Cornerstones of CRISPR–Cas in drug discovery and therapy

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Abstract | The recent development of CRISPR–Cas systems as easily accessible and programmable tools for genome editing and regulation is spurring a revolution in biology. Paired with the rapid expansion of reference and personalized genomic sequence information, technologies based on CRISPR–Cas are enabling nearly unlimited genetic manipulation, even in previously difficult contexts, including human cells. Although much attention has focused on the potential of CRISPR–Cas to cure Mendelian diseases, the technology also holds promise to transform the development of therapies to treat complex heritable and somatic disorders. In this Review, we discuss how CRISPR–Cas can affect the next generation of drugs by accelerating the identification and validation of high-value targets, uncovering high-confidence biomarkers and developing differentiated breakthrough therapies. We focus on the promises, pitfalls and hurdles of this revolutionary gene-editing technology, discuss key aspects of different CRISPR–Cas screening platforms and offer our perspectives on the best practices in genome engineering.

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The central dogma of molecular biology posits a flow of information from gene to mRNA to protein[1]. The genome serves as the blueprint of life, setting the stage for all downstream activity. Although approaches to treat human disease predominantly target the end of the information cascade (for example, by inhibiting signalling pathways, supplementing metabolites or interfering with viral polymerases), the discovery and validation of therapeutic targets often takes place at the level of genes and transcripts. The discovery of human mutations that are directly linked to disease, such as somatic breakpoint cluster region–Abelson tyrosine kinase 1 (*BCR–ABL1*) fusions in chronic myeloid leukaemia or inherited *BRCA1* mutations in breast cancer, or of mutations associated with a survival benefit, including proprotein convertase subtilisin/kexin type 9 (*PCSK9*) mutations in minimizing cardiovascular disease, is considered by many to be the 'gold standard' for drug target identification. However, the paucity of scalable genetic engineering tools in mammalian cell culture and model systems has necessitated that many discovery efforts that link genotype with phenotype are either observational, such as genome-wide association studies (GWAS), or take place in genetically malleable invertebrate models such as the fruitfly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*.

The recent development of easily programmable RNA-guided nucleases, which are derived from microbial adaptive immune systems, has revolutionized the molecular toolbox for mammalian genome engineering[2–6]. Gene-editing technologies in the form of clustered regularly interspaced short palindromic repeat (CRISPR)–CRISPR-associated protein (Cas) systems stand poised to transform many stages of drug discovery and development by enabling fast and accurate alterations of genomic information in mammalian model systems and human tissues. In addition, direct somatic editing[7] in patients will, eventually, radically change the druggable space[8] by enabling the targeting of nearly any entity, including the introduction of corrective mutations and the modification of regulatory elements or splicing patterns. Following the description of a two-component single guide RNA (sgRNA)–Cas9 complex to introduce DNA double-strand breaks (DSBs) in an RNA-guided manner[2], many studies have demonstrated ingenious applications and uncovered orthogonal immune systems, together enabling nearly unlimited genome engineering opportunities (FIG. 1).

The technological domestication of CRISPR–Cas systems and molecular mechanisms of Cas-based genome editing have been thoroughly covered elsewhere[9–11]. Briefly, a sgRNA directs the Cas9 endonuclease to induce DSBs at homologous sites[2]. During genome editing, the DSBs are fixed by cellular DNA repair mechanisms, including the predominant error-prone non-homologous end joining (NHEJ)[12–14] and the less-frequent templated homology-directed repair (HDR)[15–19] pathways. NHEJ is most often leveraged to disrupt genetic sequences,

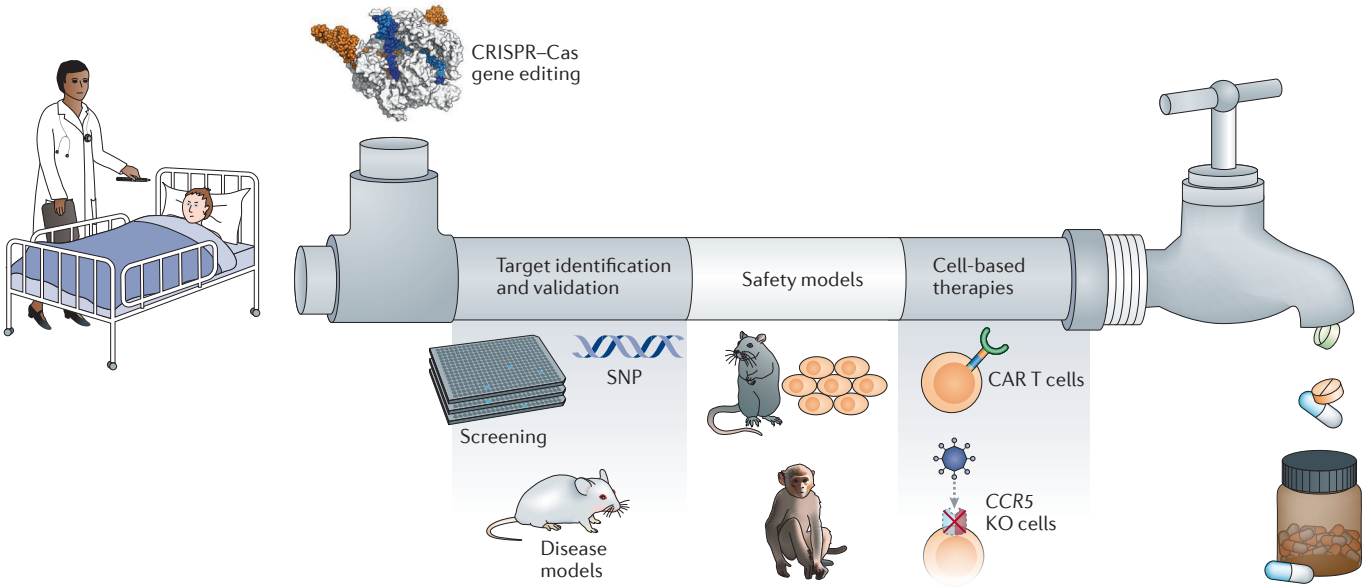

Figure 1 | **Pipeline of CRISPR–Cas-assisted drug discovery.** Unmet medical needs for numerous diseases and the rapid progress of CRISPR–Cas gene editing can feed into a drug discovery and development pipeline, which leads to improved therapies. The CRISPR–Cas system allows for improved target identification and validation as well as faster generation of safety models. CRISPR–Cas can also be used to develop cell-based therapies, such as chimeric antigen receptor (CAR) T cells for immunotherapy and C-C motif chemokine receptor 5 (*CCR5*)-knockout (KO) cells for HIV treatment. CRISPR–Cas-assisted drug discovery will yield innovative therapies and treatment paradigms for patients. SNP, single-nucleotide polymorphism.

whereas HDR can be used to introduce or alter information at a specific locus with properly designed repair templates. In addition, a catalytically inactive mutant of Cas9 can be fused to various effector domains to activate or inhibit the transcription of target genes, strategies known as CRISPRa and CRISPRi, respectively[20–22]. Most studies to date have used Cas9 from *Streptococcus pyogenes* (SpyCas9), which is the default Cas9 referenced in this Review. Cas9 molecules from other species, Cas9-like CRISPR nucleases and engineered versions of Cas9 with novel functions have also been established and can convey particular advantages in various settings (Supplementary information S1 (table)). Although we focus on SpyCas9, in particular its use in therapeutic discovery and the building of the next generation of transformational drugs, the general outline described here applies to the larger ensemble of CRISPR–Cas tools.

CRISPR–Cas as a tool for drug discovery

Precision cellular models. Advances in DNA sequencing and their large-scale application have provided insight into genetic variation across groups of patients and populations, which has expanded our understanding of the links between genetic variation and disease predisposition, disease development and the treatment response. For example, integrated information from The Cancer Genome Atlas (TCGA)[23–28], the Cancer Cell Line Encyclopedia (CCLE)[28] and the Encyclopedia of DNA Elements (ENCODE)[29,30] led to improvements in the standard of care for patients with glioblastoma, enabling stratification based on the methylation status of the O^6-methylguanine DNA methyltransferase (*MGMT*) promoter[31]. Such advances have stimulated interest in 'personalized' or 'precision' medicine, which combines classical patient information with personal genetic data to directly inform individual treatment strategies. However, hypotheses that are generated by large-scale observational 'omics' efforts often demand testing with precise genetic models, particularly to evaluate variants of unknown significance, optimize patient stratification, reassign approved drugs to new indications and develop alternative treatment paradigms.

Even when a single factor (such as the mutational status of *TP53*, *MYC* or *KRAS*) is compared between cells, there are often many confounding features that obscure a direct relationship between genotype and disease phenotype. Researchers might use matched patient samples from diseased and normal tissues to tease apart such relationships. However, large collections of matched samples can be difficult to obtain and are not available in many cases. Although overexpression of appropriate (often mutant) cDNA can partially address this issue, such constructs are often expressed at non-native levels and in the presence of the wild-type protein. The generation of mutant or knockout clones via classical homologous recombination led to a limited set of isogenic cell lines, in which a derived line differs from the parent by a minimal, defined mutation[32–35]. These resources have proved to be incredibly useful, but initial techniques for their generation were very labour intensive and time consuming, which hindered their widespread adoption for drug development.

The advent of CRISPR–Cas genome editing[2] has drastically altered this landscape (FIG. 2). The generation of isogenic knockout human (and other) cell lines for comparative genomics is now so straightforward that, in just

Non-homologous end joining
(NHEJ). The repair of double-strand DNA breaks by direct ligation of the broken ends. No homology is required to promote the end-joining reaction, and it can result in the introduction of small non-templated insertions or deletions (indels).

Homology-directed repair
(HDR). The repair of double-strand DNA breaks using an endogenous or exogenous DNA template with homology to regions flanking the break.

CRISPRa
The activation of transcription through RNA-guided recruitment of a catalytically inactive Cas9 fused to transcriptional activators.

CRISPRi
The inhibition of transcription through RNA-guided recruitment of a catalytically inactive Cas9 fused to transcriptional repressors.

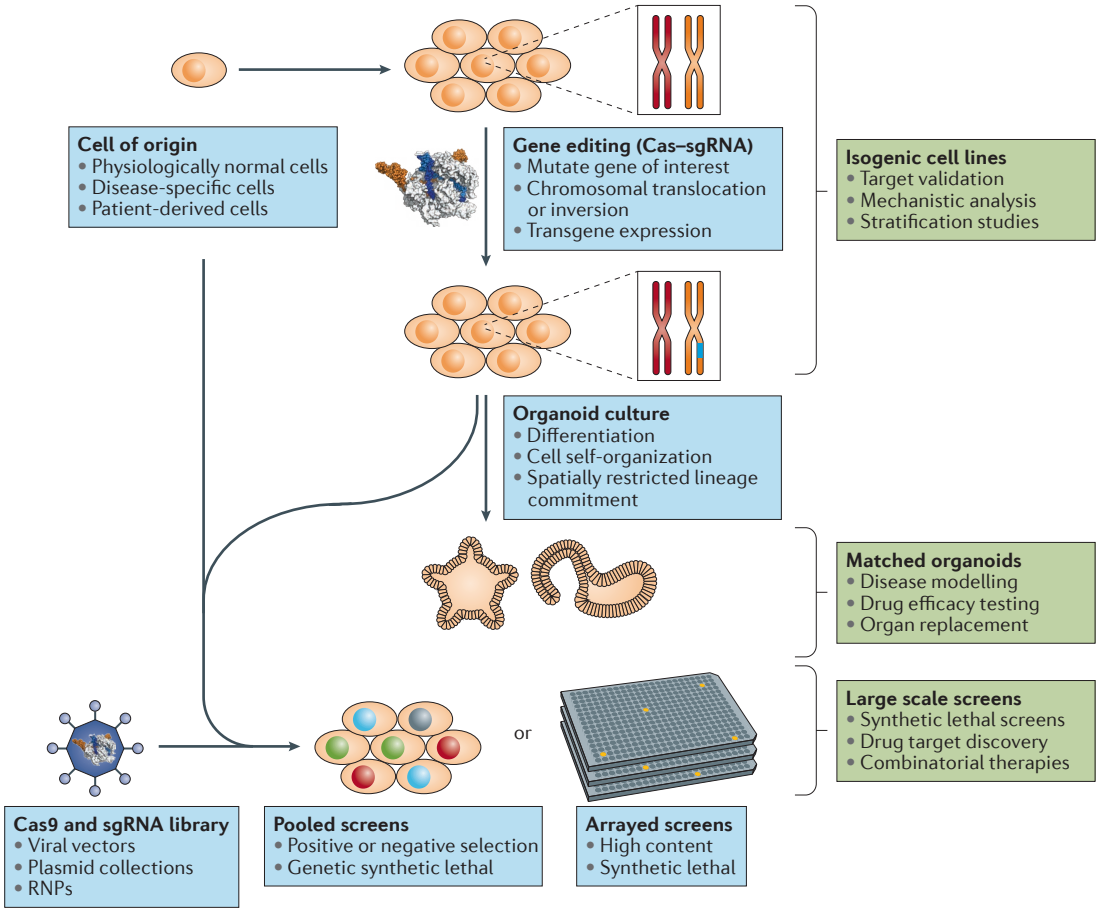

Figure 2 | **CRISPR–Cas in the generation of cellular models and large-scale screens.** CRISPR–Cas gene editing can be used to generate isogenic cell lines for drug target validation, mechanistic analysis and patient stratification studies. Isogenic cell lines can also be used to generate organoids, which are particularly useful for modelling differentiation and self-organization processes. Large-scale single guide RNA (sgRNA) libraries can be used for high-throughput pooled or high-content arrayed screens, either in unmodified or in CRISPR–Cas-edited cell lines. RNPs, ribonucleoproteins.

4 years, the practice has become commonplace[36] and is being carried out by researchers around the globe. Gene knockout via CRISPR–Cas has proved to be efficacious in virtually all cell types, including induced pluripotent stem cells (iPSCs), cancer-specific organoids and primary immune cells[37–40]. Knockout-based target discovery efforts are thus no longer limited to specialized cell lines, such as the haploid lines that were previously used for gene trap experiments[41,42], and can instead be performed in the cell type that is most appropriate for the disease of interest. For example, if a panel of tumour-derived lines are thought to be sensitized to a drug candidate via a genetic lesion, CRISPR–Cas-mediated gene knockout can directly test the hypothesis of synthetic lethality[43–45]. Such isogenic knockouts allow researchers to rapidly establish causative roles for oncogenes, tumour suppressors and other factors in a defined context, thereby removing secondary differences.

Similarly, 'knocking in' mutant alleles by HDR allows researchers to test the effects of disease-associated mutations in an isogenic background. For example, HDR can serve to generate mutant allelic series to compare the effects of each variant found across patients, as is the case for oncogenes such as *KRAS*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) and isocitrate dehydrogenase 1 (*IDH1*), or tumour suppressors including *TP53*, *RB1* and von Hippel–Lindau (*VHL*)[46,47]. More generally, isogenic series can be used to analyse the effect of mutants on disease development or to query the specificity of mutant-targeting therapeutic candidates. From a technical perspective, HDR requires delivery of the Cas9–sgRNA complex — in the form of a viral vector or plasmid (that encodes Cas9 and the sgRNA), or Cas9–sgRNA ribonucleoprotein (RNP) complexes (comprising Cas9 protein and the sgRNA) — along with a DNA repair template. The HDR template can also take on different forms, and its exact design can substantially affect repair efficiency[48,49]. In mammalian cells, short single-stranded DNA (ssDNA) oligonucleotides can be designed to take advantage of the molecular nature of the Cas9–target architecture and these oligonucleotides have been shown to effectively introduce small mutations[50]. Additional control over the efficiency of mutant introduction and zygosity can be achieved by varying the distance between the DSB and the site of the mutation on the repair template [51].

Despite this promise, although CRISPR–Cas knockouts are effective in nearly any cell, rates of HDR can vary across cell types. As one example, it has been difficult to achieve even moderate levels of HDR in non-mitotic human cells, including neurons. These barriers are particularly frustrating, because sequence insertion or replacement in these contexts could be used to model or to treat many genetic diseases. New approaches that use non-homologous or microhomology-mediated integration of cassettes[48,52–55] offer routes to bypass HDR pathways that are inactive in non-mitotic human cells and in organisms in which HDR has proved to be difficult. Another exciting new development is the engineering of Cas enzymes with additional functionalities to enable precise, template-less introduction of specific mutations by direct alteration of target bases. A first step towards this goal was the fusion of various cytidine deaminases to Cas9, which resulted in hybrid enzymes that are capable of RNA-guided 'base editing' (REFS 56,57), and one can anticipate a dramatic increase in the number of new Cas derivatives developed using similar strategies.

Functional screening with CRISPR–Cas. Large-scale functional screening with CRISPR–Cas is simultaneously expanding and evolving, as researchers uncover the advantages and disadvantages of different screening systems. Until recently, systematic loss-of-function studies focused on genome-wide RNA interference (RNAi) screens[58–60] or insertional mutagenesis screens in haploid human cell lines[41,42,61,62]. CRISPR–Cas screens have rapidly been adopted in various contexts owing to the simplicity of designing potent sgRNAs and the ability to apply the system to nearly any cell type or tissue (FIG. 2). Large-scale screens typically rely on pooled lentiviral libraries of sgRNAs, often achieving robust hit identification by including 3–10 sgRNAs per gene[20,63–67]. The procedure of CRISPR–Cas-based screens is very similar to that of short hairpin RNA (shRNA) screens. A pool of cells that co-express Cas9 and the sgRNA library is subjected to the desired phenotypic selection, and high-throughput DNA sequencing of the sgRNA cassette is used to identify sgRNAs that were enriched or depleted during the treatment.

Genome-scale CRISPR–Cas knockout, inhibition and activation screens have identified essential genes in various cancer cell lines[63,64,68–70], uncovered genes that are involved in the response to small-molecule inhibitors[60,65] and cellular toxins[20,66], and dissected the relative importance of viral host factors[71]. They have also been used in a xenograft mouse model of tumour growth and metastasis to assay gene phenotypes in cancer evolution[72]. Although CRISPR–Cas screens for cell growth or survival have been quite successful (except when targeting genetically amplified regions[64,68,69]), screens for more complex phenotypes are still in the process of being optimized. Recent comparisons with microRNA-based shRNA screens have found comparable performance[60,70], and the complementary strengths of both approaches should be carefully weighed when choosing a screening platform (TABLE 1).

In CRISPR nuclease (CRISPRn) screens, stably expressed Cas9–sgRNA complexes continue to operate on a target site until it is ablated and can therefore

Table 1 | **Comparison of screening platforms**

Characteristic	CRISPRn	CRISPRi	RNAi*	CRISPRa
Effect	Knockout	Knockdown	Knockdown	Activation
Mechanism	Mutation-causing indel	Transcriptional interference	Transcript degradation and/or translational interference	Transcriptional activation
Guide target choice	Anywhere in the genome with a PAM	TSS with a PAM	Exons	TSS with a PAM
Target selectivity	Can distinguish any target	Depends on TSS, cannot distinguish products derived from the same transcript	Can distinguish splice variants	Depends on TSS, cannot distinguish products derived from the same transcript
Highly amplified regions (genes)	Off-target effects: DSBs evoke DNA damage repair, resulting in cell cycle arrest independently of target	Can be targeted if all use the same TSS	Can be targeted	Can be targeted if all use the same TSS
Distinguish alternative TSSs	Possible	Yes	Possible	Yes
Distinguish transcript splice variants	Possible	No	Possible	No
Performance of individual sgRNAs or shRNAs	Most work	Many work	Requires good prediction tools or testing	Many work

DSB, double-strand break; indel, insertion and/or deletion; PAM, protospacer adjacent motif; RNAi, RNA interference; sgRNA, single guide RNA; shRNA, short hairpin RNA; TSS, transcription start site. *MicroRNA-based shRNAs.

CRISPR nuclease (CRISPRn). Targeting a DNA sequence with catalytically active Cas9 to generate a double-strand break or a nick.

generate homozygous knockout phenotypes at high frequency in most cell types. Conversely, high copy number genomic amplifications can be a barrier to CRISPRn screens, mainly because of the large numbers of DNA breaks that are generated in high copy number regions. The large number of DNA breaks can lead to reduced cell growth triggered by the DNA damage response and cell cycle arrest, which are activated independently of the targeted gene or genomic region (thus representing a systematic, sequence-independent off-target effect)[64,68,69]. As CRISPRn generally depends on sequence frame-shifting to generate knockouts, phenotype penetrance can be affected if in-frame deletions are preferentially created. This can be overcome by targeting functional domains[43], although this approach requires pre-existing knowledge of target proteins. Furthermore, sgRNAs that target the 5′ end of the coding region may be ineffective if alternative downstream start codons are present[68].

CRISPRi screens do not rely on frame shifting and can offer certain advantages over CRISPRn screens from a drug discovery perspective, because knocking down gene expression (using CRISPRi or RNAi) can mimic the effects of a small-molecule inhibitor more closely than does complete gene ablation[73]. CRISPRi screens can also identify the contributions of transcripts arising from different transcription start sites (TSSs), whereas RNAi screens can uniquely distinguish different splice variants[59,74].

CRISPRa screens, which assess gene targets whose overexpression leads to a given phenotype[20,21], are an emerging and particularly exciting area of recent development. They have an array of benefits and trade-offs compared with cDNA screens, which have previously been used in this area. Construction and use of cDNA screening resources are labour intensive owing to the complex nature of cDNAs. By contrast, the resources necessary to perform CRISPRa screens are similar to those required by CRISPRn or CRISPRi screens[20]. Moreover, cDNA expression screens can only interrogate the transcripts present in the library, which may lack certain genes or transcript variants. Conversely, by stimulating expression from the endogenous locus, CRISPRa screening can activate expression of alternative transcripts from secondary TSSs as easily as it activates expression of the primary transcript, and sgRNAs can be designed to target each TSS within each gene. However, CRISPRa screens are subject to their own set of false negatives. For example, CRISPRa will have no effect if the target gene contains loss-of-function mutations or is missing entirely in the cell line of interest.

A substantial technical barrier for CRISPRa screening is the activation of highly repressed genes. To overcome this challenge, a range of CRISPRa systems have been developed that recruit multiple and/or diverse transcriptional activation domains to increase the potency of gene activation[20,21,75–80]. Ultimately, an ideal CRISPRa screening platform would use the fewest necessary exogenous parts to potently activate any gene target; additional developments and systematic comparisons are needed in order to achieve this goal[81].

We expect that CRISPR–Cas-based screens will continue to improve, especially as they are used for an increasingly broad array of phenotypes. Most of the pioneering CRISPR screens simply looked for growth advantages and disadvantages, leading to the identification of genes that are essential for proliferation, or resistance or sensitivity to certain toxins. Going forward, there will be more CRISPR screens to examine the sensitivity of cancer cells to candidate therapeutics, resistance to pathogen infections, or the regulation and cellular localization of a gene or protein of interest[42,60,71]. CRISPR screens in human pathogens can also be used to identify candidate drug targets[82]. The relatively low cost of sgRNA library design facilitates creative screening approaches, such as efforts to identify non-coding sequences that control expression of B-cell CLL/lymphoma 11A (*BCL11A*), *TP53* and oestrogen receptor 1 (*ESR1*) using target-tiling CRISPRn screens[83,84], in which a genomic region is targeted with multiple guide RNAs, and we expect future screens for non-coding regulatory elements to examine even larger regions of DNA sequence. However, more systematic analyses are needed to compare CRISPRn, CRISPRi and various types of RNAi screens (including microRNA-based shRNAs)[70]. Such comparisons will define the relative strengths and weaknesses of each platform and allow researchers to choose the best type of screen to address their question (TABLE 1).

Rapid generation of animal models. Beyond cell culture applications, genome editing has dramatically altered our ability to generate animal models of disease (FIG. 3). It will soon be common for early 'go' or 'no-go' decisions in a drug development campaign to be based on results from rapidly created mutant animals of the most relevant model species for a disease. Indeed, shortly after their initial development, CRISPR–Cas tools were used to generate mice with multiple genetic lesions in a single editing step[85], as well as for one-step knock-in of reporter and conditional alleles into mouse zygotes[86].

In general, efficient CRISPR–Cas editing techniques, including NHEJ and short HDR, can be achieved by microinjection or simple electroporation of zygotes instead of proceeding through traditional embryonic stem (ES) cell manipulation[87–89]. This is a crucial development in two ways. First, as multiple genes can be targeted in a single step, double- and triple-mutant mice can be rapidly generated without the need for crossing single-mutant strains, although it must be noted that such alleles follow Mendelian segregation upon breeding. Second, genome editing in zygotes eliminates the requirement to derive, culture and edit ES cells, which has slowed the generation of mutants and has been a major barrier to widespread genetic tractability in several model organisms relevant to the process of therapeutic discovery, such as rats. Zygote editing also accelerates the generation of additional mutations in pre-existing animal models of disease by eliminating the need for ES cell derivation or lengthy backcrossing. Nevertheless, the introduction of large transgenes or complex multicomponent systems via zygote editing remains inefficient,

Backcrossing
The process of breeding a hybrid organism with an individual genetically similar to one of its parents, with the objective of diluting the genetic contribution of the other parent to subsequent generations.

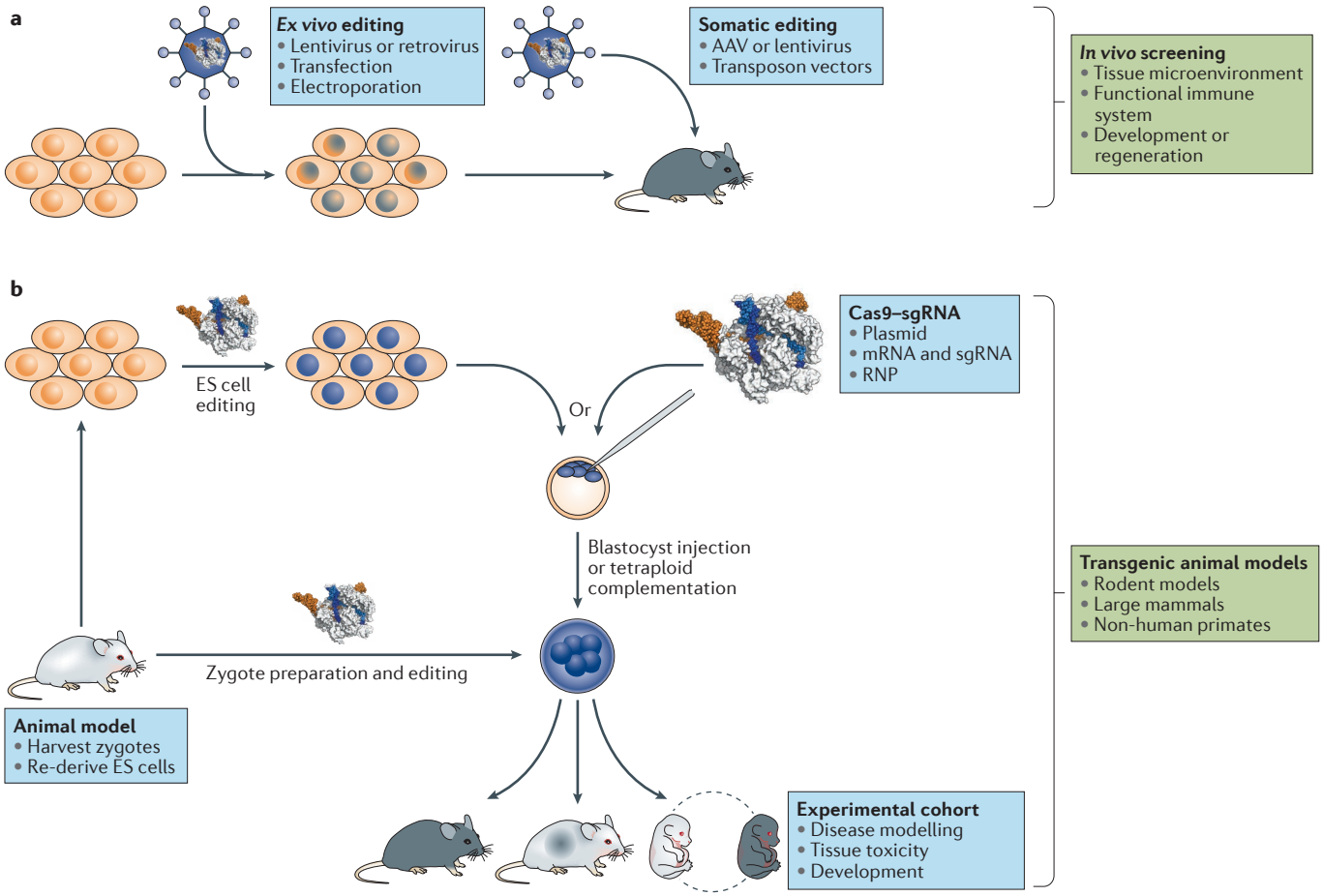

Figure 3 | **Applications of CRISPR–Cas in *in vivo* screens and the generation of animal models. a** | *Ex vivo* editing can be used to generate a library of modified cells for transplantation into recipient animals. Alternatively, editing reagents can be delivered to host animal tissues directly for somatic *in situ* editing. **b** | CRISPR–Cas has also revolutionized the generation of transgenic animal models through facile editing of embryonic stem (ES) cells for traditional gene targeting and by enabling direct zygote editing in most species. Zygote editing can be done *ex vivo* by electroporating or microinjecting zygotes with CRISPR–Cas constructs in the form of plasmids, RNA preparations or ribonucleoproteins (RNPs). AAV, adeno-associated virus; sgRNA, single guide RNA.

and for now, gene targeting in ES cells is likely to remain the method of choice for generating animals that harbour such mutations[90,91].

Founder animals from zygote editing or conventional blastocyst injection of modified ES cells can exhibit mosaicism (BOX 1). Mosaicism in ES cell injection studies can be reduced by the tetraploid complementation method[92,93] in which modified ES cells are introduced into developmentally compromised blastocysts, although this is a technically complex procedure that requires amenable ES cells. Conversely, in zygote electroporation studies, mosaicism is due to the fact that the single-cell zygote occasionally divides before editing occurs. Hence, replacing Cas9 mRNA and sgRNA or Cas9–sgRNA-encoding plasmids with Cas9–sgRNA RNP complexes, which can immediately act on their targets, increases the fraction of non-mosaic founders but does not completely solve the problem[87,88]. Overall, CRISPR–Cas promises to revolutionize mouse genetics by reducing the time that is necessary to generate targeted models from years to months or weeks. A large range of models can now be generated in a timescale relevant to early go or no-go decisions in a modern drug discovery campaign. Drug discovery implications of gene editing in additional species are discussed at the end of this section.

Pairing CRISPR–Cas with viral or transposon-based vectors has allowed researchers to directly introduce somatic mutations in certain tissues, such as lung and liver tissues, in adult animals. This approach has been used to create numerous cancer and other disease models[94–96] and to correct disease mutations and phenotypes[97–100]. One illustrative example of the power of CRISPR–Cas tools is the *in vivo* engineering of oncogenic chromosomal rearrangements that mimic fusion proteins found in patients (for example, EML4–ALK, KIF5B–RET and CD74–ROS1), which lead to *in situ* tumour initiation from edited somatic cells[101,102]. The ability to introduce disease-associated alleles in live animals is particularly transformative compared with xenograft models that require immunosuppressed recipients and mostly rely on implantation at non-native sites.

Box 1 | **Mosaicism**

Mosaicism is the presence of cells that have multiple different genotypes within a single animal or cell population.

In cell culture

In most cases, a population of edited cells will contain numerous mutations, even if all of the alleles within the cell population are edited. This is because DNA double-strand break (DSB) repair by the predominant non-homologous end joining (NHEJ) pathway can lead to different insertions and/or deletions (indels) in different cells. Depending on the experiment, mosaicism in cultured cells may or may not be problematic. If the editing efficiency is sufficiently high and all mutations cause the same phenotype (for example, loss of function due to mutations in the active site of an enzyme), the mosaicism is functionally irrelevant. In other cases, some indels might result in an in-frame deletion that has no phenotype, leading to a variegated population. Mosaicism can be eliminated by deriving single-cell clones.

In animal models

When edited embryonic stem (ES) cells are injected into a blastocyst for model generation, the resulting animal can be a mosaic of the donor ES cells and the cells of the recipient blastocyst. Tetraploid embryo complementation, a method that renders a recipient blastocyst developmentally compromised, can reduce this risk. Mosaicism can also be a result of zygote editing if editing takes place after the one-cell stage. Hence, editing methods that act on their DNA targets directly upon transduction (such as Cas9–single guide RNA ribonucleoprotein complexes) may reduce mosaicism in founder animals. Mosaicism at a given locus can be eliminated by backcrossing founder animals for a single generation, but can nonetheless be problematic if multiple genes are targeted simultaneously. For example, founder animals with mutations in three targeted genes will not necessarily carry all three mutations in every individual cell. If this is the case, multiple generations of breeding are needed to generate non-mosaic animals with mutations in all three genes.

The *in situ* introduction of mutations with CRISPR–Cas allows researchers to accurately recapitulate disease initiation, development and maintenance in an autochthonous and immunocompetent setting, including the native microenvironment and tissue structure. This ability will be transformative for many diseases, particularly for cancer, in which the interaction of tumour cells with immune cells can have a drastic effect on disease outcome[96,103].

The large and rapidly growing number of organisms targeted by CRISPR–Cas holds great promise, as traditional gene targeting has remained difficult in preclinical models other than mice. CRISPR–Cas editing has been performed in rats[104], dogs[105] and cynomolgus monkeys[106], which are all commonly used during preclinical drug discovery and development. As with mouse zygote targeting, many of the edited animals exhibit mosaicism. The generation of disease models in primates, such as a model of Duchenne muscular dystrophy in rhesus monkeys[107], further emphasizes how gene editing can not only accelerate therapeutic development but also test the efficacy and safety of therapeutic compounds. CRISPR–Cas may even drive the development of porcine xenotransplant platforms through the inactivation of endogenous retroviruses[108].

CRISPR–Cas editing will also be a boon to infectious disease research. Many human pathogens are best modelled in hosts other than mice, such as influenza (ferrets)[109], leptospirosis (hamsters)[109] and tuberculosis (guinea pigs)[110], and we expect zygote editing to be proven feasible in these organisms in the near future. The optimization of conditions for ES cell work was one of the biggest challenges in the genetic manipulation of new mammalian model organisms. CRISPR–Cas zygote editing should soon eliminate this hurdle.

Specificity of CRISPR systems

Although CRISPR-based tools are easily programmed to target basically any genomic location, they can also lead to low rates of off-target editing or sequence-independent cell cycle arrest if highly amplified loci are targeted[64,68,69]. At first glance, one might assume that a less than perfect gene-editing reagent would prevent substantial adoption of the tool. However, for non-therapeutic use, such stringency might not always be needed and can be compensated for with proper controls. Hence, the most important aspect is a thorough understanding of off-target events, their biological consequences and how these effects can be mitigated.

Sequence-dependent off-target propensities are best understood for the SpyCas9 enzyme, for which combinations of systematic and unbiased experiments have begun to shed light on potential liabilities[111–113]. Several excellent reviews have extensively discussed CRISPR–Cas off-target effects[114–116]. Nevertheless, our understanding of the molecular mechanisms through which Cas9 can sometimes inappropriately bind to and cut off-target sequences is still in its infancy, and indeed such tolerance may be built into naturally evolved CRISPR–Cas systems as part of the immunological 'arms race' between the phage and its bacterial host. For SpyCas9, phenomenological data revealed that the 8–10 nucleotides neighbouring the protospacer adjacent motif (PAM) are most stringently recognized, whereas one or two mismatches can be tolerated in the remaining nucleotides[67,117,118].

Off-target sites are determined by the nuclease and the sgRNA sequence. Thus, several algorithms have been developed to predict sgRNA efficiency and off-target sites[67,119–123]. Although comparison with unbiased genome-wide assessment of off-target sites has revealed the limited predictive power of many algorithms for distantly related off-target sites[111], likely off-target sites and clearly risky sgRNAs can still be identified. From the perspective of research use for target identification and validation, any candidate sequence that is identified through a CRISPR–Cas knockout experiment should be validated with orthogonal strategies to rule out off-target effects. These strategies might include the use of multiple sgRNAs, isolation of multiple clonal lines, validation by alternative transcript knockdown methods (for example, CRISPRi or RNAi), and cDNA or CRISPRa complementation studies. This methodology mirrors follow-up experiments that are required for comparable RNAi approaches. In a research setting, the ability to perform such validation experiments makes the extensive identification of rare off-target sites relatively superfluous. Off-target analyses and de-risking strategies are far more critical for therapeutic CRISPR–Cas gene editing than for preclinical investigations.

Much effort has been put into the development of strategies to systematically minimize CRISPR–Cas off-target effects. One tactic requires two Cas9 nickases

Protospacer adjacent motif (PAM). Short genomic sequence adjacent to the sequence targeted by the guide RNA that is required for recognition by Cas effectors. This sequence varies based on the identity of the effector (for example, Cas9 versus Cpf1) and species (for example, *Streptococcus pyogenes* versus *Francisella novicida*).

(Cas9-D10A or Cas9-H840A[2], which cleave or 'nick' only a single DNA strand) to bind at neighbouring sites, thereby increasing the effective stringency that is due to the low probability of adjacent off-target sites within a genome[124,125]. Similarly, a dimerizing FokI nuclease domain (used by other DNA-editing tools, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs)) has been fused to a catalytically inactive Cas9 to ensure that only paired binding can induce DSBs[126,127]. Although they reduce off-target events, paired-nickase strategies also reduce the targetable space because they require two sgRNAs to bind to their targets within a relatively short stretch of DNA. In pooled screening scenarios, paired nickases also require a combinatorial library or tandem sgRNA vectors.

A second strategy to reduce off-target events relies on sgRNA or protein engineering to enforce higher specificity. Truncated guide RNAs can remove a few of the relatively permissive bases from the 5′ end of the guide RNA, which results in both decreased on-target and off-target activity[111,128]. SpyCas9 has also been mutagenized to more specifically recognize only a single PAM[129] or to abrogate nonspecific binding and thereby reduce the cleavage of non-target sequences[130,131]; although, again, the mechanism of action is still under investigation.

A third strategy to reduce off-target events adds strict control over the amount of active Cas9 in cells. So far, such approaches have used tightly regulated induction of Cas9 activity[132] and even reversible small-molecule-induced or photo-induced Cas9 activity[133,134]. These methods reduce off-target effects and also enable temporal control of genome editing. In appropriate scenarios, use of carefully titrated amounts of Cas9–sgRNA RNP complexes, which are rapidly degraded, can have similar benefits. Ultimately, many of the strategies outlined here might even be modularly combined for further gains in specificity, although this has yet to be experimentally tested. SpyCas9 has naturally high fidelity, and a variety of approaches have been able to improve its specificity. It is easy to foresee how additional engineering approaches, combined with a more detailed mechanistic understanding of the conformational changes that occur during target binding and cleavage, will advance editing precision.

Using CRISPR–Cas to make therapeutics

In addition to generating powerful research tools, genome editing with CRISPR–Cas technology holds great promise to make therapeutic agents or as a therapeutic itself. In principle, any DNA-editing technology could be used for the therapeutic strategies described in this section. Although ZFNs have advanced the furthest in clinical trials to date, there is currently insufficient evidence to declare whether the clinical utility of CRISPR–Cas, ZFNs or TALENs will be superior. The fast and inexpensive reprogramming of Cas9 gives it a clear advantage in contexts in which rapid experimental iteration is beneficial or in contexts in which many different loci need to be targeted. Here, we briefly discuss the current state of therapeutic gene editing (mostly in the context of ZFNs and TALENs) and how CRISPR–Cas can contribute to the field. We focus on therapeutic applications other than *in vivo* gene editing, as this topic has been covered by several recent reviews.

Creating CAR T cell-based therapies with gene editing. The application of gene editing for somatic diseases has begun to overlap with the rapidly expanding field of cancer immunotherapy, with immediate interest centring on the production of next-generation chimeric antigen receptor (CAR) T cells. These modified T cells, which express tumour-targeting receptors, have shown promise in the treatment of various leukaemias and lymphomas, and may eventually be used to treat solid cancers[135]. CARs comprise an extracellular binding domain (currently a single-chain variable fragment), which recognizes an antigen that is strongly expressed on — and specific to — tumour cells, and an intracellular chimeric signalling domain that activates the T cell upon receptor engagement and promotes T cell-mediated killing of tumour cells. The first battery of CAR T cell-mediated therapies targeted CD19, an antigen expressed by B cells and related cancer cells; several of these therapies have entered clinical trials (Juno Therapeutics: NCT02535364 and NCT02631044; Kite Pharma: NCT02601313 and NCT02348216; and Novartis: NCT02030834 and NCT02445248).

Currently, most CAR T cells are generated by using each patient's own T cells, an expensive and time-consuming process that involves isolating, modifying and expanding T cells for every new patient. This process is limited by current manufacturing capabilities. Hence, the economics of CAR T cells are less favourable than those of antibody-based checkpoint cancer immunotherapies such as ipilimumab, pembrolizumab and nivolumab. CAR T cell therapy could become much faster and less expensive if universal donor CAR T cells could be generated, as 'off-the-shelf' cells would substantially increase the number of patients that could be treated by a single CAR T cell product. However, graft-versus-host disease (GVHD) and host rejection, caused by recognition of recipients' cells by the CAR T cells and recognition of the CAR T cells by the host, respectively, remain major barriers to an off-the-shelf approach. In this context, ZFNs and TALENs have been used to knock out endogenous T cell receptor genes in T cells, which could prevent unwanted graft-versus-host reactivity[136,137] (Servier: NCT02808442). Genome-editing strategies could also be used to prevent or delay the rejection of CAR T cells by the recipient's immune system through the elimination of or a decrease in the expression of histocompatibility antigens on the donor T cells[138].

In addition to enabling the generation of off-the-shelf CAR T cells, genome editing could be used to boost CAR T cell efficacy by knocking out the genes encoding T cell inhibitory receptors or signalling molecules, such as cytotoxic T lymphocyte-associated protein 4 (CTLA4) or programmed cell death protein 1 (PD1)[139,140]. Indeed, the US National Institutes of Health (NIH) Recombinant DNA Advisory Committee (RAC) recently approved a clinical trial that will be carried out at the University of Pennsylvania in which Cas9 will be used to knock

out the genes encoding PD1 and the endogenous T cell receptor in melanoma-targeting CAR T cells (see Further information). China recently began the first clinical trial of CRISPR–Cas. The trial uses Cas9 to knock out *PD1* in T cells of individuals with lung cancer, although no CAR will be introduced in that trial[141]. Similar trials with *PD1*-knockout T cells for prostate and bladder cancer, as well as renal cell carcinoma, are also being initiated (see Further information). In the future, gene editing might even be used to introduce the CAR itself via HDR. Site-specific knock-in would eliminate the need for randomly integrating viral delivery vectors and allow for control over where the CAR integrates[142,143]. Future CAR T cell therapies could benefit from combined modification of endogenous T cell receptor genes, histocompatibility genes and components of signalling pathways. Still, it will be important to establish that the removal of inhibitory signals does not enable uncontrolled proliferation of the CAR T cells.

Compared with other gene-editing reagents, such as ZFNs and TALENs, CRISPR–Cas allows for extremely rapid testing of any newly proposed genetic modifications. Several industry partnerships have been announced between developers of CAR T cell therapies and companies specializing in gene editing, including Novartis' collaboration with Intellia Therapeutics and Caribou Biosciences, and Juno Therapeutics' collaboration with Editas Medicine. CAR T cell producer Cellectis acquired a licence to use TALENs from the University of Minnesota.

Therapeutic* ex vivo *gene editing. Drug delivery to the appropriate cells or tissue *in situ* is challenging in many fields and is certainly a major limitation for therapeutic applications of CRISPR–Cas. *Ex vivo* manipulation of target cells circumvents this issue. The haematopoietic system is an excellent target for *ex vivo* gene editing, because cells are readily obtained from peripheral blood samples and can be re-injected after manipulation and expansion. Therapeutic *ex vivo* gene editing of haematopoietic stem cells (HSCs) has previously been explored using ZFNs and TALENs, and some of these therapies are showing promise in clinical trials. The most advanced strategy uses ZFNs to target the C-C motif chemokine receptor 5 (*CCR5*) gene in cells from patients with HIV[144]. CCR5 is a co-receptor for HIV entry, and individuals with loss-of-function mutations in *CCR5* are highly resistant to HIV infection but are otherwise healthy. Importantly, transplantation of bone marrow from a CCR5-deficient donor to an HIV-infected individual with leukaemia, known as the 'Berlin patient', reduced the patient's viral load to undetectable[145]. Although CCR5-deficient, human leukocyte antigen (HLA)-matched donors are too rare for cell transplantation to be a broadly applicable treatment, they served as a proof of principle for the targeted disruption of *CCR5* to cure HIV infection.

Researchers have used ZFNs to disrupt the *CCR5* gene in T cells that were isolated from patients with HIV, followed by expansion and re-injection of the edited T cells, to create a pool of HIV-resistant, autologous T cells within the patient[144,146]. Phase I/II clinical trials of this approach are currently underway. Although mutations in the *CCR5* gene in T cells are permanent, the T cells themselves may not be. Researchers have recently focused on disrupting *CCR5* in HSCs in order to produce long-term self-renewing HIV-resistant cells[147]. CRISPR–Cas could also be applied to the same workflow of extracting, editing and re-implanting cells, and several groups have edited *CCR5* with Cas9 (REFS 6,148,149).

The haemoglobinopathies sickle cell disease (SCD) and β-thalassaemia have been targeted for *ex vivo* gene correction instead of disruption. All patients with SCD carry the same causal mutation in the haemoglobin subunit beta (*HBB*) gene, which causes a glutamate-to-valine substitution, ultimately leading to the aggregation of haemoglobin and misshapen red blood cells. ZFNs have been used to correct the sickle allele in HSCs via HDR by using an integrase-defective lentiviral vector or a single-stranded oligonucleotide donor[150]. CRISPR–Cas has rapidly caught up to ZFNs, demonstrating correction of the sickle allele using either an adeno-associated virus 6 (AAV6) or oligonucleotide donor[151,152]. By contrast, β-thalassaemia is caused by a variety of null or hypomorphic mutations in *HBB*[153], thereby requiring a plethora of case-specific targeting complexes and repair donors. CRISPR–Cas could be superior to other nucleases in such situations, as designing new sgRNAs is much faster and cheaper than engineering new TALENs or ZFNs. The regulatory landscape that surrounds such personalized approaches is in flux. Currently, even though multiple editing reagents might revert mutations to the same sequence, they would be classified as separate investigational new drugs (INDs).

Regardless, individually correcting all disease-causing *HBB* mutations could be unnecessary, as β-thalassaemia and SCD may be correctable by reactivation of fetal γ-globin (also known as haemoglobin γ-subunit) expression. The transcription factor BCL11A represses fetal γ-globin in adults, and Sangamo and Biogen initially sought to systemically disrupt BCL11A to increase fetal globin expression in patients with β-thalassaemia. However, several groups have now used TALENs, ZFNs and CRISPR–Cas to identify an erythroid-specific enhancer that controls *BCL11A* expression[83,154,155]. Notably, tiling sgRNA libraries, in which a genomic region is targeted with many sgRNAs, took advantage of the ease with which CRISPR–Cas can be reprogrammed to probe more than 500 sites in the enhancer region, and identified a minimal target sequence for disruption[83]. As disruption of the enhancer leads to an erythroid-specific decrease in BCL11A and an increase in fetal globin production, Biogen and Sangamo have combined their *BCL11A*-targeting programmes to focus on mutating the enhancer region (see Further information)[83]. These efforts currently use ZFNs, but various companies are exploring CRISPR–Cas for clinical disruption of the *BCL11A* enhancer.

Defining a path to the clinic

The path by which new gene-editing therapies advance to the clinic will undoubtedly be shaped by Sangamo's use of ZFNs to disrupt *CCR5*. Establishing the specificity of the nuclease was an important early hurdle, and the methods used to predict and measure off-target DNA

Investigational new drugs (INDs). A designation used to describe drugs that have permission from the US Food and Drug Administration (FDA) to be shipped across state lines, thus allowing these drugs to be tested in human clinical trials. IND applications are reviewed by the FDA to ensure that testing of the drug in humans does not pose excessive risk to the patient.

breaks are similar to the tools that assess Cas9 specificity, as discussed above. However, safety testing of gene-editing therapies must extend well beyond establishing the specificity of a nuclease. IND-enabling safety studies of ZFN-treated T cells and HSCs, which will set the stage for future CRISPR–Cas therapies, aim to demonstrate that edited T cells and HSCs will not lead to adverse effects, including leukaemia. These studies have included karyotype analysis, soft-agar transformation assays and tumorigenicity studies of whole-patient doses of cells in immunodeficient mice (see ZFN-based Stem Cell Therapy for AIDS and NIH RAC on safety of gene transfer; Further information). However, the capacity of *in vitro* studies and animal models to predict adverse events in humans will always raise concerns. Ultimately, patients, clinicians and regulatory agencies must discuss the level of risk that is acceptable under each circumstance and develop appropriate safety measures.

Even when effective DNA-editing reagents are developed and the treated cells are shown to be non-tumorigenic, substantial hurdles can remain for advancing a therapy into the clinic. Producing *ex vivo* edited cells at clinical scale (a dose of 10^{10} ZFN-treated T cells was used in the first trial of *CCR5* editing to treat patients with HIV[144]) under conditions compliant with good manufacturing practice is a major challenge. It is also important that the phenotype of gene-edited cells is only changed by editing, and that epigenetic alterations are not inadvertently introduced through *ex vivo* culture. Many assays, such as those that measure the capacity of HSCs to engraft and differentiate into numerous leukocyte subtypes[147], can assess the healthy function of edited T cells and HSCs, although whether such assays can fully recapitulate behaviour in humans is unclear. It will thus be crucial to build deep phenotypic characterization protocols for all gene-editing therapies.

Last, but not least, newly expressed or corrected proteins may be recognized as foreign by the recipient's immune system. For example, individuals with haemophilia can develop neutralizing antibodies against replacement blood clotting factors[156]. Edited cells could, in principle, be recognized and eliminated by cytotoxic T lymphocytes[157]. However, rejection of edited cells due to recognition of the transgene has not yet been an issue in clinical trials of anti-sickling globin gene therapy[158,159], which suggests that this may not be a problem for all edited genes or cell types.

Conclusions

CRISPR–Cas tools have been developed for many cells and organisms in which genetic manipulation was previously relatively intractable, from human ES cells to the malaria parasite. Particularly in mammalian model systems and human cells, these technologies can accelerate functional genomics to uncover cellular mechanisms and identify or validate new drug targets. Applying CRISPR–Cas editing to animals will lead to better models of human disease, more predictive safety testing, and improved stratification and treatment regimens for patients. Rapid gene editing and regulation also promise to enable innovative therapies for non-genetic diseases through the generation of customized autologous cellular treatments, including cancer-seeking T cells and reprogrammed iPSCs. While CRISPR–Cas systems will undoubtedly improve further, and new complementary or orthogonal methods will be developed to deliver reagents and edit somatic tissues directly in humans, we believe that gene editing is ready to have an immediate impact in real-world drug discovery and development. CRISPR–Cas-aided discovery, validation and safety testing allow acceleration and improvement of known protocols and pipelines, without the need to solve delivery or redefine administrative procedures. CRISPR–Cas will be key to the next generation of transformational therapies and treatment paradigms.

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Competing interests statement
The authors declare competing interests: see Web version for details.

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FURTHER INFORMATION
Biogen and Sangamo's programme for *BCL11A*: http://investor.sangamo.com/releasedetail.cfm?ReleaseID=912987
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Encyclopedia of DNA Elements (ENCODE): https://www.encodeproject.org/
NIH RAC on clinical trials of CRISPR-edited CAR-T cells: http://osp.od.nih.gov/office-biotechnology-activities/event/2016-06-21-123000-2016-06-22-164500/rac-meeting
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