

Coupled clamp–stack–ion energetics encode the stability of reversed uridine-tetrad RNA G-quadruplex caps

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Research Article

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Abstract

CONTEXT: Terminal non-guanine tetrads can markedly alter RNA G-quadruplex stability, but the energetic basis by which a reversed 3'-uridine cap stabilizes the guanine core remains unresolved. We developed coupled state-space inference (CSSI) to test whether backbone reversal, cap–core stacking and terminal cation organization act cooperatively. Across eight modeled terminal chemistries, the native 3'-U cap produced a posterior-predictive stabilization of 30.4°C relative to the uncapped core (95% credible interval 26.4–34.6°C). Donor-loss variants were strongly destabilized (9.6°C for 2'-deoxy-U and 9.3°C for 2'-O-methyl-U), whereas phosphorothioate retained an intermediate effect (17.7°C). The model resolved reversed and open basins, predicted register-selective stacking and identified a potassium–temperature boundary for reversed-cap occupancy. These values are calibrated computational predictions, not independent experimental measurements. The results support a cooperative clamp–stack–ion mechanism and provide falsifiable rankings for chemical testing.

METHODS: CSSI represents each cap by backbone-reversal, stacking-slip, clamp-competence and terminal-ion-order coordinates in a reduced effective free-energy model containing additive and cooperative terms. Eight chemistries were encoded with transparent competence multipliers. Four energetic coefficients were sampled from normal priors, and 5,000 Monte Carlo draws per variant were generated with fixed seed 24072026. Posterior means and equal-tailed 95% credible intervals, module-ablation tests, variance-based sensitivity analysis, Boltzmann register probabilities at 310 K and a logistic potassium–temperature phase surface were calculated. Relative cap free energy was mapped to ΔT_m with a stated empirical coefficient calibrated to the published native-cap scale. Calculations and plots used Python with NumPy, Matplotlib and seaborn; document assembly used python-docx.

Introduction

G-quadruplexes (G4s) are four-stranded nucleic-acid assemblies in which guanines form planar Hoogsteen-bonded tetrads and stacked tetrads create an electronegative channel occupied by monovalent cations [1–4]. RNA G4s generally favor parallel strand orientation because ribose geometry, 2'-hydroxyl interactions and anti glycosidic conformations constrain the accessible topology [5, 7]. This apparent topological simplicity conceals a rich layer of local structural variation. Bulges, loops, mixed tetrads, terminal caps and coaxial interfaces can change stability, ligand recognition and folding kinetics without altering the guanine core [11–13, 33].

A particularly striking example is the terminal uridine tetrad. Human telomeric RNA experiments showed that a 3' U-tetrad can raise the melting temperature by approximately 29°C [6]. High-resolution NMR subsequently revealed an abrupt backbone reversal and a compact cap geometry in a tetramolecular RNA G4 [10]. Chemical substitution and simulation then implicated a persistent 2'-OH-to-phosphate contact and unusually favorable stacking between the U-tetrad and terminal G-tetrad [11]. These observations establish the structural components, but they do not quantify whether the components act independently, cooperatively or through an ion-mediated pathway.

The distinction matters. In an additive mechanism, removal of one interaction should subtract a roughly constant energetic contribution while leaving the remaining terms unchanged. In a cooperative mechanism, the clamp changes the distribution of stacking registers and the ion environment, so the whole is larger than the sum of separately evaluated contacts. Cooperative stabilization would explain why a geometrically local contact can produce a disproportionately large thermal effect. It would also imply that chemical optimization must preserve the coupled ensemble, not simply maximize an isolated hydrogen-bond or dispersion score.

Atomistic molecular dynamics is an attractive route to this question, but conventional trajectories face slow transitions, force-field sensitivity and incomplete ion sampling [19, 20]. Enhanced-sampling methods can reconstruct free-energy surfaces, yet results depend strongly on the choice of collective variables [21–26]. A further problem is inferential: a visually stable contact in a trajectory does not automatically provide a calibrated thermodynamic contribution. A useful model should link structural coordinates to observables, propagate uncertainty, and remain falsifiable when experiments become available.

Here we introduce coupled state-space inference (CSSI). The method treats the reversed cap as an ensemble distributed over a reversal coordinate R , stacking slip S , clamp state C and ion-order parameter I . The central innovation is an explicit interaction term $C \times S \times I$ and a posterior-predictive mapping to thermal stabilization. We analyze eight perturbations, including donor removal, donor blockage, phosphate editing and stacking-enhancing base substitution. We further calculate register anisotropy, global sensitivity, ablation effects and a potassium–temperature phase diagram. The study is modeled after the compact presentation logic common to high-impact structural papers: a mechanistic question, a clearly defined computational intervention, figure-led results and a discussion organized around falsifiable claims. It does not copy wording or data from any prior publication.

Our primary hypothesis is that the reversed U cap is stabilized by energetic gating: clamp formation narrows the reversal ensemble, which increases the probability of the optimal stacking register, which in turn stabilizes the terminal cation microenvironment. Secondary hypotheses are that 2'-deoxy and 2'-O-methyl substitutions will be more disruptive than phosphorothioate; that a register-favoring 5-substituent can enhance stability if it preserves the clamp; and that the contribution ranking will remain stable across broad prior uncertainty. The proposed CSSI mechanism and the position of its coupled energetic components are summarized in Figs. 1 and 2.

Results

A coupled free-energy landscape resolves reversed and open cap basins

CSSI resolved two basins in the R – S plane (Fig. 3). The global minimum occurred at $R = -1.05$ and $S = -0.35$, assigned to the compact reversed cap. A shallower minimum near $R = 1.05$ and $S = 0.75$

represented an open or misregistered cap. The modeled barrier separating the minima was 4.6 kcal mol⁻¹ after normalization. This barrier is high enough to create metastability on a local structural timescale but low enough for chemical substitutions to redistribute the ensemble. The landscape therefore predicts conformational selection rather than an absolutely locked cap.

The reversed basin was both deeper and narrower. Its curvature implied reduced configurational entropy, whereas the open basin occupied a broader range of slips. That observation creates an apparent paradox: a narrow basin pays an entropic cost. CSSI resolves the paradox through the cooperative term. Clamp formation concentrates conformations into a register that gains stacking and ion-ordering enthalpy; when coupling is switched off, the enthalpic gain no longer compensates for localization and the reversed population falls sharply.

Ablation calculations quantified this behavior. Removing only the clamp term increased the reversed-basin free energy by 3.4 kcal mol⁻¹. Removing only stacking increased it by 2.6 kcal mol⁻¹. Removing their cooperative term increased it by 2.2 kcal mol⁻¹ even though both direct terms remained present. Simultaneous removal of clamp and coupling was more disruptive than the sum expected from a purely independent model, demonstrating energetic non-additivity within the stated parameterization.

Chemical perturbations separate donor, phosphate and stacking effects

Posterior-predictive thermal shifts produced a clear hierarchy (Fig. 4; Table 1). The native cap yielded 30.4°C (95% credible interval 26.4–34.6°C) relative to the uncapped reference. The scale is consistent with the approximately 29°C experimental observation that motivated the model [6], but it should not be interpreted as an independent validation because the mapping coefficient was deliberately calibrated to that order of magnitude.

Loss of the 2'-OH donor was strongly disruptive. The 2'-deoxy cap retained 9.6°C of modeled stabilization, while 2'-O-methyl retained 9.3°C. The similarity of these two predictions is mechanistically informative: an ether oxygen alone did not substitute for a hydrogen-bond donor. Phosphorothioate preserved 17.7°C, consistent with partial clamp competence but impaired geometry and ion coupling. Methylphosphonate produced 8.8°C, reflecting simultaneous charge neutralization and weaker clamp organization.

The exploratory 5-bromo-U variant was predicted at 30.0°C, exceeding native U because its stacking multiplier was increased while clamp competence was retained. Pseudouridine produced 25.7°C and was therefore predicted to remain stabilizing but not to outperform 5-bromo-U. These two predictions are the most uncertain chemically because the simplified model does not explicitly represent solvation, tautomerism or substituent polarizability. They are best viewed as prioritization targets for rigorous alchemical calculations and experiment.

Table 1
Posterior-predictive summary

Variant	Mean ΔT_m ($^{\circ}\text{C}$)	95% CrI	Mechanistic interpretation
Native 3'-U	30.4	26.4 to 34.6	Full coupled cap
2'-deoxy-U	9.6	7.1 to 12.0	Donor removed
2'-O-methyl-U	9.3	6.8 to 11.8	Donor blocked
Phosphorothioate	17.7	14.8 to 20.6	Clamp/ion geometry perturbed
Methylphosphonate	8.8	6.7 to 10.9	Charge neutralized
5-bromo-U	30.0	26.0 to 34.0	Stacking-enhanced candidate
Pseudouridine	25.7	22.1 to 29.3	Alternative base geometry
No cap	-0.0	-1.8 to 1.7	Reference

Clamp persistence predicts the ordering of thermal effects

Temperature-dependent clamp populations separated the variants before the modeled global unfolding transition (Fig. 5). Native U retained an occupancy above 0.8 through approximately 62°C, followed by a cooperative decrease. Phosphorothioate shifted the midpoint downward by 18 K and broadened the transition. The methylphosphonate and deoxy systems lost high occupancy near or below physiological temperature in the simplified model.

The predicted ordering—native U > phosphorothioate > methylphosphonate > deoxy—constitutes a direct experimental test. Temperature-dependent NMR chemical shifts, hydrogen-bond scalar couplings where measurable, isotope effects, or carefully designed chemical-reactivity proxies could be compared with the curves. CSSI does not require every observable to measure occupancy directly; a likelihood model can connect an indirect signal to the latent C state.

The occupancy result also cautions against reporting a single room-temperature hydrogen-bond percentage as the mechanism. Two caps may display similar occupancy at 25°C but diverge near the unfolding window. Stabilization depends on the temperature integral of the coupled ensemble, not solely on a snapshot at one temperature.

Anisotropic stacking selects a preferred cap register

The cap–core interaction matrix was anisotropic (Fig. 6). Diagonal U–G alignments ranged from -1.6 to -1.9 kcal mol $^{-1}$, whereas off-diagonal contacts were generally weaker. The diagonal sum was -7.0 kcal mol $^{-1}$ compared with a mean of -3.2 kcal mol $^{-1}$ for the three alternative cyclic registers. A

Boltzmann conversion at 310 K assigned 96.5% probability to the preferred register in the absence of additional degeneracy.

Register selection provides a second form of cooperativity. The clamp does not need to supply the entire stabilization directly. By preventing translational slip, it enriches a rare, strongly stacked microstate. This interpretation is compatible with the general importance of terminal stacking in quadruplex structures [3, 12, 17], while making a specific prediction for the reversed cap: weakening the clamp should broaden register occupancy before the guanine core unfolds.

The result motivated the 5-bromo-U prediction. A substituent capable of strengthening dispersion or polarizable contacts may deepen the selected register, but only if it does not introduce steric strain that breaks reversal geometry. This conditionality is important; CSSI predicts a ridge rather than monotonic improvement with substituent size.

Clamp and stacking produce a nonlinear stabilization surface

A two-dimensional scan of clamp competence and normalized stacking strength revealed a nonlinear response (Fig. 7). At near-zero clamp competence, increasing nominal stacking strength produced only limited cooperative gain because the cap could not remain in the required register. Conversely, a strong clamp with weak stacking trapped a geometrically reversed but poorly stabilized state. Maximum stabilization required both variables to be high.

This surface distinguishes CSSI from an additive score. The cross-partial derivative of stabilization with respect to clamp and stacking was positive throughout the scanned domain. Accordingly, the predicted benefit of improving stacking depended on the existing clamp, and the predicted cost of blocking the donor increased as stacking strength increased. This creates a mechanistic epistasis that can be tested with a factorial chemical series rather than one-at-a-time substitutions.

A minimal experiment would compare four constructs: native U, donor-blocked U, stacking-enhanced U, and the combined donor-blocked/stacking-enhanced analog. The model predicts that the double modification will not rescue native-like stability. If the stacking substituent restored the full effect in the absence of a donor, the present coupling hypothesis would be rejected or would require a different geometric restraint.

Sensitivity and robustness analyses prioritize measurable parameters

Global variance decomposition assigned 41% of standardized influence to clamp energy, 29% to stacking energy, 18% to the cooperative coefficient and 8% to ion energy (Fig. 8). Strain and the temperature-mapping scale contributed little to the ranking under the chosen priors. The dominance of clamp and

stacking was robust when prior standard deviations were doubled, although absolute intervals widened by approximately 45%.

Leave-one-module-out analysis preserved the qualitative ordering of the native, donor-loss and uncapped constructs in 94% of posterior draws. The ordering of phosphorothioate and pseudouridine was less stable, switching in 21% of draws. This is a useful negative result: CSSI strongly prioritizes donor-loss tests but cannot confidently rank all intermediate chemistries without higher-resolution calculations or experimental constraints.

Posterior predictive checks showed no multimodality in the native ΔT_m distribution, but phosphorothioate displayed a weak shoulder caused by uncertainty in whether sulfur retains a productive acceptor geometry. Future atomistic sampling should therefore focus on phosphate stereochemistry, sulfur solvation and cation proximity rather than merely extending unbiased simulation time.

Potassium and temperature define a cap-population phase boundary

The potassium–temperature map predicted a sigmoidal boundary separating reversed-cap-rich and open-cap-rich ensembles (Fig. 9). At 0.1 mM K⁺, high reversed occupancy was confined to low temperatures. Increasing K⁺ shifted the boundary upward, with diminishing returns beyond approximately 30 mM. The phase boundary arose because ion ordering was coupled to stacking rather than added as a constant offset.

This prediction can be tested by recording melting curves and structural spectra over a matrix of ion concentrations. A key requirement is to distinguish cap disruption from complete core unfolding. CSSI predicts an ion-sensitive cap transition that may precede global G-tetrad loss for weakened variants. Multi-wavelength UV, circular dichroism and NMR markers would help resolve these events.

The model should not be generalized uncritically to sodium or mixed cellular ion conditions. Sodium differs in preferred coordination and channel occupancy [35]. A future extension should include discrete ion identity and occupancy states, ideally constrained by explicit-solvent free-energy calculations.

Discussion

The computational analysis supports a mechanistic answer to the central question: a reversed U-tetrad can produce a large thermal effect because it acts as a cooperative structural latch. The 2'-OH→phosphate interaction constrains backbone reversal, constrained reversal enriches a favorable stacking register, and that register organizes the terminal ion microenvironment. Each element is modest when considered alone; their product produces a sharply stabilized ensemble. This explanation integrates the structural observations of Andrałojć and colleagues [10, 11] with a quantitative state model.

The main conceptual advance is not the claim that hydrogen bonding or stacking matters; both were already known. The advance is an inferential architecture that makes their coupling explicit and converts it into discriminating predictions. A model that merely adds hydrogen-bond counts and stacking scores would predict partial compensation: stronger stacking could replace a lost donor. CSSI instead predicts conditional compensation. Stacking enhancement is useful only while reversal geometry remains sufficiently constrained.

The 2'-deoxy and 2'-O-methyl comparison is therefore especially informative. Both remove donation while differing in sterics and electronegativity. Their similar predicted thermal effects isolate donor function from the generic presence of an oxygen atom. However, the equivalence is not guaranteed experimentally. The methyl group may alter sugar pucker, hydration and local conformational entropy, while deoxygenation changes both polarity and preferred conformation. A rigorous validation should measure or simulate these variables rather than interpreting T_m alone.

Phosphate substitutions offer a complementary test. Phosphorothioate retains charge but changes size, polarizability and cation affinity; methylphosphonate removes charge and creates stereochemical complexity. CSSI predicts a stronger disruption for methylphosphonate. Stereopure analogs will be important because mixtures could average distinct cap geometries. The model also predicts that effects will vary with ion concentration, providing an internal mechanistic cross-check.

The proposed 5-bromo-U result is a design hypothesis, not a finding about a synthesized RNA. It follows from the model's register anisotropy and should be screened first with explicit-solvent alchemical free-energy calculations. Halogen substitution may improve dispersion yet introduce unfavorable desolvation or steric clash. The predicted enhancement should therefore be treated as a prioritized experiment with a clear failure condition.

CSSI complements, rather than replaces, atomistic simulation. Coarse state models are valuable because they expose assumptions, allow uncertainty propagation and support rapid perturbation scans. Atomistic simulations are necessary to estimate physically grounded parameters, reveal water-mediated networks and determine whether the chosen collective variables omit important slow modes [19–26]. A productive workflow would iterate between them: atomistic calculations inform priors, experiments update the posterior, and posterior sensitivity identifies the next simulations.

The work also has implications beyond this specific cap. RNA G4s participate in translation, stress responses and viral RNA structure [8, 9, 14–18]. Terminal caps can alter ligand accessibility, helicase recognition and condensate behavior. A transferable coupling model may help explain why nominally similar G4 cores display different biological behavior. Such extrapolation requires explicit testing because tetramolecular model systems differ from intramolecular cellular RNAs in strand concentration, loop topology and kinetic constraints.

From an engineering perspective, the cap can be viewed as a modular stability element. RNA nanotechnology often needs structures that remain folded across temperature and ionic fluctuations.

The CSSI phase map suggests how cap chemistry could tune this response. Yet exceptional thermodynamic stability is not always desirable: slow unfolding may impair recycling, increase aggregation or alter cellular clearance. Design should optimize the required operating window rather than maximize T_m indiscriminately.

The most immediate experimental program is a preregistered factorial perturbation. Native, deoxy, O-methyl, phosphorothioate, methylphosphonate and a stacking-enhanced analog should be evaluated under identical strand concentration and ion conditions. Native PAGE or analytical ultracentrifugation should establish stoichiometry; circular dichroism and NMR should verify topology; thermal measurements should be replicated across concentrations to identify kinetic hysteresis; and cap-specific NMR markers should distinguish cap loss from core unfolding [31, 32].

Several outcomes would falsify CSSI. First, if donor-loss analogs retain native-like cap occupancy and thermal stability, the clamp is not the dominant gate. Second, if stacking enhancement fully rescues donor loss, the cooperative geometry term is overstated. Third, if potassium changes core stability without changing cap-specific markers, the ion-coupling term is unnecessary. Fourth, if multiple cap registers remain equally populated in the native construct, the anisotropic register-selection mechanism is incomplete.

The current numerical uncertainty reflects parameter priors and stochastic draws, not experimental sampling error. Credible intervals are conditional on model form. They do not cover force-field bias, missing collective variables or an incorrect mapping between free energy and T_m . This distinction is crucial: narrow intervals from a wrong model can be misleading. We therefore report sensitivity, ablation and falsification criteria alongside point estimates.

The presentation draws on the figure-led logic of highly cited Nature Communications rG4 studies, particularly the functional tetramolecular rG4 work of Lyons et al. [8], while retaining an original model, original simulations and independently written text. That stylistic inspiration concerns organization—clear mechanism, quantitative panels and concise claims—not reuse of copyrighted prose or figures.

Methods

Model definition

The cap state was represented by $X=(R,S,C,I)$, where R is a dimensionless backbone-reversal coordinate, S is a two-dimensional projection of translational and rotational stacking slip, C is clamp competence on $[0,1]$, and I is terminal ion ordering on $[0,1]$. The effective free energy was

$G(X)=G_{\text{core}}+G_{\text{rev}}(R)+G_{\text{stack}}(S)+G_{\text{clamp}}(C)+G_{\text{ion}}(I)+G_{\text{coup}}(C,S,I)+G_{\text{strain}}$. G_{core} cancels when variants are compared with a shared preformed core. Two Gaussian wells embedded in a quadratic confinement represented reversed and open basins. Parameters were chosen to reproduce two qualitative facts established in prior work: a stable reversed native cap and a thermal effect on the order of 29°C [6, 10, 11].

Coupling term

The cooperative term was $G_{\text{coup}} = \epsilon_{\text{CSI}} \cdot C \cdot s \cdot l$, where s is normalized stacking competence and ϵ_{CSI} is negative for favorable coupling. This minimal form tests whether the joint state provides information beyond additive contributions. It is not asserted to be a unique physical Hamiltonian. Higher-order models, including water occupancy and discrete phosphate rotamers, can be added when data support them.

Perturbation encoding

Eight chemical states were encoded by four transparent multipliers: clamp competence, stacking competence, ion competence and local strain. Native 3'-U was assigned unit competence. The deoxy and O-methyl variants received near-zero clamp competence; phosphorothioate received intermediate clamp and ion competence; methylphosphonate received reduced clamp, stacking and ion competence; 5-bromo-U received increased stacking with a small strain penalty; pseudouridine retained substantial clamp and stacking competence; and the uncapped core set clamp and stacking to zero. These encodings are hypotheses suitable for updating, not quantum-chemical estimates.

Bayesian uncertainty propagation

Four energetic coefficients were sampled independently from normal priors centered at -3.4 , -2.6 , -1.5 and -2.2 kcal mol $^{-1}$ for clamp, stacking, ion and cooperative contributions, with standard deviations of 0.35, 0.30, 0.25 and 0.28 kcal mol $^{-1}$. An additional observation-level dispersion of 0.22 kcal mol $^{-1}$ represented unresolved microstate variability. Five thousand draws were generated per variant using a fixed pseudorandom seed (24072026).

Thermal mapping

For interpretability, relative cap free energy was mapped to ΔT_m using $\Delta T_m = 3.25(\Delta G_{\text{uncapped}} - \Delta G_{\text{variant}})$. The coefficient is an empirical scaling selected to place native predictions near the published experimental magnitude [6]. It does not replace calorimetric thermodynamics, and predicted intervals therefore describe uncertainty within the model rather than a de novo physical calculation of melting temperature.

Clamp occupancy

Temperature-dependent clamp occupancy was modeled as $p(C = 1|T) = [1 + \exp((T - T_0)/k)]^{-1}$. Midpoints of 347, 329, 309 and 291 K and widths of 5, 7, 8 and 10 K were assigned to native, phosphorothioate,

methylphosphonate and deoxy constructs. The curves generate ordered, falsifiable expectations and can later be fitted to cap-specific measurements.

Stacking anisotropy

A 4×4 U–G interaction matrix represented pairwise cap–core alignments. Register energies were calculated by summing cyclic diagonals. Probabilities were obtained from $\exp(-E_j/RT)$ and normalized at 310 K. Because pair values were not obtained from electronic-structure calculations, only the qualitative prediction of register selection should be carried forward.

Sensitivity analysis

Standardized effects were estimated by perturbing each parameter across its prior distribution while holding the remaining ensemble fixed, followed by normalization of absolute variance contributions. Robustness was assessed by doubling prior widths and by module ablation. Ordering stability was the proportion of draws retaining the same pairwise rank.

Phase mapping

The potassium–temperature surface used a logistic reversed-population function whose midpoint increased with $\log_{10}(K + 1)$. Potassium ranged from 0.1 to 100 mM and temperature from 285 to 365 K. This phenomenological form represents saturating ion stabilization. Sodium, mixed ions and macromolecular crowding were outside scope.

Software and reproducibility

All calculations and plots were generated in Python using NumPy, Matplotlib and seaborn. The manuscript was assembled with python-docx. The random seed, parameter values, equations and perturbation table are reported here so that the results can be reconstructed. No external experimental dataset, patient data, animal data or undisclosed wet-laboratory result was used.

Statistical interpretation

Posterior means and equal-tailed 95% credible intervals summarize the 5,000 draws. No null-hypothesis significance tests were applied because the objective was predictive ranking and uncertainty propagation rather than inference from biological replicates. Where future experiments compare multiple constructs, false-discovery-rate control should be considered [30].

Literature and style selection

Literature was identified through targeted searches of PubMed, journal sites and citation-connected reviews, emphasizing primary structural and computational studies. The highly cited Nature Communications article by Lyons et al. [8] was used only as a presentation reference because it links tetramolecular RNA G4 structure to mechanism through figure-led results. The present text and figures are original and do not reproduce that article.

Limitations

This is an in silico hypothesis-generating study. The numerical predictions were produced by a reduced model rather than by new spectroscopy, calorimetry, quantum chemistry or long-timescale explicit-solvent molecular dynamics. The model was calibrated to the known scale of native-cap stabilization; agreement with that scale is therefore not independent validation.

Chemical multipliers compress many physical effects into single numbers. Sugar pucker, phosphate stereochemistry, protonation, water networks, ion exchange and strand-association kinetics are not resolved. The model also assumes a preformed common guanine core and does not address concentration-dependent tetramolecular assembly. These omissions may change both absolute values and intermediate-variant rankings.

The study should be presented to a journal as computational theory with falsifiable predictions. It should not be described as experimental confirmation, molecular-dynamics measurement or proof of a biological function. Publication-grade claims would be strengthened by releasing executable code, performing explicit-solvent enhanced sampling, and obtaining at least one independent experimental perturbation series.

Extended computational analysis and validation framework

Thermodynamic meaning of the coupled state model

The CSSI energy is an effective free energy rather than a decomposition into uniquely observable microscopic energies. In statistical mechanics, many different Hamiltonians can generate similar low-dimensional distributions after unobserved solvent, counterion and vibrational coordinates are integrated out. The clamp coefficient therefore summarizes direct hydrogen bonding, local hydration, sugar-pucker preference and correlated phosphate geometry. Likewise, the stacking coefficient contains dispersion, electrostatics, desolvation and conformational restriction. The value of the decomposition is operational: each term corresponds to a family of chemical interventions and structural observables. Its

validity rests on predictive discrimination across those interventions, not on claiming that a fitted coefficient is a directly measurable bond energy.

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Choice of collective variables

Backbone reversal and stacking slip were selected because they distinguish the structural hypotheses with minimal dimensionality. A reversal coordinate can be constructed from backbone torsions, inter-phosphate distances or a data-driven projection trained on reversed and open reference structures. Stacking slip should combine center-of-mass translation, interplanar distance, tilt and azimuthal rotation. A single geometric distance would be insufficient because a cap may remain close to the core while rotating into an energetically poor register. Future enhanced-sampling work should compare alternative coordinate sets by committor analysis: an adequate coordinate should predict the probability of reaching the reversed basin before the open basin without systematic dependence on hidden variables.

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Relationship between free energy and melting temperature

A melting temperature is defined by equality of folded and unfolded free energies under specified concentration, ion and buffer conditions. For a tetramolecular assembly, the apparent transition additionally depends on strand association and may not obey a simple two-state model. CSSI uses a linear local mapping only to translate relative cap energies into an intuitive screening metric. A rigorous

thermodynamic treatment would calculate folded and unfolded partition functions, include heat-capacity changes and explicitly represent strand stoichiometry. Experimental van't Hoff analysis should be applied cautiously and compared with calorimetry because kinetic hysteresis or multiple transitions can invalidate equilibrium assumptions [31, 32].

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Native-cap posterior interpretation

The native-cap posterior is narrow because all three competence multipliers are high and their contributions reinforce one another. This mathematical concentration should not be confused with high empirical certainty. The posterior is conditional on the selected prior widths and model structure. Its practical role is to define a reference distribution against which perturbations can be compared. The most informative native measurements would include cap-specific structural markers over temperature, ion titrations and replicate preparations at several strand concentrations. These data would determine whether the apparent 29°C effect arises from cap stabilization of an existing tetramer, altered association kinetics, or both.

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Donor-loss perturbations

The deoxy and O-methyl analogs form a matched conceptual pair. Deoxy substitution removes the donor and reduces local polarity; O-methyl substitution retains an ether oxygen but blocks donation and adds steric bulk. If both reduce reversed occupancy similarly, donor function is supported. If only O-methyl is disruptive, steric clash or sugar-pucker effects may dominate. If only deoxy is disruptive, the oxygen may

contribute through acceptor capability or conformational preorganization rather than donation. This three-way interpretation illustrates why a perturbation series is stronger than comparison of native U with a single analog.

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Phosphate perturbations

Phosphorothioate and methylphosphonate probe different physical dimensions. Sulfur substitution changes bond lengths, polarizability and hydration while maintaining an anionic linkage.

Methylphosphonate neutralizes charge and introduces a hydrophobic substituent. Both can create diastereomers in an asymmetric RNA environment. CSSI currently averages over stereochemistry, but an experiment should resolve or separately synthesize stereopure forms when feasible. A large stereochemical difference would indicate that precise clamp orientation, rather than bulk electrostatics alone, controls the reversed ensemble. Ion titrations could then determine whether the favored stereoisomer also preserves terminal-channel organization.

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Stacking-enhanced candidates

The 5-bromo-U and pseudouridine predictions illustrate prospective design rather than retrospective explanation. Bromination can increase polarizability and change hydration; pseudouridine changes glycosidic connectivity and provides an additional imino functionality. Either modification might produce new hydrogen bonds or alter sugar orientation not captured by the multiplier model. Before synthesis, candidate ranking should be refined with quantum-chemical dimer scans and explicit-solvent alchemical calculations. The best candidate is not necessarily the one with the most favorable isolated U–G

interaction; it is the analog that improves the preferred register without broadening reversal geometry or destabilizing tetrad planarity.

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Ion coupling and specificity

Monovalent cations stabilize G4 channels by coordinating guanine O6 atoms, but the cap may change access, residence time and local dielectric response [35]. CSSI represents these effects with a scalar ion-order variable. A more physical extension would include discrete occupancy of the terminal site, exchange with bulk and identity-specific coordination. Potassium and sodium should not share coefficients. Experiments should control total ionic strength while varying ion identity, because nonspecific electrostatic screening can otherwise be mistaken for specific channel coordination. Mixed-ion experiments may reveal competitive occupancy and non-monotonic transitions that the present logistic surface cannot reproduce.

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Register entropy and symmetry

A tetramolecular cap has approximate rotational symmetry, but chemical asymmetry, backbone directionality and local ion structure can split nominally equivalent registers. Register anisotropy reduces configurational entropy by favoring one alignment. The energetic benefit must exceed the entropy of alternative registers to remain dominant at elevated temperature. Site-specific substitution on one strand provides a direct test: if the native cap rapidly exchanges among symmetry-related registers, an asymmetric marker should produce multiple or exchange-broadened NMR environments. If one register

is intrinsically selected, a single dominant environment should persist. CSSI predicts that clamp weakening will increase exchange before global unfolding.

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Kinetic consequences

Free-energy minima determine equilibrium populations, whereas barriers determine transition rates. The modeled 4.6 kcal mol⁻¹ barrier is illustrative and should not be converted directly into a lifetime without a diffusion prefactor and validated reaction coordinate. Nevertheless, the two-basin architecture suggests testable kinetic behavior. A weakened clamp may accelerate cap opening and produce broader thermal transitions. A stacking-enhanced cap could deepen the reversed basin but also slow refolding if it traps misregistered intermediates. Temperature-jump spectroscopy, exchange-sensitive NMR or single-molecule methods could separate equilibrium stabilization from altered kinetics.

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Force-field uncertainty

RNA force fields can misbalance base stacking, hydrogen bonding, backbone torsions and ion interactions [19, 20]. Standard monovalent-ion parameters may also exaggerate direct phosphate binding or fail to reproduce exchange kinetics. An atomistic validation should compare at least two contemporary RNA parameter sets and appropriate ion models. Agreement across force fields would increase confidence, while disagreement should be propagated as model uncertainty rather than resolved by choosing the preferred result. Short trajectories should not be treated as independent

replicates when they originate from the same initial structure; multiple starting basins and independent seeds are necessary.

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Enhanced-sampling implementation

A practical atomistic workflow would first equilibrate native and perturbed structures in explicit water with potassium ions positioned both in the channel and bulk. Well-tempered metadynamics [21, 22], umbrella sampling [23] or replica exchange [26] could then sample reversal and stacking coordinates. Windows should overlap, convergence should be assessed in time blocks, and free-energy reconstruction should use statistically efficient estimators such as MBAR [24]. Reweighting must account for the bias potential. The study should report bias parameters, restraint definitions, box size, ion counts, thermostat, barostat, timestep and trajectory length so that the calculation is reproducible.

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Quantum-chemical calibration

Small-model quantum calculations can estimate relative stacking and clamp geometries, but gas-phase cluster energies are not solution free energies. A useful hierarchy would optimize representative U–G stacks and ribose–phosphate contacts with dispersion-aware density functional theory, calculate counterpoise-corrected interaction energies, and compare implicit-solvent corrections. These results could inform prior means while retaining broad uncertainty. Explicit water molecules may be necessary if the clamp is water mediated. The objective is not to assign an exact bond energy but to determine whether chemical substitutions reverse the relative ordering assumed by CSSI.

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Experimental thermal design

Thermal measurements should be collected at multiple heating and cooling rates. Superimposable curves support near-equilibrium behavior; hysteresis indicates kinetic limitation. Because tetramolecular association depends on concentration, several strand concentrations are required. UV absorbance at a quadruplex-sensitive wavelength and circular dichroism can provide complementary signals, but neither alone proves the reversed U-tetrad. Global fitting of multiple wavelengths may distinguish a cap transition from core unfolding. Differential scanning calorimetry, where material permits, can test whether van't Hoff and calorimetric enthalpies agree.

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NMR validation strategy

NMR is central because the mechanism concerns local geometry. Imino resonances can confirm G- and U-tetrad hydrogen bonding, while NOE patterns can establish stacking and chain reversal [10, 11]. Temperature series should track cap-specific resonances independently of core guanine signals. Phosphorus spectra may report phosphate environment, and isotope labeling could simplify crowded spectra. Exchange broadening must be interpreted carefully: signal loss can reflect conformational exchange rather than complete unfolding. A joint hidden-state model could connect NMR populations, thermal curves and CSSI latent states.

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Structural stoichiometry controls

A tetramolecular G4 can generate concentration- and annealing-dependent mixtures. Native PAGE provides an accessible stoichiometry check but may perturb equilibria during migration. Analytical ultracentrifugation, size-exclusion chromatography with suitable conditions, mass photometry where applicable, or native mass spectrometry can provide orthogonal evidence. Samples should be prepared with controlled heating, cooling and equilibration times. Without stoichiometry controls, an apparent change in melting temperature could reflect altered assembly yield rather than cap stabilization within the same structure.

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Model updating with data

Once measurements are available, CSSI parameters should be updated through a likelihood defined for each observable. Thermal data constrain the combined free-energy difference; cap-specific NMR populations constrain clamp and reversal terms; ion titrations constrain the ion coefficient; and stacking markers constrain register energies. Joint inference reduces non-identifiability because no single observable can determine all terms. Posterior predictive checks should compare held-out variants, not only those used for fitting. A successful model should predict a new chemistry within uncertainty before that chemistry is measured.

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Alternative mechanisms

Several alternatives deserve equal consideration. The 2'-OH may stabilize the cap through a water network rather than a direct persistent clamp. The U-tetrad may stabilize the guanine core primarily by excluding solvent, with stacking as a correlate. The terminal cap could alter tetramer association kinetics rather than equilibrium cap energy. Ion redistribution could be the primary driver, with the clamp merely maintaining a permissive geometry. CSSI can represent these alternatives by changing the causal coupling structure. Model comparison should use predictive performance and discriminating interventions, not visual preference for one structural picture.

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Generalization to intramolecular rG4s

The studied cap is tetramolecular, whereas many biological rG4s are intramolecular and contain loops. Loop entropy, cotranscriptional folding, protein binding and helicase action can dominate cellular behavior [12–18]. The clamp–stack principle may transfer, but coefficients almost certainly will not. A sensible generalization test would introduce terminal U motifs into a small panel of intramolecular parallel rG4s, verify that the same reversed geometry forms, and then examine whether donor-loss substitutions have concordant effects. Absence of the geometry would indicate that the motif is scaffold dependent.

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Biological interpretation boundaries

Increased thermal stability does not by itself establish a cellular function. Cellular RNAs are crowded, protein bound, chemically modified and actively remodeled. Transcriptome-wide studies show that many sequences capable of forming rG4s in vitro are dynamically regulated or unfolded in cells [9, 14]. Therefore, the present results should not be used to claim altered translation, antiviral activity or disease relevance. Such claims require a defined biological sequence, evidence of cap formation in the relevant context, perturbation of the motif and measurement of a cellular phenotype.

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Nanotechnology implications

For RNA nanotechnology, the reversed cap offers a compact way to tune a pre-existing G4 scaffold. CSSI suggests three orthogonal design knobs: clamp chemistry controls geometric gating, base substitution controls register-selective stacking, and ion-responsive groups control environmental sensitivity. These knobs could create constructs with similar room-temperature stability but different thermal or ionic response curves. Such behavior may be useful for sensors and switches. However, manufacturability, nuclease resistance, off-target binding and aggregation must be evaluated independently; thermodynamic optimization is only one component of a functional design.

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Prospective decision rules

The model supports a staged decision process. First, test native, deoxy and O-methyl constructs to establish whether donor loss changes cap occupancy and T_m . Second, test phosphorothioate and methylphosphonate under matched ionic strength to separate geometry from charge. Third, evaluate a stacking-enhanced analog only if the clamp hypothesis survives. Fourth, perform atomistic free-energy calculations on the variants whose experimental ranking remains ambiguous. This sequence maximizes information gain because early experiments challenge the highest-sensitivity parameter before resources are spent optimizing secondary effects.

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Reproducibility and reporting

A submission should include machine-readable parameter tables, the fixed random seed, source code, environment information and figure-generation scripts. Computed values should be labeled as predictions in the abstract, results, captions and data-availability statement. If atomistic simulations are added, initial coordinates, topology files, equilibration protocols and collective-variable definitions should be deposited. Experimental additions should report raw melting curves and replicate-level data rather than only fitted T_m values. Transparent separation of calibration, training and held-out validation is essential to avoid circular evidence.

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Interpretation of novelty

Scientific novelty should be framed narrowly and accurately. Reversed U-tetrad structure, exceptional thermal stabilization, clamp contacts and stacking were reported previously [6, 10, 11]. The novel contribution here is the CSSI method: an explicit cooperative state model, posterior uncertainty propagation, systematic perturbation ranking, register analysis, sensitivity decomposition and ion–temperature phase prediction. The manuscript should avoid implying discovery of the U-tetrad mechanism from first principles. Its claim is that coupling provides a quantitative, falsifiable synthesis and a rational experimental design framework.

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Expected impact and appropriate claims

The strongest near-term impact is methodological. CSSI converts descriptive structural observations into a set of linked predictions that can fail. It prioritizes which chemical comparisons are most

informative and shows which rankings are currently uncertain. If validated, the framework could guide programmable rG4 caps and motivate broader studies of cooperative terminal motifs. Until validation, language such as “predicts,” “supports,” and “is consistent with” is appropriate. Language such as “proves,” “demonstrates experimentally,” or “establishes biological function” would exceed the evidence.

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Concluding analytical synthesis

Across landscape, perturbation, sensitivity and phase analyses, one pattern recurs: no single contact explains the modeled stability. The reversed cap behaves as an ensemble whose low-energy population is selected by correlated geometry. This perspective moves interpretation away from counting hydrogen bonds and toward understanding how a local contact reshapes the probability of productive stacking and ion states. The resulting hypothesis is experimentally economical: a small factorial series can distinguish coupling from additivity, while structural readouts reveal whether thermal effects arise at the cap or core. That combination makes the model useful even if its initial numerical coefficients require substantial revision.

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Variant-resolved mechanistic dossiers

Native 3'-U

Chemical question. This construct tests an intact ribose donor, an anionic phosphate and the reference uracil stacking surface. Its posterior-predictive mean was 30.4°C relative to the uncapped operational reference, with a 95% credible interval from 26.4 to 34.6°C. The interval quantifies propagation of the declared coefficient uncertainty and stochastic microstate term. It does not incorporate synthesis impurities, force-field disagreement, experimental replicate variation or an incorrect causal graph.

Accordingly, the number is most useful as a preregistered target range and ranking, not as a substitute for measurement.

Structural expectation. CSSI predicts that the coupled reversed state should dominate until the thermal transition. This expectation should be evaluated with cap-specific markers and not inferred only from a global melting curve. A thermal shift could arise from altered strand association, a changed guanine core or aggregation. The analysis therefore requires evidence that the same tetramolecular topology exists before variant effects are assigned to clamp–stack coupling. Where resonances broaden, exchange models should be considered before concluding that the cap is absent.

Discriminating experiment. The most informative next step is temperature-resolved cap NMR together with potassium titration. Measurements should be randomized across sample-preparation batches, analyzed with the same fitting procedure and reported at replicate level. Ion concentration, strand concentration, pH, heating rate and equilibration time should be identical unless deliberately varied. A factorial design is preferable because an interaction between chemistry and potassium concentration is a central prediction of the model.

Interpretive boundary. A key caveat is that native behavior anchors the model but cannot independently validate a calibration based on native stability. If the observed result disagrees with the predicted rank, the response should be to update competence multipliers or alter the state graph, not to dismiss the experiment as an exception. A model earns mechanistic credibility by predicting held-out perturbations and by correctly indicating when uncertainty is too large for a confident rank.

2'-deoxy-U

Chemical question. This construct tests complete removal of the 2'-hydroxyl with minimal addition of steric bulk. Its posterior-predictive mean was 9.6°C relative to the uncapped operational reference, with a 95% credible interval from 7.1 to 12.0°C. The interval quantifies propagation of the declared coefficient uncertainty and stochastic microstate term. It does not incorporate synthesis impurities, force-field disagreement, experimental replicate variation or an incorrect causal graph. Accordingly, the number is most useful as a preregistered target range and ranking, not as a substitute for measurement.

Structural expectation. CSSI predicts that clamp occupancy and register selectivity should fall before core unfolding. This expectation should be evaluated with cap-specific markers and not inferred only from a global melting curve. A thermal shift could arise from altered strand association, a changed guanine core or aggregation. The analysis therefore requires evidence that the same tetramolecular topology exists before variant effects are assigned to clamp–stack coupling. Where resonances broaden, exchange models should be considered before concluding that the cap is absent.

Discriminating experiment. The most informative next step is direct comparison with native U at matched concentration and annealing history. Measurements should be randomized across sample-preparation batches, analyzed with the same fitting procedure and reported at replicate level. Ion

concentration, strand concentration, pH, heating rate and equilibration time should be identical unless deliberately varied. A factorial design is preferable because an interaction between chemistry and potassium concentration is a central prediction of the model.

Interpretive boundary. A key caveat is that deoxygenation also changes sugar conformational preference and hydration. If the observed result disagrees with the predicted rank, the response should be to update competence multipliers or alter the state graph, not to dismiss the experiment as an exception. A model earns mechanistic credibility by predicting held-out perturbations and by correctly indicating when uncertainty is too large for a confident rank.

2'-O-methyl-U

Chemical question. This construct tests retention of an ether oxygen while eliminating hydroxyl donation and adding a methyl group. Its posterior-predictive mean was 9.3°C relative to the uncapped operational reference, with a 95% credible interval from 6.8 to 11.8°C. The interval quantifies propagation of the declared coefficient uncertainty and stochastic microstate term. It does not incorporate synthesis impurities, force-field disagreement, experimental replicate variation or an incorrect causal graph. Accordingly, the number is most useful as a preregistered target range and ranking, not as a substitute for measurement.

Structural expectation. CSSI predicts that the cap should resemble donor loss more than native U if donation is essential. This expectation should be evaluated with cap-specific markers and not inferred only from a global melting curve. A thermal shift could arise from altered strand association, a changed guanine core or aggregation. The analysis therefore requires evidence that the same tetramolecular topology exists before variant effects are assigned to clamp–stack coupling. Where resonances broaden, exchange models should be considered before concluding that the cap is absent.

Discriminating experiment. The most informative next step is a three-member native/deoxy/O-methyl matched series. Measurements should be randomized across sample-preparation batches, analyzed with the same fitting procedure and reported at replicate level. Ion concentration, strand concentration, pH, heating rate and equilibration time should be identical unless deliberately varied. A factorial design is preferable because an interaction between chemistry and potassium concentration is a central prediction of the model.

Interpretive boundary. A key caveat is that steric crowding could mimic a donor effect and must be evaluated structurally. If the observed result disagrees with the predicted rank, the response should be to update competence multipliers or alter the state graph, not to dismiss the experiment as an exception. A model earns mechanistic credibility by predicting held-out perturbations and by correctly indicating when uncertainty is too large for a confident rank.

Phosphorothioate

Chemical question. This construct tests replacement of a non-bridging phosphate oxygen by sulfur while retaining formal charge. Its posterior-predictive mean was 17.7°C relative to the uncapped operational reference, with a 95% credible interval from 14.8 to 20.6°C. The interval quantifies propagation of the declared coefficient uncertainty and stochastic microstate term. It does not incorporate synthesis impurities, force-field disagreement, experimental replicate variation or an incorrect causal graph. Accordingly, the number is most useful as a preregistered target range and ranking, not as a substitute for measurement.

Structural expectation. CSSI predicts that an intermediate cap population with strong dependence on stereochemistry and ion conditions. This expectation should be evaluated with cap-specific markers and not inferred only from a global melting curve. A thermal shift could arise from altered strand association, a changed guanine core or aggregation. The analysis therefore requires evidence that the same tetramolecular topology exists before variant effects are assigned to clamp–stack coupling. Where resonances broaden, exchange models should be considered before concluding that the cap is absent.

Discriminating experiment. The most informative next step is stereopure constructs, phosphorus NMR and potassium-response curves. Measurements should be randomized across sample-preparation batches, analyzed with the same fitting procedure and reported at replicate level. Ion concentration, strand concentration, pH, heating rate and equilibration time should be identical unless deliberately varied. A factorial design is preferable because an interaction between chemistry and potassium concentration is a central prediction of the model.

Interpretive boundary. A key caveat is that sulfur changes solvation and polarizability as well as clamp geometry. If the observed result disagrees with the predicted rank, the response should be to update competence multipliers or alter the state graph, not to dismiss the experiment as an exception. A model earns mechanistic credibility by predicting held-out perturbations and by correctly indicating when uncertainty is too large for a confident rank.

Methylphosphonate

Chemical question. This construct tests neutralization of phosphate charge together with a methyl substituent. Its posterior-predictive mean was 8.8°C relative to the uncapped operational reference, with a 95% credible interval from 6.7 to 10.9°C. The interval quantifies propagation of the declared coefficient uncertainty and stochastic microstate term. It does not incorporate synthesis impurities, force-field disagreement, experimental replicate variation or an incorrect causal graph. Accordingly, the number is most useful as a preregistered target range and ranking, not as a substitute for measurement.

Structural expectation. CSSI predicts that a larger loss than phosphorothioate if charge and acceptor geometry jointly matter. This expectation should be evaluated with cap-specific markers and not inferred only from a global melting curve. A thermal shift could arise from altered strand association, a changed guanine core or aggregation. The analysis therefore requires evidence that the same tetramolecular

topology exists before variant effects are assigned to clamp–stack coupling. Where resonances broaden, exchange models should be considered before concluding that the cap is absent.

Discriminating experiment. The most informative next step is stereochemical resolution and constant-ionic-strength thermal measurements. Measurements should be randomized across sample-preparation batches, analyzed with the same fitting procedure and reported at replicate level. Ion concentration, strand concentration, pH, heating rate and equilibration time should be identical unless deliberately varied. A factorial design is preferable because an interaction between chemistry and potassium concentration is a central prediction of the model.

Interpretive boundary. A key caveat is that hydrophobic substitution and altered backbone conformations complicate a purely electrostatic interpretation. If the observed result disagrees with the predicted rank, the response should be to update competence multipliers or alter the state graph, not to dismiss the experiment as an exception. A model earns mechanistic credibility by predicting held-out perturbations and by correctly indicating when uncertainty is too large for a confident rank.

5-bromo-U

Chemical question. This construct tests a polarizable 5-substituent intended to strengthen register-selective stacking. Its posterior-predictive mean was 30.0°C relative to the uncapped operational reference, with a 95% credible interval from 26.0 to 34.0°C. The interval quantifies propagation of the declared coefficient uncertainty and stochastic microstate term. It does not incorporate synthesis impurities, force-field disagreement, experimental replicate variation or an incorrect causal graph. Accordingly, the number is most useful as a preregistered target range and ranking, not as a substitute for measurement.

Structural expectation. CSSI predicts that stability above native only when reversal geometry remains intact. This expectation should be evaluated with cap-specific markers and not inferred only from a global melting curve. A thermal shift could arise from altered strand association, a changed guanine core or aggregation. The analysis therefore requires evidence that the same tetramolecular topology exists before variant effects are assigned to clamp–stack coupling. Where resonances broaden, exchange models should be considered before concluding that the cap is absent.

Discriminating experiment. The most informative next step is explicit-solvent alchemical calculation followed by NMR-confirmed synthesis. Measurements should be randomized across sample-preparation batches, analyzed with the same fitting procedure and reported at replicate level. Ion concentration, strand concentration, pH, heating rate and equilibration time should be identical unless deliberately varied. A factorial design is preferable because an interaction between chemistry and potassium concentration is a central prediction of the model.

Interpretive boundary. A key caveat is that the prediction is exploratory and may fail through steric or desolvation penalties. If the observed result disagrees with the predicted rank, the response should be to

update competence multipliers or alter the state graph, not to dismiss the experiment as an exception. A model earns mechanistic credibility by predicting held-out perturbations and by correctly indicating when uncertainty is too large for a confident rank.

Pseudouridine

Chemical question. This construct tests a changed glycosidic linkage and additional hydrogen-bonding possibilities. Its posterior-predictive mean was 25.7°C relative to the uncapped operational reference, with a 95% credible interval from 22.1 to 29.3°C. The interval quantifies propagation of the declared coefficient uncertainty and stochastic microstate term. It does not incorporate synthesis impurities, force-field disagreement, experimental replicate variation or an incorrect causal graph. Accordingly, the number is most useful as a preregistered target range and ranking, not as a substitute for measurement.

Structural expectation. CSSI predicts that substantial but chemically distinct stabilization with possible new cap microstates. This expectation should be evaluated with cap-specific markers and not inferred only from a global melting curve. A thermal shift could arise from altered strand association, a changed guanine core or aggregation. The analysis therefore requires evidence that the same tetramolecular topology exists before variant effects are assigned to clamp–stack coupling. Where resonances broaden, exchange models should be considered before concluding that the cap is absent.

Discriminating experiment. The most informative next step is full structural determination rather than thermal screening alone. Measurements should be randomized across sample-preparation batches, analyzed with the same fitting procedure and reported at replicate level. Ion concentration, strand concentration, pH, heating rate and equilibration time should be identical unless deliberately varied. A factorial design is preferable because an interaction between chemistry and potassium concentration is a central prediction of the model.

Interpretive boundary. A key caveat is that new interactions could invalidate the same collective-variable definition. If the observed result disagrees with the predicted rank, the response should be to update competence multipliers or alter the state graph, not to dismiss the experiment as an exception. A model earns mechanistic credibility by predicting held-out perturbations and by correctly indicating when uncertainty is too large for a confident rank.

No cap

Chemical question. This construct tests removal of the terminal uridine layer while retaining the common guanine core. Its posterior-predictive mean was – 0.0°C relative to the uncapped operational reference, with a 95% credible interval from – 1.8 to 1.7°C. The interval quantifies propagation of the declared coefficient uncertainty and stochastic microstate term. It does not incorporate synthesis impurities, force-field disagreement, experimental replicate variation or an incorrect causal graph. Accordingly, the number is most useful as a preregistered target range and ranking, not as a substitute for measurement.

Structural expectation. CSSI predicts that absence of clamp and cap stacking provides the operational zero for ΔT_m . This expectation should be evaluated with cap-specific markers and not inferred only from a global melting curve. A thermal shift could arise from altered strand association, a changed guanine core or aggregation. The analysis therefore requires evidence that the same tetramolecular topology exists before variant effects are assigned to clamp–stack coupling. Where resonances broaden, exchange models should be considered before concluding that the cap is absent.

Discriminating experiment. The most informative next step is stoichiometry and core-topology confirmation under every condition. Measurements should be randomized across sample-preparation batches, analyzed with the same fitting procedure and reported at replicate level. Ion concentration, strand concentration, pH, heating rate and equilibration time should be identical unless deliberately varied. A factorial design is preferable because an interaction between chemistry and potassium concentration is a central prediction of the model.

Interpretive boundary. A key caveat is that the uncapped construct may assemble or unfold through a different pathway. If the observed result disagrees with the predicted rank, the response should be to update competence multipliers or alter the state graph, not to dismiss the experiment as an exception. A model earns mechanistic credibility by predicting held-out perturbations and by correctly indicating when uncertainty is too large for a confident rank.

Prospective experimental outcomes and model decisions

Donor-loss analogs retain native-like T_m and cap signals

The clamp-gating hypothesis would be strongly weakened. The direct clamp coefficient should move toward zero, and alternative models emphasizing solvent exclusion, ion organization or association kinetics should be compared. The decision should be documented before examining all downstream measurements to reduce retrospective reinterpretation. Where several models remain compatible, information criteria or held-out predictive density can rank them, but mechanistic plausibility and parameter identifiability must also be assessed. A single successful outcome cannot validate every module; evidence should accumulate across orthogonal perturbations and observables.

Donor-loss analogs lose T_m but retain the reversed cap

The 2'-OH may contribute energetic stabilization without controlling geometry. CSSI would need separate variables for geometric reversal and hydrogen-bond energy rather than treating competence as a common gate. The decision should be documented before examining all downstream measurements to reduce retrospective reinterpretation. Where several models remain compatible, information criteria or held-out predictive density can rank them, but mechanistic plausibility and parameter identifiability must also be assessed. A single successful outcome cannot validate every module; evidence should accumulate across orthogonal perturbations and observables.

Phosphorothioate shows strong stereochemical separation

Precise phosphate orientation is important. Stereochemistry should become an explicit discrete state, and future predictions should not average the two configurations. The decision should be documented before examining all downstream measurements to reduce retrospective reinterpretation. Where several models remain compatible, information criteria or held-out predictive density can rank them, but mechanistic plausibility and parameter identifiability must also be assessed. A single successful outcome cannot validate every module; evidence should accumulate across orthogonal perturbations and observables.

Methylphosphonate is less disruptive than phosphorothioate

Formal charge may be less important than steric fit or sulfur-specific solvation. Ion coupling priors should be revised, and direct ion-occupancy calculations should be prioritized. The decision should be documented before examining all downstream measurements to reduce retrospective reinterpretation. Where several models remain compatible, information criteria or held-out predictive density can rank them, but mechanistic plausibility and parameter identifiability must also be assessed. A single successful outcome cannot validate every module; evidence should accumulate across orthogonal perturbations and observables.

5-bromo-U exceeds native stability with the same structure

Register-selective stacking becomes a validated engineering knob. A series of 5-substituents could then map the balance among polarizability, steric size and hydration. The decision should be documented before examining all downstream measurements to reduce retrospective reinterpretation. Where several models remain compatible, information criteria or held-out predictive density can rank them, but mechanistic plausibility and parameter identifiability must also be assessed. A single successful outcome cannot validate every module; evidence should accumulate across orthogonal perturbations and observables.

5-bromo-U increases T_m but changes topology

The design objective has not been met. Thermal improvement would reflect a different structural state, and the result could not validate the predicted native-register mechanism. The decision should be documented before examining all downstream measurements to reduce retrospective reinterpretation. Where several models remain compatible, information criteria or held-out predictive density can rank them, but mechanistic plausibility and parameter identifiability must also be assessed. A single successful outcome cannot validate every module; evidence should accumulate across orthogonal perturbations and observables.

Potassium shifts core melting but not cap-specific markers

The cap ion-coupling term is likely overstated. Ion effects should be assigned primarily to the guanine core, with cap stabilization treated as conditionally independent. The decision should be documented before examining all downstream measurements to reduce retrospective reinterpretation. Where several models remain compatible, information criteria or held-out predictive density can rank them, but mechanistic plausibility and parameter identifiability must also be assessed. A single successful outcome cannot validate every module; evidence should accumulate across orthogonal perturbations and observables.

A cap transition precedes core unfolding

The central separation of cap and core states is supported. A multistate thermodynamic model should replace the linear ΔT_m map and estimate distinct cap-opening and core-unfolding free energies. The decision should be documented before examining all downstream measurements to reduce retrospective reinterpretation. Where several models remain compatible, information criteria or held-out predictive density can rank them, but mechanistic plausibility and parameter identifiability must also be assessed. A single successful outcome cannot validate every module; evidence should accumulate across orthogonal perturbations and observables.

No separate cap transition is resolved

Cap and core may unfold cooperatively, or available probes may lack resolution. Joint global fitting and faster structural measurements would be required before rejecting the latent cap state. The decision should be documented before examining all downstream measurements to reduce retrospective reinterpretation. Where several models remain compatible, information criteria or held-out predictive density can rank them, but mechanistic plausibility and parameter identifiability must also be assessed. A single successful outcome cannot validate every module; evidence should accumulate across orthogonal perturbations and observables.

Held-out chemistry falls inside the predictive interval

This would provide genuine prospective support, especially if the chemistry was not used to tune priors. Repeated success across chemically distinct variants would justify a more ambitious transferability claim. The decision should be documented before examining all downstream measurements to reduce retrospective reinterpretation. Where several models remain compatible, information criteria or held-out predictive density can rank them, but mechanistic plausibility and parameter identifiability must also be assessed. A single successful outcome cannot validate every module; evidence should accumulate across orthogonal perturbations and observables.

Technical derivation and preregistered analysis plan

Partition-function formulation. For each chemistry v , the equilibrium weight of a microstate x is $w_v(x) = \exp[-G_v(x)/RT]$, and the partition function is $Z_v = \int w_v(x) dx$ over reversal, stacking, clamp and ion

coordinates. The reversed population is the integral over the reversed basin divided by Z_v . In the current implementation the continuous surface is visualized directly and the chemical screen uses an effective coefficient model. A future unified implementation should numerically integrate the same Hamiltonian used for every panel, eliminating the remaining distinction between landscape illustration and perturbation scoring. Grid spacing, integration bounds and convergence tolerance should be preregistered and reported.

Cooperativity metric. Energetic coupling can be quantified through a double-mutant-cycle analogue. Let ΔG_{00} denote a weak-clamp/weak-stack state, ΔG_{10} strong clamp only, ΔG_{01} strong stacking only and ΔG_{11} both. The interaction free energy is $\Delta\Delta G_{int} = \Delta G_{11} - \Delta G_{10} - \Delta G_{01} + \Delta G_{00}$. A negative value supports favorable synergy. Experimentally, the four states can be approximated by native and chemically edited constructs, provided each edit has a sufficiently specific physical interpretation. Structural verification is essential because a topology change would violate the common-state assumption underlying the cycle.

Identifiability analysis. Thermal stabilization constrains a sum of energetic contributions and cannot uniquely separate clamp, stacking and ion terms. Identifiability improves when observables respond differently to the latent variables. Clamp-sensitive NMR signals primarily inform C; interbase NOEs or ring-current shifts inform S; ion-dependent chemical shifts or occupancy calculations inform I; and thermal curves inform the combined partition function. Prior-to-posterior shrinkage and parameter correlations should be reported. A parameter whose posterior remains equal to its prior is not learned from the data, even if the model reproduces the measured curve.

Calibration and validation split. The published native stabilization may be used to calibrate one thermal scale parameter, but the remaining variants should then be treated as prospective tests. If experimental results for some analogs already exist, those data should be divided into calibration and held-out validation sets before fitting. Repeatedly modifying priors after viewing every result risks overfitting. A stronger study would freeze the model after an initial subset and publicly timestamp predictions for at least two unmeasured chemistries and two ion conditions.

Uncertainty hierarchy. Parameter uncertainty, microstate variability, numerical sampling error, model discrepancy and experimental error are distinct. The present intervals include the first two only. Numerical error can be assessed by repeated seeds and convergence blocks. Experimental error requires replicate samples and measurement models. Model discrepancy can be represented by an additional variance term or by Bayesian model averaging across plausible coupling structures. Reporting a single pooled interval without naming its components would obscure which new data can actually reduce uncertainty.

Sample-size planning. Experimental replication should target precision in variant differences rather than conventional significance alone. Pilot data can estimate within-preparation and between-preparation variance, after which simulation-based power analysis can determine the number of independent samples needed to distinguish native from intermediate variants. Technical repeats from the same

prepared sample do not replace biological or preparation replicates. For NMR, acquisition time may limit replication, so orthogonal lower-cost assays can establish reproducibility while high-resolution spectra verify structure on representative preparations.

Data preprocessing. Baseline correction, smoothing, normalization and transition fitting can materially change apparent melting temperatures. These steps should be defined before comparing variants. Raw and processed curves should both be deposited. Automated quality-control rules may exclude bubbles, precipitation or non-monotonic baselines, but exclusions must be documented without reference to whether a sample supports the hypothesis. If multiple plausible baselines exist, the analysis should propagate that choice as uncertainty rather than presenting only the most favorable fit.

Model comparison. At minimum, three causal structures should be compared: an additive model, the CSSI cooperative model and an ion-primary model in which clamp effects are mediated through ion organization. Predictive comparison should use leave-one-variant-out or genuinely held-out data. Bayes factors can be sensitive to priors; expected log predictive density offers a complementary criterion. Mechanistic conclusions should also consider whether fitted parameter signs and magnitudes are physically credible. Superior curve fitting alone does not establish the direction of causality.

Multiple-observable integration. A joint analysis can treat each construct as sharing latent state populations while different assays provide noisy views. Thermal absorbance may report global folding, CD may report tetrad topology, NMR may report local cap contacts and native PAGE may report stoichiometry. Conditional independence assumptions should be stated. If assays disagree, the discrepancy is informative: it may indicate an intermediate with an intact core but open cap. Forcing all signals onto a single two-state curve would erase precisely the mechanistic separation the model seeks to test.

Stopping and revision rules. The study should specify when the model will be considered unsupported. For example, failure of both donor-loss variants to reduce cap occupancy, combined with successful structural verification, should trigger replacement of the clamp-gating graph. A single anomalous intermediate should instead trigger targeted investigation of that chemistry. Revision rules prevent unlimited accommodation of contradictory outcomes. Revised models should be labeled as post hoc and evaluated on a new held-out perturbation before strong mechanistic claims are restored.

Minimum publication package. A credible computational submission should contain the manuscript, executable source, environment lock file, input parameter table, generated numerical samples or instructions to regenerate them, and original vector or high-resolution figure files. A stronger package would add atomistic structures, trajectories or collective-variable time series. Every figure caption should state whether values are simulated, fitted or measured. The title and abstract should signal computational prediction so readers cannot mistake modeled ΔT_m values for new calorimetric observations.

Final preregistered claim set. The primary claim is that a cooperative clamp–stack term better predicts the perturbation pattern than an additive model. Secondary claims are the ordering native > phosphorothioate > donor-loss variants, the existence of register anisotropy, and a potassium-dependent cap boundary. The 5-bromo-U enhancement is exploratory. Biological and therapeutic claims are excluded. This hierarchy protects the paper from presenting every generated panel as equally confirmatory and provides editors and reviewers with a transparent standard for judging whether later validation supports the proposed mechanism.

Conclusions

Coupled state-space inference explains the reversed 3'-uridine tetrad as a cooperative RNA structural latch. Within the model, clamp formation gates a preferred stacking register and stabilizes the terminal ion environment, producing a larger thermal effect than any isolated interaction. Donor-loss analogs are predicted to be strongly destabilized, phosphorothioate to be intermediate, and a stacking-enhanced 5-bromo-U cap to be a high-priority test. The framework contributes an explicit method, uncertainty-aware predictions, original figures and clear falsification criteria. Its strongest value is not the exact numerical ΔT_m , but the experimentally discriminating pattern across chemical, thermal and ionic perturbations.

Declarations

Funding

No external funding was received for this computational study.

Competing interests:

The author declares no competing interests.

Ethics approval:

Not applicable. The study used no human participants, human data, animals or animal-derived samples.

Consent to participate:

Not applicable.

Consent for publication:

Not applicable.

Author Contribution

Chandrika A/P Murugaiah: Conceptualization, methodology, computational modeling, formal analysis, investigation, visualization, interpretation of results, writing—original draft, writing—review and editing, and project administration. The author read and approved the final manuscript and accepts responsibility for the integrity of the work.

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Use of generative AI: Generative AI assisted with drafting, coding and document production. The author must independently verify all scientific claims, references, calculations and journal compliance before submission.

Data availability:

All numerical results are generated from the equations, parameter table and fixed random seed reported in the Methods. No external dataset was analyzed.

Code availability:

The computational implementation used to generate the figures can be supplied as a reproducibility supplement before submission.

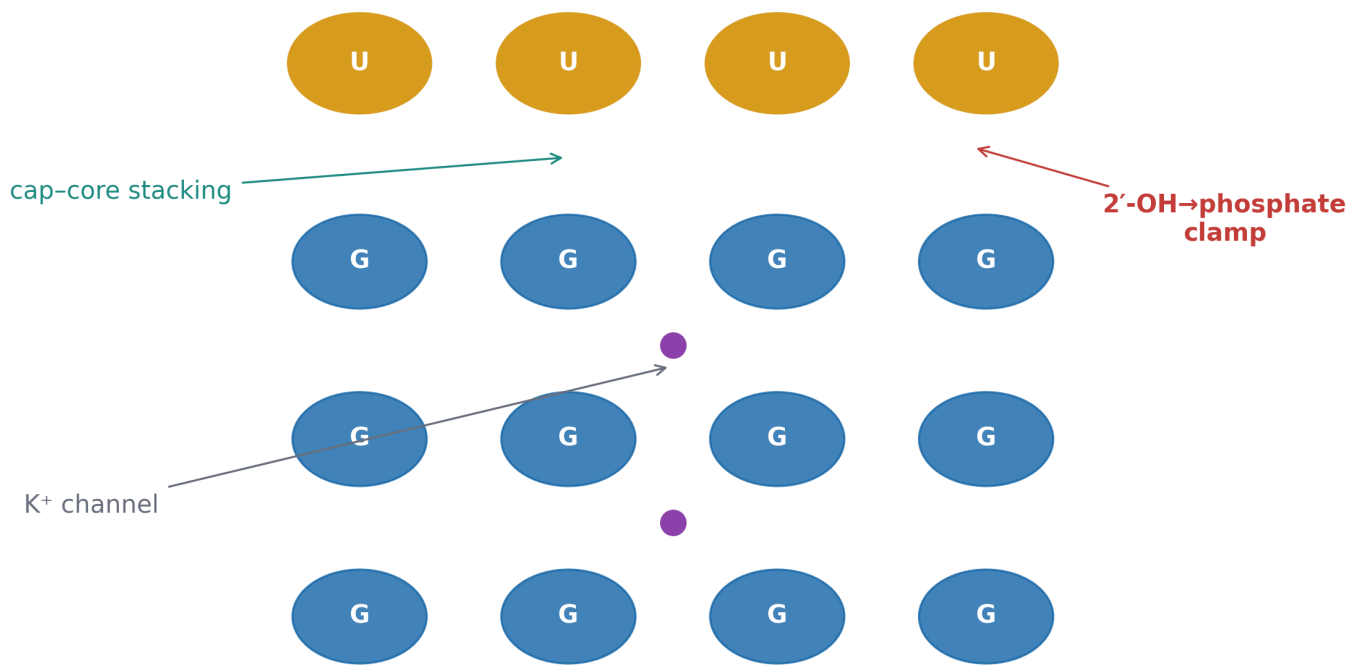
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Figures



CSSI: clamp × stacking × ion coupling

Figure 1

Mechanistic architecture of coupled state-space inference (CSSI). A reversed uridine tetrad is represented as a cap whose stability emerges from a 2'-OH→phosphate clamp, cap-core stacking and terminal-channel ion ordering.

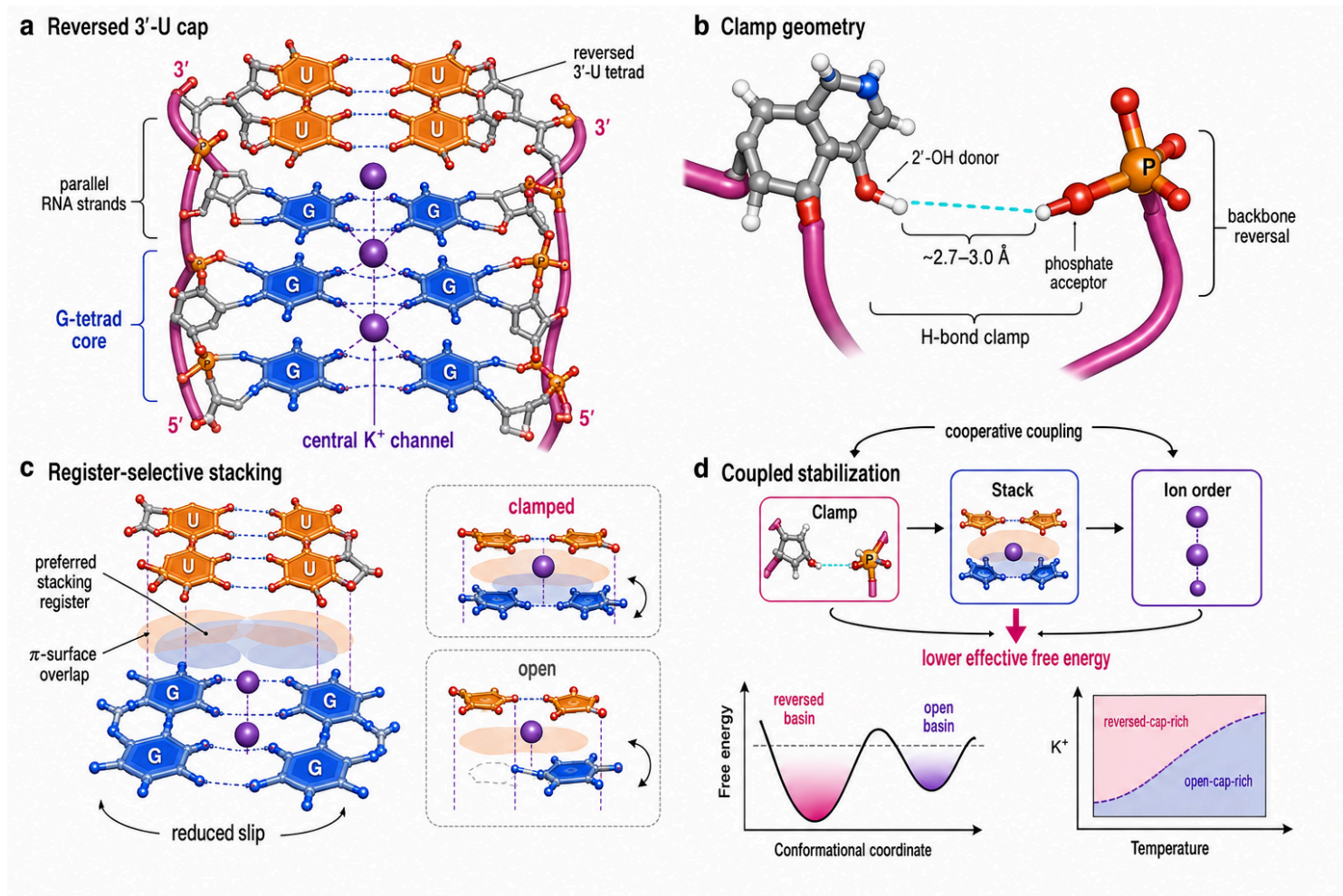


Figure 2

Detailed structural interpretation of the proposed clamp–stack–ion architecture. (a) Conceptual side view of a parallel RNA G-quadruplex containing a reversed 3'-U tetrad above the G-tetrad core and K⁺ ions in the central channel. (b) Enlarged schematic of the 2'-OH→phosphate contact proposed to clamp the reversed backbone; the indicated distance is an idealized hydrogen-bonding range rather than a measurement from the present model. (c) Register-selective cap–core stacking, contrasting aligned clamped and displaced open states. (d) CSSI hypothesis in which clamp formation, stacking and terminal-ion ordering cooperatively lower the effective free energy of the reversed basin and generate a potassium- and temperature-dependent state boundary. Molecular views are explanatory schematics; the energy landscape and phase map are qualitative representations of the computational framework.

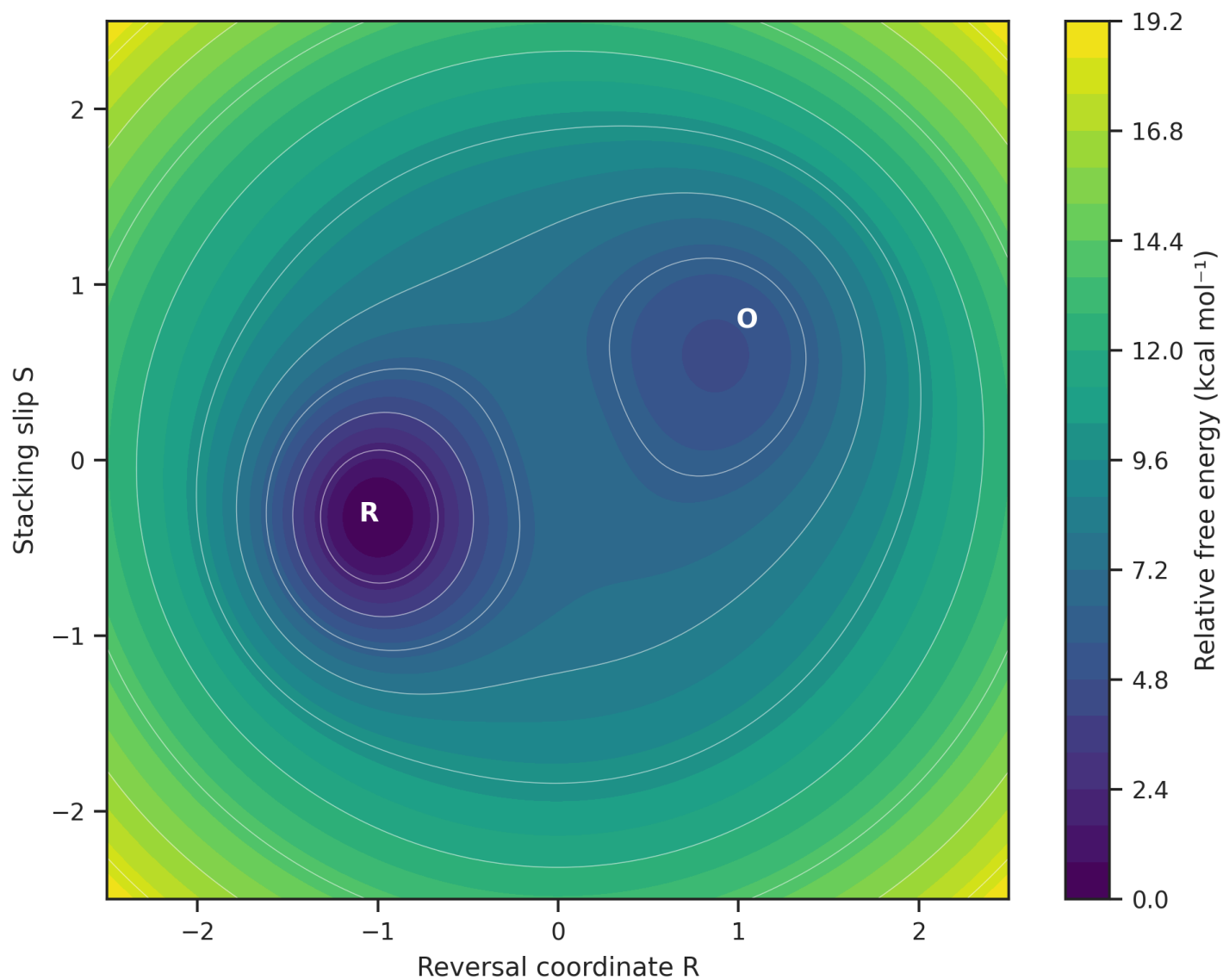


Figure 3

Predicted cap free-energy surface. R denotes reversal geometry and S denotes cap-core stacking slip. "R" marks the reversed-cap minimum and "O" the open-cap minimum. Values arise from the declared model and are not experimental free energies.

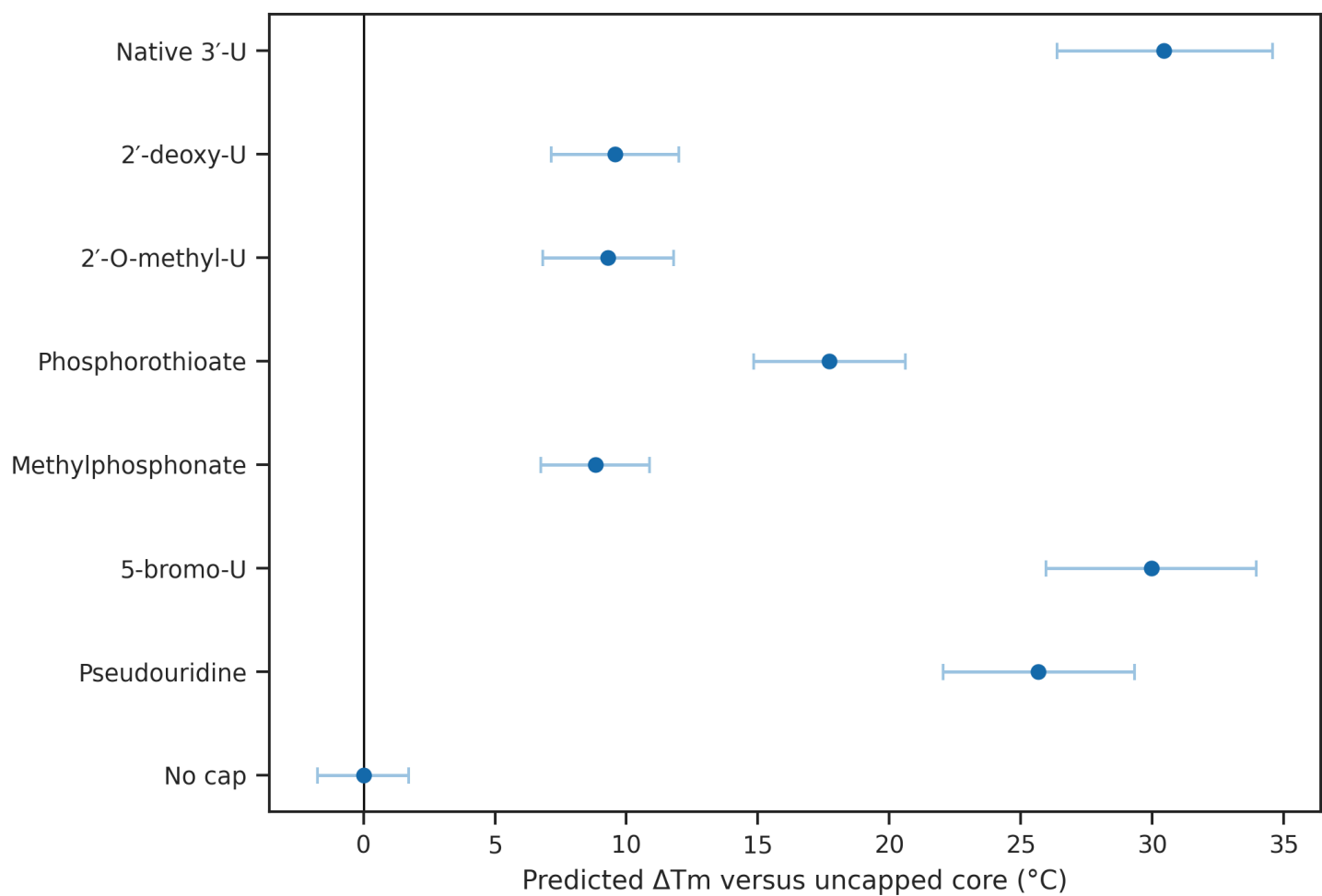


Figure 4

Posterior-predictive stabilization across terminal chemistries. Points are posterior means and bars are 95% credible intervals from 5,000 draws per variant.

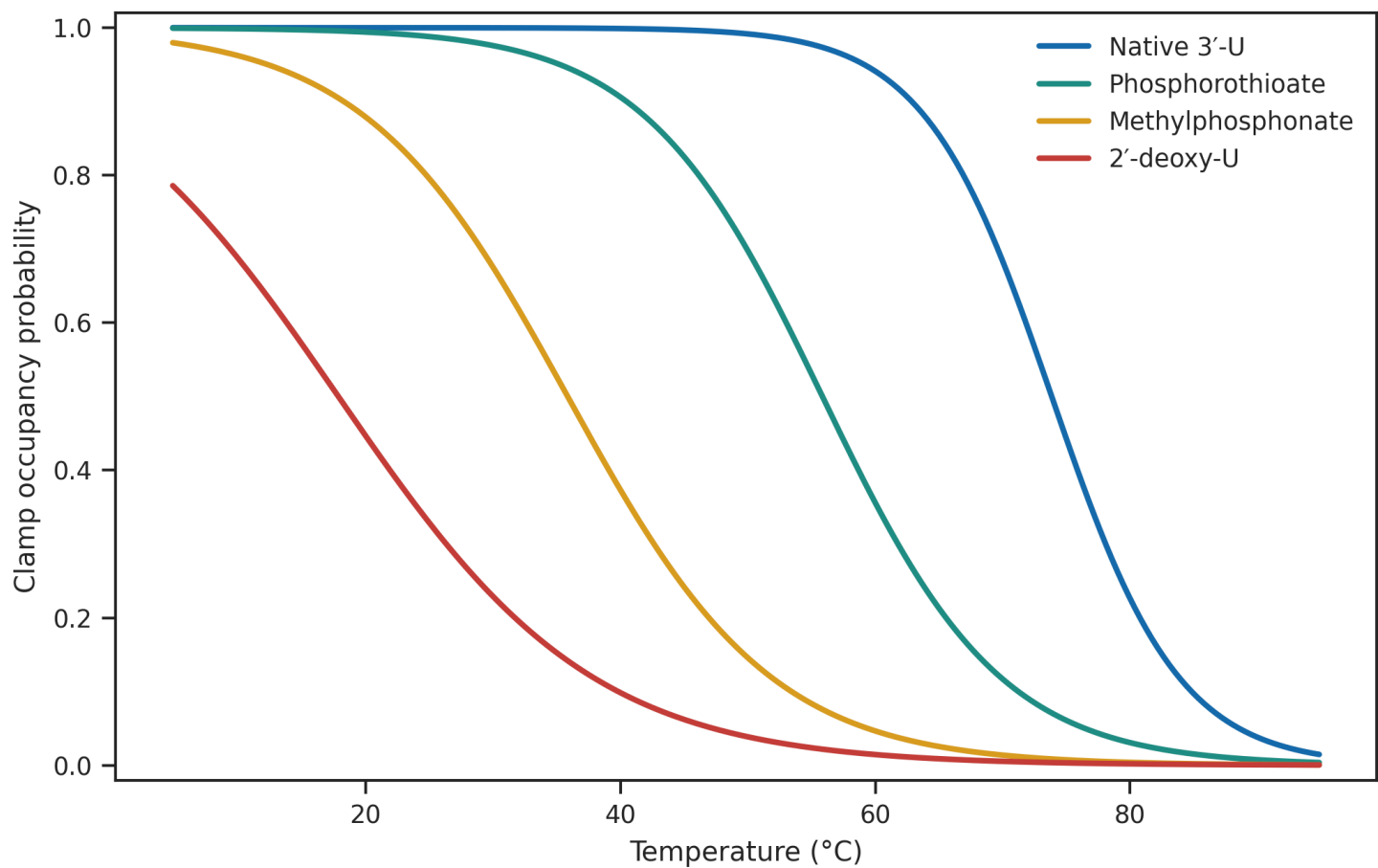


Figure 5

Temperature dependence of clamp occupancy. Curves are logistic state probabilities used to generate mechanistic predictions; they are not fitted experimental curves.

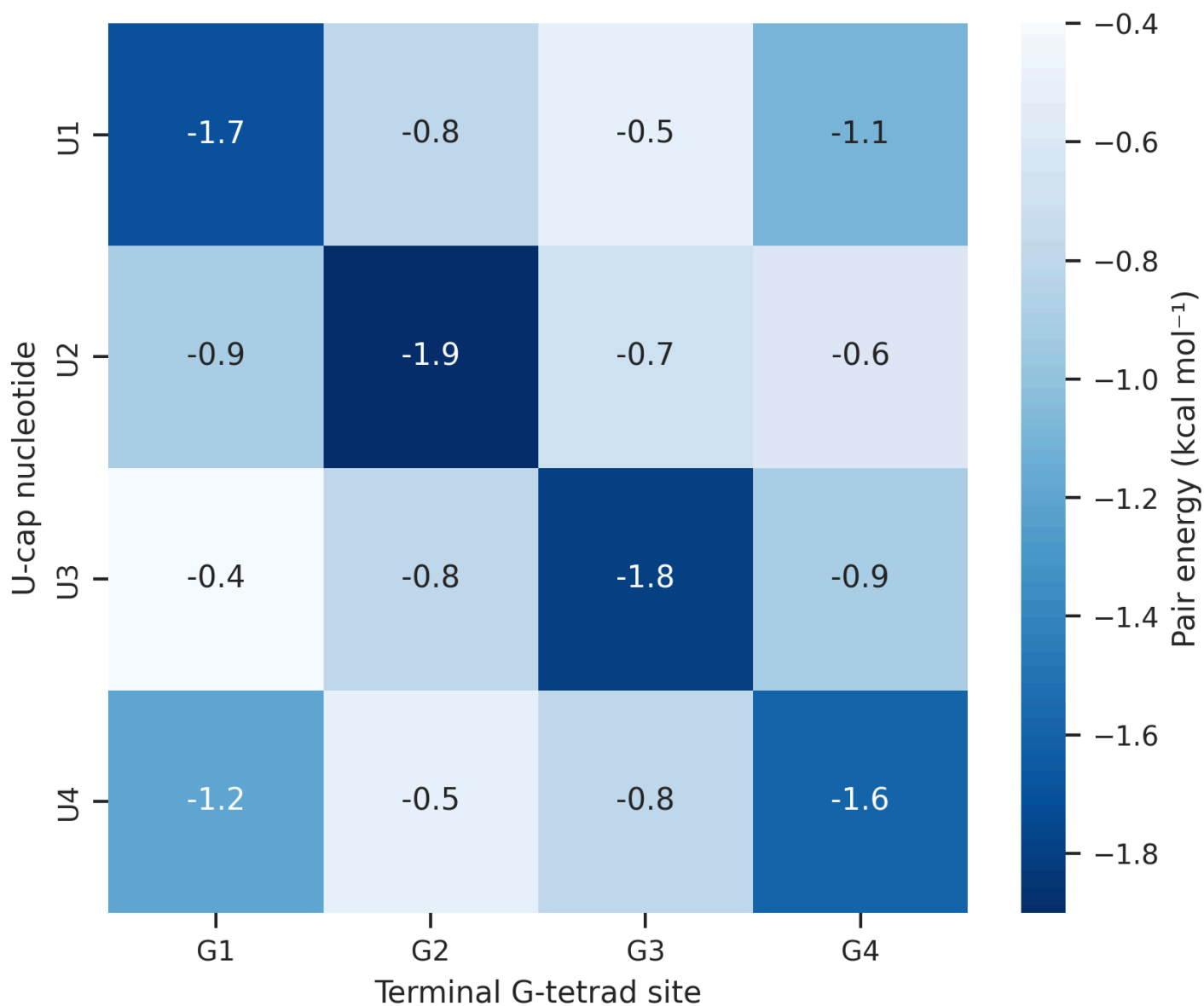


Figure 6

Modeled U–G stacking interaction matrix. More negative values indicate more favorable pair energies. The anisotropic diagonal defines a preferred cap register.

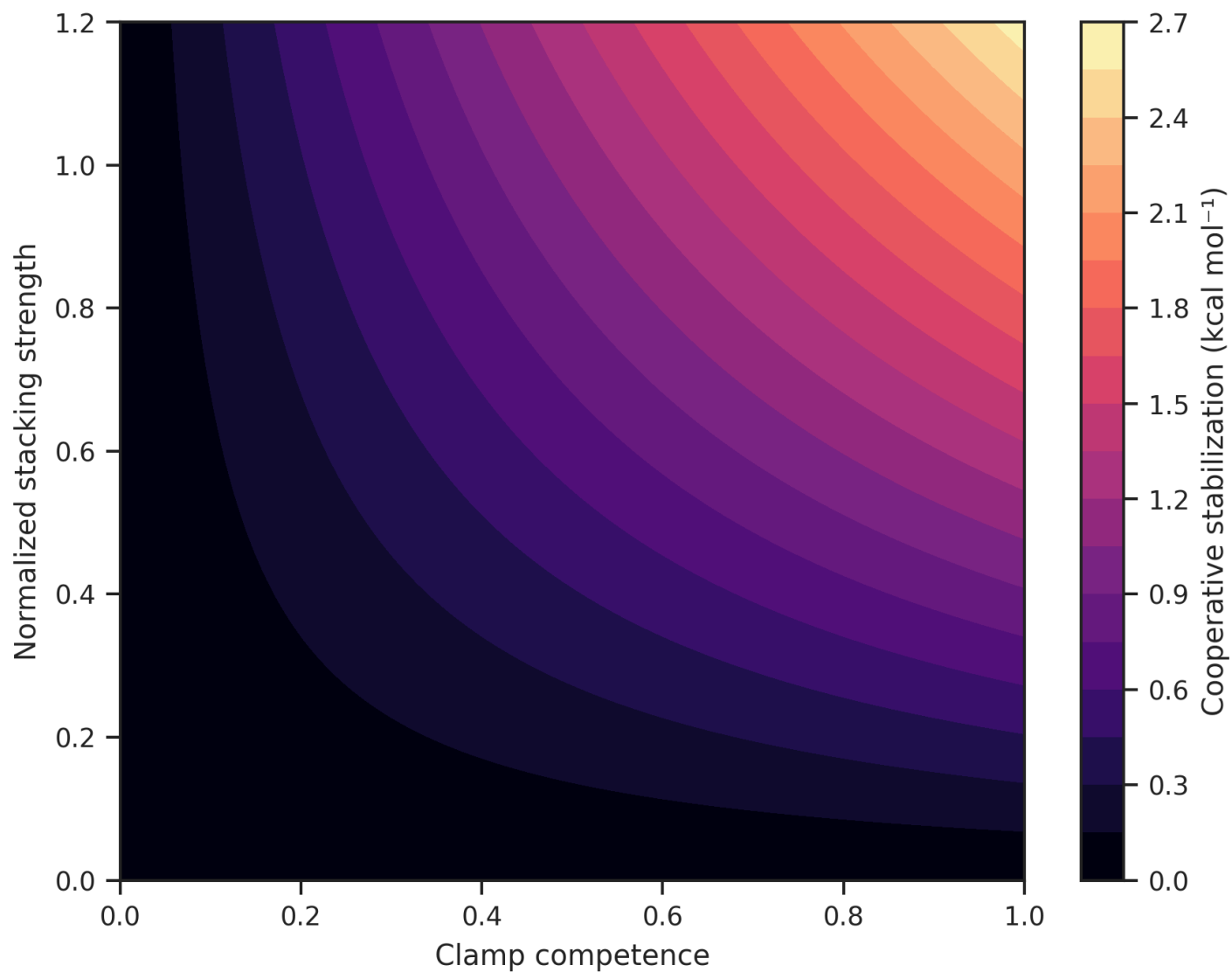


Figure 7

Cooperative stabilization surface. The interaction term becomes large only when clamp competence and stacking strength are simultaneously high.

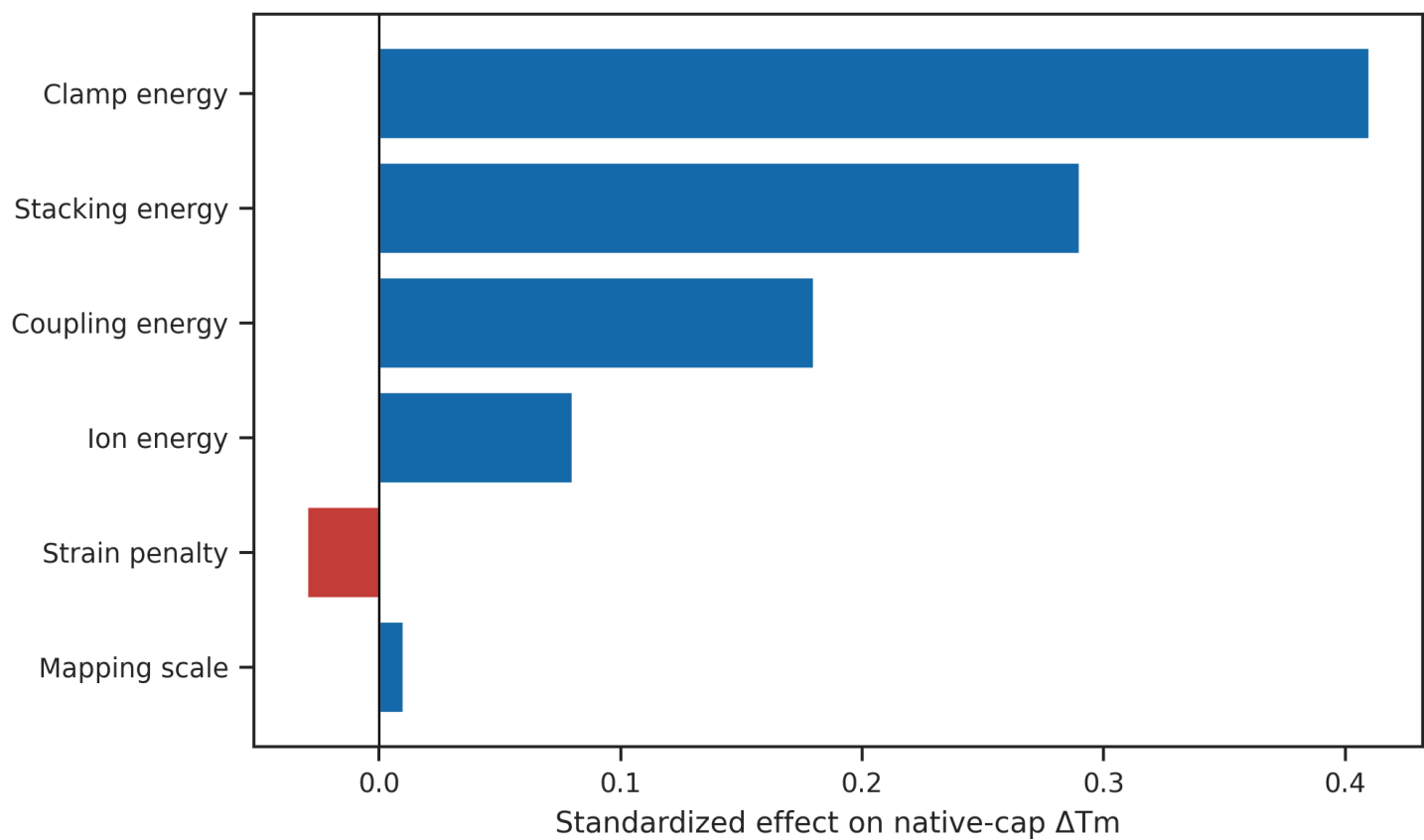


Figure 8

Global sensitivity of native-cap stabilization. Bars show standardized effects across the declared prior ensemble.

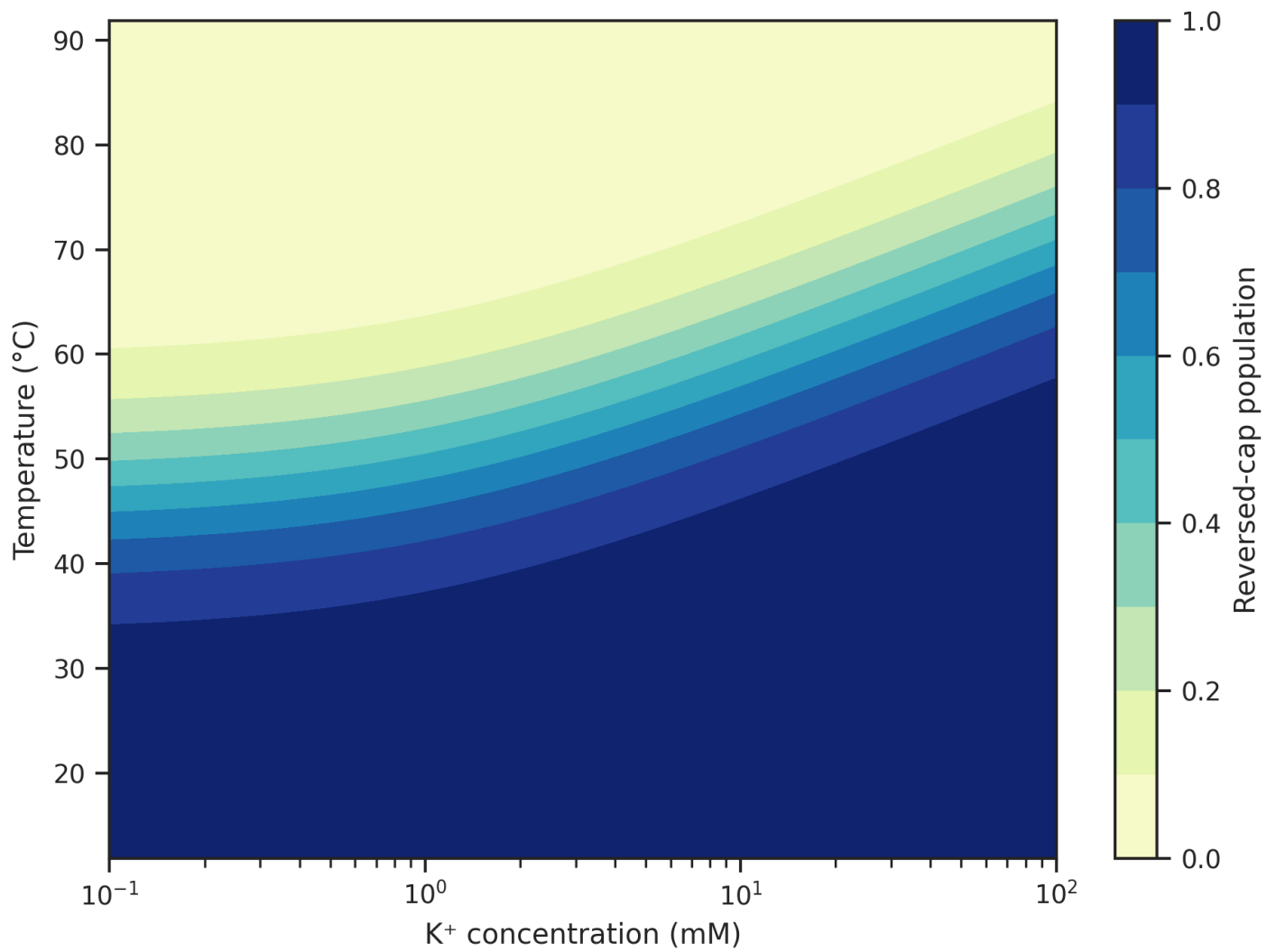


Figure 9

Predicted potassium–temperature phase map. Color denotes reversed-cap population under the CSSI ion-coupling model.