The use of CRISPR/Cas9-based gene editing strategies to explore cancer gene function in mice ☆

Louise van der Weyden[1], Jos Jonkers[2] and David J Adams[1]

CRISPR/Cas9 systems have revolutionised the field of gene editing, allowing for precise modifications to be generated *in vivo* to mimic the genetic events found in human cancer cells. These systems may be used to generate germline or somatic loss-of-function of events, and also chromosomal rearrangements, either constitutively or in a spatiotemporally controlled manner. Forward genetic screens have also been performed using CRISPR/Cas9 systems to identify new driver genes and approaches using catalytically inactive Cas9 fused to base editors have enabled genome editing with single-base precision. Here we discuss the many 'flavours' of the CRISPR/Cas9 system and give examples of their use for the generation of clinically-relevant mouse models of cancer.

Addresses
[1] Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge, CB10 1SA, United Kingdom

[2] Oncode Institute and Division of Molecular Pathology, The Netherlands Cancer Institute, 1066 CX, Amsterdam, Netherlands

Corresponding author: Adams, David J (da1@sanger.ac.uk)

Introduction

Human cancer cells carry varying numbers of genetic alterations, comprising irrelevant passenger mutations as well as mutations in cancer genes that drive tumour initiation and progression. The latter can include in loss-of-function events in tumour suppressor genes as a result of nucleotide insertions/deletions ('indels'; such as in *APC* in colorectal cancer and in *PTEN* in many cancers), or point mutations (such as *TP53* mutations in a wide range of cancers). Similarly, oncogenes may be activated by point mutations (such as $BRAF^{V600E}$ in melanoma), gene amplification (such as *HER2* gene amplification in breast cancer) or gene fusion events (such as fusions of *EWSR1*

and *FLI1* in Ewing's sarcoma). In addition, epigenetic alterations change the regulatory landscape of cancer cells.

Accurate animal models of cancer require the tumour and its microenvironment to be as genetically, physiologically and anatomically similar to the disease in humans as possible. For these studies the laboratory mouse is the most widely used model system due to its genetic similarity to humans, small size, short reproductive cycle and the ease with which its genome can be manipulated. Conventional technologies to generate genetically-modified mice, such as gene-targeting and insertional mutagenesis, may be extremely laborious with long model generation times [1], which has led to great interest in gene editing approaches (Figure 1).

Although genome-editing technologies such as designer zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and homing meganucleases have allowed for the generation of targeted genomic modifications, it is the class of engineering tools based on the RNA-guided Cas9 nuclease from the type II prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system that has made the biggest impact on our ability to rapidly and efficiently alter the mouse genome. The CRISPR/Cas9 system uses guide RNA (gRNA) targeting to direct the Cas9 endonuclease to a specific locus, where upon it induces DNA breaks, which are subsequently 'repaired' by the cellular DNA repair machinery.

Modelling germline and somatic loss-of-function events

CRISPR/Cas9 technology has been used to generate germline 'knockout' (KO) mouse models of cancer. For example, co-injection of *Cas9* mRNA and single-guide RNAs (sgRNAs) targeting the DNA methylation enzymes *Tet1* and *Tet2* into fertilized oocytes (zygotes), generated mice with biallelic mutations in both genes (at 80% efficiency) in under one month [2]. In addition, co-injection of the *Cas9* mRNA and sgRNAs with mutant oligos (representing 'donor templates' with homology arms) generated precise point mutations simultaneously in *Tet1* and *Tet2*; both genes had one allele modified with the specific base pair changes directed by the oligo (through homology-directed repair (HDR)), and the other

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Figure 1
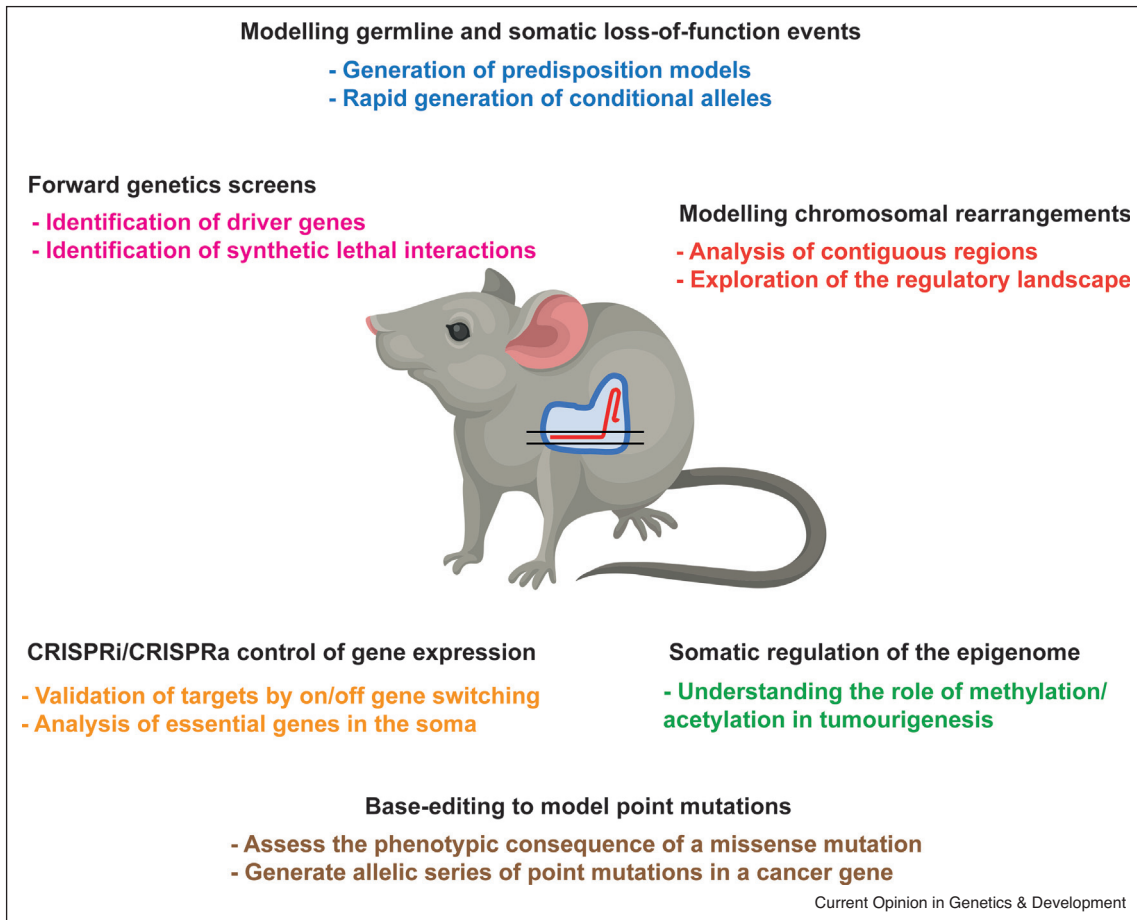
The ways that CRISPR/Cas9-gene editing may be used to understand cancer gene function in mice.

allele modified with indels (through non-homologous end-joining (NHEJ)-mediated repair) [2].

CRISPR/Cas9 technology has also been used for the generation of somatic KO mouse models of cancer. For example, hydrodynamic tail vein injection (HTVI; to deliver CRISPR/Cas9 components to the liver) of a plasmid expressing both Cas9 and sgRNAs against *Pten* and *Trp53*, resulted in the development of hepatocellular carcinomas that mimicked those seen in traditionally-generated mice with liver-specific loss of *Pten* and *Trp53* [3$^{\bullet\bullet}$]. Similarly, intraductal injections of *WapCre;Cdh1$^{flox/flox}$;Cas9* female mice with a lentivirus carrying a sgRNA for *Pten* or *Myh9* resulted in a proportion of the mice developing invasive lobular breast carcinomas (ILCs) that closely resembled human ILCs [4,5$^{\bullet}$]. To model brain cancer, wildtype mice underwent *in utero* electroporation of the developing prosencephalon with three plasmids carrying Cas9 together with sgRNAs targeting *Nf1*, *Trp53* or *Pten* which led to the development of highly aggressive glioblastomas, similar to those seen in human glioblastoma patients [6]. Extending this approach

using multiplexing, *Alb-Cre;Kras$^{LSL-G12D/+}$* mice were HTVI-administered 10 individual CRISPR-SB vectors (each vector carrying Cas9 and one of 10 different sgRNAs, flanked by Sleeping Beauty (SB) transposon repeats) and an SB transposase vector to promote genomic integration of the CRISPR-SB vectors, resulting in the development of hepatocellular carcinoma and intrahepatic cholangiocarcinoma [7]. This system allows for assessment of the oncogenic effects of multiple genes and genetic interactions in a single experiment.

CRISPR/Cas9 technology can also be used in a forward genetics approach to perform genetic *in vivo* 'screens' in mouse models of cancer, allowing for the process of tumour growth to drive enrichment of sgRNAs targeting cancer driver genes. For example, a cell line made from a mouse non-small cell cancer that carried an oncogenic Kras as well as p53 and Dicer1 loss-of-function alleles (*Kras$^{G12D/+}$;p53$^{-/-}$;Dicer1$^{+/-}$*; termed KPD), was transduced with a lentivirus carrying a Cas9 fused to green fluorescent protein, to create the 'Cas9-GFP KPD' cell line

(capable of primary tumour growth, but not metastasis, in immunocompromised mice) [8]. The Cas9-GFP KPD cells were transduced with a genome-wide sgRNA library, subcutaneously transplanted into mice, and samples collected at different timepoints. Analysis of these samples found that lung metastases and late stage primary tumours were enriched for sgRNAs that targeted only a small subset of genes, and in a validation screen, individual sgRNAs and a small pool of 624 sgRNAs targeting the top scoring genes from the primary screen, dramatically accelerated metastasis, thus effectively demonstrating that CRISPR/Cas9-based screening can be used to systematically assess the phenotypic role of genes in cancer evolution *in vivo* [8]. Similarly, blast crisis (bc) chronic myeloid leukaemia (CML) stem cells from the spleens of *BCR-ABL* mice were lentivirally infected with a genome-wide library of sgRNAs and after 48 hours of drug selection were transplanted into lethally-irradiated recipient wildtype mice. After 7 days, leukaemic cells were sorted for sequencing before the onset of full-blown disease [9]. Computational analysis revealed genes that may be essential for leukemia growth and propagation (resulting in *in vivo* depletion of the corresponding sgRNAs), known drivers of CML and bcCML, and genes that could be potential new regulators of leukaemia (such as *Stau2*, which was shown to be a key regulator of myeloid leukaemia) [9].

Conditional control of gene editing

Conditional *in vivo* genome editing allows both temporal and spatial control of the induction of genetic alterations. The mouse 'genetics toolkit' has several ways to generate conditional alleles, of which a commonly used approach is the Cre recombinase (Cre) enzyme which can inactivate or activate genes by excising *loxP*-flanked (*flox*) exons or *LoxP-Stop-LoxP* (*LSL*) transcriptional terminators, respectively [1]. The CRISPR/Cas9 system can be used in conditional mouse models of cancer to study cooperation between cancer driver genes. For example, $Kras^{LSL-G12D/+};Trp53^{flox/flox}$ (KP) mice intratracheally administered a lentivirus encoding a sgRNA targeting *Nkx2.1* or *Pten*, Cas9 and Cre, developed lung adenocarcinomas faster than KP mice treated with a lentivirus encoding Cre alone [10]. Other studies have used transgenic mice carrying a *LSL*-Cas9 cassette 'knocked in' to a safe/inert targeting site, such as *Hipp11* (*H11*) or *Rosa26* (*R26*). For example, $Kras^{LSL-G12D/+};R26^{LSL-Tom};H11^{LSL-Cas9}$ (*KT;Cas9*) mice given retrograde pancreatic ductal injections of a lentivirus carrying Cre and a sgRNA against *Lkb1* developed extensive tumour growth in the pancreas as early as 2 months after tumour initiation, with histological features that were indistinguishable from those found in $KT;Lkb1^{flox/flox}$ mice injected with a Cre lentivirus [11]. The *KT;Cas9* mice have also been used to study combinatorial tumour suppressor inactivation *in vivo*. Specifically, lung adenocarcinomas were initiated with intratracheal administration of lentiviruses (carrying Cre recombinase, and a pool of four inert sgRNAs and 11 sgRNAs targeting known and candidate

tumour suppressor genes) into *KT;Cas9* mice, $KT;Cas9;Trp53^{flox/flox}$ mice and $KT;Cas9;Lkb1^{flox/flox}$ mice [12]. As each sgRNA vector contained a unique sgID and a random barcode, it allowed quantification of individual tumour sizes via deep sequencing [13], resulting in the ability to map the tumour suppressive effects of 31 common lung adenocarcinoma genotypes (demonstrating context dependence and differential effect strengths amongst them) [12].

Another study used intratracheal delivery of an adeno-associated virus carrying Cre, a $Kras^{G12D}$ HDR donor template, and sgRNAs for *Kras*, *Trp53*, and *Lkb1*, to $R26^{LSLCas9}$ mice and found the presence of lung adenocarcinomas at 9 weeks after dosing [14]. Similarly, *p48-Cre;LSL-Cas9* 'knock-in' mice (that express Cas9 in the adult pancreas) administered adenoviruses carrying a $Kras^{G12D}$ HDR 'donor site' and multiplexed sgRNAs targeting *Trp53*, *Lkb1* and *Arid1a*, developed a spectrum of precursor lesions, such as pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasia, with eventual progression to pancreatic ductal adenocarcinoma (PDAC) [15].

An alternative system for conditional genome editing makes use of doxycycline (dox)-regulated Cas9 alleles. For example, one study utilised $R26^{rtTA/+};Col1a1^{c3GIC9/+}$ mice carrying two 'knock-ins', one allele ($R26^{rtTA}$) encoding the reverse tetracycline-controlled transactivator (rtTA) and another allele ($Col1a1^{c3GIC9}$) encoding dox-inducible Cas9 and sgRNAs targeting *Apc* and *Trp53* [16]. Dox-administered $R26^{rtTA/+};Col1a1^{c3GIC9/+}$ mice showed intestinal histopathology mirroring that seen in conditional *Apc* knockout mice, including dramatic hyperproliferation, crypt expansion, a marked reduction in differentiation and ectopic production of Paneth cells [15]. A similar approach was used to investigate the role of the E3 ubiquitin ligase RNF43 in pancreatic ductal adenocarcinoma (PDAC). Embryonic stem (ES) cells from the $Kras^{LSL-G12D};Ptf1a-Cre$ (KC) mouse PDAC model were retrofitted with an $R26^{CAGS-LSL-rtTA3-mKate2}$ (RIK) allele for Cre-inducible rtTA expression [17]. The resulting *KC-RIK* ES cells were further modified by targeting the *Col1a1* locus with c3GIC9 plasmids encoding dox-inducible Cas9 and *Rnf43*-targeting or 'control' sgRNAs, and then transmitted through the germline to produce *KC-RIK-Rnf43* or *KC-RIK-cgCR8* mice, respectively [18]. Relative to dox-treated *KC-RIK-cgCR8* mice, dox-treated *KC-RIK-Rnf43* mice exhibited reduced survival with a larger proportion of the cohort developing invasive PDAC [18].

Conditional Cas9 mice can also be used for *in vivo* genetic screens with sgRNA libraries, to identify key drivers of tumour growth and/or metastasis. For example, to test the role of significantly mutated genes (SMGs) found in genome atlases of glioblastoma multiforme (GBM), a library of sgRNAs targeting mouse homologs of the 56 top-ranked pan-cancer SMGs ('mTSG' library) were

cloned into an AAV vector encoding astrocyte-specific *GFAP*-Cre and an sgRNA targeting *Trp53* (which is frequently mutated in GBM) [19]. Analysis of brain tumours from *LSL-Cas9* mice in which AAV-mTSG library viruses had been injected into the lateral ventricle or hippocampus, showed that many genes were significantly mutated by sgRNA-induced indels in 20-50% of mice, and highlighted a critical role for epigenetic regulators in brain tumourigenesis. In addition, co-mutation analysis identified frequently co-occurring driver combinations, with the top pair being *Nf1* and *Pten* [19].

Modelling chromosomal rearrangements

The CRISPR/Cas9 system can be used to accurately recapitulate the complex genomic rearrangements seen in human cancers, such as chromosomal deletions, translocations and gene fusion events. For example, one group utilised the replication-competent avian leukosis virus splice-acceptor (RCAS)-TVA-based approach, which uses RCAS vectors to target individual cells engineered to express the TVA cell surface receptor. To create a chromosomal deletion, an RCAS virus carrying sgRNAs targeting intron 13 of *Bcan* and intron 10 of *Ntrk1* was used to infect neural stem cells (NSCs) isolated from *Gtv-a;GFAP-Cre;LSL-Cas9;Trp53^lox/lox* pups, with had brain-specific *p53*-loss and expression of TVA and Cas9 [20]. Intracranial injection of the infected NSCs into NOD/SCID mice resulted in tumours that showed histological features of high-grade glioma, including a high percentage of Ki67-positive cells, microvascular proliferation and pseudopalisading necrosis [20]. Another group used the above-mentioned KC-RIK mice to mimic a large homozygous chromosomal deletion seen on chromosome 9 in human PDAC. Two sgRNAs (sgDel-A and sgDel-B) against target sites separated by ∼1.2Mb on the syntenic region of mouse chromosome 4 were cloned into the 3GIC9 plasmid, which was then inserted into the *Col1a1* locus of KC-RIK ES cells and transmitted through the germline [18]. The pancreas of dox-treated KC-RIK-sgDel-A/B mice showed the presence of multiple foci of PDAC, of poor to moderate differentiation. The CRISPR/Cas9 system has also been used to generate chromosomal inversions *in vivo*, with one study generating a mouse model of *Eml4-Alk*-driven lung cancer; using a plasmid that simultaneously expressed Cas9 and two distinct sgRNAs (*Eml4* and *Alk*) from tandem U6 promoters. Recombinant adenoviruses expressing Cas9 and both sgRNAs (Ad-EA) were administered to mice by intratracheal instillation and at 12-14 weeks post-infection, the lungs of Ad-EA-infected mice showed the presence of multiple adenocarcinomas [21]. Importantly, the Ad-EA-induced lung tumours were sensitive to crizotinib, which is an ALK/MET inhibitor used in the clinic to treat patients with *ALK*-positive non-small cell lung cancer [21].

The use of base editing to model point mutations in cancer

Base editing using the CRISPR/Cas9 system allows for precise engineering of somatic point mutations of key cancer drivers by enabling conversion of single nucleotides without formation of DNA breaks [22,23]. The best characterized base editors to-date are the cytosine base editors (CBEs), allowing C > T transitions. 'BE3' is a CBE composed of a nuclease defective Cas9 (dCas9) fused to a cytidine deaminase [24••]. A conditional BE3 allele was introduced into the *Col1a1* locus of ES cells from *WapCre;Brca1^flox/flox^;Trp53^flox/flox^* (WB1P) mice and transmitted through the germline to produce WB1P-BE3 mice [25]. Intraductal injection of these mice with a lentivirus carrying either a non-targeting (NT) sgRNA or an sgRNA targeting the third exon of *Akt1* (in order to generate an oncogenic *Akt1^E17K^* missense mutation by base editing), as well as a *Myc*-overexpression cassette (Lenti-sg*Akt1*^E17K^-*Myc*), resulted in the development mammary tumours; however, the Lenti-sg*Akt1*^E17K^-*Myc* mice developed tumours with much shorter latency than the Lenti-sgNT-*Myc* mice [25].

The same study also generated an allelic series of missense mutations of *Pik3ca in vivo*, and showed that Lenti-sg*Pik3ca^E542K^*-*Myc*, Lenti-sg*Pik3ca^E545K^*-*Myc* and Lenti-sg*Pik3ca^E453K^*-*Myc*-dosed WB1P-BE3 females developed mammary tumours with a significantly shorter latency than Lenti-sgNT-*Myc*-dosed females, underscoring the fact that whilst the E453 K mutation is not found as commonly as the other 2 mutations in human tumours, it has similar cooperative effects in this setting [25]. The feasibility of multiplexed base editing was also demonstrated when WB1P-BE3 mice carrying a *Trp53^F/+^* allele were injected with a tandem Lenti-sg*Pik3ca^E545K^*/sg*Trp53^Q97*^*-*Myc* vector that harbours two arrayed sgRNA cassettes, to simultaneously introduce the *Pik3ca^E545K^* missense mutation and inactivate the residual wildtype copy of *Trp53*; the mice developed mammary tumours significantly faster than Lenti-sg*Trp53^Q97*^*-*Myc*-dosed mice [25].

Future directions for CRISPR/Cas9 mouse modelling of cancer

As detailed above dCas9 can be fused with base-editing enzymes to allow specific alteration of the genome. Following on from this, dCas9 can be fused with epigenetic enzymes to alter DNA methylation and histone methylation/acetylation of genomic DNA. For example, sites on the skin and in the brain of *Igdmr^Snrpn-EGFP^* methylation reporter mice that were lentivirally infected with dCas9 fused to the CpG demethylase TET1 (dCas9-*Tet1*) and *Snrpn* sgRNAs, showed demethylation of the *Snrpn* promoter and expression of the EGFP reporter [26]. Another study fused dCas9 with the DNA methyltransferase MQ1 (dCas9-MQ1), and induced CpG methylation in the differentially methylated region of the paternally imprinted *Igf2/H19* locus in mice by zygote microinjection of the methylation construct along with multiple sgRNAs [27•]. Given that abnormal DNA methylation has been observed in cancer, there is no doubt that in the future

these and other fusions of dCas9 with epigenetic enzymes will be pressed into use for cancer modelling in mice.

CRISPR interference (CRISPRi) is an alternative approach for loss-of-function studies that inhibits expression by guiding transcriptional repressors (such as *Krüppel associated box*, *KRAB*) to the transcription start-site (TSS) of sgRNA-targeted genes. For example, a CRISPRi screen in human ES cells identified molecular drivers of early mesoderm development, such as the *FOXA2* transcription factor [28]. A transgenic mouse line which carried a transgene composed of an sgRNA against *Tnfsf11* and a dCas9-KRAB, showed that high expression of the transgene resulted in a phenotype comparable to mice with germline deletion of *Tnfsf11* [29]. More recently, an *in vitro* CRISPRi screen using sgRNAs targeting the TSSs of 5,689 lncRNA loci was performed in a human GBM cell line that stably expressed dCas9-KRAB, to identify specific lncRNAs that sensitize glioma cells to radiotherapy [30].

Conversely, dCas9 fusions can be used to activate gene expression (CRISPRa). These systems involve dCas9 fused to transcriptional activators (such as *VP64* and *p65*) which are guided to promoter and enhancer regions by sgRNAs, resulting in upregulation of gene expression. For example, a CRISPRa screen of the $BRAF^{V600E}$ mutant A375 melanoma cell line to study the mechanisms of resistance to the BRAF inhibitor PLX-4720, identified both previously known resistance mechanisms, such as EGFR and ERK pathway activation, as well as novel resistance mechanisms involving G protein-coupled receptors [31]. Given that reversible control of gene expression provides better opportunities to mirror the genetics of human tumours, it will not be long before CRISPRi/a systems are utilised *in vivo* with mouse models of cancer.

Thus, CRISPR/Cas9 has been an invaluable addition to the 'genetics toolkit' to allow generation of mouse models that faithfully recapitulate the myriad of genetic and epigenetic modifications seen in human cancers.

Conflict of interest statement
Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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