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# Elucidating drug targets and mechanisms of action by genetic screens in mammalian cells

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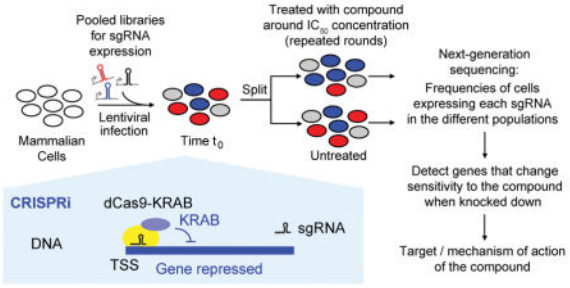
## Abstract

Phenotypic screening is a powerful approach to discover small molecules with desired effects on biological systems, which can then be developed into therapeutic drugs. The identification of the target and mechanism of action of compounds discovered in phenotypic screens remains a major challenge. This Feature Article describes the use of genetic tools to reveal drug targets and mechanisms in mammalian cells. Until recently, RNA interference was the method of choice for such studies. Here, we highlight very recent additions to the genetic toolkit in mammalian cells, including CRISPR, CRISPR interference, and CRISPR activation, and we illustrate their usefulness for drug target identification.

## Graphical Abstract




## Introduction

Small molecules are the method of choice to target biological functions for therapeutic or research purposes. The development of potent and selective small-molecule modulators of biological functions remains a challenge. One strategy is the targeted development of inhibitors or activators for a specific protein of interest, in which molecules are optimized based on their activity on the purified protein target in vitro[7].

Another strategy is the identification of molecules with desired activities in a cell-based phenotypic assay in high-throughput screens. The advantage of such phenotypic screens is that small molecules modulating a biological process of interested can be recovered without

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prior knowledge of the molecular mechanism of the process. However, it is challenging to identify the molecular target and mechanism of action of compounds identified in phenotypic screens[8].

The central problem of drug target identification has been approached in different ways. Biochemical approaches rely on physical binding of the compound to its protein target[8]. Cellular proteins binding the compound can be identified by mass spectrometry. While this approach has successfully been used for target identification, it can fail to detect the target if the experimental strategy (typically involving a modified version of the compound for immobilization or cross-linking) interferes with binding of the compound to the target. It can also lead to false-positive results, if proteins physically interact with the compound, but are not the relevant targets mediating the cellular phenotype.

A distinctly different approach to drug target identification is based on genetics. In a classic paper published 20 years ago[9], Friend and colleagues demonstrated the usefulness of determining drug sensitivities of a panel of yeast strains harboring mutations in selected genes. Yeast cells with mutations in a pathway targeted by a drug showed enhanced sensitivity to the drug. A related approach was implemented in yeast as haploinsufficiency profiling (HIP)[10]: yeast cells in which one of two copies of a gene was deleted are sensitized to drugs targeting this gene, thus enabling the identification of drug targets by screening a library of heterozygous yeast cells for sensitivity to a drug of interest. Large-scale studies of the sensitivity of mutant cells to compound panels, so-called chemical genomics, can yield insight into both the function of genes and the mechanism of action of compounds[11].

The recent development of genetic tools for mammalian cells enables us to apply similar approaches directly in human cells. In particular, the development of CRISPR-based approaches has greatly improved the quality of genetic screen in human cells, as discussed below. This is an important advance, since some therapeutically relevant phenotypes and drug targets cannot be recapitulated in model organisms. This Feature Article aims to present our personal perspective on genetics-based identification of drug targets and mechanisms of action in human cells, and describe our recent contributions to the field.

## A new toolkit for genetics in mammalian cells

Until recently, the method of choice for genetic perturbations in mammalian cells was RNA interference (RNAi, Fig. 1). RNAi relies on short double-stranded RNA molecules; either chemically synthesized short interfering RNAs (siRNAs) that are transiently introduced into cells, or stably expressed short hairpin RNAs (shRNAs). Both siRNAs and shRNAs direct cellular machinery to degrade protein-coding messenger RNAs (mRNAs) containing the complementary RNA sequence. Thereby, RNAi facilitates the investigation of the consequences of a loss of function of the encoded protein.

However, results from genome-wide RNAi-based screens were notoriously noisy[12], due in large part to the off-target effects of siRNAs and shRNAs. To overcome this limitation, we developed ultra-complex shRNA libraries targeting each human gene with 25 independent shRNAs, and containing thousands of non-targeting negative-control shRNAs. In

combination with a rigorous statistical framework, these libraries yielded robust results in genome-wide screens[3, 13]. However, the large size of such ultra-complex libraries can be prohibitive for genome-wide screens.

We were able to overcome this limitation by taking advantage of CRISPR/Cas9, a bacterial endonuclease that can be programmed by a single guide RNA (sgRNA) to cleave specific DNA sequences[14] (Fig. 1). We co-developed a genetic screening platform that exploits catalytically dead Cas9 (dCas9) to recruit transcriptional repressors or activators to endogenous genes to enable inducible and reversible repression (CRISPRi) and activation (CRISPRa) of genes in human cells (Fig. 1), enabling genome-wide loss- and gain-of-function screens[2]. CRISPRi is highly specific and thereby overcomes the problem of off-target effects that has plagued RNAi-based approaches[2]. Its performance in genome-wide screens is comparable to CRISPR cutting-based platforms[15]. Together, CRISPRi and CRISPRa make it possible to investigate the biological function of essential genes by enabling different levels of knockdown and overexpression, essentially creating an allelic series[2].

In some cases, complete loss-of-function of a gene may be required to observe the full phenotype. In such cases, CRISPR cutting-based approaches (Fig. 1) can achieve complete knockout if double-stranded DNA breaks are repaired by cellular pathways that result in a frame-shift deletion[16, 17]. However, cutting-based approaches can have other limitations, such as non-specific toxicity[18].

## Identifying targets and mechanisms of cytotoxic drugs

To identify the targets of cytotoxic compounds, a pooled screening strategy can be used, as outlined in Fig. 2. A relevant mammalian cell type is chosen – in the case of anti-cancer compounds, typically a human cancer cell line representing the targeted type of cancer. For CRISPR-based applications, the cells are engineered to express the Cas9 machinery required for the intended application. The cells are stably transduced with a lentiviral library of expression constructs for shRNAs (for RNAi applications) or sgRNAs (for CRISPR-based applications). These libraries can either target each gene in the human genome, or a selected subset of genes[13, 15]. Each gene is targeted with several independent shRNAs or sgRNAs, and the libraries contain a large number of non-targeting negative-control shRNAs/sgRNAs.

Cells are then split into two populations: one is treated with the compound of interest, the other one is an untreated control population. Typically, cells are treated with a concentration of compound that kills approximately 50% of the cells, and then allowed to recover in the absence of compound. This pulse treatment is repeated several time. At the end of the experiment, genomic DNA is isolated from the cells, and the locus of the shRNA/sgRNA expression cassette is PCR amplified. Using next-generation sequencing, the frequencies of cells expressing a given shRNA or sgRNA are quantified in the treated and untreated populations, as well as the starting population (collected at time t0). For each shRNA/sgRNA, quantitative phenotypes are calculated based on the frequencies in the different populations. A decrease in frequency between t0 and the untreated population (relative to the non-targeting negative controls) indicates a negative effect on cell growth or survival in the

absence of treatment. By comparing shRNA/sgRNA frequencies between the untreated and compound-treated populations at the end point of the experiments, compound-specific phenotypes can be monitored, while compound-independent effects on cellular growth/survival are corrected for. We named this phenotype "rho" [3], and it is the relevant phenotype for the identification of compound targets. In particular, if knockdown of a gene strongly sensitizes cells to the compound (negative value of rho), the gene is a candidate to be the direct target of the compound.

Our first application of this strategy was a collaboration with Michael Cleary (Stanford)[1]. The Cleary lab had identified a lead compound, STF-118804, with promising activity against high-risk acute lymphoblastic leukemia cells. Since the compound was discovered in a phenotypic screen, its molecular target was unknown. We applied the strategy outlined in Fig. 2 to identify genes controlling the sensitivity to STF-118804 in a human leukemia cell line, MV411. Intriguingly, knockdown of one specific gene, NAMPT, strongly sensitized cells to STF-118804 (Fig. 3). NAMPT encodes nicotinamide phosphoribosyl transferase, a rate-limiting enzyme for nicotinamide adenine dinucleotide biosynthesis. Using biochemical approaches, we validated that NAMPT is indeed the target of STF-118804[1].

Even in cases where a drug is developed to inhibit a specific protein target, and validated to inhibit the purified protein in vitro, it remains a challenge to demonstrate that cytotoxic effects of the drug are mediated by inhibition of the intended target in cells, rather than by off-target effects. We demonstrated the power of the CRISPRi/CRISPRa approach for this purposes in a collaboration with Cleave Biosciences[4]. Cleave had developed a potent inhibitor of the AAA-ATPase p97/VCP, CB-5083, which was cytotoxic in many cancer cell lines (Fig 4a). To demonstrate that cytotoxicity was mediated by p97/VCP inhibition, CB-5083 sensitivity was determined for a panel of 340 cancer cell lines. Consistent with p97/VCP as the relevant target, there was a positive correlation between IC50 and p97/VCP expression levels (Fig. 4b). However, the correlation was very weak ($R^2 = 0.02$), likely reflecting the fact that there were many differences between the cancer cell lines that affected CB-5083 sensitivity other than their p97/VCP expression levels. To evaluate the correlation of p97/VCP expression levels and CB-5083 sensitivity in an otherwise isogenic background, we generated an allelic series of sgRNAs to achieve different levels of CRISPRi-mediated p97/VCP knockdown and CRISPRa-mediated p97/VCP overexpression in a K562 leukemia cell line. In this series, the correlation between p97/VCP expression levels and CB-5083 sensitivity was extremely high ($R^2 = 0.97$), supporting p97/VCP as the relevant target of CB-5083 (Fig. 4c).

In other applications, a genetic approach may not be able to detect the direct target of a compound, but shed light on cellular mechanisms involved the compound's mechanism of action. A highly unusual cytotoxic compound identified in Jim Well's lab (UCSF) forms fibrils, and our genetic results pointed to the importance of the cellular endolysosomal system to mediate compound toxicity: knockdown of specific proteins mediating vesicular trafficking protected cells from compound toxicity, presumably by blocking transport of the compound fibrils to the lysosome[19].

## Identifying genetic modifiers of drug action, and targets for combination therapy

Genetic screens can not only reveal cellular targets of a compound, but also identify possible mechanisms of drug resistance. For anti-cancer drugs, such screens can thereby reveal biomarkers that predict the response of different patients to the drug, and uncover possible mechanisms of acquired resistance. We applied this approach to the proteasome inhibitor carfilzomib, which is effective against multiple myeloma[5]. As expected, knockdown of the known target of carfilzomib, the beta-5 subunit (gene name PSMB5) of the 20S proteasome, sensitized cells to carfilzomib (Fig. 5). Surprisingly, knockdown of subunits of the 19S proteasomal regulator protected cells from carfilzomib (Fig. 5). We found that in multiple myeloma patients, expression levels of 19S subunits were similarly predictive of response to carfilzomib (Fig. 5).

Genetic screens can also reveal potential combination therapy targets, in particular by taking advantage of synthetic lethal relationships between genes, in which combinatorial inhibition of a synthetic-lethal gene pair has synergistic effects on cancer cell survival.

Our screen for genetic modifiers of carfilzomib sensitivity in multiple myeloma cells also revealed additional genes knockdown of which sensitized cells to carfilzomib (Fig. 5), such as Hsp70 and Hsp90 chaperones. These factors are therefore potential targets for combination therapy with carfilzomib. Conversely, knockdown of mTOR protected cells from carfilzomib (Fig. 5). Even though mTOR inhibitors themselves are cytotoxic to multiple myeloma cells, our results suggest that they are not suitable for combination therapy with carfilzomib.

More recently, we used a genetic screen to identify a synergistic combination therapy target for the glucocorticoid dexamethasone in childhood B-cell precursor acute lymphoblastic leukemia (B-ALL): Phosphatidylinositol 3-kinase delta (PI3Kδ)[20]. Combinatorial treatment of a B-ALL animal model with dexamethasone and the PI3Kδ inhibitor idelasilib inhibited tumor growth in a highly synergistic manner[20].

## Target identification for compounds with complex phenotypes

Drugs with therapeutic applications other than cancer typically do not cause cell death, but have a more specific effect on a relevant cellular process. If this process can be monitored using a fluorescence-based assay, a pooled genetic screen can be used to reveal the target of a compound of interest.

We first applied this strategy to identify the target of ISRIB, a small molecule identified by Peter Walter's lab (UCSF) as an inhibitor of the integrated stress response[21] (Fig. 6a) Intriguingly, this compound enhances memory in mice, but its cellular mechanism of action was unknown. We established a fluorescent reporter cell line for the integrated stress response (Fig. 6b). Activation of the reporter by the ER stressor thapsigargin is blocked by ISRIB in a dose-dependent manner (Fig. 6c). We transduced the reporter cell line with an shRNA library targeting genes related to protein homeostasis, and exposed cells to

thapsigargin either in the absence or in the presence of ISRIB at a concentration around its $EC_{50}$ (Fig. 6d). We then separated cells based on their reporter signal using fluorescence-activated cell sorting (FACS) and used next-generation sequencing to determine the frequency of cells expressing each shRNA in the different cell populations (Fig. 6c). Our screen revealed that knockdown of subunits of the eIF2B nucleotide exchange factor diminished the cellular response to ISRIB (Fig. 6d). In biochemical experiments, we validated that eIF2B was indeed the direct target of ISRIB, and that ISRIB activated eIF2B[6]. A separate team of researchers independently came to the same conclusion[22], further supporting our findings.

## Conclusions

As illustrated in this article, genetic screening in mammalian cells is a powerful tool to identify cellular targets of compounds, and to elucidate their mechanism of action. Furthermore, it can reveal biomarkers predictive of drug responses in patients, and point to mechanisms of drug resistance as well as potential combination therapy targets.

The next frontier will be to extend this approach to other cell types and complex phenotypes. We are currently establishing our CRISPRi/CRISPRa screening platform in cell types derived from human induced pluripotent stem cells, which will enable genetic screens in non-cancerous human cell types, such as neurons. In parallel, the development of arrayed, high-content genetic screens will facilitate the evaluation of non-cell autonomous or time-resolved phenotypes, which cannot easily be captured in pooled genetic screens. Together, these new developments will expand the scope of phenotypes and compounds that can be investigated using next-generation genetic tools in mammalian cells. Ultimately, screens in organismal models of disease may provide even more physiologically relevant insights.

## Acknowledgments

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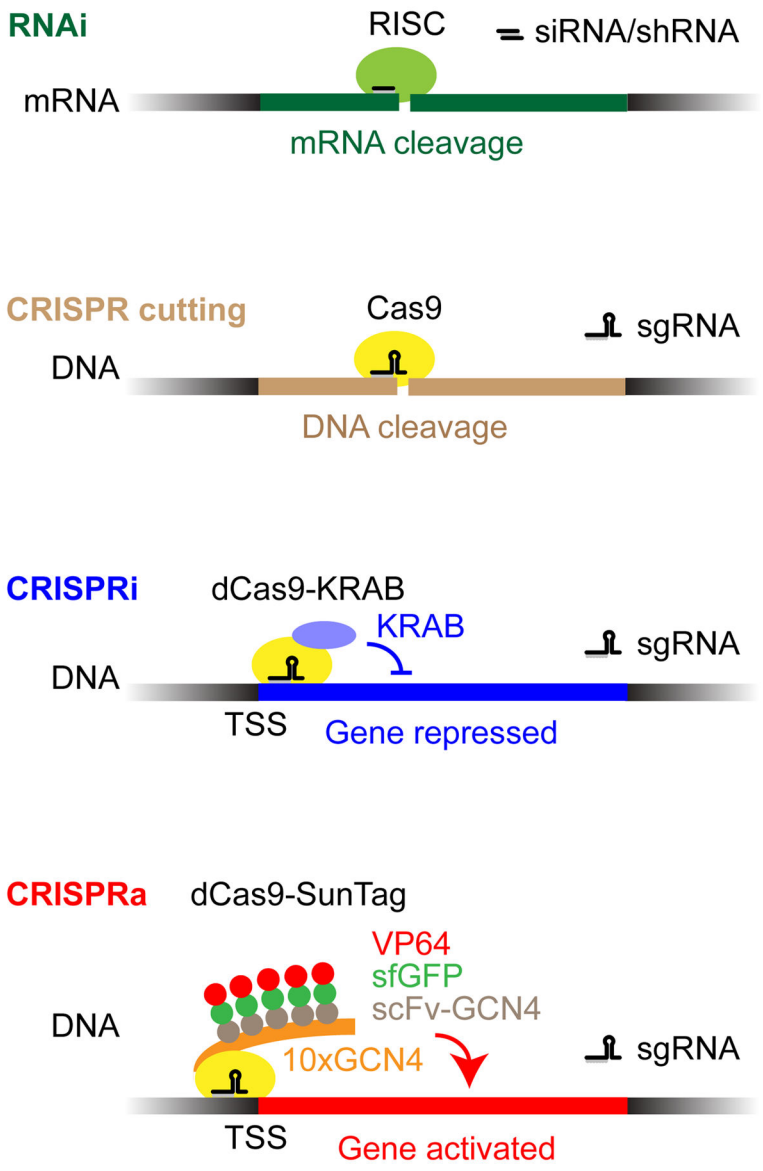


**Fig. 1.**

Pooled genetic screening strategy to identify the target of cytotoxic compounds. Mammalian cells are stably transduced with a pooled lentiviral library to integrate expression cassettes for shRNAs or sgRNAs into the genomic DNA. The library targets a large number of genes each with several shRNAs/sgRNAs and contains non-targeting negative-control shRNAs/sgRNAs. Each cell typically expresses one element of the library. This population is either cultured in the absence of treatment, or treated with several pulses of the compound of interest, at a concentration that kills approximately 50% of the cells (IC50). Then, genomic DNA is isolated and the shRNA/sgRNA expression cassette is subjected to next-generation sequencing, enabling quantification of the frequencies of cells expressing each shRNA/sgRNA in the different cell populations. Using our quantitative framework[3], genes controlling sensitivity to the compound are robustly identified. Adapted from ref. [5]

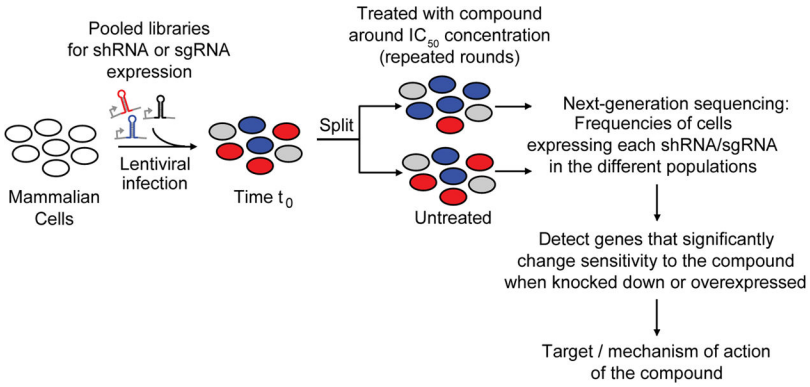


**Fig. 2.**

Genetic tools in mammalian cells. RNA interference (RNAi): short interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) direct the RISC complex to degrade protein-coding mRNAs with complementary RNA sequences, thereby leading to a loss of function of the encoded protein. CRISPR cutting: a single guide RNA (sgRNA) directs the bacterial Cas9 protein to cleave DNA. This can lead to error-prone non-homologous end-joining repair, thus inactivating the encoded gene. Catalytically dead Cas9 (dCas9) can be used to recruit transcriptional repressors or activators to endogenous genes to enable inducible and reversible gene repression (CRISPRi) or activation (CRISPRa). Our screening platform[2] uses the KRAB repressor domain, and the SunTag, in which several copies of the activator VP64 are recruited to a dCas9-fused tandem repeat of the GCN4 epitope via a superfolder GFP (sfGFP)-stabilized nanobody targeting the GCN4 epitope (scFv-GCN4).

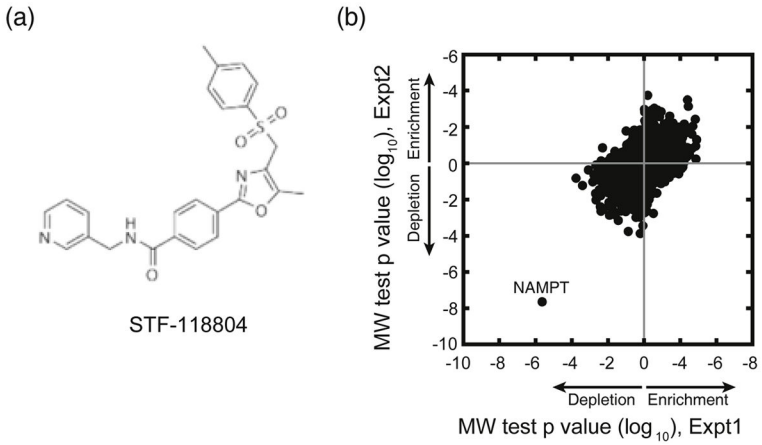


**Fig. 3.**

Investigating the relationship between drug target expression and sensitivity. (a) Structure of CB-5083. (b) Sensitivity to CB-5083 was determined in a panel of 340 cancer cell lines (each represented by a dot), and displayed as a function of the expression level of p97/VCP, the intended target of CB-5083. (c) p97/VCP expression levels were controlled in a K562 leukemia cell line using different sgRNAs to mediate knockdown via CRISPRi or overexpression via CRISPRa (each point is one sgRNA, error bars indicate the standard error of the mean for p97/VCP mRNA levels measured in triplicate by quantitative PCR). The relative resistance to CB-5083 is shown on the y axis, using the rho metric we previously defined[3]. Figure adapted, with permission, from ref. [4]

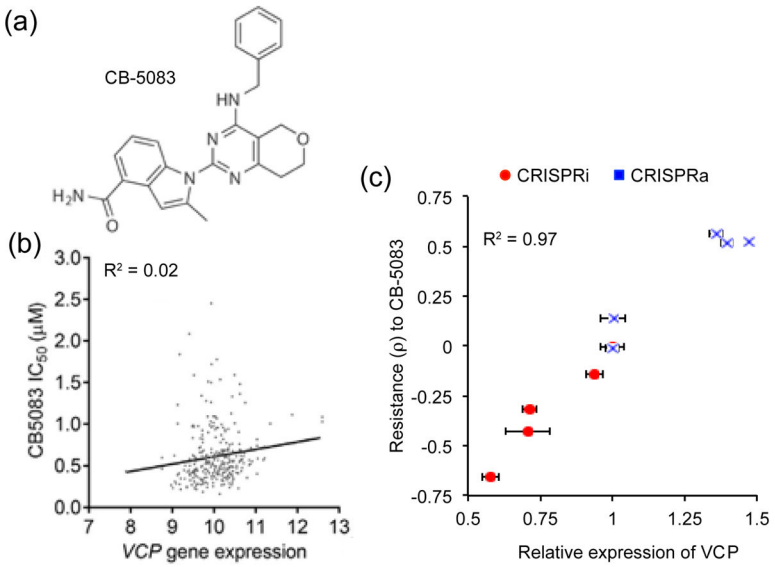


**Fig. 4.**

Identification of the target of an anti-cancer compound. (a) Structure of STF-118804. (b) An shRNA screen was conducted in MV411 leukemia cells to identify genes controlling sensitivity to the compound STF-118804, following the experimental strategy outlined in Fig. 2. Knockdown of the gene NAMPT significantly sensitized cells to the compound in two experimental replicates (P value from Mann-Whitney U test, each gene targeted by the shRNA library is shown as a dot). In follow-up experiments, we confirmed NAMPT as the target of STF-118804. Adapted, with permission, from ref. [1]

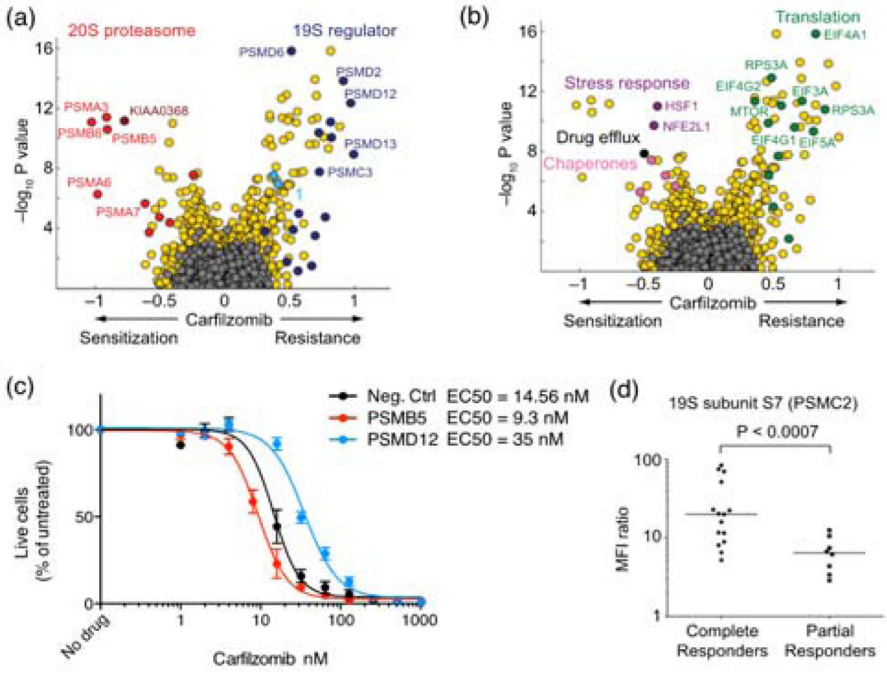


**Fig. 5.**

Genetic screen reveals biomarkers for patient response and potential combination therapy targets. An shRNA screen in the multiple myeloma cell line U266 revealed genes controlling sensitivity to the proteasome inhibitor carfilzomib. (a,b) For the genes targeted by the shRNA library, effect of knockdown on carfilzomib sensitivity and P value for the statistical significance of the effect are displayed in a volcano plot. Grey dots represent “quasi-genes” generated by grouping non-targeting negative control shRNAs. Dots in other colors represent protein-coding human genes. The same data is shown in (a) and (b), with different groups of genes highlighted and labelled in the two panels. (c) Validation of the effect of knockdown of a 20S proteasomal subunit (PSMB5) and a 19S proteasomal subunit (PSMD12) on carfilzomib sensitivity. Mean and standard deviation of two experimental replicates are shown. (d) Pre-therapy expression levels of the 19S proteasomal subunit S7 (quantified by flow cytometry in CD138 + bone marrow cells, which encompass plasma cells and multiple myeloma cells) are predictive of multiple myeloma patient response to carfilzomib. Figure adapted from ref [5].

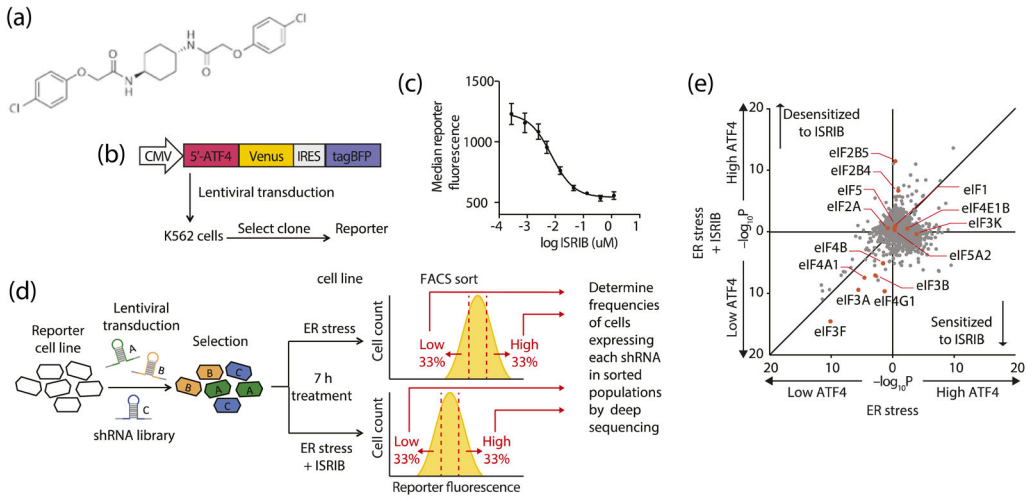


**Fig. 6.**

Identification of a drug target based on a complex phenotype. (a) Structure of ISRIB (b) A fluorescent reporter for the phenotype of interest, the integrated stress response. Activation of this stress response can be induced with ER stressors such as thapsigargin, and leads to translation of the Venus fluorescent protein, which is encoded downstream of the 5′ region of the ATF4 mRNA. We established a monoclonal reporter cell line was established in human K562 leukemia cells. (c) The reporter activity of this cell line in response to 300 nM thapsigargin is inhibited by the small molecule ISRIB in a dose-dependent manner (mean and standard deviation for experimental triplicates are shown). (d) Experimental strategy: the reporter cell line was transduced with an shRNA library targeting genes related to protein homeostasis, and exposed to thapsigargin in the presence or absence of ISRIB around its EC50 concentration. Cells with high and low levels of reporter activity were isolated by fluorescence-activated cell sorting (FACS), and the frequencies of cells expressing each shRNA were quantified in the different populations by next-generation sequencing. (e) The effect of gene knockdown on reporter activation either in the presence or absence of ISRIB are shown, each dot is a gene and P values for the statistical significance of the effect of a gene were calculated using the Mann-Whitney U test as previously described[3]. Knockdown of eIF2B4 and eIF2B5, two subunits of the eIF2B complex, blocked ISRIB action, and we validated biochemically that ISRIB is an activator of eIF2B. Figure adapted from ref [6].