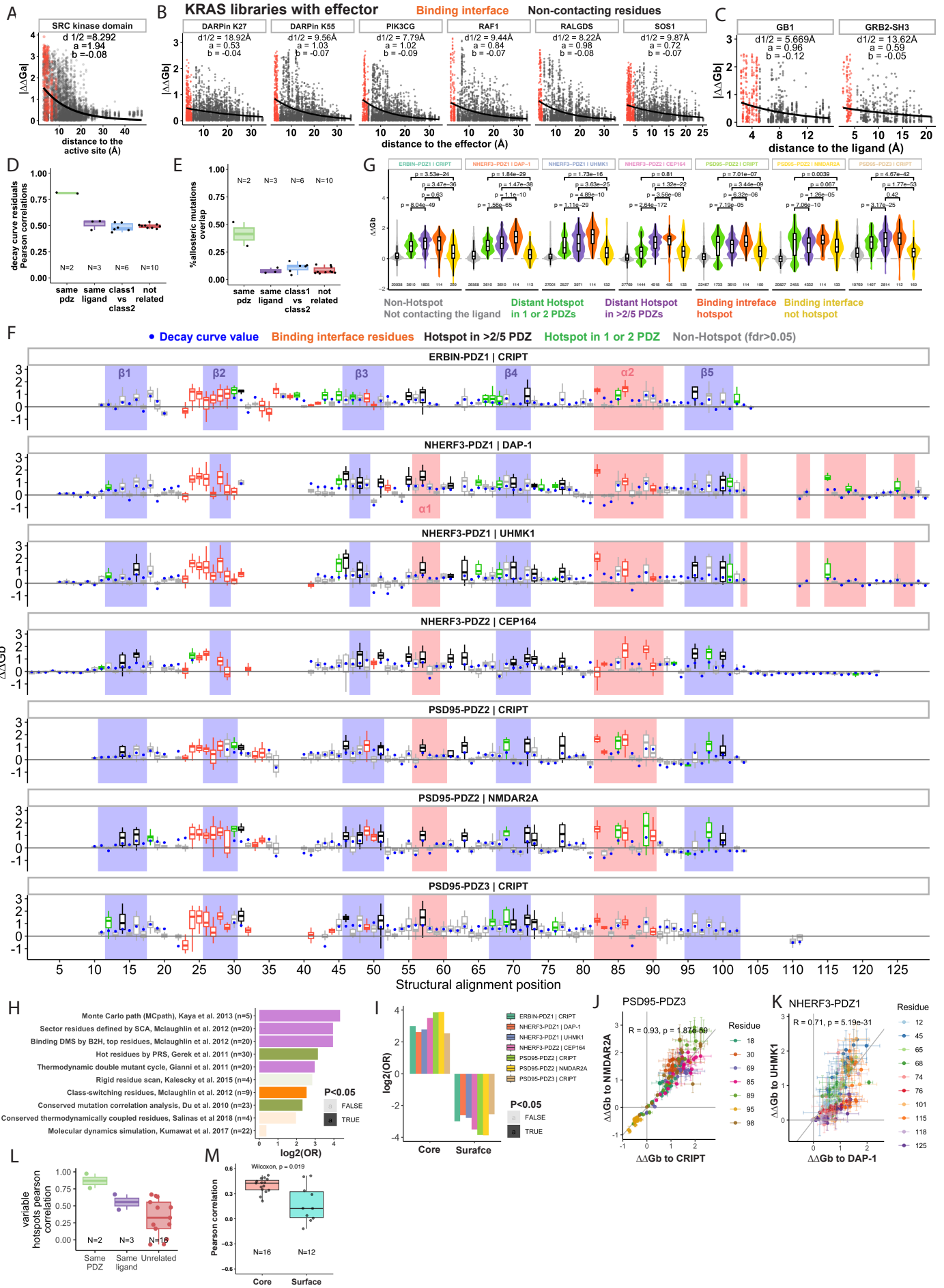
Supplementary Figure 2: From molecular phenotypes to free energy changes

a. Performance of models fit to doubleddeepPCA data for all libraries. Observed versus predicted fitness from thermodynamic models fitted. Pearson correlation is reported per library and block. **b.** Non-linear relationships (global epistasis) between observed aPCA fitness and changes in free energy of folding. Thermodynamic models fitted are shown in red. **c.** Non-linear relationships (global epistasis) between observed bPCA fitness and both changes in free energy of folding and binding. Thermodynamic models fitted are shown in red. **d.** $\Delta\Delta G_f$ distributions of core vs surface residues. P-value for two-sided Wilcoxon rank-sum test provided. Boxplots show the median of $\Delta\Delta G_f$ values in each group; hinges indicate the 25th and 75th percentiles; whiskers extend to 1.5x interquartile range (IQR). **e.** Scatter plots comparing folding and binding free energy changes for single amino acid substitutions for the same PDZ domain.



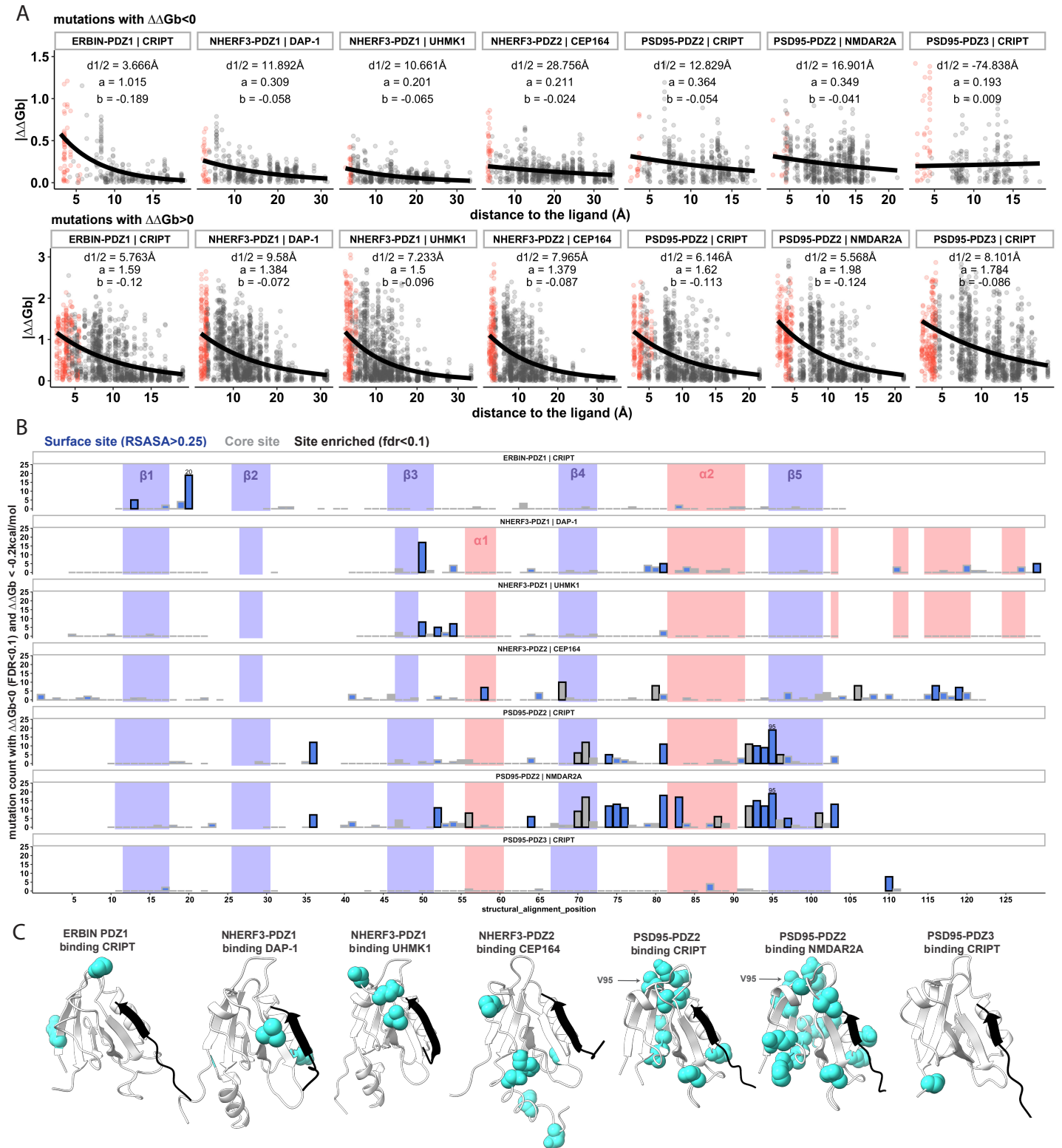
Supplementary Figure 3: Binding interface energy landscapes for seven interactions

a. Structure representation of hotspots in every interaction. All highlighted residues are ligand contacts. Non-hotspots (white), hotspots in 1 PDZ domain (purple), and recurrent hotspots in at least 3 (red). Ligand is represented in light grey. Dashed black lines show all predicted structural contacts with the ligand. **b.** Heat map of all structurally aligned residues that are classified as binding interface hotspots in at least one interaction. Letter labels refer to wild type residues in each position and interaction. Scatter points overlaid on the heat map represent ligand wild-type amino acids that make direct contact with the corresponding PDZ domain residue. Scatter points below the heat map represent the residue's minimum side chain heavy atom distance to the ligand. **c.** Same heat map for residues never classified as binding interface hotspots.



Supplementary Figure 4: Seven comparative energy maps

a. Absolute $\Delta\Delta G_a$ versus the minimum distances to the binding partner. Lines represent exponential decay fits ($y = a \cdot e^{bx}$), calculated excluding mutations within the binding interface for SRC, **b.** for KRAS and all interaction partners, and **c.** for GB1 and GRB2-SH3 domains. **d.** Pearson correlation of $\Delta\Delta G_b$ residuals across all pairwise comparisons of libraries. Boxplots show median; hinges indicate the 25th and 75th percentiles; whiskers extend to 1.5x IQR. **e.** Percentage of shared mutations with $\Delta\Delta G_b$ residuals > 0 (FDR < 0.1, one-sided Z-test) for pairwise library comparisons. Boxplots as in d. **f.** Boxplots of $\Delta\Delta G_b$ values for all residues across interactions. Blue dots indicate expected $\Delta\Delta G_b$ from distance-dependent decay fits. Black outlines denote conserved hotspots (≥ 3 PDZs; FDR < 0.05, one-sided t-test); green, variable hotspots (1-2 PDZs); red, binding-interface residues. Boxplots as in d. **g.** Distribution of $\Delta\Delta G_b$ values per interaction for non-hotspots outside the binding interface (gray), hotspots in one or two PDZs (green), conserved hotspots in three or more (purple), binding interface hotspots (orange), and binding interface non-hotspots (yellow). Two-sided Wilcoxon rank-sum P-values reported. **h.** Enrichment of hotspots in previously reported allosteric networks in PSD95-PDZ3. Bars show the most significant result among four one-sided Fisher's exact tests per dataset: PSD95-PDZ3-CRIPT hotspots excluding interface residues (blue); conserved hotspots (≥ 3 PDZs) excluding interface residues (green); PSD95-PDZ3-CRIPT hotspots including interface residues (orange); and conserved hotspots including interface residues (purple). **i.** One-sided Fisher's exact test enrichments of hotspots in core (rSASA < 0.25) and surfaces, per interaction. **j.** $\Delta\Delta G_b$ Pearson correlation for PSD95-PDZ2 binding two different partners; error bars indicate 95% confidence intervals from a Monte Carlo simulation approach (n = 10 experiments), and the same for **k.** NHERF3-PDZ1. **l.** $\Delta\Delta G_b$ Pearson's correlation for mutations in variable hotspots (1-2 PDZs). Boxplots as in d. **m.** Pearson correlation of mutations in hotspot residues located in the core versus the surface across all pairwise comparisons between different PDZ domains. Boxplots as in d.



Supplementary Figure 5: Affinity-increasing mutations.

a. Relationship between absolute $\Delta\Delta G_b$ and the minimum side-chain heavy-atom distance to the ligand for affinity-increasing ($\Delta\Delta G_b < 0$, top) and loss-of-function ($\Delta\Delta G_b > 0$, bottom) mutations. Curves show exponential decay fits ($y = a \cdot e^{bx}$), calculated excluding mutations within the binding interface.

b. Number of mutations per residue with $\Delta\Delta G_b < -0.2$ and significantly $\Delta\Delta G_b < 0$ effects (FDR<0.1, one-sided Z-test). Blue bars indicate surface residues; grey bars indicate core residues (rSASA<0.25). Black outlines mark residues significantly enriched in affinity-increasing mutations (FDR<0.1, one-sided FET).

c. Structural visualization of residues enriched in mutations with significantly $\Delta\Delta G_b < 0$ (FDR<0.1, One-sided FET) and $\Delta\Delta G_b < -0.2$.