

Critical cancer vulnerabilities identified by unbiased CRISPR/Cas9 screens inform on efficient cancer Immunotherapy

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Keywords: CRISPR, Screening, Disease Models, Cancer, Immunotherapy

## **ABSTRACT**

The mutational landscape of human cancers is highly complex. While next-generation sequencing aims to comprehensively catalogue somatic alterations in tumour cells, it fails to delineate driver from passenger mutations. Functional genomic approaches, particularly CRISPR/Cas9, enable both gene discovery and annotation of gene function. Indeed, recent CRISPR/Cas9 technologies have flourished with the development of more sophisticated and versatile platforms capable of gene knockouts to high-throughput genome wide editing of a

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1002/eji.202048712](https://doi.org/10.1002/eji.202048712).

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single nucleotide base. With new platforms constantly emerging, it can be challenging to navigate what CRISPR tools are available and how they can be effectively applied to understanding cancer biology. This review provides an overview of current and emerging CRISPR technologies and their power to model cancer and identify novel treatments. Specifically, how CRISPR screening approaches have been exploited to enhance immunotherapies through the identification of tumour intrinsic and extrinsic mechanisms to escape immune recognition will be discussed.

## **INTRODUCTION**

Global consortiums have been established to profile all somatic alterations in human cancers by deep-sequencing technologies [1-4]. Whilst these studies have been transformative in identifying the genetic landscape of human cancers, they cannot distinguish driver from passenger mutations, the latter of which fail to confer a fitness advantage to cancer cells. Alternatively, functional genomic approaches employing diverse technologies, such as CRISPR/Cas9, have been broadly applied in cancer research to understand the functional consequences of specific genetic perturbations [5]. Due to its ease to use and high efficiency, CRISPR can be applied in many cancer contexts. An array of novel CRISPR techniques have rapidly become available enabling precise, robust and versatile genetic modulations, ranging from knockout of an individual gene, to the precise editing of a single nucleotide base (**Table 1**). Together, these sophisticated systems have greatly extended our understanding of critical cancer pathways. Importantly, the scalability of CRISPR techniques enable the high-throughput identification of genes and pathways responsible for tumour initiation and growth, but also genes mediating or reversing therapy resistance. Moreover, CRISPR can be exploited to understand and potentially improve cancer immunotherapy by identifying genes in the malignant or immune cell that enhances the recognition and hence killing of the tumour cells themselves (reviewed in [6]). This review provides an overview of current and emerging CRISPR technologies and their effectiveness in modelling and identifying novel tumour drivers (oncogenes and tumour suppressors), which will aid to improve cancer treatments, in particular immunotherapies.

### **Identifying novel tumour suppressive and oncogenic genes using CRISPR**

### ***Tumour suppressor gene discovery with CRISPR/Cas9 and CRISPR inhibition***

The CRISPR/Cas9 system was revolutionary in facilitating the identification of tumour suppressor genes (TSGs). Non-functional proteins can be produced through the Cas9 endonuclease catalysing a DNA double stranded break at a specific site determined by a single guide RNA (sgRNA). The DNA double strand break leads to the activation of the DNA repair pathway to “fix” the cut by the highly error prone non-homologous end joining (NHEJ) process [7-9]. This highly erroneous repair can lead to the production of frameshift mutations, which can result in the inactivation of the gene of interest through an early stop codon or mRNA that is degraded by the nonsense mediated decay pathway [8, 9] (**Figure 1A**). In contrast to RNA interference (RNAi), previously used to identify novel cancer driving genes [10], CRISPR knockout (CRISPRko) is a permanent genetic alteration. Importantly, CRISPRko is not only more efficient, but has greatly reduced off target effects owing to the combined specificity of the sgRNA and protospacer adjacent motif (PAM) sequence [11]. Albeit, by no means are CRISPR systems free of any off-target effects as some sgRNAs are more prone to non-specifically targeting other loci [12]. While this poses a problem for clinical applications, it can be overcome in a research setting by applying the newest off-target detection methodologies such as Digenome-seq [13] and CIRCLE-seq [14] that identify off-target Cas activity *in vitro* using deep sequencing approaches or by simply using multiple sgRNAs for targeting the same gene, which will result in the same phenotypical outcome if all sgRNAs are “hitting” the same target [15].

Whilst inducing permanent and penetrant loss-of-function mutations can be advantageous in identifying novel TSGs, the complete deletion of some genes might be deleterious for the cells. Moreover, it does not faithfully recapitulate therapeutic targeting, which decreases, but also does not fully abolish activities of a specific gene. Hence, the development of a CRISPR inhibition (CRISPRi) system, which allows the suppression of transcription at specific gene loci through the usage of an enzymatic inactive Cas9 (dCas9) fused with a transcriptional repressor (e.g. dCas9-KRAB), overcomes this issue [16-18]. Directing dCas9-KRAB through a sgRNA to the promoter of a gene leads to robust reduction of gene expression by either directly blocking RNA polymerase activity or by modifying chromatin (**Figure 1B**).

The first genome-wide *in vivo* CRISPRko screen was demonstrated by Chen *et al.* identifying tumour promoting mutations and metastasis genes in a lung cancer model [19]. Since then, genome-wide or custom CRISPRko screens have been deployed in a range of cancer contexts identify novel TSGs both *in vitro* and *in vivo* [15, 20-26]. While CRISPRko has broadly been utilised for TSG identification, there are limited examples of the application of CRISPRi in this setting. One such CRISPRi screen targeted ~16 000 genomic loci encoding long non coding RNAs (lncRNA) in diverse human cell lines (six transformed and one induced pluripotent cell line) to identify lncRNAs that modulate cell growth [23]. This clearly demonstrates that CRISPR is not limited to targeting and interrogating the function of only coding regions of the genome, but indeed the whole-genome.

### ***Oncogene discovery with CRISPR activation***

A CRISPR system that enables identification of oncogenes through gene overexpression has also been developed. Similar to CRISPRi, CRISPR activation (CRISPRa) systems have been engineered by fusing dCas9 to transcriptional activation machinery and are directed to promoter regions of genes by specific sgRNAs [17, 27-30]. Several CRISPRa versions have been developed. The SUpErNova (Sun) tagged dCas9 (dCas9-Sun Tag) uses dCas9 fused to multiple repeat (10 times) GCN4 peptide sequences and co-expression of a GCN4 specific single chain antibody linked to a VP64 transcriptional activator domain [27]. Through targeting the dCas9-Sun Tag to promoter/enhancer elements, the recruitment of multiple sc-GCN4-VP64 antibodies leads to transcriptional activation. Its broad utility was demonstrated by a genome-wide activation screen for Cholera-diphtheria Toxin Complements [17]. While the dCas9-Sun Tag system relies on the recruitment of one transcriptional activator, further developments trialled the recruitment of diverse transcriptional activator domains through dCas9. One such system employs the fusion of dCas9 to a VP64, p65 and Rta tripartite transcriptional activator complex, which demonstrated reproducible and strong gene activation at multiple genomic sites [28]. Another CRISPR mediated transcriptional activator tool is the so called dCas9-synergistic activation mediator (SAM) system [29, 30]. In this approach, dCas9 is fused to VP64 and additional transcriptional activator modules are recruited along with a sgRNA, which contains RNA aptamer structures [30]. These structures are recognised by the bacteriophage MS2 peptide fused to the transcriptional activation

domains p65 and HSF1 [30]. The recruitment of multiple transcriptional activator domains in the dCas9-SAM system drives very robust target gene expression (**Figure 1C**). Konermann *et al.* demonstrated multiplexed overexpression with dCas9-SAM and constructed a genome-wide sgRNA library to assess highly expressed gene products in melanoma cell lines that confer resistance to the BRAF inhibitor PLX-4720 [30]. Few *in vivo* CRISPRa studies interrogating cancer biology have been undertaken. Braun *et al.* performed a custom *in vivo* activation screen to identify known or novel mutations in DNA damage response genes that confer resistance to therapy using dCas9-VP64 expression B-lymphoma cells [18]. Critically, they demonstrated that dCas9-VP64 can both activate gene expression when directed to a promoter and repress gene expression when directed downstream of a promoter by effectively blocking transcription [18]. Alternatively, CRISPRa was harnessed to enhance anti-tumour immunity by augmenting cancer cell antigen presentation in a triple negative breast cancer (TNBC) model, termed MAEGI (multiplexed activation of endogenous genes as an immunotherapy) [31]. Wang *et al.* demonstrated that endogenous gene overexpression in dCas9-SAM expressing TNBC cells evoked potent tumour rejection in immunocompetent mice due to increased antigen presentation and thus enhanced T cell killing [31]. Overall, the CRISPRa platform has great potential from oncogene discovery to unravelling new immunotherapy mechanisms in diverse cancer contexts.

### ***Determine specific cancer mutations to delineate driver from passenger mutations***

Recently, more sophisticated CRISPR tools were developed that provide even greater control to induce precise DNA edits, rather than simple loss or overexpression of a protein. Although base substitutions can be introduced via traditional CRISPR/Cas9 induced homology directed repair (HDR), this methodology requires the additional introduction of DNA templates, which is often inefficient resulting in very low targeting rates [9, 32]. These limitations were recently overcome by a new CRISPR methodology called Base editing, engineered by the Liu group [33, 34]. This allows the introduction of single base substitutions in the DNA code without the requirement of a DNA template. Moreover, this enables the functional assessment of single nucleotide variants in diverse genes important in different cellular processes. Two classes of base editors (BEs) exist; cytosine base editors (CBEs) and adenine base editors (ABEs) that induce C:G to T:A and A:T to G:C inversion point mutations, respectively. BEs induce mismatch repair DNA pathways by catalysing a single

stranded DNA break utilising a Cas9-D10A nickase (Cas9n) fused to an enzyme that deaminates the target base (**Figure 1D**). APOBEC1 for example, deaminates Cytosine to Thymidine through a Uridine intermediate in CBEs, while the synthetically engineered Tada enzyme (originally derived from prokaryotes) deaminates Adenine to Guanine through an Inosine intermediate in ABEs [33, 34]. To date, base editing has been only demonstrated in limited contexts, such as transformed human and mouse cell lines [34, 35]. Recently, Annunziato and colleagues demonstrated *in situ* cytosine base editing to model cooperating mutations in a conditional BE (BE3) expressing TNBC mouse model [36]. Both oncogenic missense mutations and tumour suppressive nonsense mutations were induced combined with MYC overexpression by intraductal delivery of sgRNAs, resulting in accelerated tumorigenesis [36]. Further optimisation of base editing is however required to overcome bystander editing within the target window, increase the efficiency of editing and improve desired editing outcomes in CBEs. First steps towards improving activity of BEs has recently been reached by solving the structure of different generations of ABEs while editing DNA [37]. These studies will assist in the design of BEs with higher efficiency to edit on-target bases with low off-target activities. Another major obstacle precluding the routine use of BE systems for cancer research, is their delivery into diverse cell types, which is mainly restricted by the size of the Cas9 fusion protein. Recently a split BE system deliverable by adeno-associated viruses (AAV) based vectors has been described [38]. Indeed, efficient editing was demonstrated following administering of the split system into brain, liver, retina, heart and skeletal muscle of living mice. Therefore, this system might represent an ideal platform for inducing cancer through specifically introducing tumour driving mutations in pre-clinical models mimicking real life scenarios. Moreover, within the same model, diverse immune therapeutic approaches can be trialled to identify new and efficient treatment options towards the cancers developed through mimicking real life mutations.

While BEs are not yet suited for generating accurate base substitutions, targeted mutagenesis screens can be performed exploiting their ability to induce multiple mutations within the editing window. Hess *et al.* developed CRISPR-X (dCas9-AID complex) to identify mutations that confer drug resistance to bortezomib in myelodysplastic syndrome (MDS) [39]. A custom sgRNA library tiling all coding exons of *PSMB5*, the target of bortezomib, directed CRISPR-X mutagenesis. In addition to already known mutations, they also discovered new bortezomib conferring resistance mutations in *PSMB5*, clearly

demonstrating the usefulness of base editing as a deep sequence mutagenesis tool. Similar mutagenesis screens were performed using other BE proteins to identify *BRCA1* variants that increase resistance or sensitivity to Peroxisome proliferator-activated receptors (PARP) inhibitors [32]. Hanna *et al.* also employed a CBE (BE4max) to determine drug evasion mutations and synthetic lethal mutations against BH3 mimetics and PARP inhibitors in different cancer contexts. Together, these studies demonstrate the effectiveness of BEs in targeted mutagenesis screens to identify resistance mutations and synthetic lethal interactions [40].

Furthermore, the Liu lab recently described the latest CRISPR tool called Prime Editing [41]. This tool is capable of introducing all types of targeted genetic alterations, including insertions, deletions and point mutations. The PRIME editor consists of Cas9n fused to a reverse transcriptase domain that can incorporate a template sequence provided by the prime editing sgRNA (pegRNA) into the target locus, in the absence of a DNA double stranded break (**Figure 1E**) [41]. Anzalone and colleagues demonstrated that PRIME editing can insert a sequence up to 44bp in length, delete up to 80bp, and insert targeted point mutations, both inversion (C-T, G-A, A-G, T-C) and transversion (C-G, C-A, G-T, G-T, A-T, A-C, T-G, T-A) base changes [41]. This system though versatile, requires however further characterisation of its applicability and performance before its effective use in cancer research can be guaranteed. Prime Editing once fully established represents a powerful system that can manipulate cells with ease at levels not possible before. This is particularly important for primary cell types, which are only viable in culture for a couple of days, such as primary haematopoietic cells.

### **Defining tumour suppressive and oncogenic pathways with the use of CRISPR**

Generation of genetically engineered mouse models (GEMMs) and cancer cell lines are the gold standard for assigning function to specific genes or mutations and deconvoluting the contribution of multiple genetic mutations. CRISPR/Cas9 generation of GEMMs is simpler, robust and more rapid than traditional methods [5, 42]. Simply, Cas9 and sgRNAs are co-injected into mouse zygotes producing mice with desired germline mutations enabling assessment of putative TSG or oncogene's role in cancer onset and progression [43-47].

Generating cell-type specific or somatic cancer models is more desirable than germline GEMMs as it more closely resembles human disease. One approach to generating somatic mouse models is the delivery of Cas9 and sgRNAs directly into the cell-type of interest. This is straight-forward for haematological cancers, in which haematopoietic stem and progenitor cells (HSPCs) can be modified with Cas9/sgRNAs in a dish prior to transplantation into lethally irradiated recipient mice to model cancer onset and progression [48, 49]. Alternatively, Xue *et al.* directly injected plasmids encoding Cas9 and sgRNAs into the liver of mice to assess the role of putative TSGs and oncogenes in hepatocellular carcinoma [50]. Viral vector systems have also been exploited in GEMMs of cancer to examine the potency of candidate TSGs. Sanchez-Rivera *et al.* engineered a lentiviral vector to deliver the CRISPR system (sgRNA and Cas9) and cre-recombinase (pSECC) [51]. Proof-of-concept experiments in *Kras*<sup>G12D</sup>-driven lung adenocarcinoma (LUAD) GEMMs validated the utility of this viral system, which has been utilised subsequently in lung, breast and colorectal cancer models [36, 52-55]. However, a major constraint preventing CRISPR/Cas9 utility in broad somatic cell types is the efficacy of delivery of the large Cas9 transgene. To overcome this, a cre-inducible Cas9 transgenic mouse was engineered that demonstrated efficient CRISPR/Cas9 activity in multiple tissues upon delivery of single or multiple sgRNAs [56]. Such models have been exploited in concomitant with novel lentiviral vectors that in addition to harbouring cre-recombinase contain an sgRNA tagged to a unique barcode sequence [25]. This approach has enabled the fitness of putative TSGs to be evaluated in boutique CRISPR/Cas9 screens *in vivo* [25, 57]. Together, these studies highlight the versatility of CRISPR technologies to generate sophisticated cancer GEMMs, that mimic the genomic complexity observed in the human disease.

### **Screening for cancer drug vulnerabilities or dependencies *in vitro* and *in vivo***

Identifying genes essential for the growth of cancer cells, but dispensable for their normal counterpart, represent attractive therapeutic targets with minor toxicity and/or side effects. CRISPRko screens in human and mouse cell lines have led to the identification of genotype specific cancer essential genes. For example, Tzelepis *et al.* performed a whole-genome knockout screen in five acute myeloid leukemia (AML) cell lines to generate a catalogue of cancer essential genes. They identified 492 AML-specific genes, some of which were known

and many that could be therapeutically targeted by repurposing existing inhibitors or performing screens for novel small molecule inhibitors [58]. Similar *in vitro* screens have identified novel tumour essential genes in mouse cell lines for AML and lymphoma [15, 20, 49, 59], human glioblastoma, colorectal and cervical cancer cell lines [21, 60] and 3D lung cancer spheroids [61]. Synthetic lethality is achieved when therapeutic inhibition combined with genetic deletion synergistically increase cancer cell cytotoxicity. Genome wide CRISPRko screens systematically reveal synergistic gene interactions that enhance therapy, for example synthetic lethal interactions with oncogenic Ras [62]. Szlachta *et al.* performed *in vitro* and *in vivo* CRISPRko screens in patient derived pancreatic ductal adenocarcinoma (PDAC) cancer cells to identify genes that synergise with MEK inhibition [63]. Despite reduced representation of sgRNAs *in vivo*, a number of pathways were identified that correlated with genes identified in the parallel *in vitro* screen. These assays therefore allow the discovery of cancer essential genes, which might themselves represent novel drug targets for anti-cancer therapies. Critically, these newly identified targets might not kill but increase susceptibility of the cancer cells to other drug regimes, or even more interesting, to novel immune therapy approaches.

### **The use of CRISPR screens to improve immune-based therapies**

The tumour microenvironment (TME) is a highly dynamic and complex milieu, which in addition to tumour cells, is comprised of a myriad of cell types, including blood vessels, immune cells and fibroblasts supported by the extracellular matrix (**Figure 2**). Indeed, targeting specific tumour-immune cell interactions has led to the development of immunotherapeutic approaches, such as anti-PD-1 and anti-PD-L1 inhibitors [64]. While this approach has revolutionised the treatment of solid tumours, such as melanoma and non-small cell lung cancer (NSCLC) not all patients respond, highlighting the need to better understand mechanisms tumour cells adopt to evade immune cell detection (reviewed in [65]). Novel CRISPR screens have therefore emerged to investigate intricate tumour-immune cell interactions and identify mechanisms underlying resistance to anti-PD-1 therapies. These can be broadly separated into tumour intrinsic and tumour extrinsic targets and are discussed in detail below.

## ***Utilising CRISPR screens to reveal tumour cell intrinsic mechanisms of immune evasion***

To date, only a small number of genes have been discovered to mediate tumour cell-intrinsic mechanisms of immune evasion, examples including *JAK1* and *B2M* [66, 67]. To more comprehensively interrogate the genetics of immune evasion in an unbiased manner, *in vitro* and *in vivo* CRISPR screens are now also being employed. *In vitro* screens are often genome-wide, allowing the interrogation of thousands of genes in an unbiased manner. The ease by which target cells of interest can be expanded *in vitro* is advantageous and allows for a high library coverage increasing the ability to identify robust novel genetic targets. In contrast, the library sizes utilised for *in vivo* CRISPR screens is generally reduced, restricted by the number of gene-edited cells transplantable in recipient mice. However, *in vivo* screens are often more reliable as the individual cell is growing within its “normal” tissue microenvironment, characteristics which will be discussed in greater detail below.

### ***in vitro screens***

Briefly, Cas9-expressing cancer cells of interest are transduced with an sgRNA library (whole genome or boutique) to generate a gene-edited population of cells. Genetically-modified cancer cells are then isolated, based for example on differing cell surface expression patterns (e.g. increased PD-L1 or MHC-I expression) by flow cytometry. Finally, next generation sequencing (NGS) of sorted modified-cell populations allows for the identification of candidate genes (enriched/depleted sgRNAs), that are subsequently validated in functional studies (**Figure 3A**).

Cancer cell intrinsic expression of PD-L1 is thus far, one of the most recognised mechanisms of immune evasion (reviewed in [68]). Historically, PD-L1 expression on tumour cells has been shown to correlate with response to immune checkpoint inhibitors (ICIs) across a number of cancer types (reviewed in [69]). However, tumour cells exhibit heterogenous levels of PD-L1 expression [70, 71]. It is therefore essential to identify mechanisms that regulate PD-L1 expression to not only better understand tumour biology, but to develop treatment strategies that elicit an improved anti-tumour response. PD-L1 surface expression has been employed as a readout of *in vitro* CRISPR screens, whereby

increased expression may lead to higher tumour cell clearance in response to ICIs [72]. Exploiting this concept, Burr *et al.* performed a genome-wide CRISPR screen in the BxPC-3 PDAC cell line and identified a novel protein CKLF-like MARVEL transmembrane domain containing protein 6 (CMTM6) as a regulator of PD-L1 surface expression [72]. Interestingly, CMTM6 is co-expressed with PD-L1 at the plasma membrane and in recycling endosomes and functions by protecting PD-L1 from being targeted for lysosome-mediated degradation [72]. Interestingly, CMTM6 was independently identified as a regulator of PD-L1 expression using a haploid genetic screen [73], confirming its importance in PD-L1 stability. Furthermore, in a cohort of NSCLCs, a significant correlation between CMTM6 and PD-L1 expression was detected, particularly in the stromal and macrophage compartments of the tumour. Critically, this co-expression was associated with increased overall survival in immunotherapy-treated patients [74]. Together, these findings raise the possibility that CMTM6 inhibitors may increase the efficacy of ICIs in solid tumours, such as NSCLC. Increased PD-L1 surface expression was exploited in a similar CRISPR screening approach in H358 lung adenocarcinoma (LUAD) cells [75]. In addition to identifying known regulators of PD-L1 expression, including *CMTM6* and *SMAD4*, Uroporphyrinogen decarboxylase (*UROD*) was identified as a novel regulator of PD-L1 protein expression. Interestingly, *PD-L1* mRNA expression remained unchanged following *UROD* inactivation, suggesting that loss of *UROD* regulates PD-L1 expression via post-translational mechanisms. Indeed, loss of *UROD* promoted an immunosuppressive tumour microenvironment via the integrated stress response (ISR) to increase PD-L1 translation. Activation of the ISR promoting PD-L1 was driven by the translation factor eIF5B, inhibition of eIF5B which, led to reduced expression of PD-L1 and reduced tumour burden. Interestingly, *EI5FB* is frequently overexpressed in human LUAD and correlates with poorer survival outcomes [75]. Together these studies reveal novel mechanisms of PD-L1 regulation in tumour cells, highlighting the power of CRISPR screens in uncovering alternative vulnerabilities that can be harnessed to design improved treatment modalities.

Another critical mechanism of immune evasion and acquired resistance to ICI is diminished MHC class I (MHC-I) expression [76, 77]. To identify novel negative regulators of MHC-I expression, Burr and colleagues performed a genome-wide CRISPR screen in K562 tumour cells, known to exhibit low MHC-I expression levels [78]. Interestingly, sgRNAs targeting *Eed*, *Ezh2* and *Ezh1*, components of the Polycomb Repressive Complex 2 (PRC2), were

enriched in MHC-I-high cells, highlighting that basal MHC-I expression is under strict epigenetic control. Indeed, genetic and pharmacological inhibition of either EED or EZH2 and EZH1 restored MHC-I levels in a number of cancer cell lines, including small cell lung cancer (SCLC). Critically, reversal of MHC-I expression resulted in enhanced T cell-mediated killing of SCLC cells *in vitro*, and induced a potent anti-tumour response in allogeneic transplantation studies *in vivo* [78]. Whilst MHC-I expression can be low in certain tumours, this screen highlights targetable mechanisms to increase MHC-I for antigen presentation, allowing cytotoxic T cell clearance of tumour cells.

### ***In vivo* screens**

The advantage of *in vivo* CRISPR screens is their ability to replicate the complexity and dynamic interactions that occur within the TME. *In vivo* CRISPR-Cas9 screens have therefore emerged to bridge this gap to identify regulators of immune evasion in cancer cells or inhibitors within the immune cells themselves. To date, *in vivo* screens are of a similar design as the *in vitro* screens, whereby Cas9-expressing cancer cells are transduced with an sgRNA library to generate a population of mutant cells. These cells are then transplanted via different routes into the recipient mice to allow for tumour cell growth. Commonly, the subcutaneous growth of modified tumour cells is compared between immune-competent and immune-modified/deficient mice [79-81]. Through sequencing of harvested tumours, inference can be drawn from sgRNAs depleted in the cancer cells in immune-competent animals indicating that the particular genetic hit is critical in producing a robust anti-tumour response. (Figure 3A).

Manguso *et al.* were amongst the first to employ an *in vivo* CRISPR screening approach to identify novel immunotherapy targets [80]. Specifically, Cas9-modified B16 melanoma cells were transplanted into mice and treated with either a granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting irradiated tumour cell vaccine (GVAX) alone or in combination with anti-PD-1. Importantly, sgRNAs targeting genes with known roles in immune evasion, *Stat1*, *Jak1*, *Ifngr2*, *Ifngr1* and *Jak2*, were found enriched in GVAX/anti-PD-1 treated animals, validating the robust nature of the *in vivo* screen method employed. In addition to known regulators, inactivation of the tyrosine phosphatase *Ptpn2*, was shown to increase the response to anti-PD-1 blockade [80]. Specifically, *Ptpn2* loss increased antigen

presentation by tumour cells, leading to an increased CD8<sup>+</sup> T cell infiltration and cancer cell clearance in the TME. Interestingly, the screen also revealed that the loss of *Adar1*, a double strand RNA-sensing enzyme, sensitises tumour cells to anti-PD-1 and overcomes resistance to immunotherapy [79]. Loss of *Adar1* resulted in enhanced infiltration of T cells (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> and  $\gamma\delta$ -T) and natural killer (NK) cells and decreased proportions of myeloid-derived suppressor cells and tumour-associated neutrophils in the TME. Critically, IFN $\gamma$  signalling is required to elicit an anti-tumour response in *Adar1*-deficient tumours [79], highlighting a novel checkpoint that increases tumour inflammation in a T cell independent manner, and thus overcoming resistance to immunotherapy. Other novel genes and pathways have also been investigated to improve current immunotherapies. In line with the mounting evidence implicating epigenetic regulators in the anti-tumour immune response [78, 82-85], an epigenetic sgRNA CRISPR screen was performed to identify genes that could improve the efficacy of anti-PD-1 blockade [86]. The histone chaperone *Asf1a* was identified and found to sensitise *Kras/p53* tumour cells to anti-PD-1 therapy [81]. Loss of *Asf1a* induced an inflammatory response and GM-CSF secretion, allowing for M1-like macrophage polarisation and T cell activation [81]. Together, these studies demonstrate how CRISPR screens have been exploited to interrogate molecular mechanisms that underpin tumour cell immune evasion. Moreover, novel targets and biological pathways have been revealed that may improve responses to ICIs, confirming the power and flexibility of CRISPR screening to identify novel regulators of immunotherapy resistance.

Interestingly, the majority of *in vivo* CRISPR screens have utilised subcutaneous transplantation assays, most likely due to their amenability to easily monitor tumour growth. It is however, important to consider the site of transplantation when performing a CRISPR screen. Indeed, the immune microenvironment of a subcutaneous grown tumour does not represent the TME of a tumour generated in the organ of interest (reviewed in [87, 88]). Recently, it has been shown that the TME of commonly metastasising cancers to sites such as the liver and lungs, influences the efficacy of anti-PD-1/anti-CTLA4 immunotherapies [89]. Specifically, tumours transplanted either subcutaneously, in the mammary fat pad or in the liver resolved, which contrasted tumours transplanted into the lungs of mice, that failed to respond to anti-PD-1/anti-CTLA4 blockade [89]. Importantly, lungs of mice displayed an increased immunosuppressive environment compared to mammary fat pad, with lower levels of CD8<sup>+</sup> T cells and lower NK cell activation [89]. This study therefore emphasises the

importance of faithfully replicating the TME for an individual cancer subtype when designing *in vivo* CRISPR screens.

### **Tumour Extrinsic CRISPR screens to understand the biology of immune cells within the TME**

CRISPR/Cas9 screening has also investigated mechanisms that reduce immune cell activity and cancer cell clearance within the TME. Whilst the majority of CRISPR/Cas9 screens have focused on the role of CD8<sup>+</sup> T cells within the TME [90], the basic screening approach can be extrapolated to investigate other immune cell subsets to improve immunotherapy performance, such as CD4<sup>+</sup> T cells or NK cells.

### ***Exploiting CRISPR/Cas9 screens to identify novel anti-tumour mechanisms of T cell function***

CRISPR/Cas9 screens focused on tumour extrinsic cell function are similar in their approach to tumour intrinsic screens (**Figure 3B**). In these screens, CD8<sup>+</sup> T cells are first isolated from human donors or genetically modified mice, before infection with an sgRNA library *in vitro*. Screens can be performed *in vitro*, typically in co-culture assays with tumour cells. Alternatively, modified immune cells can be injected into cancer bearing model organisms *in vivo*, such as the mouse [91, 92]. Depending on the experimental setup, the immune cells will be isolated to investigate sgRNA enrichment for genes that either boost or hinder immune cell function for cancer clearance. CD8<sup>+</sup> T cells have been the focus on numerous CRISPR/Cas9 screens to date, given that their presence within the TME has been associated with effective tumour cell clearance [93].

Dong *et al.* performed a genome-wide CRISPR screen in CD8<sup>+</sup> T cells isolated from OT-I;Cas9 double transgenic mice to investigate regulators of T cell infiltration and cytotoxicity [94]. The group injected sgRNA library transduced OT-I;Cas9 CD8<sup>+</sup> T cells into mice transplanted with E0771 TNBC cells grown either in the mammary fat pad or subcutaneously. The RNA helicase Dhx37 was discovered, which regulates CD8<sup>+</sup> T cell infiltration into the tumour. *Dhx37*-deficient CD8<sup>+</sup> T cells exhibited increased effector cytokine

production, such as granzyme B and IFN $\gamma$ , as well as increased expression of PD-1, Lag3 and Tim-3. Critically, transfer of *Dhx37*-deficient CD8<sup>+</sup> T cells into mice harbouring E0771 tumour cells, led to a potent CD8<sup>+</sup> T cell response concomitant with a reduction in tumour burden. These key results highlight the potential to develop small molecule inhibitors to target *Dhx37* in CD8<sup>+</sup> T cells to increase T cell infiltration into the TME [94]. Due to CD8<sup>+</sup> T cell exhaustion within the TME, other methods to modulate immunotherapeutic responses have explored adoptive (T) cell therapy (ACT). This CRISPR screen used again CD8<sup>+</sup> T cells from OT-I;Cas9 double transgenic mice and transduced them with a focused sgRNA library targeting enzymes and transcriptional modulators of metabolism [95]. Modified CRISPR-CD8<sup>+</sup> T cells were adoptively transferred into recipient mice harbouring B16F10 melanoma cells. sgRNAs targeting the ribonuclease *Regnase-1*, a negative regulator of CD8<sup>+</sup> T cells, emerged as the most enriched target in this screen. Validation of *Regnase-1* identified its roles in switching CD8<sup>+</sup> T cells to long-lived effector cells, with *Regnase-1* null CD8<sup>+</sup> T cells expressing higher levels of IFN $\gamma$  and granzyme B [94]. Together, these screens highlight the power of how CRISPR/Cas9 technology can be utilised to investigate and improve T cell function for immunotherapeutic applications.

While lentiviral transduction is the most common method for delivery of sgRNAs into cells, other studies have also employed alternative viral delivery systems for introduction into CD8<sup>+</sup> T cells. Ye *et al.* screened CD8<sup>+</sup> T cells utilising an adeno-associated viral CRISPR approach to investigate membrane-coding genes on CD8<sup>+</sup> T cells isolated from Cas9 transgenic mice [92]. High level of sgRNA expression was validated, and thus this methodology was further developed for an *in vivo* screen [92]. Specifically, modified CD8<sup>+</sup> T cells were orthotopically administered into the brains of recipient mice together with GL261 glioma cells. Two genes with no prior ascribed role in immune regulation in CD8<sup>+</sup> T cells, *Pdia3* and *Mgat5*, emerged as genetic targets from the screen. Critically, transcriptome analysis confirmed an enhanced effector phenotype in *Pdia3*-deficient CD8<sup>+</sup> T cells, implicating *Pdia3* as a novel target for T cell based immunotherapies. CRISPR/Cas9 screening has extended the ability to analyse processes that regulate CD8<sup>+</sup> T cell function and maturation. LaFleur *et al.* developed an *in vivo* CRISPR screening approach whereby a Cas9/sgRNA delivery system enabled the deletion of genes involved in regulating adaptive immune cells without affecting mature immune cells, termed CHimeric IMMune Editing (CHIME) [96]. This study demonstrated that loss of *Ptpn2* enhanced the CD8<sup>+</sup> T cell

response to LCMV Clone 13 infection. Whilst this study did not screen for specific tumour extrinsic functions, it provided additional evidence for *Ptpn2* as a negative regulator of T cell function and hence a novel immunotherapeutic target [80]. Together, these innovative screening approaches highlight how CRISPR screening approaches can be utilised to discover regulators of CD8<sup>+</sup> T cell function within the TME, as well as offer novel targets for immunotherapy development.

***Other immune populations offer novel avenues for CRISPR screens to identify anti-tumour targets***

While current immunotherapies are heavily focused on improving cytotoxic T cell function, other immune cell types that make up the milieu of the TME can also be exploited to improve tumour cell clearance. One such cell type is Natural Killer (NK) cells, a critical cytotoxic lymphocyte of the innate immune system. Indeed, NK cell based immunotherapies are gaining traction as a treatment approach for a multitude of cancers (reviewed in [97]). Consistent with this, high NK cell infiltration in solid tumours such as melanoma, colorectal and lung cancer has been shown to correlate with improved prognosis [98-100]. Indeed, inactivation of a number of genes specifically in NK cells, including cytokine-inducible SH2-containing protein (*Cish*) increase the cytotoxic capacity of NK cells, particularly in settings of metastatic dissemination [101, 102]. A number of CRISPR screens have explored regulatory pathways in NK cells identifying genetic aberrations that sensitise tumour cells to NK cell-mediated killing [103, 104]. Whilst, these studies have focused on genetic alternations present in the tumour cells, moving forward, CRISPR modification of mouse and/or human NK cells may unveil novel NK cell checkpoints, that can be exploited therapeutically. And whilst cancer immunotherapies have not been the primary focus for other CRISPR screens, this technology has been utilised to interrogate regulatory processes in B cells [105-107], macrophages [108-113] and dendritic cells [114-116]. Furthermore, the power this technology holds has unveiled novel gene networks controlling the function of specific subsets of T cells (e.g. Treg cells), that could be harnessed to increase anti-tumour immunity [117, 118]. These studies highlight the potential that CRISPR-Cas9 technology holds to understand tumour biology, with the ultimate aim of improving the efficacy of current immunotherapeutic approaches or unveiling novel checkpoints in other immune cell populations.

## Insights into future directions of CRISPR approaches in immunotherapeutic development and tumour biology

Standard CRISPR/Cas9 approaches remain the foremost tool implemented in cancer research despite availability of CRISPRi and CRISPRa for many years. With the recent addition of base editors and prime editors, application of these versatile CRISPR technologies will enable exploration of an untapped reservoir of knowledge. Alternate Cas9 nucleases are also being adapted to overcome and extend current capabilities and limitations of Cas9. Evolved spCas9-NG or xCas9 recognise alternate PAM sequences thus broadening the scope of targetable sequences [119-121]. Cas12 is a different class to Cas9 and has recently gained a lot of attention, as it has a high editing efficacy in cells and has the added benefit that it can process its own CRISPR RNA (i.e. guide RNA) [122, 123]. This has enabled the targeting of multiple genes with one precursor RNA and was recently demonstrated to serve as an elegant tool for complex combinatorial CRISPR screens [124-126]. Hence, while to date all the CRISPR techniques target DNA, the recent discovery of Cas13 has extended the CRISPR toolbox to either inhibit or specifically edit RNA [127].

Researchers are integrating pooled CRISPR screens with single cell RNA-sequencing (RNA-seq) to characterise the effect of gene perturbations on gene signatures, gene interactions and cell states at a single cell resolution. These approaches are endowed with the power to dissect multiple genetic disturbances and their effect on critical pathways. First generation approaches including Perturb-seq [114], CRISPR-seq [115] and CROP-seq [128], demonstrated the use of pooled CRISPRko screens combined with RNA-seq. Here, the sgRNA responsible for gene disruption is identifiable by a unique guide index (UGI) or barcode, which allows the analysis of the transcriptional profile in the same cell in which the sgRNA mediated gene disruption has occurred. Hill *et al.* developed an improved CROP-seq methodology whereby the sgRNA serves directly as the barcode. They performed a proof-of-concept study whereby CRISPRko of *TRP53* in non-transformed breast epithelial cell lines displayed a down regulated cell cycle checkpoint response gene signature by RNA-seq [129]. Even more sophisticated platforms have been developed recently, employing CRISPRi and CRISPRa, in addition to CRISPRko, and enabling combinatorial libraries (two sgRNAs per cell) to be assessed [130, 131]. CRISPRa Tracing of Clones in Heterogeneous

cell populations, or CaTCH, is another new platform that builds on lineage tracing that permits functional characterisation and comparison of founder clones with their post-selection counterparts [132]. CaTCH was utilised to discern whether melanoma cells had pre-existing resistance mechanisms to RAF and MEK inhibitors, or whether they acquired resistance during treatment *in vivo*[132]. Simply, treatment naïve melanoma cells were transduced with the transcriptional activator dCas9-VPR and unique barcodes fused to an inducible fluorescent reporter tag. Following drug selection, enriched ‘drug resistant’ clones were determined by genomic sequencing and barcode-complementary sgRNAs were transduced back into a heterogeneous population of melanoma cells [132]. The fluorescent reporter is activated in the clone of interest, which can be isolated by fluorescence-activated cell sorting and functionally characterised and compared to treatment naïve founder cells. Platforms combining CRISPR and single cell tracing or phenotyping platforms are an exciting development that will enable researchers to comprehensively dissect the inherent heterogeneity within tumours and ultimately reveal potential vulnerabilities that can be therapeutically targeted.

## **CONCLUSION**

Cancer researchers are embracing the rapidly evolving CRISPR technologies to understand cancer biology. What we hope to highlight in this review is how CRISPR screening provides the foundation for in-depth exploration into tumour biology. The flexibility and modularity of CRISPR gene technologies should demonstrate how screens can be designed to improve our understanding of tumour biology and to better develop immunotherapies to target cancers.

## **ACKNOWLEDGMENTS**

We thank all of our current and past colleagues at The Walter and Eliza Hall Institute and elsewhere. Our work was supported by grants and fellowships from the Australian National Health and Medical Research Council (NHMRC) (Project Grants 1186575 and 1145728,1143105,1159658 and Fellowship 1156095 to MJH), the Leukemia and Lymphoma Society of America (LLS SCOR 7015-18 to MJH), the Cancer Council of Victoria (project

grant 1147328 and Venture Grant to MJH), the Australian Phenomics Network (to MJH). KDS is supported by a Victorian Cancer Agency Mid-Career Fellowship (MCRF18003) and The Peter and Julie Alston Centenary Fellowship. MAP and JAM are recipients of the Research Training Scholarship, The University of Melbourne. This work was made possible through Victorian State Government Operational Infrastructure Support and Australian Government National Health and Medical Research Council Independent Research Institutes Infrastructure Support Scheme.

## **CONFLICT OF INTEREST**

The authors declare no financial or commercial conflict of interest.

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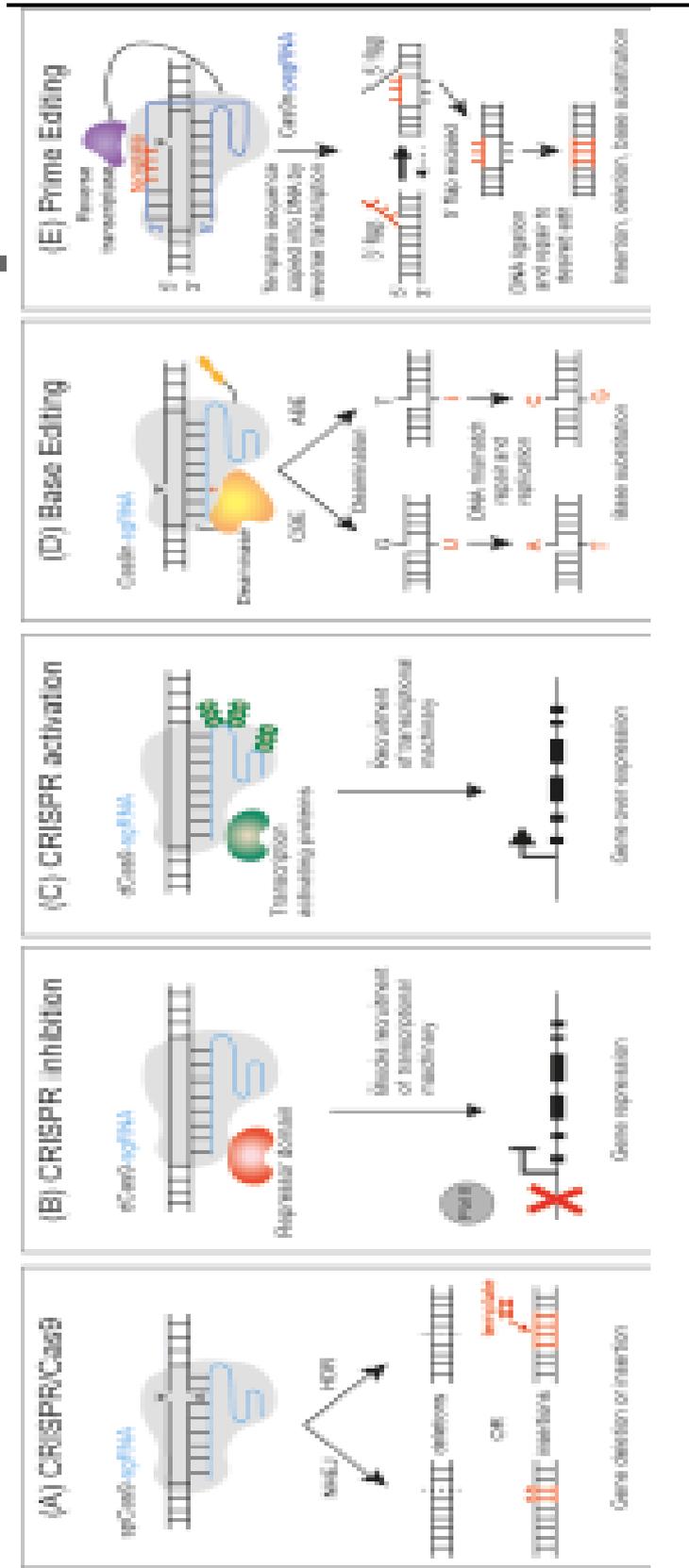
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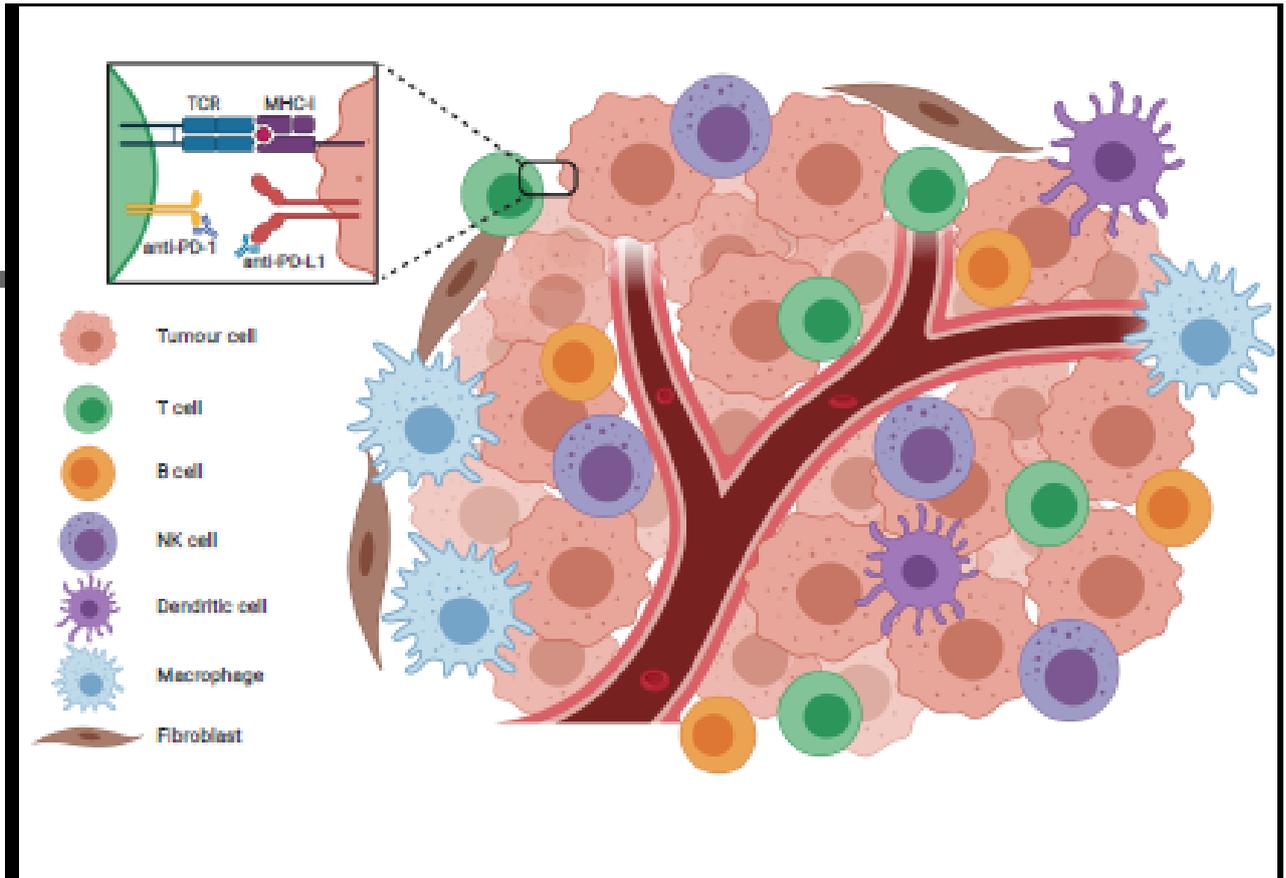
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## FIGURES

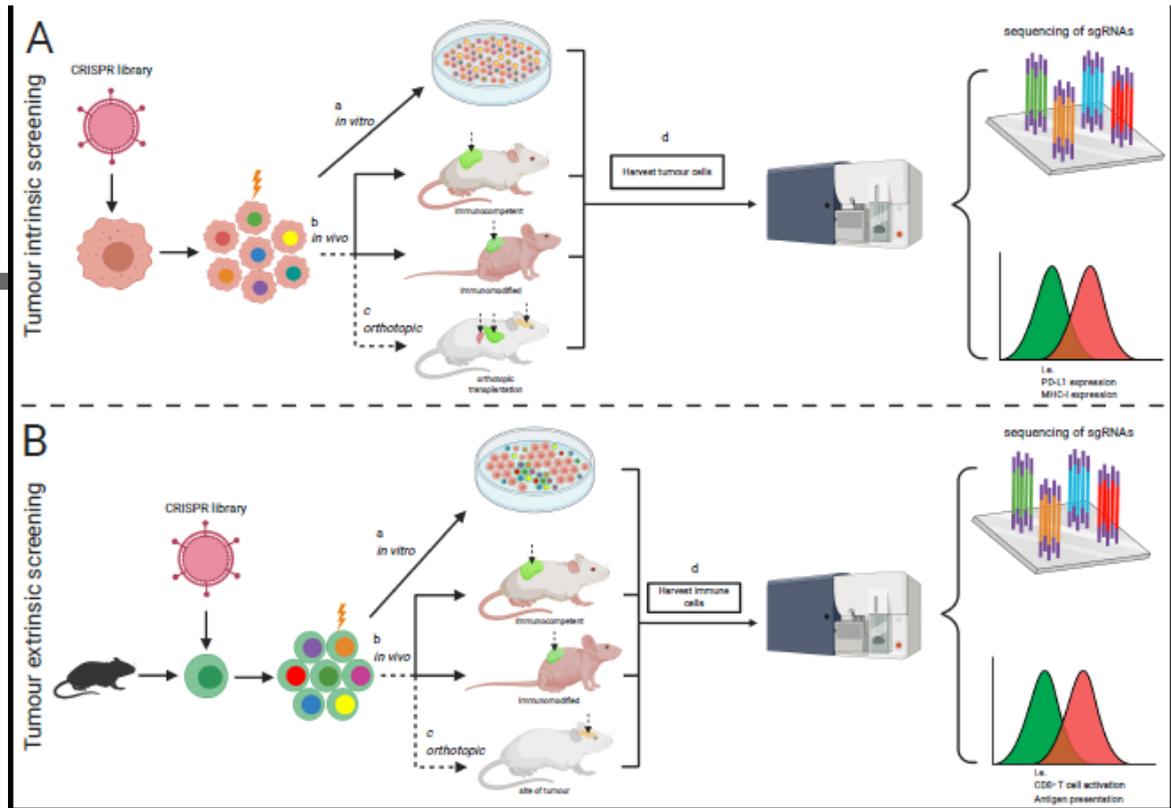


**Figure 1. Mechanisms of CRISPR technologies for inducing genetic modulations**

(A) CRISPR/Cas9 induces permanent genomic modifications including gene knockouts or targeted insertions. Cas9 endonuclease catalyses a DNA double stranded break at a specific locus as directed by complementary binding of the sgRNA and availability of appropriate protospacer adjacent motif (PAM) sequence. DNA repair pathways are activated, either error-prone non homologous end joining (NHEJ) that may introduce insertions/deletions (indels) or homology directed repair (HDR) that facilitates integration of exogenous template DNA. Delivery of two sgRNAs can induce large deletions or oncogenic translocations. (B) CRISPR inhibition induces robust directed repression of gene expression. Upon binding to the promoter region of a gene, dCas9-KRAB blocks polymerase activity or KRAB alters chromatin accessibility. (C) CRISPR activation induces constitutive overexpression of target genes. Here, dCas9 is complexed with transcriptional activating proteins such as VP64, p65, HSF1 and MS2 and directed to the enhancer or promoter regions of target genes by sgRNAs, thus assembling transcriptional machinery including Pol II to drive gene expression. (D) Base editors induce single base substitutions. Cas9 nickase (Cas9n) is tethered to a deaminase enzyme that deaminates cytidine (C) to uridine (U) (CBEs), or adenosine (A) to inosine (I) (ABEs) creating a DNA mismatch pair. The mismatch repair pathways favour altering the non-edited strand that was 'nicked' by Cas9n. Following DNA repair or replication C:G to T:A and AT: to G:C base substitutions are achieved. (E) Prime editing can induce deletions, targeted insertions and base substitutions. The editor is directed to the target DNA by a prime editor sgRNA (pegRNA), which encodes a DNA homology binding sequence and a template sequence. Cas9n nicks the target strand exposing a 3' flap that hybridises with the pegRNA target sequence. The reverse transcriptase tethered to Cas9n copies the pegRNA template sequence into the genomic DNA and this 3' flap intermediate encoding the desired mutation sequence competes with the 5' wildtype flap intermediate to be incorporated. The 5' flap is excised and DNA ligated leading to DNA heteroduplex which is resolved by a second nick to the non-edited strand favouring incorporation of the edited sequence.



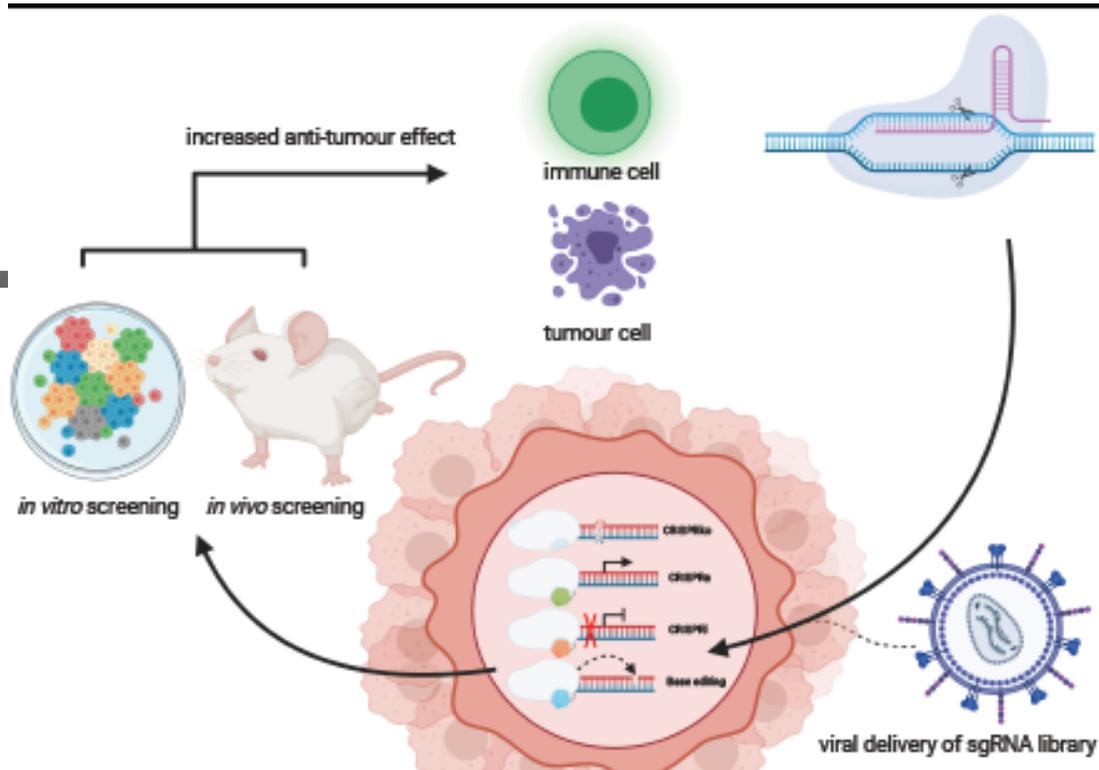
**Figure 2. A Schematic representation of the tumour microenvironment (TME).** The TME is a heterogenous environment comprised of tumour cells, immune cells (including B cells, T cells, NK cells, macrophages and dendritic cells), fibroblasts and tumour vasculature. Immune checkpoint inhibitors (ICIs) including anti-PD-1 (Programmed Death-1) and anti-PD-L1 (Programmed Death-Ligand 1) have been developed to target specific interactions between tumour and immune cells.



**Figure 3. A schematic representation of CRISPR screens designed to identify tumour intrinsic and extrinsic mechanisms of immune evasion. (A) Tumour intrinsic CRISPR screening:** Tumour cells are infected with sgRNA libraries packaged in viruses. (a) CRISPR-modified tumour cells are cultured *in vitro*, where additional selective pressures can be applied, e.g. IFN $\gamma$  stimulation. (b) Modified tumour cells are subcutaneously transplanted into immunocompetent or immune-modified recipient mice. (c) Modified tumour cells are transplanted orthotopically, recreating the dynamic interactions of the TME seen in patients. (d) Tumour cells are harvested, sorted and sequenced to identify enriched/depleted sgRNAs. **(B) Tumour extrinsic CRISPR screening pipeline:** Immune cells of interest are harvested from host animals (i.e. CD8<sup>+</sup> T cells from Cas9 transgenic mice) and an sgRNA library is introduced. (a) Isolated CRISPR-modified immune cells are co-cultured with tumour cells *in vitro*. (b) Modified immune cells are introduced *in vivo*, in immunocompetent and/or immune-modified mice. (c) CRISPR-modified immune cells are orthotopically introduced at the original tumour site, faithfully replicating the complexities of the TME. (d) CRISPR-modified Immune cells are harvested, sorted and sequenced for enriched/depleted sgRNAs, revealing underlying immune cell function.

**Table 1. Overview of current CRISPR technologies**

	<b>CRISPR/Cas9</b>	<b>CRISPR inhibition</b>	<b>CRISPR activation</b>	<b>Base editing</b>	<b>Prime Editing</b>
<b>Genetic perturbation</b>	Gene knockout or targeted insertion	Transcriptional repression	Transcriptional overexpression	Single point mutation	Targeted insertion, deletion and single point mutations
<b>Cas variant</b>	SpCas9, SaCas9, Cas12a  (DNA double stranded break)	dCas9  (catalytically inactive)	dCas9  (catalytically inactive)	Cas9 D10A nickase  (single strand DNA break)	Cas9 D10A nickase  (single strand DNA break)
<b>Target region</b>	All coding and non-coding genomic regions	Promoter regions and TSS	Enhancer and promoter regions	All coding and non-coding regions	All coding and non-coding regions
<b>Advantages</b>	<ul style="list-style-type: none"> <li>• Permanent genetic alterations</li> <li>• Precise genetic targeting</li> <li>• Lower off-target effects than RNAi approaches</li> </ul>	<ul style="list-style-type: none"> <li>• Reduced cytotoxicity compared to RNAi</li> <li>• Greater knockdown efficiency than RNAi</li> <li>• Mimics activity of drug inhibitors</li> </ul>	<ul style="list-style-type: none"> <li>• Activates endogenous gene expression</li> <li>• Simpler and more robust than cDNA overexpression approaches</li> </ul>	<ul style="list-style-type: none"> <li>• Induce point mutations without DNA double stranded break</li> <li>• Model point mutations</li> </ul>	<ul style="list-style-type: none"> <li>• Delete up to 80bp</li> <li>• Insert up to 44pb</li> <li>• Induce inversion and transversion point mutations</li> <li>• No DNA double stranded break or donor template required</li> <li>• Edit PAM site in parallel to prevent secondary editing of same site</li> </ul>
<b>Limitations</b>	<ul style="list-style-type: none"> <li>• Low efficiency of HDR</li> <li>• sgRNA design limited by PAM sequence availability</li> </ul>	<ul style="list-style-type: none"> <li>• Restricted window of sgRNA design</li> <li>• Promoter regions are not completely annotated in mice</li> <li>• DNA accessibility may hinder binding of complex</li> </ul>	<ul style="list-style-type: none"> <li>• Restricted window of sgRNA design</li> <li>• Enhancer and promoter regions are not completely annotated in mice limiting sgRNA design</li> <li>• DNA accessibility may hinder binding of complex</li> </ul>	<ul style="list-style-type: none"> <li>• Undesired editing: transversion base substitutions by CBEs, indel formation</li> <li>• Bystander mutations within the editing window</li> <li>• Base editor complexes are large thus difficult to deliver to target cells</li> </ul>	<ul style="list-style-type: none"> <li>• Low editing efficiency</li> <li>• Desired edit may not be incorporated</li> <li>• Undesired indel formation from nickase activity of Cas and pegRNA</li> </ul>



Immunotherapy is one of the most promising new anti-cancer therapies in the clinic. This review discusses the use of unbiased CRISPR screens in diverse disease models as a means to uncover cancer vulnerabilities for enhancing the immune response and eradicating the cancer cells.