Supplemental Figure captions

Table S1. Human Pumilio 1 and 2 λ-dynamics screening results to unmodified RNA.

Table S2. Human Pumilio 1 λ-dynamics screening results to modified RNA.

Table S3. Human Pumilio 2 λ-dynamics and in vitro binding results to modified RNA.

Table S4. Alchemical groups for initial λ-dynamics screen of modified RNA bases.

Fig S1. ANOVA binding statistics**.** Binding statistics of **(A)** human Pumilio 1 (hPUM1) and **(B)** human Pumilio 2 (hPUM2) in vitro binding results to wild type or modified RNA. All results were compared to wild type RNA. See the **Methods** for further details.

Fig S2. Human Pumilio 2 binding to modified RNAs in vitro was also consistent to λ-dynamics predictions in silico. **(A)** Electrophoretic Mobility Shift Assays (EMSAs) were used to estimate the binding affinity in vitro. Synthetic RNA oligos without or with the designated RNA modifications were incubated with increasing concentrations (0-1000 nM) of recombinant human Pumilio 2 (hPUM2) RNA binding domain, run on a polyacrylamide gel, and imaged for carboxyfluorescein (FAM) fluorescence. A sample without protein served as an unbound RNA control. The lower band corresponds to unbound RNA. The upper band corresponds to RNA bound to the recombinant protein. The binding affinity can be estimated by calculating the protein concentration at the binding inflection point. Shown are representative gels from hPUM2 EMSA binding experiments. **(B)** EMSAs were performed at least three times, and calculated binding dissociation constants (K_d) reported with their mean and standard deviation. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. See **Fig S1** for full statistical analyses and the **Methods** for more experimental details.

Fig S3. A thermodynamic cycle used for computing relative binding free energies ($\Delta\Delta G_{bind}$) between native and mutant RNA, labeled “RNA” and “RNA*”, respectively, bound to a protein, P. The mutant RNA* represents either a canonical RNA nucleobase or a modified base substitution at any one site along the length of the oligo. The relative difference in binding affinities between native (ΔG_{nat}) and mutant (ΔG_{mut}) RNAs can be calculated as a difference in alchemically perturbing the reference RNA sequence into the mutant sequence in the RNA-protein complex (ΔG_{Cmplx}) and solvated, unbound RNA (ΔG_{RNA}). No mutations were sampled on the protein (ΔG_P), and therefore its free energy contribution is zero.