CRISPR-Suppressor Scanning Unsticks Molecular Glues

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> CRISPR-suppressor scanning reveals potential resistance mechanisms of cancer cells to molecular glue degraders via neosubstrate alteration.

Small molecules that facilitate protein–protein associations are transformative therapeutics which can enable the targeting of difficult-to-drug oncogenic drivers. One class of association-inducers, molecular glues (MGs), engage a target protein to generate a novel binding surface (a neosubstrate) for a second protein.[1] Drugs which induce degradation of oncogenic neosubstrates through recruitment of E3 ligases and the ubiquitin-proteasome system have emerged as particularly powerful demonstrations of the MG concept.[2] Understanding the features of neosubstrates which govern degrader efficacy is a crucial step toward rational design of these next-generation therapeutics. Studying resistance to MGs driven by mutations within the neosubstrate provides an opportunity to examine directly the target features impacting degrader efficacy in a disease-relevant context. The unique interactions induced by MGs provide multiple points for cancers to escape their pharmacological effects. In this issue of *ACS Central Science*, Gosavi et al. break exciting ground by using a CRISPR scanning-mutagenesis strategy to analyze how mutations of the oncogenic drivers GSPT1 and RBM39 impact the sensitivity of acute myeloid leukemia (AML) cells to MG degraders.[3]

Molecular glue degraders such as thalidomide analogues were used to treat multiple myelomas and lymphomas before a complete understanding of their molecular mechanism of action emerged.[1,2] Extensive work on a subset of neosubstrate-MG pairs over several decades revealed the molecular features which drove degradation. Cancers might evade compound-promoted degradation through multiple mechanisms, including
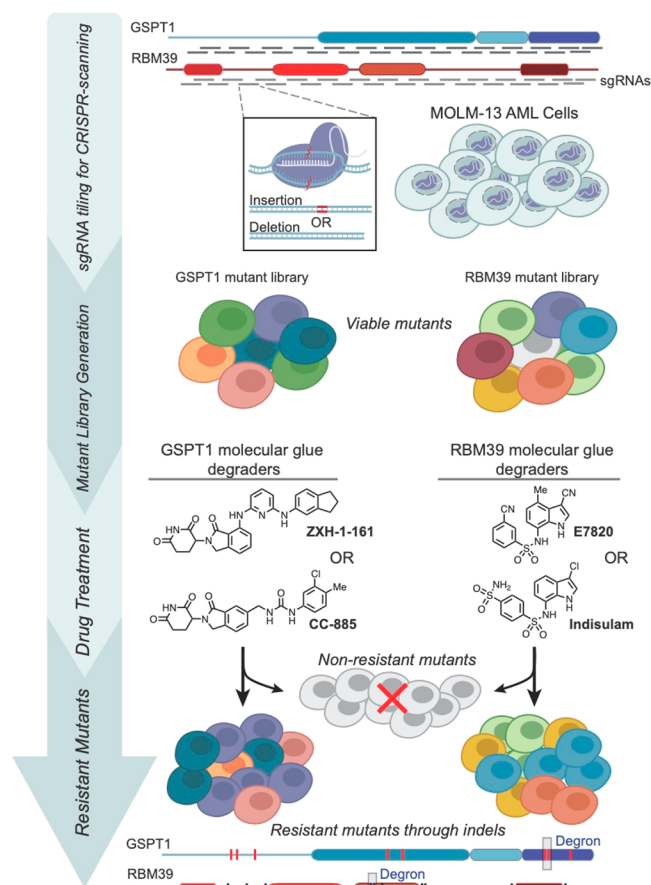
Figure 1. CRISPR-suppressor screen of oncogene neosubstrates and molecular glue degraders. A comprehensive mutagenesis strategy which spans the entire gene of a target with sgRNAs and uses Cas9 to induce double-stranded breaks in the DNA. The cell repairs double-stranded breaks to recover the WT sequence, an insertion of a new base pair, or a deletion and frameshift of the sequence. A library of sgRNA-containing cells is treated with molecular glue degrader drugs that selects for cells containing mutations of the target protein which confer resistance to the drug.
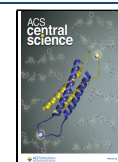
mutations in the MG-induced degron which engages E3 ligase machinery. The molecular complexity of an induced interaction surface between a neosubstrate and an E3 ligase complicates a residue-by-residue analysis of domains that might be susceptible to mutation. CRISPR-suppressor scanning can experimentally examine all domains of a target by systematically arraying sgRNAs across a gene to induce CRISPR-generated double-stranded breaks (DSBs). Cells repair DSBs to form insertions or deletions (indels) in the gene of interest to create a diverse mutational landscape targeted across a protein of interest.[4,5] Gosavi et al. applied the CRISPR-suppressor scanning method to examine mutations of GSPT1 and RBM39 (Figure 1), which are transformed into neosubstrates for E3 ligases (Figure 2) via MG degraders (CC-885 or ZXH-1-161 for GSPT1, indisulam or E7820 for RBM39).[6−9] After sgRNA library transduction into MOLM-13 cells and treatment with degraders, drug-resistant cells expressing variants of the target proteins were obtained and analyzed through both targeted and deep sequencing. Importantly, CRISPR-mediated mutations which cause cell lethality drop out prior to compound treatment, increasing the likelihood of obtaining truly drug-resistant cells.

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Resistance-conferring mutations of the target proteins disrupted degradation by altering neosubstrate binding to degradation machinery. The majority of induced GSPT1 mutations were at the β-hairpin, preventing cereblon binding. Similarly, MG treatment also induced mutations in the degron region of RBM39. For both targeted proteins, mutations which impacted neosubstrate binding were sufficient to escape MG-mediated cytotoxicity. Prior work had identified RBM39 as an anticancer target by sequencing indisulam-resistant carcinomas and found frequent mutations in the RBM39 helical degron region, consistent with the results of Gosavi et al.[8] Interestingly, RBM39 mutants lacking R150-D151-A152 (RBM39^{RDAdel}), a motif just upstream of the helical degron, exhibited reduced degradation when compared to wild type (WT) RBM39. However, the RBM39^{RDAdel} cells retained the ability to engage the E3 ligase component DCAF15, suggesting that attenuation rather than complete loss of degradation capacity is sufficient to escape compound-mediated cytotoxicity. This implies the existence

of drug resistance mechanisms that fail to fully abolish molecular glue or neosubstrate interaction with E3 ligases, partially uncoupling molecular engagement from degradation capability. Further examination of degradation in MOLM-13 and K562 cells transduced with a fluorescent reporter, which co-expressed a GFP-tagged RBM39^{RDAdel} and mCherry from a single transcript, confirmed that modest changes in target degradation levels were sufficient for resistance. These results highlight the importance of achieving sufficiently high levels of degradation to induce a phenotype. A challenge for the field remains how to predict what these levels may be for a given target. When DCAF15 is overexpressed, RBM39^{RDAdel} is degraded at levels similar to RBM39^{WT}. This observation suggests that resistance-conferring mutations might be overturned by an overexpression of a cognate E3. Conversely, cells with neosubstrate mutations which only partially impact degradation efficiency might enhance resistance through concomitant downregulation of an E3 ligase. The synergism of neosubstrate mutation and altered expression levels of relevant degradation machinery might provide an additional axis of inquiry for further studies of acquired resistance to MG degraders.

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CRISPR-suppressor scanning of RBM39 also revealed that mutations far-removed from the canonical degron and neosubstrate binding surface can confer resistance. Like the RBM39^{RDAdel} variant, distal mutations reduced levels of degradation without abolishing ternary complex formation with DCAF15. These effects were additive, as degradation was further diminished through a combination of multiple distal mutations. Understanding how additive mutations finely balance the susceptibility of a neosubstrate to degradation with the maintenance of a minimum threshold of essential biological activity is an intriguing avenue for further study. The cellular features that dictate these degradation thresholds remain to be determined but could have a significant impact on defining viable targets for small molecule-mediated degradation strategies in a number of cancers.

Drug treatments exert an evolutionary pressure on proliferating cells. In the context of cancer, numerous mutations leading to cell survival and proliferation provide potential
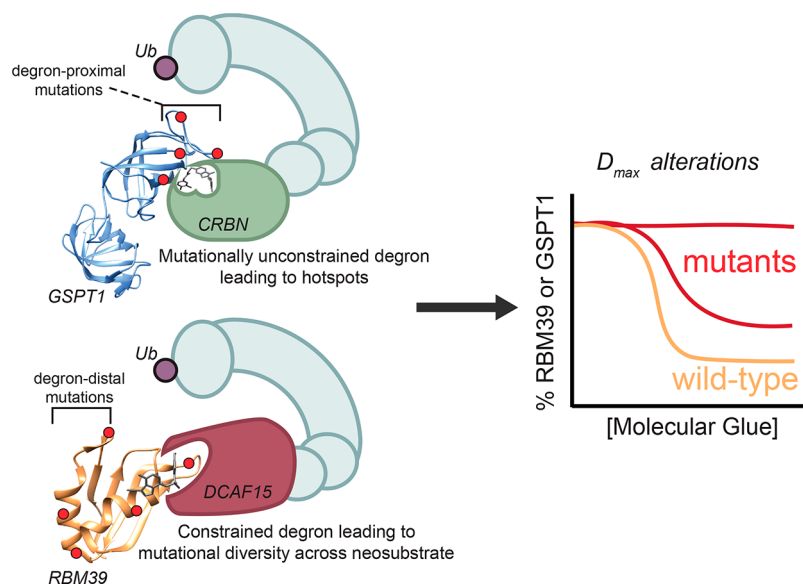
Figure 2. Neosubstrate mutations confer drug resistance through degron-proximal and distal mutations which attenuate degradation. Drug-resistant mutations (red circles) localized at the β-hairpin degron in the GSPT1 structure (blue, PDB: 5HXB) bound to molecular glue CC-885. Drug-resistant mutations in RBM39 (orange, PDB: 6UE5) were found proximal and distal to the α-helix degron. Some neosubstrate mutations did not universally abolish degradation but rather provided more subtle alterations to the level of maximal degradation (D_{max}).

routes to evade the deleterious consequences of molecular glues. Residues in the target protein which are important for function or folding would remain conserved in a resistance-developing population, while less consequential residues might be prone to a higher-frequency of mutation. Residues which were not highly conserved in the degron tags of the target proteins allowed for higher fitness through disrupting neosubstrate formation. However, fewer residues were mutated in the RBM39 degron helix, suggesting this mutational constraint drove mutations distal to the degron in the pools of surviving cells. Residue-specific conservation scores allowed for retrospective insight into the effects of mutagenesis on protein function and impact on protein degradation.

> The power of CRISPR-suppressor scanning lies in the unbiased examination of key residues and protein features for a given therapeutic mechanism that might not be obviously important for function.

This important contribution from Gosavi et al. creates a new lens to examine the potential for resistance-conferring mutations to impact MG therapeutics. Drug-resistant mutations in proliferating cells must strike a balance by retaining critical protein function while disrupting neosubstrate engagement or degradation. Determination of mutational hotspots or degradation-defining features outside of the immediate degron

region might provide a wealth of information for prospective rational design of MGs, especially when integrated with computational approaches characterizing features which promote degradation. The power of CRISPR-suppressor scanning lies in the unbiased examination of key residues and protein features for a given therapeutic mechanism that might not be obviously important for function. Beyond MG degraders, this seminal study invites exploration of other induced proximity strategies with a CRISPR-suppressor scanning approach. Broadly defining the essential molecular features of neocomplexes generated by small molecules or biologics across several modalities will further guide general principles for a prospective design of new induced proximity agents. The current CRISPR-suppressor scanning approach requires a proliferative advantage for resistance-conferring mutations to be enriched and identified after drug treatment. While proliferation-based screens are convenient, they are constrained by the nature of the drug or intervention. Integrating mutational scanning with readouts using nonlethal reporters might release the constraint for a direct antiproliferative effect caused by a drug of interest, expanding the scope of drug–protein interactions which can be characterized. Incorporation of advances in CRISPR technologies into mutational scanning approaches will further increase the scope and depth of information which can be obtained from these experiments. The rigorous application of CRISPR-suppressor scanning provides an exciting blueprint that paves the way for numerous further

studies to define the potential of next-generation therapeutic molecules.

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https://pubs.acs.org/10.1021/acscentsci.2c00216

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