A NOVEL COMBINATORIAL HIGH-THROUGHPUT ASSAY OF SMALL MOLECULES AND THEIR PROTEIN TARGETS

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ABSTRACT

Understanding the molecular determinants of substrate specificity is essential for designing molecular glue degraders that engage new targets or avoid undesired off-targets. While prior studies have characterized degron motifs and structure-activity relationships of the thalidomide scaffold, decoding selective degradation requires profiling the effects of co-varying both sides of the interaction, and these efforts have been limited because we lack a scalable method to probe chemical and protein variation combinatorially. To address this need, we leveraged single-cell RNA sequencing and designed a molecular recording circuit to enable a platform for high-throughput screens of a chemical library and protein library combinatorially in live cells. Using this system, we quantified the degradation of 117 SALL4 point mutants across 138 glutaramide-containing compounds, generating 16,146 measurements that revealed key residue-functional group interactions. Together, these results inform rational design of molecular glue degraders with improved specificity, nominate promising leads for degraders of new neosubstrates, and establish a generalizable framework for mapping chemical-genetic interaction landscapes more broadly.

1 INTRODUCTION

A central challenge in drug discovery is designing small molecules that are selective for their target. Machine learning approaches offer a promising approach to design small molecules that bind a given target (Stokes et al., 2020) or proteins that bind a given small molecule (Gainza et al., 2020; Polizzi & DeGrado, 2020). However, designing for selectivity remains difficult because we lack large-scale datasets that co-vary both sides of a biomolecular interaction. In addition to informing molecular design, such a dataset can also offer mechanistic insight as it often represents the gold standard evidence for establishing direct molecular interactions: perturbing one component alone may disrupt binding indirectly by destabilizing the protein, whereas compensatory perturbations on the opposing component can confirm a true interaction interface (Belshaw et al., 1995; Belshaw & Schreiber, 1997; Bishop et al., 2000; Knight & Shokat, 2007).

Targeted protein degradation represents one area where this question of selectivity is particularly relevant. Molecular glue degraders are small molecules that bind endogenous components of the ubiquitin-proteasome system, remodel its recognition interface, and thus recruit otherwise unrelated protein substrates for degradation. Several molecular glue degraders that degrade transcription factors implicated in cancer have achieved clinical success. Despite this progress, most known degraders were discovered serendipitously, and a major goal of the field is to generalize this therapeutic mechanism to enable programmable degradation of diverse disease-relevant targets (Schreiber, 2021; Brennan et al., 2025). Achieving this goal requires a quantitative understanding of how specific chemical features interact with specific protein determinants to drive selective degradation.

Existing experimental approaches scale in either chemical space or protein space, but not both simultaneously. High-throughput chemical screens, such as HiBiT-based assays or phenotypic screens (Ting et al., 2024), can efficiently explore large chemical space to find hits that degrade a single target. But the workflow must be repeated independently for each additional protein of interest.

Conversely, proteomics-based methods (Krönke et al., 2014) and pooled genetic screens (Sievers et al., 2018; Słabicki et al., 2025) can profile the substrate scope of a single compound across many proteins but are inherently low-throughput in chemical space. As a result, neither strategy enables systematic exploration of the joint chemical-protein landscape.

To identify general principles governing selectivity, a higher-dimensional dataset that scales simultaneously in both chemical and protein dimensions is required. The goal of this work is to develop a method that enables such combinatorial exploration. We introduce a platform that generates a pool of mammalian cells that each pairs a degrader and a degron; encodes the resulting level of degradation activity through RNA editing; and recovers this information along with the identities of the degrader and degron through an adapted single-cell RNA barcoding and sequencing workflow. While we demonstrate proof of concept in the context of targeted protein degradation, the approach is readily generalizable, providing a scalable framework for mapping chemical-genetic interaction landscapes to inform both mechanistic biology and drug discovery.

2 METHODS

To enable a screen that is scalable in both the protein dimension and the chemical dimension simultaneously, we devised Genetic-Chemical Interaction Landscape Analysis and Discovery Assay by Sequencing (GenChILADA-seq). In this workflow, a pooled library of protein variants is first delivered to cells. This pool of cells is split across a multi-well plate where each well is transfected with a uniquely barcoded recorder plasmid and then treated with a different compound from the compound library. At this point, these cells represent every pair of degron and degrader. The cells are subsequently pooled and processed as a single batch, thus enabling massive scale in both protein and chemical dimensions simultaneously.

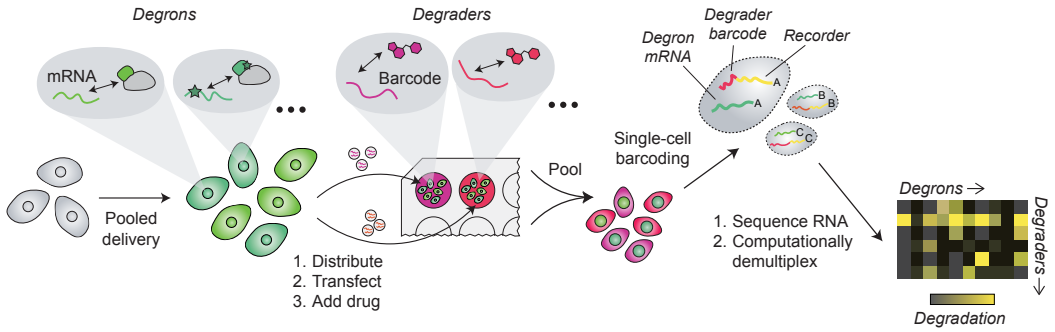

Figure 1: Schematic of overall workflow.

To recover the level of degradation activity for each degron and degrader, we envisioned a RNA sequencing-based readout from these cells. We devised a molecular recording circuit that ties the level of degradation activity to the number of RNA edits (Figure 2A, B). In the effector construct, the degron is fused to MS2 coat protein (MCP) (Bernardi & Spahr, 1972) and a hyper-active variant of ADAR2, which is an RNA editing enzyme that converts adenosine to inosine which is ultimately sequenced as guanosine (Kuttan & Bass, 2012). The effector is recruited to the recorder RNA transcript through the interaction between MCP and the MS2 stem loop, thus producing A-to-G edits on the transcript. Upon treatment with a degrader compound that is active against the degron, the effector construct is degraded, resulting in less editing. Furthermore, a barcode on the recorder is used to uniquely identify the treatment condition so that the cells can be harvested and processed in a single pool. We validated this circuit using HEK293T cells stably expressing the effector construct with SALL4 zinc finger 2 as the degron (Słabicki et al., 2025) and transfected with a plasmid encoding the recorder transcript. As expected, we observed decreased editing when treated with mezigdomide compared to DMSO vehicle (Figure 2C). Furthermore, this effect is ablated when ADAR2 is catalytically inactivated with the point mutation E396A, confirming that the effector construct is directly responsible for the observed editing. To account for subtle variation in incubation times, all subsequent experiments included these conditions, which were used to linearly normalize editing scores to [0, 1], corresponding to the mezigdomide and DMSO treatment conditions, respectively.

While the degrader identity is encoded by a barcode on the same sequencing read as the recorder that reports degradation activity, the degron identity is encoded on its mRNA transcript and captured on a separate sequencing read. To associate these reads at the single-cell level, we adapted the high-throughput PIPseq workflow to ligate single-cell barcodes onto all RNA molecules originating from the same cell (Clark et al., 2023). After barcode ligation, RNA is extracted and processed as a single pooled sample. Following sequencing, computational demultiplexing using these barcodes links reads from the same cell, thereby pairing degron identity and degrader identity with the corresponding quantitative degradation readout.

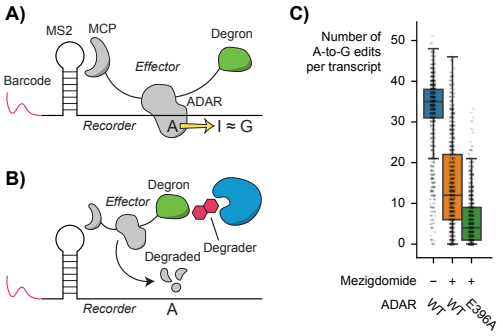

Figure 2: (A, B) Schematics of degradation reporter when the effector (A) is not degraded or (B) is degraded. (C) Bulk RNA sequencing results for SALL4 zinc finger 2 degron (amino acids 392-449). Mezigdomide is a potent degrader. The E396A mutation ablates ADAR's catalytic activity.

3 Results

3.1 Dose-response curves for individual substrates from a pooled experiment

We first applied GenChILADA-seq to obtain eight-point dose response curves for each combination of six degrader compounds and five point mutants of the SALL4 degron, yielding 240 measurements plus controls (Figure 3A). Consistent with prior reports, mutation of G416 completely ablates degradation (Sievers et al., 2018) while the H417A mutation enhances degradation (Matyskiela et al., 2020). On the chemical side, we observed that mezigdomide is the most potent compound across all substrates, in agreement with its enhanced ability to stabilize the degradation-competent conformation of cereblon (Watson et al., 2022).

Beyond effects attributable to either the protein or chemical dimension alone, a key strength of our approach is its ability to capture interaction-specific effects arising from co-variation. Indeed, we recapitulated the known interaction in which the V411Q mutation selectively disrupts degradation by thalidomide-5-OH but not by thalidomide (Furihata et al., 2020). The same mutation also disrupts degradation by DKY709, consistent with the design of this compound to avoid degradation of IKZF1, which has a glutamine at this position, while selectively targeting IKZF2, which has an aromatic residue represented here by the V411F mutation (Bonazzi et al., 2023).

To quantitatively benchmark assay performance, we performed an orthogonal degradation assay using flow cytometry to measure GFP fluorescence from the effector construct. DC_{50} values derived from this assay showed strong agreement with those obtained by GenChILADA-seq ($R = 0.9716$; Figure 3B), validating the accuracy of our method. Moreover, these results highlight the sensitivity of our assay for resolving intermediate activity levels. Unlike enrichment-based pooled screening strategies, which perform well for binary hit identification, our readout is based on the number of RNA edits per transcript, enabling more precise quantitative measurements where confidence is independent of abundance.

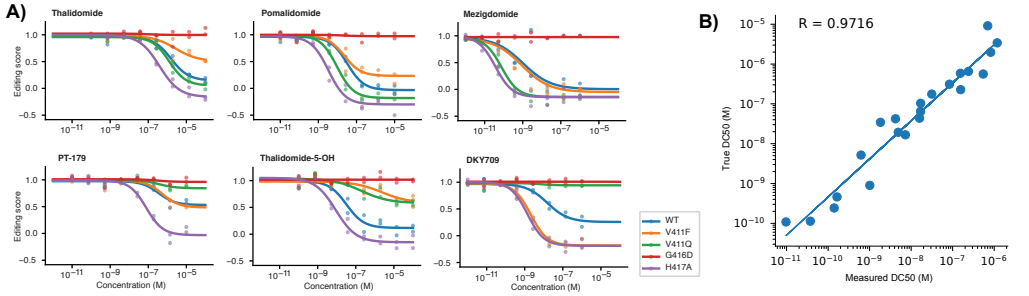

Figure 3: (A) Dose response curves. Data from two replicates are shown. (B) Correlation between measured and true DC50 values.

3.2 Structure-activity relationships

We next profiled interactions between the SALL4 degron and molecular glue degraders by systematically co-varying both components. We constructed a library of 117 SALL4 point mutants at seven residues close to the drug-binding interface and screened this library against 138 glutarimide-containing compounds from Enamine, spanning multiple scaffolds including thalidomide, avadomide, and ALV1/2. In total, this experiment yielded 16,146 degron-degrader measurements (Figure 4). Achieving this scale simultaneously across both protein and chemical dimensions is impractical with existing approaches.

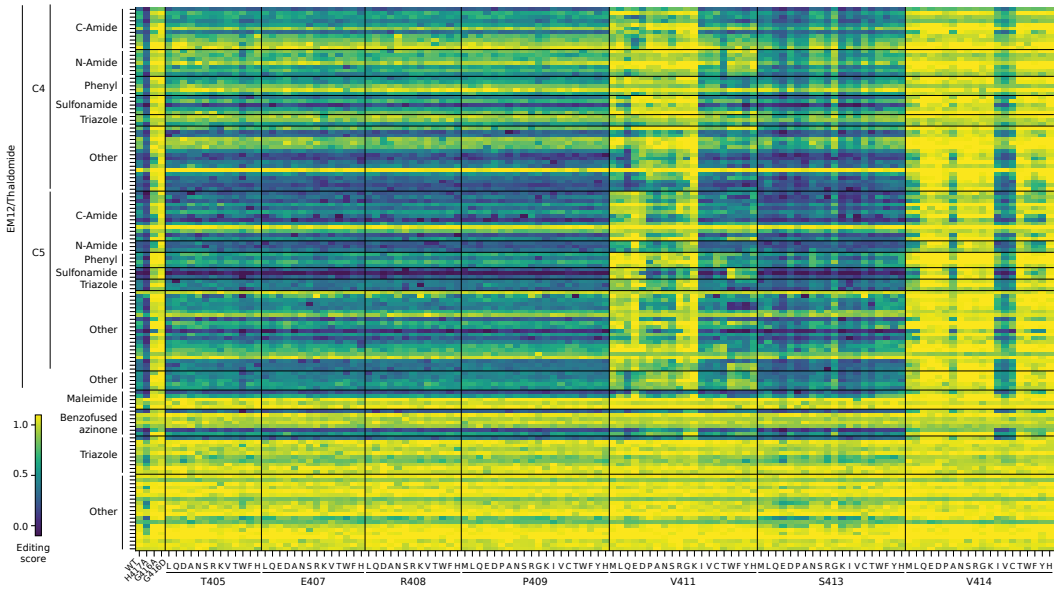

Figure 4: Heatmap of degradation activities for all pairs of 117 SALL4 point mutants and 138 degrader compounds. Data are averaged between two replicates.

This dense functional map enables mechanistic interrogation of compound selectivity that is difficult to extract from structural data alone. As a representative example, DKY709 and ALV2 were designed to selectively degrade IKZF2 over IKZF1. Although structures of the relevant complexes have been solved, the molecular basis for this selectivity has remained incompletely understood. Our functional data reveal that these compounds preferentially tolerate aromatic residues at the corresponding position, like histidine in IKZF2, while disfavoring charged or polar residues, like glutamine in IKZF1 (Figure 5).

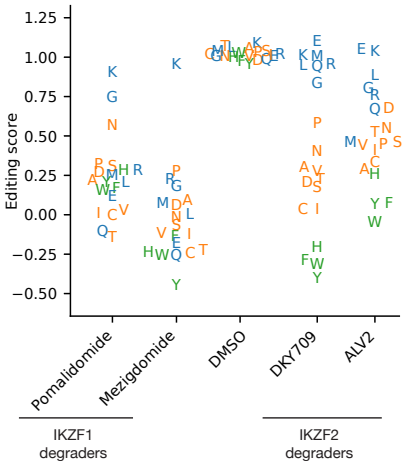

Figure 5: Swarm plot of degradation. The letters indicate the mutation at SALL4 V411. Data are averaged between two replicates.

4 Conclusion

In this work, we presented a high-throughput platform for combinatorial screening of small molecule and protein libraries in live cells. By directly profiling joint chemical-genetic variation, this approach generates functional data that provide molecular resolution evidence of interacting residues and functional groups. We are actively leveraging these datasets to train machine learning models that capture and predict interaction specificity towards enabling in silico design of molecular glue degraders and accelerating drug discovery. More broadly, this framework is readily generalizable to other systems, providing a scalable strategy for mapping chemical-genetic interaction landscapes.

Acknowledgments

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