Supplementary Information

Computational Details for 20 ns FEP simulations

The complete initial structure with dual topology was subjected to an initial relaxation for 4000 steps and then 6 ns long equilibration at 298 K was performed in two independent simulations, where the λ was set to 0 and 1. Then, the structures (saved very 4 ps) from last 5 ns of each simulation were subjected to cluster analysis, where they were categorized under 5 groups based on RMSD from the initial structure. Then, a structure from each group was extracted to be used in a subsequent FEP calculation. Therefore, the free energy differences were calculated at least 5 times for each mutation with each starting from a distinct initial structure.

Based on the preliminary considerations, we decided to perform each mutation in 60 windows and sample each window for 170 ps providing over 20 ns of total simulation time for each calculation including the forward and backward sampling. To avoid singularity at small interaction distance, when λ approaches to 0 or 1 (called endpoint-catastrophe), we used soft core Lennard-Jones potential. Although this will ensure to avoid the endpoint-catastrophe, we also used a soft core potential with our adapted 60-window scheme: [λ=0.0, 0.0001, 0.001, 0.01, 0.05, 0.1, 0.12, 0.14, 0.16, 0.18, …..0.86, 0.88, 0.90, 0.95, 0.99, 0.9999, 1.0] In addition, we ran another 10 ns-long 'backward' simulation for each simulation, where the transformation is achieved from the mutant to the wild-type. This backward simulation was set to start from exactly where the forward simulation ended.

Analysis of FEP: BAR Method

For two states A and B, the BAR method uses sampling on both A and B together with a constant C to calculate ΔG:

$$\Delta G = -kT ln \frac{\langle e^{-(U_B - U_A - C)kT} \rangle_A}{\langle e^{-(U_B - U_A + C)kT} \rangle_B} e^{-C/kT}$$

The optimal value of C, which is when it is equal to ΔG, is then iteratively optimized to convergence. This gives the maximum-likelihood of value of the free energy with the statistical error. Again, this calculation is divided into windows and by combining C values from each window, we will obtain overall ΔG with statistical error.

SI Table 1. Results from Other Webservers and 20 ns-long FEP simulations. The latter were not completed for all due to unrealistically high results obtained. *FEP technically cannot be applied to Pro-

MBD Mutants	ΔΔG (kcal/mol, negative means stabilizing)							
	Cupsat (1qk9)	**Cupsat (3c2i)**	**dFIRE (1qk9)**	**dFIRE (3c2i)**	**SDM (1qk9)**	**SDM (3c2i)**	**MuStab (Seq)**	**FEP (1qk9)**
T158M	0.56	-0.12	-1.40	-4.67	-1.55	-1.35	+	0.64
R133C	-0.26	-1.57	1.21	1.21	0.65	-0.3	+	8.31
R106W	2.32	2.33	1.16	-1.36	-2.02	-1.9	-	8.12
P152R	0.77	1.53	-0.33	-4.18	1.53	0.77	+	*
A140V	0.28	-	0.49	-2.91	1.57	1.42	+	-1.4
S134C	0.18	0.06	-0.69	-3.57	0.68	0.11	-	0.90
R106Q	2.46	5.93	1.02	-2.80	0.14	0.62	-	-
D156E	-0.06	0.81	-1.51	-3.67	-0.6	-0.11	-	18.0
R133H	-0.26	-1.5	-0.37	-2.56	0.46	-1.23	+	8.03
L100V	0.59	1.65	-1.84	-5.21	0.63	0.94	+	-0.52
F155S	1.71	3.52	-5.73	-8.72	4.88	3.88	+	5.03
T158A	0.69	0.60	-1.59	-6.64	-2.00	0.02	+	0.12
R111G	0.64	-0.99	0.43	0.37	0.77	2.61	-	-

involving mutations. MuStab conditions: pH=7 and T=25 degrees Celsius.

SI Table 2. Percent relative solvent accessibility (SASA) of wild-type and mutant structures (1QK9 and 3C2I) and the change in SASA upon each mutation. SASA was normalized by dividing the absolute SASA value obtained for the residue in question in the protein by its value in the three-residue segment from the same protein, where the residue in question is in the middle.

	SASA 1qk9	SASA Change	SASA 3c2i	SASA Change
L100	16.5		24.5	
L100V	6	-10.5	32.1	7.6
R106	11		4.2	
R106W	7	-4	2.8	-1.4
P152	29.3		12.7	
P152R	36	0.1	20	0.1
R133	44.2		60	
R133C	45	0.8	49.2	-10.7
R133H	38.4	-5.8	37.6	-22.4
S134	41.3		42.7	
S134C	64.1	22.9	42.2	-0.5
R106Q	18.6	0.1	17.4	0.1
A140	41.2		50.4	
A140V	53.1	11.8	60.4	10
F155	3.3		6.8	
F155S	32.1	28.8	7.9	1.2
D156	28.3		6.9	
D156E	40.1	11.8	4.6	-2.3
T158	37.4		45.4	
T158A	25.6	-11.8	32.1	-13.3
T158M	43.6	6.2	50.8	5.4
R111	59.4		43.7	
R111G	41	-0.2	25.8	-0.2

SI Table 3: Salt bridges in WT and mutant structures

Structures		E137-R133	D156-R106	D97-R106	D121-R111	D156-R162
WT	1QK9	✓	X	✓	✓	✓
	3C2I	✓	✓	✓	✓	✓
T158M	1QK9	✓	X	✓	✓	✓
	3C2I	✓	✓	✓	✓	✓
R133C	1QK9	X	X	✓	✓	✓
	3C2I	X	✓	✓	✓	✓
R106W	1QK9	✓	X	X	X	✓
	3C2I	✓	X	X	✓	✓
A140V	1QK9	✓	X	✓	✓	✓
	3C2I	✓	✓	✓	✓	✓
S134C	1QK9	✓	X	✓	✓	✓
	3C2I	✓	✓	✓	✓	✓
D156E	1QK9	✓	✓	✓	✓	X
	3C2I	✓	X	✓	X	✓
R133H	1QK9	X	X	✓	✓	✓
	3C2I	✓	✓	✓	✓	✓
L100V	1QK9	✓	X	✓	✓	✓
	3C2I	✓	✓	✓	✓	✓
F155S	1QK9	✓	✓	X	✓	✓
	3C2I	✓	X	✓	X	✓
T158A	1QK9	✓	X	✓	✓	✓
	3C2I	✓	✓	X	✓	✓
R111G	1QK9	✓	X	✓	X	✓
	3C2I	✓	✓	X	X	✓
R106Q	1QK9	✓	X	X	X	X
	3C2I	✓	X	X	✓	✓
P152R	1QK9	✓	X	✓	X	✓
	3C2I	✓	✓	✓	✓	✓