STUDY DESIGNS

Am I ready for CRISPR? A user's guide to genetic screens

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Abstract | Exciting new technologies are often self-limiting in their rollout, as access to state-of-the-art instrumentation or the need for years of hands-on experience, for better or worse, ensures slow adoption by the community. CRISPR technology, however, presents the opposite dilemma, where the simplicity of the system enabled the parallel development of many applications, improvements and derivatives, and new users are now presented with an almost paralyzing abundance of choices. This Review intends to guide users through the process of applying CRISPR technology to their biological problems of interest, especially in the context of discovering gene function at scale.

Confirmation bias
The tendency to focus on information that confirms a pre-existing belief to the exclusion of contradictory information. In genetic screens, this can manifest in choosing to follow up a gene that scores with marginal statistical significance in the primary screen, rather than focusing on the experimentally identified top hits.

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doi:10.1038/nrg.2017.97
Published online 4 Dec 2017

Whereas the first human genome took over 10 years to sequence, now a genome is sequenced approximately once every 10 minutes. This wealth of information on our genes — including their variance across populations and relationship to disease — has laid bare our still elementary understanding of what each gene does and how they all work together to create a wondrous spectrum of phenotypes[1]. Integrating a deep understanding of the function of genes in normal and disease contexts, and using knowledge of these differences to discover treatments, remains a major challenge in biomedical research[2].

For much of the twentieth century, the speed, scale and control of genetics in model organisms stood in contrast to the limitations of human population studies, laborious mouse models and genetically imperturbable cell culture models. Thus, the discovery and development of first RNA interference (RNAi) and then CRISPR has generated tremendous excitement because these technologies finally enabled facile manipulation of genetic information: the ability to perturb gene function and see what happens. Genetic screens, it must be stressed, rely equally on three components — a perturbation, a model and an assay — and it is important to avoid the temptation of thinking that the excitement around CRISPR as a powerful technology for the perturbation diminishes the need to think deeply about the properties of a relevant model system or to carefully optimize an appropriate assay.

In the past decade, pooled screens — that is, the simultaneous testing of thousands of individual perturbations in a single batch — have become an increasingly popular means of conducting genetic screens in mammalian cells[3–8]. Because they do not require robotics for liquid handling, pooled screens can be executed with existing infrastructure by most laboratories that are already performing cell culture (albeit at a larger scale than typical experiments) and at a reasonable cost, enabling genome-scale interrogation. Pooled screens begin with the introduction of a library of perturbations into a population of cells. Lentiviruses or other retroviruses are used for this step because they integrate into the genome of the target cells, and thus a cell's genome is permanently marked by the perturbation it received. All pooled screens then rely on an assay that physically separates cells displaying a phenotype of interest from those that do not. The identities of the perturbations that caused the phenotype are determined by retrieving the sequence from genomic DNA by PCR, followed by massively parallel sequencing, which serves to quantify the abundance of each perturbation.

A thought experiment worth performing before embarking on a screening project is to randomly select genes targeted by a library and to then consider the next steps if these were the top hits. Although there might be some well-characterized genes near the top of the list, many hits may be relatively unstudied, with no obvious link to the phenotype. Indeed, if all the top hits are genes expected to cause the phenotype, then what was learned by doing the screen? It is a mistake to assume that the identity of the hit genes will shine a light on the follow-up path, both because most genes are poorly understood and because the annotated functions of genes can introduce a confirmation bias. Thus, well before beginning a screen, one should enumerate the steps that will follow: what informative experiments cannot be done at the genome scale but can be done once candidates have been identified, regardless of the identity of those candidates? What techniques and analyses will constitute figures 2 through 7 of the resulting manuscript? While

the prospect of hitting a favourite nail with the CRISPR hammer can be exciting, motivating the start of a project as soon as possible, the genetic screen is often only the first step, and clarity of purpose at the outset can prevent intellectual paralysis that may arise after a screen when faced with a long hit list of many potential leads to pursue.

This Review aims to guide users through developing, executing and analysing a pooled screen by use of CRISPR technology, including the parameters for selection of an appropriate model system, cell-based assay and library of single-guide RNAs (sgRNAs), while highlighting common pitfalls along the way. I focus on mammalian systems, but CRISPR technology has been successfully applied to a wide range of organisms; although the details may vary, the principles described here will hold true. Likewise, pooled screens work in complement with other technologies, most notably multiplex oligonucleotide synthesis, lentiviral delivery and massively parallel sequencing. While users do not need to become experts in any of these in order to execute a genetic screen, viewing them as black boxes that do not require any understanding of their strengths and weaknesses will almost certainly lead to trouble; excellent user-oriented reviews are available for these topics[9–11]. Additionally, CRISPR reagents are now available in arrayed format, enabling screens in 96-well plates or 384-well plates, and much of the advice from small interfering RNA (siRNA)-based arrayed screens will apply directly to CRISPR-based arrayed screens[12], so it is not covered here. Finally, many different Cas9 nucleases and related proteins can be found in nature, and like restriction enzymes, the catalogue continues to grow[13–18]. This Review focuses on applications using *Streptococcus pyogenes* Cas9 because it is the most widely used and developed, as the principles of planning and executing a screen hold true regardless of the exact perturbation.

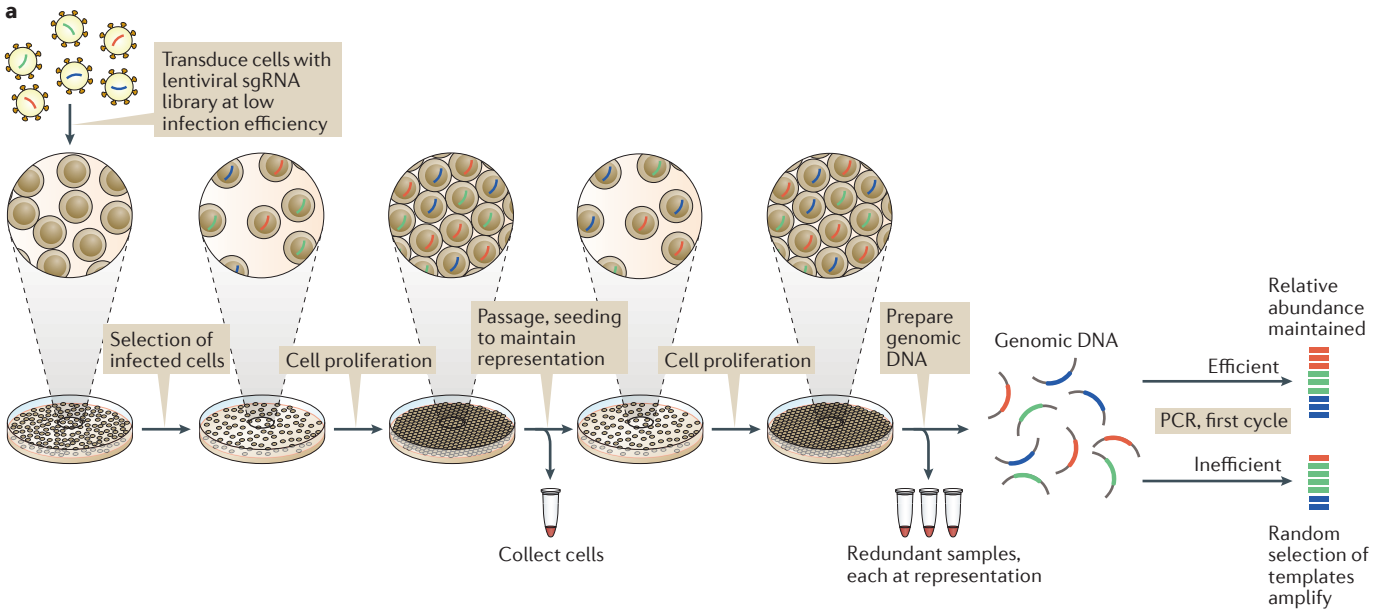
Optimizing an effective model

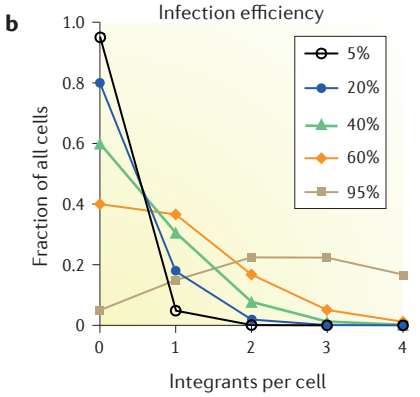
Selecting a model system for a screen should begin with a consideration of its relevance to the overarching biological question: will results from this model ultimately provide insight that peers in the field consider meaningful? Often it will be the case that the most relevant models — for example, primary cells, organoids or mice — will be at odds with practical considerations of scale and ease of use that tumour-derived cell lines can provide, as genome-scale screens necessarily require tens of millions of cells (FIG. 1). One path is thus to use a scalable model for a genome-wide primary screen and then to pursue potential hit genes in the more-relevant model. Of course, if the scalable model has only a tenuous relationship to the biology of interest, then a project may be better done by starting with a smaller-scale screen targeting fewer genes in the relevant model. Regardless of scale, confirming the technical suitability of a model for screening often requires several months of effort, often more time than executing the screen itself, but this investment upfront pays off when a meaningful hit list emerges.

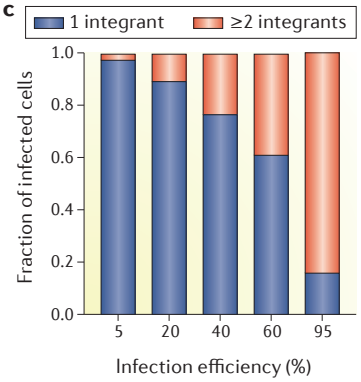
Figure 1 | **Maintaining representation in pooled screens.** Maintaining adequate representation of the library at all steps of a pooled screen is required for accurate quantification. **a** | A schematic of infection and cell passaging is presented. In the first step of a screen, a library of lentiviruses is added to a population of cells; the different colours represent different single-guide RNAs (sgRNAs) in each viral particle. Pooled screens, especially viability screens, often last several weeks. During each passage, a fraction of the cells is reseeded, and the number that is reseeded determines the representation that is maintained. The remaining cells should be saved rather than discarded. Likewise, at the end of the screen, there will probably be more cells than necessary for maintaining representation, and it is better to save multiple aliquots than a single aliquot in case a sample fails during genomic DNA preparation. Finally, sampling error can also occur after the screen, during PCR from genomic DNA. PCR conditions and genomic DNA quality should be optimized to ensure that each sgRNA integrant serves as a template for amplification and that representation is thus maintained rather than simply increasing the number of PCR cycles to achieve more product from only a fraction of the integrants. **b** | At different infection efficiencies, defined as the percentage of cells that received at least one viral particle, the range of integrants per cell will vary according to a Poisson distribution. **c** | Pooled screens aim to balance a reasonably high infection efficiency, so that large numbers of cells are not discarded because they were not infected, while also minimizing the number of cells that receive more than one perturbation, because multiple integrants can add noise to most pooled screens. Thus, 5% or 95% infection efficiencies are too low and too high, respectively, whereas an efficiency of 20–60% provides a good balance. **d** | An example calculation of the number of cells needed to maintain representation of the library, both during passaging and at the initial infection, is presented. **e** | A simulation of cell culture shows how both the number of passages and the representation can affect the distribution of the library simply owing to random chance. At a low representation (50×), the library will become more spread out, with more perturbations falling outside 2 standard deviations (SD) of the initial distribution (vertical dotted lines), leading to erroneous interpretation of dropout or enrichment. This source of noise can be greatly mitigated by achieving fold coverage of 500× or greater. **f** | From the same simulations as used in part **e**, the fraction of the library that falls outside 2 SD of the initial abundance is plotted as a function of the maintained representation. The horizontal dotted line indicates the fraction of a library that, by the definition of a normal distribution, will fall >2 SD from the mean. For further methodological detail on the simulations underlying parts **e**,**f**, see Supplementary information S1 (box).

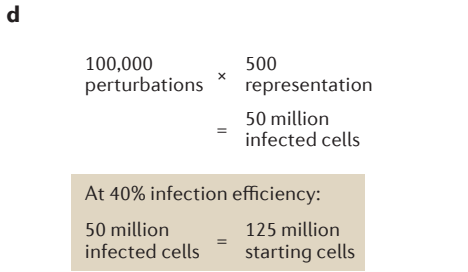
For technical optimization, various practical aspects of a pooled screen should be tested in advance (FIG. 1a). If several different cell types are all equally suitable from the standpoint of relevance, then these optimizations can be performed in parallel in case one or more cell type proves intractable. Even if the primary screen is ultimately performed in only one cell type, the conditions for subsequent validation in the other cell types will have been established, accelerating follow-up.

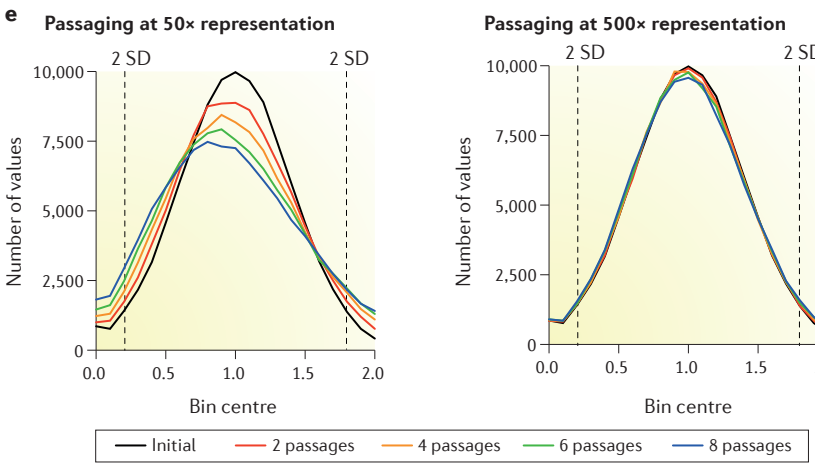
Single-guide RNAs (sgRNAs). The first CRISPR systems characterized in prokaryotes required two RNAs to program the Cas9 protein: a CRISPR RNA (crRNA) and a transactivating crRNA (tracrRNA). To simplify the system, these two independent RNAs can instead be merged into a single transcript, the sgRNA, which has practical benefits especially for ease of expression in mammalian cells.

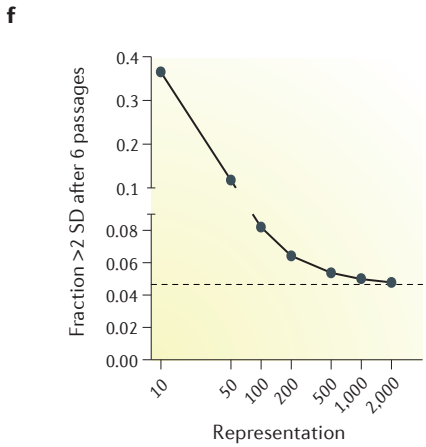












In pooled screens, lentiviral delivery of Cas9 and the sgRNA often utilizes a resistance gene to a drug-selectable marker (such as hygromycin, neomycin, blasticidin or puromycin) that is co-delivered on the lentiviral vector. Importantly, different drugs have different kinetics of selecting for a pure population — in general, puromycin kills nonresistant cells quickly, whereas neomycin can take weeks — and the appropriate dose will vary across cell types. Too little drug will result in uninfected, uninformative cells lingering in the population, whereas too much drug will kill successfully infected cells and is likely to have unpredictable

gene expression consequences on those that do survive. It is important to determine the proper dose empirically and close in time to the genetic screen; furthermore, a drug concentration should never be chosen simply by consulting the literature, as laboratory-to-laboratory and time-dependent drifts in the properties of a cell line are common. An alternative approach for selecting infected cells is to use fluorescent proteins and flow cytometry. This requires more hands-on time than drug selection but requires less time in culture to achieve purity, which may be especially helpful when using primary cells.

As the gene encoding Cas9 is large, delivering it on the same lentiviral vector that delivers the sgRNA leads to much lower lentiviral titre, and thus one common screening strategy is to first deliver the gene for Cas9 in advance of the library of sgRNAs, the latter of which can thus be made at high titre. In this approach, it is important to functionally determine Cas9 activity, as differences in expression, protein stability and localization could uncouple the Cas9 gene from its selectable marker. Reporter constructs that test Cas9 activity should be used[19], and if a Cas9-expressing cell line is kept in culture for an extended time, activity should be confirmed periodically. If maintaining a stable population of Cas9-active cells proves difficult, one potential solution is to screen with a library that delivers both Cas9 and the sgRNA at the same time. As the activity of the sgRNA requires only transient expression of Cas9 to cut DNA, even if Cas9 is subsequently silenced, sufficient DNA cutting may have already occurred. Another approach is to generate a single-cell clone of Cas9-expressing cells, which may be less prone to drift in its performance over time, although to do this appropriately, several unique clones should be isolated to ensure that stochastic properties that are specific to the selected clone do not confound subsequent biological interpretation of screening results.

When delivering the library of sgRNAs for pooled screens, very high infection efficiencies are not desirable, as this would lead to many cells with multiple different sgRNAs, which may lead to nonspecific toxic effects. Based on the Poisson distribution, an infection efficiency of 20–60% results in a majority of cells with one integrant (FIG. 1b,c) while also minimizing the number of uninfected cells, thus minimizing the number of cells needed at the start of the screen (FIG. 1d).

A seemingly pedestrian but ultimately critical step in a pooled screen is the isolation of genomic DNA. Many commercial kits and homemade protocols are readily available, although there is no one-size-fits-all solution because yields and quality will vary across cell types. Furthermore, a genomic DNA preparation method should be assessed with not only standard DNA quantification methods such as ultraviolet absorbance or fluorescence with intercalating dyes but also a functional test of PCR performance, as PCR inhibitors, which are missed by these quantification methods, may be at high levels in certain cell types and co-purify with genomic DNA, depending on the purification method. Because accurate quantification of library abundance requires that each perturbation serves as a template for PCR, simply increasing the number of cycles to overcome poor PCR efficiency is unhelpful (FIG. 1a). In sum, a particular genomic DNA preparation should be validated in advance before attempting purification of a precious screening sample.

Titre
The titre of a lentivirus is the number of infectious particles per unit of volume, and the ratio of lentiviral integrants to cells is the multiplicity of infection (MOI). Importantly, cells differ in their inherent infectivity, and thus the volume of virus that is sufficient to achieve a given infection efficiency in cell type A is not necessarily the same in cell type B.

Pooled screening assays

Viability screens. One of the most basic experiments to perform on a model system is to find genes affecting cell fitness. Perturbations that reduce cell fitness will be depleted or wholly absent by the end of the screen; hence, these screens are referred to as dropout or negative selection screens. Here, screening at a high enough representation to avoid bottlenecks is critical — that is, depletion via random chance — and thus tens of millions of cells are needed for executing such screens at the genome scale (FIG. 1d–f). Negative selection screens have been used most often in the context of cancer biology in order to identify dependencies of tumour cells that can be traced to a particular mutation, copy number alteration, expression pattern or other biomarker[8,20–26]. Another take on the negative selection screen is to combine a genetic viability assay with a small molecule that, by itself, does not substantially affect the proliferation of the cells, and thus hit genes are those that sensitize cells to the small molecule.

It is important to note the limitations of end point cell viability screens, as there are many mechanisms for a perturbation to deplete over time. For example, cells with a 25% slower doubling time will be >90% depleted after 3 weeks in culture in a pooled screen. Although this is a very different mechanism from depletion due to apoptosis, the disparate scenarios will look similar from end point analysis. Alternative means to isolate cells that are depleted by different mechanisms, whether by sampling at multiple time points or employing methods to purify cells with certain characteristics[27,28], can provide clearer distinctions, but these assays necessarily involve more hands-on time and optimization.

The alternative to the negative selection screen is the positive selection screen, in which the cells of interest are those that become enriched over time. Here, the selective pressure can be alternative culturing conditions[29,30], small molecules[31–34] or infectious agents[35–39], to name a few. The dynamic range of a positive selection screen can be much wider than a negative selection screen, as the majority of the population is eliminated, and thus the small fraction of perturbations that survive can be enriched by 100-fold or greater. Additionally, such screens will often yield only a small number of hit genes, and thus the follow-up path may be clearer by virtue of having fewer choices.

Ultimately, a single screen can provide both positive selection phenotypes and negative selection phenotypes. For example, a knockout viability screen in a cancer cell line can identify both the depletion of oncogenes and the enrichment of tumour suppressors. Likewise, intermediate doses of a small molecule can reveal both resistance genes and sensitization genes[40].

Flow cytometry. The premise of a pooled screen is the physical separation of cells on the basis of a desired phenotype, and fluorescence-activated cell sorting

(FACS) technology, when optimized, provides a powerful means of achieving this separation[41–44]. However, the flow cytometer should not be approached as a black box but rather as a sophisticated instrument with many parameters that need to be optimized in advance of the screen. Tens of millions of cells or more are required for genome-wide screens, which will necessarily require hours of sort time, and thus flow rates are particularly important if one wants to conduct a screen without adding hours to the required sort time: disassociating cells properly to avoid clumps while also preserving external epitopes, resuspending cells at a concentration that maximizes speed while avoiding drops with doublets and using an appropriate nozzle size on the sorter are all parameters that should be examined well in advance of the actual genetic screen.

Additionally, the assay should be closely examined as a function of time: as the cells sit in a tube waiting to go on the flow cytometer, do their properties change? Are they still as viable? Is the antibody still bound to the epitope, and is the fluorophore still equally bright? FACS sends cells vigorously through a fluidics system to emerge on the other end in a collection tube; do the cells start to undergo apoptosis, shredding the genomic DNA that contains the sequence of the sgRNA? If cell fixation is required, can the genomic DNA still serve as a template for PCR? Variations of these parameters over the span of hours will not be apparent from small-scale tests, and thus specific consideration of the extended time course of the sorting parameters of the genetic screen is necessary. Fortunately, these are all questions that can be answered by straightforward experiments in advance in order to ensure that the day of FACS proceeds smoothly. One simple and proven tactic is to screen with a colleague in batches: one person can prepare a fraction of the cells while the other person tends the flow cytometer.

Screens in vivo. The desire to screen for phenotypes in models that better recapitulate the diversity of environments and interactions between cell types makes *in vivo* screens, usually in mouse models, particularly attractive[45–49]. Here, the determination of representation is far more challenging, as cell numbers cannot easily be monitored in the organism over time. For example, suppose the goal is to study genes involved in tumour cell growth in a xenograft model. Although 1 million cells may be introduced into the mouse, there is no guarantee that all the cells will successfully engraft and contribute to the tumour. As the engraftment efficiency is a function of both the mouse genetic background (such as the exact combination of mutations to disable its immune system) and the human tumour cells that are being introduced, the number of cells, and thus the number of perturbations, that can successfully be assayed in a reasonable number of mice must be determined empirically[50,51]. The same considerations apply when modifying endogenous mouse cells by introducing lentivirus directly to the animal: it is important to consider the number of relevant cells — those that pertain to the biological question — that receive the perturbation (for example, distinguishing neurons from glia or tumour from stroma).

Determination of the capacity of an *in vivo* model can be done with the use of barcoded lentiviruses, such as with a library that was explicitly designed to contain a great deal of sequence diversity[52]. Alternatively, a pre-existing genome-wide sgRNA library, which is likely to contain far more perturbations than the capacity of the mouse model, can be used by simply treating the sgRNAs as barcodes, with the assumption that for estimation purposes, most sgRNAs will have minimal consequences on the phenotype of interest and thus behave as cellular barcodes. In either case, cells are harvested from the mouse, genomic DNA is prepared and the barcode or sgRNA is amplified. Sequencing can then reveal how many unique sequences are present in the sample and thus estimate the number of perturbations that can be effectively screened.

After performing such experiments, it will often be the case that genome-wide screens are not feasible, and thus a more fruitful strategy will be to create smaller, customized libraries, operating under the premise that it is better to get a good answer for a smaller number of genes than a weaker answer for many genes. Certainly, a successful screen with a customized library that validates the relevance and practicality of a model is an excellent motivator for funding and executing additional screens.

Gene expression. Combining CRISPR technology with single-cell RNA sequencing is an exciting emerging strategy, as the final output is not simply a change in sgRNA abundance but rather a partial gene expression profile of each cell[53–56]. Thus, from the primary screen, one can immediately begin to organize perturbations by similar patterns of gene expression changes implying common function. Here, a pool of sgRNAs is introduced into cells as in any other pooled screen, and then single-cell RNA sequencing methods are applied, with the notion that the sgRNA itself, or a linked barcode, is detected along with hundreds to thousands of cellular mRNAs. The approach is currently limited in scale — the largest screens to date have examined a few hundred sgRNAs — but decreased sequencing costs can be anticipated with time. The depth of information of an RNA expression profile is both daunting and powerful; a senior author of one of these studies noted that it took about two weeks to collect the data and six months to analyse it. Nevertheless, as both the infrastructure to execute such experiments and the ability to readily interpret profiling data become more widespread, this marriage of technologies will certainly become a powerful approach.

Perturbation choices: Cas9 and its many uses

Because Cas9 is natively an RNA-guided DNA-endonuclease, the out-of-the-box application in mammalian cells is cutting DNA[57–59]. The most typical outcome of a double-strand DNA break is repair via the nonhomologous end joining (NHEJ) pathway, which often introduces small insertions or deletions (indels) that can, for example, frameshift a protein-coding region or disrupt a binding site for a transcription factor. Thus, loss-of-function screens with wild-type Cas9, known as CRISPR knockout (CRISPR-ko) screens, have been

Xenograft
The transplantation of cells from one species to another. Often, it involves introducing human cancer cells into a mouse model to study their behaviour in complex microenvironments that are difficult to model in cell culture. Mice with an active immune system will recognize foreign cells and clear them out; thus, such experiments must be performed in immunodeficient mice.

widely applied[5–7]. Additional applications are enabled by inactivating the nuclease activity of Cas9 (dCas9)[60], converting it to an RNA-programmed DNA binding protein and appending functional domains that can be used to activate or inhibit transcriptional activity (CRISPRa and CRISPRi, respectively)[61–65], alter the methylation status of DNA[66,67], modify nearby histones[68–72] or directly edit specific nucleotides[73–75] (FIG. 2). Thus, genetic information can be manipulated at multiple levels.

Each of these technologies has particular advantages. For loss-of-function studies, CRISPR-ko can create truly null alleles, which may be necessary for some gene products if even residual levels are enough to maintain a phenotype. Conversely, CRISPRi and other repressive modalities may be better suited to studying genes that manifest different phenotypes at different gene doses and essential genes that cannot tolerate complete knockout or to examining non-coding regions, as discussed below. Finally, RNAi should not be dismissed for creating loss-of-function, as it provides the ability to target mRNA directly and avoids the need to co-deliver the large and exogenous Cas9 protein[76], although the much higher rate of off-target effects for RNAi than for CRISPR necessitates additional vigilance in validation[77–81]. For turning genes on, CRISPRa has the benefit of capturing the diversity of splice isoforms and does not require initial cloning of the cDNA of interest, whereas overexpression of open reading frames has practical advantages for studying variants and mapping protein function[82,83].

Given all these options for Cas9 activities and complementary perturbations, the question is not 'Which one should I use?' but rather 'Which one should I use first?'. Generally, loss-of-function libraries are the most developed and thus are the best choice when using a model and assay for the first time. The development of a relevant model system that provides meaningful insight is the bottleneck to impactful science, and once the combination of a model and an assay has proven itself successful with one class of perturbation, there is surely more to learn by conducting screens with additional technologies.

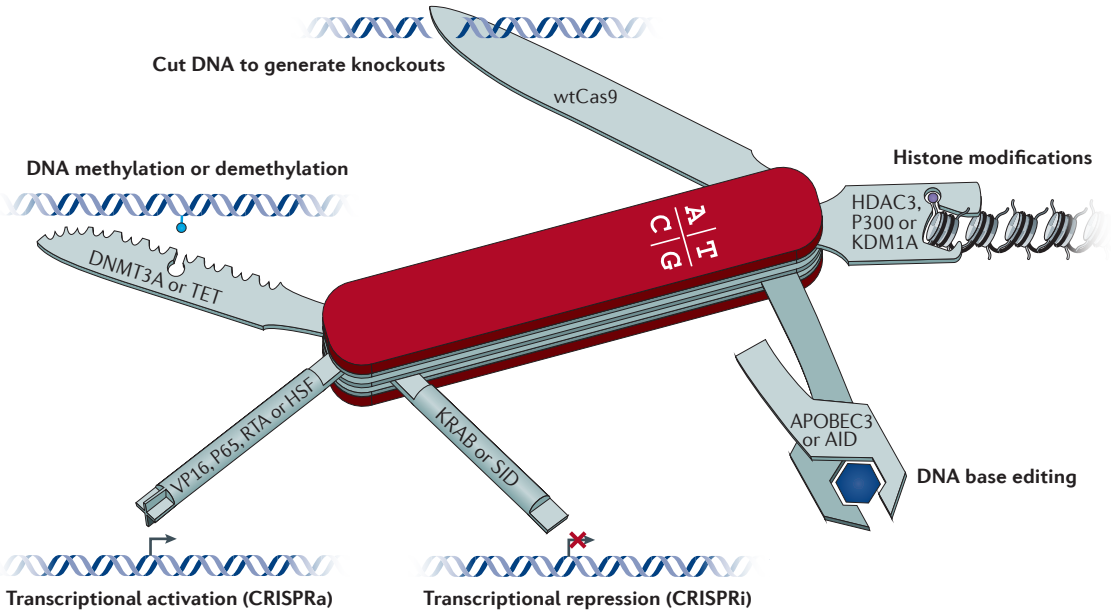


Figure 2 | **Diversity of activities with Cas9.** Cas9 can be used in its unmodified, wild-type form (wtCas9) to cut DNA and create knockouts (CRISPR-ko). After inactivating the nuclease domains to create dead Cas9 (dCas9), additional domains can be appended to extend the range of activities for CRISPR technology. Transcriptional activation (CRISPRa)[62] is achieved by tethering one or more activating domains; a recent study compared the efficacy of several approaches[129]. Conversely, dCas9 can be tethered to inhibitory domains to interfere with transcription, known as CRISPRi. Modification of DNA methylation marks is accomplished by DNA (cytosine-5)-methyltransferase 3A (DNMT3A), which adds methyl groups at CpG sites, and TET methylcytosine dioxygenase (TET) removes methylation[130]. Several domains have been shown to enable modification of histone marks[68,69]; a recent review provides additional detail[131]. Finally, fusing a cytidine deaminase domain from DNA dC→dU-editing enzyme APOBEC3 or an activation-induced cytidine deaminase (AID) domain to dCas9 allows conversion of DNA bases in the absence of double-strand DNA breaks. Depending on additional differences, the result can be conversion of bases in a quasi-random fashion (CRISPR-X)[74,75] or precise gene editing (Base Editor Cas9)[73]. HDAC3, histone deacetylase 3, to deacetylate histone H3 lysine 27 (H3K27); HSF, activation domain from human heat shock factor protein 1; KDM1A, lysine-specific histone demethylase 1A (also known as LSD1), to decrease dimethylation at H3K4); KRAB, Krüppel-associated box domain, found in many zinc-finger proteins; P300, catalytic core of the human histone acetyltransferase p300, to acetylate H3K27; P65, transcription factor p65 (also known as RELA), a component of the nuclear factor-κB (NF-κB) transcription complex; RTA, replication and transcriptional activator from gammaherpesviruses; SID, mSIN interaction domain; VP16, a domain from herpes simplex virus (the use of four tandem copies of VP16 is often referred to as VP64).

Perturbation choices: sgRNA library design

Designing a library requires balancing desired properties, such as the number of target genes compared with the cost and feasibility of the screen. If cell numbers and the subsequent assay are not limiting, then genome-scale screens have implicit appeal, especially if they allow the use of readily available, premade libraries[22,26,84–86]. Likewise, the number of sgRNAs per gene can vary. The use of more sgRNAs per gene in a primary screen (~8–10) allows greater statistical certainty for hits that emerge from a primary screen, which may be especially helpful for the initial detection of genes with weaker phenotypes. Conversely, the use of fewer sgRNAs per gene (~2–4) requires fewer cells and thus, for the same cost as screening a larger library, enables the primary screen to be performed in more models. One excellent source for browsing and comparing existing libraries is Addgene, which can also provide either plasmid DNA or, for some libraries, ready-to-screen lentiviruses.

When the model or assay dictates the use of a smaller library, choices must be made about which genes to examine. One approach is to use premade genomic subsets, targeting sets of genes organized around a biological theme, such as kinases, transcription factors or mitochondrial proteins[6,84,87]. A second approach is to use previously generated data to nominate genes of interest, such as differential expression profiling or genome-wide association studies (GWAS), followed by the creation of a customized library, which can be done in-house[88] or outsourced to commercial vendors. The creation of custom pools will also be an essential task for those interested in less-studied model organisms.

Genomic characterization of target genes. Library design requires the curation of genomic data from a number of disparate sources. For premade libraries, that work has already been done, but it is important to understand the choices that were made, both to know the strengths and weaknesses of a particular library and to understand the path for designing a customized library. Furthermore, libraries designed for general use, without prior knowledge of what cell type they will be applied to, must make some simplifying assumptions, whereas customized libraries can more explicitly use genomic characterization data. Importantly, annotation of the human genome continues to be dynamic. Changes include recognition of new genes while other genes are reclassified as pseudogenes, inclusion or exclusion of an exon in the representative transcript and redefining minor and major single-nucleotide polymorphisms (SNPs), to name a few. Thus, libraries depreciate over time, and even if a library is used that was designed several years ago, using an updated annotation of the target (or targets) of each sgRNA will improve downstream analysis.

Targeting protein-coding genes with CRISPR-ko presents an abundance of potential sgRNAs, which must be chosen wisely, as there is wide variability in the efficacy of different sgRNAs (FIG. 3a), and, as discussed below, consistency of sgRNAs targeting the same gene is the bedrock of screen analysis. Considering the structure of the target gene is important, as certain sites should generally be avoided, including sites upstream of an alternative start codon, sites that overlap with high-frequency SNPs[89] and sites close to the end of the coding region[19], as these may not produce a phenotype (FIG. 3b). In some cases, targeting conserved protein domains has been helpful, presumably because even in-frame repair products are more likely to be deleterious[90,91]. Additionally, alternative splicing information can be used to target constitutive or transcript-specific exons; here, the APPRIS database[92], which is incorporated into GENCODE, is particularly helpful. Although genome-wide libraries generally pick a single representative transcript, when designing a custom library, RNA sequencing of the specific cell type can be used to target the relevant isoforms and may also be used to exclude nonexpressed genes.

The on-target properties of sgRNAs have been the focus of several studies, and it is clear that the primary sequence of the sgRNA and the surrounding target site is a major determinant of its activity[6,19,84,93]. While computational predictions are imperfect, they greatly bias the user towards success (FIG. 3c,d), and the performance of different prediction algorithms has been compared across data sets[94]. Importantly, the relative efficacy of sgRNAs is generally concordant across cell lines, and at least part of this concordance is due to the fact that the repair products of NHEJ are consistent across cell lines[95]; in other words, sgRNAs do not cause a random set of repair products, with one-third in-frame and two-thirds out of frame; rather, DNA is repaired according to not-yet-understood rules[96]. Thus, sgRNAs can be identified that are biased towards out-of-frame repair.

The ability for a perturbation to target sites other than the intended one can confound experiments, and methods to determine the off-target activity of sgRNAs have recently been reviewed[97]. Computational prediction of off-target sites has improved over time, and different approaches have recently been compared[94,98]. There does not appear to be much, if any, relationship between on-target activity and propensity for off-target effects (FIG. 3e), and thus one can generally select sgRNAs that maximize efficacy while minimizing off-target effects. Most recent design websites provide a numerical score for each of these properties and may also aggregate them into a single ranking to identify the most desirable sgRNAs for a given gene.

For CRISPRa and CRISPRi, the location of the transcription start site (TSS) is the most important design criterion[86,99]. Here, use of the FANTOM database[100], which uses TSS annotations based on the cap analysis of gene expression (CAGE) technique, which physically captures the mRNA cap, gives rise to higher-activity sgRNAs than those designed using the TSS annotations provided by RefSeq or Ensembl[99]. Although sequence features of the sgRNA can still play a role in improving activity[84,87,93], the narrow window of optimal activity — only ~100 nucleotides — leads to fewer sgRNAs from which to choose relative to screens using CRISPR-ko. However, this also means that off-target effects may be less of a concern, as sgRNAs bound outside the critical

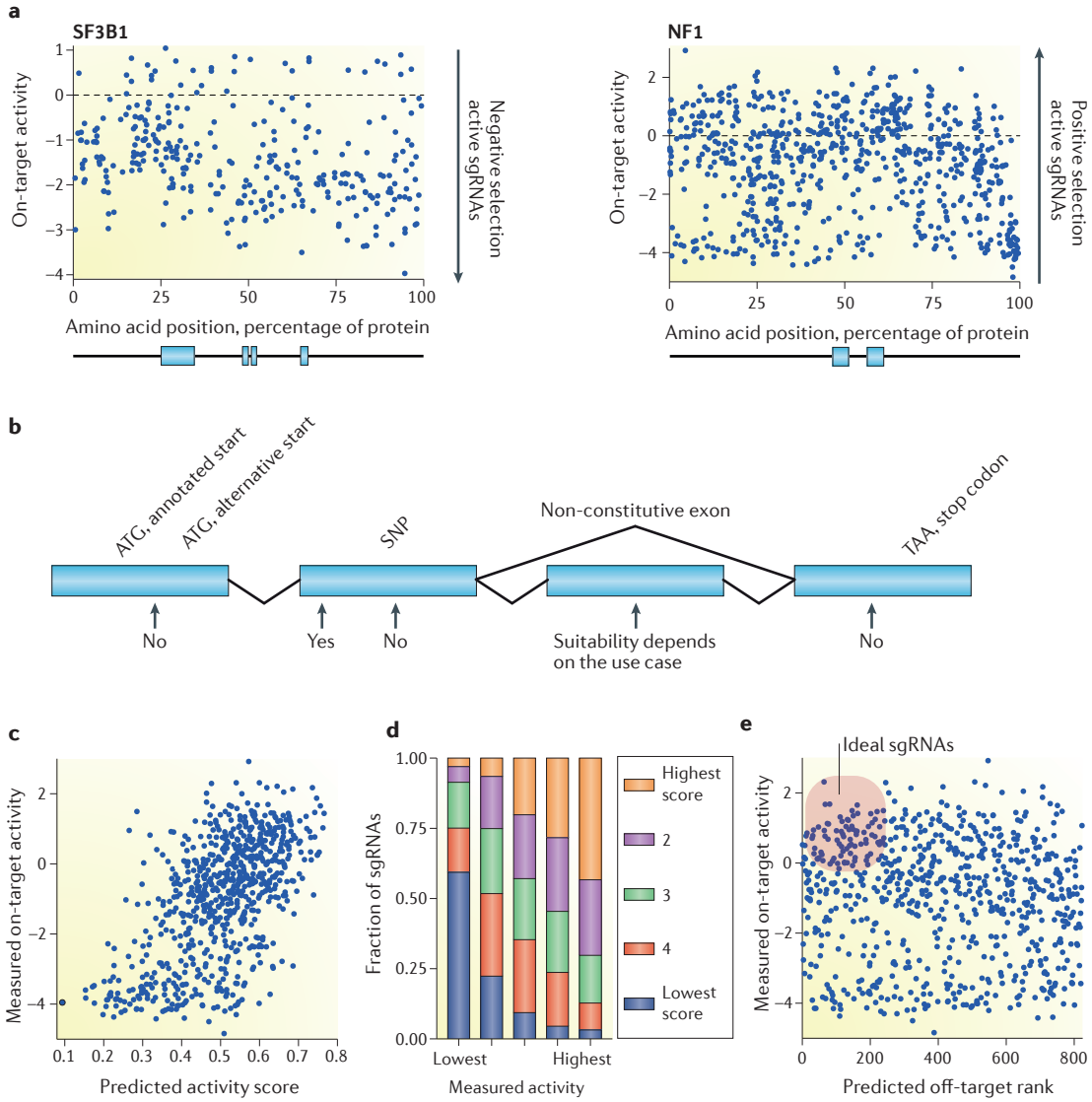


Figure 3 | **Design considerations for CRISPR-based knockout. a** | Measured activity for all single-guide RNAs (sgRNAs) targeting splicing factor 3B subunit 1 (*SF3B1*), an essential gene measured in a viability assay[91], or neurofibromin 1 (*NF1*), a resistance gene measured in an experiment with vemurafenib treatment[84]. Protein domains annotated in the Pfam database are indicated by boxes beneath the plot. **b** | Schematic of protein-coding gene and areas to avoid in choosing sgRNAs. A target site too close to the annotated amino terminus of the protein may result in the use of an alternative ATG start codon and thus potentially in maintained protein activity. Likewise, targeting too close to the carboxyl terminus may not result in a null allele, as the majority of the protein will still be translated. Sites of common single-nucleotide polymorphisms (SNPs) should be avoided as target sites, as the target cell type may contain a minor allele and thus will not be effectively targeted. Finally, although alternatively spliced exons are generally avoided in predesigned libraries, they may be targets of interest in custom libraries. **c** | Measured activity for *NF1* sgRNAs shown in part **a** compared with predicted activity scores, showing that sgRNAs with high predicted activity are indeed likely to be active. **d** | The measured activity and the predicted score binned by quintiles for the comparison shown in part **c**. **e** | Comparison of measured on-target activity to the predicted off-target activity, ranked according to the cutting frequency determination (CFD) score[84]. Generally, there is no relationship between on-target activity and off-target activity; thus, sgRNAs can be selected that are optimal by both metrics.

window are unlikely to have much activity. Similar to the selection of a representative transcript for genome-wide CRISPR-ko libraries, CRISPRa and CRISPRi libraries generally select a representative TSS for a gene, and thus customized libraries can be made more specific by designing with knowledge of the TSS used in the target cell type.

There are many websites that can assist in sgRNA design[19,94,101–104], and different features will appeal to different users: for example, the number of genomes supported; the ability to visualize results for a gene of interest; or the ability to design against many genes in batch mode (TABLE 1). Just as libraries depreciate over time owing to changing annotations, so too do design

Table 1 | **A selection of recently updated and consistently maintained websites for the design of single-guide RNAs**

Program	Website	Strengths	Refs
Cas-OFFinder	http://www.rgenome.net/cas-offinder/	Supports many different Cas9 variants and related enzymes	104
CRISPOR	http://crispor.tefor.net	Numerous genomes supported; displays multiple on-target and off-target scoring schemes	94
GPP sgRNA designer; Microsoft Azimuth	https://portals.broadinstitute.org/gpp/public/ https://www.microsoft.com/en-us/research/project/azimuth/	Detailed on-target and off-target predictions for CRISPR-ko, CRISPRa and CRISPRi for human and mouse	84
GUIDES	http://guides.sanjanalab.org/	Intuitive graphical user interface for human and mouse designs, although limited off-target search	103

Several early websites lacked comprehensive off-target search algorithms and therefore missed many potential sites; thus, they are no longer recommended for sgRNA design[64,132]. This includes one of the first and most popular CRISPR design websites, http://crispr.mit.edu, which was no longer under active development as of April 2017. CRISPRa, CRISPR-based transcriptional activation; CRISPRi, CRISPR-based transcriptional inhibition; CRISPR-ko, CRISPR-based knockout; sgRNA, single-guide RNA.

sites, and one should choose sites on the basis of consistent maintenance, not simply because they are already bookmarked.

Beyond the proteome. The use of the term 'genome-wide' to refer to libraries that modulate only protein-coding genes is common but inaccurate, selling short a very large fraction of the human genome, and CRISPR technology has shown success in interrogating DNA regulatory regions. Here, because the landscape of potential targets is both more broad and more cell-type-specific, generic libraries are not as widely applicable as they are for protein-coding genes, and instead, libraries customized to particular regions of interest have been most productive. Both CRISPRi[105] and CRISPR-ko[106,107] have been used to occlude or disrupt regulatory elements, respectively, although more direct comparisons will be needed to determine whether one approach is consistently more informative. Given that the majority of variation implicated in human disease by GWAS studies falls in non-coding regions, these types of screens will have broad applicability, although here, the challenge will not be in nominating regions or designing libraries but rather in identifying the appropriate cell type in which variants exert their effects.

Non-coding RNAs are another class of gene where CRISPR technology has shown promise as a screening technology. These RNAs are poorly targeted by RNAi, at least in part because many of them are relatively depleted in the cytoplasm, where the RNAi machinery is active. Furthermore, CRISPR-ko has limited ability to perturb non-coding RNAs because small indels may affect function only rarely (unlike the functional consequences of frameshifts in protein-coding genes). Therefore, CRISPRa and CRISPRi technologies are the methods of choice for manipulating the expression of this class of genes. A genome-scale CRISPRi screen of non-coding RNAs showed that downregulation of some non-coding RNAs leads to viability effects, which exhibited more cell-type specificity than protein-coding genes[108]. Thus, similar to the choice of different perturbational technologies, the choice of target types — protein-coding genes, regulatory regions or non-coding transcripts — is not a question of which one to screen but of which one to screen first in a proven and informative model system.

Analysis and follow-up

From screening data to a hit list. The expertise of biologists often does not extend to the command line or advanced statistics, and when confronted with a large data set, many quickly run up against the limitations of what can be accomplished with Microsoft Excel. However, good progress can still be made with minimal computational or mathematical expertise. A suitable approach is to begin by checking replicate correlation to gain an intuition of how much technical variability was present in the model and the assay during the screen. Such manual examination of the data is essential in order to identify outlier replicates, sample swaps, mislabelled conditions and other errors that may be lost if all the data are too quickly dropped into an analysis package. Next, simple averaging of the effect of multiple sgRNAs targeting the same gene can quickly provide a working 'hit list'; if multiple sgRNAs targeting a gene all rank highly, then that gene is quite likely to be a hit, and literature searches can commence to learn about the genes in the hit list and to design follow-up steps.

For more complete analysis, several fairly easy-to-implement design packages have been described that can provide more depth of information, although these analyses should not wildly alter the identity of top hit genes compared to those identified in the preliminary analysis[109–111]. Furthermore, it is important to distinguish unfamiliarity with computational approaches from disinterest in the underlying principles of the analysis; the former can be bridged with help from a colleague, whereas the latter is simply poor practice, as the responsibility for thinking should not be outsourced. The most important principle for analysis of genetic screening data is to leverage built-in redundancy, that is, the use of multiple sgRNAs targeting a gene. Ultimately, there is a strong cultural desire to produce the 'hit list' — a single, authoritative ranking of genes that appears as a table in a publication — but there are multiple ways of performing analyses, all reasonable, and the many generally equivalent permutations must not be allowed to lead to paralysis. A start-to-finish example of an effective screening, analysis and follow-up approach is provided in BOX 1.

Although many screens do not have the benefit of a positive control — indeed, discovering the genes

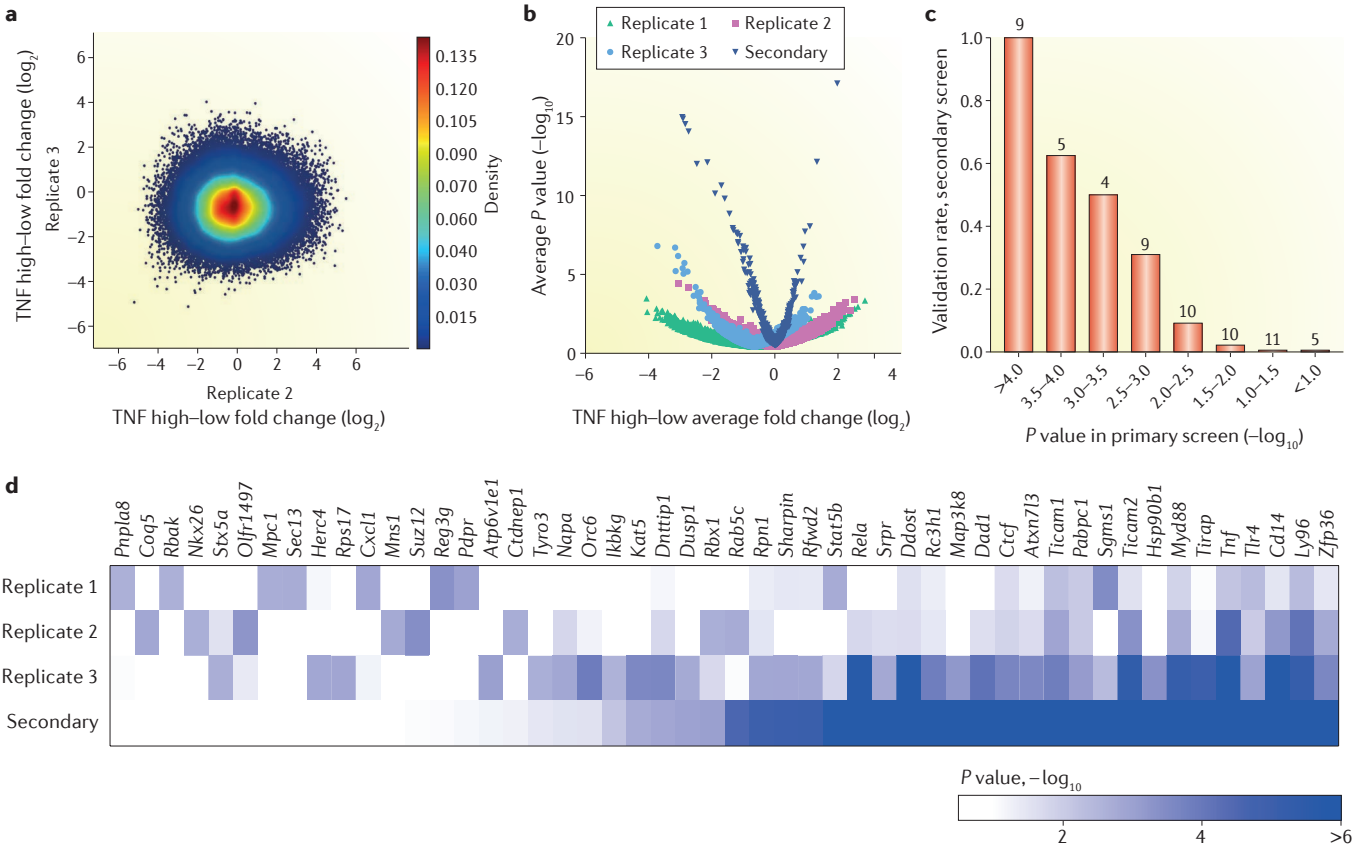
Box 1 | **Validation and analysis workflow**

The screen by Parnas *et al.* for regulators of the innate immune response provides an instructive example for the step-wise workflow of a pooled screen in a challenging model system[42]. Here, cell inputs were limiting, as the screen relied on primary cells harvested from mouse bone marrow. These cells were infected with a genome-scale library with 6 single-guide RNAs (sgRNAs) per gene for a total size of ~120,000 sgRNAs, stimulated with lipopolysaccharide and then fluorescence-activated cell sorting (FACS) was used to isolate cells with high and low levels of intracellular tumour necrosis factor (TNF).

During analysis of the screen, sgRNAs that were initially at low abundance in the library were eliminated from consideration, as were a few sgRNAs targeting control genes for which plasmid DNAs, abundant in the laboratory, contaminated the genomic DNA samples (a good warning that genomic DNA preparation and PCR set-up should be done in a physically distinct location from bacterial maxi-preps).

The abundance of sgRNAs in the TNF-low expressing population was subtracted from the TNF-high expressing population. At first glance, this produced discouraging results: pairwise correlations of 0.017, 0.003 and 0.021 across three biological replicates (see the figure, part **a**). However, after combining information for sgRNAs targeting the same gene, a signal begins to emerge (see the figure, part **b**). Notice that the first replicate produced the fewest genes scoring with high statistical significance, whereas the third replicate produced the most; the use of more cells and additional experience in executing the screen probably led to this performance improvement.

A secondary pool was then generated based on genes that scored in the primary screen, with 10 sgRNAs per gene for ~2,500 genes, and the reduction in library size allowed the collection of much cleaner data; here, pairwise replicate correlations ranged from 0.60 to 0.63, and the increase in both data quality and the number of sgRNAs per gene led to many hits scoring with very strong statistical significance (see the figure, part **b**). Comparing the primary to the secondary screen, there is a clear relationship between the statistical strength of the hit in the primary screen and the validation rate in the secondary screen (see the figure, part **c**; the total number of genes that were validated is indicated on top of the bar), and it is also clear that the secondary screen enabled clear discrimination between true and false positives from the primary screen (see the figure, part **d**). Thus, the strategy that Parnas *et al.* took has proven successful: they immediately began to validate genes that scored strongly on the basis of only primary screening data while in parallel generating a secondary pool that can be used to mine more genes from an assay, especially at a time when the relevant hands-on expertise is still present in the laboratory.



involved in the phenotype is the point of the screen — it can be useful to empirically test screen performance beyond metrics of technical reproducibility. Here, gold standard sets of essential and nonessential genes have been curated[26,85,112] and represent an important resource for benchmarking screening data for any screen employing loss-of-function approaches (perhaps for use in a control arm of an experiment, if viability was not the main objective). Strong separation between sgRNAs targeting essential and nonessential genes indicates robust Cas9 activity, ample sampling of cells and quality of sample processing and analysis after a screen.

Following a primary screen, an effective follow-up strategy is to design a custom pool, as used in the screen

discussed in BOX 1. Here, the thought process is that the primary screen is more concerned with identifying potential hit genes, and users can move forward with many genes, even those with marginal phenotypes. In other words, the concern at this stage is less about false positives, as those will be weeded out in the secondary screen, and more about making sure genes that may have scored weakly owing to ineffective perturbations are not left behind as false negatives. In this custom pool for a secondary screen, many more sgRNAs per gene can be included, which gives much greater confidence to the resulting answer for each gene. Practically, by virtue of being substantially smaller, this custom pool can be screened across several cell types and in more-relevant cell types for which screening at the genome scale proved infeasible. For example, if the initial library targeted 20,000 genes with 5 sgRNAs per gene, the secondary pool can be made to target 500 genes with 20 sgRNAs per gene and still be tenfold smaller. As the sgRNA pool is being made anew, this step also offers the opportunity to take into account cell-type-specific genomic information that may not have been leveraged if a general-use genome-wide library was used initially.

Systematic error modes. No technology is without its shortcomings and blind spots, and CRISPR is no exception. Although the intended outcome of sgRNAs used with wild-type Cas9 is the creation of loss-of-function alleles, some positive selection screens have identified fortuitous gain-of-function mutations resulting from in-frame repair[113,114]. Fortunately, these are rare enough to avoid confounding gene-level interpretation, and they can provide useful insight into protein domains when detected. A broader challenge for CRISPR-ko screens is that double-strand DNA breaks trigger a cellular response. If a particular sgRNA truly cuts the genome twice, once for each copy of the gene, there may not appear to be much of an effect on cellular fitness. However, if the target site is in an amplified region, or the sgRNA is promiscuous and has many off-target sites, the cell will pause in the cell cycle or undergo apoptosis[84,85,91,115]. The resulting dropout may appear to indicate that the target gene is essential for the cell, but the fitness defect is not due to loss of the protein product; rather, it is due to this multiplicity-of-cutting effect. Indeed, when this has been examined in detail, such as for the *BCR–ABL* and *AKT* oncogenes, CRISPR reagents will erroneously identify many genes in a copy-number-amplified region as essential, whereas RNAi reagents can correctly identify only the known driver oncogene. CRISPRi, because it does not cut the genome, avoids this error mode but has a blind spot of its own: by virtue of repressing transcription, when targeted to bidirectional promoters, CRISPRi can silence both genes[116]; CRISPRa approaches may have the same shortcoming, although this has not been studied in detail. These sources of false positives should be examined during analysis of screening results, for example, by looking for hit genes that cluster in nearby genomic locations, and further emphasize the value in examining a model system with multiple perturbational technologies.

Regardless of the perturbation used, one important limitation of pooled screens is paracrine signalling effects or other non-cell-autonomous roles of genes. For example, if a secreted growth factor that is essential for viability is knocked out of all cells in one well in an arrayed assay, then the cells in that well will die, and that gene will score. However, if that same growth factor is knocked out of a cell in a pooled population, as the other cells in that population have different genes knocked out, the growth factor will still be present in the culture, and the importance of that gene will be missed. Thus, even a genome-wide screen will not necessarily identify all genes that are relevant to a phenotype.

Validating and generalizing results: different cells, different answers. A common refrain is that the first step after the screen is to 'validate' the hits, a word that has several layers of meaning. First, the perturbation itself can be validated based on its technical reproducibility, which may or may not be necessary depending on the quality of the primary screening data. Second, the target of the perturbation can be validated to distinguish on-target effects from off-target effects, which also may or may not be strictly necessary, again depending on the quality of the primary data: indeed, we have seen screens in which all sgRNAs targeting a gene score at the very top of a hit list[5,6,84]. On-target validation can include western blots to confirm loss of protein, but not all proteins will have available antibodies. For CRISPR-ko screens, designing PCR primers that amplify across the cut site and detecting indel formation by either endonuclease assays or direct sequencing can prove on-target activity[117], whereas for CRISPRa and CRISPRi, quantitative reverse-transcription PCR (qRT-PCR) to examine RNA levels is an assay that scales well. Fundamentally, it is important to note that confirmation of on-target activity does not rule out the possibility that the phenotype is caused by an off-target effect. Here, it is critical to continue to perform phenotypic validation experiments with multiple sgRNAs per gene, not simply with the one sgRNA that gives the most on-target activity.

A step beyond technical validation is to examine the extent to which the result generalizes to other biological contexts or is specific to the one screened. A gene–phenotype relationship that does not extend to a different model is not necessarily evidence of a false positive finding. Rather, it is an observation that will require additional experiments to understand the differences across models, and it might even have valuable applications, such as gene dependencies that occur only in cells that harbour particular cancer mutations. A simple example from the literature shows that it is common for results to vary across systems. The small molecule 6-thioguanine, a purine analogue, and its target protein, hypoxanthine-guanine phosphoribosyltransferase (HPRT1), have constituted a workhorse model of mammalian genetics for half a century[118]. In two studies using CRISPR-ko screens for resistance to 6-thioguanine, one in human cells[6] and one in mouse cells[7], HPRT1 scored, as did multiple members of the mismatch repair pathway. However, another

CRISPR-ko study in different human cell lines[84], as well as a gene trap screen in haploid human embryonic stem cells[119], also found HPRT1, but failed to identify mismatch repair pathway genes, instead identifying the gene *NUDT5*, which functions biochemically upstream of HPRT1 in purine metabolism. These results demonstrate both the variability of cellular circuitry across cell types and the value of screening with multiple model systems.

Reporting guidelines. Although most manuscripts that use screening technology tend to focus deeply on a small number of genes deemed to be interesting, the ability of readers to easily browse and reanalyse the data as a whole is an important contribution of the study. The onus is on authors, reviewers and editors to ensure that the screening data are appropriately documented and made available. In its most raw form, the output of pooled screens is unprocessed sequencing reads, which are often (but not always) uploaded to a central repository such as the Sequence Read Archive of the US National Center for Biotechnology Information (NCBI). While in theory this allows anyone to fully repeat the analysis, for most users, this is too raw, as the steps required are multiple and time consuming. The user must download very large files, acquire and understand existing code to deconvolute the data or write scripts to do so and organize the data in a way that matches the experimental and analytical framework of the original publication. This can easily be a full day's work or more, especially if the user has not already gone through this process many times before.

When sequencing data are to be used for assembling genomes, identifying rare alleles or calling tumour mutations, the details about error rates and read lengths are critical, and thus reanalysis of the raw data is important. However, in genetic screens, sequencing is used solely as a means of counting sgRNAs. It would be far more applicable and useful for authors to provide such counts; in all cases these counts are represented in a simple text file that must already exist on their local hard drives. On the other hand, the data provided may be overly processed, such as when only gene-level data are given and information about individual sgRNAs is missing. In this case, numerous decisions have been set in stone by the authors, such as choices about how to combine replicate information and how to weight multiple sgRNAs targeting the same gene, and the details underlying these decisions are lost in the output data. Although it is useful for authors to provide an authoritative take on their own data set, as they know it best, future users of the data should not be constrained by these choices.

Going forward (and acknowledging that these guidelines are suggested from experience, not from this author always following them completely himself), it would be most useful if all pooled screening data were provided in each of three ways: first, raw sequencing reads on a public server; second, read counts provided as a supplementary text file (not a PDF, as this hinders extraction and processing of the data); and third, gene scores of all genes, by the authors' preferred perturbation-to-gene scoring scheme, rather than simply a table of top hits. Taking these steps will extend the reach of studies and deepen their impact, enabling mining of connections between genes and model systems that could not have been anticipated. A very useful resource in this regard is GenomeCRISPR, a browsable database that collates data from published screens[120].

Conclusions

CRISPR technology has ushered in a new wave of optimism for our ability to functionally annotate the genome. For genetic screens, progress with CRISPR has been greatly accelerated by both the successes and the lessons learned from earlier screening technologies, most notably RNAi. Although the technology continues to develop at a rapid pace, the execution of a genetic screen relies on understanding fundamental principles and optimizing experimental steps that will remain constant regardless of the exact perturbation.

For example, combinatorial screens are beginning to become possible, which will be particularly powerful in understanding paralogues and other functional redundancies that can prevent single-gene-perturbation screens from uncovering phenotypes[121–123]. This may be especially important when attempting to use genetic perturbations to uncover potential drug targets: while a small molecule will often be able to inhibit multiple paralogues so long as the binding sites are conserved, targeting only a single gene with a genetic perturbation will leave the pathway intact. Clear examples are the redundancies in the mitogen activated protein kinase (MAPK) pathway proteins, such as between MEK1 and MEK2 and between ERK1 and ERK2. Additionally, the ability to build genetic interaction maps has proved to be a powerful and unbiased approach to organizing the function of genes[124], as has been comprehensively done in yeast[125,126]. For expanding to larger genomes, the number of gene–gene combinations grows exponentially — testing 20,000 genes pairwise is 400 million combinations — hence, this is an area where even one genome-wide screen would be a herculean effort and will certainly not be feasible on a routine basis. Thus, similar to the creation of custom pools when cell numbers are limiting, screens in pairwise space will generally require focused interrogation of curated gene sets.

Another exciting application for genetic screens is dissecting the mechanism of action of small molecules that score in phenotypic assays[40,127,128]. Certainly, proteomic approaches will remain powerful and necessary for ultimate identification of the direct target of a small molecule, but the relative ease of execution of genetic screens can inform on the pathways involved by identifying genes that, when modulated, either sensitize the cells to the small molecule or lead to resistance. Here, because small molecule screens are performed in multiwell formats, the assay will need to be reconfigured to be amenable to pooled screening; for viability screens, this is easily done, but for high-dimensional readouts such as imaging, finding and validating an appropriate surrogate that can be screened in a pooled format may represent a considerable challenge.

Paralogues
Two genes that are produced by a gene duplication event and that, owing to their shared sequence, may have the same or similar functions. Thus, loss of one of them is often insufficient to manifest a phenotype, as the other paralogue can compensate.

CRISPR technology is one of the most exciting developments in biology this decade, and the fact that nearly any laboratory can approach this technology is one of its greatest strengths. Yet challenges in uptake remain. Most of the new approaches with CRISPR are developed in easy-to-use cell culture systems, and these advances do not always port seamlessly to more complex model systems. Likewise, lentiviral delivery of perturbations to cells is still challenging in some cell types, and some cellular models are difficult to scale. Thus, getting off the ground with CRISPR screens in a favourite model system may proceed slowly at first in a way that is rarely captured in the narrative of published manuscripts but is nearly always part of the story. This perseverance will be rewarded, as the proven power of CRISPR technology to annotate gene function at scale promises to accelerate many research projects.

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Acknowledgements
The author thanks K. Donovan for assistance in manuscript preparation, M. Hegde for assistance with analysis and the entire Genetic Perturbation Platform (GPP) at the Broad Institute. For insightful discussions, the author thanks J. Arroyo, O. Parnas and Z. Tothova (Broad Institute); J. Listgarten and N. Fusi (Microsoft Research); C.Wilen and R. Orchard (Washington University); J. Klappenbach (Merck); L. Brody (Desktop Genetics); and M. Fan (Addgene). This work is dedicated to Francis Edward Sheehan.

Competing interests statement
The author declares competing interests. See Web version for details.

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