A-ALPHA BIO

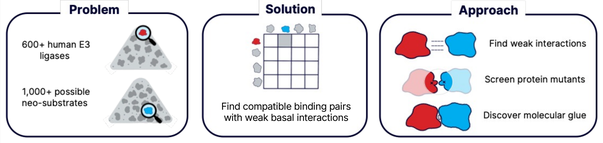
A-Alpha Bio, Inc.
Seattle, WA

Intelligent Molecular Glue Discovery by Identifying Glueable Ligase-Target Pairs at Scale

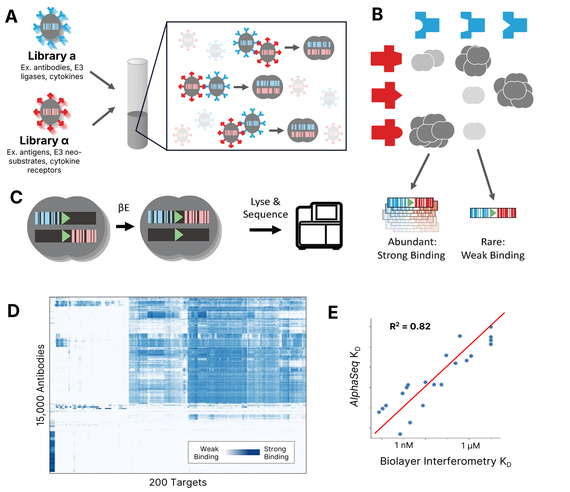
Phil Burke, Arpita Sen, David Noble, Abhishek Dogra, Ruchi Bansal, Dasha Krayushkina, Kerry McGowan, Natasha Seelam Murakowska, Charles Lin, Yeweon Lee, Randolph Lopez

Abstract

Small molecule glues binding the substrate-recognition domain of an E3 ubiquitin ligase can re-route binding and induce degradation of novel targets. However, for a molecular glue to sufficiently stabilize a ternary complex, the proteins must exhibit proper shape complementarity and contain productive amino acid contacts. Identifying novel glueable ligase-target pairs requires the discovery of weak interactions between thousands of potential pairs of human E3 ligases and target proteins, which has historically been a serendipitous process. To address this challenge, we apply AlphaSeq, a high-throughput experimental platform for measuring protein-protein interactions, to discover and structurally characterize weak ligase-target interactions. We validate our purpose-driven approach by characterizing interactions agonized by known small molecules and leverage the platform to intelligently discover molecular glues for novel ligase-target pairs.



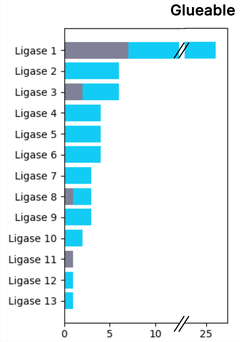
AlphaSeq platform



AlphaSeq applies synthetic biology and next-generation sequencing to measure protein-protein interactions at a library-on-library scale. **(A)** Two libraries containing barcoded protein sequences for display on the yeast cell surface are mixed in liquid culture. Interactions between surface-displayed proteins drive agglutination and cell fusion. **(B)** The number of fused cells with a given protein pair depends on protein interaction strength. **(C)** Recombination is induced with β-estradiol to consolidate DNA barcodes. Cells are then lysed and sequenced to count the abundance of each barcode pair and determine all protein interaction strengths. **(D)** Example: *AlphaSeq* dataset measuring ~3 million interaction affinities in a single assay. **(E)** *AlphaSeq*-measured affinities strongly correlate with BLI-measured affinities.

AlphaSeq discovery of glueable ligase-target pairs

Glueable pairs discovered, by ligase



Summary of identified novel protein-protein interactions (PPIs) between human E3 ligases and therapeutic target proteins. These interactions span a range of binding affinities, from 100 nM to 10 μM. Additionally, we discovered target mutants that significantly enhance binding affinity and revealed key structural features of the complex interface. A subset of these interactions have been selected towards orthogonal validation and small molecule discovery.

For more information on our platforms, pipeline, and partnerships, visit aalphabio.com or get in touch at contact@aalphabio.com

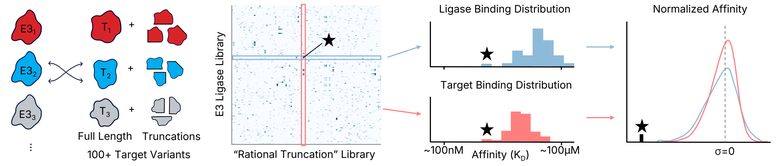
Basal and mutational screening for intelligent discovery of molecular glues

RATIONAL TRUNCATION (RT)

Measure binding between E3 ligase library and multiple truncations of each target.

High sensitivity and multi-dimensionality enables discovery of weak but highly specific interactions by identifying statistical outliers.

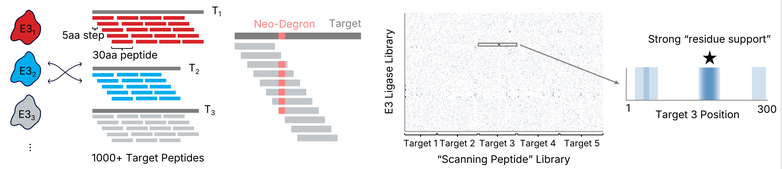
Basal screening



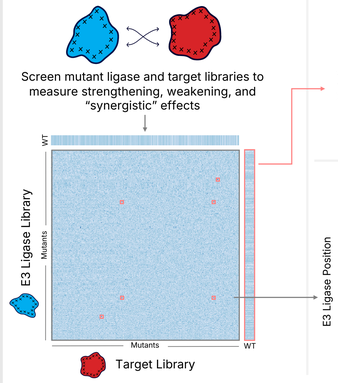
SCANNING PEPTIDE (SP)

Measure binding between E3 ligase library and overlapping peptides that tile entire target sequence.

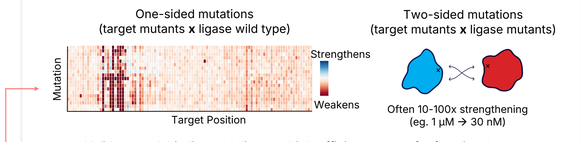
Binding signal from multiple overlapping peptides reveals precise location of linear degron domain.



Mutational screening



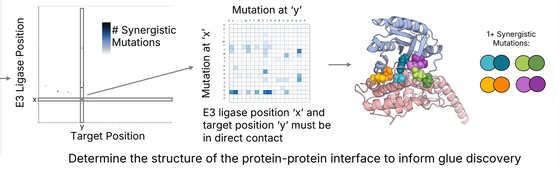
DISCOVER STRENTHENING & WEAKENING MUTATIONS



Validate weak binding and discover high-affinity reagents for functional assays

DISCOVER SYNERGISTIC MUTATIONS

'synergistic mutations' occur when a mutation in the ligase and a mutation in the target individually weaken binding, but together strengthen binding

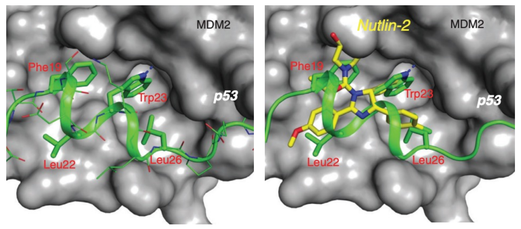


Determine the structure of the protein-protein interface to inform glue discovery

Leveraging *AlphaSeq* to discover glueable neosubstrates for MDM2

MDM2 binds to P53 and targets it for degradation

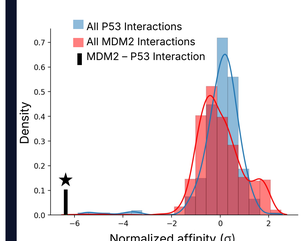
MDM2 is a human E3 ligase that recognizes a linear degron motif present in the transcription factor P53, and targets this tumor suppressor protein for degradation[1]. The MDM2-P53 interaction can be inhibited by small molecules such as nutlin, which bind to the degron-recognition pocket of MDM2, resulting in stabilization of P53.



MDM2 shown bound to the P53 degron peptide sequence (left), and the overlap of nutlin and P53 in the MDM2 degron recognition domain (right)

Figures adapted from Wang et. al 2017[1]

Binding between MDM2-P53 is captured in RT and SP basal screening



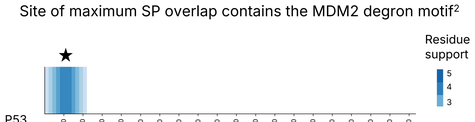
AlphaSeq assay representative of a typical RT basal screen:

- **Targets**: 130 (with multiple variants per target)
- **Ligases**: 39
- **Network size**: ~80,000 PPIs

Evidence of interaction between MDM2 and P53 in basal screen:

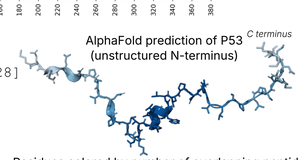
- **Affinity (K_D)**: 10 nM
- **Normalized affinity**: -6.2 σ

Site of maximum SP overlap contains the MDM2 degron motif[2]



5-peptide overlapping region:
[16] QETFSDLWKLLWK [28]

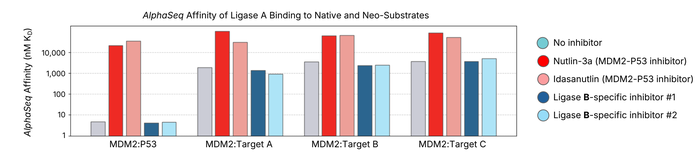
Known degron:
[19] FSDLWKLL [26]



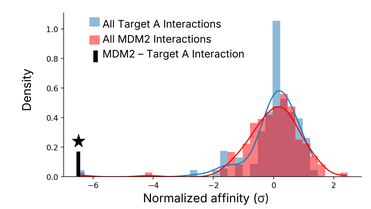
Residues colored by number of overlapping peptide hits

Basal screening identifies multiple novel MDM2 neosubstrates

AlphaSeq basal screening was performed in the presence of MDM2-P53 inhibitors nutlin-3a or idasanutlin, as well as unrelated inhibitors for other ligases. The nutlin conditions show disruption of the MDM2-P53 interaction, as well as disruption of MDM2 interactions with several other proteins. This suggests that these proteins are recognized by the MDM2 degron binding domain and may be glueable neosubstrates. Target A was chosen for follow-up studies.



Screening in the absence of small molecules shows a weak but specific MDM2-Target A interaction

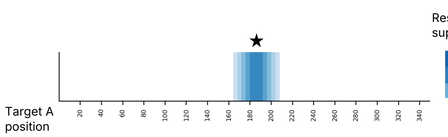


AlphaSeq RT basal screen:

- **Targets**: 220 (multiple variants per target)
- **Ligases**: 20
- **Network size**: ~360,000 PPIs

Evidence of specific interaction between MDM2 and Target A in basal RT screen:

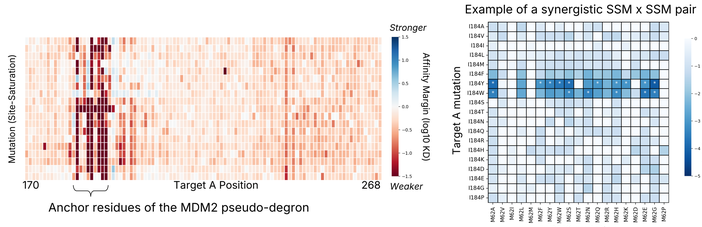
- **Affinity (K_D)**: ~350-1000 nM
- **Normalized affinity**: < -6 σ



Site of maximum SP overlap contains multiple MDM2 degron anchor residues

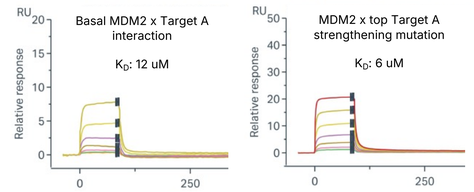
SSM characterization of the MDM2-Target A interface

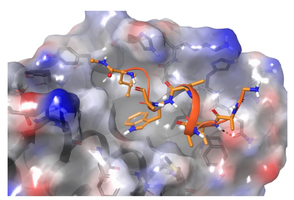
Most mutations in the region of Target A that resembles the MDM2 degron sequence result in abolished binding to MDM2 (dark red), while a few substitutions resulted in increased binding (light blue) (left). Two-sided mutagenesis revealed residue 184 in Target A and residue 62 in the MDM2 to be synergistic and likely to be in close contact at the interface (right).



Advancing the MDM2-Target A interaction toward molecular glue discovery

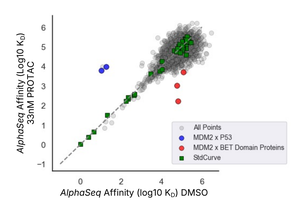
Surface Plasmon Resonance (SPR) measurements of MDM2 binding to a peptide encoding the Target A pseudo-degron (left). Strengthening mutations to the Target A peptide improved affinity by 2-fold in SPR (right).

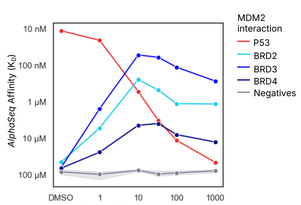




In Silico structural modeling of MDM2, in complex with a peptide encoding the degron-like region from Target A, enables virtual screening efforts.

AlphaSeq 3D: Multidimensional small molecule screening in *AlphaSeq*





AlphaSeq was performed on a network of 5,000 PPIs, in the presence of a PROTAC engineered to link MDM2 to BET domain proteins. BET domain proteins BRD2, BRD3, and BRD4 were present in this network.

This PROTAC shows enhancement of the BET domain protein interactions with MDM2 in *AlphaSeq* relative to a DMSO control condition. We observe a hook effect on *AlphaSeq* affinities at higher PROTAC concentrations. In addition, the native MDM2-P53 interaction is disrupted by the nutlin-based MDM2 recruitment ligand.

These experiments illustrate the potential for *AlphaSeq 3D* in early-stage molecular glue screening and validation.

References

1. Wang et al. Targeting the MDM2-p53 Protein-Protein Interaction for New Cancer Therapy: Progress and Challenges. Cold Spring Harb. Perspect. (2017)
2. Kumar et al. ELM-the Eukaryotic Linear Motif resource-2024 update, DEG_MDM2_SWIB_1, *Nucleic Acids Res.* (2024)