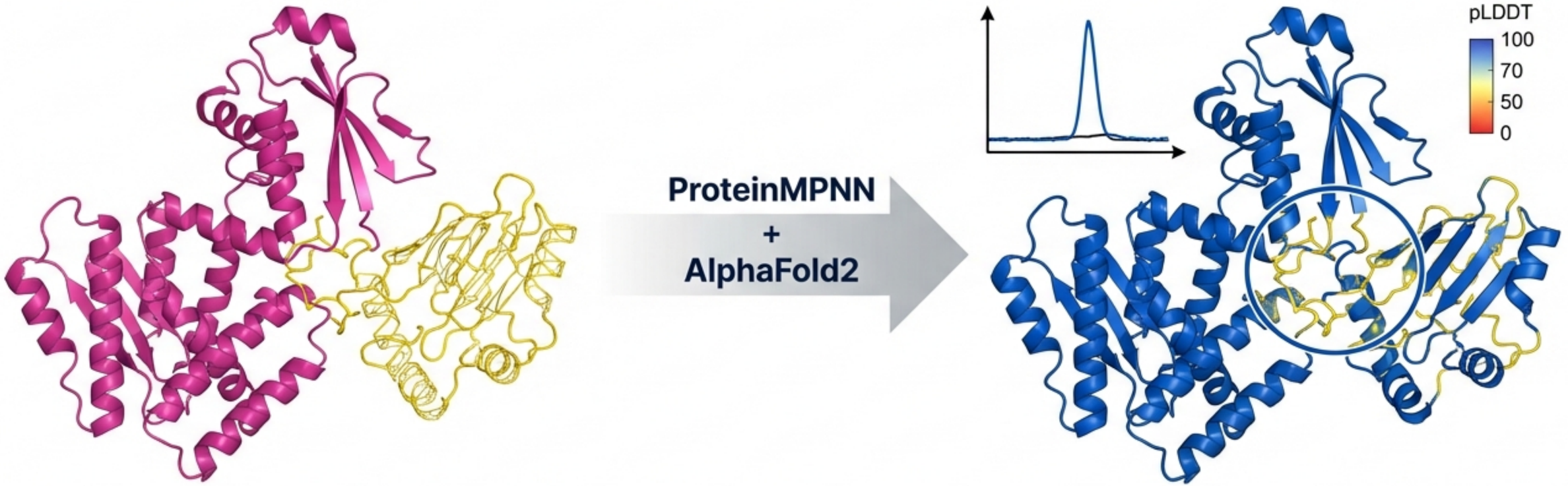
Precision Biology

Robust Design of Allosteric Activators for Rsp5 E3 Ligase via ProteinMPNN

A 3-Month Sprint from In Silico Generation to Crystal Structure Validation



Hsi-Wen Kao *et al.*, Institute of Biological Chemistry, Academia Sinica.

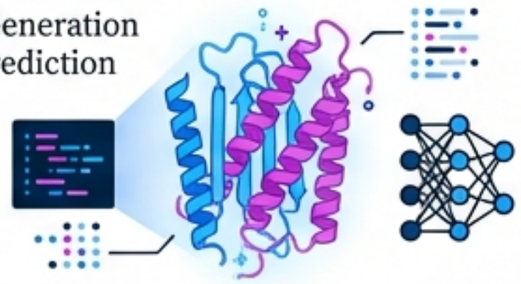
A paradigm shift from physical screening to AI-driven virtual design.

The 3-Month Sprint: Virtual Evolution at Speed

Month 1

Virtual Design

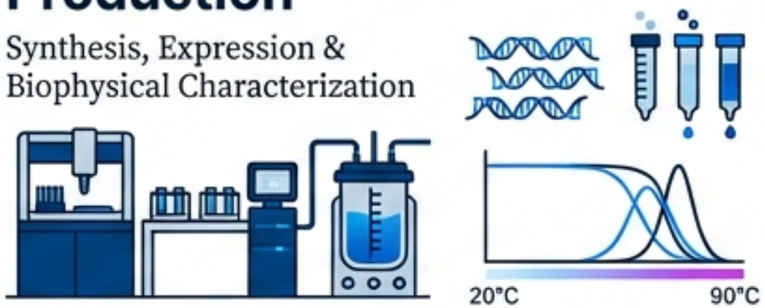
ProteinMPNN Generation
+ AlphaFold2 Prediction



Month 2

Production

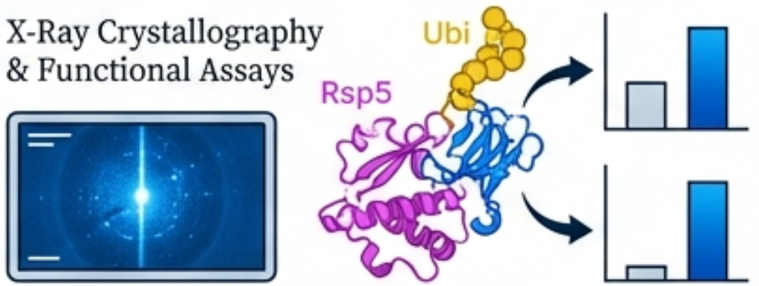
Synthesis, Expression &
Biophysical Characterization



Month 3

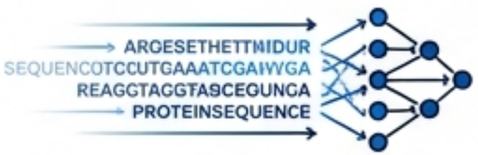
Validation

X-Ray Crystallography
& Functional Assays



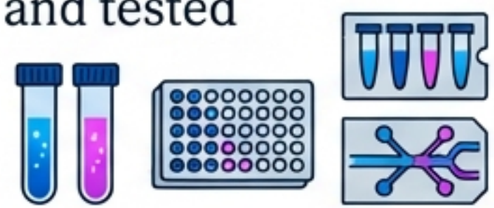
3000

Sequences generated in ~10 minutes



20

Variants synthesized and tested



100%

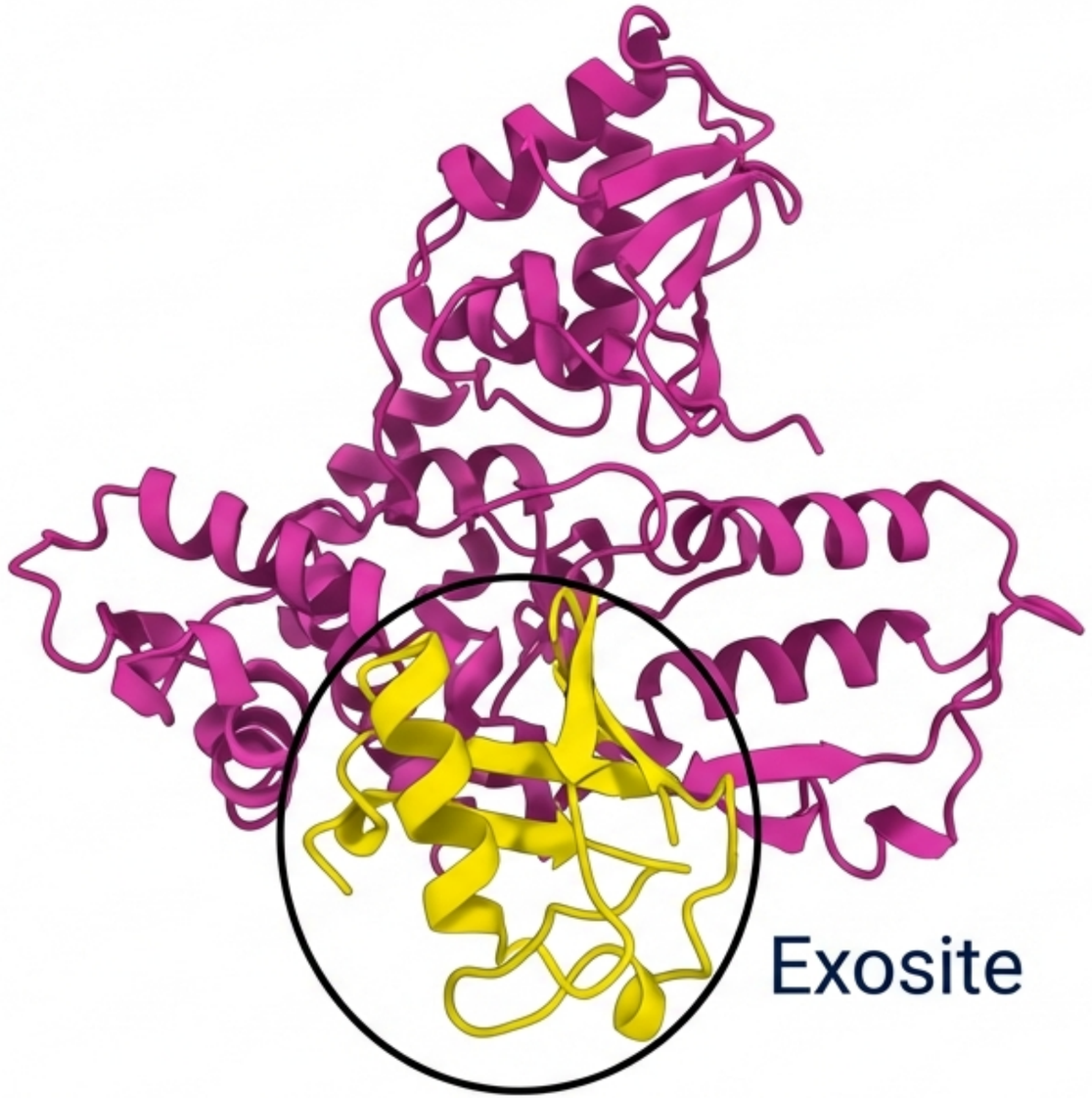
Solubility & Stability (up to 90°C)

6-Fold

Enhancement of enzymatic activity

Impact: This study achieved in 90 days what typically takes months or years using directed evolution libraries.

The Target: Rsp5 E3 Ligase and the Exosite



Role in Homeostasis

Rsp5 is a HECT E3 ligase that regulates protein homeostasis and cellular functions.

The Mechanism

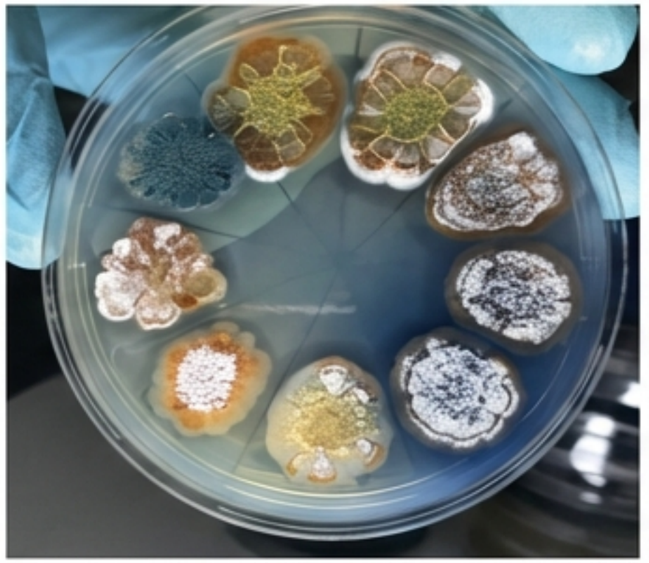
It functions via “allosteric activation.” When Ubiquitin (Ub) binds to the exosite (circled left), it unlocks the enzyme, triggering the transfer of ubiquitin to the substrate.

The Engineering Goal

Design Ubiquitin Variants (UbVs) that bind this exosite tighter and more specifically than wild-type Ub, effectively serving as a “master key” to modulate activity.

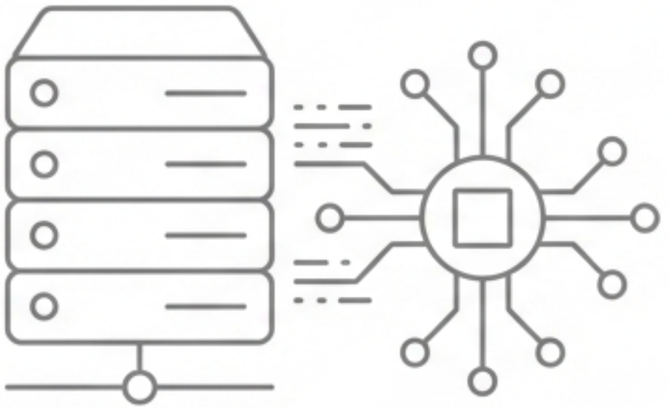
The Bottleneck: Physical Screening vs. Virtual Design

The Status Quo: Phage Display



- Laborious and costly process
- Requires extensive physical libraries
- Often yields proteins with low thermal stability
- Search limited to specific mutagenesis sites

The Innovation: ProteinMPNN

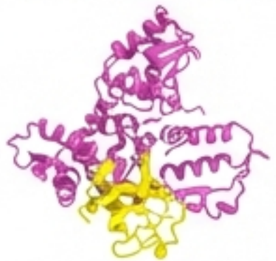


- Cost-effective and time-efficient
- Virtual screening of thousands of sequences
- Implicitly optimizes for solubility and folding
- Explores the entire sequence space (global optimization)

"A virtual solution for rapidly and cost-effectively designing UbVs... circumventing the need for time-consuming experimental screenings."

The Workflow: From Template to Sequence

Input Template

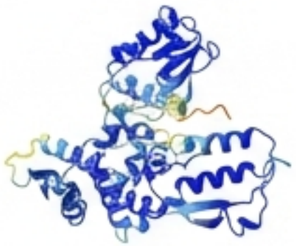


Crystal structure of Rsp5-Ub complex (PDB: 3OLM).

Generation

ProteinMPNN fixes Rsp5 sequence, redesigns Ubiquitin chain.

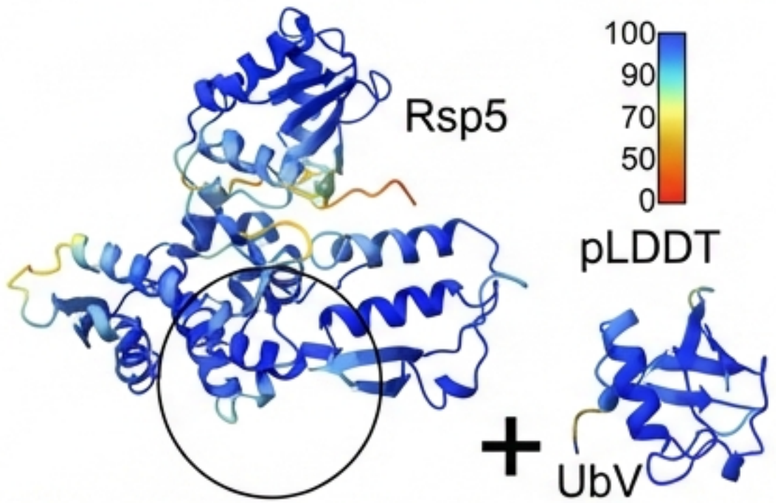
Prediction



AlphaFold2 (AF2) predicts complex structure (No templates used).

Selection

Filter by pLDDT confidence scores & physical properties.



Design Strategy: Global Optimization Yields Better Recovery

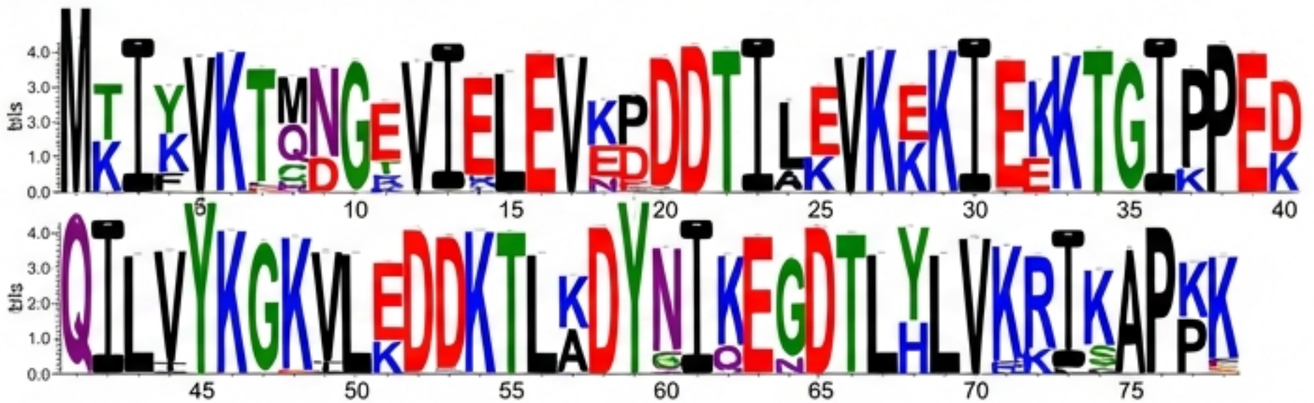
Dataset A (The Winner)

- Method: Whole sequence optimization (Residues 2-78).
- Outcome: High sequence recovery (0.7–0.9).
- Selection: 12 variants (R1-R12).

Dataset B (The Restricted)

- Method: Fixed 49 residues; mutations only at phage-display sites.
- Outcome: Low sequence recovery (0.3–0.5).
- Selection: 6 variants (R13-R18).

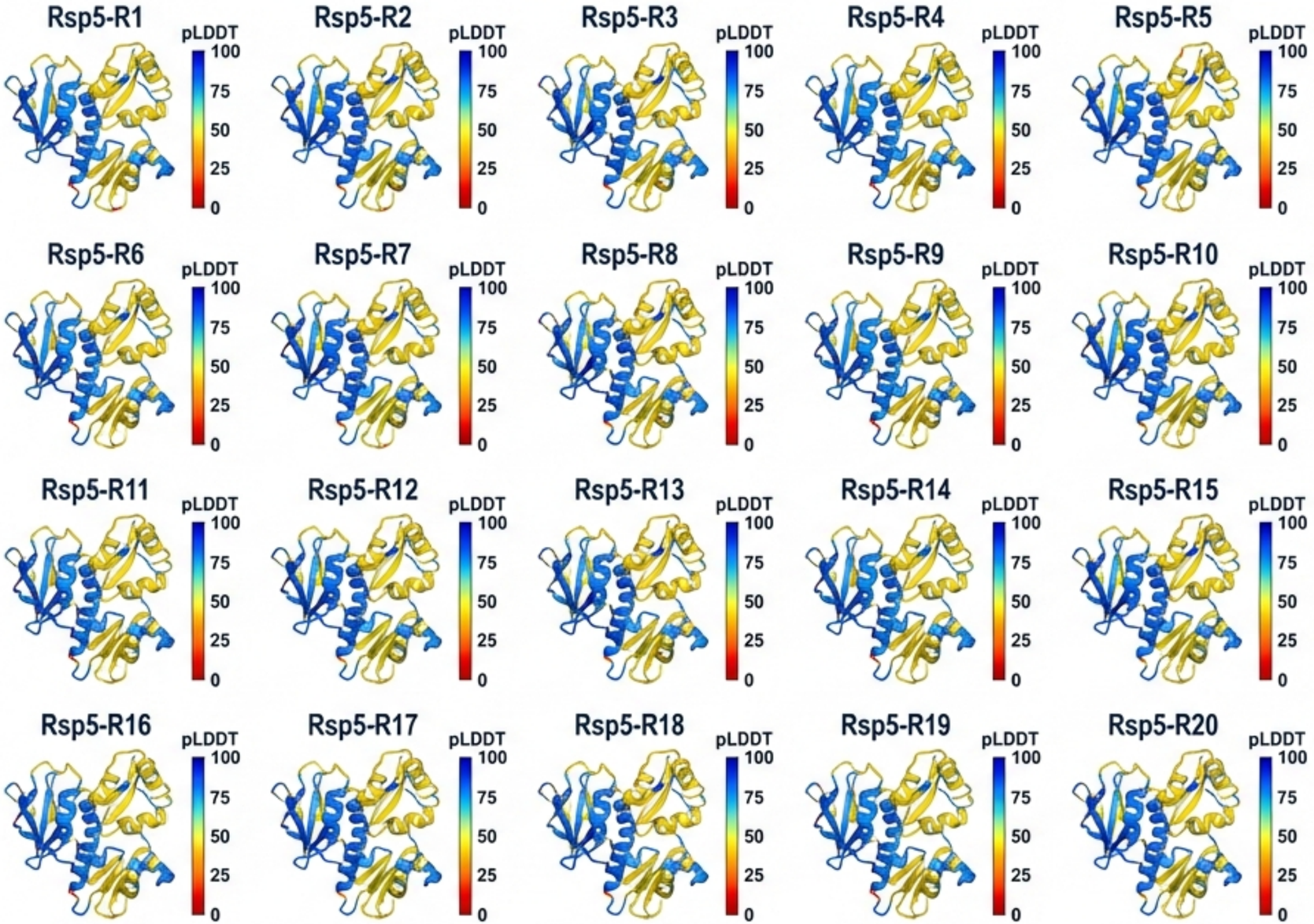
Dataset A (3000 sequences)



Dataset B (3000 sequences)

Key Insight: Restricting the AI to human-biased 'hotspots' (Dataset B) performed worse than allowing global sequence optimization (Dataset A).

In Silico Validation: AlphaFold High-Confidence Predictions



Confidence Scores

Heatmap shows >90 pLDDT confidence for the UbV backbone.

Structural Consistency

The 20 selected UbVs had an RMSD of 0.5–1.1 Å compared to the template crystal structure.

Conclusion

ProteinMPNN predicted mutations that maintain the Ubiquitin fold while altering surface chemistry for binding.

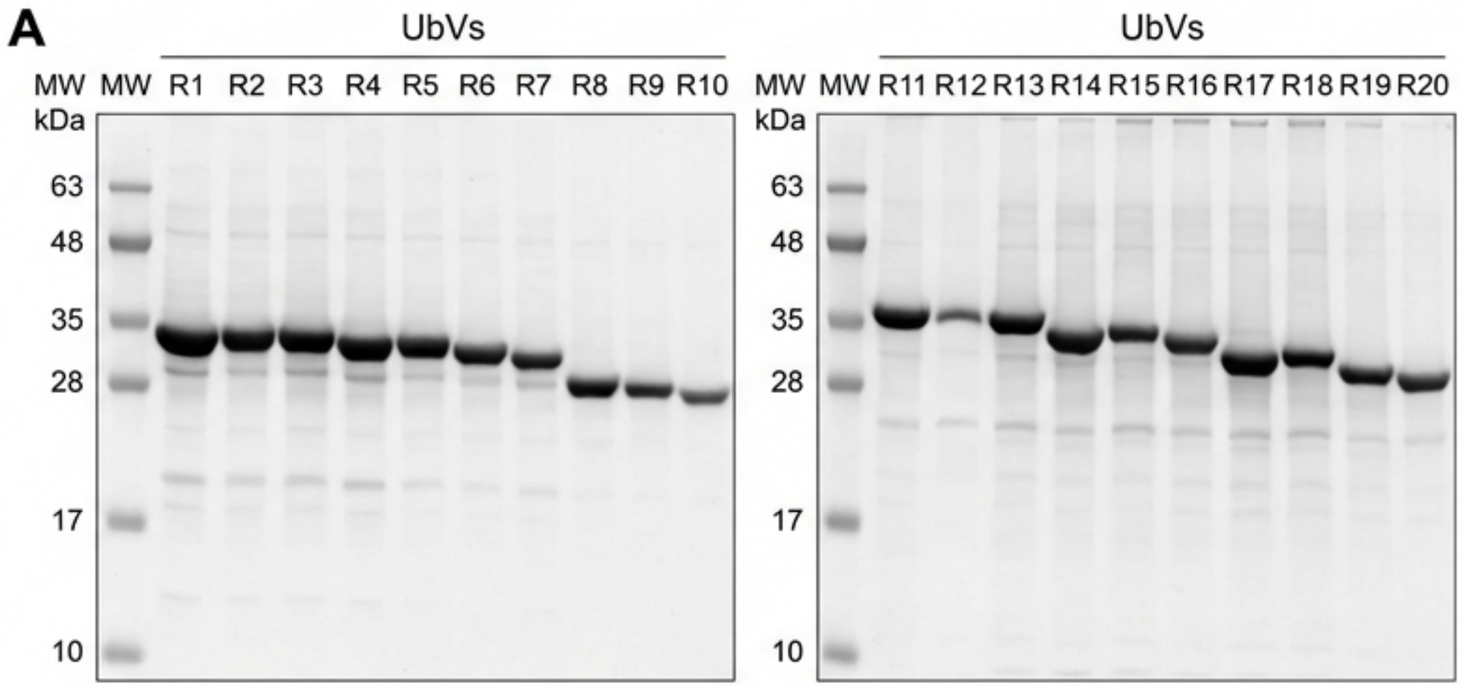
Physical Reality: Expression and Solubility

High Yield Expression

~25 mg of protein obtained from just 300 mL culture via autoinduction.

Solubility Profile

SEC-MALS data confirms variants are monomeric in solution with no aggregation.

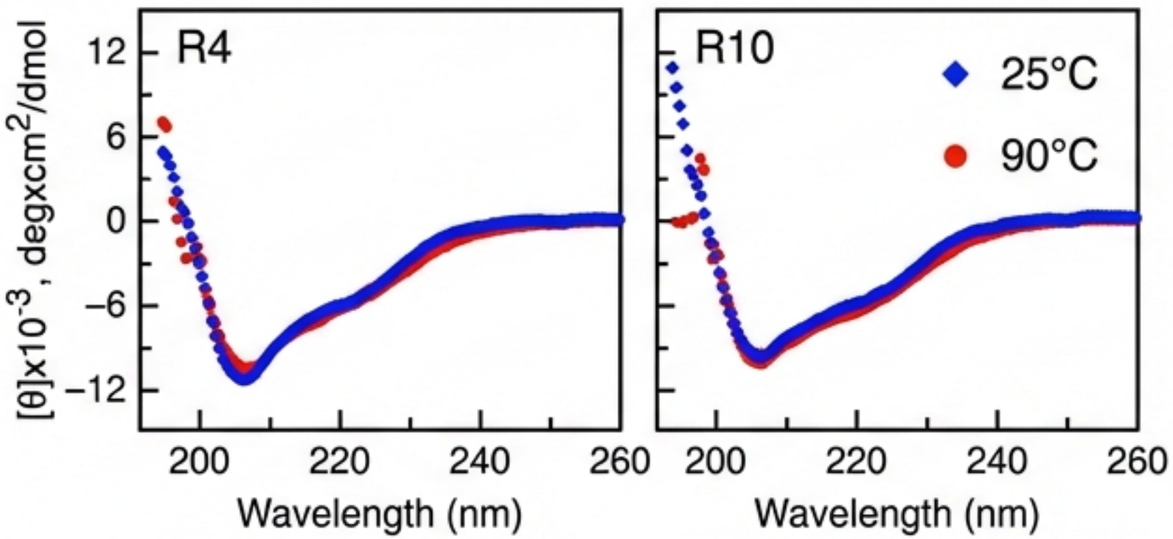


Source Serif Pro. Varying migration (despite similar mass) is due to distinct pI values (ranging from 4.7 to 9.5), confirming sequence diversity.

Thermal Stability: The 90°C Torture Test

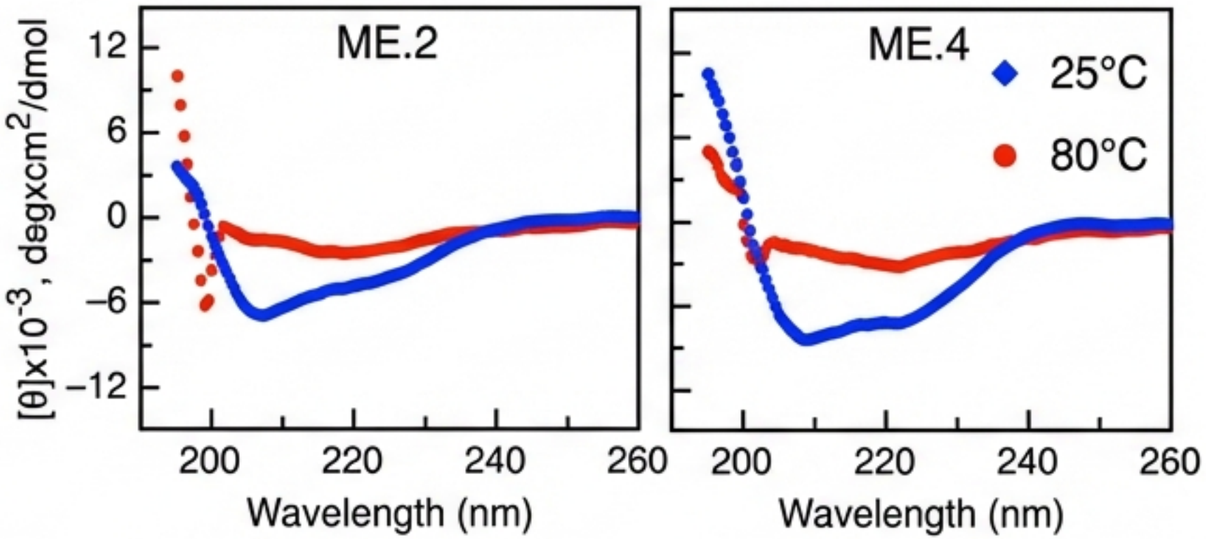
AI Design Success (R4 & R10)

(Data from In. 0)



Phage Display Failure (Reference)

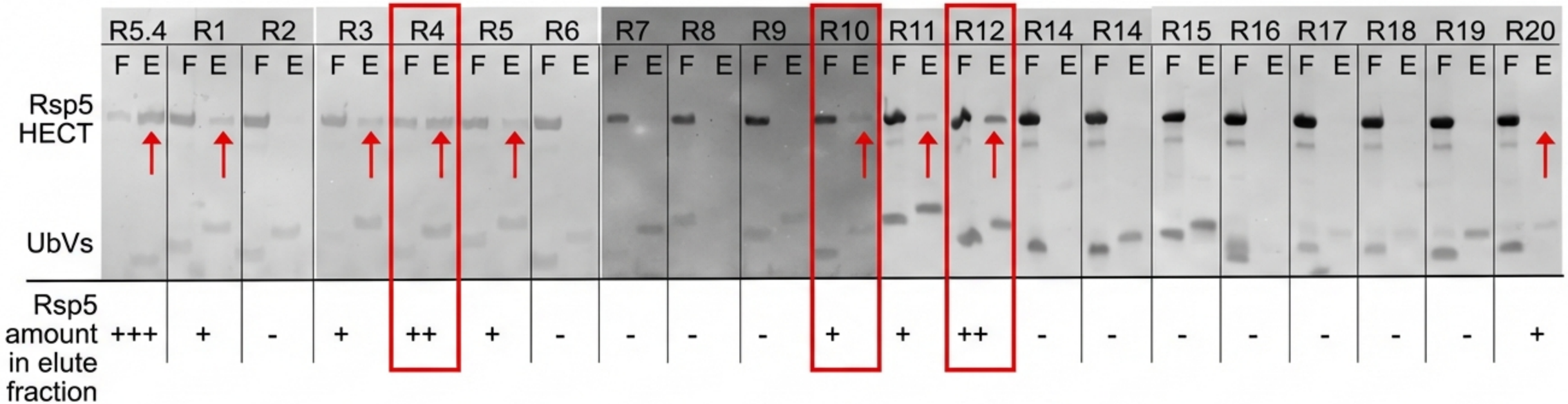
(Data from Inter_1)



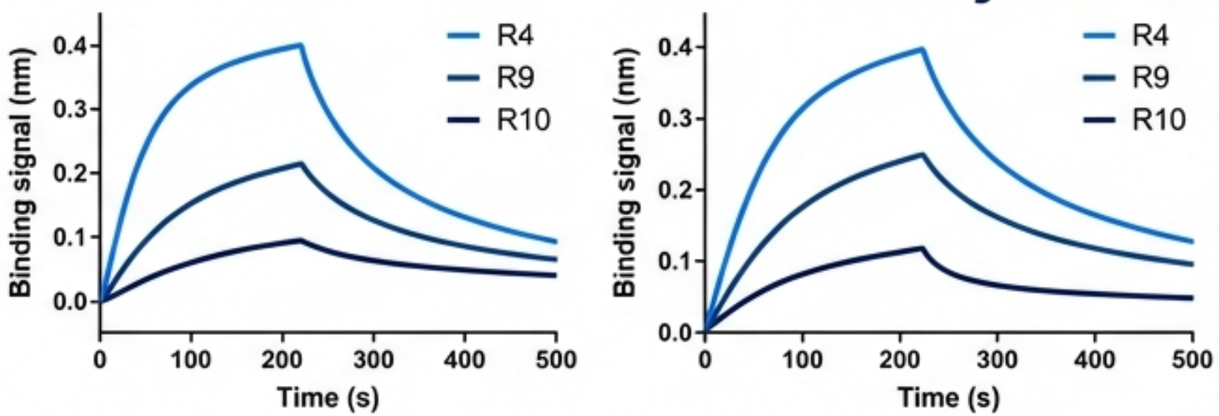
Result: ProteinMPNN designs retained the exceptional thermal **stability** of wild-type Ubiquitin, remaining folded even at 90°C, unlike many phage-display variants which unfold.

Binding Affinity: Identifying the Top Candidates

Pull-Down Assay Screening



Bio-Layer Interferometry (BLI)



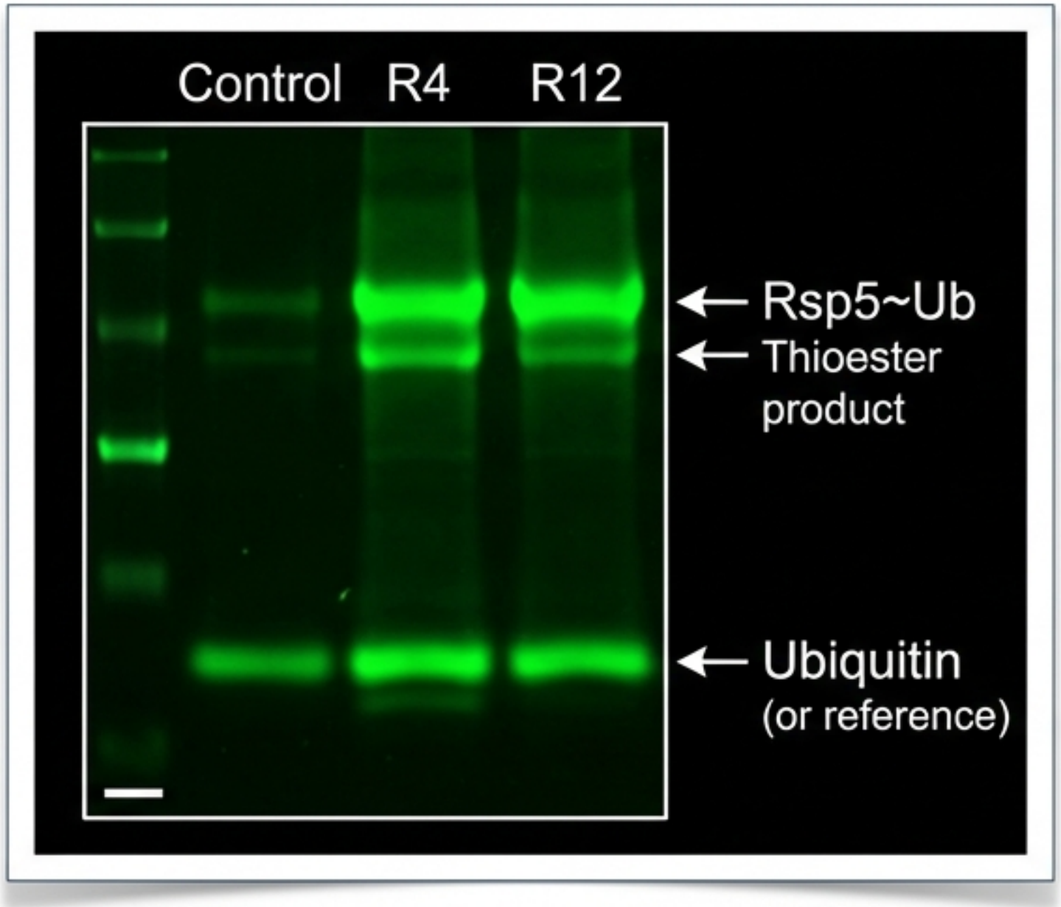
R4: $K_d = 5.5 \pm 1.1$ μM (Strongest Binder)

R12: $K_d = 7.9 \pm 0.8$ μM

R10: $K_d = 13.8 \pm 1.2$ μM

Dataset B Variants (R13-R18): Weak/No Binding

Functional Activation: Driving Enzymatic Activity



The Assay

- Monitoring the formation of Rsp5~Ub thioester (transfer from E2 to E3).

Activity Enhancement

- **R4** & **R12**: Enhanced Rsp5 activity ~6-fold compared to control.
- **R10**, **R11**, **R18**: Enhanced activity ~3-fold.

Benchmarking

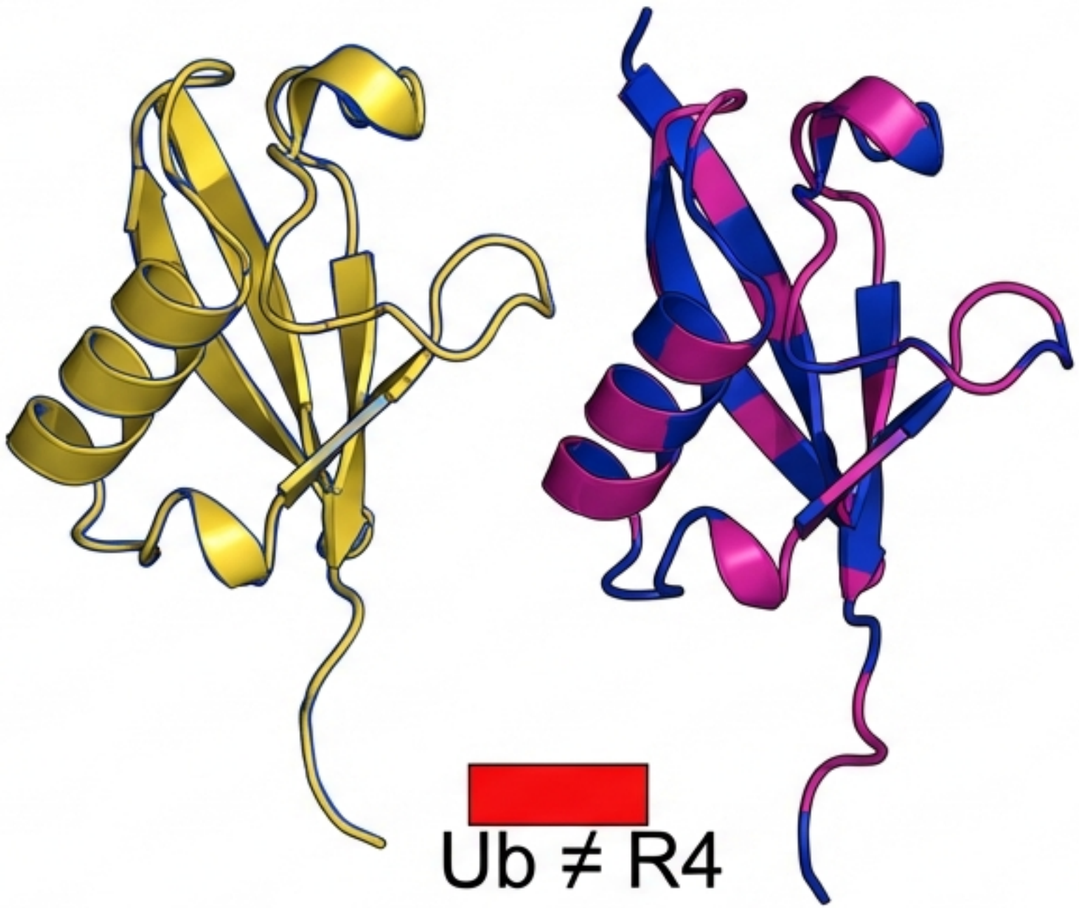
- **R4** achieves ~60-70% of the catalytic efficiency of the phage-display champion (**R5.4**).

Conclusion: The virtual designs are not just passive binders; they are functional allosteric switches.

Structural Truth: Crystal Structure of Variant R4

Resolution:
3.0 Å

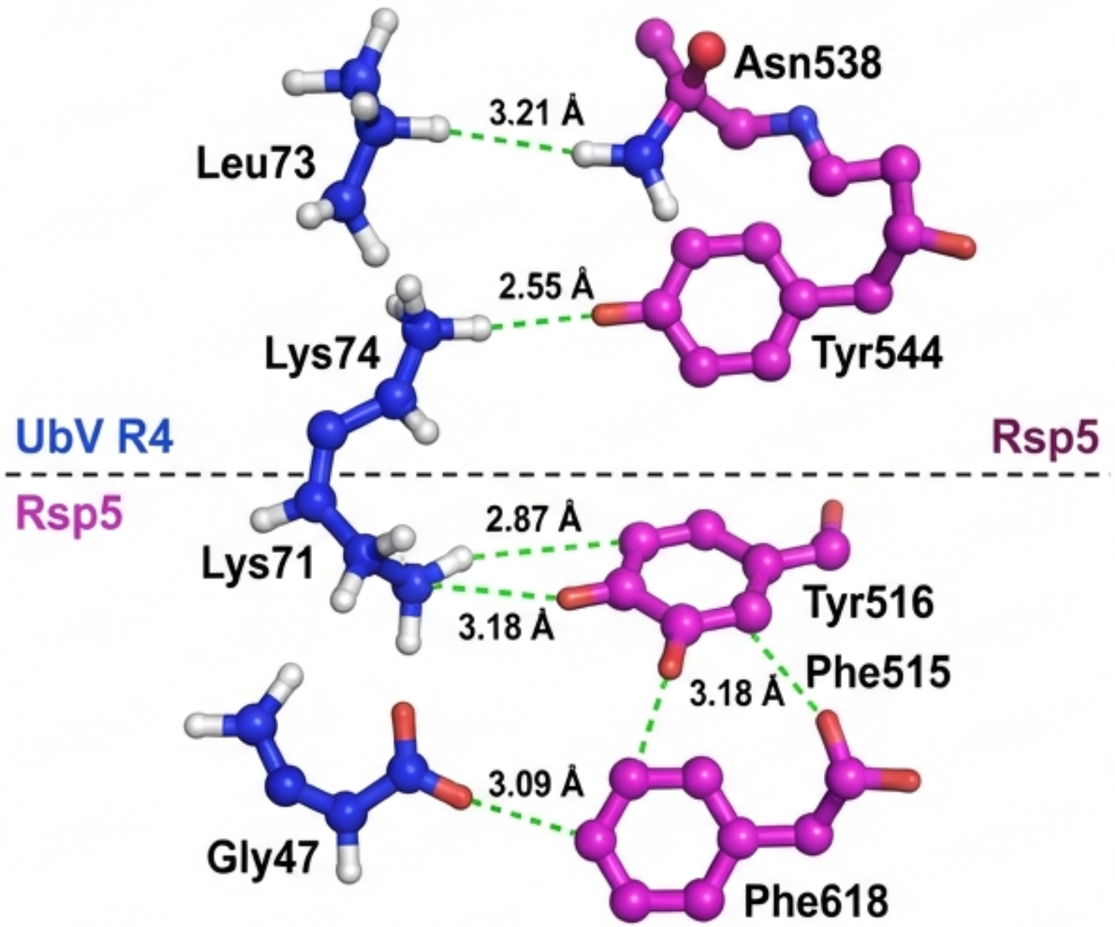
Backbone RMSD:
0.55 Å relative to WT



Mutation Count:
32 residues differed from WT

"Designed UbVs display minimal structural perturbations despite having divergent side-chain compositions."

Mechanism: The Rsp5-UbV Interface



Molecular Docking

- The β-turn (residues 8–11) orients towards the Rsp5 exosite.
- AlphaFold accurately predicted the specific hydrogen bonding networks stabilizing the complex.

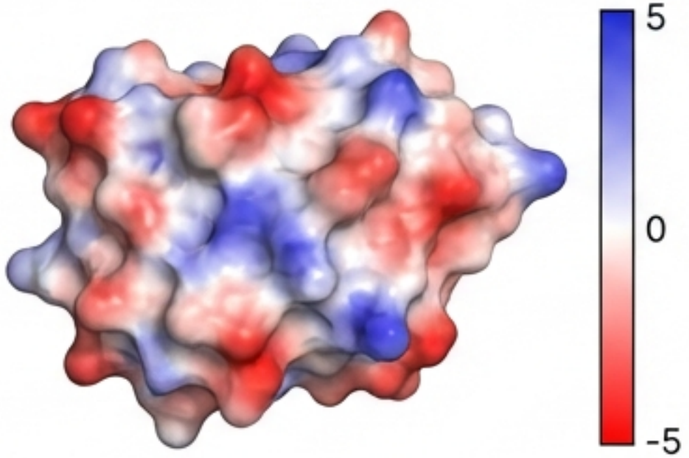
Key Interactions

- R4 Residues: Gly47, Lys71, Leu73
- Rsp5 Partners: Asn538, Tyr544, Phe515

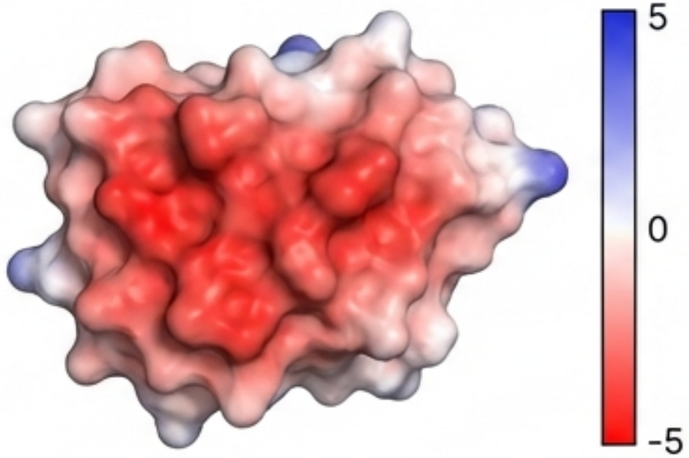
Head-to-Head: AI (R4) vs. Phase Display (R5.4)

Visual Comparison

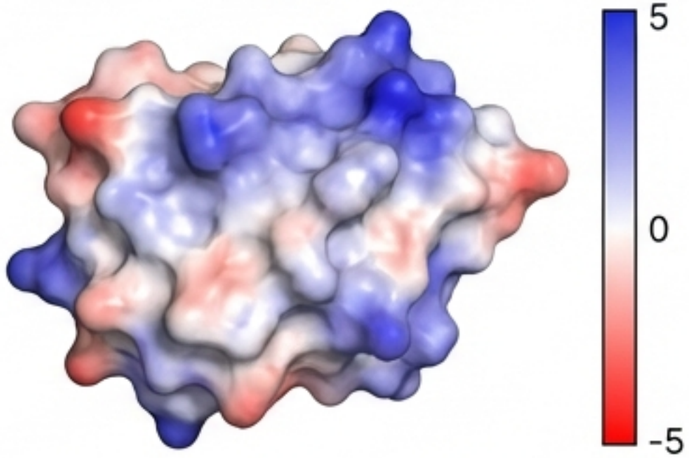
Ub



R4 (AI Variant)



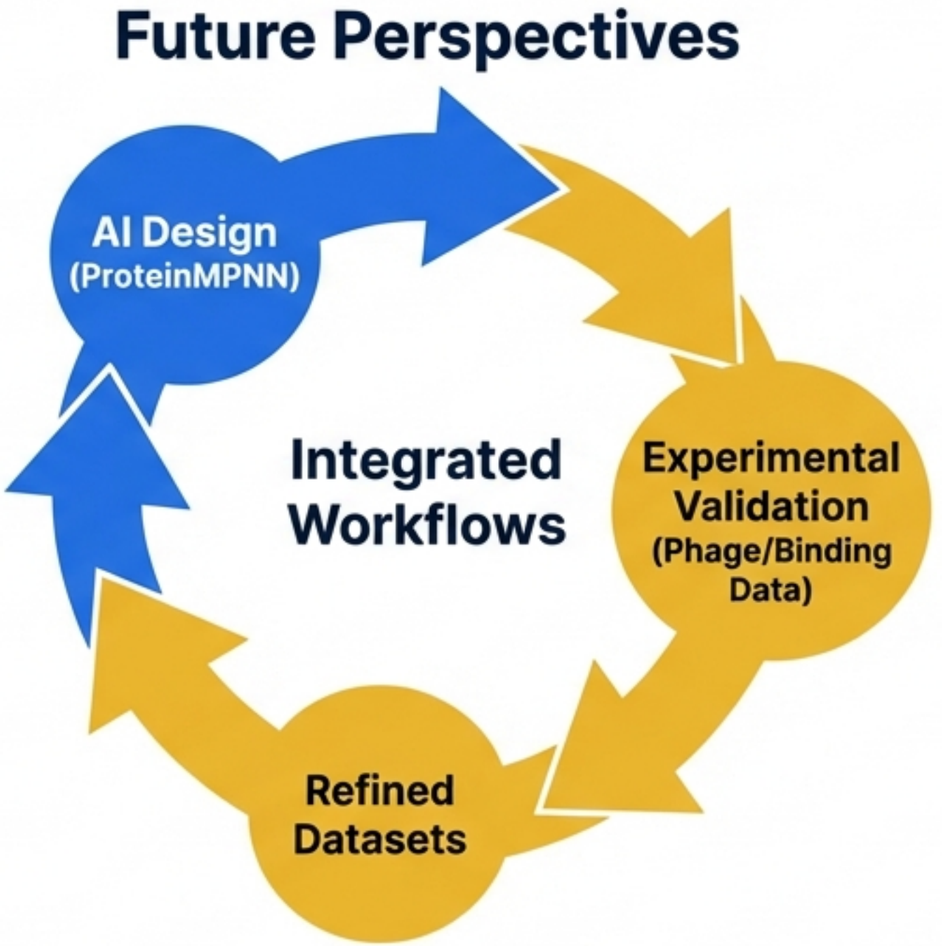
R5.4 (Phage Display)



Metric	AI Variant (R4)	Phage Display (R5.4)
Affinity (Kd)	~5.5 µM	~125 nM (Tighter)
Thermal Stability	Hyper-stable (>90°C)	Stable (varies by variant)
Electrostatics (pI)	5.11 (Acidic)	8.61 (Basic)
Time to Design	Days	Weeks/Months

The ‘Virtual Evolution’ Advantage

- ✓ **FAST:** 3 months from concept to crystal structure.
- ✓ **CHEAP:** No physical libraries or panning rounds required.
- ✓ **ROBUST:** High solubility and thermal stability (90°C).
- ✓ **FUNCTIONAL:** Validated allosteric activation of E3 ligase.



Conclusion: This pipeline transforms Ubiquitin from a passive marker into a tunable, non-covalent drug-like modulator, paving the way for similar engineering of NEDD8 and SUMO.