

Supplementary Information

Proton-coupled alternating access in a versatile mycobacterial Spns drug transporter

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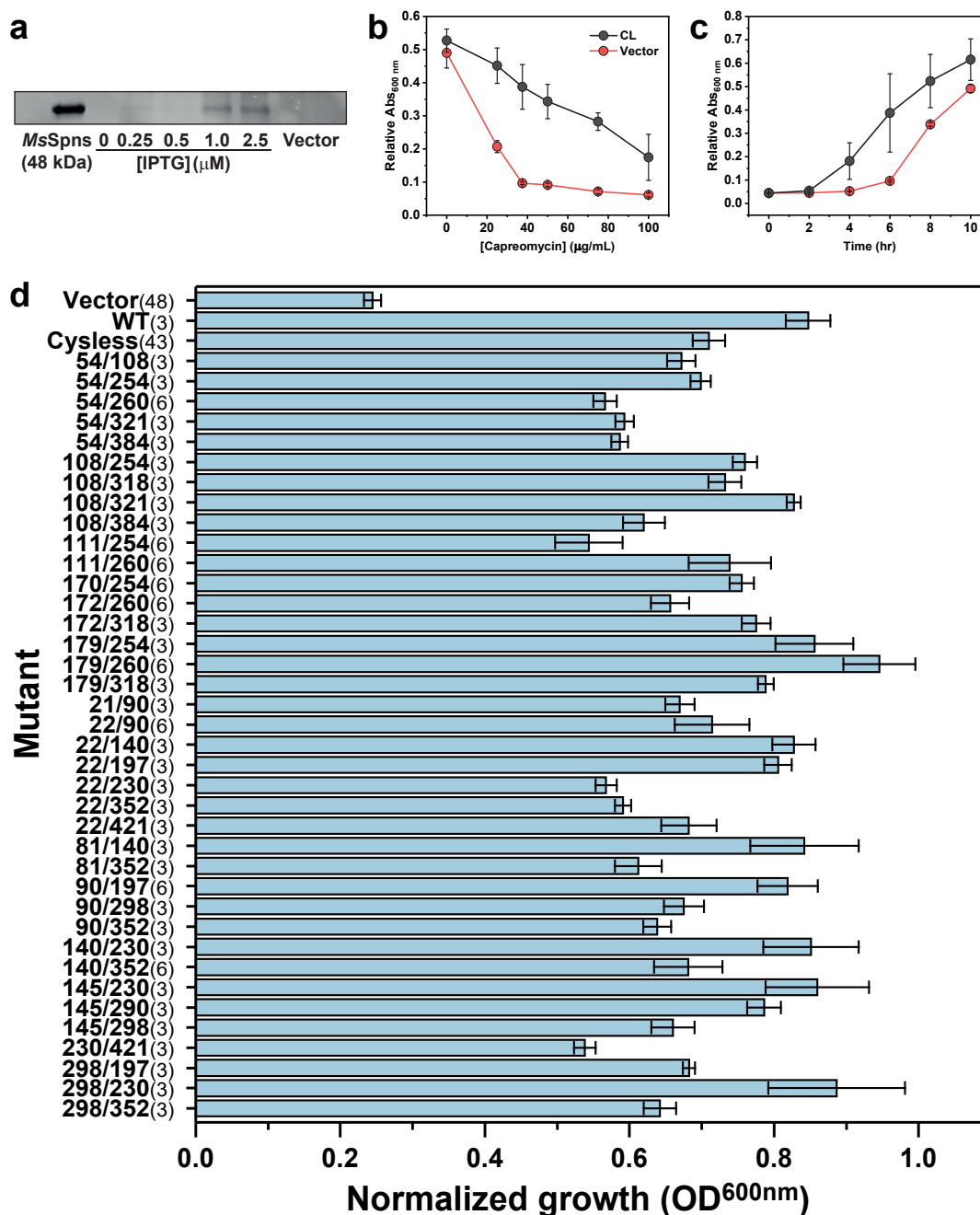
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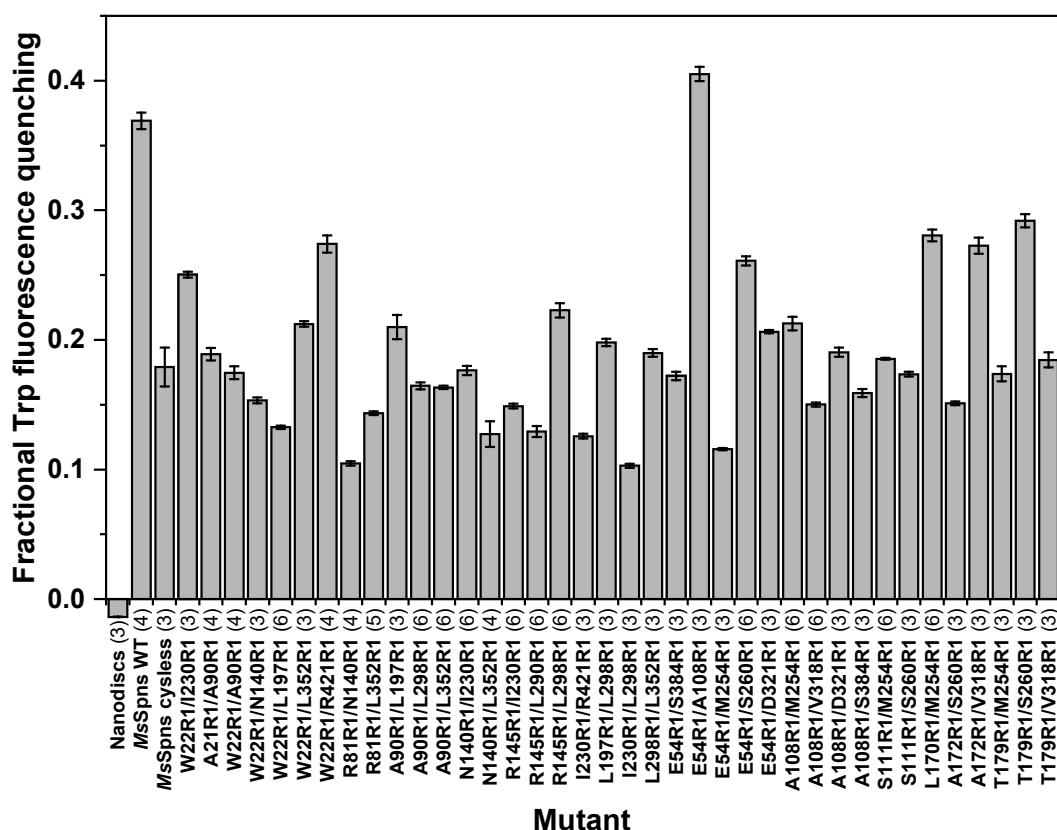
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Supplementary Figs. 1-9

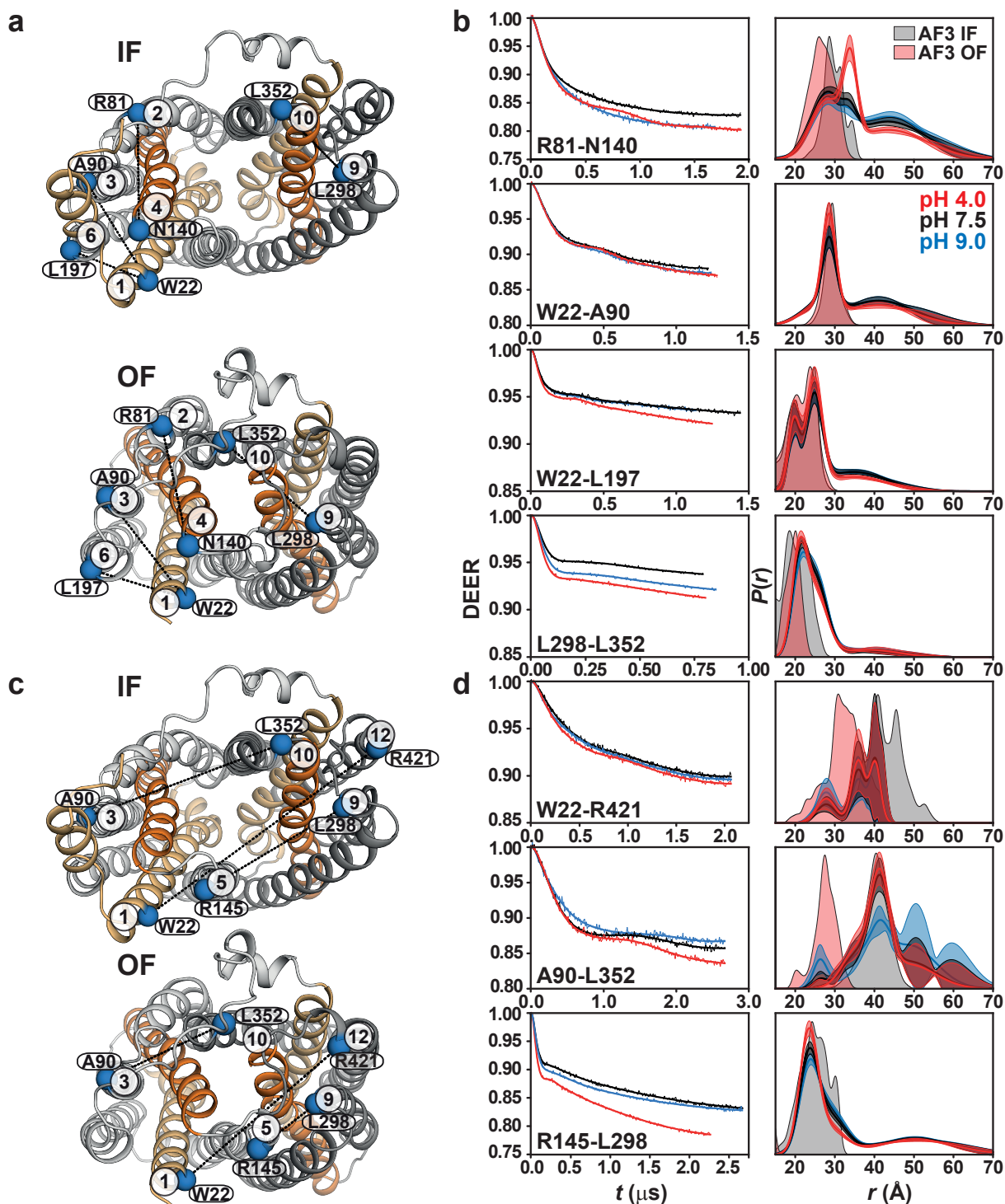


Supplementary Figure 1. Capreomycin resistance profiles of *MsSpns* mutants used for DEER spectroscopy. (a) Expression of cysteine-less (CL) *MsSpns* in cell-growth assays at different IPTG concentrations (0–2.5 μ M), visualized by SDS–PAGE and InVision His-tag staining. Purified CL *MsSpns* is shown as a standard. 1 μ M IPTG was used for protein expression in growth assays. (b) Expression of CL *MsSpns* enhances cell survival at elevated capreomycin concentrations relative to the vector control. Measurements were performed in triplicate after 8 h at 37 °C. A_{600nm} values were normalized to the 0 μ g/mL capreomycin control, and standard deviations are shown. The optimal capreomycin concentration was 37.5 μ g/mL. (c) Cells expressing CL *MsSpns* show improved growth relative to the vector control at all time points in the presence of 37.5 μ g/mL capreomycin. A_{600nm} values were normalized to the 0 μ g/mL control; data points represent mean $\pm$ s.d. from triplicate measurements, as in b. (d) Growth of *MsSpns* mutants at 37.5 μ g/mL capreomycin, normalized to the 0 μ g/mL control. Bars show mean $\pm$ S.E.M. for at least three replicates, with replicate numbers indicated in parentheses. For $n > 3$, data were collected from multiple biological replicates. One-way ANOVA showed significant differences among mutants at the 0.05 level $F(40, 234) = 28.81$, $p < 0.00001$, whereas Tukey's multiple-comparison test indicated that the double-cysteine mutants did not differ significantly from WT or CL *MsSpns*, showing that cysteine substitutions generally do not impair capreomycin resistance.

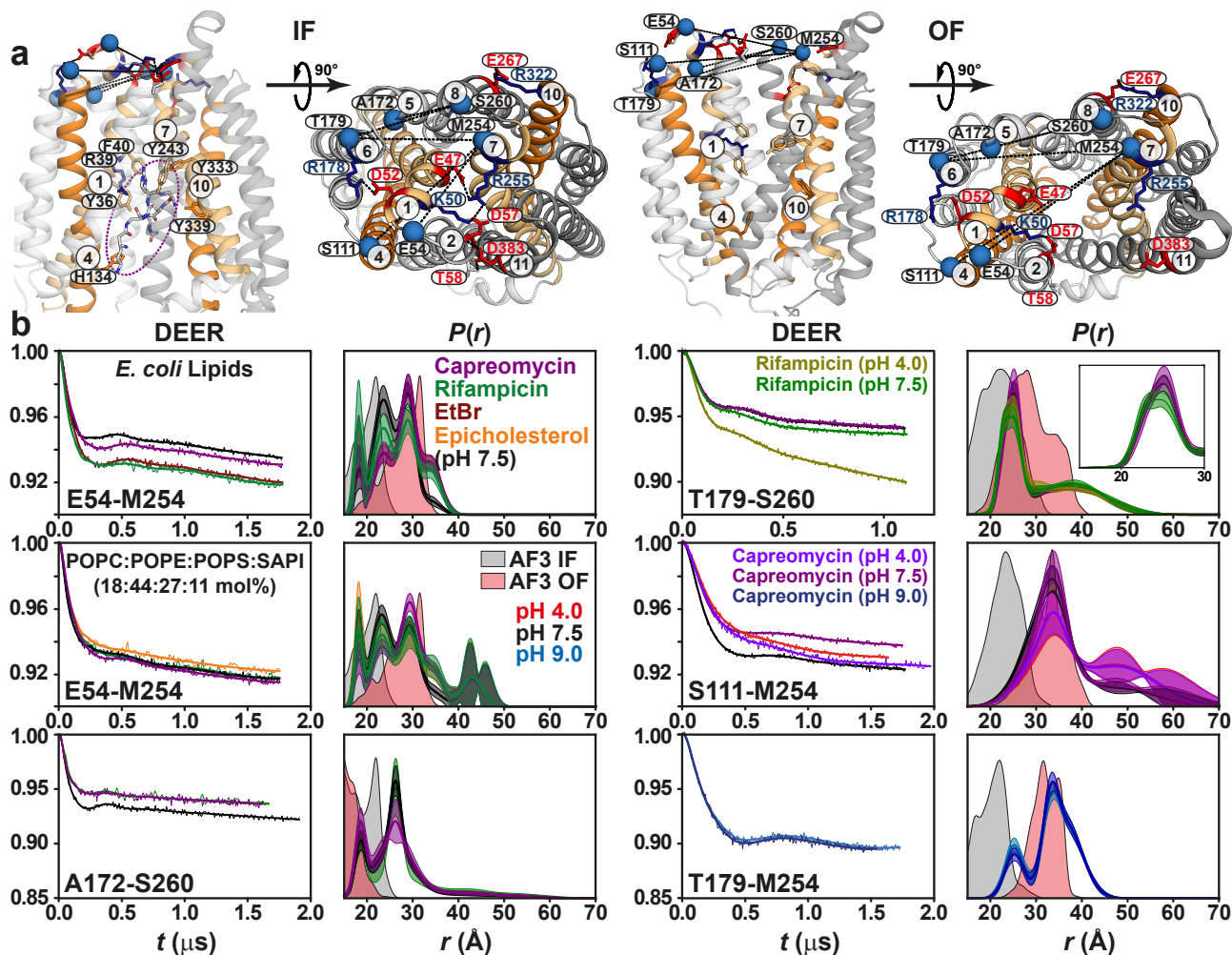


Supplementary Figure 2. Fractional fluorescence quenching of *MsSpns* mutants.

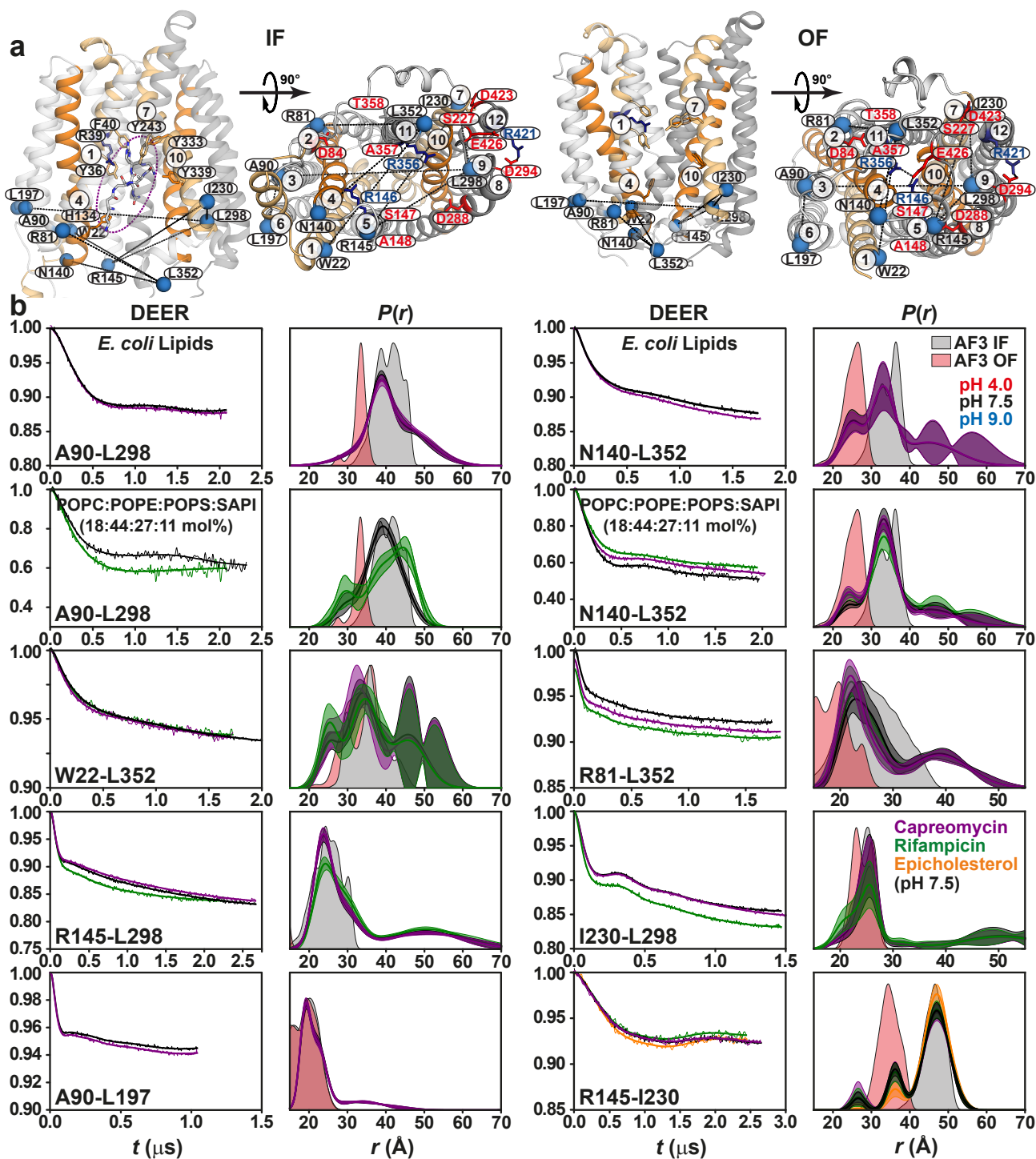
Low-pH fluorescence quenching of native tryptophan residues was used as a surrogate reporter of conformational changes in spin-labeled *MsSpns* reconstituted in nanodiscs, as described in Methods. Bars show mean $\pm$ S.E.M. for at least three replicates, with replicate numbers indicated in parentheses. For $n > 3$, data were collected from multiple biological replicates. One-way ANOVA showed significant differences among mutants $F(40,166) = 1160.9$, $p < 0.00001$, whereas Tukey's multiple-comparison test indicated that removal of native cysteines, introduction of target-site cysteines, and spin labeling did not significantly alter conformationally coupled fluorescence quenching.



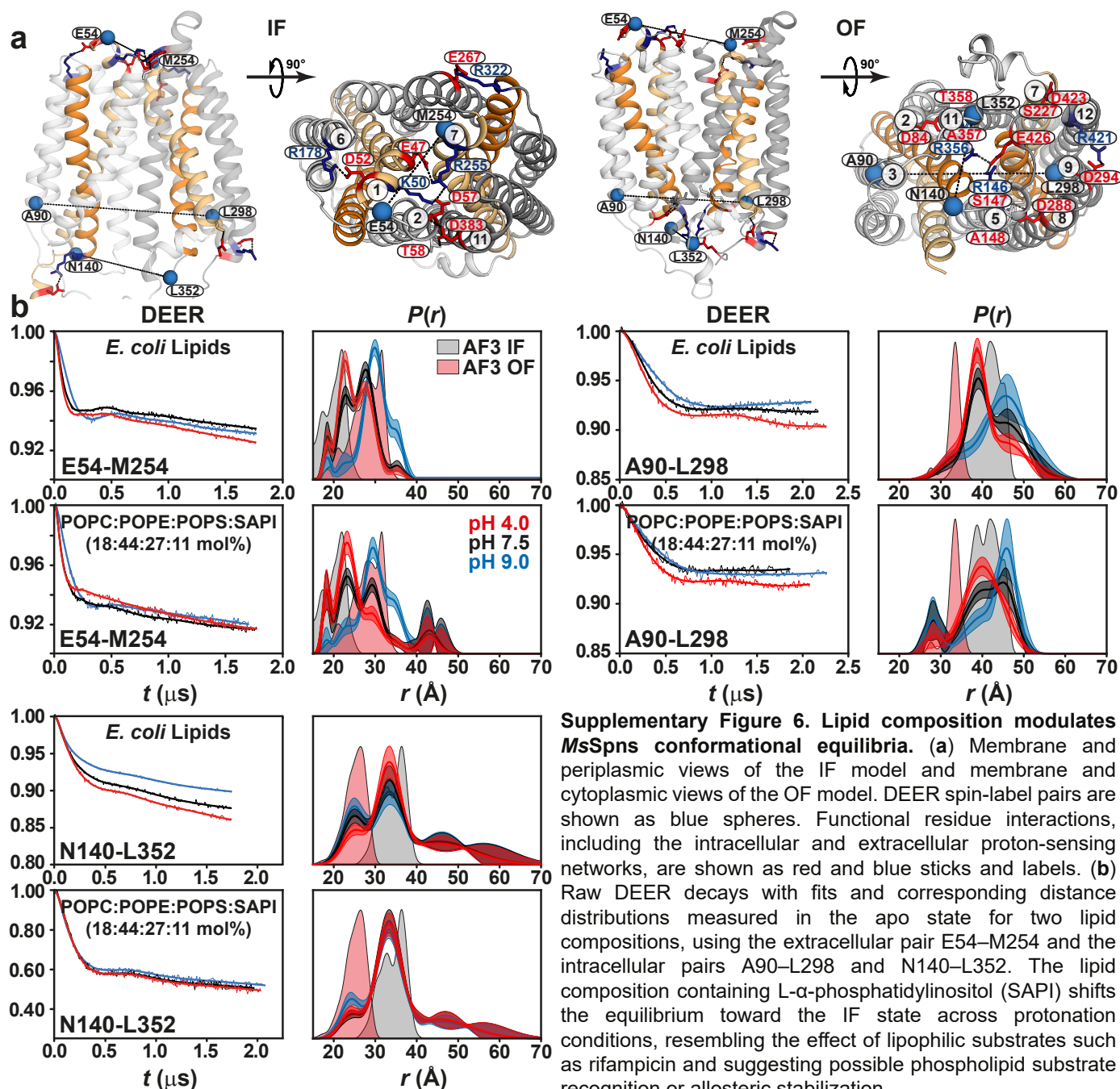
Supplementary Figure 3. Intra- and interdomain DEER distance measurements on the intracellular side. (a) Intradomain spin-label pairs are shown as blue spheres on the intracellular side of the IF and OF models. (b) Raw DEER decays with fits (left) and corresponding distance distributions, $P(r)$, measured in lipid nanodiscs (right). Distance distributions predicted from the IF and OF models are shown as gray and red shaded regions, respectively. Most intradomain pairs show limited proton-coupled conformational changes, except for R81–L352. (c) Interdomain spin-label pairs are shown as blue spheres on the intracellular side of the IF and OF models. (d) Raw DEER decays with fits (left) and corresponding nanodiscs distance distributions, $P(r)$ (right), with predicted IF and OF distributions shaded gray and red, respectively.

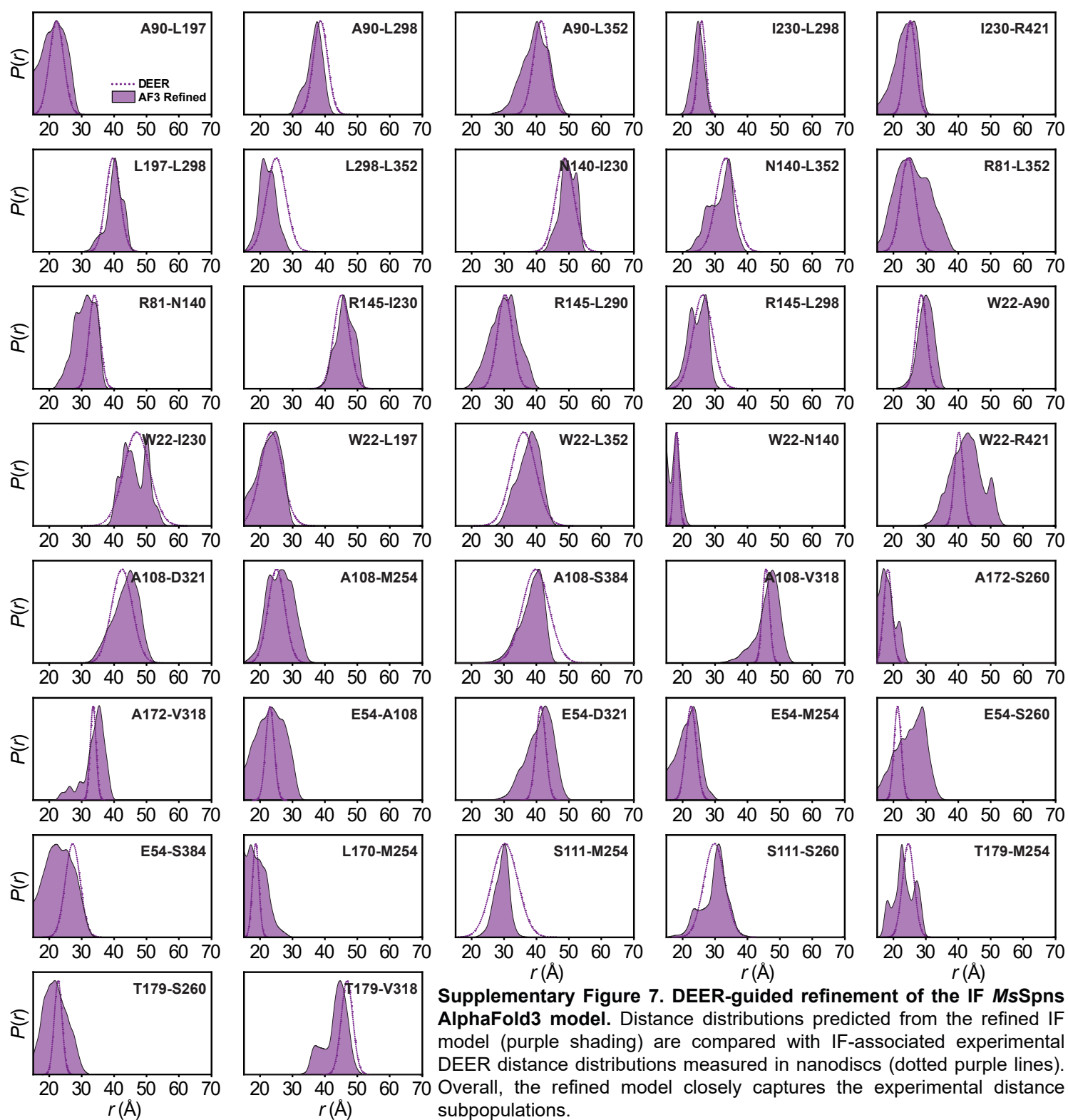


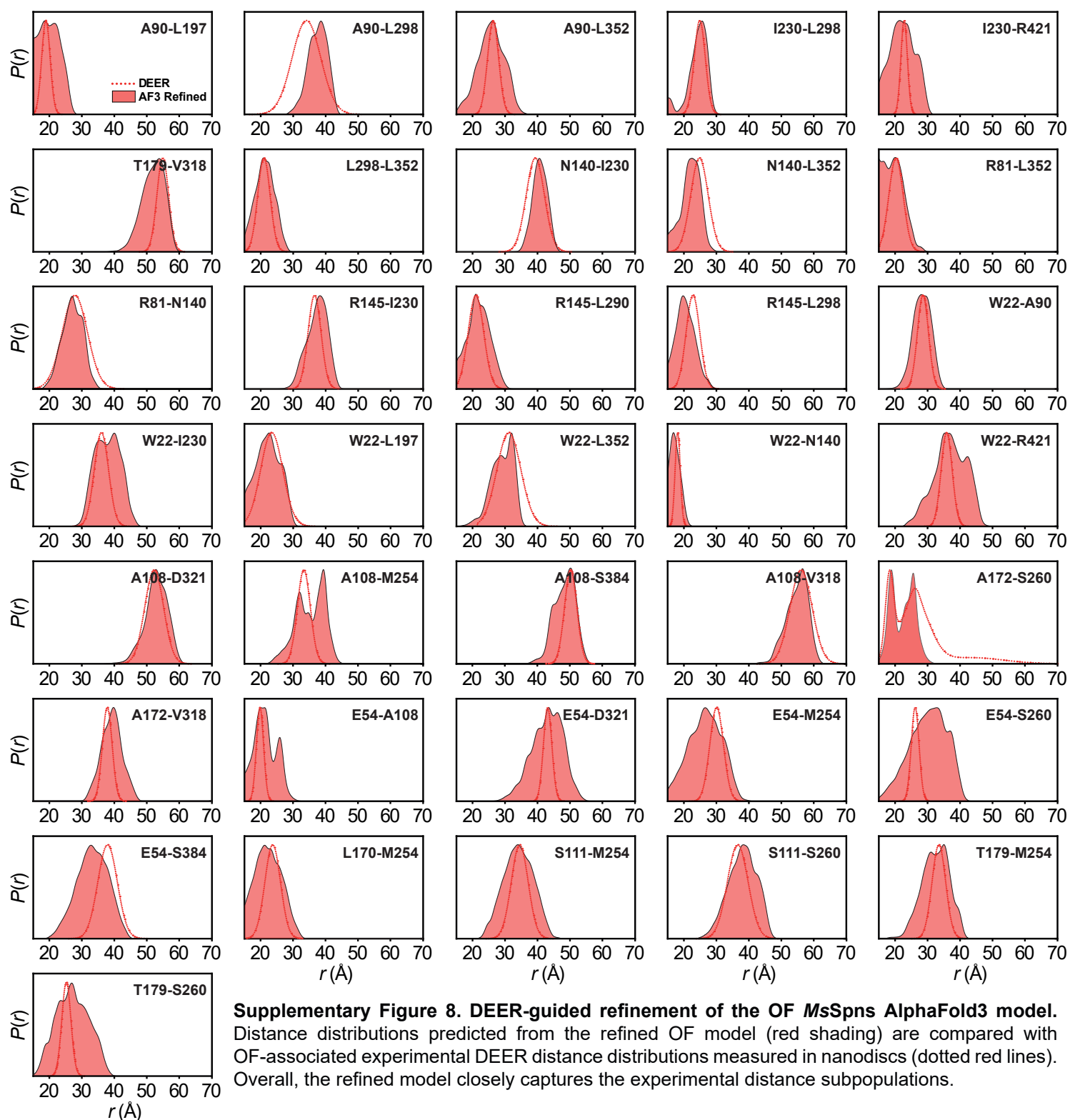
Supplementary Figure 4. Hydrophobic and hydrophilic substrates differentially shift extracellular MsSps conformational equilibria. (a) Membrane and periplasmic views of the IF and OF models. Capreomycin (circled) is docked in the IF substrate-binding cavity, with conserved aromatic residues in binding cavity shown as sticks and DEER spin-label pairs shown as blue spheres. Functional residue interactions stabilizing the IF conformation are highlighted in red and blue in the periplasmic views. (b) Raw DEER decays with fits and corresponding extracellular distance distributions measured in the apo state and in the presence of substrates. Two lipid compositions were tested for the E54–M254 pair. Water-soluble cationic substrates, including capreomycin and ethidium bromide, stabilize the OF conformation, whereas lipophilic substrates, including rifampicin and epicholesterol, shift the equilibrium toward the IF state.

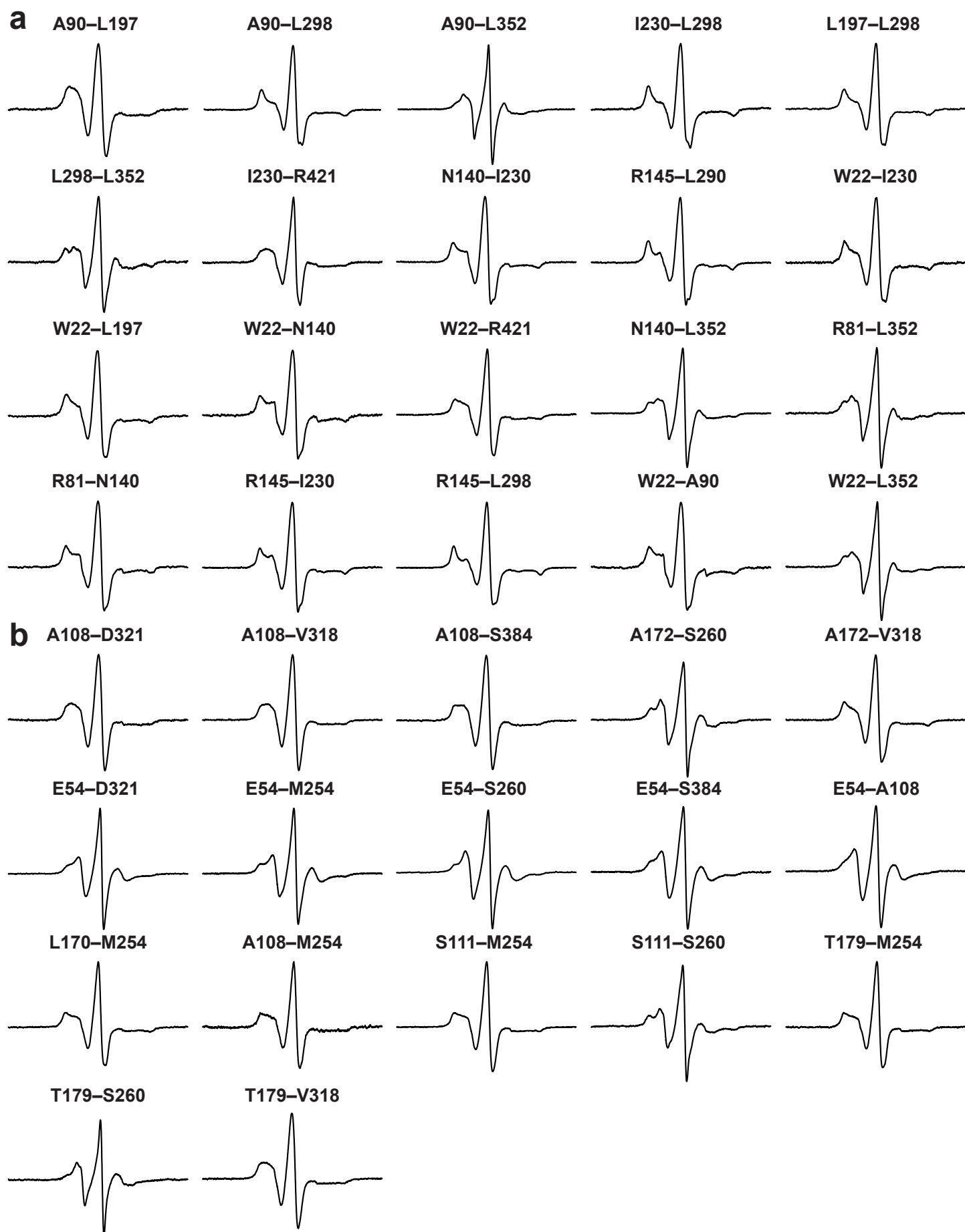


Supplementary Figure 5. Substrate hydrophobicity drives opposing shifts in intracellular *MsSpns* conformational equilibria. (a) Membrane and cytoplasmic views of the IF and OF models. Capreomycin is docked in the IF substrate-binding cavity, with conserved aromatic residues shown as sticks and DEER spin-label pairs shown as blue spheres. Functional residue interactions that stabilize the OF conformation are highlighted in red and blue in the cytoplasmic views. (b) Raw DEER decays with fits and corresponding intracellular distance distributions measured in the apo state and in the presence of substrates. Two lipid compositions were tested for the A90–L298 and N140–L352 pairs. Water-soluble cationic substrates, including capreomycin and ethidium bromide, stabilize the OF conformation, whereas lipophilic substrates, including rifampicin and epicholesterol, shift the equilibrium toward the IF state.









Supplementary Figure 9. CW EPR spectra of spin-labeled double-cysteine mutants for DEER spectroscopy.



MsSpns **0** **0.25** **0.5** **1.0** **2.5** **Vector**
(48 kDa) **[IPTG](μ M)**

Supplementary Figure 1a

