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## Cancer CRISPR screens *in vivo*

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### Abstract

CRISPR screening is a powerful toolset for investigating diverse biological processes. Most CRISPR screens to date have been performed with *in vitro* cultures or cellular transplant models. To interrogate cancer in animal models that more closely recapitulate the human disease, autochthonous direct *in vivo* CRISPR screens have recently been developed that can identify causative drivers in the native tissue microenvironment. By empowering multiplexed mutagenesis in fully immunocompetent animals, direct *in vivo* CRISPR screens enable the rapid generation of patient-specific avatars that can guide precision medicine. This review discusses the current status of *in vivo* CRISPR screens in cancer, and offers perspectives on future applications.

### Keywords

CRISPR screen; *in vivo*; functional genomics; cancer

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### Glossary Notes

Certain definitions are general concepts, modified from original definitions.

### Conflicts of Interest

The authors have no conflicts of interest related to this review.

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## CRISPR-mediated genome editing meets the complexity of the cancer genome

Over the past decade, tremendous efforts have been devoted to profiling patient cancers. Multi-institutional consortia such as The Cancer Genome Atlas have now profiled over 10,000 tumors, generating petabytes of high-dimensional data that illuminate the complexities of several cancer types [1]. Although the molecular portraits of cancer are now in higher resolution than ever before, the path to clinical translation for many cancer types still remains largely unexplored.

The central issue is that cancer genomics can inform what mutations are present, but has limited power to indicate which ones are functionally important [2,3]. Individual tumors often have hundreds, if not thousands, of molecular aberrations. While some of these alterations are found in well-established oncogenes and tumor suppressors, many novel aberrations occur in previously uncharacterized or even unannotated regions, making it difficult to reliably discern whether they are actually driving the progression of a given cancer. Furthermore, different patients can present with unique combinations of these various mutations that can drastically influence a cancer's growth pattern, tendency to metastasize, and susceptibility to therapy.

CRISPR-mediated genome editing has become a powerful tool in cancer biology due to its programmability and flexibility [4]. In addition to its canonical use for targeted gene knockouts [5,6], CRISPR has also been reengineered for a variety of purposes including transcriptional activation [7,8], transcriptional repression [9], histone modification [10], base editing [11,12], DNA methylation [13–15], and genome architecture manipulation [16]. Following in the footsteps of RNA interference (RNAi) screens, the modularity of CRISPR has naturally lent itself to high-throughput screening approaches [17]. By designing custom libraries of single guide RNAs (sgRNAs), one can simultaneously screen large collections of genomic elements (coding genes, regulatory elements such as enhancers, non-coding RNAs, or other non-coding features) in a given biological context [18]. Here, we review the development and application of *in vivo* CRISPR screens.

### ***In vivo* CRISPR screens to interrogate the complexities of cancer**

CRISPR screens were first developed by using pooled sgRNA libraries to target all annotated genes in the human genome [19,20]. High-throughput, and particularly genome-wide, CRISPR screens combine the power of forward genetics and reverse genetics. Such approaches offer an unbiased, yet precisely targeted method of identifying genes that contribute to a phenotype, such as cancer progression. Compared to random mutagenesis, CRISPR screens use customized sgRNA libraries, while simultaneously preserving the “randomness” during selection. Unlike RNAi screens, CRISPR generates precise mutations or complete knockouts instead of partial gene knockdown or silencing, which has been shown to facilitate higher between-construct concordance and lower off-target rates [21,22]. CRISPR screens have thus become the state-of-the-art approach for discovery of genetic drivers and phenotypic modulators in many biological contexts.

In cancer, CRISPR screens have been performed to identify genes involved in a wide variety of processes [23], including regulators of drug resistance [24–28], synergistic and synthetic lethal interactions [29,30], regulators of PD-L1 expression [31], and essential genes [32–34] (Figure 1). While *in vitro* studies are valuable for identifying cell-intrinsic properties of cancer cells and potential therapeutic windows (Figure 2A, Key Figure), they cannot address problems involving complex interactions between multiple cell types that reflect the *bona fide* nature of cancer as an organ, instead of a collection of isolated tumor cells [35]. As highlighted by recent advances in cancer immunotherapy, the microenvironmental milieu is constantly engaged in conversation with tumor cells, with significant clinical consequences [36–40]. To more faithfully recapitulate the human disease for enhanced translational accuracy, it is essential to investigate these phenomena *in vivo* where cancers can develop within the context of a tissue microenvironment.

To date, virtually all *in vivo* CRISPR screens have aimed to investigate phenotypes of cancer, directly demonstrating the power and simplicity of this technology in oncology. The first *in vivo* CRISPR screen investigated annotated genes in the mammalian genome and their potential to promote tumor growth and metastasis upon mutagenesis [41]. In this study, the authors introduced a genome-wide sgRNA library into a non-metastatic cancer cell line. The pool-mutagenized cell library was then subcutaneously transplanted into the back skins of nude mice, and monitored for metastasis to the lung. By sequencing the sgRNAs present in the metastases, the authors identified and subsequently validated a panel of hits from the initial screen that functionally drove lung metastasis. Multiple *in vivo* CRISPR screens have since been performed to identify tumor suppressors [42–44], oncogenes [45], synthetically lethal genes [46], and regulators of cancer immunotherapy, both in two-cell type (2-CT) co-culture systems [47] and transplant tumor models [48], among others. A common thread in these studies is their two-step workflow: the sgRNA library is first introduced to cells in culture, followed by transplantation into mice to assess phenotypes *in vivo*. After a selection phase (for instance, expression of a reporter, outgrowth of a tumor, resistance to therapy), the cells are sequenced to identify enriched and/or depleted sgRNAs (Figure 2B).

### Limitations of transplant-based *in vivo* CRISPR screens

By virtue of having a microenvironment, in comparison with *in vitro* CRISPR screens, it is anticipated that *in vivo* studies more faithfully model carcinogenesis as it occurs in humans. However, transplant-based *in vivo* screens have significant limitations. First, introducing large numbers of cancer cells into mice clearly does not resemble the normal process of tumorigenesis. Second, transplants are commonly performed subcutaneously, rather than orthotopically in the relevant organs. In addition, immunodeficient mice are often used to promote the grafting efficiency of the mutagenized cell library, limiting the applicability of these models for investigating cancer-immune interactions. Moreover, the engraftment efficiency of transferred cells can vary from cell line to cell line, and from host to host. Furthermore, there exist multiple cellular bottlenecks of tumor evolution *in vivo*, such as the circulation limit during the metastasis cascade. Finally, the native organ microenvironment casts numerous constraints on transplanted cells even in orthotopic settings. These limitations can affect the preclinical utility of cancer-cell transplant-based *in vivo* CRISPR screens.

## Direct *in vivo* CRISPR screens to more faithfully recapitulate human cancers

To better model the natural context of cancer as it occurs in humans, the following features can provide guiding principles for more accurate *in vivo* CRISPR screen in cancer: 1) the tumors being modeled derive from the endogenous target tissue; 2) the immune system remains intact; and 3) the corresponding tissue microenvironment is preserved. These challenges can be overcome by directly mutagenizing target tissues *in vivo*, rather than using a transplant approach.

A number of studies have demonstrated efficient CRISPR-mediated *in vivo* mutagenesis directly at the target organ site. In the liver, hydrodynamic injection of sgRNA-containing plasmids into the tail veins of Cas9 mice was sufficient to induce multiplexed mutagenesis in hepatocytes [49], while in the lung, intratracheal delivery of sgRNA-carrying lentiviruses was able to induce mutagenesis directly in Cas9-expressing lung epithelial cells [50]. Through the use of adeno-associated viruses (AAVs), direct mutagenesis in the mouse brain was also shown to be feasible [51]. Because Cas9 and other genome-editing RNA-guided nucleases (RGNs) are large proteins, generation of Cas9 transgenic animals [52–54] simplifies the delivery of CRISPR components and facilitates direct *in vivo* mutagenesis.

Extending on these direct *in vivo* CRISPR techniques, autochthonous CRISPR screens of varying scales have now been performed in a few organ systems (Figure 2C). Hydrodynamic injection of plasmids with sgRNA and Cas9 expression cassettes flanked by *Sleeping Beauty* (SB) inverted repeats, along with a SB-transposase vector, has been utilized to screen 10 sgRNAs for their ability to induce CRISPR-mediated tumorigenesis in a *Kras*<sup>G12D</sup>-sensitized mouse liver [55]. As another approach, lentiviral pools were utilized to screen 11 sgRNAs for their ability to drive lung tumorigenesis in Cre-driven Cas9 mice [56]. Finally, stereotaxic delivery of an AAV sgRNA library directly into the mouse brain efficiently induced glioblastomas that recapitulate the pathologic features of the human disease [57], which enabled direct and quantitative assessment of the tumorigenicity of 280 sgRNAs targeting 56 genes. Of note, the relative mutant frequencies of these genes in the AAV-based model significantly correlated with the mutant frequencies observed in human glioblastoma cohorts. Delivery of AAV-CRISPR sgRNA pools intravenously followed by captured sequencing using customized probesets [57] or molecular inversion probe sequencing [58] allows autochthonous mapping of causative functional variants directly in the targeted genomic loci, as demonstrated in the brain [57] and liver [58]. Facilitated by the high efficiency of AAV-mediated transduction, various significantly co-occurring pairs were identified, thereby pinpointing synergistic driver pairs in tumorigenesis.

Nevertheless, direct *in vivo* approaches have their share of limitations. Optimal technical parameters that are easily achievable within *in vitro* or cell line transplant settings (i.e. “indirect” *in vivo*) such as library size, coverage and multiplicity of infection (MOI), are much more challenging with a direct *in vivo* approach. Therefore, the size of an sgRNA library must be controlled to ensure adequate coverage *in vivo*. With an oversized library, random sampling errors from low viral transduction rates will invariably lead to many spurious positive and negative “hits”. Other key constraints include the number of tumor-

originating cells in the native organ, accessibility of such cells due to complexity of cellular organization in endogenous tissues, uncharacterized or unknown cell-cell interactions, viral transduction efficiency, and immune rejection. To this end, it is worth noting that AAV-mediated approaches have the major advantage of higher titer, higher direct *in vivo* transduction efficiencies, and provoke minimal immune reactions [57], thus enabling larger CRISPR libraries to be screened more efficiently in an autochthonous setting. Since AAVs usually do not integrate into the genome, the relative abundance of sgRNAs within tumors cannot be ascertained by sgRNA cassette sequencing as is commonly done for lentiviral-based screens. Instead, the AAV-mediated approach requires capture sequencing of the sgRNA target regions in order to functionally extract the mutational signatures of the tumors.

## Direct *in vivo* CRISPR pooled mutagenesis technologies for precision medicine

With further improvements and modifications, we envision that direct *in vivo* CRISPR screens could be widely applied in multiple facets of precision medicine. Using the genomic information from a patient's tumor, a personalized CRISPR library could be readily designed to directly generate disease mimics, or cancers encompassing the same set of mutations, for investigating the behaviors of those mutations in mice (Figure 3A). As a proof-of-principle, high-titer AAV libraries have been successfully used to infect single cells at high MOI *in vivo*, leading to genetically complex tumors with several mutations [57,58]. This "mouse avatar" approach would enable robust enumeration of the tumorigenic potential of each mutation present within a patient's tumor. More importantly, rapid generation of such disease mimics allow robust therapeutic testing of approved or novel treatments in genetically matched tumors. This type of approach could then be utilized to predict the outcomes of treatments in patients, to potentially anticipate which specific tumors would exhibit sensitivity or resistance to chemo-, targeted- or immune- therapies, and thus be leveraged for prioritizing therapies.

It was recently demonstrated that patient-derived tumor xenografts (PDXs) adopt divergent evolutionary courses from the human primary tumor [59]. While this effect is likely due in large part to intrinsic differences between mice and humans, a key contributing factor may lie in the method, as xenografts intrinsically require immunodeficient hosts for successful engraftment. The development and application of humanized mice [60] has helped to bridge this gap in part, particularly with regards to innate immunity, though a complete humanization of the full immune system remains to be seen. In contrast, direct *in vivo* models are designed to originate from the autochthonous tissue site in fully immunocompetent mice. For these reasons, we anticipate that direct *in vivo* CRISPR screens will more faithfully recapitulate the behavior and evolutionary course of human cancers.

Intratumoral heterogeneity is increasingly being recognized as an important feature of human cancers, potentially contributing to drug resistance and relapse [61,62]. In addition to cell-type heterogeneity and microenvironmental variations, intratumoral heterogeneity is often characterized by distinct subclonal mutation signatures that differentially influence

cellular behavior [63]. To this end, AAV-mediated direct *in vivo* CRISPR screens are particularly well equipped to model the heterogeneity present within human cancers. As demonstrated in the brain and liver, AAV-CRISPR approaches can rapidly create genetically complex multi-clonal tumors within individual mice [57,58]. This immense heterogeneity inherent to AAV-mediated CRISPR screens could be exploited to investigate clonal dynamics in tumors, serving as a discovery platform for how tumor subclones interact in controlled experimental settings in otherwise wildtype organisms.

## Concluding Remarks

Recent development and applications of *in vivo* CRISPR screens have showcased their power for unbiased identification of functional genetic elements. Particularly, direct *in vivo* CRISPR screens offer a high-throughput strategy to interrogate candidate genes for their ability to drive tumorigenesis from the autochthonous tissue. Unlike *in vitro* or transplant-based approaches, direct *in vivo* CRISPR studies retain the endogenous tissue microenvironment and can be performed in fully immunocompetent animals at high efficiency. Future studies will need to explicitly evaluate whether direct *in vivo* CRISPR models better recapitulate human disease (see outstanding questions). A key experiment would be to compare the *in vivo* evolutionary trajectory of a primary patient tumor and the corresponding mouse avatar, with or without therapeutic selection pressure.

### Outstanding Questions

- Does a corresponding CRISPR-engineered mouse avatar mimic the drug response of a genetically matched tumor from a patient?
- Can *in vivo* CRISPR screens be effectively leveraged for the study of complex genetic interactions in cancer?
- How can direct *in vivo* CRISPR screens be efficiently coupled with other high-throughput approaches such as single-cell sequencing and high-content imaging to enable the dissection of highly heterogeneous cancers?
- Can direct *in vivo* CRISPR screens be utilized to deduce mutations that are associated with sensitivity to immunotherapy, as well as those conferring primary or acquired resistance?
- How can *in vivo* CRISPR screens be effectively used in immune cells to identify novel regulators of the tumor microenvironment?

The design philosophy behind most CRISPR screens is to study the effects of single gene mutations on a desired phenotype. However, many biological processes are driven by interactions between multiple genes. In cancer, the precise combinations of mutations within individual tumors can lead to strikingly diverse behaviors, influencing tumor aggressiveness and clinical response. In this regard, *in vivo* CRISPR screens can be readily applied to identify phenotypic modulators on specific sensitized backgrounds. For instance, direct *in vivo* CRISPR screens could be used to identify factors that influence the metastatic properties of mutant *Kras*-driven tumors. An exciting avenue for further work is the



adaptation of Cpf1 (CRISPR-associated endonuclease in *Prevotella* and *Francisella* 1) [64,65] to *in vivo* cancer modeling. Like Cas9, the Cpf1 RGN can be used for precisely targeted genome editing, yet it has unique multiplexing capabilities due to its independence from a tracrRNA [64,65]. Cpf1 has just begun to emerge for higher-dimensional genetic screening of tumor growth and metastasis *in vivo* [66], and yet to be more broadly applied for other aspects of cancer. The development of this technology would enable high-dimensional screening of different mutation combinations, potentially leading to the identification of novel synergistic or synthetically lethal genetic interactions in cancer (Figure 3B).

Additionally, the application of dCas9-activator mice could enable the functional identification of oncogenes directly *in vivo*, providing an orthogonal perspective to preexisting tumor suppressors screens [67]. Similarly, mice engineered to express CRISPR-targeted base editors might offer an approach to screen specific point mutations that drive tumorigenesis from the autochthonous tissue. It would also be interesting to apply single cell RNA-seq (scRNA-seq) to tumors generated by direct *in vivo* CRISPR screens [68–70]. Together, these technologies would enable the creation and subsequent dissection of cancer heterogeneity at ultra-high resolution (Figure 3C). To further provide spatial information, multiplexed fluorescent *in situ* hybridization (i.e. MERFISH) [71] could potentially be adapted for the purpose of sequencing sgRNA pools directly in tissue sections. These data would allow for matched comparisons between CRISPR-induced mutational signatures and histopathological phenotypes (Figure 3D).

Finally, direct *in vivo* CRISPR screens can be readily applied for studies in cancer immunity, as these models are fully functional in immunocompetent animals. A key question on the forefront of immuno-oncology is to understand why only a fraction of patients respond to immunotherapy, such as checkpoint inhibitors. Multiplexed AAV-CRISPR screens could be applied in the context of checkpoint blockade in order to deduce which mutations are associated with sensitivity to immunotherapy, as well as those conferring primary or acquired resistance. With further technological development, direct *in vivo* screens on primary immune cell populations may also become feasible, allowing for high-throughput interrogation of factors that regulate immune responses against tumors.

As these technologies continue to develop and mature, they can be adapted for personalized cancer modeling, tumor driver profiling, as well as the identification of novel and relevant therapeutic targets. Emerging new technologies such as direct *in vivo* CRISPR screens continue to transform oncology discovery.

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## Glossary Box

### Genome editing

Genome editing, or genome editing with engineered nucleases (GEEN), is a type of genetic engineering in which DNA is inserted, deleted or replaced in the genome of an organism using engineered nucleases, or “molecular scissors.”

### CRISPR

Clustered regularly interspaced short palindromic repeats (CRISPR) are segments of prokaryotic DNA containing short repetitions of base sequences. Each repetition is followed by short segments of “spacer DNA” from previous exposures to a bacteriophage virus or plasmid. The CRISPR/Cas system is a prokaryotic immune system that confers resistance to foreign genetic elements such as those present within plasmids and phages. The Cas protein(s) use the CRISPR spacers to recognize and cut these exogenous genetic elements in a manner analogous to RNA interference in eukaryotic organisms. By delivering the Cas9 nuclease and appropriate guide RNAs into a cell, the cell’s genome can be cut at a desired location, allowing existing genes to be removed and/or new ones added.

### Cas9

An endonuclease that is directed to specific sites in the genome by CRISPR spacers, where it induces double stranded breaks in the target DNA.

### dCas9

Catalytically dead Cas9 nuclease that cannot generate double stranded breaks in DNA. dCas9 is commonly tethered to other proteins to enable programmable targeting of the tethered enzymes.

### gRNA

Guide RNA, a short synthetic RNA composed of a “scaffold” sequence necessary for Cas9-binding and a user-defined ~20 nucleotide “spacer” or “targeting” sequence which defines the genomic target to be modified.

### sgRNA

Single-guide RNA, a term generally interchangeable with gRNA in genome editing with CRISPR/Cas9 system.

### crRNA

CRISPR RNA, the short RNA sequence that directly guides CRISPR nucleases to target sites in the genome that is independent of the scaffold sequence present in full-length sgRNAs.

### Cpf1: CRISPR-associated endonuclease in *Prevotella* and *Francisella* 1

Cpf1 (also known as Cas12a) is a CRISPR-guided endonuclease that can be utilized for targeted genome editing in diverse species. Unlike Cas9, Cpf1 does not require a tracrRNA for DNA cleavage, and has the capability to autonomously process crRNA arrays.

### *In vivo* CRISPR screen



By generating libraries of sgRNAs targeting different genes, a CRISPR screen can be performed to assess the importance of these genes towards a given phenotype. With *in vivo* CRISPR screens, the selection phase occurs inside of a living organism, for instance a mouse.

**Driver mutation**

A driver mutation is causally implicated in oncogenesis. It confers a growth advantage on the cancer cell and is favored in the microenvironment of the tissue in which the cancer arises.

**Oncogene**

An oncogene is a gene that has the potential to cause cancer. In tumor cells, oncogenes are often mutated or expressed at high levels.

**Tumor suppressor gene**

A tumor suppressor gene, or anti-oncogene, is a gene that protects a cell from one step on the path to cancer. Mutations that inactivate tumor suppressors will contribute to cancer progression.

**Double knockout CRISPR screen**

A CRISPR screen in which two genes are simultaneously interrogated, rather than a single gene.

**High-dimensional genetic screen**

A CRISPR screen in which three or more genes are simultaneously interrogated, enabling the investigation of complex multigenic phenotypes.

**Reporter gene**

A gene that researchers attach to a regulatory sequence to facilitate readout of gene regulatory activity. Common examples include green fluorescent protein (GFP) and firefly luciferase.

**Synthetic lethality**

Synthetic lethality arises when a combination of mutations in two or more genes leads to cell death, whereas a mutation in only one of these genes does not. In a synthetic lethal genetic screen, it is necessary to begin with a mutation that does not kill the cell, although may confer a phenotype (for example, slow growth), and then systematically test other mutations at additional loci to determine which confer lethality. Synthetic lethality indicates functional relationships between genes.

**Tumor microenvironment**

The stroma and supporting milieu surrounding the tumor, usually consisting of fibroblasts, immune cells and endothelial cells.

**Tumor immunity**

Tumor immunity refers to the interaction between the host immune system and a tumor. In different contexts, the immune system can have opposing roles towards cancer progression.

Cancer immunotherapies aim to tip the scales towards immune-mediated elimination of the tumor.

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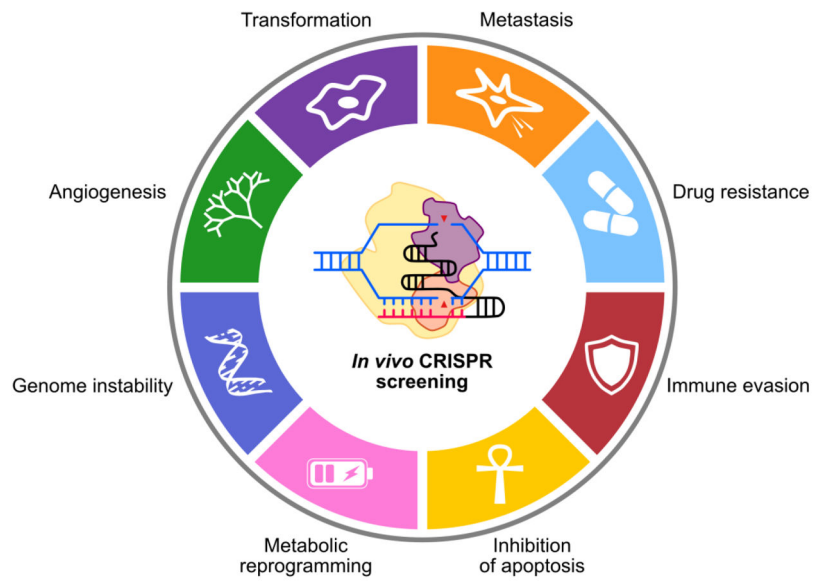
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**Trends Box**

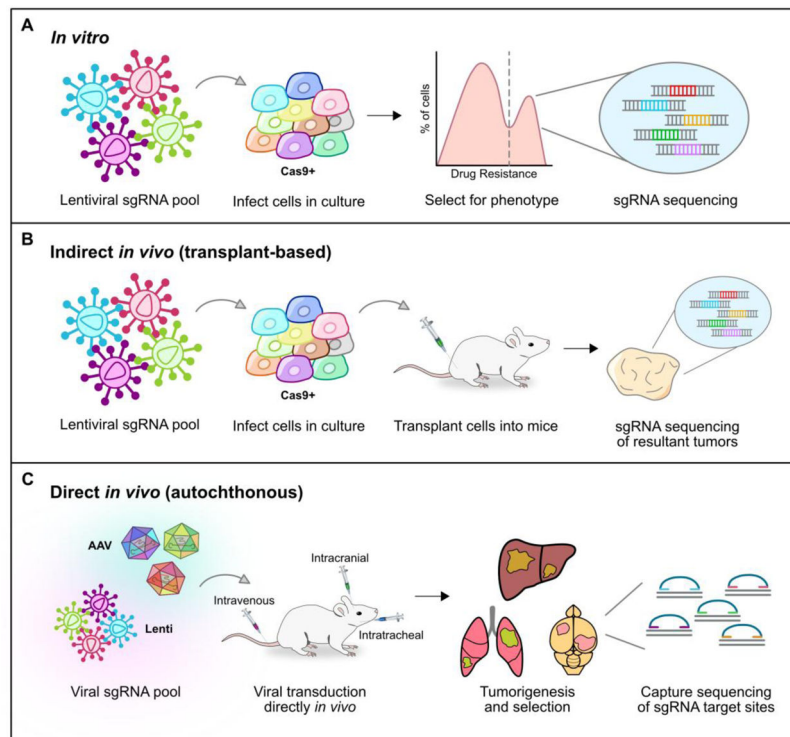
- *In vivo* CRISPR screens enable high-throughput interrogation of complex processes in cancer.
- Direct autochthonous models recapitulate human cancer by maintaining the native microenvironment.
- Direct *in vivo* CRISPR technologies can empower patient-specific cancer modeling for precision medicine.



**Figure 1. Functional cancer genomics with *in vivo* CRISPR screening**

*In vivo* CRISPR screening is a powerful, flexible tool to dissect important processes in cancer. Genome-wide CRISPR screens have illuminated novel regulators of metastasis, malignant transformation, immune evasion, and drug resistance. Moving forward, *in vivo* CRISPR screens may improve our understanding of other processes, such as angiogenesis, genome instability, and metabolic programming.



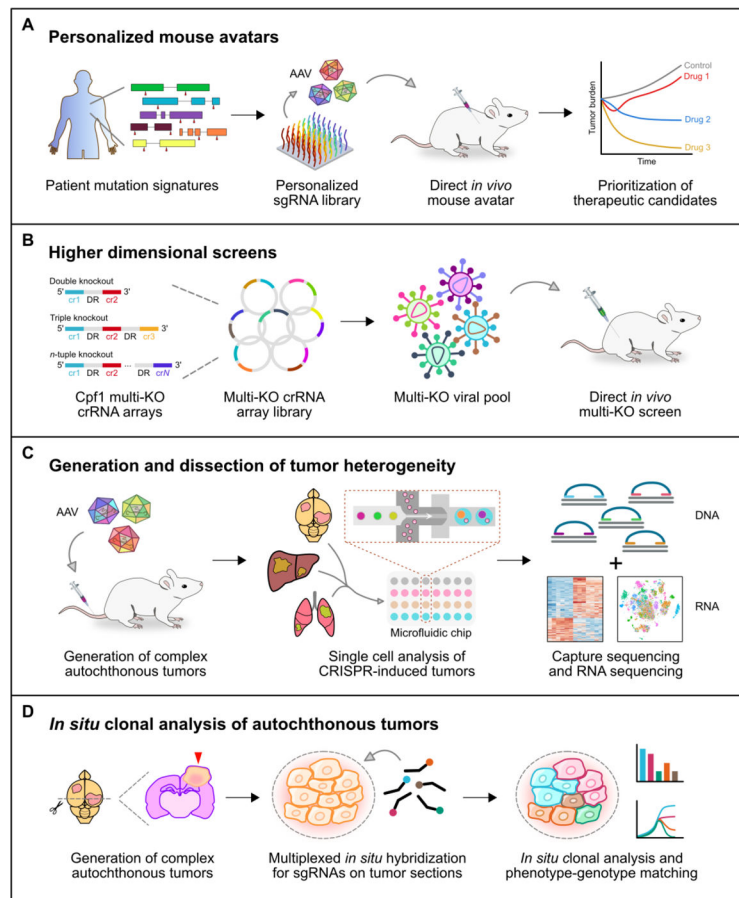


**Figure 2 Key Figure. Three modes of CRISPR screening**

**A.** To perform an *in vitro* CRISPR screen, the desired sgRNA library must first be cloned into expression vectors. Lentiviral vectors are commonly used, as they can stably integrate into the host genome. After a selection phase to enrich for a desired phenotype, the sgRNA cassettes are amplified from genomic DNA and sequenced to identify the top candidate genes.

**B.** Indirect *in vivo* screens follow the same steps as *in vitro* studies, but the selection phase occurs within a recipient animal. Following transplantation of the mutagenized cell pool into mice, different mutants will become enriched. In the case of a tumorigenesis screen, highly abundant sgRNAs within the resultant tumors would be brought forward as candidate tumor suppressors.

**C.** For direct *in vivo* screens, CRISPR mutagenesis occurs at the autochthonous target organ site instead of in culture. Lentiviral and adeno-associated viral (AAV) approaches have both been successfully used for multiplexed direct *in vivo* mutagenesis. Intravenous, intracranial, and intratracheal viral injections can drive tumorigenesis from the liver, brain, and lung, respectively. Since AAVs do not integrate into the genome, capture sequencing must be performed to readout the results of the screen (i.e. highly abundant indel variants).



**Figure 3. Applications and extensions of direct *in vivo* CRISPR screens**

**A.** Direct *in vivo* CRISPR screens can be readily used to generate personalized mouse avatars. CRISPR libraries can be customized to the mutations present within a given patient's tumor. Following autochthonous mutagenesis, therapeutic candidates can be evaluated using the mouse avatars, informing clinical decision-making.

**B.** Higher dimensional screens (i.e. double, triple, *n*-tuple knockouts) using CRISPR-Cpf1 offer an elegant high-throughput approach to investigate genetic interactions. Such studies could uncover synergistic driver mutations and synthetically lethal combinations, which may help to inform patient prognostication and to identify novel therapeutic vulnerabilities.

**C.** Direct *in vivo* CRISPR mutagenesis drives the formation of genetically complex multi-clonal tumors. Coupled with single-cell capture sequencing and RNA sequencing, direct *in vivo* CRISPR screens offer a powerful approach for the generation and subsequent dissection of tumor heterogeneity.

**D.** *In vivo* CRISPR screens thus far have lacked spatial resolution, as sgRNA sequencing and/or capture sequencing is performed on genomic DNA extracted from dissociated cell suspensions. By combining direct *in vivo* CRISPR mutagenesis with multiplexed *in situ* hybridization, it may be feasible to perform clonal analysis and phenotype-genotype matching on tissue sections.