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Minireview

A roadmap toward genome-wide CRISPR screening throughout the organism

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https://doi.org/10.1016/j.xgen.2025.100777

Genome-wide CRISPR screening in the organism has tremendous potential to answer long-standing questions of mammalian physiology and disease. However, bringing this powerful technology *in vivo* presents unique challenges, including delivering a genome-wide sgRNA library to the appropriate cell type, achieving sufficient coverage of the library, and selecting for the phenotype of interest. In this review, we highlight recent advances in sgRNA delivery, library design, and phenotypic readout that can help overcome these technical challenges and thereby bring high-throughput genetic dissection to an increasing number of tissues and questions. We are excited about the potential for ongoing innovation in these areas to ultimately enable genome-wide CRISPR screening in any cell type of interest in the organism, allowing for unprecedented investigation into diverse questions of mammalian physiology and disease.

INTRODUCTION

Mammalian physiology and disease is best studied in its native context: the organism. In this regard, mouse models offer many physiologic parallels to humans alongside improved experimental tractability relative to non-human primates. However, comprehensive and unbiased genetic dissection in mice has historically been limited by the availability of high-throughput tools. For decades, investigating genotype-phenotype relationships in mice required generating single-gene knockout animals, effectively precluding unbiased, high-throughput investigation of phenomena in the organism. Fortunately, over the last decade, CRISPR has revolutionized our capacity to perform high-throughput genetic manipulation, including genome-wide screens, in mammalian cells.[1,2] This powerful technology can be extended to mouse models, and indeed there is now precedent for performing genome-wide CRISPR screens directly within mouse tissues.[3–5] These recent advances raise the exciting possibility of bringing high-throughput functional genomics to any cell type of interest in the organism, offering unprecedented investigative power to diverse questions of mammalian physiology and disease.

Early *in vivo* genetic screens using various genetic perturbation strategies demonstrate the exceptional power of unbiased, genome-wide screening in the organism. Prior to the advent of CRISPR-based screens, Beronja et al. performed a genome-wide RNA interference screen in mouse skin, discovering several unexpected genes playing key roles in both skin cancer oncogenesis and healthy epidermal development.[6] Following the development of CRISPR screening *in vitro*, Wertz et al. performed a genome-wide CRISPR screen in the mouse central nervous system (CNS) to investigate genetic regulators of CNS development and genetic dependencies in Huntington's disease.[4] Together, these flagship studies established the possibility for genome-wide screens in mouse tissues and the unique capacity of such techniques to reveal novel and physiologically relevant regulation of mammalian phenomena. To identify novel genetic dependencies at this scale, however, these pioneering screens required cohorts of at least several dozen mice. More recently, genome-wide screens have been successfully performed in smaller experimental cohorts. For example, Noguchi et al. performed a genome-wide screen in the testis, discovering novel regulators of mouse spermatogenesis in a cohort of only 11 mice.[3] Additionally, we recently demonstrated the ability to perform a genome-wide CRISPR screen in a single mouse liver, identifying genetic determinants of cell fitness that had not been observed in cell culture.[5] Collectively, these examples underscore the power of *in vivo* genome-wide screens to reveal novel insights into previously inaccessible questions and highlight the growing potential to expand this technology across cell and tissue types in a manner that is accessible to diverse research contexts.

Although this recent progress brings the promise of accessible genome-wide screening throughout the mouse into the realm of possibility, a handful of technical hurdles must be overcome to make this a reality. A typical genome-wide screen involves delivering a CRISPR effector and genome-wide sgRNA library to a cell type of interest, applying a selective pressure to enrich for a desired phenotype, and comparing sgRNA representation in cells with and without that phenotype. Bringing this approach into the organism presents unique challenges: namely, delivering sgRNAs to the cell type of interest, achieving sufficient coverage of sgRNAs at genome scale, and selecting for the phenotype of interest. In this review, we will discuss recent advances and ongoing efforts to overcome these challenges and bring the power of *in vivo* genome-wide screening to a growing number of cell types, contexts, and questions.

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Table 1. Possible mechanisms for delivering an sgRNA library *in vivo*

Delivery mechanism	Targetable cell types	Advantages	Disadvantages
Lentivirus	liver (high efficiency),[5] CNS (low efficiency),[4] mammary (low efficiency)[12]	rapid and stable sgRNA expression; large packaging size (8–10 kb)	difficult to specifically target most extrahepatic sites at high efficiency
AAV (±transposon)	liver,[15] CNS,[16,17] T cells,[18] muscle,[19] more[20]	broader tropisms; when coupled with a transposon, stable sgRNA expression is possible	without transposon-mediated integration, sgRNA expression may be transient; sgRNA expression may be slow; small packaging size (5–6 kb)
Direct delivery of sgRNA with transposon	liver[21]	efficient delivery to liver with stable sgRNA expression; eliminates need for viral vector	limited to hepatic delivery; requires hydrodynamic tail vein injection

ACHIEVING EFFICIENT sgRNA DELIVERY

The first step toward performing a genome-wide screen in the mouse is delivering a CRISPR effector and a genome-wide sgRNA library to the specific cell type under investigation. An array of CRISPR effectors have been established for distinct genetic perturbations, including Cas9 and Cas12a to knockout genes or modified endonuclease dead Cas9 (dCas9) variants to alter gene expression. Fortunately, there are now a variety of commercially available transgenic mice that can constitutively or inducibly express CRISPR effectors including Cas9,[7] Cas12a,[8] dCas9 fused to transcriptional activators,[9] and dCas9 fused to transcriptional repressors.[9] The increasing availability of these transgenic mice can simplify the delivery challenge to only sgRNAs.

The current gold standard for Cas9-based knockout screening *in vitro* is to have at least four unique sgRNAs targeting each gene to ensure each gene is effectively perturbed. To resolve true hits from background noise, each unique sgRNA is traditionally represented—or "covered"—in at least 250 cells (250× coverage).[10] That said, retrospective down-sampling analysis in a recent T cell screen suggests that fitness phenotypes may be resolvable at less than 250× coverage.[11] The minimum coverage required is likely to be specific to each screen, driven by screen-specific parameters such as the heterogeneity of the cell population and the strength of phenotypic selection. If we assume a coverage requirement of 250× and a genome size of 20,000 genes, performing a genome-scale screen requires delivering sgRNAs to 20 million cells, a nontrivial number to target *in vivo*. Moreover, these sgRNAs are ideally delivered to the organism in a manner that specifically targets the cell type of interest and persists in the target cell for the duration of the screen. Fortunately, these technical challenges are benefiting from widespread efforts to improve transgene delivery in the organism for both research and therapeutic purposes. Ongoing innovation in delivery methods is expanding our capacity to deliver sgRNAs effectively to different cell types in the mouse, increasing the tissue types and phenotypic contexts amenable to *in vivo* genome-wide screening.

Lentiviral vectors, the preferred delivery modality for *in vitro* genome-wide CRISPR screens, have the advantage of integrating sgRNAs into the target cell genome, thereby ensuring the persistence of sgRNAs throughout the screen. Traditional lentiviral vectors pseudotyped with the vesicular stomatitis virus glycoprotein (VSVG) can efficiently target hepatocytes upon intravenous delivery and select other cell types upon intraorgan injection[4,5,12] (Table 1). However, delivering VSVG-pseudotyped lentivirus to most extrahepatic sites at the efficiencies required for genome-wide screening remains challenging. Ongoing efforts to engineer the lentiviral envelope to target other cell types in the organism offers the promise of efficiently and stably delivering genome-scale sgRNA libraries to a broader range of cell types.[3,13,14]

Adeno-associated viral vectors (AAVs) can target a wider range of cell and tissue types compared to lentiviral vectors (Table 1). However, upon delivery, the AAV genome typically remains episomal and does not replicate during cell division.[22] As such, sgRNAs delivered via AAV can be lost over time, especially when introduced into highly proliferative cells. Additionally, AAV vectors can sometimes take weeks to achieve maximal expression, limiting their applicability in screens where immediate sgRNA expression is necessary. To resolve the issue of sgRNA persistence, Ye et al. engineered a hybrid system which packages both an sgRNA and a transposon into an AAV vector, enabling persistence of the sgRNA via transposon-mediated genome integration.[18] Additionally, Zheng et al. performed a screen of 86 unique AAV vectors and identified particular serotypes that achieve more rapid (days rather than weeks) transgene expression, results which work in tandem with transposon-mediated genome integration for rapid, long-lasting expression.[16] Like lentiviral vectors, several groups are working to expand the tropism of AAVs even further by engineering the viral capsid.[23–27] Collectively, these recent and ongoing innovations bolster the potential for AAVs to offer a realistic path toward expanding genome-wide screening to other tissues.

In some cases, sgRNA delivery can be achieved without viral vectors. Several groups have delivered sgRNAs stably to hepatocytes by hydrodynamic tail vein injection of a transposable element containing sgRNAs alongside a transposase[21,28,29] (Table 1). Although this method is currently limited to the liver, it highlights the possibility of delivering sgRNAs independent of traditional viral vectors.

Delivering CRISPR effectors and sgRNAs exclusively to the cell type of interest is essential for effective screening, as bystander perturbations in surrounding cell types can obscure phenotype readouts. Improvements in delivery modalities will continue to benefit screen resolution in this way. However, in cases where specific sgRNA delivery is not yet possible, a variety

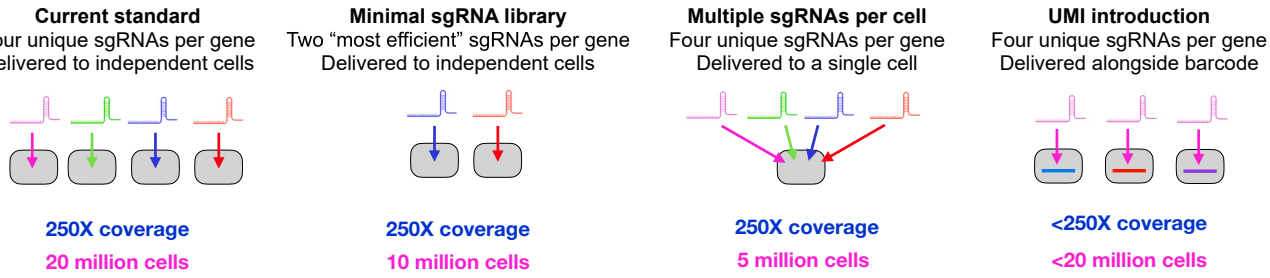

Figure 1. Recent developments to reduce the number of cells required for a genome-wide CRISPR screen

The approximate coverage required per sgRNA is written in blue. The estimated cell number required for a genome-scale screen is written in magenta.

of transgenic approaches are available that can effectively restrict the delivery or expression of either the CRISPR effector or sgRNA to a cell type of interest.[30–32]

OPTIMIZING sgRNA COVERAGE

Despite significant improvements in sgRNA delivery, delivering sgRNAs to the cell type of interest at the coverage required for genome-wide screening remains a key barrier to applying this technology throughout the organism. Moreover, even if sgRNAs could be delivered to every single cell, some cell types simply do not exist in the necessary numbers in a single animal. These realities motivate the search for means of reducing the number of cells required for an effective genome-wide screen. One straightforward approach is to expand the screen across multiple mice. This can be achieved either by dividing the genome-wide library into sub-libraries, each of which can be sufficiently covered in a single animal, or by delivering the same genome-wide library across multiple individuals.[11] However, this solution increases cost and introduces organism-to-organism variation.

An alternative means of reducing the number of cells is to decrease the number of unique sgRNAs per gene in the library (Figure 1). By leveraging the growing pool of data from hundreds of recent *in vitro* CRISPR screens, researchers have optimized sgRNA library design through selection of sgRNAs with robust efficacy across contexts. By incorporating this information about sgRNA efficiency, researchers have reduced the number of unique sgRNAs per gene to as few as two. These minimal sgRNA libraries can reduce the number of cells required by over 50% while still retaining the capacity to identify hits.[33] This approach presents a promising potential for improving screen throughput, but it remains to be independently verified in diverse screening contexts.

Alternatively, it is possible to consolidate all sgRNAs targeting a gene into a single construct that targets a single cell (Figure 1). To achieve this, Anvar et al. leveraged and optimized an alternative CRISPR system, Cas12a in4mer, in which multiple sgRNAs can be expressed from a single promoter as an array.[34] Their Cas12a in4mer platform combines four sgRNAs targeting any given gene into a single array that is introduced into a single cell, substantially reducing library size and decreasing the number of cells that must be targeted 4-fold. Further work is required to assess the relative levels of off-target effects and DNA damage caused by introducing multiple guides in a cell relative to the traditional approach using Cas9 and a single sgRNA. In a similar study performed with a Cas9 effector, Arnan et al. suggest that targeting a single cell with two sgRNAs also enables higher Cas9 editing efficiency, offering an alternative method to decrease the necessary cell numbers for those with preference toward the more widely studied Cas9 enzyme.[35]

Another way to potentially decrease the number of cells required for a screen is to improve the resolution of phenotypic effects by incorporating information about each occurrence of any given sgRNA sequence. By introducing random nucleotide barcodes, or unique molecular identifiers (UMIs), nearby the sgRNA sequence in the library, one can resolve the performance of any given sgRNA across the different cells into which it integrated (Figure 1). The resolution of individual integration events can improve the ratio of signal to noise and offers robustness against population bottlenecks and cell-cell heterogeneity.[11,36] The ability of UMIs to improve screen resolution makes it theoretically possible to lower sgRNA coverage without compromising screen performance.

CHOOSING A PHENOTYPIC READOUT

Assuming that efficient sgRNA delivery can be achieved for a cell type of interest, one must then determine the proper phenotype readout for the specified question. Recent advances have made it possible to select for a variety of phenotypes in the organism (Figure 2). Perhaps the most common and straightforward approach is assessing cellular fitness over a given period of time or in the setting of a specific perturbation, which is easily assessed by comparing sgRNA abundance in the population of cells before and after the screen.[4,5] Alternatively, one can select for single-cell phenotypes such as RNA or protein expression through *in situ* hybridization or immunostaining followed by fluorescence-activated cell sorting.[37] While both abovementioned approaches offer the benefit of throughput, they are limited in the information offered about each genetic perturbation. To increase the information gleaned from each genetic perturbation *in vivo*, Jin et al. pioneered *in vivo* Perturb-seq, which employs single-cell RNA sequencing to identify transcriptomic changes caused by each sgRNA.[17] Beyond transcriptomic states, new techniques aim to harness the spatial information that is uniquely preserved in *in vivo* screening. It is now possible to perform optical screening—where sgRNAs are identified through *in situ* hybridization or sequencing approaches—in tissues. This approach offers a promising avenue

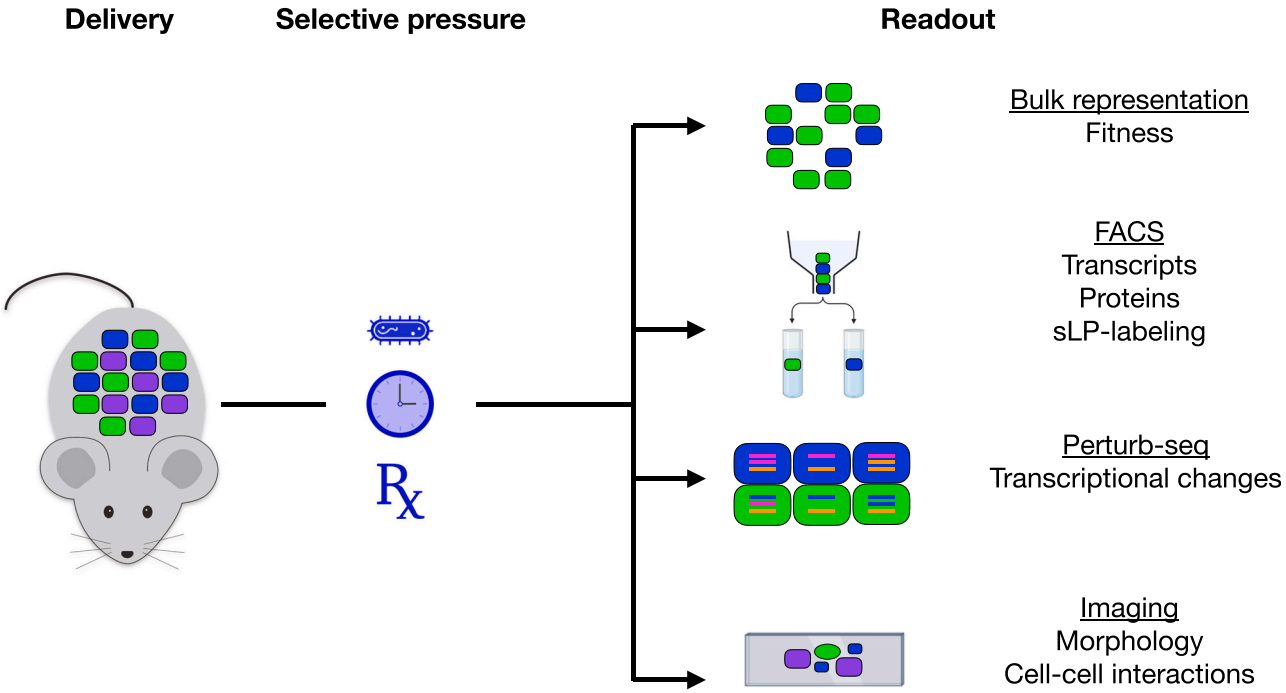

Figure 2. Summary of phenotypic readouts possible for an *in vivo* CRISPR screen

to evaluate multiple image-based phenotypes from perturbed cells while preserving their native morphology and evaluating their extracellular context.[38] Going beyond cell-autonomous phenotypes, it is possible to screen for non-autonomous phenotypes by implementing reporters that can be transferred between cells, using technology like the sLP-labeling system.[39] This creative approach was recently leveraged to study the role of host tissue factors in metastatic seeding.[40]

OUTLOOK

Genetic screening in the organism enables investigation into diverse questions of mammalian physiology and disease with the benefits of both preserved physiologic context and experimental throughput. Although targeted screening approaches can investigate genes predicted to be associated with a given phenotype and do not require as many cells for sufficient coverage, such approaches rely on existing knowledge and may overlook important but unexpected mechanisms. In contrast, genome-wide screens benefit from an unbiased and comprehensive approach, eliminating the need for prior knowledge and enabling unanticipated discoveries. Although the organism presents unique challenges for genome-wide screening, recent developments and ongoing work toward improving sgRNA delivery, decreasing sgRNA library size, and improving screen resolution are making these screens possible in a growing number of cell types and contexts. The ongoing development of new approaches for phenotypic selection are constantly expanding the types of questions that can be asked. Additionally, modifying sgRNA libraries to perturb separate genes and/or paralogs within the same cell provides the exciting opportunity to investigate genetic interactions and redundancy within the organism.[34,41] By building on recent progress with continued innovation, we envision a future where high-throughput functional genomics can be applied in any cell type and context in the mouse, empowering unprecedented genetic dissection of myriad questions of organismal physiology and disease.

ACKNOWLEDGMENTS

Figures were created using BioRender. T.K.F. is supported by funding from the National Science Foundation Graduate Research Fellowship Program (2141064). Any opinions, findings, and conclusions or representations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. Work in the Knouse laboratory has been supported by the National Institutes of Health NIH Director's Early Independence Award (DP5-OD026369) to K.A.K., the MIT Stem Cell Initiative, and the National Cancer Institute Koch Institute Support Grant (P30-CA14051).

DECLARATION OF INTERESTS

K.A.K. is a co-inventor on a patent filed by the Whitehead Institute related to our publication that we cited in this manuscript.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xgen.2025.100777.

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